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Original Article

A New RP-HPLC Method for the Simultaneous Estimation of Azithromycin and Levofloxacin in it's Pure and Pharmaceutical Dosage Form as per ICH Guidelines

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ABSTRACT

The Present work was to develop a simple, fast, accurate, precise, reproducible, Reverse Phase High Performance Liquid Chromatographic Method for simultaneous estimation of Azithromycin and Levofloxacin in pure and combined dosage form marketed as Loxof-AZ. Chromatographic separation was done using Symmetry C18 column having dimension of 4.6×150mm having particle size of 5.0 µm, with mobile phase consisting of Ammonium acetate buffer pH 6 ±0.02 pH and methanol (30:70 %v/v), flow rate was adjusted to 1ml/min and detection wavelength at 262nm. The retention times of Azithromycin and Levofloxacin was found to be 4.862 and 3.515 mins. The proposed method has been validated for accuracy, precision, linearity; robustness and range were within the acceptance limit according to ICH guidelines. Linearity for Azithromycin and Levofloxacin was found in range of 20µg-100µg and 2µg-10µg and correlation coefficient was found to be 0.999 and 0.999% RSD for intermediate precision was found to be 0.1 and 0.2, for repeatability was 0.4 and 0.1, % mean recovery for Azithromycin and Levofloxacin was found to be 99.56% and 99.47% respectively. The method was found to be robust even by change in the mobile phase +10% and in more flow condition. The developed method can be successfully employed for the routine analysis of Azithromycin and Levofloxacin in API and Pharmaceutical dosage forms.

Keywords: Azithromycin, Levofloxacin, RP-HPLC, Method development, Validation, Combined dosage form.

1. INTRODUCTION

Chromatographic Methods[1] are developed for new products when no official methods are available. Alternate methods for existing (Non-Pharmacopoeias) products are developed as per ICH[2,3] guidelines to

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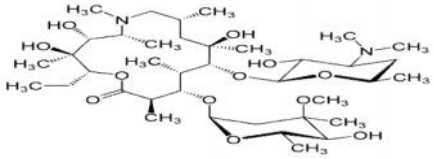
reduce the cost and time for better precision and ruggedness. Trial runs are conducted, method is optimized and validated. When alternate method proposed is intended to replace the existing procedure, comparative laboratory data including merits/demerits should be made available.

An assay for a major component requires a different approach and acceptance criteria than a method for a trace impurity. A final method[4] may be performed at different sites around the world. Differences in HPLC instrumentation, laboratory equipment and reagent sources and variations in the skills and background of personnel may require specific features in the HPLC method[5]. In addition, the development of different formulations of the same drug with varying strengths or physical forms may require flexibility in method procedures.

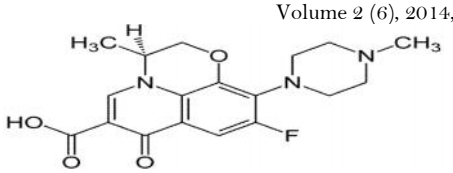
Method validation[6] study include system suitability, linearity, precision, accuracy, specificity, robustness, limit of detection, limit of quantification and stability of samples, reagents, instruments.

AZITHROMYCIN drug profile[7]



1. IUPAC name : (2R,3S,4R,5R,8R,10R,11R,12S,13S,14R)-11-{[(2S,3R,4S,6R)-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
2. Molecular formula : $C_{38}H_{72}N_2 \cdot O12$,
3. Molecular weight : 748.984g/mol
4. Melting point : 168-170ºc
5. Category : Antibiotic. (semi synthetic macrolide

LEVOFLOXACIN drug profile[8]



1. IUPAC name : (2S)-7-fluoro-2-methyl-6-(4-methylpiperazin-1-yl)-10-oxo-4-oxa-1-azatricyclo [7.3.1.0{5,13}]trideca-5(13),6,8,11-tetraene-11-carboxylic acid.
2. Molecular formula : $C_{18}H_{20}\,FN_3 \cdot O4$,
3. Molecular weight : 748.984g/mol
4. Melting point : 134ºc
5. Category : Antibiotic. (synthetic fluoroquinone)

2. MATERIALS AND METHODS

The materials, chemicals and instrument used below mentioned.

