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Determination of Residues of Quinolones in Poultry Products by High Pressure Liquid Chromatography

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Abstract: In this study, High Performance Liquid Chromatographic method was used for the determination of ciprofloxacin, enrofloxacin, levofloxacin, norfloxacin, ofloxacin, flumequine, oxolinic acid and nalidixic acid. These compounds were extracted with meta phosphoric acid : acetonitrile (3:7), followed by a bond elute resins and n-hexane was used to remove fates. The detection limits of quinolones were between 1 to 4 μg on U.V detector. Good linearity was observed from the calibration the graph at concentrations from 2 to 400 μg. A comparative was made between the winter and summer seasons. It was found that the amount of residual quinolones in liver and kidneys were more than those in muscles and eggs.

Key words: Quinolones, high performance liquid chromatography, residual analysis, solvent extraction, cartridges

INTRODUCTION

During the last ten years, the poultry industries have grown to an enormous scale. These need large amounts of Quinolones antibacterial to prevent the infectious diseases of respiratory, digestive and urinary systems therein. Quinolones show antibacterial activity owing to their ability to inhibit DNA gyrase[1,2] which are a type 2 topoisomerase, an essential enzyme for forming DNA supercoils[3].

Quinolones are series of synthetic antibacterial derived from nalidixic acid. There common structure is composed by 1-substituted-1, 4-dihydro-4-oxopyridine-3-carboxylic moiety and aromatic groups (single or multiple rings). Quinolones are classified as the first, second and third generation based on their antibacterial spectrum, potency and pharmacology, there is no widely accepted classification at present. Based on chemical structures, Quinolone antibacterial are divided into two categories. The first category includes antibacterial containing pyridone-carboxylic acid, such as flumequine, oxolinic acid, nalidixic acid, which have good antibacterial activity against gram-negative bacteria. Their antibacterial effect is no longer good as drug resistant bacteria have evolved. The second category (second and third generation) includes the floroquinolones containing fluorine at C-6 position and piprazinyl group at C-7 position such as ciprofloxacin, enrofloxacin, levofloxacin, ofloxacin, which has a broad antibacterial spectrum against gram- negative, gram positive bacteria and mycoplasma, So their antibacterial activity is better. Its multipurpose activities have shown progressive increase and diversity from its 1st to 4th generation. Recently, it is used in veterinary medicine; meat-producing animals resulted in faster growth.

Eleven thousand people were infected with fluoroquinolone resistant campylobacter from eating chicken in 1999 and eight thousand such cases in 1998[4]. In October 2000, FDA proposed banning the use of fluoroquinolones in poultry claiming that these cause infections of resistant bacteria[5]. Studies have demonstrated unmistakable links between the use of antibiotics as sub-therapeutic growth promotants and the prevalence of resistant bacteria against fluoroquinolones, which are used in poultry feed[6]. FDA is looking to ban two poultry antibiotics that are members of the family of drugs also used in humans. These are sarafloxain and enrofloxacin. The concern is mainly due to the rise of fluoroquinolones resistant pathogens called campylobacter bacteria. Pathogens that are transferred to humans when they eat undercooked poultry[7].

Effect of cooking on the decomposition and concentration of Flumequine and oxolinic acid was observed. It showed that cooking temperature had no effect but concentration of these quinolones increased by diffusion from the kidney and liver[8].

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Flumequine is used to treat poultry cholera but it does not dissolve in water sufficiently in stomach and intestinal. But it dissolves in water at pH 10. To dissolve it under digestive and assimilative condition of the food, Kitasamycin is added which reacts with it[9].

The heavy metals form complexes with the fluoroquinolones[10] This is due to their multidentacy arising from the electron donor nitrogen, oxygen and quinoid nucleus. The National Academy of Sciences provided the recommended limits for several toxic substances in water for poultry and livestock[11]. Hard water carries calcium and magnesium which effect feed efficiency and well being of broilers. The varying underground water table varies the contents of metals and non-metal impurities.

