Comparison of xenobiotic metabolism in phase I oxidation and phase II conjugation between rats and bird species

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ABSTRACT

There have been many reports regarding toxic chemicals in birds. Chemicals are mainly metabolized in the liver through phase I oxidation by cytochrome P450 (CYP) and phase II conjugation by conjugated enzymes, such as UDP-glucuronosyltransferase (UGT), sulfotransferase (SULT), glutathione-S-transferase (GST), etc. Xenobiotic metabolism differs among bird species, but little detailed information is available. In the present study, the four-ring polycyclic aromatic hydrocarbon (PAH), pyrene, was used as a model xenobiotic to clarify the characteristics of xenobiotic metabolism in birds compared with laboratory animals by *in vivo* and *in vitro* studies. Plasma, bile, and excreta (urine and feces) were collected after oral administration of pyrene and analyzed to clarify xenobiotic metabolism ability in chickens and quails. Interestingly, pyrenediol-glucuronide sulfate (PYDOGS) and pyrenediol-diglucuronide (PYDOGG) were present in chickens and quails but not in rats. In addition, the area under the curve (AUC), maximum plasma concentration (C_{max}), and time to maximum plasma concentration (T_{max}) of pyrene-1-sulfate (PYOS) were higher than those of the parent molecule, pyrene, while the elimination half-life ($t_{1/2}$) and mean residence time (MRT) were faster than those of the parent pyrene. With regard to sulfation of 1-hydroxypyrene (PYOH), the maximum velocity (V_{max}) and Michaelis constant (K_m) of rat liver cytosol were greater than those of chicken and quail liver cytosol. Furthermore, V_{max}/K_m of UGT activity in rat liver microsomes was also greater than those of chicken and quail liver microsomes. Characterization of xenobiotic metabolism revealed species differences between birds and mammals, raising concerns about exposure to various xenobiotics in the environment.

Keywords: birds, species differences, pyrene, conjugated metabolites, kinetics

1. Introduction

Since the 1950s, there have been increasing reports of injuries to wild birds worldwide due to the influence of various xenobiotics, such as dichlorodiphenyltrichloroethane (DDT), coumarin-derived anticoagulant rodenticides, and non-steroidal anti-inflammation drugs (NSAIDs), such as diclofenac (Bowerman et al., 1995; Elliott et al., 1988; Erickson et al., 2004; Norstrom and Hebert, 2006; Prakash et al., 2007). These xenobiotics cause secondary poisoning that could affect and damage the reproductive system, liver, and kidney in scavenging and raptorial birds (Albers et al., 2003; Albert et al., 2010; Erickson et al., 2004; Prakash et al., 2007).

Polycyclic aromatic hydrocarbons (PAHs) are a ubiquitous group of several chemically related compounds produced naturally and by human activities. They also persist in the environment and show varied toxic effects on organisms through various actions causing carcinogenic and mutagenic effects. Generally, PAHs enter the environment and animal bodies through various routes, and are usually found as mixtures containing two or more of these compounds (Armstrong et al., 2004). In birds, the mechanism of toxicity is considered to involve interference with the function of cellular membranes as well as with enzyme systems associated with the cell membrane. Toxic effects have been documented mostly in embryos, young birds, and adult birds. There have also been reports of reduced egg production, hatching, and growth (Albers et al., 2003). Although PAHs influence absorption to organic material or degradation in the environment, some are persistent and bioaccumulate in the food chain (Jiang et al., 2011). Wild and domestic birds may be exposed to PAHs and accumulate them in their bodies. Xenobiotics, including PAHs, are generally absorbed and distributed in the body, and are metabolized based by phase I oxidation (mainly enzymes as cytochrome P450s) and phase II conjugation enzymes, such as UDP-glucuronosyltransferase (UGT), sulfotransferase (SULT), and glutathione-S-transferase (GST). The more water-soluble metabolites are excreted in the urine and feces. However, there have been reports of interspecies differences in xenobiotic metabolic activity among bird species *in vitro* (Watanabe et al., 2010). However, insufficient information is available to clarify the xenobiotic metabolism ability in phase I oxidation and phase II conjugation in bird species *in vivo*, especially with regard to their kinetic parameters.

Pyrene, a four-ring PAH, was selected as a xenobiotic model to observe the metabolic activity in birds in comparison with laboratory animals following *in vivo* and *in vitro* studies. In addition, pyrene and it metabolites are typical phenolic xenobiotic models. To understand xenobiotic metabolism in bird species, we used pyrene as a xenobiotic model and analyzed pyrene metabolites in urine. The excretion of urine containing pyrene metabolites is useful to characterize differences in phase II xenobiotic conjugation reactions between species (Saengtienchai et al., 2016).

To clarify the roles of phase I oxidation and phase II conjugation reactions of pyrene in bird species in comparison with laboratory animals, an *in vivo* exposure study was performed. An important factor in pharmacokinetics of pyrene and its conjugated metabolites were observed to complete the absorption, distribution, metabolism, and excretion in birds. To assess the efficiency of phase II conjugation enzymes *in vitro*, 1-hydroxypyrene (PYOH) was chosen as a substrate to measure UGT-dependent and SULT-dependent activities.

2. Materials and methods

2.1.Animals

Nine-week-old male Wistar rats (*Rattus norvegicus*) ($n=3$) were obtained from SLP (Hamamatsu, Japan). Eight-week-old male White Leghorn chickens (*Gallus gallus*) ($n=3$) were obtained from Hokudo Co., Ltd. (Sapporo, Japan). One-year-old quails (*Coturnix japonica*) (male, $n=1$; female, $n=2$) were obtained from a local commercial supplier in Sapporo, Japan. The animals were acclimated for one week in the laboratory and kept under conditions of 40%–70% humidity at 23 °C ±2 °C in a temperature-controlled room with a 12-h light/dark cycle. They were given laboratory food and clean water *ad libitum*. All animal experiments were performed under supervision and with the approval of the Institutional Animal Care and Use Committee of Hokkaido University (approval no. 100067), in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

2.2.Chemicals

Pyrene, PYOH, 3'-phosphoadenosine 5'-phosphoslfate (PAPS), sulfatase (from limpet Type V; 34 U/mg), β-glucuronidase (from bovine liver, Type B-1; 1,240 U/mg), β-glucosidase (from almond; 3.4 U/mg), and bovine serum albumin were obtained from Sigma-Aldrich Co. (St. Louis, MO). Methanol, acetonitrile, acetic acid, and ammonium acetate were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Pyrene-1-sulfate (PYOS) was obtained from TOPU Bio (Toyama, Japan). Uridine-diphosphate-glucuronic acid (UDP-GA) was purchased from Wako (Osaka, Japan). All chemicals used for high-performance liquid chromatography (HPLC) and mass spectrometry (MS) were of HPLC or MS grade, respectively, and were obtained from Kanto Chemical Co. Inc.

