STABILITY INDICATING RP-HPLC PDA ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF BESIFLOXACIN HYDROCHLORIDE IN BULK AND FORMULATION

Subhash G. Chate[a]* and Arunadevi S. Birajdar[b]

ABSTRACT

The goal of the study was to provide an overview of the technique development and validation of a stability-indicating HPLC approach for routine analysis of besifloxacin hydrochloride in pharmaceutical product (ophthalmic suspension). HPLC method was development on octadecasilyl silica (C_{18}, 250 mm x 4.6 mm x 5µ) column at 37 °C and isocratic mode with a running solvent (phosphate buffer: methyl alcohol 40:60 % V/V) using flow rate (1.0 mL minute^{-1}) and UV wavelength 292 nm. Proposed method was validated for specificity, linearity, accuracy, precision, range and robustness according to ICH Q2 (R1) standards. The collected results attest to the validated method's compliance with the set acceptance standards. Besifloxacin hydrochloride was subjected to hydrolytic, oxidative, thermal and photolytic stress conditions. These samples were then examined using our suggested approach. Hence, this method can be used for routine use for determination assay of besifloxacin hydrochloride drug substance (API) and drug product (ophthalmic suspension).

INTRODUCTION

Besifloxacin hydrochloride (BFHC) is a 4th generation fluoroquinolone antibiotic used as an ophthalmic formulation to prevent infectious complications in patients receiving laser treatment for cataracts, bacterial conjunctivitis brought on by aerobic, facultative gram-positive microorganisms, and aerobic and facultative gram-negative microorganisms. *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus hominis*, *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus pneumoniae*, and *Streptococcus salivarius* are among the bacteria that can cause skin infections, etc[1-2]. It is a disease that is extremely contagious and has the potential to spread quickly. The antibiotics used to treat this pathology are crucial because they reduce the spread of the infection, time missed from work or school, infection recurrence, and the danger of further, perhaps irreversible ocular damage[2-4].

Besifloxacin functions by adhering to the bacterial enzymes DNA gyrase and topoisomerase IV, which are necessary for bacterial DNA replication[5]. When these enzymes are inhibited, bacterial chromosomal DNA cannot be transcriptionally transcribed, replicated, or divided during cell division (Central Dogma)[6-8]. Bactericidal activity is a property of besifloxacin. The most frequent mechanisms of besifloxacin resistance include changes to the bacterial membrane outflow pumps and point mutations in the genes that code for DNA gyrase and topoisomerase IV[9]. Besifloxacin, however, has certain hypothetical advantages over other fluoroquinolones in the case of resistance[6-8]. High affinity for DNA gyrase and topoisomerase IV, in contrast to nearly all fluoroquinolones, which only have sympathy for one of them and correlate with the occurrence of resistance[10]. Therefore, for a bacteria to acquire besifloxacin resistance, besifloxacin-encoding genes must undergo the evolution of voluntary mutations, which is a less frequent occurrence[9,11]. Additionally, only topical ophthalmic usage of besifloxacin is available, which restricts systemic exposure and, as a result, slows the development of resistance[12-13]. In 2009, Bausch & Lomb

a   Department of Pharmaceutical Chemistry, Dr. D.Y. Patil Institute of Pharmaceutical Sciences & Research, Pimpri, Pune - 411 018, Maharashtra, India

b   Quality Assurance Department, K T Patil College of Pharmacy, Osmanabad - 413 501, Maharashtra, India

*For Correspondence: E-mail: chatesubhash123@gmail.com

received FDA approval for BEX. HCl, a medication used to treat non viral bacterial inflammation of the conjunctiva of the eye. BFHC is 7-[(3*R*)-3-aminoazepan-1-yl]-8-chloro-1-cyclopropyl-6-fluoro-4-oxo-1,4-hydroquinoline-3-carboxylic acid hydrochloride (IUPAC Name)[1,11,14]. Structure of besifloxacin HCl, containing basic a basic ring and side side structural components as shown in Fig. 1.
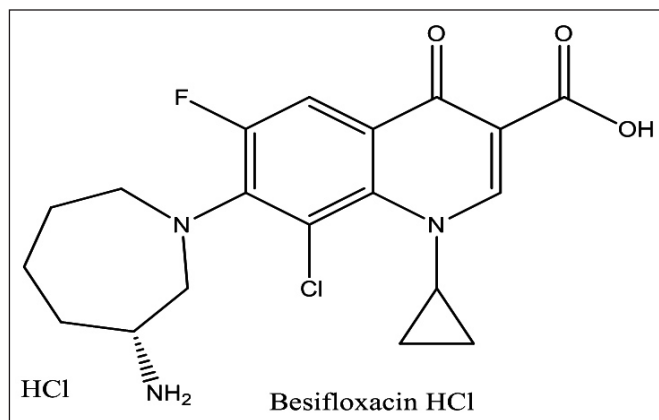

Fig. 1: Structure of besifloxacin HCl

Method development was performed using different column chemistry like C_8 and C_{18}, but C_{18} column shows good resolution and peak compared to C_8 column[15]. Hence C_{18} column of two different brands and particle size, Agilent Eclipse XDB-C_{18} column (150x4.6mm i.d., 3.5 µm particle size) and Kromasil C_{18} (250mm×4.6mm×5µ) were used. The best option for producing a symmetrical peak with good resolution was a Kromasil manufacture column. To optimize chromatographic conditions, different mobile phase ratios with organic solvent trial were taken and final ratios of running phase (buffer phosphate pH 3.0: methyl alcohol 40:60 V/V) using flow rate (1.0 mL minute^{-1}) and UV lambda max 292 nm showing highest sensitivity[16]. The above method validated according to International Conference on Harmonization standards and proved to be specific, rapid, accurate, precise, reproducible; robust method for assay of besifloxacin hydrochloride drug substance (API) and drug product (ophthalmic suspension)[15-17].

