The use of liver slices from the Cape vulture (*Gyps coprotheres*) to better understand the role of liver toxicity of Non-steroidal anti-inflammatory drugs (NSAIDs) in vultures

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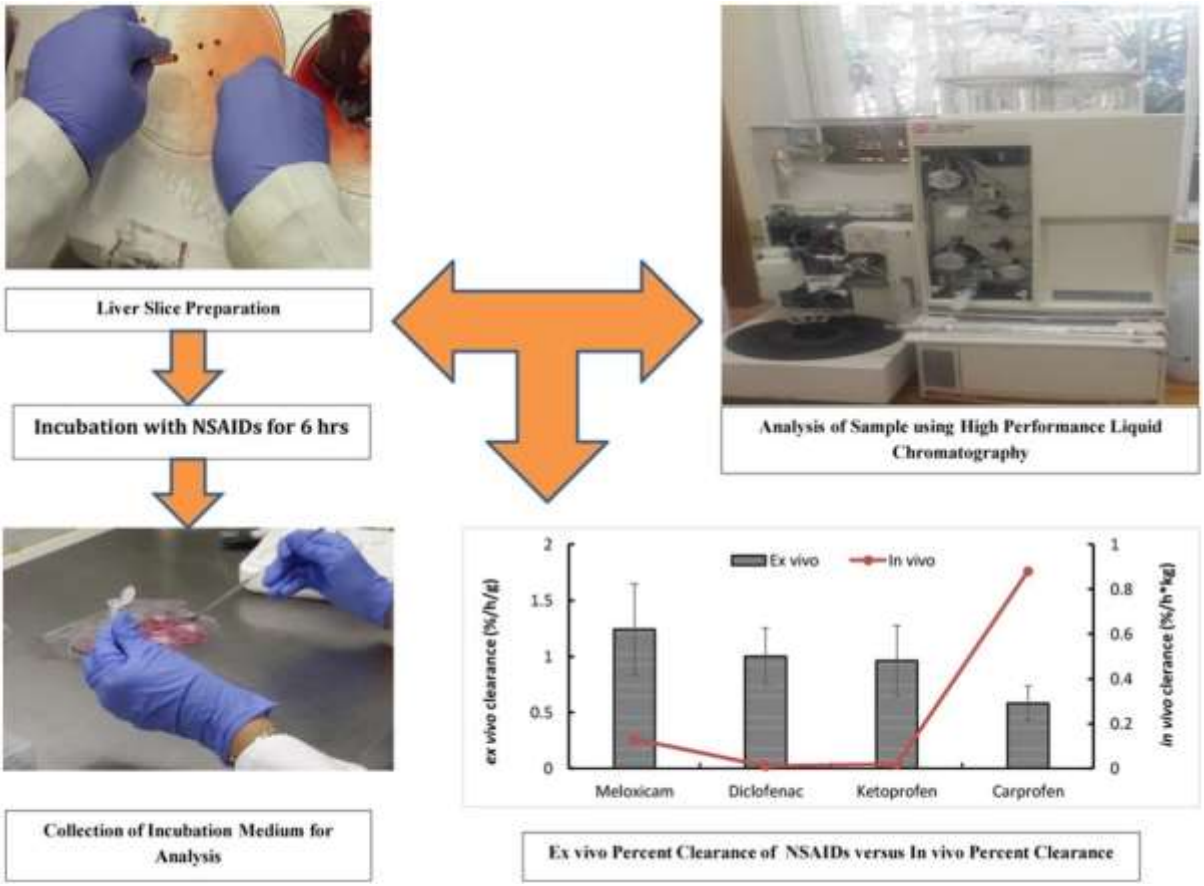
Highlights

- Liver slices cultures can be established from euthanized vulture.
- The vulture liver had a total lower protein content than for an animal of its size.
- While *ex vivo* metabolism resulted, no correlation to previous *in vivo* studies were present.
- All tested NSAIDs were toxic, including meloxicam which was likely due to the residence time.
- The results show that vultures liver slice cultures are a poor predictor of NSAID susceptibility.

Graphical abstract



Abstract

Diclofenac, a non-steroidal anti-inflammatory drug (NSAID) was responsible for the death of millions of vultures on the Asian subcontinent, following the consumption of diclofenac contaminated carcasses. The aim of this research was to establish if liver slices could serve as an alternate means of predicting the toxicity of NSAIDs in Gyps vultures. The Cape vulture liver slices was prepared and incubated with four NSAIDs for 6 h. A percent clearance of 1.0 ± 0.253, 0.58 ± 0.153, 0.961 ± 0.312 and 1.242 ± 0.406 (%/h*g) was attained for diclofenac, carprofen, ketoprofen and meloxicam respectively. Both meloxicam and diclofenac exerted toxic effects on the hepatic cells. Protein content indicated that the vulture tissue had lower enzyme levels than expected for an animal of its size. The poor distinction

between the *ex vivo* hepatic percent clearance of meloxicam and diclofenac indicates that liver slices is not an ideal model to investigate NSAIDs toxicity in Cape vulture.

Keywords

Diclofenac; Meloxicam; Liver slices; Cape vulture; Percent clearance; Toxicity

1. Introduction

Vultures are large raptors that feed predominately on carrion, thereby playing an important role in cleaning the environment as well as mitigating the spread of infectious diseases from putrefying carcasses (Markandya et al., 2008,Vijaikumar et al., 2002). Unfortunately with the global decline in vulture population, carcasses end up rotting in the environment producing an unpleasant smells with increased pest numbers resulting in the spread of infectious diseases. Tlike anthrax, leptospirosis, brucellosis and tuberculosis (Markandya et al., 2008,Swan et al., 2006b). Globally, the population of this group of bird species has declined due to a multiple of threats from loss of habitat, direct persecution, poisoning (both malicious and intentional), electrocutions, muti trade and most recently from the exposure to diclofenac on the Asian subcontinent (Ogada et al., 2012,Angelov et al., 2013,Angelov et al., 2013,Virani et al., 2011,Boshoff et al., 2011,Boshoff et al., 2011,Sarans and Purohit, 2012,Sarans and Purohit, 2012,Oaks et al., 2004,MaMing and Xu, 2015,Naidoo et al., 2011,Naidoo et al., 2011,Naidoo et al., 2017). Of all these, the latter has received the most attention after three vulture species, the long-billed vulture (LBV) (*Gyps indicus*), the slender-billed vulture (SBV) (*G. tenuirostris*) and oriental white-backed vulture (OWBV) (*G. bengalensis*) in India, Pakistan and Nepal were brought to the brink of extinction, with a cumulative decline from 1990 to 2007 of 96.7 to 99.9% (Prakash et al., 2007). All three

species are now listed as critically endangered by the International Union for the Conservation of Nature (IUCN) (Hilton-Taylor and Mittermeier, 2000).

