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1 **Comparative metabolism as a key driver of wildlife species sensitivity to human and**
2 **veterinary pharmaceuticals.**

3

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18

19

20 **Abstract**

21

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

43

44 [abstract word count = 249]

45

46 Keywords: medicines, environment, exposure, birds, fish, invertebrates

47

48 **1.0 Introduction**

49

50 Investigation of a pharmaceutical's absorption, distribution, metabolism, elimination and
51 toxicology (ADMET) play a central role in the pre-clinical and clinical safety assessment of
52 human medicines [1]. Likewise, Active Pharmaceutical Ingredients (APIs) used in veterinary
53 medicine are evaluated for their ADMET profile in the species of interest (for example,
54 poultry or ruminants) [2, 3]. Metabolism of endogenous and exogenous molecules (eg plant
55 toxins, pesticides and pharmaceuticals) is normally classified into Phase I enzymatic
56 reactions (which add or expose -OH, -SH, -NH₂ or -COOH functional groups on xenobiotics)
57 and Phase II reactions (resulting in xenobiotic conjugation with large water-soluble, polar
58 molecules). Additionally, lipophilic xenobiotics, or their metabolites, can be pumped out of
59 cells by specific transporter proteins and this efflux pump activity is often termed Phase III
60 metabolism [4]. For approximately 5-7% of human drugs, Phase I metabolism may be
61 responsible for conversion of a prodrug into the API [5]. More broadly, many Phase I
62 biotransformations of lipophilic xenobiotics are carried out by microsomal monooxygenases,
63 located in the endoplasmic reticulum of the liver and other organs [6]. The haem protein
64 cytochrome P450 provides the active centre of these enzymes and has huge diversity, with
65 37 cytochrome P450 families currently identified across many animal species [7]. It is
66 hypothesized that the P450 superfamily has undergone repeated rounds of expansion by

67 genome duplication, whereby approximately one and a half billion years ago, the first
68 expansion gave rise to the P450 families primarily involved in metabolising endogenous
69 fatty acids, cholesterol and its derivatives (CYP4 and CYP11 families) which likely played a
70 key role in maintaining the eukaryotic cell membrane integrity. A later expansion of the
71 P450 family nine hundred million years ago may have led to several endogenous steroid-
72 synthesizing cytochrome P450 lineages (including CYP19, CYP21 and CYP27 gene families;
73 whereby the CYP21 family later diverged to give rise to the CYP1 and CYP2 families). A final
74 major expansion of several P450 families involved in xenobiotic metabolism (including CYP2,
75 CYP3, CYP4 and CYP6), began about four hundred million years ago. This most recent
76 expansion is thought to have been driven by first the emergence of aquatic organisms onto
77 land associated eating toxic plant allelochemicals ('animal – plant warfare'), together with
78 exposure of terrestrial organisms to hydrocarbon-based combustion products in the
79 atmosphere [7-11].

80 Much data exists on the metabolism of pharmaceuticals and other xenobiotics by the liver
81 microsomes of mammals, birds and other species, with rates of microsomal oxidative
82 metabolism determined across a range of vertebrates [2, 12, 13]. For example, Abass and
83 colleagues [14] studied the metabolism of the insecticide benfuracarb by hepatic
84 microsomes taken from seven mammalian species to investigate species-specific metabolic
85 pathways. Benfuracarb is metabolised via sulphur-oxidation and nitrogen-sulphur bond
86 cleavage (producing carbofuran which is further metabolised). Clearance rates for the seven
87 species ranged from 1.4 (monkey) to 3.5 (rat), these differences being due to variability in
88 CYP enzyme expression [14]. Among herbivorous and omnivorous mammals, there is a clear
89 inverse correlation between the microsomal monooxygenase activity and body weight [15,
90 16]. When hepatic monooxygenase activities are expressed in terms of body weight, much
91 higher values are found in small rodents than in large mammals. This observation is
92 consistent with the concept of a co-evolutionary arms race between plants and herbivorous
93 animals. In this context, small mammals need to consume more food per unit body weight
94 than do large ones in order to maintain body temperature due to their high surface area to
95 volume ratios. In contrast to the mammalian species studied by Walker and colleagues, the
96 carnivorous (piscivorous or raptorial) species showed distinctly lower microsomal
97 monooxygenase activities than did herbivorous or omnivorous birds (an observation also
98 explicable in terms of 'animal-plant arms race' theory). Predatory mammals (eg cats) and
99 birds (eg raptors) eat very little, if any, plant material and therefore do not incur major
100 pressure to drive the evolution of enzymes to metabolise plant toxins [16-19]. Interestingly,
101 zebrafish (a widely used model in pharmaceutical research) show a dramatic increase in
102 Phase I and II enzyme activity at the juvenile life stage in association with being fed plant
103 based diets [20].

