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METHOD DEVELOPMENT AND VALIDATION FOR THE DETERMINATION OF PRULIFLOXACIN IN BULK FORM AND TABLET DOSAGE FORM BY RP-HPLC

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ABSTRACT
A new, economical, simple, rapid, precise, accurate and reproducible RP-HPLC method for determination of Prulifloxacin in bulk form and marketed pharmaceutical formulation. Separation of Prulifloxacin was successfully achieved on a Symmetry ODS (C18) RP Column, 250 mm x 4.6 mm, 5µm column in an isocratic mode of separation utilizing Phosphate Buffer: Methanol in the ratio of 46:54% v/v (pH-3.2) at a flow rate of 1.0mL/min and the detection was carried out at 206nm. The method was validated according to ICH guidelines for linearity, sensitivity, accuracy, precision, specificity and robustness. The response was found to be linear in the drug concentration range of 0-140mcg/mL for Prulifloxacin. The correlation coefficient was found to be 0.9993 for Prulifloxacin. The LOD and LOQ for Prulifloxacin were found to be 0.08µg/mL and 0.24µg/mL respectively. The proposed method was found to be good percentage recovery for Prulifloxacin, which indicates that the proposed method is highly accurate. The specificity of the method shows good correlation between retention times of standard solution with the sample solution. Therefore, the proposed method specifically determines the analyte in the sample without interference from excipients of pharmaceutical dosage forms.

KEYWORDS: Prulifloxacin, RP-HPLC, Accuracy, Precision, Linearity, ICH Guidelines.

INTRODUCTION
Prulifloxacin is an older synthetic antibiotic of the fluoroquinolone class undergoing clinical trials prior to a possible NDA (New Drug Application) submission to the U.S. Food and Drug Administration (FDA). It is a prodrug which is metabolized in the body to the active compound Ulifloxacin.[1] It was developed over two decades ago by Nippon Shinyaku Co. and was patented in Japan in 1987 and in the United States in 1989. It has been approved for the treatment of uncomplicated and complicated urinary tract infections, community-acquired respiratory tract infections in Italy and gastroenteritis, including infectious diarrheas, in Japan. Prulifloxacin has not been approved for use in the United States. Prulifloxacin is a quinolone antibiotic and a fluoroquinolone antibiotic. Prulifloxacin has been investigated for the treatment of Urinary Tract Infection. Prulifloxacin, the prodrug of Prulifloxacin, is a broad-spectrum oral fluoroquinolone antibacterial agent. After absorption, Prulifloxacin is metabolized by esterases to Prulifloxacin. The drug has a long elimination half-life, allowing once-daily administration.[2] Like other fluoroquinolones, Prulifloxacin prevents bacterial DNA replication, transcription, repair and recombination through inhibition of bacterial DNA gyrase. Prulifloxacin is used in the treatment of urinary tract infections (UTIs). It may also be used in infections of tonsils, sinus, nose, throat, female genital organ, skin & soft tissues and lungs (pneumonia). Prulifloxacin is an antibiotic. It works by stopping the action of a bacterial enzyme called DNA-gyrase.[3] The IUPAC name of Prulifloxacin is 6-fluoro-1-methyl-7-[4-[(5-methyl-2-oxo-1, 3-dioxol-4-yl) methyl] piperazin-1-yl]-4-oxo-1H-[1, 3] thiazeto [3, 2-a] quinoline-3-carboxylic acid. The Chemical Structure of Prulifloxacin is shown in following figure-1.

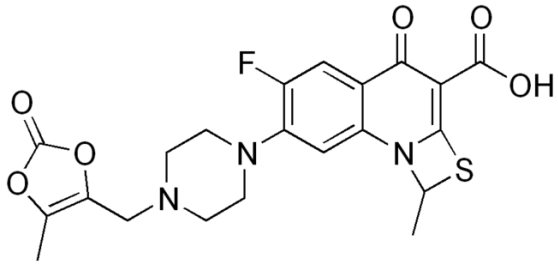

Fig-1: Chemical Structure of Prulifloxacin.