At first the cause for the vulture deaths on the Asian subcontinent was unknown, with clinical signs of neck drooping, depression, elevated serum uric acid, and visceral gout lesion on the liver and kidney being the only signs (Oaks et al., 2004,Shultz et al., 2004,Green et al., 2004). After substantial investigation, the source of toxicity was eventually linked to residues of diclofenac present in the vulture food chain as a result of livestock being on palliative diclofenac treatment at the time of their death (Oaks et al., 2004,Shultz et al., 2004,Green et al., 2004). Diclofenac (2-[2-(2,6-dichlorophenyl amino) phenyl]acetic acid) is a non-steroidal anti-inflammatory drug (NSAID), developed in the late 1970s for the treatment of pain, fever and inflammatory conditions such as osteoarthritis, rheumatoid arthritis, and ankylosing spondylosis in humans (Scully et al., 1993,Burke et al., 2006). The drug also has merit in veterinary medicine for the management of livestock disease due to its analgesic and anti-inflammatory properties. In India specifically, where there are large populations of livestock due to their religious significance, diclofenac was commonly used from a combination of its cost effectiveness, pharmacological effectivity and ease of acquisition as it could be acquired without veterinary prescription. This in combination with the absence of proper facilities for the disposal of dead animal's carcasses resulted in these carcasses being left out in the field to putrefy or to feed canine feeders. This practice over time contributed to a large number of vultures being exposed to toxic levels of diclofenac.

In an attempt to prevent the extinction of these species of vulture, the veterinary use of diclofenac was banned in India, Pakistan, Nepal, and most recently in Bangladesh (Prakash et al., 2007,Save, 2014). Efforts were also made into searching for alternative drugs such as meloxicam (Swan et al., 2006a), as well as to establish viable breeding colony of birds that would later be introduced when the threat of diclofenac was removed (Save, 2014,Bowden et

al., 2012). In addition, further research was undertaken to characterise the mechanism of diclofenac toxicity in the Asian Gyps. From pharmacological studies, the pharmacokinetics of diclofenac was established in numerous species. Of the numerous studies undertaken, the two specific studies undertaken in South Africa with two vulture species, showed them to be highly susceptible to diclofenac toxicity, with the toxicity being associated with an extended elimination half-life of 12.24 h in the Cape vulture (*Gyps coprotheres*) and 16.78 hours in African white-backed vulture (*Gyps africanus*) (Naidoo et al., 2009). The determined half-life was in marked contrast to the Pied crow (*Corvus albus*), which showed no signs of toxicity, with the diclofenac being barely detectable in the plasma with a half-life of elimination estimated to be in the region of 2.33 hours (Naidoo et al., 2011). Furthermore, 25 mg/kg of diclofenac administered to Turkey vulture (*Cathates aura*) showed no sign of toxicity and had an elimination half-life of 6 hours in (Rattner et al., 2008). This led to the speculation that toxicity in the Vulture was linked to capacity specific metabolism.

From a pharmacological point of view, the half-life of a drug is dependent on the rate of absorption, the volume of distribution, metabolism and excretion (clearance) (de Graaf et al 2006; De Kanter et al 2004; de Graaf et al 2010). A drug with a high clearance rate will have a short half-life, which is highly dependent on the kidney as well as other organs such as the liver, lungs and gastrointestinal tract. Clearance of a drug is also affected by the volume of distribution, amount bound to plasma protein, blood flow to the excretory organs, rate of biotransformation and pharmacogenomic capability of the metabolizing enzymes (phase 1 or phase 2 enzymes) (Kasperek et al., 2015). However, in contrast the clearance of drug in ex vivo model such as liver slices, specialised cell lines (for example renal or hepatocytes), cytosolic (S9 fraction) or microsomal fraction would depend on the functionality of the metabolizing enzymes (Cytochrome P450 enzymes). Variations in the functionality of these enzymes determine the differences observed with drug clearance among animal species

(Watanabe et al., 2013,Yan and Caldwell, 2001). This makes liver slice models an attractive model for the evaluation of toxicity as a direct effect of metabolic capacity or limitation can be ascertained.

(Naidoo et al., 2011)For this study the ex vivo half-life of diclofenac was determined in a liver slice model as a means to evaluate metabolism in the absence of external factors. For comparative purposes the study included meloxicam, ketoprofen and carprofen as they have been shown to be toxic in old world vultures with the exception of meloxicam, together with all having different half-lives of elimination (Naidoo et al., 2017,Naidoo et al., 2010,Naidoo et al., 2008). The latter drugs were thus used to evaluate the appropriateness of the model in relation to differential metabolic rates. To ensure metabolism was not related to harvest site specific variation in enzyme content, microsomal protein content was determined for equalisation. The latter also allowed for inter species comparison of total protein content as a surrogate in determining differences in enzyme content.

Tissue slices are a viable multicellular explant of tissue that can be cultured *ex vivo* in suitable culture medium under certain conditions. They consist of all cell types with intercellular and cellular matrix interactions remaining intact. Tissue slices have been prepared from the liver, heart, kidney, lungs, spleen, intestine, brain, prostrate and several tumours (de Graaf et al., 2010,Graaf et al., 2007,de Graaf et al., 2006). They have been used to study biochemical functions such as endogenous metabolism, biotransformation and the induction and transport of drugs and other xenobiotics. They are also used in toxicological studies as well as efficacy studies of drugs on diseased tissues. In addition, tissue slices have been used to study ischaemia/reperfusion damage and determine the specificity of viruses as carriers for gene therapy agents (de Graaf et al., 2010,t Hart et al., 2005,Langdale et al., 2003). Based on these previous reports, the current study intends to establish the usage of liver tissue slices as a model to evaluate the toxicity of NSAIDs in vultures.

