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Environmental Toxicology

CYTOCHROME P450–MEDIATED WARFARIN METABOLIC ABILITY IS NOT A CRITICAL DETERMINANT OF WARFARIN SENSITIVITY IN AVIAN SPECIES: IN VITRO ASSAYS IN SEVERAL BIRDS AND IN VIVO ASSAYS IN CHICKEN

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Abstract: Coumarin-derivative anticoagulant rodenticides used for rodent control are posing a serious risk to wild bird populations. For warfarin, a classic coumarin derivative, chickens have a high median lethal dose (LD50), whereas mammalian species generally have much lower LD50. Large interspecies differences in sensitivity to warfarin are to be expected. The authors previously reported substantial differences in warfarin metabolism among avian species; however, the actual in vivo pharmacokinetics have yet to be elucidated, even in the chicken. In the present study, the authors sought to provide an in-depth characterization of warfarin metabolism in birds using in vivo and in vitro approaches. A kinetic analysis of warfarin metabolism was performed using liver microsomes of 4 avian species, and the metabolic abilities of the chicken and crow were much higher in comparison with those of the mallard and ostrich. Analysis of in vivo metabolites from chickens showed that excretions predominantly consisted of 4′-hydroxywarfarin, which was consistent with the in vitro results. Pharmacokinetic analysis suggested that chickens have an unexpectedly long half-life despite showing high metabolic ability in vitro. The results suggest that the half-life of warfarin in other bird species could be longer than that in the chicken and

that warfarin metabolism may not be a critical determinant of species differences with respect to warfarin sensitivity.

Keywords: Avian, Cytochrome P450, Species difference, Warfarin, Pharmacokinetics

All Supplemental Data may be found in the online version of this article.

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INTRODUCTION

Coumarin-derivative anticoagulant rodenticides, such as bromadiolone and brodifacoum, have been reported to cause secondary poisoning of scavenging and raptorial bird populations [1–8]. Whereas toxicity tests with poultry species have suggested that they are relatively resistant to a classic coumarin-derivative anticoagulant, warfarin. The median lethal doses (LD50) of a single dose of orally administered warfarin have been reported for chicken (*Gallus gallus*, 942 mg/kg), mallard (*Anas platyrhynchos*, 620 mg/kg), and northern bobwhite (*Colinus virginianus*, >2150 mg/kg); these are much higher than those for rat and mouse (2.5–680 mg/kg) [9].

Cytochrome P450 (CYP)–mediated warfarin metabolic ability is well established in mammalian species and has been employed as a determinant of warfarin

sensitivity. The isoforms of CYP catalyze the first step of warfarin metabolism by producing hydroxywarfarins (Figure 1). In rats, CYP1A, CYP2B, CYP2C, and CYP3A isoforms are known to metabolize warfarin [10]. In humans, CYP2C9 is a major isoform metabolizing the *S* isomer of warfarin, a more potent inhibitor of human vitamin K epoxide reductase activity in comparison with *R*-warfarin; thus, the single-nucleotide polymorphism of CYP2C9 can affect the clearance of *S*-warfarin [11]. Consequently, the single-nucleotide polymorphism of CYP2C9 explains approximately 15% of individual variance in dose requirements for therapeutics [12]. In warfarin-resistant rats, elevated CYP-mediated warfarin metabolism is reported to be 1 of the warfarin resistance mechanisms [13].

We previously reported in vitro warfarin metabolic activity using liver microsomes from several bird species [14]. A large interspecies difference in warfarin metabolic activity among bird species was observed, although only a single high concentration of warfarin was used at the time of measurement. To clarify the role of metabolism as a determinant of in vivo warfarin sensitivity, it is necessary to take into account CYP-mediated metabolism, an important factor in absorption, distribution, metabolism, and excretion and the actual pharmacokinetics of warfarin in birds. In the present study, we sought to clarify differences in the in vitro warfarin metabolic ability

among birds by kinetic analysis and to more completely elucidate the in vitro metabolism and in vivo pharmacokinetics of warfarin in chickens.

MATERIALS AND METHODS

Chemicals

Bovine serum albumin, sulfatase, β-glucuronidase, and racemic warfarin sodium were purchased from Sigma-Aldrich. Glucose 6-phosphate, glucose 6-phosphate dehydrogenase, and β-reduced nicotinamide adenine dinucleotide phosphate were purchased from Oriental Yeast. Magnesium chloride was obtained from Wako Pure Chemical Industries. Warfarin metabolites (4′-hydroxywarfarin [4′-OH], 6-hydroxywarfarin [6-OH], 7-hydroxywarfarin [7-OH], 8-hydroxywarfarin [8-OH], and 10-hydroxywarfarin [10-OH]) were obtained from Ultrafine Chemicals.

Animals

Wistar rats were purchased from Japan SLC (male, $n = 3$). Rats were housed for 1 wk in plastic cages at 22 ± 1 °C with a 12:12-h light;dark cycle and fed laboratory chow and tap water ad libitum before being sacrificed. White leghorn chickens (*Gallus gallus*, $n = 3$, male) were purchased from Hokudo. They were similarly housed with a 12:12-h light:dark cycle with a normal diet (Nihon Haigo Shiryo for <5-wk-old chickens, Nihon Nosan Kogyo for 5-wk-old chickens) and water ad libitum. Animals were sacrificed by

carbon dioxide inhalation, and liver samples were immediately collected and frozen in liquid nitrogen. The liver samples were stored at –80 °C until preparation of liver microsomes. At the time of sacrifice, rats and chickens were 10 wk and 4 wk to 5 wk old, respectively.

For the in vivo study of warfarin metabolism, 3 male and 3 female chickens were purchased from Hokudo. They were used for the assay at the age of 6 wk.

