

Comparative study of residues of Levofloxacin and Spiramycin in normal and infected tissues of chicken by HPLC

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Abstract

The purpose of this study was to compare the residues of Levofloxacin and Spiramycin in chicken plasma, kidney and breast muscle tissues by HPLC assay in (normal and infected tissues with *E. coli*) at (0,3,5,8) day. After the last therapeutic dose. Sixty broiler chicken with 30 days old of mixed sexes were used in the study. They were divided into 5 groups: control group (non-infected – non treated), normal birds received levofloxacin at 10 mg per kg body weight orally for five days, birds received Spiramycin (15mg/ kg) body weight orally for five days, Birds were challenged with *E. coli* and treated orally with Levofloxacin, Birds were challenged with *E. coli* and treated orally with Spiramycin. and placed in the animal house at Faculty of Veterinary Medicine, Kufa University. The result of residue concentration of two drugs were gradually decreased in all tissue samples from day zero to day eight after the last dose. Concentration in kidney more than muscle tissue for two drugs levofloxacin and Spiramycin and concentration in infected tissue more than normal tissue ($\mu\text{g/gm}$), levofloxacin concentration were ($0.069 \pm 0.002, 0.106 \pm 0.004$) ($\mu\text{g/gm}$). in normal and infection kidney respectively in day 5 after the last therapeutic dose 10mg/kg /B.W orally for 5 days, which less than maximum residue limit (MRL), ($0.2 \mu\text{g/gm}$). Will Spiramycin concentration in infected and normal kidney tissue in day five after the last dose with therapeutic dose 60000 IU/kg/B.W were $0.720 \pm 0.06, 0.349 \pm 0.03$ respectively, more than MRL ($0.30 \mu\text{g/gm}$), therefore the levofloxacin, less residue in tissues and low withdrawal time. Than Spiramycin. **Aim of the study:** This work study the safety of two drug by estimation residue in plasma and tissue and withdrawal time for normal case and infected animals with avian *E. coli*. Following oral administration at the therapeutic dose for five days in the chicken.

Key words: Levofloxacin, Spiramycin, residue, chicken, *E. coli*, HPLC.

دراسة مقارنة لمخلفات الدوائين الليفوفلوكساسين والسبائراميسين في الأنسجة الطبيعية والمصابة تجريبيا (بالعصيات القولونية) في الدواجن بواسطة جهاز كروماتوكرافيا السائل ذات الاداء العالي

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الخلاصة:

اشتملت هذه التجربة على حساب مخلفات الدوائين في الأنسجة الطبيعية والمصابة (بالعصيات القولونية) بواسطة جهاز كروماتوكرافيا السائل ذات الاداء العالي، حيث قسم 60 طير الى 5 مجاميع متساوية، الاولى مجموعة السيطرة الطبيعية، الثانية طبيعية ومعالجة بالليفوفلوكساسين 10 ملغم/كغم من وزن الجسم ولمدة 5 ايام متتالية، الثالثة طبيعية ومعالجة بالسبائراميسين 15 ملغم/كغم من وزن الجسم ولمدة 5 ايام متتالية، المجموعتان الرابعة والخامسة اصيبتا بالعصيات القولونية وعولجت الرابعة بالليفوفلوكساسين والخامسة بالسبائراميسين حيث كانت التراكيز في نسيج الكلية أعلى من بقية الأنسجة لكلا الدوائين والتراكيز في الأنسجة المصابة أكثر من الأنسجة الطبيعية وأظهرت الدراسة إن متبقيات علاج السبائراميسين في جميع الأنسجة أعلى من الكميات المحددة المسموح بها من قبل الاتحاد الأوربي في اليوم الخامس بعد العلاج وتبقى المخلفات حتى اليوم الثامن بعد اخر جرعة اما تركيز عقار الليفوفلوكساسين في الأنسجة كان ضمن الكميات المحددة المسموح بها من قبل الاتحاد الأوربي في اليوم الخامس بعد اخر جرعة، لذلك يفضل استخدام الليفوفلوكساسين في الاصابات البكتيرية وخاصة العصيات القولونية لفعاليتها العلاجية وسرعة التخلص من المخلفات في الأنسجة.

