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DEVELOPMENT AND EVALUATION OF STABILITY INDICATING RP- HPLC METHOD FOR ANALYSIS OF BESIFLOXACIN IN PHARMACEUTICAL FORMULATION

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ABSTRACT

In the present work RP-high performance liquid chromatography (RP-HPLC) method was developed and validated for the determination of besifloxacin in pure form and also in pharmaceutical formulations. The analyzed drug was separated on a reversed phase column with octadecyl silane (ODS-3) Inertsil® C_{18} column (250mm×4.6mm×5µm) by using a mobile phase containing 0.5% triethylamine (pH=3) and methanol- acetonitrile mixture (70:30 v/v) with UV detection at 289 nm. The calibration standards were prepared in the range of 0.05-20 µg/ml, where regression coefficient (r^2) was found to be 0.999. The limit of detection and quantitation were 0.43 µg/ml and 1.29 µg/ml respectively. Results of proposed method were validated as per ICH guidelines. The stability studies included forced and non-forced degradation that was also performed to demonstrate the stability indicating power of developed method. Thus developed method was found to be simple, accurate, precise and robust to analyze the besifloxacin in different pharmaceutical formulations.

KEYWORDS: Besifloxacin., RP-high performance liquid chromatography (RP-HPLC)., ICH- guidelines., Pharmaceutical formulations., Stability indicating validation., Force and non-force degradation.

Bacterial conjunctivitis is an inflammation of the conjunctiva, characterized by persistent mucopurlent discharge and redness of the eye[1-2]. The most common type of bacteria that causes bacterial conjunctivitis are *Aerococcus viridans, CDC coryneform group G, Corynebacterium striatum, Haemophilus influenzae, Moraxella catarrhalis, Moraxella lacunata, Pseudomonas aeruginosa Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus hominis, Staphylococcus lugdunensis, Staphylococcus warneri, Streptococcus mitis group, Streptococcus oralis, Streptococcus pneumoniae, Streptococcus salivariu*[3-4] besifloxacin ophthalmic suspension 0.6% was recently approved for the treatment of bacterial conjunctivitis[5]. Besifloxacin ophthalmic suspension 0.6% provides safe and efficacious treatment for bacterial conjunctivitis[6-10].

Chemically, besifloxacin (BSF) is known as- [(3*R*)-3-aminoazepam-1-yl]-8-chloro-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (Fig. 1). The literature reported the quantitative evaluation of BSF by HPLC[11] in formulation, estimation of BSF in human tears[12], determination of entiomeric impurities in BSF by Chiral HPLC[13]. The literature also revealed other analytical technique like microbiological, MS, ^{1}H-NMR, and ^{13}NMR analysis. The aim of the present method was to develop and validate the simple, fast, sensitive and precise UV-HPLC for the assay of BSF in bulk and in different pharmaceutical dosages form.

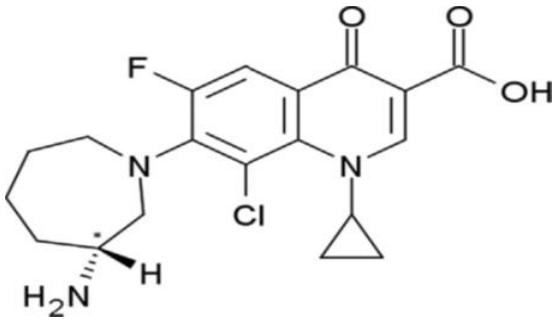

Figure1. Chemical structure of besifloxacin.

MATERIALS AND METHODS

Chemicals

The API standard of besifloxacin hydrochloride was obtained as a kind gift from Indoco Remedies Ltd, Mumbai that was arranged by KPS Clinical Services Greater Noida, UP; While besifloxacin ophthalmic suspension 0.6% w/v (Besix® eye drops Batch no- AD0082L) containing 5 ml of product were procured from a local pharmacy. Acetonitrile, orthophosphoric acid and sodium hydroxide were purchased from RFCL, New Delhi, where

methanol, triethylamine and water of HPLC grade were procured from Fisher scientific (Qualigen) Ltd. Mumbai, India.

