CHAPTER 4: The pharmacokinetics of meloxicam in vultures

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The pharmacokinetics of meloxicam in vultures

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4.1 Abstract

Vulture populations across the Asian subcontinent have declined dramatically in the last fifteen years and are now on the verge of extinction. Although the cause of the population decline was initially unknown, the decrease has recently been conclusively linked to the use of the non-steroidal anti-inflammatory drug diclofenac in cattle that inadvertently ended up in the vulture food chain. With the vulture numbers continuing to decline by up to 48% a year, the Indian, Nepali and Pakistan governments have recently banned the manufacture and importation of veterinary diclofenac. They have also suggested meloxicam as an alternate anti-inflammatory for use in cattle. This recommendation was based on extensive acute safety studies in the African White-backed Vulture (*Gyps africanus*) which evaluated worst case scenarios of maximum intake based on a once in

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three day feeding pattern. However, the possible cumulative pharmacokinetic and pharmacodynamic effects in vultures receiving multiple daily doses of meloxicam over time were not assessed. At present very little pharmacokinetic or pharmacodynamic information is available to add further support for the safety of meloxicam in this animal species. This article discusses the oral and intramuscular pharmacokinetics of meloxicam in Cape Griffon vultures (*Gyps coprotheres*). Therapeutic drug monitoring was also undertaken in White-backed, Egyptian (*Neophron pernopterus*) and one Lappet Faced Vulture (*Torgos tracheliotos*). In all these species meloxicam was characterised by a short half-life of elimination. The rapid metabolism of meloxicam in combination with a short duration of effect in the studied *Gyps* vulture species shown in this study makes it unlikely that the drug could accumulate. This confirms the safety of repeated exposure to meloxicam in vultures of this genus.

Keywords: Meloxicam, Vultures, Safety, Diclofenac, NSAID, COX

4.2 Introduction

Three species of vultures endemic to South Asia are in grave danger of extinction across the Indian subcontinent. Populations of Oriental white-backed vultures (*Gyps bengalensis*), long-billed vultures (*G. indicus*) and slender-billed vultures (*G. tenuirostris*) have declined by more than 97% in India and Pakistan (Shultz *et al.*., 2004) and continue to decline at rates of 22-48% per year (Green *et al.*., 2004). In 2004, Oaks *et al.*., linked the population crash to the veterinary use of diclofenac, a non-steroidal anti-inflammatory drug (NSAID). In this study, Oaks *et al.*. (2004) was able to demonstrate that diclofenac residues in the carcasses of dead cattle, treated prior to their deaths, were highly toxic to scavenging vultures. From subsequent modelling data Green *et al.* (2004) were able to show that residues in few as one in approximately two hundred carcasses would be sufficient to cause the decline rates reported. Diclofenac has also been shown to have an approximate LD_{50} of 0.098 to 0.225 mg/kg in vultures (Swan *et al.*., 2006b), making it more lethal than aldicarb, which is regarded as one of the most toxic pharmaceutical compound in animals (Pesticide Information Profiles, 1996).

To protect the remaining vulture populations, the governments of India, Pakistan and Nepal have taken steps to phase out the veterinary use of diclofenac, including bans on the manufacture and importation of the drug in addition to recommending the use of vulture safe alternatives such as meloxicam for livestock treatment (Mo, 2006). The recommendation to use meloxicam was based on an extensive safety-testing study, in which *Gyps* vultures were exposed to oral meloxicam as either pure drug in formulation or residues in tissues from livestock dosed with meloxicam (Swan *et al.*., 2006a; Swarup *et al.*. 2007). While these studies have demonstrated the safety of meloxicam (in comparison to diclofenac) following single exposure to the drug, the safety of meloxicam following repeated exposure was not documented. Swan *et al.*. (2006a) estimated the maximum levels of exposure on the basis of meloxicam residues in livestock liver tissues shortly after dosing and for a vulture consuming enough liver tissue at one sitting (1.02kg) to provide the estimated energetic requirements for three days: a plausible maximum duration between meals based on observations of wild and captive vultures (Mundy *et al.*., 1994). This does not, however, consider the cumulative effect of multiple exposures to meloxicam over a short time period, which may occur for birds consuming livestock tissues on a daily basis.

