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Validation of Developed Analytical Method for Balofloxacin Floating Tablets by Reverse Phase High Performance Liquid Chromatography

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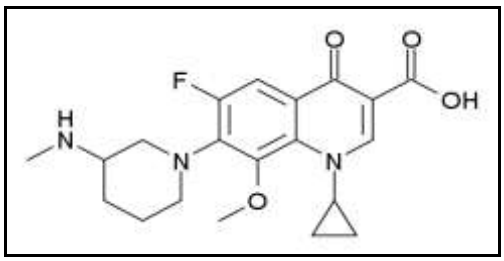
Abstract : Aim of the present investigation was to validate a new analytical, simple, sensitive, selective and precise High Performance Layer Chromatograpic (HPLC) method for the estimation of Balofloxacin in tablet dosage form. Balofloxacin chemically 1 cyclopropyl-6-fluoro-8-methoxy-7- (3-methylamino piperidine -1-yl)-4- oxoquinoline-3-carboxalic acid used as an Antibacterial agent. The mobile comprised of Methanol:water (350:650) and set at a flow rate of 1.2ml/minute. Detection was carried out at 293nm using pre-packed Symmetry C_{18}; 250x4.6mm, 5µm particle size column. The retention time of Balofloxacin was found to be 2.978. The assay was linear over concentration range of 12.5µg/ml to 75µg/ml (R=0.9998). The limit of detection and the limit of quantification were found to be 1.47µg/ml and 4.46µg/ml respectively. The amount of Balofloxacin was found to be 100.88±0.89 and the accuracy of Balofloxacin was found to be 99.60% to 101.60%. The statistical analysis of the data showed that the method is reproducible and selective for the estimation of Balofloxacin in tablet dosage form during routine analysis.
Keywords : Balofloxacin, RP-HPLC, Validation, Method development.

Introduction:

Balofloxacin chemically 1 cyclopropyl-6-fluoro-8-methoxy-7- (3-methylamino piperidine -1-yl)-4-oxoquinoline-3-carboxalic acid used as an Antibacterial agent. Fluoroquinolones inhibit the topoisomerase II ligase domain, leaving the two nuclease domains intact. This modification, coupled with the constant action of the topoisomerase II in the bacterial cell, leads to DNA fragmentation via the nucleasic activity of the intact enzyme domains. Recent evidence has shown eukaryotic topoisomerase II is also a target for a variety of quinolone-based drugs. Thus far, most of the compounds that show high activity against the eukaryotic type II enzyme contain aromatic substituents at their C-7 positions. Fluoroquinolones can enter cells easily via porins and, therefore, are often used to treat intracellular pathogens such as *Legionella pneumophila* and *Mycoplasma pneumoniae*. For many Gram-negative bacteria, DNA gyrase is the target, whereas topoisomerase IV is the target for many Gram-positive bacteria. Some compounds in this class have been shown to inhibit the synthesis of mitochondrial DNA. The literature survey [1-9] reveals that there is some Spectroscopic and HPLC methods have been reported. In this paper we describe a simple, inexpensive, sensitive and validated HPLC method for the determination of Balofloxacin in tablet dosage form.



Structure of Balofloxacin

Experimental Work:

Working standard of Balofloxacin, HPLC grade Methanol, Sodium hydroxide, 0.45µm PVDF membrane filter and Milli-Q water were procured from the market. The separation was carried out on isocratic HPLC system Shimadzu with UV detector with pre-packed Symmetry C18 250 x 4.6mm, 5.0µ m particle size using filtered and degassed mixture of Methanol:Water (350:650) as mobile phase.

