International Journal of Pharmacy
Journal Homepage: http://www.pharmascholars.com

Research Article **CODEN: IJPNL6**

DEVELOPMENT AND APPLICATION OF LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF AZITHROMYCIN IN FIXED DOSAGE FORM

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ABSTRACT
A simple, precise, rapid and accurate reverse phase HPLC method was developed for the estimation of Azithromycin in tablet dosage form.Xterra RP 18 (250x4.6mm, 5µ particle size), with mobile phase consisting of *ortho*-phosphori acid: methanol 70:30 V/V was used. The flow rate 1.0 ml/min and the effluents were monitored at 205 nm. The retention time& Recovery time was 12 minutes. The detector response was linear in the concentration of 25–275 µg/ mL. The respective linear regression equation being *Y= 1228.302 x+5230.5524.* The limit of detection and limit of quantification was 12.5mcg and 37.5mcg/ml respectively. The percentage assay of Azithromycin was 98.0%. The method was validated by determining its accuracy, precision and system suitability.The results of the study showed that the proposed RP-HPLC method is simple, rapid, precise and accurate, which is useful for the routine determination of Azithromycin in bulk drug and in its pharmaceutical dosage form.

Keywords: Azithromycin, RP-HPLC, Estimation, and Tablets

INTRODUCTION

Azithromycin trade name Azalid-Kit®, Custodian Pharma pvt ltd) is an antibiotic useful for the treatment of bacterial infections. It is an azalide, a subclass of macrolide antibiotic. It is derived from erythromycin, with a methyl-substituted nitrogen atom incorporated into the lactone ring, thus making the lactone ring 15-membered. Azithromycin is somewhat more potent against certain bacterial species than erythromycin, but its widespread popularity arises primarily from its slow elimination from the body, which allows many infections to be treated with 3–5 days of once-daily administration, compared to 3-4 times a day for up to two weeks for erythromycin. The method developed results of the study showed that the proposed RP-HPLC method is simple, rapid, precise and accurate which is useful for the routine determination of Azithromycin and in its pharmaceutical dosage form.

EXPERIMENTAL

Instrumentation: Quantitative HPLC was performed on liquid Chromatograph, Waters separation 2996, PDA detector module equipped with automatic injector with injection volume 20 µL, and 2693 pump. Xterra RP 18 (250x4.6mm, 5µ particle size was used. The HPLC system was equipped with Empower Software.

Chemicals and solvents: Azithromycin was provided as gift sample byHetro Labs, Hyderabad, India. All the chemicals potassium dihydrogen phosphate*ortho*phosphoric acids were of AR grade and acetonitrile of HPLC grade were purchased from Merck Specialities Pvt. Ltd., Mumbai, India. Commercial tablets of Azithromycin were purchased from local market. HPLC grade water obtained from Milli-Q water purification system was used throughout the study.

Preparation of the mobile phase and diluent: 0.02M Potassium Dihydrogen Orthophosphate and 0.02M Dipotassium hydrogen Orthophosphate in water and pH adjusted to 7.0 with *ortho*-phosphoriacid: methanol was taken in 70:30 (v/v) ratios. The resultant solution was thoroughly mixed and filtered througha poly-tetra-fluoro ethanol (PTFE) filter of 0.45 µm pore size using vacuum pump and degassed by sonication to expel the dissolved gases in solvent system.

Preparation of System suitability solution (standard solution): Standard solution of the three active ingredients of the drug was prepared in the following manner: Transfer 250 mg of azithromycin, working standards into a 100 ml volumetric flask, dissolve and dilute with 0.02M Potassium dihydrogen *ortho*-phosphate and 0.02M dipotassium. Hydrogen *ortho*-phosphate in water and pH adjusted to 7.0 with *ortho*-phosphoric acid: methanol was taken in70:30 (v/v) ratios as diluent. 5 ml of the resulting solution is further diluted up to 50 ml in volumetric flask with diluents. The resulting solution contains 250 µg/mL of azithromycin as working standard solution. The prepared stock solutions were stored at 4 ^{0}C and protected from light.

Preparation of sample solutions: Twenty tablets were weighed and their average weight was calculated. The tablets were crushed to a homogeneous powder and a quantity equivalent to 100 % standard solution was weighed and transferred in to a 100-mL volumetric flask, extracted in diluent by sonication, and filtered through Whatman no. 41 filter paper. The filtrate (5 mL) was quantitatively transferred to a 50-mL volumetric flask, and solution was dilutedto volume with the diluents. The resulting solution contains 250 µg/mL of azithromycin as working test or sample solutions. The prepared stock solutions were stored at 4 ^{0}C and protected from light.

Methodology: The HPLC system was stabilized for thirty minutes by passing mobile phase, detector was set at 205 nm, flow rate of 1.0 mL/min to get a stable base line. One blank followed by six replicates of a single standard solution was injected to check the system suitability. Six replicates of each standard solutions 25,50,100,150,200,250,275µg/mL were injected. Calibration graph was plotted by concentration of Azithromycin on X-axis and peak area on Y-axis and linearity curve was shown in Figure 1. The amount of drug present in sample was computed by calibration graph. Chromatographic conditions for estimation of Azithromycin were described in Table 1.