Irrespective of the duration of exposure, when evaluating the safety of a NSAID, one other factor which must be considered is the duration of cyclo-oxygenase (COX) enzyme inhibition i.e. should COX inhibition be reversible, the time required for complete enzyme recovery is important (Boothe, 2001). As an illustration, carprofen is effective for once a day pain management in the dog despite the drug having a half-life of just 8 hours (Clark, 2006). As such it is plausible that a drug with a short pharmacokinetic half-life (non-cumulative) could still result in toxicity if successive doses lead to prolonged enzyme inhibition mechanistically.

In this study we characterise the intramuscular and oral bioavailability of a single dose of meloxicam in adult *G. corportheres* vultures to determine the elimination half-life of the drug and if possible to extrapolate the plasma profile for multiple exposures over time. The safety of meloxicam was also evaluated by monitoring birds treated on a daily basis.

4.3 Material and Methods

4.3.1 *Pharmacokinetic Study*

The pharmacokinetics (PK) of meloxicam in adult Cape Griffon vultures was evaluated using a single dose, two phase parallel study consisting of six birds per treatment group (Table 4-1). The birds were allowed an acclimatization period of one week. To facilitate the management of the study the intramuscular (i.m.) and oral dosing was separated by one week. The vultures were housed within the University of Pretoria's Biomedical Research Centre (UPBRC) in single aviaries of 1x1x2.5 metres. During the study the birds were fed twice weekly with one kilogram of beef each, bought from a commercial butchery. The meat was assumed to be free of meloxicam and other NSAIDs as South Africa follows the minimum residue limit guidelines proposed by the joint expert committee on food additives of the FAO and WHO (JECFA) in the determination of withdrawal periods (Act 54/72). At the end of the study, the birds were returned to De Wildt Cheetah and Wildlife Centre from where they were sourced. All captive animals used in this study were in captivity for at least one year prior to inclusion in the study.

The birds were given a single dose of meloxicam (Melonex 0.5%m/v, India) at 2 mg/kg by either intramuscular injection or oral gavage. Dose rates of 2 mg/kg were selected, as this is the estimated maximum level of exposure to meloxicam used in previous safety testing (Swan *et al.*., 2006). For gavage a small diameter tube was passed directly into the crop. Once the drug was dosed, the tube was flushed with 2 ml of sterile water. Once the tube was removed, a further 2 ml of water was squirted into the mouth of the vulture. Intramuscular injections were administered directly into the pectoral muscle.

Blood samples were collected by means of a 5ml syringe and immediately transferred into 5ml lithium heparinised vacutainer. Samples were collected generally from the tarsal vein or when necessary the wing vein, before drug administration and at 4 and 30 minutes and at 1, 1.5, 2, 6, 8 and 10 hrs after treatment. Within two hours of collection the blood samples were centrifuged at ca 3000*g* and 4°C for 15 min and the supernatant of each sample transferred to labelled polycarbonate tubes.

Plasma concentration data for all animals were analysed using WinNonLin 4.2 (sponsored by the Pharsight Corporation). The plasma curve for meloxicam for both intramuscular and oral routes were best fitted to a one compartment open model (model 3) and were best described by the equation 1:

$$C = \frac{\text{Dose} \times K_a}{V_d/F \times (K_a - K_e)} \times (e^{-kat} - e^{-ket}) \qquad (1)$$

where C is the plasma concentration at time t, K_a the absorption constant, K_e the elimination constant and V_d/F the apparent volume of distribution. The relative bioavailability ($F_{relative}$) was calculated according to equation 2.

$$F_{relative} = \frac{AUC_{oral}}{AUC_{im}} \times 100 \qquad (2)$$

where AUC represents the area under curve to the last time point for the oral and i.m. routes of administration.

4.3.2 *Liquid chromatography tandem mass spectrometry*

Sample extraction and preparation was done using a method developed in our laboratory and reported previously (Swan *et al.*, 2006a). Briefly, 2 ml acetonitrile was added to 200 µl of plasma, mixed for 4 min on a Lab-tek multitube vortexer and subsequently centrifuged at 1200*g*. The supernatants were transferred to clean glass tubes and evaporated to dryness at 60 °C under a stream of nitrogen gas in a Zymak TurboVap® LV Evaporator. Reconstitution was performed with 50 µl 100% methanol, followed by addition of 100 µl 0.4% acetic acid in methanol/dH_2O (60:40).

