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Research Article

# RP-HPLC Method Development and Validation of Pazufloxacin in their Bulk and formulation

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**Abstract**

The present work was the development of a simple, efficient, and reproducible reverse-phase high performance liquid chromatographic (RP-HPLC) method for determination of Pazufloxacin (PFX) in bulk and its injection dosage form. The solvent system and wavelength were optimized in order to maximize the sensitivity of the proposed method and detection wavelength was carried out at 249 nm. The separation was achieved on HPLC binary gradient system equipped with HPLC 3000 series UV detector and Agilent Zorbax column C18 (4.6mm×50mm×5µm). The optimized mobile phase composition was methanol: phosphate buffer pH 4 (50:50%v/v). The separation of PFX was carried out on Kromasil C-18 (250 × 4.6 mm×5 $\mu$m) column using phosphate buffer pH 4 and methanol by linear gradient program. Flow rate was 1.0 ml/min with a column temperature of 30°C. The method was validated in terms of accuracy, precision, linearity, LOD & LOQ of sample solution as per ICH guidelines. Linearity was observed in the concentration range of 5-25 µg/ml & gave mean correlation coefficient 0.998. The developed RP-HPLC method was found to be accurate, precise and was successful applied to a Pazufloxacin bulk powder and its marketed formulation for qualitative estimation of Pazufloxacin.

**Keywords:** Pazufloxacin, RP-HPLC, Methanol, Phosphate buffer, Validation, ICH guidelines

## Introduction

RP-HPLC involves the separation of molecules on the basis of hydrophobicity. Excellent resolution that can be achieved under a wide range of chromatographic conditions. Chromatographic selectivity can be manipulated through changes in mobile phase characteristics. High recoveries and high productivity. Excellent reproducibility of repetitive separations carried out over a long period of time.

Pazufloxacin(-)-(S)-10-(1-aminocyclopropyl)-9-fluoro-3-methyl-7-oxo-2,3-dihydro-pyrido[2,3-de][1,4]benzoxazine-6-carboxylic acid monomethanesulfonate[1-2] (Figure 1). Pazufloxacin is a fused tricyclic quinolone derivative with a 1-aminocyclopropyl substituent at C-10 position. The presence of aminoacyl group at C-10 is a unique feature of the molecule imparting potent broad spectrum activity against gram-positive and gram-negative bacteria including variety of resistant strains and anaerobic bacteria[3-6].

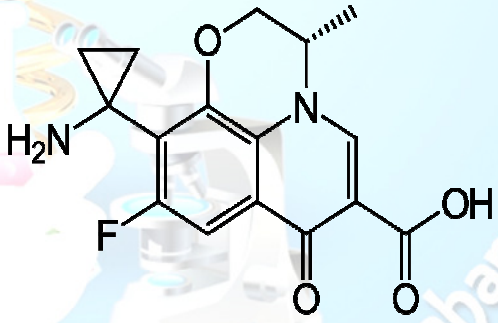



**Figure 1:** Chemical structure of Pazufloxacin

This drug has good *in vitro* and *in vivo* activity against a broad range of bacteria, especially Gram-negative bacteria[7-8]. Clinical trials showed its intravenous injection formula was effective in treating respiratory infections[9].

Pazufloxacin is not yet the subject of a monograph

in any pharmacopoeia. Reviewing the literature revealed that, few methods have been reported for the determination of pazufloxacin in raw material, pharmaceutical formulation and/or human plasma. These methods include spectrophotometric methods[10-13], spectroflurimetric methods[14-16], electrochemical method[17], capillary electrophoresis[18-20], HPLC methods[21-25]. Also there is some reported stability indicating method for the determination of pazufloxacin in pharmaceutical formulation and in presence of its degradation products[26].

Only limited analytical methods have been reported for the determination of Pazufloxacin in their bulk and dosage form which includes HPLC, LC-MS and LC-ESI-MS. While not a single method reported using RP-HPLC method for the analysis of same. Based on literature study, our aim was to develop and validate simple, precise and accurate RP-HPLC method for the quantitative estimation of Pazufloxacin in bulk and marketed injection formulation.