MATERIALS AND METHODS

Details of chemicals/ reagents used for research study with their make and grade mentioned as in the Table I.

Table I: Chemicals/ reagents details

Sr. No	Chemicals/ reagents	Make	Grade
01.	Phosphate buffer	Merck	HPLC and equivalent
02.	acetonitrile	Merck	HPLC and equivalent
03.	Methanol	Merck	HPLC and equivalent
04.	Water	-	Milli Q

Details of product used, Batch No and making, for research study as mentioned in the Table II.

Table II: Sample details

Sr. No	Product name	Batch No.	Make
01.	Besifloxacin ophthalmic suspension 30 mg mL^{-1}	01A110 and 1BA821	Ajanta Pharma

Details of instrument on which research carried out with their making as mentioned in the Table III.

Table III: Instrument details

Sr. No.	Instrument name	Mode	Make
01.	HPLC Shimadzu manual sampler with PDA detector	HPLC (LC20AD) and detector (LC20AD)	Shimadzu Instruments

RESULTS AND DISCUSSION

Method development and chromatographic condition optimization

Initial method development was performed on the C_8 column at 0.8 mL minute^{-1} flow using different ratios of buffer and acetonitrile, no peak elution was observed. Numerous modifications were made to the mobile phase's composition and chromatographic settings to increase the possibility that chromatographic systems' selectivity would alter[15-17]. This modification included the change like column chemistry C_8 replace with C_{18}, organic modifier acetonitrile replaced by methanol, flow change to 1.0 mL minute^{-1}, running phase ratio (buffer phosphate pH 3.0: methyl alcohol 40:60 V/V) and column oven temperature to 37 °C. Wavelength and injection volume finalize based on peak response and sensitivities at 292 nm of besifloxacin hydrochloride.

Besifloxacin HCl is slightly soluble in methanol and it is sparingly soluble in aqueous solutions. We diluted the organic solvent solution with aqueous buffers to increase aqueous solubility; as a result, the diluent was chosen as the mobile phase[18].

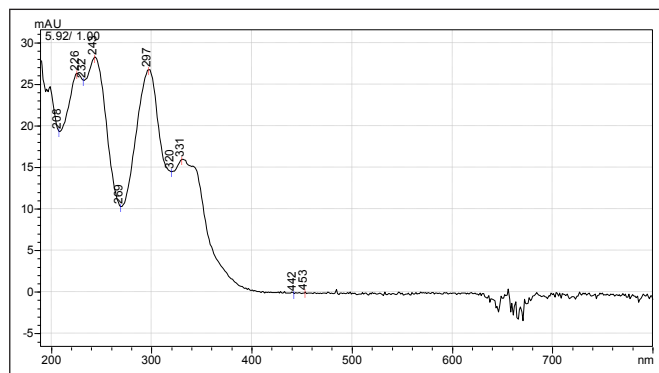UV spectrum of besifloxacin HCl indicating lambda max is as shown in Fig. 2.

Fig. 2: UV spectrum of besifloxacin hydrochloride

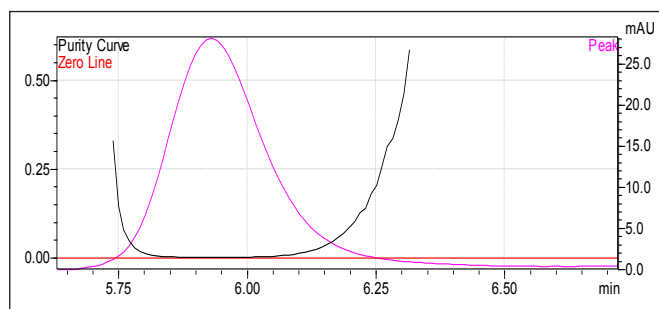Spectrum of besifloxacin HCl indicating peak purity is shown in Fig. 3.

Fig. 3: Peak purity

Details of impurity, peak purity index, single point threshold and minimum peak purity index of besifloxacin hydrochloride used for research study are mentioned in the Table IV.

Table IV: Peak purity

01.	Impurity	Not detected
02.	Peak purity index	0.999998
03.	Single point threshold	0.999881
04.	Minimum peak purity index	117

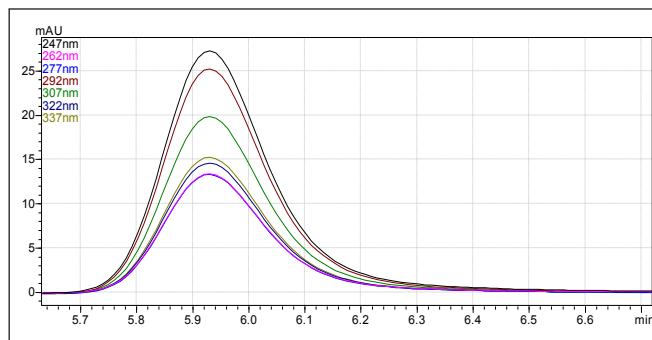Spectra of besifloxacin HCl indicating peak profile is shown in Fig. 4.

Fig. 4: Peak profile of besifloxacin hydrochloride

Final chromatographic condition follows

Preparation of buffer for mobile phase

3.4 g of potassium dihydrogen phosphate was weighed and dissolved in 1000 mL of water. Ortho phosphoric acid solution was used to adjust the pH between 3.0 ± 0.05 and was sonicated to dissolve the gas.

Preparation of mobile phase and diluent

Mobile phase buffer and methanol was prepared in the ratio of 40:60 V/V.

Details of optimized conditions of developed method of besifloxacin hydrochloride used for research study are mentioned in the Table V.

Table V: Conditions for method development

01.	Column	Kromasil ODS, C_{18} (250m, m × 4.6mm × 5μ)
02.	Optimized flow rate	1.0 mL minute^{-1}
03.	Temperature of a column oven	37 °C
04.	Run time	10 minutes
05.	Injection volume	10 μL
06.	Detection wavelength	292 nm
07.	Pump mode	Isocratic mode

Preparation of standard solutions

3 mg of BFHC standard was weighed and dissolved in 50 mL of mobile phase. Mobile phase mixed into it, to make up the volume 100 mL (Concentration of besifloxacin hydrochloride 30 μg mL^{-1}) and was sonicated to dissolve the gas.

