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Development and Validation of Stability indicating RP-HPLC Method for the estimation of Azithromycin Suspension

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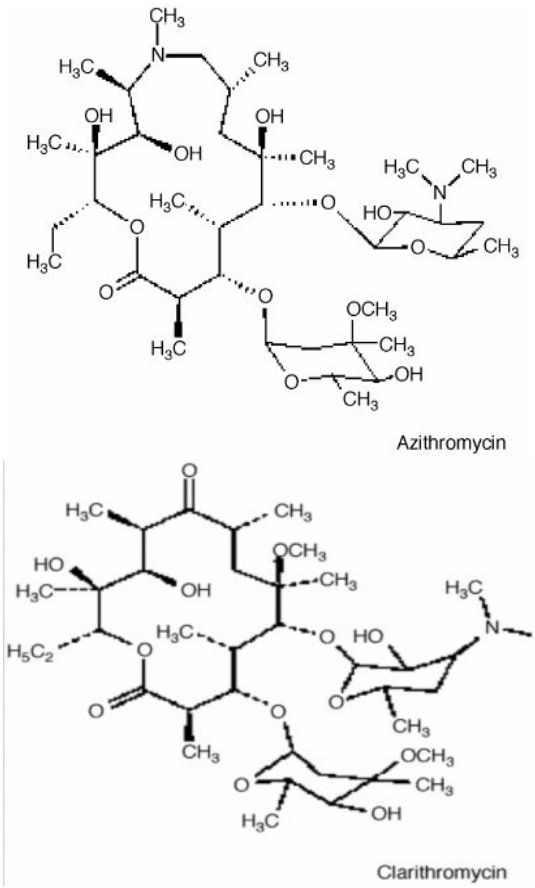
Abstract: A RP-HPLC method was developed and validated for quantitative determination of azithromycin in pharmaceutical suspension dosage forms. The chromatography was carried out on a Phenomenex C_{18} (150 x 4.6 mm i.d., 5µ) column with Acetonitrile: 0.5 % Formic acid as mobile phase (Isocratic A: B = 40: 60 % v/v), at 215 nm detector wave length with a flow rate of 1 ml/min. Clarithromycin was used as an internal standard. The linearity was established in the range of 20 - 600 ng/ml for HPLC. The HPLC method was accurate and precised for azithromycin suspension with a recovery of 98.75 to 99.44%. The spiked sample solutions were stable upto 1 month. This validated method can be used for estimation of azithromycin in pharmaceutical suspension.
Key words: Azithromycin, Reversed Phase High Performance Liquid Chromatography, Analytical method validation, Pharmaceutical solid dosage forms.

Introduction

Azithromycin[1] is chemically (2*R*, 3*S*, 4*R*, 5*R*, 8*R*, 10*R*,11*R*,12*S*,13*S*,14*R*)-13-[(2,6-dideoxy -3 - *C* - methyl - 3 - *O* - methyl - α- *L* - *ribo* - hexopyranosyl) oxy]-2-ethyl-3, 4, 10-trihydroxy-3, 5, 6, 8, 10, 12, 14-heptamethyl - 11 - [[3, 4, 6-trideoxy-3-(dimethylamino)-β-*D*-*xylo*-hexopyranosyl]oxy]-1-oxa-6-azacyclopentadecan-15-one. It is derived from erythromycin; however, it differs chemically from erythromycin in that a methyl-substituted nitrogen atom is incorporated into the lactone ring. Azithromycin, like all macrolyte antibiotics, prevent bacteria from going by interfering with their ability to make protein. Due to the differences in the way proteins are made in bacteria and humans, the macrolyte antibiotics do not interfere with humans' ability to make proteins. It binds to the 50s rRNA subunit of the 70s bacterial ribosome's, therefore inhibits RNA-dependent protein synthesis. It inhibits the translation of mRNA in bacterial cells at the chain elongation step; result in the blockage of transpeptidation. Nucleic acid synthesis is not affected. Various analytical methods[2-19] like UV, HPLC, HPTLC and LC/MS have been reported for the determination of azithromycin in its tablet or capsule dosage forms either in single or in combination.

There is however no reported HPLC method for the analysis of azithromycin in suspension dosage forms. This paper describes a validated HPLC method for the quantitative determination of azithromycin suspension in reverse phase mode using Clarithromycin as an internal standard. The proposed HPLC method fulfilled the requirements of analytical parameters necessary to be applied to the content uniformity tests for finished pharmaceutical products in the study and hence can be successfully applied for routine quality control.

