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RP-HPLC Method Development and Validation for simultaneous estimation of Azithromycin and Levofloxacin in bulk and Pharmaceutical Dosage form

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ABSTRACT: Background: The literature survey revealed that few analytical methods have been developed for stability analysis of Azithromycin and Levofloxacin in Pharmaceutical solid dosage form. **Aim:** A new simple, accurate, economic, rapid and precise reverse phase high performance liquid chromatographic method has been developed for the validation of Azithromycin and Levofloxacin, in its pure form as well as in pharmaceutical dosage form. **Method:** Chromatography was carried out on X bridge C18 (4.6 × 150 mm) 5 µ column using a mixture of Methanol: Phosphate Buffer of pH 3.6 (30:70 v/v) as the mobile phase at a flow rate of 1.0 ml/min. The detection was carried out at 260 nm. **Results:** The retention time of the Azithromycin and Levofloxacin was 2.669 and 3.855 min respectively. The method produces linear responses in the concentration range of 10 to 50 µg/ml of Azithromycin and 10 to 50 µg/ml of Levofloxacin. The method precision for the determination of assay was below 2.0 % RSD. **Conclusion:** The method is useful in the quality control of bulk and pharmaceutical formulations.

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INTRODUCTION:

Analytical chemistry is a scientific discipline used to study the chemical composition, structure and behavior of matter. Validation is a necessary and important step in both framing and documenting the capabilities of the developed method [1,2]. The utility of the developed method to determine the content of drugs in commercial formulation was also demonstrated. Validation of the method was done in accordance with USP and ICH guidelines for the assay of active ingredients [3,4]. The method was validated for parameters like system suitability, linearity, precision, accuracy, specificity, ruggedness, and robustness, limit of detection and limit

of quantification [5,6]. This method provides means to quantify the component. This proposed method was suitable for the analysis of Pharmaceutical dosage forms [7,8]. The primary objective of proposed work is to develop a new simple, sensitive, accurate and economical analytical method for the simultaneous estimation of Azithromycin (AZI) and Levofloxacin (LFI). To validate the proposed method in accordance with USP and ICH guidelines for the intended analytical application i.e., to apply the proposed method for analysis of the Azithromycin (AZI) and Levofloxacin (LFI) in dosage form.

MATERIALS:

The Azithromycin and Levofloxacin pure drug (API) were procured as gift samples from Cadila Pharmaceutical Ltd, Mumbai, India. The tablets containing a combination of Azithromycin and Levofloxacin were purchased from the Local market of Moniabad. Methanol, ortho phosphoric acid and acetonitrile (CAN) were purchased from Merck, India. All other chemicals used in this study were of analytical grade and procured from authorized dealers.

METHODS:

Mobile Phase Optimization:

Initially the mobile phase used were Water: Methanol and ACN: Methanol with varying proportions. Finally, the mobile phase was optimized to phosphate buffer (pH 3.6) and methanol in proportion of 70:30 v/v respectively.

Optimization of Column:

The method was performed with various columns like C18 column ODS column, Zodiac column, and Xterra C18 column. Xbridge C18 (4.6 × 150 mm, 5µm) was found to be ideal as it gave good peak shape and resolution at 1 ml/min flow (Table 1) [9].

Table 1. The chromatographic study parameters.

Parameter	Values
Mobile phase	Methanol: Phosphate Buffer pH3.6 (30: 70 v/v)
Column	X bridge (4.6×150 mm, 5 µ)
Flow rate	1.0 ml/min
Wavelength	260 nm
Column temp	Ambient
Injection Volume	10 µl
Run time	8 min

Preparation of Phosphate buffer (pH 3.6):

About 1.1998 g of anhydrous di hydrogen phosphate was dissolved in sufficient HPLC Grade water to produce 1000 ml. The pH was adjusted to 3.6 by using ortho phosphoric acid.

Preparation of mobile phase:

Accurately 300 ml (30 %) of methanol and 700 ml of Phosphate buffer (70 %) were measured, mixed and degassed in digital Ultrasonicater (Grant MXB6 Ultrasonic Bath, , Space Tech Scientifique, Nigeria) for 10 min and then the solution was filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation:

The Mobile phase was used as the diluent.

Method of Precision [10-12]:

***Preparation of Standard Solution*:**

Accurately 10 mg of Azithromycin and 10 mg of Levofloxacin were weighed as working standard and transferred separately into a 10 ml clean dry volumetric flask. To the flask about 10 ml diluent was added. The mixture was sonicated to dissolve it completely. Finally the volume was made up to the mark with the same solvent (Stock solution). Further, about 0.3 ml of Azithromycin and Levofloxacin from stock solution was pipetted out into a 10 ml volumetric flask and diluted up to the mark with diluents.

