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# DETERMINATION OF ENANTIOMERIC IMPURITY IN BESIFLOXACIN HYDROCHLORIDE BY CHIRAL HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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## ABSTRACT

A sensitive simple rapid and robust new chiral normal phase HPLC method is developed for determination of Enantiomeric impurity (Impurity-A) in Besifloxacin Hydrochloride by using amylose tris (3,5-dichlorophenylcarbamate) as stationary phase with column dimension of 4.6 mm I.D × 250 mm length and 5 µm particle size (Chiralpak IE). Mobile phase consists of a mixture of Methylene dichloride, n-Hexane, Isopropyl alcohol, diethylamine and trifluoroacetic acid in the ratio (65:25:10:0.2:0.2). Isocratic elution mode is used for the separation using a flow rate of 0.8 mL/min and column oven temperature kept at 25°C. Detection is carried out at wavelength 295 nm. Developed method is validated as per ICH guideline and found out to be linear, accurate, specific, selective, precise, and robust. Test solution was found to be stable for 24 hrs. The method can be successfully applied for the determination of enantiomeric impurity in Besifloxacin Hydrochloride for routine and stability samples.

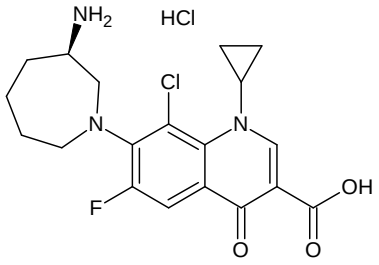
**KEYWORDS:** Besifloxacin, Enantiomer, HPLC, method development, validation.

## 1. INTRODUCTION

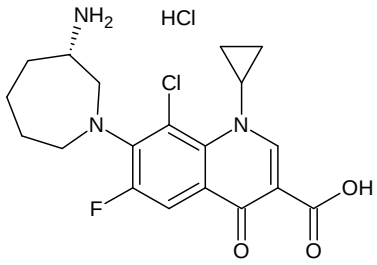
Besifloxacin Hydrochloride chemical known as 7-[(3R)-3-aminoazepan-1-yl]-8-chloro-1-cyclopropyl-6-fluoro-4-oxoquinoline-3-carboxylic acid; hydrochloride is a fourth-generation

fluoroquinolone antibiotic used as eye drops to treat bacterial conjunctivitis caused by aerobic and facultative gram-positive microorganisms[1].

The present work described in this paper is related to the development and validation of a isocratic normal phase HPLC method which is capable of determination of enatiomeric impurity Impurity-A of Besifloxacin Hydrochloride (Fig.1). A thorough literature survey was done and found out that there are HPLC method for determination of Enantiomer in Besifloxacin Hydrochoride by derivatisation[2] and one method in patent on cyclodextrin column with less separation and higher limit of detection[3]. One of chiral method published for Besifloxacin is using conventional coated column is having a higher detection limit and low resolution for peak due to Besifloxacin and Impurity-A (Enantiomer)[4]. Hence, the goal was set to separate the Enantiomer and Besifloxacin with good resolution, low detection, without the use of expensive derivatisation reagent thus avoiding the derivatisation process so that method can be used in routine analysis.




Besifloxacin Hydrochloride




Impurity-A (Enantiomer)

**Figure. 1. Structure of Besifloxacin and Impurity-A**

**Table 1. Chemical name of Besifloxacin Hydrochloride and Impurity-A**

| Component | Chemical name |
|---|---|
| Besifloxacin Hydrochloride | 7-[(3R)-3-aminoazepan-1-yl]-8-chloro-1-cyclopropyl-6-Fluoro-4-oxoquinoline-3-carboxylic acid; hydrochloride |
| Impurity-A (Enantiomer) | 7-[(3S)-3-aminoazepan-1-yl]-8-chloro-1-cyclopropyl-6-fluoro-4-oxoquinoline-3-carboxylic acid; hydrochloride |

## 2. MATERIALS

### 2.1 Reagent and Chemicals

Besifloxacin Hydrochloride sample and working standard, along with Impurity-A (Enantiomer) as Besifloxacin racemic working standard were received from Analytical research and development Department of Indoco research centre (Navi Mumbai). HPLC grade Methylene dichloride, n-Hexane, Isopropyl alcohol and Analytical grade Diethylamine

and Trifluoroacetic acid were purchased from Merck (India). The potency (% w/w) of Besifloxacin Hydrochloride working standard and Besifloxacin racemic working standard were 99.38 and 50.18 (enantiomer) respectively.

