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ESTIMATION OF SELECTED RESIDUAL ANTIBIOTICS IN MUSCLE, KIDNEY, LIVER, AND EGG OF LAYER CHICKEN

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Abstract: The present studies were conducted for the estimation of quinolone residues in our local poultry products. The poultry products included muscle, kidney, liver and egg (yolk and white). The quinolones included in this study were, oxolonic acid, flumequine, enrofloxacin, and ciprofloxacin. An assessment of the variation of each analyte (quinolones antibiotic residues) in these products was made. A comparison was made among the analyte (quinolones) concentrations in different tissues/organs and their internationally permissible safer maximum residue limits (MRLS'). Infra Red spectra were used to detect the presence and identification of different quinolones. HPLC with ODS Column and U.V. detector was used for the quantification.

Keywords: Antibiotic residues, Infra Red spectroscopy, U.V. spectroscopy and High Pressure Liquid Chromatography

Introduction

Pakistan is one of the developing states of the third world. Similarly its frontiers of industry, trade and economy are in a developing state. One of these industries is the poultry industry which, though not advanced, yet has an investment of Rs.65 billion that will come up to Rs.71 by the next year. We have 15000/- poultry farms, producing 370000/- metric tonnes poultry meat and 48.60 million poultry eggs. Seventy percent of the total antibiotics produced today are fed to poultry birds and livestock. U.S senate has passed a bill No. S-2508 for the preservation of antibiotics for human treatment through an act of 2002, with the risk from pathogenic resistance against the antibiotics. 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 (ENRF). The concern is mainly due to the rise of fluoro-quinolone resistant pathogens called campylo-bacter bacteria. Pathogens that are transferred to humans when they eat undercooked poultry [1].

Quinolones are applied to prevent infectious diseases. When the lung, urinary or digestive system of an animal is infected, it can be treated by quinolone anti-bacterials by inhibition of DNA gyrase [2,3] which is a type II topoisomerase, essential enzyme for forming DNA supercoils [4].

The quinolones have been classified according to their antibacterial spectrum; potency and pharmacology. There is no widely accepted classification of these at present [4,5]. These are divded into two categories; the first generation quinolones include, nalidixic acid (NAL acid), oxolinic acid (OXOLN acid), Flumequine (FLUM) and piromidic acid (PIRM acid), which have good antibacterial activity against gram-

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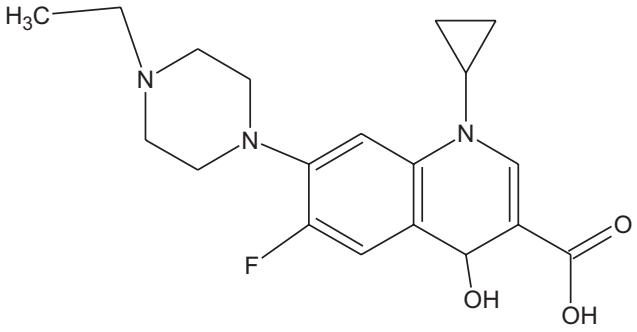
negative bacteria [6]. Their antibacterial effect is no longer good as drug resistant bacteria have evolved [7]. The second category; (the second and third generation) includes fluoroquinolones containing fluorine at C-6 position and piperazinyl at C-7 position, such as marbofloxacin (MARBF), ofloxacin (OFLX), danofloxacin (DNF), ENRF, CPRF, sarafloxacin (SRF), norfloxacin (NORF), and levofloxacin (LEVOF). These have broad antibacterial spectrum. They are safe drugs. These are effective against gram positive, gram-negative bacteria and mycoplasma. So their antibacterial activity is better [2,7,8].