Chemicals	Manufacturer Name	Grade
Water	Merck	HPLC grade
Methanol	Merck	HPLC grade
Ammonium acetate	Merck	G.R
Glacial acetic acid	Merck	G.R
0. 22µ Nylon filter	Advanced lab	HPLC grade
0.45µ filter paper	Millipore	HPLC grade
LOXOF-AZ	Hetero labs limited	Tablet form
Azithromycin and Levofloxacin	In – House	In- House

Instrument name	Model number	Soft ware	ManufacturersName
HPLC-auto sampler –UV detector	Separation module2695, UV.detector2487	Empower-software version-2	Waters
U.V double beam spectrometer	UV 3000+	U.V win soft ware	Lab India
Digital weighing balance(sensitivity 5mg)	ER 200A	-	Ascoset
pH meter	AD 102U	-	ADWA

Sonicator	SE60US	-	Enertech

Design of the experiment

The present study reported was aimed to develop a new method development and validation of estimation of azithromycin and levofloxacin by RP-HPLC. On the literature survey [9-15] most of literatures related to LC/MS and HPLC methods have been reported determination of azithromycin alone. Few methods reported related to HPLC in tablet dosage form but is time of analysis more. Hence an attempt has made to develop a HPLC method for the determination of azithromycin and levofloxacin in API and pharmaceutical dosage forms

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 azithromycin and levofloxacin was obtained and the isobestic point of azithromycin and levofloxacin showed absorbance's maxima at 262nm. The spectrums are shown in Fig. No. 14-16

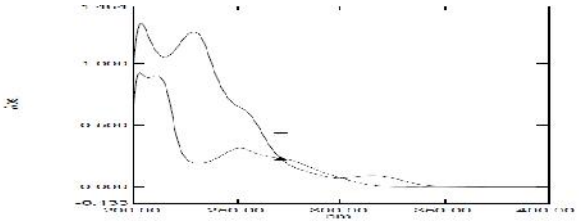


Fig 1: Spectrum showing overlapping spectrum of AZY and LEV

No.	Wavelength	Absorbance	Description
1.	262.00	0.233	Azth&Levo

The chromatographic method development for the simultaneous estimation of azithromycin and levofloxacin 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 azithromycin and levofloxacin in API and pharmaceutical dosage form by RP-HPLC method.

Optimized chromatographic conditions for simultaneous estimations of azithromycin and levofloxacin by RP-HPLC method

Mobile phase : Ammonium acetate buffer6pH: Methanol (30:70%v/v)

Column : symmetry C18 5µm (4.6*150mm) 5 µ

Flow rate : 1 ml/min

Wavelength : 262 nm

Column temp : Ambient

Sample Temp : Ambient

Injection Volume : 10 µl

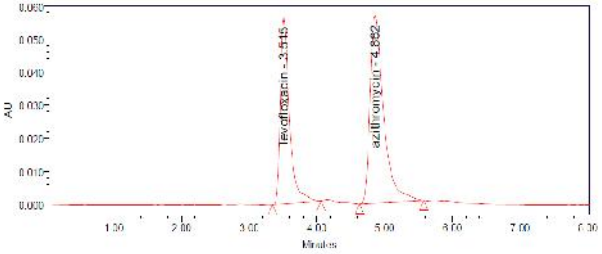


Fig 2: Chromatogram showing standard injection

	Name	RT	Area	Height	Resolution	Tailing	Plate count	Inj
1.	Levo	3.515	524516	56491		1.58	3765	1
2.	Azithro	4.862	794125	56792	4.48	1.67	3118	1

3. RESULTS AND DISCUSSION

Assay Calculation for Azithromycin And Levofloxacin:

The assay study was performed for the azithromycin and levofloxacin. Each three injections of sample and standard were injected into chromatographic system and results are tabulated.

