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Development and Validation of a Precise LC Method for the Determination of Drug Release during Dissolution profile in Pharmaceutical Formulation

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1.0 Abstract: A simple, short, rapid, sensitive and robust reversed phase-HPLC method was developed and validated to measure the amount of Azithromycin in dissolution profile. An isocratic elution of filtered sample was performed on cosmosil RP18 (50mm x 4.6mm), 5µm column with phosphate buffer (pH 7.5) & methanol (35:65 v/v) as mobile phase and UV detection at 210 nm. Mobile phase was delivered at flow of 2.0mL/min and maintaining the column temperature at 50ºC and quantification was achieved with reference to the external standards. The total run time is about three minutes only. The Azithromycin and two degradable impurities are separated with good resolution within three minutes. The linearity for concentrations between 70µg/mL and 840µg/mL (12.5% to 150.0%) for Azithromycin was established. The percentage RSD during intra and inter-day precision was about 2.4 & 3.7% which is less than 6.0%. The method was successfully applied for the determination of Azithromycin in a pharmaceutical formulation without any interference from common excipients, diluent and dissolution medium. In addition, standard and sample solutions stability and robustness were demonstrated. A statistical design of experiments was used for the robustness evaluation of HPLC method. All results were accepted and confirmed that the method is suitable for its intended applications. All the validation parameters were within the acceptance range, and concordant to ICH guidelines.
Key words: Azithromycin, Dissolution, In-vitro determination, High Performance Liquid Chromatography, Pharmaceutical dosage forms.

2.0 Introduction

Dissolution test is used to guide development of new drug product and to assess lot-to-lot variability of drug product after its oral administration. Dissolution methods, as well as other analytical methods, are validated to ensure that they are suitable for their intended use and give accurate and reliable data. Guidance on validation characteristics and considerations has been published.

Azithromycin prevents bacteria from growing by interfering with their protein synthesis. Azithromycin binds to the 50S sub-unit of the bacterial ribosome, and thus inhibits translation of mRNA. Nucleic acid synthesis is not affected.

Azithromycin is a macrolide antibiotic related to erythromycin. It is used primarily to treat various bacterial infection caused by respiratory pathogens, such as aerobic gram-positive microorganisms and aerobic gram-negative microorganisms. Azithromycin prevents bacterial cells from manufacturing specific proteins necessary for their survival. Azithromycin is rapidly absorbed and is widely distributed to tissues and concentrated in cells. Peak plasma concentration is achieved within 2 to 3 hours [1]. Available dosage forms are 250 mg capsule, 125, 250 and 500 mg tablets and powder for oral administration as a suspension.

The chemical (IUPAC) name of azithromycin is (2*R*,3*S*,4*R*,5*R*,8*R*,10*R*,11*R*,12*S*,13*S*,14*R*)-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*- hexopyranosyl] oxy}-1-oxa-6-azacyclopentadec-13-yl 2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranoside.

