

RESIDUES OF ENROFLOXACIN AND ITS MAIN METABOLITE CIPROFLOXACIN IN BROILER CHICKENS

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The presence of enrofloxacin residues in muscle and liver after peroral administration of enrofloxacin (10 mg/kg bw/day) to chickens was studied. Microbiological method – plate pH 8.0 with Escherichia coli NCIMB 11595 and HPLC with fluorescence detection were used for the detection of enrofloxacin residues. During the 5 days dosing period, enrofloxacin concentrations in breast muscle and liver greatly exceeded the EU MRL values. Ciprofloxacin was not detected in muscles after 2 days of dosing, but it was detected in the liver in concentrations over the MRL. During the post treatment period, enrofloxacin and ciprofloxacin concentrations in the breast muscle and liver exceeded the MRL values until the 4th day of the withdrawal period. Ciprofloxacin was not detected in the breast muscle after 4 days post dosing, but it was detected in the liver at concentrations below MRL. Four days withdrawal period is the allowed time for the drug concentration to decrease to an acceptable level in the meat and liver, prior to slaughter (below MRL). Microbiological method – plate pH 8.0 with Escherichia coli NCIMB 11595 gave positive results in all the samples which had residues above MRL values.

Key words: chickens, enrofloxacin, HPLC, microbiological method, residues

INTRODUCTION

Enrofloxacin is an antimicrobial agent developed in the late 1980s exclusively for use in veterinary medicine. Enrofloxacin, C₁₉H₂₂FN₃O₃ or (1-cyclopropyl-6-fluoro-1,4-dihydro-4oxo-7-[4-ethyl-1-piperazinyl]-3-quinoline carboxylic acid) belongs to the early second or second generation of fluoroquinolone antimicrobials, which have bactericidal activity against *Enterobacteriaceae* and other Gram-negative bacteria and have some activity against certain Gram-positive cocci (Martindale, 2005; Martinez *et al.*, 2005; Cupic *et al.*, 2002). Fluoroquinolones damage bacterial DNA and lead to defects in negative supercoiling. This effect is linked to the inhibition of DNA gyrase activity, an enzyme found in all bacteria (Prescott *et al.*, 2000). Enrofloxacin is approved for

therapeutic and prophylactic usage in poultry in Serbia and Montenegro (Jezdimirovic, 2002). Common poultry infections, such as mycoplasmal infections, colibacillosis and pasteurellosis, frequently are treated with enrofloxacin (Report WHO, 1998; Jezdimirovic, 2002; Martinez *et al.*, 2005).

After oral application, enrofloxacin is well absorbed, distributed into tissues and excreted in the urine and feces at high concentrations (Prescott *et al.*, 2000; Less and Aliabadi, 2002). Enrofloxacin is metabolised in the liver to its main metabolite ciprofloxacin and some minor metabolites (EMEA, 1998). Ciprofloxacin has also a bactericidal activity, as the parent compound it is approved in human medicine.

Phototoxic skin reactions in humans (Klecak *et al.*, 1997), chondrotoxic effects in young animals (Stahlmann *et al.*, 2000) and tendon rupture (Pierfitte *et al.*, 1995) can be induced by fluoroquinolone antimicrobials. Facultative anaerobic human intestinal microflora can be strongly suppressed with low levels of ciprofloxacin (Cerngila and Kotarski, 1999). Ho *et al.* (2003) had also reported 15 cases of allergic reactions to ciprofloxacin, some of occurred after the first usage of this quinolone.

Safe food is free of residues, meaning that levels of residues in tissues is lower than MRL according to the regulations. MRL values for fluoroquinolone antimicrobials are not established in Serbia and Montenegro. MRL values for the total sum of enrofloxacin and ciprofloxacin in EU are 100 ng/g for chicken meat and 200 ng/g for the liver (Consleg, 2002).

The aim of this study was to investigate the target tissue residues of enrofloxacin and its main metabolite ciprofloxacin, according to MRL values to eliminate health risk for the consumers. Presence of enrofloxacin and ciprofloxacin in breast muscle and liver, were detected by microbial inhibition assay and by HPLC method.

