ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

ANSInet
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorijps@gmail.com

International Journal of Poultry Science 5 (2): 104-111, 2006
ISSN 1682-8356

Hepatic CYP Isoforms and Drug-Metabolizing Enzyme Activities in Broiler Chicks

Joseph C. Kawalek[1]*, Michael J. Myers[1], Karyn D. Howard[1], Dorothy E. Farrell[1] and Badar Shaikh[2]
[1]Division of Animal Research, [2]Division of Residue Chemistry, Office of Research,
Center for Veterinary Medicine, Food and Drug Administration, 8401 Muirkirk Road, Laurel, MD 20708, USA

Abstract: Day-old chicks were raised for four - six weeks in pens using different bedding materials (wire, paper, corn cobs, hardwood, rice hulls, cedar, and pine) to determine if these beddings have any effect on hepatic drug metabolizing enzyme activities, cytochrome P450 isoforms, and the disposition of enrofloxacin, a drug previously approved for use in chickens. Cytochrome P450 (CYP) isoforms from the major P450 isoform families (1A, 2A, 2B, 2C, 2D, 2E, 3A, and 4A) and their corresponding hepatic drug metabolizing enzyme activities were measured in subcellular fractions obtained from 4-and 5-week-old birds. Five week-old birds were treated with medicated water containing Baytril at 50 ppm enrofloxacin for five consecutive days and then withdrawn for two days prior to sacrifice. There were no significant differences in P450-mediated reactions or levels of CYP isoforms between any of the different test groups. The marker residue for enrofloxacin was barely detectable in the marker tissue (breast muscle) of the treated birds. The major metabolites of enrofloxacin, namely, ciprofloxacin, desethylenyl-enrofloxacin, and desethylenyl ciprofloxacin, were detectable in extracts of liver homogenates of the treated birds. However, the levels were below the level of quantification.

Key words: Chickens, CYP isoforms, litter, bedding, enrofloxacin

Introduction

There is no information on environmental factors that might affect hepatic drug metabolizing enzyme activities in chickens. Broilers and free-ranging laying hens are exposed to different sources of litter/bedding depending on the location of the production facilities. In rodents, some litter-types, such as cedar chips, can induce hepatic drug metabolizing enzyme (DME) activities (Ferguson, 1966), whereas other bedding materials, such as corn cobs and hardwood shavings do not (Vesell, 1968, Weichbrod *et al*., 1988). If chickens are similarly affected, then induction of hepatic DME activities by litter/bedding, such as pine shavings, would be expected to alter the metabolism of drugs administered to broilers. This effect might be observed as a reduction in the drug residues within the required withdrawal period. Conversely, non-inducing litter types could result in decreased hepatic DME activities with drug residues increased within the withdrawal period. Except for birds raised in brooders for experimental studies, one would expect that the majority of chickens would be exposed to some type of bedding. At the present time, pine shavings are the most common litter used. Therefore one might predict that DME in birds raised in this way would be "induced", if we use rodents as a comparison. Therefore any other bedding, except cedar chips, would be expected to act as a "non-inducer" and hence, birds raised under these conditions might be expected to have lower levels of enzymatic activity.

Since producers would be expected to use whatever litter-type(s) readily available in their region, it is conceivable they could use a different litter than that used in studies conducted to approve a therapeutic drug used to treat some disease. If the drug approval studies were conducted with litter-types that induce drug metabolism (e.g. pine shavings), then use of litter-types that do not induce hepatic DME activities may actually result in increased drug residues in treated animals. Consequently the drug depletion times would have to be increased relative to that of animals used during the approval studies. If the converse occurs, i.e. inducing litter-types are used by the producer when the approval is based on studies using non-inducing litter, then this situation might result in decreased drug bioavailability, and possibly loss of efficacy, if the drug level is below the minimal therapeutic concentration.

We hypothesized that chicks raised on "non-inducing" litter types, such as, wire, paper, corn cobs, hardwood, or rice hulls would have lower hepatic DME activities and that the marker residue of the drug, enrofloxacin, would be higher in these animals when compared with birds raised on either pine or cedar shavings. The overall goal of these studies was to determine if environmental factors - in this case, the litter - can affect the metabolism and disposition of drugs used in chickens.

Materials and Methods

Litter types: Besides raised 3/8" wire mesh, pine shavings (King Forest Industries Inc., Wentworth, NH), cedar shavings (LM Animal Farms, Inc. Pleasant Plain, OH), aspen shavings (Beta-Chip, heat-treated hardwood, Quality Lab Products, Elkridge, MD), corn

cobs (Bed-O'Cobs - ¼ inch, Quality Lab Products, Elkridge, MD), unground rice hulls (Riceland Foods, Stuttgart, AR), and paperchip pellets (Quality Lab Products, Elkridge, MD) were used as beddings/litter. Each of the different litter types was applied to the pen floors at a depth of ~3 inches. The bedding was top-dressed with about 1 inch of bedding as needed.