Introduction:

Fluoroquinolone are an important group of antibacterial drugs used in veterinary medicine. **levofloxacin** is a synthetic broad-spectrum of fluoroquinolone group and is used to treat severe bacterial infections which failed to respond to other antibiotic classes (1) Levofloxacin with molecular formula $C_{18}H_{20}FN_3O_4 \cdot 2H_2O$ It is a yellowish white to yellow powder (2). act by inhibiting the DNA gyrase, thus interfering with the DNA-rejoining reaction and the inhibition of the resealing leading to the liberation of fragments that are subsequently destroyed by the bacterial exonucleases. Levofloxacin are active against some gram-negative bacteria, including *E. coli*, *Enterobacter* species, *Klebsiella* species, *Pasteurella* species, *Proteus* species, and *Salmonella* species, including activity against some gram-positive bacteria, chlamydia, mycobacteria, and mycoplasma. In some regions, the use of fluoroquinolones is approved for the treatment of colibacillosis of chickens and turkeys, fowl cholera in turkeys, and bovine. the consumption of animal products with fluoroquinolone residues may result in transmission of resistant bacteria. (3)

Spiramycin

Spiramycin is an antibiotic macrolide whose molecular structure is formed by a ring lactone of 16 members, with a molecular weight of 843.1 Da and pKa of 7.9. Three components spiramycin called spiramycin I, which has a group hydroxyl in C-3 of the aglycone, spiramycin II, in which the hydroxyl groups are acetylated, and spiramycin III, in which this same position is propionilada (4)

The mechanism of action

The mechanism of action of spiramycin is not set completely. It is known to bind reversibly to the 50 S subunit of ribosomes bacterial, leading to blocking reactions transpeptidation and ribosomal translocation, inhibiting protein synthesis and subsequent cell growth. Initially, it acts as bacteriostatic agent, but may be bactericidal against sensitive strains when administered at high concentrations. Spiramycin also accumulates in high concentrations in bacterial cells. Unlike erythromycin, spiramycin not produce stimulation of gastrointestinal motility.

Materials and methods

present study was carried out to evaluate the residues of levofloxacin and Spiramycin

after repeated oral administration for five days in normal and infected chicken thirty days old broiler chicken housed individually with constant environment in temperature ($25^{\circ}\text{C} \pm 2^{\circ}\text{C}$) and 45 to 65 per cent relative humidity. The food and water were provided ad libitum and experimental chicken divided as following groups.

Group I (n=12) birds served as control, the tissue samples of which were used for the calibration of, Liquid chromatography for two drug.

Group- II (n=12) birds received levofloxacin at 10 mg / kg body weight orally for five days.

Group- III (n=12) birds received Spiramycin (15 mg/ kg body weight orally for five days.

Group IV- Birds were challenged with *E. coli* and treated orally with Levofloxacin (10 mg/ kg, B.W), orally for five days.

Group V-. Birds were challenged with *E. coli* and treated orally with Spiramycin (15mg/ kg, BW) orally for five days.

The birds were immediate exsanguinations on day 0, 3, 5, and 8 after the administration of the last dose of levofloxacin and Spiramycin (n=3). Samples of plasma, kidney and breast muscle were collected and the tissue samples were stored at (-45°C) until assayed for concentrations of two drugs by HPLC.

