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RP-HPLC Method for Detection and Quantification of Residual Fluoroquinolones in Buffalo Meat

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ABSTRACT

A reverse-phase liquid chromatographic method for simultaneous quantification of enrofloxacin and its active metabolite ciprofloxacin in buffalo meat matrix is described. The method entails simple liquid-liquid extraction of meat samples. After extraction of the meat samples for the two fluoroquinolones, the analysis of the drugs was carried out using a reverse phase C18 column at an oven temperature of 40ºC. The chromatographic separation was accomplished with mobile phase consisting of 0.1% aqueous formic acid and 0.1% formic acid in acetonitrile (80:20, v/v) in an isocratic elution mode. The flow rate of the mobile phase was maintained at 1.2 ml/min and injection volume was 20 µl. A fluorescent detector was operated at an excitation and emission wavelength of 280 and 450 nm. The assay was linear in the concentrations ranging from 10 to 200 µg/kg. Mean extraction recoveries of enrofloxacin and ciprofloxacin were in the range of 70-88 %. The limits of quantification for enrofloxacin and ciprofloxacin were 36 and 30 µg/kg, respectively. This RP-HPLC method has highly applicability for routine analysis of residual fluoroquinolones in buffalo meat below Codex MRL.

Keywords: *Residues, Enrofloxacin, Ciprofloxacin, Buffalo meat, HPLC method*

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INTRODUCTION

Enrofloxacin, the second-generation fluoroquinolone is an exclusive veterinary antimicrobial. This antimicrobial is widely prescribed in pursuit of countering clinically important diseases like Bovine Respiratory Disease, acute E. coli mastitis and digestive diseases in ruminants including buffaloes (Hoeben *et al.* 2000). Ciprofloxacin is the pharmacologically active metabolite of enrofloxacin after partial N de-ethylation (Prescott *et al.,* 2000 and Grobbel *et al.,* 2007) which is a critical antimicrobial in human Campylobacteriosis therapy (Taccetti et al., 2008 and Deckert *et al.,* 2013). The use of antimicrobials may result in drug residues in the edible tissues, especially if they are not used according to label directions or if withdrawal times. The presence of fluoroquinolone residues in meat and it's by products may pose a health threat to consumers, ranging from allergic reactions in sensitive individuals and emergence of fluoroquinolone resistant Salmonella, Campylobacter and resistant E. coli strains (Ho *et al.,* 2003 and Giguere *et al.,* 2007). To prevent consumers from suffering with the possible health problems, regulatory agencies like EU, Codex Alimentarius Commission have established maximum residue level (MRL) of 100 µg/kg for these fluoroquinolones in animal derived food products. Even though lots of confirmatory analysis using sophisticated LC-MS/MS (Liu *et al.,* 2019; Zheng *et al.,* 2019) ,LC-MS methods (Lolo *et al.,* 2016) for fluoroquinolone (FQ) residues like enrofloxacin are available in different food matrices using complex extraction process, we are still in need of simpler and cost effective quantitative methods using HPLC that could be used for routine monitoring of FQ residues. This fact marks the need to have a simple and accurate techniques for detection and quantification fluoroquinolone residues in meat and meat products. Therefore, the present study describes a liquid chromatographic method using fluorescence detector for simultaneous quantitation of enrofloxacin and its active metabolite ciprofloxacin in buffalo meat in compliance with Codex MRL, to check violative levels if any.

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MATERIALS AND METHODS

Chemicals and reagents: The analytical standards enrofloxacin and ciprofloxacin hydrochloride were obtained from Sigma-Aldrich (St. Louis, MO, USA). Formic acid 98% were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade methanol (MeOH), acetonitrile (ACN), n-hexane from Merck (Darmstadt, Germany), were used. Water from Milli-Q system (Millipore, Bedford, MA, USA) was used.

