



CODEN [USA]: IAJ PBB

ISSN : 2349-7750

INDO AMERICAN JOURNAL OF PHARMACEUTICAL SCIENCES

SJIF Impact Factor: 7.187

Available online at: <http://www.iajps.com>

Research Article

METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF LEVOFLOXACIN AND AZITHROMYCIN BY RP-HPLC IN TABLET DOSAGE FORM

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Article Received: January 2023

Accepted: February 2023

Published: March 2023

Abstract:

The aim of the study was method development and validation for simultaneous estimation of levofloxacin and azithromycin by rp-hplc in tablet dosage form. To evaluate system suitability parameters such as retention time, tailing factor and USP theoretical plate count, the mobile phase was allowed to flow through the column at a flow rate of 1.0 ml/min to equilibrate the column at ambient temperature. Analysis was carried out at 265 nm. Chromatographic separation was achieved by injecting a volume of 20 µL of standard into Agilent Zorbax C18, 250x4.6, 5µ, the mobile phase of composition Phosphate Buffer: Methanol (40:60 % v/v) was allowed to flow through the column at a flow rate of 1.0 ml per minute. The proposed method will be validated as per ICH guidelines. The objective of the proposed work is to develop a new, simple, sensitive, accurate and economical analytical method and validation for the Simultaneous estimation of Levofloxacin, Azithromycin in pharmaceutical dosage form by using HPLC. To validate the developed method in accordance with ICH guidelines for the intended analytical application i.e., to apply the proposed method for analysis of the drug in its dosage form.

Keywords: Levofloxacin, Azithromycin, Method Development, Validation, Accuracy, Precision.

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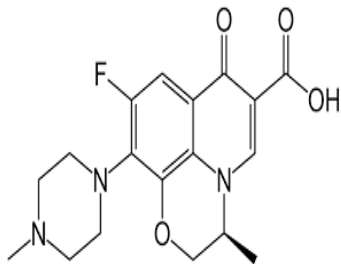
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Please cite this article in press Salgar pushpa et al, *Method Development And Validation For Simultaneous Estimation Of Levofloxacin And Azithromycin By RP-HPLC In Tablet Dosage Form.*, Indo Am. J. P. Sci, 2023; 10 (03).

INTRODUCTION:

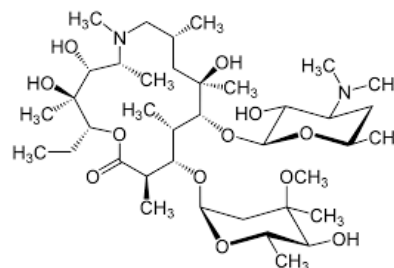
Levofloxacin is a fluoroquinolone antibiotic used to treat infections caused by susceptible bacteria of the upper respiratory tract, skin and skin structures, urinary tract, and prostate, as well as for post-exposure treatment of inhaled anthrax and the plague. Levofloxacin, like other fluoroquinolone antibiotics, exerts its antimicrobial activity via the inhibition of two key bacterial enzymes: DNA gyrase and topoisomerase IV. Both targets are type II topoisomerases, but have unique functions within the bacterial cell. DNA gyrase is an enzyme found only in bacteria that introduces negative supercoils into DNA during replication - this helps to relieve torsional strain caused by the introduction of positive supercoils during replication, and this negative supercoils are essential for chromosome condensation and the promotion of transcription initiation. It is comprised of four subunits (two A subunits and two B subunits) of which the A subunits appear to be the target of fluoroquinolone antibiotics. Bacterial topoisomerase IV, in addition to contributing to the relaxation of positive supercoils, is essential at the terminal stages of DNA replication and functions to "unlink" newly replicated chromosomes to allow for the completion of cell division.¹⁻³ IUPAC name (2S)-7-fluoro-2-methyl-6-(4-methylpiperazin-1-yl)-

**Figure 1: Structure of Levofloxacin**

The literature survey revealed that There are very few methods reported in the literature for analysis of Levofloxacin and Azithromycin alone or in combination with other drugs in the pure form and pharmaceuticals formulations⁸⁻¹⁷. In view of the need for a suitable, cost-effective HPLC method for routine analysis of Levofloxacin and Azithromycin Simultaneous estimation of in pharmaceutical dosage form. Attempts were made to develop simple, precise, accurate and cost-effective analytical method for the estimation of Levofloxacin and Azithromycin. The proposed method will be validated as per ICH guidelines. The objective of the proposed work is to develop a new, simple, sensitive, accurate and economical analytical method and validation for the Simultaneous estimation of Levofloxacin, Azithromycin in pharmaceutical dosage form by

10-oxo-4-oxa-1-azatricyclo[7.3.1.0[^]{5,13}]trideca-5(13),6,8,11-tetraene-11-carboxylic acid. Molecular Formula is C₁₈H₂₀FN₃O₄. Molecular Weight is 361.3.

