Research Article

Quantitative Bio-Analysis of Ciprofloxacin in Poultry Plasma by UHPLC to Characterise Disposition Kinetics

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Abstract

Ciprofloxacin is a fluoroquinolone antimicrobial drug used in human, animals and birds. It is also an important metabolite and residue marker of enrofloxacin, a veterinary exclusive drug. In the present study, a simple, rapid and economical UHPLC (Ultra High Performance Liquid Chromatography) method for the quantitative estimation of ciprofloxacin in broiler plasma was validated for its utility in studying disposition behaviour of the drug. A short length reverse-phase C-18 column (100 mm length with 4.6 mm diameter, 2 µ pore size) was used for the separation and elution was detected at wavelength of 279 nm using UV detector. Mobile phase was comprised of phosphate buffer and acetonitrile in the ratio of 80:20 and run at flow rate of 0.5 ml/min on isocratic mode. Retention time was 4.1 ± 0.1 minute. Moxifloxacin was used as an internal standard in the present method. Liquid-liquid extraction was followed using acetonitrile for the extraction of ciprofloxacin from broiler plasma and the mean extraction recovery was not less than 90.30 %. At any concentration studied (0.1, 1 and 10 µg/ml), accuracy was not less than 90 %. For precision, the calculated highest intraday and inter-day co-efficient of variance were 7.40 % and 7.32 %, respectively. LOD (Limit of detection) and LOQ (Limit of quantification) of the present analytical method were calculated as 0.101 and 0.307 µg/ml, respectively. The validated method was applied successfully to study disposition kinetics of the ciprofloxacin in poultry.

Keywords: Ciprofloxacin, Method validation, Plasma sample, Poultry, UHPLC

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Introduction

Ciprofloxacin is a synthetic second-generation fluoroquinolone with a broad-spectrum antibacterial activity. It acts as bactericidal drug by inhibiting DNA gyrase enzyme involved in bacterial DNA replication [1]. Ciprofloxacin is one of the fluoroquinolones that are approved for humans and are of potential interest in veterinary medicine [2]. Among veterinary species, ciprofloxacin is licensed mainly for use in swine and poultry in many Asian and Latin American countries. However, it is not licensed for use in food-producing animals in the USA, Europe, Japan and Australia [3]. Ciprofloxacin is an active drug against both Gram-negative and Gram-positive bacteria including pseudomonal and staphylococcal species. Among second-generation compounds, ciprofloxacin exhibits the strongest sensitivity against Gram-negative bacteria. Ciprofloxacin is a carboxy fluoroquinolone which contains a fluorine atom at position-6 and a carboxylic acid moiety at position-3 of the 4-quinolone nucleus (**Figure 1**). Fluorinated quinolones have a wider spectrum of activity and more potency as compared to non-fluorinated quinolone. The piperazine group at position-7 of the 4-quinolone nucleus is responsible for the antipseudomonal activity of ciprofloxacin. The drug also contains a cyclopropyl group at position-1, which enhances its antimicrobial activity [4-7].

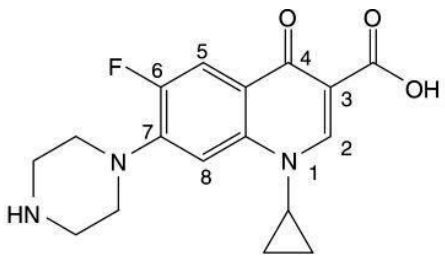

Figure 1 Chemical structure of ciprofloxacin

Ciprofloxacin is also a residue marker for another fluoroquinolone *viz*. enrofloxacin which is a veterinary exclusive and most used drug of its class in veterinary medicine. Metabolic *in vivo* conversion (N-dealkylation) of enrofloxacin into ciprofloxacin has been reported in significant amounts *i.e.* about 10 to 64% in different species [8]. Important physico-chemical characteristics of ciprofloxacin [8-10] are summarized in **Table 1**.