2.3.Pyrene exposure and sample collection

Chickens, quails, and rats were fasted for 24 h before exposure to pyrene as the test chemical. Pyrene was dissolved in corn oil and administered orally at a dose of 4 mg/kg body weight.

Plasma was collected from chickens at 0, 1, 2, 3, 6, 9, 12, 24, and 48 h after pyrene exposure for pharmacokinetic analysis. In rats, plasma samples were collected at 24 h after oral administration of pyrene. In the case of quails, plasma samples were collected after 2 h exposure, which showed a high distribution of pyrene metabolites. The excreta, such as urine, feces, urine-feces mixed (in chickens and quails), bile (in chickens), and urine contained in the ureter (in chickens) were obtained from chickens, quails, and rats after 24 h of pyrene exposure. Samples were kept at –20 °C until analysis. Animals were then sacrificed by carbon dioxide inhalation at 48 h after pyrene exposure. The livers were removed and perfused with cold 1.15% potassium chloride to remove blood, and samples were immediately placed in liquid nitrogen and kept at –80 °C.

2.4. Extraction of pyrene metabolites in samples from in vivo *study*

The method of Boocook et al. (2007) was used in this study. Briefly, aliquots of 10 μL of plasma from each animal species were extracted with 90 μL of 100% methanol. The mixed samples were stored at –20 °C for 50 min to precipitate protein. After melting at room temperature, samples were centrifuged at 13,000*g* for 15 min at room temperature. Aliquots of 10 μL of the supernatant were subjected to HPLC with fluorescence detection (FD).

Urine and feces samples were extracted and cleaned up according to the protocol described by Saengtienchai et al. (2015). Briefly, 1 mL of urine was extracted with the same amount of 70% methanol (50:50, v:v). The mixtures were extracted by vortexing for 1 min and centrifugation at 9,000*g* for 10 min at room temperature. The supernatants were then filtered with a 0.2-μm syringe filter (SupraPure; Recenttec, Tokyo, Japan). Aliquots of 1 μL of the filtrates were subjected to HPLC/FD.

Samples of approximately 1 g of feces or urine-feces mixture were homogenized with 20 mL of 70% methanol. The mixtures were then extracted by sonication for 20 min and centrifuged at 9,000*g* for 10 min at room temperature. The supernatants were transferred into 50-mL Falcon tubes. The residual samples were extracted again. The pooled supernatants were filtered with a 0.2-μm syringe filter and kept at –20 °C until analysis. Then, 5 μL of each pooled supernatant was subjected to HPLC/FD.

Aliquots of bile samples were diluted 400-fold with 70% methanol. The samples were then centrifuged at 10,000*g* for 5 min at room temperature. Then 5 μL of each supernatant was subjected to HPLC/FD.

The urine contained in the ureter samples were extracted with 500 μL of 70% methanol. The mixtures were then extracted by sonication for 20 min and centrifuged at 10,000*g* for 5 min at room temperature. Then, 5 μL of each supernatant was analyzed by HPLC/FD.

2.5. Analysis and identification of pyrene metabolites

The supernatant was then injected into the HPLC/FD system (pump: LC-20AD, auto sampler: SIL-20A, column oven: CTO-20A, controller: CEM-20A; Shimadzu) equipped with an ODS column (ODS-120T 4.6 mm × 300 mm; Tosoh, Tokyo, Japan). HPLC was performed according to the method of Beach et al. (2010) with slight modifications. Mobile phase A consisted of a mixture of 10 mM ammonium acetate buffer (pH 5) and mobile phase B (9:1, v/v). A mixture of methanol:acetonitrile:water (38:57:5, v/v/v) was used as mobile phase B. The solvent gradient was 100% mobile phase A at 0 min, followed by a linear gradient to 100% of mobile phase B from 0 to 35 min. The gradient was held at 100% of mobile phase B for 5 min until 40 min. Then, the gradient was held at 100% of mobile phase A from 40.1 to 47 min. The solvent flow rate was set at 0.5 mL/min, and a column temperature of 45 °C was used throughout. The excitation and emission wavelengths for fluorescence detection were 343 and 385 nm, respectively. Furthermore, each separated peak was identified by electrospray ionization ion-trap mass spectrometry (ESI/ion-trap/MS, LTQ XL; Thermo Fisher Scientific, Waltham, MA). The ESI conditions were fully scanned (*m/z* 80 – 800) in negative mode, with an ion source voltage and temperature of –4.2 kV and 420 °C, respectively.

2.6. Deconjugation of pyrene metabolites

Deconjugation was performed using the method described by Ikenaka et al. (2007). Briefly, the enzymes sulfatase, β-glucuronidase, and β-glucosidase were dissolved in 0.1 M sodium acetate buffer and the pH was adjusted to 5.0 with acetic acid. The enzyme

concentrations were 10, 4000, and 17 U/mL, respectively. Aliquots of 30 μL of urine sample extracts containing pyrene metabolites were mixed with 270 μL of buffer, and each deconjugation enzyme (200 μL) was added. As a control, the same quantity of bovine serum albumin (1 mg/mL) was added, and reactions were performed under the same conditions as used for the deconjugation enzymes. All samples were incubated at 37 °C for 8 h. The reaction was stopped by adding 500 μL of methanol. The deconjugation solutions were analyzed by HPLC/FD.

2.7. Preparation of liver microsomal and cytosolic fractions

Liver microsomal and cytosolic fractions were prepared according to the method of Omura and Sato (1964). The liver samples were homogenized in potassium phosphate buffer (KPB; 0.1 M, pH 7.4) on ice. The homogenates were transferred to tubes and centrifuged at 9,000*g* for 20 min at 4 °C. The supernatants (S9 fraction) were decanted into ultracentrifugation tubes and centrifuged at 105,000*g*, 4 °C for 70 min. Each homogenate consisted of two parts, with the supernatant containing the cytosolic fraction. The pellets were homogenized on ice with KPB and washed by centrifugation at 105,000*g*, 4 °C for 70 min. The microsomal pellets were homogenized with KPB again. The microsomal and cytosolic fractions were transferred to 1.5-mL tubes and stored at –80 °C. The cytosolic fractions were used to measure SULT activity; microsomal fractions were used to determine UGT activities. The protein concentrations in the microsomes and cytosol were determined using the method of Lowry et al. (1951).

