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Comparative metabolism as a key driver of wildlife species sensitivity to human and veterinary pharmaceuticals

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Human and veterinary drug development addresses absorption, distribution, metabolism, elimination and toxicology (ADMET) of the Active Pharmaceutical Ingredient (API) in the target species. Metabolism is an important factor in controlling circulating plasma and target tissue API concentrations and in generating metabolites which are more easily eliminated in bile, faeces and urine. The essential purpose of xenobiotic metabolism is to convert lipid-soluble, non-polar and non-excretable chemicals into water soluble, polar molecules that are readily excreted. Xenobiotic metabolism is classified into Phase I enzymatic reactions (which add or expose reactive functional groups on xenobiotic molecules), Phase II reactions (resulting in xenobiotic conjugation with large water-soluble, polar molecules) and Phase III cellular efflux transport processes. The human–fish plasma model provides a useful approach to understanding the pharmacokinetics of APIs (e.g. diclofenac, ibuprofen and propranolol) in freshwater fish, where gill and liver metabolism of APIs have been shown to be of importance. By contrast, wildlife species with low metabolic competency may exhibit zero-order metabolic (pharmacokinetic) profiles and thus high API toxicity, as in the case of diclofenac and the dramatic decline of vulture populations across the Indian subcontinent. A similar threat looms for African Cape Griffon vultures exposed to ketoprofen and meloxicam, recent studies indicating toxicity relates to zero-order metabolism (suggesting P450 Phase I enzyme system or Phase II glucuronidation deficiencies). While all aspects of ADMET are important in toxicity evaluations, these observations demonstrate the importance of methods for predicting API comparative metabolism as a central part of environmental risk assessment.

1. Introduction

Investigations of a pharmaceutical's absorption, distribution, metabolism, elimination and toxicology (ADMET) play a central role in the pre-clinical and clinical safety assessment of human medicines [1] and potentially in environmental risk assessment (see [2]). Likewise, Active Pharmaceutical Ingredients (APIs) used in veterinary medicine are evaluated for their ADMET profile in the species of interest (e.g. poultry or ruminants) [3,4]. Metabolism of endogenous and exogenous molecules (e.g. plant toxins, pesticides and pharmaceuticals) is normally classified into Phase I enzymatic reactions (which add or expose –OH, –SH, $–NH_2$ or –COOH functional groups on xenobiotics) and Phase II reactions (resulting in xenobiotic conjugation with large water-soluble, polar molecules). Additionally, lipophilic xenobiotics, or their metabolites, can be pumped out of cells by specific transporter proteins and this efflux pump activity is often termed Phase III metabolism [5]. For approximately 5–7% of human drugs, Phase I metabolism may be responsible for conversion of a prodrug into the API [6]. More broadly, many

Phase I biotransformations of lipophilic xenobiotics are carried out by microsomal monooxygenases, located in the endoplasmic reticulum of the liver and other organs [7]. The haem protein cytochrome P450 provides the active centre of these enzymes and has huge diversity, with 37 cytochrome P450 families currently identified across many animal species [8]. It is hypothesized that the P450 superfamily has undergone repeated rounds of expansion by genome duplication, whereby approximately one and a half billion years ago, the first expansion gave rise to the P450 families primarily involved in metabolizing endogenous fatty acids, cholesterol and its derivatives (CYP4 and CYP11 families) which likely played a key role in maintaining the eukaryotic cell membrane integrity. A later expansion of the P450 family 900 Ma may have led to several endogenous steroid-synthesizing cytochrome P450 lineages (including CYP19, CYP21 and CYP27 gene families; whereby the CYP21 family later diverged to give rise to the CYP1 and CYP2 families). A final major expansion of several P450 families involved in xenobiotic metabolism (including CYP2, CYP3, CYP4 and CYP6), began about 400 Ma. This most recent expansion is thought to have been driven by first the emergence of aquatic organisms onto land, associated with eating toxic plant allelochemicals ('animal–plant warfare'), together with exposure of terrestrial organisms to hydrocarbon-based combustion products in the atmosphere [8–12].

