

Analytical Stability Indicative Method Development and Validation by High Pressure Liquid Chromatography for Assay in Ciprofloxacin hydrochloride Drug Substances

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Abstract

Ciprofloxacin is an antibiotic generic drug that can treat a number of bacterial infections. It is a second generic fluoroquinolone. This work presents a simple, sensitive and stability indicative method for assay determination of drug substances ciprofloxacin hydrochloride by high pressure liquid chromatography (HPLC). Separation of impurities from the main drug substances and accurate assay quantification with a simple method is difficult by Spectrophotometry. The separation of co-eluent and impurities from the ciprofloxacin drug substances was achieved by HPLC with simple mobile phase 0.15% orthophosphoric acid adjusted pH to 3.0 with triethylamine and acetonitrile using a gradient program, column inertsil C18, 250 mm × 4.0 mm, 5 μm. Flow rate was 0.7 mL/min, the column temperature and injection volume were 35°C and 10.0 μL. Chromatographic analysis was carried out at wavelength 278 nm. The developed method was validated according to the international conference on harmonization (ICH) guidelines regarding: Precision, specificity by degradation, linearity, accuracy, range and robustness. The proposed method showed good linearity (correlation coefficient and regression coefficient were not less than 0.999 and 0.998) in the range of 50% to 150% of working concentration. The recovery at 50%, 100% and 150% of working concentration level was within 98.0% to 102.0%. The range of the method is concluded that developed method is from 50% to 150% of target concentration for ciprofloxacin hydrochloride. The applicability of the proposed method was verified through the analysis of the samples and percentage recoveries in the range of 98% to 102% were obtained without any interference detected at the main peak in the chromatogram. The validated method is highly selec-

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tive, simple, accurate, cost effective, and it is applicable for stability studies and routine quality-control analysis in the pharmaceutical industries.

Keywords

Ciprofloxacin Hydrochloride, Fluoroquinolone, HPLC

1. Introduction

Ciprofloxacin is a synthetic chemotherapeutic antibiotic of the fluoroquinolone drug [1]-[3]. It is a second-generation fluoroquinolone antibacterial inhibitor [4] [5]. It inhibits pathogenic bacteria by interfering with the enzymes that cause DNA to rewind after being copied; it can prevent the synthesis of DNA and protein molecule. Therefore, Ciprofloxacin hydrochloride drug molecule can treat bacterial infections [6]-[8]. Ciprofloxacin was first patented in 1983 by Bayer A.G. and then accepted by the United States Food and Drug Administration (FDA) in the year 1987. Ciprofloxacin is 1-cyclopropyl-6-fluoro-1, 4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolonecarboxylic acid. Its empirical formula is $C_{17}H_{18}FN_3O_3$ and its molecular weight of this molecule 331.4 g/mol. It is slightly yellowish to light yellow crystalline product. Ciprofloxacin hydrochloride (USP) is the monohydrochloride monohydrate salt of ciprofloxacin. It is a faintly yellowish to light yellow crystalline substance with a molecular weight of 385.8 g/mol. $C_{17}H_{18}FN_3O_3 \cdot HCl \cdot H_2O$ is the empirical formula of Ciprofloxacin hydrochloride monohydrate [9]. The structure of studied ciprofloxacin hydrochloride salt showed in **Figure 1**. Quinolones have two types of ring structure, a naphthyridine nucleus with nitrogen atom at 1 and 8 positions, a nucleus with nitrogen atom in position 1 and it is referred as the quinoline moiety. It contains the keto oxygen functional group at carbon -4 and a carboxylic side chain at carbon -3. In addition, the ciprofloxacin has a piperazinyl group at carbon -7. The presence of both carboxyl and amine groups makes the acid-base behavior of the drug influenced by the physico-chemical properties of the solvent [10]. Hence, the presence of aromatic delocalized π -electrons and electronegative heteroatoms, the molecules are highly polarizable and liable to a different specific interactions with polar solvents. Ciprofloxacin is used to cure a wide variety of infections, including infections at joints and bones, endocarditis, gastroenteritis, respiratory tract infections, cellulitis, urinary tract infections, prostatitis, anthrax, and chancroid [11].

In addition, ciprofloxacin is available as a generic drug under a variety of different brand names and is also available for limited use in veterinary medicine. These may be either new entities or partial structural modifications of the existing one. Very often there is a time delay from the date of introduction of a drug into the market to the date of inclusion in pharmacopoeias. This happens because of the likely uncertainties in the continuous and wider usage of the drugs, results of new toxicities, growth of patient resistance and introduction of better drugs by contenders. Under such conditions, finalized analytical procedures for these drugs may not be available in the pharmacopoeias. It becomes necessary to develop newer analytical methods for such drugs.