Extraction procedures of levofloxacin from tissue for HPLC assay:

The procedures of extraction of the Levofloxacin from the tissue samples were performed as reported by (5). Five grams of the tissue samples were extracted using of 15ml of (1M phosphoric acid) and (0.3% acetonitrile) (75:25v/v). After homogenizing for 3 min, the mixtures were centrifuged at 3000 rpm (10 min) at room temperature and the whole liquid extract was filtered through a 0.45 μm membrane nylon filter concentration And Purification of the samples The concentrated material was

dissolved in 10 ml of water then loaded to bound elut C-18 cartridge that was previously activated with 5 ml of methanol and rinsed with 10 ml of water. The original concentration bottle was washed twice with 5 ml of 10% methanol. The washing solution was loaded to a cartridge and the flow-through was discarded. Finally, the bottle was washed twice with 5 ml of methanol: 0.05 M NaH_2PO_4 (pH 2.5) (7:3, v/v). This washing solution was loaded to cartridge for elution. The eluent was collected and dried by depressurized concentration at 40°C . The residue was ready for HPLC analysis after dissolved in 1 mL of mobile phase, then filtered by 0.45 μm membrane.

Analytical condition:

The column for separating quinolones was ODS, C-18 (5 μm , 4.6 mm I.D. X 250 mm tekknokroma, Japan. The detector was UV Visible with scan range 200 to 800 nm. Mobile phase was acetonitrile: 0.05 M NaH_2PO_4 (pH 2.5) (35: 65, v/v) containing 3.5 mM sodium dodecylsulphate. Flow rate was 1.0 mL/min. The injected volume of samples was 20 μL .

Extraction procedures of Spiramycin from tissue for HPLC assay:

Chicken tissues (kidney, muscle) were minced and homogenized in the mincer for 1 min. 5 gm of homogenate was accurately weighted into a polypropylene centrifuge tube. 40 ml of 0.5 M phosphoric acid and methanol (3:7) was added. The solid residue was discarded. The water phase was washed 2 times 40 ml n-hexane Centrifugation at 8000 rpm for 10 min. Evaporation at 45°C water bath to 3 ml. The 3 ml sample was subjected to SPE cleanup. Analytical method was done by estimation of macrolide antibiotic spiramycin by HPLC (high performance liquid chromatography) in the collected tissue samples according to (6).

Operating conditions of Liquid chromatography :

Injection volume, 50 µl; flow rate, 1mL/min; wave length, 232 nm; column temperature, 35c; stop time, 15 min; post time, 6 min.; mobile phase 0.05 M phosphoric acid : acetonitrile = 75:25 (PH3.0 v/v).

Preparation of standard solutions of Levofloxacin:

(Figure1).

The standard solution of levofloxacin was prepared by dissolving 1000µg in 10 ml D.W to obtain a concentration of 100µg /ml Working standard concentration were made by further dilution in 0.1m phosphate buffer to obtain concentration of (0.01 -25) µg/ml