All experiments using animals were performed under the supervision and with the approval of the Institutional Animal Care and Use Committee of Hokkaido University (permission no. 10-0067).

Fresh livers of ostrich (*Struthio camelus*, *n* = 3, male, 2–6 yr old), mallard (*Anas platyrhynchos*, *n* = 3, male, 9 wk old), and jungle crow (*Corvus macrorhynchos*, *n* = 3, 2 female and 1 male, age unknown) were gifts from Hokkaido Ostrich Farm Kuroda, Hokuseien Farm, and Yubari City, respectively. The sex of crows was determined by chromo-helicase-DNA binding protein (CHD1) genes following previously detailed methods [15].

Warfarin metabolism assay in liver microsomes

Liver microsomal fractions were prepared with potassium phosphate buffer using standard procedures described by Omura and Sato [16]. Microsome protein

concentrations were measured using the BCA Protein Assay Reagent Kit (Thermo Fisher Scientific). The CYP content was estimated using the method detailed by Omura and Sato [16].

Warfarin metabolic activity was measured using liver microsomes of chicken, ostrich, crow, mallard, and rat, following a previously reported method [14]. Racemic warfarin sodium was dissolved in distilled water and used as the substrate. Substrate concentrations for kinetic analysis were 25 μM, 50 μM, 100 μM, 200 μM, 400 μM, and 800 μM. The metabolites (4′-hydroxylated, 6-hydroxylated, 7-hydroxylated, 8-hydroxylated, and 10-hydroxylated warfarin) were quantitatively analyzed using high-performance liquid chromatography separation and ultraviolet detection (HPLC-UV). The mobile phase comprised 55% KH_2PO_4 and 45% methanol:acetonitrile (2:1). A TSKgel ODS-120T column (250 × 4.6 mm, 5 μm; Tosoh) was used for separation at a flow rate of 0.3 mL/min. A UV detector set at 308 nm monitored the effluent. Limits of quantification (LOQ) of each metabolite were settled as 0.2 pmol, 0.05 pmol, 0.05 pmol, 0.2 pmol, and 0.01 pmol for 4′-hydroxylated, 6-hydroxylated, 10-hydroxylated, 7-hydroxylated, and 8-hydroxylated warfarin, respectively. The method was found to be highly accurate with <5.3% (within-run precision and between-run precision) at each metabolite. We estimated maximum velocity (V_{max}) and

the Michaelis constant (K_m) were estimated using Graph Pad Prism 5 (Graph Pad Software).

In vivo analysis: Pharmacokinetics and metabolite composition in fecal samples

Chickens were fasted for 12 h prior to oral administration of warfarin. The administered compound consisted of 3 mg/mL racemic warfarin sodium dissolved in distilled water and was administered at a dose of 1.5 mg/kg body weight. At each sampling time (0 h, 0.5 h, 1 h, 2 h, 3 h, 6 h, 9 h, 12 h, 24 h, and 72 h after oral administration), 200 µL of blood collected via the wing vein was transferred into an Eppendorf tube containing 2 µL heparin. Samples were centrifuged for 30 min at 1000 *g* within 30 min of collection, and supernatant plasma was collected and stored at −20 °C until extraction.

Warfarin was extracted from plasma using a previously reported method with slight modifications [17]. In brief, 50 µL of plasma were mixed with 10 µL of 7-hydroxycoumarin (10 µg/mL) as an internal standard. After storage for 12 h at 4 °C for equilibration, 190 µL of distilled water containing 0.2% formic acid and 1 mL of acetonitrile containing 0.2% formic acid were added and mixed by vortexing. After incubation at 4 °C for 30 min, the mixture was centrifuged for 15 min at 12 000 *g*. After the centrifugation, 1 mL of the organic layer was taken and evaporated. Residues were

dissolved in 200 μL of the mobile phase used in HPLC analysis. After another centrifugation at 12 000 *g* for 15 min, an aliquot of 50 μL was taken for further HPLC analysis. Plasma concentrations of warfarin were analyzed by HPLC-UV with the same procedure as used in the warfarin metabolism assay.

Fecal samples were collected in 20 mL of methanol 9 h after administration. Samples were homogenized and sonicated for 10 min. After centrifugation at 2000 *g* for 20 min at 4 °C, the supernatant was collected into another tube and another 20 mL of methanol was added to the pellet. The pellet was again homogenized and centrifuged, and the supernatant was collected.

The collected supernatant was hydrolyzed by 10 IU/mL sulfatase and 4000 IU/mL β-glucuronidase and analyzed with a liquid chromatography–tandem mass spectrometry (LC-MS/MS) system (Shimadzu).

The metabolite concentration in the samples was calculated using a calibration curve, and the disposition of metabolites was estimated at the 9 h time point.

A Prominence HPLC system (Shimadzu) equipped with an LCMS-8040 (Shimadzu) was used for LC-MS/MS analysis with an electrospray ionization interface. A TSKgel ODS-120T LC column was used (Tosoh). Mobile phases were water containing 1% acetic acid (A) and acetonitrile containing 1% acetic acid (B). Gradient

separation was performed at 10% (B) from 0 min to 2 min, followed by a linear gradient from 10% (B) to 90% (B) from 2 min to 27 min, followed by 10% (B) from 27 min to 30 min. Flow rate was 0.3 mL/min. The ionization mode was negative in the multiple reaction monitoring mode. Collision energies and other optimized MS parameters are shown in Supplemental Data, Table S1. Nebulizing gas flow was 3 L/min, drying gas flow was 15 L/min, desolvation line temperature was 250 °C, and heat block temperature was 400 °C. Samples and standards were injected at volumes of 10 μL. Column temperature was maintained at 50 °C. The LOQ was settled as 10 μg/L for each metabolite based on the signal-to-noise ratio. The method was found to be highly accurate, with a relative percentage standard deviation of 1.3% (for 4′-OH) to 4.5% (for 7-OH) within-run precision.

