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HPLC METHOD DEVELOPMENT AND VALIDATION FOR AZITHROMYCIN IN ORAL SUSPENSION

Singh AP*[1], Chauhan I[1], Bhardwaj S[1], Gaur P[1], Kumar SS[1], Jayendra[2]

[1]I.T.S College of Pharmacy, Muradnagar, Ghaziabad, Uttar Pradesh, India

[2]School of Pharmacy, Monad University, Hapur, Uttar Pradesh, India

ABSTRACT

Introduction: Azithromycin a semi-synthetic, azalide congener of erythromycin indicated in the treatment of respiratory tract infections. Various methods available for determination of Azithromycin, but HPLC are most versatile one. **Objective:** the present study is based on the development and validation of a rapid, simple high performance liquid chromatography (HPLC) method equipped with UV detector for quantitative analysis of Azithromycin (AZN) in suspension. **Material and methods**: The Method was performed by using Hypersil BDS-C18 (250 mm × 4.6 mm i.d.) column MS-II, with an isocratic mobile phase of methanol, acetonitrile and phosphate buffer pH 8 (60:30:10; v/v) with run time 15 minutes. The determinations were performed at a flow rate of 1.0ml/min, and UV detector set at 212 nm. **Result and Discussion**: The method was found to be specific with relative standard deviation (RSD) less than 2.09%. The method showed accuracy with RSD less than 1.34% and precision in repeatability with RSD less than 1.42%. The method was found to be linear over a wide range of concentration from 1.0 to 50.0 µg/mL (R^2 = .995). Limit of detection and limit of quantification were found to be 14.40 ng/mL and 43.66 ng/mL respectively. **Conclusion**: It was advantageous to use UV detector over other methods employing electrochemical, photodiode array etc. as the detector, because of cheap and easy availability. The developed method fulfilled all validation parameters as per ICH and can be successfully applied to quantify percent drug content in marketed oral AZN suspension.

Keywords: Trueness, Precision, HPLC, Azithromycin, Suspension.

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Correspondence:
Alok Pratap Singh
I.T.S College of Pharmacy, Muradnagar, Ghaziabad (201206), Uttar Pradesh, India.
Phone: +91-9318357335
Email: alokvats13@gmail.com

INTRODUCTION

Azithromycin (2R, 3S, 4R, 5R, 8R, 10R, 11R, 12S, 13S, 14R)-2-ethyl-3,4,10-trihydroxy 3,5, 6, 8,10,12,14-heptamethyl-15-oxo-11-{[3,4,6-trideoxy-3-(dimethylamino)-β D-xylohexapyranosyl] oxy}-1-oxo-6-azacyclopendadec-13-yl 2,6 dideoxy-3-C-methyl-3-O-methyl-α-L-ribo-hexopyranside), a semi-synthetic, azalide congener of erythromycin (extended spectrum), usually indicated in the treatment of respiratory tract infections such as pharyngitis, pneumonia, chronic bronchitis, bronchopneumonia, skin related problems and some sexually transmitted disorders like gonorrhea. It differs chemically from erythromycin in that a methyl-substituted nitrogen atom is incorporated into the lactone ring as seen in Figure 1.

Depending on dose it is bacteriostatic (at low concentration) and bactericidal (at higher concentration) in nature. It acts by inhibiting bacterial protein synthesis. It binds to the 50S subunit of the bacterial ribosome, and thus inhibits translocation of peptide chain from acceptor A to acceptor P in the 50S subunit in turns results in formation premature protein. [1] Nucleic acid synthesis is not affected. AZN is official in Indian, British and United States Pharmacopoeia. [2]

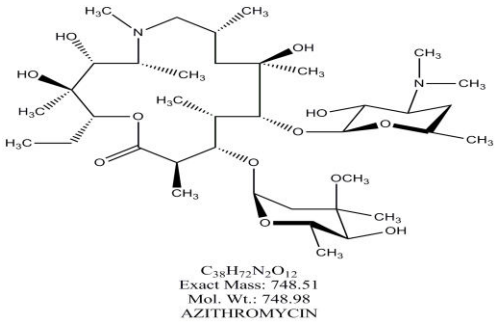

Figure 1: Chemical Structure of Azithromycin

Amongst various methods available for determination of AZN, HPLC is a most versatile one. HPLC methods earlier mentioned in literature used electrochemical, [3] mass spectrometry [4] and fluorescence detector. [5] These detectors are time consuming, not readily available at laboratory scale and require complicated procedure like pretreatment of the sample. Methods official in IP

and USP utilize a high pH mobile phase. Besides this column used in USP technique is highly explicit, [6] expensive and not readily available. Other spectroscopic methods as reported earlier are either complex [7] or need alteration in API that is pretreatment. [8] Hence there arises a need for the versatile, simple and easy method for determination of AZN by HPLC instrument equipped with UV spectroscopy as the detector.

