Acta Veterinaria (Beograd), Vol. 59, No. 5-6, 547-555, 2009.

DOI: 10.2298/AVB0906547P UDK 619:637.5.04/.07

DETERMINATION OF FLUMEQUINE RESIDUES IN BROILER CHICKENS WITH HPLC AND SCREENING METHOD

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(Received 25th April 2009)

Chickens were used to examine tissue depletion of flumequine after multiple oral doses (12 mg/kg bw every 24 h for 5 days). The presence of residues was detected using the microbiological screening method – plate pH 8.0 with E. coli NCIMB 11595 as a test microorganism. The tissue (muscle and liver) concentrations of flumequine were determined using HPLC/Fl method. During the 5 days dosing period, flumequine concentrations in breast muscle and liver exceeded the European Union MRLs (maximal residue limits). After the end of oral administration, hepatic concentrations of flumequine (1760-90 ng/g) persisted for 3 days; at that time, flumequine residues were also detected in muscle tissue (980-40 ng/g). Flumequine concentrations in breast muscle and liver exceeded the MRL values only on the first day of the withdrawal period. Microbiological method - plate pH 8.0 with E. coli NCIMB 11595 revealed positive results in all samples with residue concentrations above MRL values. Two days of withdrawal period allowed time for the drug concentrations in meat and liver to decrease to an acceptable level prior to slaughter (below MRL).

Keywords: chickens, flumequine, residue, HPLC, microbiological method

INTRODUCTION

The fluoroquinolone and 4-quinolone compounds, termed (fluoro)-quinolones, belong to a class of *semi*-synthetic antimicrobial agents that is important in both human and veterinary medicine. Flumequine is a fluoroquinolone antimicrobial developed exclusively for the use in veterinary medicine, which was patented in 1973. Flumequine $C_{14}H_{12}NFO_3$ (9-fluoro-6,7-dihydro-5-methyl-1-oxo-1H,5H-benzo-quinolizine-2-carboxylic acid) is a member of the halogenated quinoline carboxylic acid group of antibacterial agents. Flumequine belongs to the early second or second generation of fluoroquinolone antimicrobials, which have bactericidal activity against *Enterobacteriaceae* and other Gram-negative bacteria and some activity against certain Gram-positive

cocci (Appelbaum and Hunter, 2000; Ćupic *et al*., 2004; Martindale, 2005; Martinez *et al*., 2006). Fluoroquinolones damage bacterial DNA and lead to defects in negative supercoiling. This effect is linked to inhibition of DNA gyrase activity, an enzyme found in all bacteria (Prescott *et al*., 2000).

Flumequine is approved for therapeutic and prophylactic usage in poultry in Serbia (Jezdimirovic, 2002). Common poultry infections, such as mycoplasmal infections, colibacilosis and pasteurelosis, frequently are treated with flumequine (Report WHO, 1998; Jezdimirovic, 2002; Martinez *et al.,* 2006).

Flumequine is well absorbed when administered orally or parenterally to calves, sheep, pigs, poultry and trout and is excreted in the urine and faeces as the parent drug (80%), glucuronide conjugates (12.5%) and 7-hydroxyflumequine (6%). Whatever the administration route used, about 90% of the drug is excreted within 7 days, about 55% via urine, and 35% via faeces. The major portion (98%) of the drug is excreted within 24 hours (EMEA, 1996).

Flumequine is not genotoxic, carcinogenic, cannot act through interference with mammalian topoisomerases; however, high doses of flumequine increase the incidence of hepatocellular tumors due to hepatotoxicity (EMEA, 1996). 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. The widespread use of (fluoro)-quinolone compounds as therapeutic and prophylactic agents, particularly in intensive poultry production, has become a matter of great concern in recent years due to the identification of resistant *Campylobacter* and *Salmonella* strains in meat and possible transfer to humans via the food chain (Petrović *et al*., 2008).