Pharmaceutical formulations: Prepared dilution of Azithara-XP®formulation 1000mgis injected and the procedure described under bulk samples was followed. The amount of drug present in sample was computed in calibration graph. The assay results in commercial formulations of Azithromycin were described in Table 2.

RESULTS AND DISCUSSION

The objective of the present work is to develop simple, precise and reliable HPLC method for the analysis of Azithromycin in bulk and pharmaceutical dosage forms. This is achieved by using the most commonly employed Xterra RP 18 (250x4.6mm, 5µ particle size) column detection at 205nm. The representative chromatogram indicating Azithromycin is shown in Figure 2.

P**arameter fixation:** In developing this method, a systemic study of effects of various parameters was under taken by varying one parameter at a time and controlling all other parameters. The following studies were conducted for this purpose.

S**tationary phase characteristics:** Based on nature and solubility characteristics of, reverse phase mode of HPLC was selected Azithromycin for chromatography. Among different RP-HPLC stationary phases tried Xterra RP 18 (250x4.6mm, 5µ particle size was found to be optimum.

M**obile phase characteristics**: In order to get sharp peak with base line separation from interfering peaks carried out a number of experiments by varying the composition of solvents and mobile phase flow rate. To have an ideal separation of the drug under isocratic conditions, mixtures of solvents like Methanol with different buffers in different combinations were tested as mobile phase. A mixture of *ortho*-phosphoric acid: methanol 70:30(v/v) was proved to be the most suitable of all the combinations, since the chromatographic peak obtained was better defined and resolved and almost free from tailing.

Linearity: Aliquots of standard Azithromycin stock solution were taken in different 10 ml volumetric flasks and diluted up to the mark with the mobile phase such that the final concentrations of Azithromycin are in the range of 25-275µg/ml. Each of these drug solutions (20 µL) was injected three times into the column, and the peak areas and retention times were recorded. Evaluation was performed with PDA detector at 205 nm and a

Calibration graph was obtained by plotting peak area versus concentration of Azithromycin (Fig 1). The plot of peak area of each sample against respective concentration of Azithromycin was found to be linear in the range of 25-275 µg/ml with correlation coefficient of 0.9999. Linear regression least square fit data obtained from the measurements are given in table 3. The respective linear regression equation being Y= 1228.302 x+5230.5524The regression characteristics, such as slope, intercept, and %RSD were calculated for this method and given in Table 3.

Intra-day precision: To study the intra-day precision, six replicate standard solutions (300 ppm) of Azithromycin were injected. The percent relative standard deviation (%RSD) was calculated and it was found to be 0.7 which are well within the acceptable criteria of not more than 2.0.

Inter-day precision: To study the inter-day precision, six replicate standard solutions (300 ppm) of Azithromycin were injected. The percent relative standard deviation (%RSD) was calculated and it was found to be 1.6 which are well within the acceptable criteria of not more than 2.0.

Specificity: The effect of wide range of excipients and other additives usually present in the formulation of Azithromycin in the determinations under optimum conditions were investigated. Chromatographic parameters maintained are specific for Azithromycin.

Ruggedness: The ruggedness of the method was determined by carrying out the experiment on different instruments like Shimadzu HPLC, Agilent HPLC and Water's Breeze HPLC by different operators using different columns of similar type like XDB C18, Hibar C18, Kromasil C18 and Symmetry C18 didn't show any significant change.

Limit of detection and limit of quantification: The detection limit of the method was investigated by injecting standard solutions Azithromycin into the HPLC column. By using the signal-to-noise method the peak-to-peak noise around the analyte retention time is measured, and subsequently, the concentration of the analyte that would yield a signal equal to certain value of noise to signal ratio is estimated. A signal-to-noise ratio (S/N) of 3 is generally accepted for estimating LOD and signal-to-noise ratio of 10 is used for estimating LOQ. This method is commonly applied to analytical methods that exhibit baseline noise. The limit of detection (LOD) and limit of quantification (LOQ) for Azithromycin were found to be 12.5µg/ml and 37.5 µg/ml respectively.

Accuracy: The accuracy of the method was determined by standard addition method. A known amount of standard drug was added to the fixed amount of pre-analyzed standard solution. The standard addition method was performed at 80%, 100%, and 120%. The solutions were analyzed in triplicate at each level as per the proposed method. The percent recovery and %RSD was calculated and results are presented in Table 4. Satisfactory recoveries ranging from 92.8% 99.78% to 97.8% were obtained by the proposed method. This indicates that the proposed method was accurate.

Robustness: Robustness of the method was determined by making slight changes in the chromatographic conditions. It was observed that there were no marked changes in the chromatograms, which demonstrated that the RP-HPLC method developed is robust

System suitability: A system suitability test was performed to evaluate the chromatographic parameters (number of theoretical plates, tailing of the peak) before the validation runs. The results of system suitability parameters were given in Table 4. The analytical method validation was carried out as per ICH method validation guidelines.