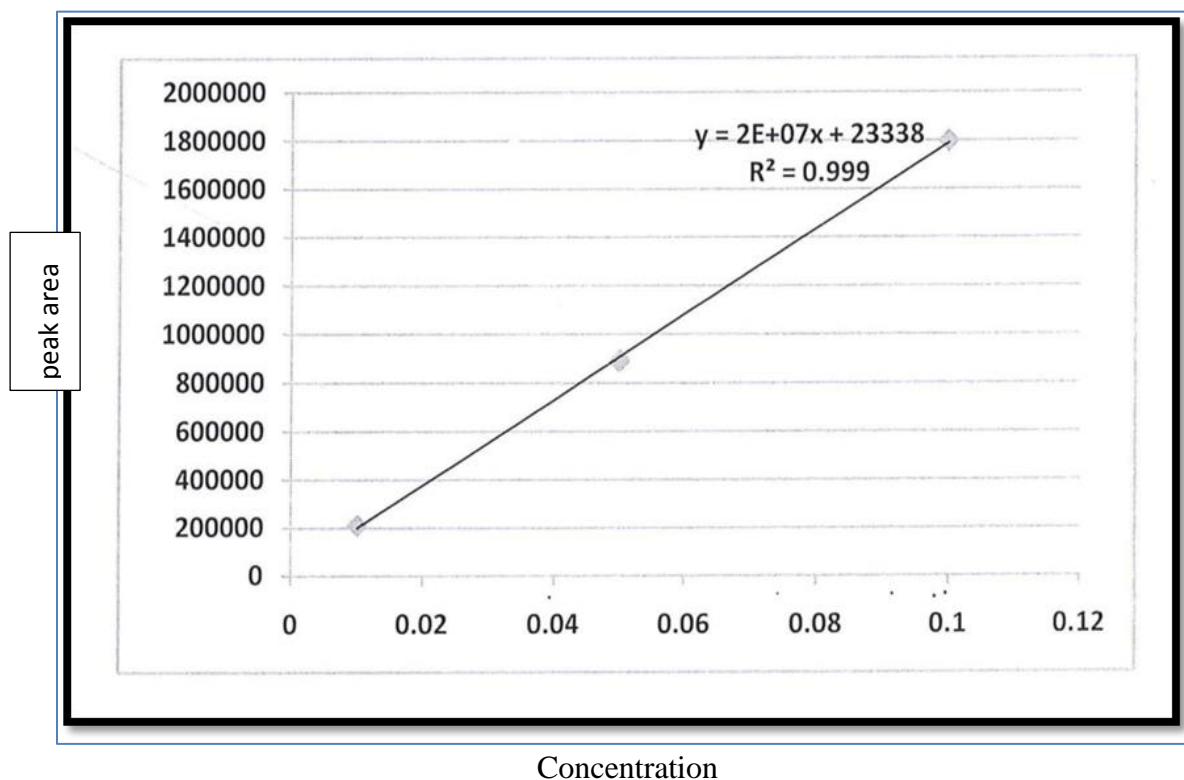
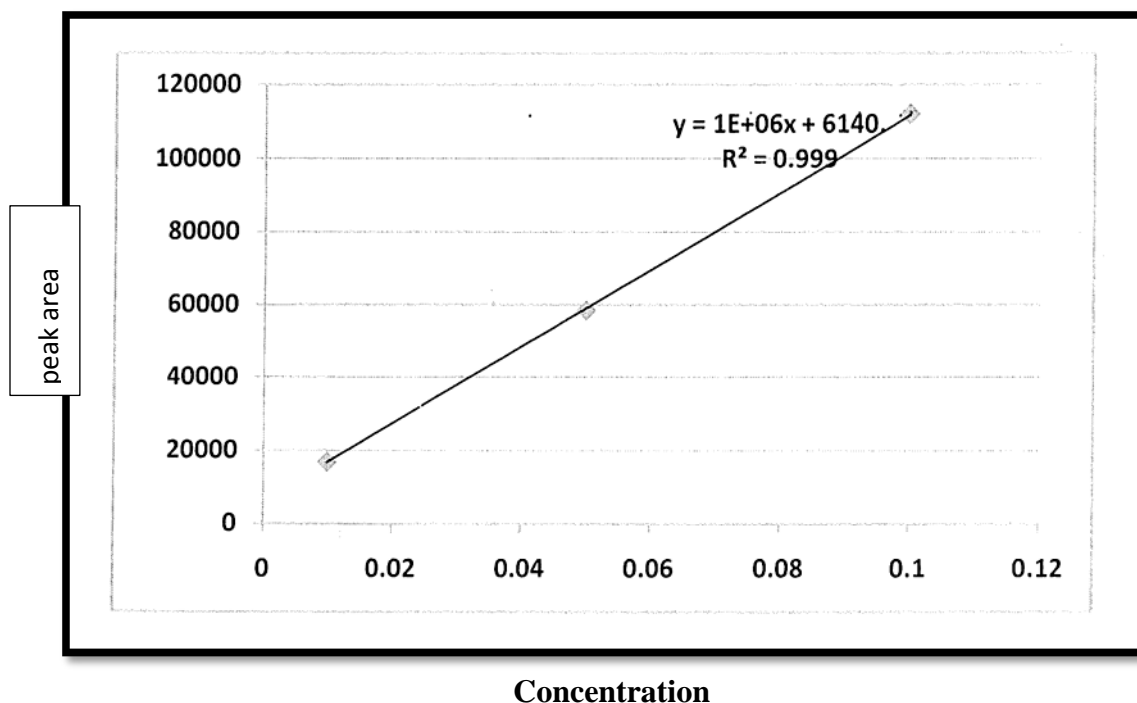


