

## DEVELOPMENT AND VALIDATION OF A RP- HPLC METHOD FOR DETERMINATION OF DIFLOXACIN RESIDUES IN DIFFERENT RABBIT TISSUES

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**Abstract:** The occurrence of antibiotic residues in food of animal origin is attributed to incorrect drug administration or neglecting withdrawal times. These residues cause a lot of human health hazards, so it is important to control their presence. The aim of our study to evaluate a reversed phase- high performance liquid chromatography (RP- HPLC) method for determination of difloxacin residues in different rabbit tissues and serum post intramuscular injection of 5 mg/kg b.wt for 5 days and to investigate the withdrawal period of difloxacin in different rabbit tissues to be safe for human consumption. This method was validated to meet United States Pharmacopeia (USP) and the International Council for Harmonisation of Technical Requirements for Pharmaceuticals (ICH) guidelines. The separation was processed using buffer of triethylamine (pH:3.5) and acetonitrile (80 to 20, v.to v.) solvent on RP- C18 column at 35°C to obtain the ideal peak at 6.752 min. the separation was achieved at wavelength of 280 nm. Validation of analytical method has been done to keep up specifications such as specificity, linearity, precision, accuracy, sensitivity, repeatability, and robustness. The study showed high recovery, low detection limit, good precision. In addition, the rapid steps of the extraction process to be more economical and meet the requirements of green chemistry through minimum use of chemicals during sample extraction with little chemical waste production. Finally, residues of difloxacin were less than recommended maximum residue limits (MRL); 300, 600, and 800 ppb for muscle, kidneys, and liver; respectively on 1<sup>st</sup> day after treatment. Thus, Rabbit`s meat could be eaten safely on the 1<sup>st</sup> day after the end of treatment with 5 mg difloxacin/kg for 5 days.

**Key words:** difloxacin; residues; rabbit; tissues; HPLC; validation

### Introduction

Fluoroquinolones (FQs) are an imperative synthetic antibiotics group, newly invented, that treats numerous types of infection in veterinary and human medication. This group of antibiotics shows great action against a wide range of Gram negative and positive microbes via inhibition of DNA gyrase or topoisomerase II enzymes (1). In the veterinary field, these systemic antibiotics treat systemic troubles including respiratory, gastro-intestinal, urinary tract, and skin infections (2).

Difloxacin (DIF) is a fluoroquinolone carboxylic acid that was industrialized in the 1980s. It is stated as the perfect antibiotic in treatment of definite bacterial infections in veterinary medicine (3-4), due to its low toxicity, and high antibacterial activity against an extensive variety of Gram-negative and positive anaerobes and aerobes. Its antibacterial activity is better than ciprofloxacin and enrofloxacin (5). It is used in chickens and turkeys for treatment of chronic respiratory infections caused by *Escherichia coli* and *Mycoplasma gallisepticum*, in turkeys for the treatment of *Pasteurella multocida* infections, In Cattle for the treatment of bovine respiratory

disease (calf pneumonia, shipping fever) caused by single or mixed infections with *Pasteurella haemolytica*, *Pasteurella multocida*, and/or *Mycoplasma spp.*, and in dogs used for the treatment of acute uncomplicated urinary tract infections caused by *Escherichia coli* or *Staphylococcus spp.*, and for the treatment of superficial pyoderma caused by *Staphylococcus intermedius* (6).

Antibiotic residues may appear in animal tissues if the antibiotics are administered incorrectly or if appropriate withdrawal times are not taken into account (7). To avoid health hazards due to the presence of residues of quinolones in food of animal origin, committee for veterinary medicinal products recommends the MRL of difloxacin in Annex I of Council Regulation (EEC) No.2377/90. Concentrations of 300, 600, and 800 ppb for muscle, kidneys, and liver; respectively were recommended (8).

Reversed phase- High performance liquid chromatography (RP- HPLC) reflects the furthestmost mutual apparatus in analytical chemistry as it categorized by its competence in separation followed by identification and quantification of the analytes existing in food materials. The HPLC usage is upward day by day in the residue study arena. The obtainability of varied choice of column filling, the variability of mobile phases, and the difference in operations methods are the causes for its great request (9). It is an automatic process with high accuracy, sensitivity, specificity, and spends shorter time to obtain results. Owing to the accelerating request to increase the speed in addition to cost-effect of the foods monitoring procedure, there is a need for multi-residue and multi-class investigative methods (10).