Safe food is free of residues, i.e. the level of residues in the tissues is lower than MRL pursuant to the relevant regulations. MRL values for fluoroquinolone antimicrobials have not yet been established in Serbia. MRL values for flumequine in EU are 400ng/g for chicken meat and 800ng/g for liver (Council Regulation 2377/90).

The aim of this study was to examine the target tissue residues of flumequine, according to MRL values, to eliminate health risks for the consumers. The presence of flumequine in breast muscle and liver was detected by microbiological inhibition assay and HPLC method.

MATERIALS AND METHODS

Animals, drug and protocol of study

The study was performed on 65 healthy chickens (Arbor acres); 1-day old chicks were included in the experiment. At the age of two weeks the chickens were randomly divided in two groups. Group A (30 animals) was the control group, which was not treated with antimicrobials. At the age of 28 days the chickens in group B (35 animals) were given a daily dose of flumequine (12 mg/kg bw/day) via drinking water, for five consecutive days. In our work we used the preparation Flumekvin® pulv ad us.vet. (Hemovet - Serbia), 100 g of powder contains 10 g of flumequine.

The chickens were euthanized two days before starting the therapy, during therapy, during the withdrawal period and after the end of the withdrawal period. At each sampling three chickens were euthanized and tissue samples of breast muscle and liver were obtained. The samples were stored at –20°C until assayed for the presence and concentrations of flumequine.

Qualitative analysis: microbiological method

Flumequine standard was purchased from Krka Company, Slovenia. Test agar pH 8.0 was seeded with *Escherichia coli* NCIMB 11595. Working solution of *E. coli* NCIMB 11595 was made of freshly prepared culture. The culture was diluted in peptone-salt solution to give an 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 a quality control. Paper disks containing 0.003 ciprofloxacin μg/disk (Mast Diagnostic, Mereyseyside, UK) were placed in the center of the Petri dish. The detection level of plate pH 8.0 *E. coli* NCIMB 11595 was previously determined (unpublished data): 400 ng/g flumequine. 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 in a refrigerator for 2 hours and than incubated at 37°C for 24 h. After incubation the plates were inspected for inhibition zones around the meat disks and inhibition zones (IZ) for all 12 replicates were 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. Flumequine analytical standard was purchased from Sigma Company, USA. All the solvents were of HPLC purity.

Liquid chromatography method (Ramos *et al.*, 2003) with fluorescence detection at excitation wavelength of 312 nm and emission wavelength of 366 nm was used for determination of flumequine residues in meat and liver. The detection limit was 20 ng/g and quantification limit was 50 ng/g.

Flumequine was detected by gradient elution in 20 minutes. Waters "Sunfire" column, C18, 150 x 4.6 mm, 3.5 mm particle size was used for separation at flow rate of 0.7 mL/min. Mobile phase (0.01M phosphoric acid (pH)/acetonitrile; 80:20 v/v - 1-10. min and 60:40 - 10-20 min) was used for the elution of flumequine. Quantification was performed using external standard method and the results were obtained from the calibration curve of blanks fortified at four levels.

Statistical analysis

Statistical analysis was performed with the program Microsoft Office Excel 2000 and statistical program SPSS for Windows 8.0.0. ANOVA were analyzed according to Hadžić (1992). Screening method data were analyzed by the use of descriptive statistic methods. Differences in IZ diameters were analyzed for

statistical significance by use of ANOVA and Student's t – test. The differences of $p<0.05$ were considered significant.

RESULTS

Breast muscle and liver samples from two days before therapy, days 1 to 5 of dosing and days 1 to 3 post dose, were analyzed by the microbiological and HPLC methods for flumequine concentrations and the results are shown in Tables 1-4 and Figures 1-2.

