Research Article

Analytical Method for Simultaneous Determination of Ofloxacin and Pefloxacin in Poultry Meat by High Performance Liquid Chromatography with Photo Diode Array Detection

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ABSTRACT

An analytical method using high performance liquid chromatography was developed for simultaneous analysis of residues of ofloxacin and pefloxacinin chicken tissues. The drugs were extracted from homogenized tissues using amine buffer, followed by extraction with acetonitrile. The separation was achieved by reversed phase chromatography on a C-18 column (250mm x 4.6mm, i.d.-5µm) held in thermostat at 30°C. The mobile phase consisted of a mixture of triethyl amine solution (pH 4.5) and acetonitrile (85:15, v/v), with a flow rate of 0.750 ml/ min. The analytes were detected using PDA detector at the wavelength of 282 nm. The retention times for ofloxacin and pefloxacin were about 7.0 and 10.0 minutes, respectively. The linearity, recovery, detection limits and other parameters of the method were evaluated from spiked tissue samples and results were found good and within range. An excellent linearity was revealed in the investigation concentration range from 10 to 1000 ng/ml with correlation coefficients of the curve over 0.97 for both analytes. Recoveries from chicken tissues ranged from87 to 97%and the limits of quantification for ofloxacin and pefloxacin were 35 and 30 µg/Kg in chicken tissues.

Keywords: Fluoroquinolones, Ofloxacin, Pefloxacin, High Performance Liquid Chromatography, Poultry meat.

INTRODUCTION

Fluoro quinolones (FQNs) antibiotics are a group of relatively new and synthetic antibiotics. FQNs, derived from 3-quinolone carboxylic acid, have a fluorine substituent at the R6 position and show a broad spectrum of microbiological potency as well as rapid absorption following the oral administration route [1].Fluoroquinolones have been used in the treatment of a variety of bacterial infections in human and veterinary medicine. In infectious diseases the use of these drugs has become a serious problem, as they are substances that leave residues in edible tissue which may be directly toxic or cause resistant human pathogens and possible allergic hypersensitivity reactions in humans [2].

Ofloxacin and pefloxacin are second generation fluoroquinolones with a broad spectrum of antibacterial activity. Both have good bioavailability after oral administration and good to excellent tissue distribution and mostly used in veterinary practice for treatment of various diseases.

Ofloxacin is a synthetic broad spectrum antibacterial agent. Chemically ofloxacin is a fluorinated carboxyquinolone. It is a racemate, (±)- 9-fluro-2, 3-dihydro-3-methyl-10- (4-methyl-1-piperazinyl)-7-oxo-7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid [3]. This synthetic antibiotic is widely used in the treatment of urinary infections with good localised action on infected sites [4].

Pefloxacin[l-ethyl-6-fluoro-7-(4-methyl-l-piperazinyl)-4-oxo-l,4-dihydro-3-quinoline carboxylic acid] in the form of its methane sulphonate (mesylatedihydrate) is a new synthetic antibiotic from the group of 4-quinolones, which makes possible the successful treatment of serious infections caused by resistant groups of bacteria. The drug has a wide antimicrobial spectrum and its oral use has simplified the treatment of infections which had been previously dealt with only parenterally[5].

The widespread use of fluoro quinolones in human and in veterinary medicine has led to a significant increase in antibacterial resistance, having therefore important consequences for public health[6]. To minimize risks in human health by the consumption of FQs residues in foods, the European Union by the Council Regulation No.2377/90 has established maximum residue limits (MRLs) of veterinary products in food stuffs of animal origin[7].

Many chromatographic techniques had been reported for determination of fluoroquinolones in foods of animal origin. High Performance Liquid chromatography (HPLC) is the important analytical technique and mostly applied for the determination of Fluoroquinolones, and both UV and fluorescence detections are usually employed. High-performance liquid chromatography (HPLC) with fluorescence[8-16], ultraviolet[17], or mass spectrometric [18, 19] detection were analytical methods for the determination of fluoroquinolone antibiotics in chicken tissues.

Fluoroquinolones have been developed many times, and only a few reports have described the determination of both FQs in chicken tissues by HPLC[8, 9, 17, 18]. The determination of ofloxacin and pefloxacin with other FQs has been described several times, but always with some

clear short comings, such as full series of FQs but not only simultaneous determination of ofloxacin and pefloxacin, the use of complicated and time-consuming sample preparation procedure and require large amounts of organic solvents and other chemicals for chromatographic analysis. For this reason, our objective has been to improve the sample preparation process and to develop an economic, rapid, and sensitive HPLC method for simultaneous determination of ofloxacin and pefloxacinin chicken tissues without the need for large amounts of organic solvents and other chemicals.Chemical structures of ofloxacin and pefloxacin are shown in figure 1.