Spectrophotometric analytical assay methods [12] [13] involve the measurement of absorbance of samples containing only one absorbing analyte. The pharmaceutical analyst frequently faces the situation where the concentration of one or more substances is required in samples known to contain other absorbing substances, which potentially interferes in the same measuring wavelength. Due to the interferences, Simple Spectrophotometric methods may not get accurate assay for the sample but separation of the interferences in a single experiment and

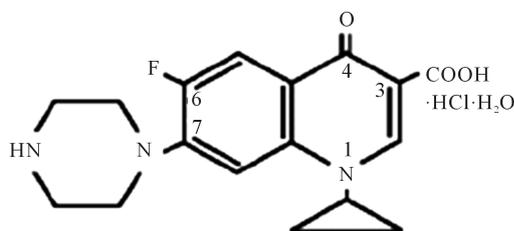


Figure 1. Chemical structure of Ciprofloxacin Hydrochloride monohydrate.

accurate, precise with good reproducibility of assay determination achieved by using High Pressure Liquid Chromatographic method [14]-[18].

In view of the above facts, it encourages us to investigate an analytical stability indicative method development and validate by high pressure liquid chromatography for assay in ciprofloxacin hydrochloride drug substances. The conformed Reverse Phase-High Pressure Liquid Chromatographic method procedure is a finely resolved, simple and good stability indicative method to determine the assay of Ciprofloxacin hydrochloride for routine quality-control analysis in pharmaceutical testing laboratories.

2. Experimental [19]-[22]

2.1. Materials and Reagents

Ciprofloxacin hydrochloride drug substances and related impurities was gifted by local Pharmaceutical industry, acetonitrile (HPLC grade) was purchased from Rankem Lab, HPLC grade water (Millipore), orthophosphoric acid (AR grade) from spectrochem (India), hydrochloric acid (AR grade) from sd-fine chemicals, hydrogen peroxide (AR grade) from CDH fine chemicals, sodium hydroxide (AR grade) from SDFCL and triethylamine (AR grade) was purchased from spectrochem (India).

2.2. Instrumentation

UV-Visible spectrophotometer Perkin Elmer's LAMDA 25. For Chromatography an Agilent 1100 series Quaternary pump with diode array detector/UV detector with chemstation software and separations of analytes and determination of the compound was performed with Inertsil C18 column (250 mm × 4.6 mm I.D; particle size 5 µm) maintained at 35°C. Weigh the sample done on Sartorius analytical balance with sensitivity of 0.01 mg. Mobile phase pH adjustments made with digital pH Meter Elico.

2.3. Chromatographic Conditions

The Reversed phase High Pressure Liquid Chromatography analysis was carried out at 35°C temperature. The components were eluted by gradient method programme (**Table 1**) with a mobile phase-A (pipette and transfer 1.5 mL of orthophosphoric acid to 1000 mL of water and mix well then adjust pH to 3.0 with triethylamine.) and mobile phase-B (acetonitrile). The mobile phases were filtered through 0.45 µm membrane filter (Millipore). Flow rate is 0.7 mL/min and the injection volume for standard and sample was 10.0 µL. The eluents were measured at a wavelength of 278 nm.

2.4. Analytical Method Development

For developing a new chromatography method the standard and sample solubility was checked in water, acetonitrile and mixture of mobile phase-A and mobile phase-B in the ratio 80:20. The standard and sample was freely soluble with the above mixture of mobile phase-A and mobile phase-B. Then a solution of standard was scanned on UV Spectrophotometer to identify the maximum absorbance of wavelength. Maxima UV absorbance observed at Wavelength 278 nm. Hence this wavelength has been chosen for detection in analysis of Ciprofloxacin hydrochloride. Since ciprofloxacin hydrochloride was freely soluble in mixture of mobile phase-A and

Table 1. The gradient method programme.

Time in minutes	Mobile Phase A (In %)	Mobile Phase B (In %)
0.0	82	18
5.0	82	18
6.0	95	5
12.0	95	5
13.0	82	18
30.0	82	18

mobile phase-B in the ratio 80:20 was kept as diluent for sample and standard preparations. Gradient mobile phase elution programme was finalized for better resolution. Equilibrated the column with initial composition of the mobile phases A and mobile phases B in the gradient method. Injected Blank (Diluent) one injection, Standard solution five injections and checked the system suitability, the relative standard deviation for ciprofloxacin hydrochloride peak in standard solution is not more than 2.0%. Its theoretical plates for peak are not less than 2000; the tailing factor of peak is not more than 2.0. The system suitability parameters pass and then injected the sample solution in duplicate, recorded main peak response.

2.5. Standard Solution Preparation

Weigh and transfer quantitatively about 100.0 mg of ciprofloxacin hydrochloride standard to 10.0 mL volumetric flask, dissolve and dilute to volume with diluent. Further dilute 1.0 ml of above solution to 100 ml with the diluent and mix.

2.6. Sample Solution Preparation

Weigh and transfer accurately about 12.0 mg of ciprofloxacin hydrochloride sample to 25.0 mL volumetric flask, dissolve and dilute to volume with diluent. Further dilute 2.0 ml of above solution to 10.0 ml with the diluent and mix.

