Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Vol 6 [9] August 2017: 58-63

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD
Global Impact Factor 0.533
Universal Impact Factor 0.9804

ORIGINAL ARTICLE **OPEN ACCESS**

Residue Level Analysis of Levofloxacin In Thigh Muscle Dual Purpose Chicken By Liquid Chromatography And Mass Spectrometry (LC-MS) Method

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ABSTRACT
Levofloxacin, a third-generation fluoroquinolone, is the S-isomer of ofloxacin and possesses excellent activity against gram-positive, gram-negative and anaerobic bacteria. There is a paucity of information on levofloxacin residue levels in thigh muscle tissues of broiler chicken. Further no fixed MRL level and withdrawal period for levofloxacin for broiler chicken by any regulatory authorities. The estimation of the residue levels of levofloxafin in thigh muscle tissues samples of chicken was studied using Liquid Chromatography Mass Spectrometry (LC-MS) analytical Technique .The study was conducted in 30 to 35 days old (n= 90) Indian Rock-3(IR-3) chicken,a strain of White Plymouth Rock were administered with levofloxacin at the dose rate of 10 mg/kg body weight for five days. The residue analysis was conducted for 10 days after the administration of last dose of levofloxacin. In the present study, high residue concentration of levofloxacin was 364.64 ± 0.78 µg / kg on day one and concentration reduced to 4.72 ± 0.42µg/kg on day nine after last dose of levofloxacin administration in thigh muscle tissue of the dual purpose chicken. It is essential to generate tissue depletion data in order to arrive at conclusion regarding Maximum Residue Limit (MRL) and withdrawal period for levofloxacin in chickens. So the present study is supporting for the fixing MRL and withdrawal period for levofloxacin in thigh muscle of dual purpose chicken.
Keywords — *Thigh muscle, Levofloxacin, Residue level, Dual Purpose Chicken.*

Received 20.06.2017 Revised 11.07.2017 Accepted 29.07.2017

INTRODUCTION

Levofloxacin is a third generation fluoroquinolone broad spectrum antibacterial agent and is a levo isomer of ofloxacin [1], [2]. It's spectrum of activity includes most strains of gram positive and gram negative anaerobic bacterial pathogens responsible for respiratory, urinary tract, gastrointestinal tract, skin and soft tissue infections [3].

With the widespread and adequate use of fluoroquinolones for animal growth and production, there is lack of recommended withdrawal period for fluroquinolones, so accumulation of drug residues in the animal tissues. To ensure safe foods to consumers, withdrawal period for drugs must be respected according to the maximum residual limits established by regulatory agencies. The administration of fluoroquinolones to food-producing animals without an adequate withdrawal period (WDT) may lead to violative concentrations of residues in foods destined for human consumption. These residues represent a risk to public health, including promoting the bacterial resistance [4].

There were few studies in the assessment of residual status of levofloxacin in thigh muscle tissues of broiler chicken. There is no MRL level and withdrawal period fixed for the levofloxacin by European Economic Comminity (EEC) and Food Safety and Standard Authority of India (FSSAI) for dual purpose chicken. It is essential to generate tissue depletion data in order to arrive at conclusion regarding Maximum Residual Limit (MRLs) of levofloxacin drug. The present study was planned to determine the residue level of levofloxacin following oral administration at the dose of 10 mg/kg body weight for five days in Indian Rock-3 chicken, a dual purpose chicken using LC-MS analytical technique

MATERIALS AND METHOD

The residue analysis of levofloxacin after the repeated oral administration was carried out in dual purpose chicken.

The study was conducted in 30 to 35 days old (n= 210) healthy Indian Rock-3(IR-3), a strain of White Plymouth Rock dual purpose chicken developed by Karnataka Veterinary Animal and Fisheries Sciences University, Bidar. The study was performed at the Department of Poultry Science, Veterinary College, Hebbal, Bangalore. The birds were kept under observation for one week prior to commencement of experiment and subjected to clinical examination in order to exclude the possibility of disease. The birds were provided antibiotic-free standard broiler feed for fourteen days. The poultry house was maintained at temperature (25±2^{0}C) and at 45 to 65 per cent relative humidity. Feed and water were supplied *ad libitum* and standard managemental practices were followed to keep the birds free from stress. The prior approval of the Institutional animal Ethics Committee (IAEC) was obtained before the commencement of the experiment (LPM/IAEC/181/2014, Date: 10/01/2014).

