

DETECTION OF ENROFLOXACINE RESIDUES BY MICROBIOLOGICAL SCREENING METHOD

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Abstract: The usage of microbiological screening tests is widespread in control of presence of antimicrobial drug residues in meat samples. Screening tests must be capable to detect antimicrobial drug residue of interest and detection limits must comply with MRL (Maximum Residue Limit). The aim of this study was to examine the performance of a microbiological screening test with *E. coli* as test microorganism: capability of detecting enrofloxacin and its main metabolite ciprofloxacin at MRL levels in both fortified and incurred chicken tissue samples. Detection limits of microbiological screening test with *E. coli* was 50 ng/g for enrofloxacin and 25 ng/g for ciprofloxacin. Screening test had positive results in all samples of fortified and incurred meat with residue concentrations above MRL level. The results of this examinations shows that microbiological screening test with *E. coli*, as simple and cost effective test, is capable to detect enrofloxacin and its metabolite ciprofloxacin in treated poultry at MRL level ie test is capable to detect unsafe poultry meat.

Key words: enrofloxacin, residue, microbiological screening test

Introduction

Antimicrobial drug treatment is widespread in food producing animals breeding. Meat for human consumption should be safe i.e. residues of antimicrobial drugs in meat should be below MRL (Maximum Residue Limit). Safety of poultry meat is very important in Serbia because this type of meat and poultry products are often consumed in Serbia (Tolimir *et al.*, 2016)

Fluoroquinolone antimicrobial drugs are *semi-synthetic* antimicrobial agents. Fluoroquinolones are used both in human and veterinary medicine.

Enrofloxacin is fluoroquinolone antimicrobial developed exclusively for the use in veterinary medicine. Intensive poultry production, housing systems, management practices have influence on poultry health (Lolly et al., 2013). Common poultry infections, such as mycoplasmal infections, colibacillosis and pasteurellosis, frequently are treated with enrofloxacin (Martinez et al., 2006). Ciprofloxacin is the main metabolite of enrofloxacin and also has bactericidal activity, as parent compound is approved in human medicine.

The widespread use of fluoroquinolone compounds as therapeutic 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 food chain (Petrovic et al., 2008). MRL values as sum of enrofloxacin and ciprofloxacin are 100 ng/g for chicken meat and 200 ng/g for liver are regulated by the Commission Regulation (EU) No 2377/2009 and amending annexes. In Serbian legislation MRL values are defined as “quantities that can be proven by known or recognized methods”, only for sulfonamide residues MRL value is defined as 100 ng/g for meat (Anonymous, 1992).

Presence of antimicrobial drug in meat usually is controlled by screening methods and confirmation of residue (identification and quantification) in suspected samples is performed by physico-chemical methods (EC, 2002). There is broad range of methods for detection of fluoroquinolone drug residues: microbiological tests, ELISA, immunoassay and Biosensor tests as screening methods (Song et al., 2015, Chen et al., 2017). Microbiological screening methods are capable to detect broad spectrum of antimicrobial drug presence but these methods are not capable to identify and quantify the exact antimicrobial drug residue present in the sample. For confirmation more precise and reliable methods are used such as HPLC with ultraviolet and fluorescence detection (Marschiello et al., 2001), liquid chromatography tandem mass spectrometry (Zhang et al., 2019).

Screening methods must satisfy the following requirements: they must detect antibiotics of interest, detection limits must comply with the requirements (MRLs), they must be easy to perform and cost effective, test results are to be obtained rapidly, and the tests must be standardized (low variability within and between batches/laboratories) (Chafer-Perices et al., 2010). Microbiological inhibitory tests are widely used as a standard for screening purposes. The test principle is based on measurement of the inhibition zone, which presents the inhibition of multiplication of test microorganism in presence of antibiotics. These tests can serve as rapid tests as the result can be obtained within 24 hours (Petrovic et al., 2008). Systematic control of residues is one of important steps which helps broiler production in Serbia to reach European standards. According to Petrovic et al. (2012) it is necessary to build efficient livestock production that can compete in the European market contributing to the growth of farmers and national income.

