RESEARCH ARTICLE

Stability Indicating Analytical Method Development and Validation of Ciprofloxacin By RP-HPLC with Fluorescence Detector

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ABSTRACT

Ciprofloxacin is an anti-microbial agent, member of the quinolone class compound with activity was found against gram-positive and negative bacteria as well as wide clinical applicability. A simple and sensitive nanomolar concentration for the quantification of ciprofloxacin (QoC) using reverse phase high-performance liquid chromatography (HPLC) method with fluorescence detection. The method was performed using a C18 column at 40ºC temperature and 0.1% orthophosphoric acid and methanol with a ratio of 70:30 (v/v). The mobile phase was used for the separation of ciprofloxacin. The wavelength of measurement was set at excitation 278 nm and emission 455nm. The method was developed in a 50 to 250 ng/mL concentration with a correlation coefficient of 0.9918. The limit of detection (LoD) and limit of quantification (LoQ) for ciprofloxacin was found to be 0.3215 ng/mL. and 0.9743 ng/mL respectively. The method was sensitive, stability-indicating, and rapid for nanomolar determination of ciprofloxacin in the pure and pharmaceutical dosage form. The analytical procedure was specific and validated as per the ICH guideline (Q2R1).

Keywords: Ciprofloxacin HCl, Fluorometric method, ICH guidelines, RP-HPLC.

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INTRODUCTION

Ciprofloxacin is a widely used antibiotic for both human and veterinary use. It was patented in 1980 and introduced in 1987. The safest and effective medicine needed in the healthcare system is on the world health organization's essential medicine list. This molecule is most effective against wide gram-positive and gram-negative organisms. This drug comes under the class of Fluoroquinolones antibiotics that are commonly used to treat various illnesses, for example, pneumonia; asexually transmitted diseases like gonorrhea, typhoid fever, infections related to diarrhea, and the skin, bones, joints, abdomen and prostate gland. It is also used to the prevention of a serious infection, plague, and anthrax.[1-2]

A literature review for ciprofloxacin studies has been performed, followed by many methods developed for ciprofloxacin in biological samples by HPLC and UV detection.[3-6] Stability indicating method has been developed to determine ciprofloxacin hydrochloride and its related compounds in film-coated formulations (Tablets) using UV detection.[7-12] Some studies reported on the bioanalytical HPLC method for the simultaneous quantification of ofloxacin, ciprofloxacin, and moxifloxacin in the human plasma of the selected patients.[3] A depletion study of enrofloxacin and its metabolite ciprofloxacin in edible tissues and feathers of white Leghorn hens by liquid chromatography with tandem mass spectrometry[13] and some study related to the ophthalmic delivery of ciprofloxacin hydrochloride from different polymer formulations: *in vitro* and *in vivo* studies.[14] Some report explores on QoC concentrations in human serum and urine by HPLC with UV and fluorescence detection.[15] The analytical methods reported for ciprofloxacin and its combinations review,[16] it is found that there is no study has been carried for the analytical method of development for ciprofloxacin using a fluorescence detector. The method developed in terms of parameters like accuracy, precision, linearity, the limit of quantification and detection, quantitation limit, robustness, and stability is as per the procedure described in International Council for Harmonisation (ICH) guidelines.[17]

MATERIALS & METHODS:

Instrument and Liquid Chromatographic Conditions

For the current study HPLC RF-20A (fluorescence detector Shimadzu), the separation was achieved by using Phenomenex

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Luna C-18 column (250 mm length X 4.60 mm diameter with the particle size of 5µ). Run time was set to 10 minutes. Methanol and 0.1% orthophosphoric acid 70:30 (v/v) was used as a mobile phase with a flow rate of 0.6 mL/min. The temperature of the column was maintained at 40°C. The excitation and emission wavelength were set at 278 nm and 455nm respectively. PHENEX PTFE 0.22 µm syringe filter was used for filtration of the prepared standards and sample solutions.

Chemicals and Reagents

Ciprofloxacin hydrochloride procured from Turtle Pharma Private Limited Mumbai, Maharashtra, as a gift sample, and all of the solvents were used as an analytical grade, HPLC grade methanol and HPLC grade Millipore water. Ciprofloxacin-500 mg tablets were used in the study and purchased from the local pharmacy.