Animals: The study protocol was approved by the CVM/OR Animal Care and Use Committee. Day-old, male chicks (Hubbard X Hubbard; 37-43g) obtained from Hubbard ISA (Statesville, NC) were raised in an environmentally-controlled facility (~25°C) in open pens containing either wire-mesh (3/8") or one of the various litter-types identified above. The pens were arranged so that those containing aromatic wood shavings (pine and cedar) were separated by an empty pen on either side to prevent cross contamination. There were two phases to this study. The first was to determine the effects of litter on hepatic DME activities and CYP isoforms in broilers and the second was to determine if the litter used had an effect on drug residue levels. In this case the drug used was an oral formulation of enrofloxacin. For the second phase, one side of the building was assigned to those pens destined to be the untreated control groups and the opposite side to the treated groups. At the beginning of the experiment, each pen held at least 40 chicks. At weekly intervals, two chicks per pen were removed to measure body weight and for collection of livers for determination of hepatic DME activities. Liver and total body weights of the pooled groups were recorded.

Diet: All chicks received the same basal chick diet provided *ad libitum*. The diet was composed of the following ingredients as a percentage of the total: ground corn (58%), soybean meal (48%; 31% of diet), fish meal (5.0%), soybean oil (2.5%), dicalcium phosphate (2.0%), limestone (0.5%), salt (0.4%), dl-methionine (0.2%), mineral mix (0.2%), vitamin mix (0.1%), and choline-HCl (50%, 0.1% of diet). Water was also available *ad libitum* via use of autowaterers.

Chemicals and Reagents: Enrofloxacin, supplied as Baytril 3.23% Concentrate Antimicrobial Solution (T&H Distributors, Harrisonburg, VA), was administered to all the birds on one side of the building (designated as the test group) at a concentration of 50 ppm in drinking water after the fifth week of age for five consecutive days followed by a two-day withdrawal. Aliquots of the dosed drinking water were tested using both UV spectra and HPLC analyses to verify proper concentration of dosage; drinking water from the untreated birds was used as the negative control.

All of the following chemicals were of reagent grade and obtained from Sigma Chemical Co. (St. Louis, MO) (NADPH, NADP, NADH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, cytochrome c, phenacetin, 7-ethoxy, 7-methoxy, and 7-pentoxy resorufins, coumarin, tolbutamide, DL-propranolol, dextromethorphan, chlorzoxazone, testosterone and 6ß-hydroxytestosterone, androstenedione, cortisol and 6ß-hydroxycortisol, erythromycin, lauric acid and 12-hydroxylauric acid, 7-ethoxycoumarin and 7-methoxy-4-methylcoumarin, 7-hydroxycoumarin, 4-methyl-7-hydroxycoumarin, formaldehyde, aminopyrine, 4-nitrophenol, 4-nitrocatechol, 4-aminophenol, and aniline hydrochloride). 7-Benzyloxyresorufin was supplied by Molecular Probes, Inc (Eugene, OR). Lancaster Synthesis, Inc. (Windham, NH) supplied the 7-ethoxy-4-methyl coumarin. Gentest (Woburn MA) was the source for the following metabolite standards: 4'-hydroxydiclofenac, 4-hydroxymephenytoin, 4'-hydroxybufuralol, and 6-hydroxychlorzoxazone. Others, such as dextrorphan and 3-methoxymorphinan, were generously supplied by Hoffmann-LaRoche, Inc. (Nutley, NJ); Bayer Corp. (Shawnee Mission, KS) supplied chromatography standards for enrofloxacin, ciprofloxacin, desethylenyl-ciprofloxacin and desethylenyl-enrofloxacin. Bicinchoninic acid reagents for protein determinations were supplied by Pierce Chemicals Inc.(Rockford, IL). Anti-human 2A6 and the corresponding recombinant human CYP2A6 standard, in addition to anti-rat 2E1 and liver microsomes from acetone-treated rats were obtained from Gentest (Woburn MA) and Zenotech (Kansas City, KS), respectively. Gentest also supplied polyclonal goat anti-rat 2C6 and rat CYP2C6, anti-CPY2B1/2 and CYP3A1/2 antibodies and liver microsomes from phenobarbital-, and dexamethasone-treated rats, and a recombinant human CYP2B6 standard. Oxford Research Laboratories (Oxford, MI) also supplied anti-rat CYP2B1/2 antibodies, liver microsomes from phenobarbital-treated rats and a recombinant human CYP2B6 standard. The specificity of these antibody preparations has been documented previously (Myers *et al.*, 2001).

Tissue preparation and assay procedures: The birds were killed by cervical dislocation. The entire liver was taken and the gall bladder removed, and then the pooled livers were put on ice. After the week of drug treatment (6 week-old chicks), sections of breast muscle (~30g/chick) were also collected. Supernatant fractions (10,000x*g*, S10) prepared from 20% liver homogenates were stored in 2ml aliquots at -80°C. Microsomal and cytosolic fractions were prepared by differential centrifugation at 10,000x*g* then at 100,000x*g* followed by re-precipitation with PEG8000 as described previously (Kawalek and El Said, 1994). Aliquots from each subfraction were analyzed for protein concentration using bicinchoninic acid with bovine serum albumen as a standard

according to the method of the manufacturer (Pierce Biochemical Co.).

In addition to measuring levels of the hepatic DME proteins as described before (Kawalek and El Said 1994, Myers *et al.*, 2001), *in vitro* metabolism of classical DME substrates specifically associated with CYP (P450) isoforms 1A (methoxy- and ethoxyresorufin O-dealkylases), 2A (coumarin hydroxylase), 2B (methoxy- and ethoxy-methyl coumarin O-dealkylases, pentoxy-, and benzyloxyresorufin O-dealkylases), 2C (diclofenac hydroxylase), 2D (propranolol and bufuralol hydroxylases, and dextromethorphan O-demethylase), 2E (*p*-nitrophenol hydroxylase), 3A (erythromycin and dextromethorphan N-demethylases, and cortisol 6ß-hydroxylase), and 4A (lauric acid 12- hydroxylase) were tested (Nelson *et al.*, 1996; Omiecinski *et al.*, 1999, Soucek and Gut, 1992; Wrighton and Stevens, 1992). Multiple substrates were assayed for most of the isoforms to provide alternative substrates in the event one or the other were not actively metabolized. Samples from each of the 5-week-old test groups were used to measure levels of CYP isoforms by Western blotting.