EXPERIMENTAL

Table 1: List of Instrument Used.

S. No.	Instruments/Equipments/Apparatus
1.	**HPLC** with Empower2 Software with Isocratic with UV-Visible Detector (Waters).
2.	**T60-LAB INDIA** UV – Vis spectrophotometer
3.	Electronic Balance **(SHIMADZU ATY224)**
4.	Ultra Sonicator **(Wensar wuc-2L)**
5.	Thermal Oven
6.	Symmetry ODS RP C_{18},5µm, 15mm x 4.6mm i.d.
7.	P^{H} Analyzer **(ELICO)**
8.	Vacuum filtration kit **(BOROSIL)**

Table 2: List of Chemicals Used.

S.No.	Name	Specifications		Manufacturer/Supplier
		Purity	Grade	
1.	Doubled distilled water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
2.	HPLC Grade Water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
3.	Methanol	99.9%	HPLC	Loba Chem; Mumbai.
4.	Hydrochloric Acid	99.9	A.R.	Sd fine-Chem ltd; Mumbai
5.	Acetonitrile	99.9%	HPLC	Loba Chem; Mumbai.
6.	Sodium Hydroxide	99.9	A.R.	Sd fine-Chem ltd; Mumbai
7.	Ethanol	99.9	A.R.	Sd fine-Chem ltd; Mumbai
8.	Octanol	99.9	A.R.	Sd fine-Chem ltd; Mumbai

Method Development

Selection of Wavelength

The Standard & Sample Stock Solutions were prepared separately by dissolving standard & sample in a solvent in mobile phase diluting with the same solvent. (After optimization of all conditions) for UV analysis. It scanned in the UV spectrum in the range of 200 to 400nm. This has been performed to know the maxima of Prulifloxacin, so that the same wave number can be utilized in HPLC UV detector for estimating the Prulifloxacin.[4] The scanned UV spectrum is attached in the following page.

Optimization of Chromatographic Conditions: The chromatographic conditions were optimized by different means. (Using different column, different mobile phase, different flow rate, different detection wavelength & different diluents for sample preparation etc.

Table 3: Summary of Process Optimization.

Column Used	Mobile Phase	Flow Rate	Wave length	Observation	Result
Symmetry ODS (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm	Methanol : Acetonitrile = 20 : 80	0.80ml/min	206nm	Very Low response	Method rejected
Symmetry ODS (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm	Methanol : Water = 70 : 30	0.9ml/min	206nm	Low response	Method rejected
Symmetry ODS (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm	Acetonitrile: Water = 50 : 50	1.0ml/min	206nm	Tailing peaks	Method rejected
Symmetry ODS (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm	Phosphate Buffer : Acetonitrile = 85:15 (pH-4.8)	1.0ml/min	206nm	Resolution was not good	Method rejected
Symmetry ODS (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm	Phosphate Buffer : Methanol = 65:35	1.0ml/min	206nm	Tailing peak	Method rejected

	(pH-4.0)				
Symmetry ODS (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm	Phosphate Buffer : Methanol = 46:54 (pH-3.2)	1.0ml/min	206nm	Nice peak	Method accepted

Preparation of 0.01M Potassium Dihydrogen Orthophosphate Solution

About 1.36086grams of Potassium dihydrogen orthophosphate was weighed and transferred into a 1000ml beaker, dissolved and diluted to 1000ml with HPLC Grade water.[5] The pH was adjusted to 3.20 with diluted orthophosphoric acid.

Preparation of Mobile Phase

460ml of Phosphate buffer (0.05M) pH 3.20 and 540ml of HPLC Grade Methanol were mixed well and degassed in ultrasonic water bath for 15 minutes.[6] The solution was filtered through 0.45 µm filter under vacuum filtration.