Much data exist on the metabolism of pharmaceuticals and other xenobiotics by the liver microsomes of mammals, birds and other species, with rates of microsomal oxidative metabolism determined across a range of vertebrates [3,13,14]. For example, Abass *et al*. [15] studied the metabolism of the insecticide benfuracarb by hepatic microsomes taken from seven mammalian species to investigate species-specific metabolic pathways. Benfuracarb is metabolized via sulfur-oxidation and nitrogen–sulfur bond cleavage (producing carbofuran which is further metabolized). Clearance rates for the seven species ranged from 1.4 (monkey) to 3.5 (rat), these differences being due to variability in CYP enzyme expression [15]. Among herbivorous and omnivorous mammals, there is a clear inverse correlation between the microsomal monooxygenase activity and body weight [16,17]. When hepatic monooxygenase activities are expressed in terms of body weight, much higher values are found in small rodents than in large mammals. This observation is consistent with the concept of a coevolutionary arms race between plants and herbivorous animals. In this context, small mammals need to consume more food per unit body weight than do large ones in order to maintain body temperature due to their high surface area to volume ratios. In contrast to the mammalian species studied by Walker and co-workers, the carnivorous (piscivorous or raptorial) species showed distinctly lower microsomal monooxygenase activities than did herbivorous or omnivorous birds (an observation also explicable in terms of 'animal–plant arms race' theory). Predatory mammals (e.g. cats) and birds (e.g. raptors) eat very little, if any, plant material and therefore do not incur major pressure to drive the evolution of enzymes to metabolize plant toxins [17–20]. Interestingly, zebrafish (a widely used model in pharmaceutical research) show a dramatic increase in Phase I and II enzyme activity at the juvenile life stage in association with being fed plant-based diets [21].

In contrast to terrestrial vertebrates, Phase I enzyme activities in fish are generally lower and there is only a weak correlation with body weight (whereas individual avian species show a correlation between body weight and hepatic microsomal monooxygenase activity across species) [13]. For fish, this has been explained on the grounds that they can excrete as well as take up (see [22]) many xenobiotics by diffusion across gills into the large volume of ambient water and it has been argued that there has not been a strong pressure for the evolution of highly active detoxification enzymes as seen in mammals [14,23]. A similar situation is thought to apply to aquatic invertebrates [24–26]. Nonetheless, as molecular and biochemical methods have advanced, there is growing evidence of both Phase I and II enzyme activity in fish [21,27,28] and recent studies have addressed how dietary and trophic variables may affect enzyme activity in fish [29]. There are also a growing number of studies on the metabolism of pharmaceuticals in fish [30–40] and to a far lesser extent invertebrates [41]. Veterinary pharmaceuticals have also been studied from a comparative metabolism perspective [42,43]. Table 1 summarizes Phase I pathways of pharmaceutical and xenobiotic metabolism in mammals and other vertebrates, adapted from Parkinson and Ogu & Maxa [44,45] and updated with examples from the DrugBank online database (http://www.drugbank.ca/) established by Wishart *et al*. [46].

2. *In vitro* and *in silico* methods to understand comparative metabolism

In vitro systems are widely used for the investigation of xenobiotic metabolism in mammals [1], birds [47] and fish [38,40]. Systems include: (i) whole liver tissue slices which retain an accurate, structural framework of the liver; (ii) whole isolated hepatocytes where the endoplasmic-reticulum-bound and cytosolic enzymes are present but the structural integrity of liver network lost; (iii) after centrifugation at 9000*g*, the S9 fraction supernatant from liver (or other tissue) homogenate containing both cytosolic (predominantly Phase II) and microsomal (predominantly Phase I) enzymes; and (iv) microsomes comprising endoplasmic-reticulum-bound enzymes that have been separated from cytosolic enzymes (P450 enzymes are concentrated in this subcellular fraction). These methods are routinely used to determine the rate and extent of metabolism and mass-spectroscopic analysis of specific metabolites. Results for clearance rates obtained from *in vitro* metabolism experiments can then be extrapolated to the *in vivo* situation using scaling factors (e.g. number of hepatocytes per liver; weight of microsomal protein per gram of liver, etc.). Allometric methods can also be used to scale *in vitro* results between different species (used in drug development for scaling from pre-clinical species to man). Where such values are known for wildlife species, this may allow for approximations between different species [16,17] and form a basis for models to aid in environmental risk assessment using fish [31,48,49], invertebrates [41] and plants [50].