Preparation of sample solutions

For drug substance (API)

3 mg of besifloxacin hydrochloride API was weighed and dissolved in 50 mL of mobile phase. Mobile phase mixed into it, to make up the volume 100 mL (Concentration of besifloxacin hydrochloride 30 µg mL^{-1}).

For drug formulation (ophthalmic suspension)

Transferred 0.5 mL of ophthalmic suspension (Equivalent 3 mg mL^{-1} besifloxacin hydrochloride) in 100 mL flask, mobile phase was added into it, to make up the volume 100 mL and mixed well (Concentration of besifloxacin hydrochloride 30 µg mL^{-1}).

Method validation

The experiments were carried out to verify the method's validity, and based on the results, the method's suitability for its intended use[19-20].

The following parameters were validated by the aforementioned analytical assay method;

1. System suitability / system precision
2. Specificity (diluent interference and forced degradation study)
3. Method precision
4. Accuracy
5. Detection Limit
6. Linearity
7. Range
8. Robustness

System suitability and system precision

System suitability testing is a vital component of the analytical method that entails injecting five replicated injections of standard solutions into the system, % RSD-
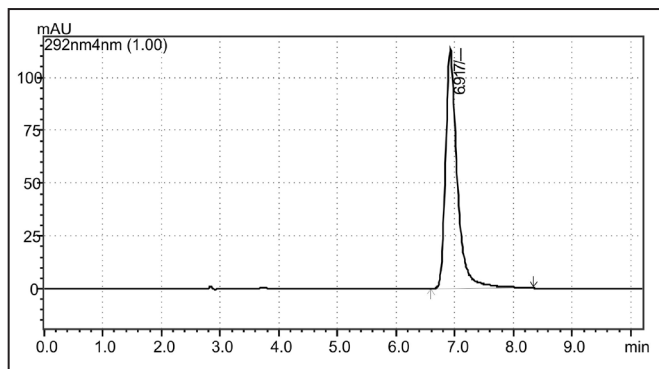
1.5 (should be less than 2.0), tailing factor-1.548 (should be less than 2.0) and plate count-6002.088 (should be more than 2000) of main peak. Chromatogram of besifloxacin HCl indicating, retention time for working standard is shown in Fig. 5.

Details of standard peak (retention time, peak area, theoretical plate and tailing factor) of besifloxacin hydrochloride used for research study are mentioned in Table VI.

Table VI: Standard peak besifloxacin hydrochloride

Sr. No.	Ret. time	Peak area	Theoretical plates	Tailing factor
01.	6.917	1214503	6002.088	1.548

Details of system suitability parameters (ret. time, USP resolution (Rs), theoretical plates, no. of theoretical plates (N), capacity factor (k' prime) and tailing factor (T)) of besifloxacin hydrochloride meeting criteria are mentioned in Table VII.

Table VII: System suitability parameters

Sr. No.	Parameter	Besifloxacin peak
01.	Retention time (R$_t$)	6.917 minutes
02.	USP resolution (Rs)	1.69
03.	Tailing factor (T)	1.548
04.	No. of theoretical plate (N)	6002.088
05.	Capacity factor (k' prime)	1.46

Specificity

At the retention time of the main peak from the diluent, no peaks were seen and all disturbances and peak purity cleared. Blank chromatogram indicating, zero interference as shown in Fig. 6.
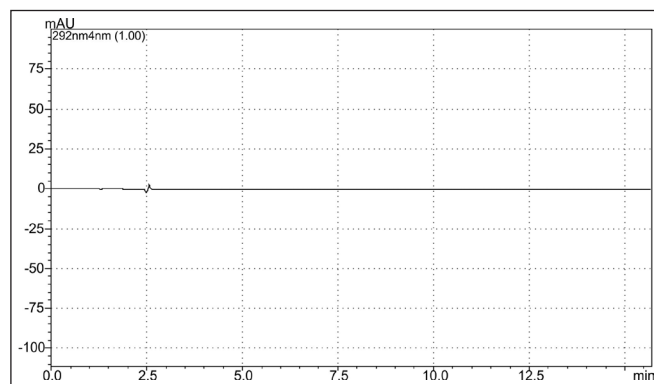

Fig. 5: Chromatogram for working standard of besifloxacin hydrochloride

Fig. 6: Blank chromatogram

Details of forced degradation study (acid stress, base stress, oxidation stress, thermal stress and U V stability) are mentioned in the Table VIII.

Table VIII: Details of forced degradation study

Sr. No	Forced degradation study conditions	
01.	Acid stress	Uni Normal HCl at 40 °C heat, for 30 minutes
		Uni Normal HCl at 40 °C heat, for 60 minutes
02.	Base stress	1/10 M NaOH for 30 minutes at 40 °C heat
		1/10 M NaOH for 45 minutes at 40°C heat
		1/10 M NaOH for 60 minutes at 40°C heat
		1/10 M NaOH for 1 h 30 minutes at 40°C heat
03.	Oxidation stress	3% H_2O_2, 40 °C heat, for 30 minutes
		3% H_2O_2, 40 °C heat, for 60 minutes
		3% H_2O_2, 40 °C heat, for 1 h 30 minutes
04.	Thermal stress	60 °C heat, for 30 minutes
		60 °C heat, for 60 minutes
05.	U V stability	U V chamber at 292 nm, for 24 h