Many methods of RP- HPLC were available for determination of difloxacin quantitatively. But there is no in-vivo study was performed till now for estimation of difloxacin distribution in different rabbit tissues after its IM injection.

The current study is the 1<sup>st</sup> to demonstrate tissues depletion of difloxacin residues that centered on verifying a cost-effective, authorized, and subtle way for the determination of difloxacin in different tissues with high recovery and estimation of withdrawal time of difloxacin in rabbits after IM injection for 5 successive days.

## Materials and methods

### *Drug*

Dicural<sup>®</sup> (100 mg per ml), Obtained from Fort Dodge Animal Health, Holland. It is administered via intramuscular (IM) route at a dosage of (5 mg per kg) for 5 days (11).

### *Experimental animals and design*

Twenty-five male healthy New Zealand rabbits (2 to 2.5 kg b.wt) have been used, fed on antibiotics-free feed, received ad libitum water and housed in batteries under similar conditions in a postgraduate research laboratory, Faculty of Veterinary Medicine, Zagazig University. Blank samples were obtained from 4 rabbits (control) for spiked samples preparation in method validation and verification. Twenty-one rabbits were injected difloxacin intramuscular (5 mg/kg) for 5 successive days. Three rabbits were slaughtered in each experiment at the 1<sup>st</sup>, 3<sup>rd</sup>, 5<sup>th</sup>, 7<sup>th</sup>, 9<sup>th</sup>, 15<sup>th</sup>, and 21<sup>st</sup> day post the final dosage. The samples from blood, muscles, liver, and kidneys were collected for quantification of difloxacin residues using HPLC assay according to Sharma et al. (12). The procedure of animal experiments was illustrated in Figure (1).

### *Sampling section*

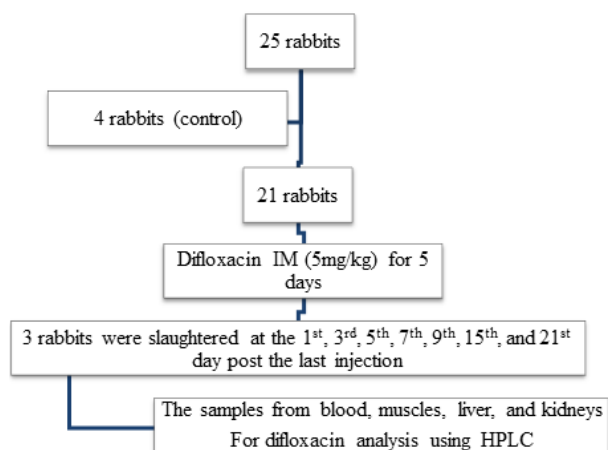
Animals that are subject to procedures exposed for a minimal pain do not require the use of pain-relieving drugs. The blood samples were taken from ear vein immediately before slaughtering in clean tubes for separation of serum, and the samples of muscle, kidneys, and liver were taken in polypropylene tubes after slaughtering using knife to cut a major blood vessel without using anesthesia (Halal slaughter).

### *Ethical approval*

All applicable governmental rules and organizational procedures for animal welfare were followed during this research. (ZU-IACUC Committee, ZU-IACUC/2/F/115/2020 and 22-10-2020).

### *Apparatus and chromatographic conditions*

HPLC; Agilent Series 1200 quaternary gradient pump, Series 1200 autosampler, Series 1200 UV Vis detector, and HPLC 2D chemistation software were used. The chromatographic column was RP-C18 column (4.6 mm, 100 mm, 5 µm), surveyor, Thermo scientific company, USA. Volume of injection: 20 µl, and rate of flow: 0.8 ml per min. and temperature of column: 35°C, UV- Detector: 280 nm and the mobile phase: buffer of triethylamine at pH 3.5 to acetonitrile (80 to 20, v to v).



**Figure 1:** Flowchart of experimental design

#### *Chemical reagents*

Deionized water and acetonitrile of HPLC-grade (Fisher Scientific Co.). Sodium hydroxide (Honeywell Co. Germany). Triethylamine and Orthophosphoric acid (Merck Specialties Pvt. Ltd., India). Reference standard of Difloxacin HCL (Sigma-Aldrich, USA).