Novel *in silico* tools may also be useful to predict metabolism, this approach tending to focus on the semi-quantitative prediction of potential metabolites and identification of the specific enzymes responsible for the metabolism. Prediction of metabolic rates of drug metabolism remains a key challenge, especially with regard to identification of potential metabolites (which may be associated with specific toxicities) and identification of the enzymes responsible (combined with knowledge of different enzyme expression in different species). Kirchmair *et al*. [51] provide an overview of *in silico* tools for predicting key factors associated with metabolism (including sites of metabolism

Table 1. Summary of vertebrate metabolic pathways with examples of pharmaceutical and xenobiotic substrates and inhibitors.

enzyme	localization	substrate	inhibitor
Phase I—hydrolysis reactions			
esterase	microsomes and cytosol	trandolapril	tamoxifen
peptidase	lysosomes	—	alogliptin
epoxide hydrolase	microsomes and cytosol	diazepam	valproate
Phase I—reduction reactions			
azo- and nitro-reduction	microsomes and cytosol	prontosil	clofibrate
carbonyl reduction	microsomes and cytosol	loxoprofen	befunolol
disulfide reduction	cytosol	captopril	—
sulfoxide reduction	cytosol	—	dimethylsulfoxide
quinone reduction	microsomes and cytosol	trenimon	warfarin
reductive dehalogenation	microsomes	chloramphenicol	—
Phase I—oxidation reactions			
alcohol dehydrogenase	cytosol	ethanol	fomepizole
aldehyde dehydrogenase	mitochondria and cytosol	acetaldehyde	disulfiram
aldehyde oxidase	cytosol	aldehyde	raloxifene
xanthine oxidase	cytosol	xanthine	allopurinol
monoamine oxidase	mitochondria	monoamine	moclobemide
diamine oxidase	cytosol	diamine	phenformin
prostaglandin H synthase	microsomes	arachidonic acid	ibuprofen
flavin-monooxygenases	microsomes	riboflavin	nitric oxide
cytochrome P450:	microsomes	—	—
CYP1A1	microsomes	7-ethoyxyresorufin	galangin
CYP1A2	microsomes	clozapine propranolol	cimetidine citalopram
CYP2C19	microsomes	citalopram diazepam	fluoxetine ketoconazole
CYP2C9	microsomes	diclofenac ibuprofen	fluconazole fluoxetine
CYP2D6	microsomes	metoprolol tramadol	fluoxetine sertraline
CYP2E1	microsomes	acetaminophen ethanol	disulfiram water cress
CYP3A4	microsomes	carbamazepine simvastatin	flavonoids ketoconazole
Phase II—enzyme reactions			
glucuronide conjugation	microsomes	Phase I metabolites	valproic acid
sulfate conjugation	cytostol	Phase I metabolites	harmol
glutathione conjugation	microsomes and cytosol	Phase I metabolites	tannic acid
amino acid conjugation	microsome	Phase I metabolites	kinetin
acetylation	mitochondria and cytosol	Phase I metabolites	garcinol
methylation	microsomes and cytosol	Phase I metabolites	5-A-2′deoxycytidine

within a molecule; potential metabolites; cytochrome P450 (CYP) binding affinity/inhibition and prediction of CYP induction). Table 2 shows a representative software tool for each of these categories, however, many other tools are available [51].