104 In contrast to terrestrial vertebrates, Phase I enzyme activities in fish are generally lower
105 and there is only a weak correlation with body weight (whereas individual avian species
106 show a correlation between body weight and hepatic microsomal monooxygenase activity
107 across species) [12]. For fish, this has been explained on the grounds that they can excrete
108 many xenobiotics by diffusion across gills into the large volume of ambient water and it has
109 been argued that there has not been a strong pressure for the evolution of highly active
110 detoxification enzymes as seen in mammals [13, 21]. A similar situation is thought to apply
111 to aquatic invertebrates [22-24]. Nonetheless, as molecular and biochemical methods have
112 advanced there growing evidence of both Phase I and II enzyme activity in fish [20, 25, 26]

113 and recent studies have addressed how dietary and trophic variables may affect enzyme
114 activity in fish [27]. There are also a growing number of studies on the metabolism of
115 pharmaceuticals in fish [28-38] but to far lesser extent invertebrates [39]. Veterinary
116 pharmaceuticals have also been studied from a comparative metabolism perspective [40,41].
117 Table 1 summarizes Phase I pathways of pharmaceutical and xenobiotic metabolism in
118 mammals and other vertebrates, adapted from [42, 43] and updated with examples from
119 the DrugBank on-line database <http://www.drugbank.ca/> established by Wishart *et al.* [44].

120

121 Inset Table 1.

122

123 **2.0 In Vitro & In Silico Methods To Understand Comparative Metabolism**

124

125 *In vitro* systems are widely used for the investigation of xenobiotic metabolism in mammals
126 [1], birds [45] and fish [36, 38]. Techniques include: (a) whole liver tissue slices which retain
127 an accurate, structural framework of the liver; (b) whole isolated hepatocytes where the
128 endoplasmic reticulum bound and cytosolic enzymes are present the structural integrity of
129 liver network lost; (c) after centrifugation at 9000g, the S9 fraction supernatant from liver
130 (or other tissue) homogenate contains both cytosolic (predominantly Phase II) and
131 microsomal (predominantly Phase I) enzymes; and (d) microsomes comprising of
132 endoplasmic reticulum bound enzymes that have been separated from cytosolic enzymes
133 (P450 enzymes are concentrated in this subcellular fraction). These methods are routinely
134 used to determine the rate and extent of metabolism and mass-spectroscopic analysis of
135 specific metabolites. Results for clearance rates obtained from *in vitro* metabolism
136 experiments can then be extrapolated to the *in vivo* situation using scaling factors (e.g.
137 number of hepatocytes per liver; weight of microsomal protein per gram of liver, etc).
138 Allometric methods can also be used to scale *in vitro* results between different species (used
139 in drug development for scaling from preclinical species to man). Where such values are
140 known for wildlife species, this may allow for approximations between different species [15,
141 16] and form a basis for models to aid in environmental risk assessment using fish [29,46,47],
142 invertebrates [39] and plants [48].

143

144 Novel *in silico* tools may also be useful to predict metabolism, this approach tending to
145 focus on the semi-quantitative prediction of potential metabolites and identification of the
146 specific enzymes responsible for the metabolism. Prediction of metabolic rates of drug
147 metabolism remains a key challenge, especially with regard to identification of potential
148 metabolites (which may be associated with specific toxicities) and identification of the
149 enzymes responsible (combined with knowledge of different enzyme expression in different
150 species). Kirchmair *et al.* [49] provide an overview of *in silico* tools for predicting key factors
151 associated with metabolism (including sites of metabolism (SOM) within a molecule;
152 potential metabolites; cytochrome P450 (CYP) binding affinity / inhibition; and prediction of
153 CYP induction). Table 2 shows a representative software tool for each of these categories,
154 however, many other tools are available [49].