Analytical Method Development

Mobile Phase Selection

Different mobile phase combinations of various ratios were checked for the selection of the mobile phase. The standard ciprofloxacin drug was injected with various combinations of mobile phases at different ratios and flow rates for peak optimization. The procedure was continued until obtaining a sharp peak. The sharp peak was obtained at 70:30 (v/v) of methanol and 0.1% orthophosphoric acid as a mobile phase.

Preparation of Standard Stock Solution

Accurately weighed 10mg of pure ciprofloxacin HCl into 10 mL volumetric flask, and dissolved HPLC grade Millipore water, diluted the resultant solution up to mark (Stock solution). Then pipette out 1 mL from the prepared stock solution and made up to 10 mL followed by pipette out 0.1 mL of the drug solution to 10 mL volumetric flask and makeup to get 1000 ng/mL. From the above solution, 50, 100, 150, 200, 250 ng/mL solutions were prepared and diluted with HPLC grade water to get the desired concentration.

Preparation of Tablet Dosage Form

Twenty tablets of the formulation containing ciprofloxacin (each tablet contains 500mg) were accurately weighed to obtain the average tablet weight. Tablets were crushed using mortar pestle to obtain a fine powder. A one tablet equivalent amount of the powder was weighed and transferred a volumetric flask of 100 mL capacity. The powder was dissolved initially in 50 mL of the water. The mixture and sonicated and powder were dissolved completely using a sonicator. Further, the solution was filtered through a syringe filter and diluted up to the volume. Further dilution was made to obtain a concentration of 1000 ng/mL.

ANALYTICAL METHOD VALIDATION

The developed method was validated according to standard ICH guideline for linearity, accuracy, precision, LoD, LoQ, robustness, ruggedness, and stability studies (ICH Guidelines).[17]

Linearity

It consisted of matrix samples processed without analyte and matrix samples with calibration standards. It shows good linearity over the range of 50 to 250 ng/mL with a correlation coefficient of 0.9918. The overlaid chromatogram and linearity graph was shown in Figures 1 and 2, respectively.

Precision

The repeatability of the process was evaluated by using different concentrations of the drug 50, 150, and 250 ng/mL. The above solutions have been prepared from the stock solution and used to inject in interday and intraday for the evaluation of precision. The concentrations were rendered at three different times in a day for intraday studies. The results were shown for the intraday and inter-day precision in Tables 1 and 2, respectively.

Accuracy

The accuracy of an analytical procedure is the similarity of the obtained value to the true value of the sample. To check the method's accuracy, formulations were spiked with 50, 100, and 150% of ciprofloxacin standard. The results were analyzed to find the %recovery of the ciprofloxacin (Table 3).

Limit of detection (LOD) and Limit of quantification (LOQ)

The LOD and LOQ is the smallest concentration of the analyte that gives a response that can be detected and measured,

