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Cytochrome P450-dependent metabolism of midazolam in hepatic microsomes from chickens, turkeys, pheasant and bobwhite quail

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In vitro putative cytochrome P450 3A mediated activity, and inhibition thereof, were measured in four avian species using midazolam (MDZ) as a substrate and ketoconazole as an inhibitor. All species produced 1-hydroxymidazolam (1-OH MDZ) to a much greater extent than 4-hydroxymidazolam (4-OH MDZ). Calculated $V\text{max}_{apparent}$ values for formation of 1-OH MDZ were 117 ± 17, 239 ± 108, 437 ± 168, and 201 ± 55 pmol/mg protein*min and $K\text{m}_{apparent}$ values were 2.1 ± 0.8, 2.4 ± 1.6, 6.7 ± 5.1 and 3.2 ± 2.1 μM for chicken, turkey, pheasant and bobwhite quail, respectively. For the formation of 4-OH MDZ the $V\text{max}_{apparent}$ values were 21 ± 10, 94 ± 46, 144 ± 112, and 68 ± 30 pmol/mg protein*min and $K\text{m}_{apparent}$ values for 4-OH MDZ formation were 12.4 ± 10.1, 18.0 ± 10.8, 38.6 ± 34.7 and 29.1 ± 10.1 μM for chicken, turkey, pheasant and bobwhite quail, respectively. In all four species, ketoconazole inhibited the production of both major metabolites of MDZ, with 4-OH MDZ formation more sensitive to inhibition than 1-OH MDZ. Pheasant and bobwhite quail appeared most sensitive to ketoconazole inhibition.

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INTRODUCTION

Comparatively little is known about the ability of commercially raised poultry and gamebirds to metabolize therapeutic drugs as compared with other species. In particular, their ability to metabolize substrates via oxidative pathways, such as those mediated by the family of enzymes known as the cytochrome P450 enzymes, is not as well understood. A need for basic research in this area stems from real-world situations in which poultry are routinely exposed to feed additives and therapeutic drugs, many of which are metabolized via these enzymes. Species differences in drug metabolism affect not only therapeutic efficacy but also drug residues of concern to consumers. The current study was undertaken to obtain an estimation of the putative cytochrome P450 3A (CYP3A) activities in these species using a marker drug.

In humans approximately 50% of therapeutic drugs are metabolized by the CYP3A family (Hardman, 2001) and this may be true in avian species as well. The presence of CYP3A has been confirmed in the chicken and there is supportive evidence for its existence in other poultry species. An isoform of CYP3A, designated CYP 3A37, has been cloned and functionally expressed in the chicken (Ourlin *et al.*, 2000). In addition, the chicken genome was recently sequenced, which should help identify more avian cytochrome P450 isoforms (Hillier *et al.*, 2004). Previous studies have demonstrated CYP3A-like activities in chickens, turkeys, pheasants and bobwhite quail using compounds known to be metabolized by CYP3A in mammalian species (Ronis *et al.*, 1994; Giorgi *et al.*, 2000; Klein *et al.*, 2000; Nebbia *et al.*, 2001). Another piece of supportive evidence for the existence of CYP3A enzymes in avian species is their detection using antibodies raised against CYP3A enzymes from mammalian species (Lorr *et al.*, 1989; Ronis *et al.*, 1994; Coulet *et al.*, 1996).

It is difficult to determine how similar poultry species are with regard to CYP3A capabilities as none of the studies reported to date directly compared all four species using the same substrate. The current study compared the *in vitro* hepatic microsomal metabolic capabilities of avian food species with regard to a compound known to be a marker substrate for CYP3A in mammals, midazolam (MDZ). MDZ is a benzodiazepine that undergoes oxidative metabolism via cytochrome P450 in the liver to the 1-hydroxy, 4-hydroxy or 1,4-dihydroxy metabolites (minor), which are then conjugated with glucuronic acid and excreted. It has been reported to be specific, although not selective, for the CYP3A isoform in humans (Kronbach *et al.*, 1989) and rats [4-hydroxymidazolam (4-OH MDZ) moreso than 1-hydroxymidazolam (1-OH MDZ)], with a smaller contribution from 2C11 (Kobayashi *et al.*, 2002). While this has not been confirmed in avian species it is a logical place to start. Another

reason for choosing MDZ is that it is used to sedate wild quail for short-term handling (Day & Roge, 1996) and to anesthetize or stop seizures in pet birds in clinical medicine settings (Forbes, 1998; Machin & Caulkett, 1998). Thus, information gained from this study may be useful to a wide range of researchers/clinicians.

