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Research

Analytical method development and validation for the simultaneous estimation of azithromycin and levoflaxacin by RP-HPLC

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Abstract

A reverse phase high performance liquid chromatographic method was developed for simultaneous estimation of Azithromycin and Levoflaxacine. The chromatographic conditions were successfully developed for the separation of Azithromycin and Levoflaxacine by using ZODIAC –SIL RP C18 column 4.6×100 mm 3.0µm, flow rate was 1.0 ml/min, mobile phase ratio was (75:25 v/v) (KH 2 PO 4 and K 2 HPO 4) pH 9, detection wave length was 292 nm. The precision study was precise, robust, and repeatable. LOD value was 2.17 and 6.60, and LOQ value was 0.032 and 0.1125 respectively. Hence the suggested RP-HPLC method can be used for routine analysis of Azithromycin and Levoflaxacin in API and Pharmaceutical dosage form.

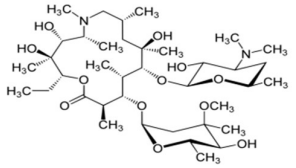
Keywords: Azithromycin, Levoflaxacin, RP HPLC, Validation Parameters

INTRODUCTION [1-3]

High Performance Liquid Chromatography originally indicated the fact that high pressure was used to generate the flow required for liquid chromatography in packed columns. In the beginning, pumps only had a pressure capability of 500psi. This was called high pressure liquid chromatography, or HPLC. New HPLC instruments could develop upto 6,000psi of pressure, and incorporated improved injectors, detectors, and columns. With continued advances in performance during this time (smaller particles, even higher pressure), the acronym HPLC remained the same, but the name was changed to high performance liquid chromatography. HPLC is the method of choice in the field of analytical chemistry, since this method is specific, robust, linear, precise and accurate and the limit of detection is low and also it offers the following advantages. Speed(min), Greater sensitivity, Improved resolution (wide variety of stationary phases) Re-usable columns Needs a sample with a high accuracy and precise easy sample recovery, handling and maintenance, Reproducibility of +/- 1% (not so for LC) Non-destructed sample during operation compared to GC Controls and automates chromatography instrumentation Provides data management, security features, and reporting and instrument validation.

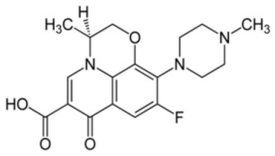
Analytical MethodValidation[11-13]: Validation is defined by the International Organization for Standardization (ISO) as "verification, where the specified requirements are adequate for an intended use", where the term verification is defined as "provision of objective evidence that a given item fulfills specified requirements". The objective of validation of an analytical procedure is todemonstrate thatitissuitableforitsintendedpurpose.According to ICH, typical analytical performance characteristics that should be considered by FDA, USP, and ICH are as follows Accuracy, Precision (Repeatability, Intermediate precision and reproducibility) Specificity, Linearity, Range, DetectionlimitQuantizationlimit, a n d Robustness.

Drug Profile: Azithromycin



Chemical formula: $C_{38}H_{72}N_2O_{12}$, Molecular weight: 748.9845, Description: A white or slightly yellow, odorless, crystalline powder, Solubility: Soluble 1 in 2 of water, 1 in 1.5 of alcohol, in 1.5 of chloroform, 1 in 15 of acetone and practically insoluble in ether, Melting point**:** 168^0 C-170^0 Category: macrolide antibiotics, it inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit of the bacterial 70S ribosome.

Levoflaxacin



Chemical formula: C18H20FN3O4, Molecular Weight: 361.367.Melting point131.5^{0}C-134.5^0 C
Description:White or yellow Solid-crystals, Odorless, slightly bitter taste.
Solubility: soluble 1 in 16 of water,1 in 2 of chloroform,1 in 39 of ether; and practically insoluble in water.
Category:A synthetic fluoroquinolone (fluoroquinolones) antibacterial agent that inhibits the super coiling activity of bacterial DNA gyrase, halting DNA replication.