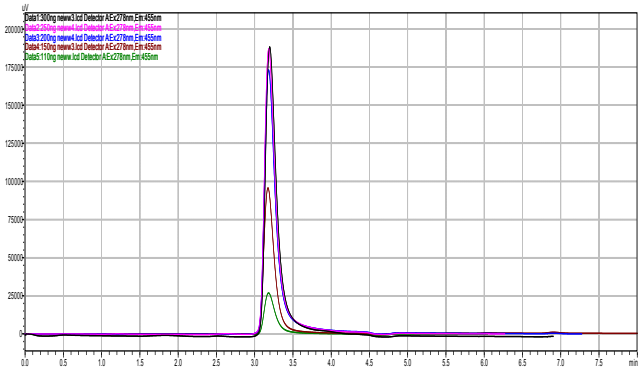

Figure 1: Overlaid chromatogram of standard ciprofloxacin 50 to 250 ng/mL

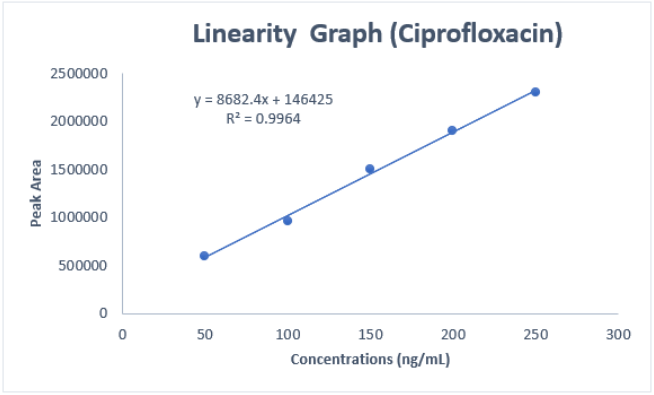

Figure 2: Linearity graph of ciprofloxacin

Table 1: Intraday precision for ciprofloxacin

Conc. (ng/mL)	*Peak area*	*Conc. (ng/mL)*	*Peak area*	*Conc. (ng/mL)*	*Peak area*
50	599081	150	1437318	250	2158465
50	596254	150	1429528	250	2194037
50	591658	150	1441167	250	2138502
50	596916	150	1406536	250	2097184
50	594522	150	1483667	250	2198853
50	594521	150	1431128	250	2195633
Avg	595492		1438224		2163779
STD Deviation	2312.021		23110.79		37109
% RSD	0.3882		1.6068		1.7150

Table 2: Interday precision for ciprofloxacin

Conc. (ng/mL)	*Peak area*	*Conc. (ng/mL)*	*Peak area*	*Conc. (ng/mL)*	*Peak area*
50	595624	150	1418965	250	2193468
50	596985	150	1404563	250	2185524
50	596685	150	1408265	250	2099835
50	599863	150	1418167	250	2122558
50	598467	150	1405837	250	2192538
50	596997	150	1418462	250	2184935
Avg	597436.8		1412377		2163143
Std deviation	1366.062		6254.223		37448.89
%RSD	0.2286		0.4428		1.7312

Table 3: Recovery studies of ciprofloxacin

Level of recovery	*Amount of formulation (ng/mL)*	*Amount of pure drug (ng/mL)*	*Total amount of drug (ng/mL)*	*Peak area*	*Difference*	*% Recovery*	*Mean*
50	100	50	150	56850121	56251040	98.94621	
50	100	50	150	56084631	55485550	98.93183	98.93183
50	100	50	150	46081461	45482380	98.69995	
100	100	100	200	52902599	51947911	98.19539	
100	100	100	200	55856329	54901641	98.29081	98.29081
100	100	100	200	54527849	53573161	98.24917	
150	100	150	250	78184526	76705870	98.10876	
150	100	150	250	79976485	78497829	98.15114	98.15114
150	100	150	250	78976352	77497696	98.12772	

respectively. LoD and LoQ was calculated by using the following equation,

$$LOD = \frac{3.3 \times \text{Standard deviation (SD)}}{\text{Slope of calibration curve}}$$

$$LOQ = \frac{10 \times \text{Standard deviation (SD)}}{\text{Slope of calibration curve}}$$

Robustness

It is the ability of a method to remain unimpaired when slight variations are applied. The robustness of the proposed techniques was checked by increasing and decreasing the detection wavelength, flow rate, column temperature. The results of robustness are shown in Table 4.

Ruggedness

An analytical method's ruggedness is its ability to remain unimpaired by a small but intentional change in method parameters. The ruggedness of the proposed methods is validated by changing analysts and instruments. The results are shown in Table 5.

STRESS DEGRADATION STUDIES

The degradation procedure was followed out according to ICH guidelines performed in Q1A (R2). Stability study of a new drug substance use a validated analytical method, and results were shown in Table 6 with the following procedure.

Table 4: Robustness study results

Wavelength (nm)		*Conc. (ng/mL)*	*Peak area*	*Wavelength (nm)*		*Conc. (ng/mL)*	*Peak area*
Excitation	Emission	150	1298567			150	1598567
276	453	150	1252897	300	455	150	1585694
		150	1299826			150	1555694
		Average	1283763			Average	1579985
		STD DEV	21831.84			STD DEV	17962.33
		%RSD	1.7006			%RSD	1.1368

Table 5: Ruggedness of the developed method

Conc. (ng/mL)	*Peak area*	*Peak area*	*Mean*	*SD*	*%RSD*
By changing the analyst					
0	0	0	0	0	0
50	592522	582253	587387.5	7261.28	0.925648
100	958998	959826	959412	585.4844	0.765426
150	1499856	1498564	1499210	913.582	0.896253
200	1899875	1885946	1892911	9849.29	0.925625
250	2292689	2287596	2290143	3601.295	0.826526
By changing the instrument					
0	0	0	0	0	
50	582659	591256	586957.5	6078.997	1.035679
100	969523	959682	964602.5	6958.638	0.7214
150	1568268	1425699	1496984	100811.5	0.862546
200	1875862	1885664	1880763	6931.061	0.965246
250	2326588	2289665	2308127	26108.5	0.786259