Chromatogram indicating stability of besifloxacin hydrochloride for acid stress; 1 N HCl at 40 °C heat, for 30 minutes as shown in Fig. 7.
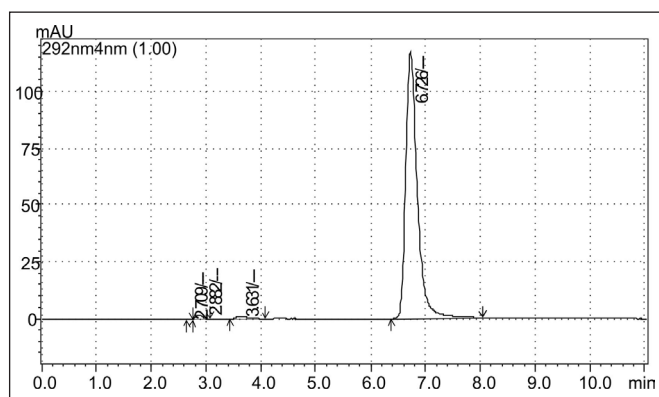

Fig. 7: Chromatogram for 1 N HCl at 40 °C, for 30 minutes

Details of acid stress study of besifloxacin hydrochloride; 1 N HCl at 40 °C, for 30 minutes are mentioned in Table IX.

Table IX: Peak retention times & peak areas for 1 N HCl at 40 °C, for 30 minutes

Sr. No.	Ret. time (Minutes)	Peak area
01.	2.709	1292
02.	2.882	12083
03.	3.631	11435
04.	6.726	1716296

Chromatogram indicating stability of besifloxacin hydrochloride for 1 N HCl at 40 °C heat, for 60 minutes is shown in Fig. 8.
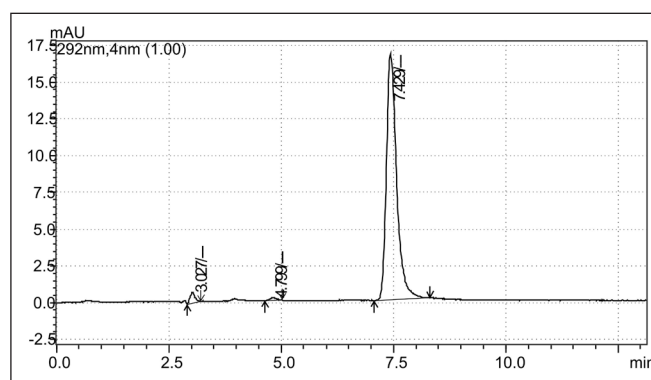

Fig. 8: Chromatogram for 1 N HCl at 40 °C, for 60 minutes

Details of acid stress study of besifloxacin hydrochloride; 1 N HCl at 40 °C, for 60 minutes are mentioned in Table X.

Table X: Peak retention times & peak areas for 1 N HCl at 40 °C, for 60 minutes

Sr. No.	Ret. time (Minutes)	Peak area
01.	3.027	5627
02.	4.799	1794
03.	7.429	1063903

Chromatogram indicating degradation (base stress) study, of besifloxacin hydrochloride; 30 minutes with 0.1 M NaOH at 40 °C is shown in Fig. 9.

Details of degradation (base stress) study of besifloxacin hydrochloride; 0.1 M NaOH at 40 °C, for 30 minutes are mentioned in Table XI.
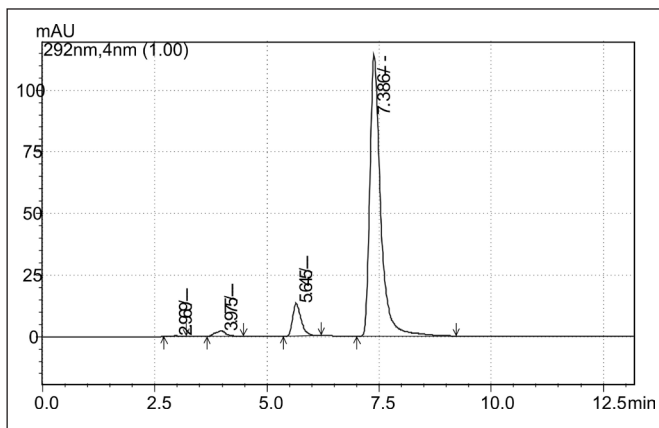
Fig. 9: Chromatogram for 30 minutes with 0.1 M NaOH at 40 °C

Table XI: Peak retention times and peak areas after 30 minutes of heating with 0.1 M NaOH at 40 °C

Sr. No.	Ret. time (Minutes)	Peak area
01.	2.969	3031
02.	3.975	34128
03.	5.645	175108
04.	7.386	1903301

Chromatogram indicating stability (base stress) of besifloxacin hydrochloride for 45 minutes with 0.1 M NaOH at 40 °C heat is shown in Fig. 10.
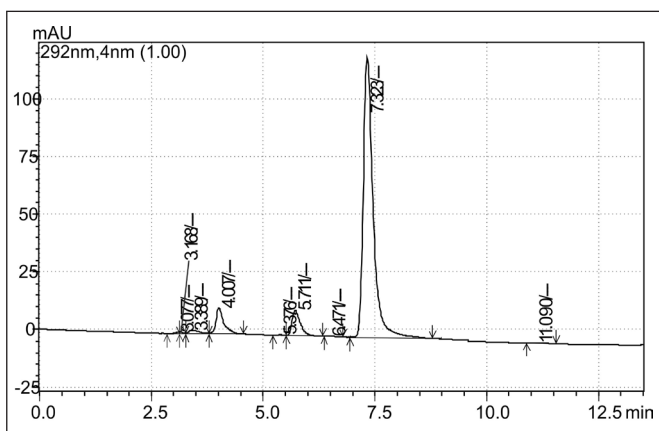

Fig. 10: Chromatogram for 45 minutes with 0.1 M NaOH at 40 °C heat

Details of degradation study (base stress) of besifloxacin hydrochloride; 0.1 M NaOH at 40 °C, for 45 minutes as mentioned in the Table XII.

