OPEN ACCESS Freely available online

ISSN: 2161-1009
Biochemistry & Analytical Biochemistry

Research Article

Analytical Method Development and Validation for Determination of Assay of Antibacterial Drugs Besifloxacin Hydrochloride and Phenoxyethanol in Gel Formulation

Pradeep Kumar[1], Neelam Pawar[1*], Neha Minocha[2], Kavita Bahmani[3], Asha Poonia[4]

[1]Department of Pharmaceutical Sciences, University of Chaudhari Bansi Lal, Haryana, India;[2]School of Medical & Allied Sciences, K.R. Mangalam University, Haryana, India;[3]Department of Pharmaceutical Sciences, Guru Jambheshwar University of Science & Technology, Haryana, India;[4]Department of Life Sciences, University of Chaudhari Bansi Lal, Haryana, India

ABSTRACT

Aim: The aim of the present work is to develop an analytical method and validate it for determination of assay of an antibacterial dug in gel formulation.

Background: Analytical method validation is a process involving confirmation studies that procedure/method/system/analyst provides precise and reproducible outcome recognized by research laboratory studies that the performance features of the technique follows the necessities required for the analytical applications.

Objective: To improve the conditions and parameters which should be followed in the development and validation by developing a new sensitive and accurate RP-HPLC method? Validating the proposed newly developed methods in accordance with the analytical parameters mentioned in the IP, USP, BP and ICH guidelines.

Methods: HPLC method was validated to indicate that the analytical Procedure used is suitable for intended use by using various parameters like specificity, linearity, LOD, LOQ, precision, accuracy, range, robustness, stability in analytical solution and system suitability.

Results: The Retention times for Drug Besifloxacin standard were found to be 7.781 min and sample was at 7.731 respectively. The Area of standard besifloxacin was 1828547 and sample area was 1825315. The Assay of sample was 98%. The Retention times for Drug Phenoxyethanol standard were found to be 2.010 min and sample was at 2.004 respectively. The Area of standard Phenoxyethanol was 438025 and sample area was 438103. The Assay of sample was 97.04%. In System suitability, The RSD for 5 replicate injections for each peak is 0.33%. In specificity peaks of Diluent, Placebo and Impurities are not interfering with the Besifloxacin peaks. Peaks of Besifloxacin were found to be pure. Degradation products were found to be well separated from besifloxacin peak. The peak purity factor was NLT 0.9995.In precision study System Precision RSD of the Retention time for Besifloxacin obtained from six replicate injections 0.33%. The RSD of the Area of Besifloxacin obtained from six replicate injections is 0.46%. Method precision RSD was calculated on 6 determinations assay value of Drug besifloxacin is 0.56%. The RSD calculated on 6 determinations for assay value of Drug besifloxacin is 0.50%. In Intermediate precision RSD was calculated on 6 determinations for assay value of Drug besifloxacin is 0.50%. The RSD calculated on 12 determinations (Method precision and Intermediate precision) for assay value is 0.50%. Stability in analytical solution for the standard and sample, the area difference of besifloxacin peak were found to be within ± 2.0% from initial Linearity. The correlation coefficient and regression coefficient (R square) should be not less than 0.995 for Besifloxacin Correlation Coefficient is 0.998 Regression coefficient is 1.000. The % intercept should be within ± 5.0% of the response at 100% level Precision at 50% and 150% level: the RSD is 0.01%. Precision at 50% and 200% level: the RSD found to be NMT 2.0%. Accuracy mean % recovery at each level found to be between 98 to 101%

Correspondence to: Neelam Pawar, Department of Pharmaceutical Sciences, University of Chaudhari Bansi Lal, Haryana, India, Tel: +91-8529002677; E-mail: neelampawar5555@gmail.com

Received: 26-Feb-2022, Manuscript No. BABCR-22-15709; **Editor assigned:** 28-Feb-2022, Pre QC No. BABCR-22-15709 (PQ); **Reviewed:** 14-Mar-2022, QC No. BABCR-22-15709; **Revised:** 21-Mar-2022, Manuscript No. BABCR-22-15709 (R); **Published:** 28-Mar-2022, DOI: 10.35248/2161-1009.22.11.426

Citation: Kumar P, Pawar N, Minocha N, Bahmani K, Poonia A (2022) Analytical Method Development and Validation for Determination of Assay of Antibacterial Drugs Besifloxacin Hydrochloride and Phenoxyethanol in Gel Formulation. Biochem Anal Biochem. 11:426.

of drug besifloxacin. RSD on 9 (3 levels x^3) determinations is 1.2 which is following the criteria of NMT 2% Range. Correlation was found 1.0% for the accuracy and linearity parameters.

Conclusion: The recovery is in between 98% to 101% and the % RSD for all recovery values is 1.41% which is in limits. In HPLC method, the conditions were optimized to obtain an adequate separation of eluted compounds.

Keywords: Analytical method validation; Antibacterial; High performance liquid chromatography; Quantification,;Topical gel

INTRODUCTION

Analytical Method Validation is a process involving confirmation studies that procedure method/system/analyst provides precise and reproducible outcome recognized by research laboratory studies that the performance features of the technique follows the necessities required for the analytical applications. The composition of validated standards in terms of its analyte concentration and matrix used should be much relatable to the sample. High Performance Liquid chromatography is a best method to separate, analyze, and purify virtually any sample; and is used for chemical analysis. It is liquid chromatography in which the mobile phase and sample are pumped (up to 10000 psi) to affect quicker and superior resolution of drug in mixture [1]. Gels are defined as semi rigid systems in which the movement of the dispersing medium is restricted by an interlacing three-dimensional network of particles or solvated macromolecules of the dispersed phase. The word 'gel' is derived from 'gelatin' [2]. The interlinking of particles gelling agent forms a rigid network. Antibacterial agents are a group of materials that fight against pathogenic bacteria. They work either by killing microorganisms or stopping them from replicating, this allows the body's natural protection system to eradicate the pathogens. Acne vulgaris is multifactorial, a commensal skin bacteria (acnes) plays a chief role in the formation of acne lesion. It is a contamination of pilosebacious glands, oil glands in the skin [3]. The anti-bacterial drugs used in this research process were Besifloxacin hydrochloride and Phenoxyethanol and their properties (Table 1).