Table 1: Showing results for assay of Azithromycin

s.no	Name	RT	Area Std.	Area Sample	Tailing	Plate count	% Purity
1.	AZY	4.981	747698	790333	1.46	3149	98.1

2.	**AZY**	**4.878**	**741850**	**790053**	**1.43**	**3348**
3.	**AZY**	**4.878**	**741850**	**795078**	**1.43**	**3348**

Table 2: Showing results for assay of Levofloxacin

s.no	Name	RT	Area Std.	Area Sample	Tailing	Plate count	%Purity
1.	Levo	3.549	508691	518397	1.42	3923	98.4
2.	Levo	3.520	519881	518072	1.46	3946	
3.	Levo	3.520	519881	521948	1.46	3946	

Validation Parameters:

Linearity

The linearity study was performed for the concentration of 20ppm to 100ppm and 2ppm to 10ppm level. Each level was injected into chromatographic system. The area of each level was used for calculation of correlation coefficient. The results are tabulated.

Table 3: Showing linearity results for azithromycin level -1 to level-5

	Conc.	RT	Area	Height	Resolution	Tailing	Plate count
1.	20ppm	4.879	293040	22681	4.7	1.5	3350.9
2.	40ppm	4.870	556608	40046	4.5	1.7	3769.3
3.	60ppm	4.864	793595	56502	4.5	1.7	3098.5
4.	80ppm	4.860	1026070	70526	4.4	1.8	2984.9
5.	100ppm	4.856	1289059	87378	4.3	2.0	2865.2
	Mean		791674.4		4.5	1.7	3093.8
	Std. Dev		389303.5				
	%RSD		49.17				

Table 4: Showing linearity results for levofloxacin level -1 to level-5

	Conc.	RT	Area	Height	Plate count	Tailing
1.	2ppm	3.514	186304	20781	3792.9	1.5
2.	4ppm	3.515	370858	41209	3733.1	1.6
3.	6ppm	3.515	527219	56214	3714.6	1.7
4.	8ppm	3.513	731149	77961	3681.4	1.7
5.	10ppm	3.513	887938	93619	3612.7	1.8
	Mean		540693.6		3706.9	1.6
	Std. Dev		278962.4			
	%RSD		51.5			

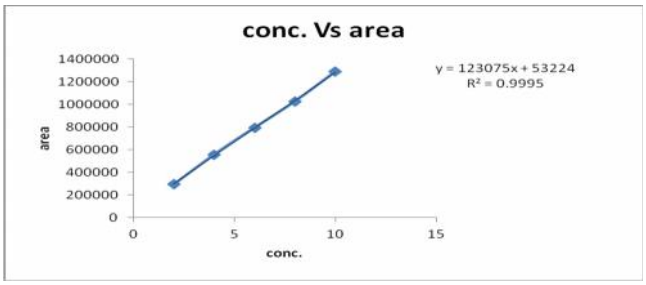


Fig 3: Showing calibration graph for Azithromycin

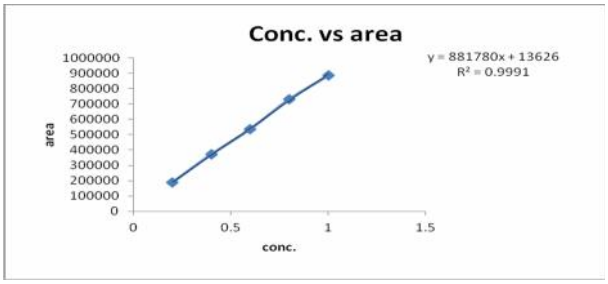


Fig 4: Showing calibration graph for levofloxacin

Accuracy

The accuracy study was performed for 50%, 100% and 150% for Azithromycin and Levofloxacin. Each level was injected in triplicate into chromatographic system. The area of each level was used for calculation of % recovery and results are tabulated

Table 5: Showing peak results for accuracy 50%

S.no	Name	RT	Area	Height	Tailing	Plate count	Recovery
1.	Azithro	4.855	1425312	96264	1.99	2850	99.91
2.	Azithro	4.856	1435604	96724	1.98	2844	
3.	Azithro	4.857	1424941	96279	1.98	2847	
1.	Levo	3.515	852858	90288	1.79	3645	99.53
2.	Levo	3.516	854502	90471	1.74	3685	
3.	Levo	3.515	853960	89576	1.78	3629	