Figure1. Standard curve of levofloxacin in blood plasma by HPLC .

Preparation of standard solutions of spiramycin

The standard solution of spiramycin was prepared by dissolving 1mg in 10 ml methanol to obtain a concentration of 100µg /ml Working standard concentration were made by further dilution in methanol to obtain concentration of (0.01 -25) µg/ml .prepared at the time of use at room temperature in the dark .(figure 2).



**Figure -2. Standard curve of Spiramycin in blood plasma by HPLC .
Escherichia coli strain and inoculation**

Escherichia coli strain was provided by poultry pathology section of Veterinary hospital of Kufa ,that was isolated from the diseased air sacs of chick with a field case of colisepticaemia, pathogenic *E. coli* was further sub-cultured for identification and characterized by using the method described by (7).

After finding out the viable cell count, the broth was diluted to have approximately (3×10^8) bacteria per 0.25 ml and was used for inducing the infection. The diluted broth culture of pathogenic strain of *E. coli* having of (3×10^8) bacteria per 0.25 ml was inoculated to (IV,V groups) intra-peritoneally as described by (7). All the groups were kept under keen observation for the development of clinical signs of *E.coli*

Pathogenicity of *E. coli*

The purified strain of *E. coli* was injected intraperitoneally In 5 chicks and another 5 Chicks were kept as Control group. The inoculated group of chicks was kept under observation till the appearance of clinical

signs. The clinical signs appeared with in twelve hours after the inoculation of *E. coli* and mortality started within 16 – 24 h (8)

Statistical Analysis

The obtained results were statistically evaluated by application of Analysis of Variance (ANOVA) The statistical programmer that was used for analysis of the data is SPSS version, (2006).

Results

A- Identification of *E .coilby* Cultural and colony characteristics

1- Mac Conkey's agar

The colonies were pin pointed, smooth, glossy and translucent and were rose pink in color. The size of the colony varied from 2 - 3 mm in diameter after 24 h of incubation at 37°C.

2-Nutrient agar

The colonies were dome shaped, round, convex, colorless and smooth. The size

of colonies varied from 1 - 2 mm in diameter after 24 h of incubation at 37°C. In nutrient broth, a slimy deposit was developed in the bottom of the tube which was slight pellicle after 24 h of incubation and by shaking the tube. A uniform turbidity appeared in the tube.

3- Eosin Methylene Blue agar

The colony developed after 24 h of incubation at 37°C were 2 - 3 mm in diameter and exhibited greenish metallic shine by the reflected light and dark purple centers by transmitted light.

4- Chromeagar: colony with dark pink to reddish.

Staining and motility

The smears were stained with gram's staining and microscopy was performed. All the isolates were Gram negative rods and motile.

4.5. Biochemical characteristics

Reactions Acid and gas were produced by the fermentation of various sugars like, lactose, glucose and sucrose within one day incubation at 37°C. Methyl red test was performed which was positive ,while no hydrogen sulphide was produced.

Pathogenicity of E. coli

The pure strain of E. coli was injected intra peritoneal in 5 chicks and another 5 chicks were kept as control group. The inoculated group of chicks was kept under observation. The clinical signs appeared within 12 hours after the inoculation of E. coli and mortality started within 16 - 24 h. The clinical signs observed were rise in temp, inappetence, dullness, depression with closed eyes. Postmortem lesions of the dead chicks showed slight congestion of liver and heart and whitish inflammatory fluid accumulation in thoracic and peritoneal cavities. The stained impression smear prepared from heart blood and liver of the dead chicks was examined for the presence of organisms. The organism

showed typically morphological and staining reaction of E. coli.

