THE INHIBITORY EFFECTS OF THE FLUOROQUINOLONE ANTIMICROBIALS NORFLOXACIN AND ENROFLOXACIN ON HEPATIC MICROSOMAL CYTOCHROME P-450 MONOOXYGENASES IN BROILER CHICKENS

A. Shlosberg, E. Ershov*, M. Bellaiche, V. Hanji, Y. Weisman and S. Soback

Kimron Veterinary Institute, P.O.B. 12, Bet Dagan 50250, Israel

SUMMARY

The fluoroquinolone antimicrobials norfloxacin and enrofloxacin were found to inhibit hepatic microsomal cytochrome P-450 monooxygenases in the livers of broiler chickens using dosages as given in commercial flocks. Norfloxacin inhibited the process of N-demethylation of aminopyrine to a greater degree, while enrofloxacin more markedly inhibited hydroxylation of aniline.

KEY WORDS

fluoroquinolone antimicrobials, microsomal cytochrome P-450 monooxygenases, liver microsomes, broiler chickens

*Author for correspondence

INTRODUCTION

Fluoroquinolone antimicrobials are widely used in both human and veterinary medicine due to their good antibacterial activity against gram-negative bacteria and their efficacy at low dosages /1/. The first modern fluoroquinolone in veterinary practice was enrofloxacin, and today additional drugs from this group are in use for treatment of calves, pigs, dogs and poultry /2,3/. As the usage of fluoroquinolones has expanded, some reports on side effects which may have severe clinical consequences have appeared. In particular, damage to juvenile cartilage and bone was observed in dogs and horses /3/, and this was the reason for limitation of usage of a fluoroquinolone (enrofloxacin) in these species /3/. Drug interactions of quinolones, such as prolongation of prothrombin time during warfarin therapy in combination with ofloxacin, norfloxacin and ciprofloxacin (the major metabolite of enrofloxacin), inhibition of the metabolic clearance of methylxanthines, and induction of convulsions by the non-steroidal anti-inflammatory drug fenbufen, have been recorded /4/. Incompatibility of some fluoroquinolones with penicillins (flucloxacillin, amoxycillin) has also been observed /4/.

The mechanisms involved in these incompatibilities have not yet been completely determined, and these phenomena have stimulated research aimed at revealing the effects of fluoroquinolone in various species. The interactions of fluoroquinolones with some methylxanthines (theophylline, caffeine) in man and laboratory animals, whereby there is a delayed biotransformation and elimination of methylxanthines, have been the most investigated /5-7/. The interaction between fluoroquinolones and theophylline is the result of cytochrome P-450 inhibition /8/. Fluoroquinolones selectively inhibit only some of the cytochrome P-450 isozymes. For example, they are not effective inhibitors of the N-demethylation of ethylmorphine (CYP3A) but do inhibit alternative substrate metabolism by CYP1A2, the primary isozyme responsible for N3-demethylation of fluoroquinolones /9-11/. The most informative approach to assessment of the inhibitory effect of fluoroquinolones related to cytochrome P-450 1A has been made using 7-ethoxyresorufin and caffeine as substrates /12/. Besides inhibition of methylxanthine metabolism, fluoroquinolones also cause reduction in the antipyrine metabolism rate (associated with CYP1A2) and the R-warfarin oxidation rate /13,14/.

The differences in the level of inhibition of metabolism of differing substrates depend on their structural peculiarities. Alkyl substitution in the 7-piperazine ring of fluoroquinolones lowers their inhibitory activity against caffeine metabolism /15/. The planar conformations and the basicity of the 4′-nitrogen atom promote the interaction between fluoroquinolones and theophylline. The planarity is a favorable characteristic for approaching the catalytic center of CYP1A, and the basicity is a positive factor for binding to the heme iron of the enzyme /16/. The fluoroquinolone-induced inhibition of metabolism of some drugs, especially theophylline, is of a competitive nature /15/. It is known that quinolones interact at the active site of the CYP1A2 /10/. Amongst the fluoroquinolones, enoxacin, ciprofloxacin and pefloxacin interact with methylxanthines more potently than ofloxacin and norfloxacin, and almost no inhibitory effect is seen with lomefloxacin, fleroxacin, and sparfloxacin /16/. Quinolones with a low inhibitory effect (lomefloxacin, ofloxacin) were substituted at the 8-position of the aromatic ring system and had methyl substitutes in the piperazinyl ring /17/.