The aim of this study was to examine the performance of screening test microbiological method with *E. coli* as test microorganism: capability to detect enrofloxacin and ciprofloxacin at MRL levels in both fortified and incurred chicken tissue samples. LOD of diffusion method were determined in tissue samples fortified with enrofloxacin and ciprofloxacin. Incurred samples were obtained in experimental design where chickens were treated with therapeutical doses of enrofloxacin. The presence of fluoroquinolones in breast muscle and liver was detected by microbiological inhibition test and HPLC method.

Material and methods

Chemicals and reagents

Enrofloxacin and ciprofloxacin analytical standards was purchased from Sigma Company, USA. In experiment was used preparation Enrocin[®] 10% ad us. vet. (Hemovet - Serbia and Montenegro), 1 ml of solution contains 100 mg enrofloxacin.

Microbiological method: test agar pH 8.0 was prepared in our laboratory (Caseine hydrolysat 2%, dextrose 0.4%, NaCl 1%, agar agar 1.6%). *Escherichia coli* NCIMB 11595 was used as test microorganism. Paper disks containing 0.003 ciprofloxacin µg/disk (Mast Diagnostic, Mereyseaside, UK) were used as positive control on each plate.

Liquid chromatography method with fluorescence detection: methanol, acetonitrile, n-hexane and phosphoric acid were purchased from J. T. Baker, Holland. All the solvents were of HPLC purity. Waters "Sunfire" column, C18, 150x4.6mm, 3.5µm particle size was used for separation at flow rate of 0.8 mL/min. Mobile phase was 0.01M phosphoric acid (pH 3)/acetonitrile; 80:20 v/v1-10. min and 60:40 - 10-20 min.

Determination of LOD – fortified samples

Detection limit of qualitative screening techniques must have a percentage of false negative results below 5% (β error) at MRL value (Decision 2002/657/EC). The limit of detection (LOD) of the microbiological method was determined by the method recommended by Reichmuth *et al.*, (1997). Series of 7 concentrations of each antibiotic were analyzed in 12 replicates. Meat without antibiotics and meat fortified with 2-3 times higher concentration of antibiotics then expected limit of detection were used as negative and positive controls, respectively. Expected LOD was determined in preliminary examinations. Three different concentrations between the negative control sample and expected positive sample were analyzed. The following concentrations were examined (ng/g) 0.00, 0.78, 1.56, 3.12, 6.25,

12.50, 25.00, 50.00, 100.00, 200.00 and 400.00. The results are shown in the form of dose-response curve. For this examination LOD is defined as that concentration, where 95% of the results were evaluated positive. LOD was determined by plotting the line for 95% positive responses. The place where the line cuts the dose-response curve presents LOD.

Animals, drug and protocol of study – incurred samples

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 the chickens were randomly divided into 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 daily doses of enrofloxacin (10 mg/kg bw/day), via drinking water, for five consecutive days.

The chickens were euthanized day before starting the therapy and during the withdrawal period. At each sampling three chickens were euthanized. The samples of breast muscle and liver were obtained. The samples were stored at -20°C until assayed for the presence and concentrations of enrofloxacin and ciprofloxacin.

Qualitative analysis: microbiological method

Test agar pH 8.0 was seeded with *Escherichia coli* NCIMB 11595. Working solution of *E. coli* was made of 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 plates were subjected to a quality control. Paper discs containing 0.003 ciprofloxacin $\mu\text{g}/\text{disk}$ were placed in the center of the Petri dish. 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 discs. Four discs of meat were placed on opposite ends of the plate. Each sample was examined in 12 replicates. The plates were kept in refrigerator for 2 hours and then incubated on 37°C for 24 h. After incubation the plates were inspected for inhibition zones around the meat discs and inhibition zones (IZ) for all 12 replicates were recorded (2 mm width was considered positive result).