In silico tools have a number of potential advantages and provide complementary techniques to *in vitro* methods. One area where information from both fields can be combined to build improved predictions is in physiologically based pharmacokinetic (PBPK) modelling. In this method, an organism is divided into a sequence of physiological compartments (e.g. brain, liver, lungs, etc.). The models integrate compound-specific data (e.g. physico-chemical properties, such as log P, pK_a or solubility; these values may be measured or predicted using *in silico* techniques) and species- (or even subject-) specific data (e.g. physiological factors such as body or organ weights, volumes or blood flow rates). Subject to validation, these models are potentially of high value in predicting concentration-time profiles for pharmaceuticals in wildlife species [31,36,48]. Understanding inter-species differences in metabolism is essential for reliable PBPK models, especially in non-mammalian species. For example, Ohyama *et al.* [47] studied methoxychlor (MXC) metabolism in rat, mouse, Japanese quail and rainbow trout using liver slices. Each species showed differences in metabolism, considered due to the substrate specificity of CYP450s involved. MXC was metabolized to bis-OH-MXC which was then glucuronidated (with only rats producing the bis-OH-MXC 4 O-sulfate 4-O-glucuronide). In mice and Japanese quail, mono-OH-MXC

Table 2. Representative examples of computational tools for predicting factors associated with mammalian metabolism (programs may have additional capabilities).

factor predicted	software	summary of method	website or key citation
(i) site of metabolism	METAPRINT2D	predicts sites of Phase I metabolism in dog, human and rat through data-mining and statistical analysis of published metabolic transformations	http://www-metaprint2d.ch.cam.ac.uk/metaprint2d
(ii) potential metabolites	METEOR NEXUS	uses expert knowledge rules for metabolism to predict metabolites which are presented in metabolic trees	http://www.lhasalimited.org/products/meteor-nexus.htm
(iii) CYP binding affinity/inhibition	ISOCYP	predicts the predominant human cytochrome P450 isoform by which a compound is metabolized	http://www.molecular-networks.com/products/isocyp
(iv) CYP induction	VIRTUALTOXLAB	predicts binding affinities to Aryl hydrocarbon receptor (and other targets) using flexible docking and quantitative structure–activity relationships	http://www.biograf.ch/index.php?id=projects&subid=virtualtoxlab

(and glucuronide conjugate) were the main metabolites and little bis-OH-MXC glucuronide was formed (dechlorinated mono-OH-MXC glucuronide was found only in mice). Rainbow trout liver slices formed similar amounts of both metabolites. In conclusion, rat and trout liver slices were able to metabolize both MXC and mono-OH MXC, whereas only MXC could be metabolized in mouse and Japanese quail [47].

3. *In vivo* approaches in studying comparative metabolism

The overall effect a xenobiotic has on any organism is ultimately the result of its intrinsic activity and its concentration at the target site. Concentration at a given target site is determined by the ADMET properties of the compound. The history of studying the time course and concentration of xenobiotics at different sites within the body has been developed predominantly within the pharmaceutical industry, with respect to drug effects on humans. However, the techniques are applicable to diverse chemical space and across diverse species. *In vivo* measurements determining the pharmacokinetic profiles of xenobiotics in environmental species are largely unavailable, hence extrapolation and predictive models (combining *in silico* and *in vitro* methods) become essential tools in determining organ-level concentrations [52]. Metabolism is one of the key factors to consider when modelling the time course of a xenobiotic within an organism, not only as it can determine the overall period of exposure, but also because the metabolite(s), rather than the parent drug, may be responsible for the toxic effect [1,53]. In the non-mammalian area, where much less is known about metabolic profiles of drugs in animals, *in vivo* experiments still have a major role to play to derive reliable environmental risk assessments (for case studies with freshwater fish, see [35,36,54]) and also in wildlife forensic studies (see following case study on birds).

4. Case study: vulture toxicity to non-steroidal anti-inflammatory drug (a process of zero-order metabolism)

The dramatic impact of diclofenac (a non-steroidal anti-inflammatory drug or 'NSAID') on Asian vulture populations represents one of the most serious ecological catastrophes of recent times. In just over a decade, diclofenac has been responsible for the deaths of millions of vultures of the Asian white-backed (*Gyps bengalensis*), long-billed (*Gyps indicus*), slender-billed (*Gyps tenuirostris*), Egyptian (*Neophron percnopterus*) and red-headed (*Sarcogyps calvus*) species across the Indian subcontinent [55,56] (also see [57]). In addition to the scale of the toxicity, the exposure route to the product was probably highly unconventional as these birds were inadvertently being poisoned by the oral route even though diclofenac was only available as an injectable cattle formulation. Whereas previous veterinary medicines and pesticides had caused their negative effects by ending up in the water, soil or general environment of the species affected, these vultures were being exposed to this product as residues in the meat of the dead cattle carcasses upon which they fed. This unique mode of exposure was linked to cultural and religious practices in the region, whereby sick and old cattle were routinely treated in a palliative manner with diclofenac, a cheap and effective NSAID. The net effect of this practice was an unfortunate high occurrence of diclofenac residues in the tissues of recently dead cattle.