To aid in determining CYP3A's role in MDZ metabolism in these species, inhibition studies were conducted using ketoconazole, an imidazole anti-fungal drug that is an inhibitor of steroidogenic cytochrome P450 in rats and humans (Baldwin *et al.*, 1995; Khan *et al.*, 2002). It is a mechanism-based inhibitor and as such inhibits many different isoforms of CYPs but with more specificity toward CYP3A (usually an order of magnitude lower IC_{50} than other isoforms) (Baldwin *et al.*, 1995). For instance, a recent study reported that ketoconazole is more specific for CYP3A than other isoforms but there is some inhibition of CYP2C11 in rats (Kobayashi *et al.*, 2003). It has been shown to be a potent inhibitor of MDZ hydroxylation in humans, as demonstrated by its low K_i value ($K_i = 15–110$ nM), and in mice (Wrighton & Ring, 1994; Gibbs *et al.*, 1999b; Perloff *et al.*, 2000). In bobwhite quail ketoconazole has been shown to inhibit testosterone hydroxylation in kidney (Ronis *et al.*, 1994, 1998). There have been no reports of ketoconazole effects on CYP3A-mediated metabolism in chickens, turkeys or pheasants to date. Comparing the inhibition of MDZ metabolism by ketoconazole in each species to the *in vitro* kinetic results aids in analyzing the relative contribution of CYP3A.

MATERIALS AND METHODS

Reagents

Clonazepam, MDZ maleate and 4-OH MDZ were kindly donated by Roche, Inc. 1-OH MDZ was purchased from Lipomed, Inc. All solvents were high performance liquid chromatography (HPLC) grade from EMD Chemicals, Inc. (Gibbstown, NJ, USA) Nicotinamide adenine dinucleotide phosphate, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA). All other reagents were purchased from Fisher Scientific Company (Pittsburgh, PA, USA).

Animals and preparation of microsomal fractions

All protocols for animal use had the approval of the University of California, Davis, Animal Use and Care Committee. Chickens, turkeys, pheasants and bobwhite quail of both sexes were obtained for the study from commercial producers. Broiler chickens, 6 weeks of age, Foster Farms Ross cross, were bred at Foster Farms facility in Turlock and raised by the poultry research unit at the University of California, Davis. Turkeys, white broad-breasted, were purchased from Phinney Hatchery, Inc. (Walla Walla, WA, USA) as 1-day olds and raised to 6 weeks of age. Ring-necked pheasants, 10 week-old, were purchased from McClellan Brothers Ringnecks of Oroville, CA, USA. Bobwhite quail, 16 weeks of age, were purchased from K and L Quail of Oroville. Six birds of each species were used, except 10 quail were used to obtain five samples consisting of two pooled livers.

Birds were euthanized via cervical dislocation, the livers immediately removed and washed in ice-cold Tris/KCl (0.02 M/0.2 M) buffer, pH 7.4, blotted dry and weighed. All subsequent steps were performed at 4 °C. Microsome preparation was adapted from Boyd *et al.* (1978). Livers were homogenized with 3–4 volumes of Tris/KCl buffer using a Potter–Elvehjem type tissue homogenizer. Homogenates were centrifuged at 9 000 ***g*** for 20 min and the supernatants further centrifuged at 145 000 ***g*** for 65 min. Supernate was decanted and the microsomal pellets resuspended in homogenization buffer and centrifuged again at 145 000 ***g*** for 65 min to remove hemoglobin. Microsomes were then resuspended in 0.1 M sodium phosphate buffer, pH 7.4 containing 0.8 mM EDTA, 1 mM dithiothreitol and 20% glycerol and stored at −80 °C until use. Immediately prior to substrate incubations, microsomes were centrifuged at 145 000 ***g*** for 65 min at 4 °C to remove the storage buffer and were resuspended in 0.2 M phosphate buffer, pH 7.4. Protein concentration (Bradford, 1976) and total cytochrome P450 content (Omura & Sato, 1964) were also determined immediately prior to substrate incubations.

Midazolam assays

Activity and inhibition assays were conducted in 1.5 mL microcentrifuge tubes in a shaking water bath at 41 °C (avian body temperature). Kinetic assay conditions were: 100 μg hepatic microsomal protein, final MDZ concentrations of 1.25, 2.5, 5, 12.5, 25 and 50 μM, with incubations proceeding for 10 min (optimized in pilot studies). Assays were considered at saturation if loss of substrate was <10%. Assay cofactors in 0.2 M phosphate buffer, pH 7.4, were added to the tubes at twice their final concentration (0.5 mM $NADP^+$, 5.67 mM glucose-6-phosphate, 0.3 U/mL glucose-6-phosphate dehydrogenase and 15 mM $MgCl_2$) and mixed well. All reaction components except substrate were allowed to warm for 5 min at 41 °C. Reactions were initiated with MDZ (5, 10 or 20 μL of 0.1 mM, or 4, 8 or 10 μL of 1 mM, both diluted in deionized water) for a final volume of 0.2 mL. Reactions were stopped with 200 μL of cold methanol containing 1 ng/mL clonazepam as internal standard and tubes were immediately placed on ice. Inhibition assays were conducted as above except using a single concentration of MDZ, 25 μM, (due to sample limitations) with 0, 3.125, 6.25, 12.5, 25, 50 and 100 μM ketoconazole, for 15 min. The inhibition assays included a 10-min preincubation at 41 °C of microsomes and cofactors with ketoconazole before addition of substrate in order to determine maximum inhibition at each inhibitor concentration. After termination of the reaction, all samples were vortexed for 30 sec and frozen at −20 °C overnight. Samples were analyzed for MDZ, 1-OH MDZ, 4-OH MDZ and clonazepam.