Unless specified otherwise, all assays were run with 1 mL reaction mixtures containing: 0.1M potassium phosphate buffer (pH 7.4), an NADPH-generating system (0.1mM NADP, 1mM glucose-6-phosphate, 1 IU glucose-6-phosphate dehydrogenase, and 3mM $MgCl_2$); substrate concentrations were all saturated (0.1 - 1mM). All enzymatic assays were conducted using 0.5-2.5 mg 10,000 x g supernatant (S10) fractions in triplicate under conditions that were linear with respect to protein concentration and time. Previous studies have indicated that there were no significant differences when the P450-mediated reactions measured here were run using either microsomes or S10 preparations (Myers *et al.*, 2001). Those assays that required spectrophotometric or fluorometric measurements were analyzed using a Spectramax (Molecular Devices, Inc.) or an HTS7000 (PE Biosystems, Inc.) micro-titre plate reader, respectively. HPLC analyses were run using either one of two HPLC systems: a) an HP1090M with diode array detector and an HP1046 fluorescence detector with system control and data analysis by an HP Chemstation (version A.06), or b) a ThermoSeparation Products modular HPLC with both UV and fluorescence detection in which instrument control and data analysis were under control of Chromquest chromatography data system (version 3.5).

p-Nitrophenol hydroxylase assays (Koop, 1986), aminopyrine and erythromycin N-demethylases and aniline hydroxylase assays, and the O-dealkylase activities with alkoxycoumarins and alkoxy-resorufins were run as described previously (Kawalek and El Said, 1994). HPLC analysis conditions for the other assays have been described in detail previously (Myers *et al.*, 2001).

Absorbance and fluorescence units, which were the units of measure for the spectroscopic and fluorometric assays were converted to nmoles product formed using concurrently generated external standard curves. Area unit integration data from HPLC analyses were also compared against external calibration curves which were run with each set of analyses. Nmoles products formed per unit time were normalized by dividing by the amount of S10 protein assayed to determine the specific activity (nmol/min/mg). Data from replicates were averaged. Results are reported as means±S.D. for individual groups.

Western blot analysis: Chicken liver microsomes (10µg protein/lane) were subjected to Western Blot analysis as previously described (Myers *et al.*, 2001). The primary antibodies were used at final titers of 1:500 or 1:1000, diluted in Dulbecco's PBS (Ca^{++} & Mg^{++} free) containing 5% BSA (5%BSA-PBS), according to the manufacturers' recommendations. The secondary alkaline phosphatase conjugated antibodies (KPL, Gaithersburg, MD) were used at a concentration of 1: 1000, diluted in 5%BSA-PBS. The developed Western Blots were subjected to densitometric analysis to determine relative intensity of staining using a Molecular Dynamics gel scanner (Model PD, Sunnyvale CA). The resulting images were quantitated using ImageQuant (Molecular Dynamics). The raw densitometry analysis results are in relative absorbance units (RAU). To facilitate comparison of the Western Blot analyses with the enzymology results, the RAU values were normalized to mg of S10 protein.

Tissue residue analyses: Enrofloxacin residues in the target tissue, breast muscle, were measured using a method supplied by the manufacturer, Bayer Corporation. In short the method involved homogenization/extraction of a 2 g muscle sample with 15 mL of a mixture containing ethanol/water/acetic acid (98:1:1) at medium speed for 1 min using a Tekmar Tissumizer® Microprobe. After centrifugation at 1700 x g, the supernatant was applied to a previously - conditioned SCX-Bond-Elute® (1g, 6 mL) cartridge. The cartridges were washed successively with 10 mL methanol, 20 mL water, and again with 20 mL methanol. The cartridges were eluted with 10 mL concentrated aqueous ammonia/methanol (1:3). An aliquot of the eluate (0.5 mL) was dried under nitrogen at 35C to remove the organic solvent. Acetonitrile (0.5 mL) was added and the sample taken to dryness. The dried sample was reconstituted with 2 mL HPLC mobile phase (20% acetonitrile in 10 mM triethylamine (TEA) buffer, pH 3 (adjusted using phosphoric acid). Samples were separated on a Zorbax Rx-C8 column (5µm, 250*4.6mm + 12.5*4mm guard) using 20% acetonitrile in 10mM TEA, pH 3 at a flow rate of 1 mL/min. Detection

Table 1: Effect of litter-type on Body and Organ Weights and Drug Metabolizing Enzyme Activities in Five Week-old Chicks