The sample extracts were analysed by LC/MS/MS using an Agilent 1100 series high pressure liquid chromatograph with temperature controlled autosampler and diode array detector (collecting the cumulative absorbance from 210 to 400nm) coupled to an Applied Biosystems API4000 QTrap mass spectrometer fitted with a "Turbo V" electrospray ionisation (ESI) source. The HPLC column used was a Phenomenex Prodigy ODS(3) C18 column (4.6x100 mm, 3 µm particle size) and the mobile phase a 20:80 mixture of A: 0.1% formic acid and B: 60% acetonitrile in 0.1% formic acid at a flow rate of 1000 µl/min for 6.5 minutes (Wiesner *et al.*, 2003). The sample injection volume used was 2 µl. The ion

source was operated in the positive mode at 450˚C with the source-specific nebuliser and source gasses set at the optimal pressures as determined during FIA optimisation. Analytes were detected and quantitated by means of characteristic ion transitions from protonated parent ions to fragment ions generated by collisionally activated dissociation (CAD) utilizing the multiple reaction monitoring mode (MRM). The collision gas was nitrogen at the high setting (using a CMC nitrogen generator), and collision energies were optimised for each analyte as listed in Table 2. Additionally the extracted wavelength diode array chromatograms (350±20nm) were used to confirm the retention times of the meloxicam metabolites. The method was shown to have no interference when plasma from untreated birds were injected and demonstrated a limit of detection (LOD), defined at a signal-to-noise ratio 3:1, was less than 30 ng/ml for meloxicam while the limit of quantitation (LOQ) was less than 125 ng/ml in spiked plasma. Calibration curves for meloxicam were linear over a range of 125 – 12500 ng/ml with regression coefficients of at least 0.99. Average accuracy over the concentration range analysed was 96% and precision varied from 13% to 0.5% depending on the concentration with the highest variation observed at the lowest concentrations.

Studies on metabolite ion fragmentation were conducted by direct infusion of plasma extracts, known to contain the metabolites, dissolved in 30% acetonitrile and 0.1% formic acid, into the ESI source. In order to elucidate the origin of the fragment ions and potential dissociation pathways, MS3 experiments were performed on samples containing the metabolites (Table 4-2).

4.3.3 *Meloxicam Clinical and Therapeutic Monitoring*

Some of the birds included in this study were injured (soft tissue injuries) and showed signs of pain such as drooping heads, decreased feed intake and reluctance to place weight on their injured limb or fly (Table 1). In total 11 vultures from four difference species were monitored for signs of toxicity following treatment with meloxicam at the dose of 2mg/kg, by intramuscular administration into the pectoral muscle. With the exception of the White-backed vultures, which received only one dose of meloxicam, all the birds received multiple treatments at 24h intervals.

Of the treated vultures one Cape vulture (*G. coprotheres*) and White-backed vulture (*G. africanus*) were monitored for clinical signs of toxicity for their 14 and 5 days of treatment respectively, without quantifying plasma concentrations. For the other birds, prior to each 24 hour treatment, plasma samples were collected and analysed for meloxicam concentrations as described above. This included one Lappet Faced vulture (*T. tracheliotos*) from which samples were collected at 0, 24, 48, 72, 96 and 120 hours; one Egyptian vulture (*N. pernopterus*) from which samples were collected at 0, 4, 18 and 24 hours and five African White-backed Vultures (*G. africanus*) from which samples were collected at 0, 4, 12, 24 hours.

4.4 Results

4.4.1 *Pharmacokinetics*

The pharmacokinetic parameters obtained are listed in Table 4-3 for both routes and illustrated in Figure 4-1. Oral absorption of meloxicam in the vultures was characterised by a relative bioavailability of 107% compared to intramuscular absorption. Meloxicam was also rapidly absorbed with C_{max} being achieved within approximately 0.5 hours of administration for both routes of administration. The absorption half life ($T_{1/2\alpha}$) of 0.41 ± 0.33h and 0.33 ± 0.17h for the intramuscular and oral routes respectively was very similar to their elimination half life ($T_{1/2\beta}$) of 0.42 ± 0.1h and 0.32 ± 0.17h respectively. A small apparent volume of distribution (V_d/F) was observed following both oral and intramuscular administration.