Table 1: Properties of besifloxacin hydrochloride and phenoxyethanol.

Property	Besifloxacin hydrochloride	Phenoxyethanol
Molecular formula	C19H22Cl2FN3O3	C8H10O2
Category	Broad spectrum fourth-generation fluoroquinolone antibiotic	Antimicrobial agent used as a preservative in cosmetics
Mechanism of action	Inhibits bacterial enzymes, DNA gyrase and topoisomerase IV; impairing cell division.	Phenoxyethanol has antibacterial properties and is effective against strains of pseudomonas aeruginosa.
Appearance	White to light brown powder	Colorless liquid with a pleasant odor
Solubility	Ethanol (<1 mg/ml at 25°C) and methanol (sparingly); DMSO: 2 mg/Ml	Miscible with acetone, with ethanol (95 per cent) and with glycerol; slightly soluble in water; Chloroform
Water solubility	0.143 mg/mL	24 g/L at 20°C
pKa (Strongest Acidic)	5.64 fg 0074	15.1
pKa (Strongest Basic)	9.67	-2.8

MATERIALS AND METHODS

Present research work was done in the Analytical Research and Development Department of Vyome Therapeutics Ltd., Delhi. Potassium dihydrogen phosphate and Ammonium dihydrogen phosphate were of grade AR/GR and bought from Merck. Acetonitrile, Triethylamine, Orthophosphoric acid, Methanol, Isopropyl Alcohol, Dichloromethane were of HPLC grade and procured from Merck. Dimethyl form amide (HPLC grade) was procured from Merck. Purified water was procured from Millipore and of Milli-Q grade. Vehicle gel and drug X gel 1% procured from of Vyome Therapeutics Ltd.

Method HPLC was then validated to specify that the analytical process used is appropriate for projected use by using several parameters like specificity, linearity, LOD, LOQ, accuracy, stability, precision, robustness and range in analytical solution and system suitability.

The RP-HPLC method was used for determination of assay and validation of anti-bacterial drug in gel formulation. Diluent was selected depending upon on the solubility of impurities, degradation products, starting materials, intermediates and the analyte. Selected diluent should be compatible with the mobile phase to get a better peak shape of analyte [4,5].

Experimental work

Diluent trial for preparation of sample and standard: Five trials were run by using *viz* water: acetonitrile (30:70), water: dimethyl formamide (50:50), methanol and dichloromethane (75:25), water: methanol: dichloromethane (25:50:25), water: methanol (50:50). Different standard stock solutions were prepared:

Standard solution A: (1 mg/ml of Besifloxacin): 27.0 mg of Besifloxacin hydrochloride working standard (equivalent 25.0 mg Besifloxacin) was weighed and transferred in a 25.0 ml of volumetric flask. 10 ml of diluent was added and dissolved by vortexing. Mixture is diluted by adding more diluent with proper mixing.

Standard solution B: (0.35 mg of Phenoxyethanol): 35.0 mg of Phenoxyethanol working standard was transfered in a 100 ml of volumetric flask. Drug was dissolved by adding 50 ml of diluent using vortexing. Mixture is diluted by adding more diluent with proper mixing.

Final standard solution: (0.1 mg of Besifloxacin and 0.035 mg of Phenoxyethanol): 5.0 ml of Solution A and 5.0 ml of Solution B were pipette out in a 50 ml of volumetric flask; to this 25 ml of diluent was added and diluted up to the mark with diluent with proper mixing.

Preparation of test solution: 1000 mg of VTL001 gel sample equivalent to 10 mg of standard was transferred into 100 ml volumetric flask and about 70 ml of diluent was added, vortexes for 5 minutes with recurrent trembling and sonication. Final volume was make-up with diluent. Further dilution of 5 ml test solution in to 10 ml volumetric flask was done. Solution was filtered through 0.45 µm PVDF (Poly Vinylidene Fluoride) syringe filter after discarding 1 ml of filtrate (Table 2) [6,7].

Table 2: Description of trials for mobile phase.

Description	Trial 1	Trial 2	Trial 3
Buffer Solution	1.36 gm potassium dihydrogen phosphate in 1 lt water	5 g m ammonium citrate in 1 lt water	
Mobile Phase	buffer solution: acetonitrile-55:45	buffer solution: acetonitrile-55:45	Mobile phase A-3.45 g ammonium dihydrogen phosphate, 1 ml trimethylamine and 500 ml water. Mobile Phase B-acetonitrile and methanol
Diluent	water: methanol-50:50 v/v	water: methanol-50:50 v/v	water: methanol-50:50 v/v
	Diluent is used as blank.	Diluent is used as blank.	Diluent is used as blank.

RP-HPLC method trials for mobile phase

In each trial, different solutions were injected into the system following the sequence of blank, standard solution, sample solution and bracketing standard with number of injections 1, 5, 2 and 1 respectively. Chromatogram was recorded for every trial.

For isocratic operation solvent used was mobile phase A: mobile phase B (600:400 ml) [7-9].

Assay determination method for validation of RP-HPLC

The following parameters were considered for the Analytical method validation of title ingredients [10].

- System suitability
- Initial assay
- Accuracy
- Precision
- Specificity
- Linearity and range
- Limit and quantification and detection
- Robustness and ruggedness.

(A) System suitability: To check that the analytical system is running appropriately and can provide accurate and precise results the system suitability parameters are to be set. Chromatographic conditions, mobile phase, diluent and standard preparation should be done as per test methodology as selected by above trials. For verify the system suitability standard was injected and results of 5 injections were checked respectively [11].

