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Der Pharma Chemica, 2013, 5(4):166-172
(http://derpharmachemica.com/archive.html)

ISSN 0975-413X
CODEN (USA): PCHHAX

Method development and validation of HPLC method for determination of azithromycin

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ABSTRACT

A simple, selective, precise and accurate High Performance Liquid Chromatographic Method for the analysis of Azithromycin in its formulations was developed and validated in the present study. The mobile phase consist a mixture of 0.0335M Phosphate Buffer (pH 7.5) and Methanol in the proportion 20:80. This was found to give sharp peak of Azithromycin at a retention time of 8.35min. HPLC analysis of Azithromycin was carried out at a wavelength of 210nm with a flow rate of 1.2ml/min. The linear regression analysis data for the calibration curve showed a good linear relationship with a regression coefficient 0.997. The linear regression equation was y = 18930x - 10493. The developed method was employed with a high degree of precision and accuracy for the analysis of Azithromycin. The method was validated for accuracy, precision, robustness, ruggedness, specificity. The precision, accuracy, sensitivity, short retention time and composition of the mobile phase indicated that this method is better for the quantification of Azithromycin.

Keywords: Azithromycin, RP-HPLC Method Development, Validation.

INTRODUCTION

Azithromycin is a semi-synthetic macrolide antibiotic of the azalide class. Azithromycin inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit of the bacterial 70S ribosome. It inhibits peptidyltransferase activity and interferes with amino acid translocation during the process of translation. Its effect may be bacteriostatic or bactericidal depending on the organism and the drug concentration. Its long half life, which enables once daily dosing and shorter administration durations, is a property distinct from other macrolides[1].

C. Barbas and L. Miguel developed a LC method for analysis of impurities in Azithromycin Tablet [3]. S. Supattanapong and J. Konsil developed a HPLC method with electrochemical detection for analysis of Azithromycin in Human plasma[4]. High performance liquid chromatography-electrospray ionization-tandem mass spectrometry, LCMS/MS, Fourier-transform, Infrared transmission spectroscopy, UV, RPHPLC-UV and Reverse Phase Ultra Performance Liquid Chromatographic techniques and amperometric electrochemical detector[2] were developed for analysis of Azithromycin but they are expens[5-10].

The RP-HPLC method described here is simple, sensitive, and reproducible for Azithromycin determination in Formulations with low background interference. An attempt has been made to develop and validate to ensure their accuracy, precision and other analytical method validation parameters as mentioned in various gradients. One method reported for the HPLC determination for developed based on the use of a C-8 column, with a suitable mobile phase, without the use of any internal standard. For Tablet formulation the proposed method is suitable for their analysis with virtually no interference of the usual additives presented in Tablet formulations.

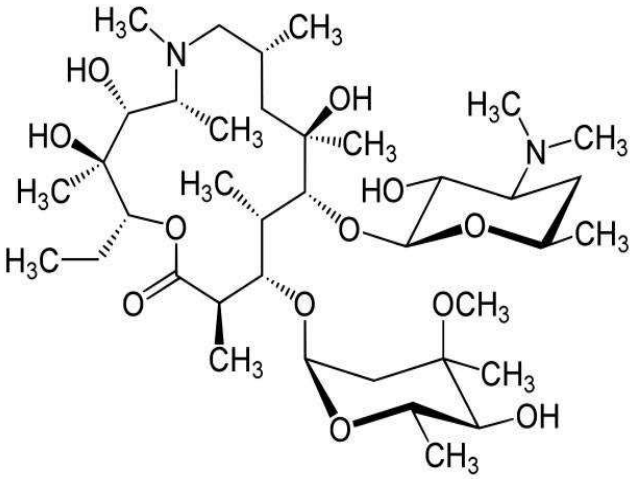


Fig. I: Structure of Azithromycin

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-xylo-]oxy}-1-oxa-6-azacyclopentadec-13-yl 2,6-dideoxy-3-C-methyl-3-O-methyl-α-L-ribo-hexopyranoside[2]

MATERIALS AND METHODS

a. Instruments:

HPLC series consisting Spectra system 2004, Spectro system P4000 pump, Autosampler AS3000, Spectro system UV2000 detector, Thermostat column compartment connected with Win Chrome2004 software.

b. Methodology:

HPLC method is carried out by using the following conditions.