HPLC analysis

The HPLC analytical method for MDZ analysis is based on a modification of Kronbach (Kronbach *et al.*, 1989). The chroma-

tographic system consisted of a Waters 600 controller with a 717 plus Autosampler with Model 996 photodiode array detector. The chromatographic column used was a Luna® (Phenomenex, Torrance, CA, USA) ODS C18 column, 4.6 × 150 mm set to 30 °C with a Phenomenex C18 ODS guard column 4 × 3 mm. Quantitative analysis was performed using Millenium 32® software (Millenium Software, LLC, Dublin, CA, USA). The mobile phase was 40:35:25 10 mM potassium phosphate buffer:methanol:acetonitrile run in isocratic mode at 1 mL/min. Samples were removed from storage at −20 °C and centrifuged at 18 300 ***g*** for 10 min at 4 °C (Fisher Marathon 21000R centrifuge; Fisher Scientific, Pittsburgh, PA, USA). Supernate, 150 μL, was then transferred to chromatography insert vials and 100 μL was injected for ultraviolet detection at 220 nm. The run time for samples from the kinetic studies was 20 min, while the run time for samples from the inhibition study (containing ketoconazole) was 30 min. Retention times were 5.1, 6.0, 6.7, 9.8 and 26 min for clonazepam (internal standard), 4-OH MDZ, 1-OH MDZ, MDZ, and ketoconazole (KTZ), respectively. Baseline separation was achieved for all analytes. Stock solutions of 1 mM clonazepam, MDZ, 1-OH MDZ and 4-OH MDZ for standard curves were prepared weekly. Final calibration curve concentrations were 0.0039–1 μg/mL for 1-OH MDZ and 4-OH MDZ and 0.039–10 μg/mL for MDZ.

Calibration curves were accepted if they were linear with an R^2 value of >0.99 and standards were within 10% of the actual value. Average recovery percentages were 104, 108 and 109 for 1-OH MDZ, 4-OH MDZ and MDZ, respectively. The limit of detection (LOD) and limit of quantification (LOQ) were determined as the baseline noise plus three times the standard deviation of the baseline noise for LOD and baseline noise plus ten times the standard deviation of the baseline noise for LOQ. The LOD and LOQ for 1-OH MDZ were 0.013 and 0.036 μg/mL, respectively. For 4-OH MDZ the LOD and LOQ were 0.007 and 0.021 μg/mL, respectively. Average intra-day precision for 1-OH MDZ and 4-OH MDZ was 0.42% and 0.5%, respectively. Average intra-day accuracy for 1-OH MDZ and 4-OH MDZ was 4.3% and 12.6%, respectively. Inter-day precision (across all four species) for 1-OH MDZ and 4-OH MDZ was 13.8% and 13.7%, respectively. Inter-day accuracy 1-OH MDZ and 4-OH MDZ was 4.3% and 8.1%, respectively.

Statistics

Estimations of the apparent Michaelis–Menten kinetic parameters, $Vmax_{apparent}$ and $Km_{apparent}$ (V_{max} and K_m), for each metabolite were obtained using two different methods to compare their utility for studies such as this. The first approach consisted of a standard two-stage approach, wherein kinetic parameters were calculated for each experiment and then averaged to get an estimation of variability in the parameters in the population. The second involved using the mean values of product formation at each substrate concentration to estimate kinetic parameters. Parameters were calculated with Winnonlin® software (Pharsight Corporation, Mountain View, CA, USA), using a simple Emax model, and with Hanes-Woolf plots for confirmation. In addition, metabolite data for each individual bird at each concentration of substrate (times four species) were first subjected to four-way analysis of variance (ANOVA) and then any pairings that were significantly different ($P < 0.05$) were subjected to an *F*-test.