(B) Initial assay: Initial assay had been carried out 3 times by the same analyst with the same instrument and same column. The resolution between the standard drug was more than 2.0. The difference between individual assays was not more than 1.0% (RSD). The average of three-assay values was taken for further validation process. Repeatability was demonstrated by taking the RSD of retention time and AUC of chromatogram of 6 replicate injections of standard, which were computed and was within 1%. The average of three assay values was taken for further validation process [12-14].

- **Preparation of working standard stock solution:** 27 mg of standard drug was transmitted to 100 ml volumetric flask, 60 ml of diluent was poured and sonicated (maintain sonicator water temperature to 25 +2°C) for 5 minutes to thaw it completely and make up the volume with diluent and mix well [15].

- **Preparation of final working standard solution:** 5 ml of working standard stock solution was poured in to 50 ml volumetric flask and diluted with diluent.

- **Preparation of Test solution:** 1000 mg of VTL001 gel formulation sample equivalent was transferred to 10 mg of Besifloxacin into 100 ml volumetric flask and 70 ml of diluent was added, vortexed for 5 minutes with intermittent shaking and sonicated for 20 minutes. Final volume make up was done with diluent. Further dilution of 5 ml test solution in to 10 ml volumetric flask was done. The solution was filtered through 0.45 µm PVDF syringe filter after discarding 1 ml of filtrate [16].

(C) Precision: The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple sampling of homogeneous sample. The precision of analytical method is usually expressed as the standard deviation or relative standard deviation coefficient of variation of series of measurement. This had been validated by three types: System precision, Method precision and Intermediate precision. The system precision is checked by using standard Besifloxacin ensure that the analytical system is precise. The retention time and area of six determinations was measured and RSD was calculated. In method precision, a homogenous sample of a single batch should be analyzed six times. This indicates whether a method is giving consistent results for a single batch. The intermediate precision should be carried out to ensure that the analytical results will remain unaffected with change in instrument, analyst, and column and on different days. The method and intermediate precision was performed on Besifloxacin injection [17-19].

(D) Specificity: Photo Diode Array detector may be useful to show that analyte chromatographic peak is not attributable to more than one component. For the determination of specificity up slope similarity, down slope similarity, and 3-point peak purity should be estimated from the sample injection [20-23].

(E) Linearity and range: The linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration

of analyte in samples within a given range. Individually samples equivalent to 70%, 80%, 90%, 100%, 110%, 120% of the stated amount of sample were weighed individually and the assay was carried out. A graph of weight taken versus chromatographic area was plotted for both drug peaks. The regression line obtained was linear. From the data obtained, co-relation coefficient, slope and yintercept were calculated. Ideally co-relation coefficient should be around 1. Weight taken of the sample for 70%, 80%, 90%, 100%, 110% were 52.5 mg, 60 mg, 67.5 mg, 75 mg and 82.5 mg respectively.

For the preparation of standard stock solution: Weighed individual quantities of the sample were dissolved in diluents sonicate and volume made up to 50 ml with diluent.

For the preparation of stock solution B: 10 ml of the above solution was diluted to 50 ml with diluents [24-27].

(F) Detection limit: The Limit Of Detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected. The detection limit is determined by the analysis of sample with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

For standard dilution: 50 mg of Besifloxacin was added to 50 ml volumetric flask. The contents were dissolved using diluents, sonicate, later the volume was made up to 50 ml with diluent.

For sample dilution: 50 mg of the sample (B.NO: VTL001 vial) was weighed into a 50 ml volumetric flask. The contents were dissolved using diluents, sonicate, later the volume was made up to 50 ml with diluent.

For sample stock solution II: 10 ml filtrate of the above solution was diluted to 50 ml with diluents [28-31].

(G) Quantitation limit: Carried out in order to determine that lowest concentration of analyte which it can be estimated with acceptable precision, accuracy under the stated experimental conditions. For this the sample solution can be further diluted and the minimum concentration at which the sample can be reliably quantified should be found.

Standard dilution: 50 mg of Besifloxacin was weighed and transferred to 50 ml volumetric flask. The contents were dissolved using diluents and sonicate for 5 minutes at room temperature. Later the volume was made up to 50 ml using diluent.

Sample dilution: 50 mg of the sample (B.NO: VTL001 vial) was weighed into a 50 ml volumetric flask. The contents were dissolved using diluents, sonicate, later the volume was made up to 50 ml with distilled water.

Sample stock solution II, III, IV and V were prepared by dilution [32-34].

(H) Robustness and ruggedness: Robustness is the capability of method to remain unchanged by little intentional variations in method parameters. The variations may include change of organic solvent in mobile phase, pH of buffer in mobile phase, column oven temperature and flow rate. According to ICH, the robustness should be initiated in the early phase of method development. In addition, if the results are susceptible to variation in method parameters, the parameters should be controlled and the precautionary statement should include in the documentation of the method.

Determination of ruggedness: Carry out the assay as described above using different analyst and different instrument.

Different instrument: Test for ruggedness was performed on HPLC system VTL-LC-002

Different column: Determination of robustness was performed by use new column of same parameter as selected above [35-37].

(I) Accuracy: The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. The accuracy of an analytical method should be established across its range. Performed accuracy in three different levels for Drug Besifloxacin and spiked known quantity of Besifloxacin Standard at 50%, 100% and 150% level [38-41].

RESULTS AND DISCUSSION

Diluents trial for preparation of sample and standard

In trial 1, standard dissolved in the Water and Acetonitrile (50:50) but the gel formulation was found to be un dissolved. In trial 2, Standard and gel formulation both was found to be very Hazy in Water: Dimethylformide (50:50). In trial 3, Standard dissolved in the Methanol and Dichloromethane (75:25) and almost the gel formulation was dissolved but some un dissolved little lumps of gel components were observed to be suspended in sample. In trial 4, standard and gel formulation both were un dissolved in water, methanol and dichloromethane (25:50:25). In trial 5, Standard and gel formulation both were dissolved in the water and methanol in 50:50.