Table 1 Physico-chemical properties of ciprofloxacin

S.N.	Properties	Values / Descriptions
1	Chemical name	1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid
2	IUPAC name	1-cyclopropyl-6-fluoro-4-oxo-7-piperazin-1-ylquinoline-3-carboxylic acid
3	Empirical formula	$C_{17}H_{18}FN_3O_3$
4	Molar mass	331.347 g/mol
5	Serum protein binding	20-40 % (Mainly bound to albumin fraction)
6	Octanol / water partition	Lower than 1 (log P =0.28)
7	Acid dissociation constant (pKa)	pKa_1 = 6.1 (carboxyl group), pKa_2 = 8.62 (piperazinyl group); Isoelectric point = 7.14
8	Solubility	Water solubility: 30 g/L (at 20 °C)
9	Melting point	313–315°C
10	Density	~1.5 g/cm

The disposition kinetics or pharmacokinetics, residue studies, therapeutic drug monitoring, bioavailability and bioequivalence studies in target species requires a validated and simple but sensitive method of quantification of a drug in biological matrices (plasma, urine, tissues *etc*.) of that particular species. For fluoroquinolones like ciprofloxacin, analytical methods with LC-MS/MS or HPLC or UHPLC (ultra high performance liquid chromatography) with fluorescent detector are considered most sensitive, and there are many such reports available.

But due to advanced instrumentation, more cost, and complex extraction procedures, deployment of such analytical methods for disposition studies is always challenging. Looking to the fact, current paper explores the possibility of the simple rapid and economical analytical method for ciprofloxacin with UHPLC machine equipped with UV detector suitable for conducting disposition and pharmacokinetic experiments in poultry. There are many chromatographic methods reported for the bio-quantification of ciprofloxacin in human plasma using HPLC with UV detector [11-15] but only a few methods are found when literature search is done for the estimation of ciprofloxacin in poultry plasma [16, 17]. Among the available HPLC methods for estimation of ciprofloxacin concentration from plasma in any species, regular length reverse phase C18 columns were used and no report on use of short length RP (reverse-phase) C18 column is available. Use of short length column could make the analysis more rapid and economical due to small run time and use of mobile phase in lesser volume and slower flow rate. Therefore, the present study was undertaken to design a suitable, simple, rapid and economical method for bio-quantification of ciprofloxacin in poultry plasma using UHPLC-UV method involving short length RP C18 column as a stationary phase.

Materials and Methods

Chemicals and reagents

Ciprofloxacin and moxifloxacin powders of certified EP (European Pharmacopoeia) grade were procured from Sigma-Aldrich Chemicals Pvt. Ltd., Bengaluru, India. Ciprofloxacin E.P. grade powder was used for making reference standards for validation of UHPLC (Ultra High Performance Liquid Chromatography) analytical method. HPLC grade water, acetonitrile and orthophosphoric acid were procured from S.D. Fine-Chem Limited, Mumbai, India. Di-Sodium hydrogen phosphate and triethylamine of HPLC grade were procured from Merck, Mumbai, India.

Collection of poultry plasma

The drug-free blank poultry plasma was obtained from the broiler chicken birds procured from Department of Livestock Management and Production, College of Veterinary Science and AH, SDAU, Sardarkrushinagar, Gujarat, India. The experimentation protocol of the present study was approved by the Institutional Animal Ethics Committee (IAEC) of the same institute (Approval No. VetColl/ IAEC/16/ 11/ Protocol-03).

Instrumentation

UHPLC machine (Dionex ultimate 3000®, Thermo Fisher, Germany) with ultraviolet (UV) detector, gradient solvent delivery pump and manual injector with the fixed loop of 20 microlitres, was used in the present study. Chromatographic separation was performed by using a short length C-18 column (100 - 4.6 mm) *viz*. Chromolith® RP-18 at room temperature (~28°C). The data integration was performed by software 'Chromeleon™ version 6.8' chromatography data system. The effluents were monitored at the wavelength of 279 nm using a UV detector.