METHODOLOGY

Materials and Methods and Chemicals and standards used: Water Methanol Acetonitrile Ortho phosphoric acid KH_2PO_4 $K_2HPO_4$0. 22µ Nylon filter0.45µ filter paper Azithromycin, Instruments used: HPLC-auto sampler-Separation module-2695, UV-detector 2487 –UV detector U.V double beam spectrometer Digital weighing balance (sensitivity 5mg) Digital weighing balance (sensitivity 5mg) pH meter Sonicator

Method Development for the Simultaneous Estimation of Levoflaxacin and Azithromycin by Using RP-HPLC.

Selection of mobile phase: pH 9 phosphate buffer: Acetonitrile (25: 75% v/v)**,** Buffer pH should be between 6 to 9.Below 2: siloxane linkages are cleaved. Above 8: dissolution of silica.
pH selected: 9 ±0.05 pH controls the elution properties by controlling the ionization characteristics.Reasons: To decrease the retention and improve separation. Good Response, Area, Tailing factor, Resolution.

Selection of wavelength:500 mg of Levofloxacin and Azithromycin was dissolved in mobile phase. The solution was scanned from 200-400 nm the spectrum was obtained. The overlay spectrum was used for selection of wavelength for Levofloxacin and Azithromycin. The isobestic point was taken as detection wavelength. The overlay spectrums are shown in fig.

Selection of column: Heart of HPLC made of 316 grade stainless steel packed with stationary phase.Silica based columns with different cross linking's in the increasing order of polarity are as follows:

←------- Non-polar----------moderately polar--------Polar----------→
C_{18}< C_8< C_6< Phenyl < Amino <Cyano< Silica

In reverse phase chromatography, hydrophobic interaction between drug molecule and the alkyl chains on the column packing material. Column is selected based on solubility, polarity and chemical differences among analytes and Column selected: i.e. Zodiac sil-C18 column 100×4.6 mm 3.0 µm.Reasons: Better separation, Good tailing factor.

Selection of solvent delivery system: Always preferable solvent delivery system. More chance of getting reproducible result on retention time of analytes. More economic than gradient technique.

Selection of flow rate: Acceptable limit: - Not more than 2.5 ml/min, Flow rate selected was 0.5ml/min, Flow rate is selected based on 1. Retention time,2. Column back pressure 3. Peak symmetry 4.Separation of impurities. Reasons: For earlier elution of analyte and elution of all impurities within 6.0 min. Information from the reference method in literature.

Selection of diluents: Selection of diluent is based on the solubility of the analyte, Diluents' selected: Acetonitrile: phosphate buffer pH 9 (75: 25v/v)
Reason: Analyte is soluble in methanol and acetonitrile.

Selection of column temperature: Preferable temperature is ambient or room temperature.
Reasons: To elute all impurities along with analyte with in 6.0 min of run time, Less retention time, Good peak shape, Higher theoretical plates, Good resolution.

Selection of test concentration and injection volume: Test concentration is finalized after it is proved that API is completely extractable at the selected test concentration. Test concentration is fixed based upon the response of API peak at selected detector wavelength.Levofloxacin and Azithromycin label claimed 500 and 500 mg.And the test concentration selected is 150 ppm.Injection volume selected was 20µL.Reason: good peak area, retention time, peak symmetry.

Analytical Method Validation
Validation parameters are: Specificity, Linearity, Range, Accuracy, Precision, Repeatability, Intermediate Precision, Detection Limit, Quantitation Limit, and Robustness.

RESULTS AND DISCUSSIONS

The present investigation reported in the thesis was aimed to develop a new method development and validation for the simultaneous estimation of Levofloxacin and Azithromycin by RP-HPLC method. Literature reveals that there are less analytical methods reported for the simultaneous estimation Levofloxacin and Azithromycin by RP-HPLC method. Hence, it was felt that, there is a need of new analytical method development for the simultaneous estimation of Levofloxacin and Azithromycin in pharmaceutical dosage form.