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

Treatment day		$\bar{x}$	SD	SE	Cv	Iv	t	% posit.
-1	M	0.00	-	-	-	-	-	0
	L	0.00	-	-	-	-		0
2	M	2.81	0.753	0.266	26.77	4.00	0.33	50
	L	2.94	1.129	1.063	36.18	3.00		100
3	M	2.81	0.655	0.134	23.34	2.00	3.87*	100
	L	5.06	2.23	0.559	44.07	6.00		100
5	M	4.33	1.945	0.398	45.00	5.00	4.21*	100
	L	6.33	1.274	0.261	20.13	5.00		100

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	% posit.
1	M	3.71	1.429	0.292	38.53	2.00	3.59*	100
	L	5.46	1.911	0.390	35.00	7.00		100
2	M	0.00	-	-	-	-	-	0
	L	0.00	-	-	-	-		0
3	M	0.00	-	-	-	-	-	0
	L	0.00	-	-	-	-		0

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

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

Day	Meat			Liver		
	2^{T}	3^{T}	5^{T}	2^{T}	3^{T}	5^{T}
1^{PT}	2.28*	2.69*	1.25	5.23*	0.59*	1.85
5^{T}	3.18*	3.54*	-	8.33*	2.06	-
3^{T}	0.00	-	-	3.39*	-	-

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

Table 4. Flumequine residues in chicken muscle and liver determined by HPLC method

Tissue	Flumequine residues (ng/g) by days					
	-1	2^{T}	3^{T}	1^{PT}	2^{PT}	3^{PT}
Meat	0	220	710	980	60	40
Liver	0	420	840	1760	100	90

^{T} – treatment day, ^{PT} – post treatment day

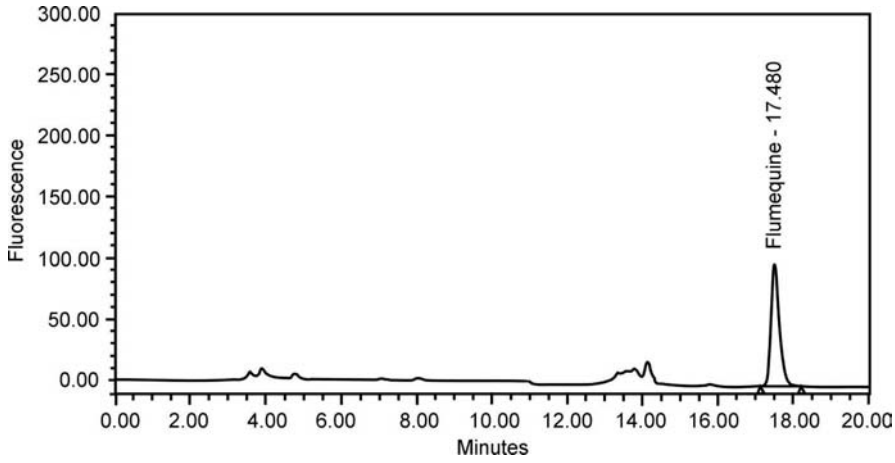

Figure 1. Chromatographic determination of flumequine residues in chicken muscle – the third day of therapy

Residues were not detected in the chicken breast muscle and liver with the microbiological and HPLC method, before beginning of the therapy. Residues in meat and liver were detected on plate pH 8.0 *E. coli* NCIMB 11595, during the

treatment period. Flumequine residues were detected by HPLC method, in breast muscle and liver in ascending concentrations during therapy until first day post treatment (Figures 1-2). Positive results in all muscle and liver samples were found on the first post treatment day with the microbiological assay, but on the second day no positive results were found on this assay. From the first post treatment day (peak), the descending concentrations of flumequine residues were found in the meat and liver, by the HPLC method.