Chromatographic conditions

The mobile phase was run on isocratic mode at the flow rate of 0.5 ml/min and consisted of aqueous phosphate buffer and acetonitrile (organic solvent part) in the ratio of 80:20. Phosphate buffer for the mobile phase was 0.02-M-Disodium hydrogen phosphate (Na_2HPO_4) prepared in HPLC water. Also, 0.005-M-triethylamine was added to the buffer part. After filtration and sonication, pH of the buffer part of the mobile phase was adjusted to 2.7 with orthophosphoric acid. During the sample run, intermittent washings of manual microsyringe were done with washing solution (80 acetonitrile: 20 HPLC water) to avoid carry-over effect.

Extraction protocol

A liquid-liquid extraction protocol was used in the present study using acetonitrile [15] for the determination of ciprofloxacin in a microquantity of poultry plasma (300 µL). Exactly 300 µL of plasma sample was taken into 2 ml Eppendorf® micro-centrifuge tube, and 20 µL strong 0.1-M-Phosphate (Na_2HPO_4) buffer having pH 2.7 was added to it and then 450 µL ice-cold acetonitrile was added and vortex mixed for 3 minutes. Then the mixture was centrifuged at 10000 RPM for 10 minutes at 4°C. The upper organic phase was transferred into evaporation vials and completely dried using an N_2 evaporator (50°C, 10 psi pressure, 30 minutes). For making reconstitution solution, 30 µL of internal standard (40 µg/ml moxifloxacin dissolved in HPLC water) was mixed with 120 µL mobile phase for each extracted and evaporated sample. Thus, dried residues were reconstituted with a total of 150 µL solution. The prepared sample was vortexed for 90 seconds, and then finally centrifuged (10000 RPM, 5 minutes, 4°C) and 20 µL clear supernatant was manually injected into UHPLC.

Calculation of validation parameters

Important validation parameters like linearity, accuracy and precision (intraday and inter-day) and extraction recovery were calculated as per standard guidelines [18, 19] for the present method for analysis of ciprofloxacin in poultry plasma. The limit of detection (LOD) and limit of quantification (LOQ) were calculated using the following formulae based on the standard deviation of responses (SD) and the slope value of the calibration curve [20]:

$$LOD = 3.3 \times (SD / Slope) \text{ and } LOQ = 10 \times (SD / Slope)$$

Statistical Analysis

All the values of validation parameters were expressed as the mean ± standard deviation. Precision C.V. (Co-efficient of variance) was calculated using the following equation:

$$CV\ (\%) = (\text{Standard Deviation} / \text{Mean of observed concentration}) \times 100$$

Results and Discussion

HPLC methods are considered more precise and reliable assay for the bio-quantification of antimicrobial drugs like ciprofloxacin as compared to the microbiological bio-assays [11]. Optimization of the UHPLC method was done to achieve acceptable and reproducible peaks with good resolution and shape and without any interfering background peaks. Detector responses were generated as chromatograms using '*Chromeleon*™ version 6.8' software at the data collection rate of 2.5 Hz and a time constant of 0.6 seconds. The values of optimised system parameters are given in **Table 2**.

All the previously reported methods for HPLC assay of ciprofloxacin in plasma use regular length reverse phase C-18 column (250 mm length), however, in the present study, short length C-18 column (100 - 4.6 mm) *viz.* Chromolith® RP-18 was used. Use of short column and slow flow rate of mobile phase (0.5 ml/minute) allow the present analytical method to be economical as less volume of solvents per sample was consumed. Faster flow rates

ranging from 1.0 to 2.0 ml/minute were used in other studies [11-17]. The short retention time of ciprofloxacin (4.1 minutes) optimised in the present method was feasible due to a short column. Higher retention times like 6.4 min [11], 12 min [12], 6.4 min [11], 8.3 min [13] and 6.5 min [1 in 4] were reported in other studies. A shorter retention time of 3.26 was reported for human plasma [15] where phosphate buffer and acetonitrile were used in the mobile phase in the ratio of 77:23 whereas, in the present study, it was 80:20. A shorter retention time below 4 minutes was not attempted in the present method due to the presence of interfering background peaks between 2 to 4 minutes in chromatograms obtained.