155

156 Insert Table 2.

157

158 *In silico* tools have a number of potential advantages and provide complementary
159 techniques to *in vitro* methods. One area where information from both fields can be
160 combined to build improved predictions is in physiologically-based pharmacokinetic (PBPK)
161 modelling. In this method an organism is divided into a sequence of physiological
162 compartments (e.g. brain, liver, lungs, etc.). The models integrate compound-specific data
163 (e.g. physico-chemical properties, such as log P, pK_a or solubility, these values may be
164 measured or predicted using *in silico* techniques) and species (or even subject)-specific data
165 (e.g. physiological factors such as body or organ weights, volumes, or blood flow rates).
166 Subject to validation, these models are potentially of high value in predicting concentration-
167 time profiles for pharmaceuticals in wildlife species [29,34,46]. Understanding inter-species
168 differences in metabolism is essential for reliable PBPK models, especially in non-
169 mammalian species. For example, Ohyama *et al.* [45] studied methoxychlor (MXC)
170 metabolism in rat, mouse, Japanese quail and rainbow trout using liver slices. Each species
171 showed differences in metabolism, considered due to substrate specificity of CYP450s
172 involved. MXC was metabolised to bis-OH-MXC which was then glucuronidated (with only
173 rats producing the bis-OH-MXC 4 O-sulphate 4-O- glucuronide). In mice and Japanese quail,
174 mono-OH-MXC (and glucuronide conjugate) were the main metabolites and little bis-OH-
175 MXC glucuronide was formed (dechlorinated mono-OH-MXC glucuronide was found only in
176 mice). Rainbow trout liver slices formed similar amounts of both metabolites. In conclusion,
177 rat and trout livers slices were able to metabolise both MXC and mono-OH MXC, whereas
178 only MXC could be metabolised in mouse and Japanese quail [45].

179

180 **3.0 In Vivo Approaches in Studying Comparative Metabolism**

181

182 The overall effect a xenobiotic has on any organism is ultimately the result of its intrinsic
183 activity and its concentration at the target site. Concentration at a given target site is
184 determined by the ADME properties of the compound. The history of studying the time
185 course and concentration of xenobiotics at different sites within the body has been
186 developed predominantly within the pharmaceutical industry, with respect to drug effects
187 on humans. However the techniques are applicable to diverse chemical space and across
188 diverse species. *In vivo* measurements determining the pharmacokinetic profiles of
189 xenobiotics in environmental species are largely unavailable, hence extrapolation and
190 predictive models (combining *in silico* and *in vitro* methods) become essential tools in
191 determining organ-level concentrations [50]. Metabolism is one of the key factors to
192 consider when modelling the time course of a xenobiotic within an organism, not only as it
193 can determine the overall period of exposure, but also because the metabolite(s), rather
194 than the parent drug, may be responsible for the toxic effect [1,51]. In the non-mammalian
195 area where much less is known about metabolic profiles of drugs in animals, *in vivo*
196 experiments still have a major role to play to derive reliable environmental risk assessments
197 [for case studies with freshwater fish see references 33,34,52] and also in wildlife forensic
198 studies (see following case study on birds).

199

200

201

202 4.0 Case Study - Vulture toxicity to NSAIDs (A Process of Zero-Order Metabolism)

203

204 The dramatic impact of diclofenac (a non-steroidal anti-inflammatory drug or 'NSAID') on
205 Asian vulture populations represents one of the most serious ecological catastrophes of
206 recent times. In just over a decade, diclofenac has been responsible for the deaths of
207 millions of vultures of the Asian White-backed (*Gyps bengalensis*), Long-Billed (*G. indicus*),
208 Slender-billed (*G. tenuirostris*), Egyptian (*Neophron percnopterus*) and red-headed
209 (*Sarcogyps calvus*) vulture species across the Indian subcontinent [53,54]. In addition to the
210 scale of the toxicity, the exposure route to the product was probably highly unconventional
211 as these birds were inadvertently being poisoned by the oral route even though diclofenac
212 was only available as an injectable cattle formulation. Whereas previous veterinary
213 medicines and pesticides had caused their negative effects by ending up in the water, soil or
214 general environment of the species affected, these vultures were being exposed to this
215 product as residues in the meat of the dead cattle carcasses upon which they fed. This
216 unique mode of exposure was linked to cultural and religious practices in the region
217 whereby sick and old cattle were routinely treated in a palliative manner with diclofenac, a
218 cheap and effective NSAID. The net effect of this practice was an unfortunate high
219 occurrence of diclofenac residues in the tissues of recently dead cattle.