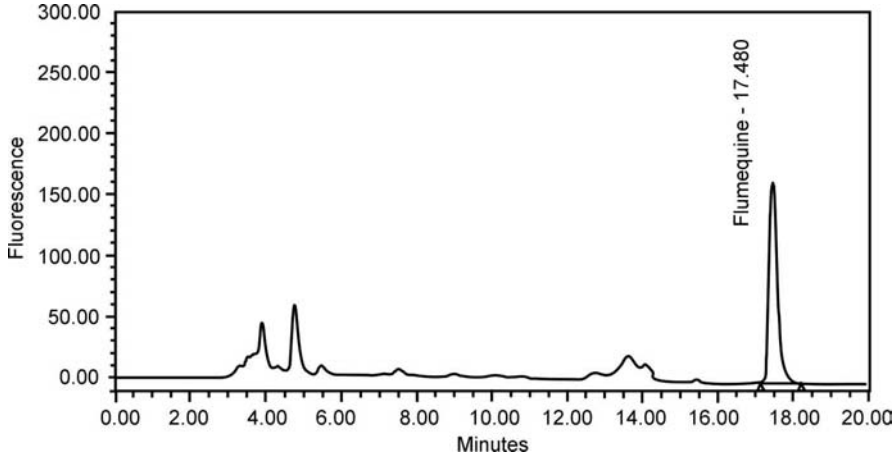

Figure 2. Chromatographic determination of flumequine residues in chicken liver – the first day of withdrawal period

DISCUSSION

High concentrations of flumequine in the meat and liver 48 h after the beginning of treatment are a consequence of rapid absorption from chicken intestines (Prescott *et al.,* 2000). Peak serum concentrations of fluoroquinolones in poultry occur within 1.5-3h (Anadon *et al*., 1990). Fluoroquinolones are well distributed from plasma into tissues (Anadon *et al*., 1995). Flumeqine concentrations were nearly two times (1.90) higher in the liver than in muscles 48h after the beginning of treatment. This correlation was recorded during the whole experiment. Similar distribution data for other fluoroquinolone-enrofloxacines were reported by Anadon *et al*. (1995) and Petrović *et al*. (2006). Enrofloxacine concentrations were 2-4 times higher in the liver during treatment. Our results point on active metabolism of flumequine in the liver during the treatment, but less intensive than enrofloxacine metabolism. Flumequine is glucuronidated in the liver and to a lesser extent hydroxylated to 7-hydroxyflumequine. This metabolite exhibits approximately one-eight of the antimicrobial activity of flumequine (Schuppan *et al*., 1985), but flumequine supports the main microbiological activity in all edible tissues, therefore it was retained by EMEA (1999) as a marker residue.

The ratios of flumequine towards total microbiologically active residues is 0.81 in muscle, 0.50 in skin/fat, 0.72 in liver and 0.79 in kidney (EMEA, 1999).

A rapid decline of flumequine residues in broiler edible tissues was found in our experiment. Similar data were published in the EMEA report (1996), chickens were treated with equal flumequine doses like in our experiment (12 mg/kg/day for five days), 6 hours after cessation of the treatment, the concentrations of flumequine were significant: 1500 ng/g in muscle, 720 ng/g in skin/fat and 2450 ng/g in the liver. Flumequine was gradually eliminated from the chicken's body, after the treatment was finished (EMEA, 1996; Prescott *et al.,* 2000). After 48 hour withdrawal period, the concentrations of flumequine were below 170 ng/g in our experiment, as well as in EMEA report (1999).

The results of the screening method are manifested by the presence of an inhibition zone which is proportional to the quantity of antibiotics in the sample. Therefore, the results of the screening method are not only qualitative, but also semi-quantitative. The statistically significant differences ($p<0.05$) in the inhibition zone width between the samples taken at different periods of therapy and withdrawal were found in the experiment (Table 3). Inhibition width had a constant significant increase ($p<0.05$) during therapy. Also, the screening method discovered significantly higher ($p<0.05$) quantities of residues in the liver. These results were proved by HPLC method (Table 4). The screening method was used for examination of individual samples of meat and liver, in every sacrificed chicken. A big variation interval (Iv = 2-7 mm) in the inhibition zone width (Table 1) was a consequence of great variations in quantity of residues in some chickens. Anadon *et al*. (1995) and Garces *et al*. (2006) have also found big individual differences in tissue concentrations after treatment of chicken with fluoroquinolones via food or water (even 400 ng/g). Differences in measured concentrations can be caused by many factors, such as different metabolic speed, different weight of animals and, consequently, different distribution volume, etc. Another factor might be a different amount of water intake because the same water intake had not been ensured, resulting in a possibly uneven intake of the drug between the animals.