#### *Preparation of standard solutions*

Stock standard solution of difloxacin complex was formulated at a 1 mg/ml (1000 ppm) concentration in deionized water and stored in amber glass at 4°C. It was diluted with triethylamine buffer solution (0.5%, pH 3.5) to obtain the fortification solution at a concentration of 10 ppm, which should be freshly prepared daily. The calibration curve was created by fortifying blank rabbit tissues, blank serum with various volumes of fortification solution to yield a concentration range of 200–4000 ppb (calibration samples) and spike blank tissues to prepare quality control (QC) samples at 150, 300 and 600 ppb for muscle, at 400, 800 and 1600 ppb for liver, at 200, 600 and 1200 ppb for kidneys and for serum at a low level of 200 ppb, moderate level 2000 ppb and at a high level 4000 ppb.

#### *Analytical procedures*

Extraction of difloxacin was performed according to Sharma et al. (12); two grams of tissue were precisely weighted into a polypropylene centrifuge tube. Four ml water (deionized) was incorporated. Samples were homogenized, two millili-

ters of them (0.5 ml serum) were taken, then add 1.0 ml of amine buffer (0.5 ml for serum) and left for fifteen min. The mixture has been filtered and kept aside for fifteen min. The tube was firmly closed and vortexed for 5 minutes after adding 2.0 ml of acetonitrile (1 ml for serum). This mixture has been centrifuged for half an hour at 4000 rpm. Then this supernatant has been taken to other tube and centrifuged one more time at 8000 rpm for half an hour. Then the supernatant has been filtered using 0.2  $\mu\text{m}$  syringe filter.

#### *Validation of the method*

It is the procedure of determining the required performance through an analytical method and ensuring that the method under study has performance abilities with the requirements of application (13).

This method has been validated to match requirements of ICH and USP (14, 15). Linearity and range, intra-day precision and inter-day precision, recovery, limits of detection and quantification (LOD and LOQ), robustness, system suitability testing (SST), and specificity were determined using fortified samples and quality control (QC) samples.

#### *Statistical analysis*

This obtained result was demonstrated as mean  $\pm$  standard deviation (SD). These parameters have been analyzed statistically using descriptive statistics via SPSS Inc., version 20.0, Chicago, IL, USA (16).

## **Results**

#### *Results of method validation*

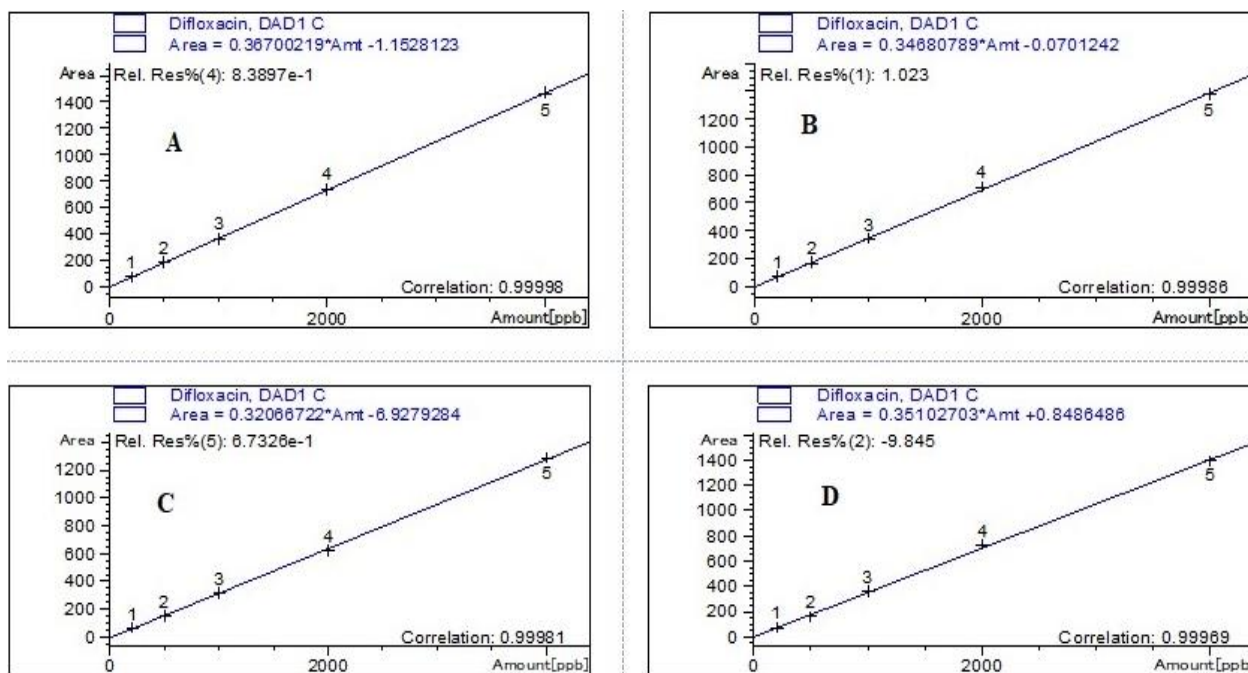
Results in Table (1) summarize the parameters of method validation in different matrixes (serum, muscle, liver, and kidneys) which demonstrated that all the obtained results meet the acceptance criteria set by the specialized agencies (14, 15) as showed in Figures (2).