Investigations into the mechanisms of action of the fluoroquinolones, including hepatic microsomal cytochrome P-450 monooxygenase inhibition, have been performed mainly with laboratory animals and human liver microsomes. The study of the inhibitory effect of the fluoroquinolones on methylxanthine metabolism gave results of a surprising similarity in humans and rats. It was suggested /16/ that the rat model is the best predictor of the degree of these interactions in humans.

As broiler chickens in their short lifespan are often dosed with many drugs, sometimes simultaneously, the topic of drug interaction is of considerable veterinary, financial and scientific importance in this animal. The potential effect of fluoroquinolones on hepatic cytochrome P-450 containing microsomal enzymes in broiler chickens has not been studied sufficiently, partially due to the difficulties encountered with the specificity of their microsomal cytochrome P-450 monooxygenases. Birds have much smaller amounts of microsomal enzyme protein and cytochrome P-450 than mammals, their hepatic microsomal enzyme activity is also much less, and there are large differences between different avian species /18-21/. This study investigated the effects of the fluoroquinolone antimicrobials nor-

floxacin and enrofloxacin on hepatic microsomal cytochrome P-450 monooxygenase activity in broiler chickens.

MATERIALS AND METHODS

Chemicals

Norfloxacin nicotinate (36%, QuinAbic, Teva-Abic Ltd., Netanya, Israel) and enrofloxacin (10%, Baytril, Bayer, Germany) were the two fluoroquinolones used. Aminopyrine, aniline, nicotinamide adenine dinucleotide phosphate (reduced form), acetyl acetone, ammonium acetate and bovine serum albumin were obtained from Sigma Chemical Company (St. Louis, MO, USA).

Animals, treatments and microsomes

One day-old male commercial broiler chickens were housed in electrically heated battery brooders. A broiler starter feed was supplied *ad libitum* throughout. When the birds were 25 days old, they were weighed and birds in a weight range of 560-650 g were put into four randomly constituted groups. Group 1 served as a control and received no drug treatment. Group 2 was administered norfloxacin, delivered *per os* at a dosage of 20 mg/kg body weight daily for a 5 day period. Group 3 was administered enrofloxacin, given *per os* at a dosage of 10 mg/kg body weight daily for a 3 day period. At 1, 3, 5, 7 and 9 days after the last dose, and 24 hours after the last feed was given, six birds in the appropriate group were killed for the determination of liver microsomal cytochrome P-450 monooxygenase activity. Livers were immediately perfused with ice-cold 1.15% KCl solution from the cranial vena cava caudally, until the efferent perfusion fluid was blood-free. Determination of aminopyrine N-demethylase (AD) activity, aniline hydroxylase (AH) activity and protein was made in the 9000 *g* supernatant liver fraction. Group 4 birds were administered norfloxacin *per os* at a dosage of 20 mg/kg body weight daily for a 5 day period, and 3 days after the last treatment were given xylazine (5 mg/kg, intramuscularly) and ketamine (15 mg/kg, intramuscularly), and the duration of sleeping time was measured, as the time from loss of the righting reflex to the time the animal regained the reflex and returned to sternal recumbency /22/.

Enzyme assays

The concentration of microsomal protein was measured using bovine serum albumin as a reference standard /23/. The determination of AD activity was made by measuring the production of formaldehyde /24/, and AH activity was assayed by measuring the formation of p-aminophenol /25/.

Statistical analysis

Data were analyzed to test the difference between treatment and control groups using Student's t-test; $P<0.05$ was taken as the significance level. All tests and standard errors are based on inter-assay variation.

RESULTS

Both enrofloxacin and norfloxacin were found to inhibit microsomal cytochrome P-450 monooxygenases, with enrofloxacin being a stronger inhibitor, significantly lowering AH activity up to 9 days post treatment (Fig. 1). The activity of AH was reduced by 35, 81, 31 and 56%, respectively, 1, 3, 5 and 9 days post treatment. The inhibitory effect of AD activity developed more slowly, and restoration of the enzyme activity was more rapid. The activity of AD was significantly reduced, by 55 and 40%, respectively, 3 and 5 days post treatment. By the 9th day the AD activity rose to 42% higher than in the control birds.

Norfloxacin was seen (Fig. 2) to cause significant reductions in AD and AH activity, by 46 and 53%, respectively, only on the first day after the last treatment. In contrast to enrofloxacin, norfloxacin administration led to more rapid (by the third day) restoration of AH activity, and AD activity was restored by the 7th day.

No change in relative liver weight of chickens was observed with enrofloxacin or norfloxacin action (Table 1). The microsomal cytochrome P-450 monooxygenase inhibition caused by norfloxacin led to prolongation of xylazine-ketamine anesthesia, from 89 ± 17 minutes to 109 ± 12 minutes; one of the six birds in the norfloxacin group died.