Residues of Levofloxacin and Spiramycin in chicken plasma, kidney and breast muscle tissue by HPLC in normal tissues and infected at zero time ,3 ,5,8 days.

Following repeated oral administration of levofloxacin 10 mg/kg B.W. and Spiramycin, 60000 IU/kg B.W. of once daily in normal and experimentally infected chicken for five consecutive days. the decreasing concentrations of residues of levofloxacin and Spiramycin were found in all investigated tissues up to the 8th day after treatment .

Concentration in kidney more than muscle tissue for two drugs levofloxacin and Spiramycin ,and concentration in infected tissue more than normal tissue different within group. Spiramycin concentration in infected and normal kidney and muscle tissue in(5d) more than MRL(0.3,0.2µg/g)respectively. In particular levofloxacin indicated the reduced possibility of finding residues of antimicrobial in broiler chicken a few days after treatment and necessity of shorter withdrawal time for this antimicrobial ,in 5 day concentration in kidney and muscle equal to MRL(0.2 ,0.1µg/g) The obtained results was shorter than that recorded of spiramycin at(60000IU) per kg body weight for 5 days, a pre-slaughter withdrawal time of more than 5days is needed to ensure that the drug is eliminated from the tissues. Table (1,2).

Groups	Time (day)	Plasma	Kidney	Muscle
Infection	0	0.267±0.017 c D	1.057±0.10 c D	0.850±0.03 c E
	3	0.153±0.008 b C	0.377±0.0 b B	0.208±0.01 b B
	5	0.066±0.001 a A	0.106±0.004 a A	0.097±0.004 a A
	8	0.049±0.003 a A	0.070±0.003 a A	0.060±0.009 a A
Normal	0	0.286±0.01 C D	0.720±0.01 c C	0.654±0.02 c D
	3	0.114±0.008 b B	0.326±0.002 b A	0.120±0.01 b C
	5	0.063±0.001 a A	0.069±0.004 a A	0.061±0.01 a A
	8	0.052±0.001 a A	0.051±0.003 a A	0.048±0.007 a A

Table (1) Concentration of Levofloxacin (µg/g) in normal and infected tissue of chicken by HPLC.

Values represent mean ± S.E. small letter denote different within group, capital letter denote different between groups

Table (2) Concentration of Spiramycin (µg/g) in normal and infected tissue of chicken. by HPLC assay.

Groups	Time (day)	Plasma	Kidney	Muscle
Infection	0	2.45±0.19 c C	1.577±0.03 c C	0.800±0.08 c D
	3	1.43±0.06 b B	0.870±0.08 b B	0.507±0.04 b C

	5	0.38±0.03 Aa	0.720±0.06 b B	0.390±0.06 b B C
	8	0.14±0.006 a A	0.201±0.006 a A	0.185±0.01 a A
Normal	0	1.37±0.32 c B	0.651±0.22 b B	0.442±0.10 b B C
	3	1.19±0.43 b c B	0.395±0.03 ab A	0.366±0.04 b BC
	5	0.327±0.05 ab A	0.349±0.03 ab A	0.270±0.01 ab A B
	8	0.196±0.03 a A	0.191±0.01 a A	0.101±0.004 a A

Value s represent mean \pm S.E small letter denote different within group ,capital letter denote different between groups

Discussion

Estimation of residues of Levofloxacin and Spiramycin In chicken plasma, kidney and breast muscle in normal tissues and infected by HPLC in poultry, antibiotics and antiparasitics are used extensively for disease prevention and treatment. the antibiotics are also used for growth promotion, although this type of use has been prohibited in the European Union since 2006 (9,10). Edible tissues containing veterinary drug residues can pose risks to human health, including direct toxic effects, allergic reactions and increased bacterial resistance to common antibiotics (11,12).