RESULTS AND DISCUSSION

Analytical Method Development

Selection of Wavelength

Sample & Standard Preparation for the UV-Spectrophotometer Analysis

25 mg of Prulifloxacin standard was transferred into 25 ml volumetric flask, dissolved & make up to volume with mobile phase. Further dilution was done by transferring 0.5 ml of the above solution into a 10ml volumetric flask and make up to volume with mobile phase.

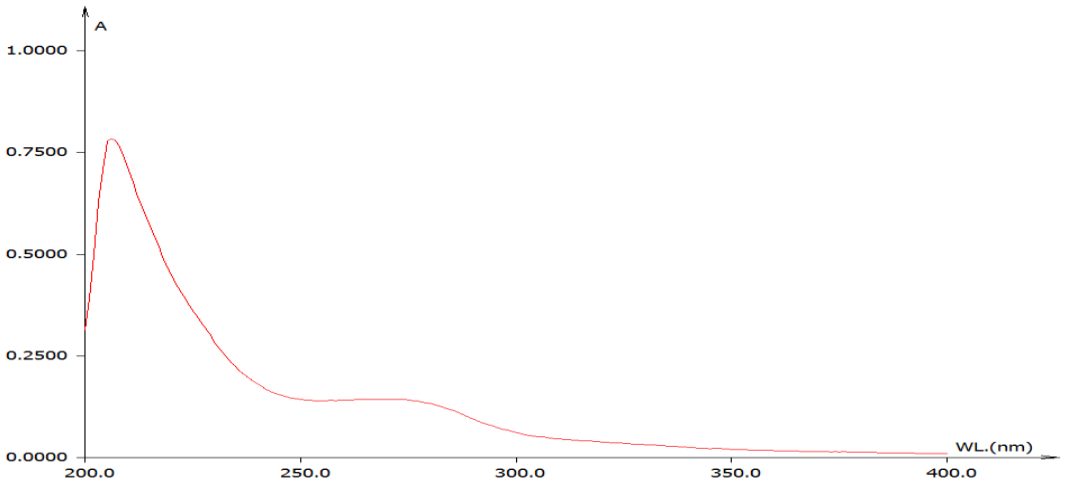

Fig-2: UV Spectrum for Prulifloxacin (206nm)

Observation: While scanning the Prulifloxacin solution we observed the maxima at 206nm.

Summary of Optimized Chromatographic Conditions

The Optimum Chromatographic conditions obtained from experiments can be summarized as below.

Table 4: Summary of Optimised Chromatographic Conditions.

Mobile phase	Phosphate Buffer : Methanol = 46:54 (pH-3.2)
Column	Symmetry ODS (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm
Column Temperature	Ambient
Detection Wavelength	206 nm
Flow rate	1.0 ml/ min.
Run time	08 min.
Temperature of Auto sampler	Ambient
Diluent	Mobile Phase
Injection Volume	20µl
Type of Elution	Isocratic
Retention time	3.523 minutes