Apparatus

Reversed-phase high performance liquid chromatography (RP-HPLC) determinations were performed with Agilent Technologies (1120, Compact) G4288A low pressure binary gradient pump consisting of vacuum in built degasser unit, non-PDA, VWD-UV detector, column oven, manual injector equipped with 50 µL injector loop, having analytical syringe (50 µL Germany) and EZChrom Elite (3.0.1) with LMD software controlled. Ultrasonic bath degasser and cleaner were used (Lansany, Chandīgarh, India). All weights were taken on electronic balance (Vibra, DJ-150S-S, Shinko Denshi, Japan) with 1 mg sensitivity.

Chromatographic conditions

Chromatographic separation was carried out at 30 ^{0}C on an Inertsil® ODS-3, C18 column (250 mm × 4.6 mm × 5 µm) particle sizes, GL Sciences, Inc. USA, www.glsciencesinc.com). The BSF was separated gradient with a mobile phase consisting of inorganic phase-water in 0.5% triethylamine (pH=3) and organic phase (mixture of methanol and acetonitrile in 70:30 v/v) at 80:20 v/v ratio. The pH of inorganic phase (water) was adjusted with diluted orthophosphoric acid and sodium hydroxide. The mobile phase was filtered and degassed for five minutes in a bath sonicator prior to use. To reach equilibrium the analysis was usually started after the passage of 60-70 ml of mobile phase. The flow rate was 2.25 ml/min. The injection volume was 50 µL, and the eluted analytes for drug was traced by UV-detection at 289 nm.

Standard drug solution

The standard stock solution of BSF (50 µg/ml) was prepared by dissolving 5 mg of standard drug in 100 ml of volumetric flask having mobile phase in composition of organic: inorganic (55:45). The working standard solution 0.05-20 µg/ml was prepared by dilution of the stock solution with mobile phase. The stock solution (60 µg/ml) of Besix® marketed product was prepared by dissolving 1 ml of 0.6% suspension 100 ml of mobile phase.

Construction of the calibration curve

The working standard dilution of 0.05, 0.5, 1, 2, 5, 15, 20 µg/ml prepared by standard stock of BSF (60 µg/ml) with mobile phase. The mobile phase was pumped for about 10 minutes to saturate the column to get the baseline correction. Then each dilution was injected

(triplicates) and read for peak elution. The average (n=3) peak area of BSF was plotted with concentration of BSF in µg/ml. This information was used to compute regression equation.

METHOD VALIDATION

The presented RP-HPLC method was validated according to ICH guidelines[14-15].

Specificity (selectivity)

The method was studied for their interaction with different solvent and excipients used in method development and formulation.

Linearity and Range

The linearity of the calibration curve was studied on the basis of the correlation coefficient (r^2) at specific range 0.05-20.00 µg/ml. The specified range for this method was established in triplicate (n=3) by analyzing and assuming 5 µg/ml as 100% concentration where 4 µg/ml as 80% and 6 µg/ml as 120% respectively. The result was expressed as recovery of samples with SD and %RSD.

Accuracy

The accuracy of the method was established by standard addition method. Where standard stock solution (10 µg/ml) were added with 2, 3 and 4 µg/ml of sample stock solution respectively to get final concentration of 12, 13 and 14 µg/ml after diluting with mobile phase in separate 10 ml volumetric flask. The accuracy was reported as % recovery of sample stock with SD.

Precision

The repeatability precision of the proposed method was calculated by analyzing 5 µg/ml standard solutions at 100% in six determinations and also analyzing three independent drug standard solutions of 7, 8 and 9 µg/ml in triplicate. The precision was expressed as percentage recovery with SD and %RSD.

Limit of detection and limit of quantification

The limit of detection (LOD) and limit of quantification (LOQ) were calculated and it was based on the slope of the calibration curve and standard deviation of intercept of regression line.

Stability

This paper describes as stability indicating RP-HPLC-UV method in both forced and non-forced conditions to determine the drug concentration in bulk. The forced condition includes acid hydrolysis, base hydrolysis, oxidation and thermal where as non-forced condition refers to room temperature stability.

Robustness

The robustness of the proposed method was examined by changing chromatographic parameter such as mobile phase composition, pH of mobile phase, temperature of column, flow rate, and $\lambda_{max.}$ For this study three consecutive injection of standard drug solution (10 µg/ml) was selected and injected.