Quantitative analysis – HPLC with fluorescence detection

Liquid chromatography method with fluorescence detection at excitation wavelength of 280 nm and emission wavelength of 455 nm was used for determination of enrofloxacin and ciprofloxacin residues in meat and liver (*Ramos*

et al., 2003). Detection limit was 10 ng/g and quantification limit was 20 ng/g. Enrofloxacin and ciprofloxacin were detected isocratically in 7-10 minutes. 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 using the Microsoft Office Excel 2000 and statistical software SPSS for Windows 8.0.0. Screening method data were analyzed by the use of descriptive statistic methods. Differences in IZ diameters were analyzed for statistical significance by the use of Student's t – test. The differences of $p < 0.05$ were considered significant.

Results and discussion

Limit of detection (LOD) is the basic parameter in determining the test sensitivity. Test sensitivity is the probability of obtaining positive test result in truly positive samples. In a view of antimicrobial residue detection in food, a positive sample is the sample that contains residues at level above the MRL. This value is the basic parameter for sample assessment, since samples containing residues below MRL level are considered negative, i.e. safe. An ideal screening test would yield a LOD exactly at MRL level for each particular antimicrobial. However, performing of such tests is not always feasible in daily practice. Thus, the test is considered enough sensitive if the detection limit is at or below the MRL level, an never above the MRL. The LOD of a microbiological test depends of the innate sensitivity of the test bacterium, pH and thickness of growth medium (*Gaudin et al.*, 2010).

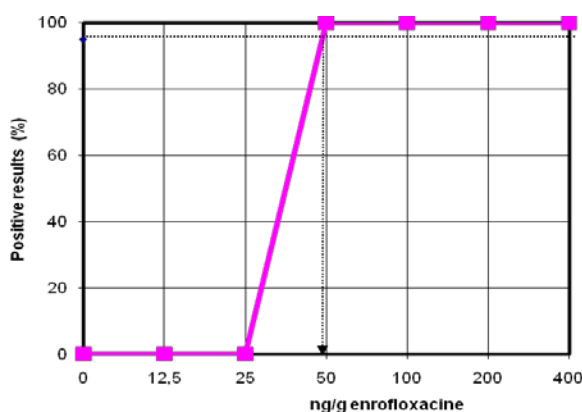


Figure 1. LOD of microbiological method for enrofloxacin

Figures 1 and 2 demonstrate the results of the examination of the microbiological method sensitivity to enrofloxacin and ciprofloxacin in the form of dose-response curve. Concentrations 0.78 - 25.00 ng/g of enrofloxacin did not have any positive response, while the concentrations 50 ng/g and above gave 100% positive responses. For this examination, LODs were defined as concentrations, where 95% of the results were evaluated as positive. LODs can be derived from figures 1 and 2 as 50 ng/g for enrofloxacin and 25ng/g for ciprofloxacin.