Mobile phase preparation

Diluent:. Mix Methanol: Water in the ratio of 300:700

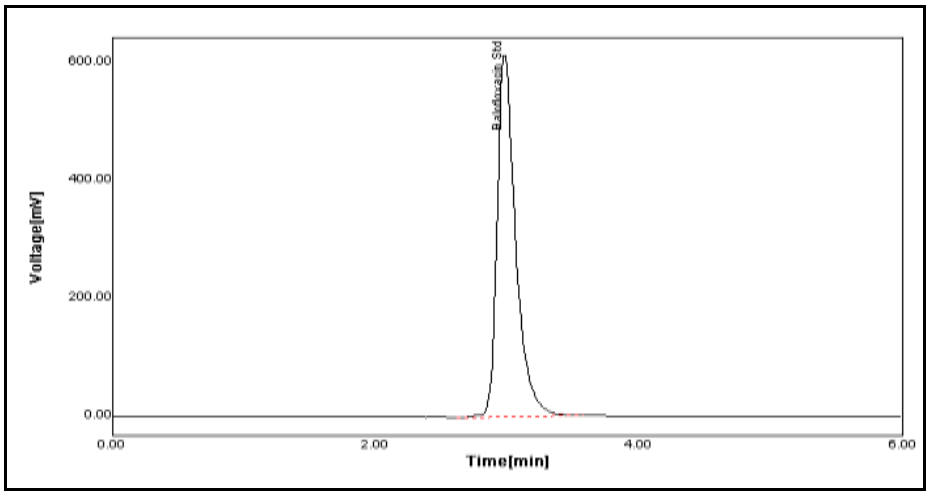
Mobile phase: Filtered and degassed mixture of Methanol: Water in the ratio of 350:650

Standard preparation: Weighed accurately about 0.100g of Balofloxacin working standard into a 100ml volumetric flask, added 70ml of diluent, shake and sonicated to dissolve the content, made up the volume with diluent. Pipetted out 5ml of resulting solution to 100ml volumetric flask made up with diluent. Filtered through 0.45 micron membrane filter. Collected the filtrate after discarding the few ml of the filtrate.

Assay preparation: Weighed 20 tablets, triturate to a fine powder. Weighed accurately about 0.300g powdered tablets (equivalent to 0.100g of Balofloxacin) in to a 100ml volumetric flask. Added 70ml of diluent shake for 15 minutes and sonicated for 15minutes, and made up the volume with diluent, pipetted out 5ml of filtrate to 100ml with diluent. Filtered the solution through 0.45micron membrane filter. Collected the filtrate after discarding the first few ml of the filtrate.

Chromatographic conditions: Flow rate 1.0ml/min; detection wavelength 293nm; injection volume 20µl; column used symmetry column (5µm, 250x4.6mm); column temperature: 25^{o}C; mobile phase: methanol:water (350:650).

Method development [10-22] : Working standard of various concentrations was prepared by taking aliquots of standard solution and diluted to get required concentration for calibration plot and which was injected.



Chromatogram of Balofloxacin

Validation of developed method [10-22]:

System Suitability Preparation: Weighed 20 tablets, triturate to a fine powder. Weighed accurately about 0.300g powdered tablets (equivalent to 0.100g of Balofloxacin) in to a 100ml volumetric flask. Added 70ml of diluent shake for 15 minutes and sonicated for 15minutes, and made up the volume with diluent, pipetted out 5ml of filtrate to 100ml with diluent. Filtered the solution through 0.45micron membrane filter. Collected the filtrate after discarding the first few ml of the filtrate.

Procedure: Separately injected equal volumes (about 20 µl) of the standard preparation and the assay preparation into the chromatograph, recorded the chromatograms, and measured the responses for the Balofloxacin peak.

Precision: To establish the precision of the analytical method by using the following two methods.

System Precision: Establish the repeatability of the analytical method by estimating the assay for six different sample preparations of the same batch. Calculate the assay for all six-sample preparations and report the %RSD for the same.

Preparation of Blank: Use diluent as blank**.**

Preparation of standard solution: Weighed accurately about 0.100g of Balofloxacin working standard into a 100ml volumetric flask, added 70ml of diluent, shacked and sonicated to dissolve the content, made up the volume with diluent. Pipetted out 5ml of resulting solution to 100ml volumetric flask made up with diluent. Filtered through 0.45 micron membrane filter. Collected the filtrate after discarding the few ml of the filtrate.

Preparation of sample solutions: Weighed 20 tablets, triturate to a fine powder. Weighed accurately about 0.300g powdered tablets (equivalent to 0.100g of Balofloxacin) in to a 100ml volumetric flask. Added 70ml of diluent shake for 15 minutes and sonicated for 15minutes, and made up the volume with diluent, pipetted out 5ml of filtrate to 100ml with diluent. Filtered the solution through 0.45micron membrane filter. Collected the filtrate after discarding the first few ml of the filtrate.

Procedure: Injected separately 20µl of blank, standard and sample preparations into the chromatograph and measured the peak responses for the major peak. Calculated the content of Balofloxacin in the individual solutions.

Intermediate precision (Ruggedness):

A different analyst using a different HPLC system with a different similar column on a different day should carry out this experiment.

Estimating the assay for six different sample preparations of the same batch. Calculate the assay for all six-sample preparations and report the %RSD for the same.

Blank preparation: Use diluent as blank.