3. Results and Discussion

3.1. Chromatographic Method Development

As per the spectrum of UV spectrophotometer, ciprofloxacin hydrochloride has been showed wavelength was at 278 nm (λ max). Hence 278 nm was selected for determination of ciprofloxacin hydrochloride (**Figures 2(a)**). The method was optimized to separate major impurity and degradation product peaks formed under different stress conditions. The main target of the chromatographic method is to separate closely eluting peaks of the sample, mainly ethylenediamine compound (ciprofloxacin impurity-C) and 7-chloro-1-cyclopropyl-4-oxo-6-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid hydrochloride salt (Ciprofloxacin Related compound-A) peaks. The degradation samples were run using different columns with same stationary phase like C18 and mobile phases containing buffers like Acetate and phosphate.

The Gradient mode method has been worked out to separate very closely eluting impurity peaks. To improve response, peak shape and resolution between the peaks the method was tried at different column temperatures. But the separation, response of peaks and peak shapes were satisfactory in the adopted chromatographic conditions only, mainly the ciprofloxacin impurity-C at RRT 0.74 and ciprofloxacin related compound-A at RRT 1.17. It indicated that the gradient mode method with acetonitrile as an organic modifier in mobile phase was successful in separating the main component from all other chromatographic degradation products (**Figures 2(b)-(g)**).

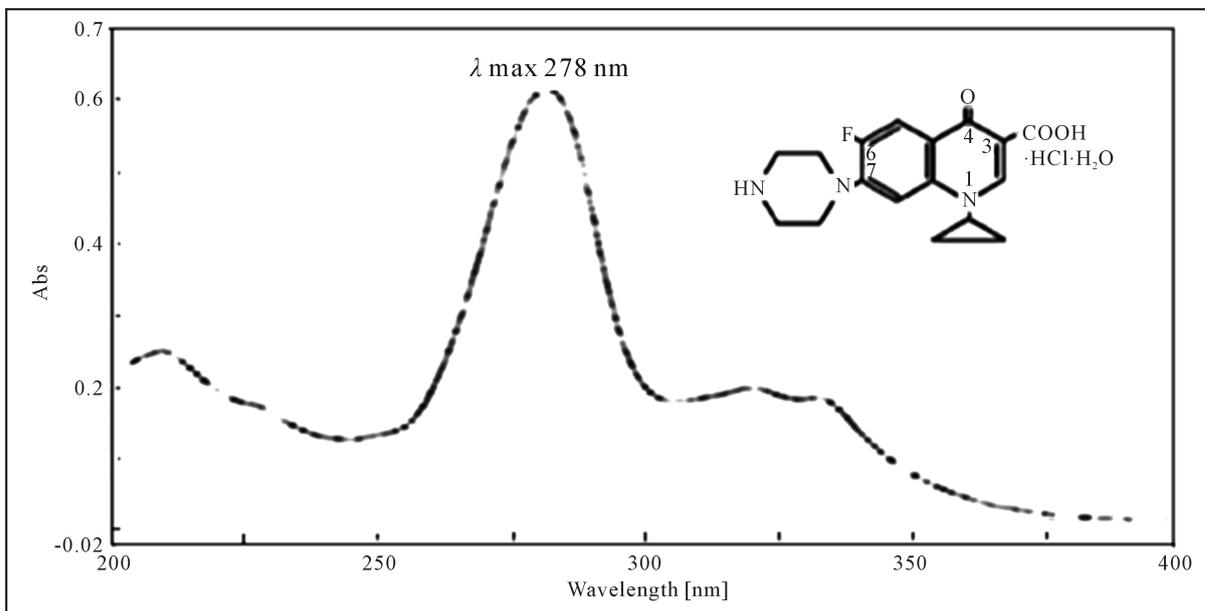
3.2. Column Selection

Based on the retention, better peak shape of the compound, good column life on usage and easy availability with low cost, Inertsil C18 (250 × 4.0 mm; 5 μ m) column was selected as suitable column for analysis of Ciprofloxacin Hydrochloride.