Parameter	p<0.05	Wire[a]	Paper	Corncobs	Hardwood	Rice Hulls	Cedar	Pine	Pooled (N=28)
Body Wt[b]	NSD	1437±420	1579±350	1544±444	1476±571	1627±462	1669±411	1700±484	1576±410
Liver Wt[c]	NSD	36±9	46±6	52±12	48±14	38±9	50±16	43±14	45±12
S-10[d]	NSD	21.4±3.6	19.6±2.0	19.2±2.2	20.5±4.8	20.7±3.1	19.5±2.5	20.5±3.2	20±2.9
Microsomes[e]	NSD	3.9±1.6	3.4±1.0	3.3±1.2	3.1±1.0	3.2±0.9	3.4±1.2	3.5±1.5	3.4±1.1
Cytosol[f]	NSD	11.4±5.5	10.5±4.4	10.3±4.6	10.7±4.8	11.0±4.6	11.2±4.9	10.7±4.7	10.8 ±4.2
b5g,[#]	NSD	0.17±0.02	0.14±0.04	0.11±0.03	0.17±0.06	0.17±0.04	0.12±0.04	0.19±0.05	0.15±0.05
P450 Red[h]	NSD	73±21	68±16	62±20	85±12	80±20	59±14	74±26	72 ±19
b5 Red[i]	NSD	1.2±0.9	1.6±1.0	1.2±0.4	1.2±0.7	1.2±0.8	1.1±0.6	1.1±0.3	1.2±0.7
GSH-S-transferase[j]	NSD	716±239	811±360	611±233	868±410	1010±614	1070±646	638±140	818±404

[a]Mean±SD (n=4); [b]average weight (gm) of four birds (two/pen) for each group; [c]average liver weight (gm) of four birds (two/pen) for each group; [d]mg protein/mL 10 000xg supernatant; mg [e]protein/mL microsomes; mg [f]protein/mL cytosol; nmol b5/mg; nmol/min/mg microsomes; [i]mol/min/mg microsomes; [j]nmol DNCB-SG /min/mg cytosol. [#]P450 was not detected in these microsomal suspensions

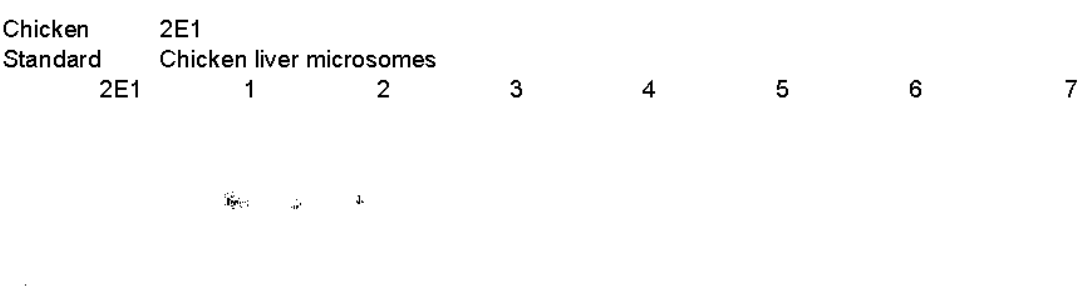

Fig. 1: Western Blot of Chicken Liver Microsomes against anti-CYP2E. Ten :g aliquots of microsomes from five week-old chicks were treated with anti-CYP2E prepared against rat CYP2E1. Lanes 1-7 represent samples from chicks raised on wire (1), pine (2), corncobs (3), hardwood (4), cedar (5), rice hulls (6), and paper (7).

was based on fluorescence (Ex280nm/Em440nm). Chromatography was performed on a modular HP1050LC system using HP Chemstation version A.06 for instrument control and data acquisition.

Enrofloxacin and its metabolites were also analyzed in portions of liver homogenates left over from preparation of subcellular fractions. The samples (1 mL) were treated with 0.2 mL of 10% perchloric acid then incubated at 60°C for one hour. Afterwards, 0.2 mL 2M KCl was added, and tubes put in a freezer (-20°C) for at least 15 min to initiate precipitation of excess perchlorate ion as potassium perchlorate. After centrifuging to remove protein/perchlorate salt precipitates, the supernatants were analyzed by HPLC (Thermo Separations described above) using a Hypersil-ODS column (5 :m (150 x 4.5 mm) + (20 x 4.5mm) guard) at room temperature. The column was eluted with a mobile phase containing 15% acetonitrile in 0.45% triethylamine/ 50mM phosphate buffer, pH 3, with a flow rate of 1 mL/min. Detection was at Ex275nm/Em440nm. Retention times were 3.84 min for desethylene ciprofloxacin, 4.82 min for desethylene enrofloxacin, 5.62 min for ciprofloxacin, and 7.33 min for enrofloxacin.

Data evaluation and statistical analysis: Data transformations were performed using Excel 97. One- and two-way ANOVA and Tukey's multiple comparison tests ($p < 0.05$) were used to evaluate differences between treated groups using SigmaStat 2.03.

Results and Discussion

Specimens from only the four and five week-old animals were analyzed for CYP isoforms and their associated activities and the results pooled. Previous experiments indicated that there were no significant differences in some hepatic CYP-mediated reactions between 4- and 5-week-old chicks (Kawalek, unpublished observations). The metabolic data from the individual groups, along with data, pooled from all the test groups, are summarized in Table 1-3. There was considerable variation in measured levels of CYP isoforms in samples from the various litter/bedding groups (Table 2). Examples of this variability were observed in the protein levels of CYPs 2E and 2C (Fig. 1 and 2, respectively). Also, there were large variances within the litter-type test groups for all the measured parameters and as a consequence there were no statistically significant differences among these test groups. Therefore we could not draw any conclusions about the effects of litter on hepatic DME activities or on the distribution of various CYP isoforms in broilers.