4.4.2 *Biotransformation Pathways*

We identified three peaks as potential metabolites due to their time dependent increase in concentration in conjunction with meloxicam's decline (Figure 4-2). Based on molecular mass they correspond to two hydroxy-methyl-metabolites together with one glucuronide conjugate (Table 4-2).

4.4.3 *Meloxicam Clinical and Therapeutic Monitoring*

No signs of toxicity were seen in the Egyptian (*N. pernopterus*), Lappet Faced (*T. tracheliotos*), African White-backed (*G. africanus*) or Cape Griffon (*G. corprotheres*) vultures following repeated treatment with parenteral meloxicam. While no drug was detectable for the Lappet Faced vulture, the plasma concentrations in the Egyptian vulture was 5 µg/ml at 4 hours. Meloxicam (0.22 ± 0.20µg/ml) was detectable for all the dosed White-backed vultures in which a 4 hour sample was collected. None of the other time points, had detectable drug concentrations.

The Cape Griffon Vulture that was treated with meloxicam for 14 days was eventually terminated due to non-recovery for an injured leg. From the full post-mortem examination of this bird, no gross pathological lesions were evident, except muscle atrophy for the injured limb.

4.5 Discussion

4.5.1 *Pharmacokinetics*

Although the reason for the higher oral bioavailability (107%) relative to the intramuscular route is unknown it may be an artefact as absorption was more rapid following oral administration resulting in the increased oral C_{max} . In our opinion this is the reason for the higher AUC_{oral} and is a reflection of the sampling intervals rather than drug effect. This supported by Toutain *et al.* (2004), who stated that small differences of this nature are usually non-significant and may result from the predetermined sampling interval. However, to determine the actual significance of the higher oral bioavailability the absolute bioavailability for both routes will have to be determined.

With the absorption and elimination half-lives being almost identical for each route, this tends to suggest that absorption is the limiting factor in the rate of elimination of the drug. It is therefore possible that a degree of flip-flop kinetics is being seen and requires further investigation following intravenous administration. A V_d/F was observed following both oral and intramuscular administration. The result was similar to results previously reported

for the pigeon and a consistent finding for most NSAIDs (Boothe, 2001; Baert, 2003). With the NSAIDs being highly plasma protein bound in birds, the low V_d/F likely results from extensive macro-molecular binding and possibly rapid metabolism. (Lees *et al.*., 2004; Boothe, 2001; Baert *et al.*., 2002; Baert *et al.*., 2003).

This rapid half-life of elimination is extremely important as it can prevent drug accumulation and delayed toxicity. With the assumption that 99% of all drug is eliminated in ten elimination half-lives, these birds will be virtually free of the drug 5-7 hours post exposure (Brown, 2001). The main objective of this study was to assess the potential for meloxicam's accumulation following multiple feedings: given the observed time to elimination a vulture will have to consume numerous meals in one day for accumulation to occur. Ecological studies of vultures in the wild indicate that it is normal for birds to engorge themselves at one session if sufficient food is available, making it unlikely that a bird will be able to consume more than one meal a day (Mundy *et al.*., 1992). Moreover, the dose of 2.0 mg/kg administered in this study, is the likely maximum level of exposure based upon a bird consuming three days of food (1.02 kg) at just one sitting, and consuming only liver tissues from an animal dosed with meloxicam in the hours immediately prior to death. Given this scenario, it is very unlikely that birds can take in larger concentrations of meloxicam at levels able to cause accumulation.

With this study making use of the drug in formulation instead of residues in meat, it may be possible that the presence of meat could adversely influence the process of absorption. i.e. food decreasing the rate of absorption. Although this has never been specifically found with meloxicam, the consumption of food in people is known to slow the rate of absorption of ketoprofen (Busch *et al.*, 1990; Bannwarth *et al.*, 2004). Unfortunately we are doubtful that the influence of a meal on meloxicam's absorption can ever be properly determined in the vulture as unlike mammals, one of the defence mechanisms of the vulture is to regurgitate its meal at the first sign of a threat (Mundey *et al.*, 1992). This is believed to have two important effects, firstly to scare off the threatening animal and secondly to make the body lighter and quicken escape times. As a result it is impossible to get close to these birds soon after feeding for blood collection, as they immediately regurgitate the ingested meal. Realistically we don't believe that will have a major influence on the safety of the

product as slower absorption should promote lower plasma concentrations and lower the drug's toxic potential.