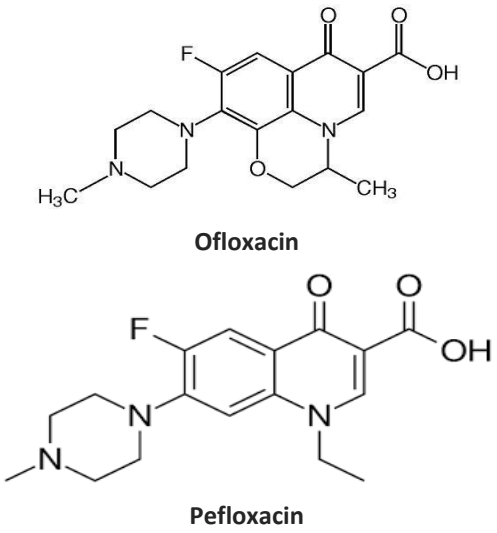


Figure1: Chemical structure of ofloxacin and pefloxacin.

MATERIALS AND METHODS

Sample Preparation

The chicken tissue samples were obtained from the healthy birds that were not treated with any veterinary drugs. The tissue samples were deep-frozen until analysis for the simultaneous determination of both drug residues. The tissue samples were thawed to room temperature and then cut into small pieces. An accurately weighed 0.5 gm of this tissue sample was placed in 15.0 ml polypropylene centrifuge tube and homogenized using Polytron Homogenizer (PT 1600 E) with 2.0 ml of amine buffer solution. After that, added 1.0 ml of KCl solution and kept for 10 minutes. After that, 2.0 ml of acetonitrile was added to it and the tube was tightly capped and vortexed for 5 minutes using Spinix Vortex (Tarsons products pvt. Ltd., India). The mixture was then sonicated and left undisturbed for 15 minutes. The mixture was centrifuged for 15 minutes at 5000 rpm using Research Centrifuge R-24 (Remi, India). The supernatant was decanted into another tube and centrifuged once again at 8,000 rpm for 30 minutes using cooling centrifuge CM-12 (Remi, India). The finally supernatant was filtered using 0.45µm (nylon + prefilter) mdi syringe filter (Advanced Microdevices Pvt. Ltd., India). 20µl of this filtrate was then injected into the HPLC system for analysis. The tissue extracts were then analyzed by HPLC system using the developed chromatographic conditions.

Instrumentation

The High Performance Liquid Chromatography (HPLC) was carried out on a Shimadzu system (Shimadzu, Kyoto, Japan). The system was equipped with a Quaternary gradient pump (LC-10ATvp), a Diode array detector (SPD-M10Avp), a Column oven (CTO-10ASvp), a System controller (SCL-10Avp), a Degasser (DGU-14Avp) and an Auto injector (SILL-10ADvp). The CLASS VP Software package was used for instrument control, data acquisition, and data analysis. A reverse phase C18 column Purospher Star, 250mm x 4.6mm with the particle size of 5µm (Merck) was used as stationary phase for separation of the compounds.

A glass vacuum filtration apparatus was employed for the filtration of the buffer solution using 0.2 µm nylon membrane filter obtained from Borosil, India. Prior to use solvents were degassed by sonication in ultrasonic bath Rivotech (Riviera glass pvt.ltd. India). Micro analytical balance CPA225D (Sartorius weighing technology, Germany) was used to weighing reference standards and Cyberscan pH meter (Eutech instruments, Malaysia) used to adjust pH of buffer solution. A tissue homogenizer Polytron PT 1600E (Kinematica AG, Switzerland) was used to homogenize tissue samples during pretreatment. A vortex mixer, Spinix Vortex (Tarsons products pvt. Ltd., India) was used to mix tissue samples employed for the sample preparation and a Research centrifuge R-24 (REMI India) as well as Cooling microfuge (REMI India) were used to perform the extractions.

