Research Article

RP-HPLC Analytical Method Development and Validation for Azithromycin and Levofloxacin in Tablet Dosage Form.

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ABSTRACT

A simple, rapid, and accurate reversed phase high-performance liquid chromatographic (RP-HPLC) method has been developed and subsequently validated for the simultaneous determination of azithromycin and levofloxacin in pharmaceutical dosage form. The HPLC analysis was performed on the C18 column (250 mm x 4.6 mm id, 5 µm particle size) in isocratic mode, at 30°C temperature using a water consisting of methanol: potassium dihydrogen phosphate buffer (60:40, v/v) at a flow rate of 1 mL/min. The detection was carried out at 279.6 nm for azithromycin and levofloxacin. The retention time for azithromycin and levofloxacin were found to be 5.08 min, and 2.90 min, respectively. The method was validated for precision, recovery, robustness, specificity, and detection and quantification limits, in accordance with ICH Q_2R_1 guidelines. Linearity was observed in the concentration range from 500-1500 µg/mL (r^2=0.99) for azithromycin and levofloxacin (r^2=0.99), the limit of detection and quantification of AZT were 2.68 µg/mL and 8.94 µg/mL, respectively. While for LFC it was 2.42 mg/mL and 8.08 mg/mL, respectively. The % recovery was found to be within 97-103% for Azithromycin and 98-102% for Levofloxacin. The % RSD below 2.0 showed the high precision of proposed method.

Keywords: Azithromycin, levofloxacin, high performance liquid chromatography and validation.

INTRODUCTION

Azithromycin (AZT) is semi synthetic macrolide antibiotic (Figure 1). Chemically it is (2R,3S,4R,5R,8R,10R,11R,12S,13S,14R)-13-{[(2,6dideoxy-3-o-methyl-∞-L-ribo hexopyranosyl) oxy]-2-ethyl-3,4,10-tri hydroxyl-3,5,6,8,10,12,14-hepta methyl-11-[(3,4,6-trideoxy-3-(dimethyl)amino)-β-D-xylo hexo pyranosyl)]oxy]-1-oxa-6-azacyclopentadecan15-one.
Levofloxacin (LFC) is broad spectrum, fluorinated quinolone antibacterial (Figure 2).

Chemically it is 9fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido[1,2,3-de]-1,4-benzaxazine-6-carboxilic acid.[1]

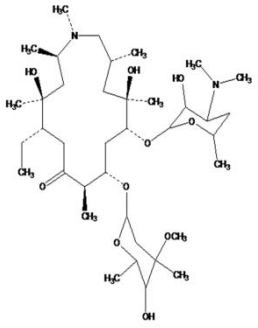

Figure 1: Structure of AZT.

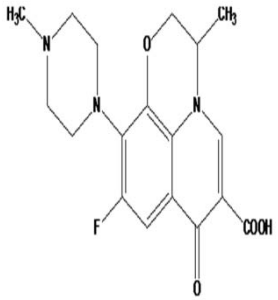

Figure 2: Structure of LFC.

A comprehensive literature survey revealed that various methods were reported for estimation of AZT and LFC individually or in combination with other drugs.

A number of assay methods have been used for analysis of AZT in bulk drug, pharmaceutical preparations, and serum using different techniques including spectrophotometer[2], LC-MS[3], HPTLC[4], UPLC[5]. LFC stability indicating assay method[6] and estimation in human plasma by HPLC-UV method[7] were also reported. LFC is also assayed with its combination of other drugs by spectro-photometry[8,9], RP-HPLC[10,11] and HPTLC method[12]. AZT and LFC were estimated with ambroxol HCl by HPLC[13], HPTLC[14] respectively.

No method had been reported for simultaneous estimation of these two drugs using HPLC in pharmaceutical dosage forms. Present work describes method development and validation of AZT and LFC as per ICH guidelines[15].

MATERIALS AND METHODS

Chemicals and Reagents

Working standard of AZT and LFC were obtained as a generous gift samples from Lara Labs Pvt. Ltd, Hyderabad were used without purification. Fixed dose combination tablet (Levomac-AZ) containing 500 mg Azithromycin and 500 mg Levofloxacin was purchased from Local medical, Pune. The present study all the reagents and chemicals of HPLC grade were used. Fischer scientific water was used.

Instrument

HPLC experimentation performed using Waters alliance e2695 separations model, equipped with photodiode array (PDA) detector. The method was conducted using isocratic reverse phase technique using Auto sampler, C18 column (250 mm x 4.6 mm id, 5 µm particle size), data acquisition and processing were performed using Empower Pro software.