In the vulture, diclofenac is highly toxic with rapid mortality resulting from a single meal of 1 kg of meat rich in residue, with an estimated LD_{50} of 0.1–0.2 mg kg^{-1} [58]. Toxicity following exposure is also fairly predictable with birds showing signs of depression and head drooping as early as 24 h post exposure. Death is the typical endpoint with birds literally being described as falling dead from their perches. Based on the results from controlled toxicity studies, it has been shown that death after a single exposure consistently resulted within 48 h of exposure, with related massive increases in plasma uric acid and potassium concentrations and increased alkaline phosphatase activity. Necropsies are also very typical with signs of severe nephrosis, dehydration and accompanying diffuse visceral and articular gout. Histopathology indicated toxicity was characterized by necrosis of hepatocytes and the renal tubular epithelial cells (RTE) of the proximal convoluted tubules with associated uric acid tophi accumulation. While the mechanism of toxicity of diclofenac remains incompletely described, toxicity has been linked to RTE cell damage in a time-related manner, subsequent accumulation of uric acid, acidosis and terminal hyperkalaemia [59]. Results from various pharmacokinetics studies of diclofenac in different bird

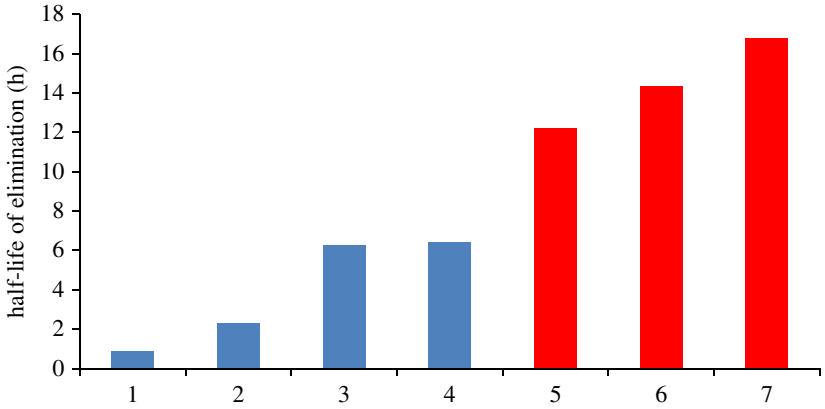

Figure 1. Estimated half-life of elimination for various avian species dosed with diclofenac in controlled toxicity studies. The half-lives have be ranked from fastest to slowest and represent: 1—*G. domesticus* (0.8 mg kg^{-1} oral); 2—*C. albus* (10 mg kg^{-1} oral); 3—*C. aura* (25 mg kg^{-1} oral); 4—*C. aura* (8 mg kg^{-1} oral); 5—*G. coprotheres* (0.8 mg kg^{-1} IV); 6—*G. domesticus* (5 mg kg^{-1} oral); 7—*G. africanus* (0.8 mg kg^{-1} oral). The red bars (5 to 7) indicate those doses associated with mortality. (Online version in colour.)

species, compared to the pharmacokinetic profiles of ketoprofen and meloxicam, which exhibit comparatively lower toxicity in the vulture, clearly indicate that toxicity is related to diclofenac's pharmacokinetics (figure 1).