### 2.2 Instrumentation

Waters, Alliance 2695 series HPLC system (Milford) comprising a quaternary pump, an autosampler, a thermostatted column compartment, a solvent cabinet with degasser along with photodiode array (PDA) 2998 and ultraviolet (UV) 2487 detectors were used for separation and detection. Data acquisition and calculations were carried out using Waters Empower3 software (Milford). Sartorius (Germany) analytical balance was used for weighing of the materials.

## 3. METHODOLOGY

### 3.1 Method optimization

Various chiral columns such as Chiralpak IA, Chiralpak IB, Chiralpak AD were used with various dimensions, but best separation was achieved on Chiralpak IE (Make-Daicel, Japan) 250 mm x 4.6 mm, 5 µm. Also, various combinations of mobile phase along with additive were tried and finally optimized by using solvent Methylene dichloride, n-Hexane, Isopropyl alcohol, Diethylamine and Trifluoroacetic acid in the ratio (65:25:10:0.2:0.2) respectively. Flow was optimized to 0.8 ml/min. Details of other optimized chromatographic parameters are given in Table 2. For suitability of system, resolution between Impurity-A (Enantiomer) and Besifloxacin peak was set to be greater than 4.0 obtain with system suitability solution and percent relative standard deviation (RSD) for the peak area of Besifloxacin for five replicate injections of a reference solution (a) was kept to be not more than 5.0.

**Table 2. Chromatography condition**

| | |
|---|---|
| Column Temperature | 25°C±2°C |
| Flow Rate | 0.8 mL/min |
| Injection Volume | 20 µL |
| Detector Wavelength | 295 nm |
| Run Time | 40 minutes |
| Retention Time | Besifloxacin, about 19.0 minutes |
| Relative retention time | Impurity-A about 0.7 relative to Besifloxacin |
| Needle wash | Methylene dichloride: Isopropyl alcohol (80:20) |

### 3.2 Preparation of solutions

**Preparation of Mobile Phase**

Transferred 650 mL of Methylene dichloride, 250 mL of n-Hexane, 100 mL of Isopropyl alcohol, 2.0 mL of Diethylamine and 2.0 mL of Trifluoroacetic acid into 1L bottle. Mixed well and degassed by sonication for 2 minutes

**Diluent**

Mixed Mobile phase and Methanol in ratio (70: 30).

**Preparation of Blank**

Diluent solution is used as blank.

**System suitability solution**

Transferred about 10 mg of Besifloxacin racemic working standard into 20.0 mL of volumetric flask, dissolve in 10.0 mL of diluent and made it upto the mark with diluent.

**Reference solution (a)** (Besifloxacin 0.5 % w.r.t. sample)

Weighed 25.0 mg of Besifloxacin Hydrochloride working standard into 25.0 mL of volumetric flask, dissolve in 10.0 mL of diluent and made upto the mark with diluent. Transfer 1.0 mL of this solution to 20 mL volumetric flask and made upto the mark with diluent. Transfer 5.0 mL of this solution to 50 mL volumetric flask and made upto the mark with diluent**.**

**Test solution**

Prepared 1.0 mg/mL solution by accurately weighing 25.0 mg of Besifloxacin Hydrochloride test sample and transfering into 25.0 mL volumetric flask. Dissolved in 10.0 mL of diluent and made upto mark with diluent.

Calculate Impurity-A content by following formula and report the content:

$$\% \text{ Impurity-A content} = \frac{A \times W_S}{B \times W_T} \times 0.5$$

Where, A is peak area of Impurity-A obtained in test sample, B is peak area of Besifloxacin in Reference solution (a), Ws is weight of Besifloxacin Hydrochloride working standard

taken for Reference solution (a) preparation and $W_T$ is weight of Besifloxacin Hydrochloride test sample taken for Test solution preparation and 0.5 is dilution factor.

## 4 ANALYTICAL METHOD VALIDATION

The developed method is subjected to analytical method validation, which is conducted according to the International council for Harmonisation (ICH) guidelines[5-10]. The parameter with which analytical method is validated is specificity, limit of detection, limit of quantitation, linearity, accuracy, precision, robustness and solution stability.