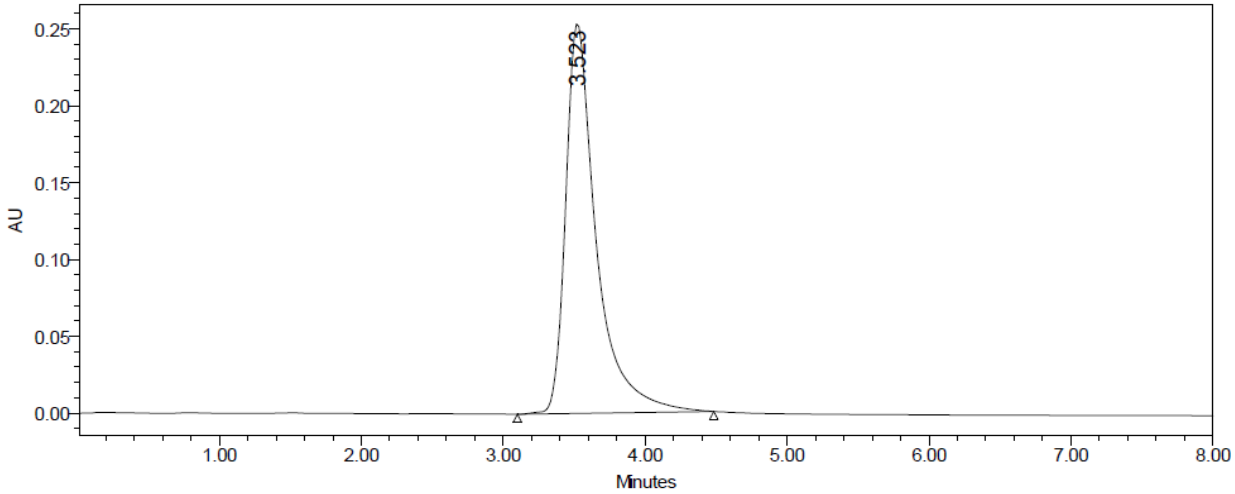

Fig-3: Chromatogram of Prulifloxacin in Optimized Condition.

Table 5: Peak Results of Optimized Condition.

Drug Name	RT	Peak Area	Tailing Factor	Plate Count
Prulifloxacin	3.523	4893218	1.27	2995

Observation: The selected and optimized mobile phase was Phosphate Buffer: Methanol = 46:54% v/v (pH-3.2) and conditions optimized were flow rate (1.0 ml/minute), wavelength (206nm), Run time was 08 mins. Here the peaks were separated and showed better resolution, theoretical plate count and symmetry.[7] The proposed chromatographic conditions were found appropriate for the quantitative determination of the drug.

Analytical Method Validation

Validation of the optimized method was performed according to the ICH Q2 (R) guidelines.[11,12,25,30]

1. System Suitability Parameter

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analysed constitute an integral system that can be evaluated as such. Following system suitability test parameters were established.[8-9] The data are shown in Table-6.

Table 6: Data of System Suitability Parameter.

S. No.	Parameter	Limit	Result
1	Resolution	Rs > 2	9.34
2	Asymmetry	T ≤ 2	Prulifloxacin=0.16
3	Theoretical plate	N > 2000	Prulifloxacin=3065
4	Tailing Factor	T<2	Prulifloxacin=1.55

2. Linearity & Range

The calibration curve showed good linearity in the range of 0 – 140 µg/ml, for Prulifloxacin (API) with correlation coefficient (r^2) of 0.999 (Fig-4). A typical calibration curve has the regression equation of y = 48313x + 71968 for Prulifloxacin.[10-11]

Table-7: Results of Linearity.

CONC.(µg/ml)	MEAN AUC (n=6)
0ppm	0
60ppm	3059294
80ppm	3979280
100ppm	4919463
120ppm	5859590
140ppm	6770480