2. Materials and Methods

2.1. Materials

Sodium pentobarbital (Euthapent®), Williams medium E (WME), containing L-glutamine, gentamicin sulphate, amphotericin β, D-glucose monohydrate, sodium hydroxide (NaOH), formalin 4% (formaldehyde solution 3.8-4.2% v/v), diclofenac sodium, ketoprofen, meloxicam and carprofen, bovine serum albumin, and Bradford Reagent were manufactured by Sigma-Aldrich (St Louis, MO, USA). A 6mm biopsy punch was supplied by Lakato (Johannesburg, South Africa). Cell culture multi-well plates, (96- and 6 well), cryogenic vial storage boxes, 50 ml centrifuge tubes and 2 ml cryogenic tubes (Greiner Bio-One, Frickenhausen, GE) and HPLC grade methanol and acetonitrile (Merck, Darmstadt, GE) were used. Carbogen gas (5% CO2/ 95% O2) was obtained from Afrox (Johannesburg, South Africa). Equipment comprised of a BioTek Synergy HT spectrophotometer (BioTek, Winooski, VT, USA), a Beckman Allegra TM FX-22R centrifuge (Beckman Coulter, Palo Alto, CA, USA), a Beckman XL 90 ultracentrifuge equipped with a Ti50 rotor (Beckman Coulter, Palo Alto, CA, USA), and a Beckman System Gold high performance liquid chromatograph (HPLC), equipped with a 126 Solvent Module, a 168 PDA detector, a 508 autosampler, and 32 Karat 8.0 software (Beckman Coulter, Fullerton, CA, USA). Chromatographic separation was achieved with a Thermo Scientific BDS HYPERSIL C18 HPLC column (Thermo Scientific, Runcorn, UK), dimensions: 250mm X 4.6mm X 5µm.

2.2. Methods

2.2.1. Preparation of liver tissue slices

The use of vulture liver for this study was approved by the Animal Ethics Committee (AEC) of the University of Pretoria, South Africa, with project number V093-15. A two-year old male cape vulture with the history of lameness of the left leg (for eight weeks) was

brought to the University of Pretoria Biomedical Research Centre (UPBRC) for euthanasia. The bird was otherwise in good health. The bird was euthanized by intravenous injection of pentobarbital. The dead bird was transferred into the laminar hood to aseptically remove the liver thus, briefly, the abdominal cavity was opened, the ribs were severed and the breast bone raised and maintained in this position to permit access to the liver. The liver was excised from the abdominal cavity and manually sliced with modification to the method of Graaf et al (2010) as described below.

The William medium E (WME) was prepared by mixing 1.375 g of D-glucose monohydrate with 500 µL gentamicin (50 mg/mL), and 5 µL of amphotericin β (250 µg/mL) to 500 mL of commercial WME (containing L-glutamine). The resulting solution was oxygenated with 95% oxygen and 5% carbon dioxide and stored for 24 hours at 4^{o}C prior to liver slices incubation. The freshly excised liver was transferred into a sterile sample bottle containing ice-cold WME solution on ice under sterile condition in a laminar hood. The liver was cut into segments using a surgical blade and the surface kept wet by pouring WME solution on it. Cores were made by pressing a biopsy punch (6 mm diameter) perpendicularly into the tissue until it reached the bottom of the petri dish to produce a 5 mm tissue core. The cores were transferred to a petri dish containing ice cold WME solution on ice using a spatula and the medium was oxygenated continuously. The tissue cores were later cut into thin slices of 5 mm diameter width. The tissue slices were then transferred into pre-weighed vials of WME solution and then reweighed to get the exact weight (ranging from 5.6 to 8.2 mg) of the tissue slice. Sliced liver tissues were transferred into fresh ice-cold WME solution continuously bubbled with 95% oxygen and 5% CO_2 and placed on ice prior to tissue slice assay. Individual liver slices were incubated in 6 well plates filled with William medium E (3.2 ml/well) and supplemented with glucose (25 µM), and gentamicin (50 µg/ml). The medium was pre-warmed inside an incubator at 37^{0}C and quickly transferred into the 6 well

plates measuring 3.2 ml using a sterile pipette under the laminar hood. The plates were placed in an incubator and shaken every 10 minutes to ensure slices are continuously submerged in the medium at 37 ^{0}C under constant flow of humidified 95% O_2 / 5% CO_2. The tissue slices were allowed 3 hours to equilibrate prior to the incubation of the slices with the drugs.

2.2.2. Drug treatment and sample preparation

The NSAIDs were added into the incubation medium using an optimal concentration (1 µM), that was considered to be non-cytotoxic. This concentration was based on previous dose-determination studies using chicken and mouse liver slices for which no signs of toxicity were evident (results not shown). The positive control groups contained NSAIDs and the incubation medium without liver slices while the negative control groups contained tissue slices and incubation medium. Liver tissue slices were incubated with NSAIDs for 6 hours. Following addition of the specific NSAIDs, aliquots of the medium (250 µL) were collected at 0, 1, 2, 3, 4, 5 and 6 hours respectively. Aliquots of the sampled medium (250 µL) were mixed with 250 µL ice-cold methanol and stored at -80^{0}C until analysis. Diclofenac-exposed samples were centrifuged at 8000 x *g* for 10 minutes at 4 ^{0}C and mixed with equal amount of the mobile phase and acidified with 2 M HCl at a ratio of 1:100. Carprofen, meloxicam and ketoprofen were quantified without any prior sample preparation as the case of diclofenac (Bort et al., 1999,De Kanter et al., 2004).

2.2.3. High performance liquid chromatography (HPLC) analysis of collected samples

The samples were analysed for depletion of NSAIDs using high performance liquid chromatography (HPLC). For the separation of the various drug compounds, an isocratic method was used comprising of two mobile phases namely sodium dihydrogen phosphate (A) (pH 4.85-4.89) and acetonitrile (B). For the diclofenac analyses, the mobile phases were mixed at the ratio of 42.5 (A): 57.5 (B) while the remaining NSAIDs were analysed at a

mixture of 60 (A) : 40 (B). Fifty microliter (50 µL) of each sample was injected onto the HPLC column at 1000 µL/min with a total runtime of 8 minutes. The detection of the NSAIDs was undertaken at 275 nm (Naidoo et al., 2007).

2.3. Evaluation of tissue slice viability

The basal cellular viability of cultured liver slices are investigated using parameters such as ATP content, Na^+/K^+ ATP-ase activity and integrity of morphology. The most important of these parameters is the morphology of tissue slice with the demerit of using skilled personnel to interpret the results (de Graaf et al 2010). Therefore, the viability of liver slices were evaluated histomorphologically for the untreated and treated slices preincubated in William Medium E without drugs for 3 hours and those exposed to NSAIDs for 6 hours. Liver slices were fixed in formalin and embedded in paraffin wax. Wax blocks were subsequent cut and stained with Haematoxylin and Eosin for microscopic evaluation.

