Development of a Simple RP-HPLC, UV-VIS, TGA, XRD Method for Validation and Azithromycin in Bulk and Pharmaceutical Dosage forms as an Alternative to the USP Method

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***Abstract*-** Using a Photodiode Array UV detector, researchers were able to develop a simple liquid chromatographic method for analyzing azithromycin in bulk and medicinal dosage forms. In this study, we used a C18 Column with dimensions of 25cm x 4.6mm and L1 packing at 50 + 1°C, a flow rate of 2ml/min, and an injection volume of 100µl each injection. Drugs can be separated quickly and precisely using a mobile phase that has been optimized. This experiment used a flow rate of 1.5 mL/min, a wavelength of 210 nm, and an injection volume of 500 L. The best separation and resolution were achieved with a 90:10 v/v isocratic methanol/buffer mobile phase. Over a wide range of azithromycin concentrations the suggested method proved accurate, precise, sensitive, and linear In contrast to the USP method, which makes use of an electrochemical detector, the new approach utilizes a UV detector. We also performed the Validation parameters like Linearity, Repeatability, Reproducibility, Precisions, Accuracy and Robustness with the same specs as we mentioned for other analysis. Analysis of azithromycin in both bulk and pharmaceutical dose forms were successful when using the tested method.

***Index Terms*-** Azithromycin Monohydrate, UV-VIS, XRD, RP-HPLC, Validation

I. INTRODUCTION

As the first macrolide, Erythromycin has a similar effect on Gram positive bacteria and Gram negative cocci, particularly Neisseria gonorrhoeae. However, there are some extra antimicrobial properties. In particular, activity against soil-dwelling anaerobic bacteria was revealed withstanding susceptibility to benzylpenicillin and microorganisms with thin cell walls like Inherent resistance to Mactam medicines in Mycoplasma pneumoniae In addition, Several new erythromycin targets have been discovered over the years elucidating the relationship between known illnesses and undiscovered diseases Chlamydia trachomatis, for example, has been found to be a new and dangerous bacteria[1]. Chlamydia pneumoniae, Helicobacter pylori, Legionella pneumophila, and Campylobacters jejunipylori are pathogenic bacteria. Outpatient oral antibiotics such as Erythromycin A and its derivatives are widely prescribed therapy, as well as treatment for children Erythromycin, on the other hand, appears to be useful[2]. A lack of regular blood levels and the acidic atmosphere have impeded this research Limited ability to take action due to gastrointestinal difficulties It was published by Washington and Wilson in 1985[3].

Azithromycin (9-deoxo-9a-aza-9a-methyl-9a-homoerythromycin) is generated structurally from erythromycin A by substituting the 9a carbonyl in the aglycone ring with methyl-substituted nitrogen and expanding the ring to 15 members[4]. Because of this structural difference, the internal process that forms the hemiketal is impeded, and the acid hydrolysis of the ether bond leading to the neutral cladinose sugar is the major avenue through which the hemiketal can be broken down[5]. Azithromycin degrades by 10% in 20.1 minutes in solution at 37°C, pH 2, and an ionic strength of j = 0.02, whereas erythromycin declines by 3.7 seconds. Cladianose and azithromycin had activation energies of 25.3kcal/mol for the hydrolysis of the ether bond, compared to 15.6kcal/mol in erythromycin for internal dehydration[6]. It appears that azithromycin is 300 times more acid stable than erythromycin in the stomach pH range, according to these findings[7]. Azithromycin appears to be mostly found in lysosomes, particularly those found in tissue fibroblasts. Animal models,

on the other hand, have shown that azithromycin is effective against infections produced by intracellular and extracellular organisms when tissue or extravascular concentrations surpass the MICs and blood concentrations are substantially lower[8].

The goal of this study is to validate the method for analyzing azithromycin at various parameters in order to determine its stability at various dose forms and temperatures.