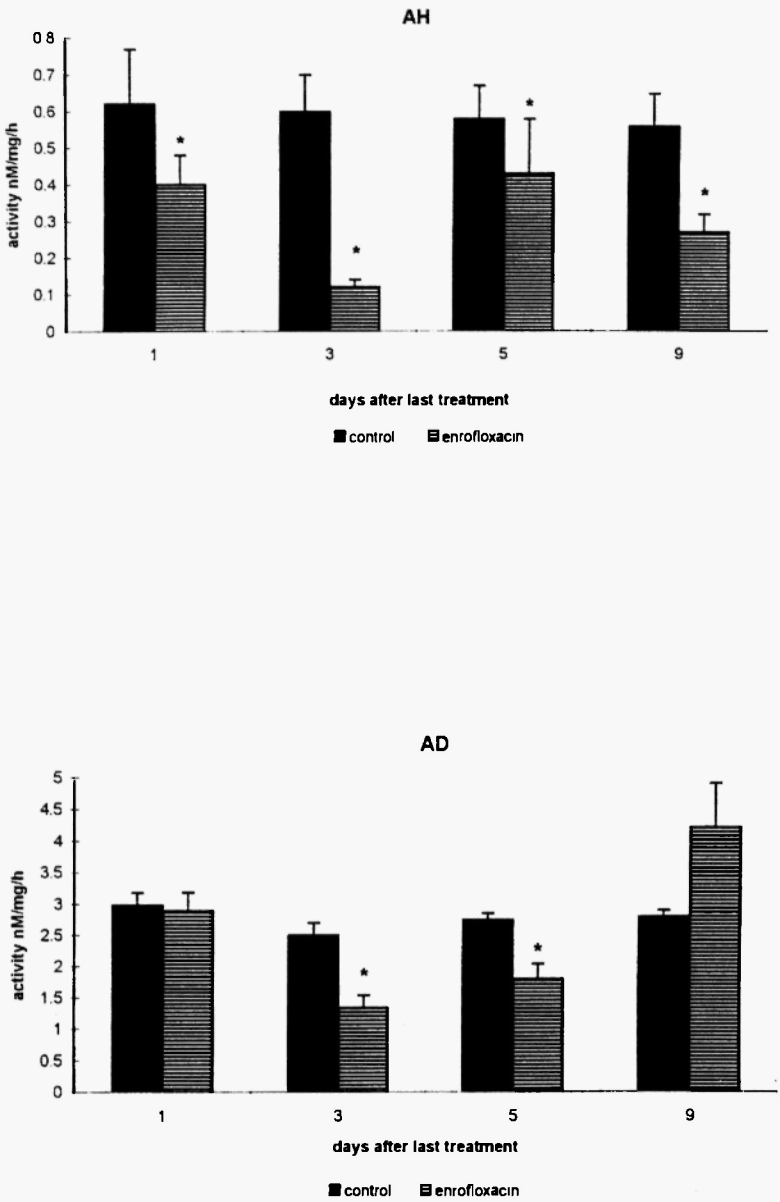

Fig. 1: Effect of enrofloxacin on aniline hydroxylase (AH) and aminopyrine N-demethylase (AD) activity in broiler chickens. * signifies significant ($p<0.05$) change.