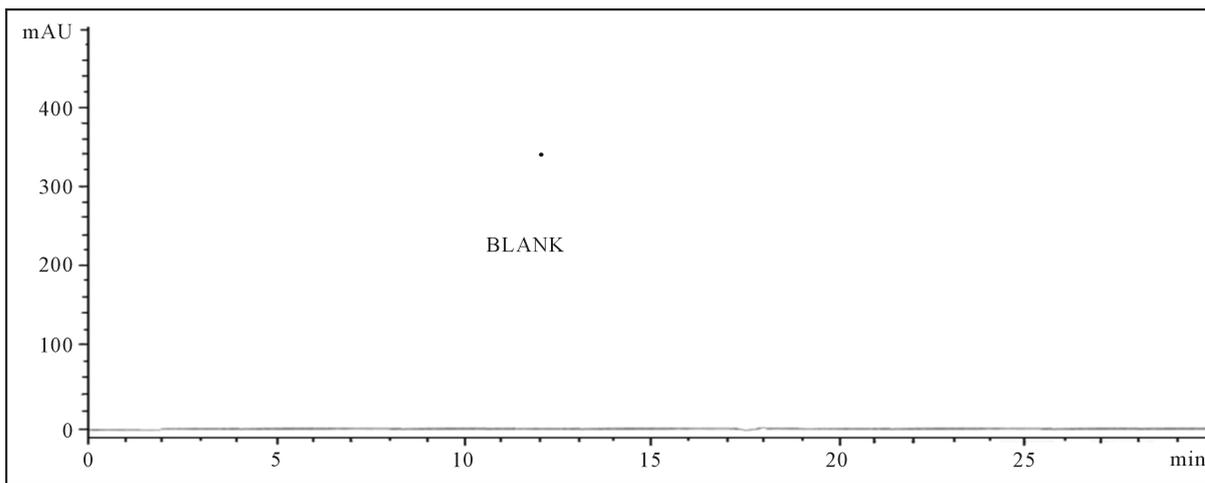
4. Method Validation

Validation is a process of proving or documenting, analytical method provides analytical data, for the intended use. There are many reasons for the need to validate analytical procedures. To assuring the Quality and achieving Quality control requirements, to achieve acceptance of the product by international agencies, mandatory require purpose for accreditation as per ISO 17025 guidelines. The Code of Federal Regulations (CFR) 311.165c explicitly states that “the specificity, sensitivity, recovery and reproducibility of test methods employed by the firm shall be established and documented”.

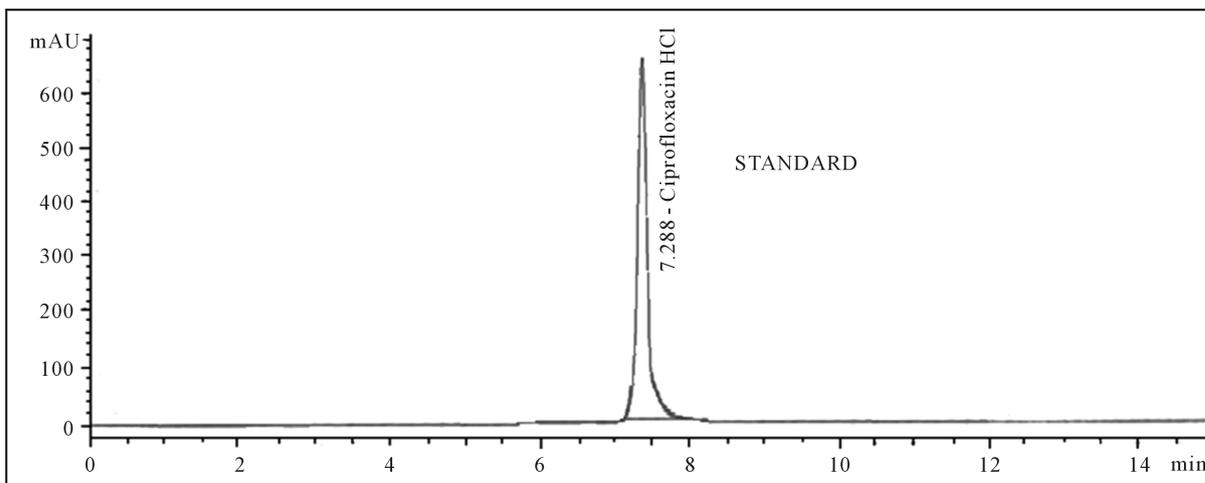
According to FDA guidance document, a stability-indicating method is “a validated quantitative analytical procedure that can detect the changes with time in the pertinent properties of the drug substances and finished