Statistical analysis

Data analyses were performed using the pharmacokinetic software Phoenix WinNonLin (Certara). Pharmacokinetic parameters were estimated with the following conditions: 1 compartment, no lag time, and first-order input and elimination rate. Statistical analyses were performed based on a Student *t* test of sex differences in pharmacokinetic parameters of the chicken, and Tukey's honestly significant difference test for species differences in warfarin metabolism using JMP Ver 7.0 (SAS Institute). *P*

< 0.05 was considered significant.

RESULTS

CYP contents in liver microsomes

The CYP contents are shown in Figure 2. The rank order of average CYP contents was as follows: crow (0.78 ± 0.04 nmol/mg protein) > rat (0.75 ± 0.07 nmol/mg protein) > ostrich (0.42 ± 0.08 nmol/mg protein) > chicken (0.39 ± 0.03 nmol/mg protein) > mallard (0.20 ± 0.01 nmol/mg protein). Crow showed the greatest CYP content and was 3.9 times higher than that of mallard, which had the lowest CYP content. The CYP content in chicken liver microsomes was consistent with previous reports, with values half or less than half of values for rat [18,19]. Ostrich and mallard also had lower contents than rat. Interestingly, only crow had a similar CYP content to rat.

Kinetic parameters of in vitro warfarin metabolism in birds

Table 1 shows kinetic parameters for each species and each metabolite. Metabolic ability was normalized by CYP content. The range of average V_{max} in avian species was as follows: 225.2 pmol/min/nmol CYP (mallard) to 1162.1 pmol/min/nmol CYP (crow) for 4′-OH, 50.1 pmol/min/nmol CYP (crow) to 399.6 pmol/min/nmol CYP (chicken) for 6-OH, 13.4 pmol/min/nmol CYP (crow) to 217.7 pmol/min/nmol CYP

(chicken) for 7-OH, 11.2 pmol/min/nmol CYP (crow) to 127.9 pmol/min/nmol CYP (chicken) for 8-OH, and 26.0 pmol/min/nmol CYP (crow) to 30.8 pmol/min/nmol CYP (chicken) for 10-OH. The range of average K_m was as follows: 33.2 μM (crow) to 104.8 μM (mallard) for 4′-OH, 94.7 μM (chicken) to 346.2 μM (crow) for 6-OH, 85.9 μM (crow) to 277.4 μM (chicken) for 7-OH, 150.8 μM (ostrich) to 606.5 μM (mallard) for 8-OH, and 23.5 μM (crow) to 232.6 μM (chicken) for 10-OH. Figure 3 shows cumulative intrinsic clearance of warfarin metabolic activity. The rank order of cumulative enzymatic efficiency was as follows: crow (36.6 ± 3.9 mL/min/nmol CYP) > chicken (24.5 ± 2.3 mL/min/nmol CYP) > ostrich (10.3 ± 0.5 mL/min/nmol CYP) > mallard (3.3 ± 0.8 mL/min/nmol CYP). A significant difference (Tukey's honestly significant difference test) in enzymatic efficiency was detected for every species.

Major metabolites commonly found in bird species

The major metabolite was 4′-OH in all examined bird species. The composition of 4′-OH in the cumulative intrinsic clearance of all metabolites ranged from 66.7% to 94.0%; in comparison, rat had only 30.2%. The K_m for 4′-OH was lower in birds than in rat. The lowest K_m among metabolites was found for 4′-OH, with the exception of the crow (10-OH).

Pharmacokinetic parameters in chickens

The average plasma concentration of warfarin in chickens is indicated in Figure 4, with males and females indicated separately. The results of the pharmacokinetic analysis are shown in Table 2. We observed several sex differences in chickens. Maximum plasma concentration (C_{max}) was significantly higher in females. Although the differences in time to maximum concentration (T_{max}) were not significant, T_{max} was lower in females, half-life ($t_{1/2}$) was shorter in males, and the area under the curve was lower in males. The $t_{1/2}$ in chickens was generally longer than in most mammalian species with the exception of humans (Table 3).

In the analysis of warfarin content in plasma, 4′-OH was detected (Figure 5). The shape of 4′-OH implies the possibility of hepatic–intestinal circulation. The clearance of 4′-OH was found to be slower than that of warfarin.

Metabolite composition in in vivo excretions

Metabolite compositions including unmetabolized warfarin found in the fecal samples are indicated in Figure 6. Unmetabolized warfarin accounted for approximately 20% and 40% of all excreted warfarin-related compounds in male and female chickens, respectively. Other than warfarin, 4′-OH and 6-OH were prevalent; whereas 7-OH was identified only in excretions of female chickens. The metabolites 4′-OH and 6-OH accounted for 87.2% and 12.8% of the 5 metabolites in male chickens and for 84.0%

and 11.1% in female chickens, respectively.

DISCUSSION

Species difference in in vitro warfarin metabolic activity

We previously reported a large interspecies difference in warfarin metabolic activity based on a single high substrate concentration of 400 μM [14]. However, the actual concentration of warfarin in vivo is lower. For example, the C_{max} of a female dosed with 1.5 mg/kg body weight at 4.5 μg/mL is approximately 15 μM. In the present study, we performed a kinetic analysis of warfarin metabolism to determine enzymatic efficiency, which reflects the warfarin metabolic ability at very low concentrations. The results showed that chicken and crow had higher enzymatic efficiency than other birds and that the enzymatic efficiency of crow was 11-fold higher than that of mallard. Because crow with the highest enzymatic efficiency also showed the highest CYP contents used as a normalizer, we suggest that the species difference in warfarin metabolic ability in vivo may be even larger than the species difference in the enzymatic efficiency determined in the present study.