Table 6: Stress degradation study of ciprofloxacin

Sl.No	*Stress condition*	*% assay of ciprofloxacin*	*% degradation*
1	Acid (0.1HCl)	93.96	6.04
2	Alkali (0.1N NaOH)	94.235	5.765
3	Oxidation (10% H_2O_2)	96.189	3.811
4	Thermal (80%)	97.939	3.069

Acid Degradation Study

A 2 mL (20 ng/mL) of ciprofloxacin solution was transferred to a 10 mL volumetric flask, followed by 2 mL of standard solution 0.1 N hydrochloric acid was added and refluxed for a period of 30 minutes at 60ºC on a water bath. Later, the solution was neutralized with 2 mL of a standard solution of 0.1 N sodium hydroxide, then diluted to 10 mL with acetonitrile. The resultant solution was filtered through a syringe filter, and 10 µL of the solution was injected, and the corresponding chromatogram was shown in Figure 3.

Basic Degradation Studies/alkali Degradation Studies

2 mL (20 ng/mL) solution of ciprofloxacin was taken in 10 mL volumetric flask, followed by 2 mL of a standard solution of 0.1 N sodium hydroxide was added, and the solution was neutralized with 2 mL of 0.1 N hydrochloric acid, refluxed on a water bath for 30 minutes at 60ºC. The resultant solution was filtered through a syringe filter (0.22 µm), and 10 µL of the solution was injected and the corresponding chromatogram as shown in Figure 4.

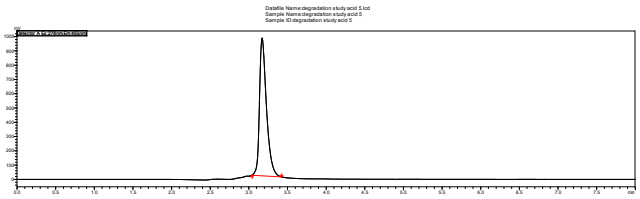

Figure 3: Chromatograph of ciprofloxacin stressed under 0.1 N HCl

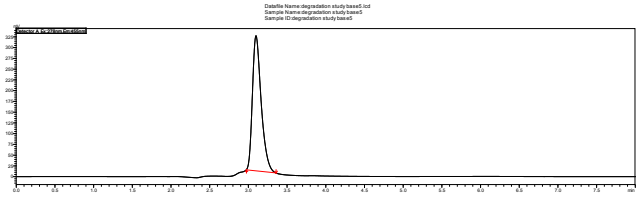

Figure 4: Chromatograph of ciprofloxacin stressed under 0.1 N NaOH

Oxidation

2 mL (20 ng/mL) solution of ciprofloxacin was transferred to 10 mL volumetric flask, then 2 mL of 3% v/v solution hydrogen peroxide was added it and refluxed for 30 minutes at a temperature of 60°C on a water bath; further, it was diluted

to 10 mL with acetonitrile. The resultant solution was filtered through a syringe filter (0.22 µm) and 10 µL of the solution was injected, and the respective chromatogram was showed in Figure 5.

Photolytic studies

This study was performed by exposing 16 ng/mL solution in UV light by placing the beaker in the UV chamber for 4 days**.** The entire solution was filtered using a syringe filter, then the 10 µL of the sample was injected and the chromatograms have been recorded as showed in Figure 6.

Thermal Degradation

Dry heat studies were contacted by holding the sample in the oven (80º C) for a period of 4 hours and the sample was taken after 4 hours, and the stock 1 solution was prepared. From the stock -1, 125 µL of the solution ware transferred to the 5 mL pre-calibrated volumetric flask, and volume was made up to 5 mL with an equivalent volume of HPLC grade water. The solution was vortexed and filtered by a 0.22 µm syringe filter. A filtered solution was analyzed for ciprofloxacin by using prevalidated HPLC method (Figure 7).

RESULTS AND DISCUSSIONS

Optimization of the Method

Our study aimed at developing an accurate, precise, and simple method for the development and validation of ciprofloxacin. Along these lines, initial trials were conducted using varying ratios of methanol and 1% orthophosphoric acid. The best mobile phase ratio, i.e., methanol and 1% orthophosphoric acid in the ratio 70:30 v/v with buffer pH 3 could effectively give excellent peak shapes at the wavelength excitation 278 nm and emission 455 nm respectively.