MATERIAL AND METHODS

Materials

AZN standard (99.54%) was supplied by Synokem Pharmaceuticals Ltd. Ranipur, Haridwar, India as gift sample. Methanol and acetonitrile (HPLC-grade), potassium dihydrogen phosphate, hydrochloric acid, and sodium hydroxide were acquired from Merck (Darmstadt, Germany). Distilled water was passed through 0.45 µm Millipore filter (Millipore Company, USA) before use. All other reagents used in experiment were of AR and HPLC grades.

Instrumentation and conditions

All Chromatographic analysis was performed on a binary gradient Jasco HPLC system with UV Visible (JASCO UV-2075 plus) detector. The obtained signal's acquisition, analysis, and reporting were performed by using Borwin Chromatographic software. Hypersil BDS-C18 (250 mm × 4.6 mm i.d.) column MS-II was used for separation.

Determination of appropriate wavelength

The suitable wavelength for determination of AZN was determined in the mobile phase by scanning 1.0 microgram sample solution over the range of 200-400 nm with Shimadzu UV-1800 (Shimadzu, Japan) as presented in Figure 2.

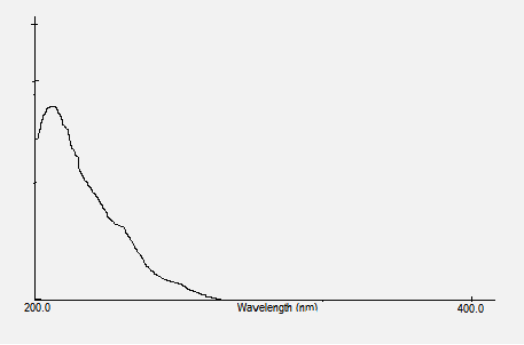

Figure 2: UV spectrum of Azithromycin

Preparation of Mobile Phase

A water-based solvent, organic solvent, or a mixture of the two is mainly used as the mobile phase for HPLC. A buffer solution is often used as the aqueous solvent. After hit and trial, two organic solvents methanol and acetonitrile along with Phosphate buffer pH 8 (in a ratio of 60:30:10 respectively) was chosen as the mobile phase. The solution mixture was degassed in an ultrasonic water bath for 10 minutes and filtered through 0.22 micron pore size membrane filter under vacuum condition.

Preparation of standard solution

A stock standard solution of 500 µg/mL was prepared by dissolving 1mg AZN in 2.0 mL of mobile phase and subsequently diluted with mobile phase to get ten standard solutions (1.0, 2.0, 4.0, 6.0, 8.0, 10.0, 20.0, 30.0, 40.0 and 50.0 µg/mL).The pH of the solution was maintained at 8. The mobile phase was filtered through a 0.22 µm pore size filter (Millipore, Bedford, USA) prior to injection.

Chromatographic conditions

A chromatographic examination was performed in the isocratic mode. The mobile phase was pumped at a flow rate of 1.0mL/min. The sample injection volume was 20 µL, and the UV Vis detection wavelength was set at 212 nm. The method run time was 15 min, and all investigations were performed at 25°C. A simple chromatogram of Azithromycin is indicated in Figure 3 and the chromatographic conditions are presented in Table 1.

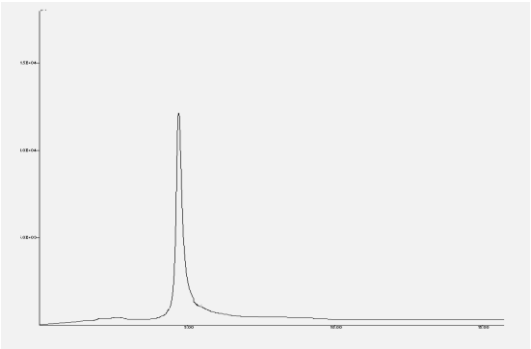

Figure 3: Azithromycin HPLC chromatogram

Table 1: Chromatographic Condition

S. No.	Optimized parameters	Optimized Outcomes
1	Drug used	Azithromycin
2	Column Stationary phase	Hypersil BDS-C18 column MS-II
3	Mobile Phase	Methanol, Acetonitrile and Phosphate buffer (60:30:10)
4	pH	8
5	Flow Rate	1.0 mL/min
6	Run Time	15 min
7	Retention time (Rt)	4.8 min
8.	Column Temperature	25°C
9.	Volume of injection	20 µL
10.	Detection wavelength	212 nm

Method validation

This process ensures the suitability of the analytical procedure used for specific test and its intended use. The method was validated as stated by the International Conference on Harmonization (ICH) guidelines Q2 (R1) 2005 with respect to characteristics like specificity, linearity, accuracy, precision, Limit of detection, Limit of quantification, and its applicability.