4′-OH as a common major metabolite in bird species

Based on the kinetic analysis, the common dominant metabolite in the examined bird species was 4′-OH. In humans, 4′-OH is commonly produced by

CYP2C8, CYP2C9, CYP2C18, and CYP2C19, in contrast to CYP2B1 and CYP2C11 in rats [10,20]. In chickens, we previously showed the dominance of CYP2C genes in a comparative mRNA study of chicken liver and suggested that CYP2Cs are the dominant enzymes in the xenobiotic metabolism in the chicken [21]. No CYP2B genes for these bird species could be found in GenBank. We therefore may speculate that avian CYP2C isoforms are a major contributor to 4′-hydroxylation of warfarin.

In guinea pigs 4′-OH not only inhibits human CYP2C9 but also shows anticoagulant activity [22,23]. Further investigation is needed to clarify the characteristics specific to avian species in terms of in vivo pharmacokinetics of warfarin and 4′-OH, as well as the CYP-mediated 4′-hydroxylation in birds.

In vivo analysis in parallel to in vitro analysis

The rank order of enzymatic efficiency in vitro for each metabolite in chicken was 4′-OH > 6-OH > 7-OH > 8-OH > 10-OH. The ranking of prevalence of in vivo metabolites in assessed excretions showed that 4′-OH is the dominant metabolite, followed by 6-OH and 7-OH, the latter of which was observed only in female chickens. In contrast, in vitro metabolism produced 8-OH and 10-OH, which were not detected in in vivo excretions. This discrepancy can be explained by the high K_m of the 8-OH and 10-OH pathways in vitro as the in vivo warfarin concentrations were much lower.

Moreover, the major metabolite in chicken (4′-OH) was the only metabolite observed in plasma after oral administration of warfarin. The results from the in vitro and in vivo assays were consistent in terms of metabolite patterns and relative quantity. We therefore were able to confirm that in vitro warfarin metabolism in liver tissue of the 4 avian species studied is consistent with in vivo warfarin metabolism in chickens.

Sex difference in chicken warfarin metabolism in vivo

The sex difference in warfarin pharmacokinetics suggested rapid absorption in female chickens and rapid metabolism and elimination in male chickens. The CYP contents and activity, as indicated by ethoxy-resorufin-*O*-deethylase, coumarin 7-hydroxylase, hexobarbital hydroxylase, and ethoxy-coumarin deethylase, were higher in male chickens [24]. This may suggest that warfarin metabolic ability is greater in male chickens.

A similar case was also observed in rats. Significant sex differences were observed in area under the curve and terminal half-life [25]. This was likely because of sex differences in CYP isoforms as CYP2C11 and CYP3A2 are male-specific and CYP2B1 is also dominant in male rats.

CYP as a determinant of sensitivity in birds

The LD50 of warfarin in chicken is reported to be 942 mg/kg, suggesting that it

is unlikely that chicken would die from warfarin ingestion at environmentally realistic concentrations [26]. We previously clarified the resistance mechanism with respect to 2 aspects: CYP enzymes mediating warfarin metabolism and the target enzyme of warfarin, vitamin K epoxide reductase [14]. That study presented both “high warfarin metabolic ability” (~60-fold that of owls) and a “low inhibitory effect of warfarin on chicken vitamin K epoxide reductase activity.” However, no study has compared in vivo pharmacokinetics between birds and mammals.

A pharmacokinetic analysis of the chicken was performed in the present study, with the expectation that warfarin might display a shorter half-life in chicken than in rat (Table 3). Surprisingly, the half-life of warfarin in chicken was longer than in most mammalian species despite the high warfarin metabolic ability in vitro. In humans, a very large proportion of warfarin in plasma is bound to albumin (~99%), in contrast to rats, where albumin has only 1 warfarin-binding site [27,28]. Free warfarin can be subject to CYP metabolism and renal excretion; thus, the low amount of free warfarin with respect to warfarin binding to albumin can result in a longer half-life in chickens. This is analogous in terms of warfarin toxicity. Free warfarin can inhibit the target enzyme, vitamin K epoxide reductase. This suggests that chicken albumin may have a greater warfarin-binding capacity, resulting in a longer half-life and less toxicity despite

high metabolic ability, although the albumin concentration of the birds including chicken is generally lower (0.2–2.4 g/dL) than that of mammals (normal concentration in human is 4–5 g/dL) [29,30]. In contrast to this, the tissue half-life of another anticoagulant, diphacinone, in American kestrel is shorter than that of rat. Further study is needed to clarify the pharmacokinetic difference of free warfarin and protein-bound warfarin separately in other birds as well as the difference of pharmacokinetics among the anticoagulant rodenticides in birds.

CONCLUSION

Although warfarin is readily metabolized by birds, it was found that the half-life of warfarin in chickens is relatively long in comparison with other mammalian species. Other bird species may have much longer warfarin half-lives, with the implication that a single dose could be sufficient to cause toxicity, depending on the inhibition rate constant of vitamin K epoxide reductase. Further study is needed to clarify in vivo warfarin pharmacokinetics and detailed albumin binding of warfarin in avian species.

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Data availability—Please contact the corresponding author for additional data (ishizum@vetmed.hokudai.ac.jp).

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Figure 1. The warfarin metabolic pathway in the human and rat. CYP = cytochrome P450.