Azithromycin is a macrolide antibiotic used to treat a variety of bacterial infections. In order to replicate, bacteria require a specific process of protein synthesis, enabled by ribosomal proteins. Azithromycin binds to the 23S rRNA of the bacterial 50S ribosomal subunit. It stops bacterial protein synthesis by inhibiting the transpeptidation/translocation step of protein synthesis and by inhibiting the assembly of the 50S ribosomal subunit Label. This results in the control of various bacterial infections, Label. The strong affinity of macrolides, including azithromycin, for bacterial ribosomes, is consistent with their broad-spectrum antibacterial activities.⁴⁻⁷ IUPAC name is 4-(dimethylamino)-3-hydroxy-6-methyloxan-2-yl]oxy}-2-ethyl-3,4,10-trihydroxy-13-[(2R,4R,5S,6S)-5-hydroxy-4-methoxy-4,6-dimethyloxan-2-yl]oxy}-3,5,6,8,10,12,14-heptamethyl-1-oxa-6-azacyclopentadecan-15-one. Molecular formula is C₃₈H₇₂N₂O₁₂. Molecular weight is 748.9 g/mol.

**Figure 2: Structure of Azithromycin**

using HPLC. To validate the developed method in accordance with ICH guidelines for the intended analytical application i.e., to apply the proposed method for analysis of the drug in its dosage form.

MATERIALS AND METHODS:**Chemicals and Reagents:**

Levofloxacin and Azithromycin were Purchased from Hetero drugs. NaH₂PO₄ was analytical grade supplied by Finerchem limited, Orthophosphoric acid (Merck), and Water and Methanol for HPLC (Lichrosolv (Merck).

Equipment and Chromatographic Conditions:

The chromatography was performed on a Waters 2695 HPLC system, equipped with an auto sampler, UV detector and Empower 2 software. Analysis was

carried out at 265 nm with column Agilent ZorbaxC18, 250x4.6, 5 μ . dimensions at 30 $^{\circ}$ C temperature. The optimized mobile phase consists of K₂HPO₄: Methanol (400:600). Flow rate was maintained at 1 ml/min.

Preparation of solutions:**Preparation of mobile phase:**

Transfer 500ml of HPLC water into 500ml of beaker and DiPotassium hydrogen phosphate adjust pH 3.5 using O-phosphoric acid. Transfer the above solution 400ml of K₂HPO₄, 600ml of Methanol is used as mobile phase. They are mixed and sonicated for 20min.

Preparation of the levofloxacin and azithromycin standard and sample solution:**Preparation of standard solution:**

Accurately weigh and transfer 100mg of Levofloxacin and Azithromycin into 100ml of volumetric flask and add 10ml of Methanol and sonicate 10min (or) shake 5min and make with water. Transfers the above solution into 5ml into 50ml volumetric flask dilute to volume with water.

Preparation of sample stock solution:

Commercially available 20 tablets were weighed and powdered the powdered equivalent to the 500mg of Levofloxacin and Azithromycin of active ingredients were transfer into a 100ml of volumetric flask and add 10ml of Methanol and sonicate 20min (or) shake 10min and makeup with water. Transfers above solution 5ml into 50ml of the volumetric flask dilute the volume with Methanol. And the solution was filtered through 0.45 μ m filter before injecting into HPLC system.

Sample preparation:

10 tablets were weighed and crushed, from the powdered tablets, weighed accurately about 500mg(500mg Levofloxacin and 500mg Azithromycin) into a 100 ml volumetric flask and 50 ml of mobile phase was added. The mixture was subjected to sonication for 20 min with intermediate shaking for complete extraction of drugs. Filtered and cooled to room temperature and solution was made

up to mark with mobile phase. From the above solution 5 mL is taken and further diluted in 25 ml volumetric flasks with mobile phase. To acquire a concentration of 500mg Levofloxacin and 500mg Azithromycin.