Table XII: Peak retention times and peak areas after 45 minutes of heating with 0.1 M NaOH at 40 °C

Sr. No.	Ret. time (Minutes)	Peak area
01.	3.077	5050
02.	3.168	3324
03.	3.389	27111
04.	4.007	139000
05.	5.376	2969
06.	5.711	144091
07.	6.471	2567
08.	7.323	1874457
09.	11.090	5049

Chromatogram indicating stability (base stress) of besifloxacin hydrochloride for 45 minutes with 0.1 M NaOH at 40 °C heat is shown in Fig. 11.
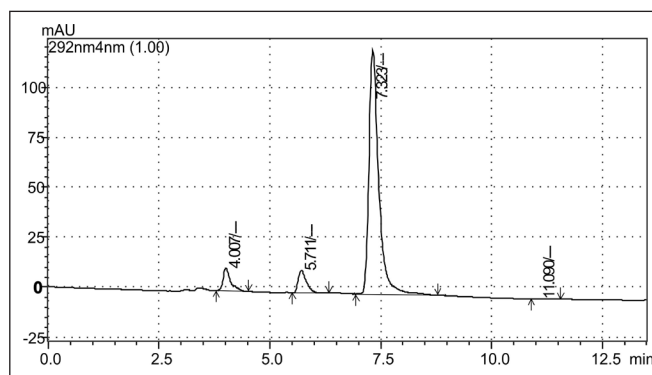

Fig. 11: Chromatogram for 60 minutes with 0.1 M NaOH at 40 °C heat

Details of degradation study (base stress) of besifloxacin hydrochloride; 0.1 M NaOH at 40 °C, for 60 minutes are mentioned in Table XIII.

Table XIII: Peak retention times and peak areas after 60 minutes of heating with 0.1 M NaOH at 40 °C

Sr. No.	Ret. Time (Minutes)	Peak area
01.	4.007	130284
02.	5.711	144091
03.	7.323	1174457
04.	11.090	5049

Chromatogram indicating stability (base stress) of besifloxacin hydrochloride for 1 h 30 minutes with 0.1 M NaOH at 40 °C is shown in Fig. 12.
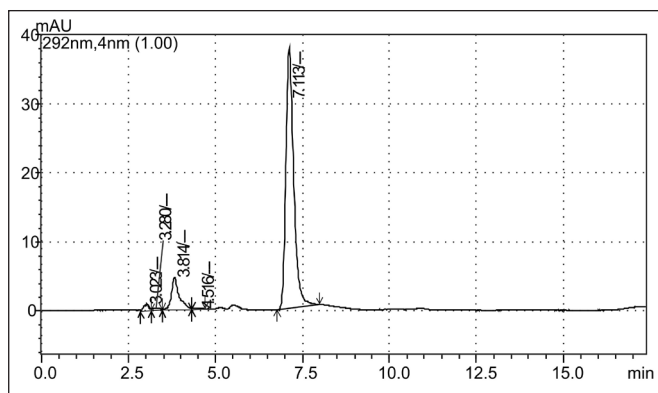
Fig. 12: Chromatogram for 1 h 30 minutes with 0.1 M NaOH at 40 °C heat

Details of degradation study (base stress) of besifloxacin hydrochloride; 0.1 M NaOH at 40 °C, for 1 h 30 minutes is mentioned in Table XIV.

Table XIV: Peak retention times and peak areas after 1 h 30 minutes of heating with 0.1 M NaOH at 40 °C

Sr. No.	Ret. time (Minutes)	Peak area
01.	3.023	10674
02.	3.280	6825
03.	4.516	74167
04.	7.274	2591
05.	7.113	601444

Chromatogram indicating stability (peroxide stress) of besifloxacin hydrochloride for 3% H_2O_2, 40 °C, for 30 minutes is shown in Fig. 13.
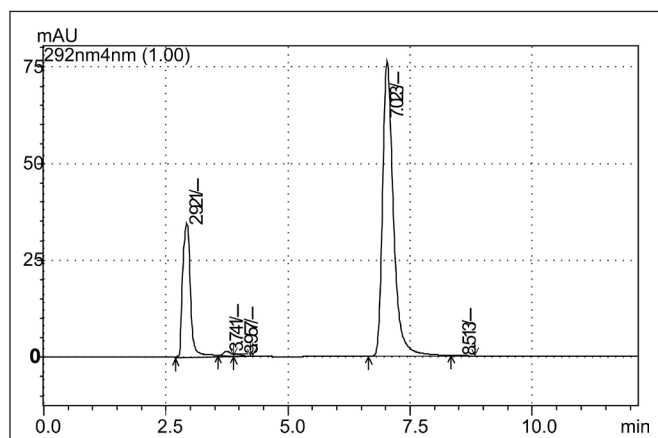

Fig. 13: Chromatograms for 3% H_2O_2, 40 °C, for 30 minutes

Details of forced degradation study (peroxide stress) of besifloxacin hydrochloride; 3% H_2O_2 at 40 °C, for 30 minutes are mentioned in the Table XV.

Table XV: Peak retention times & peak areas for 3% H_2O_2 at 40 °C, for 30 minutes

Sr. No.	Ret. time (Minutes)	Peak area
01.	2.921	383424
02.	3.741	13233
03.	3.957	5313
04.	7.023	1201894
05.	8.513	2736

Chromatogram indicating stability (peroxide stress) of besifloxacin hydrochloride for 3% H_2O_2, 40 °C, for 60 minutes is shown in Fig. 14.
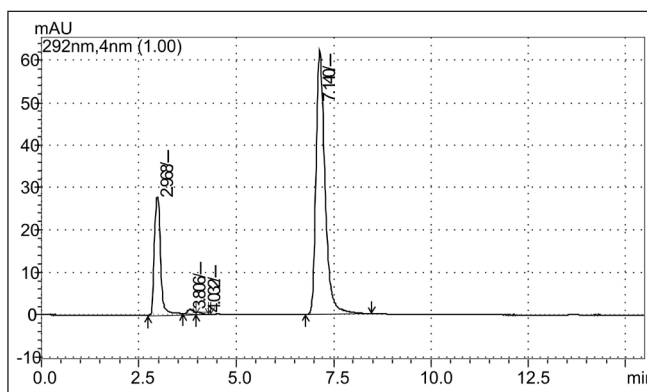

Fig. 14: Chromatograms for 3% H_2O_2, 40 °C, for 60 minutes

Details of forced degradation study of besifloxacin hydrochloride; 3% H_2O_2 at 40 °C, for 60 minutes are mentioned in the Table XVI.