Chromatographic Conditions:

Column	:	C-8, 250mm X 4.6mm, 5µ,
Flow rate	:	1.2 ml /min
Wavelength	:	210nm
Column temperature	:	45°C
Injection volume	:	20 µ L
Run time	:	15 minutes
Diluent	:	Mobile phase
Elution	:	Isocratic
Needle wash	:	Water: Methanol 90:10 (v/v)

c. Preparation of Mobile Phase:

The content of the Mobile Phase was prepared from filtered and degassed mixture of Phosphate buffer (4.5590gm of Potassium dihydrogen orthophosphate in 1.0 litre Water and pH was adjusted to 7.5) and Methanol in the ratio of 20:80v/v.

d. Preparation of Azithromycin Standard Stock solution:

Weigh and transfer 50mg of Azithromycin powder into 50ml volumetric flask add 30ml of diluents and sonicate and further filter the solution through 0.45µ filter paper and make up with diluent.

e. Preparation of Sample solution:

Take 10Tablets, each containing 500mg of Azithromycin. The tablets were crushed to fine powder and amount of powder equivalent to 100mg of Azithromycin were weighed and transferred to 100ml dried volumetric flask dissolve the content by shaking rapidly and 100ml volumetric flask previously containing 20ml of diluents. Make up the volume with diluent and mix well and inject immediately.

f. Procedure:

Inject 20µl of blank solution, placebo solution, six times of Standard solution, Disregard peaks due to blank and placebo.

System suitability requirements from SST solution:

Tailing factor : NMT 2.0

Theoretical Plates **:** NLT 2000

g. Precision (Repeatability): Preparation of precision solution:
Dilute the 10ml of standard stock solution to 100ml and make-up to volume with diluent. The same procedure is repeated to remaining six preparations. %RSD for the RT and Area are tabulated as below in Table I and II.

Table I: System Precision

S. No.	RT	Area
1	8.35	123179.43
2	8.35	125458.40
3	8.35	124739.43
4	8.35	121780.51
5	8.35	123135.37
6	8.41	124150.46
Avg.	8.36	123740.6
St. dev.	0.02449	1314.5076
%RSD	0.293	1.062

Table II: Method Precision

S. No.	RT	Area
1	8.41	124209.66
2	8.41	122306.29
3	8.41	123446.69
4	8.41	121256.00
5	8.41	122487.94
6	8.44	122453.83
Avg.	8.415	122693.4017
St. dev.	0.01225	1018.0443
%RSD	0.146	0.830

Acceptance criteria:
The %RSD of areas from six preparations precision level should not be more than 2.0%.

h. Accuracy:
The accuracy of the test method was demonstrated by preparing recovery samples (i.e. test sample with known quantities of at the level of 115%, 125% and 150% of target concentration)

The observations of Area are tabulated as below in Table III.

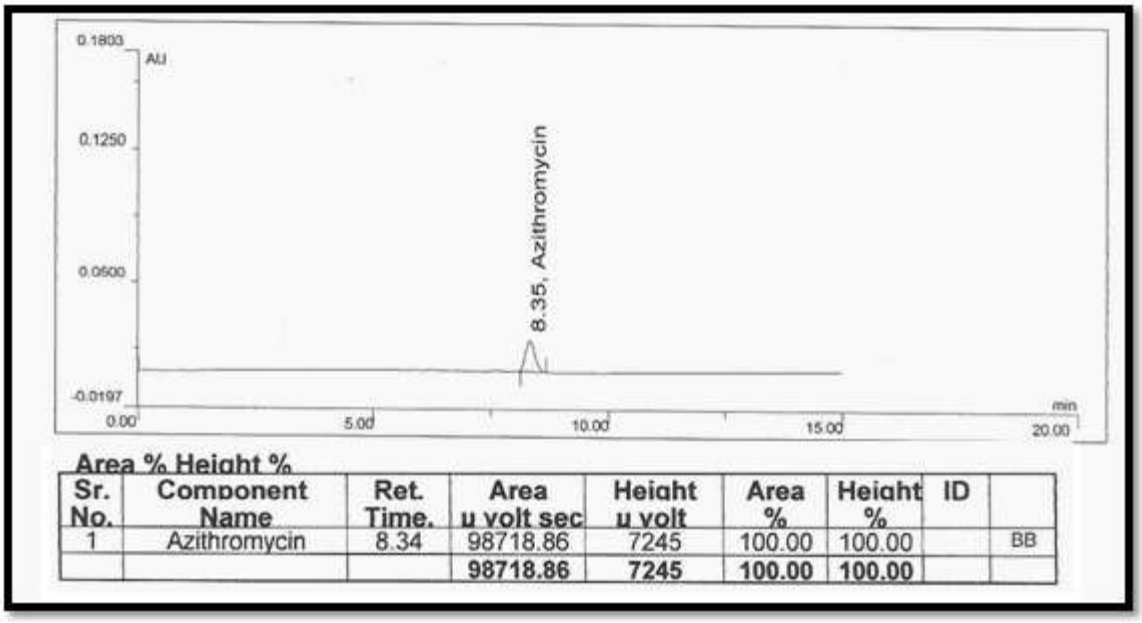
Table III: Accuracy Study

Accuracy	Std.	115%Spike	125%Spike	150%Spike
Trial-1	123409.55	141617.83	156607.41	190120.12
Trial-2	124160.52	142379.43	156320.97	190108.89
Trial-3	124646.87	142077.31	156421.72	190077.31
Avg.	124072.3133	142024.8567	156450.0333	190102.1067
Amt. Recovered (mg)	99.97	114.88	124.86	149.62
% of Recovery	99.97	99.90	99.89	99.75