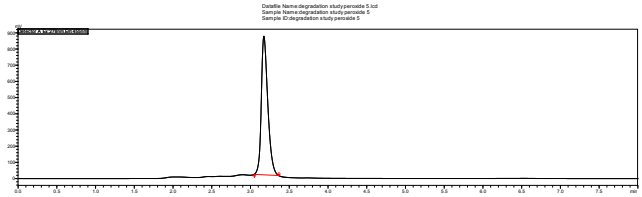

Figure 5: Chromatograph of ciprofloxacin stressed under 10% H_2O_2

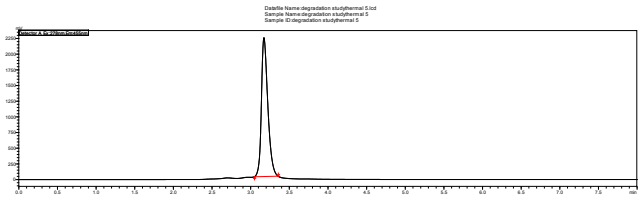

Figure 6: Chromatograph of ciprofloxacin stressed under UV light

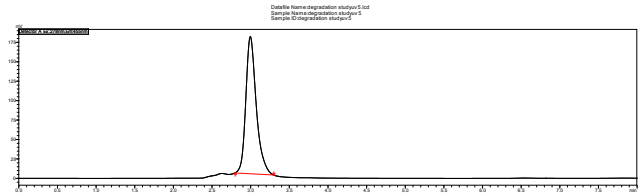

Figure 7: Chromatograph of ciprofloxacin stressed under thermal

Method Validation

The method was linear in the concentration of 50 to 250 ng/mL, and the regression coefficient of the linearity equation was found to be 0.9918 for ciprofloxacin. For intraday precision, % RSD was found to be 0.388254, and interday precision, % RSD was found to be 0.228654, shown in Table 1 and Table 2, respectively. The percentage recovery of ciprofloxacin at each level was within limits of 98%; hence for the current work, accuracy was established, and the method was found to be accurate (Table 3). The LoD for ciprofloxacin was found to be 0.32154 ng/mL. The LoQ for ciprofloxacin was found to be 0.97437ng/mL. The robustness study was revealed that less than 2% variation in %RSD (Table 4), and so method is robust.

Stress Degradation Studies

The ciprofloxacin degradation studies were performed as per the procedure described. It is found that the percentage of ciprofloxacin in the acidic and basic medium was found to be 93.96 and 94.235%. In the acidic and basic medium, the degradation for ciprofloxacin was found to be 6.04 and 5.765%, which is stable as per stability guidelines. Interestingly, with optimum condition followed in the case of oxidative and photolytic studies, there were no additional peaks were observed, and the drug percentage was found to be 96.189, and 96.611% and the percentage of degradation was found to be 3.811 and 3.389%, and for heat or thermal degradation, the percentage of standard ciprofloxacin was found to be 97.937%. The percentage of degradation was found to be 3.069%. According to standard values the degraded % values are in the acceptable range. The details of the results for the degradation study were shown in Table 6.

Significance of the Developed Method

Developed method for indicating analytical stability has many advantages over reported methods. The method was simple since the cost of the mobile phase is economic, easy to access, and the selected drug was eluted within 3.2 minutes of 10 minutes of the total run time. The method is highly sensitive as it is developed in nanogram level which was confirmed in the limit of detection and limit of quantification and hence it is useful for the determination of ciprofloxacin in biological samples. The LoD for ciprofloxacin 0.3215 ng/mL and the LoQ was 0.9743 ng/mL indicates that the process was sensible and quick. And the analysis of forced degradation (Table 6) shows that the process was very accurate, stable in the proposed method. The degradants do not produce in the study and confirm the method is stable and robust.

However, the study of forced degradation was neither performed nor published elsewhere, so it was the first stabilizing LC approach for quality control of ciprofloxacin.. The validation of the developed method in accordance with ICH guidelines indicates that the method was highly precise, rapid, simple, economical, and sensitive to the accurate pharmaceutical dosage form.

CONCLUSION

The chromatographic developed method is quite simple, rapid, and sensitive to the extent of nanomolar concentrations. The

method was validated according to standard ICH guidelines regarding accuracy, precision, linearity, the limit of detection, and LoQ, robustness, and stress degradation studies. It was concluded that the liquid chromatographic together with the fluorescence method for ciprofloxacin, was found to precise, accurate, rapid, specific, and economical.

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