Preparation of standard solution: : Weighed accurately about 0.100g of Balofloxacin working standard into a 100ml volumetric flask, added 70ml of diluent, shacked and sonicated to dissolve the content, made up the volume with diluent. Pipetted out 5ml of resulting solution to 100ml volumetric flask made up with diluent. Filtered through 0.45 micron membrane filter. Collected the filtrate after discarding the few ml of the filtrate.

Preparation of sample solutions: Weighed 20 tablets, triturate to a fine powder. Weighed accurately about 0.300g powdered tablets (equivalent to 0.100g of Balofloxacin) in to a 100ml volumetric flask. Added 70ml of diluent shake for 15 minutes and sonicated for 15minutes, and made up the volume with diluent, pipetted out 5ml of filtrate to 100ml with diluent. Filtered the solution through 0.45micron membrane filter. Collected the filtrate after discarding the first few ml of the filtrate.

Procedure: Separately injected 20µl of blank, standard and sample preparations into the chromatograph and measured the peak responses for the major peak. Calculated the content of Balofloxacin in the individual solutions

Linearity & Range

Objective: To establish the linearity of the analytical method for assay using the following two methods.

Linearity & range for Balofloxacin working standard: Demonstrate the linearity of the analytical method for assay by injecting the various concentrations of standard preparations prepared in the range of 25% to 150% into the chromatograph, covering 6 different concentrations. Draw a plot between the Concentrations vs. peak response of Balofloxacin. Report the slope, intercept and regression coefficient from the plot obtained for concentration vs. Peak response of Balofloxacin in standard preparation.

Preparation of analytical solutions for linearity & range for Balofloxacin standard preparations:

a) Blank preparation: Use diluent as blank.
b) Standard stock solution preparation: Transfer an accurately weighed quantity of about 100 mg of Balofloxacin working standard into 100 ml volumetric flask , add 20ml of diluent, sonicated for 10 minutes to dissolve and made to volume with mobile phase. From the stock solution 10 ml was pippeted out into the 100 ml volumetric flask and made to volume with mobile phase.
c) 25%Linearity Standard solution preparation (12.5 ppm): Pipette out 6.25 ml of Standard stock solution into 50 ml volumetric flaks and make up to volume with diluent.
d) 50%Linearity Standard solution preparation (25.0 ppm): Pipette out 12.50 ml of Standard stock solution into 50 ml volumetric flaks and make up to volume with diluent.
e) 75% Linearity Standard solution preparation (37.5 ppm): Pipette out 18.75 ml of Standard stock solution into 50 ml volumetric flaks and make up to volume with diluent.
f) 100% Linearity Standard solution preparation (50 ppm): Pipette out 25 ml of Standard stock solution into 50 ml volumetric flaks and make up to volume with diluent.
g) 125% Linearity Standard solution preparation (62.5 ppm): Pipette out 31.25 ml of Standard stock solution into 50 ml volumetric flaks and make up to volume with diluent.
h) 150% Linearity Standard solution preparation (75.0 ppm): Pipette out 37.5 ml of Standard stock solution into 50 ml volumetric flaks and make up to volume with diluent.

Calculations: Draw a plot between the concentration vs. the average peak responses of Balofloxacin peak for all the above studies. Calculate slope, intercept and regression coefficient from the plot obtained.

Accuracy / Recovery

Objective: To establish the accuracy of the analytical method is the closeness of sample results obtained by method to the true value by using recovery study.

Procedure: Perform the recovery studies by adding known quantity of Balofloxacin working standard to known quantity of placebo (Balofloxacin Tablet 100 mg excipient mixture) in the range of 50% to 150% of the sample concentration. Report the percentage recovery in relative standard deviation for all the values of % recovery.

a) Blank preparation: Use diluent as blank.
b) Standard preparation: Weighed accurately about 0.100g of Balofloxacin working standard into a 100ml volumetric flask, added 70ml of diluent, shacked and sonicated to dissolve the content, made up the volume with diluent. Pipetted out 5ml of resulting solution to 100ml volumetric flask made up with diluent. Filtered through 0.45 micron membrane filter. Collected the filtrate after discarding the few ml of the filtrate.
c) 50% recovery solution preparation: Weighed 20 tablets, triturate to a fine powder. Weighed accurately about 0.300g powdered tablets (equivalent to 0.100g of Balofloxacin) in to a 100ml volumetric flask containing 50 mg of Balofloxacin add 70ml of diluent. Sonicate for 10 minutes to dissolve and make up to the volume with diluent & mix. Filter through 0.45µ membrane filter. Diluted the above solution as 10ml to 50 ml with diluent. Repeat this procedure for another two sample preparations.
d) 100% recovery solution preparation: Weighed 20 tablets, triturate to a fine powder. Weighed accurately about 0.300g powdered tablets (equivalent to 0.100g of Balofloxacin) in to a 100ml volumetric flask containing 100 mg of Balofloxacin add 70ml of diluent. Sonicate for 10 minutes to dissolve and make up to the volume with diluent & mix. Filter through 0.45µ membrane filter. Diluted the above solution as 10ml to 50 ml with diluent. Repeat this procedure for another two sample preparations.