Figure 2. Total cytochrome (CYP) contents in liver microsomes. Total CYP contents were measured using the CO difference spectrum method and normalized by the amount of microsomal protein. *n* = 3 for each species. Values are mean ± standard deviation. CYP = cytochrome P450.

Figure 3. Cumulative enzymatic efficiency of warfarin metabolism in liver microsomes. Cumulative enzymatic efficiencies were calculated as the sum of maximal velocity and the Michaelis constant of each of the 5 metabolites. Error bars indicate the standard deviation of total enzymatic efficiency. *n* = 3 for each species. CYP = cytochrome P450; 10-OH = 10-hydroxywarfarin; 8-OH = 8-hydroxywarfarin; 7-OH = 7-hydroxywarfarin; 6-OH = 6-hydroxywarfarin; 4′-OH = 4′-hydroxywarfarin.

Figure 4. Plasma concentration of warfarin after oral dose (1.5 mg/kg). Plasma concentration of warfarin (ng/μL) after a single oral dose is shown as average ± standard deviation (*n* = 3 for both male and female). Blood samples were collected at 0.5 h, 1 h, 2 h, 3 h, 6 h, 9 h, 12 h, 24 h, and 72 h.

Figure 5. Plasma concentration of 4′-OH after oral administration of warfarin (1.5 mg/kg). Typical data of each metabolite in male and female chickens are shown. Only

4′-OH was detected in plasma out of the 5 metabolites. The shape of the curve suggests enterohepatic circulation of 4′-OH in both sexes. 4′-OH = 4′-hydroxywarfarin.

Figure 6. The composition of warfarin metabolites in fecal samples. Fecal samples were collected 9 h after administration and analyzed for warfarin and its metabolites. Unmetabolized warfarin accounted for 20% and 40% of excreted warfarin and hydroxywarfarins in male and female chickens, respectively. Also, 4′-hydroxywarfarin (4′-OH) was the major metabolite in fecal samples, followed by 6-OH; 7-OH was detected only in female chickens. WF = warfarin; 7-OH = 7-hydroxywarfarin; 6-OH = 4′-hydroxywarfarin; 4′-OH = 4′-hydroxywarfarin.

Figure 1

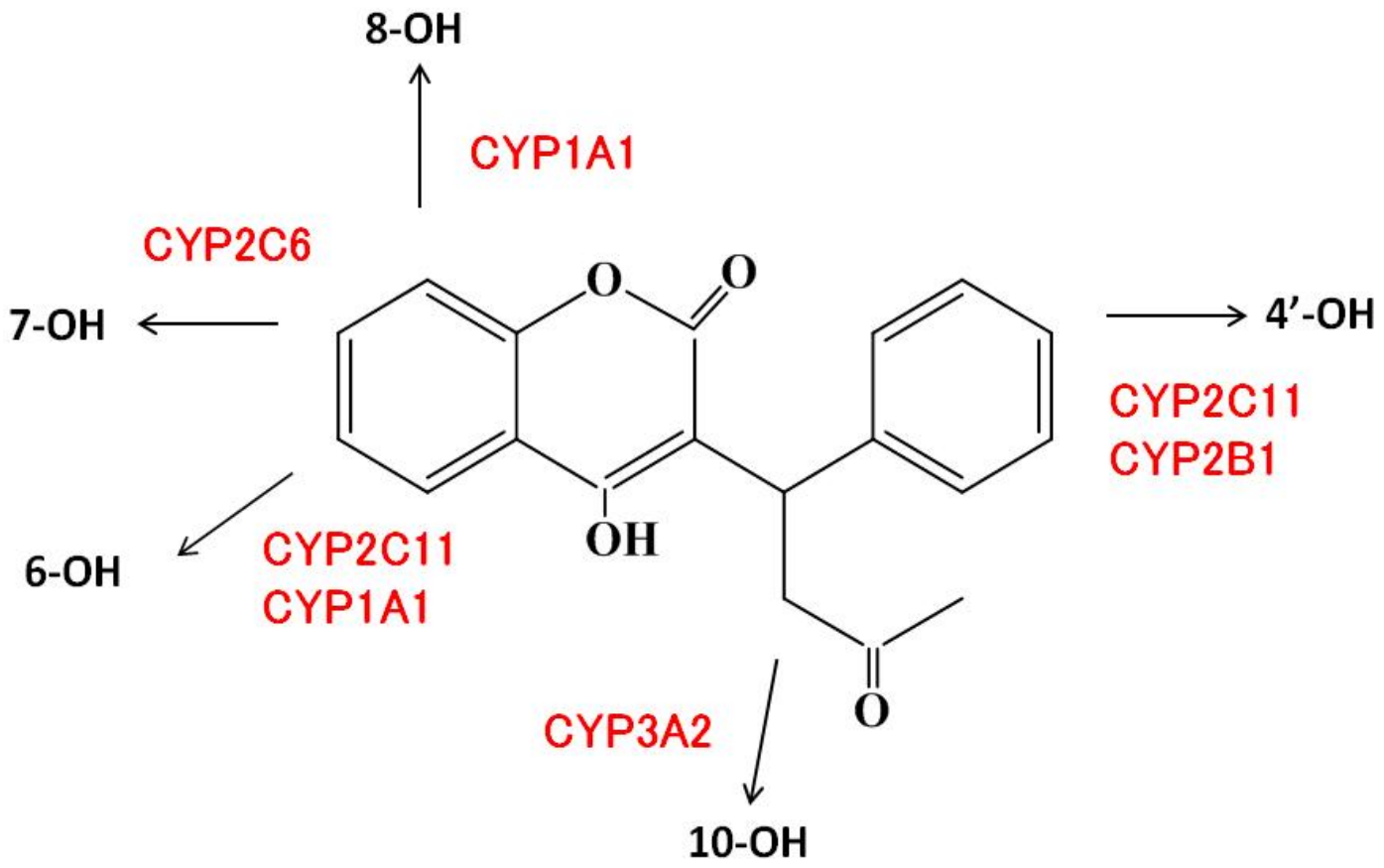
8-OH
CYP1A1
CYP2C6
7-OH
4'-OH
CYP2C11
CYP2B1
6-OH
CYP2C11
CYP1A1
CYP3A2
10-OH
O
O
OH
O