Method Development: The detection wavelength was selected by dissolving the drug in mobile phase to get a concentration of 10µg/ml for individual and mixed standards. The resulting solution was scanned in U.V range from 200-400nm. The overlay spectrum of Levofloxacin and Azithromycin was obtained and the isobestic point of Levofloxacin and Azithromycin showed absorbance's maxima at 271nm. The spectrums are shown in Fig.

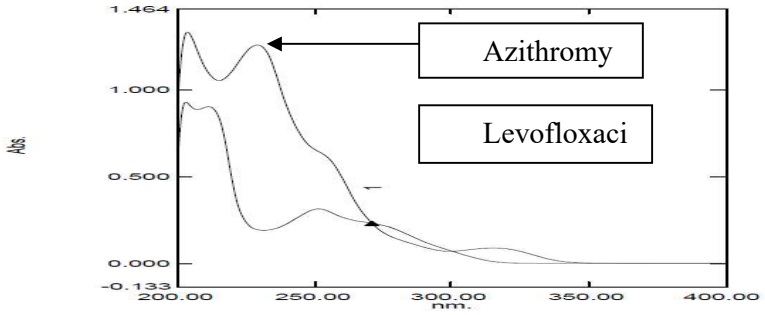


Fig 1: Spectrum showing overlapping spectrum of IMP and DIZ

The chromatographic method development for the simultaneous estimation of Levofloxacin and Azithromycin were optimized by several trials for various parameters as different column, flow rate and mobile phase, finally

the following chromatographic method was selected for the separation and quantification of Levofloxacin and Azithromycin in API and pharmaceutical dosage form by RP-HPLC method.

Optimized chromatographic conditions for simultaneous estimations of Levofloxacin and Azithromycin by RP-HPLC method

Column	:	Zodiac sil RP C184.5×100 mm 3.0 µm
Column temperature	:	Ambient
Wavelength	:	292 nm
Mobile phase ratio	:	75:25% V/v Acetonitrile: phosphate Buffer pH adjusted to 9 with ortho phosphoric acid
Flow rate	:	1 min/ml
Auto sampler temperature	:	Ambient
Injection volume	:	20µl
Run time	:	8 minutes

Peak Results

	Name	RT	Area	Height	USP Resolution	USP Tailing	USP Plate Count	Injection
1	levofloxacin	3.516	518397	57148		1.47	3828	1
2	azithromycin	4.870	790333	57188	4.56	1.56	3149	1
3	levofloxacin	3.514	518072	56951		1.51	3789	2
4	azithromycin	4.867	790053	56762	4.52	1.58	3118	2
5	levofloxacin	3.514	521948	57230		1.50	3861	3
6	azithromycin	4.864	795078	57114	4.53	1.61	3112	3

Peak Results

	Name	RT	Area	Height	USP Resolution	USP Tailing	USP Plate Count	Injection
1	levofloxacin	3.549	508691	56817		1.42	3923	2
2	azithrimycin	4.981	747698	55296	4.79	1.46	3149	2
3	levofloxacin	3.520	519881	57981		1.46	3946	3
4	azithrimycin	4.878	741850	57648	4.71	1.43	3348	3
5		3.520	519881	57981		1.46	3946	3
6		4.878	741850	57648	4.71	1.43	3348	3

S.No	Name of compound	Label claim	Amount taken	%purity
1	Levofloxacin	500	161.8	98.24
2	Azithromycin	500	161.8	100.27

The retention time of Levofloxacin and Azithromycin was found to be 3.549 mins and 4.981 mins respectively. The system suitability parameters for Levofloxacin and Azithromycin such as theoretical plates and tailing factor were found to be 3923, 1.43 and 3348, 1.46. Resolution was 4.71. The % purity of Levofloxacin and Azithromycin in pharmaceutical dosage form was found to be 98.24 and 100.27% respectively.