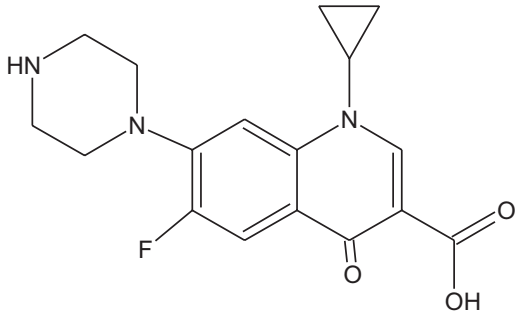
In October 2000, FDA proposed banning the use of fluoroquinolones in poultry claiming that these cause infections by resistant bacteria [9]. Studies have demonstrated unmistakable links between the use of antibiotics as sub-therapeutic growth promoters and the prevalence of resistant bacteria against fluoroquinolones, which are used in poultry feed [10].

Different international fora have set up maximum permissible and safer antibiotic residue limits [11,12,13]. Hard water carries calcium and magnesium which affect feed efficiency and well being of layers. The varying underground water table changes the contents of metals and non-metal impurities.

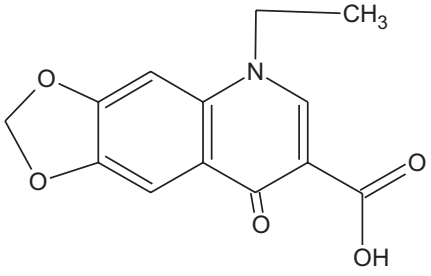
The effect of cooking on the decomposition and concentration of FLUM and OXLN acid was observed. It showed that cooking temperature had no effect but concentration of these quinolones increased by diffusion from the kidney and liver [14]. Flumequine is used to treat poultry cholera but it does not dissolve in water sufficiently at



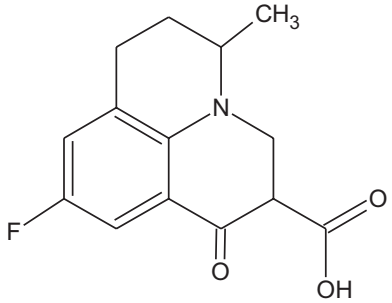
*Enrofloxacin



*Ciprofloxacin



Oxolinic Acid



*Flumequine

Fig. 1. The Chemical structure of quinolone (*=fluoroquinolones).

the stomach and intestinal pH. 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 [15]. Minimum inhibitory concentrations (MIC) of 21 antimicrobial agents including FLUM, NAL, OXOLN, Oxytertracycline (OTC), ENRF were estimated against the avian mycoplasma, in Taiwan. All of the field isolates were highly sensitive with (MIC 50μg/ml) [16]. OXOLN, FLUM and OTC reduced the transformation of fatty acids in marine fish farm sediment from a simulated abandoned site. These change humus into natural less fertile soil [17]. The residual amounts of antibiotics in poultry habitualize the microorganisms against themselves. This potential effect is transferred to the poultry product users in the environment and the ecological system contaminated first with the residues and then with the dangerously resistant pathogens. Moreover in the tropical and sub-tropical type of environment such as in Pakistan, high temperature is unable to disintegrate the fluoroquinolones and quinolones, even cooking cannot modify them, hence the increasing no of resistant pathogens is a growing danger to humans as well as to the other animal health. So, it stifles the economy and trade if breakouts of the diseases occur.

Materials and Methods

The samples were collected randomly from local markets situated in Lahore. The layer birds and their eggs were obtained from local markets in Lahore. The samples were collected during the summer of 2004.

HPLC systems consisted of LC- 9A pump (Shimadzu), SPO-6AB, UV-visible spectro-photometric detector (Shimadzu), CTO 6A oven (Shimadzu), SCL -6B system controller, and CSW-32 software. The ultrasonic cleaner (EYELA Tokyo Rikakai Co-Ltd) was used for degassing the samples, standard and mobile phase. Sartorius filtration assembly (Germany) was used for filtration with nylon filter of 0.45ìm pore size (Germany). PH of the mobile phase was set by pH meter (Hanna HI-8418). The extractants, solvents and mobile phase constituents were of HPLC grade.