The difloxacin chromatograms either in serum and different tissues were demonstrated at a specific retention time at 6.572 min. There is no intervention between peaks of any matrix impurities and the intended peak (Figures 3).

**Table 1:** Validation sheet of difloxacin by HPLC

Parameter	Serum	Muscle	Liver	Kidney	Acceptance criteria
Retention time (min.)			6.752		
Range (ppb)			200- 4000		
Slope	0.367	0.3468	0.3207	0.351	
Intercept	- 1.153	- 0.07	- 6.927	0.849	
Correlation coefficient	0.99998	0.99986	0.99981	0.99969	≥ 0.99
LOD (ppb)	6.11	15.9	17.2	24.3	
LOQ (ppb)	18.3	47.7	51.5	72.8	
Recovery (%)	98.1-100.3	91.8-96.1	81.7-87.4	86.9-98	85- 115 %
Intra-day precision (CV %)	0.23	0.37	0.57	0.22	≤ 1%
Inter-day precision (CV %)	0.71	0.84	0.92	1.2	≤ 2%
Pooled robustness (CV %)	0.95	1.04	1.5	0.89	≤ 2%
Tailing factor	1.02±0.01	1.01±0.01	1.01±0.01	1.03±0.02	≤ 2
SST* Symmetry	0.91±0.01	0.92±0.01	0.93±0.01	0.91±0.01	≤ 1
Theoretical plate	6800±41	6640±32	6851±35	6440±60	≥ 2000

\* Results expressed as mean ± SD

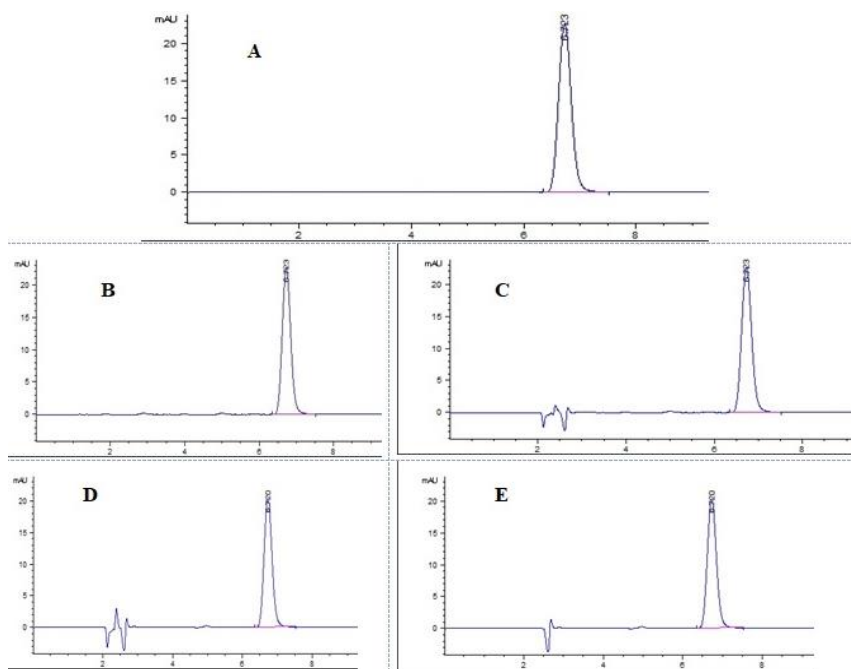


**Figure 2:** Calibration curves for the analytical method for difloxacin quantification in rabbit serum (A), muscle (B), liver (C), and kidneys (D) in the range of 200 to 4000 ppb

#### Results of difloxacin residues in different rabbit tissues

The concentrations of difloxacin antibiotic residues in serum, muscle, liver, and kidneys were illustrated in the Table (2) and Figure (4) after intramuscular injection in healthy rabbits for 5 successive days showing that difloxacin remained

within detectable limit till 3<sup>rd</sup> day in rabbit's serum and muscle and till 5<sup>th</sup> day in kidneys, but still detected in liver until the 9<sup>th</sup> day following intramuscular injection of difloxacin. All obtained concentrations of difloxacin in analyzed tissues were below the maximum residue limits recommended by EMEA (8) at 1<sup>st</sup> day following last intramuscular injection of difloxacin.

