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Original Article

RP-HPLC METHOD AND ITS VALIDATION FOR ANALYSIS OF AZITHROMYCIN AND LEVO FLOXACIN HCL IN BULK AND PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

High-performance liquid chromatography (HPLC) is a sophisticated and widely used technique for pharmaceutical analysis. In this study, an RP-HPLC method was developed and validated for the simultaneous estimation of Azithromycin and Levofloxacin HCl in pharmaceutical formulations. The mobile phase was optimized consisting of Methanol and Phosphate buffer (pH 3) in a 30:70% v/v ratio. An Xbridge C18 column (4.6 x 150 mm, 5 µm) was employed as the stationary phase. The chromatographic separation was performed at a constant flow rate of 1.0 mL/min. The linearity of the method was found to be in the range of 10-50 µg/mL for both Azithromycin and Levofloxacin, with correlation coefficients (r^2) of 0.999 for both drugs. The method demonstrated good precision, with % RSD values less than 2%, indicating its accuracy and reliability. The percentage recovery for both drugs was in the range of 98-102%, confirming the method's accuracy. The Limit of Detection (LOD) and Limit of Quantification (LOQ) were found to be within acceptable limits. Validation of the method was conducted as per ICH and USP guidelines, confirming that the method is accurate, precise, linear, and suitable for routine analysis. This RP-HPLC method offers a simple, reliable, and effective approach for the routine analysis of Azithromycin and Levofloxacin HCl in bulk and pharmaceutical dosage forms with high accuracy and precision.

Keywords: *Azithromycin and Levofloxacin, RP-HPLC, Validation, Accuracy.*

INTRODUCTION

Every country has legislation on bulk drugs and their pharmaceutical formulations that sets standards and obligatory quality indices for them. These regulations are presented in separate articles relating to individual drugs and are published in the form of book called "Pharmacopoeia" (e.g. IP, USP, and BP). Quantitative chemical analysis is an important tool to ensure that the raw material used, and the intermediate products meet the required specifications. Every year number of drugs is introduced into the market. Also, quality is important in every product or service, but it is vital in medicines as it involves life [1-3]

There is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, report of new toxicities and development of patient resistance and introduction of better drugs by the competitors. Under these conditions standard and analytical procedures for these drugs may not be available in Pharmacopoeias. In instrumental analysis, the physical property of the substance is measured to determine its chemical composition. Pharmaceutical analysis comprises those procedures necessary to determine the identity, strength, quality and purity of substances of therapeutic importance [4-7].

Pharmaceutical analysis deals not only with medicaments (drugs and their formulations) but also with their precursors i.e. with the raw material on which degree of purity and quality of medicament depends. The quality of the drug is determined after establishing its authenticity by testing its purity and the quality of pure substance in the drug and its formulations [8-11].

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Quality control is a concept which strives to produce a perfect product by series of measures designed to prevent and eliminate errors at different stages of production. The decision to release or reject a product is based on one or more types of control action. With the growth of pharmaceutical industry during last several years, there has been rapid progress in the field of pharmaceutical analysis involving complex instrumentation. Providing simple analytical procedure for complex formulation is a matter of most importance. So, it becomes necessary to develop new analytical methods for such drugs. In brief the reasons for the development of newer methods of drugs analysis are 12-16:

1. The drug or drug combination may not be official in any pharmacopoeias.

2. A proper analytical procedure for the drug may not be available in the literature due to Patent regulations.

3. Analytical methods for a drug in combination with other drugs may not be available.

4. Analytical methods for the quantitation of the drug in biological fluids may not be available.

5. The existing analytical procedures may require expensive reagents and solvents. It may also involve cumbersome extraction and separation procedures, and these may not be reliable [17-19].

HPLC is also called high pressure liquid chromatography since high pressure is used to increase the flow rate and efficient separation by forcing the mobile phase through at much higher rate. The pressure is applied using a pumping system. The development of HPLC from classical column chromatography can be attributed to the development of smaller particle sizes. Smaller particle size is important since they offer more surface area over the conventional large particle sizes. The 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 [20-24].

1. Improved resolution of separate substances

2. Column packing with very small (3,5 and 10 µm) particles

3. Faster separation times (minutes)

4. Sensitivity

5. Reproducibility

6. Continuous flow detectors capable of handling small flow rates

7. Easy sample recovery, handling and maintenance. 6

The basic liquid chromatograph consists of six basic units. The mobile phase supply system, the pump and programmer, the sample valve, the column, the detector and finally a means of presenting and processing the results.