Figure 1: Structure of Azithromycin and Clarithromycin (Internal Standard)



Materials and Methods

Materials

Azithromycin and Clarithromycin working standard were kindly supplied by Dr Reddy's Labs Ltd., Hyderabad, India. Acetonitrile (HPLC grade) and Formic acid (AR grade) were purchased from SD Fine Chem., Mumbai, India. Pharmacopoeial grades of excipients were procured from Nehal Traders, Hyderabad, India. In-house triple distilled water and distilled water was used for HPLC method respectively. Azithromycin suspension containing 200 mg/10mL of Azithromycin was obtained from PFIZER G, Germany.

Analytical Conditions

The HPLC method was performed on a Shimadzu HPLC system equipped with LC-10ATVP pump, SPD-10ADVP UV detector, and Rheodyne injector system fitted with 20 µl loop. The HPLC analysis was performed on reversed phase high-performance liquid chromatographic system with isocratic elution mode using a mobile phase of Acetonitrile: 0.5% formic acid (40:60% v/v), on Gemini C-18 column (Phenomenex, 150×4.6 mm, 5 µm particle size) with 1ml/min flow rate at 215 nm using UV detector. Spinchrom software was used for the data interpretation.

Preparation of Standard Solutions

The different concentrations of standard solutions were prepared to contain 20.0 to 600.0 ng/mL of Azithromycin containing 40.00 µg/mL of internal standard. These solutions were analysed and the peak areas and response factors were calculated. The calibration curve was plotted using response factor Vs concentration of the standard solutions. All solutions were filtered through 0.45 µ membrane filter prior to use.

Preparation of the Sample Solutions

The contents of azithromycin suspension were taken. The Suspension containing equivalent to 25 mg of azithromycin was accurately weighed and transferred into a 50 ml volumetric flask. To this, 30 ml of water was added and sonicated for 10 min with occasional shaking to disperse and dissolve the contents. The volume was made up to 50 ml with water to give 500 ng/ml of azithromycin solution. This solution was filtered through 0.45 µ membrane filter
and diluted suitably using mobile phase to obtain 40 ng/ml solutions.

Method Validation

The methods were validated according to International Conference on Harmonisation (ICH) guidelines for validation of analytical procedures[20, 21].

Specificity

The specificity of the methods was evaluated by comparing the chromatograms (HPLC) obtained from the standard solution and sample solutions of azithromycin.

Linearity

Eight concentrations of the standard solutions in 20-600 ng /ml range were analyzed by HPLC. Calibration curves were constructed by plotting average peak areas *vs* Concentrations. Calibration curves were constructed by plotting average absorbance *versus* concentrations. Linearity was determined by regression equations for both methods. This experiment was repeated six times (Table 1).

Accuracy (by internal standard method)

Accuracy of the method was determined by relative and absolute recovery experiments. The absolute recovery of Azithromycin was determined by comparing the response factor of the drug obtained from the plasma with response factor obtained by the direct injection of Azithromycin in mobile phase at three different levels. Recovery studies were carried

out for three levels at six times and the % recovery, mean, standard deviation and % CV was calculated and presented in Table.2.

Precision

The precision of the method was determined by intraday precision and interday precision studies. The intraday precision was evaluated by analysis of blank plasma sample containing Azithromycin at three different concentrations of LQC (lower quantified concentration), MQC (middle quantified concentration) and HQC (higher quantified concentration) using nine replicate determinations for three occasions. The interday precision was similarly evaluated over two-week period. The mean concentration, standard deviation and % CV were calculated and presented in Table 3.

Limit of detection (LOD) and limit of quantification (LOQ)

The parameters LOD and LOQ were determined using signal to noise ratio.

Stability studies

The stability of triplicate short term samples spiked with Azithromycin was kept at room temperature for 1.00 to 48.00 hours. The samples of the long term stability were stored in the deep freezer at -70 ^{0}C until the time of analysis. The mean concentrations of the stability samples were compared to the theoretical concentrations. The stability of the Azithromycin suspension standard solution at room temperature for 48 hrs and freezed condition for four weeks were demonstrated by comparing a freshly prepared standard solution. The stability of the internal standard stock solution was also performed by comparing a freshly prepared standard solution containing internal standard. The results were presented in Table 4.