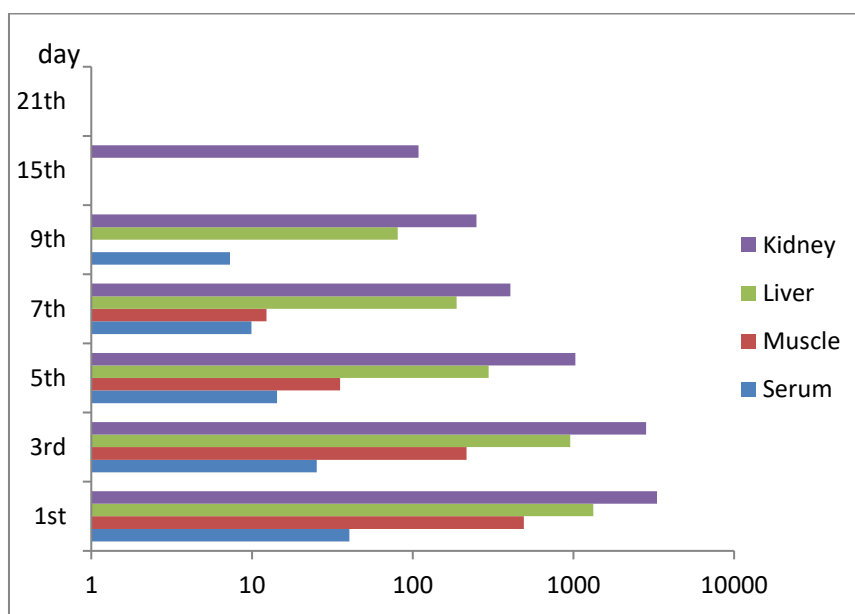


**Figure 3:** Chromatograms of difloxacin at a concentration of 1000 ppb (A: pure standard, B: serum, C: liver, D: kidney, E: muscle) at retention time 6.752 min

**Table 2:** Difloxacin concentrations (ppb) in different rabbit's tissues and serum after intramuscular injection for 5 days (n=3)

	1 <sup>st</sup>	3 <sup>rd</sup>	5 <sup>th</sup>	7 <sup>th</sup>	9 <sup>th</sup>	15 <sup>th</sup> - 21 <sup>st</sup>	MRL
Serum	120±2.5	62±4.4	Nd	Nd	Nd	Nd	
Muscle	146±8.5	58.3±3.5	Nd	Nd	Nd	Nd	300
Liver	730.7±7	317.3±2.5	101.3±2.1	188±9.2	58.7±2.1	Nd	800
Kidneys	246.7±5.1	121±3.6	77.3±2.5	Nd	Nd	Nd	600

Results expressed as mean ± SD



**Figure 4:** Semilogarithmic plot showing difloxacin residues in different rabbit tissues and serum at various intervals following intramuscular dose (5 mg/kg b.wt) for 5 days

## Discussion

The obtained data of validation and verification were found to meet all acceptance criteria of validation recommended by ICH and USP (14, 15). The tailing factor was found to be more than 2 and theoretical plate's  $\geq 2000$  that indicate ideal peak of difloxacin with retention time at 6.572 min. Validation of new analytical method was finished to match with specifications like specificity, sensitivity linearity, accuracy, repeatability, precision, and robustness studies.

Antibiotics are being used on wide scale for treating and preventing many types of disease in animals and humans. Difloxacin supplies important empirical treatment of a wide spectrum of Gram negative and positive microbes and also *Chlamydia*, *Rickettsia*, and *Mycoplasma*. In veterinary medication, difloxacin appears having a strong ability to treat recurrent or severe infections in urinary tract, skin, or soft tissue. The abuses of these antibiotics cause drug-resistance to bacteria and removing it from your vet's arsenal of antimicrobial agents (17-19). The proper use of potent antibiotics, like difloxacin, in food-producing animals is required to maintain the safety and efficacy of difloxacin in future.