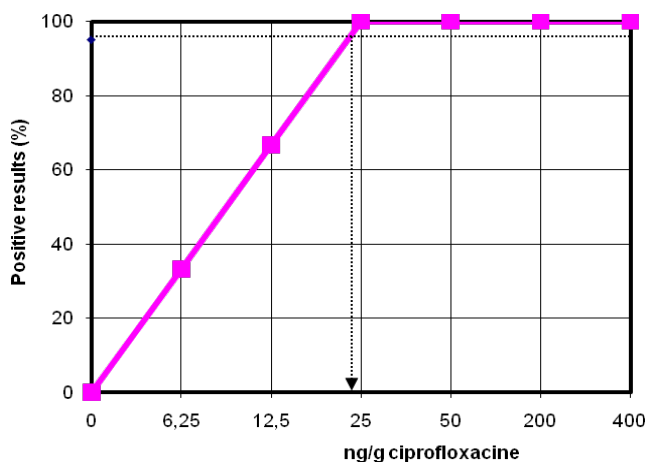


Figure 2. LOD of microbiological method for ciprofloxacin

The results obtained in this research corresponds to the reports of *Choi et al.*, (1999) on detection limits for *E. coli* strain 128 ranging from 35 to 50 ng/g for enrofloxacin and 30 ng/g for ciprofloxacin. According to *Okerman et al.*, (1998) detection limits of the pH6 plate *E. coli* ATCC 11303 were 50 ng/g for enrofloxacin and 30 ng/g for ciprofloxacin. In 2001, the same authors investigated sensitivity of another strain of *E. coli*- Bayer 14 and established detection limits of 150 ng/g and 30 ng/g for enrofloxacin and ciprofloxacin, respectively. Lower LOD was determined in milk compared to meat: in milk enrofloxacin and ciprofloxacin had LOD of 20ng/g and 10 ng/g respectively and in meat LOD was 200 ng/g. These results were obtained by STAR test, microbiological screening method with *E.coli* as test microorganism in pH8 plate (*Gaudin et al.*, 2010). Sensitivity differences that occur in various authors are mainly related to diverse strains of *E. coli* as well as to differences with respect to test-design (nutritive medium, incubation temperature).

Examination of negative control samples did not revealed any false positive response. The established detection limit corresponds with MRL values for enrofloxacin and, ciprofloxacin in poultry meat and liver. Within MRL for examined fluoroquinolones microbiological inhibition method revealed 100% positive results. The demands of Serbian national regulative are also fulfilled, because residues could be detected at unsafe level.

After oral application, fluoroquinolones are well absorbed, distributed into tissues and excreted in urine and feces at high concentrations (*Prescott et al., 2000*). Enrofloxacin is metabolised in liver to main metabolite ciprofloxacin and some minor metabolites (*EMEA, 1998*). Breast muscle and liver samples from day 1 before dosing and day 1 and 4 of withdrawal period and day 1 post withdrawal were analyzed by the microbiological and HPLC method (Table 1).

Table 1. Determination of residues before and after enrofloxacin administration

Treatment day		Microbiological method (IZ in mm)						HPLC (ng/g)		
		x	SD	SE	Cv	Iv	t	% posit	Enro	Cipro
-1	M	0	-	-	-	-	-	0	0	0
	L	0	-	-	-	-		0	0	0
1 ^W	M	15.0	1.5	0.4	9.7	4.0	2.3*	100	580	50
	L	16.2	1.3	0.3	7.9	3.0		100	1200	820
4 ^W	M	1.4	-	-	-	9.0	-	16.7	30	<10
	L	8.0	0.9	0.2	2.4	3.0		100	50	70
1 ^{PW}	M	0.0	-	-	-	-	-	0	20	<10
	L	6.7	1.0	0.2	14.6	4.0		100	50	<10

M- meat; L- liver; -1- before therapy; W- withdrawal; PW -day after the end of withdrawal period; * – significant difference ($p < 0.05$), enro- enrofloxacin; cipro- ciprofloxacin; /- not examined; SD- standard deviation; SE- standard error; CV- coefficient of variation; Iv- interval of variation; t- t test value

During the withdrawal period, enrofloxacin and ciprofloxacin concentrations in breast muscle and liver exceeded the MRL values on day 1 of withdrawal period (Figure 3). Ciprofloxacin was not detected in muscle on day 4 of withdrawal period, but it was detected in liver in concentrations below MRL. During the withdrawal period, all muscle samples gave positive results in 100% microbiological method examinations on day 1, while on day 4 only 16.7% muscle samples were positive. One day after the end of withdrawal period, enrofloxacin was detected by HPLC method, i.e. in meat 20 ng/g and 50 ng/g in liver, while there was no ciprofloxacin. Similar results were obtained by *Schneider (2001)*, on day 3 of withdrawal period there was 38.2 ng/g of enrofloxacin and 0.9 ng/g

ciprofloxacin in meat, but in liver there were 142.0 ng/g of enrofloxacin and 51.0 ng/g of ciprofloxacin. More recent data from the same author, using lower doses of enrofloxacin (50 ng/g) for the same period, revealed the following: in meat there were 28.8 ng/g enrofloxacin and 0.0 ng/g of ciprofloxacin, and in liver 70.8 ng/g of enrofloxacin and 25.1 ng/g of ciprofloxacin (Schneider and Donoghue, 2002). In the *EMEA (1998 a,b)* reports, three days after withdrawal period 42 ng/g of enrofloxacin were found in chicken liver.