## 5 RESULTS AND DISCUSSION

### 5.1 System suitability

The System suitability test represents an integral part of the method and are used to ensure adequate performance of the chromatographic system. For this system suitability solution was injected and resolution between Impurity-A and Besifloxacin was observed, recorded the data in Table 3 Similarly, five replicate injections of Reference solution (a) were injected and percent relative standard deviation for the peak area of Besifloxacin is calculated and recored the data in Table 3.

System suitability was checked before each validation parameter. Throught validation resolution between Impurity-A and Besifloxacin peak was more than 4.0 and percent relative standard deviation was less than 5.0.

**Table 3. System suitability data**

| Sample ID | Injection number | Peak Area |
|---|---|---|
| Reference solution (a) | Injection-1 | 228269 |
| | Injection-2 | 218007 |
| | Injection-3 | 214812 |
| | Injection-4 | 223586 |
| | Injection-5 | 221845 |
| % Relative standard deviation | | 2.34 |
| Resolution between Besifloaxacin and Impurity-A | | 5.03 |

### 5.2 Specificity

Specificity is the capability of the method to measure the analyte response in the presence of impurities. Figure 2 shows the typical chromatograms of the blank solution, system suitability solution, reference solution (a) and Besifloxacin hydrochloride spiked test sample. The

results indicated that the two isomers are well separated under the current chromatographic conditions. The retention times were about 13.5 and 19.0 min for Impurity-A and Besifloxacin, respectively. No interference peaks from the blank solution and the samples were observed within the retention time range of Impurity-A. Peak purity for Besifloxacin and Impurity-A is passing for all solution Table 4.

**Table 4. Peak purity for spiked test solution**

| Sample ID | Peak name | Retention time (min) | Purity angle | Purity threshold | Peak purity |
|---|---|---|---|---|---|
| Reference solution (a) | Besifloxacin | 18.657 | 1.897 | 1.946 | Pass |
| System suitability solution | Impurity-A | 13.445 | 0.043 | 0.251 | Pass |
| | Besifloxacin | 18.940 | 0.053 | 0.245 | Pass |
| Besifloxacin spiked sample | Impurity-A | 13.624 | 0.848 | 1.020 | Pass |
| | Besifloxacin | 18.321 | 0.035 | 0.301 | Pass |

