DETECTION OF FLUOROQUINOLONE RESIDUES BY MICROBIOLOGICAL SCREENING METHOD – FLUMEQUINE

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Abstract: Systematic control of antibiotic residues includes analysis and selection of large number of samples, and requires a wide range of screening methods. Screening tests should satisfy the following requirements: they must be capable to detect antibiotics of interest, detection limits must comply with the requirements (MRL- maximum residue limit), they must be easy to perform and cost effective, test results are to be obtained rapidly, and the tests must be standardized. The aim of this study was to examine the performance of screening test microbiological method with \textit{E. coli} as test microorganism: capability to detect fluoroquinolone – flumequine at MRL levels in both fortified and incurred chicken and fish tissue samples. LOD (limit of detection) of microbiological method were determined in tissue samples fortified with flumequine. Incurred samples were obtained in experimental design where chickens were treated with therapeutical doses of flumequine. The presence of fluoroquinolones in muscle and liver was detected by microbiological and HPLC method. Examination results revealed detection limits of the microbiological method ranging from 300 ng/g to 400 ng/g for flumequine. Examination of treated animals using microbiological screening method gave positive results in all samples where the residues content was above MRL level. The results of examination of the flumequine residues in tissues of treated animals using screening microbiological method entirely fulfill the demands of a qualitative method.

Key words: fluoroquinolones, flumequine, residue, screening

Introduction

The fluoroquinolone antimicrobials belongs to a class of \textit{semi}-synthetic agents that are important in both human and veterinary medicine. Flumequine is fluoroquinolone antimicrobial developed exclusively for the use in veterinary
medicine. Common poultry infections, such as mycoplasmal infections, colibacilosis and pasteurellosis, frequently are treated with this drug as well as fish infections caused by *Aeromonas salmonicida*, *Vibrio anguillarum*, *Yersinia ruckeri*, *Renibacterium salmoninarum*, *Pasterurella piscida* (della Roca et al., 2004; Martinez et al., 2006).

The widespread use of fluoroquinolone 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 food chain (Petrovic et al., 2008). MRL values for flumequine in EU are 400ng/g and 800ng/g for chicken meat and liver, 600ng/g for fish meat (Council Regulation 2377/90).

A number of methods have been developed for the analysis of fluoroquinolones in animal tissues, some of them rely on HPLC with ultraviolet and fluorescence detection (Marschiello et al., 2001), automated microdialysis-liquid chromatography (Schneider, 2001) and LC-MS (Schneider and Donoghue, 2002). Systematic control of antibiotic residues in European Union includes analysis and selection of large number of samples, and requires a wide range of screening methods. Samples that are positive or suspect as to the presence of residues are further analyzed using confirmatory methods (HPLC, LC/MS). Introduction of systematic control of residues is one of important steps which will help our broiler and fish production 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.

Screening tests 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) (Suhren and Heeschen, 1996). Microbiological inhibition 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).

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

**Material and Methods**

**Chemicals and reagents.** Flumequine analytical standards was purchased from Sigma Company, USA. In our experiment we used preparation Flumekvin® pulv ad us.vet. (Hemovet - Serbia), 100 g of powder contains 10 g of flumequine.

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, Mereyseyside, UK) were used as positive control.

HPLC/Fl: 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 for flumequine of 0.7 mL/min. Mobile phase (0.01M phosphoric acid (pH 3)/acetonitrile; 80:20 v/v1-10. min and 60:40 - 10-20 min) was used for the elution.

**Determination of LOD – fortified samples.** 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 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): flumequine 0.00, 3.12, 6.25, 12.50, 25.00, 100.00, 200.00, 400.00, 800.00 and 1600.00. 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. Fish samples were fortified with ¼ MRL, ½ MRL, 1MRL, 2 MRL, 4MRL, MRL is for fish 600ng/g.

**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 and group B (35 animals) was treated group. At the age of 28 days the chickens in group B were started with therapy (12 mg/kg bw/day). Drug
were given via drinking water, for five consecutive days. The chickens were euthanized 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 flumequine.

**Qualitative analysis: microbiological method.** 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 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 µg/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/liver 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 than incubated on 37°C for 24 h. After incubation the plates were inspected for inhibition zones around the meat/liver discs and inhibition zones (IZ) were recorded (2 mm width was considered positive result).

**Quantitative analysis – HPLC with fluorescence detection.** HPLC method with fluorescence detection at excitation wavelength of 312 nm and emission wavelength of 366 nm (*Ramos et al., 2003*) was used for determination of flumequine residues in meat and liver. The detection for flumequine limit is 20ng/g and quantification limit is 50ng/g. Flumequine was detected by gradient elution in 20 minutes. Quantification was performed using external standard method and the results were obtained from the calibration curve of blanks fortified at four levels of flumequine.

**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**

Figure 1 demonstrates the results of the examination of the microbiological method sensitivity in chicken muscle towards flumequine in the form of dose-response curve. Concentrations 0.00 - 100.00 ng/g of flumequine did not have any positive response, while the concentrations 200 and above gave 50-100% positive responses. For this examination, LOD was defined as concentrations, where 95%
of the results were evaluated as positive. LOD of flumequine can be derived from figure 1 as 400 ng/g for flumequine. For fish muscle samples similar LOD was found because concentration of 300 ng/g had 100% of positive responses in Table 1.

Chicken breast muscle and liver samples from chickens from experiment sampled during withdrawal period were analyzed by the microbiological and HPLC methods for flumequine presence and concentrations, and the results are shown in Table 2 and Figure 2.