Even though there were no significant differences detected among the different test groups, several of the

Table 2: Effect of litter-type on CYP Isoforms in Five Week-old Chicks

CYP Isoform	p<0.05	Wire[a]	Paper	Corncobs	Hardwood	Rice Hulls	Cedar	Pine	Pooled *
CYP1A1	NSD	2882,890	1953,1144	910,1289	992,816	2532,919	1180,1234	1225,2326	1449±681
CYP1A2	NSD	4599,4092	5248,1423	1594,3459	1195,2824	2471,2595	1288,4721	1372,3649	2895±1415
CYP2B1	NSD	945, 0	1087,478	418,84	477,0	1126,0	260,1235	0, 1019	5095±479
CYP2B2	NSD	2512,3571	3143,1746	1440,2457	1428,2855	2312,3133	1155,3410	2321,2082	2398±768
CYP2C6	NSD	1528,998	1169,381	746,501	746,1057	1185,846	428,926	458,695	833±335
CYP2C8/9	NSD	887,706	582,77	827,361	1001,400	894,437	518,983	527,966	655±283
CYP2C11	NSD	479,768	799,285	239,345	310,694	363178	95,526	487,324	421±216
CYP2C13	NSD	334,526	33,258	92,350	222,109	331,141	205,320	279,53	232±138
CYP2E	NSD	4001,664	1846,1573	1024,742	1394,1033	3151,1311	997,2001	687,2706	1652±1006
CYP3A-1	NSD	1507,1774	2279,1104	506,1384	161,953	995,488	382,2230	932,1330	1145±655
CYP3A-2	NSD	562,953	856,515	393,769	250,765	564,752	309,1016	584,639	638±230

[a]Units = Relative Volume/mg S10 protein; averaged data from two birds pooled from individual pens; NSD = no significant differences. *Data averaged from all seven test groups.

Fig. 2: Western Blot of Chicken Liver Microsomes against anti-CYP2C. Ten µg aliquots of microsomes from five week-old chicks were treated with anti-CYP2C prepared against rat CYP2C. Lanes 1-7 represent samples from chicks raised on wire (1), pine (2), corncobs (3), hardwood (4), cedar (5), rice hulls (6), and paper (7).

measured CYP-mediated reactions exhibited much higher levels of activity than the majority of the others. Specifically, COH (21.2±8.4 nmol/min/mg S10 protein), ETOC (605±271 nmol/min/mg S10 protein), ETOMC (243±170 nmol/min/mg S10 protein), and MOMC (248±166 nmol/min/mg S10 protein) were at least one order of magnitude greater than any of the other (0.05 - 2.2 nmol/min/mg S10 protein) CYP-mediated reactions measured (Table 3). It was also interesting to note that diclofenac was hydroxylated to form the three major metabolites, namely, 3-OH, 5-OH, and 4'-OH diclofenates, in the same manner as rats. In humans, 4'-OH-diclofenate is the major CYP2C8/9 metabolite. In pigs, formation of the 4'-metabolite is minimal, whereas the 3-OH- and 5-OH- metabolites are formed at much higher levels (Myers *et al.*, 2001).

In the second phase of this study, we were expecting to show that different litter types would have an effect on the residue levels of a therapeutically-administered drug. Marker tissue samples (breast muscle) were taken after a five-day therapeutic regimen with enrofloxacin. Only trace amounts of the marker residue, enrofloxacin, were detectable in the target tissue; these residue levels were well below the level of quantitation. Analysis of liver homogenates using a procedure used to measure enrofloxacin metabolism indicated the presence of peaks corresponding to enrofloxacin metabolites. These peaks which co-chromatographed with desethylene-ciprofloxacin and desethylene-enrofloxacin were detectable but not quantifiable (Fig. 3). These metabolites were only present in the "treated" group and were not seen in the untreated "control" groups.

Much of the earlier work related to measurement of CYP isoforms in this species has relied on studies using inducers of hepatic drug-metabolizing enzymes (primarily TCDD, ß-naphthoflavone, and phenobarbital) (Gupta *et al.*, 1990; Gupta and Abou-Dania, 1992 and 1998) and in many instances have centered on the use of chick embryos or hepatocytes derived from this developmental state (Mahajan and Rifkind, 1999, Ourlin *et al.*, 2000; Baader *et al.*, 2002; Ourlin *et al.*, 2002). There are several instances where adult animals were studied and these usually entailed species comparisons (Dalvi *et al.*, 1987; Souhili El Amri *et al.*, 1986; Govindwar and Adav, 1999; Verbrugge *et al.*, 2001; Nebbia *et al.*, 2001; Carre *et al.*, 2002; Khalil *et al.*, 2001). Other studies (Coulet *et al.*, 1996; Khalil *et al.*, 2001) demonstrated the ability of subfractions from chicken liver to metabolize various classical substrates of hepatic DME. The ontogenic development of these enzyme systems in 3, 6, 9, and 12 week-old males showed that CYP1A2, CYP2B4, CYP2C7, and CYP3A4

Table 3: Effect of litter-type on CYP-mediated Activities in Five Week-old Chicks