RP-HPLC method trials

In trial 1, Equilibrate the HPLC system with the mobile phase until a steady baseline is obtained. Inject all solutions as per injection sequence into the chromatograph and record the chromatograms. Chromatogram of Besifloxacin and its symmetry were not good. And the chromatogram was found to be merging with the chromatograms of impurities. To increase the resolution, next trail was taken. In trial 2, chromatogram of besifloxacin and its symmetry were not good. The tailing factor found in this buffer is more than 2. To decrease the tailing, next trail was taken. In trial 3, chromatogram of Besifloxacin and its symmetry and peak shape were good. The chromatogram was found to be clearly separated with the chromatograms of impurities. The system suitability is passed in this trial. The method used in this trial is appropriate for Besifloxacin (Tables 3 and 4).

Table 3: Results of diluents trial for preparation of sample and standard.

Trial number	Observation
Trial 1	Standard dissolved in the diluent but the gel formulation was un dissolved.
Trial 2	Standard and sample gel formulation both was found to be very hazy in diluent.
Trial 3	Standard dissolved in the diluent and almost the gel formulation was dissolved but some un dissolved little lumps of gel components were observed to be suspended in sample.
Trial 4	Standard and gel formulation both were un dissolved in the diluent.
Trial 5	Standard and gel formulation both were dissolved in the diluent

Table 4: Results of RP-HPLC method trials.

Trial	Retention time of Besifloxacin	Retention time of Phenoxyethanol	Chromatogram appearance	Conclusion
Trial 1	9.12	4.3	Chromatogram was found to be merging with the chromatograms of impurities.	Next trial was taken to increase the resolution.
Trial 2	14.258	6.2	The tailing factor found in this buffer is more than 2.	Next trial was taken to decrease the tailing.
Trial 3	7.7	2.01	Chromatogram was good.	Appropriate for both the drugs

Chromatogram of blank, placebo, standard and sample are given as respectively (Figures 1-4). Assay of Besifloxacin was found to be 98% and assay of phenoxyethanol was found to be 97.4%. Information about the area and retention time of standards and sample (Table 5).

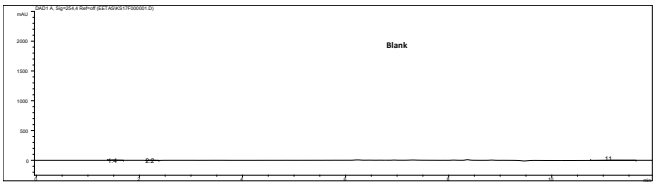

Figure 1: Chromatogram of blank sample.

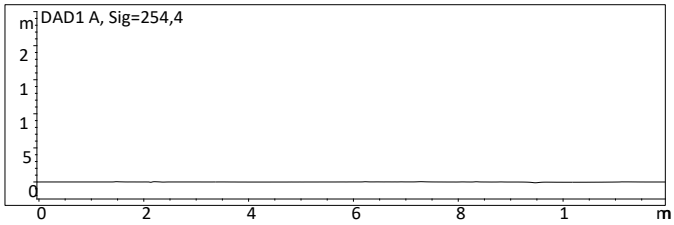

Figure 2: Chromatogram of placebo sample.

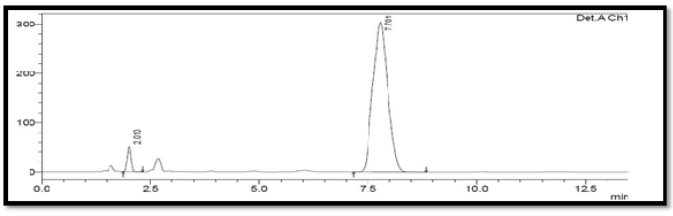

Figure 3: Chromatogram of standard sample.

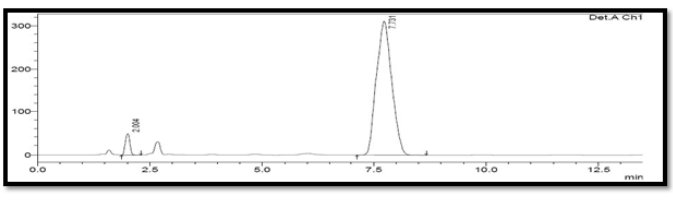

Figure 4: Chromatogram of sample.

Table 5: Area and retention time of standards and sample.

Area	Retention time
Besifloxacin standard-1828547	Besifloxacin standard-7.781
Sample-1825315	Sample-7.731
Phenoxyethanol standard-438025	Phenoxyethanol standard-2.010
Sample-438103	Sample-2.004

Validation of RP-HPLC

System suitability: Standard solutions of Besifloxacin and Phenoxyethanol were injected and standard area and retention time were noted for 5 replicated injections of samples; of which average standard area and retention time were calculated. Data generated with the sample runs are tabulated, which showed that system suitability parameter meets the requirement of method validation (Tables 6 and 7) (Figure 5).

Table 6: Data of standard solution of besifloxacin and phenoxyethanol.

Observation/Result	Besifloxacin		Phenoxyethanol	
S. No.	Standard area	Retention time	Standard area	Retention time
1	1828547	8.157	438034	2.022
2	1828918	8.004	438243	2.027
3	1829084	7.746	438103	2.027
4	1828556	7.986	438042	2.01
5	1825315	7.944	437560	2.019
Avg.	1828084	7.9	437996	2.019
RSD	0.1		0.1	
Tailing factor	1.2		1.1	
Column efficiency	5286		6103	
% Correlation	99.5		100.6	

Table 7: System suitability parameters.

Sr. No.	System suitability parameters	Results
1	Tailing factor for both drug peaks should be NMT 2%	1.20%
2	% Relative standard deviations should be not more than 2% for 5 replicate injections for each peak	0.10%

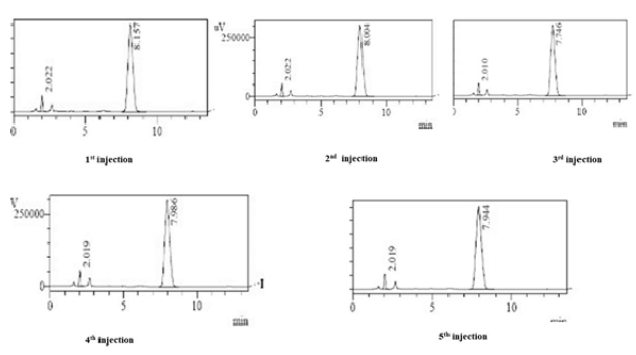

Figure 5: Chromatograms of samples for recording system suitability parameter.