2.4. Protein determination of liver slices

The protein content of Cape vulture liver slices was used as suitable surrogate in allometric comparison to other animal species. The protein content of pre-weighed tissue slices was determined with the Bradford reagent dye and a bovine serum albumin (BSA) standard curve using a 96 well method. Liver slices were homogenised in 0.1M NaOH under ice. The protein content of the slices was determined by a spectrophotometer at 595 nm wavelength. A stock solution of BSA (2 mg/mL) was serially diluted to a range of 1 - 0.1 mg/mL using 0.1M NaOH buffer. The homogenised liver slices were diluted to a stock concentration of 10 mg/mL. In addition, 100 µL of the stock samples were diluted with 900 µL of 0.1M NaOH to give a working concentration of 1 mg/mL. Five microliters of the BSA and homogenised liver slices were placed into different wells in triplicate and 250 µL of the Bradford reagent added to each well. To the blank wells, 5µL of 0.1 M NaOH was placed

into them, as well as 250µL of the Bradford Reagent. After the addition of the Bradford Reagent, the samples were mixed on a shaker for 30 seconds and allowed to incubate at room temperature for 5-45 minutes. The absorbance of samples was measured at 595 nm. The protein concentration of the homogenised liver slices was determined by plotting the net absorbance versus the protein concentration of the standard BSA and comparing the net absorbance values against the standard curve (Bradford, 1976).

2.5. Preparation of microsomal liver fraction

Microsomes from Cape vulture liver slices were compared to other bird and mammalian species to investigate the reduced metabolic capability of old world vultures to NSAIDs. Ten (10) liver slices stored in cyrotubes at -80 ^{0}C, weighing between 22.6-55.8 mg were homogenised in 0.15M KCl; 0.02 M HEPES; pH 7.5 under ice. The homogenised liver samples were transferred into Beckman 10.4 mL polycarbonated ultracentrifuge tubes, and loaded into the Beckman Allegra TM FX-22R centrifuge and centrifuged at 10,000 x *g* for 20 minutes at 2^{o}C. After centrifugation, the supernatant was carefully transferred into another clean ultracentrifuge tube to avoid the transfer of fat along with the supernatant. The supernatant from the first spin was properly balanced using homogenising buffer on a balance scale and loaded into the Beckman XL90 ultracentrifuge. The samples were centrifuged for 60 minutes at 100,000 x *g* at 2^{o}C. Hereafter, the supernatant was discarded and the tube cleaned to remove fat particles. The microsomal pellets at the bottom of the ultracentrifuge tube was homogenized using the homogenizer pestle with the homogenizing buffer in the same ultracentrifuge tube, and centrifuged for the second time as described above. After, a third centrifuge, the supernatant was discarded and the tube cleaned to get rid of fat particles after which the microsomal pellets were resuspended in 0.3 mL of resuspension buffer (0.05 M tris; 1 mM EDTA; 20% glycerol; pH 7.4) , transferred into a cryotube and stored at -80^{o}C

for further analysis (Papp et al., 2005,Martinez and Alonso, 2014). The protein content of the liver microsomal fractions was determined using the Bradford method as above.

2.6.Statistical analysis of results

The percent clearance per hour (%CL/h) of the non-steroidal anti-inflammatory drugs were calculated by subtracting the concentration of the NSAIDs remaining after six hours of incubation in WME from the initial percent concentration of 100 divided by six hours of incubation as shown in equation 1(Kumar et al., 2002).

$$\%CL/h = \frac{A-B}{C} \quad \text{----------Equation 1}$$

Where %CL/h is the percent clearance of NSAID per hour, A is NSAID initial percent concentration of 100, B is the NSAID concentration in percentage remaining after six hours of incubation and C is the entire time of incubation of 6 hours. Clearance was subsequently corrected by the quantity of protein per slice (see below). Difference in the ex vivo clearance of NSAIDs used in this study was determined by means of a one way analysis of variance (ANOVA) using GraphPad Prism 6.0 (Prism, 2014). Results were considered to be significant when $p < 0.05$. (Bradford, 1976)(Papp et al., 2005,Martinez and Alonso, 2014)

2.7. Allometric scaling

The protein content of the Cape vulture liver was scaled to reported protein content of other mammal species using the liver weight of these species and their protein content (mg/g liver) (Hunter and Isaza, 2008,Cox et al., 2004,Mahmood, 2007,Riviere et al., 1997). The weight of the different animal species, weight of the liver and protein content of the liver was obtained from published articles and compared to the weight of the liver and bird used for this study. The best fit equation was subsequently obtained from the logarithmic conversion of both tissue weight and protein content. The total protein content of the liver (mg) was

obtained by multiplying the weight of the liver (g) with the protein content (mg/g liver) and the logarithm of the liver weight and total protein content.

3. Results

3.1. Percent clearance of NSAIDs after incubation with liver tissue slices

The analytical method used for the quantification of NSAIDs in Cape liver slices was fully validated prior to analysis of the incubation media samples. The following validation results were obtained (table 1). In all cases the method showed good linearity (>99%). The limit of quantification was 0.5µM for meloxicam, ketoprofen and carprofen; and 0.25 µM for diclofenac. The positive control groups showed a constant amount of NSAIDs over the entire incubation period.

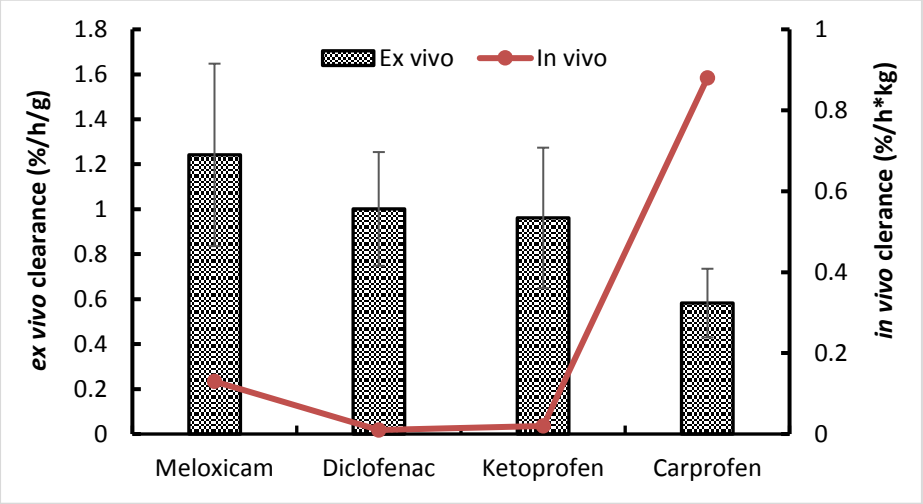
Table 1: Validation of the analytical method