Extraction of quinolones from layer meat and eggs

Samples of egg yolk and white, liver and muscle (10g of each) were used extraction of quinolones. Phosphate buffer with acetonitrile was added to each sample and magnetic stirring was performed for extraction. The samples were filtered by Whatman filter paper. This procedure was repeated thrice for each sample. Then activated charcoal was used for decoloration and anhydrous sodium sulphate was used for dehydration of the sample. The defatting was done by n-hexane saturated with acetonitrile in a separation flask. Each sample was made up to 50ml by addition of de-ionized water and cleaning was performed with cation and anion exchange bond elute cartridges. The cleaned samples were dried on a water bath and reconstituted with each mobile phase up-to 2 ml followed by high pressure liquid chromatographic analysis.

Extraction of fluoroquinolones from egg

Egg yolk and white (10g) was weighed and homogenized with 2% acetic acid in CH_3CN. Anhydrous sodium sulphate was used for dehydration of the samples before centrifugation. The supernatant was filtered through anhydrous sodium sulphate and washed with acetonitrile. Acidic quinolones were extracted in the same manner as above but without the addition of acetic acid. Acetonitrile was evaporated to dryness and redissolved in 0.05 M disodium hydrogen phosphate (pH 4).

Cleaning of samples

A Bond Elute strong cation exchange (SCX 500mg, Varian) solid phase extraction (SPE) cartridge was conditioned with acetonitrile-glacial acetic acid (95+5 v/v). More acetic acid (5ml) was added to the extract which was then applied to the cartridge. The cartridge was washed sequentially with acetone (2.5ml), (methanol 5ml) and (acetonitrile 5ml). The cartridge was eluted with methanol-35% ammonia solution (95+5 v/v, 5ml) into a test tube. The eluate was dried and reconstituted with HPLC mobile phase. Acid quinolones were cleaned with AGMP-1 resin (Bio-Rad, Richmond (CA, USA) by slurring it in water and packed into empty column and poultry sample extracts were applied to the cartridge. The cartridge was washed with water, methanol, and acetonitrile 5ml each, sequentially it was then eluted with 2x5 ml methanol-acetic acid (95+5 v/v). The eluate was dried and redissolved in mobile phase [18].

HPLC determination

HPLC mobile phase for ENRF and quinolone acids consisted of acetonitrile phosphate buffer with 3.5mM SDS (35:65) at pH 3. The mobile phase for CPRF consisted of CH_3CN: phosphoric acid: deionised water (200:2:800). The ODS column was used. The injection volume was 20ìl. The UV detector at ambient temperature was used. The flow rates and detection wave lengths for CPRF and ENRF were 1.5ml and 278 nm and 1ml and 284 nm, respectively. The quinolone carboxylic acid OXOLN acid and FLUM were determined with the same apparatus as that for basic quinolones but the mobile phase consisted of 0.01 M oxalic acid: acetonitrile: methanol (6:3:1). The wavelengths of detection were 241 nm and 267 nm for FLUM and OXOLN acid, respectively.

Identification and Quantification

Quinolones (CPRF, ENRF, OXOLN acid and FLUM) were identified in the sample by

Table 1.
Maximum Residue Limits (MRL's) for quinolones in force in Europe [11,12,13].

Substance	Marker Residue	Species	MRL's (µg/kg)	Tissue
Enrofloxacin	Sum of enrofloxacin & ciprofloxacin	Bovine Porcine Poultry	30 30 30	Muscle Liver Kidney
Ciprofloxacin	Sum of enrofloxacin & ciprofloxacin	Bovine Porcine Poultry	30 30 30	Muscle Liver Kidney
Flumequine	Flumequine	Bovine Ovine Porcine Poultry Salamander	50 100 300 50 150	Muscle Liver Kidney Fat, Skin Muscle, Skin

comparing IR spectra, and retention times of standards with those of unknown substances in the samples using their respective mobile phases in the case of HPLC with U.V. detection. Estimations were made (1) by using standard calibration curves, and (2) by using the relation of peak area per ppm calculations from the standard calibration curve values of peak area/ppm calculation from the standard calibration and concentrations.