Mobile phase (solvent) reservoirs and solvent degassing

The mobile phase supply system consists of number of reservoirs (200 mL to 1,000 mLin capacity). They are usually constructed of glass or stainless-steel materials which are chemically resistant to mobile phase.

Mobile phase

Mobile phases in HPLC are usually mixtures of two or more individual solvents. The usual approach is to choose what appears to be the most appropriate column, and then to design a mobile phase that will optimize the retention and selectivity of the system. The two most critical parameters for nonionic mobile phases are strength and selectivity25.

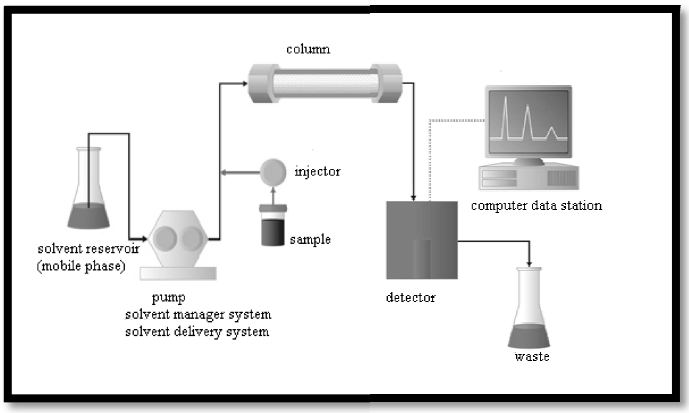


Fig 1: Components of HPLC instrument block diagram.

Mobile phase preparation: Mobile phases must be prepared from high purity solvents, including water that must be highly purified. Mobile phases must be filtered through ≤1 µm pore size filters and be degassed before use.

Degassing of solvents: Many solvents and solvent mixtures (particularly aqueous mixtures) contain significant amounts of dissolved nitrogen and oxygen from the air. These gases can form bubbles in the chromatographic system that cause both serious detector noise and loss of column efficiency. These dissolved gases in solvent can be removed by the process of degassing. Every solvent must be degassed before introduction into pump as it alter the resolution of column and interfere with monitoring of the column effluent[26].

Degassing is done in many ways:

1. By warming the solvents

2. By stirring vigorously with a magnetic stirrer

3. By subjecting to vaccum filtration

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4. By ultra sonication (using ultrasonicator)

5. By bubbling the gas through the solvent reservoir.

Levofloxacin inhibits bacterial type II topoisomerases, topoisomerase IV and DNA gyrase. Levofloxacin, like other fluoroquinolones, inhibits the A subunits of DNA gyrase, two subunits encoded by the gyrA gene. This results in strand breakage on abacterial chromosome, supercoiling, and resealing; DNA replication and transcription is inhibited.

Azithromycin, a semisynthetic antibiotic belonging to the macrolide subgroup of azalides, is used to treat STDs due to chlamydia and gonorrhea, community-acquired pneumonia, pelvic inflammatory disease, pediatric otitis media and pharyngitis, and Mycobacterium avium complex (MAC) in patients with advanced HIV disease. Similar in structure to erythromycin. azithromycin reaches higher intracellular concentrations than erythromycin, increasing its efficacy and duration of action. The aim of this study is to develop and validate a reverse phase high-performance liquid chromatography (RP-HPLC) method for the simultaneous determination of azithromycin and levofloxacin HCI in bulk and pharmaceutical dosage forms. The method will be optimized for precision, accuracy, and sensitivity, ensuring its applicability in routine quality control analysis and compliance with pharmacopeial standards [27, 28].

Table 2: Physical properties of common HPLC solvents.

Solvent	MP Melting point	BP Boiling point	RI (25oC) Refractive index	UV Cutoff (nm)	Density g/Ml (25oC)	Viscosity (25oC)	Dielectric Constant
Acetonitrile	41.0	82	1.342	190	0.787	0.358	38.8
Dioxane	88.1	101	1.420	215	1.034	1.26	2.21
Ethanol	46.1	78	1.359	205	0.789	1.19	24.5
Ethyl acetate	88.1	77	1.372	256	0.901	0.450	6.02
Methanol	32.0	65	1.326	205	0.792	0.584	32.7
CH2Cl2	84.9	40	1.424	233	1.326	0.44	8.93
Isopropanol	60.1	82	1.375	205	0.785	2.39	19.9
n-propanol	60.1	97	1.383	205	0.804	2.20	20.3
THF	72.1	66	1.404	210	0.889	0.51	7.58
Water	18.0	100	1.333	170	0.998	1.00	78.5

Materials: Azithromycin and Levofloxacin received as kind gift from Hetero Drugs and Koye Pharmaceuticals, citric acid, anhydrous dihydrogen phosphate purchased from Finar Chemicals, all other Organic solvents are of HPLC grade.