e) 150% recovery solution preparation: Weighed 20 tablets, triturate to a fine powder. Weighed accurately about 0.300g powdered tablets (equivalent to 0.100g of Balofloxacin) in to a 100ml volumetric flask containing 150 mg of Balofloxacin add 70ml of diluent. Sonicate for 10 minutes to dissolve and make up to the volume with diluent & mix. Filter through 0.45µ membrane filter. Diluted the above solution as 10ml to 50 ml with diluent. Repeat this procedure for another two sample preparations.

Procedure: Separately inject 20µl of standard and sample preparations of recovery solutions into the chromatograph and measure the peak responses for the major peak. Calculate the % recovery in recovery solutions using the following expression.

Recovery Calculation:

$$\text{Assay Percentage} = \frac{\text{peak area of spl} \times \text{wt of std} \times 5 \times 100 \times 100 \times 99.8 \times 100 \times \text{avg wt}}{\text{std area} \times 100 \times 100 \times \text{wt of sample} \times 5 \times 100 \times 100}$$

$$\% \text{ of recovery} = \frac{\text{mg of Balofloxacin working standard recovered}}{\text{mg of Balofloxacin working standard added}} \times 100$$

Stability of Analytical solutions

Objective : To establish the stability of analytical solutions by injecting the standard and sample solutions at periodic intervals up to 32hrs.

Preparation of analytical solutions

a) Blank preparation: Use diluent as blank.

b) Standard solution preparation: Weighed accurately about 0.100g of Balofloxacin working standard into a 100ml volumetric flask, added 70ml of diluent, shacked and sonicated to dissolve the content, made up the volume with diluent. Pipetted out 5ml of resulting solution to 100ml volumetric flask made up with diluent. Filtered through 0.45 micron membrane filter. Collected the filtrate after discarding the few ml of the filtrate.

c) Sample solution preparation: Weighed 20 tablets, triturate to a fine powder. Weighed accurately about 0.300g powdered tablets (equivalent to 0.100g of Balofloxacin) in to a 100ml volumetric flask. Added 70ml of diluent shake for 15 minutes and sonicated for 15minutes, and made up the volume with diluent, pipetted out 5ml of filtrate to 100ml with diluent. Filtered the solution through 0.45micron membrane filter. Collected the filtrate after discarding the first few ml of the filtrate.

Procedure: Inject 20µl of blank, resolution solution, standard preparation and sample preparations into the chromatograph and record the chromatograms. Measure the peak responses for major peak for all solutions. Continue the chromatography with periodic injections in duplicate for standard and sample preparations in the interval of 4hrs or suitable interval depending on the instrument utilization and sequence of injections.

Calculation: Calculate the average peak response and %RSD for initial 5 replicate injections of standard preparations. Calculate the %RSD for average peak responses of standard and sample preparations for periodical intervals.

Results and Discussion:

Table no 1: Data for system suitability

Injections	RT	Peak area	USP Plate count	USP Tailing
1	2.976	2526.5756	3552	1.35
2	2.988	2526.8732	3534	1.35
3	2.894	2530.3781	3535	1.34
4	3.041	2529.8461	3563	1.35
5	3.027	2527.4909	3554	1.33
6	2.944	2529.9548	3537	1.34
Mean	**2.978**	**2528.520**	**3545.833**	**1.343**
Std deviation	**0.054**	**1.722**	**12.123**	**0.008**
% RSD	**1.82**	**0.07**	**0.34**	**0.61**

The % RSD of all the parameters like retention time, area, theoretical plates and tailing factor was within the limit. So the method passes these system suitability parameters.