Parameter	**Diclofenac**	**Meloxicam**	**Ketoprofen**	**Carprofen**
Best Fit Equation	Y = 26122x + 1856.7	Y = 30749x - 1135.3	Y = 25189x – 1361.4	Y = 14919x - 530.46
Goodness of fit	R^2 = 0.9996	R^2 = 0.9989	R^2 = 0.9974	R^2 = 0.9988
LOD (µM)	0.0625	0.125	0.125	0.0625
LOQ (µM)	0.25	0.5	0.5	0.5
Retention Time (hour)	4.88	5.66	5.56	5.65

The *ex vivo* percent clearance of diclofenac, meloxicam, ketoprofen and carprofen after incubation for 6 hours in Cape vulture liver slices in comparison to published *in vivo* clearance of the same drug in Cape vulture are presented in Fig. 1. Following correction for protein content, the corrected percent clearance and standard error of the four non-steroidal anti-inflammatory drugs was 1.0±0.253 (%/h*g), 0.582±0.153 (%/h*g), 0.961±0.312 (%/h*g) and 1.242±0.406 (%/h*g) for diclofenac, carprofen, ketoprofen and meloxicam respectively. In general, the degree of *ex vivo* clearance was almost the same for meloxicam and

diclofenac, followed by ketoprofen and lastly by carprofen. No statistical differences were observed between the samples after statistical analysis (p=0.5043).

Fig.1. *Ex vivo* liver slices percent clearance of meloxicam, diclofenac, ketoprofen and carprofen (1.242±0.406 %/h*g , 1.0±0.253 %/h*g, 0.961±0.312 %/h*g and 0.582±0.153 %/h*g) in comparison to *in vivo* clearance (0.13 L/ h*kg, 0.01-0.02 L/ h*kg, 0.02 L/ h*kg and 0.88 L/ h*kg) of same drugs in Cape vulture (Naidoo et al. 2010, Naidoo et al. 2008, Naidoo et al. 2009, Fourie 2015).



To determine the validity of the *ex vivo* clearance, the percent clearance from the liver slices was compared to their corresponding *in vivo* clearance of diclofenac sodium, carprofen, ketoprofen and meloxicam as presented in Fig.1. The *ex vivo* metabolism of the drugs did not clearly predict the high *in vivo* clearance of meloxicam in comparison to diclofenac and ketoprofen with the exception of carprofen which was reported to have a high clearance (0.88 L/h*kg) in the Cape vulture but in the African white-backed vulture a low clearance of 0.015 L/kg (Naidoo et al., 2017,Fourie, 2015). A trend for faster clearance was however evident for meloxicam in comparison to the other drugs. The clearance of carprofen appeared to be completely non-predictable.

3.2. Histomorphological viability of liver slices

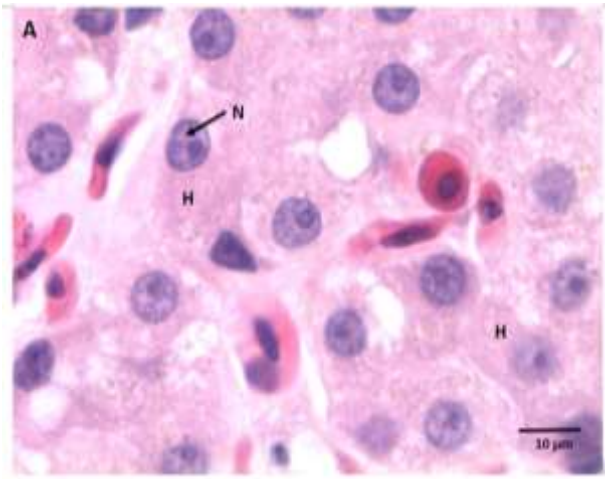
The non-toxic concentration of 1 µM NSAIDs were validated prior to the commencement of the study using liver slices incubated with the drugs from chicken and

mouse. The histomorphological features of the liver slices of chicken and mouse had a normal nuclear structure with no sign of toxicity.

Following the termination of the experiment, the tissue slices were histologically evaluated (Fig.2). The tissue from the control group and those exposed to media showed no signs of toxicity with all the hepatocytes appearing healthy with normal nuclear structure (Fig.2-A and B). For the tissues exposed to the NSAIDs, the slices showed morphological lesions of toxicity, characterised as cytoplasmic vacuolation, nuclear shrinkage, nuclear basophilia and loss of internal structure (Fig.2-C). In addition, the slices exposed to meloxicam, showed fragmentation of the hepatic cords, cytoplasmic eosinophilia, nuclear pkynosis and karyorrhexis (Fig.2-D); while the carprofen slices at 1 µM showed cytoplasmic vacuolation, loss of internal cytoplasmic structures, nuclear pkynosis and nuclear karyorrhexis (Fig.2-E). Finally, slices exposed to ketoprofen showed cytoplasmic vacuolation, nuclear pkynosis, nuclear karyorrhexis and cytoplasmic eosinophilia (Fig.2-F).

Fig.2. Light microscope photographs of liver slices of Cape vulture treated with NSAIDs (H&E).

A. Control. H: hepatocyte; N: nucleus of hepatocytes.
B. Liver slice in William's medium E only for 6 hours. H: hepatocyte; N: nucleus of hepatocytes.
C. Liver slice incubated with diclofenac (6 hours). V: vacuolation; SN: pkynosis of nucleus; TLN: total loss of nucleus.
D. Liver slice incubated with meloxicam (6 hours). V: vacuolation; EN: eosinophilic nucleus; KN: karyorrhexis of nucleus; PN: pkynosis of nucleus.
E. Liver slice incubated with carprofen (6 hours). V: vacuolation; KN: karyorrhexis of nucleus; PN: pkynosis of nucleus.
F. Liver slice incubated with ketoprofen (6 hours). V: vacuolation; EN: eosinophilic nucleus; KN: karyorrhexis of nucleus; PN: pkynosis of nucleus.