2.8. UGT-dependent PYOH glucuronidation activity

UGT activity of PYOH was assessed using the method described by Ueda et al. (2011) with slight modifications. Initially, microsomes were diluted to 4 mg/mL protein with 0.1 M phosphate buffer (pH 7.4), and then 2.5 μL of 1% sodium cholate solution was added and incubated on ice for 30 min. After treatment, 50 μL of microsome solution was mixed with 41.5 μL of buffer, 5 μL of 100 mM $MgCl_2$, and 1 μL of PYOH. The final concentrations of PYOH were 10, 20, 25, 38, 50, 100, and 200 μM. Samples were preincubated at 37 °C for 5 min. The reaction was started with 2.5 μL of a 50 mM UDP-GA in a final volume of 100 μL. The reaction

was allowed to proceed for 10 min, and 400 μL of ice-cold methanol was added to stop the reaction. The reaction solutions were placed on ice for 20 min, and then centrifuged at 1,600*g* for 15 min. The supernatants were transferred to HPLC vials and subjected to HPLC/FD (Shimadzu). Samples were analyzed with an Inertsil ODS-3 column (2.1 mm × 150 mm; GL Sciences). Mobile phase A was 10 mM ammonium acetate buffer (pH 5) and mobile phase B consisted of methanol:acetonitrile:water (38:57:5, v/v/v). The linear gradient was 17 min in length with 90% phase B. injection volume was 10 μL, the flow rate was 0.5 mL/min, and the column temperature was 45 °C. Excitation and emission wavelengths were 343 and 385 nm, respectively. Kinetic parameters, including maximal velocity (V_{max}), Michaelis–Menten constant (K_m), and V_{max}/K_m ratio (enzyme efficiency), were determined by the Michaelis–Menten equation using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA).

2.9. SULT-dependent PYOH sulfation activity

The SULT activity for PYOH was determined using a modification of the method of Ueda et al. (2011). The final protein concentration in the liver cytosol was 250 μg protein/mL, which was adjusted with 100 mM Tris-HCl buffer (pH 7.4). Then, 50 μL of cytosol solution was mixed with 10 μL of 100 mM $MgCl_2$, 10 μL of 50 mM Na_2SO_3, and 1 μL of 1-hydroxypyrene. Tris-HCl buffer (100 mM, pH 7.4) was added to make up the volume to 97.5 μL. The final concentrations of 1-hydroxypyrene were 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, and 0.5 μM. The mixtures were preincubated at 37 °C for 5 min. The reaction was initiated by adding 2.5 μL of 1 mM PAPS and the final volume was 100 μL. After 10 min of incubation, 400 μL of ice-cold methanol was added to stop the reaction. The samples were placed on ice for 20 min, and then centrifuged at 1,600*g* for 15 min. The supernatants were transferred into HPLC vials and subjected to HPLC/FD (Shimadzu). Samples were analyzed with an Inertsil ODS-3 column (2.1 mm × 150 mm: GL Sciences, Inc.). Mobile phase A was 10 mM ammonium acetate buffer (pH 5) and mobile phase B consisted of methanol:acetonitrile:water (38:57:5, v/v/v). The linear gradient was 17 min with 90% phase B. Injection volume was 10 μL, the flow rate was 0.5 mL/min, and the column temperature was 45 °C. Excitation and emission wavelengths were 343 and 385 nm, respectively. Kinetic parameters were determined using the Michaelis–Menten equation, as described above.

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238 *2.10. Statistical analysis*

239 The results were subjected to Tukey's HSD test (JMP 7.0; SAS Institute Inc., Raleigh,
240 NC). All kinetic parameters (Michaelis–Menten equation) were analyzed using GraphPad Prism
241 Version 5.01. In all analyses, $p < 0.05$ was taken to indicate statistical significance.

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3. Results

3.1.Identification of pyrene metabolites in chicken

The chromatograms of pyrene metabolites in chicken bile are shown in Fig. 1. A total of six pyrene metabolites were detected, peak-a to peak-f, with retention times (RT) of 11.6, 14.8, 15.0, 17.4, 22.9, and 25.9 min, respectively. Table 1 presents a summary of each RT, results of deconjugation enzymes treatment, and estimated metabolites. PYOH and pyrene were eluted at RT of 36 and 41.8 min, respectively. The other unidentified peaks were determined by LC/MS/MS. The ESI negative mass spectra of peak-a (RT 11.6 min) had a parent ion of mass to charge ratio (*m/z)* 489 (MS) and MS^2 of *m/z* 409 (M-80; considered as sulfate moiety), 313 (M-176; glucuronide moiety), and 233 (M-256; both sulfate and glucuronide moieties). Peak-b eluted at 14.8 min and contained a major ion at *m/z* 585 (MS) with MS^2 of *m/z* 409 (M-176; glucuronide moiety). Peak-c was eluted at RT 15.0 min and had *m/z* 393 (MS) including MS^2 of *m/z* 313 (M-80; sulfate moiety) and 233 (M-160; di-sulfate moieties). The metabolite from peak-d (RT 17.4 min) consisted of an ion at *m/z* 313 (MS) and MS^2 at *m/z* 233 (M-80; sulfate moiety). Peak-e eluted at 22.9 min with parent ion at *m/z* 393 (MS) and MS^2 at *m/z* 217 (M-176; glucuronide moiety) it was pyrene-1-glucuronide (PYOG). Furthermore, peak-f was eluted at 25.9 min with *m/z* of 297 (MS) and MS^2 217 (M-80; sulfate moiety) and it was suspected to be PYOS. These results suggested that pyrenediol (PYDOH) (*m/z* 233) could be a conjugation product of peak-a, c, and d. In addition, peak-e and f also had PYOH (*m/z* 217) as a conjugation product. Interestingly, the conjugation product of peak-b seemed to differ from the other peaks. The ion of *m/z* 409 may have been similar to PYDOG.

Deconjugation conditions were used to identify each pyrene metabolite peak (peak-a to peak-f) in chicken bile after collection of each peak by fraction collectors. The fractions were treated with the specific enzymes used to digest the conjugated metabolites. Sulfatase, β-glucuronidase, and β-glucosidase were used for deconjugation (Table 1). Peak-a, c, d, and f disappeared after sulfatase treatment. In the case of β-glucuronidase treatment, peak-a disappeared completely, while this enzyme reduced peak-e by about 10%. However, all six peaks did not respond to β-glucosidase treatment. These results indicated that peak-a to peak-f were pyrene conjugated metabolites in chicken bile. Peak-a was suspected to be a PYDOGS, peak-b to be a PYDOGG,

peak-c to be a PYDOSS, peak-d to be a PYDOS, peak-e, to be a PYOG, and peak-f , to be a PYOS.