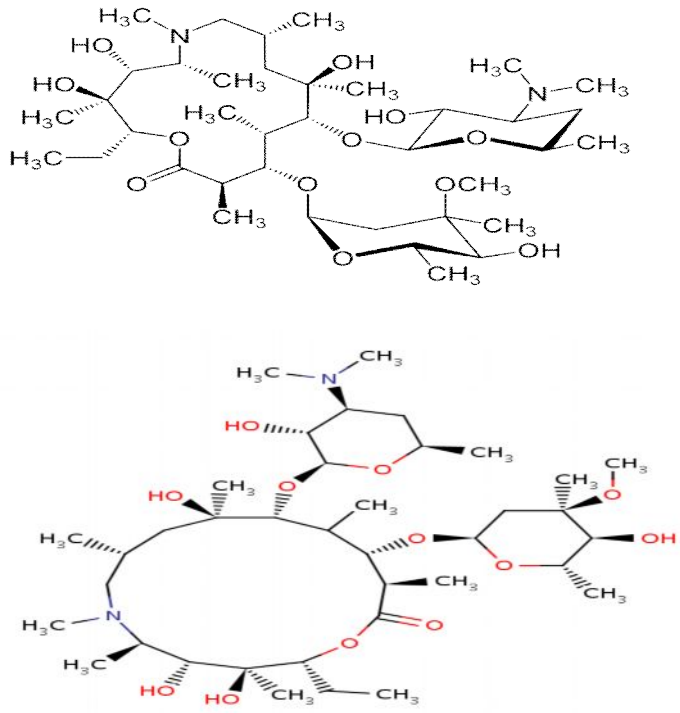


Fig.-1 Structure of Azithromycin

Its molecular formula is $C_{38}H_{72}N_2O_{12}$, molecular weight is 748.98 g/mol. Azithromycin consists of a two major degradable impurity desosaminylazithromycin and N-demethylazithromycin.

Azithromycin has been analyzed by HPLC[2,3] using electrochemical[2,3], fluorescence[4], mass spectrometry[5] and UV spectroscopy[6,7] for the detection in bulk materials and pharmaceutical forms. The USP, and BP method describes the use of high pH (about 9-11) of mobile phase which required specific column which is expensive and difficult to obtain commercially. HPLC with UV detector is a good selection as UV detector is available in the most laboratories.

The objective of the work is the development and validation of a HPLC method with UV detector for the determination of azithromycin in dissolution solution with short runtime about three minute with high resolution between azithromycin and its degradable major products. Validation of the method will be performed to the requirements of dissolution determination which including accuracy, precision, specificity, linearity and range. Additionally, in order to meet the regulatory guidance of the Federal Drug Administration (FDA)/International Conference of Harmonization (ICH). Azithromycin will be forcibly degraded in acidic, basic and strong oxidizing agent's solution.

3.0 Experimental

3.1 Materials and reagents:

All experiment was performed using 'A class' volumetric glassware and pharmaceutical grade Azithromycin. Analytical grade potassium dihydrogen orthophosphate (27011006, Finar chem, Ahmedabad, India) was used for dissolution media preparation. Using HPLC grade methanol (R009J06, Rankem, India), and highly pure HPLC grade Milli Q water (Millipore, Bedford, MA, USA) mobile phase was prepared and employed. Mobile phase was filtered through 0.45µm membrane filter (Millipore, Barcelona) and degassed under vacuum by filtering assembly, prior to use. The pharmaceutical preparations, declaring to contain Azithromycin (250mg, 500mg) with other excipients was obtained from M/s Cadila Pharmaceuticals Ltd., Gujarat, India.

3.2 Chromatographic system:

The liquid chromatography consists of an Agilent system, equipped with an automatic sample injector and PDA detector. For data collection and calculation chemstation software was used.

Buffer preparation: Dissolved 4.55 g of potassium dihydrogen orthophosphate in 1000 mL of Milli-Q water. Adjusted the pH 7.5 with diluted sodium hydroxide solution.

The chromatographic condition was optimized using a column cosmosil RP-18 50mm x 4.6 mm, 5.0µ. The mobile phase consisted of methanol: buffer (65:35, v/v) mixture. The mobile phase was filtered through a 0.22 µ membrane filter (Millipore, Barcelone) and degassed under vacuum prior to use. The flow rate was 2.0 mL/min. The monitoring wavelength was 210 nm and the injection volume was 20 µL with maintaining column oven temperature at 50ºC. Peak area was measured and HPLC analysis was conducted at room temperature. The phosphate buffer was used a diluent.

3.3 Dissolution parameter, standard and Sample preparation:

Dissolution parameters: Carry out the dissolution test using USP Apparatus II dissolution apparatus. Use paddle-stirring assembly and maintain the parameters as follows:

Medium : Phosphate buffer solution having pH-6.0

Volume : 900 ml.

RPM : 75

Temperature: 37°C ± 0.5°C.

Time : 30 minutes

Standard preparation: Standard solution of Azithromycin was prepared with phosphate buffer solution (of 6.0 pH) as a diluent. Standard solution contains 555 mcg/mL of Azithromycin.