Figure 2

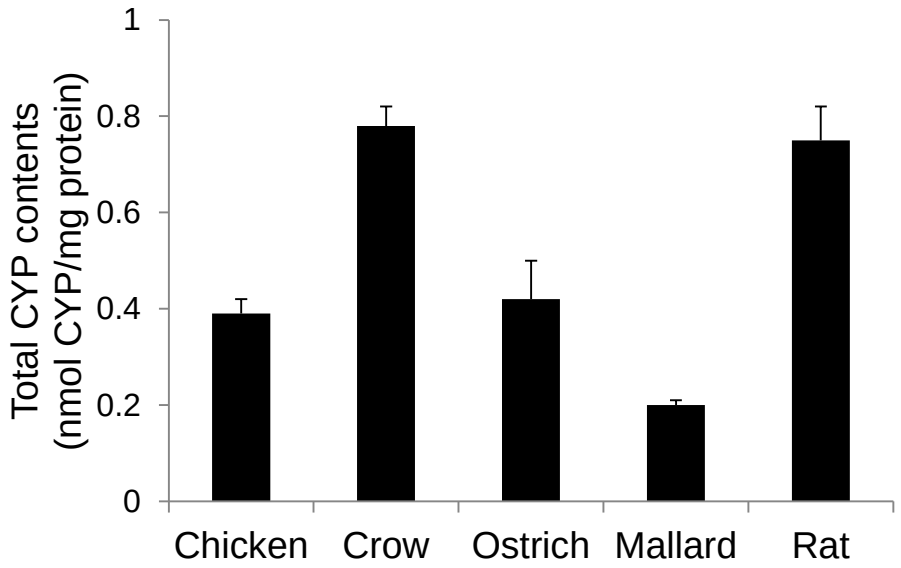
Total CYP contents
(nmol CYP/mg protein)
1
0.8
0.6
0.4
0.2
0
Chicken
Crow
Ostrich
Mallard
Rat

Figure 3

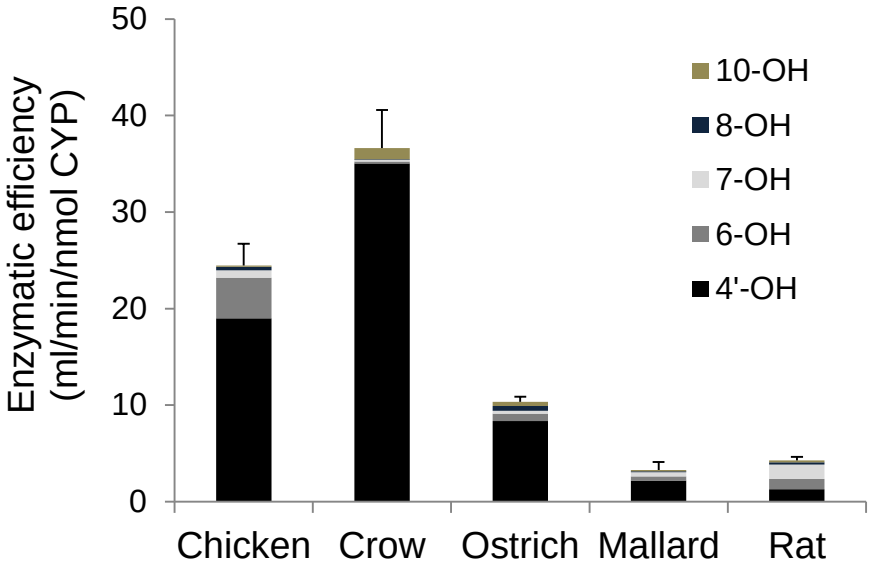
Enzymatic efficiency (ml/min/nmol CYP)
50
40
30
20
10
0
Chicken
Crow
Ostrich
Mallard
Rat
10-OH
8-OH
7-OH
6-OH
4'-OH