Table no 2: Data for System Precision

Injections	Retention Time	Peak Area
1	2.835	2514.1294
2	2.891	2514.9712
3	2.886	2517.4893
4	2.863	2514.6069
5	2.884	2517.9929
Mean	**2.872**	**2515.83794**
Std deviation	**0.023**	**1.77176**
% RSD	**0.599**	**0.07042**

The % RSD of five replicate injections of standard solution is within the specified acceptance criteria.

Table no 3: Data for Method Precision

Samples	Peak Area	Weight of sample	Assay	
			in mg	in %
1	2564.2756	256.25	101.99	102.0
2	2518.6352	256.32	100.14	100.1
3	2506.0352	256.35	99.63	99.6
4	2555.6316	256.25	101.64	101.6
5	2533.5845	256.22	100.78	100.8
6	2541.3588	256.27	101.07	101.07
Mean			**100.88**	**100.9**
Std deviation			**0.89**	**0.89**
% RSD			**0.88**	**0.88**

The assay values for Balofloxacin tablets 100mg obtained from six samples were found to be within the acceptance criteria. The RSD of assay values from 6 samples is not more than 2.0%. Therefore, the method is considered precise.

Table no 4: Data for Intermediate Precision

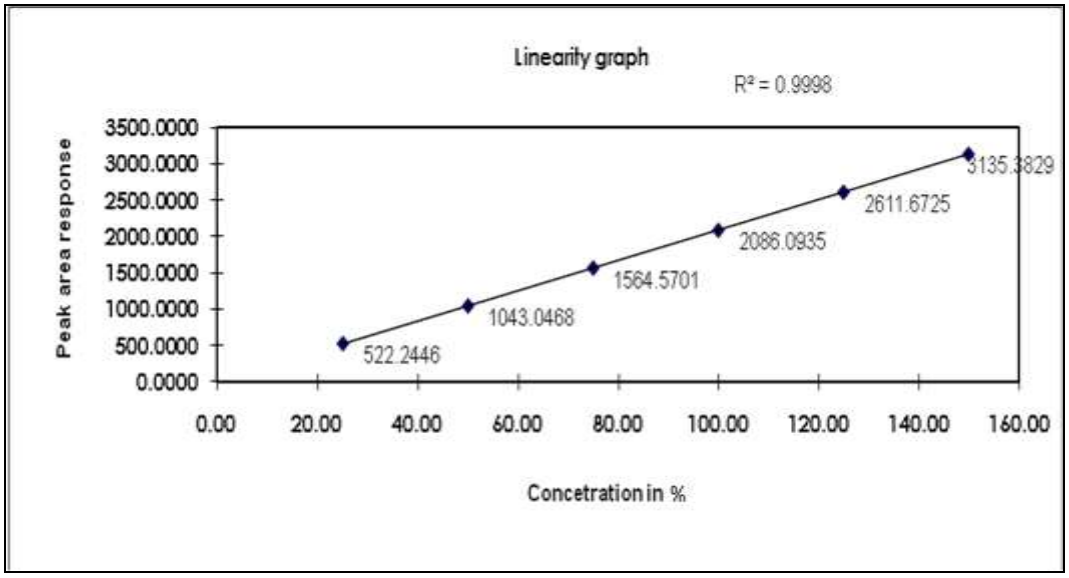
Day	Sample Inj	Peak Area	Weight Of Sample	Assay	
				in mg	in %
1	1	2541.2756	256.32	101.05	101.0
	2	2509.6352	256.27	99.81	99.8
2	3	2532.0352	256.22	100.72	100.7
	4	2525.6216	256.35	100.41	100.4
3	5	2545.5845	256.27	101.24	101.2
	6	2539.5388	256.21	101.02	101.02
Mean				**100.71**	**100.7**
Std deviation				**0.53**	**0.53**
% RSD				**0.52**	**0.52**

The assay values for Balofloxacin tablets 100mg obtained from six samples were found to be within the acceptance criteria. The RSD of assay values from 6 samples is not more than 2.0%. Therefore, the method is considered precise.