Parameter	CYP Isoform	p<0.05	Wire[a]	Paper	Corn cobs	Hardwood	Rice Hulls	Cedar	Pine	Pooled *
ETOC[b]	1A2, 2A	NSD	794±283	620±355	591±258	468±187	558±349	581±302	626±251	605±271
ERF	1A	NSD	2.7±2.6	2.1±1.8	2.1±2.0	1.8±1.6	1.6±1.3	1.9±1.8	2.2±2.1	2.1±1.7
PHC	1A2	NSD	0.4±0.2	0.5±0.2	0.5±0.2	0.3±0.1	0.3±0.1	0.4±0.2	0.4±0.1	0.4±0.2
COH	2A	NSD	29.7±4.6	23.4±9.0	21.4±7.8	15.7±1.3	15.9±7.1	21.3±13.9	21.3±6.1	21.2±8.4
AMP	2B1/2, 3A	NSD	0.2±0.1	0.1±0.1	0.2±0.1	0.1±0.1	0.8±1.5	0.2±0.2	0.2±0.1	0.2±0.6
MRF	2B1/2	NSD	3.0±3.2	2.0±1.9	2.3±2.7	2.2±2.3	1.4±1.3	2.4±2.4	2.6±2.5	2.3±2.2
PRF	2B1/2	NSD	0.6±0.7	0.6±0.6	0.7±0.6	0.6±0.4	0.6±0.5	0.7±0.6	0.7±0.6	0.7±0.5
BRF	2B1/2	NSD	1.6±1.6	2.2±2.2	2.2±2.3	2.4±2.7	2.5±2.6	2.2±2.7	2.1±2.4	2.2±2.1
ETOMC	2B3	NSD	338±252	219±139	236±184	191±145	201±142	253±207	264±201	243±170
MOMC	2B3	NSD	351±251	227±130	244±182	193±134	206±146	250±193	265±190	248±166
4'-OH-DIC	2C8/9	NSD	0.2±0.0	0.2±0.1	0.1±0.0	0.1±0.0	0.2±0.1	0.2±0.1	0.2±0.0	0.1±0.1
3/5-OH-DIC	2C8/9	NSD	0.5±0.1	0.6±0.0	0.4±0.0	0.5±0.3	0.4±0.2	0.3±0.3	0.4±0.1	0.2±0.3
BUF-OH	2D	NSD	0.1±0.0	0.1±0.0	0.1±0.0	0.04±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0
Dextrorphan	2D	NSD	0.6±0.1	0.4±0.2	0.4±0.2	0.3±0.1	0.4±0.2	0.5±0.4	0.4±0.2	0.4±0.2
4-OH PPL	2D	NSD	0.5±0.5	0.4±0.4	0.4±0.4	0.3±0.2	0.4±0.4	0.4±0.4	0.4±0.3	0.4±0.3
DIP-PPL	2D	NSD	1.6±1.9	0.7±0.8	0.7±0.8	0.6±0.6	0.8±0.9	0.9±1.3	0.8±0.9	0.9±1.0
PNP	2E	NSD	0.1±0.0	0.2±0.2	0.1±0.1	0.1±0.1	0.04±0.0	0.1±0.1	0.1±0.1	0.1±0.1
3-MM	3A	NSD	0.4±0.3	0.2±0.1	0.2±0.1	0.1±0.1	0.2±0.1	0.2±0.2	0.2±0.1	0.2±0.2
ERY	3A	NSD	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1
6-OH-cortisol	3A	NSD	0.03±0.0	0.1±0.0	0.1±0.0	0.04±0.0	0.04±0.0	0.1±0.0	0.04± 0.0	0.04±0.0
12-OH-LA	4A	NSD	0.8±0.8	0.5±0.5	0.8±0.6	0.6±0.4	0.4±0.3	0.5±0.4	0.4±0.5	0.6±0.5

[a]Units = nmol/min/mg S10 protein; Mean±SD (n=4); [b]ETOC = 7-ethoxycoumarin-O-deethylase, ERF = 7-ethoxyresorufin-O-deethylase, PHC = phenacetin-O-deethylase, COH = coumarin-7-hydroxylase, AMP = aminopyrine-N-demethylase, MRF = 7-methoxyresorufin-O-demethylase, PRF = 7-pentoxyresorufin-O-dealkylase, BRF = 7-benzyloxyresorufin-O-dealkylase, ETOMC = 7-ethoxy-4-methylcoumarin-O-deethylase, MOMC = 7-methoxy-4-methylcoumarin-O-demethylase, 4'-OH-DIC = diclofenate-4'-hydroxylase, X-OH-DIC = diclofenate-3-and/or 5-hydroxylases, BUF-OH = bufuralol-4'-hydroxylase, 4-OH-PPL = propranolol-4-hydroxylase, DIP-PPL = propranolol-N-desisopropylase, 3-MM = dextromethorphan-N-demethylase, dextrorphan = dextromethorphan-O-demethylase, ERY = erythromycin-N-demethylase, 12-OH-LA = lauric acid. 12-hydroxylase. *Data averaged from all seven test groups.

isoforms were constitutively expressed (Coulet *et al.*, 1996). In contrast to these results, we were able to detect isoforms corresponding to essentially all the major human or rat CYP isoforms (CYPs 1A1, 1A2, 2B1, 2B2, 2C6, 2C8/9, 2C11, 2C13, 2E, 3A1, and 3A2) using commercially available antibodies. Since the source of the anti-CYP preparations used by Coulet *et al.*, 1996, was not identified, it is difficult to ascertain if lack of correlation with our results is simply a matter of using a different antibody. We have previously shown in swine that one can obtain different responses depending on the antibody preparation used (Myers *et al.*, 2001).