Standard preparation:

Accurately weighed quantity of 500mg Levofloxacin and 500mg Azithromycin was taken in a 100 ml volumetric flask and 50 ml of mobile phase was added. The mixture was subjected to sonication for 20 min with intermediate shaking for complete extraction of drugs. Filtered and cooled to room temperature and solution was made up to mark with mobile phase. From the above solution 5 ml is taken and further diluted in 25 mL volumetric flasks with mobile phase. To acquire a concentration of 500mg Levofloxacin and 500mg Azithromycin.

Procedure:

Separately injected both the standard (2 injections) and sample preparations (2 injections) into the chromatographic system and recorded the peak area responses.

RESULTS AND DISCUSSION:**METHOD:**

The developed chromatographic method was validated for system suitability, linearity accuracy, precision, ruggedness and robustness as per ICH guidelines.

System suitability parameters:

To evaluate system suitability parameters such as retention time, tailing factor and USP theoretical plate count, the mobile phase was allowed to flow through the column at a flow rate of 1.0 ml/min to equilibrate the column at ambient temperature. Chromatographic separation was achieved by injecting a volume of 20 μ L of standard into Agilent ZorbaxC18, 250x4.6, 5 μ , the mobile phase of composition Phosphate Buffer : Methanol (40:60 % v/v) was allowed to flow through the column at a flow rate of 1.0 ml per minute. Retention time, tailing factor and USP theoretical plate count of the developed method are shown in table 1.

Table 1: System suitability parameters

parameter	Levofloxacin	Azithromycin	Acceptance criteria
Retention time	3.154	3.436	+10
Theoretical plates	14206	11075	>2500
Tailing factor	1.35	1.55	<2.00
% RSD	0.3	0.2	<2.00

Assay of pharmaceutical formulation:

The proposed validated method was successfully applied to determine Levofloxacin and Azithromycin in their tablet dosage form. The result obtained for was comparable with the corresponding labeled amounts and they were shown in Table-2.