220

221 In the vulture, diclofenac is highly toxic with acute death resulting from a single meal of 1kg
222 of meat rich in residue, with an estimated LD₅₀ of 0.1 to 0.2 mg/kg [55]. Toxicity following
223 exposure is also fairly predictable with birds showing signs of depression and head drooping
224 as early as 24 hours post exposure. Death is the typical end-point with birds literally being
225 described as falling dead from their perches. Based on the results from controlled toxicity
226 studies, it has been shown that death after a single exposure consistently resulted within 48
227 hours of exposure, with related massive increases in plasma uric acid and potassium
228 concentrations and increased alkaline phosphatase (ALP) activity. Necropsies are also very
229 typical with signs of severe nephrosis, dehydration and accompanying diffuse visceral and
230 articular gout. Histopathology indicated toxicity was characterised by necrosis of
231 hepatocytes and the renal tubular epithelial cells (RTE) of the proximal convoluted tubules
232 with associated uric acid tophi accumulation. While the mechanism of toxicity of diclofenac
233 remains incompletely described, toxicity has been linked to RTE cell damage in a time
234 related manner, subsequent accumulation of uric acid, acidosis and terminal hyperkalaemia
235 [56]. Results from various pharmacokinetics studies of diclofenac in different bird species,
236 and the pharmacokinetic profiles of ketoprofen and meloxicam in the vulture, clearly
237 indicate that toxicity is related to the drug's pharmacokinetics (Figure 1).

238

239 Insert Figure 1

240

241 For the first of these studies, the pharmacokinetics of diclofenac was evaluated in the Cape
242 Griffon Vulture (*Gyps coprotheres*) [57]. While environmental toxicity has not been seen in
243 this vulture, the species was specifically validated as a suitable model for further
244 mechanistic studies on the toxicity of diclofenac and other NSAIDs. The choice of this
245 species was two-fold, firstly the easier availability to the study site as well as being less
246 endangered than the Indian vulture species. From this controlled acute toxicity study, the
247 Cape Griffon was shown to be equally susceptible to diclofenac as the Oriental White-
248 backed at 0.8 mg/kg i.v. with exactly the same clinical signs, clinical pathological and

249 histopathological changes. Non-compartmental analysis revealed a half-life of elimination
250 ($T_{1/2}$) of 12.24 ± 0.99 hours, area under curve to the last quantifiable time point (AUC_{last}) of
251 80.28 ± 51.26 $\mu\text{g/ml/hour}$, a mean residence time (MRT) of 15.11 ± 4.13 hours. To evaluate
252 the importance of the obtained pharmacokinetic profile obtained, parameters were
253 previously compared by [58] to that published for other bird species (Figure 2). This included
254 the African-white backed vulture (*G. africanus*), the Pied crow (*Corvus albus*), the turkey
255 vulture (*Cathartes aura*) and the domestic chicken (*Gallus domesticus*). For these studies no
256 mortalities were reported for the Pied Crow (0.8 and 10mg/kg), Turkey Vulture (8 and 25
257 mg/kg) and the domestic chicken (0.8 mg/kg), while toxicity was reported in the Cape
258 Griffon (0.8 mg/kg), the African white-back (0.8 mg/kg) and one chicken at a higher dose (5
259 mg/kg). An important finding from these comparisons was a tentative link between the $T_{1/2}$
260 and the occurrence of toxicity with a $T_{1/2}$ above 12 hours being associated with death.
261 Furthermore zero order metabolism was seen as a feature of toxicity as the $T_{1/2}$ was
262 increased in the one chicken that died, from 0.89 hours at 0.8 mg/kg to 14.34 hours at 5
263 mg/kg.

264
265 Insert Figure 2.

266
267 While diclofenac has received wide attention in published literature as a result of its
268 environmental toxic effect it is not, however, the only NSAID evaluated in vultures in terms
269 of safety and pharmacokinetics. In an attempt to have diclofenac removed from the Indian
270 veterinary market, a replacement for the drug needed to be found for use in cattle, as
271 diclofenac was of valuable cultural benefit to the sick cattle being treated. Following an
272 international survey, meloxicam and ketoprofen were identified as potentially replacement
273 i.e. they were effective in cattle with some evidence of safety in captive vulture species
274 [59,60]. Subsequently both these drugs were evaluated in extensive safety studies including
275 full characterisation of their pharmacokinetics once again in Cape Griffon as the model, with
276 vastly contrasting results.