**Material and Methods**

The pure drug Pazufloxacin was obtained from reputed firm with the certificate of analysis. Pazufloxacin (IV) injection (Pezflow, 500 mg/100 ml; CIPLA Ltd., Mumbai, India) were purchased from Local market, Ahmedabad, India. HPLC grade methanol was purchased from S.D.Fine Chemicals, Mumbai, India. Standard and sample solution was prepared in diluents of methanol: phosphate buffer pH 4 (50:50) %v/v. All others chemicals/reagents used were of HPLC grade. The HPLC system used was HPLC binary gradient system equipped with HPLC3000 series, UV detector and Agilent Zorbax column C18 (4.6×50mm×5µm). Analytical balance of SHIMADZU (ATY 64) and ScienTech (SE-366) Sonicator were used in study.

**Preparation of standard stock solution:**

An accurately weigh amount of 10mg of Pazufloxacin was transferred to volumetric flask of 10 ml volume. Small amount of methanol was added to dissolve the sample and further the volume made using methanol: phosphate buffer (pH 4) (50:50% v/v). The resultant solution was filtered using Whatman filter paper to remove the undissolved matter. From this stock solution, series of aliquots were pipette out and placed into 10 ml of volumetric flask. The volume was made upto mark with mobile phase to give a desired concentration of solution (5-50 µg/ml). Further the absorbance of resulting solution was determined at 249 nm.

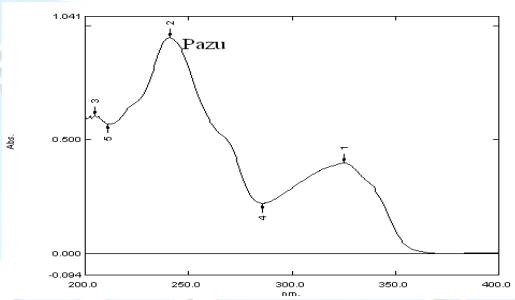



**Figure 2: UV spectra of Pazufloxacin (Selected wavelength; 249 nm)**

**Chromatographic conditions**

The mobile phase used was prepared by mixing methanol and phosphate buffer pH 4 (50:50 %v/v). The mobile phases were filtered by vacuum filtration through 0.45 µm filter and degassed by ultrasound sonication for 30 minutes prior to study. The column was equilibrated with the mobile phase. Chromatographic separation was performed on a reverse phase Hexon C8 shield. The mobile phase was a mixture of methanol and water (80:20 % v/v). The flow rate of the mobile phase was adjusted to 1.0 ml /min. The detection was carried out at wavelength 249 nm (Figure 2). The injection volume of the standard and sample solution was set at 20 µl.

**Analysis of Marketed Formulation:**

The developed method was applied to the assay of Pazufloxacin in IV injection (Pezflow Injection, 500mg/100ml; Cipla Ltd.). The average percent recoveries of different concentrations were calculated. It was calculated on the average of three replicate determinations. The accuracy of the proposed methods was assessed by applying the standard addition technique. Known amounts of the drug were added to the pharmaceutical product. The procedure stated under linearity was then applied. The concentrations, standard deviations and relative standard deviation were calculated for each added concentration.

**Method validation**

The method was validated for linearity, accuracy,

precision and limit of detection, and limit and quantitation.

**Linearity:** The linearity of response for Pazufloxacin was determined in the range of 5-25 µg/ ml for standard drug for HPLC methods.

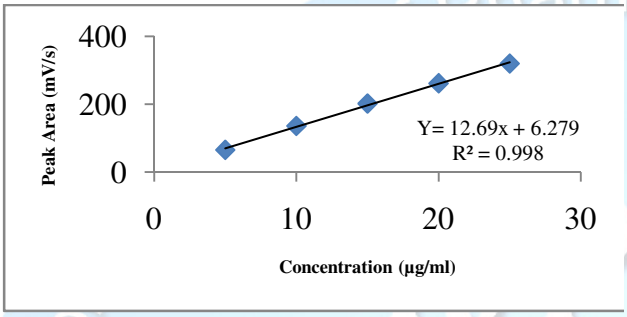



**Figure 3: Calibration curve of Pazufloxacin (5-25 µg/ml)**

**Accuracy:** The accuracy of the analytical method was assessed by determination of the recovery of the method at 3 different concentrations (50%, 100% and 150% concentration) by addition of known amount of standard to the placebo. For each concentration three sets were prepared. To test the validity of the method it was applied to the determination of pure samples of pazufloxacin over the concentration range of 5-50 µg ml-1 for HPLC method. It was expressed as percent recovery [mean back-calculated concentration/theoretical concentration×100]