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Preparation of Solutions

Standard Stock Solutions

The accurately weighed (500 mg) reference standard of each of AZT and LFC were added to volumetric flask containing water and sonicated for 15min. Makeup the volume with water to obtain the concentration of 10 mg/mL each of AZT and LFC. The solution was filtered through 0.45µm, 47mm membrane filter. An aliquot was diluted up to the mark with water to obtain the final concentration of AZT and LFC to 1000µg/mL. This dilution is considered as 100% solution. The working standard solution of azithromycin and levofloxacin were prepared from suitable aliquots of standard stock solution.

Sample Stock Solution

To determine the content of AZT and LFC in tablets, twenty tablets were weighed and average weight was determined. The accurately weighed powder equivalent to 500mg each of AZT and LFC were added to volumetric flask containing water and sonicated for 15min. Makeup the volume with water to obtained the concentration of 10 mg/mL each of AZT and LFC. The solution was filtered through 0.45µm, 47mm membrane filter. An aliquot was diluted up to the mark with water to obtain the final concentration of AZT and LFC to 1000µg/mL. This dilution is considered as 100% solution. Further dilutions were made accordingly.

Mixed Standard Stock Solution of AZT and LFC

Accurately weighed AZT (10 mg) and LFC (10 mg) were transferred to 10mL volumetric flask, dissolve and dilute up to the mark with water.

Optimization of HPLC Method

The RP-HPLC method was optimized with a view to develop simultaneous assay method for AZT and LFC. The mixed standard stock solution of drug AZT and LFC were injected and run in different solvent system. Phenomenex C18 (5 µm, 250 mm x 4.6 mm id) column thermostated at 30°C was used for chromatographic separation. The mobile phase composed of methanol: potassium di hydrogen phosphate buffer in ratio (60:40, v/v). The flow rate and injection volume was 1 mL/min and 10 µL respectively. The scanning wavelength selected was 279.6 nm.

Validation of the Method

Validation of the optimized RP-HPLC method was carried out with respect to the following parameters.

Linearity

From above standard stock solutions of AZT and LFC (10mg/mL) were further diluted to obtain the concentration of the drugs in the range of 500-1500 mg/mL (50-150%) each of AZT and LFC. The solutions of 50, 75, 100, 125, 150 % (10 µl) were injected into column. All measurements were repeated six times for each concentration. The calibration curves of the area under curve Vs concentration were recorded for both drugs.

Precision

Precision of the method was verified by repeatability. It is performed by injecting 100% sample solution for 6 times.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

LOD and LOQ were calculated as 3.3 C/σ and 10 C/σ, respectively as per ICH guidelines, where C is concentration of solution σ is s/n ratio.

Robustness of the Method

To evaluate robustness of HPLC method, few parameters were deliberately varied. The parameters included variation of flow rate and temperature. Robustness of the method was done at temperature of 25°C, 35°C and 0.8 mL, 1.2 mL flow rate per min.

Accuracy

Accuracy method was carried out by injecting 50, 100, 150% concentrations of sample solutions. 3 concentrations and 3 replicates of each concentration was injected, total of 9 determinations were performed.

Analysis of a Marketed Formulation

To determine the content of AZT and LFC in conventional tablet (Brand name: Levomac-AZ, Label claim: 500 mg AZT and 500 mg LFC, average weight of tablet equivalent to 500 mg AZT and 500 mg LFC was transferred into a 50 mL volumetric flask containing 25 mL water, sonicated for 10 min and diluted to 50 mL with water. The resulting solution was centrifuged at 3000 rpm for 5 min. The above stock solution was further diluted to get sample solution containing 1000 µg/mL AZT and LFC. A 10 µl volume of sample solution was injected into HPLC. The analysis was repeated in triplicate. The possibility of excipient interference with the analysis was examined.

RESULTS AND DISCUSSION

Linearity

Table 1: Linear regression data for calibration curve of AZT and LFC. (n=6)

Parameters	AZT	LFC
Linearity Range	500-1500 µg/mL	500-1500µg/mL
r^2	0.993	0.999
Slope	93182	58336
Intercept	29456	49396

The response for the detector was determined to be linear over the range of 500-1500 µg/mL for AZT and LFC. Calibration curve was constructed by plotting peak area vs. the drug concentration (%). The linear regression data for the calibration curves showed good linear relationship over the above mentioned concentration range. Linear regression equation was determined. Results expressed

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in the **Table 1.** The linearity curve of both AZT and LFC was showed in **Figure 3 and 4.**

Precision

The results of the repeatability experiment were shown in **Table 2.** The developed method was found to be precise as the RSD values for repeatability were < 2 % as recommended by ICH guidelines.

LOD and LOQ

Signal-to-noise ratios of 3:1 and 10:1 were obtained for the LOD and LOQ respectively. The LOD of AZT and LFC found to be 2.6 µg/mL and 2.4 µg/mL respectively.

The LOQ of AZT and LFC found to be 8.94 µg/mL and 8.08 µg/mL respectively.