277
278 In the first ketoprofen study Cape Griffon vultures treated at 1 mg/kg showed no indications
279 of toxicity on both clinical and clinical pathological evaluations [61]. However, when a
280 second group of vultures were treated at increased dose of 5 mg/kg, the study resulted in
281 mortalities in seven of the 11 birds treated with the characteristic signs of toxicity seen in
282 the diclofenac treated birds. The most interesting finding for this study was a difference in
283 the $T_{1/2}$ between these two dose levels but also between the birds that died or survived at
284 the 5 mg/kg dose. At 1 mg/kg the half-life was 2.66 ± 0.46 hours. In the four birds that
285 survived at 5 mg/kg the half-life was marginally higher at 3.24 ± 1.59 hours. For the birds
286 that died at the 5 mg/kg dose, the half-life had increased to 7.38 ± 1.72 hours. With regards
287 to AUC_{last} , the four birds that survived had an AUC_{last} five-fold higher as expected for the 5-
288 fold increase in dose (9.79 ± 3.23 $\mu\text{g/ml/hour}$ versus 50.31 ± 17.71 $\mu\text{g/ml/hour}$, respectively).
289 However, the birds that died at 5 mg/kg had an increased AUC_{last} of 156.51 ± 33.14
290 $\mu\text{g/ml/hour}$ and C_{max} of 21.0 ± 1.88 $\mu\text{g/ml}$ in comparison to 10.77 ± 3.26 $\mu\text{g/ml}$ to the birds
291 that survived. This once again supported previous findings that toxicity is related to zero
292 order metabolism. In addition, the increase in the AUC_{last} and C_{max} also indicated that
293 toxicity resulted in saturation of presystemic elimination pathways [61].

294

295 In the last of the described pharmacokinetic studies, meloxicam was administered to Cape
296 Griffon vultures in a two-way cross over study at a dose of 2 mg/kg by either oral or
297 intramuscular route, without any signs of toxicity or changes in the monitored clinical
298 pathology parameters [59]. Meloxicam was characterised by a short half-life of elimination
299 of 0.33 ± 0.167 hours and 0.42 ± 0.11 hours for the oral and intramuscular routes
300 respectively. This study further attempted to characterise the metabolites produced via LC-
301 MSMS analysis. Two CYP metabolites, hydroxymethyl meloxicam (87%) and an unknown
302 hydroxylated metabolite (7%), and one glucuronide (0.56%) metabolite were identified
303 (Figure 3). Based on literature in laboratory animals, it is suspected that the CYP most likely
304 involved in metabolism was predominantly CYP2C9.

305

306 Insert Figure 3.

307

308 While the metabolic pathway for diclofenac in the vulture is yet to be evaluated, the current
309 pharmacokinetic information available allows for some conclusions to be drawn. The first of
310 these is that toxicity is clearly linked to zero order kinetics. For the NSAIDs, this deficiency
311 could be at the level of the Phase I enzyme (CYP) system or Phase II glucuronidation, both of
312 which have been previously described. Decreased CYP2C9 activity in people has been
313 associated with resultant longer half-life of metabolised NSAIDs, while the absence of
314 glucuronidation (UGT1A6) has been described as an important mechanism in the toxicity of
315 paracetamol in the cat [18]. Limited glucuronide activity has also been described in people
316 in association with aspirin toxicity. Based on the presence of a glucuronide metabolite for
317 meloxicam, it is likely that toxicity in humans is not due to a complete absence of Phase II
318 processes as in the cat. In addition, it is also doubtful that limited glucuronidation plays a
319 role in human toxicity [62]. As a result, the rate limiting step in avian metabolism is most
320 likely at the level of cytochrome P450 enzyme system. From medical literature, meloxicam is
321 metabolised predominantly by the CYP2C9 and, to a much lower extent CYP3A4); diclofenac
322 predominantly by the CYP2C9, with some metabolism by CYP3A4 and CYP2C8 [63,64]; and
323 ketoprofen by CYP2C9 [65]. When the half-life of elimination of diclofenac, ketoprofen and
324 meloxicam in people is compared to the vulture, an important difference is present. In
325 humans the half-life of elimination of diclofenac, ketoprofen and meloxicam is typically 1-2
326 hours, 2 hours and 15-20 hours, respectively [66], while as reported above this is ± 14 hours,
327 ± 3 hours and 0.33 hours, respectively for the vulture, with the metabolism of ketoprofen in
328 vultures also being zero order. With the CYP2C9 being the one common enzyme in
329 metabolism, it is most probably that this is the rate limiting enzyme. With the rapid
330 metabolism of meloxicam in vultures in contrast to humans, it may even be possible that
331 the vulture is reliant on a Phase I system other than CYP2C9 for metabolism (in vultures
332 CYP3A4 seem a possibility). If this is the case, then the extreme sensitivity of the vulture to
333 NSAID toxicity may be associated with the hepatotoxicity of diclofenac in humans which is
334 tentatively linked to CYP3A4 metabolism [67].