For the first of these studies, the pharmacokinetics of diclofenac was evaluated in the Cape Griffon vulture (*Gyps coprotheres*) [60]. While environmental toxicity has not been seen in this vulture, the species was specifically validated as a suitable model for further mechanistic studies on the toxicity of diclofenac and other NSAIDs. The choice of this species was twofold, firstly the easier availability to the study site, as well as being less endangered than the Indian vulture species. From this controlled acute toxicity study, the Cape Griffon vulture was shown to be equally susceptible to diclofenac as the oriental white-backed vulture at 0.8 mg kg^{-1} (intravenous (IV) dose) with exactly the same clinical signs, clinical pathological and histopathological changes. Non-compartmental analysis revealed a half-life of elimination ($T_{1/2}$) of 12.24 $\pm$ 0.99 h, area under curve to the last quantifiable time point (AUC_{last}) of 80.28 $\pm$ 51.26 µg ml h^{-1}, and a mean residence time of 15.11 $\pm$ 4.13 h. To evaluate the importance of the pharmacokinetic profile obtained, Naidoo *et al*. [61] compared it to those published for other bird species (figure 2). This included the African white-backed vulture (*Gyps africanus*), the pied crow (*Corvus albus*), the turkey vulture (*Cathartes aura*) and the domestic chicken (*Gallus domesticus*). For these studies, no mortalities were reported for the pied crow (0.8 and 10 mg kg^{-1} oral), turkey vulture (8 and 25 mg kg^{-1} oral) and the domestic chicken (0.8 mg kg^{-1} oral), while toxicity was reported in the Cape Griffon (0.8 mg kg^{-1} IV), the African white-back (0.8 mg kg^{-1} oral) and one chicken at a higher dose (5 mg kg^{-1} oral). An important finding from these comparisons was a tentative link between the $T_{1/2}$ and the occurrence of toxicity, with a $T_{1/2}$ above 12 h being associated with death. Furthermore, zero-order metabolism was seen as a feature of toxicity as the $T_{1/2}$ was increased in the one chicken that died, from 0.89 h at 0.8 mg kg^{-1} to 14.34 h at 5 mg kg^{-1} oral.

While diclofenac has received wide attention in published literature as a result of its environmental toxic effect, it is not, however, the only NSAID evaluated in vultures in terms of safety and pharmacokinetics. In an attempt to have diclofenac removed from the Indian veterinary market, a replacement for the drug needed to be found for use in cattle, as diclofenac

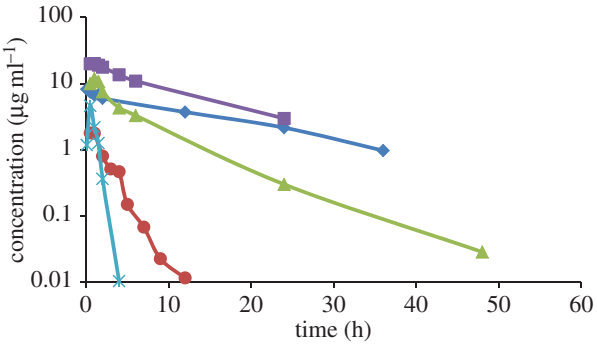

Figure 2. Plasma versus time profiles for diclofenac at 0.8 mg kg^{-1} IV in *G. coprotheres* (rhomboid); ketoprofen at 5 mg kg^{-1} oral for *G. coprotheres* that died (square); ketoprofen at 5 mg kg^{-1} oral in *G. coprotheres* that survived (triangle); diclofenac in chickens at 0.8 mg kg^{-1} oral (circle) and meloxicam in *G. coprotheres* at 2 mg kg^{-1} oral/intramuscular (cross). (Online version in colour.)

was of valuable cultural benefit to the sick cattle being treated. Following an international survey, meloxicam and ketoprofen were identified as potential replacements: they were effective in cattle with some evidence of safety in captive vulture species [62,63]. Subsequently, both these drugs were evaluated in extensive safety studies including full characterization of their pharmacokinetics, once again in Cape Griffon as the model, with vastly contrasting results.