MATERIALS AND METHODS

Animals

The study was performed on 65 healthy chickens (Arbor acres), 1-day old chickens were included in the experiment. At the age of two weeks chickens were randomly divided in two groups. Group A (30 animals) was the control group. This group was not treated with antibiotics. At the age of 28 days the chickens in group B (35 animals) were given daily doses of enrofloxacin (10 mg/kg bw/day) via drinking water, for five consecutive days. In our work we used Enrocin® 10% ad us. vet. (Hemovet - Serbia and Montenegro), 1 ml of solution containing 100 mg enrofloxacin.

A number of chickens was euthanized two days before starting therapy, during therapy, during the withdrawal period and after the end of the withdrawal period. At each sampling time three chickens were euthanised, and samples of breast muscle and liver were obtained. The samples were stored at -20°C, until assayed for presence and concentration values of enrofloxacin and ciprofloxacin.

Qualitative analysis: microbiological method

Standards were purchased from Sigma Company – USA (enrofloxacin) and Bayer- Germany (ciprofloxacin). Test agar pH 8.0 was seeded with *Escherichia coli* NCIMB 11595. A working solution of *E. coli* NCIMB 11595 was made with a freshly prepared culture. The culture was diluted in peptone-salt solution to give optical density of 0.452 at 620 nm in a 10 mm cell, with the use of peptone-salt solution as a reference. Sterile Petri dishes were filled with inoculated test agar. All the plates were subjected to quality control. Paper disks containing 0.003 ciprofloxacin µg/disk (Mast Diagnostic, Mereyseaside, UK) were placed in the center of the Petri dish. Detection levels of plate pH 8.0 *E. coli* NCIMB 11 595 were previously determined (unpublished data): 50 ng/g enrofloxacin and 25 ng/g ciprofloxacin. Meat and liver were sampled while still frozen. An 8 mm diameter cork borer was used to remove a cylinder of frozen meat. The meat cylinders were cut into 2 mm thick disks. Four disks of meat were placed on opposite ends of the plate. Each sample was examined in 12 replicates. The plates were kept refrigerated for 2 hours and then incubated on 37°C for 24 h. After incubation the plates were inspected for inhibition zones around the meat disks and the average inhibition zone (IZ) for all 12 replicates was recorded (2 mm width was considered as positive).

Quantitative analysis – HPLC with fluorescence detection

Methanol, acetonitrile, n-hexane, and phosphoric acid were purchased from J. T. Baker (Holland). Enrofloxacin and ciprofloxacin analytical standards were purchased from Sigma Company – USA. All the solvents were of HPLC purity.

Liquid chromatography (Ramos *et al.*, 2003) with fluorescence detection at excitation wavelength of 280 nm and emission wavelength of 455 nm was used for the determination of enrofloxacin and ciprofloxacin residues in meat and liver. Detection limit is 10 ng/g and quantification limit is 20 ng/g.

Enrofloxacin and ciprofloxacin were detected isocratically in 7 to 10 minutes. Waters "Sunfire" column, C18, 150x4.6 mm, 3.5 µm particle size was used for separation at a flow rate of 0.8 mL/min. The mobile phase (0.01M phosphoric acid (pH 3)/acetonitrile; 80:20 v/v) was used to elute both compounds. Quantification was performed using the external standard method and the results were obtained from the calibration curve of blanks fortified at four levels.

Statistical analysis

Statistical analysis was performed by Microsoft Office Excel 2000 and statistical program SPSS for Windows 8.0.0. ANOVA were analyzed by Hadžić (1992). Screening method data were analyzed by the use of descriptive statistical methods. Differences in IZ diameters were analyzed for statistical significance by the use of ANOVA and Student's t-test. Differences of $p < 0.05$ were considered significant.