Initial assay: Initial assay had been carried out 3 times to check RSD of Retention Time and Area of standard drug Besifloxacin and the results are tabulated, which showed that RSD of Retention Time and Area of six replicate is within 2%, which proves repeatability (Table 8).

Table 8: Area and retention time of standard drug besifloxacin.

S. No.	Besifloxacin		Phenoxyethanol	
	Standard area	Retention time	Standard area	Retention time
1	1828547	7.781	438030	2.022
2	1828918	7.817	438216	2.027
3	1829084	7.778	438106	2.027
4	1828556	7.806	438025	2.01
5	1825315	7.821	437568	2.019
6	1827876	7.879	434539	2.007
Avg.	1828049	7.813	437414	2.018
RSD	0.1		0.3259	

Chromatograms of 3 runs of initial assay with standard and sample initial assay 1, Initial assay 2 and Initial assay 3 are represented in respectively (Figures 6-8).

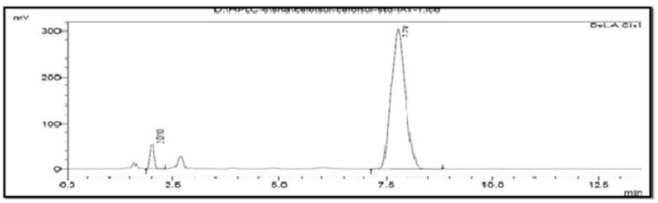

Figure 6: Chromatograms of initial assay 1.

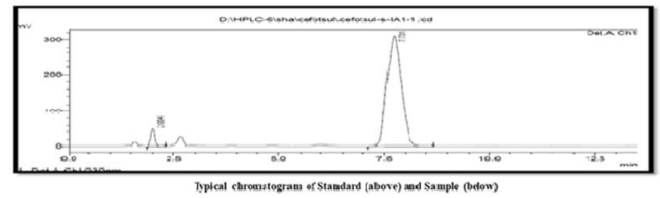

Figure 7: Chromatograms of initial assay 2.

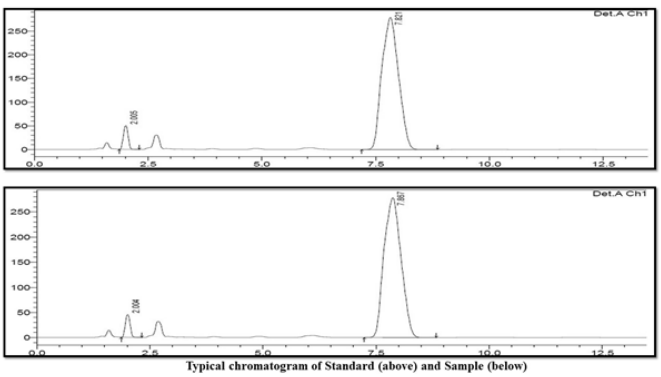

Figure 8: Chromatograms of initial assay 3.

The results of the three trials of Initial assay are summarized in, which showed that RSD of 3 initial Assays performed in different timings are within 2% and this complies with ICH guidelines and thus, initial assay parameter meets the requirement of method validation (Table 9).

Table 9: Summation of initial results (Repeatability).

Assay	Drug (Besifloxacin)
1	101.33%
2	101.65%
3	101.31%
Average	101.43%
% RSD	0.7048

Precision: Results obtained from system precision studies are tabulated in, concluding that retention time and area response are consistent as evidenced by the values of relative standard deviation and thus, system precision parameter meets the requirement of method validation (Table 10).

Table 10: Results of injecting besifloxacin.

Injection No.	Drug Besifloxacin	
	Area	Retention time (min.)
1	1828237	7.781
2	1828920	7.817
3	1829087	7.778
4	1828556	7.806
5	1825252	7.821
6	1827456	7.879
Mean	1827918	7.813
% RSD	0.07	0.4695

Results of method precision and intermediate precision are tabulated in Table 11 collectively. Results of Table 10 and concluded that method is precise and rugged. Chromatograms of 6 samples of precision are presented (Table 11) (Figures 9 and 10).

Table 11: Data interpretation of method precision and intermediate precision.

S. No.	Method precision	Intermediate precision
	Percentage of drug Besifloxacin	Percentage of drug Besifloxacin
1	101.6	99.06
2	101.5	99.85
3	100.41	100.41
4	100.05	100.05
5	99.48	99.48
6	99.63	99.63
Mean	99.75	99.75
% RSD	0.5679	0.5079

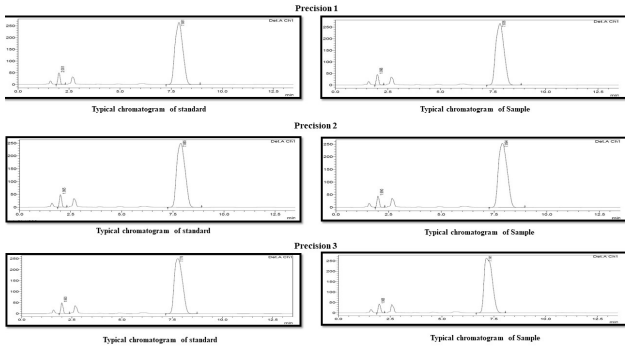

Figure 9: Chromatograms of first three precision assays.