Table no 5: Linearity study for Balofloxacin

Sample No	%Level	Concentration (µg/ml)	Area
1	25	12.5	522.2446
2	50	25.0	1043.0468
3	75	37.5	1564.5701
4	100	50.0	2086.0935
5	125	62.5	2611.6725
6	150	75.0	3135.3829

Linearity curve:



Linearity Curve of Balofloxacin

Table No 6: Method accuracy study of Balofloxacin

Sample No	Theoretical (%)	Mean Peak area	Recovery		Mean (%) Recovery	%RSD
			In (mg)	In (%)		
1	50	3771.19409	49.90	99.8	100.4	0.50
2	50	3782.24135	50.34	100.7		
3	50	3781.88954	50.32	100.6		
1	100	5029.94238	99.93	99.9	101.6	1.49
2	100	5104.21035	102.88	102.9		
3	100	5082.22235	102.01	102.0		
1	150	6293.72315	150.16	100.1	99.6	0.50
2	150	6255.98853	148.66	99.1		
3	150	6273.36326	149.35	99.6		

The assay values for the range of recovery levels from 50%-150% of the Balofloxacin working concentration (25micron/ml) conform to the acceptance criteria. The percentage Balofloxacin recovered at each of the levels falls between 99.6%-101.6% and the %RSD of all determinations at each level was not more than 2.0% .therefore the method is considered accurate.

Table No 7: Data for LOD and LOQ

Sample No	%Level	Concentration (µg/ml)	AREA
1	25	12.5	522.2446
2	50	25.0	1043.0468
3	75	37.5	1564.5701
4	100	50.0	2086.0935
5	125	62.5	2611.6725
6	150	75.0	3135.3829
Slope			20.88
Standard Deviation			46.7707
Correlation co-efficient			0.9998
LOD			1.47 µg/ml
LOQ			4.46 µg/ml

The LOD and LOQ of the Balofloxacin was calculated from the following formula,

LOD = 3.3σ / S

Where the σ = the standard deviation of the response,

S = the slope of the calibration curve

LOQ = 10 σ / S

Where the σ = the standard deviation of the response,

S = the slope of the calibration curve

Table No 8: Data for standard solution stability

S. No.	Time In Hours	RT	Peak Area
1	0	2.967	2548.7249
2	4	2.906	2531.0928
3	8	3.023	2565.4893
4	12	2.898	2634.5463
5	16	2.924	2545.2384
Mean		**2.94**	**2565.0**
Std deviation		**0.05**	**40.75**
% RSD		**1.76**	**1.59**

The RSD of obtained standard area is not more than 2.0%. Therefore, the solution is considered stable.

Table no 9: Data for sample solution stability

S. No.	Time In Hours	RT	Peak Area
1	0	2.987	2532.7249
2	4	2.355	2532.7249
3	8	2.924	2587.3893
4	12	2.991	2623.2463
5	16	2.964	2553.5384
Mean		**2.84**	**2565.9**
Std deviation		**0.27**	**39.07**
% RSD		**9.66**	**1.52**

The RSD of obtained sample area is not more than 2.0%. Therefore, the solution is considered stable.

Summary and Conclusion:

The validated method was to quantitatively estimate the amount of Balofloxacin in Pharmaceutical tablet dosage form using HPLC method. The calibration curve for Balofloxacin was found to be linear in the range of 12.5µg/ml to 75µg/ml (r2=0.9998) indicating a good linearity. The percentage recovery of sample was found to 99.40 to 101.60 %w/w for Balofloxacin indicating the good accuracy of the method. To evaluate the validity and reproducibility of the method, known amount of pure drug was added to previously analysed samples and these samples were reanalysed by proposed method, the percentage recovery was found to be close to 100% for all the methods. The limit of detection and limit of quantification was done by using linearity data, slope and standard deviation of the linearity samples were found to 1.47µg/ml and 4.46µg/ml respectively. The % relative standard deviation (%RSD) values for method precision was 0.88% and system precision was 0.07% found to be less than 2% and so the method is said to be precise.

Table no 10: Data of validation parameters for Balofloxacin

Parameters	Balofloxacin
Specificity	No interference between blank, standard and sample peak
System Suitability	
Retention time	2.978
Peak Area	2528.5198
Theoretical Plates	3546
Tailing Factor	1.34

Precision	
System precision (% RSD)	0.07%
Method precision (% RSD)	0.88%
Intermediate Precision (% RSD)	0.52%
Linearity and Range	12.5 µg/ml to 75.0 µg/ml
Slope	20.88
Standard deviation	46.7707
Correlation co-efficient	0.9998
% Recovery	
50%	100.40%
100%	101.60%
150%	99.60%
LOD	1.47 µg/ml
LOQ	4.46 µg/ml
Solution Stability	
Standard (% RSD)	1.59%
Sample (% RSD)	1.52%

The developed RP-HPLC method is simple and selective for estimation of Balofloxacin in tablet dosage form was found to be accurate, rapid and sensitive. The values of coefficient of variance were satisfactory low and recovery was close to 100% indicating reproducibility of the method. The linearity was observed within limit hence method is linear.

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