Statistical Analysis

The statistical parameters of range, range size and confidence level are all important in this analysis. The variation of the residual amounts of quinolones in different samples of the bird's organ/tissue differ very widely. These have to be described precisely and comprehensively. Hence the mean residue concentration of quinolones is described at 95% confidence level. For example layer liver concentration of ENRF was 1357.15 ± 342.32 at this confidence level. It means that ENRF residues were found in the narrower range deviating positively and negatively by 342.32 μg from the mean value 1357.15 μg / Kg with 95% probability. The ranges described in the Tables are significant because these give information of minimum and maximum limits of residues found actually in the samples. The range sizes of different quinolones residues give a patch of concentration irrespective of there upper and lower limits. It also is an index of the bulk of variance. The range is related to standard deviation by the following relation:

$$Sr = RK_R$$

Sr, R and K_R are standard deviation of the population, range and range deviation factor respectively. As the number of samples decreases, the efficiency of the range decreases relative to the standard deviation. These parameters are also an index of the depository and excretory behaviour of the residues of quinolones in the poultry birds. The confidence limits could be calculated using Sr obtained from the range. The confidence limit, mean concentration and range are interrelated as follows.

$$\text{Confidence limit} = x \pm Rt_r$$

Where x, R and t_r are mean concentration, range and range confidence factor respectively. As all these statistical values are interrelated, these affect one another. To describe the analytical data having some degree of variance like that of quinolone residues, with full precision, we have to use the above parameters [19].

Results and Discussion

Tables 2-7 show that ENRF occurs most abundantly and widely in the poultry products. The order of abundance of the quinolones in decreasing order as found tissue/organ wise was: ENRF > CPRF > FLUM> OXOLN acid.

The concentration of the three residues according to tissue/organ was ENRF: Layer Kidney > Layer Liver > Layer ''egg'', > Layer Muscle and CPRF: Layer Liver > Layer Kidney > Layer Muscle > Layer ''Egg'' and FLUM: Layer Kidney > Layer Liver. FLUM was absent in layer muscle & ''egg''. OXOLN Acid was not detected in any of the samples.

The higher values of RSD (relative standard deviation) for kidney and ''egg'' (43.784 and 56.852%) in case of ENRF show widely varying concentrations in kidneys and ''eggs'' of the layers (Table 2).

The highest value (13.47%) of RSD for kidney in case of CPRF shows that CPRF (Table 3) concentration differs most widely in kidney than in liver muscle and ''egg''. The FLUM

Table 2.
Various parameters of enrofloxacin in layer tissues/organs and "egg".

Sample	Range Size R (µg/kg)	Standard Deviation (S)	Coefficient of variation	Mean residue conc. (µg/kg) at 95% Confidence level
Layer Liver	742.93	342.00	25.223	1357.15 ± 342.32
Layer Kidney	2932.70	1224.002	43.784	2795.57 ±1224.00
Layer Muscle	576.71	206.997	29.618	698.90 ± 206.99
Layer "Egg"	1279.476	496.237	56.852	872.86 ± 496.24

Table 3.
Various parameters of ciprofloxacin in layer tissues/organs and "egg".

Sample	Range Size R (µg/kg)	Standard Deviation (S)	Coefficient of variation	Mean residue conc. (µg/kg) at 95% Confidence level
Layer Liver	72.06	28.67	12.27	233.57 ± 28.669
Layer Kidney	48.31	18.95	13.47	140.67 ± 18.948
Layer Muscle	16.62	6.644	7.59	87.43 ± 6.644
Layer "Egg"	9.98	4.214	5.93	71.06 ± 4.214

Table 4.
Various parameters of flumequine in layer tissues/organs and "egg".

Sample	Range Size R (µg/kg)	Standard Deviation (S)	Coefficient of variation	Mean residue conc. (µg/kg) at 95% Confidence level
Layer Liver	70.00	29.07	126.33%	23 ± 29.07
Layer Kidney	100.08	41.55	117.94%	35.23 ± 41.55
Layer Muscle	Nil	Nil	Nil	Nil
Layer "Egg"	Nil	Nil	Nil	Nil

Table 5.
Enrofloxacin in layer tissues/organs and "egg".