Analysis of marketed Azithromycin suspension by RP- HPLC

Azithromycin suspensions were analyzed by optimized RP-HPLC method. The suspension was analyzed by six independent determinations.

Table 1: Calibration curve of Azithromycin (Linearity and Range)

Drug Concentration (ng /mL)	Internal Standard Concentration (µg/mL)	Response Factor (RSD)
0	50	0
20	50	0.0188
100	50	0.0734
150	50	0.1083
250	50	0.1782
400	50	0.2841
500	50	0.3458
600	50	0.4157

Table 2: Accuracy – Recovery Studies

Level	Concentration of drug added ng/mL	Amount of Drug recovered (%) in Mobile phase	Relative Recovery (%)
Level-I (LQC)	100.0	Mean : 99.44 CV : 0.82 N : 6	97.76
Level-II (MQC)	400.0	Mean : 98.98 CV : 1.32 N : 6	98.32
Level-III (HQC)	600.0	Mean : 98.75 CV : 0.29 N : 6	98.53

Table 3: Precision Studies

Nominal Concentration (ng/mL)			
S.N	**LQC**	**MQC**	**HQC**
	100.00	**400.00**	**600.00**
1	99.78	394.02	582.91
2	98.33	392.47	590.71
3	99.26	395.58	593.54
4	98.81	392.26	592.39
5	97.66	393.33	598.42
6	97.70	394.63	594.21
Mean	**98.59**	**393.72**	**593.92**
S.D (+/-)	**0.852**	**1.343**	**1.40**
C.V. (%)	**0.864**	**0.341**	**0.236**
N (no. of replicates)	**6**	**6**	**6**

Table 4: Stability Studies of sample solution and standard stock solution

Nominal Concentration (ng /mL)			
Short Term at	**LQC**	**MQC**	**HQC**
Room Temperature	**100.00**	**400.00**	**600.00**
Recovery after 1 hr	95.32	362.54	594.71
Recovery after 2 hr	92.84	361.99	598.74
Recovery after 3 hr	92.67	360.88	595.58
Recovery after 48 hr	92.67	361.94	596.56
Mean	**93.38**	**361.84**	**596.40**
S.D (+/-)	**1.30**	**0.694**	**1.734**
C.V. (%)	**1.39**	**0.192**	**0.291**
N (no. of replicates)	**3**	**3**	**3**
Nominal Concentration (ng /mL)			
Long Term	**LQC**	**MQC**	**HQC**
Sample at -70º	**100.00**	**400.00**	**600.00**
Recovery After 1 week	94.08	372.26	580.12
Recovery After 2week	92.15	374.08	579.88
Recovery After 4 week	97.11	371.27	582.04
Mean	**94.11**	**372.54**	**580.68**
S.D (+/-)	**0.980**	**1.425**	**1.183**
C.V. (%)	**1.041**	**0.383**	**0.204**
N (no. of replicates)	**3**	**3**	**3**

Nominal Concentration (ng /mL)			
Standard Stock solutions	**LQC**	**MQC**	**HQC**
	100.00	**400.00**	**600.00**
Recovery after 3 hr	99.78	396.99	598.42
Recovery after 48 hr	98.84	398.69	599.07
Recovery after 4 Week	99.08	399.78	599.39
Mean	**99.233**	**398.487**	598.960
S.D (+/-)	**0.4884**	**1.4061**	**0.4943**
C.V. (%)	**0.49**	**0.28**	**0.07**
N	**3**	**3**	**3**

Table 5: System Suitability Studies

S. No	Parameters	INT STD	DRUG
1	Theoretical Plate	46789	50148
2	Resolution factor	0.5	
3	Asymmetric factor	1.00	1.00
4	LOD (ng/ml)	5.00	1.00
5	LOQ (ng/ml)	10.0	5.0