4.5.2 *Biotransformation Pathway*

Generally in mammals meloxicam is metabolised by CYP2C9 during the Phase I reactions and by glucuronide transferase in PhaseII. The predominant Phase I metabolites in laboratory animals and man are the 5-hydroxymethyl derivative and a 5-carboxy metabolite (Busch, 1998; Chesne, 1998). With two hydroxy-methyl-metabolites together with one glucuronide conjugate being tentatively identified, it is likely that vultures make use of the cytochrome P450 enzyme system, perhaps even CYP2C9 as in man, for initial metabolic transformation and glucuronidation for the synthetic reaction (Chesne *et al.*., 1998). The presence of a glucuronide conjugate also indicates that this species appears to use the standard metabolic pathways for NSAID metabolism as described in other animals (Baert and De, 2003; Busch *et al.*., 1998; Kumar *et al.*., 2002). Unlike in mammals the carboxy-metabolite (m/z = 391) was absent. Since the hydroxyl metabolite is converted to a carboxy metabolite by a non-cytochrome dependant pathway, the absence of the peak suggests the absence of these pathways in the vulture (Chesne *et al.*., 1998). More work is, however required to confirm these finding.

4.5.3 *Meloxicam Clinical and Therapeutic Monitoring*

For the Egyptian vulture in which drug was detectable at only 4 hours, we estimate a $T_{1/2\beta}$ of below 2 hours. More birds will have to be evaluated to confirm the half-life of the drug in the Egyptian vulture. As for the Egyptian vulture, the terminal half-life could be established for the White-backed vulture as only one time point had detectable drug concentrations. None the less the plasma concentration for this species fits the equation described for meloxicam disposition in the *G. corprotheres* where the concentration is 0.29 ±0.27 µg/ml at 4h. This tends to suggest that the half-life and absorption of meloxicam is likely to be similar in the two species.

The lack of notable pathology in the Cape vulture treated for two weeks in addition to the absence of clinical signs of toxicity in any of the birds receiving repeated therapy was considered a significant finding as this clearly shows that toxicity will not result from the cumulative exposure to the product.

4.6 Conclusion

The anti-inflammatory drug, meloxicam appears to be rapidly metabolised and excreted in vultures. The rapid excretion in the vulture species tested may indicate that the metabolism is similar in all four of these vulture species. This study therefore, goes some extent towards explaining the safety of the drug in this species and implies that meloxicam is unlikely to have a toxic effect in birds feeding once a day. For vultures being treated therapeutically it is possible that with a rapid half-life birds may require more frequent therapy than the daily regime used at present. Although twice a day dosing may be more helpful, dosage intervals should also be based on the duration of apparent analgesic effect.

4.7 Acknowledgements

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Table 4-1: A list of the different birds included in this study. All the listed birds were in captivity following attempted rehabilitation

Vulture Species	**n**	**Route**	**Status**	**Health Status**	**Analysis**
G. coprotheres	6	Im	Captive	Healthy	Meloxicam PK
G. coprotheres	6	Oral	Captive	Healthy	Meloxicam PK
G. africanus	8	Oral	Captive	Healthy	Meloxicam TDM
N. pernopterus	1	Im	Captive	Injured leg	Meloxicam TDM
G. africanus	1	Im	Wild	Injured Wing	Meloxicam TDM
T. tracheliotos	1	Im	Captive	Injured leg	Meloxicam TDM
G. coprotheres	1	Im	Wild	Injured leg	Monitored for response to meloxicam

Table 4-2: The analytical and mass spectrometer parameters used in identifying the metabolites of meloxicam in *G. corprotheres* plasma samples

Metabolite	MW (Da)	Characteristic transitions (% abundance)			CE (eV)	RT (min)
Meloxicam parent	351.4	352>115 (100)*	352>141 (40)	352>153 (5)	65	5.08
Mono-hydroxy 1	367.4	368>115*			50	3.51
Mono-hydroxy 2	367.4	368>115*			50	2.83
Glucuronide	543	544>115*			65	1.61
Carboxy	381	382>115*			65	ND