335

336 5.0 Conclusions

337

338 Pharmaceuticals provide many important health and economic benefits in the context of
339 their capacity to generate desired and specific therapeutic effects in the target species
340 (namely humans or in some cases, domestic animals and companion animals). In some
341 cases, however, environmental exposures of wildlife to pharmaceutical residues can have

342 dramatic consequences on non-mammalian species, as seen in the case of diclofenac and
343 vultures [54,55] or fish populations in ecosystems exposed to synthetic oestrogens [68].
344 These notable examples, together with evidence of the widespread presence of
345 pharmaceuticals in the environment, have been widely recognized to support the need for
346 predictive environmental risk assessments [69-72] and consider API residues in cattle and
347 other livestock species [73].

348
349 A fundamental aspect of this challenge relates to the need to consider comparative
350 metabolism for a range of non-mammalian species. Specifically, it is clear that there remain
351 major knowledge gaps regarding the comparative metabolisms of human and veterinary
352 pharmaceuticals in non-mammalian species and this situation needs to be addressed in
353 order to develop reliable environmental risk assessments for these important groups of
354 medicines. It is proposed that this knowledge gap could be addressed in an efficient and
355 ethical manner through the use of *in vitro* methods to define metabolism of reference APIs
356 (selected from Table 1) in hepatocytes from carnivorous birds compared with omnivorous
357 bird species, for example cormorants *Phalacrocorax auritus* and chickens *Gallus domesticus*,
358 respectively [74,75]. For fish, the same approach is feasible using *in vitro* hepatocyte assays
359 for mainly carnivorous salmonid species such as rainbow trout (*Oncorhynchus mykiss*)
360 versus the mainly herbivorous cyprinid species such as zebrafish (*Danio rerio*) or carp
361 (*Cyprinus carpio*) [20, 25]. For invertebrates, an *in vivo* approach would seem the best
362 option and should be extended to both freshwater and marine species as part of an Adverse
363 Outcome Pathways approach [39, 76-78]. Subsequently, the *in vitro* avian and fish
364 metabolic data and the *in vivo* invertebrate data for reference APIs could be used to develop
365 and validate *in silico* tools to better predict which enzymes are responsible for API
366 metabolism. If the measured or predicted metabolism of a human or veterinary drug in
367 mammalian or non-mammalian wildlife species raised concerns, further work could be done
368 to evaluate the *in vitro* metabolites data through computational toxicology or metabolic
369 pathway analysis [50, 79, 80].

370
371 In the wider context, where predicted regional increases in drug use occur or measurements
372 of APIs in the environment raise concerns, the availability of validated *in silico* and *in vitro*
373 methods to predict comparative metabolism will be of immense use in conducting
374 environmental risk assessments. Specifically, together with prioritisation through the
375 Predicted Exposure Concentration (PEC) approach, an understanding of ADMET can play an
376 important role in defining Predicting No-Observed Effect Concentrations (PNECs) for
377 freshwater, terrestrial and other environmental compartments, including predators
378 [70,71,81]. In addition to this predictive aspect of pharmaceutical risk assessment, an
379 understanding of ADMET can provide an important role for targeted monitoring of wildlife
380 species of concern (eg vultures and other ultra-carnivorous species) [72,77].