RESULTS

Comparative kinetics

All four avian species produced 1-OH MDZ to a much greater extent than 4-OH MDZ (1-OH MDZ accounted for 77–100% of total metabolism). The production of metabolites demonstrated Michaelis–Menten kinetics in most, but not all, cases. There were two birds of each species for which the production of 4-OH MDZ was sigmoidal (this sigmoidicity was lost when averaging data). The apparent *in vitro* kinetic parameters calculated for each species using Winnonlin® are shown in Table 1. Overall, there was considerable intra-species variability in rates of MDZ metabolism. Despite this variability, analysis of variance (ANOVA, $P < 0.05$ using *F*-statistic) showed significant differences between species with regard to V_{max} but not K_m as follows: chicken significantly lower than all other species with regard to 1-OH MDZ and 4-OH MDZ and pheasant significantly higher than all other species with regard to 1-OH MDZ. The K_m values for 1-OH MDZ for all species, except turkey, were much lower than their corresponding K_m values for 4-OH MDZ.

Table 1. Michaelis–Menten parameters calculated from hepatic microsomal metabolism of midazolam using Winnonlin models

Species	Total P450 (nmol/mg)	1-hydroxymidazolam			4-hydroxymidazolam		
		V_{max} (pmol/mg*min)	K_m (μM)	Clin (V_{max}/K_m)	V_{max} (pmol/mg*min)	K_m (μM)	Clin (V_{max}/K_m)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Chicken ($n = 6$)	0.26 ± 0.05	116.8 ± 17.4[b,c,d]	2.1 ± 0.8	60.1 ± 22.0	20.7 ± 10.2[b,c,d]	12.4 ± 10.1	4.4 ± 5.8
Turkey ($n = 7$)	0.48 ± 0.07	239.2 ± 108.0[a,c]	2.4 ± 1.6	128.4 ± 107.0	93.6 ± 46.1	18.0 ± 10.8	5.7 ± 2.1
Pheasant ($n = 7$)	0.47 ± 0.12	436.8 ± 167.9[a,b,d]	6.7 ± 5.1	95.7 ± 73.4	143.7 ± 112.0	38.6 ± 34.7	4.5 ± 3.3
Quail ($n = 5 \times 2$ pooled)	0.51 ± 0.10	201.2 ± 54.7[a,c]	3.2 ± 2.1	108.8 ± 77.9	68.2 ± 30.1	29.1 ± 10.1	2.6 ± 0.5

Analysis of variance single factor using *F*-statistic, $P < 0.05$; a, significantly different from chicken; b, significantly different from turkey; c, significantly different from pheasant; d, significantly different from quail. Rates expressed as pmole product per milligram of protein per minute.