Specificity

The parameter is used to identify the absolute, expected to be present analyte in the presence of components. Typically these might include impurities, degradants, matrix, etc. The specificity was evaluated by comparing the representative chromatograms of samples containing possible interfering substances and samples containing AZN. Additionally, specificity was demonstrated by performing four conditions of stress i.e., photo stability, pH variation, temperature, and oxidation [9] as indicated in Table 2 and a chromatogram is presented in Figure 4.

Table 2: Specificity as recovery of proposed method

Experimental Conditions	Concentration			% RSD
	Sample (µg/ml)	Drug Recovered (µg/ml)±SD	% drug Recovered	
Reference	30	30.80± 0.218	102.6	0.70
Sunlight	30	26.98± 0.564	89.9	2.09
Acidic pH	30	24.12± 0.489	80.4	2.02
Alkaline pH	30	29.56± 0.246	98.5	0.89
Temperature	30	30.18± 0.128	100.6	0.42
Oxidation	30	27.68± 0.297	92.2	1.07

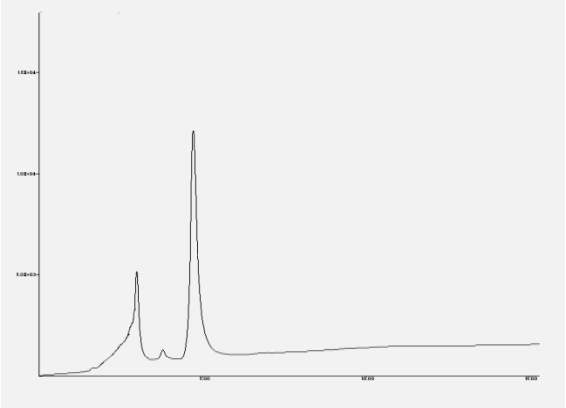

Figure 4: Azithromycin HPLC chromatogram

Photo stability

Pure solid drug (in 1mm thick layer in a petri plate) was exposed to sunlight for 8 hours. The sample was analyzed by HPLC after suitable dilutions.

pH variation

***Acid hydrolysis*:** 3.0 mL of stock solution was taken in 10 mL volumetric flask and 1 mL of 0.1 N Hydrochloric acid was added to it, the volume was makeup with methanol. The solution was kept aside for 90 min in normal condition. After 30 min time interval 1 mL of sample was taken and diluted to obtain a concentration of 30 µg/mL. The sample was neutralized by 0.1N NaOH before injecting in the column.

***Alkali hydrolysis*:** 3.0 mL of stock solution was taken in 10 mL volumetric flask and 1 mL of 0.1 N NaOH was added to it, the volume was makeup with methanol. The solution was put aside for 90 min in normal condition. After 30 min, 1 mL of sample was taken and diluted to get a concentration of 30 µg/mL. Before injecting into the column the solution was neutralized by 0.1N HCl.

Temperature

Powdered drug was kept in Petri plate at 70 °C for 48 hrs. The sample was analyzed by HPLC after suitable dilutions.

Oxidative degradation

3.0 mL of AZN Solution and 1 mL of 30% (v/v) of hydrogen peroxide was mixed in a 10 mL volumetric flask and diluted up to the mark with methanol. The solution was left overnight at room temperature.

Linearity

This parameter is used explains the direct proportionate relations between the test results and concentration of analyte in sample within given range. Linearity was determined by calculating a regression line from the plot of peak area vs. concentration for the ten standard solutions in mobile phase (i.e. 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, 20.0, 30.0, 40.0 and 50.0 µg/mL) using the linear least squares methodology. Linearity plot for Azithromycin was presented in Figure 5.

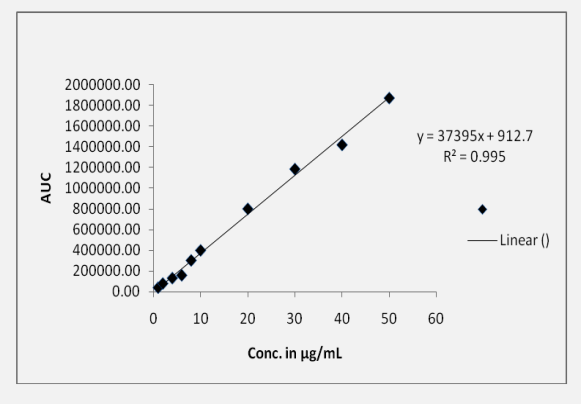

Figure 5: Linearity plot for Azithromycin

Trueness

The trueness/accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. The accuracy was tested by calculating the percent recovery of the mean concentration of AZN at different concentration levels in triplicate, and the relative standard deviation (RSD) was determined. [10] The mean concentration value obtained for each level was compared to the theoretical value, which was considered to be 100 %. Accuracy as a measure of recovery of proposed method was presented in Table 3.