**Precision:** The instrument precision was evaluated by determining the absorbance of the standard solution six times repeatedly. The results are reported in terms of relative standard deviation. The intra-day precision was evaluated through replicate analysis of three concentrations of pazufloxacin in pure form on three successive times. The inter-day precision was also evaluated through replicate analysis of three concentrations for a period of three successive days. The precision of the methods was expressed in terms of S.D. and CV%.

**Limit of detection and limit of quantitation:** The limit of detection (LOD) and limit of quantitation (LOQ) of the Pazufloxacin by the proposed method was determined using calibration standards close to the expected LOD and LOQ. LOD and LOQ were calculated as 3.3 σ/s and 10 σ/s, respectively, where σ is the standard deviation of y-intercept of regression equation and s is the slope of the calibration curve[27].

**Robustness:** The robustness is a measure of method capacity to remain unaffected by small but deliberate variations in method parameters. For HPLC, the influences of small changes in mobile phase composition (Methanol±3.00%), pH (±0.20), wavelength of detection (±2.00), flow rate (±0.1) and column Pheonomenex-C18 (4.6×250 mm) column was used.

**Results and discussion**

**Optimization of chromatographic conditions**

The developed RP-HPLC method has been applied for the determination of Pazufloxacin in bulk and marketed injection formulations. To optimize the HPLC parameters, several mobile phase composition were tried. It is apparent that the retention increases when the pH of the mobile phase is increased. This is because the compounds are less ionized at high pH and thus have more affinity for the stationary phase. By the use of Methanol: Water (70:30 %v/v), the retention time increased to 1.5 minutes while with methanol: phosphate buffer (50:50 %v/v) showed retention time decreased. The use of water instead of phosphate buffer pH 4 as modifier led to further increase in the retention of pazufloxacin to 4.2 minutes.

On the basis of above results, the chosen mobile phase was methanol: phosphate buffer pH 4(50:50 %v/v) with a flow rate 1ml/min.

Figure 4 show the chromatograms of pazufloxacin. The average retention times with SD, for 3 replicate injections of Pazufloxacin were found to be 3.500 ± 0.021 (5 ppm), 3.563±0.053 (15 ppm) and 3.567± 0.043 (25 ppm); respectively.

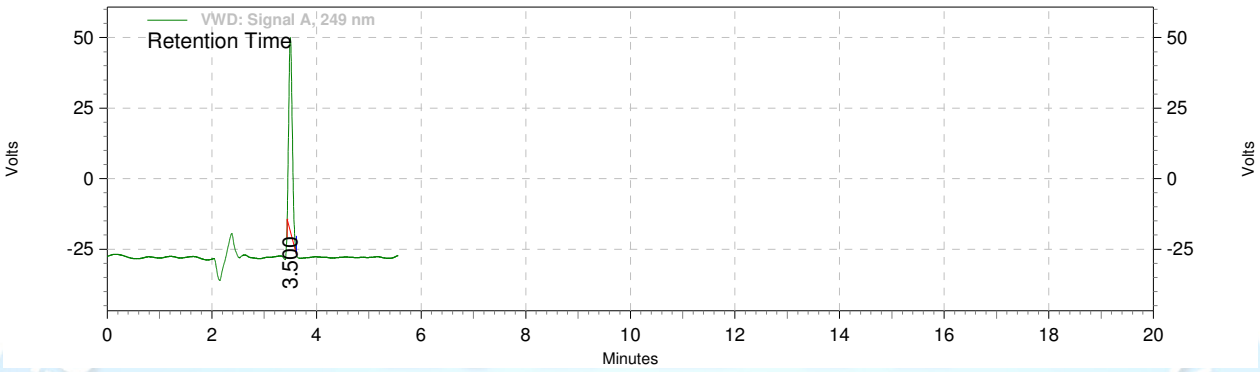
The average retention times, for 3 replicate injections of Pazufloxacin was found to be 3.590±0.035. The concentration estimated by proposed method for marketed injection of Pazufloxacin was 99.98 %±0.141 with % RSD of 1. These results indicate that the developed method could be used for estimation of Pazufloxacin in injection formulation.