Robustness

None of the alterations in experimental conditions caused a significant change in tailing factor and theoretical plates of AZT and LFC. Table 3 indicated robustness of the method.

Accuracy

As shown from the data in Table 4 good recoveries of the AZT and LFC in the range from 97 to 103 and 98 to 102 were obtained respectively at various added concentrations.

System Suitability Studies

The retention time, theoretical plate count, tailing factor, resolution were calculated for the standard solutions. The values obtained demonstrated the suitability of system for analysis of above drug combination.

Analysis of Azithromycin and Levofloxacin in Tablet

Using the proposed chromatographic methods the analysis of AZT and LFC in tablet was carried out. The peaks of AZT and LFC were observed in chromatograms at the retention time (t_R) of 2.918 and 5.213 minute, respectively **(Figure 5).** The in detail summary of analytical method validation is given in **Table 5.**

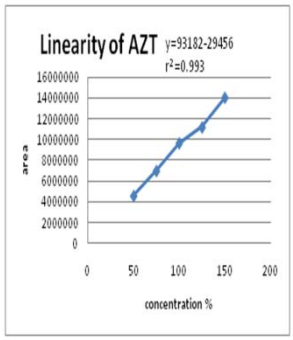

Figure 3: Linearity curve for AZT

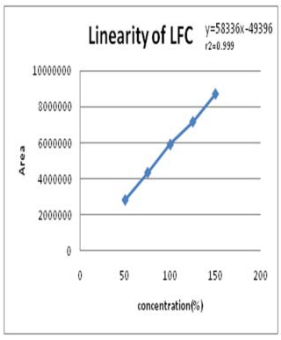

Figure 4: Linearity curve for LFC

Table 2: Precision results for AZT and LFC.

Sr. No.	Sample weight	AZT area	LFC area	AZT %Assay	LFC %Assay
1	1340.00	9659666	6000589	101	102
2	1340.00	9780727	6040214	102	102
3	1280.00	8956886	5752278	98	102
4	1340.00	9678630	5921893	101	100
5	1340.00	9640306	5992518	100	102
6	1340.00	9472925	5856800	99	99
Avg. Assay	-	-	-	100	101
% RSD	-	-	-	1.54	1.16

Table 3: Robustness results for AZT and LFC. (n=3)

Drug	Results	0.8mL/min Flow-1	1.2mL/min Flow-2	25°C Temp-1	35°C Temp-2
AZT	Tailing	1.389	1.355	1.38	1.38
	Resolution	12.552	12.85	13.02	12.81
	Theoretical plates	8104	7963	8433	9247
LFC	Tailing	1.639	1.539	1.518	1.535
	Resolution	-	-	-	-
	Theoretical plates	8183	8975	9424	9770

Table 4: Accuracy results for AZT and LFC.

Spiked Level (%)	Azithromycin			Levofloxacin		
	µg/ml added	µg/ml found	% Recovery	µg/ml added	µg/ml found	% Recovery
50%	451.95	452.28	100	456.52	459.19	101
100%	954.13	977.32	102	963.76	970.19	101
150%	1443.5	1460.2	101	1458.09	1429.6	98

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Table 5: Summary of Analytical Method validation.

Validation parameter	Azithromycin	Levofloxacin
Retention time (Rt) min	5.213	2.918
Tailing factor	1.417	1.643
Theoretical plate count	7910	7941
LOD (µg/mL)	2.68	2.42
LOQ (µg/mL)	8.94	8.08
Repeatability (avg. assay)	100	101
Linearity range (µg/mL)	500-1500	500-1500
% Recovery	100-102	98-101
Robustness	Robust	Robust

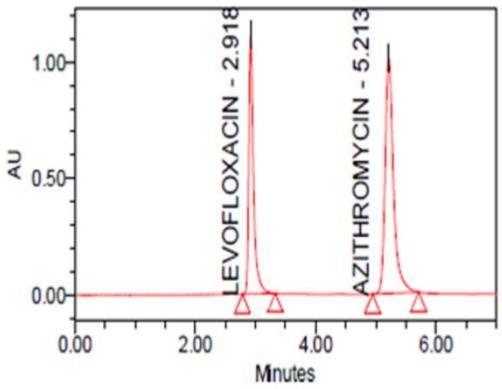

Figure 5: Typical chromatogram for AZT and LFC.

CONCLUSION

This method is simple, specific, and easy to perform and requires short time to perform to analyse the samples. The developed method was validated in terms of Linearity, precision, robustness, LOD, LOQ and accuracy. A good linear relationship was observed. The precision results were good enough to indicate that the proposed method was precise and reproducible. The assay experiment showed that the contents of AZT and LFC estimated in tablet dosage form were free from the interference of excipients. This demonstrated that the developed HPLC method could be conveniently adopted for the routine quality control analysis of AZT and LFC simultaneously from its pharmaceutical formulation and bulk drug.

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