Table XVI: Peak retention times & peak areas for 3% H_2O_2, 40 °C, for 60 minutes

Sr. No.	Retention Time (Minutes)	Peak Area
01.	2.968	323641
02.	3.806	11472
03.	4.032	4385
04.	7.140	985867

Chromatogram indicating stability (peroxide stress) of besifloxacin hydrochloride for 3% H_2O_2, 40 °C, for 1 h 30 minutes is shown in Fig.15.

Details of degradation study (peroxide stress) of besifloxacin hydrochloride; 3% H_2O_2 at 40 °C, for 1 h 30 minutes are mentioned in Table XVII.
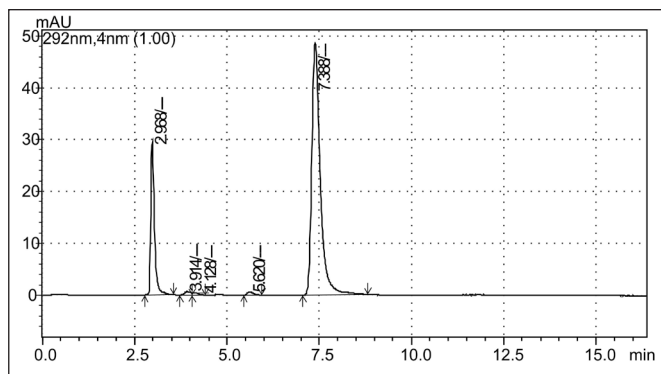
Fig. 15: Chromatogram for 3% H$_2$O$_2$, 40 °C, for 1 h 30 minutes

Table XVII: Peak retention times & peak areas for 3% H$_2$O$_2$, 40 °C, for 1 h 30 minutes

Sr. No	Ret. Time (Minutes)	Peak area
01.	2.968	208582
02.	3.914	7624
03.	4.128	4107
04.	5.620	7034
05.	7.388	773342

Chromatogram indicating stability (thermal stress) of besifloxacin hydrochloride for 60 °C heat, for 30 minutes is shown in Fig.16.

Fig. 16: Chromatogram for degradation at 60 °C, for 30 minutes

Details of degradation study (thermal stress) of besifloxacin hydrochloride; at 60 °C heat, for 30 minutes are mentioned in the Table XVIII.

Table XVIII: Peak retention times & peak areas at 60 °C heat, for 30 minutes

Sr. No.	Ret. Time (Minutes)	Peak area
01.	2.715	6312
02.	6.286	1630909

Chromatogram indicating stability (Thermal Stress) of besifloxacin hydrochloride; 60 °C heat & 60 minutes is shown in Fig. 17.
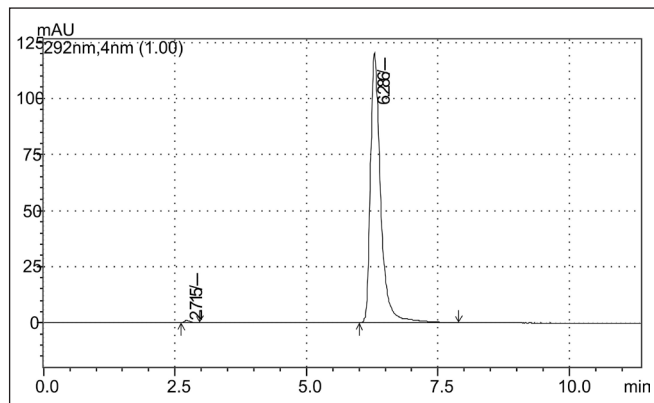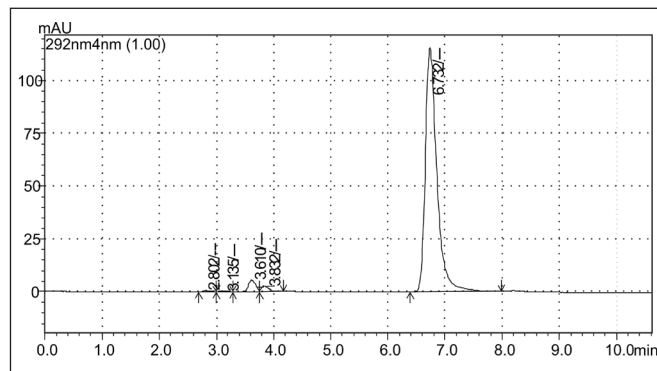

Fig. 17: Chromatogram for degradation at 60 °C, for 60 minutes

Details of degradation study (thermal stress) of besifloxacin hydrochloride; 60 °C heat, for 60 minutes are mentioned in Table XIX.

Table XIX: Peak retention times & peak areas for 60 °C, for 60 minutes

Sr. No.	Ret. Time (Minutes)	Peak area
01.	2.802	6229
02.	3.135	1620
03.	3.610	51359
04.	3.832	26987
05.	6.732	1043545

Chromatogram indicating stability (photolytic stress) of besifloxacin hydrochloride; U V chamber at 292 nm, for 24 h is shown in Fig. 18.
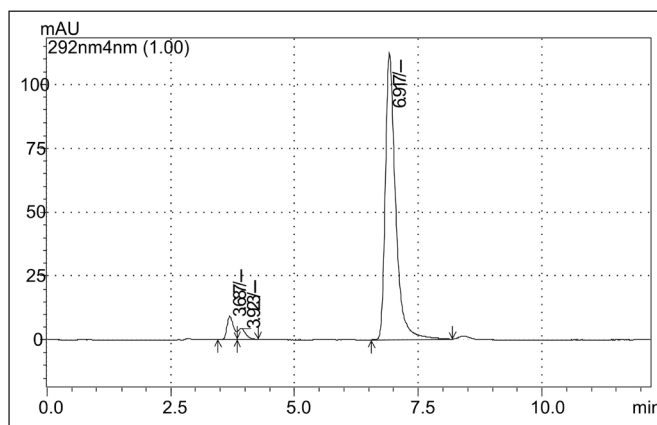

Fig. 18: Chromatogram for U V chamber at 292 nm, for 24 h

Details of degradation study (photolytic stress) of besifloxacin hydrochloride; UV chamber at 292 nm, for 24 h are mentioned in Table XX.