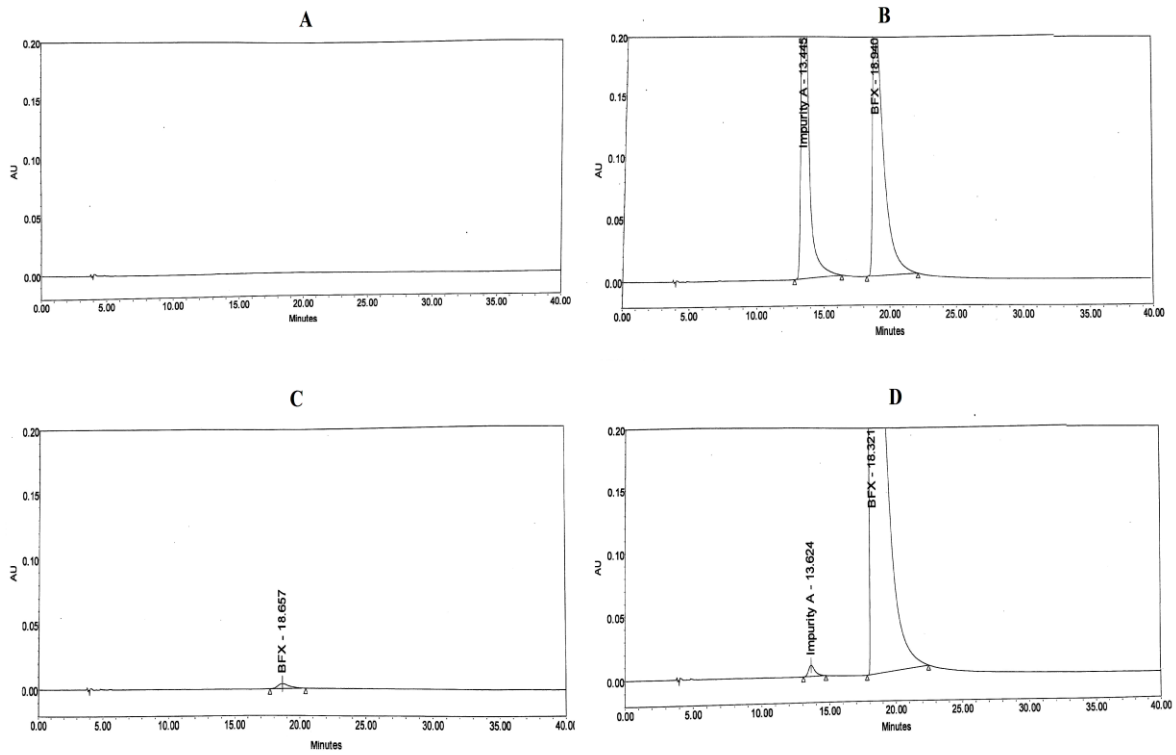



**Figure. 2. A) Blank, B) System suitability solution, C) Reference solution (a) and D) Besifloxacin Hydrochloride spiked test sample.**

### 5.3 Limit of detection and limit of quantiation

Series of standard solutions of Besifloxacin and Impurity-A were prepared in concentration ranging from 50% to 150% of target concentration (0.5% w.r.t. sample). Limit of detection (LOD) and Limit of quantitation (LOQ) was calculated based on residual standard deviation

of regression line and slope. Both LOD and LOQ calculated were well below 0.02% for the Impurity-A and Besifloxacin (Table 5).

**Table 5. Limit of detection and quantitation**

| Parameter | Besifloxacin | Impurity-A |
|---|---|---|
| LOD (%) | 0.006 | 0.018 |
| LOQ (%) | 0.005 | 0.015 |

### 5.4 Linearity

Series of linearity solution of Besifloaxcin and Impurity-A solution were prepared from limit of quantification (LOQ) to 150% of target concentration (0.5% w.r.t. sample). Linearity curves were drawn by plotting the peak areas of Besifloaxacin and Impurity-A against its corresponding concentration of linearity solution (Figure 3). Regression coefficient, slope and % y intercept are calculate and reported in Table 6. The observed regression coefficient was greater than 0.999 and % y intercept was less than 5.0%.

**Table 6. Linearity for Besifloxacin and Impurity-A**

| Linearity | Besifloxacin | Impurity-A |
|---|---|---|
| Regression coefficient | 0.9998 | 0.9994 |
| % y intercept | 1.12 | 1.12 |
| Slope | 404227.92 | 367417.02 |
| % Lower range | 0.015 | 0.015 |
| % Upper range | 0.754 | 0.759 |