Selected enzymatic activities of CYPs 1A, 2C9, 2C19, 2D, and 3A were compared in adult chickens and dogs (Khalil *et al.*, 2001). Each of the substrates used in their study were actively metabolized by the chicken microsomal fractions. The most notable observation was the very high levels of tolbutamide hydroxylation.

The finding that residue levels of enrofloxacin in phase 2 were barely detectable (Fig. 3) was quite puzzling. Sufficient quantities of medicated drinking water

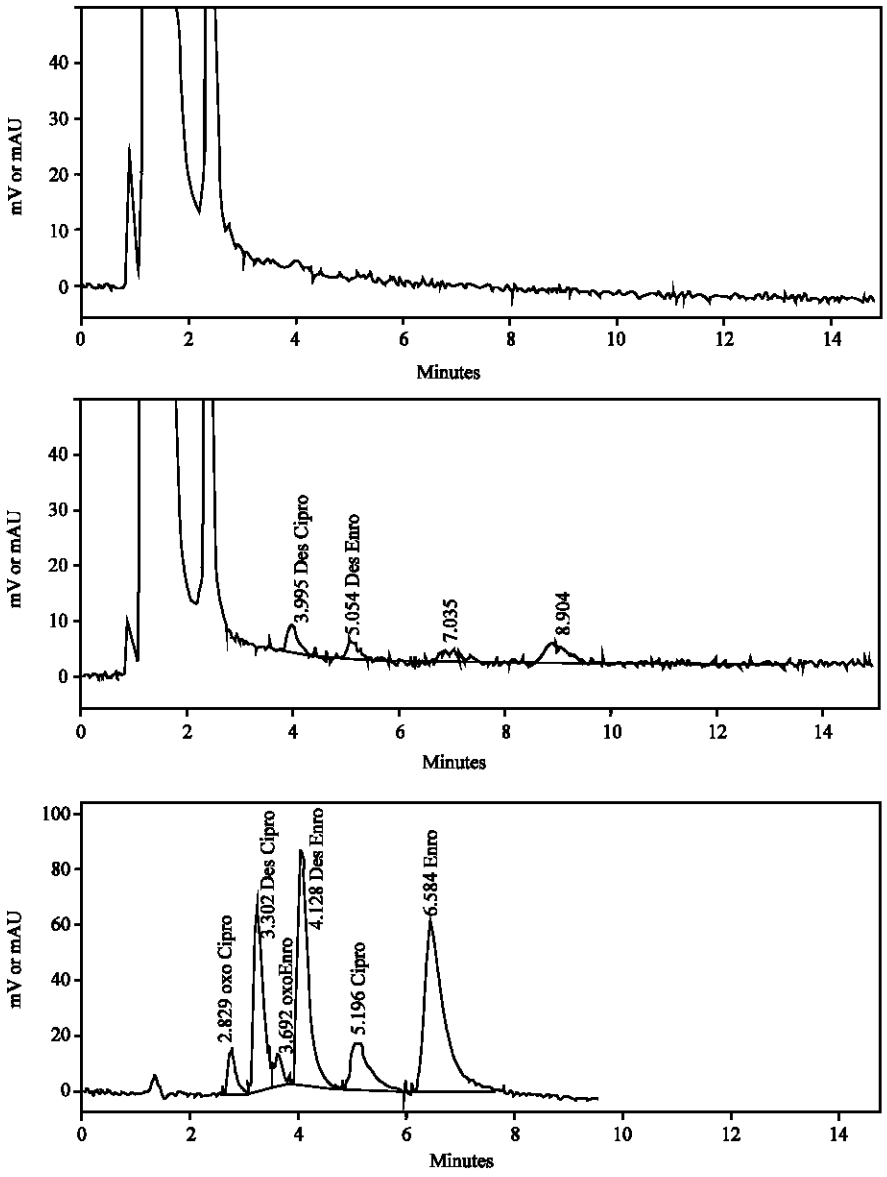

Fig. 3: High-pressure liquid chromatographs which are representative of extracts of liver homogenates from six week-old chicks. Top chromatogram is from untreated "control" chicks. Middle chromatogram is from chicks treated with Baytril in their drinking water for five days followed by untreated water for two days. Lower chromatogram is of a mixture of standards. Chromatography was performed using a Thermo Separations Modular liquid chromatograph that is described in the Methods Section.

were prepared each morning and it was consumed during the course of the day with no evidence of loss or spillage. Preparation of the correct dosage was verified using samples taken daily for HPLC analysis. Since we followed label instructions as per dosing and withdrawal time, we can only assume that the birds used in this study were able to efficiently eliminate the drug with resultant residues being far below established tolerances.

Acknowledgments

We would like to thank Mark Henderson, Mark McDonald and Sam Howard for their assistance in providing daily care for the birds and during the necropsy procedures.

References

Baader, M., C. Gnerre, J.J. Stegeman and U.A. Meyer, 2002. Transcriptional activation of cytochrome P450 CYP2C45 by drugs is mediated by the chicken xenobiotic receptor (CXR) interacting with a phenobarbital response enhancer unit. J. Biol. Chem., 277: 15647-15653.

Carre, W., E. Bourneuf, M. Douaire and C. Diot, 2002. Differential expression and genetic variation of hepatic messenger RNAs from genetically lean and fat chickens. Gene, 299: 235-243.

Coulet, M., C. Eeckhoutte and P. Galtier, 1996. Ontogenic development of drug-metabolizing enzymes in male chicken liver. Can. J. Physiol. Pharmacol., 74: 32-37.