381
382 **[word count = 4269]**

383

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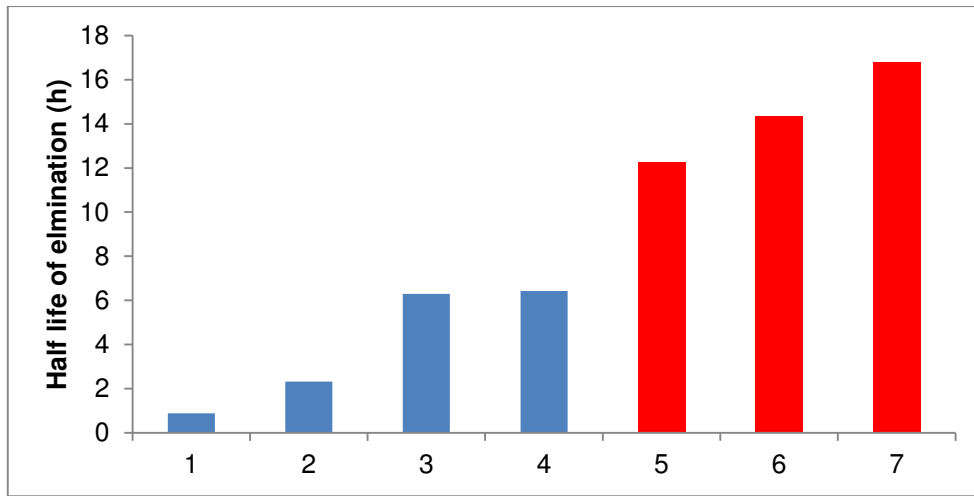
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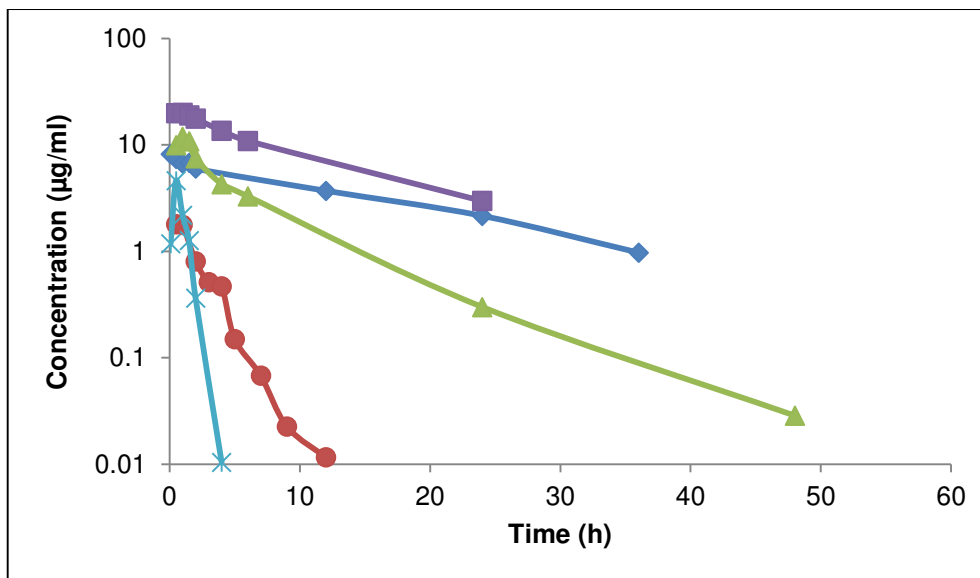
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Figure 1. Estimated half-life of elimination for various avian species dosed with diclofenac in controlled toxicity studies. The half-lives have been ranked from fastest to slowest and represent; 1- *Gallus domesticus* (0.8 mg/kg); 2- *Corvus albus* (10 mg/kg); 3- *Cathartes aura* (25 mg/kg); 4- *Cathartes aura* (8 mg/kg); 5- *Gyps coprotheres* (0.8 mg/kg); 6- *Gallus domesticus* (5mg/kg); 7- *Gyps africanus* (0.8 mg/kg). The red bars, indicate those doses associated with mortality.