RESULTS

Breast muscle and liver samples from day 1 to day 5 of dosing and from day 1 to day 22 (post dose) were analyzed by the microbiological and HPLC methods for enrofloxacin and ciprofloxacin concentrations, and the results are shown in Tables 1-5 and figures 1-2. During the 5 days dosing period, enrofloxacin concentrations in the breast muscle and liver greatly exceeded the MRL values. Ciprofloxacin was not detected in the muscles on day 2 of dosing, but it was detected in the liver in concentrations over MRL. All the muscle and liver samples during the treatment gave positive results on plate pH 8.0 *E. coli* NCIMB 11595.

Table 1. Determination of residues by microbiological method during enrofloxacin administration (IZ in mm)

Treatment day		\bar{x}	SD	SE	Cv	Iv	t	% positive
-1	M	0	-	-	-	-	-	0.00
	L	0	-	-	-	-		0.00
2	M	12.62	1.088	0.272	8.62	3.00	9.350*	100.00
	L	15.87	0.806	0.202	5.05	2.00		100.00
5	M	13.58	1.349	0.275	9.93	5.00	5.645*	100.00
	L	15.75	1.032	0.211	6.55	3.00		100.00

M - meat; L - liver; *- significant difference ($p < 0.05$)

Table 2. Determination of residues by microbiological method after the end of treatment (IZ in mm)

Post treatment day		\bar{x}	SD	SE	Cv	Iv	t	% positive
1	M	15.00	1.461	0.365	9.74	4.00	3.721*	100.00
	L	16.19	1.276	0.319	7.88	3.00		100.00
2	M	10.46	0.658	0.134	6.29	3.00	4.338*	100.00
	L	11.96	1.301	0.266	10.88	3.00		100.00
4	M	1.42	-	-	-	9.00	-	16.67
	L	8.04	0.955	0.195	2.42	3.00		100.00
6	M	0.00	-	-	-	-	-	0.00
	L	6.75	0.989	0.202	14.65	4.00		100.00
8	M	0.00	-	-	-	-	-	0.00
	L	6.00	0.000	0.000	0.00	0.00		100.00
9	M	0.00	-	-	-	-	-	0.00
	J	6.67	2.099	0.428	31.48	5.00		100.00
11	M	0.00	-	-	-	-	-	0.00
	L	3.75	0.447	0.112	11.92	1.00		100.00
17	M	0.00	-	-	-	-	-	0.00
	L	0.00	-	-	-	-		0.00
22	M	0.00	-	-	-	-	-	0.00
	L	0.00	-	-	-	-		0.00

M - meat; L - liver; *- significant difference ($p < 0.05$)

Table 3. Statistical significance of differences IZ (mm) in chicken meat

Day	4 ^{PT}	2 ^{PT}	1 ^{PT}	5 ^T
2 ^T	2.25*	10.93*	6.39*	9.76*
5 ^T	2.23*	10.75*	15.16*	-
1 ^{PT}	11.22*	11.22*	-	-
2 ^{PT}	12.63*	-	-	-

^T - treatment day, ^{PT} - post treatment day, * - significant difference ($p < 0.05$)

Table 4. Statistical significance of differences IZ (mm) in chicken liver

Day	11 ^{PT}	9 ^{PT}	8 ^{PT}	6 ^{PT}	4 ^{PT}	2 ^{PT}	1 ^{PT}	5 ^T
2 ^T	42.27*	16.73*	48.99*	30.26*	32.32*	14.36*	1.32	1.86
5 ^T	47.98*	18.72*	46.28*	31.18*	36.25*	8.73*	2.40*	-
1 ^{PT}	29.75*	21.64*	31.92*	20.47*	32.69*	10.30*	-	-
2 ^{PT}	53.03*	13.18*	22.43*	19.35*	10.05*	-	-	-
4 ^{PT}	15.29*	3.48*	10.48*	3.95*	-	-	-	-
6 ^{PT}	10.42*	0.18	3.81*	-	-	-	-	-
8 ^{PT}	20.12*	1.56	-	-	-	-	-	-
9 ^{PT}	5.85*	-	-	-	-	-	-	-