Dalvi, R.R., V.A. Nunn and J. Juskevich, 1987. Studies on the induction and activity of microsomal drug-metabolizing enzymes in quail, ducks, geese, chickens, turkeys and rats. Comp. Biochem. Physiol. C Pharmacol. Toxicol., 87: 421-424.

Ferguson, H.C., 1966. Effect of red cedar chip bedding on hexobarbital and pentobarbital sleep time. J. Pharm. Sci., 55: 1142.

Govindwar, S.P. and S.S. Adav, 1999. Effect of aflatoxin B1 on the hepatic microsomal mixed function oxidase system during phenobarbital and benzo (a) pyene treatment in chickens. Vet. Human Toxicol., 41: 210-212.

Gupta, R.P. and M.B. Abou-Dania, 1992. Ethanol-inducible microsomal aniline hydroxylase activity and cytochrome P-450 isozymes in adult hen liver. Comp. Biochem. Physiol. C, Comp. Pharmacol. Toxicol., 101C: 505-512.

Gupta, R.P., D.M. Lapadula and M.B. Abou-Dania, 1990. Purification and characterization of cytochrome P450 isozymes from ß-naphthoflavone-induced adult hen liver. Arch. Biochem. Biophys., 282: 170-182.

Gupta, R.P. and M.B. Abou-Donia, 1998. Cytochrome P450 enzymes in chickens: characteristics and induction by xenobiotics. Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol., 121: 73-83.

Kawalek, J.C. and K.R. El Said, 1994. Maturational development of drug-metabolizing enzymes in sheep. Am. J. Vet. Res., 51: 1736-1741.

Khalil, W.F., T. Saitoh, M. Shimoda and E. Kokue, 2001. *In vitro* cytochrome P450-mediated hepatic activities for five substrates in specific pathogen free chickens. J. Vet. Pharmacol. Ther., 24: 343-348.

Koop, D.R., 1986. Hydroxylation of *p*-Nitrophenol by Rabbit Ethanol-Inducible Cytochrome P-450 Isozyme 3a, Molecular Pharmacology, 29: 399-404.

Mahajan, S.S. and A.B. Rifkind, 1999. Transcriptional activation of avian CYP1A4 and CYP1A5 by 2,3,7,8-tetrachlorodibenzo-p-dioxin: differences in gene expression and regulation compared to mammalian CYP1A1 and CYP1A2. Toxicol. Appl. Pharmacol. 155:96-106.

Myers, M.J., D.E. Farrell, K.D. Howard, J. Derr and J.C. Kawalek, 2001. Identification of multiple constitutive and inducible hepatic cytochrome P450 enzymes in market weight swine. Drug Metab. Disp., 29: 908-915.

Nebbia, C., L. Ceppa, M. Dacasto, C. Nachtmann and M. Carletti, 2001. Oxidative monensin metabolism and cytochrome P450 3A content and functions in liver microsomes from horses, pigs, broiler chicks, cattle and rats. J. Vet. Pharmacol. Ther. 24: 399-403.

Nelson, D.R., L. Koymans, T. Kamataki, J.J. Stegeman, R. Feyersisen, D.J. Waxman, M.R. Waterman, O. Gotoh, R.W. Estabrook, I.C. Gunsalus and D.W. Nebert, 1996. P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. Pharmacogenetics, 6: 1-42.

Omiecinski, C.J., R.R. Remmel and V.P. Hosograhara, 1999. Forum. Concise Review of the cytochrome p450s and their roles in toxicology. Toxicol. Sci., 48: 151-156.

Ourlin, J.C., C. Handschin, M. Kaufmann and U.A. Myer, 2002. A link between cholesterol levels and phenobarbital induction of cytochrome P450. Biochem. Biophys. Res. Commun., 291: 378-384.

Ourlin, J.C., M. Baader, D. Fraser, J.R. Halpert and U.A. Myer, 2000. Cloning and functional expression of a first inducible avian cytochrome P450 of the CYP3A subfamily (CYP3A37). Arch. Biochem. Biophys., 373: 375-384.

Soucek, P. and I Gut, 1992. Cytochrome P-450 in rats: structures, functions, properties and relevant human forms. Xenobiotica, 22: 83-103.

Souhili, El Amri, H., A.M. Batt and G. Siest, 1986. Comparison of cytochromes P450 content and activities in liver microsomes of seven animal species, including man. Xenobiotica, 16: 357-358.

Verbrugge, L.A., J.P. Giesy, D.A. Verbrugge, B.R. Woodin and J.J. Stegeman, 2001. Catalytic and immunochemical properties of hepatic cytochrome P450 1A in three avian species treated with beta-naphthoflavone or isosafrole. Comp. Biochem. Physiol. C Toxicol. Pharmacol., 130: 67-83.

Vesell, E.S., 1968. Genetic and environmental factors affecting hexobarbital metabolism in mice. Ann. N.Y. Acad Sci., 151: 304-369.

Weichbrod, R.H., C.F. Cisar, J.G. Miller, R.C. Simmonds, A.P. Alvares and T-H Ueng, 1988. Effects of cage beddings on microsomal oxidative enzymes in rat liver. Lab. Anim. Sci., 38: 296-298.

Wrighton, S.A. and J.C. Stevens, 1992. The human hepatic cytochromes P450 involved in drug metabolism. Crit. Rev. Toxicol., 22: 1-21.