Table 2: Assay results for Levofloxacin and Azithromycin

	Label Claim (mg)	% Assay
Levofloxacin	100	99.1
Azithromycin	100	99.7

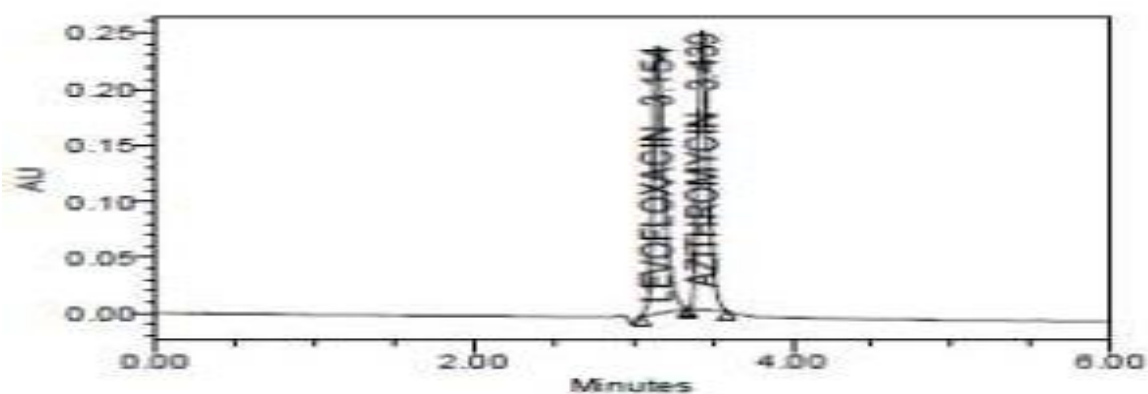


Figure 3: Standard chromatogram

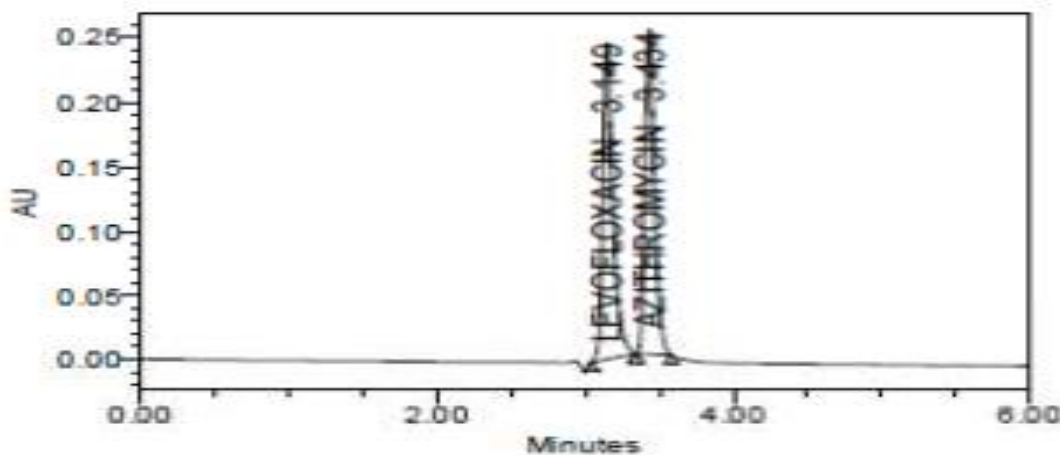


Figure 4: Sample chromatogram

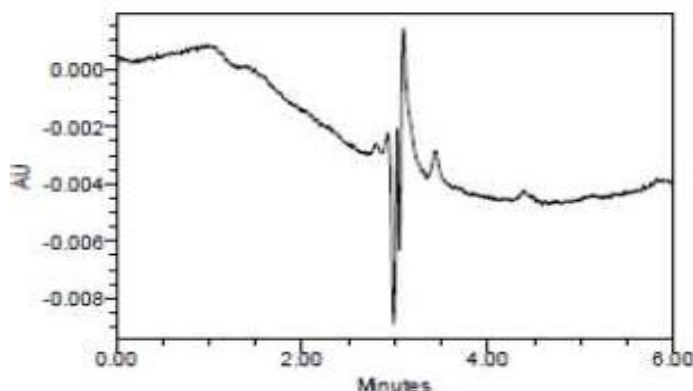


Figure 5: Blank chromatogram

Validation of Analytical method:

Linearity: The linearity study was performed for the concentration of 50 ppm to 150 ppm and 50 ppm to 150 ppm level. Each level was injected into chromatographic system. The area of each level was used for calculation of correlation coefficient. Inject each level into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient. The results are shown in table 3,4.

Table 3: Linearity results of Levofloxacin

s.no	Conc($\mu\text{g/ml}$)	RT	Area
1.	50	3.155	568388
2.	75	3.146	852253
3.	100	3.136	1131849
4.	125	3.139	1427500
5.	150	3.137	1700737
Std.dev			
Slope			
Intercept			
Correlation coefficient (r^2)			0.999