Sample Preparation: Instrument was set as mention in the test method and degassed the medium prior to use. Transferred one tablet in each of six different vessels used 900 ml of medium and operated the apparatus for exactly 45 minutes. At the end of 45 minutes, withdraw 10 ml of the test solution from zone midway between the surface of medium and top of the rotating paddle not less than 1 cm from the vessel wall. Filtered the solution through 0.45 µm nylon filter and discarded initial 5 ml of the filtrate and use the collected filtrate as a sample solution.

3.4 System suitability:

Injected five replicate injections of standard solution and checked the system suitability as follows.

1) The % RSD of the area due to azithromycin in five replicate injection was not more than 2.0
2) The theoretical plate count of azithromycin was not less than 800 and tailing factor was not more than 2.0.

3.5 Validation study:

Specificity / Selectivity: Specificity of the method was demonstrated by prepared the solutions like mobile phase, diluent, standard, sample, placebo solution, placebo spiked with API and placebo spiked with impurity. Injected each solution on to the chromatograph equipped with detector and recorded the chromatograms.

Precision: The precision is the parameter that expresses the closeness of agreement between a series of measurements obtained from multiple analysis of the same sample under the prescribed conditions. In our study the repeatability was evaluated as follows:

Instrumental precision (System suitability): Checked system suitability as mentioned in section no.-3.4.

Method precision: Prepared six consecutive sample preparations and injected into chromatography system and chromatograms were recorded.

Calculate the % drug release of Azithromycin with respected to the standard solutions. The % RSD of six drug released value of sample preparation was not be more than 6.0.

Intermediate Precision: The aim of the study consists of establishing the effect of the random events on the analytical method and the intermediate precision was evaluated by analyzing a sample by different analysts with different columns on two different days.

Accuracy (recovery method): Accuracy of a method is defined as the closeness of the measured value to the true value for the sample. The recovery method was studied at concentration levels 25%, 100% and 150% of the claimed content, in presence of placebo. Prepared three sets for each concentration level and injected them in duplicate. The recovery was calculated with respect to the standard solutions.

Linearity: The linearity study verifies that the sample solutions are in a concentration range where an analyte response is linearly proportional to the concentration. This study was performed by evaluating the system and method linearity. For the system linearity, standard solutions of Azithromycin at six concentration levels (12.5%, 25%, 37.5%, 50%, 125% and 150%) were prepared and each standard solution was injected in duplicate. The experimental results were plotted to obtain the calibration curve and carrying out the necessary statistical study.

Stability in analytical solution: The purpose of this experiment is to demonstrate the stability of standard and sample solution used in this method at room temperature (about 25ºC). Prepared the standard solution and sample solution as aforementioned and injected both the solutions on to the chromatograph and recorded the chromatograms up to 24 hours for standard solution and sample solution. Measured the peak response for the major peak and evaluate the percentage deviation in the peak response from initial for both standard and sample solution.

Robustness: The standard solutions of azithromycin were analyzed for the robustness using different chromatographic conditions as listed below:

(1) Change the temperature of column temperature by ± 5ºC (i.e 45ºC to 55 ºC)
(2) Change the flow rate of mobile phase ± 0.2 (i.e. 1.8mL/min to 2.2 mL/min)
(3) Change the wavelength of detector by ± 2 nm (i.e. 208nm to 212nm)
(4) Change the p^H of the phosphate buffer solution by ± 0.2 unit (i.e. 7.3 to 7.7)

4.0 Results and discussion:

4.1 Method development:

The introduction of new HPLC methods for a routine quality control of pharmaceutical preparations begins with a series of preliminary investigations, which enables in establishing the optimal experimental

conditions and provide maximum relevant information by analyzing the experimental data. In this study, a new RP-HPLC method for the determination of Azithromycin was developed and validated. An ease of sample preparation and shorter time of separation was considered when the study started.