3.2.Qualification and distribution ratio of pyrene metabolites in chickens, quails, and rats

Chickens, quails, and rats were exposed to pyrene, and plasma was collected at 24 h after oral administration. In chicken and quail plasma, the conjugated metabolites detected were PYDOGS, PYDOGG, PYDOSS, PYDOS, PYOG, and PYOS (Fig. 2). Although these three-species had similar types of conjugated pyrene metabolites, the composition of pyrene metabolites which are derived from PYDOH (such as PYDOSS and PYDOS) were seemed to be higher in rat plasma than that in chicken and quail. On the other hand, more than 90% of pyrene metabolites in both chicken and quail plasma were PYOS, while a smaller proportion of glucuronide conjugation was detected. Interestingly, sulfate conjugates were the main metabolites present in plasma of both bird species and rats. In addition, only PYDOGG and PYDOGS were detected in bird plasma but not in rat plasma.

The type of conjugated metabolites in the excreta (urine and feces) in both chicken and quail, such as PYDOSS, PYDOS, PYDOGS, PYDOGG, PYOS, and PYOG, were similar as observed in plasma. On the other hand, the proportion of pyrene metabolites between plasma and excreta in chicken and quail were different (Fig. 2). Although more than 90% of the conjugated metabolites were found as PYOS in plasma, various types of the metabolites were equally detected in excreta. Interestingly, only PYOH was present in rat feces.

Moreover, bile and urine in the ureter from chickens were collected to examine pyrene metabolites. The results are summarized in Fig. 2. These samples showed similar conjugated pyrene metabolites present as sulfate and glucuronide conjugates. Only PYOH was present in bird excreta but not in plasma, bile, or urine contained in the ureter. The order for percentages of PYDOSS and PYDOS was as follows: excreta＞urine contained in the ureter＞bile and plasma. PYOS was mainly present in chicken samples in the order: plasma＞urine contained in the ureter ＞bile and excreta. In addition, PYDOS, PYDOGG, and PYOG were detected at high levels in bile, excreta, urine contained in the ureter, and plasma, respectively.

3.3.Pharmacokinetic evaluation in chickens

The distributions of each peak area of pyrene metabolite-time profiles of pyrene following a single oral administration are shown in Fig. 3. Both glucuronide and sulfate conjugated metabolites were detected, and PYOS was the dominant metabolite among all conjugated pyrene metabolites. Corresponding exposure data in terms of area under the curve (AUC), maximum plasma concentration (C_{max}), time to maximum plasma concentration (T_{max}), elimination half-life ($t_{1/2}$), mean residence time (MRT), and total clearance of the drug from plasma after oral administration (CL/F) of pyrene and its metabolite, PYOS, in chicken plasma are summarized in Table 2. Pyrene was rapidly absorbed after oral administration, the peak concentration occurring faster than 1 h, whereas the peak concentration of PYOS (C_{max}, 2524 ± 265 ng/mL) was observed at 1.7 ± 0.6 hours. Furthermore, the AUC of PYOS was also larger than that of pyrene (12677 ± 799 and 3375 ± 84 ng*h/mL, respectively). The $t_{1/2}$ of pyrene was longer than that of its metabolite, PYOS (91.2 ± 68.2 and 3.6 ± 0.9 h, respectively). However, MRT (4.2 ± 0.8 h) and CL/F (0.2 ± 0.01 L/h) of PYOS were shorter than those of the parent compound, pyrene (MRT, 134.5 ± 94.4 h; CL/F, 0.3 ± 0.1 L/h).

3.4.Comparison of pyrene metabolizing ability among bird species and rats

The enzyme kinetics activities were examined by *in vitro* study to observe sulfation (in cytosol) and glucuronidation (in microsome) activities in chickens, quails, and rats using PYOH as a substrate. The results for sulfotransferase activity are presented in Fig. 4A and Table 3. The hepatic intrinsic clearance (V_{max}/K_m), the maximum reaction rate (V_{max}), and the Michaelis constant (K_m) were examined to estimate conjugated enzyme affinities. The sulfotransferase activity of PYOH was detected in both chicken and quail cytosols. Interestingly, interspecies differences between rats and bird species were found, with V_{max} and K_m of rat cytosol being significantly greater than those of chicken and quail cytosols. Although, the V_{max}/K_m in quail cytosol was greater than those in chicken and rat cytosols (830 ± 352, 608 ± 472, and 429 ± 51.1 μL/min/mg protein, respectively), the differences among species were not significant. Furthermore, UGT activity of PYOH is also shown in Fig. 4B and Table 4. The V_{max} of rat and quail microsomes were similar and were significantly higher than that of chicken microsomes. Surprisingly, the K_m of quail microsomes was significantly faster than those of rats and chicken.

Moreover, the V_{max}/K_m of chicken and quail microsomes were significantly lower than that of rat microsome (21.2 ± 1.39, 33.1 ± 7.76, and 80.8 ± 25.6 μL/min/mg protein, respectively).

4. Discussion

Glucuronide and sulfate conjugated metabolites of pyrene were identified and detected in the plasma and their excreta, including bile, of birds. The main conjugated metabolites were PYOG and PYOS, which were detected at levels similar to those in various mammals, fish, reptiles, amphibians, and marine snails (Beach et al., 2010; Ikenaka et al., 2013; Oroszlany et al., 2013; Saengtienchai et al., 2014; Ueda et al., 2011). Interestingly, PYDOGG and PYDOGS were detected in excreta and bile of birds, but not in urine or feces of rats. Although there have been reports of glucuronide and sulfate conjugates of 1,6-PYDOH and 1,8-PYDOH, and *trans*-4,5-PYDOH in urine of rats, rabbits, fish, reptiles, amphibians, and marine snails treated with pyrene (Beach et al., 2010; Boyland et al., 1964; Ikenaka et al., 2013; Oroszlany et al., 2013; Saengtienchai et al., 2014; Ueda et al., 2011), PYDOGG and PYDOGS were first reported in birds. It has been reported that genetic polymorphism of conjugated enzymes, such as UGT1A isoforms, differed among birds and mammals (Kakehi et al., 2015; Hong et al., 2007). The present study showed interspecies differences in pyrene metabolites.