Validation report

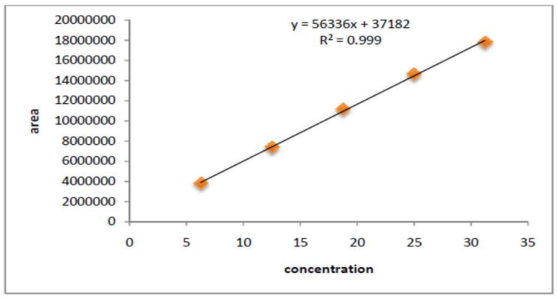
Specificity: The system suitability for specificity was carried out to determine whether there is any interference of any impurities in retention time of analytical peak. The study was performed by injecting blank.

Peak Results

	Name	RT	Area	Height	USP Resolution	USP Tailing	USP Plate Count	Injection
1	levofloxacin	3.549	508691	56817		1.42	3923	2
2	azithrimycin	4.981	747698	55296	4.79	1.46	3149	2

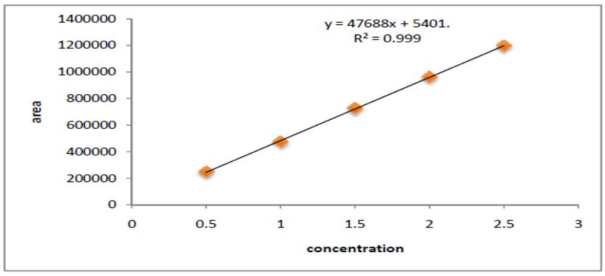
The specificity test was performed for Levofloxacin and Azithromycin. It was found that there was no interference of impurities in retention time of analytical peak.

Linearity: The linearity study was performed for the concentration of 50 ppm to 250 ppm and 50 ppm to 100 ppm level. Each level was injected into chromatographic system. The area of each level was used for calculation of correlation coefficient. The chromatograms are shown in Fig. and results are tabulated in Table. Calibration graphs are shown



Levofloxacin $r^2 = 0.999$

Fig 2: Calibration graph for Levofloxacin



Azithromycin $r^2 = 0.999$

Fig 3: Calibration graph for Azithromycin

The linearity study was performed for concentration range of 50µg-250µg and 50µg-250 µg of Levofloxacin and Azithromycin and the correlation coefficient was found to be 0.999 and 0.999.(NLT 0.999).

Accuracy: The accuracy study was performed for 50%, 100% and 150 % for Levofloxacin andAzithromycin.Eachlevelwasinjected intriplicate into chromatographic system. The area of each levelwas use d for calculation of % recovery. Chromatograms are shown in Fig and results are tabulated in Table.

Chromatogram showing accuracy-50% injection-1,2,3.

Peak Results

	Name	RT	Area	Height	USP Resolution	USP Tailing	USP Plate Count	Injection
1	levofloxacin	3.515	852858	90288		1.79	3645	1
2	azithromycin	4.855	1425312	96264	4.32	1.99	2850	1
3	levofloxacin	3.516	854502	90471		1.74	3685	2
4	azithromycin	4.856	1425604	96724	4.34	1.98	2844	2
5	levofloxacin	3.515	853960	89576		1.78	3629	3
6	azithromycin	4.857	1424941	96279	4.32	1.98	2847	3

Accuracy -100% :Chromatograms showing accuracy -100% injection-1,2,3.

Peak Results

	Name	RT	Area	Height	USP Resolution	USP Tailing	USP Plate Count	Injection
1	levofloxacin	3.521	1119197	116736		1.84	3637	1
2	azithromycin	4.863	1499388	100942	4.32	2.04	2860	1
3	levofloxacin	3.517	1119910	117508		1.80	3643	2
4	azithromycin	4.860	1500972	100747	4.33	2.02	2833	2
5	levofloxacin	3.515	1118239	117424		1.86	3627	3
6	azithromycin	4.859	1499296	100606	4.31	2.03	2852	3

Accuracy 150%: Chromatogram showing accuracy -150 % injection-1,2,3.