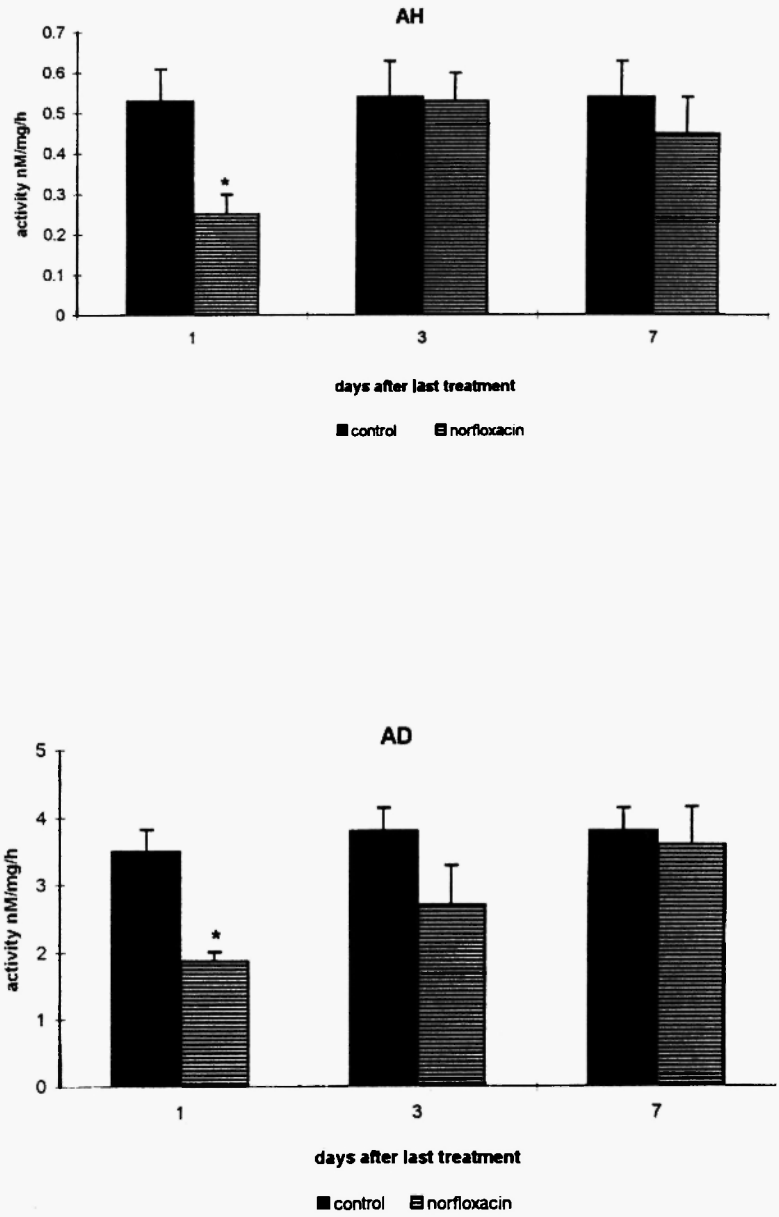

Fig. 2: Effect of norfloxacin on aniline hydroxylase (AH) and aminopyrine N-demethylase (AD) activity in broiler chickens. * signifies significant ($p<0.05$) change.

TABLE 1

Effect of enrofloxacin and norfloxacin on relative liver weight (mean ± SE) in broiler chickens (N=6 for each group)

Days after last treatment	Relative liver weight (% body weight)			
	Enrofloxacin		Norfloxacin	
	Control	Treatment	Control	Treatment
1	3.35 ± 0.10	3.17 ± 0.08	2.86 ± 0.12	2.85 ± 0.20
3	3.30 ± 0.12	3.57 ± 0.14	2.92 ± 0.10	2.91 ± 0.18
5	3.35 ± 0.10	3.28 ± 0.20	-	-
7	-	-	2.86 ± 0.12	2.80 ± 0.10
9	3.50 ± 0.13	3.40 ± 0.22	-	-

DISCUSSION

The results of this study performed with broiler chickens are in accordance with investigations in other species which showed that most of the fluoroquinolones inhibit the monooxygenase liver system. In our study, enrofloxacin and norfloxacin inhibited the processes of aminopyrine N-demethylation and aniline hydroxylation. In mammals these reactions are indices of CYP2C3 and 2E1 activities, respectively /26/. In birds the monooxygenase liver system differs significantly from that of other species /20/.

Recently the monooxygenase system of the avian liver has been more intensively studied. The interest in research on cytochrome P-450 from avian liver microsomes may be explained by the fact that avians are a convenient model for the study of cytochrome P-450 evolution, and the multiple forms of cytochrome P-450 apparently came from the same ancestor. Regions with similar sequences exist in enzymes of diverse species including prokaryotes /27/. Divergence between birds and mammals during evolution took place millions of years ago when mammalian genomes already possessed representatives of the diverse families of P-450 genes, P-450I, P-450II and P-

450III /28,29/. It has been suggested that the birds' genome possesses representatives of all three families of P-450 genes /30/.