Chromatographic Conditions

The isocratic mobile phase consisting of buffer (0.025M triethylamine, pH 4.5 adjusted with ortho-phosphoric acid) and acetonitrile in the ratio of (85:15, v/v) was used throughout the analysis. The mobile phase was mixed and filtered through a 0.2 µm nylon membrane filter (Mdi, Advanced Microdevices Pvt. Ltd. India) using glass vacuum filtration apparatus, and was degassed by sonication for 5 minutes. The flow rate of the mobile phase was maintained at 0.750 ml/min and injection volume was 20 µl. A photodiode array detector was operated at a wavelength of λmax = 282 nm. The retention times for ofloxacin and pefloxacin were about 7 and 10 min, respectively. Column was carried out at an oven temperature of 30º C and total run time for the analysis of both drugs was 20 min.

Chemicals and Reagents

Reference standard of Ofloxacin (Batch # SLBB1877V) and Pefloxacin mesylatedihydrate (Batch # SLBC5834 V) were obtained from Sigma-Aldrich, USA. Acetonitrile (HPLC-grade), Methanol (HPLC-grade), Triethylamine and ortho phosphoric acid were procured from Merck Specialties Pvt. Ltd., India. Water was purified by Milli-Q water system (Millipore, France) and this water was used throughout analysis.

Stock solutions of 1 mg/ml of ofloxacin and pefloxacin were prepared in few drops of water and diluted with HPLC grade methanol. All stock solutions were stored refrigerated at 4°C. Individual working solution of both drugs were prepared daily from the stock solutions by diluting with methanol. The working solutions, used to spike the tissue samples, were prepared by mixing the individual stock solutions. The triethylamine buffer solution (0.025 M) was prepared from triethyl amine and water. 1.7 ml of triethyl amine was dissolved in 500 ml of water. The pH of the buffer solution was adjusted to 4.5 using diluted solution of ortho- phosphoric acid.

RESULT AND DISCUSSION

Linearity

The linearity of the developed method was evaluated using the squared correlation coefficients (r2) of nine-point matrix matched calibration curves obtained via the analysis of blank tissue extracts with the analytes in a range from 10-1000 ng/ml for both analytes. Favourable linearity was achieved within the investigation concentration range, with a correlation coefficient (r^2) of 0.9707 for ofloxacin and 0.9928 for pefloxacin. Linearity curve for ofloxacin and pefloxacin using poultry tissue homogenate is shown in figure 2 and results of the linearity studies are summarized in table 1.

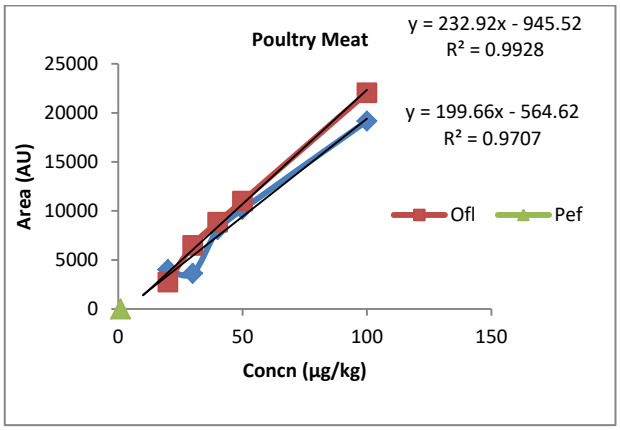


Figure2: Linearity curve for ofloxacin and pefloxacin using chicken tissues homogenate with linearity range of 10-1000 ng/ml.

Table1: Linearity parameters for ofloxacin and pefloxacin using chicken tissues homogenate**.**

Analytes	Biometric	Linearity Range (ng/ml)	Shape	Regration Equation	Correlation Co-efficient
Ofloxacin	Poultry Meat	10-1000	Linear	y = 199.66x - 564.62	0.9707
Pefloxacin	Poultry Meat	10-1000	Linear	y = 232.92x - 945.52	0.9928

Recovery

The recovery has been measured by taking into account the values corresponding to the analyzed concentrations. For the studies of recovery, blank chicken tissues was spiked with both drugs at four levels of 50, 100, 250 and 500 ng/ml and extracted as described in sample treatment procedure. Tissue samples of each level were prepared and analyzed under the same experimental conditions. A good recovery for both drugs was obtained from chicken tissues indicating the extraction procedure was very effective and recovery of ofloxacin was in the range of 86.96 to 90.68 % as well as pefloxacin was in the range of 93.68 to 96.89 %. Recoveries of ofloxacin and pefloxacin from chicken tissues are shown in table 2.

Table 2: Recoveries of ofloxacin and pefloxacin from chicken tissues at different concentration levels.