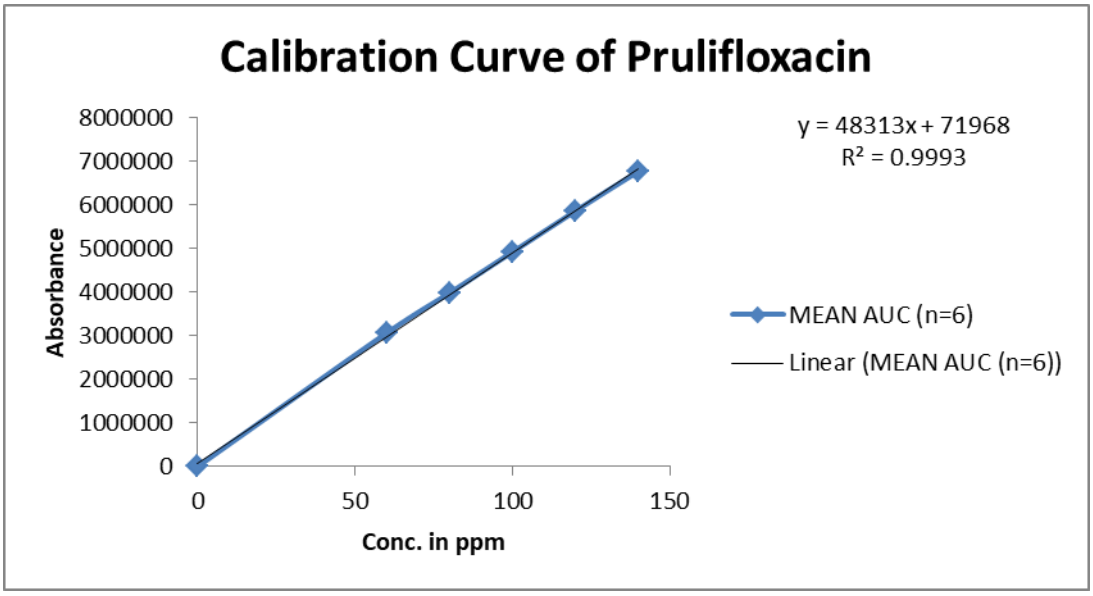

Fig-4: Calibration Curve of Prulifloxacin (API).

3. Accuracy

Recovery Study: To decide the exactness of the proposed strategy, recuperation contemplates were completed by including diverse sums (80%, 100%, and 120%) of unadulterated medication of PRULIFLOXACIN were taken and added to the pre-examined plan of fixation 100µg/ml. From that rate recuperation esteems were figured.[12-14] The outcomes were appeared in table-8.

Table 8: Readings of Accuracy.

Conc. In ppm	Conc. Found	Peak Area		% Recovery
80	80.461	3959294		100.576
80	80.095	3941634		100.118
80	80.194	3946409		100.242
			Avg.	100.312
			S.D	0.236888
			%RSD	0.236151
Conc. In ppm	**Conc. Found**	**Peak Area**		% Recovery
100	100.932	4948323		100.932
100	99.879	4897463		99.879
100	100.030	4904741		100.030
			Avg.	100.2803
			S.D	0.569388
			%RSD	0.567796
Conc. In ppm	**Conc. Found**	**Peak Area**		% Recovery
120	120.019	5870480		100.015
120	119.907	5865040		99.922
120	119.794	5859590		99.828
			Avg.	99.92167
			S.D	0.0935
			%RSD	0.093574

4. Precision

4.1. Repeatability

The precision of each method was ascertained separately from the peak areas & retention times obtained by actual determination of six replicates of a fixed amount of drug. Prulifloxacin (API).[15-17] The percent relative standard deviation was calculated for Prulifloxacin are presented in the table-9.

Table 9: Readings of Repeatability.

HPLC Injection Replicates of Prulifloxacin	Retention Time (Minutes)	Peak Area
Replicate – 1	3.639	3948323
Replicate – 2	3.622	3935751
Replicate – 3	3.575	3979135
Replicate – 4	3.525	3971013
Replicate – 5	3.526	3919463
Replicate – 6	3.523	3974741
Average		**3954738**
Standard Deviation		**24108.89**
% RSD		**0.609621**

4.2. Intermediate Precision

4.2.1. Intra-Assay & Inter-Assay

The intra & inter day variation of the method was carried out & the high values of mean assay & low values of standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Prulifloxacin revealed that the proposed method is precise.[18]

Table 10: Results of Intra-Assay & Inter-Assay.

Conc. of Prulifloxacin (API) (µg/ml)	Observed Conc. of Prulifloxacin (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
80	79.35	0.88	80.36	0.56
100	100.57	0.65	99.86	0.36
120	119.87	0.93	120.18	0.87

5. Specificity

Specificity can be determined by comparing the chromatograms obtained from the drugs with the chromatogram obtained from the blank solution. Blank solution was prepared by mixing the excipients in the mobile phase without drug. Drug solutions were prepared individually and the sample containing one drug was also prepared.[19] Now these mixtures were filtered by passing through 0.45 µ membrane filter before the analysis. In this observation no excipient peaks were obtained near the drug in the study run time. This indicates that the proposed method was specific.[20]

The chromatograms representing the peaks of blank, Prulifloxacin and the sample containing the one drug was shown in following figures respectively.