Table XX: Peak retention times & peak areas for U V chamber at 292 nm, for 24 h

Sr. No.	Ret. time (Minutes)	Peak Area
01.	3.687	86856
02.	3.923	46249
03.	6.917	9823638

Method precision

Process represents the closeness of scatter between the results. Six different sample solutions were prepared for API and ophthalmic suspension, calculated % Assay was well within acceptance criteria and % RSD was found to be less than 2.0.

Details of system suitability parameters of besifloxacin hydrochloride are mentioned in Table XXI.

Table XXI: System suitability parameters

Sr. No.	% Assay of API Sample	% Assay of ophthalmic suspension	
		B. No: 01A110	B. No: 1BA821
01.	101.5	101.5	101.5
02.	101.5	101.5	101.5
03.	101.5	101.5	101.5
04.	101.0	101.0	101.0
05.	101.0	101.0	101.0
06.	101.0	101.0	101.0
Mean	101.3	101.3	101.3
SD	0.274	0.274	0.274
% RSD	0.3	0.3	0.3

Table XXII: Accuracy (recovery) of besifloxacin hydrochloride

Compound	Recovery level (%)	Qty. spiked (µg mL^{-1})	Qty. recovered (µg mL^{-1})	Recovery (%)	RSD (%)
Besifloxacin	80	24.0	23.93	99.70	0.51
	100	30.0	29.77	99.23	0.83
	120	36.0	35.89	99.69	0.33

Table XXIII: Linearity, range and method sensitivity

Standard Conc. (µg mL^{-1})	10	20	30	40	50
Replicates	Peak area				
01.	409834	811668	1214503	1658337	2095172
02.	405321	809895	1213108	1638759	2100058
03.	402988	810082	1223247	1595237	2089764
04.	399896	810983	1216189	1667819	2077674
05.	410243	810334	1199876	1658971	2085764
Mean	405656	810592	1213384	1643824	2089686
SD	4441	728	8499	29164	8621
% RSD	1.094	0.0898	0.7004	1.777	0.4125

Accuracy

For getting accuracy, added known quantity of besifloxacin and calculated the recovery at 80%, 100% and 120% level.

Details of accuracy (recovery) of besifloxacin hydrochloride are mentioned in the Table XXII.

Linearity

Obtained test results are directly proportional to the quantity of determinands in the sample.

To check out the linearity range, serial dilutions of besifloxacin hydrochloride at 10 µg mL^{-1}, 20 µg mL^{-1}, 30 µg mL^{-1}, 40 µg mL^{-1} and 50 µg mL^{-1} respectively were used in mobile phase and injected.

Details of linearity, for range of 10 µg mL^{-1} to 50 µg mL^{-1} and method sensitivity of besifloxacin hydrochloride are mentioned in the Table XXIII.

Graph indicating linearity curve, value of R^2 and y of besifloxacin hydrochloride is shown in Fig. 19.
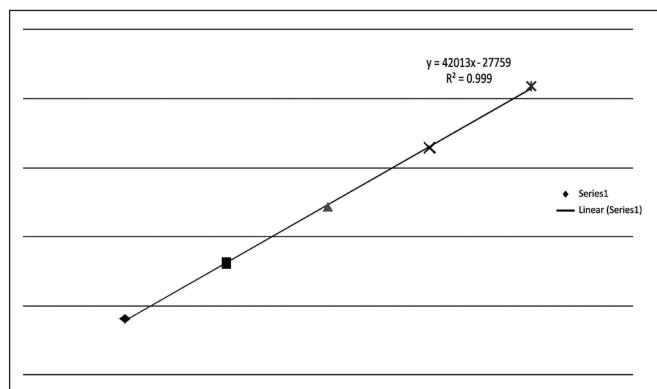

Fig. 19: Linearity curve

Detection limit (LOD)

During the practical lowest amount of analyte detected by, LOD = $(3.3 \times \sigma)/b$. Calculated values were confirmed by injection.

Range

A range for this method was established with adequate level of precision, accuracy, and linearity.

Robustness

Developed method is robust with respect to assess its propensity to persist unaltered by subtle but deliberate changes in the variables.

Details of robustness study of besifloxacin hydrochloride with respect to change in flow rate, Buffer pH, organic ratio, Column temperature and Wavelength on acceptance requirements of system suitability parameters (Tailing factor, Theoretical Plates, % RSD etc) as mentioned in the Table XXIV.