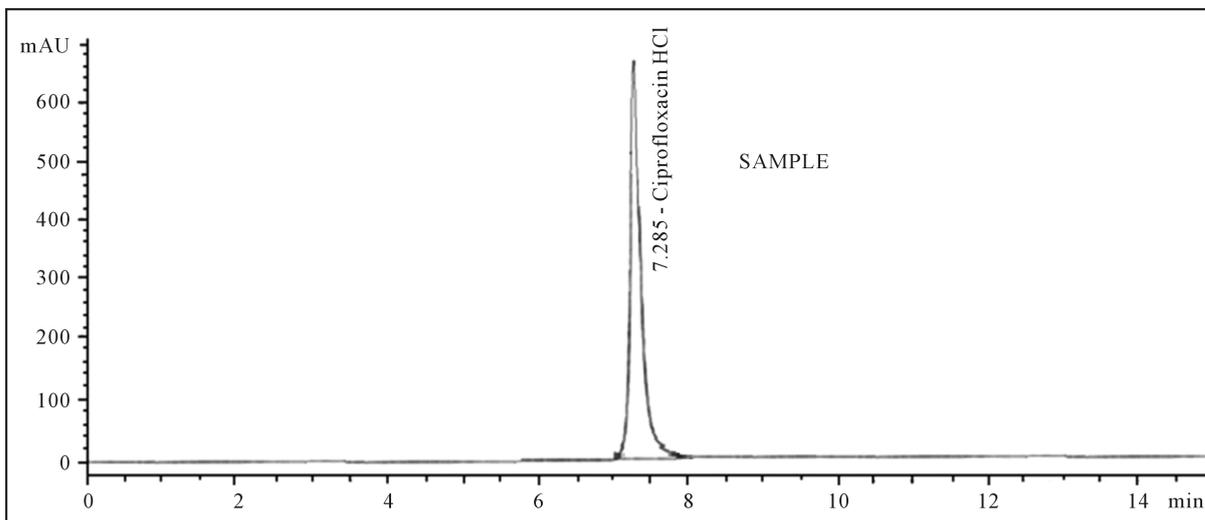
(a)



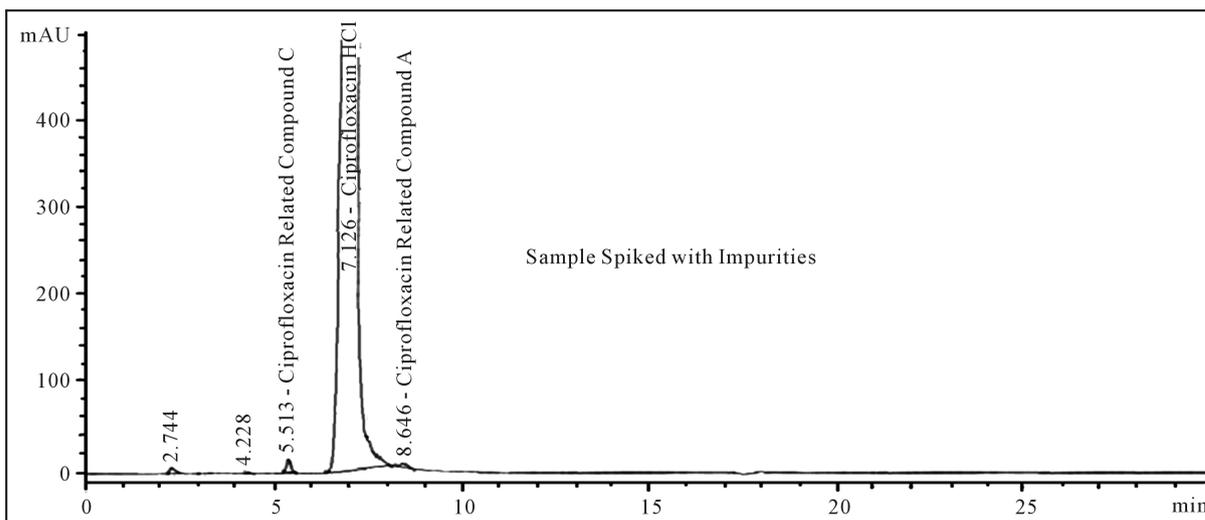
(b)



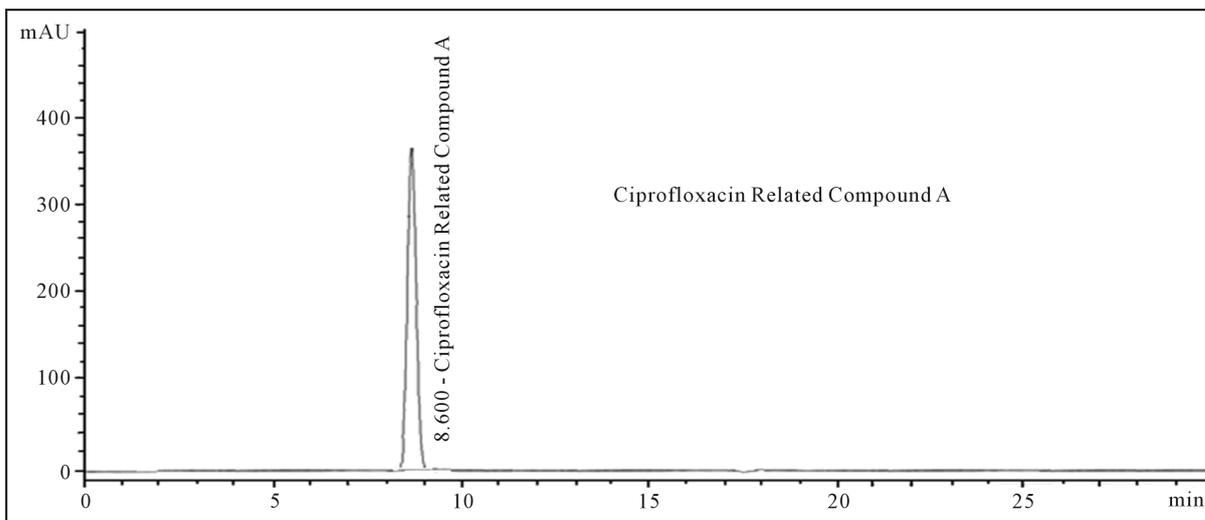
(c)



