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Original Article

Simultaneous Determination of Danofloxacin and Difloxacin Residues in Poultry Meat using High Pressure Liquid Chromatography with PDA Detection

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ABSTRACT

A liquid chromatography with PDA detection method was developed for simultaneous determination of danofloxacin and difloxacin in chicken tissues. Tissue sample preparations were carried out by adding amine buffer, followed by extraction with acetonitrile. The analytes were separated on a reversed phase column C-18 (250mm x 4.6mm, 5µm) with an isocratic mobile phase consisting of a mixture of triethylamine solution (pH 3.5) and acetonitrile (80:20, v/v). Fluoroquinolones were monitored at a wavelength of 280 nm with PDA detection and retention times for danofloxacin and difloxacin were about 8.0 and 15.0 min, respectively. The method was determined by spiking blank chicken tissues while the linearity, recovery and detection limits were checked. Extraction recoveries of danofloxacin and difloxacin from chicken tissues were in the range of 91 to 97%. The limits of quantification for danofloxacin and difloxacin were 35 and 40 µg/Kg in chicken tissues.

Keywords: Fluoroquinolones, Danofloxacin, Difloxacin, Poultry meat, High Pressure Liquid Chromatography, PDA

1. INTRODUCTION

Fluoroquinolones (FQs) constitute an important group of synthetic antibiotics, developed in recent years, which are used to treat various infections in both human and veterinary medicine. These compounds exhibit high activity against a broad spectrum of gram-negative and gram-positive bacteria through inhibition of their DNA gyrase or topoisomerase II [1]. Their common skeleton is 4-oxo-1,4-dihydroquinoline, where the pharmacological unit consists of a pyridine ring with a carboxyl group, a piperazinyl group, and a fluorine atom placed at position 3,7, and 6, respectively.

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Fluoroquinolones are used in the treatment of systemic infections including urinary tract, respiratory, gastro-intestinal and skin infections. In the veterinary field, they are used not only for the treatment of diseases but also as feed additives to increase the animal mass [2].

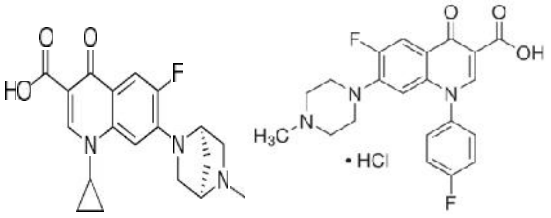
Residues of FQs may occur in animal tissues if the adequate withdrawal times are not observed or if the FQ compounds are improperly administered [3]. To minimize risks in human health by the consumption of quinolones residues in foods, the European Union by the Council Regulation No.2377/90 has established maximum residue limits (MRLs) of veterinary medicinal products in food-stuffs of animal origin and among them are some quinolones. The MRLs according to this regulation in chicken muscle are 200 µg/kg for danofloxacin and 300 µg/kg for difloxacin [4, 5].

Danofloxacin, 1-Cyclopropyl-6-fluoro-1,4-dihydro-7-[(1S,4S)-5-methyl-2,5 diazabicyclo[2.2.1] hepta-2-yl]-4-oxo quinolinecarboxylic acid, is a synthetic antibiotic of the fluoroquinolone group developed specifically for use in veterinary medicine [6] .It is an important fluoroquinolone with broad spectrum antibacterial activity and used in the treatment of respiratory disease in chickens, cattle and pigs.

Difloxacin, [6-fluoro-1-(fluorophenyl)-1,4-dihydro-7-(-4-metyl-1-piperazinyl)- 4-oxo-3-quinolone carboxylic acid], is an antimicrobial agent with activity against several pathogens [7]. It is used in the treatment of various diseases such as Genitor urinary, respiratory, gastrointestinal, skin and soft tissue infections.