In the first ketoprofen study, Cape Griffon vultures treated at 1 mg kg^{-1} oral showed no indications of toxicity on both clinical and clinical pathological evaluations [64]. However, when a second group of vultures were treated at the increased dose of 5 mg kg^{-1} oral, the study resulted in mortalities in seven of the 11 birds treated, with the characteristic signs of toxicity seen in the diclofenac-treated birds. The most interesting finding for this study was a difference in the $T_{1/2}$ between these two dose levels but also between the birds that died or survived at the 5 mg kg^{-1} dose. At 1 mg kg^{-1}, the half-life was 2.66 $\pm$ 0.46 h. In the four birds that survived at 5 mg kg^{-1}, the half-life was marginally higher at 3.24 $\pm$ 1.59 h. For the birds that died at the 5 mg kg^{-1} dose, the half-life had increased to 7.38 $\pm$ 1.72 h. With regards to AUC_{last}, the four birds that survived had an AUC_{last} fivefold higher, as expected for the fivefold increase in dose (9.79 $\pm$ 3.23 µg ml h^{-1} versus 50.31 $\pm$ 17.71 µg ml h^{-1}, respectively).

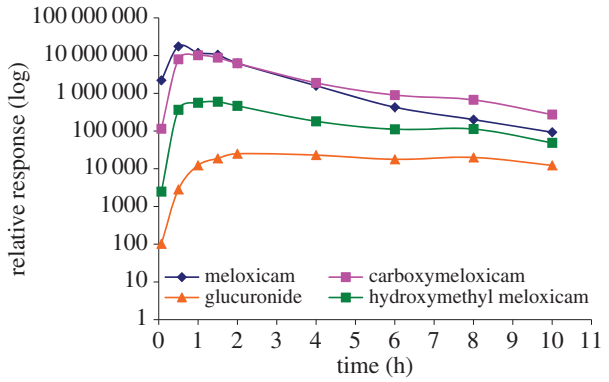

Figure 3. Relative response (AUC per peak on LC–MSMS chromatograms) versus time profiles for parent meloxicam and its three metabolites: hydroxymethyl meloxicam, an unidentified hydroxymethyl metabolite carboxymeloxicam and the glucuronide metabolite, following treatment at 2 mg kg^{-1} oral/intramuscular in *G. coprotheres*. (Online version in colour.)

However, the birds that died at 5 mg kg^{-1} had an increased AUC_{last} of 156.51 ± 33.14 μg ml h^{-1} and C_{max} of 21.0 ± 1.88 μg ml^{-1} in comparison with 10.77 ± 3.26 μg ml^{-1} for the birds that survived. This once again supported previous findings that toxicity is related to zero-order metabolism. In addition, the increase in the AUC_{last} and C_{max} also indicated that toxicity resulted in saturation of presystemic elimination pathways [64].

In the last of the described pharmacokinetic studies, meloxicam was administered to Cape Griffon vultures in a two-way cross-over study at a dose of 2 mg kg^{-1} by either oral or intramuscular route, without any signs of toxicity or changes in the monitored clinical pathology parameters [62]. Meloxicam was characterized by a short half-life of elimination of 0.33 ± 0.167 h and 0.42 ± 0.11 h for the oral and intramuscular routes, respectively. This study further attempted to characterize the metabolites produced via liquid chromatography-tandem mass spectrometry (LC-MSMS) analysis. Two CYP metabolites, hydroxymethyl meloxicam (87%) and an unknown hydroxylated metabolite (7%), and one glucuronide (0.56%) metabolite were identified (figure 3). Based on literature for laboratory animals, it was suspected that the CYP most likely involved in metabolism was predominantly CYP2C9.