Table 2: Accuracy results

Levofloxacin					
%Concentration (at specification level)	**Average area**	**Amount added (mg)**	**Amount found (mg)**	**% Recovery**	**Mean recovery**
50%	7371253	5	4.9	99.91%	
100%	14634226.7	10	9.98	99.18%	99.56%
150%	2243270.7	15	14.89	99.60%	
Azithromycin					
50%	484733	5.0	4.9	99.53%	
100%	967998	10.0	9.59	99.38%	99.47%
150%	145437	15.0	14.85	99.52%	

The accuracy study was performed for % recovery of Levofloxacin and Azithromycin. The % recovery was found to be 99.18% and 99.91% respectively (NLT 98% and NMT 102%).

Precision-Repeatability **Table 3: % RSD results**

Peak Name: levofloxacin

	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	levofloxacin	3.517	520512	55970	3743.5	1.5
2	levofloxacin	3.514	521717	56909	3777.3	1.5
3	levofloxacin	3.513	521846	57014	3776.0	1.5
4	levofloxacin	3.514	522710	56972	3822.2	1.5
5	levofloxacin	3.514	523284	56798	3770.1	1.5
Mean			522013.9		3777.8	1.5
Std. Dev.			1057.0			
% RSD			0.2			

Peak Name: azithromycin

	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing	USP Resolution
1	azithromycin	4.864	787229	56504	3081.3	1.6	4.5
2	azithromycin	4.864	789690	56992	3144.1	1.6	4.5
3	azithromycin	4.863	790187	56962	3118.1	1.6	4.5
4	azithromycin	4.862	791763	56934	3147.3	1.6	4.5
5	azithromycin	4.862	794118	57105	3101.8	1.6	4.5
Mean			790597.2		3118.5	1.6	4.5
Std. Dev.			2553.9				
% RSD			0.3				

Method precision study was performed for the %RSD of Levofloxacin and Azithromycin was found to be 0.27 and 0.34 (NMT 2).

Intermediate precision/Ruggedness: The intermediate precision study was performed for five injections of Levofloxacin and Azithromycin.Each standard injection was injected into chromatographic system. The area of each standard injection was used for calculation of % RSD. The chromatograms are shown in

Table4: Intermediate precision injections-1 to 3(day1, analyst-1).

Peak Name: azithromycin

	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing	USP Resolution
1	azithromycin	4.863	790742	56569	3075.9	1.6	4.5
2	azithromycin	4.860	794791	56512	3043.2	1.7	4.5
3	azithromycin	4.862	796445	56415	3029.9	1.6	4.4
Mean			793992.9		3049.7	1.6	4.5
Std. Dev.			2934.1				
% RSD			0.4				

Peak Name: levofloxacin

	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	levofloxacin	3.513	521817	56358	3704.2	1.6
2	levofloxacin	3.515	522684	56384	3696.0	1.5
3	levofloxacin	3.516	522921	56456	3716.3	1.5
Mean			522473.9		3705.5	1.6
Std. Dev.			581.3			
% RSD			0.1			

Table 5: Intermediate precision injection -1 to 3(day 2,analyst 2).

Peak Name: azithromycin

	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing	USP Resolution
1	azithromycin	4.863	790947	56665	3097.3	1.6	4.5
2	azithromycin	4.863	791835	56696	3066.2	1.7	4.5
3	azithromycin	4.862	792125	56645	3083.0	1.7	4.5
Mean			791635.7		3082.2	1.6	4.5
Std. Dev.			613.4				
% RSD			0.1				

Peak Name: levofloxacin

	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	levofloxacin	3.515	522263	56267	3713.3	1.5
2	levofloxacin	3.514	523696	56496	3753.0	1.6
3	levofloxacin	3.516	523942	56039	3720.2	1.6
Mean			523300.4		3728.8	1.6
Std. Dev.			906.8			
% RSD			0.2			

The intermediate precision was performed for %RSD of Levofloxacin and Azithromycin was found to be 0.2 and 0.1 respectively (NMT 2).