Preparation of Standard and working drug solutions: Analytical grade standard of enrofloxacin and ciprofloxacin hydrochloride with 99.5% purity were used for this study. Standard stock solution of 1mg/ml concentration of enrofloxacin and ciprofloxacin were prepared in methanol containing 1% acetic acid. Working stock solution of combined enrofloxacin and ciprofloxacin were prepared by diluting the stock with 0.1% aqueous formic acid to achieve the following concentrations viz. 10, 25, 50, 75, 100, 150 and 200 ng/ml. All stock and working standard solutions were stored at 4 ºC in the refrigerator.

Extraction Procedure: The buffalo meat samples were extracted using liquid-liquid extraction (LLE) method. To two gram of meat homogenate were added 1ml Ammonium acetate buffer and 2 ml acetonitrile and vortexed for 2 minutes followed by centrifugation at 4000 rpm for 20 minutes. Solvent layers were pooled and evaporated using nitrogen gas at 50 ºC. The extract was reconstituted with 200 µL of mobile phase. After passing through a 0.25 µ nylon membrane filter, 20 µl of this filtrate was then injected into the column for HPLC analysis. Fortification study was done using the same protocol spiked at MRL level with six replicates.

High Performance Liquid Chromatography Conditions: Tissue concentrations of enrofloxacin and its active metabolite ciprofloxacin were assayed by reversed-phase high–performance liquid chromatography with fluorescence (FLD) detector. The UFLC system (Shimadzu Corporation, Kyoto, Japan) comprised of an LC 20AD pumps, an auto injector SIL-20AC, a RF-20A fluorescence detector and LC solutions software. The separation of two analytes was accomplished using a C18 reverse phase column (Phenomenex, 4.6 x 250 mm; 5^{o}m) as a stationary phase. The mobile phase consisting 0.1% aqueous formic acid and 0.1% formic acid in acetonitrile (80:20, v/v) as used for elution purpose in an isocratic mode. A flow rate of 1.2 ml/min was used and column oven temperature was maintained at 40^{o}C. FLD detector wave length was adjusted to have an λmax of 280 and 450 nm for excitation and emission, respectively.

Calibration curve: Calibration curves were prepared by adding to blank samples, the corresponding volume of working solution to obtain the following concentrations of enrofloxacin and its active metabolite ciprofloxacin: 10 µg/kg (0.1 MRL), 25 µg/kg (0.25 MRL),50 µg/kg (0.5 MRL), 75 µg/kg (0.75 MRL), 100 µg/kg (MRL), 150 µg/kg (1.5MRL) and 200µg/kg (2 MRL). Enrofloxacin and its active metabolite ciprofloxacin were quantified in µg/Kg based on peak area measurements using external calibration method which was analysed in triplicate.

Statistical analysis: The recovery and precision data were evaluated with an in-house statistical software program making use of Snedecor and Cochran concepts.

RESULTS AND DISCUSSION

The presence of veterinary antimicrobial residues in foods of animal origin is an important issue in public health. There were many reports on screening methods for enrofloxacin and ciprofloxacin in foods of animal origin like biosensor assay in milk (Fernandez *et al.,* 2011) and chicken muscle (Marchesini *et al.,* 2008 and Huet *et al.,* 2008); microbial methods (Petrovic *et al.,* 2006) which were only qualitative and semi quantitative in nature. Never the less tangible information on exact quantity of residues in always required for making any policy decisions. Reports on quantitative methods employing various HPLC methods for enrofloxacin/ciprofloxacin in different biomatrices like bovine milk using fluorescence detector (Idown & Peggins, 2004 and Liang *et al.,* 2019), ion paring chromatography (Tyczkowka *et al.,* 1994), in fish matrix using UV detector (Ramamohanarao, 2012), in shrimp matrix using UPLC-MS/MS (Susakate *et al.,* 2019 and Gros *et al.,* 2019) were also available. Majority of work was in poultry meat using fluorescence detector (Masakazu *et al.,* 1997; Samanidou et al., 2005 and Cho et al., 2008) using complex multistep extraction process with varying recovery % and LOQ. Therefore, a simple RP- HPLC method using FLD detector was studied to quantify the concentration of enrofloxacin and its active metabolite ciprofloxacin residues in compliance with Codex MRL.