Drugs and Chemicals

Levofloxacin Hemihydrate oral solution 10% (Meriflox®, Vetoquinol India Animal Health Private Limited, Mumbai, India) were used for the residue analysis study. The Levofloxacin and Indomethacin technical grade powder were obtained from Vetoquinol, India Animal Health Private Limited, Mumbai and Sigma Aldrich, (Poole, UK) respectively were used for the standardization and calibration of the LC-MS equipment for residue analysis study. Formic acid, acetic acid, methanol and acetonitrile (HPLC grade) were obtained from E-Merck (Germany). HPLC grade water was prepared in-house using a Millipore Direct-QTM 5Water System (Millipore, Watford, UK). Filtration of HPLC mobile phase was performed using Sartorius membrane filters [0.45µm] obtained from Sartorius (Epsom, UK) and Solid Phase Extraction cartridges (Orochem Company, India).

Residue analysis

The residue analysis for levofloxacin was conducted as per European Economic Community [5]

The experimental birds were randomly allotted into two groups, Group I (n=10) birds served as control (without any treatment) for the standardization and calibration of the LC-MS equipment. Group II (n=80) birds were orally administered with levofloxacin at the dose rate of 10 mg/kg body weight for five days. Feed was withheld for 12 h before oral dosing but water provided *ad libitum*. The residue analysis study was conducted for 10 days after the administration of last dose of levofloxacin .

All birds in Group I and II were sacrificed by cervical dislocation (exsanguination) on day 1,2, 3,4,5,6, 7,8,9 and 10 after the administration of the last dose of levofloxacin (n=8/day). The birds were defeathered and manually eviscerated, then samples of thigh muscle were collected, stored at -45^{0}C until analysis.

Preparation of standard solution

A series of working standard solution of Indomethacin (1000-10,000 ng/ml) were prepared by suitable dilution of stock solution. These solutions were kept at 4°C and renewed weekly. These were used to calibrate the liquid chromatography (LC) detector response and recovery studies. The acetonitrile, water and methanol were of HPLC grade, concentrated formic acid, Ultra-pure water was produced from distilled water using a Milli-Q water purification system (Milli-Q gradient, Millipore) were used for preparation of standard solution.

Extraction and clean up tissue samples

A simple method was developed for simultaneous detection and quantification of levofloxacin residues in thigh muscle of dual purpose chicken. Each one gram of defrosted thigh muscle weighed and homogenized with 4 ml of normal saline in blender (Heidolph Silent Crusher M) at 10,000 rpm for 10 min and centrifuged (Eppendorf Centrifuge 5810R, Germany; fixed angle rotor) at 8000 rpm for 10 min. 750 µl of above mixture was collected in a micro centrifuge tube and 100 µl of indomethacin internal standard was added and vortexed to mix well. 250 µl of 1% v/v formic acid in water was added to the above mixture and tubes were subsequently capped and vortexed for about 10 min by using cyclomixer at 80 motor speed briefly to mix the content of tube. Take the supernatant, add 250µl of saturated hexane with acetonitrile for dissolving of the fat component present in the samples. Then tubes were centrifuged at 5000 rpm for 10 min. The supernatant was collected in separate tube and samples were loaded to previously conditioned (conditioned with 1ml of 5 % methanol and 2 ml of HPLC grade water) SPE HLB cartridges (OROCHEM, 30 mg/ml, DVB-LP with particle size 15 µm and average pore size 180-200 Å).The cartridges were washed with 2 ml of HPLC water, followed by 1 ml of 10% v/v methanol in water. The cartridges were eluted with 0.5ml of elution solution (Mobile phase - acetonitrile: 0.1% v/v formic acid in water) and collect in the tubes, then transferred to auto sampler HPLC vials and a 20 µl of the extract was injected into LC- MS system equipped with reverse phase C-18 column (Thermo Scientific BDS Hypersil C-

18 RP, 100x4.6 mm, 5µm) with a flow rate of 0.7 ml. The mobile phase consisted of acetonitrile, 0.1% v/v formic acid in water (70:30) with run time 3.5 min[6].