^T - treatment day, ^{PT} - post treatment day, * - significant difference ($p < 0.05$)

Table 5. Enrofloxacin residues (ng/g) in chicken muscle and liver determined by HPLC method

Day	ENROFLOXACIN		CIPROFLOXACIN	
	meat	liver	meat	liver
-1	0.0	0.0	0.0	0.0
2 ^T	134.4	507.5	0.0	187.1
1 ^{PT}	577.8	1196.1	49.5	817.8
4 ^{PT}	28.2	54.7	0.0	68.4
6 ^{PT}	24.9	45.4	0.0	0.0
9 ^{PT}	24.8	45.2	0.0	0.0
22 ^{PT}	0.0	0.0	0.0	0.0
29 ^{PT}	/	0.0	/	0.0

^T - treatment day, ^{PT} - post treatment day, / - not examined

During the post treatment period, enrofloxacin and ciprofloxacin concentrations in breast muscle and liver exceeded the MRL values until 4 days of the withdrawal period. Ciprofloxacin was not detected in the breast muscle on 4 days post dosing, however it was detected in the liver in concentrations below MRL. All muscle samples during the post treatment gave positive results on plate pH 8.0 *E. coli* NCIMB 11595 until day 4, on that day only 16.67% muscle samples were positive. Liver samples were 100% positive until 17 days post treatment on plate pH 8.0 *E. coli* NCIMB 11595. Ciprofloxacin residues were not detected by HPLC in muscle and liver samples on day 6 post treatment and after, but enrofloxacin residues were detected in these samples until 22 days post treatment.

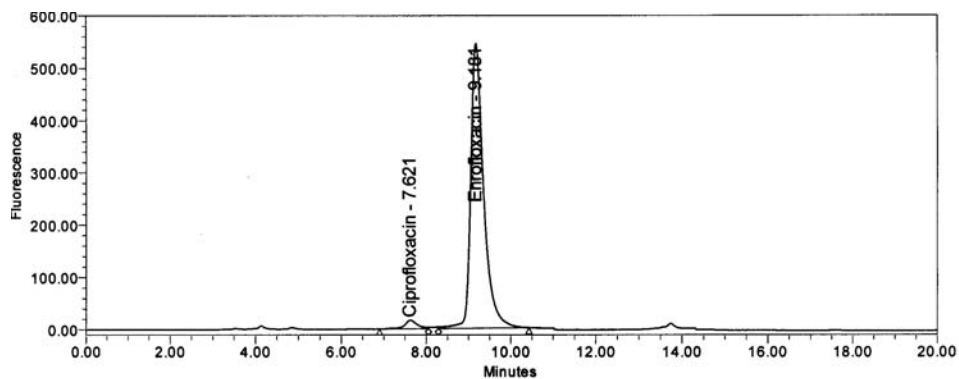


Figure 1. Chromatographic determination of enrofloxacin residues in chicken muscle – the first day of withdrawal period

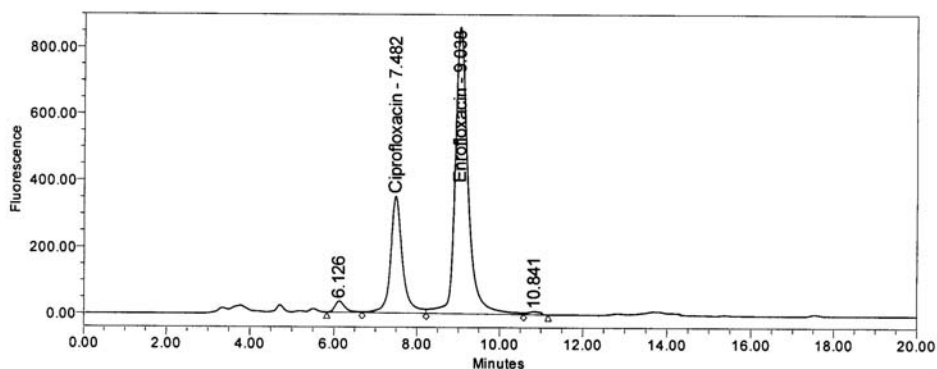


Figure 2. Chromatographic determination of enrofloxacin residues in chicken liver – the first day of withdrawal period