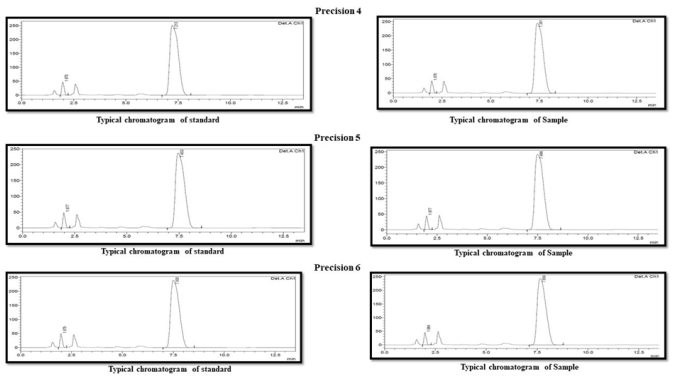

Figure 10: Chromatograms of fourth, fifth, sixth precision assays.

Specificity: Chromatogram of specificity is schematically represented in fig 10 and the values of peak point are tabulated (Table 12 and Figure 11).

Table 12: Three-point peak purity.

Peak	3 Point peak purity	Up slope similarity	Down slope similarity
Besifloxacin	0.99998	0.99997	0.99999

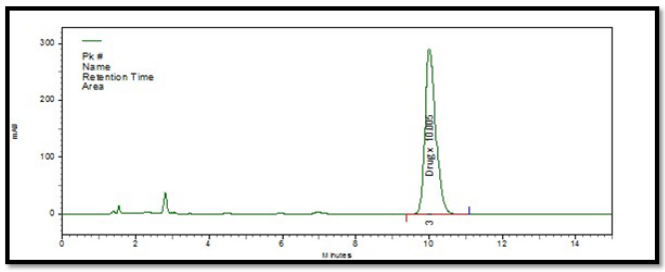

Figure 11: Typical chromatogram of peak purity.

Linearity and range: A Graph of concentration versus chromatographic response (area) is plotted. From the graph it can be seen that the correlation co-efficient is almost equal to 1 and regression line obtained is linear the linearity plot concentration (Figure 12).

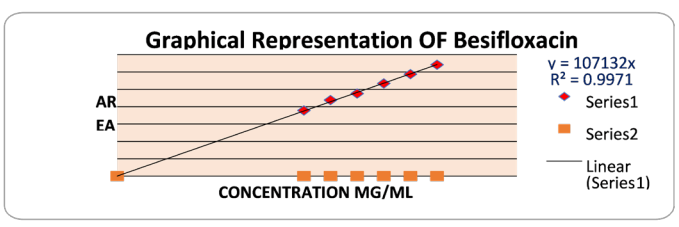

Figure 12: Linearity plot concentration of besifloxacinvs area. Note:(◆) Series1, (■)Series2.

Detection limit: Chromatograms of the detection limit. Here Besifloxacin cannot be detected because the Signal vs. Noise Ratio (S/N) should be 3/1 but here the area of signal is similar to area of noise (Figure 13).

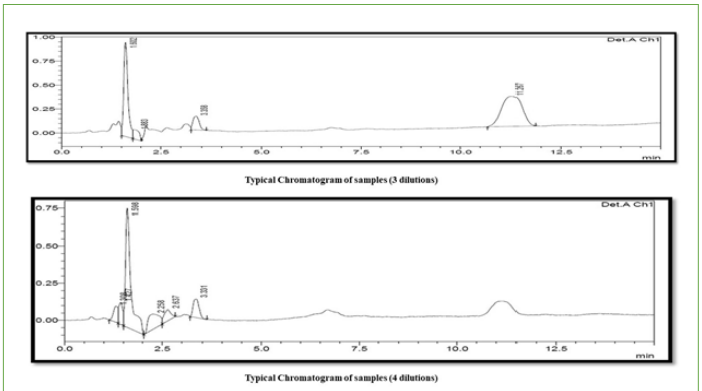

Figure 13: Chromatograms of detection limit.

From it can be seen that lowest concentration (0.058 µg/ml) of an analyte in a sample can be detected. But it couldn't be quantified (Table 13).

Table 13: Area for limit of detection of besifloxacin after 5 times dilution.

S. No.	Standard Besifloxacin area	Test Area after 5 times diluted Besifloxacin area
1	6932439	234200
2	6923691	233760
Average	6928065	233980

Quantitation limit: Chromatograms of quantitation limit test and assay of Besifloxacin is tabulated. From the above data it can be seen that sample recovery of Besifloxacin is 100.74%. Hence sample concentration up to 117 µg/ml can be quantified with the acceptable accuracy (Figure 14 and Table 14).

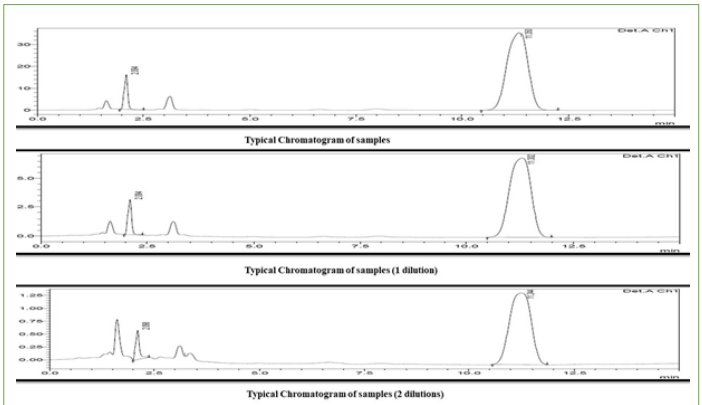

Figure 14: Chromatograms for quantitation limit.

Table 14: Results of assay of besifloxacin for quantitation test.

S. No.	Standard Besifloxacin area	Test Besifloxacin area	Assay
1	5812174	5822721	100.74%
2	5811658	5836507	99.44%
Average	5811916	5829614	100.10%

Robustness and ruggedness: Chromatogram of robustness, which was carried out by assay using different column and assay

of Besifloxacin is tabulated in from the above results, it can be concluded that the recovery is well within the limit collectively showed the data of robustness and ruggedness (Figure 15) (Tables 15 and 16).