i. Linearity:
The Linearity of detector response for was demonstrated by prepared solutions of over the range of 100 to 800% level of the target. Observations are tabulated in Table IV.

Table IV: Linearity Study (Preparation at 100% to 800% Level)

S. No.	Linear solutions (%)	Stock solution taken in (ml)	Diluted to volume (ml) with diluent	Area
1	100	10	100	98718.86
2	200	20	100	263076.8
3	400	40	100	458476.51
4	600	60	100	635686.46
5	800	800	100	858944.4



Area % Height %

Sr. No.	Component Name	Ret. Time.	Area u volt sec	Height u volt	Area %	Height %	ID	
1	Azithromycin	8.34	98718.86	7245	100.00	100.00		BB
			98718.86	**7245**	**100.00**	**100.00**		

Fig. II: Chromatogram of Azithromycin

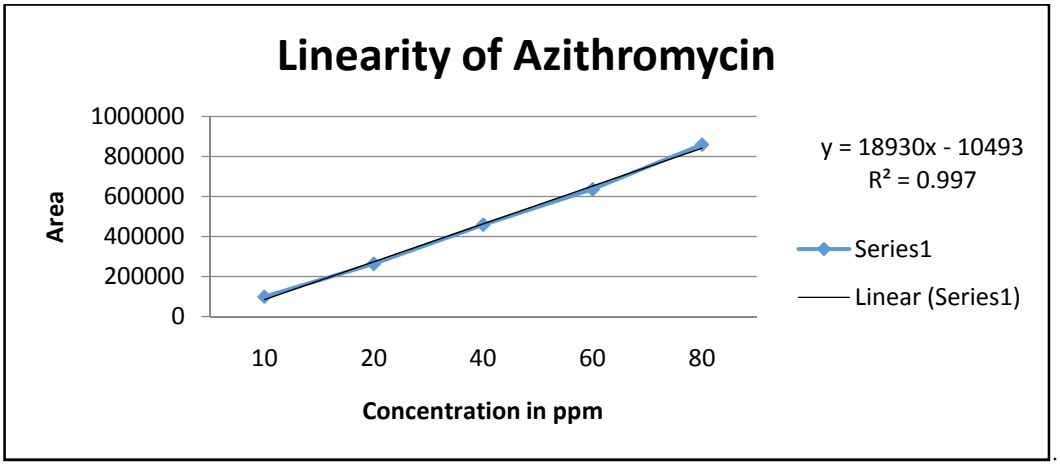


Fig. III: Linearity of Azithromycin

j. Assay:

10ml of Standard stock solution dilute to 100ml volume with diluent. Repeat the same procedure for remaining three preparations and observations are tabulated in Table V.

Table V: Assay Study of Azithromycin

Std-1	124209.66
Std-2	122306.29
Std-3	123446.69
Average weight	123320.88
Spl-1	124150.46
Spl-2	125458.40
Average	124804.43
LC	500mg
Standard weight	50.2mg
Sample weight	148.8mg
Standard factor	0.002
Sample factor	0.001
Standard purity	98.77%
Average weight	730.09mg
Amount in mg	498.54mg
%assay	99.71%

k. Ruggedness:
The ruggedness of test method was demonstrated by carrying out precision study in six preparations of sample on a single batch sample by different analysts. The results of the intermediate precision study are tabulated as below in Table VI.

Table VI: Ruggedness Study

S. No.	RT	Area
1	8.35	100093.09
2	8.36	100558.17
Avg.	8.355	100325.63
Std. dev.	0.0071	328.8612
%RSD	0.085	0.328

l. Robustness:
The robustness of test method was demonstrated by carrying out Mobile phase variation ±2.0% i.e. (22:78-18:82), flow variation ±10% i.e. (1.1ml to 1.3ml/min) and Column temperature variation ±5.0°C i.e. (40°C -50°C) the results are tabulated as below in Table VII.