Rabbit meat is valued for its nutritional properties because it is lean, rich in proteins of high biological value, low in cholesterol content and high in linolenic acid. It plays an important role in the national economy and offers excellent nutritive and dietetic properties. It can be concluded that rabbit meat is healthier over other meats frequently used in human nutrition (20). The accessibility of extra safe medicines for minor food spp. for example the rabbit is vital for consumer health. When novel therapeutics become registered, off-label and illegal usages of these therapeutics are mostly declined (21, 22) and frequencies of antibiotic resistance are less periodic (23, 24). The abuse of fluoroquinolones in food-producing animals results in drug-resistance marvels (25) and severe pathological alterations in the kids and teens joints (26).

Tissue depletion of difloxacin post IM daily injection of difloxacin (5 mg per kg BW for 5 successive days) has been studied. As far as we know, this is the first work to discuss the depletion of difloxacin residues in tissue.

Difloxacin was rapidly absorbed and distributed into different organs from injection site, these findings agree with Abd El-Aty et al. (11) who found that difloxacin was rapidly absorbed in rabbits after single intramuscular injection of 5 mg/kg body weight with short absorption half lifetime ( $t_{1/2\text{ ab}}$ ) of 0.5 hour.

Concentrations of difloxacin in liver, kidneys, and muscles tissues were initially high and over time decreased, still detected to 3<sup>rd</sup> day in serum and muscle, to 5<sup>th</sup> day in kidneys, but to 9<sup>th</sup> day in liver. The mean concentrations of difloxacin in the checked tissues were less than the MRL at 1<sup>st</sup> day after last IM injection. These results in line with Ligabue et al. (27) who investigated the elimination and distribution of the fluoroquinolone antibiotic marbofloxacin in rabbit liver, muscles, and kidneys after administration of marbofloxacin at 2 mg/kg b.wt for 5 successive days. The concentration of residuals in muscles, liver, and kidneys tissues were determined post treatment by HPLC. Marbofloxacin was distributed rapidly and eliminated rapidly from tested tissues of rabbits. The concentrations were more in the kidneys and liver than in muscles. However, 48 hours post the treatment, the concentrations of marbofloxacin decreased under the MRL determined for this antibiotic in pigs and cattle. Similarly, the withdrawal period for chicken and turkey was 24 hours (6).

Difloxacin recommended for treatment of the major diseases of rabbits, due to its elimination short time as demonstrated in the current work, use of this antibiotic can be extended to rabbit's therapeutic treatments as recommended by Ligabue et al. (27).

Highest concentrations of difloxacin were detected in liver and kidneys, these findings agree with Ligabue et al. (27) who mentioned that marbofloxacin was rapidly distributed and eliminated from rabbit tissues. Concentrations of marbofloxacin were greater in the liver and kidneys than in muscle after intramuscular injection of marbofloxacin at 2 mg/kg of body weight for five days, as the remaining levels in liver, kidneys, and muscular tissues were analyzed post-treatment using high-performance-liquid-chromatography attached with fluorescence-detector.

These findings agreed with that recorded by Abou El-Nil (28) who observed that following

intramuscular injection of 10 mg pefloxacin /kg b.wt in rabbits for 5 succeeding days, the highest level was detected in kidneys at 12 hours from last dose and then not detected on 144 hours post treatment followed by the liver, then muscle and its level showed significant decrease 72 hours. Also, Abdel El-Aziz et al. (29) mentioned that the tissue concentrations of fluoroquinolones were higher in kidneys and liver following oral treatment of infected chicken. On the same line Shams et al. (30) found that post oral daily administrations of 10 mg pefloxacin /kg once for five sequential days in chickens, kidneys and liver have the highest level of the drug.

### Conclusion

Finally, the tissue residues of difloxacin were lower than the recommended MRL in the kidneys, liver and muscles of rabbits at the 1<sup>st</sup> day post-treatment. Thus, Rabbit's meat could be consumed safely without any health hazards from the 1<sup>st</sup> day after end of treatment with 5 mg difloxacin/kg for 5 days.

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Sherief S. Abd Elhafeez carried out the experimental design, HPLC analysis of drug residues, statistical analysis of data, and wrote the article. Ahmed A. Said, Abd El-Alim F. Abd El-Alim and Sameh M. El-Nabtity critically revised the manuscript and served as scientific advisors.

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