Table 2 Optimized chromatographic conditions for ciprofloxacin

S. No.	Chromatographic conditions	Optimized values
1	Mobile phase components and ratio	80 (Phosphate Buffer) : 20 (Acetonitrile)
2	Flow mode	Isocratic
3	Flow rate	0.5 ml/min
4	Effluent monitoring wavelength (λ_{max})	279 nm (UV detector)
5	Injection volume (loop)	20 µL
6	Run time	12 min
7	Internal standard	Moxifloxacin
8	Retention time (RT)	Ciprofloxacin: 4.1 min Moxifloxacin: 7.9 min

Many studies used phosphate buffer in the mobile phase [11, 13, 15] which was also used in the present study, however, an acetic acid aqueous solution is also used as buffer part of mobile phase for ciprofloxacin [12, 14]. Due to satisfactory separation and resolution in the present study, acetic acid was not tried. The organic part of the mobile phase used was acetonitrile. Most of the studies used acetonitrile or methanol or a mixture of both as an organic part of the mobile phase [11-17]. In the present study, acetonitrile alone was preferred as it is stronger solvent and has higher elution strength than methanol for reversed-phase chromatography, therefore a shorter retention time could be achieved for ciprofloxacin. For the UV detection of ciprofloxacin in effluent, the most common range of wavelength (λ_{max}) used in various methods is 277 to 280 [11-17]. All the four wavelengths *i.e.* 277, 278, 279 and 280 were tried in the present study and 279 was found to produce maximum height and area of the peak, therefore, the UV detection of column effluent was monitored at a wavelength of 279 nm.

An internal standard, as a part of quality control, is used to aid quantification of an analyte. The difference between retention times of the target compound and the internal standard may be used to know the relative retention time which can be further used to compensate for small retention time shifts. An internal standard should have similar physico-chemical properties to the analyte [21]. Moxifloxacin, a fluoroquinolone compound similar to ciprofloxacin, was found to be a suitable internal standard in the present method. Other internal standards used for the HPLC method of ciprofloxacin include lomefloxacin [12], ofloxacin [13], umbelliferone [14], sulfadimidine [15] and sarafloxacin [21]. Ofloxacin was also tried as an internal standard in a similar study but there was a weak separation of two compounds [15]. The present study is the first report to use moxifloxacin as an internal standard of ciprofloxacin.

The specificity of the method was indicated by the retention time of ciprofloxacin which was recorded between 4.022 to 4.173 minutes with a mean of 4.098 (~ 4.1) minutes. The chromatograms of blank plasma with internal standard moxifloxacin (**Figure 2**) and spiked plasma with ciprofloxacin (**Figure 3**) had interferences from the plasma matrix between 2 to 4 minutes interval, but chromatograms were free from any interference by endogenous substances, particularly around the retention times of ciprofloxacin (4.1 minutes) and moxifloxacin (7.9 minutes).