**Method validation**

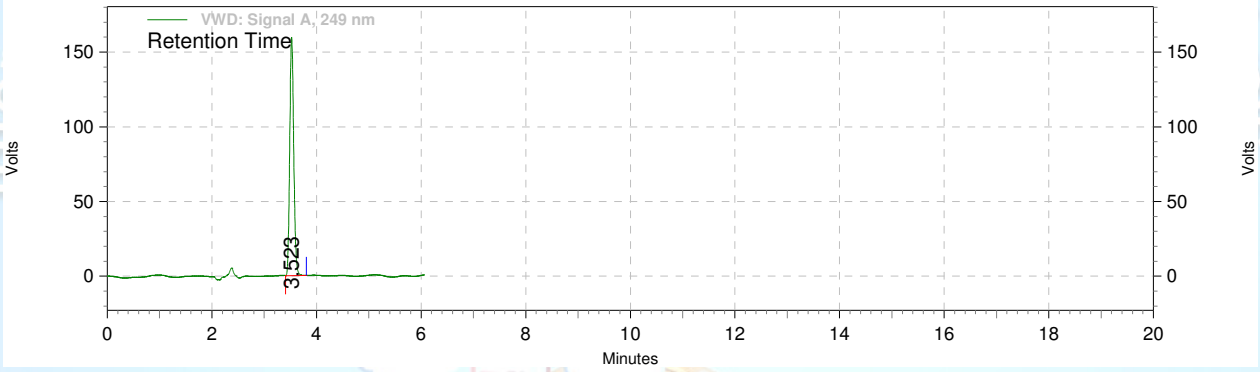
**Linearity:** The linear regression data for the calibration curves (n=5) showed good linear relation-

ship over the concentration range of 5-25 µg/ml for standard drug for HPLC methods.

Characteristic parameters for regression equations and correlation coefficients were given in table 1. The linearity of the calibration graphs were validated by the high value of correlation coefficients of the regression.




(A)

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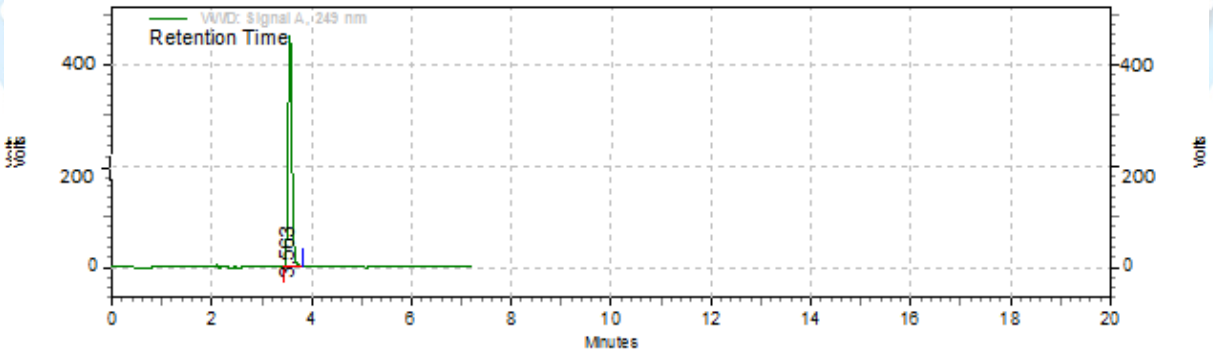


(C)

**Figure 4:** Typical chromatogram of Pazufloxacin (A) 5µg/ml (B) 15µg/ml (C) 25µg/ml

**Accuracy and precision:** The accuracy and precision of the developed methods were expressed in terms of recovery% and %RSD, respectively (Table 2). Table 3 summarizes the accuracy, intra and inter-day precision of pazufloxacin. The low values of SD and % RSD demonstrate excellent precision of the methods.

**Limit of detection and limit of quantitation:** For calculation of LOD and LOQ, the standard deviation of response method based on the standard deviation of intercept was used. LOD and LOQ of Pazufloxacin were found to be 0.8 µg and 2.4 µg for HPLC method which indicates the adequate sensitivity of the methods.