A
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H
H
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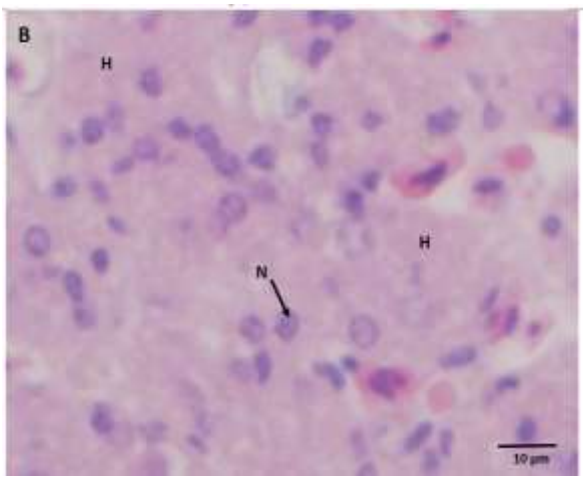
B
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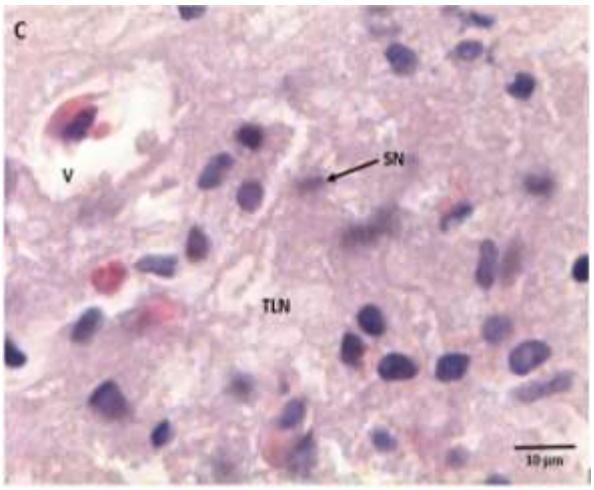
C
V
SN
TLN
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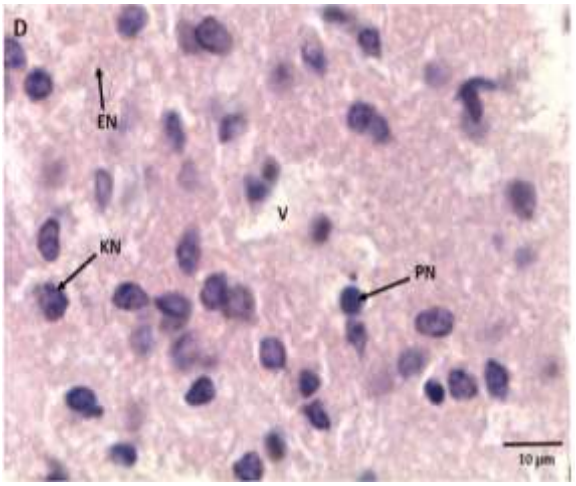
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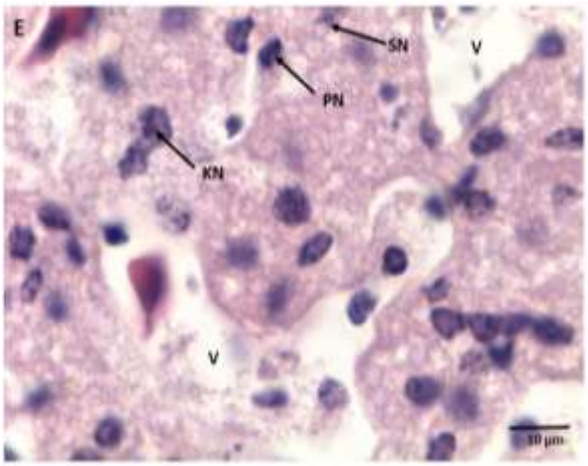
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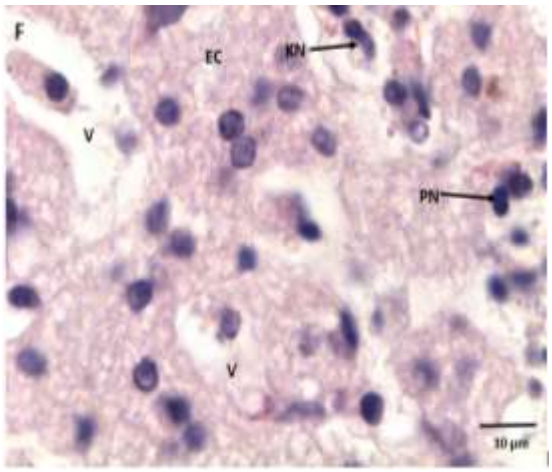
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10 µm

3.3. Allometric scaling of liver protein

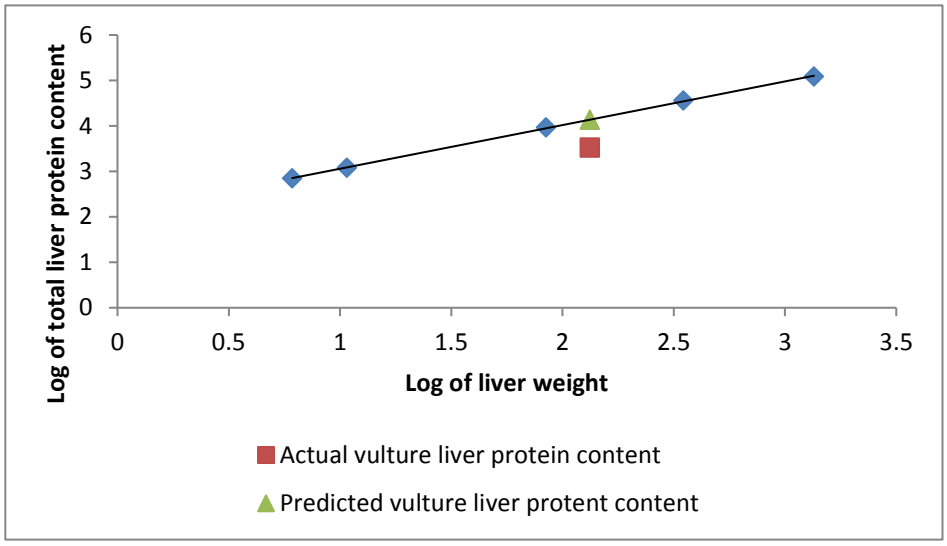
The total liver protein content of the Cape vulture was 3325 mg/g liver, obtained by multiplying the liver weight of the Cape vulture (133 g) and average protein content of the whole liver slices (25 mg). For the allometric scaling the liver weights from humans, dogs, rabbits, rats, and mice used in the study are presented in Table 2, while the protein content of the listed species are presented on a double logarithmic plot in Figure 4. For the latter, the best fit equation is 0.9604x + 2.0975 with a goodness of fit of 0.99. Based on the equation for animal of the size of the study vulture, one would have expected a liver protein content of 13716.62 mg/g liver. Thus based on weight of the animal, the actual liver protein content was 24% lower than expected.