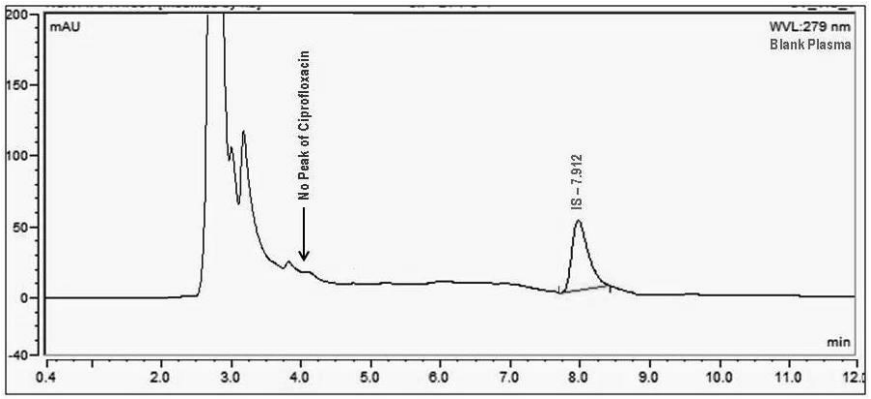

Figure 2 Chromatogram of blank plasma with internal standard only

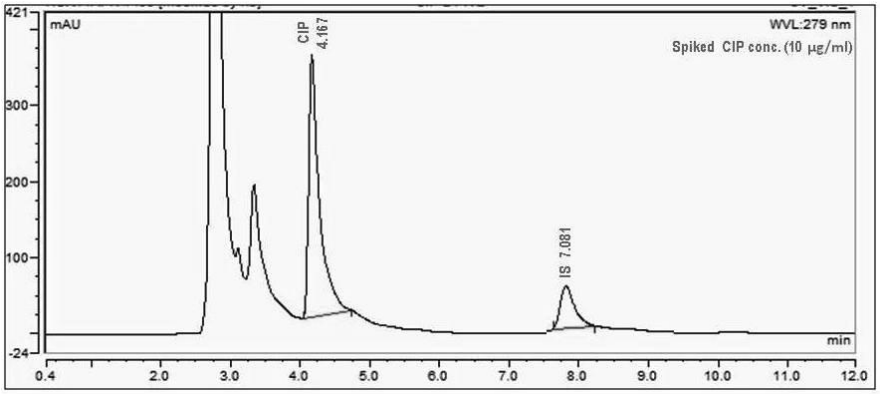

Figure 3 Chromatogram of plasma spiked with ciprofloxacin (10 µg/ml)

For assessment of linearity, seven ciprofloxacin concentrations *i.e.* 0.05, 0.10, 0.50, 1.00, 2.50, 5.00, and 10.00 µg/ml were prepared in the drug-free poultry plasma and then extracted and analysed five times (n=5) with UHPLC to construct calibration curves. The calibration curves covered the entire range of expected concentrations for the disposition study and so, the generated calibration curve was further utilized for calculation of ciprofloxacin concentration in unknown plasma samples. Good linearity was shown by the calibration curves prepared from spiked plasma samples for ciprofloxacin concentrations ranging from 0.05 to 10.00 µg/ml with values of correlation coefficients (R^2) ranging from 0.9997 to 0.9999. Thus, the goodness-of-fit (r^2) was found at least 0.9997 among all five curves, indicating a functional linear relationship between the plasma ciprofloxacin concentration and resultant area under the peak.

The extraction recovery for ciprofloxacin was calculated at three different concentrations of 0.10, 1.00 and 10.00 µg/ml for which respective mean recoveries were obtained as 91.24, 94.84 and 90.30 % (**Table 3**). Acetonitrile is the most common organic solvents which has been used to extract ciprofloxacin from plasma samples with good extraction recoveries like 88-123 % [11], 96.1-98.4 % [12], 93.8 % [14], 96.9 % [15] and 94 % [16]. Other than this, dichloromethane was also used to extract ciprofloxacin [13] but the extraction recovery was lower than 80 %. In another study, a mixture of phosphoric acid and perchloric acid was also used to extract ciprofloxacin but the sample was poultry serum instead of plasma [17]. In the present study, plasma protein precipitation with acetonitrile was preferred as it is lesser polar (as compared to methanol) and thus, expected to be more effective at precipitating proteins [15].

The accuracy (%) was observed at three different concentrations (0.1, 1 and 10 µg/ml) in triplicates (n=3). At any concentration, accuracy was not less than 90% (**Table 4**).

The highest intraday and inter-day C.V. % calculated were 7.40 % and 7.32 % (both at 0.50 µg/ml), respectively (**Table 5**). Both intra-day and inter-day C.V. was below 10 % which shows good precision of the optimized and validated method.