Detection limit:LOD's can be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (S) at levels approximating the LOD according to the formula. The standard deviation of the response can be determined based on the standard deviation of y-intercepts of regression lines.

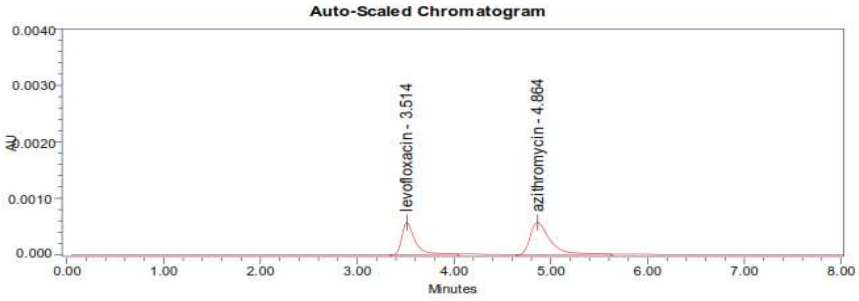


Table 6: Results for Limit of Detection

Drug name	Standard deviation(σ)	Slope(s)	LOD(µg)
Levofloxacin	371827.90	563365963	2.17
Azithromycin	5401.60	479884400	0.0372

The LOD was performed for Levofloxacin and Azithromycin was found to be 2.17and 0.0372 respectively.

Quantitation limit: LOQ's can be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (S) according to the formula. Again, the standard deviation of the response can be determined based on the standard deviation of y-intercepts of regression lines.

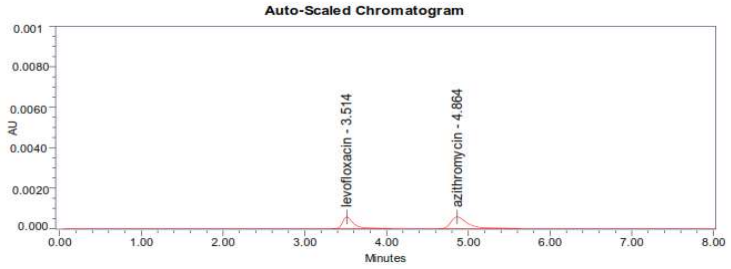
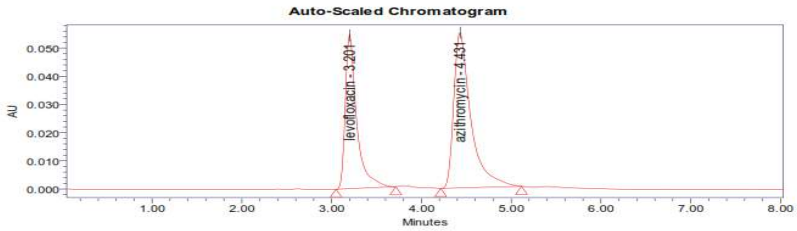


Table 7: Results for Limit of Quantitation

Drug name	Standard deviation(σ)	Slope(s)	LOQ(µg)
Levofloxacin	371827.90	563365963	6.60
Azithromycin	5401.60	479884400	0.112

The LOQ was performed for Levofloxacin and Azithromycin was found to be 6.60 and 0.112 respectively.

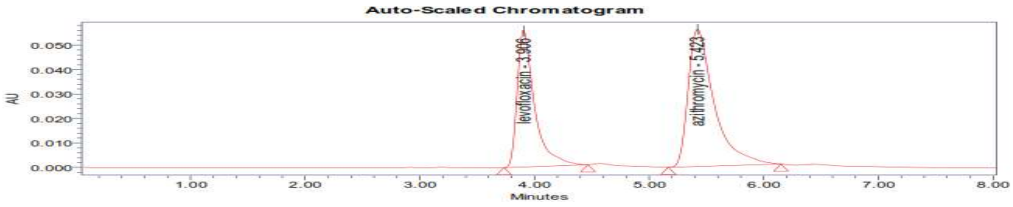
Robustness: The robustness was performed for the flow rate variations from 1.2 ml/min to 0.8 ml/min and mobile phase ratio variation from more organic phase to less organic phase ratio for Levofloxacin and Azithromycin. The method is robust only in less flow condition and the method is robust even by change in the Mobile phase ±5%.