(d)



(e)



(f)

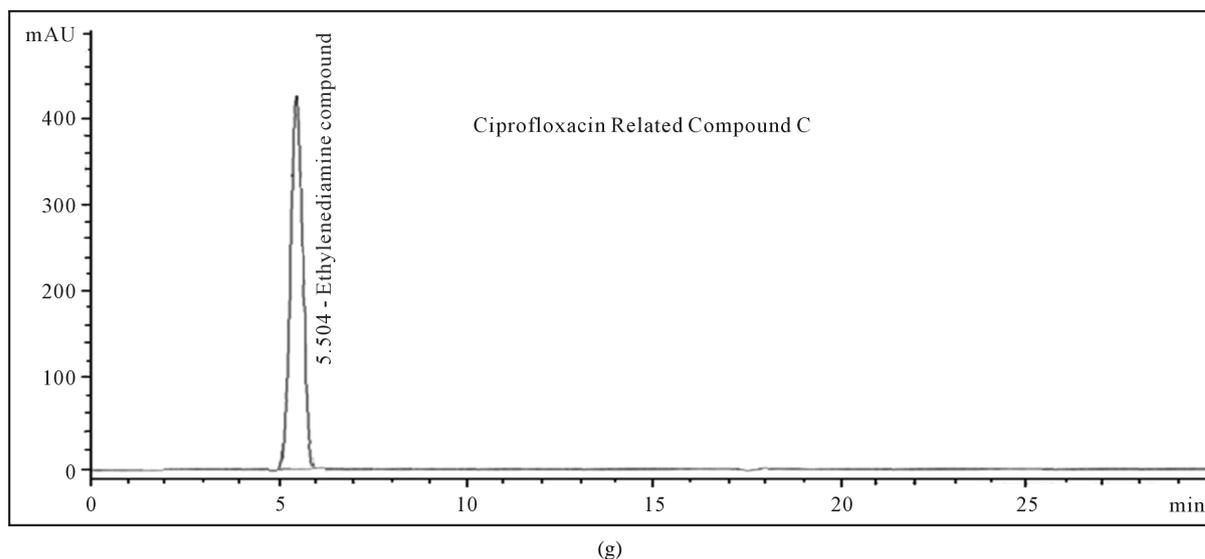


Figure 2. (a) A typical UV spectrum of ciprofloxacin hydrochloride; (b) A typical chromatogram of blank; (c) A typical chromatogram of standard; (d) A typical chromatogram of sample; (e) A typical chromatogram of sample spiked with impurities; (f) A typical chromatogram of ciprofloxacin related compound A; (g) A typical chromatogram of ethylenediamine compound.

product. A stability-indicating method measures accurately the active ingredients, without interference from the stressed products, process impurities, excipients or other potential impurities". Hence the stability-indicating method must be validated. The developed RP-HPLC assay method of ciprofloxacin hydrochloride drug substances was extensively validated as per ICH guidelines using the following parameters.

4.1. System Suitability

To verify that the analytical system is working properly and can give accurate and precise results, the system suitability parameters are to be checked by injecting the one injection of blank solution and five injections of standard solution into chromatographic system. Recorded chromatograms, checked system suitability. The Relative standard deviation of ciprofloxacin hydrochloride peak area for five injections of standard solution was 0.5%. The tailing factor and theoretical plates was 1.2 and 12150. From these results, it concluded that the system is suitable for analytical method validation.

4.2. Specificity

Specificity is the ability of analytical method to assess unequivocally the analyte in the presence of component that may be expected to be present, such as impurities, degradation products and matrix components. Performed the specificity parameter of the method by injecting diluent (Blank), standard and sample solution into the HPLC system, then recorded the retention time of each injected solution. Observed no interferences at diluent peak and also peak of ciprofloxacin hydrochloride was pure.

4.3. Stress Studies

Stress testing of drug substances can help to identify the likely degradation products, which can in turn help establish the degradation pathways and the intrinsic stability of the molecule. All stress decomposition studies were performed at a 100 ppm concentration of ciprofloxacin hydrochloride sample. Acid hydrolysis was performed in 0.1N and 1.0N Hydrochloric acid at 80°C for 4 hours. The study in basic condition was carried out in 0.1N and 1.0N sodium hydroxide solution at 80°C for 4 hours. For neutral stressed condition, drug substances dissolved in water and refluxed at 80°C for 4 hours, oxidation stressed study were carried out on water bath at 80°C for 4 hours in 3% hydrogen peroxide solution. Sample exposed to sun light for 8 hours, photo degradation studies were carried out by exposing the sample to UV light for 8 hours (254 nm and 356 nm), The drug sub-

stances was exposed to dry heat by keeping the sample in oven at 80°C for 6 hours. Sample were withdrawn at appropriate time and subjected to RP-HPLC analysis after suitable dilution (Table 2).