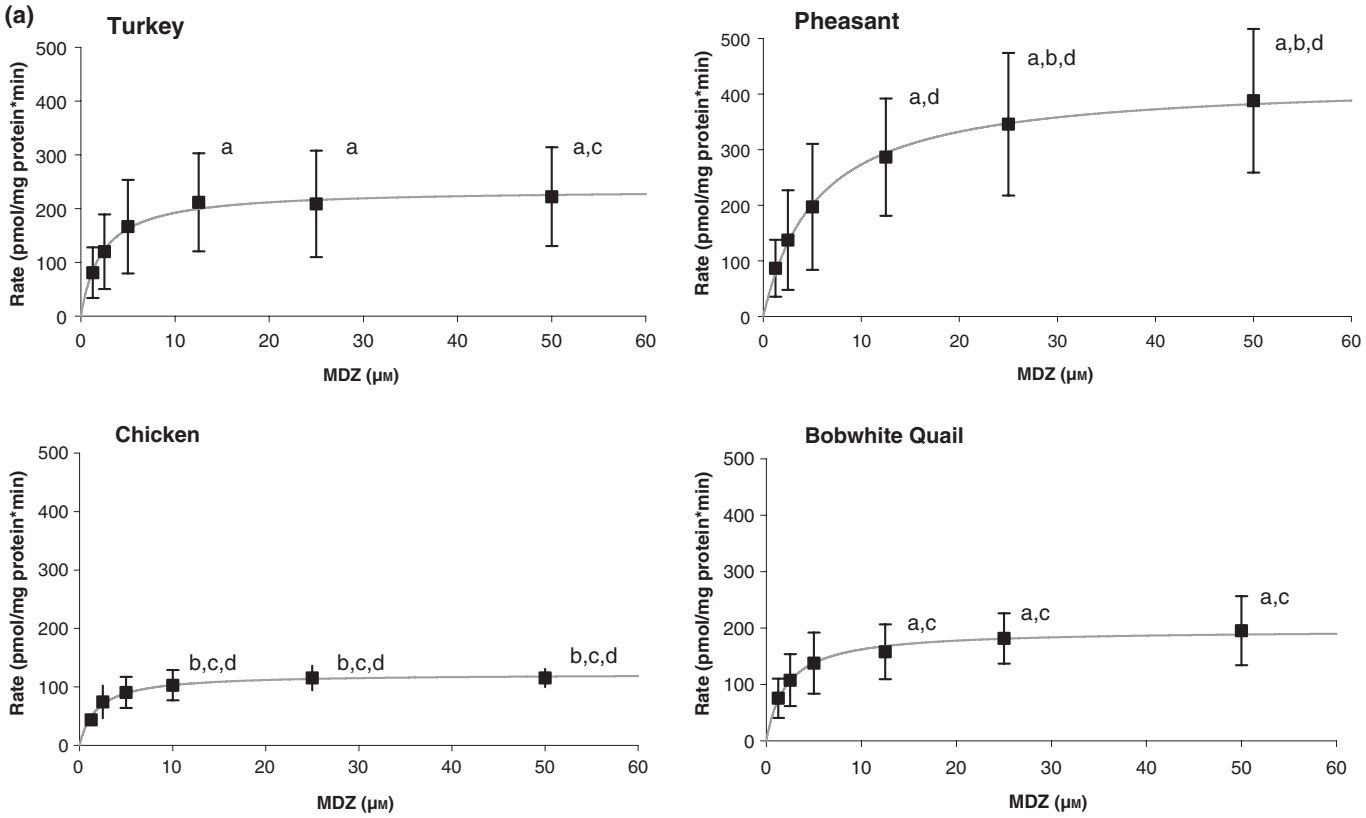

Fig. 1a. Initial rate of 1-hydroxymidazolam formation in hepatic microsomes incubated with 1.25–50 μM midazolam. Values are raw data means ± standard deviation. Chicken $n = 6$, turkey $n = 7$, pheasant $n = 7$, bobwhite quail $n = 5 \times 2$ pooled. Statistical differences using ANOVA (single factor, $P < 0.05$) as follows: a, b, c, d = significantly different from chicken, turkey, pheasant and quail, respectively.

Rates of metabolite formation from 1.25–50 μM MDZ, along with the best-fit curves, are shown in Fig. 1a,b. ANOVA showed significant differences between species from 10–50 μM MDZ, but no significant differences between any of the species at the three lowest MDZ concentrations used in the study. The first-order rate constants, used as an estimate of internal clearance for use in scaling to *in vivo*, are also shown in Table 1 (Houston & Kenworthy, 2000).

In vitro inhibition of midazolam with ketoconazole

Ketoconazole inhibited the production of both major metabolites of MDZ in all four species studied. Table 2 shows the calculated IC_{50} values (inhibitor concentration at which 50% inhibition is observed compared with control) for ketoconazole inhibition of MDZ. There was considerable intra-species variability in response to ketoconazole inhibition. Single factor ANOVA ($P < 0.05$) of the IC_{50} values revealed no significant differences for inhibition of 1-OH MDZ formation. However, a general sensitivity ranking based on mean IC_{50} values for 1-OH MDZ formation shows quail = turkey > pheasant > chicken. Ketoconazole inhibited 4-OH MDZ to a greater extent than 1-OH MDZ in all four species. Single factor ANOVA ($P < 0.05$) of the IC_{50} values for 4-OH MDZ formation revealed pheasant to be significantly lower than turkey, and quail significantly lower than turkey and chicken. Pheasant and bobwhite quail appeared most sensitive to ketoconazole inhibition, as reflected by their lower calculated IC_{50} values and overall lower production of MDZ metabolites at all ketoconazole concentrations used.

DISCUSSION AND CONCLUSIONS

In vitro kinetic estimations for midazolam

METHOD COMPARISON

Kinetic parameters calculated using only the means of product formation at each substrate concentration yielded similar values to those of the two-stage approach. However, in this case the only variability one would calculate is the ability of the model to predict the best-fit line, which gives no information on the likely variability of the kinetic parameters in a population. Thus the two-stage approach was used, in which kinetic parameters are estimated for each bird and then averaged. This approach is simple but one limitation is that it may overestimate the variability in a population (Ette & Williams, 2004). Hanes-Woolf plots (S/V vs. S) were also used to calculate the kinetic