Figure 4

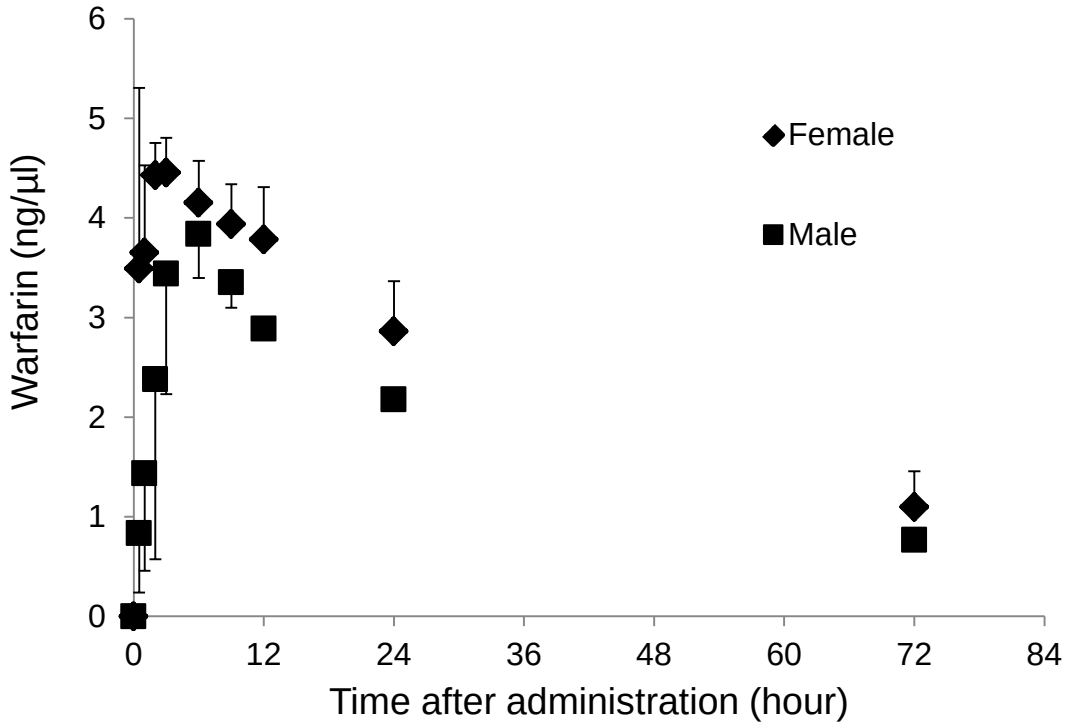
Warfarin (ng/μl)
6
5
4
3
2
1
0
0
12
24
36
48
60
72
84
Time after administration (hour)
Female
Male

Figure 5

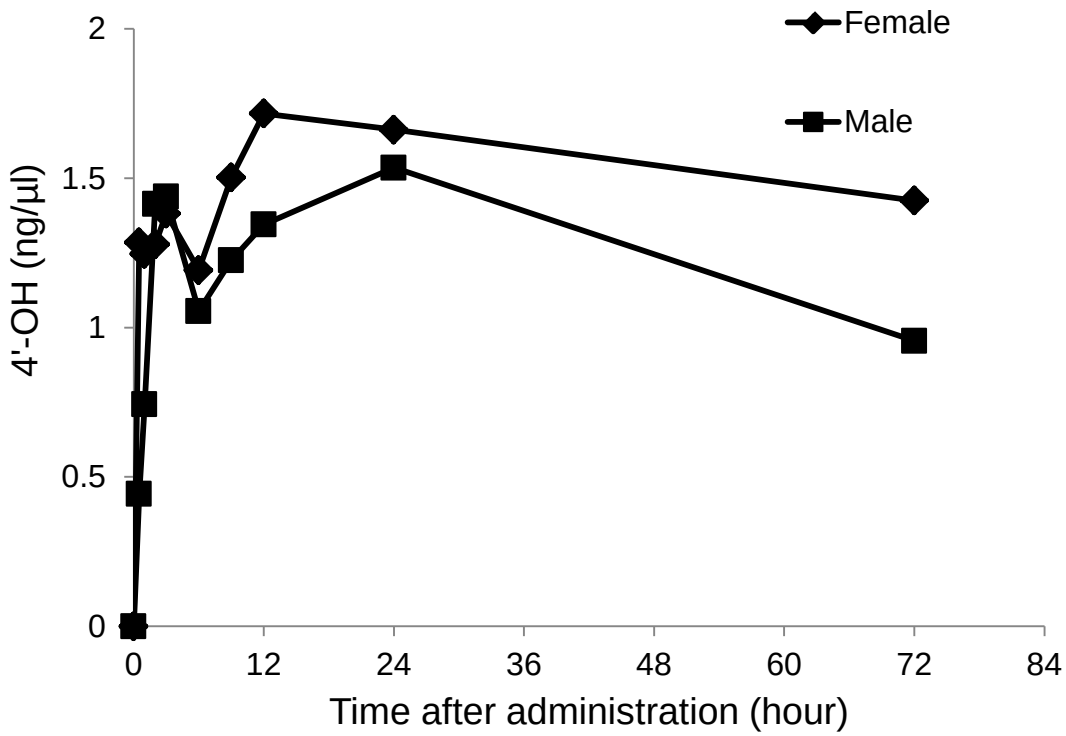
Female
Male
4'-OH (ng/μl)
2
1.5
1
0.5
0
0
12
24
36
48
60
72
84
Time after administration (hour)

Figure 6

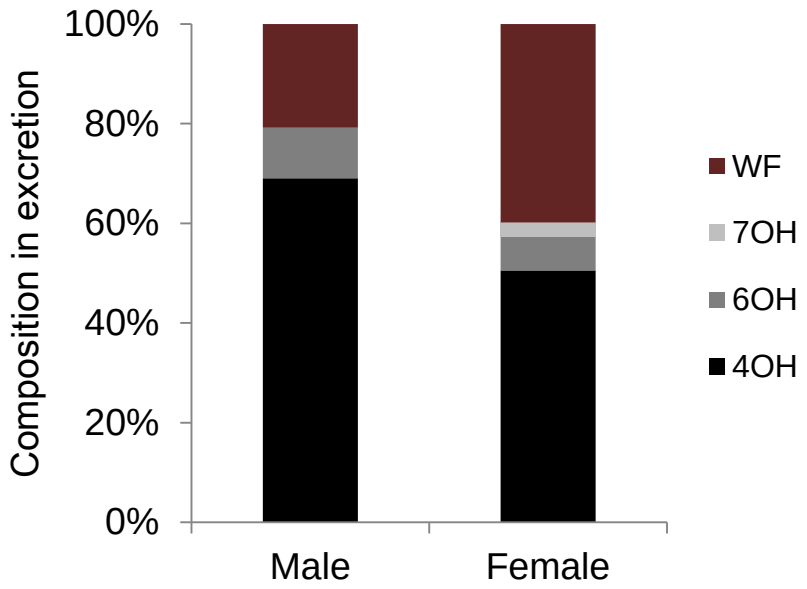
100%
80%
60%
40%
20%
0%
Composition in excretion
Male
Female
WF
7OH
6OH
4OH