Peak Results

	Name	RT	Area	Height	USP Resolution	USP Tailing	USP Plate Count	Injection
1	levofloxacin	3.201	481064	54727		1.82	3657	1
2	azithromycin	4.431	729496	55125	4.42	1.91	3032	1

Fig. Chromatogram showing more flow rate 1.2 ml/min



Peak Results

	Name	RT	Area	Height	USP Resolution	USP Tailing	USP Plate Count	Injection
1	levofloxacin	3.906	584732	56135		1.82	3696	1
2	azithromycin	5.423	884751	56302	4.52	1.88	3108	1

The results are summarized on evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly. Hence it indicates that the method is robust even by change in the flow rate ±0.2ml/min.The method is robust only in less flow condition.

Table 8:System suitability results for Levofloxacin

S. No	Flow rate (ml/min)	System suitability results	
		USP Plate Count	USP Tailing
1	0.8	3696	1.8
2	**1.0**	**3646**	**1.4**
3	1.2	3657	1.8

Table 9:System suitability results for Azithromycin

S. No	Flow rate (ml/min)	System suitability results	
		USP Plate Count	USP Tailing
1	0.4	3108	1.8
2	**0.5**	**3348**	**1.4**
3	0.6	3057	1.9

Table 10: System suitability results for Levofloxacin

S. No	Change in organic composition in the mobile phase	System suitability results	
		USP Plate Count	USP Tailing
1	5 % less	3706	1.75
2	***Actual**	**3646**	**1.4**
3	5 % more	3627	1.8

Table11: System suitability results for Azithromycin

S. No	Change in organic composition the mobile phase	System suitability results	
		USP Plate Count	USP Tailing
	% less	309	86
	Actual	**348**	**4**
	% more	220	9

SUMMARY AND CONCLUSION

A new method was established for simultaneous estimation of Azithromycin and Levoflaxacin by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of Azithromycin and Levoflaxacin by using ZODIAC –SIL RP C18 column 4.6×100 mm 3.0µm, flow rate was 1.0 ml/min, mobile phase ratio was (75:25 v/v) acetonitrile : phosphate buffer (KH_2PO_4 and K_2HPO_4) pH 9 (pH was adjusted with orthophosphoric acid), detection wave length was 292 nm. The instrumentusedwasWATERSHPLCAutoSampler, Separation module 2695, PDA detector 2487, Empower-software version-2. The retention times were found to be 4.878mins and 3.420mins. The % purity of Azithromycin and Levoflaxacin was found to be 100.27% and 99.87% respectively. The system suitability parameters for Azithromycin and Levoflaxacin such as theoretical plates and tailing factor were found to be 3396, 1.4and 3696 and 1.4, the resolution was found to be 8.67. The analytical method was validated according to ICH guidelines (ICH, Q2 (R1)). The linearity study for Azithromycin and Levoflaxacin was found in concentration range of 6.25µg-37.5µg and 0.5µg-3.0µg and correlation coefficient (r^2) was found to be 0.999 and 0.999, % recovery was found to be 99.56% and 99.48%, %RSD for repeatability was 0.27 and 0.40, % RSD for intermediate precision was 0.27 and 0.94 respectively. The precision study was precise, robust, and repeatable. LOD value was 2.17 and 6.60, and LOQ value was 0.032 and 0.1125 respectively.Hence the suggested RP-HPLC method can be used for routine analysis of Azithromycin and Levoflaxacin in API and Pharmaceutical dosage form.

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