***Preparation of Sample Solution*:**

Accurately 10 combination tablets were weighed and crushed in mortar and pestle. The powder equivalent to 10 mg of Azithromycin, Levofloxacin (marketed formulation-dose of Azithromycin is 250 mg, Dose of Levofloxacin is 250 mg in combination tablet) was transferred as sample into a 10 ml clean dry volumetric flask, into which about 7 ml of diluents was added and sonicated to dissolve it completely. The volume was made up to the mark with the same solvent (Stock solution).

Further, about 0.3 ml of Azithromycin and Levofloxacin from stock solution was pipetted out into a 10 ml volumetric flask and diluted up to the mark with diluents.

The standard and sample solutions of 30 µg/ml of Levofloxacin and 30 µg/ml of Azithromycin were injected for five times and the peak areas were recorded in HPLC. The mean and percentage relative standard deviation were calculated from the peak areas.

***Preparation of stock solution*:**
Accurately 10 mg of Azithromycin and 10 mg of Levofloxacin were weighed as working standard and transferred separately into a 10 ml clean dry volumetric flask. To the flask about 10 ml diluent was added and sonicated to dissolve it completely. The volume was made up to the mark with the same solvent (Stock solution).
Further, about 0.3 ml of Azithromycin and Levofloxacin from stock solution was pipetted out into a 10 ml volumetric flask and diluted up to the mark with diluents. The concentration Levofloxacin and Azithromycin in standard and sample solutions was 30 µg/ml. The standard solution was injected for five times and the area was measured for all five injections in HPLC. The % RSD for the area of five replicate injections was found to be within the specified limits.

Accuracy [10-12]:

***Preparation of Standard stock solution*:**
Accurately 10 mg of Azithromycin and 10 mg of Levofloxacin were weighed as working standard and transferred separately into a 10 ml clean dry volumetric flask. To the flask about 10 ml diluent was added and sonicated to dissolve it completely. The volume was made up to the mark with the same solvent. The strength of the stock solution I was 1000 µg/ml. Further, about 0.3 ml of Azithromycin and Levofloxacin from stock solution was pipetted out into a 10 ml volumetric flask and diluted up to the mark with diluents.

***Preparation Sample (50, 100 and 150 %) solutions*:**
Accurately 10 mg of Azithromycin and 10 mg of Levofloxacin were weighed as working standard and transferred separately into a 10 ml clean dry volumetric flask. To the flask about 10 ml diluent was added and sonicated to dissolve it completely. The volume was made up to the mark with the same solvent (Stock solution). Further from the above stock solution, 0.15, 0.30 and 0.45 ml of solutions were pipetted out into another three different 10 ml capacity volumetric flasks and all three flasks were diluted up to the mark with the diluent. The strength of standard and sample solutions were 50, 100 and 150 %.

***Procedure*:**
All the standard solutions of accuracy 50, 100 and 150 % were injected in the HPLC. The amount of Azithromycin and Levofloxacin which were added to the solution was calculated. The individual recovery and mean recovery values were also determined. These solutions were filtered through 0.45 µ membrane and then each concentration of solution was determined. The chromatograms were recorded and the peak responses were measured.

Linearity [10-12]:

***Preparation of stock solution*:**
Accurately 10 mg of Azithromycin and 10 mg of Levofloxacin were weighed as working standard and transferred separately into a 10 ml clean dry volumetric flask. To the flask about 10 ml diluent was added and sonicated to dissolve it completely. The volume was made up to the mark with the same solvent. The strength of the stock solution I was 1000 µg/ml. Further, about 0.3 ml of Azithromycin and Levofloxacin from stock solution was pipetted out into a 10 ml volumetric flask and diluted up to the mark with diluents.

***Procedure*:**
Each level of solution was injected into the chromatographic system and the peak area was measured. A graph was plotted by taking peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and the regression coefficient correlation was calculated.

Robustness [10-12]:
The analysis was performed in different conditions to find the variability of test results. The following conditions are checked for variation of results.

***Preparation of sample solution*:**
Accurately 10 mg of Azithromycin and 10 mg of Levofloxacin were weighed as working standard and transferred separately into a 10 ml clean dry volumetric flask. To the flask about 7 ml diluent was added and sonicated to dissolve it completely. The volume was made up to the mark with the same solvent. The strength of the stock solution was 1000 µg/ml. Further, about 0.3 ml of Azithromycin and Levofloxacin from stock solution was pipetted out into a 10 ml volumetric flask and diluted up to the mark with diluents. The strength of Azithromycin and Levofloxacin was 30 µg/ml.