**Table 1. Fish muscle fortified fish with flumequine**

<table>
<thead>
<tr>
<th>Flumequine MRL 600ng/g</th>
<th>Microbiological method (IZ in mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>x</td>
</tr>
<tr>
<td>4 MRL</td>
<td>8.29</td>
</tr>
<tr>
<td>2 MRL</td>
<td>8.25</td>
</tr>
<tr>
<td>1 MRL</td>
<td>7.38</td>
</tr>
<tr>
<td>1/2 MRL</td>
<td>3.67</td>
</tr>
<tr>
<td>1/4 MRL</td>
<td>0</td>
</tr>
</tbody>
</table>

**Figure 1. LOD of microbiological method for flumequine**
Table 2. Determination of residues during flumequine administration

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Microbiological method (IZ in mm)</th>
<th>HPLC (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>x</td>
<td>SD</td>
</tr>
<tr>
<td>1&lt;sup&gt;PT&lt;/sup&gt;</td>
<td>M</td>
<td>3.71</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>5.46</td>
</tr>
<tr>
<td>2&lt;sup&gt;PT&lt;/sup&gt;</td>
<td>M</td>
<td>0.00</td>
</tr>
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<td></td>
<td>L</td>
<td>0.00</td>
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</tbody>
</table>

M- meat; L- liver; -PT- day after the end of therapy; * – significant difference (p < 0.05),

Limit of detection 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 (Petrovic 2006).

The results obtained in this research indicated detection limits of microbiological inhibition test towards flumequine being between 300 and 400 ng/g, respectively in fish and chicken muscle. According to Okerman et al. (1998 a,b) detection limits of the pH6 plate E. coli ATCC 11303 were 1000 ng/g towards flumequine. In 2001, the same authors investigated sensitivity of another strain of
*E. coli*- Bayer 14 and established detection limits of 150 ng/g. Similar data were reported by other researchers. Kibis and Marinsek (2004) applied microbiological inhibition method with *E. coli* ATCC 25922 as a test microorganism. Examination of poultry meat containing 400 ng/g flumequine revealed positive result in 80% samples, whereas concentration of 450 ng/g resulted in 93.3% positive responses. 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 toward flumequine in poultry meat and liver and fish meat. Samples with residue concentration within of above MRL examined by microbiological inhibition method revealed 100% positive results.

After oral application, fluoroquinolones are well absorbed, distributed into tissues and excreted in urine and feces at high concentrations (Prescott et al., 2000). Flumequine is excreted in the urine and faeces as the parent drug (80%), glucuronide conjugates (12.5%) and 7-hydroxyflumequine (6%), (EMEA, 1996).

During the post treatment period, flumequine concentrations in breast muscle and liver exceeded the MRL values until 2-d of withdrawal period. Rapid decline of flumequine residues in broiler edible tissues was found in our experiment. Similar data were presented in the EMEA report (1996), chickens were treated with equal flumequine doses like in our experiment (12mg/kg/day for five days), 6 hours after cessation of treatment, the concentrations of flumequine were significant: 1 500 ng/g in muscle, 720 ng/g in skin/fat and 2 450 ng/g in 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 170ng/g in our experiment, as well as in EMEA report (1999a,b). On the second day no muscle and liver samples had positive response in microbiological assay. Low levels of flumequine residues were detected in these samples, much below MRL as well as much below the level of detection of microbiological method. On the first day after the end of withdrawal period flumequine was detected by HPLC method in meat (40ng/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.

Two-day withdrawal period for flumequine allowed time for the residue concentration in meat and liver to decrease to an acceptable level prior to slaughter (below MRL).
Conclusion

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

Acknowledgment

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Detekcija rezidua fluorohinolona primenom mikrobiološke skrining metode – flumekvin

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Rezime

Sistemska kontrola rezidua antibiotika uključuje analizu i selekciju velikog broja uzoraka i zahteva primenu različitih skrining metoda. Skrining testovi treba da ispune sledeće zahteve: moraju imati sposobnost detekcije antibiotika od interesa, prag detekcije mora biti saglasan sa zakonskim okvirima (MDK- maksimalno dozvoljena koncentracija), moraju biti jednostavni za izvođenje i isplativi, rezultati testa treba da se dobijaju brzo, testovi moraju biti standardizovani. Cilj ovog rada je da ispita performanse mikrobiološke skrining metode sa E. coli kao test mikroorganizmom: sposobnosti da detektuje antibiotik od interesa fluorohinolon – flumekvin u MDK količinama u obogaćenim i u uzorcima iz ogleda (tkiva pilica i riba). Prag detekcije (LOD) mikrobiološke metode je određen u uzorcima tkiva obogaćenim sa flumekvinom. Uzorci tkiva iz ogleda su dobijeni eksperimentalnim ispitivanjima na pilicima koji su tretirani propisanim terapijskim dozama flumekvina. Prisustvo fluorohinolona u mesu i jetri je ispitano mikrobiološkom i HPLC metodom. Ispitivanjima je ustanovljen prag detekcije mikrobiološke metode u opsegu od 300 ng/g do 400 ng/g flumekvina. Ispitivanjem lečenih životinja mikrobiološkom metodom su dobijeni pozitivni rezultati za sva tkiva u kojima je sadržaj rezidua bio iznad MDK. Rezultati
ispitivanja rezidua flumekvina u tkivima lečenih životinja primenom mikrobiološke skrining metode u potpunosti ispunjavaju zahteve za kvalitativne skrining metode.

References