* MRM transition signal used for quantitation of the compound.; ND-non detected

Table 4-3: Pharmacokinetics parameters for meloxicam following intramuscular and oral administration in *G. corprotheres* using a one compartmental analysis

Parameter	**Unit**	**IM**		**Oral**	
		Mean (n=6)	**%CV**	**Mean (n=6)**	**%CV**
Ka	h^{-1}	1.77	27.28	2.56	45.10
$T_{1/2\alpha}$	H	0.41	24.14	0.33	53.19
C_{max}	µg/mL	3.58	44.33	5.25	33.51
T_{max}	H	0.60	25.31	0.47	52.78
Ke	h^{-1}	1.75	29.55	2.57	0.01
$T_{1/2\beta}$	H	0.42	26.56	0.32	52.37
AUC	µg/mL*h	5.86	58.50	6.29	41.78
Cl/F	mL/kg/h	130.20	130.79	56.82	62.52
V_d/F	L/kg	0.26	0.00	0.15	0.00

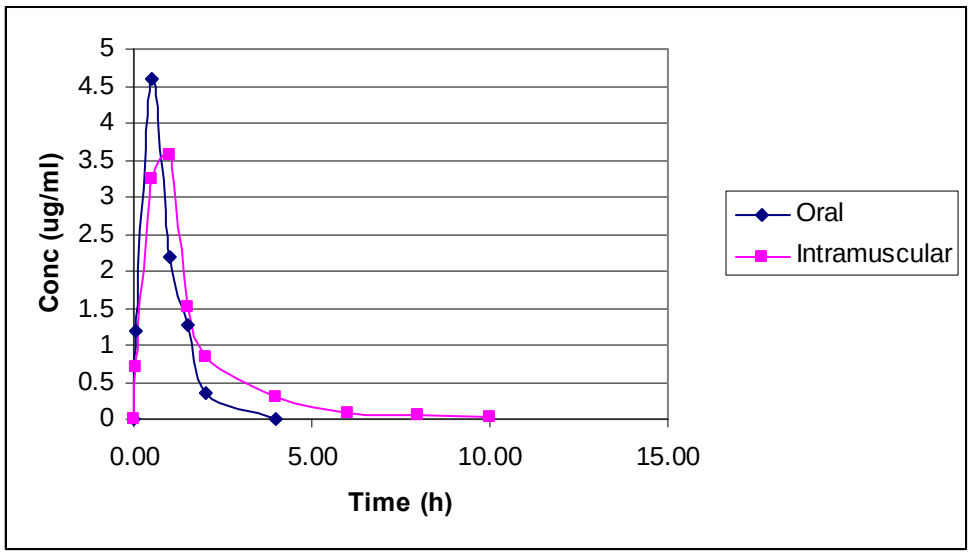

Figure 4-1: Mean plasma concentration versus time curve following oral and intramuscular meloxicam administration in adult *G. corprotheres* vultures.