Table 1: Analytical conditions

System	**UHPLC**
Column	C18
	4.6 X 250 mm 5µ
Injection volume	20 µl
Oven temperature	40 oC
Mobile phase	MP:0.1% aqueous formic acid & 0.1%
	Formic acid in Acetonitrile (80:20, v/v)
Flow rate	1.2 ml/min
Detector	FLD λmax Ex 280; Em 450nm

The procedure entails a simple Liquid-liquid extraction of buffalo meat followed by liquid chromatographic determination of enrofloxacin and its active metabolite, ciprofloxacin. These fluoroquinolones were detected using fluorescence detector at 280 nm excitation and 450 nm emission wavelength. Good chromatographic separation between enrofloxacin and ciprofloxacin was achieved using 0.1% aqueous formic acid: 0.1% formic acid in acetonitrile (80: 20, v/v) as the mobile phase (Table 1). The proposed method allows identification and quantification of the studied analytes in a single analytical run within a total run time of 10 minutes.

Table 2: Performance parameters

	Buffalo Meat	
Performance parameters	**Enrofloxacin**	**Ciprofloxacin**
Selectivity	No co-eluting interference found	
Linearity Range (µg/kg)	10-200	10-200
Co-relation Coefficient	0.999	0.999
Recovery (%) at MRL level n=6	83.3-88.2	70.1-77.3
LOD (µg/kg)	10.88	10.02
LOQ (µg/kg)	36.27	30.38

The performance parameters studied, demonstrated complete adequacy of the method for detection and quantification of residual fluoroquinolones, in buffalo meat incompliance with Codex MRL (Table 2). Analysis of blank muscle samples showed that there were no interfering compounds at the retention times of analytes of our interest, demonstrating the selectivity of the method. The retention times for enrofloxacin and ciprofloxacin were 5.9 and 7.6 minutes respectively (Fig 1). The assay was linear from 10 to 200 µg/ kg (Fig 2). The coefficients of determination (R2) values of the calibration curves were higher than 0.99, indicating a good fit of the data to the regression line. Values higher than 0.99 for linearity tests are recommended by the European Commission (CD 2002/657/EC). The correlation coefficient values (R2 > 0.999) indicated appropriate correlations between the investigated analyte concentrations and their peak area within the test ranges (Table 2). The recoveries were investigated at MRL level (100µg/Kg). The percentage recovery from buffalo meat was adequate for method studied, they ranged from 83% to 88% (enrofloxacin) and 70% to 77% (ciprofloxacin).

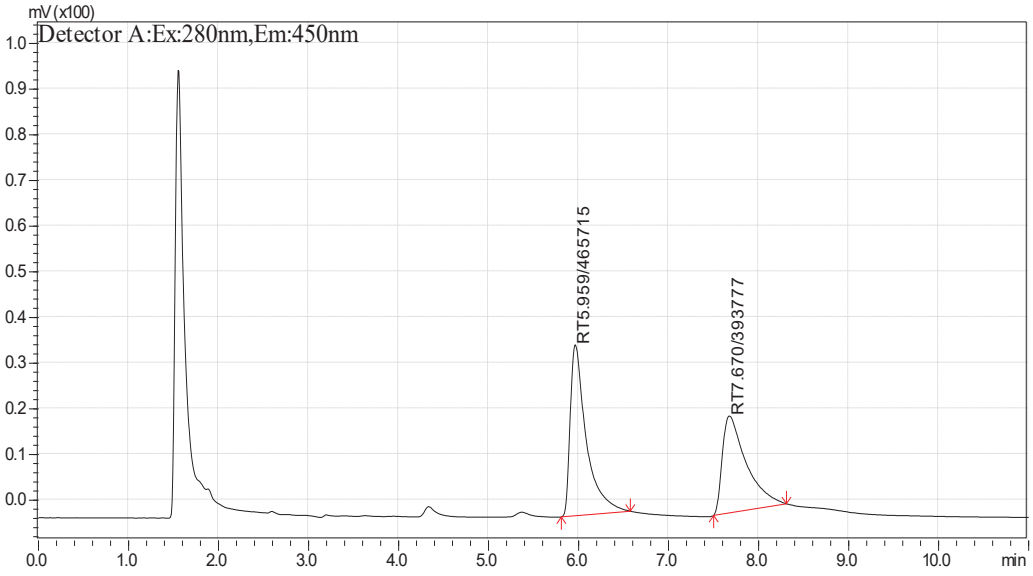

Fig. 1 Representative optimized chromatogram spiked with 100 µg/Kg of enrofloxacin and ciprofloxacin.