RESULTS AND DISCUSSION

The specificity of BSF drug method was studied by scanning the drug solution in wavelength interval of 200- 400 nm for its best UV absorption detection that was found at 289 nm. The selected mobile phase was appropriate because it provided satisfactory result in regard to peak symmetry, drug and formulation solubility and short time of analysis (Fig. 2) and Chromatogram of blank(Fig. 3). The finding of drug chromatogram retention time was around 4 minutes that proves short time of analysis.

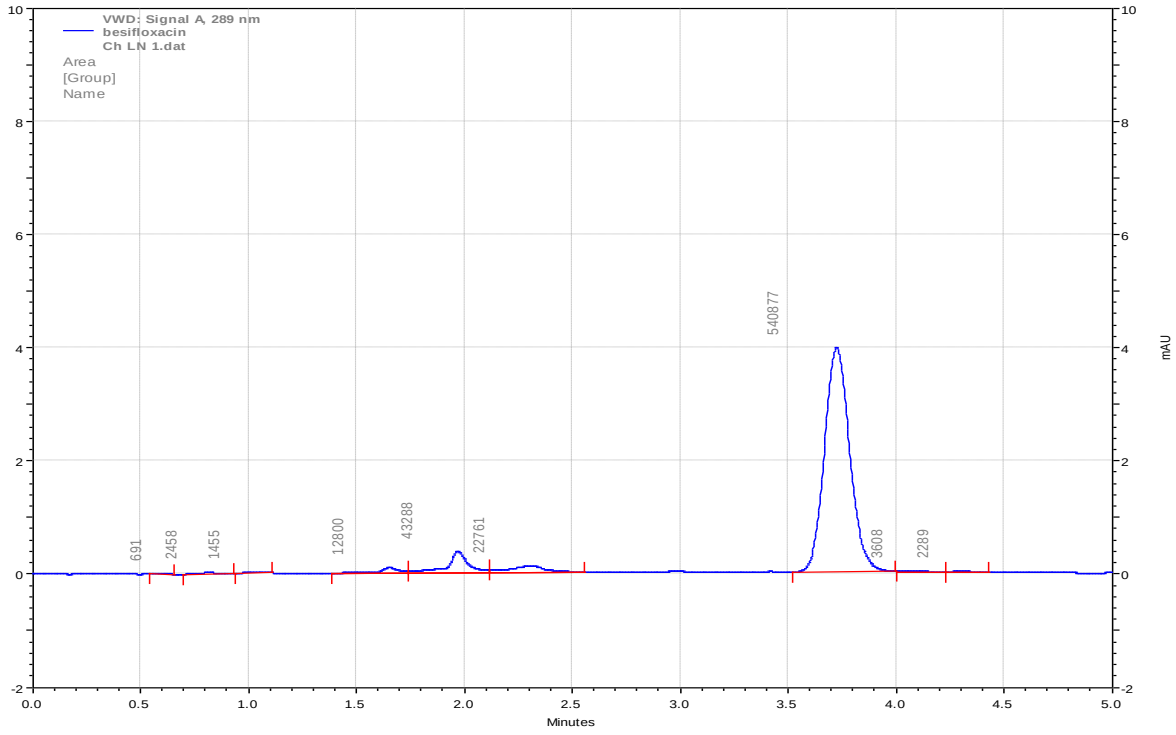

Figure2. Chromatogram of pure drug.