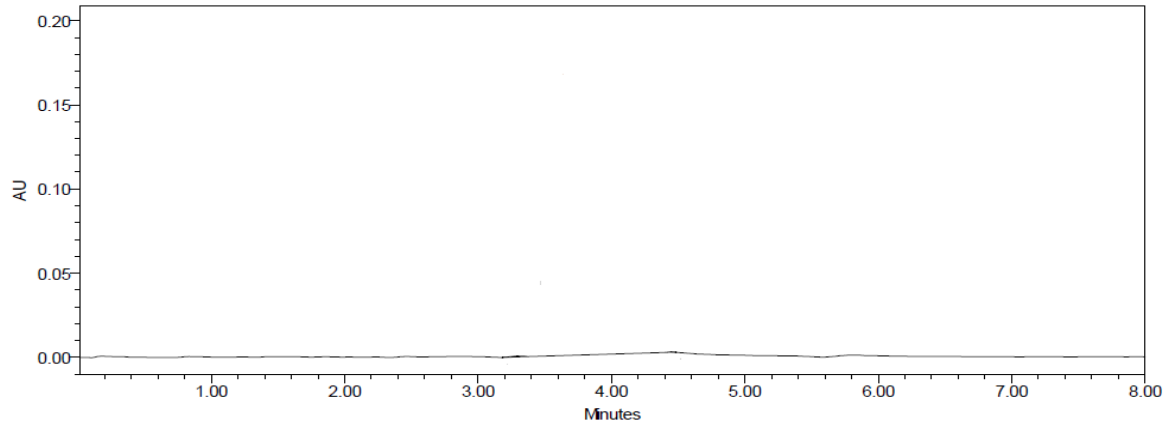

Fig-5: Chromatogram for Blank Solution.

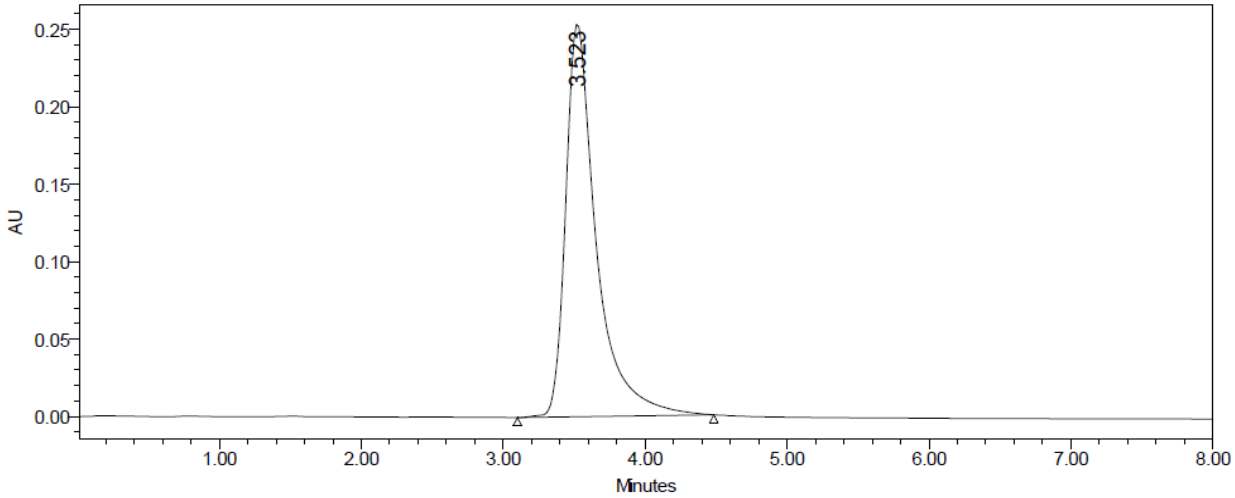

Fig-6: Standard Solution of Prulifloxacin.