Table XXIV: Robustness study

Sr. No.	Parameter	System suitability criteria parameter			
		Tailing factor	Theoretical Plates	% RSD	Retention time (Minutes)
	Acceptance requirements	NMT 2.0	NLT 2000	NMT 2.0	
01.	Low flow rate (0.9 mL minute^{-1})	1.7	6742	0.2	6.914
02.	High flow rate (1.1 mL minute^{-1})	1.7	6524	0.1	6.911
03.	Low MP buffer pH 2.9	1.7	6524	0.1	6.917
04.	High MP buffer pH 3.1	1.7	6742	0.2	6.921
05.	Low organic ratio (45:55)	1.7	6517	0.1	6.919
06.	High organic ratio (65:35)	1.8	6642	0.7	6.916
07.	Low column temp. 30 °C	1.7	6516	0.1	6.915
08.	High column temp. 40 °C	1.7	6578	0.1	6.910
09.	Low wavelength 290 nm	1.7	6550	0.1	6.917
10.	High wavelength 293 nm	1.7	6550	0.6	6.916

CONCLUSION

Result of each individual method validation study meets its predefined acceptance criteria of ICH guidelines, which is described in respective study. It is determined that the analytical approach is precise, specific, linear, tough (rugged), and resilient (robust) based on the analytical data and findings of each investigation. As a result, the RP-HPLC method described above, which indicates stability, is appropriate for "Determination of assay of besifloxacin hydrochloride in drug substance (API) and drug product" (ophthalmic suspension). Due to the fact that all peaks are clearly separated, the procedure is very straightforward, quick, and economical, making it particularly appropriate for routine quality control analytical work.

REFERENCES

1. Tótoli E. G. and Salgado H. R. N .: Besifloxacin A Critical Review of Its Characteristics, Properties, and Analytical Methods, **Crit. Rev. Anal. Chem.,** 2018, 48(2), 132-142.

2. Khimdas S., Visscher K. L. and Hutnik C. M. L.: Besifloxacin Ophthalmic Suspension Emerging Evidence of its Therapeutic Value in Bacterial Conjunctivitis, **Ophthalmol. Eye Dis.,** 2011, 3, 7-12.

3. Karpecki P., Paterno M. R. and Comstock T. L.: Limitations of current antibiotics for the treatment of bacterial conjunctivitis, **Optom. Vis. Sci.,** 2010, 87(11), 908-919.

4. Saher O., Ghorab D. M. and Mursi N. M.: Levofloxacin hemihydrate ocular semi-sponges for topical treatment of bacterial conjunctivitis: formulation and *in vitro/in vivo* characterization, **J. Drug Deliv. Sci. Technol.,** 2016, 31, 22-34.

5. Deschênes J. and Blondeau J.: Besifloxacin in the management of bacterial infections of the ocular surface, **Can. J. Ophthalmol.,** 2015, 50(3), 184-191.

6. Arnalich F., Rodriguez A. E., Luque-Rio A. and Alio J. L.: Solid platelet rich plasma in corneal surgery, **Ophthalmol. Ther.,** 2016, 5(1), 31-45.

7. Cambau E., Matrat S., Pan X. S., Roth Dit Bettoni R., Corbel C., Aubry A., Lascols C., Driot J. Y. and Fisher L. M.: Target specificity of the new fluoroquinolone besifloxacin in *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Escherichia coli*, **J Antimicrob. Chemother.,** 2009, 63(3), 443-450.

8. Singh C. L., Singh A., Kumar S. and Majumdar D. K.: Besifloxacin The Fourth Generation Fluoroquinolone A Review, **J. Drug Deliv. Ther.,** 2014, 4 (6), 39-44.

9. Bhattacharjee A., Das P. J., Dey S., Nayak A. K., Roy P. K., Chakrabarti S., Marbaniang D., Das S. K., Ray S. and Chattopadhyay P. J. C.: Physicochemical S A Aspects E Development and optimization of besifloxacin hydrochloride loaded liposomal gel prepared by thin film hydration method using 32 full factorial design, **Colloids Surf. A Physicochem. Eng. Asp.,** 2020, 585, 124071.

10. Chang M. H. and Fung H. B.: Besifloxacin A topical fluoroquinolone for the treatment of bacterial conjunctivitis, **Clin. Ther.,** 2010, 32 (3), 454-471.

11. Askarkara S. S., Ganorkara A. V. and Gupta K. R. J. B. J. O. S.: Research T Design of Experiment Based Validated Stability Indicating Method for Determination of Besifloxacin Hydrochloride in Bulk Drug and Formulation, **Biomed. J. Sci. Technol. Res.,** 2018, 4 (4), 4011-4019.

12. Mah F. S.: Fourth-generation fluoroquinolones: new topical agents in the war on ocular bacterial infections, **Curr. Opin. Ophthalmol.,** 2004, 15 (4), 316-320.

13. Blondeau J. M.: Fluoroquinolones: mechanism of action, classification, and development of resistance, **Surv. Ophthalmol.,** 2004, 49 (2), S73-S78.

14. Tótoli E. G. and Salgado H. R. N. J. T.: Miniaturized turbidimetric assay: A green option for the analysis of besifloxacin in ophthalmic suspension, **Talanta,** 2020, 209, 120532.

15. Dey S., Patro S. S., Babu N. S., Murthy P. and Panda S.: Development and validation of a stability-indicating RP–HPLC method for estimation of atazanavir sulfate in bulk, **J. Pharm. Anal.,** 2017, 7 (2), 134-140.

16. Bandla J. and Ganapaty S.: Stability indicating RP-HPLC method development and validation for the simultaneous determination of Sofosbuvir and Velpatasvir in tablet dosage forms, **Indian J. Pharm. Biol. Res.,** 2017, 5 (04), 10-16.

17. Hadad G. M., Emara S. and Mahmoud W. M.: Development and Validation of a stability-indicating RP-HPLC method for the determination of paracetamol with dantrolene or/ and cetirizine and pseudoephedrine in two pharmaceutical dosage forms, **Talanta,** 2009, 79 (5), 1360-1367.

18. Karunakaran K., Navaneethan G. and Elango K.: Development and Validation of a stability-indicating RP-HPLC method for simultaneous determination of paracetamol, tramadol HCl and domperidone in a combined dosage form, **Trop. J. Pharm. Res.,** 2012, 11 (1), 99-106.

19. ICH Q2 (R1) Validation of Analytical Procedures: Text and Methodology, International Conference on Harmonization, IFPMA, Geneva, Switzerland 2005.

20. ICH Q2 (R2) Validation of Analytical Procedure: Methodology, International Conference on Harmonization, IFPMA, Geneva, Switzerland 2005.