The average inhibition width was decreased, but not significantly ($p>0.05$) on the first day of the withdrawal period. On the second day no muscle and liver samples had a positive response, on microbiological assay. Low level of flumequine residues were detected in these samples, much below MRL, as well as much below the level of detection of the microbiological method. On the first day after the end of the withdrawal period flumequine was detected by HPLC method in meat (40 ng/g) and liver (90 ng/g). Similar results are found in EMEA reports (1996), where seventy two hours after the end of the treatment only the traces of flumequine could be detected in all broiler tissues.

CONCLUSIONS

1. After oral therapy with flumequine, residue levels are nearly two times higher in the liver than in the meat.

2. Two days withdrawal period allowed time for the drug concentration in the meat and liver tissue to decrease to an acceptable level prior to slaughter (below EU MRL for flumequine).

3. The examination results of the residue presence in treated animals' tissues with screening method Plate pH 8 *E. coli* NCIMB 11595 entirely fulfill the demands for qualitative methods. Examination of treated animals tissues using this screening method had positive results in all samples where the residues concentrations were above the MRL level.

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REFERENCES

1. *Anadon A, Martinez-Larranaga MR, Diaz MJ, Velez C, Bringas* P, 1990, Pharmacokinetics and residue studies of quinolone compounds and olaquindox in poultry, *Ann Res Vet*, 1, 137-44.
2. Anadon A, Martinez-Larranaga MR, Diaz MJ, Bringas P, Martinez MA, Fernandez-Cruz ML *et al*., 1995, Pharmacokinetics and residues of enrofloxacin in chickens, *Am J Vet Res*, 56, 4, 501-6.
3. *Appelbaum PC, Hunter PA*, 2000, The fluoroquinolone antibacterials: past, present and future perspectives, *Int J of Antimicrobial Agents*, 16, 5-15.
4. Council Regulation (EEC) No 2377/90, 1990, Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin, *Official J of the EU*, 224, 1-110.
5. EMEA, Comittee for Veterinary Medicinal products,1996, Flumequine. Summary report (1), EMEA/MRL/104/96-FINAL, 1-7.
6. EMEA, Comittee for Veterinary Medicinal products, 1999, Flumequine. Summary report (2), EMEA/MRL/429/99-FINAL, 1-9.
7. *Garces A, Zerzanov A, Kucera R, Barron D, Barbosa J*, 2006, Determination of a series of quinolones in pig plasma using solid-phase extraction and liquid chromatography coupled with mass spectrometric detection. Application to pharmacokinetic studies, *J of Chromatography A,* 1137, 22-9.
8. *Hadžić O*, 1992, Disperziona analiza, In: Hadžić O, urednik, Numeričke i statističke metode u obradi eksperimentalnih podataka, Drugo izdanje, Novi Sad: Univerzitet u Novom Sadu, Institut za matematiku, Štamparija za grafičku delatnost, 194-206.
9. *Jezdimirović M*, 2002, Hinoloni (Kvinoloni), U: Jezdimirović MB, urednik, Osnovi farmakoterapije i gotovi lekovi ad us. vet., Beograd: Fakultet veterinarske medicine, Beograd, AD štamparija "Kultura" Bački Petrovac, 197-9.
10. *Klecak G, Urbach F, Urwyler H*, 1997, Fluoroquinolone antibacterials enhance UVA-induced skin tumors, *J Photochem Photobiol B: Biology*, 37, 174-81.
11. *Martindale*, 2005, In: Sweetman S, editor, The Complete Drug Reference, Thirty fourth edition, London: Pharmaceutical Press.
12. *Martinez M, McDermott P, Walker R*, 2006, Pharmacology of the fluoroquinolones: a perspective for the use in domestic animals, *Vet J*, 172, 10-28.
13. *Petrović J, Petrović T, Kovačević M*, 2008, Food Safety Trends: Antimicrobial Resistance in Food Borne Pathogens, Viruses in Food and Safe Disposal of Animal Wastes. Proceedings of the

Second Joint PSU-UNS International Conference on BioScience: Food, Agriculture and the Environment, Novi Sad, Serbia, June 22-24, 14.