DISCUSSION

High concentrations of enrofloxacin in the meat and liver 24 h after the beginning of the treatment are a consequence of rapid absorption from chicken intestines (Prescott *et al.*, 2000). Peak serum concentrations occur within 72h (Anadon *et al.*, 1990). Enrofloxacin is well distributed from plasma into tissues (Anadon *et al.*, 1995) and its concentration was higher in the liver than in breast muscle in all our examinations. Enrofloxacin concentration was 3.78 times higher in the liver than in muscles 24h after the beginning of treatment. This correlation slowly decreases on the first day post treatment – 2.07 times and on day 4 post treatment 1.94 times. The decrease stopped after this period and correlation was equalized at 1.82 times. Similar results on distribution of enrofloxacin in the liver

and muscle were reported by Schneider (2001), enrofloxacin concentrations were 4-4.7 times higher in the liver during treatment and on the first day post treatment this correlation decreased 2.5 times. These results point on intensive metabolism of enrofloxacin in the liver during treatment. The drug was gradually eliminated from the chicken's body. Enrofloxacin is metabolised in the liver and transformed into the main metabolite ciprofloxacin and some minor metabolites such as: oxociprofloxacin, enrofloxacin amide, dioxociprofloxacin, desethyleneciprofloxacin, destethyleneciprofloxacin, N-formyl ciprofloxacin, oxoenrofloxacin and hydroxy oxoenrofloxacin (EMEA, 1998; Prescott *et al.*, 2000). The level of enrofloxacin transformation to ciprofloxacin is not so high. We have found that most of the residue in the liver and muscles at the end of treatment was enrofloxacin.

Anadon *et al.* (1995) treated chickens with enrofloxacin 10 mg/kg/day for four days, on the fifth day they have determined 540 ng/g enrofloxacin and 650 ng/g ciprofloxacin in the meat and 990 ng/g enrofloxacin and 960 ng/g ciprofloxacin in the liver. These authors concluded that ciprofloxacin level is always similar or greater than enrofloxacin level. In our experiment we found lower levels of ciprofloxacin in the liver and significantly lower in the breast muscle. Our findings are similar to the findings of Schneider (2001). She treated chickens with enrofloxacin 11 mg/kg/day for seven days and found 2-10 times lower levels of ciprofloxacin in the meat and liver. Knoll *et al.* (1999) reported that ciprofloxacin concentrations in chicken's organs were about 4% of enrofloxacin concentrations, except in the liver 38% and kidney 11%. Our results are close to the data published by EMEA report (1998): 85% residue in chicken meat and 66% in chicken liver is enrofloxacin.

Results of the screening method are manifested by the presence of the inhibition zone, that is proportional to the quantity of antibiotics in the sample. Therefore, results of the screening method are not only qualitative but also semi-quantitative. Statistical data processing of the inhibition zone width showed that there are significant differences in the inhibition zone width between the samples taken at different times of the chicken's life - cycle (Tables 3-4). Twenty four hours after the beginning of therapy until the first day after the end of therapy, inhibition width expressed a constant and significant ($p < 0.05$) increase. Also, the screening method discovered a significantly ($p < 0.05$) higher quantity of residues in the liver, and meat. These results were proved by HPLC method (Table 5). The screening method was used to examine individual samples of meat and liver in every sacrificed chicken. The variation interval showed in the results (0.806-1.349 mm) is a consequence of great variation in the quantity of residues in some chickens. Anadon *et al.* (1995) after treatment of chickens with enrofloxacin, discovered that individual differences can be great; 110 ng/g enrofloxacin or even 670 ng/g of ciprofloxacin in the liver. It may be assumed that these variations are a consequence of uneven intake of water, which results in an uneven intake of the drug.