Accuracy: Accuracy testing showed % RSD for all recovery values is 1.31%. There is clear difference between the assay carried out under different conditions and that of Accuracy is within ± 2%. This proves that lack of influence operational and environmental variables does not effect on the test results by using this method (Table 17).

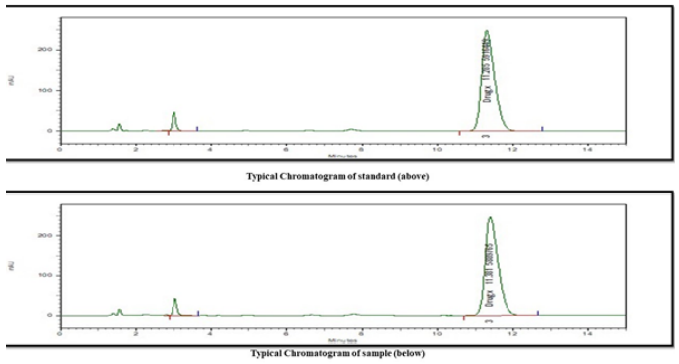

Figure 15: Chromatogram of standard and sample for robustness.

Table 15: Assay of besifloxacin by using different column.

S. No.	Standard Besifloxacin area	Test Besifloxacin area	Assay
1	5916449	5889765	99.60%
2	5942631	5996881	99.30%
Average	5926259	6008419	99.50%

Table 16: Results of robustness and ruggedness.

Condition	Assay Besifloxacin %	Difference with assay obtained in accuracy %
Different analyst	99.81	-1.67
Different instrument	100.02	-1.46
Different column	99.68	-1.8

Table 17: Accuracy data.

S. No.	Level in percentage	Amount added (ppm)	Amount recover (ppm)	% Recovery	Mean %	% RSD
1	50%	51.643	51.79	100.3	100.09	1.95
2	50%	51.643	50.62	98.04		
3	50%	51.643	52.64	101.93		
4	100%	103.28	102.32	99.07	100.03	1.07
5	100%	103.28	104.22	100.9		
6	100%	103.28	104.24	100.93		
7	150%	159.34	156.78	98.39	98.7	0.91
8	150%	159.34	158.95	99.75		
9	150%	159.34	156.25	98.06		

CONCLUSION

From the above results, it can be concluded that the recovery is well within the limit. Hence, the method is accurate. The recovery is in between 98% to 101% and the % RSD for all recovery values is 1.41% which is in limits. In HPLC method, the conditions were optimized to obtain an adequate separation of eluted compounds. Initially, various mobile phase compositions were tried, to separate title ingredients. Mobile phase and flow rate selection was based on peak parameters (height, tailing, theoretical plates, capacity or symmetry factor), run time, resolution. The system with Acetonitrile: methanol: buffer with 1.0 ml/min flow rate is quite robust. Since all the acceptance criteria's of the parameters selected for validation are satisfied, the method stands validated.