14. *Petrović J, Baltić M, Ćupić V, Stefanović S, Stojanović D*, 2006, Resuidues of enrofloxacine and its main metabolite ciprofloxacine in broiler chickens, *Acta Veterinaria* (*Belgrade*), 56, 5-6, 497-506.
15. *Pierfitte C, Gillet P, Royer R,* 1995, More on fluoroquinolone antibiotics and tendon rupture, *N Engl J Med*, 332, 193, 3, 414-6.
16. *Prescott J, Baggot J, Walker R*, 2000, Fluoroquinolones, In: Prescott J, Baggot J, Walker R, editors, Antimicrobial therapy in veterinary medicine, Third edition, Ames: Iowa State University Press, 315-39.
17. *Ramos M, Aranda A, Garcia E, Reuvers T, Hooghuis H,* 2003, Simple and sensitive determination of five quinolones in food by liquid chromatography with fluorescence detection, *J of Chromatography B*, 789, 2, 373-81.
18. Report and Proceedings of a WHO Meeting, 1998, Quinolone use in animals and resistance in animal bacteria. Use of quinolones in food animals and potential impact on human health, Geneva, Switzerland, 2-5 June, 9-13.
19. *Stahlman R, Kuhner S, Shakibaei M, SchwabeR, Flores J, Evander SA et al*., 2000, Chondrotoxicity of ciprofloxacin in immature beagle dogs: Immunohistochemistry, electron microscopy and drug plasma concentrations, *Arch Tochicol*, 73, 10-11, 564-72.
20. *Schuppan D, Harrison L, Rohlfing S, Miller H, Funk M, Hansen C, Ober R*, 1985, Plasma and urine levels of flumequine and 7-hydroxyflumequine following single and multiple oral dosing, *J of Antimicrob Chemother*, 15, 337-43.
21. *Ćupić V, Dobrić S, Trailović D, Pejčić Z*, 2004, Antimicrobial drugs in veterinary medicine, *Veterinarski glasnik*, 58, 5-6, 577-94.

ISPITIVANJE REZIDUA FLUMEKVINA KOD BROJLERA HPLC I SKRINING METODOM

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SADRŽAJ

U ovom radu je ispitivano prisustvo rezidua flumekvina u mesu i jetri pilića posle peroralnog aplikovanja ovog leka u dozi od 12 mg/kg/dan. Za ispitivanje rezidua korišćene su: skrining mikrobiološka metoda i HPLC metoda sa fluorescentnom detekcijom za kvantifikaciju rezidua. Tokom petodnevnog perioda aplikovanja leka, koncentracije flumekvina u grudnoj muskulaturi i jetri su bile iznad MDKR (maksimalno dozvoljene količine rezidua) propisanih u EU. Posle završetka oralnog aplikovanja leka tokom tri dana izmerene su značajne koncentracije flumekvina u jetri (1760-90 ng/g) i u mišićima (980-40 ng/g). Rezidue flumekvina u grudnoj muskulaturi i jetri su bile iznad MDKR samo prvog dana perioda karence. Mikrobiološkom metodom, na pločama pri pH 8.0 sa *Escherichia coli* NCIMB 11595, dobijeni su pozitivni rezultati u svim uzorcima u kojima je sadržaj rezidua bio iznad MDKR vrednosti. Na osnovu ovih rezultata se može zaključiti da dva dana propisanog perioda karence omogućava da sadržaj rezidua u mesu i jetri opadne do prihvatljivog nivoa pre klanja, ispod MDKR.