The vigorosity of investigation of ofloxacin has much more increased due to the resistance of a broad spectrum of microbes against it, especially when it is used as growth promotant (sub-therapeutic use)[10,11]. Thus the traces have become even more important than the drug itself; the sophisticated dose of this medicine is one of the major tools to control the trace amounts of the drug in various food organisms. The methods for analyzing quinolones include thin layer chromatography, HPLC with UV or fluorescence detection, capillary electrophoresis, HPLC-mass spectrometry and gas chromatography-mass spectrometry. Among these methods HPLC is the most popular one and cheap. The purpose of this research is to establish data of eight quinolones and create awareness about excess use of antibiotic for rapid augmentation. Results can be provided to health authorities as a reference for administration and regulation.

This broad spectrum antibacterial, fluorinated quinolone (Ofloxacin) was first of all synthesized by Hayakawa *et al.*[12]. Hence the residual amounts of antibiotics in poultry habitualize the microorganisms against themselves. This potential effect is transferred to the poultry products' users in the environment and the ecological system contaminated first with the residues and then with the dangerously resistant pathogens. More over in the tropical and subtropical type of environment like that of Ours, high temperature is unable to disintegrate the fluoroquinolones and quinolones, even cooking can not modify them, hence the increasing no of resistant pathogens is a growing danger to humans as well as the other animal health. So it dwindles the economy and trade if breakouts of the diseases occur.

MATERIALS AND METHODS

Procedure: There were 150 samples (120 samples of broiler's meat (liver, kidney and muscles) and 30 samples of layer's egg) randomly purchased from super and local markets in Lahore Pakistan. All samples were stored at -4°C until analyzed. Ten gram of each of the samples of egg, liver, muscle and five gram of kidney samples were used for extraction of quinolones. The samples other than those of egg were of broiler's meat. Phosphate buffer with acetonitrile was added to each sample and magnetic stirring was used for extraction of above cited quinolones. The samples were filtered through whatman filter paper. This procedure was repeated thrice for each sample. Then activated charcoal was used for decolorization and anhydrous sodium sulphate was used for dehydration of the sample. The defatting was done by n-hexane saturated with acetonitrile in a separating flask. Each sample was made upto 50 mL by addition of de-ionized water and cleaning was performed with cation and anion exchange bond elute cartridges. Then the cleaned sample were dried on a water bath and reconstituted with each mobile phase up to 2 mL. Then high-pressure liquid chromatographic analysis was performed.

Chemicals: Reference standards ciprofloxacin (99.13%), enrofloxacin (99.45%), levofloxacin (99.5%) and ofloxacin (99.25%) were purchased from sigma Chem. Flumequine, oxolinic acid, nalidixic acid were provided local industries. The chemical structures of all eight quinolones are shown in Fig. 1. Methanol, Acetonitrile and n-hexane of HPLC grade were purchased from Merck. Sodium hydroxide, sodium hydrogen phosphate, meta phosphoric acid and sodium dodecylsulphate of ultra high purity grade purchased from Merck.

Instrument and apparatus

HPLC: A Shimadzu (shimadzu corporation, Kyoto, Japan) instrument includes shimadzu LC-9A pump system, SPD-6AV UV-Vis Detector and HPLC monitor. The data processing system is class-LC9 control and integration software from Shimadzu.