Furthermore, the portion of pyrene conjugated metabolites in plasma, excreta, and bile showed interspecies differences between birds and rats. In plasma, sulfate conjugated metabolites were mainly detected in both birds and rats. However, more than 90% of sulfate conjugated metabolites observed in birds was the sulfate conjugate for PYOH, whereas the around 90% of the metabolites observed from rats were for PYDOH. These results seemed to parallel those of the *in vitro* study indicating that V_{max}/K_m of PYOH sulfation in hepatic cytosol of birds was higher than that of rats.

In urine and feces, conjugated metabolites of pyrene had various forms and unique characteristics among species, including birds. Glucuronide conjugated metabolites were mainly detected in the excreta of birds, whereas sulfate conjugated metabolites and PYOH were mostly excreted into urine and feces of rats, respectively. In contrast, the V_{max}/K_m of PYOH glucuronidation in hepatic microsomes of birds was lower than that of rats *in vitro*. This discrepancy may be explained by the potentially divergent biotransformation in birds and rats due to differences in the pattern of xenobiotic elimination. With regard to xenobiotic metabolism, the metabolizing organs above the liver, such as the kidney, lung, and intestines,

also showed phase II conjugation reactions with rapid passage of xenobiotics and/or their metabolites from the circulation into the tissues (Saengtienchai et al., 2015). Moreover, the ability of the transporters expressed in various tissues, such as multidrug resistance protein 2 and breast cancer resistance protein, may be other factors that could drive excretion of sulfate and glucuronide conjugate into urine and feces (Fink-Gremmels, 2010). On the other hand, differences in biliary excretion could also explain the observed species-related differences in xenobiotic metabolism, and the molecular weight has been suggested as an important factor in biliary excretion (Abou-El-Makaren et al., 1967; Hirom et al., 1972). High molecular weight compounds (in the range $>355-550$) could cause an apparent increase in excretion through biliary excretion in rats, dogs, guinea pigs, rabbits, hen, and turkeys (Abou-El-Makaren et al., 1967; Hirom et al., 1972; Smith et al., 1995; Smith et al., 2000). Meanwhile, the conjugated metabolites with higher molecular weights were present in the bile of birds. However, the percentage of conjugated pyrene metabolites was altered in the excreta associated with biodegradation of pyrene conjugated metabolites by normal flora in the intestine (Kanaly and Harayama, 2000; Saengtienchai et al., 2015). Conjugated pyrene metabolites present in urine and feces of rats were markedly different from those in birds. Furthermore, Watanabe et al. (2010, 2015) previously reported a large interspecies difference in CYP-mediated metabolism in phase I oxidation following treatment with warfarin. This may be because birds have unique xenobiotic metabolism occurring in both phase I oxidation and phase II conjugation.

The pharmacokinetics findings provided an understanding of the influence of a single pyrene exposure in chickens on absorption, metabolism, and elimination. In addition, the $t_{1/2}$ of pyrene in chickens was longer than that of its metabolite, PYOS. The results of the present study indicated a longer $t_{1/2}$ of pyrene than that reported in rats (4.9 h) (Bouchard et al., 1998; Withey et al., 1991). This may have been because pyrene showed persistence in plasma with prolongation of MRT and CL/F. Therefore, pyrene could be accumulated in various tissues and its metabolism may also occur in these tissues (Saengtienchai et al., 2015). Meanwhile, PYOS was produced rapidly and was eliminated with less residue in the chicken body. Moreover, Watanabe et al. (2015) reported that the $t_{1/2}$ of warfarin in chickens was longer than in mammalian species. The results of the present study indicated that xenobiotics seemed to accumulate in bird tissues following a single exposure, which could be sufficient to cause toxicity.

5. Conclusions

In the present study, interspecies differences in xenobiotic metabolism were clarified among bird and mammalian species. Pyrene was readily metabolized with conjugated enzymes from birds upon single exposure, although pyrene itself had a longer elimination half-life in birds than in rats. Moreover, conjugated metabolites, such as PYDOGG and PYDOGS, were first reported in the present study. Further studies are required regarding the bioaccumulation of xenobiotics. This may be related to the decreases in bird populations and may affect the ecosystem in the near future.

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519 **Figure Legends**

520

521 Fig. 1. Chromatograms of pyrene metabolites in chicken bile after oral administration and
522 analysis by HPLC/FD. Peak-a, b, c, d, e, and f represent the pyrene metabolites detected, and
523 included PYOH (peak-g) and pyrene (peak-h). Peak-a was a PYDOGS, peak-b was a PYDOGG,
524 peak-c was a PYDOSS, peak-d was a PYDOS, peak-e was a PYOG, and peak-f was a PYOS.
525 Excitation (Ex) and emission (Em) wavelengths for FD were 343 and 385 nm, respectively.

526

527 Fig. 2. Percentages of each pyrene metabolite in plasma, excreta, bile, and urine contained in the
528 ureter in chickens ($n = 3$); plasma and excreta in quails ($n = 3$); plasma, urine, and feces in rats (n
529 $= 3$). These percentages were obtained from the area of each metabolite peak detected by
530 HPLC/FD. Each pyrene metabolite structure is indicated in color.

531

532 Fig. 3. Distribution profiles between pyrene and PYOS and time (h) in chicken plasma. Each
533 point represents the average peak area ± SD, $n = 3$.