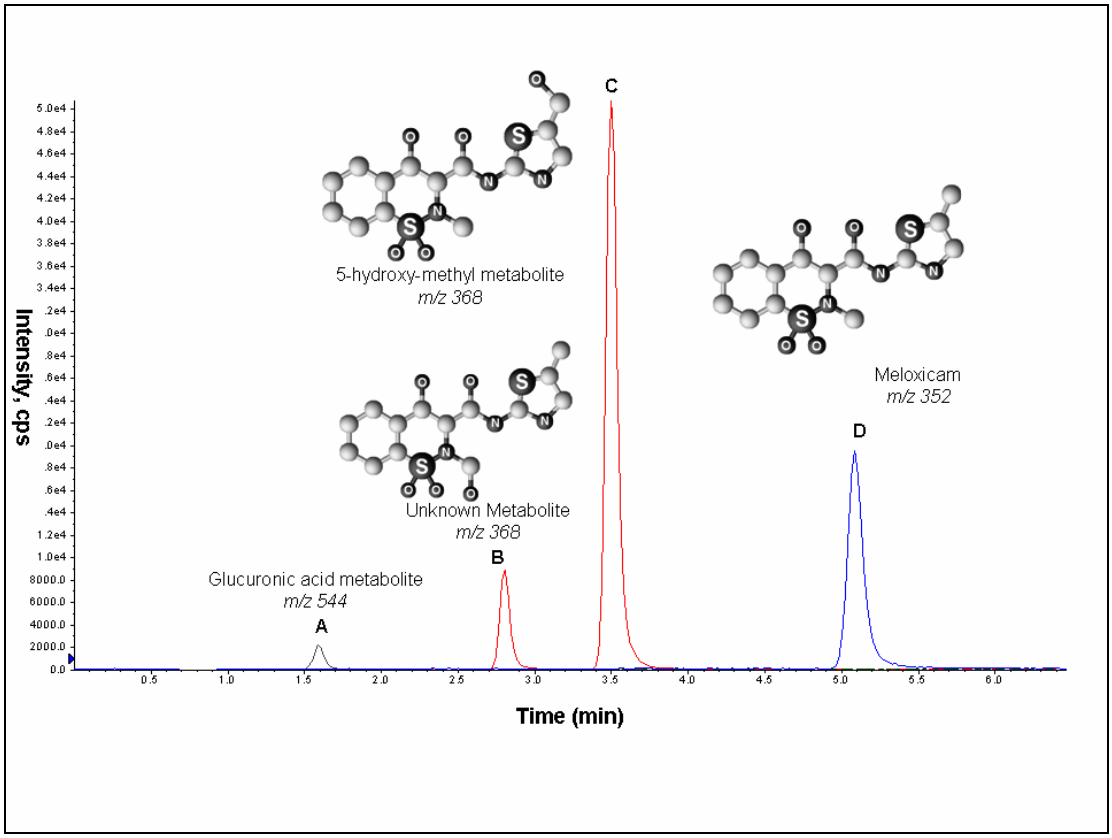

Figure 4-2: Identified meloxicam metabolites as determined by LCMSMS. a) Glucuronide metabolite, b) hydroxyl metabolite 1 c) the unknown metabolite indentified as potential second hydroxyl metabolite, d) meloxicam

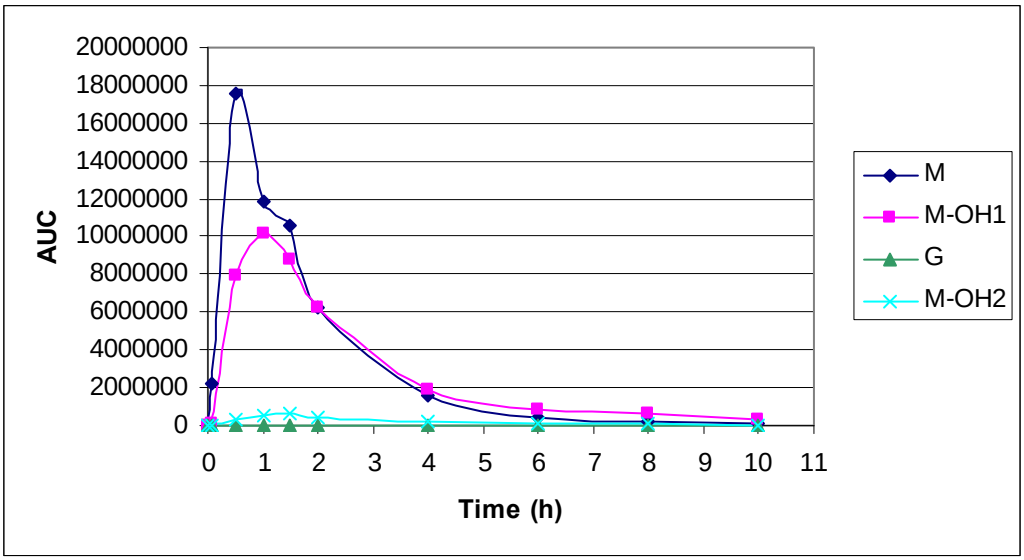

Figure 4-3: Change in the average area under curve over time for each metabolite following the oral administration of meloxicam, using diode-array detection. The initial increase over time corresponded to a decrease in plasma meloxicam concentrations (M: Meloxicam parent, M-OH1- Hydroxy metabolite 1, G-glucuronide metabolite, M-OH2- Hydroxy metabolite 2)