Preparation of Standard: To prepare a standard solutions, 10 mg of ciprofloxacin, enrofloxacin, levofloxacin, ofloxacin, flumequine, oxolinic acid, nalidixic acid were weight and dissolved in 0.01 N NaOH: methanol (2:8, v/v),

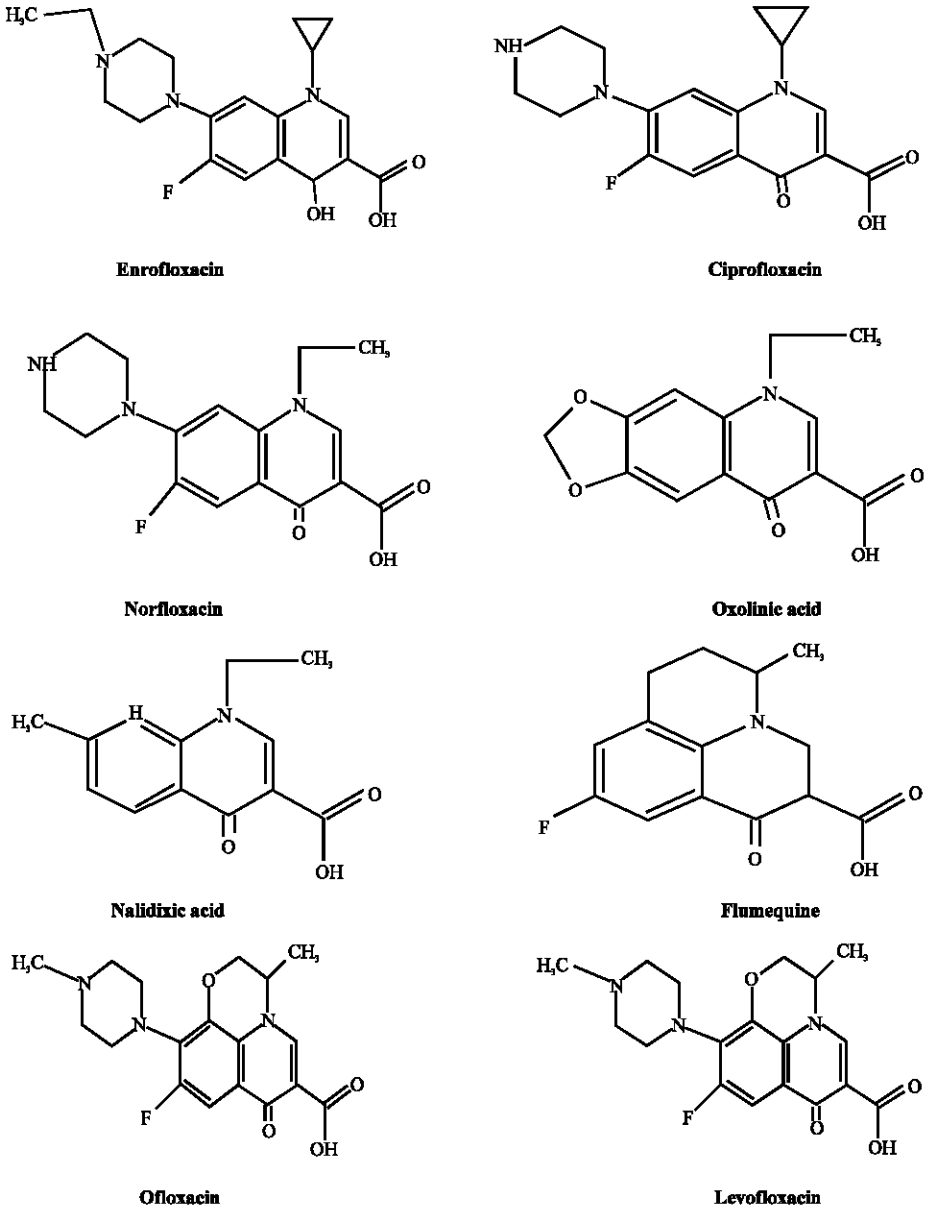

Fig. 1: Chemical structures of the fluoroquinolones/quinolones antibiotics important for residues in poultry industry[10]

added 3.5m M sodium dodecylsulphate and diluted to the volume with mobile phase. Flow rate was 1.0 mL/min. The injected volume of samples was 20 mL.