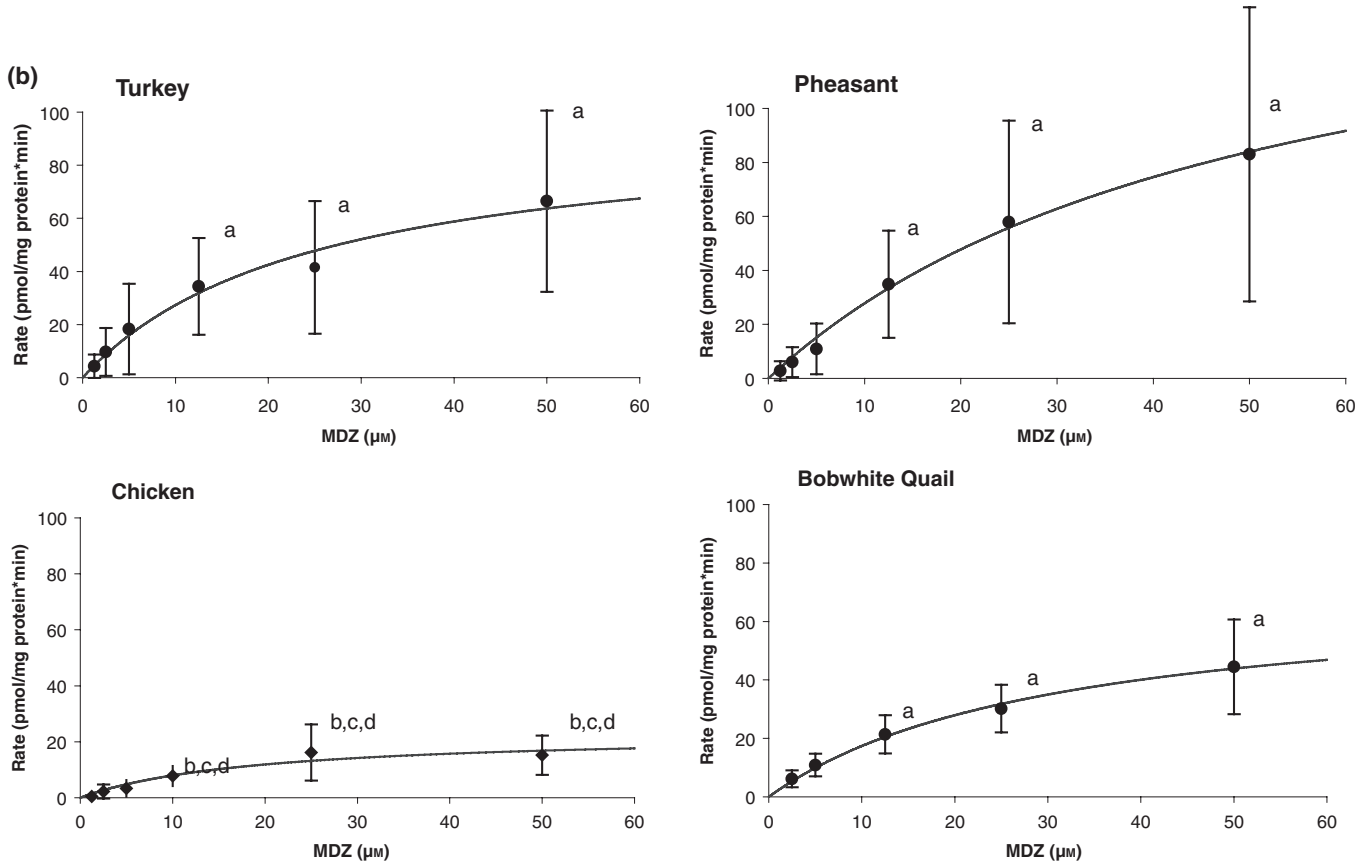

Fig. 1b. Initial rate of 4-hydroxymidazolam formation in hepatic microsomes incubated with 1.25–50 μM midazolam. Values are raw data means ± standard deviation. Chicken $n = 6$, turkey $n = 7$, pheasant $n = 7$, bobwhite quail $n = 5 \times 2$ pooled. Statistical differences using ANOVA (single factor, $P < 0.05$) as follows: a, b, c, d = significantly different from chicken, turkey, pheasant and quail, respectively.

Table 2. Calculated IC_{50} values for ketoconazole (0–100 μM) inhibition of midazolam (25 μM) metabolism

	IC_{50} (μM)	
Species	1-hydroxymidazolam (Mean ± SD)	4-hydroxymidazolam (Mean ± SD)
Chicken ($n = 6$)	40 ± 31	18 ± 8[d]
Turkey ($n = 6$)	18 ± 7	27 ± 6[c,d]
Pheasant ($n = 6$)	22 ± 14	10 ± 9[b]
Quail ($n = 5 \times 2$ pooled)	17 ± 8	9 ± 3[a,b]

Analysis of variance single factor using *F*-statistic, $P < 0.05$; a, significantly different from chicken; b, significantly different from turkey; c, significantly different from pheasant; d, significantly different from quail.

parameters and give a more linear visual inspection. These were used instead of the more common Lineweaver-Burke plots (1/V vs. 1/S) because the errors in S/V provide a more accurate relfection of errors in velocity measurements (Cornish-Bowden, 1995). The Hanes-Woolf plots generally yielded kinetic parameter estimates that were in close agreement with those obtained using Winnonlin® (data not shown), although they could not be used in those cases where the substrate vs. product formation curves were sigmoidal.