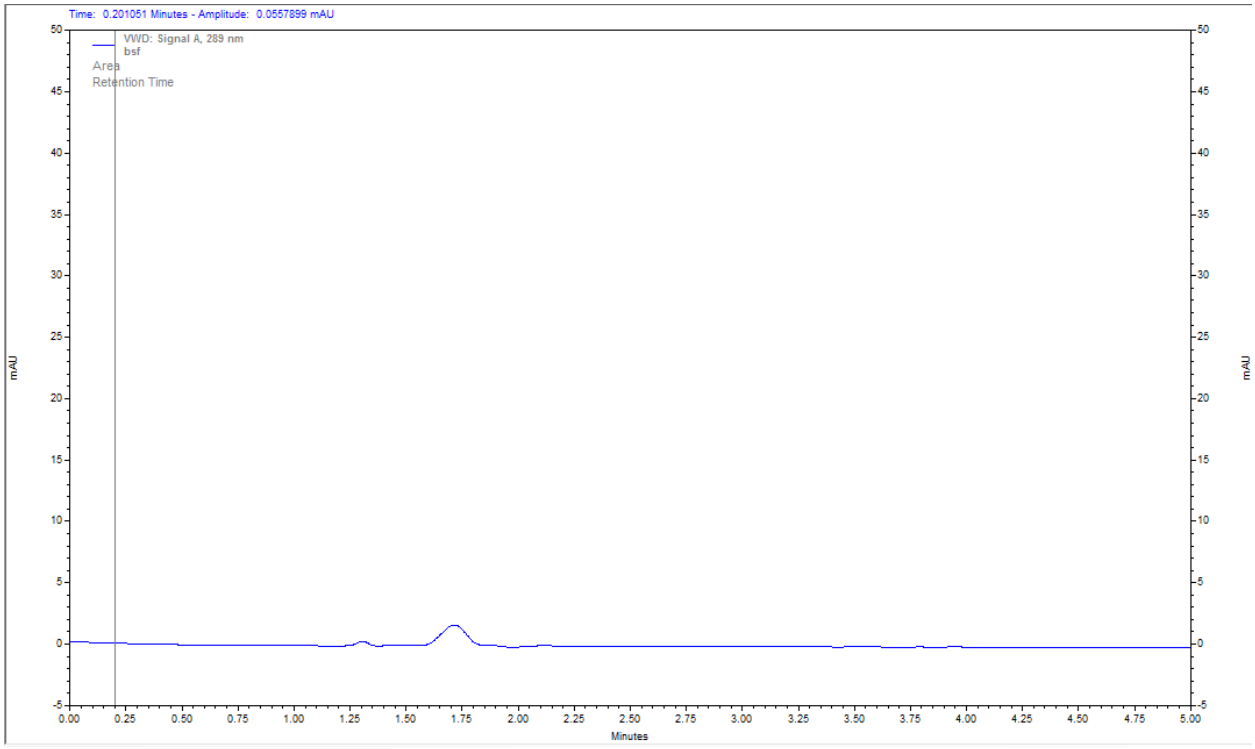

Figure 3. Chromatogram of blank.

The linearity test i.e. the regression analysis of standard drug curve was revealed appropriate linearity (r^2=0.999) in the concentration interval of 0.05- 20.00 µg/ml Table 1. The linear regression data for tested components was listed in Table 2. The specified range test that was analyzed at 80%, 100% and 120% was found to be high recovery with less than one %RSD showed high range of the method (Table 3).

The accuracy of the proposed method by standard addition method was determined and the mean recovery of added samples were found to be around 100% that indicates high degree of accuracy, Table 4. The repeatability precision of six determinates of 5 µg/ml at 100% concentration was found high with less %RSD Table 2. In another set of experiment excellent percentage recovery of nine determinates of three different concentrations were found very high with low value of %RSD confirms high degree of precision Table 5.

The detection limit (DL) and quantification limit (QL) of the method was analyzed and found to be 0.43 µg/ml and 1.29 µg/ml respectively that were good and high that increases range and utility of method (Table 1).

The data obtained after different stress conditions shows various impact on stability. The drug stability was more in acidic environment as compare to forced base and oxidation. However

% recovery in non-forced condition was more as compared to forced-condition like acid hydrolysis, base hydrolysis, oxidation and thermal stress Table 6.

The robustness of the method was determined by deliberately changing chromatographic parameter and found that each parameter shows slightly impact within limit on peak area, theoretical plates, plates/meter and retention time that confirm that proposed method was specific and robust Table 7.

Table 1: Regression analysis of linearity data of standard drug

Parameters	Values
*Regression equation	Y= 526752.24 X + 129671.10
Linearity (µg/mL)	0.05- 20.00
Slope	129671.10
Intercept	526752.24
Standard error of slope	68313.16
Standard error of intercept	7060.72
Standard error of estimate	138417.02
Correlations coefficient (r^2)	0.999
DL (µg/mL)	0.43
QL(µg/mL)	1.29

DL- Limit of detection

QL- Limit of quantitation

*Regression linearity was based on ratio of peak area of besifloxacin (analyte)

Table 2: System suitability parameters

Parameters	Besifloxacin (API)
*System precision (% RSD), n= 6	0.287
Retention time (min)	3.50
Area	290694
Height	37489
Peak width	0.69
Theoretical plate (USP)	5133
Asymmetry	1.04
Plates /meter (USP)	20532
USP width	0.20
Width at 5%	0.28
Width at 10%	0.22
Width at 50%	0.11
Area percent	14.92