Identification and quantitative analysis: Quinolones were identified by comparing the retention time, area and spectra of peaks of unknown substance with standard substance. Samples and mixed standard solutions were analyzed by HPLC with UV detector. Quantitative analysis of each quinolones was calculated by the formula as below:

$$\text{Amount of each quinolone in samples } (\mu g) = \frac{\text{AUC of Sample X purity of STD}}{\text{AUC of STD}} \times 100$$

AUC: Area under curve of sample
AUS: Area under curve of STD.

Preparation of samples

Extraction: After homogenized, 10 g of sample was weighed and then transferred to the homogenizer with 30

mL of 0.3% m-phosphoric acid: acetonitrile (1 : 10, v/v), followed by homogenizing for 3 min. The mixture was filtered under suction by Buchner funnel and then shaken for 5 min with 50 mL of hexane, saturated by acetonitrile in a separatory funnel.

Clean up: 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 (nylon, micron separations Inc., west Borough, MA, USA).

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.

RESULTS AND DISCUSSION

The present studies were conducted for the estimation of quinolones in poultry products purchased from local market. The quinolones included in this study were ciprofloxacin, enrofloxacin, levofloxacin, norfloxacin, ofloxacin, flumequine, oxolinic acid and nalidixic acid. The poultry products included [broiler's meat (liver, kidney, muscle) and layer's egg].

Many poultry growers are using antibiotics quinolones for the rapid augmentation of chickens and to decrease the occurrence of diseases. The usage of these antibiotics in poultry feed industry is increasing day by day particularly quinolones are ahead. And people use chicken as daily food, so people get small doses of the drugs, when they eat chicken it weakens the effectiveness of those drugs in people and also cause some side effects (gastrointestinal pain and stomach problems etc.)[13]. Wide spread use of antibiotics such as quinolones in poultry food industry results in germs that don't respond to antibiotic treatment. People become infected with these bacteria when they eat contaminated chicken. This research shows that poultry is considered to be the food most often contaminated with disease-causing organisms. So, chicken should be free from these drugs for the safety of human health. Quinolones is the main class of antibiotics, which is used abundantly in poultry industry (Fluoroquinolones is the sub-group of quinolones). In Fluoroquinolones, Ciprofloxacin and Enrofloxacin are used at large scale as growth promoters in chicken.

Table 1: Detection limits of eight quinolones with UV detector

Quinolones	Wavelength (max)	Detection limit (μg)
Ciprofloxacin	278	2
Enrofloxacin	286	2
Levofloxacin	294	2
Norfloxacin	275	2
Ofloxacin	294	2
Flumequine	241	4
Oxolinic acid	267	1
Nalidixic acid	258	1

High performance liquid chromatographic method was used to determine the residues of antibiotics. Enrofloxacin, ciprofloxacin, norfloxacin, levofloxacin and ofloxacin have same detection limit 2 μg, flumequine has 4 μg and oxolinic acid Nalidixic acid has 1 μg limit, respectively (Table 1).

According to the method described by Horie *et al.*[16] various concentrations of SDS (2 to 5 mM) were added to acetonitrile: 0.05 M NaH_2PO_4 (pH 2.5) (35:65, v/v) to study its effect of separating quinolones. The first generation quinolones are more hydrophobic because ion-pairing reagents do not affect them. On the other hand, the second-generation quinolones contain two ionizable groups, carboxylic acid and piperazine, so they are in the form of cation in acidic condition and their polarity is stronger. The polarity factor will increase along with SDS concentration.

Chicken liver contains the grater level of enrofloxacin, ciprofloxacin, ofloxacin and Flumequine then the rest of the four quinolones. These results are different from drug levels found in animal tissues, among which liver usually contains a higher amount. Subsequently, chicken samples (liver, kidney, muscle and egg) were stored at 4°C for 1, 4 and 8 days to test the stability of enrofloxacin, ciprofloxacin and ofloxacin. The residual level of Enrofloxacin in samples did not decrease after eight days. That is the residual enrofloxacin in chicken muscle samples did not easily degrade during refrigerated transportation and storage (4°C) process.