Table VII: Robustness Study

S. No.	RT	Area
Mobile Phase-1	8.54	122487.94
Mobile Phase-2	8.35	121780.51
Flow rate-1	8.54	125087.71
Flow rate-2	8.30	124189.52
Column temp-1	8.33	124998.84
Column temp-2	8.35	124050.82

m. Limit of Detection (LOD):
LOD = 3 x STDEV / SLOPE
= 52.246 µg/ml

n. Limit of Quantization (LOQ):
LOQ = 10 x STDEV / SLOPE
=158.321 µg/ml

o. Specificity Studies:
The sample was analyzed in Specific conditions i.e. Placebo Study. Chromatogram of placebo doesn't show interference at the retention time of Azithromycin. Therefore this method is specific for determination of Azithromycin.

RESULTS AND DISCUSSION

The appropriate wavelength in UV region has been selected for the measurement of active ingredient in the proposed method. This method was validated by linear fit curve and all the other parameters were calculated.

Parameters Fixation:
In developing methods, systematic study of the effects of various parameters was undertaken by varying one parameter at a time and controlling all other parameters. The following studies were conducted for this purpose.

a) Mobile Phase Characteristics:
In order to get sharp peaks and base line separation of the components, carried out number of experiments by varying different components like percentage of Organic phase in the mobile phase, p^H of the aqueous phase, total p^H of the selected mobile phase, flow rate and column temperature by changing one at a time and keeping all other parameters constant. The optimum conditions obtained were included in the procedure proposed.

b) Detection Characteristics:
To test whether Azithromycin has been linearly eluted from the column, different amounts of Azithromycin were taken and analyzed by the above mentioned procedures. The peak area ratios of component areas were calculated and the values are graphically represented in Fig. III. The linear fit of the system was illustrated graphically. Least square regression analysis for the method was carried out for the slope, intercepts and correlation coefficient. The

results are presented in table IV.

c) Performance Calculations:
To ascertain the system suitability for the proposed method, a number of statistical values have been calculated with the observed readings and the results are recorded in Table VIII.

Table VIII: Performance Calculations

Parameter	RP-HPLC Method
Retention time (t) min	8.35
Theoretical plates (n)	7706.76
Linearity Range (µg/ml)	49.32-148.69
LOD (µg/ml)	52.246
LOQ (µg/ml)	158.321
Regression equation (y* =bc-a)	
Slope (b)	18930
Intercept (a)	10493
Correlation coefficient	0.997
Method Precision Relative Standard Deviation (%RSD)	0.830
System Precision Relative Standard Deviation (%RSD)	1.062

d) Method Validations:
The UV absorption maximum for Azithromycin was fixed at 210nm respectively. As the final detection was made by the UV absorption spectrum, each method was validated by linear fit curve.

e) Precision:
The Precision of the method and system was ascertained separately from the peak area ratios obtained by actual determination of a fixed amount of powder. The percentage of relative standard deviation is calculated for Azithromycin and readings presented in Table I and Table II. The Precession of the assays was also determined in terms of dilution variation in the peak areas for a set of powder solution was calculated in terms of %RSD and the results are presented in Table V.

f) Accuracy:
To determine the accuracy of the proposed methods, different amount of samples of Azithromycin within the linearity limits were taken and analyzed by the proposed method. The results (%RSD error) are recorded in Table III.

g) Ruggedness:
The ruggedness of test method was demonstrated by carrying out precision study in six preparations of sample on a single batch sample by different analysts. The results of the precision study are recorded in Table VI.

h) Robustness:
The robustness of test method was demonstrated by carrying out Mobile Phase variation ±2.0% i.e. (22:78 and 18:82), flow variation ±10%, i.e. (1.1ml and 1.3ml/min) and Column temperature variation ± 5.0°C i.e. (40°C-50°C). Results of this study are recorded in Table VII.

i) Specificity Studies:
The Specificity Studies are carried out by varying specific conditions i.e. Placebo study. Chromatogram of placebo doesn't show interference at the retention time of Azithromycin. Therefore this method is specific for determination of Azithromycin.

CONCLUSION

The method was found to be accurate and precise, as indicated by recovery studies close to 100 and %RSD is not more than 2. The summary of validation parameters of proposed HPLC method is given in tables.

The simple, accurate and precise RP-HPLC method for the determination of Azithromycin as Technical and formulation has been developed. The method may be recommended for routine and quality-control analysis the investigated drug in formulations. The analytical solution hence, it is concluded that the analytical method is validated and can be used for routine analysis.

Acknowledgements
Thanks to Department of QA, SND College of Pharmacy, Babhulgaon, Yeola, Nashik and Kaytross ACG Lifescience Ltd, Ambad, Nashik for providing laboratory facilities.

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