The proposed method was applied to the quantitative analysis of Pazufloxacin in marketed formulation Pezflow Injection (500 mg/100 ml; Cipla Ltd.). The assay results were found to be within acceptable limits (99.98 %±0.141; 1.0% RSD). The results are shown in Table 5.

**Table 1: Result of calibration reading of Pazufloxacin (5-25µg/ml)**

| Concentrations (µg/ml) | Mean of Area(mV/s)* | Mean of concentration* ± SD | %RSD |
|---|---|---|---|
| 5 | 65.08 | 4.63 ± 0.122 | 2.0 |
| 10 | 135.49 | 10.18 ± 0.134 | 1.5 |
| 15 | 201.79 | 15.40 ± 0.120 | 0.99 |
| 20 | 261.4 | 20.10 ± 0.153 | 1.0 |
| 25 | 319.37 | 24.67 ± 0.122 | 0.64 |

*n=3 readings

**Table 2: Accuracy data of Pazufloxacin**

| Concentration of Std. Solution used (µg/mL) | Concentration of Sample Solution added (µg/mL) | Amount recovered* (µg/mL) | % Recovery ± SD | %RSD |
|---|---|---|---|---|
| 10 | 5 | 5.02 | 100.4±0125 | 1.2 |
| 10 | 10 | 10.15 | 101.5±0.14 | 1.5 |
| 10 | 15 | 15.09 | 100.6±.0.125 | 0.95 |

**Table 3: Repeatability and Intermediate data of Pazufloxacin**

| Concentration (µg/ml) | | Area | | Mean conc. ±SD (µg/ml) | | %RSD (NMT 2) | |
|---|---|---|---|---|---|---|---|
| Intra | Inter | Intra | Inter | Intra | Inter | Intra | Inter |
| 5 | 5 | 64.91 | 63.55 | 4.62±0.12 | 4.51±0.21 | 2.0 | 0.40 |
| 5 | 5 | 64.94 | 63.44 | | | | |
| 5 | 5 | 64.96 | 63.78 | | | | |
| **Mean area** | | **64.93** | **63.59** | | | | |
| 15 | 15 | 201.01 | 201.22 | 15.37±0.14 | 15.36±0.16 | 0.99 | 1.0 |
| 15 | 15 | 201.98 | 201.16 | | | | |
| 15 | 15 | 201.10 | 201.31 | | | | |
| **Mean area** | | **201.36** | **201.23** | | | | |
| 25 | 25 | 319.70 | 319.11 | 24.68±0.21 | 24.65±0.14 | 0.97 | 0.63 |
| 25 | 25 | 319.50 | 319.02 | | | | |
| 25 | 25 | 319.28 | 319.20 | | | | |
| **Mean area** | | **319.49** | **319.11** | | | | |

**Table 4: LOD and LOQ data of Pazufloxacin**

| Drug | LOD (µg/ml) | LOQ (µg/ml) |
|---|---|---|
| Pazufloxacin | 0.8 | 2.4 |

**Table 5: Assay of Marketed Formulation**

| Drug & Brand Name | Label claim mg/injection | Conc. estimated* (mg) | % Conc. estimated*±SD | % RSD |
|---|---|---|---|---|
| Pazufloxacin (**Pezflow Inj. 500 mg/100 ml; CIPLA)** | 500mg | 499.92 | 99.98 %±0.141 | 1.0 |

## Conclusion

A HPLC method has been developed for estimation of Pazufloxacin in bulk and injection formulation. The developed method for quantitative estimation of Pazufloxacin and its marketed injection formulation reverse phase chromatographic method utilizing $C_{18}$ column and methanol: phosphate buffer pH 4 as mobile phase. Detection of the eluent was carried out using UV detector. The run time per sample was 1min. The result of analysis of Pazufloxacin standard drug powder and its injection formulation using this developed method was found close to 100%. Values of standard deviation were satisfactorily low indicating developed method is highly accurate and reproducible. Results of recovery study were satisfactory and show that there is no interference of excipients in marketed injection. The developed method was found to be simple, rapid, and accurate and can be used for routine analysis of Pazufloxacin in injection dosage form.

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