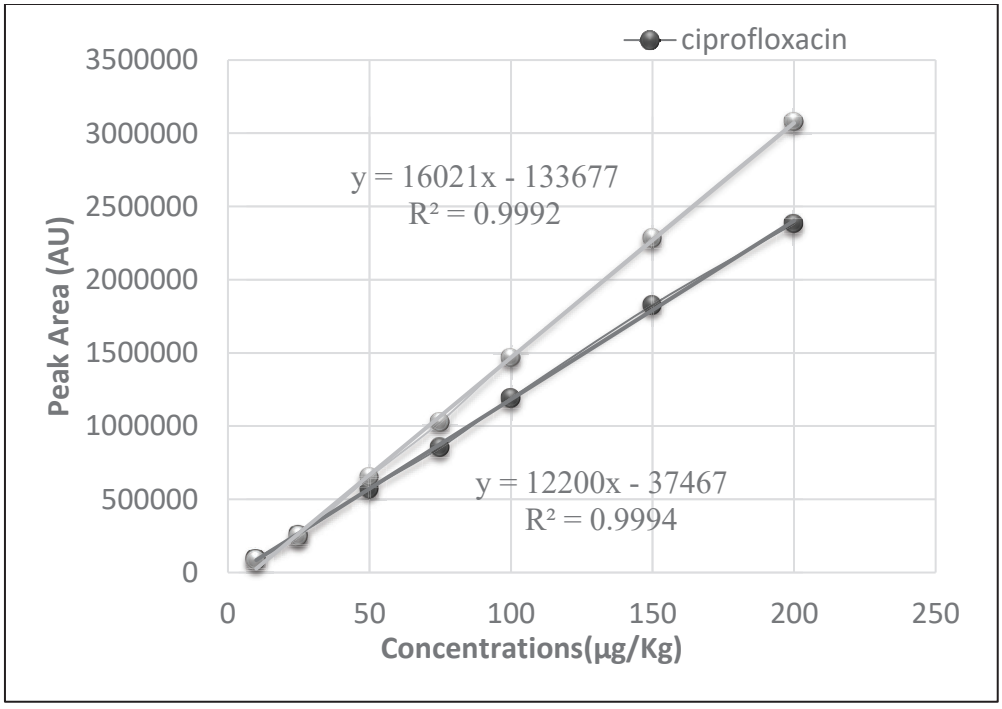

Fig. 2 Calibration curve in the concentration range 10-200 µg/Kg of enrofloxacin and ciprofloxacin.

The limit of quantification (LOQ) of enrofloxacin and its active metabolite ciprofloxacin were 36 and 30 µg/kg in buffalo meat. The obtained values were remarkably lower than the MRL established for the said analytes (Table 2). This method may be used to reliably quantify the residue concentrations lower than those allowed by Regulation (EU/ 37/2010).Results show that the proposed RP-HPLC method is simple characterized by good recovery, linearity and low LOQ values, adequate for quantification of residual fluoroquinolones in buffalo meat below Codex MRL.

CONCLUSION

A simple HPLC method displaying high specificity and sensitivity has been obtained with good recoveries and linearity accuracy to quantitate simultaneously, enrofloxacin and its active metabolite ciprofloxacin in buffalo meat matrix. The limit of quantification demonstrated for enrofloxacin and ciprofloxacin in this assay is found to be far lower than the established codex maximum permissible limit. Therefore, this method seems, to be applicable to quantify these fluoroquinolones, below Codex MRL (100µg/ Kg) in buffalo meat samples.

COMPETING INTERESTS: No

ETHICS STATEMENT: Not Applicable

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