Diluent peaks were not interfering with ciprofloxacin hydrochloride peak. Degradation was observed in Acid, Alkali, Peroxide stressed conditions and sunlight exposed condition. The degradation products generated are well separated from ciprofloxacin hydrochloride peak. The ciprofloxacin hydrochloride peak is pure. Hence, the assay method was considered specific and stability indicating.

4.4. Precision of Test Method

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple sampling of homogeneous sample. The precision of analytical method is usually expressed as the standard deviation or relative standard deviation (Coefficient of variation) of series of measurements.

4.4.1. System Precision

The system precision is checked by using standard chemical substance to ensure that the analytical system is working properly. The retention time and area response of six determinations should be measured and calculate relative standard deviation. Then, the injected a blank solution of one injection and standard solution six injections into chromatograph. Recorded chromatograms, calculated the relative standard deviation of the retention time of ciprofloxacin hydrochloride peak at 7.2 minute is 0.5% and relative standard deviation of the area response of ciprofloxacin hydrochloride peak obtained from six injections of standard solution is 0.2%. Hence it was concluded that the system precision parameter meets the requirement of validation.

4.4.2. Intra-Day and Inter Day Precision

The precision of the assay method was evaluated by carrying out six times of same sample as per analytical procedure against qualified standard. The percentage of relative standard deviation of six assay values was calculated. Different analyst from the same laboratory evaluated the intermediate precision of the method (Table 3).

Table 2. The results table of stress study.

Conditions	Peak purity*	Assay in %	Purity factor
As such sample preparation	P	101.0	999.899
Stressed with 0.1N HCl at 80°C for 4 hours and neutralized with 0.1N NaOH	P	99.4	999.920
Stressed with 1.0N HCl at 80°C for 4 hours and neutralized with 1.0N NaOH	P	87.0	999.872
Stressed with 0.1N NaOH at 80°C for 4 hours and neutralized with 0.1N HCl	P	98.7	999.922
Stressed with 1.0N NaOH at 80°C for 4 hours and neutralized with 1.0N HCl	P	94.2	999.934
Stressed with 3% hydrogen peroxide at 80°C for 4 hours and neutralized with water	P	96.5	999.890
Exposed to sunlight for 8 hours and cooled	P	100.9	999.906
Exposed to UV light for 8 hours (254 nm and 356 nm)	P	96.0	999.930
Exposed to Dry heat at 80°C for 6 hours	P	90.5	999.937
Stressed with water at 80°C for 4 hours	P	96.0	999.931

Peak purity*: "p" indicates ciprofloxacin hydrochloride peak is pure, which conformed by diode array detector and Agilent chemstation software.

Table 3. The results table of precision.

Sr.No	% Assay	
	Intra-day precision	Inter-day precision
1	100.6	100.5
2	100.4	101.4
3	100.3	101.5
4	100.4	101.6
5	101.6	102.2
6	100.5	102.1
Average	100.6	101.6
% RSD	0.5%	0.6%

4.5. Stability in Analytical Solution

The stability in analytical solution is evaluated by injecting the standard and sample solution at regular intervals. The standard solution is stable up to 38 hours at Room temperature (25°C) with the percent difference 0.4% and sample solution is stable up to 35 hours at Room temperature (25°C) with the percent difference 1.7%.

4.6. Linearity

The linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range. Linearity test solution for the assay method were prepared from stock solution at seven different concentration levels (50%, 80%, 90%, 100%, 110%, 120% and 150%) of the target assay concentration of ciprofloxacin hydrochloride (100 ppm) and 10 µL of each solution was injected into the high pressure liquid chromatography system and recorded the peak area response of the chromatogram, also precision has been performed at lower and higher concentration levels. A graph was plotted to concentration in ppm on X-axis versus Peak area response on Y-axis. Calculated slope, intercept, correlation coefficient and regression coefficient (R square) for the linearity level solutions. Linearity graph is showed in **Figure 3**. Linearity results of the method are presented in **Table 4**.

From the statistical treatment of the linearity data of ciprofloxacin hydrochloride, it is clear that the response of ciprofloxacin HCl is linear between 50% to 150% level. The Correlation coefficient and regression coefficients were more than 0.998. In addition, the analysis of residuals show that the values are randomly scattered around zero, which shows good fit to the linear model. To evaluate whether the y-intercepts were significantly different from zero, the P-value was determined. The P value is > 0.05 then intercept is statistically equal to zero. For Ciprofloxacin hydrochloride P value is 0.20. Hence it is statistically equal to zero. In addition, the origin is within the lower and the upper limit of the 95% of Confidence interval, that gives high degree of confidence to the value obtained for intercept. Moreover, the value of the intercept is within the $\pm 2.0\%$ of the area response at 100% level.