%RSD- Percentage relative standard deviation

*Data was based on peak area of besifloxacin (analyte)

Table 3: Range (known concentration)

Percentage Conc.	Mean recovery ± SD	Percentage Recovery	Percentage RSD
80	3.98 ± 0.00	99.65	0.22
100	4.59 ± 0.01	97.86	0.24
120	5.96 ± 0.03	99.45	0.61

SD- Standard deviation

%RSD- Percentage relative standard deviation

Table 4: Accuracy analysis by standard addition method

Sample Conc. (µg/mL)	Conc. of Added Solution (µg/mL)	Final Conc. (µg/mL)	Mean Recovery ± SD	Percentage Recovery	Percentage RSD
10	2	12	12.78 ± 0.058	106.51	0.45
10	3	13	13.17 ± 0.002	101.37	0.02
10	4	14	14.43 ± 0.079	103.14	0.07

SD- Standard deviation

%RSD- Percentage relative standard deviation

Table 5: Precision by recovery

Conc. (µg/mL)	Mean recovery ± SD	Percentage Recovery	Percentage RSD
7	6.92 ± 0.031	98.99	0.45
8	8.23 ± 0.023	102.99	0.28
9	8.96 ±0.020	99.59	0.22

SD- Standard deviation

%RSD- Percentage relative standard deviation

Table 6: Stability study of besifloxacin in forced and non-forced condition

Storage Conditions	Mean Percentage Recovery ± SD	Remark Significant Degradation Found: Yes/No
Acid hydrolysis (1 N HCl, at 8 ^{0}C), Forced	92.66 ± 0.18	No
Base hydrolysis (1 N NaOH, at 8 ^{0}C), Forced	81.23 ± 0.49	Yes
Oxidation (H_2O_2 at 8 ^{0}C), Forced	88.69 ± 0.24	Yes
Thermal at 8 ^{0}C	101.39 ± 0.12	No
Room temperature (25 ^{0}C), Non-forced	96.40 ± 0.66	No

SD- Standard deviation

Table 7: Chromatographic condition investigations during robustness testing

Variables	Range	Area	Mean Retention Time	Theoretical Plates (USP)	Plates/meter (USP)	% Mean Recovery	%RSD**
Flow rate (mL/min)	2.37	224323	3.69	2942	21354	96.34	0.27
	2.50***	290694	3.50	5133	20532	99.36	0.03
	2.62	254567	3.63	3436	22314	97.23	1.32
pH	2.80	245343	3.70	2865	21584	69.38	0.23
	3.00***	290694	3.50	5133	20532	99.36	0.03
	3.20	288324	3.52	3987	15353	75.62	0.68
Mobile phase ratio (Organic: inorganic)	53.90: 46.10	237997	3.56	3347	23389	97.27	0.14
	75:25***	290694	3.50	5133	20532	99.36	0.03
	56.10: 43.90	285449	3.18	3319	15221	94.68	0.63
Temperature (^{0}C)	27	248200	3.40	3548	25825	96.32	0.24
	30***	290694	3.50	5133	20532	99.36	0.03
	35	253672	4.32	4749	26973	104.02	0.29
Wavelength (nm)	287	224254	3.36	4102	26408	98.89	0.31
	289***	290694	3.50	5133	20532	99.36	0.03
	291	285225	3.62	5193	26770	102.35	0.21

RT- Retention time

%RSD- Percentage relative standard deviation

*Data was based on peak area of besifloxacin (analyte)

** %RSD was based on recovery of peak area ratio of drug and internal standard of concentration 10 µg/mL

*** Data based on optimized metho

To conclude the developed method was found to be very simple, sensitive, accurate and economical. In this study an effective RP-HPLC method was developed for determination of BSF with high accuracy and precision. Thus, it can be concluded that the proposed method was sufficiently sensitive, reproducible and specific for analysis of BSF in pharmaceutical formulations within a short analysis of time. The above method was validated by evaluating different parameter according to ICH guidelines like linearity, precision, accuracy, robustness and stability. Robustness study concluded that developed method was highly robust and accurate. Stability study in forced and non-forced condition imparts that the appropriate storage condition must be provided during storage and handling of drug and drug products.

Thus the developed method was accurate and precise for the analysis of besifloxacin in pharmaceutical formulation and in bulks.

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CONFLICTS OF INTEREST

Authors have no conflict of interest.

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