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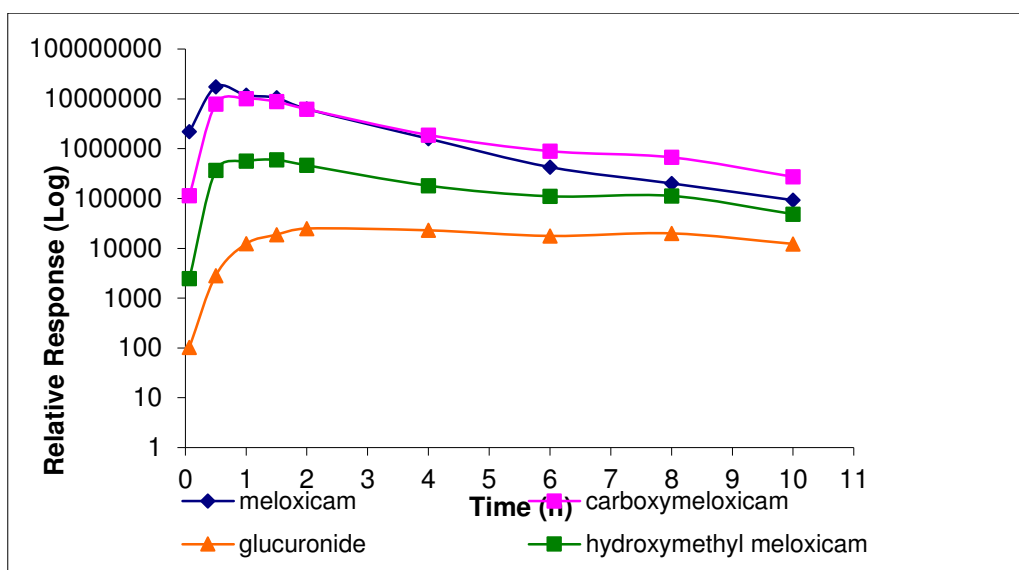


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Figure 2. Plasma versus time profiles for diclofenac at 0.8 mg/kg in the *G. coprotheres* (Rhomboid); ketoprofen at 5 mg/kg for the *G. coprotheres* that died (Square); ketoprofen at 5 mg/kg in *G. coprotheres* that survived (Triangle); Diclofenac in chickens at 0.8 mg/kg (circle) and meloxicam in *G. coprotheres* at 2 mg/kg (cross).

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658 **Figure 3.** Relative response (AUC per peak on LC-MSMS chromatograms) versus time
659 profiles for parent meloxicam and its three metabolites hydroxymethyl meloxicam; an
660 unidentified hydroxymethyl metabolite carboxymeloxicam and the glucuronide metabolite
661 following treatment at 2 mg/kg in *G. coprotheres*.

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

Enzyme	Localization	Substrate	Inhibitor
Phase I – Hydrolysis Reactions:			
Esterase	Microsomes, cytosol	trandolapril	tamoxifen
Peptidase	Lysosomes	-	alogliptin
Epoxide hydrolase	Microsomes, cytosol	Diazepam	valproate
Phase I – Reduction Reactions:			
Azo- and nitro-reduction	Microsomes, cytosol	Prontosil	clofibrate
Carbonyl reduction	Microsomes, cytosol	Loxoprofen	befunolol
Disulphide reduction	Cytosol	captopril	-
Sulphoxide reduction	Cytosol	-	dimethylsulfoxide
Quinone reduction	Microsomes, cytosol	Trenimon	warfarin
Reductive dehalogenation	Microsomes	Chloramphenicol	-
Phase I – Oxidation Reactions:			
Alcohol dehydrogenase	Cytosol	ethanol	fomepizole
Aldehyde dehydrogenase	Mitochondria, 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-monoxygenases	Microsomes	riboflavin	nitric oxide
Cytochrome P450:	Microsomes	-	-
CYP1A1	Microsomes	7-ethoxyresorufin	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
Sulphate conjugation	Cytosol	Phase I metabolites	harmol
Glutathione conjugation	Microsomes, cytosol	Phase I metabolites	tannic acid
Amino acid conjugation	Microsome	Phase I metabolites	kinetin
Acetylation	Mitochondria, cytosol	Phase I metabolites	garcinol
Methylation	Microsomes, cytosol	Phase I metabolites	5-A-2'deoxyctidine

669

670 Table 1 = 220 words

671 Table 2. Representative examples of computational tools for predicting factors associated
 672 with mammalian metabolism (note - 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 metabolised	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

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 674 Table 2 = 121 words
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