***Effect of Variation of flow*:**
The sample was analyzed at 1.1 and 1.3 ml/min instead of 1.2 ml/min, remaining conditions were the same. About 10 µL of the above sample solution was injected twice and chromatograms were recorded.

***Effect of variation of mobile phase composition*:**
The sample was analyzed by variation of mobile phase i.e. methanol: Phosphate buffer of pH 3.6 was taken in

the ratios of 35:65 and 25:75 instead 30:70. Remaining conditions were the same. About 10 µL of the above sample was injected twice and chromatograms were recorded.

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

$LOD = 3.3 \times \sigma / s$ ……. (1)

Where, σ is Standard deviation of the response and S is Slope of the calibration curve.

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined.

$LOQ = 10 \times \sigma / S$ …….. (2)

RESULTS AND DISCUSSIONS:

High performance liquid chromatography is at present one of the most sophisticated tools of the analysis. The estimation of Azithromycin and Levofloxacin was done by RP-HPLC [13].

The Phosphate buffer was pH 3.6 and the mobile phase was optimized with Methanol: Phosphate buffer (pH 3) mixed in the ratio of 30: 70 % v/ v. An Xbridge column C18 (4.6 × 150 mm, 5 mm) or equivalent chemically bonded to porous silica particles was used as a stationary phase. The solutions were chromatographed at a constant flow rate of 1.0 ml/min.

This trial shows improper separation sample peaks, baseline and shows very less plate count in the chromatogram. So it's required more trials to obtain good peaks.

From the above chromatogram it was observed that the Levofloxacin and Azithromycin peaks are well separated and they show proper retention time, resolution, peak tail and plate count. So it's an optimized trial. The retention time of Levofloxacin and Azithromycin was found to be 2.669 and 3.855 min respectively (Table 2 and 3).

Table 2. The chromatographic (HPLC) peak data of Azithromycin and Levofloxacin.

SN	R_t	Area	Height	USP Res.	USP Tailing	USP PC
A1	2.669	986574	128672	--	1.5	3551.0
L2	3.855	5365216	562209	1.7	1.4	4675.7

A1 – Azithromycin, L2 – Levofloxacin, Res. – Resolution, Rt – Retention time, PC – Plate count. SN – Serial Number.

Table 3. Results of system suitability parameters for Azithromycin and Levofloxacin.

Sl No	R_t	Area (µV sec)	Height (µV)	USP Res.	USP Tailing	USP PC
A1	2.669	979867	129658	--	1.6	3854
L2	3.855	5356471	587452	1.8	1.9	4796

A1 – Azithromycin, L2 – Levofloxacin, Res. – Resolution, Rt – Retention time, PC – Plate count.

The linearity range of Azithromycin and Levofloxacin were found to be from 10 to 50 mg/ml (Table 4 and 5). The linear regression coefficient was not more than 0.999 (Fig 1 and 2).

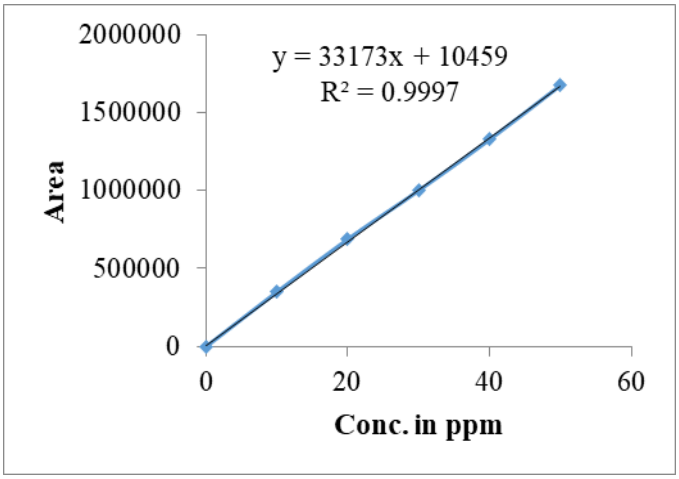

Fig 1. Calibration graph for Azithromycin.