Table 6: Showing peak results for accuracy 100%

S.no	Name	RT	Area	Height	Tailing	Plate count	Recovery
1.	Azithro	4.863	1499388	100942	2.04	2860	99.81
2.	Azithro	4.860	1500972	100747	2.02	2833	
3.	Azithro	4.859	1499296	100606	2.03	2852	
1.	Levo	3.521	1119197	116736	1.84	3637	99.53
2.	Levo	3.517	1119910	117508	1.80	3643	
3.	Levo	3.515	1118239	117424	1.86	3627	

Table 7: Showing peak results for accuracy 150%

	Name	RT	Area	Height	Tailing	Plate count	Recovery
1.	Azithro	4.853	2024658	132459	2.09	2678	
2.	Azithro	4.852	2021564	131567	2.09	2705	99.60
3.	Azithro	4.853	2021860	131786	2.09	2694	
1.	Levo	3.517	1038871	108527	1.87	3623	
2.	Levo	3.516	1038553	107528	1.86	3611	99.52
3.	Levo	3.516	1037242	108249	1.89	3631	

Precision:

Repeatability

The standard solution was injected for three times and measured the area for all three injections in HPLC. The %RSD for the area of three replicate injections was found to be within the specified limits.

Intermediate precision/Ruggedness

The standard solution was injected for three times and measured the area for all three injections in HPLC. The %RSD for the area of three replicate injections was found to be within the specified limits.

Repeatability

The precision study was performed for three injections of azithromycin and levofloxacin. Each standard injection was injected in to chromatographic system. The area of each Standard injection was used for calculation of % RSD. The results are tabulated.

Table 8: Showing% RSD results for azithromycin

	Peak name	RT	Area	Height	USP Resolution	Tailing	Plate count
1.	Azithro	4.863	790742	56569	3075.9	1.6	4.5
2.	Azithro	4.860	794791	56512	3043.2	1.7	4.5
3.	Azithro	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				

Table 9: Showing %RSD results for levofloxacin

	Peak name	RT	Area	Height	Plate count	Tailing
1.	Levo	3.513	521817	56358	3704.2	1.6
2.	Levo	3.515	522684	56384	3696.0	1.5
3.	Levo	3.516	522921	56456	3716.3	1.5
	Mean		522473.9		3705.5	1.6
	Std. Dev		58.1			
	%RSD		0.1			

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.

Formula:

$$LOD = 3.3 \times \frac{\sigma}{S}$$

Where σ - Standard deviation (SD)

S - Slope

Table 10: Showing results for Limit of Detection

Drug name	Standard deviation(σ)	Slope(s)	LOD(µg)
Azithromycin	389303.5	12307	0.01
Levofloxacin	278962.4	88178	0.001

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.

Formula:

$$LOQ = 10 X \frac{\sigma}{S}$$

Where

σ - Standard deviation

S - Slope

Table 11: Showing results for Limit of Quantification

Drug name	Standard deviation(σ)	Slope(s)	LOQ(µg)
Azithromycin	389303.5	12307	0.03
Levofloxacin	278962.4	88178	0.003

Robustness

The robustness was performed for the flow rate variations from 0.8ml/min to 1.2ml/min and mobile phase ratio variation from more organic phase to less organic phase ratio for azithromycin and levofloxacin. The method is robust only in more flow condition and the method is varies even by change in the Mobile phase ±2%.

4. CONCLUSION

A new method was established for simultaneous estimation of azithromycin and levofloxacin by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of azithromycin and levofloxacin by using symmetry C18 column[16] 4.6×150 mm 5.0µm, flow rate was 1.0ml/min, mobile phase ratio was (70:30 v/v) methanol : ammonium acetate buffer pH 6 (pH was adjusted with ammonia or acetic acid), detection[17] wave length was 262nm. The analytical method was validated according to ICH guidelines (ICH, Q2 (R1)). The linearity study for azithromycin and levofloxacin was found in concentration range of 20µg-100µg and 2µg-10µg 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 0.4 and 0.1, % RSD for intermediate precision was 0.1 and 0.2 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.

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