Midazolam in vitro metabolism

It should be emphasized that MDZ was chosen as a marker substrate for the CYP3A family because it is used as such in mammals but this has not been confirmed in avian species. However, the existence of CYP3A37 has been confirmed in the chicken, as demonstrated by its 6β-hydroxylation of steroid hormones and its recognition by a polyclonal antibody against rat CYP3A1 (Ourlin *et al.*, 2000) and there is supportive evidence for the existence of CYP3A isoforms in closely related avian species. In addition, MDZ was chosen because it is used in wildlife and clinical medicine settings.

The current investigation found that chickens, turkeys, pheasant and bobwhite quail form 1-OH MDZ as the major metabolite, which is similar to humans, dogs and mice and in contrast to rats (Heizmann & Ziegler, 1981; Ghosal *et al.*, 1996; Perloff *et al.*, 2000; Day *et al.*, 2005). This finding was somewhat unexpected and suggests structural differences in the rat and avian enzyme with regard to substrate binding but not necessarily differences in enzyme regulation. For example, Ourlin *et al.*, 2000 noted that rifampicin is a potent inducer of CYP3A in humans and rabbits but not in rats or chickens, suggesting that the CYP3A of the latter species may be regulated in a similar manner. In contrast, Ronis noted that while clotrimazole is a

potent inducer of CYP3A in rats, as judged from erythromycin N-demethylase activity and induction of a protein recognized by rabbit anti-rat CYP3A2, it has no effect in bobwhite quail (Ronis *et al.*, 1994).

All four species demonstrated Michaelis–Menten kinetics for the 1-OH MDZ metabolite. In contrast, while most birds of each species demonstrated Michaelis–Menten kinetics for 4-OH MDZ, two birds of each species exhibited sigmoidal kinetics. This could be due to the presence of either one enzyme with two binding sites or two enzymes, one of which is competitively inhibited (Segel, 1993). In mice, but not humans, it is recognized that CYP2C (via antibodies against rat CYP2C11) plays a significant role in metabolism of MDZ to 1-OH MDZ (<40%) (Perloff *et al.*, 2000; Hamaoka *et al.*, 2001). Alternately, CYP3A4 has been reported to exhibit both homotropic and heterotropic cooperativity and likely contains two binding sites in its active site (Domanski *et al.*, 2000). In a detailed study involving active-site mutants of CYP3A4 in E. coli, Khan *et al.* concluded that the two proposed binding sites for MDZ in CYP3A4 may overlap. To complicate matters, there could be two isozymes present. For example, in humans the presence of both CYP3A4 and CYP3A5 results in a higher ratio of 1-OH MDZ:4-OH MDZ compared with those with only CYP3A4 (Gorski *et al.*, 1994). Further study is needed in avian species regarding the presence of multiple isoforms of CYP3A.

With respect to 1-OH MDZ, the calculated V_{max} values for all four avian species fell within the low end of the reported range for humans (190–4380 pmol/mg protein*min) and well below that reported in rats, dogs and monkeys (2000, 1110 and 2481 pmol/mg protein*min, respectively) (Sharer *et al.*, 1995; Ghosal *et al.*, 1996). The K_m values for 1-OH MDZ in all avian species were within the range of values reported for the K_m in humans (2.5–8.6 μM) but below that reported for rats (32.3 μM) (Kronbach *et al.*, 1989; Sharer *et al.*, 1995; Ghosal *et al.*, 1996; Perloff *et al.*, 2000). With respect to 4-OH MDZ, the calculated V_{max} for all four avian species was again within the low end of the range reported in humans (0–3430 pmol/mg protein*min) and much lower than that reported in rats (5900 pmol/mg protein*min) but this is expected as 4-OH MDZ is the major metabolite in rats (Ghosal *et al.*, 1996). The K_m range for 4-OH MDZ metabolism in the avian species (12.4–38.6 μM) was similar to humans (11–60 μM) and to rats (24.5 μM), (Kronbach *et al.*, 1989; Ghosal *et al.*, 1996; Perloff *et al.*, 2000). This similarity between birds and mammals with regard to K_m for both metabolites suggests conservation of the enzyme(s) binding site. However, caution should be used when comparing K_m values across studies because different protein concentrations will have different effects on mutual depletion, and thus affect the K_m values (Gibbs *et al.*, 1999a).