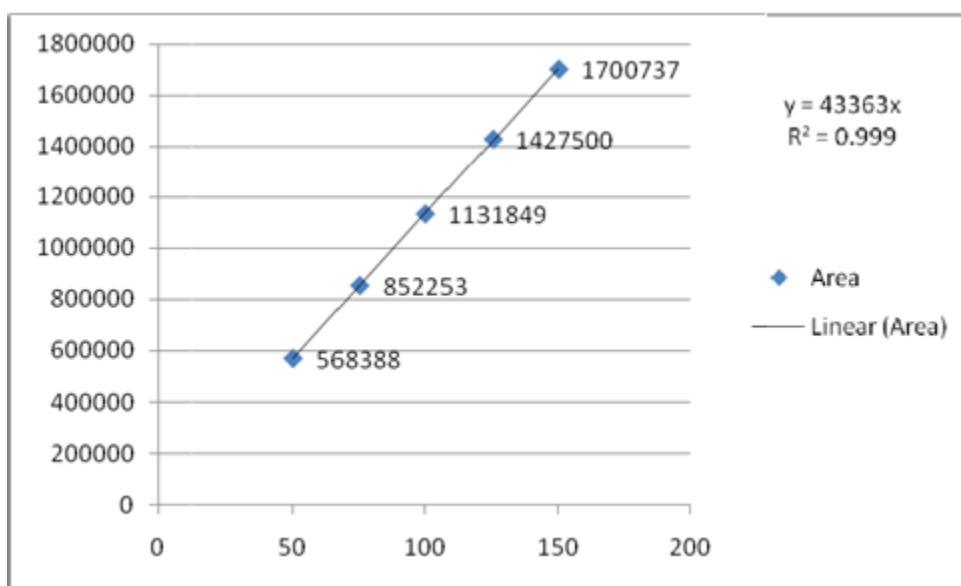
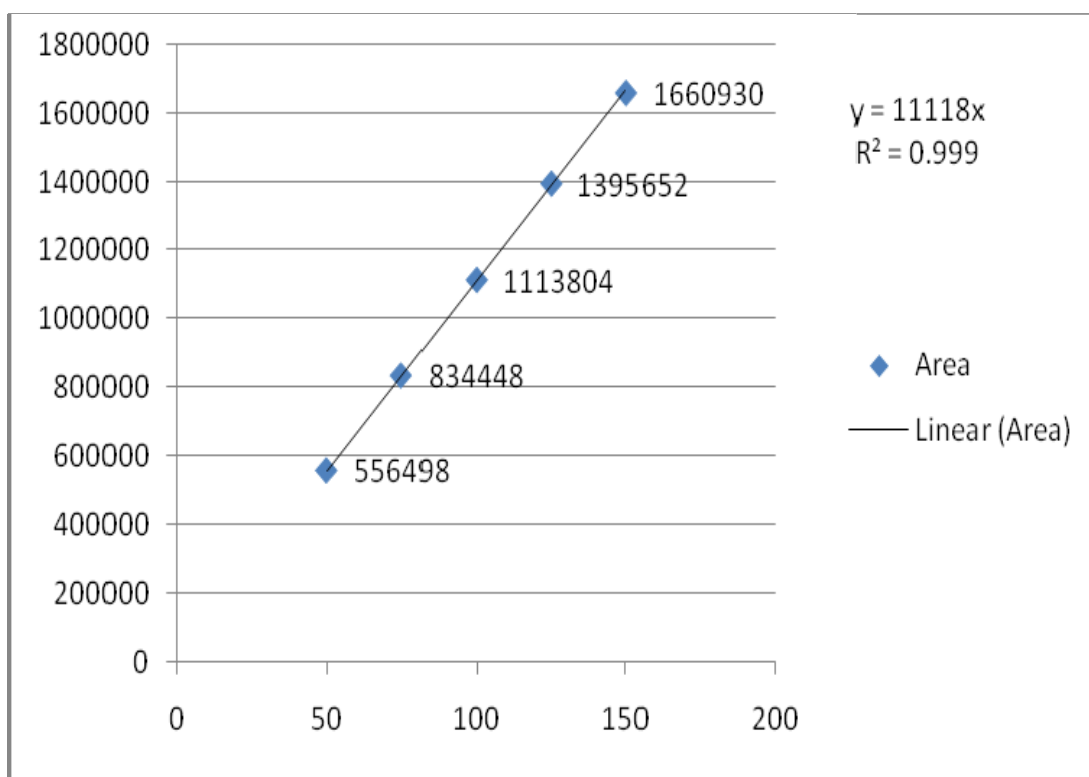


Figure 6: Linearity graph for Levofloxacin

Table 4: Linearity results of Azithromycin

s.no	Conc($\mu\text{g/ml}$)	RT	Area
1.	50	3.443	556498
2.	75	3.433	834448
3.	100	3.426	1113804
4.	125	3.431	1395652
5.	150	3.428	1660930
Std.dev			
Slope			
Intercept			
Correlation coefficient (r^2)			0.999

**Figure 6: Linearity graph for Azithromycin****Accuracy studies:**

The accuracy was determined by help of recovery study. The recovery method carried out at three level 50%, 100%, 150% and 50%, 100%, 150% Inject the standard solutions into chromatographic system. Calculate the Amount found and Amount added for Levofloxacin and Azithromycin and calculate the individual recovery and mean recovery values. The results are shown in table 5,6.

Table 5: Showing accuracy results for Levofloxacin

S.NO	Accuracy level	Sample name	Sample weight	µg/ml added	µg/ml found	% Recovery	% Mean
1	50%	1	691.50	49.500	49.53	100	100
		2	691.50	49.500	49.58	100	
		3	691.50	49.500	49.57	100	
		4	691.50	49.500	49.53	100	
		5	691.50	49.500	49.55	100	
		6	691.50	49.500	49.53	100	
2	100%	1	1383.0	99.000	98.65	100	100
		2	1383.0	99.000	98.49	99	
		3	1383.0	99.000	98.50	99	
3	150%	1	2074.50	148.500	148.82	100	100
		2	2074.50	148.500	148.25	100	
		3	2074.50	148.500	148.84	100	
		4	2074.50	148.500	148.20	100	
		5	2074.50	148.500	148.94	100	
		6	2074.50	148.500	148.37	100	