Figure 2: Calibration curve of Azithromycin

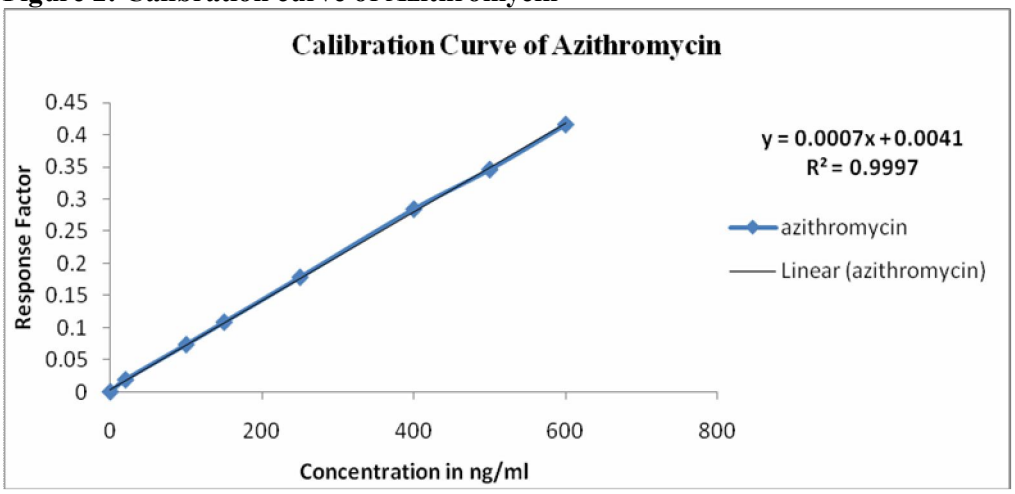
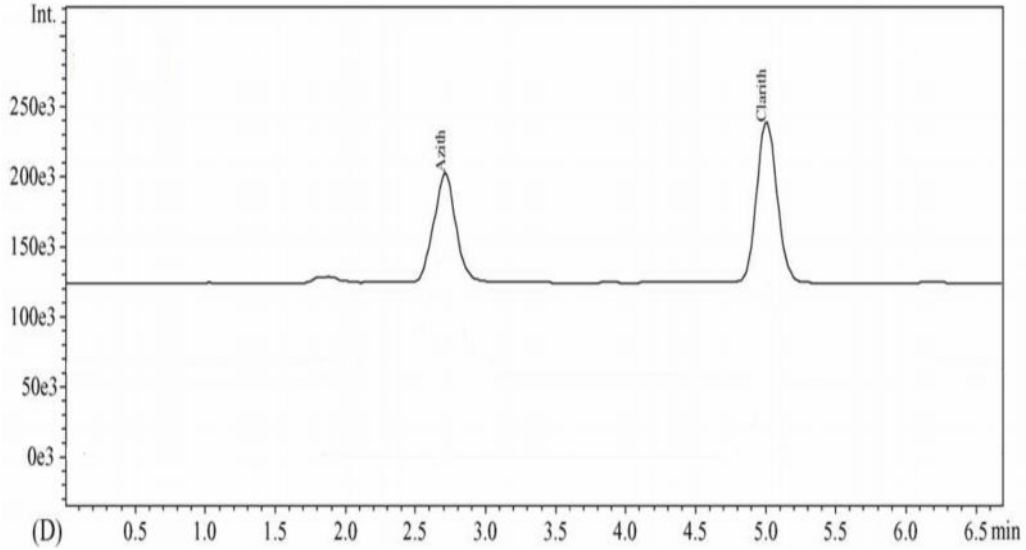


Figure 3: Typical chromatogram of Azithromycin and Clarithromycin (Internal Standard)



Results and Discussions

Optimization of HPLC Method

Optimization of mobile phase was performed based on peak symmetry, peak width, and run time. The mobile phase of Acetonitrile and 0.5% formic acid (40:60% v/v) was found to be satisfactory. The Fig.1 shows typical chromatograms obtained from the analysis of a standard and sample solutions of azithromycin using the proposed method. The retention time observed (2.7 mins) permits a sharp and rapid determination of the drug, which is important for routine analysis. System suitability parameters for this method are reported in Table 5. The parameters were within the acceptance limits.

Validation of HPLC Method

The method was found linear over the range 20 to 600 ng/ml (Fig 2). The described reversed-phase HPLC method was found to be specific for azithromycin, as none of the excipients interfered with the estimation of azithromycin (Fig. 3). The LOD were found to be 4.0 ng/ml for internal standard and 1.0 ng/ml for drug and LOQ were found to be 8.0 ng/ml for internal standard and 4.0 ng/ml for drug respectively, indicating a high sensitivity of the method. The results for accuracy and precision are summarized in Table 2 and Table 3. The results of recovery studies indicate a high agreement between the true value and the estimated value. The standard and sample solutions were stable upto 4 weeks (Table 4).

Conclusions

The HPLC method for the determination of azithromycin suspension was found to be simple, rapid, precise, accurate and sensitive. The validated HPLC method can be used for the drug analysis in routine quality control for azithromycin suspension.

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