Preparation of calibration standard solutions and quality control stocks

The primary stock solution of levofloxacin for calibration standard and quality control (QC) samples were prepared in methanol. From the primary stock solution, appropriate dilutions were made using methanol : water (50:50% v/v) as a diluents to produce working standard solutions of 2000, 4000, 10000, 20000, 40000, 80000, 120000, 160000 and 200000 ng/ml. These solutions were used to prepare relevant calibration curve (CC) standards. Another set of working solutions of levofloxacin was prepared in the diluents (from primary stock) at concentrations of 2000, 6000, 100000 and 180000 ng/ml respectively for QC samples (LLOQC, LQC, MQC and HQC). The calibration standards and quality control samples were prepared by spiking 0.01 ml of the spiking stock solution (levofloxacin) into 0.190 ml of screened blank chicken tissue sample. The calibration samples were made at concentrations of 100, 200, 500, 1000, 2000, 4000, 6000, 8000 and 10000 ng/ml. Quality control samples were prepared at concentrations of 100 ng/ml (Lower limit of quality control, LLOQC), 300 ng/ml (lower quality control, LQC) 5000 ng/ml (Medium quality control, MQC) and 9000 ng/ml (Higher quality control, HQC) .

The linearity of the standard calibration curve for levofloxacin in chicken tissues (Fig 1), product ion spectra of levofloxacin (Fig.2) and the chromatograms of thigh muscle were carried out for residue analysis study (Fig.3).

Assay of levofloxacin in tissue samples

The residue concentration of levofloxacin in thigh muscle were analysed by LC-MS equipment (Fig.4).

RESULTS AND DISCUSSIONS

The mean residue concentration of levofloxacin (µg/kg) in thigh muscle (Table II) after the administration of the last dose of levofloxacin with the accuracy of the $AUC_{0\text{-}t/\ 0\text{-}\infty\_obs}$ was0.98. The highest residue level of levofloxacin was observed on day one and gradually decreased up to day nine in thigh muscle samples in after the final dose of administration (Table.1 and Fig.5)

The MRL set for the fluoroquinolones like enrofloxacin and ciprofloxacin in chicken muscle tissue sample was 100 µg/kg (EEC,1990).

High residue concentration of levofloxacin in thigh muscle observed was 364.64 ± 0.78 µg / kg on day one and concentration reduced up to 4.72 ± 0.42µg/kg on day nine after last dose administration of levofloxacin in dual purpose chicken. The residue concentration was not detected on day 10 in the thigh muscle sample because of complete elimination of levofloxacin residues from the thigh muscle tissues.

There are only less data available regarding the residue level of levofloxacin in the thigh muscle of the birds. The present findings are in agreement with following findings, The higher residue level of norfloxacin was 1.14± 0.03 µg/g or 114 ± 0.032 µg/kg/day one and decreased in the concentration up to 0.02± 0.01 µg/g or 20±0.01µg/kg on day five after the final dose administration of norfloxacin in broiler chicken [6]. The high residue level of the levofloxacin in the breast muscle was 428±253 µg/kg on day one and gradually decreased in the residue concentration up to 56±15 µg/kg on day eight after the treatment [7]. The higher levofloxacin residue concentration in breast muscle was 230±0.04µg/kg or 0.23±0.04 µg/g on day one and decreased in residue concentration up to 0.05±0.002 µg/g or 50 ± 0.002 µg/kg on day five after the treatment [8] The residues of ciprofloxacin in broiler chickens at the dose of 8 mg/ kg bw for three days through oral route and were estimated by HPLC technique. The mean tissue concentrations of ciprofloxacin in muscle samples were 0.74± 0.07 µg/kg and 0.02± 0.008 µg/kg on day one and five respectively [9]. The residue depletion of difloxacin and its major metabolite sarafloxacin in chicken tissues after multiple oral doses at 10 mg /kg bw daily for five days using HPLC method. The mean concentrations of difloxacin and sarafloxacin in muscle ranged between 604.8±132.5 and 368.1±52.5 µg/kg [10].