REFERENCES

1. Buszewski B, Dziubakiewicz E, Pomastowski P, Hrynkiewicz K, Ploszaj-Pyrek J. Analytical method development and validation: A concise review. J Anal Bioanal Tech. 2015;6(1):2.
2. Gupta V, Singh J. A novel drug delivery system: Floating microspheres in the development of gastroretentive drug delivery system. Rese J Topical Cosm Sci. 2021;12(2):86-92.
3. Keshari S, Kumar M, Balasubramaniam A, Chang TW, Tong Y, Huang CM. Prospects of acne vaccines targeting secreted virulence factors of Cutibacterium acnes. Expert Rev Vaccines. 2019;18(5):433-437.
4. Askarkara SS, Ganorkara AV, Gupta KR. Design of experiment based validated stability indicating method for determination of besifloxacin hydrochloride in bulk drug and formulation. Biomed J Sci Tech Res. 2018;4(4):4011-4019.
5. Jung KH. Continuous enzymatic synthesis of phenoxyethanol galactoside. Biotechnol J Bioindu. 2020;8:57-63.
6. Mahapatra A, Sreedhar C. HPLC and analytical method validation: A Review. Indian J Pharm Sci. 2018;5(1)1399-1414.
7. Hotha K, Phani S, Reddy K, Ravindranath LK. Forced degradation studies: Practical approach-overview of regulatory guidance and literature for the drug products and drug substances. Int Res J Pharm. 2013;4(5):78-85.
8. Sharma S, Wakode S. Quality by design approach for analytical method development. World J Pharm Pharm Sci. 2017;354-368.
9. Ravichandran V, Shalini S, Sundram KM, Harish R. Validation of analytical methods–strategies & importance. Int J Pharm Pharm Sci. 2010;2(3):18-22.
10. Ravisankar P, Navya CN, Pravallika D, Sri DN. A review on step-by-step analytical method validation. IOSR J Pharm. 2015;5(10):7-19.
11. Gupta V, Jain AD, Gill NS, Guptan K. Development and validation of HPLC method-a review. Int res J pharm Appl Sci. 2012;2(4):17-25.
12. Saimalakondaiah D, Kumar VR, Reddy TR, Ajitha A, Rao VU. Stability indicating HPLC method development and validation. Int. J. Pharma Res. Rev. 2014;3:46-57.
13. Kachave R, Mandlik P, Mundhe A. Stability indicating assay method for determination of Febuxostat by RP-HPLC. Cur Chin Chem. 2021.
14. Bhawani S, Nageshwari HG, Mamatha G, Venu M, Krishna SM, Krishna MK. Analytical method development and validation of Bendamustine in bulk using RP-HPLC. Pharm Res. 2018;2(2):158.
15. Sharma B, Singh LR. Pharmaceutical gels for topical drug delivery: An overview. Int J Pharm Pharm Sci. 2018;2:19-24.
16. Rathod HJ, Mehta DP. A review on pharmaceutical gel. Int J Pharm Res. 2015;1(1):33-47.
17. Gollnick H, Cunliffe W, Berson D, Dreno B, Finlay A, Leyden JJ, et al. Management of acne: A report from a global alliance to improve outcomes in acne. J Am Acad Dermatol. 2003;49(1):S1-37.
18. Bhate K, Williams HC. Epidemiology of acne vulgaris. Br J Dermatol. 2013;168(3):474-485.
19. TP A, Rajasekaran A. Forced degradation studies-a tool for determination of stability in pharmaceutical dosage forms. Int J boil Pharm res. 3(5):699-702.
20. Blessy MR, Patel RD, Prajapati PN, Agrawal YK. Development of forced degradation and stability indicating studies of drugs—A review. J Pharm Anal. 2014;4(3):159-165.
21. Indrani B, Basak S, Ghosh SK. Isolation and characterization of acid and base degradation products in atenolol and hydrochlorothiazide and a validated selective stability-indicating HPLC–UV method for their quantification. Int J Adv Pharm Res. 2015;5(2):36-41.
22. Atici EB, Karlıga B. Identification, synthesis and characterization of process related impurities of benidipine hydrochloride, stress-testing/stability studies and HPLC/UPLC method validations. J Pharm Anal. 2015;5(4):256-268.
23. Laha TK, Sen S. A Validated stability indicating reversed phase high performance liquid chromatographic method of leflunomide and characterization of its degradation products through retro-synthesis. J appl Pharm Sci. 2017;7(05):12-17.
24. Rao RN, Naidu CG, Prasad KG, Santhakumar B, Saida S. Development and validation of a stability indicating assay of doxofylline by RP-HPLC: ESI-MS/MS, 1H and 13C NMR spectroscopic characterization of degradation products and process related impurities. J Pharm Biomed Anal. 2013;78:92-99.
25. Ware AL, Pekamwar SS. Development and validation of bioanalytical uv-spectrophotometric method for determination of candesartan and development and validation of uv-spectrophotometric method for determination of candesartan in bulk drug and formulation. Asian J Pharm Anal. 2021;11(2).
26. Kaushik D, Bansal G. Four new degradation products of doxorubicin: An application of forced degradation study and hyphenated chromatographic techniques. J Pharm Anal. 2015;5(5):285-295.
27. Rao RN, Ramakrishna K, Sravan B, Santhakumar K. RP-HPLC separation and ESI-MS, 1H, and 13C NMR characterization of forced degradants including process related impurities of carisbamate: Method development and validation. J Pharm Biomed Anal. 2013;77:49-54.
28. Yang Y, Zhu X, Zhang F, Li W, Wu Y, Ding L. Stability-indicating HPLC method development and structural elucidation of novel degradation products in posaconazole injection by LC–TOF/MS, LC–MS/MS and NMR. J Pharm Biomed Anal. 2016;125:165-177.
29. Abbas SS, Elghobashy MR, Shokry RF, Bebawy LI. Stability indicating HPLC and spectrophotometric methods for the determination of bupropion hydrochloride in the presence of its alkaline degradates and related impurity. Bull Pharm. 2012;50(1):49-59.
30. Ramisetti NR, Kuntamukkala R, Lakshetti S, Sripadi P. Identification and characterization of stress degradants of lacosamide by LC–MS and ESI-Q-TOF-MS/MS: Development and validation of a stability indicating RP-HPLC method. J Pharm Biomed Anal. 2014;95:256-264.
31. Ye Q, Ding W, Rinaldi F, Huang Y, Miller SA, Bolgar M. Structural characterization of low level degradants in aztreonam injection and an innovative approach to aid HPLC method validation. J Pharm Biomed Anal. 2016;124:358-364.
32. Bakshi M, Ojha T, Singh S. Validated specific HPLC methods for determination of prazosin, terazosin and doxazosin in the presence of degradation products formed under ICH-recommended stress conditions. J Pharm Biomed Anal. 2004;34(1):19-26.

33. Sonune DP, Mone MK. Isolation, characterization of degradation products of sitagliptin and development of validated stability-indicating HPLC assay method for sitagliptin api and tablets. Int J Pharm Sci Res. 2013;4(9):3494.

34. Karmarkar S, Yang X, Garber R, Szajkovics A, Koberda M. Quality by design (QbD) based development and validation of an HPLC method for amiodarone hydrochloride and its impurities in the drug substance. J Pharm Biomed Anal. 2014;100:167-174.

35. Ganorkar SB, Dhumal DM, Shirkhedkar AA. Development and validation of simple RP-HPLC-PDA analytical protocol for zileuton assisted with Design of Experiments for robustness determination. Arab J Chem. 2017;10(2):273-282.

36. Inglot T, Mączka P, Rutkowska E. New HPLC method with experimental design and fluorescence detection for analytical study of antihypertensive mixture, amlodipine and valsartan. Am J Analyt Chem. 2013;4(1).

37. Cela R, Ordoñez EY, Quintana JB, Rodil R. Chemometric-assisted method development in reversed-phase liquid chromatography. J Chromatogr A. 2013;1287:2-2.

38. El-Gindy A, Emara S, Shaaban H. Development and validation of chemometrics-assisted spectrophotometric and liquid chromatographic methods for the simultaneous determination of two multicomponent mixtures containing bronchodilator drugs. J Pharm Biomed Anal. 2007;43(3):973-982.

39. Bhatt DA, Rane SI. QbD approach to analytical RPHPLC method development and its validation. Int J Pharm Pharm Sci. 2011;3(1):179-187.

40. Venkatesan S, Kannappan N, Mannemala SS. Stability-indicating HPLC method for the simultaneous determination of HIV tablet containing emtricitabine, tenofovir disoproxil fumarate, and rilpivirine hydrochloride in pharmaceutical dosage forms. Int Sch Res Notices. 2014;2014.

41. Kumar L, Reddy MS, Managuli RS, Pai G. Full factorial design for optimization, development and validation of HPLC method to determine valsartan in nanoparticles. Saudi Pharm J. 2015;23(5):549-555.