4.7. Accuracy

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value (Standard value). The study of recovery of ciprofloxacin hydrochloride was evaluated in triplicate at three concentration levels, *i.e.* 50%, 100% and 150% of working concentration (100 ppm) of the sample. The percentage of recoveries were calculated and found it be as within the limits (**Table 5**).

4.8. Range

The range of Analytical Method is the interval between the upper and lower levels of analyte that has been

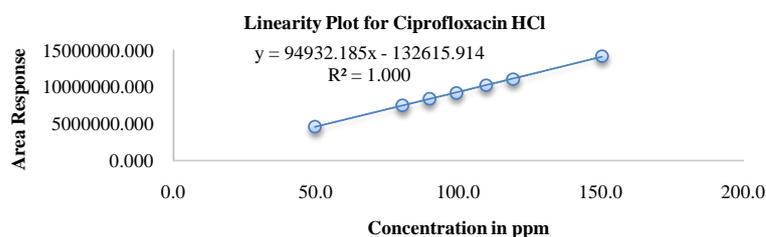


Figure 3. Linearity graph.

Table 4. The results table of Linearity.

% Level	Concentration in ppm	Area response
50	49.6598	4628113.962
80	80.4016	7523524.857
90	89.8606	8408472.042
100	99.3197	9213073.152
110	109.7246	10275208.117
120	119.1836	11106062.170
150	150.3983	14231940.974
Correlation coefficient		1.000
Regression coefficient		1.000
Slope		94932.185
Intercept		-132615.9141
% Intercept		-1.4

Table 5. The results table of accuracy.

Sr.No.	Levels	Mean mg Added	Mean mg Recovered	Mean % Recovery	% RSD
1	50%	5.7091	5.7648	101.0	0.3
2	100%	11.3061	11.4258	101.1	0.5
3	150%	16.9937	17.2446	101.5	0.2

demonstrated to be determined with a suitable Accuracy and Linearity. Derived the specified ranges from accuracy and linearity studies and observed results are presented in **Table 6** and **Table 7**. Linearity range graph and accuracy range graph is showed in **Figure 4** and **Figure 5**.

4.9. Robustness

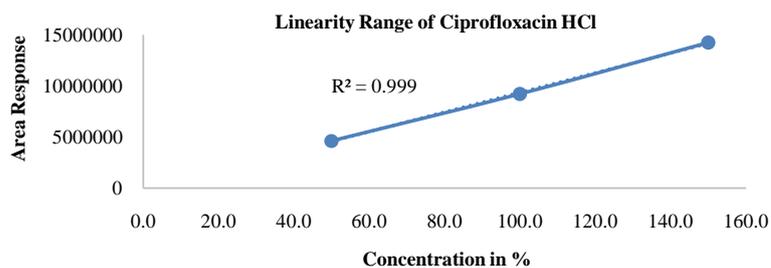
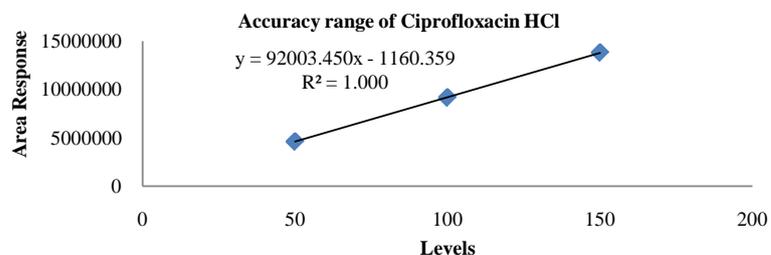
The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. To determine the robustness of the method the experimental conditions were deliberately altered by change in column temperature $\pm 5^\circ\text{C}$, change in flow rate ± 0.2 mL/min and change in mobile phase pH ± 0.2 units. The system suitability parameters are passed for all the conditions and the results for assay were evaluated.

Table 6. The results table of Accuracy range.

Accuracy range	
Level (Concentration in %)	Mean area response
50	4620089.731
100	9157029.499
150	13820434.750
Correlation Coefficient	1.000
RSD	0.3%

Table 7. The results table of Linearity range.

Linearity range	
Level (Concentration in %)	Mean area response
50	4628113.962
100	9213073.152
150	14231940.97
Correlation Coefficient	1.000

**Figure 4.** Linearity range graph.**Figure 5.** Accuracy range graph.

5. Conclusion

The proposed reversed phase high pressure liquid chromatographic method with a novel gradient mode was found to be a simple, specific, linear, precise, robust and accurate. The method was completely validated showing satisfactory data for all the method validation parameters tested. The developed method is stability indicative and can be used for quantitative determinations of the Ciprofloxacin hydrochloride in presence of degradation products in stability by the Pharmaceutical industries.

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