In residue analysis, the persistence of levofloxacin in chicken tissues is attributed to the liphophilicity of the drug. Because of liphophilicity, slow elimination and tissue perfusion rate, residual concentration of levofloxacin was higher in the on day one and gradual reduced to MRL level in breast and thigh muscle tissue samples[11],[12].

CONCLUSION

In the present study, high residue concentration of levofloxacin in thigh muscle were observed was day one day and reduced to Maximum Residue Limit (MRL) on day nine after last dose administration in dual purpose chicken. In order to arrive MRL and withdrawal period, it is suggested to generate tissue depletion data for levofloxacin in birds. The MRL Level and withdrawal period are important in estimating the residue level of the levofloxacin in the chicken tissues and avoid the antibiotic residue

problem in the poultry meat consumers. So the present study is helpful for the fixing MRL and withdrawal period for levofloxacin in birds.

Table 1 . Mean residue concentration of levofloxacin in thigh muscle

Days	Levofloxacin concentration(µg/kg) (Mean ±SE)
1	364.64 ± 0.78
2	191.86 ± 0.70
3	138.79± 0.90
4	85.68 ± 0.84
5	42.38 ± 0.65
6	32.85 ± 0.46
7	24.79 ± 0.95
8	12.83 ± 0.50
9	4.72 ± 0.42
10	ND

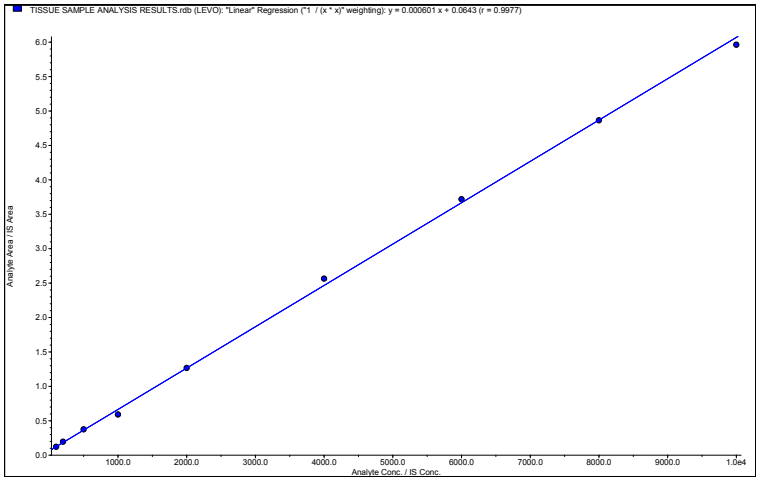

Fig.1. Represents the linearity for the standard calibration curve of levofloxacinin chicken tissue sample (1000 - 10000 ng/ml)