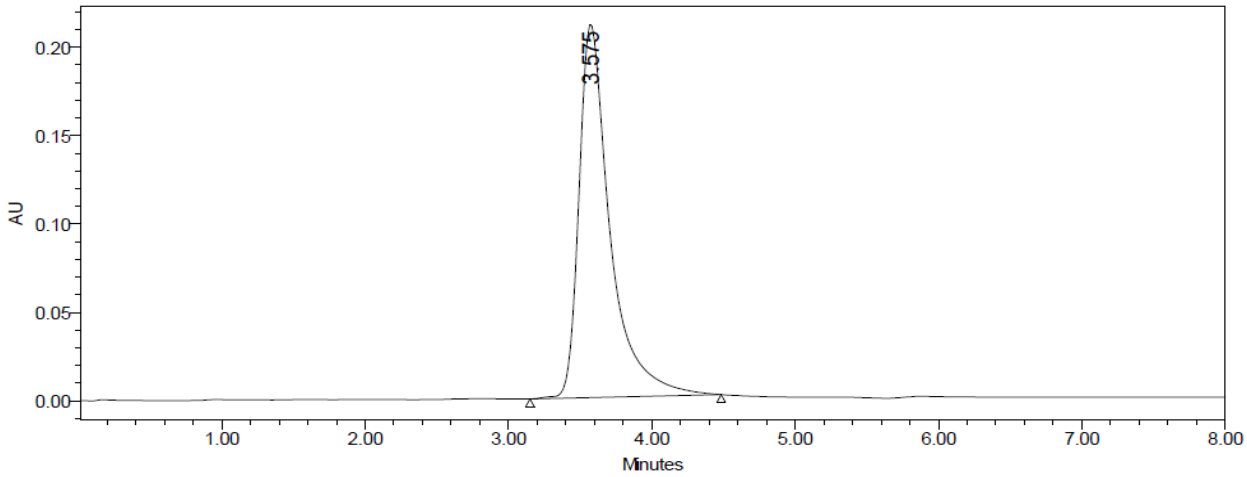

Fig-7: Sample Solution of Prulifloxacin.

OBSERVATION

In this test method blank, standard solutions were analyzed individually to examine the interference. The above chromatograms show that the active ingredient was well separated from blank and their excipients and there was no interference of blank with the principal peak. Hence the method is specific.

6. LOD & LOQ

The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 0.08 & 0.24µg/ml respectively.[21-23]

7. Method Robustness

Impact of little changes in chromatographic conditions, for example, change in Flow rate (± 0.1ml/min), Wavelength of location (±2nm) and organic phase content in mobile phase (±5%) concentrated to decide the Robustness of the technique are additionally for (Table-11, % RSD < 2%) the created RP-HPLC strategy for the examination of Prulifloxacin (API).[24-26]

Table 11: Result of Method Robustness Test.

Change in Parameter	% RSD
Flow (1.1 ml/min)	0.61
Flow (0.9 ml/min)	0.75
More Organic	0.69
Less Organic	0.81
Wavelength of Detection (208 nm)	0.89
Wavelength of detection (204 nm)	0.99

8. Estimation of Prulifloxacin in Pharmaceutical Dosage Form

Label claim: 600mg

Each tablet contains: 600 mg

Twenty pharmaceutical dosage forms were taken and the I.P. technique was taken after to decide the normal weight. Above measured tablets were at long last powdered and triturated well. An amount of powder comparable to 25 mg of medications were exchanged to 25 ml volumetric jar, make and arrangement was sonicated for 15 minutes, there after volume was made up to 25 ml with same dissolvable. At that point 10 ml of the above arrangement was weakened to 100 ml with

mobile phase. The arrangement was separated through a film channel (0.45 µm) and sonicated to degas.[27] The arrangement arranged was infused in five repeats into the HPLC framework and the perceptions were recorded.