The determination of danofloxacin and difloxacin has been described several times, but always with some clear short comings, such as time-consuming sample preparation protocol, complex sample treatment procedure, expensive methods and full series of FQs but not only simultaneous determination of danofloxacin and difloxacin. For this reason, our objective has been to develop a simple, sensitive and rapid HPLC method with PDA detection for simultaneous determination of danofloxacin and difloxacin in chicken muscle using simple sample treatment procedure.

A liquid chromatographic method with fluorescence detection (LC-FD) reported by **Z**eng *et al.*, 2005 [8], permitted the determination of nine fluoroquinolones (FQs) in egg white and yolk using a simple pretreatment of the samples. Recoveries for nine FQs were 74.7-91.2 % and limit of quantification were 5-20 ng/gm [9], determined five fluoroquinolones in muscle samples (cattle and poultry) by liquid chromatography-fluorescence detection and used dispersive solid-phase extraction for the cleanup of the muscle extract. Detection capabilities were from 132.8 to 421.0 µg/Kg and recoveries ranged from 51.7 to 80.91%, depending on the analyte. [10] Chang *et al.*, 2008, used SPE with HLB cartridges for sample extraction and reported multiresidue method for the determination of 11 quinolones in chicken, pork, fish and shrimp by liquid chromatography with fluorescence detection with gradient elution program. Mean recoveries of eleven QNs from different tissues were 71.7-105.3 % and limits of quantification ranged from 5 to 28 ng/gm. [11] Stoilova *et al.*, 2010, developed a HPLC-method with fluorescence detection for determination of nine quinolone residues in chicken muscle with gradient program for eluting that required a complex solid-phase extraction (SPE)-procedure with HLB cartridges for sample cleanup and extraction. Detection capabilities were from 10.72 to 100.79 µg/Kg and analytical recoveries ranged from 48 to 123%, depending on the compound. [12] Hassouan *et al.*, 2007, proposed a LC-FD procedure with gradient program for the simultaneous determination of five fluoroquinolones in bovine milk using simple sample treatment protocol. Quantification limits were 3 to 19 ng/ml and recoveries ranged from 93 to 101%, depending on the analyte. [13] Canada *et al.*, 2012, described a liquid chromatography method with photometric and fluorescence detection for the determination of fourteen quinolone residues in fish samples with gradient program that required a complex solid-phase extraction (SPE)-procedure with ENV + Isolute cartridges for sample cleanup and extraction. The detection and quantification limits were between 0.2-9.5 and 0.7-32 μ g/kg, respectively as well as mean recoveries of fluoroquinolones ranged from 50% to 102%, depending on the analyte.



Danofloxacin **Difloxacin**

2. MATERIALS AND METHODS

2.1 Sample Preparation

The chicken muscle 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 danofloxacin and difloxacin drug residues. The tissue samples were thawed to room temperature and then cut into small pieces. An accurately weighed 2.0 gm of this tissue sample was placed in 15.0 ml polypropylene centrifuge tube and homogenized using Polytron Homogenizer (PT 1600 E) with 4.0 ml of milli-Q-water. From this resultant homogenate, 2.0 ml was taken into a 15.0 ml polypropylene centrifuge tube and to this added 1.0 ml of amine buffer and kept for 15 minutes. The mixture was then sonicated and left undisturbed for 15 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 centrifuged for 30 min at 4000 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 0.2µm (nylon + prefilter) mdi syringe filter (Advanced

Microdevices Pvt. Ltd., India). 20µl of this filterate was then injected into the HPLC system for analysis.

2.2 Equipment

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 Hypersil BDS, 250mm x 4.6mm with the particle size of 5µm (Thermo Scientific) was used as stationary phase for separation of the compounds.

A glass vacuum filteration apparatus was employed for the filteration 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.