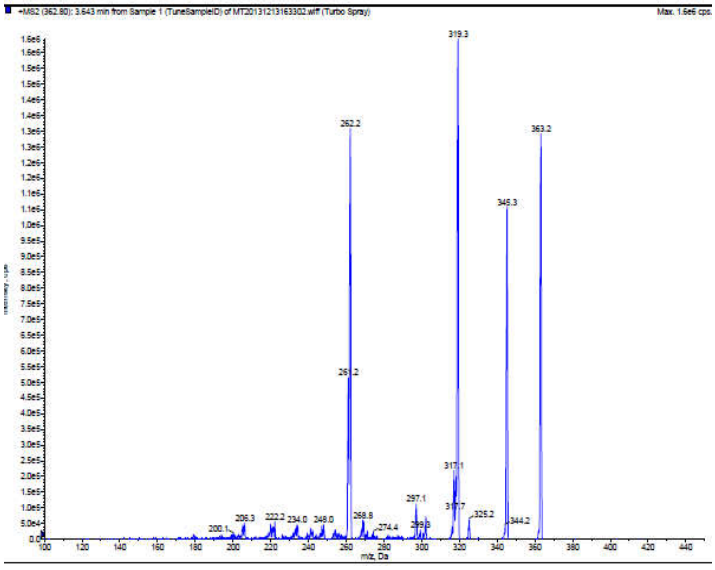

Fig. 2. Product ion mass spectra of levofloxacin

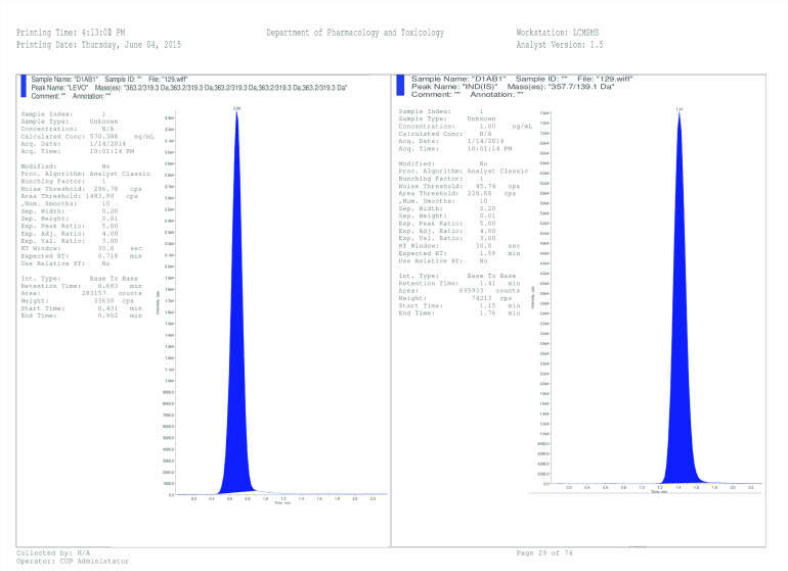

Fig.3. Represents the chromatogram of extracted thigh muscle for levofloxacin

Fig.4. LC-MS EQUIPMENT

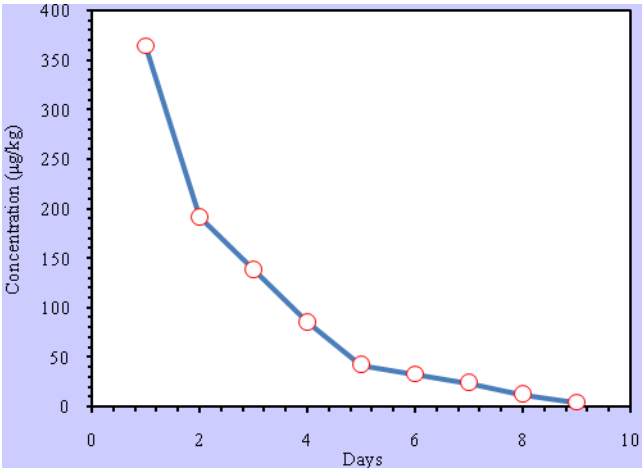

Fig .5. Mean residue concentration of levofloxacin in thigh muscle

ACKNOWLEDGMENT

We would like to thank Vetoquinol, India Animal Health Private Limited, Mumbai for providing Levofloxacin standard and Department of Poultry Science, Veterinary College, Bangalore for providing the birds and space to conduct the experiment.

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CITATION OF THIS ARTICLE

Ravi Kumar,C., Jagadeesh S .Sanganal, Shivashankar,B.P., Shridhar .N.B., Ramachnadra, U., Narayanaswamy, H.D..Sunilchandra, U. and Santhosh C.R. Residue Level Analysis of Levofloxacin In Thigh Muscle Dual Purpose Chicken By Liquid Chromatography And Mass Spectrometry (LC-MS) Method **.** Bull. Env. Pharmacol. Life Sci., Vol 6 [9] August 2017: 58-63