Table 2: Total liver protein content (mg) of different species of animals

Species	Weight of animal (kg)	Weight of liver (g)	Logarithm of liver weight	Total protein content of liver (mg)	Protein content (mg/g liver)	Logarithm of total protein content
Human [e]	62	1350[e]	3.13	121500	90±17[a]	5.08
Dog [a]	17.5	349.2	2.54	35967.6	103±6[a]	4.56
Rabbit [a]	1.3	84.3[d]	1.93	9188.7	109±9[a]	3.96
Rat [b]	0.28	10.76[b]	1.03	1204.56	112±10[a]	3.08
Mouse [c]	0.02	6.1[c]	0.79	701.04	115±7[a]	2.85
Cape Vulture	8.7	133	2.12	3325	25±1.7	3.52

[a](Sohlenius-Sternbeck 2006), [b](Piao, Liu & Xie 2013) , [c] (Kendall et al. 2014)[d](Brown, Pearce & Van Allen 1925)

Fig. 3: The allometric scale of liver protein from human, dog, rabbit, rat, mouse and Cape vulture.



4. Discussion

It has been widely accepted that old world vultures are highly sensitive to the toxic effects of various NSAIDs, with the exception of meloxicam for an unknown reason (Oaks et al 2004; Swan et al 2006; Naidoo et al 2007; Naidoo et al 2010; Naidoo et al 2011). While the mechanism behind toxicity remains elusive, various pharmacokinetic studies have shown that the faster the clearance, the lower the toxic potential of the NSAIDs (Naidoo et al 2011). From previous studies in *Gyps coprotheres*, vulture safe meloxicam at a dose of 2 mg/kg was characterised by a half-life of elimination of 0.42 ± 0.1 hour and clearance of 0.13 ±0.00 L/h*kg, while diclofenac at the dose of 0.8 mg/kg had a longer elimination half-life of 12.24 ± 0.99 hours and a clearance of 0.01 ± 0.006 L/h* kg (Naidoo et al., 2009,Naidoo et al., 2008). The same trend was seen with toxic doses of ketoprofen at 5 mg/kg which had a half-life of elimination of 7.38 ± 1.72 hours and a clearance of 0.02 ± 0.01 L/h*kg. Carprofen had a longer half-life of elimination of 13.26± 5.08 hours and a high clearance of 0.88± 0.85 L/h*kg (Naidoo et al., 2011,Naidoo et al., 2010,Naidoo et al., 2010). This raised the question as to why the meloxicam was so rapidly cleared in comparison to the other two drugs. From

pharmacokinetic theory, the rapid clearance may result from rapid biotransformation and/or rapid excretion of the unchanged molecule. Of these two, both are likely based on information available from other species, albeit mammalian.

In the majority of mammalian species meloxicam is rapidly metabolised and excreted by the kidneys, as 5'-hydroxymethyl and 5'-carboxymethyl metabolites. In mice, 60-65% of the metabolites are excreted through the urine (5'-hydroxymethyl metabolites (51%), 5'-carboxymethyl metabolites (4.5%) and parent drug (0.2%)), while 35-40% are eliminated via the faeces in the rat, renal excretion accounts for 70% of meloxicam elimination (5'-carboxymethyl metabolite (30%), 5'-hydroxymethyl metabolite (40%) and parent drug (0.2%)); while in the mini-pig, 5'-hydroxymethyl metabolite accounts for 50% of the metabolites in the urine, while 5'-carboxylmethyl metabolites is 10%. In addition, in the Baboon, 4% of the parent drug is found in the urine while 75% of the other metabolites are found in the urine (Busch et al., 1998). The only species that appears to be different is the cat, which shows a high degree of excretion of unchanged drug, with up to 50% of the administered dose being excreted unchanged in the bile. At present it has been suggested that latter may result from the cat's inability to glucuronide drugs in comparison to other animal species (Lascelles et al., 2007,Grudé et al., 2010)

Despite the difference in the manner of clearance, the cat and dog (5-hydroxymethyl and 5'-carboxymethyl metabolites) has similar half-lives of excretion at 15 and 12 hours respectively. This would indicate that as a drug, meloxicam is still well excreted in the presence of poor or deficient metabolism.

The aim of this study was to establish if there was a clear difference in the metabolism of diclofenac, meloxicam, carprofen and ketoprofen under *ex vivo* conditions, in the absence of possible influence from other pharmacokinetic factors such as blood supply and the excretion of the non-metabolized drug. The drugs selected were based on previous evidence

of toxicity (diclofenac, carprofen and ketoprofen) or safety (meloxicam) of the specific drugs in old world vulture species and the *in vivo* pharmacokinetic information available. The *in vitro* clearance of tissue slices has previously been used by various researchers to predict the *in vivo* clearance of different drug models (De Kanter et al., 2004,Kumar et al., 2002,Houston, 1994,Scott et al., 2011,Rane et al., 1977). The theory behind the extrapolation of *in vivo* clearance from *in vitro* data is based on the determination of the intrinsic clearance of the drug, which measures liver enzyme activity not influenced by other physiological determinant of liver clearance such as hepatic blood flow or drug binding within the haemoproteins (Houston, 1994).

From the results of the hepatic slice culture, the positive control group showed constant level of the drugs for the entire incubation period of 6 hour, indicating non-degradation of the drugs in the incubation medium. Furthermore, all the drugs showed a non-significantly different rate of clearance for which the following trend was evident: meloxicam > diclofenac = ketoprofen > carprofen, with relatively poor correlation to the *in vivo* clearance. These results differ to a similar study that used intestinal slices of the rat for the toxicity ranking of five non-steroidal anti-inflammatory drugs (diflunisal > diclofenac = indomethacin > Naproxen > aspirin) for which a strong correlation was established between the published *in vitro* and *in vivo* data (Niu et al., 2014). This therefore would indicate that the vulture liver is either unable or poorly able to metabolise the NSAIDs in comparison to mammals. With this said, one of the problems with tissue cultures is that tissue from different individuals are known to have different hepatic enzyme concentrations, while different lobes of the liver may also differ in their metabolic capacity (Tottmar et al., 1973). Taking this into account we used tissue from the same donor animal and liver slices from the same lobe. To further demonstrate that limitation in metabolism played a role, the protein content in the liver was 3325 mg/g, which was 24% lower than we predicted for an animal the size of the

vulture (8.7 kg). While it may be argued that the use of mammalian data could have skewed the results, current allometric scaling indicates that mammals and bird can be grouped together (Cox et al., 2004). Based on the protein content, we are confident that the limitation in metabolic capacity is responsible for NSAIDs toxicity in old world vultures.