CONCLUSION

The proposed HPLC method is rapid, sensitive, precise and accurate for the estimation of Azithromycin and can be reliably adopted for routine quality control analysis of Azithromycin in its tablet dosage forms.

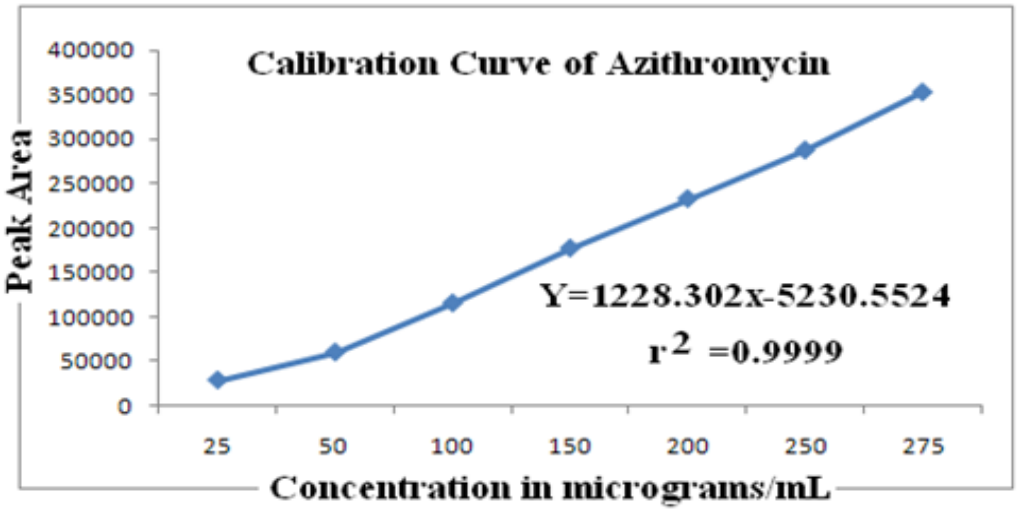

***Figure 1*: Linearity curve of Azithromycin**

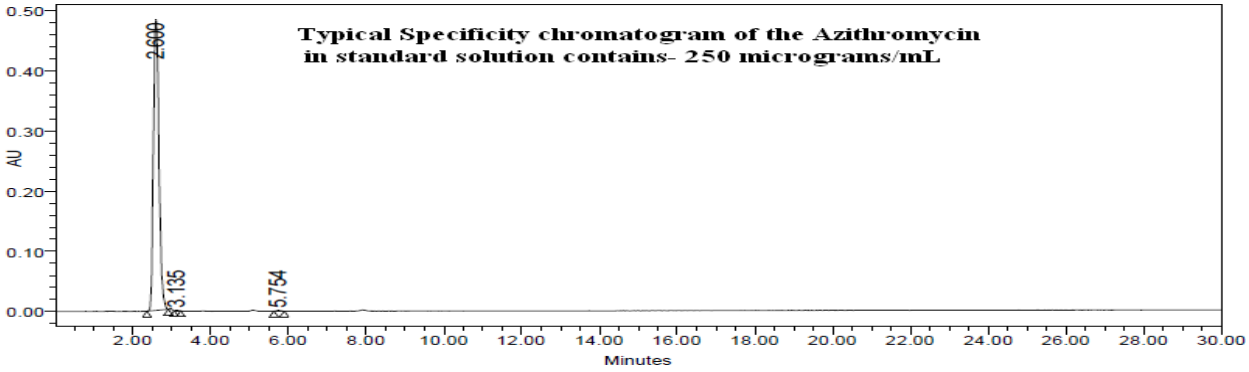

***Figure 2*: Typical chromatogram of Azithromycin**

***Tabel 1*: Optimized chromatographic conditions of Azithromycin Parameters:**

Table 1: Optimized chromatographic conditions of Azithromycin Parameters	Condition
Mobile phase	*ortho*-phosphori acid: methanol 70:30
pH	4.0
Diluent	methanol
Column	Xterra RP 18 (250x4.6mm, 5µ particle size)
Column temperature	30°C
Wave length	205 nm
Injection volume	20 µL
Flow rate	1.0 mL/min
Run time	12 min
Retention time	2.563 min

***Tabel 2*: Assay results of Azithromycin Formulation**

Azithra-XP® is a fixed-dose tablet	Label claim	Amount found	%Assay
azithra	**1000mg**	**92**	**99.12**

***Table 3*: Linearity results of Azithromycin**

Concentration (µg/mL)	Area
25	28519
50	56582
100	115250
150	176992
200	232610
250	287268
275	352482

***Table4*: Validation parameters of Azithromycin**

Parameter	Azithromycin
Retention time (min)	2.563
Theoretical plates	2850.95
Tailing Factor	1.02
HETP	8.7688×10^{-5}
USP plates/meter	11403.8
Resolution	----
Peak area	284105
% of Peak area	1.69

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