Table 1. Kinetic parameters of warfarin metabolism in 4 avian species

Metabolite		Chicken	Crow	Ostrich	Mallard	Rat
4'-OH	V_{max}	917.8 ± 87.3^{a}	1162.1 ± 189.2^{a}	530.0 ± 90.8^{b}	225.2 ± 56.6^{c}	139.4 ± 15.3^{c}
	K_m	48.4 ± 3.9^{bc}	33.2 ± 4.4^{c}	64.0 ± 12.1^{b}	104.8 ± 7.9^{a}	110.2 ± 19.1^{a}
	V_{max}/K_m	19.0 ± 1.7^{b}	35.0 ± 3.5^{a}	8.3 ± 0.9^{c}	2.2 ± 0.6^{d}	1.3 ± 0.1^{d}
6-OH	V_{max}	399.6 ± 166.7^{a}	50.1 ± 21.4^{b}	106.5 ± 35.6^{b}	63.1 ± 34.8^{b}	97.8 ± 9.4^{b}
	K_m	94.7 ± 38.4	346.2 ± 324.7	139.5 ± 4.0	161.1 ± 129.8	94.9 ± 25.6
	V_{max}/K_m	4.2 ± 0.4^{a}	0.2 ± 0.1^{c}	0.8 ± 0.3^{bc}	0.4 ± 0.1^{bc}	1.1 ± 0.2^{b}
7-OH	V_{max}	217.7 ± 91.8^{a}	13.4 ± 4.3^{b}	49.5 ± 27.5^{b}	52.6 ± 18.5^{b}	64.0 ± 10.8^{b}
	K_m	277.4 ± 23.6^{a}	85.9 ± 10.6^{cd}	159.4 ± 44.3^{b}	122.8 ± 10.6^{bc}	42.3 ± 5.8^{d}
	V_{max}/K_m	0.8 ± 0.3^{b}	0.2 ± 0.1^{c}	0.3 ± 0.1^{c}	0.4 ± 0.1^{bc}	1.5 ± 0.1^{a}
8-OH	V_{max}	127.9 ± 48.5^{a}	11.2 ± 3.6^{b}	79.9 ± 35.4^{ab}	50.6 ± 31.1^{ab}	38.8 ± 31.1^{ab}

	K_m	350.2 ± 100.1	182.3 ± 50.7	150.8 ± 28.8	606.5 ± 441.4	181.7 ± 132.4
	V_{max}/K_m	0.4 ± 0.04^{ab}	0.1 ± 0.04^{c}	0.5 ± 0.1^{a}	0.1 ± 0.04^{c}	0.2 ± 0.04^{bc}
10-OH	V_{max}	30.8 ± 6.2	26.0 ± 1.4	26.9 ± 7.9	26.7 ± 8.8	38.9 ± 4.6
	K_m	232.6 ± 32.0^{a}	23.5 ± 8.5^{c}	68.1 ± 21.3^{bc}	186.5 ± 74.4^{ab}	221.1 ± 60.3^{a}
	V_{max}/K_m	0.1 ± 0.05^{b}	1.2 ± 0.3^{a}	0.4 ± 0.2^{b}	0.1 ± 0.03^{b}	0.2 ± 0.03^{b}

Values are indicated by mean ± standard deviation.

Different letters indicate the significant difference among the bird species by Tukey's honestly significant difference test.

K_m = Michaelis constant (μM); V_{max} = maximum velocity (pmol/min/nmol cytochrome P450).

Table 2. Pharmacokinetic parameters of warfarin in male and female chickens following a single oral dose of 1.5 mg/kg (male and female, each $n = 3$)

Chicken	Area under the curve (μg h/mL)	K10_HL (h)	CL_F (mL/min/kg body weight)	T_{max} (h)	C_{max} [a] (μg/mL)
Female					
1	249	34.5	0.008	4.7	4.55
2	166	25.0	0.012	1.0	4.47
3	282	42.6	0.007	1.3	4.50
Male					
1	175	30.5	0.011	4.7	3.57
2	173	30.4	0.012	5.1	3.51
3	142	21.4	0.014	15.4	2.79
Average (SD)	232 (60)	34.0 (8.8)	0.009 (0.003)	2.3 (2.0)	4.51 (0.004)
	163 (19)	27.4 (5.3)	0.012 (0.002)	8.4 (6.1)	3.29 (0.44)

[a] Only C_{max} showed a significant difference between male and female chickens, by Student's *t* test.

K10_HL = plasma half-life; CL_F = oral clearance; T_{max} = time to maximum concentration; C_{max} = maximum concentration; SD = standard deviation.

Table 3. Comparison of half-life of warfarin between chicken and mammalian species

Species	*n*	Half-life (h)	Reference
Chicken	3 (male)	27.4	Present study
	3 (female)	34.0	
Rat	10	11.6	Yacobi et al. [31]
	13	7.1	Sawada et al. [32]
Human	10	42.0	O'Reilly et al. [33]
	12	36.3	Vessell et al. [34]
	10	34.0	Sawada et al. [32]
Dog	4	18.4	Bachmann et al. [35]
Monkey	4	10.9	Scott et al. [36]
Opossum	8	11.9	Eason et al. [37]
Cat	10	26.2	Smith et al. [38]