While the metabolic pathway for diclofenac in the vulture is yet to be evaluated, the current pharmacokinetic information available allows for some conclusions to be drawn. The first of these is that toxicity is clearly linked to zero-order kinetics. For the NSAIDs, this deficiency could be at the level of the Phase I enzyme (CYP) system or Phase II glucuronidation, both of which have been previously described. Decreased CYP2C9 activity in people has been associated with resultant longer half-life of metabolized NSAIDs, whereas the absence of glucuronidation (UGT1A6) has been described as an important mechanism in the toxicity of paracetamol in the cat [19]. Limited glucuronide activity has also been described in people in association with aspirin toxicity. Based on the presence of a glucuronide metabolite for meloxicam, it is likely that toxicity in humans is not due to a complete absence of Phase II processes as in the cat. In addition, it is also doubtful that limited glucuronidation plays a role in human toxicity [65]. As a result, the rate-limiting step in avian metabolism is most likely at the level of the cytochrome P450 enzyme system. From medical literature, meloxicam is metabolized predominantly by CYP2C9 and, to a much lower extent, CYP3A4; diclofenac predominantly by CYP2C9, with some metabolism by CYP3A4 and CYP2C8 [66,67]; and ketoprofen by CYP2C9 [68]. When the half-life of elimination of diclofenac, ketoprofen and meloxicam in people is compared with the vulture, an important difference is noticed. In humans, the half-life of elimination of diclofenac, ketoprofen and meloxicam is typically 1–2, 2 and 15–20 h, respectively [69], while (as reported above) this is ±14, ±3 and 0.33 h, respectively for the vulture, with the metabolism of ketoprofen in vultures also being zero order. With CYP2C9 being the one common enzyme in metabolism, this is most probably the rate-limiting enzyme. With the rapid metabolism of meloxicam in vultures in contrast to humans, it may even be possible that the vulture is reliant on a Phase I system other than CYP2C9 for metabolism (in vultures, CYP3A4 seems a possibility). If this is the case, then the extreme sensitivity of the vulture to NSAID toxicity may be associated with the hepatotoxicity of diclofenac in humans, which is tentatively linked to CYP3A4 metabolism [70].

5. Conclusion

Pharmaceuticals provide many important health and economic benefits in the context of their capacity to generate desired and specific therapeutic effects in the target species (namely humans or in some cases, domestic animals and companion animals). In some cases, however, environmental exposures of wildlife to pharmaceutical residues can have dramatic consequences on non-mammalian species, as seen in the case of diclofenac and vultures [56–58] or fish populations in ecosystems exposed to synthetic oestrogens [71] (also see [72]). These notable examples, together with evidence of the widespread presence of pharmaceuticals in the environment, have been widely recognized to support the need for predictive environmental risk assessments [73–76] and consideration of the API residues in cattle and other livestock species [77].

A fundamental aspect of this challenge relates to the need to consider comparative metabolism for a range of non-mammalian species. Specifically, it is clear that there remain major knowledge gaps regarding the comparative metabolism of human and veterinary pharmaceuticals in non-mammalian species and this situation needs to be addressed in order to develop reliable environmental risk assessments for these important groups of medicines. It is proposed that this knowledge gap could be addressed in an efficient and ethical manner through the use of *in vitro* methods to define metabolism of reference APIs (selected from table 1) in hepatocytes from carnivorous birds compared with omnivorous bird species, for example, cormorants (*Phalacrocorax auritus*) and chickens (*G. domesticus*), respectively [78,79]. For fish, the same approach is feasible using *in vitro* hepatocyte assays for mainly carnivorous salmonid species such as rainbow trout (*Oncorhynchus mykiss*) versus the mainly herbivorous cyprinid species such as zebrafish (*Danio rerio*) or carp (*Cyprinus carpio*) [21,27]. For invertebrates, an *in vivo* approach would seem the best option and should be extended to both freshwater and marine species as part of an Adverse Outcome Pathways approach [41,80–82]. Subsequently, the *in vitro* avian and fish metabolic data and the *in vivo* invertebrate data for reference APIs could be used to develop and validate *in silico* tools to better predict which enzymes are responsible for API metabolism. If the measured

or predicted metabolism of a human or veterinary drug in mammalian or non-mammalian wildlife species raised concerns, further work could be done to evaluate the *in vitro* metabolites data through computational toxicology or metabolic pathway analysis [52,83,84].

In the wider context, where predicted regional increases in drug use occur or measurements of APIs in the environment raise concerns, the availability of validated *in silico* and *in vitro* methods to predict comparative metabolism will be of immense use in conducting environmental risk assessments. Specifically, together with prioritization through the predicted exposure concentration approach, an understanding of ADMET can play an important role in defining and predicting no-observed effect concentrations for freshwater, terrestrial and other environmental compartments, including predators [74,75,85]. In addition to this predictive aspect of pharmaceutical risk assessment, an understanding of ADMET can provide an important role for targeted monitoring of wildlife species of concern (e.g. vultures and other ultra-carnivorous species [76,81]; also see [86]).

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