Table 3 Recovery (extraction) for ciprofloxacin from spiked poultry plasma

S. No.	Spiked Plasma Concentration (µg/ml)	Recovery (%)	Range (%)	Mean Recovery (%)
1	0.10	91.07	89.98 – 92.16	91.24
2	0.10	91.74		
3	0.10	92.16		
4	0.10	89.98		
5	0.10	91.89		
6	1.00	91.56	91.56 – 99.57	94.84
7	1.00	91.66		
8	1.00	99.57		
9	1.00	96.57		
10	1.00	97.07		
11	10.00	91.48	88.91 – 91.48	90.30
12	10.00	90.35		
13	10.00	88.91		
14	10.00	90.47		
15	10.00	89.36		

Table 4 Accuracy of the UHPLC method for ciprofloxacin analysis in poultry plasma

S. No.	Spiked Plasma Concentration (µg/ml)	Observed Concentration (µg/ml)	Inaccuracy (%)	Accuracy (%)
1	0.100	0.112	1.2	98.80
2	0.100	0.113	1.3	98.70
3	0.100	0.113	1.3	98.70
4	1.000	0.992	-0.84	99.16
5	1.000	1.0761	7.61	92.39
6	1.000	1.044	4.4	95.60
7	10.000	10.0312	3.12	96.88
8	10.000	9.9017	-9.83	90.17
9	10.000	10.045	4.5	95.50

Table 5 Intraday and inter-day precision of the method for UHPLC - UV detection of ciprofloxacin in poultry plasma

S. No.	Spiked Concentration (µg/ml)	Mean Observed Area (mAU*min)	Standard Deviation	Precision C.V. (%)
Intraday values				
1	0.05	0.2716	0.0087	3.20
2	0.10	0.5447	0.0123	2.26
3	0.50	2.4928	0.1844	7.40
4	1.00	5.4983	0.2207	4.01
5	2.50	13.4431	0.2892	2.15
6	5.00	26.7853	1.1970	4.47
7	10.00	54.3493	1.4843	2.73
Interday values				
1	0.05	0.2862	0.0122	4.26
2	0.10	0.5788	0.0179	3.09
3	0.50	2.9563	0.2163	7.32
4	1.00	6.0103	0.3939	6.55
5	2.50	14.9767	1.0838	7.24
6	5.00	29.4017	1.7278	5.88
7	10.00	57.9924	2.5741	4.44

For knowing the sensitivity, the calculated LOD and LOQ of the present analytical method for ciprofloxacin were 0.101 and 0.307 µg/ml, respectively. However, peaks at 0.05 µg/ml concentrations were also acceptable based on signal: noise ratios which were found to be more than three. The calculated LOD (101 ng/ml) in present method is considered as good sensitivity looking at the small injection volume (loop volume) equal to 20 µL. Such a small value of LOD and LOQ are also adequate to study the disposition kinetics of ciprofloxacin in target animals.

Application

Venous blood samples were collected periodically at ten time-points (including pre-dosing collection) from two poultry birds (broiler chickens) following single-dose intravenous administration of ciprofloxacin at the dose rate of 10 mg/kg body weight. This part of the research was done to check the applicability of the present validated method for disposition kinetics study. Plasma was harvested from the collected blood samples, and then extracted and assayed with the present validated UHPLC method to know the drug plasma concentrations at various time points. The plasma concentrations observed are presented in **Table 6**.

Table 6 Plasma concentration of ciprofloxacin (µg/kg) following single dose intravenous administration in poultry birds

Time	5 min	15 min	30 min	1 h	2 h	4 h	8 h	12 h	24 h
Bird 1	8.293	4.053	3.316	1.788	1.025	0.612	0.485	0.384	0.131
Bird 2	9.614	5.131	3.073	1.695	0.908	0.651	0.505	0.325	0.137

Conclusion

The present simple, rapid (short elution time) and economical (using short column) UHPLC method was found sensitive, specific, accurate and precise for analysis of ciprofloxacin in poultry plasma with good recovery rate. The present method using moxifloxacin as an internal standard was appropriate and can be applied to study the disposition kinetics of the ciprofloxacin in poultry birds.

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