Parameters	Layer Liver	Layer Kidney	Layer Muscle	Layer "Egg"
Mean Residue level (µg/kg)	1357.15	2795.57	698.898	872.86
No of Obs. (n)	5	5	5	5
Deviating % age from MRL	100	100	100	100
Range (µg/kg)	974.23-1717.16	1629.41-4562.11	425.00-1001.71	393.426-1672.902

Table 6.
Ciprofloxacin in layer tissues/organs and "egg".

Parameters	Layer Liver	Layer Kidney	Layer Muscle	Layer "Egg"
Mean Residue level (µg/kg)	233.568	140.666	87.434	71.066
No of Obs. (n)	5	5	5	5
Deviating % age from MRL	100	100	100	100
Range Size (µg/kg)	190.08-262.14	124.23-172.54	80.18-96.80	66.05-76.03

Table 7.
Flumequine in layer tissues/organs and "egg".

Parameters	Layer Liver	Layer Kidney	Layer Muscle	Layer "Egg"
Mean Residue level (µg/kg)	23.002	35.226	Nil	Nil
No of Obs. (n)	5	5	5	5
Deviating% age from MRL	0.00	0.00	0.00	0.00
Range Size (µg/kg)	0.0-70	0.0-100.08	Nil	Nil

occurs almost equally widely (RSD's 126.33% and 117.94%) in liver and kidney, respectively with smaller mean concentration values of 23.002 and 35.23 ìg/kg for liver and kidney respectively (Table 4). ENRF and CPRF show 100% deviation from the internationally accepted MRL's (Maximum Residue Limits) (Table 5-6). Hence, these require longer washing out periods and dose sophistication for poultry chicken during summer. FLUM residues occur only in liver and kidney but only in 60% samples and to the extent that is less than MRL's. (Table7). OXOLN acid residues were not detected in any layer sample. Range sizes of the residual antibiotics depend mostly on the water intake, nature of water, its metallic content and pH of the poultry water and feed. The least concentrations of FLUM residues show that working pH of kidney and urine is most favorable for the acidic FLUM secretion. On the other hand, the larger values of range, range sizes and residual concentrations for basic fluoroquinolones; CPRF and ENRF are due to their (1) heavy doses, and (2) decreased renal secretions due to their larger lipophylicities [20]. Large range sizes and concentration of kidney residues show that basic quinolones are not ionized properly due to unfavourable renal pH and for elimination from the kidneys these have to be in ionized form. The larger the difference in residual range sizes between the liver and the kidney, the greater the decrease in their elimination. Owing to their smaller residual amounts in the kidney compared to the liver, the CPRF show increased secretion in contrast to ENRF whose concentration in the kidney exceeds that in the liver. This is also due to more lipophilicity of ENRF than that of CPRF. To decrease kidney, liver and muscle residues, the washing out time periods should be increased along with the intake of Vitamin C which will render the kidney to work in still more acidic medium and increase the secretion of ENRF and CPRF [21]. The absence of FLUM in muscle and "egg" shows an ideal condition which should also be achieved in case of CPRF and ENRF. The pH, the heavy metals and washing out times should be monitored rigorously. Dose sophistication and nature of the drug should also be accounted for.

Out of the detected analytes, ENRF and CPRF residues show excessive concentration levels (Tables 2,3). These must be monitored in the poultry industry. Washing out period must be enhanced to satisfactory limits. Dose sophistication must be ensured with respect to seasonal variation. Vitamin C, feed and secretion pH must be optimized. FLUM and OXOLN acid residues obey international regulations. But if the monitoring of the ENRF and CPRF concentration levels are neglected in our poultry industry, it is likely to undergo resistance against pseudomonas, staphylococcus aureus, *P. aeruginosa,* Enterobacteriaceae and *Salmonella*.

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