The inhibition zone width has a significant ($p < 0.05$) increase compared to the measurements at the beginning of therapy, and all the meat and liver samples had a positive response. From the second day of the withdrawal period in muscle

samples the inhibition zones were significantly ($p < 0.05$) reduced. On the fourth day of the withdrawal period only 16.67% of muscle samples had a positive response.

The presence of residues in the liver was determined up to the third day after the withdrawal period with a significant ($p < 0.05$) decrease of inhibition zones. However, there was a 100% positive response.

Two days after the end of the withdrawal period enrofloxacin was detected by HPLC method in meat (24.8 ng/g) and in liver (45.2 ng/g), while ciprofloxacin was not detected. Similar results were discovered by Schneider (2001) and only three days after the end of therapy there was 38.2 ng/g of enrofloxacin and 0.97 ng/g ciprofloxacin in the meat, but in the liver there was 142.0 ng/g of enrofloxacin and 51.0 ng/g of ciprofloxacin. Recent data from the same author, with a supplied lower dose of enrofloxacin (50 µg/ml) for the same period, are as follows: in the meat there were 28.8 ng/g enrofloxacin and 0.00 ng/g of ciprofloxacin, and in the liver 70.8 ng/g of enrofloxacin and 25.1 ng/g of ciprofloxacin (Schneider and Donoghue, 2002). In EMEA (1998) reports three days after the withdrawal period 42 ng/g of enrofloxacin were found in chicken liver.

Residues could not be detected using HPLC 15 days after the withdrawal period. Similar data are found in the reports of EMEA (1998) where 15 days after the end of therapy an extremely low quantity of residues was measured in the liver (11 ng/g of enrofloxacin).

CONCLUSIONS

1. After oral therapy with enrofloxacin, residue levels are 2-4 times higher in the liver than in meat. Residues mostly consist of enrofloxacin and a smaller quantity of ciprofloxacin.

2. Four days withdrawal period allowed time for the drug concentration, in both meat and liver, to decrease to an acceptable level prior to slaughter (below EU MRL for enrofloxacin and ciprofloxacin).

3. The results of examining the residues in tissues in treated animals using screening method on Plate pH 8 *E coli* NCIMB 11595 entirely fulfill the demands of a qualitative method. Examination of tissue samples of treated animals by the screening method gave positive results in all the samples where the residue content was above MRL level.

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**REZIDUE ENROFLOKSACINA I NJEGOVOG GLAVNOG METABOLITA
CIPROFLOKSACINA KOD BROJLERA**

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SADRŽAJ

Prisustvo rezidua enrofloksacina u mesu i jetri pilića ispitivano je posle peroralnog aplikovanja enrofloksacina (10 mg/kg t.m./dan. Za ispitivanje rezidua su korišćene: mikrobiološka metoda – ploča pH 8.0 sa *Escherichia coli* NCIMB 11595 i HPLC metoda sa fluorescentnom detekcijom. Tokom pet dana primene terapije, koncentracije enrofloksacina u grudnoj muskulaturi i jetri su bile značajno iznad EU maksimalno dozvoljenih koncentracija (MDK). Ciprofloksacin nije detektovan u muskulaturi drugog dana primene terapije ali je detektovan u jetri u koncentracijama iznad MDK. Posle prekida terapije, koncentracije enrofloksacina i ciprofloksacina u grudnoj muskulaturi i jetri su bile iznad MDK nivoa do četvrtog dana karence. Ciprofloksacin nije detektovan u muskulaturi četvrtog dana karence ali je detektovan u jetri u koncentracijama ispod MDK. Četiri dana karence su period koji omogućuje da koncentracija leka u mesu i jetri padne na prihvatljiv nivo pre klanja (ispod MDK). Mikrobiološka metoda – ploča pH 8.0 sa *Escherichia coli* NCIMB 11595 daje pozitivne rezultate u svim uzorcima u kojima je sadržaj rezidua bio iznad MDK.