Enrofloxacin occur most abundantly and widely in the poultry products. The decreasing order of abundance of the quinolones as found tissue wise was as follows.

Liver > Kidney > Muscle > egg

Liver

Enrofloxacin > Ciprofloxacin > Ofloxacin = Flumequine > Levofloxacin > Norfloxacin > Oxolinic acid =Nalidixic acid

Oxolinic acid and Nalidixic acid was not detected of all liver samples.

Kidney

Ciprofloxacin > Enrofloxacin > Ofloxacin > Flumequine > Levofloxacin > Norfloxacin > Oxolinic acid =Nalidixic acid

Oxolinic acid and Nalidixic acid was not detected of all kidney samples.

Muscle

Enrofloxacin > Ciprofloxacin > Ofloxacin > Flumequine > Norfloxacin > Levofloxacin =Oxolinic acid =Nalidixic acid

Oxolinic acid, Flumequine and Nalidixic acid was not detected of all muscle samples.

Egg

Ciprofloxacin > Enrofloxacin > Ofloxacin > Levofloxacin > Flumequine > Norfloxacin > Oxolinic acid = Nalidixic acid

Oxolinic acid and Nalidixic acid was not detected of all egg samples.

The ranges of ciprofloxacin, enrofloxacin, norfloxacin and ofloxacin were found in liver 2.45 to 245 μg kg^{-1}, 3.10 to 364 μg kg^{-1}, 2.20 to 31 μg kg^{-1} and 2.05 to 22 μg kg^{-1} in summer season respectively Table 2. The highest value 345.13 μg kg^{-1} for kidney in case of ciprofloxacin show that ciprofloxacin concentration differs most widely in kidney then in liver, muscle and egg. The flumequine was found 14 and 13 μg kg^{-1} in liver and kidney in summer season respectively and 6 μg kg^{-1} for muscle and egg. Enrofloxacin and ciprofloxacin show 85 and 92%, 82 and 78%, 62 and violation from the internationally accepted MRL's (Maximum Residue Limits)[15-17]. Hence these required more washing out periods and dose sophistication for poultry chickens during summer. The residues of oxolinic acid and nalidixic acid were not detected in any sample.

Seasonal variation were also observed and shown in Table 2. A comparison between two seasons (winter and summer) was made. Antibiotic residues were estimated more in summer season then winter. The average values in Table 3 and 4 of quinolones were also shown that antibiotics were administrated more in summer season then winter. Form data in Table 2, liver and Kidney was most contaminated part of poultry products in both seasons. The residues of antibiotic were found in lower

Table 2: Quinolones residues in chicken muscle, liver, kidney and egg purchased from various markets of Lahore