The mammalian cytochrome P-450 isoforms have been depicted at molecular levels but the identification of this system in birds has been insufficiently studied /26/, therefore precluding the possibility of extrapolation of data obtained with mammals to birds. Little is known about the influence of chemically diverse compounds on the microsomal cytochrome P-450 monooxygenases of chickens. Immunoblot analysis of chicken hepatic microsomes by MAb 2-13-2 against rat pregnenolone-16-α-carbonitrile P-450 (PCN-P-450) showed that in the embryonic and neonatal periods two profiles could be seen /30/. It was suggested that these profiles may correspond to the development of two P-450 classes, one related to the rodent P-450II forms and another to the rodent P-450III forms. The proteins resembling the corresponding rodent P-450 isoforms were revealed in chicken microsomes with phenobarbital (PB) and methylcholanthrene (MC) used as microsomal cytochrome P-450 monooxygenase inducers /31-35/. When chicken liver was treated with polychlorinated biphenyls, two cytochrome P-450 forms were induced, P-448H and P-448L /36/. The P-448L form had a high 7-ethoxyresorufin O-deethylase activity and a low AD activity and corresponded to P-450c in rats and LM_6 in rabbits (closer to cytochrome LM_6 in rabbits). The second form, P-448H, was similar to P-450d in rats. PB caused induction in chicken hepatocytes of two P-450 isozymes, 2H1 and 2H2 (IIF1 and IIF2), and it was difficult to compare them to specific mammalian isoforms /37/. These isozymes have a 51-55% amino acid sequence similarity with some PB-induced and constitutive mammalian CYP2C (2C7) forms, but chicken CYP2H1 and 2H2 forms were induced in a response to PB-like inducing agents, as with CYP2B1 and 2B2 in rats. In contrast to 2B1 and 2B2 in rat hepatocytes, they were not inhibited by cycloheximide. Four forms of cytochrome P-450 that were induced in chicken liver by acetone or ethanol were purified and characterized /38/. Two of these forms may be attributed to CYP2H1 and CYP2H2. The third form was similar to mammalian CYP2E in its catalytic activity. It should be noted that the N-terminal amino acid sequence of this form had only a 30-33% amino acid sequence similarity with CYP2E purified from rat, rabbit and human livers. The fourth form did not correspond to any mammalian cytochrome P-450 reported to be induced by acetone according to its amino acid sequence and

enzymic activities. Most of the cytochrome P-450 isoforms induced in birds are not mammalian orthologues because they do not react immunospecifically with monoclonal antibodies directed against corresponding mammalian isozymes /29/.

Species differences exist in substrate specificity of P-450 isoenzymes induced by different compounds. For example, in hepatic tissue from chicken embryos the treatment by PB-type inducers caused induction of ethoxycoumarin and aminopyrine dealkylase activities but not of pentoxy- and benzyloxyresorufin dealkylase, as was noted with other species /39/. In experiments with rats it was shown /12/ that 7-ethoxyresorufin and caffeine may be used as marker substrates for selective inhibiton of hepatic oxidative metabolism by quinolones (namely, CYP1A2 isoenzymes inhibition). The most specific test for chicken CYP1A2 activity is considered to be uroporphyrinogen oxidation /40/.

One of the substrates used in this work was aminopyrine, which, as with some other substrates, has been routinely used with rodents and other species for measuring cytochrome P-450b activity. Unlike pentoxyresorufin, it is not a highly specific substrate for determining this P-450 isozyme, and its activity is similar to that of benzphetamine and ethylmorphine. When the AD activity of birds was determined in the embryonic and postnatal periods /41,42/, it was found that the activity peak was at the first day post-hatching and relatively high levels were also found at the 10th and 36th days post-hatching. AD activity was induced by PB treatment to a higher degree than by treatment with TCB (3,4,3′,4′-tetrachlorobiphenyl), 3-MC (3-methylcholanthrene), or TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) /41/. In our experiments AD activity seemed to be a sensitive reaction in broilers treated with enrofloxacin and norfloxacin.

The other substrate used in this work was aniline, which is also not considered to be specific because its metabolism may be connected with some cytochrome P-450 forms including cytochrome P-450 IIE. Birds have relatively high AH activity, which exceeds that of aldrin epoxidase or aminopyrine demethylase /43/. In our investigations AH activity was a sensitive indicator in broilers treated with enrofloxacin and norfloxacin. More intensive and prolonged inhibition of AH activity was observed under the influence of enrofloxacin.

The inhibiting effects of enrofloxacin and norfloxacin on the microsomal cytochrome P-450 monooxygenases may have negative

consequences with regard to a change in the metabolism of other substances. The combined action of monensin and certain sulfonamides produced such effects /44/. A similar effect was seen for xylazine-ketamine anaesthesia with various species, including broilers, when hepatic microsomal enzyme inducers and inhibitors were used /45/. Our demonstration of a tendency for norfloxacin to prolong xylazine-ketamine sleeping times gives additional data to the studies mentioned and shows the potential of modification of the metabolism of drugs used in sequence or together with norfloxacin in broilers.

These results indicate the distinct possibility of undesirable side effects of fluoroquinolone use in broilers, such as incompatibility with other drugs, change of endogenic and exogenous substrate metabolism, and even toxic effects.

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