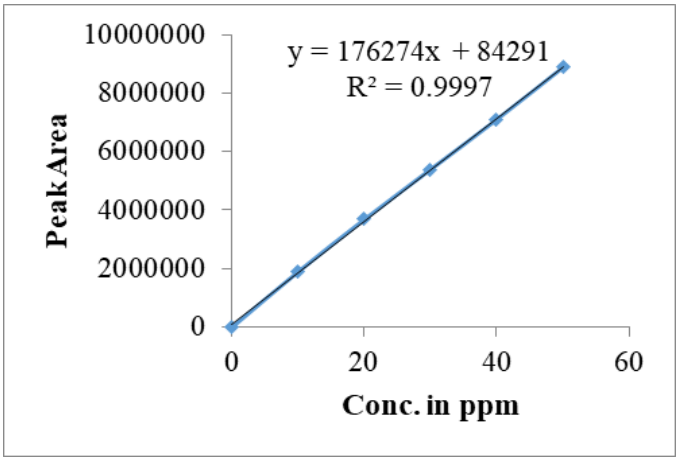

Fig 2. Calibration graph for Levofloxacin.

The % purity of Azithromycin and Levofloxacin in pharmaceutical dosage form was found to be 99 and 100 % respectively (Table 6). The percentage recovery varies from 98 to 102 % of Azithromycin and Levofloxacin. The values of % RSD are less than 2 % indicating accuracy and precision of the method (Table 7 and 8). LOD and LOQ were found to be within limit. The LOD was found to be 1.4 and 1.5 µg/ml for Azithromycin and Levofloxacin. The LOQ was found to be 4.2 and 4.7 µg/ml for Azithromycin and Levofloxacin.

Table 4. The data of assay standard results for Azithromycin and Levofloxacin.

Sl. No.	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP PC	Injection
1	Azithromycin	2.669	986587	127854	--	1.5	3552	1
2	Levofloxacin	3.855	5387451	561414	1.7	1.4	4654	1
3	Azithromycin	2.669	987824	126985	--	1.5	3571	2
4	Levofloxacin	3.855	5378475	568951	1.7	1.4	4635	2
5	Azithromycin	2.654	986541	127894	--	1.5	3841	3
6	Levofloxacin	3.849	5369875	568475	1.7	1.4	4684	3

Rt – Retention time, PC – Plate count.

Table 5. The data of assay sample results for Azithromycin and Levofloxacin dosage form.

Sl. No.	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP PC	Injection
1	Azithromycin	2.669	988626	127854	--	1.6	3561	1
2	Levofloxacin	3.855	5387547	568541	1.7	1.4	4874	1
3	Azithromycin	2.651	989685	127841	--	1.5	3658	2
4	Levofloxacin	3.849	5392435	563524	1.7	1.4	4641	2
5	Azithromycin	2.621	989874	127856	--	1.5	3854	3
6	Levofloxacin	3.840	5389854	565412	1.7	1.4	4365	3

Rt – Retention time, PC – Plate count.

Table 7. Results of Intermediate precision for Azithromycin.

Sl. No.	Name	Rt	Area	Height	USP PC	USP Tailing
1	Azithromycin	2.669	978985	128874	3686	1.5
2	Azithromycin	2.529	975686	128365	3654	1.5
3	Azithromycin	2.669	969876	128471	3536	1.5
4	Azithromycin	2.569	975487	128698	3682	1.5
5	Azithromycin	2.569	978546	128365	3598	1.5
6	Azithromycin	2.669	976898	128241	3536	1.5
Mean	--	--	975913	--	--	
Std. Dev.	--	--	3286.897	--	--	
% RSD	--	--	0.336802	--	--	--

Rt – Retention time, PC – Plate count.

Table 8. Results of Intermediate precision for Levofloxacin.

Sl. No.	Name	Rt	Area	Height	USP PC	USP Tailing	USP Resolution
1	Levofloxacin	3.845	5352141	563658	4685	1.4	1.7
2	Levofloxacin	3.795	5365847	564587	4665	1.4	1.7
3	Levofloxacin	3.855	5378412	563652	4654	1.4	1.7
4	Levofloxacin	3.840	5378543	563547	4641	1.4	1.7
5	Levofloxacin	3.855	5363598	565811	4669	1.4	1.7
6	Levofloxacin	3.855	5386879	562541	4658	1.4	1.7
Mean	--	--	5370903	--	--	--	--
Std. Dev	--	--	12656.43	--	--	--	--
% RSD	--	--	0.235648	--	--	--	--

Rt – Retention time, PC – Plate count.

CONCLUSION:

The results obtained on the validation parameters met ICH and USP requirements. It inferred the method found to be simple, accurate, precise and linear. The method was found to be having suitable application in routine laboratory analysis with high degree of accuracy and precision.

Table 6. The assay results of Azithromycin and Levofloxacin.

Sl. No.	Name of compound	% purity
1	Azithromycin	99
2	Levofloxacin	100

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