2.3 Chromatographic Conditions

The mobile phase was a mixture of triethylamine buffer (pH 3.5) and acetonitrile (80:20, v/v). The mobile phase was mixed and filtered through a 0.2 µm nylon membrane filter (Mdi, Advanced Microdevices Pvt. Ltd. India) using glass vacuum filteration apparatus, and was degassed by sonication for 5 minutes. The flow rate of the mobile phase was maintained at 0.6 ml/min and sample injection volume was 20 µl. A photodiode array detector was operated at a wavelength of λ_{max} = 280 nm. The retention times for danofloxacin and difloxacin were about 8 and 15 min, respectively. Column was carried out at an oven temperature of 30º C and total run time for the analysis of both drug was 20 min.

2.4 Chemicals and Reagents

Reference standard of Danofloxacin (Batch # 7302Z) and Difloxacin HCL (Batch # 6067X) 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 danofloxacin and difloxacin were prepared in few drops of water and diluted with HPLC grade Methanol. All stock solutions were stored refrigerated at 4°C. Individual working solutions of both drug 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 (0.5%) was prepared from triethyl amine and water. 2.5 ml of triethyl amine was dissolved in 500 ml of water. The pH of the buffer solution was adjusted to 3.5 using dilute solution of ortho phosphoric acid.

3. RESULT AND DISCUSSION

3.1 Linearity

Nine-point calibration curves were plotted in a concentration range 10-1000 ng/ml for both analyte. The calibration curves were found to be linear in the investigation range studied (10, 20, 30, 40, 50, 100, 250, 500, 1000 ng/ml levels were used). The results indicate that correlation coefficients for danofloxacin and difloxacin are 0.9972 and 0.9991, respectively. The regration equation, correlation coefficient, and linearity range for both analyte are presented in Table 1 and Calibration curve for Danofloxacin and Difloxacin in poultry meat is shown in figure 1.

Table1: The linearity parameters of calibration curve for danofloxacin and difloxacin using chicken tissue homogenate.

Analytes	Retention Time (min.)	Linearity Range (ng/ml)	Shape	Regration Equation	Correlation Co-efficient
Danofloxacin	8.5	10-1000	Linear	y = 223.41x - 1179.3	0.9972
Difloxacin	15.5	10-1000	Linear	y = 189.93x - 271.24	0.9991

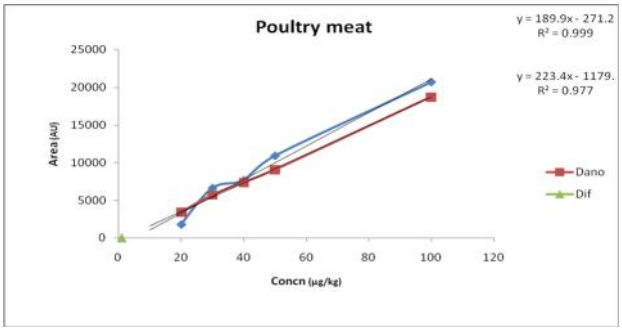


Fig 1: The Calibration curve for Danofloxacin and Difloxacin using tissue homogenate with linearity range of 10-1000 ng/ml.

3.2 Recovery

The recovery of the method was assessed by fortification of chicken tissue samples for both drug at four spike levels of 50, 100, 250 and 500 ng/ml. The percentage of recovery (%) was calculated and average recovery of danofloxacin was between 91.09 and 94.57 % as well as difloxacin was between 93.08 to 97.49 % from chicken tissues. The recoveries for both drug from chicken tissues are shown in Table 2.

Table 2: The recoveries of danofloxacin and difloxacin from chicken tissues at different concentration levels.