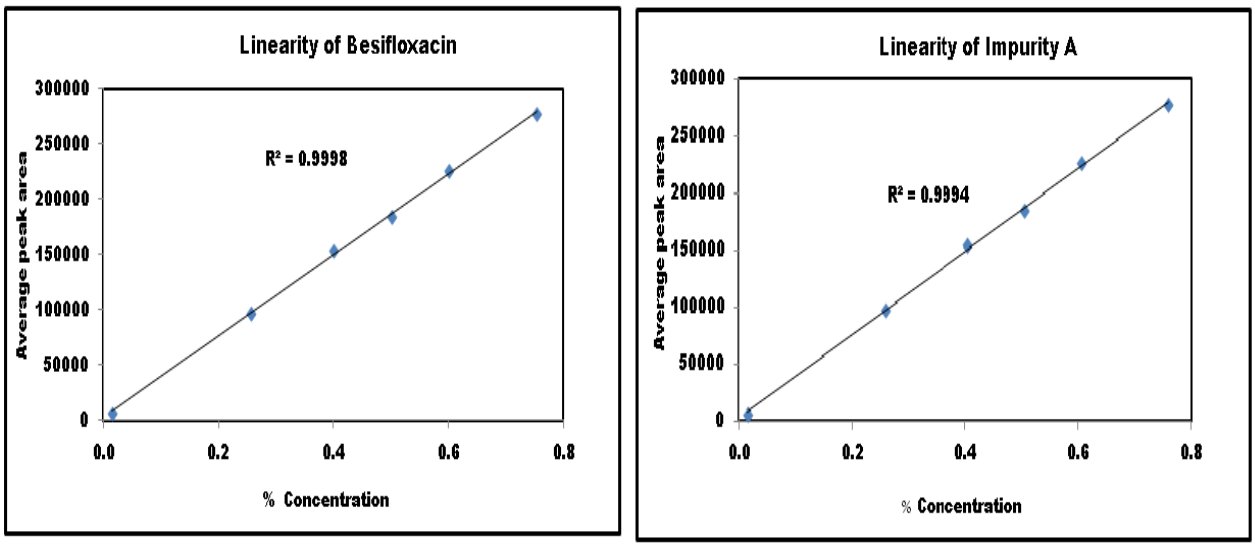



**Figure 3. Linearity of Besifloxacin and Impurity-A**

### 5.5 Precision

System precision was carried out by analysing six reference solutions (a) of Besifloxacin Hydrochloride at a limit level concentration (0.5%). Relative standard deviation for the peak area of Besifloxacin was calculated and found to be 2.34 %. Precision at LOQ was calculated by preparing Impurity-A solution at LOQ concentration and injecting six times. Relative standard deviation for Impurity-A peak area was 1.35 % (Table 7).

**Table 7. System precision and precision at LOQ**

| Parameter | Peak name | % RSD for peak area |
|---|---|---|
| System precision | Besifloxacin | 2.34 |
| Precision at LOQ | Impurity-A | 1.35 |

For repeatability and intermediate precision (different day different system), prepared six test solutions. Injected and calculated relative standard deviation (RSD) for Impurity-A content. RSD observed for both the sets were less than 5.0 % (Table 8).

**Table 8. Repeatability and Intermediate precision**

| Parameter | Peak name | % RSD for Impurity-A content |
|---|---|---|
| Repeatability | Impurity-A | 4.01 |
| Intermediate Precision | Impurity-A | 0.73 |

### 5.6 Accuracy

Accuracy of the method is established by carrying out the recovery studies of Impurity-A. Test solution was spiked with Impurity-A at concentrations LOQ, 80%, 100% and 120% of the specification level of Impurity-A (0.5%) in triplicate. Each spiked test solution was analysed for recovery of Impurity-A. Recovery obtained for Impurity-A is between 80% to 120% (Table-9).

**Table 9. Recovery of Impurity-A**

| Level | % Recovery of Impurity-A |
|---|---|
| LOQ | 99.85 |
| 80% | 99.88 |
| 100% | 103.80 |
| 120% | 109.65 |

### 5.7 Robustness

For robustness, deliberate changes were done with respect to flow rate, column temperature and mobile phase composition as given in Table 10. Each change consists of one upper set and one lower set except for column temperature were only upper limit can be set above

ambient temperature. For each set, three test preparations were done and analysed. Relative standard deviation for Impurity-A content was observed, which was less than 5.0 % (Table 11).

**Table 10. Robustness parameter changes**

| Changes | Lower set | Upper set |
|---|---|---|
| Mobile phase flow rate | 0.72 mL/min | 0.88 mL/min |
| Mobile phase composition change | 675:225:100:2:2 | 625:275:100:2:2 |
| Column Oven Temperature by 5°C | NA | 30°C |

Composition ratio is for (Methylene dichloride: n-Hexane: IPA: Diethylamine: Trifluroaceticacid).

**Table 11. Robustness results**

| Changes | % RSD for Impurity-A content | |
|---|---|---|
| | Lower set | Upper set |
| Mobile phase flow rate | 1.69 | 4.46 |
| Mobile phase composition change | 0.13 | 1.22 |
| Column oven temp. increase by 5°C | 0.86 | |

### 5.8 Solution stability

Test solution stability was established by injecting the same test solution after every six hours time interval for 24 hours. Relative standard deviation for the content of Impurity-A was determined which was found out to be less than 5.0 %, thus solution stability was established up to 24 hours at 25 °C(Table 12).

**Table 12. Solution stability of Besifloxacin Hydrochloride**

| Time | % Impurity-A content |
|---|---|
| 0 Hr | 0.247 |
| 6 Hrs | 0.250 |
| 12 Hrs | 0.252 |
| 18 Hrs | 0.250 |
| 24 Hrs | 0.253 |
| **%RSD** | **0.86** |

## 6.0 CONCLUSION

The normal phase simple, sensitive HPLC method is developed for quantitative determination of enantiomer (Impurity-A) of Besifloxacin Hydrochloride. This method is validated and found out to be linear, accurate, precise, robust and specific. Acceptable data for all method validation parameters tested and found out to be satisfactory. The developed method can

suitably use by quality control department to determine the Enantiomer (Impurity-A) in commercial and stability test samples of Besifloxacin Hydrochloride.

## 7.0 ACKNOWLEDGMENT

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