Table 6: Showing accuracy results for Azithromycin

S.NO	Accuracy level	Sample name	Sample weight	$\mu\text{g/ml}$ added	$\mu\text{g/ml}$ found	% Recovery	% Mean
1	50%	1	691.50	50.000	49.85	100	100
		2	691.50	50.000	49.82	100	
		3	691.50	50.000	49.88	100	
		4	691.50	50.000	49.87	100	
		5	691.50	50.000	49.88	100	
		6	691.50	50.000	49.84	100	
2	100%	1	1383.00	100.000	99.93	100	100
		2	1383.00	100.000	99.86	100	
		3	1383.00	100.000	99.75	100	
3	150%	1	2074.50	150.000	148.87	99	100
		2	2074.50	150.000	148.99	99	
		3	2074.50	150.000	149.17	99	
		4	2074.50	150.000	148.76	99	
		5	2074.50	150.000	149.56	100	
		6	2074.50	150.000	148.87	99	

Precision Studies:

precision was calculated from Coefficient of variance for six replicate injections of the standard. The standard solution was injected for six times and measured the area for all six Injections in HPLC. The %RSD for the area of six replicate injections was found. The results are shown in table 7,8.

Table 7: Precision results for Levofloxacin

S.no	RT	Area	%Assay
injection1	3.144	1130289	98
injection2	3.141	1135307	99
injection3	3.142	1136018	99
injection4	3.145	1131067	99
injection5	3.142	1130362	99
injection6	3.138	1133569	99
Mean			99
Std. Dev.			0.22
% RSD			0.22

Table 8: Precision results for Azithromycin

S.no	RT	Area	%Assay
injection1	3.431	1112555	100
injection 2	3.429	1116367	100
injection 3	3.428	1113656	100
injection 4	3.432	1113585	100
injection 5	3.430	1114698	100
injection 6	3.428	1114883	100
Mean			100
Std. Dev.			0.12
%RSD			0.12

Robustness: As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method. The flow rate was varied at 0.8 ml/min to 1.2 ml/min. The results are shown in table 9,10.

Table 9: Flow variation results for Levofloxacin

parameter	RT	Theoretical plates	Asymmetry
Decreased flow rate(0.8ml/min)	4.178	12562	1.52
Actual flow rate(1.0ml/min)	3.154	11143	1.49
Increased flow rate(1.2ml/min)	2.516	10342	1.40
Decreased temperature(20 ⁰ c)	4.179	12660	1.52
Actual temperature(25 ⁰ c)	3.154	11143	1.49
Increased temperature(30 ⁰ c)	4.183	12750	1.52

Table 10: Flow variation results for Azithromycin

parameter	RT	Theoretical plates	Asymmetry
Decreased flow rate (0.8ml/min)	4.564	14378	1.30
Actual flow rate (1.0ml/min)	3.439	13998	1.30
Increased flow rate (1.2ml/min)	2.746	12574	1.32
Decreased temperature(20 ⁰ c)	4.564	14785	1.28
Actual temperature(25 ⁰ c)	3.439	13998	1.30
Increased temperature(30 ⁰ c)	4.568	14978	1.30

LOD and LOQ:

The sensitivity of RP-HPLC was determined from LOD and LOQ. Which were calculated from the calibration curve using the following equations as per ICH guidelines. The results are shown in table 11.

$$\text{LOD} = 3.3\sigma/S \text{ and}$$

$$\text{LOQ} = 10 \sigma/S, \text{ where}$$

σ = Standard deviation of y intercept of regression line,

S = Slope of the calibration curve

Table 11: LOD, LOQ of Levofloxacin and Azithromycin

Drug	LOD	LOQ
Levofloxacin	2.8	9.4
Azithromycin	2.94	9.8

CONCLUSION:

The study is focused to develop and validate HPLC methods for estimation of Levofloxacin and Azithromycin in tablet dosage form. For routine analytical purpose it is desirable to establish methods capable of analyzing huge number of samples in a short time period with good robustness, accuracy and

precision without any prior separation steps. HPLC method generates large amount of quality data, which serve as highly powerful and convenient analytical tool. The method shows good reproducibility and good recovery. From the specificity studies, it was found that the developed methods were specific for Levofloxacin and Azithromycin.

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