Methodology:

HPLC METHOD DEVELOPMENT:

Mobile Phase Optimization: Initially the mobile phase tried was Water: Methanol and ACN: Methanol with varying proportions. Finally, the mobile phase was optimized to phosphate buffer (pH 3.6), Methanol in proportion 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 x 150mm, 5µm) was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

OPTIMIZED CHROMATOGRAPHIC CONDITIONS:

Instrument used: Waters HPLC with auto sampler and PDA detector 996 model.

Column: X bridge C18 (4.6×150mm) 5 µ

Buffer: Phosphate buffer (pH-3.6) - Dissolve 1.1998g of anhydrous di-hydrogen phosphate in sufficient water to produce 1000ml. Adjust the pH 3.6 by using ortho phosphoric acid.

pH: 3.6

Mobile phase: Methanol: Phosphate Buffer pH-3.6 (30:70v/v)

Flow rate: 1.0 ml per min

Wavelength: 260 nm

Injection volume: 10µl

Run time: 10 min.

Preparation of Phosphate buffer (pH-3.6): Dissolve 1.1998g of anhydrous di hydrogen phosphate dissolved in sufficient HPLC Grade water to produce 1000mL. Adjust the pH 3.6 by using ortho phosphoric acid.

Preparation of mobile phase: Accurately measured 300 ml (30%) of Methanol and 700 ml of Phosphate buffer (70%) were mixed and degassed in digital ultrasonicater for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation: The Mobile phase was used as the diluent.

VALIDATION PARAMETERS:

Method Precision:

Preparation of Standard Solution: Accurately weigh and transfer 10 mg of Azithromycin and 10mg of Levofloxacin working standard into a 10 ml and 10 ml of clean dry volumetric flasks add about 10ml and 10 ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).Further pipette 0.3ml of Azithromycin and Levofloxacin from stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Preparation of Sample Solution: Accurately weigh 10 combination tablets crush in mortor and pestle and transfer equivalent to 10 mg ofAzithromycin, Levofloxacin (marketed formulation-dose of Azithromycin is 250 mg, Dose of Levofloxacin is 250mg in combination tablet) sample into a 10mL clean dryvolumetric flask add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).Further pipette

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0.3ml of above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.The standard and sample solutions of 30 µg/ml of Levofloxacin, 30 µg/ml of Azithromycin are injected five times and the peak areas were recorded. The mean and percentage relative standard deviation were calculated from the peak areas.

Intermediate Precision/Ruggedness: To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day by using different make column of same dimensions.

Preparation of stock solution: Accurately weigh and transfer 10 mg of Azithromycin and 10mg of Levofloxacin working standard into a 10 ml and 10 ml of clean dry volumetric flasks add about 10ml and 10 ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).Further pipette 0.3ml of above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.The standard and sample solutions of containing concentrations were 30µg/ml of Levofloxacin, 30 µg/ml of Azithromycin.

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

Accuracy:

Preparation of Standard stock solution: Accurately weigh and transfer 10 mg of Azithromycin and 10mg of Levofloxacin working standard into a 10 ml and 10 ml of clean dry volumetric flasks add about 10mL and 10 ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution-I 1000ppm).Further pipette 0.3ml of Azithromycin and Levofloxacin from stock solution into a 10ml volumetric flask and dilute up to the mark with diluents

LINEARITY:

Preparation of stock solution: Accurately weigh and transfer 10 mg of Azithromycin and 10mg of Levofloxacin working standard into a 10 ml and 10 ml of clean dry volumetric flasks add about 10ml and 10 ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution, 1000ppm).

ROBUSTNESS: 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 (30µg/ml of Levofloxacin & 30µg/ml of Azithromycin)

Accurately weigh and transfer 10 mg of Azithromycin and 10mg of Levofloxacin working standard into a 10 mL and 10 ml of clean dry volumetric flasks add about 7mL and 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution- 1000ppm). Further pipette 0.3 ml of Azithromycin and Levofloxacin from stock solution into a 10ml volumetric flask and dilute up to the mark with diluents

Results & Discussion:

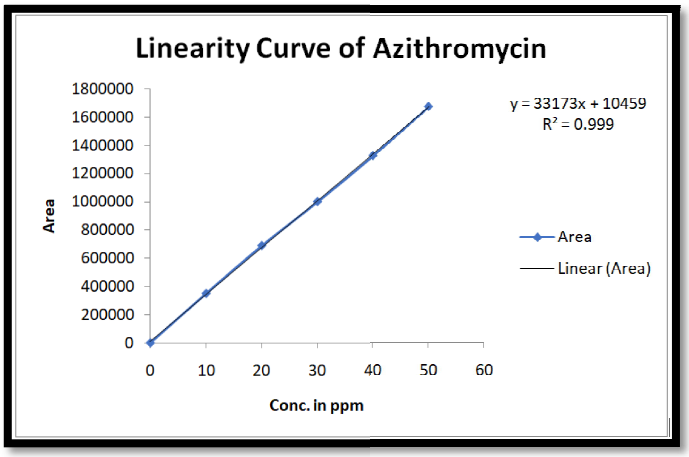


Fig 1: Calibration graph for Azithromycin

Table 1: Azithromycin Linearity Results

Concentration(ppm)	Area
10	349877
20	688574
30	999895
40	1326522
50	1673877

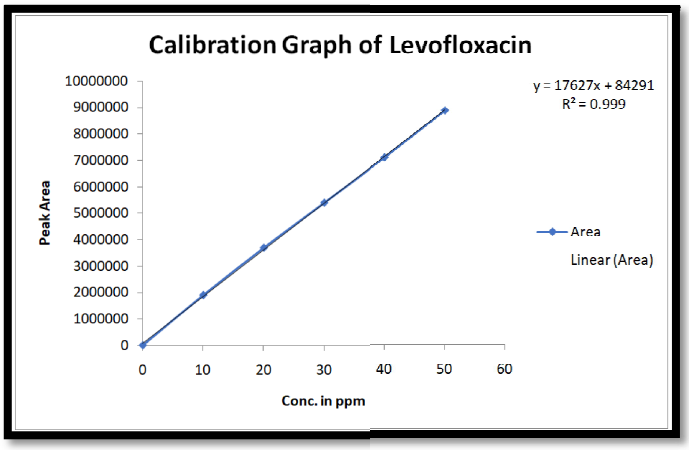


Fig 2: Calibration graph for Levofloxacin

Table 2: Levofloxacin Linearity Results

Concentration (ppm)	Area
10	1896545
20	3685798
30	5389557
40	7096443
50	8878478

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SYSTEM SUITABILITY:

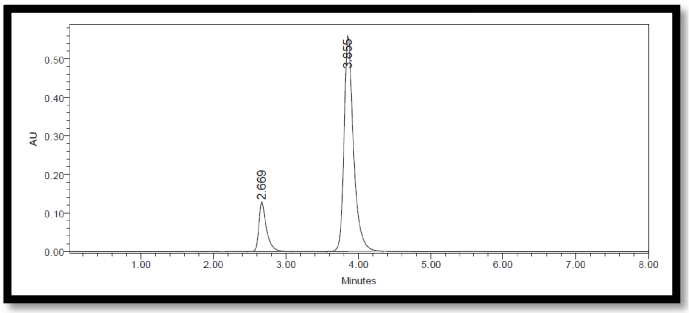


Fig 3: Chromatogram for system suitability

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

S.No	Name	Retention time(m)	Area (µV sec)	Height (µV)	USP resolution	USP tailing	USP plate count
1	Azithromycin	2.669	979867	129658		1.6	3854
2	Levofloxacin	3.855	5356471	587452	1.8	1.9	4796

Table 4: Results of method precision for Azithromycin

S.No.	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Azithromycin	2.669	986857	128231	3653	1.5
2	Azithromycin	2.659	987854	129852	3541	1.5
3	Azithromycin	2.671	985474	128145	3635	1.5
4	Azithromycin	2.669	986589	129611	3595	1.5
5	Azithromycin	2.669	985213	128321	3698	1.5
Mean			986397.4			
Std. Dev			1075.302			
% RSD			0.109013			

Table 5: Results of method precession for Levofloxacin

S.no	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Levofloxacin	3.855	5378559	565621	4675	1.4	1.7
2	Levofloxacin	3.842	5386231	564587	4696	1.4	1.7
3	Levofloxacin	3.850	5385411	563651	4684	1.4	1.7
4	Levofloxacin	3.845	5369874	563544	4763	1.4	1.7
5	Levofloxacin	3.855	5389745	578547	4954	1.4	1.7
Mean			5381964				
Std. Dev			7880.279				
% RSD			0.14642				

Accuracy standard:

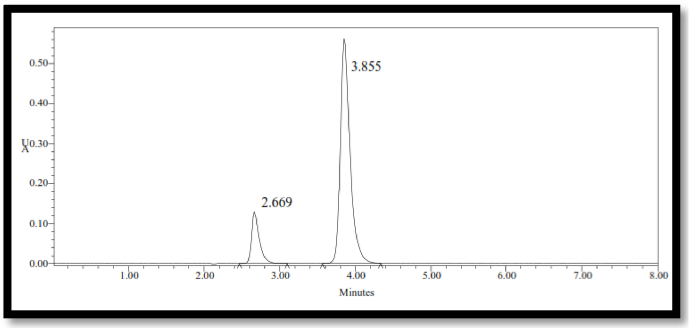


Fig 4: Chromatogram for Accuracy std. Injection

TABLE 6: Results of Accuracy standard values

Sno	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Azithromycin	2.669	509688	127412		1.5	3253	1
2	Levofloxacin	3.855		562635	1.7	1.4	4145	1
3	Azithromycin	2.658	508759	127854		1.5	3321	2
4	Levofloxacin	3.849		563651	1.7	1.4	4534	2
5	Azithromycin	2.661	509867	127854		1.5	3536	3
6	Levofloxacin	3.851		568519	1.7	1.4	4642	3

Linearity:

The linearity range was found to lie from 10µg/ml to 50µg/ml of Azithromycin, 10µg/ml to 50µg/ml of Levofloxacin and chromatograms are shown below.

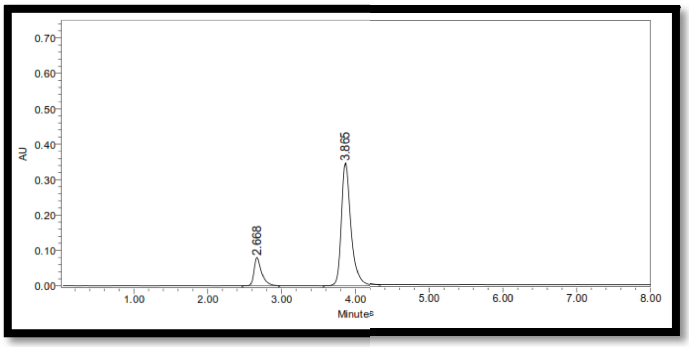


Fig 5: Chromatogram for linearity concentration 10 µg/ml of Azithromycin & 10 µg/ml of Levofloxacin

Table 7: Analytical performance parameters of Azithromycin and Levofloxacin

Parameters	Azithromycin	Levofloxacin
Slope (m)	33173	17627
Intercept (c)	10459	84291
Correlation coefficient (R2)	0.999	0.999

Robustness

Variation in flow

Table 8: System suitability results forAzithromycin

S.No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.9	3569.2	1.5
2	1.0	3551.0	1.5
3	1.1	3584.4	1.5

* Results for actual flow (1.0 ml/min) have been considered from Assay standard.

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Table 9: System suitability results for Levofloxacin

S.No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.9	4864.2	1.4
2	1.0	4675.7	1.4
3	1.1	4524.9	1.4

* Results for actual flow (1.0ml/min) have been considered from Assay standard.

Discussion: In this study, a Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) method was developed and validated for the simultaneous estimation of Azithromycin and Levofloxacin HCl in pharmaceutical dosage forms. The mobile phase was optimized, consisting of Methanol and Phosphate buffer (pH 3) in a 30:70% v/v ratio, and chromatographic separation was achieved using an Xbridge C18 column (4.6 x 150 mm, 5 µm) at a flow rate of 1.0 mL/min. The method demonstrated a linear response over the concentration range of 10-50 µg/mL for both Azithromycin and Levofloxacin, with excellent correlation coefficients (r^2 = 0.999). Precision was validated with % RSD values of less than 2%, indicating high accuracy and reproducibility. The method showed a percentage recovery of 98-102%, further confirming its reliability. Limit of Detection (LOD) and Limit of Quantification (LOQ) were determined to be within the required limits. The method was validated according to ICH and USP guidelines, ensuring its compliance for routine use in pharmaceutical analysis.

Conclusion:

The developed RP-HPLC method for the simultaneous estimation of Azithromycin and Levofloxacin HCl proved to be simple, accurate, precise, and linear, making it highly suitable for routine pharmaceutical analysis. With excellent linearity, accuracy, precision, and recovery, the method meets the standards for both ICH and USP guidelines. It offers a reliable approach for the analysis of these drugs in bulk and pharmaceutical dosage forms, providing a cost-effective solution for quality control in pharmaceutical laboratories. The method's robust validation ensures its applicability in the routine analysis of Azithromycin and Levofloxacin HCl, supporting the consistent quality and potency of these drugs in their final pharmaceutical forms.

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