4.2 validation of the developed method:

Specificity / selectivity: From the study, it was observed that the drug eluted at a retention time of 2.3. The study of the purity of Azithromycin major responses at 2.3 min the peak showed that the five spectrums were obtained at different times were within the established threshold for the peak.

No interferences with the analyte peaks due to presence of placebo, blank and impurities have been observed. On the basis of that, the method results specific for the qualitative analysis of Azithromycin dissolution study.

The peak purity angle was less than peak purity threshold or peak purity of analyte peak was more than 990, indicating that all peaks were pure. From the areas, it can be concluded that all were stable in these conditions. The purity factor for the drug assures that there was no co-elution of other peaks. Therefore, the method was specific and suitable for routine work.

Precision:

Instrumental precision (System suitability): Injected five replicate injections of standard preparation observed and recorded in the Table-1:

Table 1: System suitability observation:

% RSD of area	Tailing factor	Theoretical plate
0.7%	1.0	1105

Method precision: In this study, RSD of 2.4 % was observed, by injecting six sets of sample solution. The percentage drug released results of six sample preparations should not be more than 6.0. The low %RSD has been observed for the six dissolution results. Hence, it is concluded that the method is precise and reproducible for the analysis of percentage drug released in the dissolution media of this formulation.

Intermediate precision: The method can be found rugged if the difference between percentage drug released results of normal condition and altered condition is not more than 6.0%. The percentage drug release of each sample was calculated and demonstrated the precision by evaluating percentage relative standard deviation of drug released results, for which % RSD observed was 3.7% and the difference observed between two conditions was 1.7%. Comparison of this results complied the mentioned criteria and method found very much rugged for analysis.

Accuracy by recovery: The results obtained for the accuracy study in the samples have Azithromycin concentration among 0.140, 0.555 and 0.830 mg/mL and being the 100% corresponding to 0.555 mg/mL (n=3 for 25%, 100% and 150%), indicating that the recovery percent was between 97.4 and 99.7% of recovery. The % recovery of target concentration has been found within the acceptance criteria with acceptable % RSD of NMT 2.0 at each level. The recovery at each level should be 97.0% to 103.0%. This indicates that the method is accurate for the analysis of Azithromycin percentage drug released in dissolution of Azithromycin tablets.

Linearity: The linearity was determined as linear regression with least square method. Concentration levels of standard solution were 12.5, 25, 37.5, 50, 125 and 150% of the claimed analyte concentration, corresponding to the range of about 70-840 mcg/mL. The calibration curve obtained by plotting the azithromycin peak area versus the concentration of standard solution and was linear in the mentioned concentration range of 12.5% to 150%. For acceptance of linearity correlation coefficient of linearity curve can not be less than 0.9990. This indicates that the method is linear for the specified range of concentrations. Observations were recorded in the Table-2.

Table – 2: Linearity:

Parameter	Benzalkonium chloride
Correlation coefficient	0.999
Slope	0.678

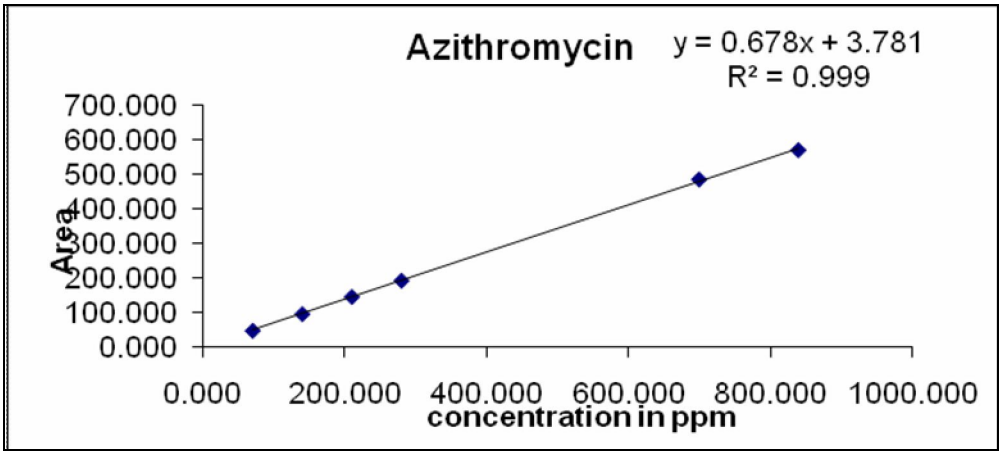


Fig. 2: Linearity curve of azithromycin

Stability of analyte solution: The solution stability was checked for the sample preparation for the beginning and up to 24 hours. The results obtained were agreed within the acceptance criteria up to 24 hours at room temperature. Both the standard solution and the sample solution thus prepared can be used within this period of time.

Robustness: The quantification was carried out with minor but deliberate changes in the parameters of the analytical method such as, detection wavelength, column temperature, p^H of mobile phase, and flow rate of mobile phase. The system suitability parameters like, theoretical plates, tailing, %RSD were observed and found good agreement with all the altered conditions.

5.0 Conclusions

A dissolution method with HPLC analysis for azithromycin tablets has been fully validated to meet global regulatory requirements. The methodology was evaluated for specificity, linearity, precision, accuracy and range in order to establish the suitability of the analytical method. Stability of analytical solution was also observed for the suitability of the method. Robustness of the dissolution method as well as robustness of the HPLC analysis method was evaluated using statistical experimental designs. In addition, intermediate precision as per regulatory requirements was performed and showed that there were no significant differences among the different "intermediate conditions" evaluated.

A precise and accurate method was successfully developed and validated for simultaneous determination of azithromycin. The total run time is 3.0min, within which the drug and their degradation products were eventually separated. Method validation results have proved the method to be selective, precise, accurate, and robust. This method can be successfully applied for the routine analysis as well as stability study.

Table 3: Method Precision and Intermediate Precision:

Sr. No.	Method precision	Intermediate precision
1	99.6	92.5
2	96.3	102.2
3	100.0	97.7
4	97.3	102.7
5	94.6	98.9
6	94.9	98.7
Mean	97.1	98.8
% RSD	2.4	3.7
% Difference		1.7

Table 4: Accuracy by Recovery:

Sr. No.	Sample	% Recovery of Azithromycin	Average of % Recovery	% RSD
1	25 % set-1	97.84	97.60	0.2
	25 % set-2	97.55		
	25 % set-3	97.40		
2	100 % set-1	99.16	99.60	0.0
	100 % set-2	99.13		
	100 % set-3	99.20		
3	150 % set-1	99.59	99.51	0.2
	150 % set-2	99.65		
	150 % set-3	99.28		

Table 5: Robustness study:

Conditions	% RSD of azithromycin	Tailing factor of azithromycin	Theoretical plate count azithromycin
Normal condition	0.7	1.0	1105
Column temperature (45ºC)	0.06	1.0	971
Column temperature (55ºC)	0.11	1.0	1163
Flow rate (1.8ml)	0.07	1.0	1166
Flow rate (2.2ml)	0.05	1.0	986
Wavelength (208nm)	0.12	1.0	1075
Wavelength (212nm)	0.15	1.0	1066
p^H of buffer (7.3)	0.09	1.0	936
p^H of buffer (7.7)	0.34	1.0	1065

Table 6: Solution stability study:

Time in Hours	Area of standard	Deviation from initial	Area of sample	Deviation from initial
Initial	70942	_	68831	_
2 Hours	72083	1.6	69957	1.6
8 Hours	71135	0.3	69125	0.4
16 Hours	70632	0.4	68791	0.1
24 Hours	71970	1.4	69028	0.3

Figure-3: Standards:

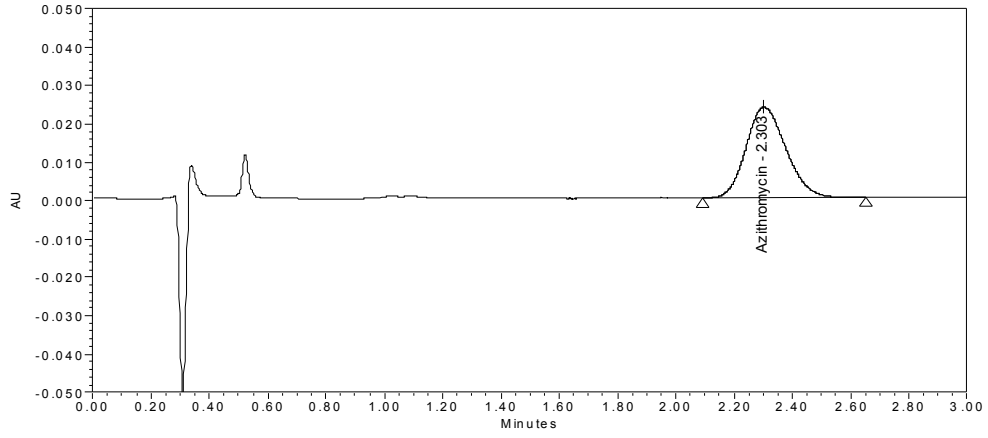


Figure-4: Azithromycin and Diluent :

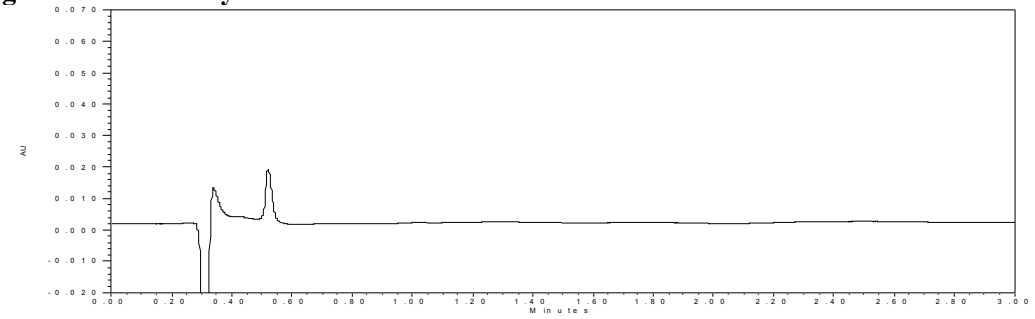


Figure-5 : Azithromycin with Placebo :

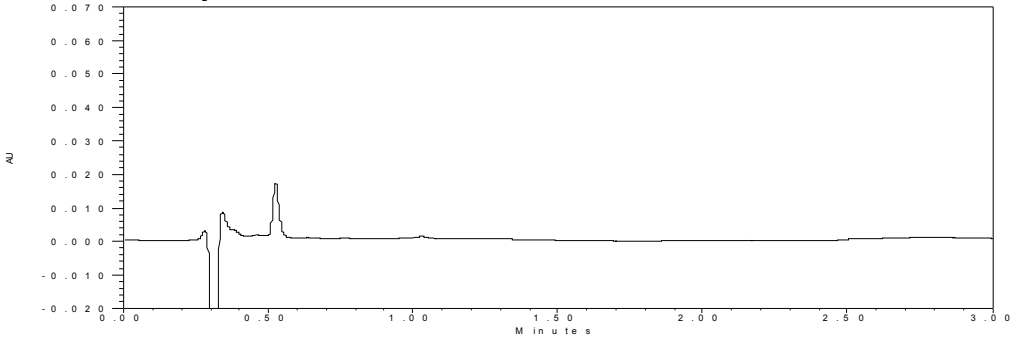


Figure-6 : Desosaminylazithromycin:

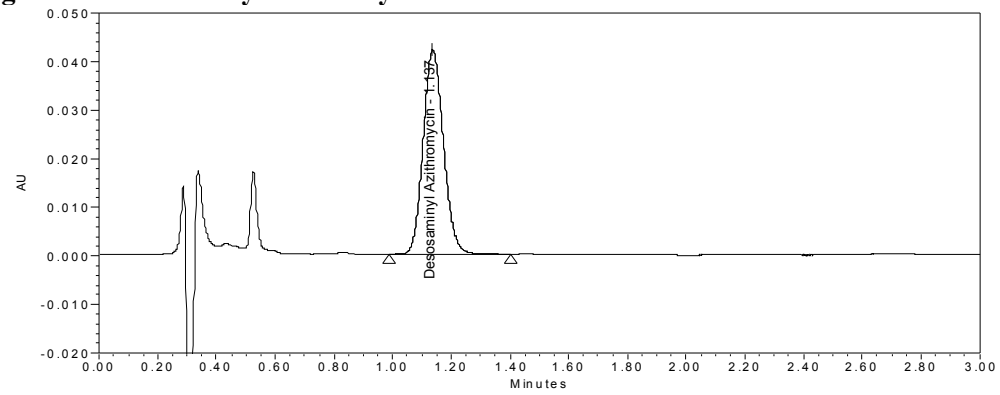


Figure-7 : N-Demethylazithromycin:

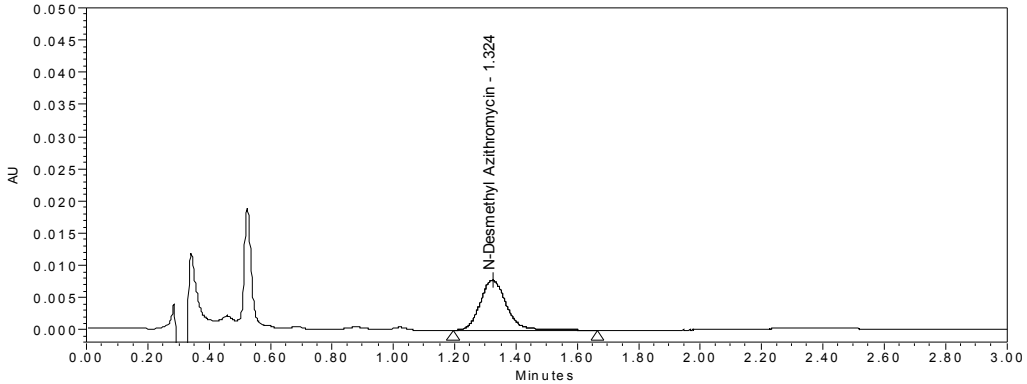
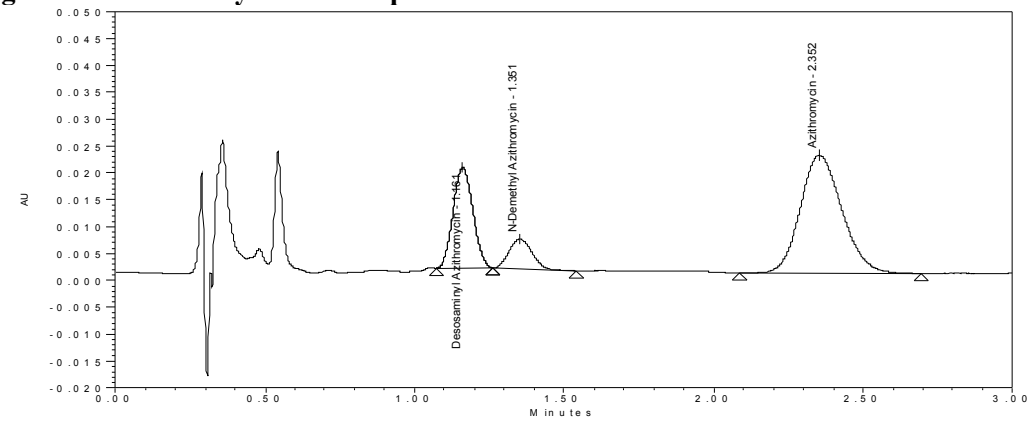


Figure- 8 : Azithromycin with impurities :



6.0 Acknowledgement

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