The estimated V_{max} parameters for the metabolism of MDZ in the avian species studied here were low compared with those reported for mammalian species. However, the relative metabolic capabilities of birds vs. mammals is highly substrate and species dependent (Dalvi *et al.*, 1987; Amsallem-Holtzman & Ben-Zvi, 1997; Olkowski *et al.*, 1998; Khalil *et al.*, 2001). Comparing only the birds in this study, chicken were significantly lower ($P < 0.05$) than all other species in formation of both 1-OH MDZ and 4-OH MDZ, and pheasant were significantly higher in their production of 1-OH MDZ, from 10–50 μM MDZ. A crude estimate of internal clearance ranks turkey > quail > pheasant > chicken for metabolism of 1-OH MDZ. This ranking shifts to turkey > pheasant > chicken > quail for 4-OH MDZ.

Although there were significant differences in calculated V_{max} values between the avian species considered here, it should be emphasized that these originated largely at the higher substrate concentrations used. All four species' metabolic capabilities were relatively similar from 1.25–12.5 μM MDZ. Of course, when extrapolating to whole animals factors such as distribution to fat, phase II metabolism and other considerations such as blood flow and hepatic vs. renal clearance will affect therapeutic efficacy and tissue drug residues. However, preliminary calculations with data from pharmacokinetic studies of MDZ in these species in our laboratory suggest concentrations initially reaching the liver could potentially be above the concentration range used in the current *in vitro* studies but would quickly drop to within the range used, and thus these studies may be relevant to the *in vivo* situation.

Ketoconazole inhibition of in vitro midazolam metabolism

Of note is that the IC_{50} values for ketoconazole inhibition of 1-OH MDZ were higher than those for the 4-OH MDZ metabolite, although not significantly. This is expected based on the lower K_m values for 1-OH MDZ vs. 4-OH MDZ and the high substrate concentration used. However, given that the average K_m values for 1-OH MDZ were ~5–9 × lower than for 4-OH MDZ, one might expect even less inhibition of 1-OH MDZ by ketoconazole, and thus even higher IC_{50} values. This suggests that 1-OH MDZ metabolism may be a better marker for CYP3A activity in these species than 4-OH MDZ, which is the case for humans but not for rats. The higher K_m values in avian species appear to be a species difference from humans, mice and dogs with respect to 4-OH MDZ. However, the magnitude of the difference for both metabolites is likely influenced by assay conditions, such as protein concentration and especially relative substrate and inhibitor concentrations (Gibbs *et al.*, 1999a).

The IC_{50} values reported here for KTZ inhibition of MDZ hydroxylation are higher than those reported for ketoconazole inhibition of MDZ in humans ie. 0.044–10 μM for 1-OH MDZ (Wrighton & Ring, 1994; Perloff *et al.*, 2000) and 0.038 μM for 4-OH MDZ, respectively (Perloff *et al.*, 2000). For mice IC_{50} values of approximately 10 and 0.025 μM have been reported for 1-OH MDZ and 4-OH MDZ, respectively (Perloff *et al.*, 2000). In beagle dogs an IC_{50} value of 0.18 μM has been reported for KTZ inhibition of 4-OH MDZ (Kuroha *et al.*, 2002). There is also the possibility that MDZ metabolism in the avian species is mediated by more than one CYP enzyme, which may not be as susceptible to inhibition by ketoconazole.

In conclusion, the current study lends support for the existence of CYP3A in avian food species because of *in vitro* metabolism of the CYP3A marker substrate, MDZ, and its inhibition by ketoconazole. All four avian species produced

1-OH MDZ as the major metabolite and demonstrated lower V_{max} values than rodents or humans for both metabolites. Differences in the calculated V_{max} values between chickens, turkeys, pheasant and quail were largely a result of differences at the highest MDZ concentrations used. Chickens appear to metabolize MDZ to both metabolites at a lower rate than the other birds, while pheasant appear to metabolize MDZ to 1-OH MDZ at a higher rate than the other species. This may have implications for attempts at species grouping with regard to therapeutic drug treatment and metabolism. Values calculated for the K_m for each species with regard to both metabolites varied but were similar in range to those reported in rats and humans, suggesting some evolutionary conservation of the enzyme(s) across species.

Ketoconazole inhibited the production of both metabolites in all four species but, taking relative K_m values into account, was more efficient at inhibiting the formation of 1-OH MDZ, suggesting that 1-OH MDZ metabolism may be a better marker for CYP3A activity in these species than 4-OH MDZ. Although care should be exercised in interpreting results used with so-called cytochrome P450 isozyme 'specific' substrates and inhibitors when using them in a new species, the metabolism of MDZ and its inhibition by ketoconazole lend support to the presence of CYP3A isoform(s) in the commercial avian food species studied here. The differences between species observed in this study were largely quantitative and not qualitative but have implications for effective disease treatment and adequate drug residue withdrawal times.

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