Theoretical Spiked Conc.(ng/ml)	Name of the analyte			
	Ofloxacin		Pefloxacin	
	Experimentally Detected Conc.(ng/ml)	Recovery from Matrix (%)	Experimentally Detected Conc.(ng/ml)	Recovery from Matrix (%)
50	45.27	90.53	48.28	96.55
100	86.96	86.96	96.89	96.89
250	226.70	90.68	234.20	93.68
500	448.20	89.64	472.30	94.46

Limit of Detection and Limit of Quantification

The limit of detection (LOD) and limit of quantification (LOQ) are determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected or quantified with suitable accuracy and precision.

The LOD and LOQ were evaluated by response of both drugs in a concentration range from 10 to 100 ng/ml. The limits of detection (LOD) were 10.50 and 9.0 µg/kg as well as the limit of quantification (LOQ) were 35 and 30 µg/kg, respectively for ofloxacin and pefloxacin in chicken tissues. Detection limits for both drugs in chicken tissues are shown in table 3.

Table 3: The values of limit of detection (LOD) and limit of quantification (LOQ) for ofloxacin and pefloxacin in chicken tissues.

Analytes	Biometric	Retention Time (min.)	Detection Range (ng/ml)	LOD (µg/Kg)	LOQ (µg/Kg)
Ofloxacin	Poultry Meat	7.0	10-100	10.5	35
Pefloxacin	Poultry Meat	10.0	10-100	9.0	30

Representative chromatograms for blank chicken tissues homogenate, mixture of both drug standards and spiked chicken tissues homogenate are shown in figure 3 and chromatograms for blank determination, spiked chicken tissues homogenate for ofloxacin and pefloxacin, separate ofloxacin and separate pefloxacin are shown in figure 4.

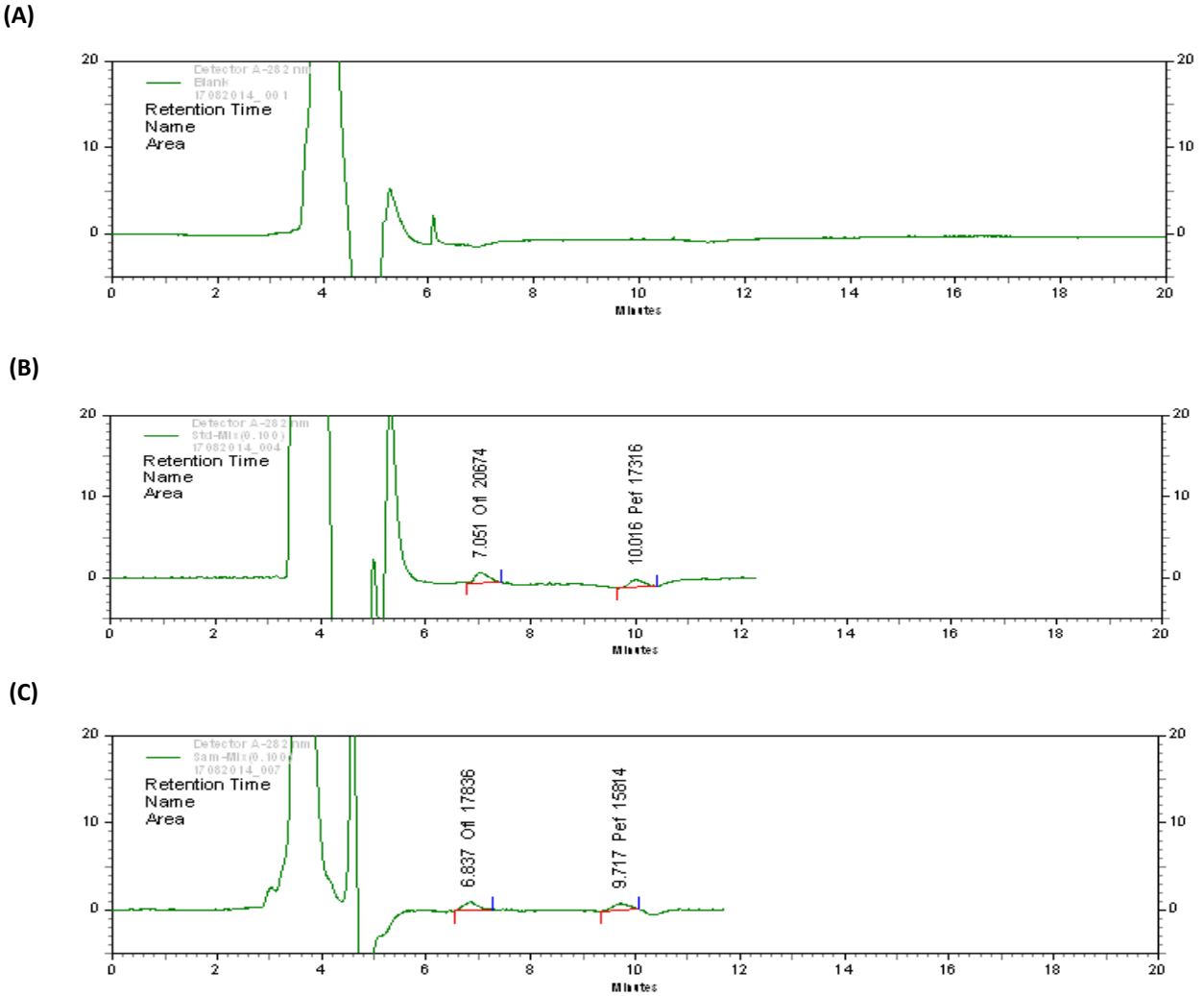
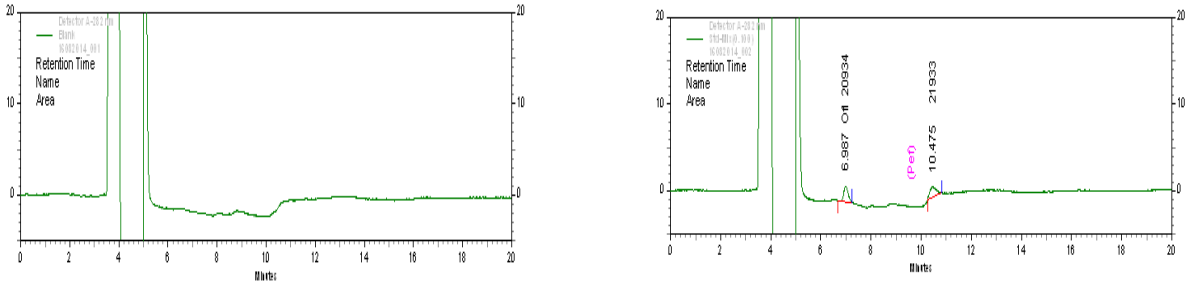
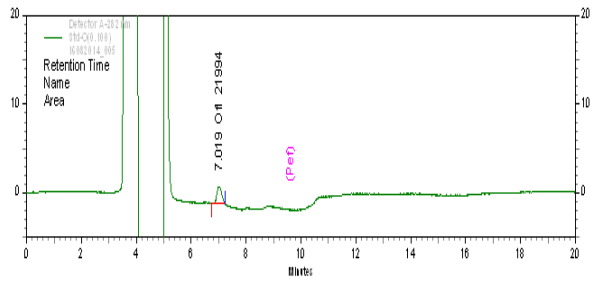