Name of the analyte					
Danofloxacin			Difloxacin		
Theoretical Spiked Conc.(ng/ml)	Experimentally Detected Conc.(ng/ml)	Recovery from Matrix (%)	Theoretical Spiked Conc.(ng/ml)	Experimentally Detected Conc.(ng/ml)	Recovery from Matrix (%)
50	46.90	93.79	50	48.74	97.48
100	94.57	94.57	100	93.08	93.08
250	227.73	91.09	250	243.73	97.49
500	462.75	92.55	500	467.65	93.53

3.3 Limit of Detection and Limit of Quantitation

The limit of detection (LOD) and limit of quantification (LOQ) were determined by tissue homogenate spiking with serial dilutions for both analyte and observed at various concentration levels. The limit of detection and limit of quantification were evaluated by response of both drug in a concentration range from 10 to100 ng/ml. The limit of detection (LOD) for danofloxacin and difloxacin in chicken tissues were 10.50 and 12 µg/kg, respectively as well as the limit of quantification (LOQ) for danofloxacin and difloxacin in chicken tissues were 35 and 40 µg/kg, respectively which are well below the Maximum Residue limit (MRLs) for both drug. The MRLs according to European Union in chicken muscle are 200 µg/kg for danofloxacin and 300 µg/kg for difloxacin.

HPLC-chromatograms of a blank sample, representative standard and spiked sample of chicken muscle are shown in Figure 2,3 and 4respectively.

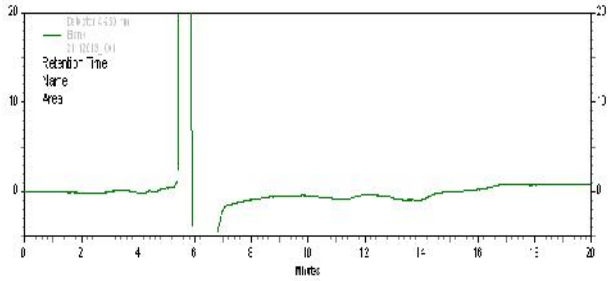


Fig 2: Optimized chromatogram of blank chicken tissues homogenate for determination of danofloxacin and difloxacin.

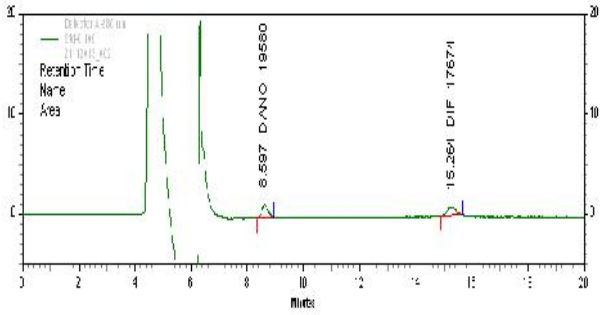


Fig 3: Representative chromatogram of danofloxacin and difloxacin using mixture of both drug standard (100 ng/ml concentration level).

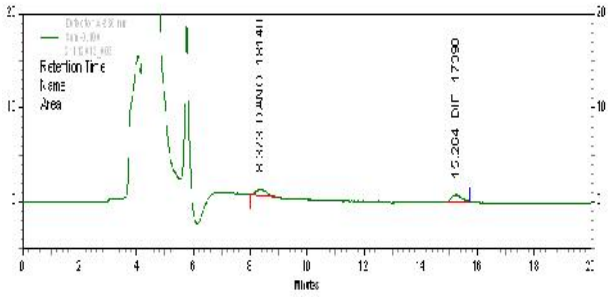


Fig 4: Chromatogram of spiked chicken tissue homogenate at 100 ng/ml concentration level for danofloxacin and difloxacin.

4. CONCLUSION

A high performance liquid chromatographic (HPLC) method for simultaneous determination of danofloxacin and difloxacin in chicken meat was developed. A rapid and simple treatment was used in order to extract the fluoroquinolones from the tissue samples with average recoveries from 91 to 97%. The limit of detection (LOD) for danofloxacin and difloxacin in chicken tissues were 10.50 and 12 µg/kg, respectively as well as the limit of quantification (LOQ) for danofloxacin and difloxacin in chicken tissues were 35 and 40 µg/kg, respectively which are well below the Maximum Residue limit (MRLs) for both drug. The separation results and parameters developed in this method are good and within range to determine fluoroquinolones in chicken tissue samples. The proposed method can be applied for routine determination of the two fluoroquinolones in chicken tissue samples.

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