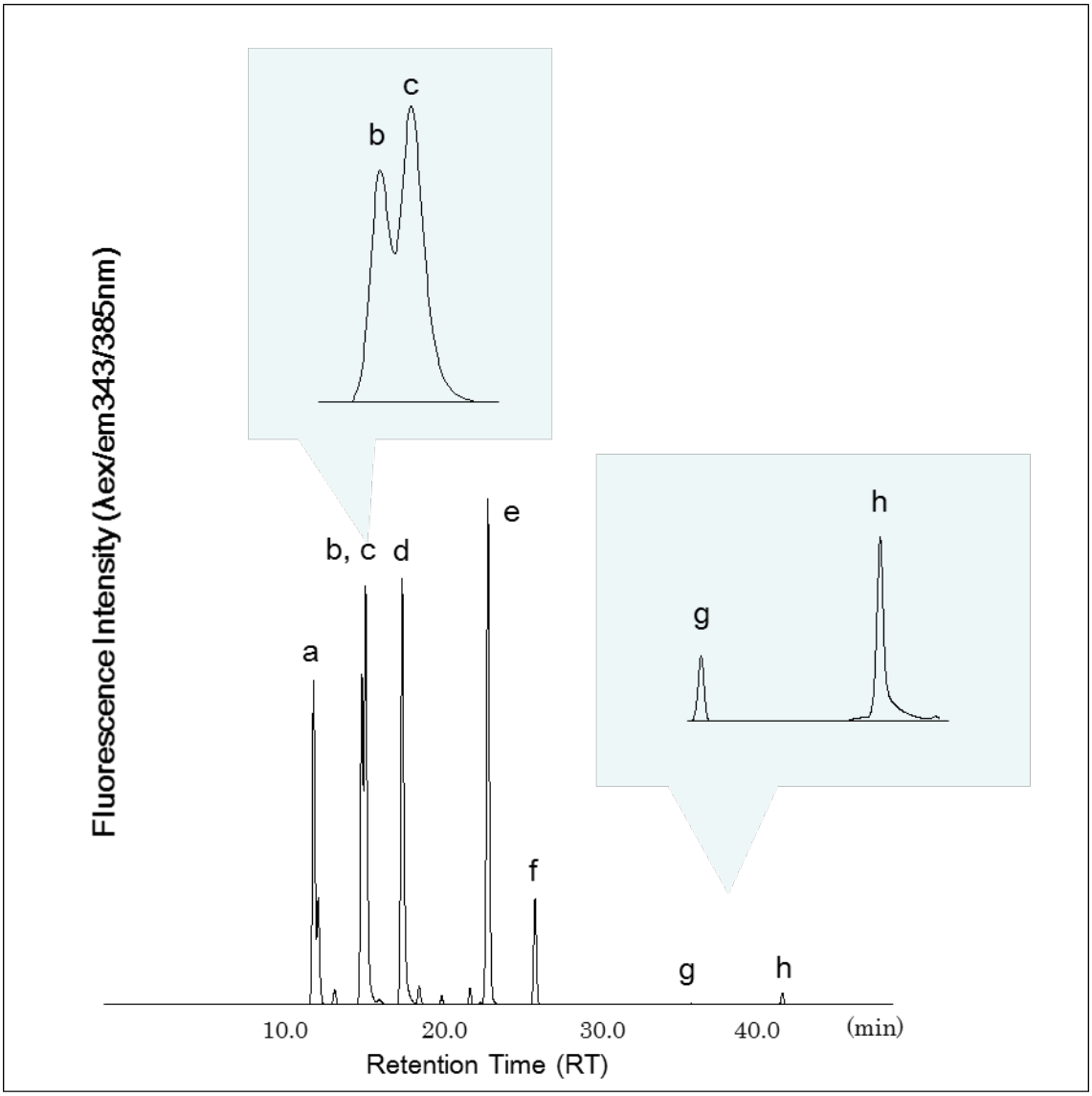
534

535 Fig. 4. Enzyme kinetics of SULT activity (4A) and UGT activity (4B) in rats and bird species.
536 The Michaelis–Menten equation was fitted and compared between rats, chickens, and quails.
537 Plots show the average ± SD, $n = 3$, each species.

538

539

Fig. 1.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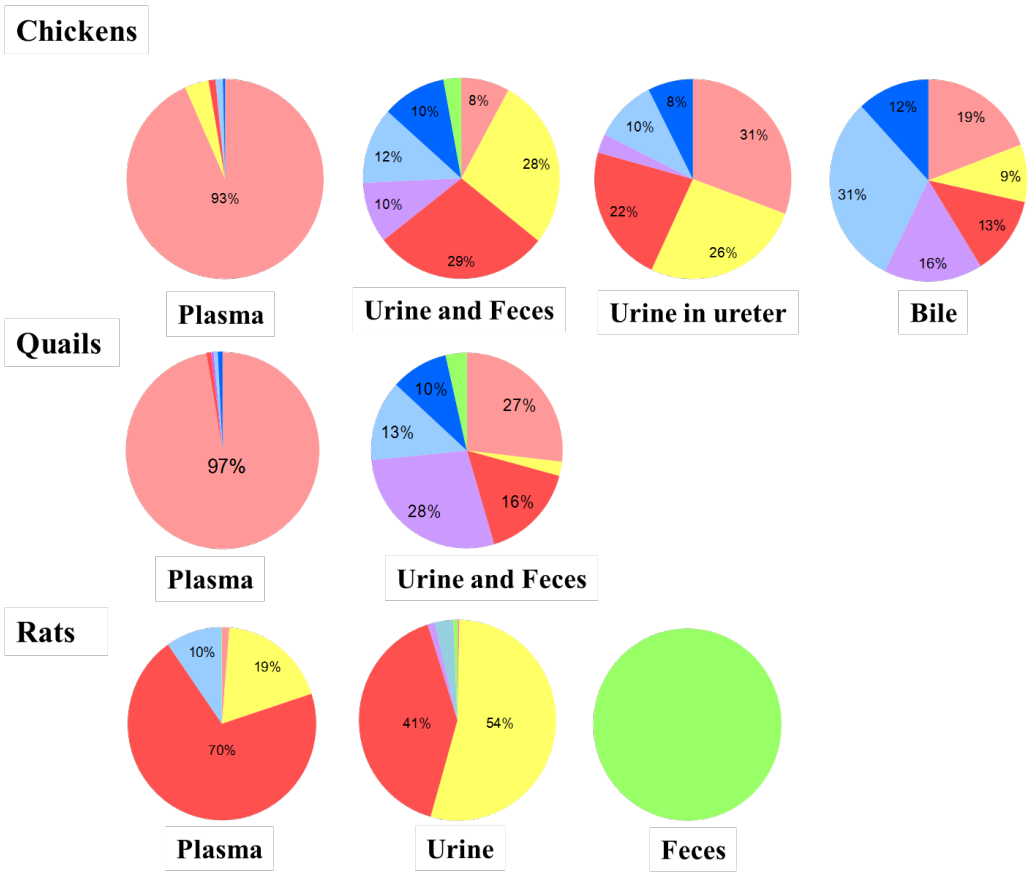
543 Fig. 2.