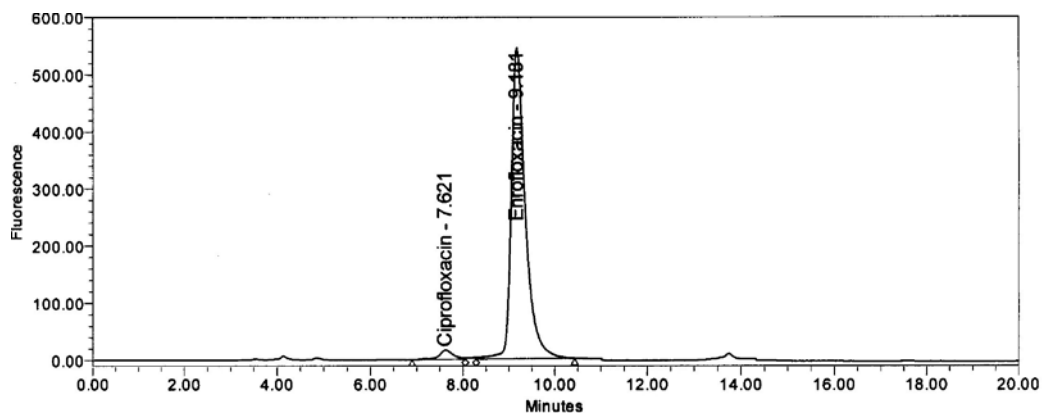


Figure 3. Chromatographic determination of enrofloxacin residues in chicken muscle on the first day of withdrawal period

A four-day withdrawal period for enrofloxacin allowed enough time to decrease drug concentration in meat and liver to an acceptable level prior to slaughter (below EU MRL).

Conclusion

The results of examining the residues in tissues of treated animals using screening microbiological method fulfill the demands for a qualitative method. Examining of treated animals using screening method gave positive results in all samples where the residues content was above MRL level.

Detekcija rezidua enrofloksacina primenom mikrobiološke skrining metode

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Rezime

Mikrobiološki skrining testovi se često koriste u kontroli prisustva rezidua antimikrobnih lekova u mesu. Skrining testovi moraju detektovati rezidue antimikrobnih lekova od interesa a granica detekcije mora biti u saglasnosti sa MDK rezidua (maksimalno dozvoljena koncentracija). Cilj rada je ispitivanje performansi mikrobiološkog skrining tesa sa *E. coli* kao test mikroorganizmom: mogućnost testa da detektuje enrofloksacin i njegov glavni metabolit u koncentracijama bliskim MDK vrednostima, u uzorcima u kojima su veštački dodati antimikrobni lekovi i u uzorcima dobijenim od tretiranih životinja. Granica detekcije mikrobiološkog skrining testa sa *E. coli* je 50 ng/g za enrofloksacin a 25 ng/g za ciprofloksacin. Skrining test je dao pozitivne rezultate u svim uzorcima u koje su veštački dodati enrofloksacin i ciprofloksacin, takođe i u uzorcima lečenih pilića u kojima je koncentracija rezidua bila znad MDK nivoa. Rezultati ovih ispitivanja pokazuju da je mikrobiološki skrining test sa *E. coli*, kao jednostavan i finansijski isplativ test, može detektovati enrofloksacin i njegov glavni metabolit ciprofloksacin iznad MDK u jestivim tkivima lečenih pilića odnosno mogu detektovati meso koje nije bezbedno za ishranu.

Ključne reči: enrofloxacin, rezidue, mikrobiološki skrining test

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