Antibiotics are widely used in all farm animals species and residues are often found in meat, and they should not exceed the maximal residue limits (13) present study was planned to investigate the residues of antibiotic Following repeated oral

administration of levofloxacin 10 mg/kg b.wt. and Spiramycin, 15mg/kg b.wt of once daily in normal and experimentally infected chicken for five consecutive days. Estimation of residue of Levofloxacin and Spiramycin in chicken plasma, kidney and breast muscle tissue by HPLC at zero time ,3day ,5day,8day after the last dose . the decreasing concentrations of residues of levofloxacin were found in all investigated tissues up to the 8th day after treatment ,concentration in kidney more than muscle by. similar finding were previously reported for broiler chickens (14 , 15 ,16,17)

With the HPLC assay on the five day the levels of kidney and muscle were below the MRL for Levofloxacin 0.097 ± 0.004 , 0.069 ± 0.004 ($\mu\text{g/gm}$) respectively in normal tissue , and in infected tissue 0.106 ± 0.004 , 0.081 ± 0.004 ($\mu\text{g/gm}$) but concentration in kidney ,and

muscle more than normal tissue but there are insignificant change which is agreed with (18) where the residue levels in muscles, were below MRL on the fourth day. (0.068 ± 10) ($\mu\text{g/gm}$), (17) results showing high concentration of levofloxacin in five day (1.05 ± 0.003) ($\mu\text{g/gm}$) in kidney but in muscle is similar to our study (0.05 ± 0.002) ($\mu\text{g/gm}$).

high levofloxacin concentration in different organ indicate its excellence for treating urinary and respiratory tract infection caused by susceptible organism. Similar results showing high concentration of moxifloxacin in different tissues of chickens were reported by (19).

It could be concluded that oral administration of levofloxacin at 10 mg/kg b.wt. may be highly efficacious against susceptible bacteria in broiler chicken. Withdrawal period was calculated based on the residual concentration of the levofloxacin in tissue of chicken, The MRLs were 0.2 and 0.1 ($\mu\text{g/kg}$) kidney and breast muscles respectively, Chicken must not be slaughtered before 5 days of stopping levofloxacin administration, (20) reported the withdrawal period of levofloxacin at five days in normal catfish, (17), reported the withdrawal period of four days for enrofloxacin in muscle and liver in broiler chicken. also our results were in agreement with (22).

The residual concentration of the Spiramycin in tissue of chicken were high values and persisted for eight day, Decreasing concentrations of residues were found in all investigated tissues upto the 8th day after treatment, (2.45 ± 0.19), (1.577 ± 0.03), (0.800 ± 0.08) ($\mu\text{g/gm}$) concentration of Spiramycin in zero time of plasma, kidney, muscle respectively by HPLC assay and decreases in 8 day for (0.196 ± 0.03) (0.191 ± 0.01), (0.101 ± 0.004) ($\mu\text{g/gm}$) in infected tissue, will in normal

tissue the concentration less of infected tissue, but they were more than (MRLs were 0.3 and $0.2 \mu\text{g/gm}$) in kidney and breast muscles respectively at 5 day. At eight days after the stop of administration of Spiramycin the levels in kidney and muscle were below the MRL.

Our results were in agreement with (23) Determination of spiramycin and tylosin in muscle were high concentration, and validated analytical method for determining the concentrations of Spiramycin and neospiramycin in the edible tissues of pigs.

In other investigation, (24) (25) recorded that Spiramycin concentration in Liver, Kidney, Spleen, and Heart and especially Lungs were high. The drug had along retention time in these tissues the long retention in tissues was caused by its relatively slow metabolism and by tissue binding (25) Depend on above results and our study, Chicken must not be slaughtered before 8 days of stopping Spiramycin administration.

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