A copy infusion of the standard arrangement was likewise infused into the HPLC framework and the pinnacle zones were recorded. The information is appeared in Table-12.

ASSAY

$$\text{Assay \%} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \text{Avg. Wt} = \text{mg/tab}$$

Where:
AT = Peak Area of medication acquired with test readiness
AS = Peak Area of medication acquired with standard readiness
WS = Weight of working standard taken in mg
WT = Weight of test taken in mg
DS = Dilution of Standard arrangement
DT = Dilution of test arrangement
P = Percentage virtue of working standard

Table 12: Recovery Data for Estimation Prulifloxacin in Punox 600 Tablet.

Brand name of Prulifloxacin	Labelled amount of Drug (mg)	Mean (± SD) amount (mg) found by the proposed method (n=6)	Assay % (± SD)
Punox 600 Tablet (Dr. Reddy's Laboratories Ltd)	600mg	599.395 (± 0.278)	99.574 (± 0.694)

RESULT AND DISCUSSION

The amount of drug in Punox 600 Tablet was found to be 599.395 (± 0.278) mg/tab for Prulifloxacin & % assay[28] was 99.574 %.

Stability Studies

Results of Stability Studies: The results of the stress studies indicated the **specificity** of the method that has been developed.[29-30] Prulifloxacin was stable in acidic and photolytic stress conditions.

Table 13: Results of Forced Degradation Studies of Prulifloxacin API.

Stress Condition	Time	Assay of Active Substance	Assay of Degraded Products	Mass Balance (%)
Acid Hydrolysis (0.1 M HCl)	24Hrs.	97.32	2.68	100.0
Basic Hydrolysis (0.1M NaOH)	24Hrs.	87.64	12.36	100.0
Thermal Degradation (50 ^{0}C)	24Hrs.	88.65	11.35	100.0
UV (254nm)	24Hrs.	93.42	6.58	100.0
3 % Hydrogen Peroxide	24Hrs.	91.04	8.96	100.0

SUMMARY

To develop a precise, linear, specific & suitable stability indicating RP-HPLC method for analysis of Prulifloxacin, different chromatographic conditions were applied & the results observed are presented in previous chapters. Isocratic elution is simple, requires only one pump & flat baseline separation for easy and reproducible results. So, it was preferred for the current study over gradient elution. In case of RP-HPLC various columns are available, but here Symmetry ODS (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm column was preferred because using this column peak shape, resolution and absorbance were good. Detection wavelength was selected after scanning the standard solution of drug over 200 to 400nm. From the U.V spectrum of Prulifloxacin it is evident that most of the HPLC work can be accomplished in the wavelength range of 206 nm conveniently. Further, a flow rate of 1.0 ml/min & an injection volume of 10µl were found to be the best analysis. The result shows the developed method is yet another suitable method for assay and stability related impurity studies which can help in the analysis of Prulifloxacin in different formulations.

CONCLUSION

A sensitive & selective stability indicting RP-HPLC method has been developed & validated for the analysis of Prulifloxacin in bulk and pharmaceutical dosage form. Based on peak purity results, obtained from the analysis of samples using described method, it can be concluded that the absence of co-eluting peak along with the main peak of Prulifloxacin indicated that the developed method is specific for the simultaneous estimation of Prulifloxacin in the bulk and pharmaceutical dosage forms. Further the proposed RP-HPLC method has excellent sensitivity, precision and reproducibility. The specific Retention time for Prulifloxacin are found to be 3.523min. The tailing factor was found to be 1.27 with theoretical plates 2995 for Prulifloxacin. The %Recoveries was determined as 100.171% for

Prulifloxacin in Accuracy. The %RSD in Repeatability is 0.609 with Intermediate Precision (Intra & Inter Day) are 0.820 & 0.596 for Prulifloxacin in Precision respectively. In Linearity, the correlation coefficient was found to be 0.9993 for Prulifloxacin. The LOD for Prulifloxacin was 0.08 and LOQ for Prulifloxacin are 0.24 respectively.

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