Table 3: Accuracy as recovery of proposed method

A	Concentration			% drug Recovered	% RSD
	B	C= A+B	Drug Recovered (µg/ml)±SD		
50	30	45	45.12± 0.115	100.2	0.25
100	30	60	61.08± 0.819	101.8	1.34
150	30	75	74.90±0.631	99.8	0.84

*(n=3); **A:** % of Standard added to sample; **B:** Sample (µg/ml); **C:** Total drug (Sample + Standard) (µg/ml).

Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability (expresses the precision under the same operating conditions over a short interval of time), intermediate precision (expresses the precision within-laboratories variations: different days, different analysts, different equipment, etc.) and reproducibility (expresses the precision between laboratories-collaborative studies, usually applied to standardization of methodology). Here the precision was assessed at two levels: repeatability or intra-day variability and intermediate precision or inter-day variability. The repeatability was assessed by injecting the standard solution of AZN at three different concentrations (2.0, 4.0 and 6.0µg/mL) three times in succession. Intraday variability was performed by the same analyst over one day and intermediate precision was carried out by another independent analyst over 3 days [10, 11]. The data were expressed as %RSD in Table 4.

Limit of detection and Limit of quantification

The limit of detection for an individual procedure represents the minimum amount of analyte in test sample. This amount can be detected but not importantly quantified as an exact value.

The LOD and LOQ were determined from the specific calibration curve obtained using different standard solutions ranging from 50.0 ng/mL to 300.0 ng/mL that was the closest to the LOQ as presented in Figure 6 and calculated by using following equation according to ICH (2005). [9] [11]

$$LOD = 3.3/\sigma*S$$

$$LOQ = 10/\sigma*S$$

(Where σ and S stands for standard deviation of the response and slope of calibration curve respectively)

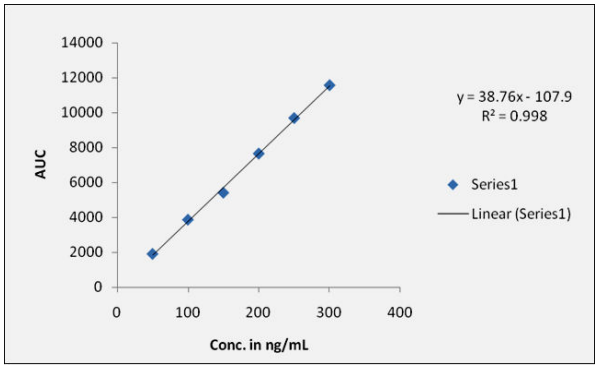

Figure 6: Calibration curve of Azithromycin for LOD and LOQ

Application of Developed method

Assay of Marketed Formulation by the proposed method

***Preparation of Sample solution*:** A suspension containing AZN was purchased from local market. An accurately measured amount of the suspension, equivalent to about 200 mg (as labeled claim) of AZN was transferred to a 200 mL volumetric flask, and by serial dilution, with mobile phase, the concentration of 2.0 µg was taken for analysis. The prepared solution was filtered through a 0.22 µm pore size filter (Millipore, Bedford, USA) prior to injection.

***Assay Procedure*:**

Equal volumes (about 20 µL) of the AZN standard preparation and the sample preparations (marketed product) of same concentration (2.0 µg/mL) were separately injected into the chromatograph, and the peak area responses (n=3) were measured for all the peaks (table 5). Then % content of AZN was calculated by using the following formula:

% Content = (Peak Area of sample/Peak Area of standard) ×100

RESULTS AND DISCUSSION

Selection of Wavelength: The UV spectra of AZN revealed maximum absorbance at 212 nm (Figure 2). As a result, the study was performed at a wavelength of 212 nm to achieve the maximum sensitivity for this analytical study.