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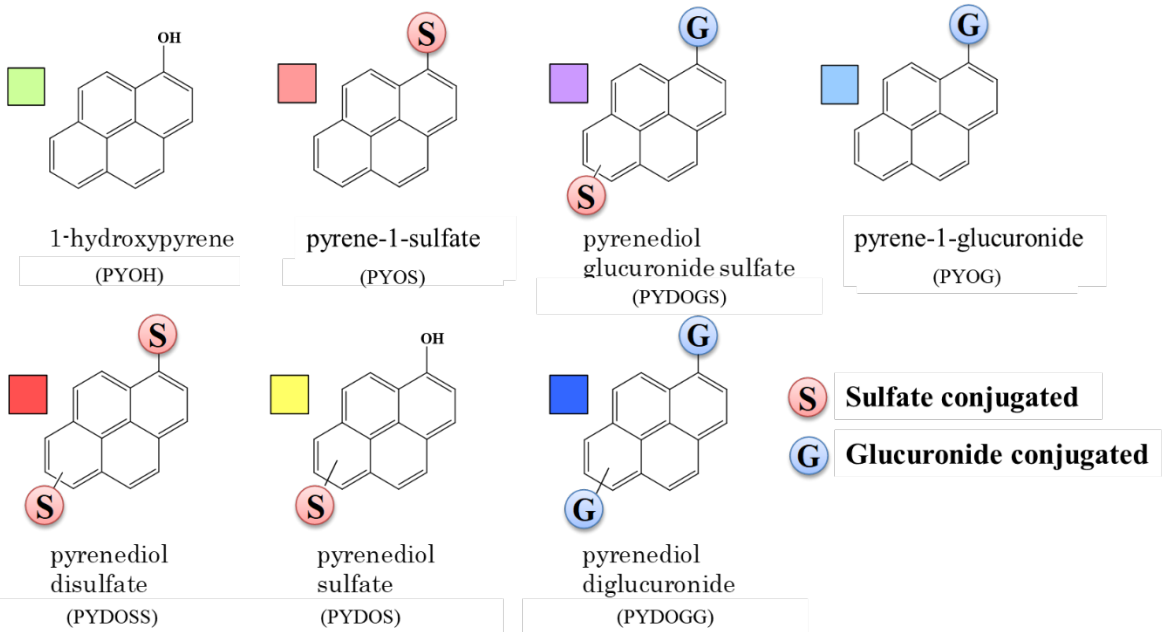

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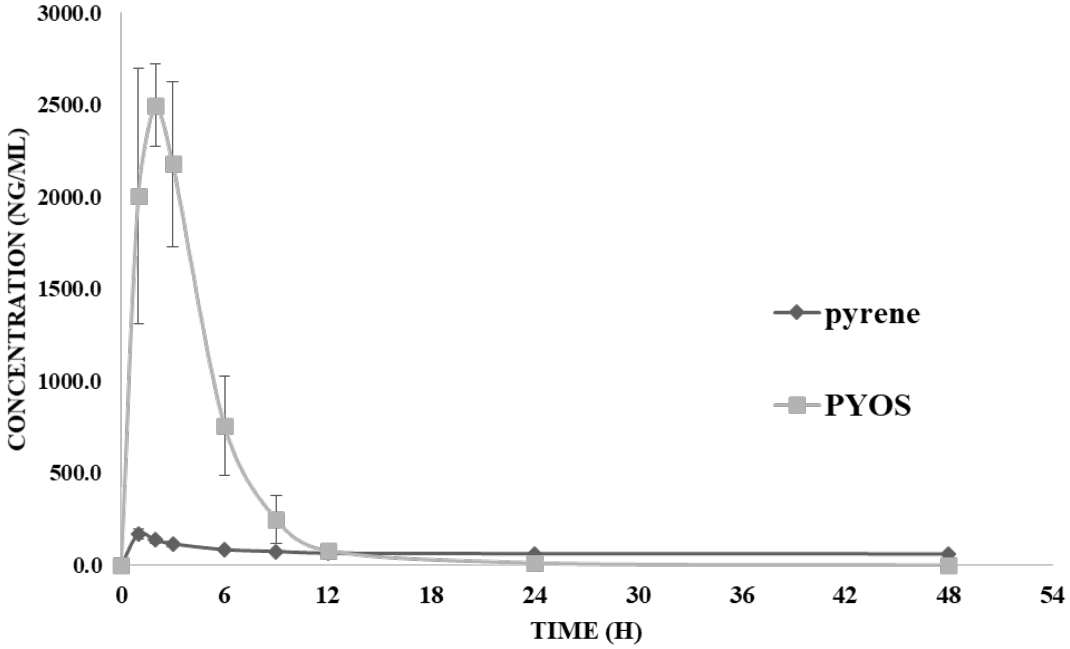
546 Fig. 3.



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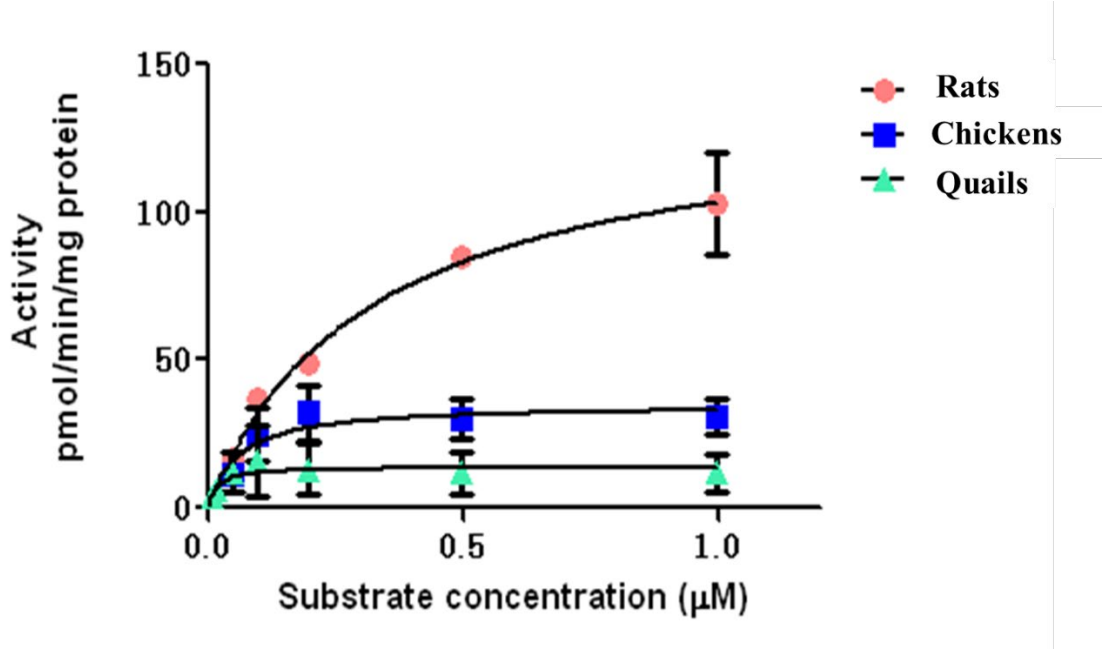
550 Fig. 4.