To further investigate the vulture restriction in hepatic capacity in comparison to mammals, we also evaluated the total amount of enzymes being expressed in the sampled bird. For this we used protein content as a surrogate, expressed in grams of albumin, as previously validated (Ronis and Walker, 1989). The actual enzyme capacity would then be a percentage of total protein content. Previous studies from nine mammalian species, showed that the cytochrome P450 content of liver microsomes was in the range of 48-114 mg CYP enzymes per gram of protein equivalents, while the CYP450 content of ten (10) Cape vulture liver slices was within the range of 12.2-26.5 mg (result not shown) which is 10.7-55.2% lower than those in mammals. Nonetheless, the microsomal protein content of 10 Cape vulture liver slices was within the range of 2.78-12.31 mg/g liver weight compared to passeriformes (12-30.6 mg/g), galliformes (10.8-27 mg/g), pelecaniformes and Charadriformes (fish eaters) (9-17.4 mg/g) (Ronis and Walker, 1989). This would indicate that birds in general have a lower metabolic capacity than mammals. However, at this stage we were unable to determine how the vulture differs from other bird in their enzyme content, as thus far all other birds exposed to diclofenac have demonstrated a much quicker half-life of elimination than old world vulture species (Mompati, 2012) i.e. the evaluation of protein content while a surrogate for total enzyme expression, cannot differentiate between the ratio of enzymes being expressed. Hence it would be important to ascertain if all the mentioned bird groups do express the same number and ratio of enzyme or whether the vulture is deficient in the NSAIDs metabolising enzymes system.

In man, it has been shown that diclofenac is metabolised by CYP2C9 while meloxicam is metabolised by both CYP2C9 and CYP3A4 (Bort et al., 1999,Busch et al., 1998,Smith and Jones, 1992,Chesne et al., 1998,Leemann et al., 1993). Interestingly, population based studies in people, have indicated that in meloxicam metabolism major pathway is via the involvement of the CYP3A4 enzymes, as persons with poorly functional CYP2C9 enzyme due to gene polymorphism, do not experience any altered pharmacokinetics (Chesne et al., 1998). Of the two, CYP2C9 is also responsible for the metabolism of most NSAIDs while CYP3A4 is known to metabolise 50% of clinically used drugs. In birds, at present the metabolic pathways for the various species and clades remains poorly studied, with much focus being on the chicken. Nonetheless, of these two enzyme systems, the CYP2 family were reported to be less represented in birds than mammals as evidenced by Western blotting and the response to inducers in the domestic chicken, Japanese quail, duck and cormorant (Ronis and Walker, 1989). Based on phenobarbital (PB) induction studies, PB caused the induction of only two CYP450 isoforms in the chicken compared to the rat and the rabbit which had four and five induced CYP isoforms respectively. Because of the low level of the inducible form of CYP450 in avian species, it is speculated that the CYP2 family are less represented (Althaus et al., 1979,Darby et al., 1986,Nebert and Gonzalez, 1987). With the CYP2 subfamily being known to be responsible for the metabolism of diclofenac in man and their lower representation in avian species, constraints at the level of this enzyme system may be reason for the vulture's susceptibility to diclofenac (Bort et al., 1999,Ronis and Walker, 1989,Rettie et al., 1992,Goldstein and de Morais, 1994).

Another important finding from this study was that while differing clearances were noted for ketoprofen and carprofen in comparison to diclofenac, the same was not the case for meloxicam. In an unexpected manner, the *ex vivo* clearance of diclofenac was not substantially lower to that of meloxicam (Fig.1). Firstly, this would indicate that the *ex vivo*

liver slice model is not an appropriate model to predict the capability of old world vultures in metabolising NSAIDs as meloxicam is the most rapidly metabolised from all the evaluated drugs. More so, it is important to note that the liver tissue for the diclofenac and meloxicam also showed marked hepatic degeneration after a short incubation of only 6 hours, despite meloxicam being known to be safe *in vivo*, thereby indicating the role of adverse influence of cell death on drug metabolism and clearance. The exposure doses (1 µM) used in this study were also relatively low compared to *in vivo* total in vivo exposure of 6.29 µg/mL*h (± 20µM*h) at 2 mg/kg meloxicam. While we are unable to completely explain the reason for toxicity seen with meloxicam, we believe that this shows the importance of mean residence time in toxicity. When looking at the *in vivo* exposure (3.524 µg/mL*h), with a half-life of 0.42 min, the liver was not exposed to high concentration of meloxicam for more than 2.1 hours, which represents five elimination half-lives. For this study, with exposure being 6 hours, the liver was being exposed to the meloxicam for 3 times as long. This also supports previous speculations that the rapid in vivo clearance of meloxicam played a major role in the safety of the molecule, as seen with *ex vivo* renal cultures by Naidoo and Swan, (2009) (Naidoo and Swan, 2009). From this study, they were able to demonstrate that renal tubular cell cultures were equally susceptible to diclofenac and meloxicam, with 40% cell death reported at 0.05µMol of meloxicam after static exposure for 12 hours.

This study indicates that liver slices are not a useful tool to investigate the biotransformation of non-steroidal anti-inflammatory drugs in the vulture due to the toxicity of the drugs on the tissue as other factors such as direct cell toxicity can influence the results. Under in vivo conditions, the toxicity of the drug is likely mitigated by the ratio of total active metabolic cells in the whole organ in relation to drug plasma concentration, as well as by the rate of blood supply to the metabolically active tissues. It is also possible that clearance of non-metabolised meloxicam as seen in the cat, may also be involved.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Contribution of Authors

Emmanuel Oluwasegun Adawaren was responsible for carrying out the research work, data analysis and study write-up.

Lilian Mukandiwa was responsibilities for study write-up.

Emmanuel Mfotie Njoya assisted with the liver slice culture assay and write-up of the study.

Lizette Bekker assisted with analytical evaluation of the drugs and study write-up.

Neil Duncan was responsible for the histopathological interpretation of the liver slides and write-up of the study.

Vinny Naidoo is the primary grant holder who conceptualizes the study and also was responsible for data analysis and study write-up.

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