Category	Quinolones	Violated samples (%)	Quinolones in summer Min	(μg kg^{-1}) Season Max	Quinolones in winter Min	(Mg kg^{-1}) Season Max
Liver	Ciprofloxacin	85	2.45	245	2.08	200
	Enrofloxacin	92	3.10	364	3.02	289
	Levofloxacin	42	3.45	25	0.00	20
	Norfloxacin	30	2.20	31	2.93	11
	Ofloxacin	56	2.05	22	2.32	32
	Flumequine	12	4.15	31	0.00	4.45
	Oxolinic acid	0	N.D	N.D	N.D	N.D
	Nalidixic acid	0	N.D	N.D	N.D	N.D
Kidney	Ciprofloxacin	82	3.15	345.13	2.68	211
	Enrofloxacin	78	3.03	321.02	3.01	180
	Levofloxacin	23	0.00	12	0.00	10
	Norfloxacin	10	2.05	8	0.00	9
	Ofloxacin	41	2.90	48.25	2.22	21.25
	Flumequine	9	2.05	26.25	2.95	23.02
	Oxolinic acid	0	N.D	N.D	N.D	N.D
	Nalidixic acid	0	N.D	N.D	N.D	N.D
Muscle	Ciprofloxacin	62	2.15	180	3.86	169
	Enrofloxacin	55	3.08	190	3.90	185
	Levofloxacin	24	0.00	10.04	0.00	12.34
	Norfloxacin	11	2.09	12.02	2.03	9.05
	Ofloxacin	45	3.20	25	3.07	12.23
	Flumequine	10	2.80	13.65	2.62	9.04
	Oxolinic acid	0	N.D	N.D	N.D	N.D
	Nalidixic acid	0	N.D	N.D	N.D	N.D
Egg	Ciprofloxacin	58	2.15	115.02	2.35	102.96
	Enrofloxacin	70	3.45	110.29	3.05	85.56
	Levofloxacin	9	0.00	5.45	0.00	5.63
	Norfloxacin	8	2.11	1.25	2.04	2.03
	Ofloxacin	40	2.01	6.23	2.02	5.64
	Flumequine	19	2.8	2.25	2.04	2.69
	Oxolinic acid	0	N.D	N.D	N.D	N.D
	Nalidixic acid	0	N.D	N.D	N.D	N.D

Table 3: Residual antibiotics (quinolones) in broiler's meat (liver, kidney, muscle) and layer's egg in summer season

Quinolones	Detection (λ max nm)	Liver Ave. value (μg kg^{-1})	Kidney Ave. value (μg kg^{-1})	Muscle Ave. value (μg kg^{-1})	Egg Ave. value (μg kg^{-1})
Ciprofloxacin	278	122	172	90	56
Enrofloxacin	286	185	166	96	63
Levofloxacin	294	22	5	6	4
Norfloxacin	275	15	5	5	6
Ofloxacin	294	10	24	12	8
Flumequine	241	14	13	6	6
Oxolinic acid	267	N.D	N.D	N.D	N.D
Nalidixic acid	258	N.D*	N.D	N.D	N.D

N.D* Not detected

Table 4: Residual antibiotics (quinolones) in broiler's meat (liver, kidney, muscle) and layer's egg in winter season

Quinolones	Detection (λ max nm)	Liver Ave. value (μg kg^{-1})	Kidney Ave. value(μg kg^{-1})	Muscle Ave. value (μg kg^{-1})	Egg Ave. value (μg kg^{-1})
Ciprofloxacin	278	100	106	82	42.0
Enrofloxacin	286	151	98	95	40.2
Levofloxacin	294	10	6	6.2	4.2
Norfloxacin	275	6	5	5.4	3.0
Ofloxacin	294	15	10	6.5	3.6
Flumequine	241	2.64	11	5.0	2.2
Oxolinic acid	267	N.D	N.D	N.D	N.D
Nalidixic acid	258	N.D	N.D*	N.D	N.D

N.D* Not detected

level in muscles and egg than rest of the samples. As a result ciprofloxacin, enrofloxacin, ofloxacin and flumequine were determined in higher concentration then the rest of the four antibiotics in both seasons.

CONCLUSION

High Performance Liquid Chromatographic (HPLC) method was used for the determination of quinolones. Ciprofloxacin and enrofloxacin residues were found in higher level in poultry tissues of liver and kidney. The results indicated that 58 to 85% samples of ciprofloxacin and 55 to 92% samples of enrofloxacin violated the regulation. The residues of oxolinic acid, nalidixic acid were not detected but the residues of flumequine, levofloxacin and norfloxacin were found in liver and kidney in higher quantity then muscle and egg. Liver and kidney are the most contaminated part of chicken than muscle and egg. Antibiotics are administrated more in summer season than winter. These must be monitored in the poultry industry. Doze sophistication must be ensured with respect to seasonal variation. The residues of flumequine, levofloxacin and norfloxacin in most of the samples followed the international regulations.

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