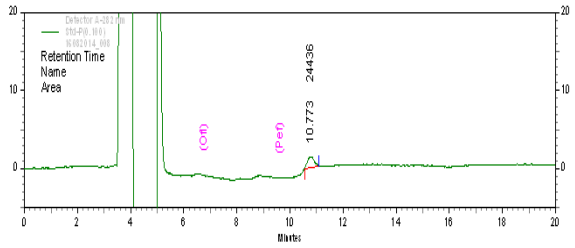
Figure 3: Chromatogram for the: (A) blank chicken tissues homogenate (B) Mixture of both drug standards at100 ng/ml concentration level and (C) Spiked chicken tissues homogenate at same concentration level.



(A) **(B)**



(C)



(D)

Figure 4: Chromatogram for the: (A) blank determination (B) Spiked chicken tissues homogenate at 100 ng/ml concentration level for ofloxacin and pefloxacin (C) for ofloxacin and (D) for pefloxacin.

CONCLUSION

A LC-UV method was successfully performed that was able to simultaneously identify and quantify of ofloxacin and pefloxacinin chicken tissues. PDA was chosen as detection method because this detection method is more sensitive and selective. A simple and fast sample treatment was used in order to extract the both drugs from the tissue samples with average recoveries from 87 to 97%.The detection and quantification limits were found to be very low i.e. the limit of detection (LOD) were 10.50 and 9 µg/kg as well as the limit of quantification (LOQ) were 35 and 30 µg/kg for ofloxacin and pefloxacin in chicken tissues. The analytical performance of the developed method was completed with satisfactory results. The proposed method has been successfully applied for routine determination of both fluoroquinolones in chicken tissue samples.

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