551 A.



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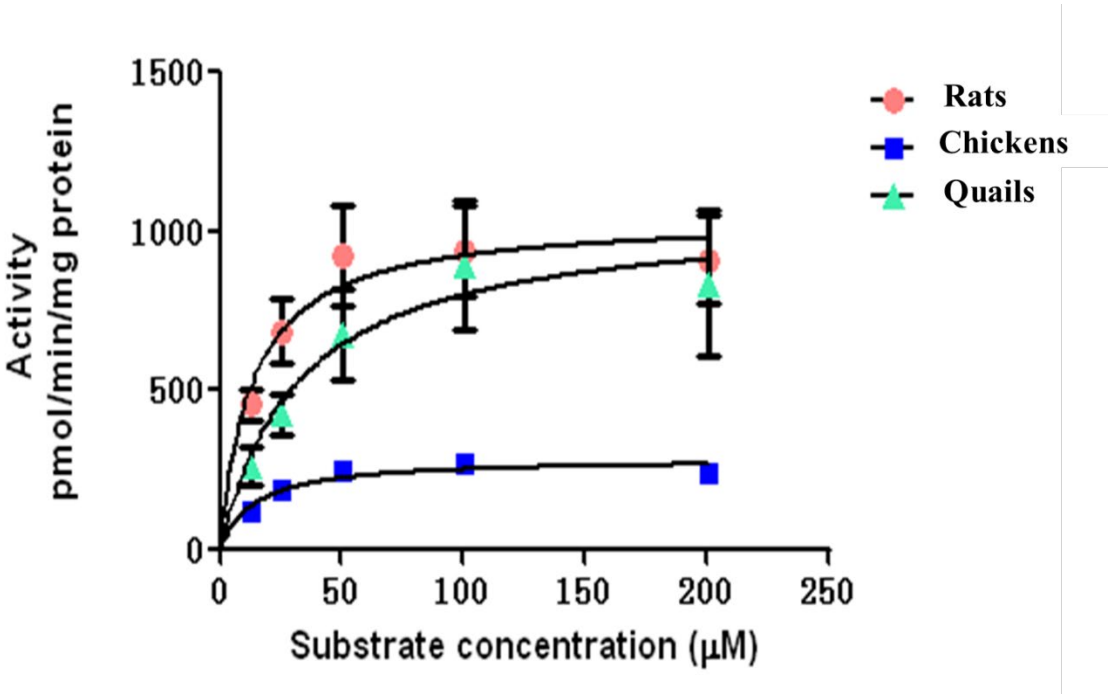
553 B.



554

555

556 Table 1. Pyrene and its metabolites under MS, MS/MS, and deconjugation conditions in chicken
557 bile

558

				Deconjugation reaction		
	RT (min)	**MS (*m/z*)**	**MS^2 (*m/z*)**	**Sulfatase**	**β-glucuronidase**	**Metabolites**
Peak-a	11.6	489	409	++	++	PYDOGS
			313			
			233			
Peak-b	14.8	585	409	–	++	PYDOGG
Peak-c	15.0	393	313	++	–	PYDOSS
			233			
Peak-d	17.4	313	233	++	–	PYDOS
Peak-e	22.9	393	217	–	+	PYOG
Peak-f	25.9	297	217	++	–	PYOS
PYOH	36.0		217	–	–	
Pyrene	41.8			–	–	

559

560 –: no change

561 +: peak reduction 10%

562 ++: peak disappearance

563

564 Table 2. Pharmacokinetic parameters of pyrene and PYOS after oral administration of pyrene (4
565 mg/kg) in chickens (mean ± SD), $n = 3$

566

Parameters	Pyrene	PYOS
AUC (ng*h/mL)	3375 ± 84	12677 ± 799
C_{max} (ng/mL)	169 ± 28	2524 ± 265
T_{max} (h)	–	1.7 ± 0.6
$t_{1/2}$ (h)	91.2 ± 68.2	3.6 ± 0.9
MRT (h)	134.5 ± 94.4	4.2 ± 0.8
CL/F (L/h)	0.3 ± 0.1	0.2 ± 0.01

567

568 AUC, area under the curve; C_{max}, maximum plasma concentration; T_{max}, time to maximum
569 plasma concentration; $t_{1/2}$, elimination half-life; MRT, mean residence time; CL/F, total clearance
570 of the drug from plasma after oral administration

571

572 Table 3. Kinetic analysis of hepatic cytosol sulfotransferase activity toward PYOH in rats,
573 chickens, and quails

574

	V_{max} (pmol/min/mg protein)	K_m (μM)	V_{max}/K_m (μL/min/mg protein)
Rats	**136 ± 30.8[a]**	**0.32 ± 0.11[a]**	**429 ± 51.1[a]**
Chickens	**34.9 ± 8.04[b]**	**0.057 ± 0.03[b]**	**608 ± 472[a]**
Quails	**13.9 ± 8.89[b]**	**0.017 ± 0.004[b]**	**830 ± 352[a]**

575

576 Mean ± SD, $n = 3$

577 [a,b]Tukey's HSD, $p < 0.05$

578 V_{max} = maximum velocity (pmol/min/mg protein); K_m = Michaelis constant (μM); V_{max}/K_m (μL
579 /min/mg protein)

580

581 Table 4. Kinetic analysis of hepatic microsome UDP-glucuronosyltransferase activity toward
582 PYOH in rats, chickens, and quails

583

	V_{max} (pmol/min/mg protein)	K_m (μM)	V_{max}/K_m (μL/min/mg protein)
Rats	**1,044 ± 175[a]**	**13.1 ± 5.57[b]**	**80.8 ± 25.6[a]**
Chickens	**287 ± 11.6[b]**	**13.5 ± 0.92[b]**	**21.2 ± 1.39[b]**
Quails	**1,057 ± 308[a]**	**31.9 ± 9.46[a]**	**33.1 ± 7.76[b]**

584

585 Mean ± SD, $n = 3$

586 [a,b]Tukey's HSD, $p < 0.05$

587 V_{max} = maximum velocity (pmol/min/mg protein); K_m = Michaelis constant (μM); V_{max}/K_m (μL
588 /min/mg protein)