Selection of Mobile Phase and Chromatographic condition: Amongst various combination, methanol, acetonitrile, and Phosphate buffer pH 8(0.2M) in a ratio of 60:30:10 respectively were selected as mobile phase exhibiting a rapid separation with retention time 4.8 min (Figure 3). Retention time was short because of aqueous solubility and larger portion of the polar component in Mobile phase. pH was maintained at 8 as low pH favors the instability of AZN and the stability of the column by preventing dissolution of silica in the column. [10]

Method validation

Specificity: Specificity of the analytical test was done under four stress conditions of photostabiliy, pH variation, temperature, and oxidation. Since the abovementioned conditions are helpful tools in establishing degradation pathways, the possible interference of degraded AZN in the above conditions (if any) should be taken into consideration. The percent drug recovered and percent RSD was found to be 89.9 and 2.09 in sunlight, 80.4 and 2.02 in acidic pH, 98.5 and 0.89 in alkaline pH, 100.6 and 0.42 in temperature and for oxidation stress, it was 92.2 and 1.07. (table2) The data revealed that AZN was not affected by these conditions although the value of percent RSD was more than 2 for sunlight and in acidic condition. % drug recovered on exposure to sunlight and acidic condition were found less it might be due to some degradation products of AZN in these conditions, this statement is strengthened by the additional RT at 3.0 min in Chromatogram presented in Figure 4.

Table 4: Precision of proposed method

Concentration (µg/ mL)	Repeatability (intraday precision)		Intermediate precision (interday)		
	Measured concentration± SD (µg/ ml)	% RSD	Days	Measured concentration± SD (µg/ ml)	% RSD
2.0	2.11±0.019	0.90	1	2.17±0.035	1.58
			2	2.09±0.023	1.10
			3	1.94±0.027	1.39
4.0	4.08±0.058	1.42	1	4.11±0.039	0.94
			2	3.89±0.042	1.07
			3	4.05±0.028	0.69
6.0	5.82±0.067	1.15	1	6.08±0.079	1.29
			2	5.91±0.062	1.04
			3	5.79±0.047	0.81

***(n=3)**

Linearity: Peak area responses were plotted against the concentration of AZN. The plot revealed good linearity over a concentration range of 1 to 50 µg/mL with regression equation $Y = 3.73\times10^4 X + 9.12\times10^2$ and a correlation coefficient of 0.995 (Fig. 6) with confidence interval at P = 0.05.

Trueness: The results for trueness or accuracy were expressed as percentage drug recovered and percentage RSD, after addition of 50 percent (15 µg), 100 percent (30 µg) and 150 percent (45 µg) of standard solution to sample solution (30 µg) (Table 3).Results depicted percent recoveries range from 99.8 to 100.2 percent and percent RSD range from 0.25 to 1.34 percent, which complies with the acceptance criteria proposed. Percent recovery should be in a range of 80 to 120 percent as per USP. [6]

Precision: Precision was assessed at two levels: repeatability or intra-day variability and intermediate precision or inter-day variability. Results for the interday and intraday precision study were expressed as %RSD (Table 4). Result revealed RSD bellow 1.42 percent for intra-day variability and for inter-day variability it was less than 1.58 percent which complies with the acceptance criteria proposed. [6]

Limit of detection and Limit of quantification: In the present study, limit of detection (LOD) and limit of quantification (LOQ) with acceptable precision and accuracy was calculated from standard deviation of the response i.e. AUC and the slope of linear regression($Y = 38.76X - 107.9$ and $r^2 = 0.998$) obtained from specific calibration curve of six different concentrations i.e. 50.0, 100.0, 150.0, 200.0, 250.0 and 300.0 ng/mL,(Figure 6) in the low end region of proposed range(9). LOD was found to be 14.40 ng/mL while LOQ was 43.66 ng/mL.

Application of Developed method: The developed method was applied to find out percent content of the marketed AZN oral suspension. AUC of both standard and suspension sample was taken with the help of chromatograph and the percent was found to be 90 percent labeled claim as shown in Table 5.

Table 5: AUC of Standard and Sample identified through chromatograph

Conc. (µg/ mL)	Peak Area of Sample ± SD	Peak Area of Standard ± SD	% Content
2.0	72224.31± 1.52	80247.33 ± 2.19	90

***(n=3)**

CONCLUSION

A new, fast, simple, and specific HPLC method using UV detection at 212 nm for determining percent content in Azithromycin oral suspension was developed and validated according to ICH guidelines. The developed method was observed

to be accurate, precise, sensitive and linear as well as superior LOD and LOQ. The run time of the method was 15 minutes which further can be reduced to 10 minutes in light of the fact that the retention time is 4.8 minutes just enabling analysis of a large number of samples in a brief timeframe. The developed method can be used to analyze AZN at various concentrations as well as in different formulation and raw materials also. In addition, the lesser concentration of acetonitrile allowed a cost-effective analytical process.

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CONFLICT OF INTEREST

No conflict of interest.

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