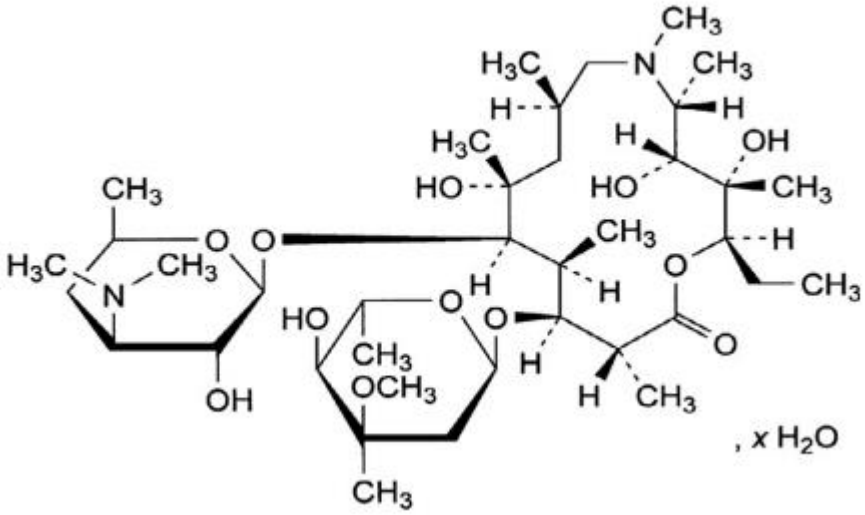


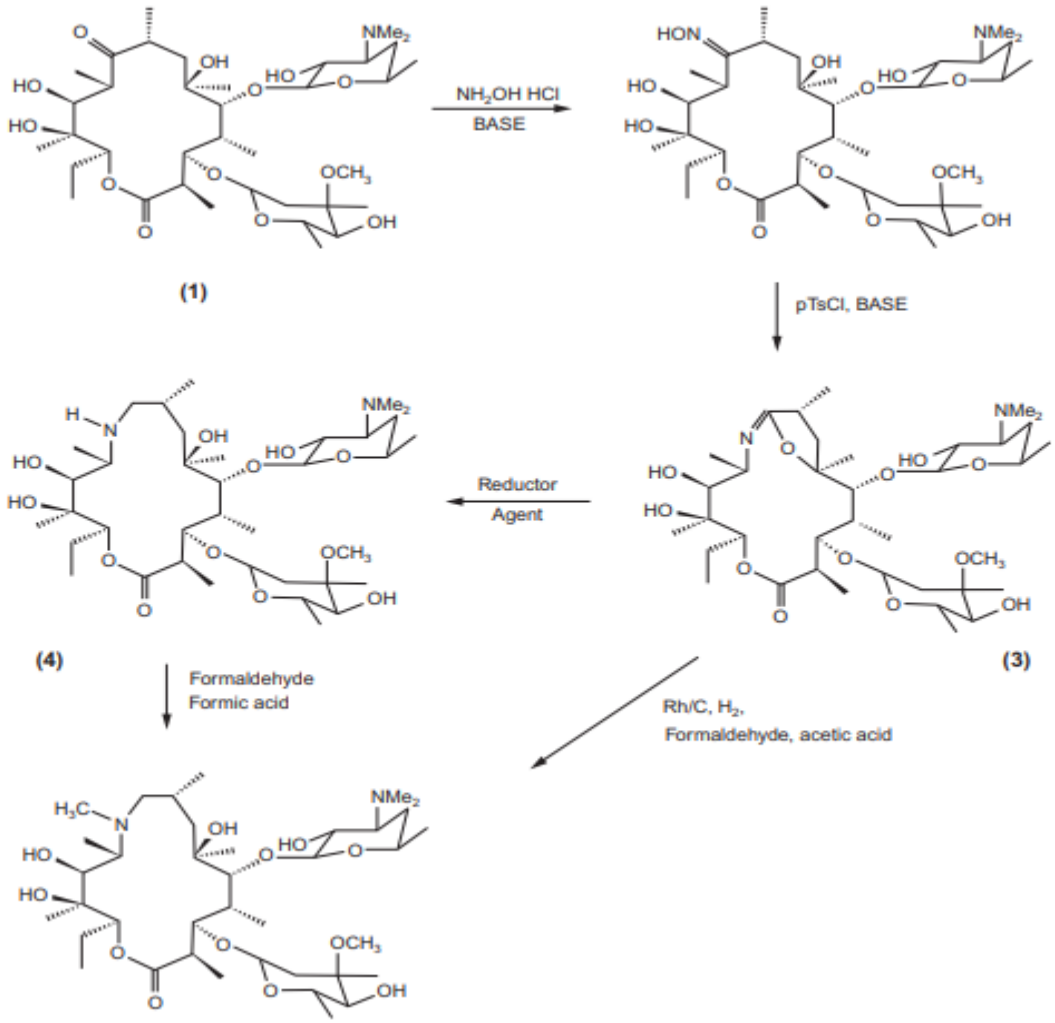
Fig. 1 Chemical Chem Draw structure of Azithromycin Monohydrate

Elemental analysis:

The theoretical elemental composition of azithromycin is as follows: Carbon: 60.94 %; Hydrogen: 9.69%; Nitrogen: 3.74%; Oxygen: 25.63%

Methods of Preparation:

Azithromycin was created by treating erythromycin A [1] in methanol with hydroxylamine hydrochloride and a base at reflux temperature for 10 hours to make oxime. The oxime was separated, purified, and subjected to Beckmann's rearrangement in aqueous acetone in the presence of p-toluenesulfonyl chloride and base for 2 hours at 5 C to give the interme diary (6,9-iminoether) (3) (Scheme 1.1). (And 2 h more at room temperature). With sodium borohydride in methanol[9] or by catalytic hydrogenation in the presence of platinum dioxide and acetic acid as solvents, the iminoether was reduced to the secondary amine[10]. Another synthetic approach for azithromycin has been published. The iminoether was synthesized in a single step (Scheme 1.1) from erythromycin A by treating the erythromycin A solution in acetone with O-methylene-sulfonylhydroxylamine, forming the mesitylenesulfonyloxime "in situ" from erythromycin A, which was then treated with an aqueous base (sodium bicar bonate) at 0 C, and the intermediary To get azithromycin, the iminoether was reduced with reductive methylation using standard procedures[11].



Scheme 1.1 Beckmann's rearrangement to obtain the intermediary (6, 9-iminoether) for Azithromycin preparation

UV-VIS Spectra:

The ultraviolet spectrum of azithromycin dihydrate in methanol and mobile phase (methanol: acetonitrile: phosphate buffer pH 6.7: tetrahydrofuran, 15:25:60:2.5, v/v). The figures were recorded using a double beam Model GBC 916UV VIS spectrophotometer (GBC Scientific Equipment Pty Ltd., Melbourne, Victoria, Australia). The values of wavelength maximum in nanometer (λmax) are 201.6 nm on methanol and 199.2 nm on mobile phase as shown in figure 2.

Thermogravimetric analysis (TGA) was carried out using TGA (Mettler, LMI JM Switzerland) apparatus. The samples (1–5 mg) were placed in aluminum pans and heated up to 250C at a rate of 1C/min under nitrogen purge (40 ml/min). The TGA thermogram obtained with the AZC sample is given in Figure 2.

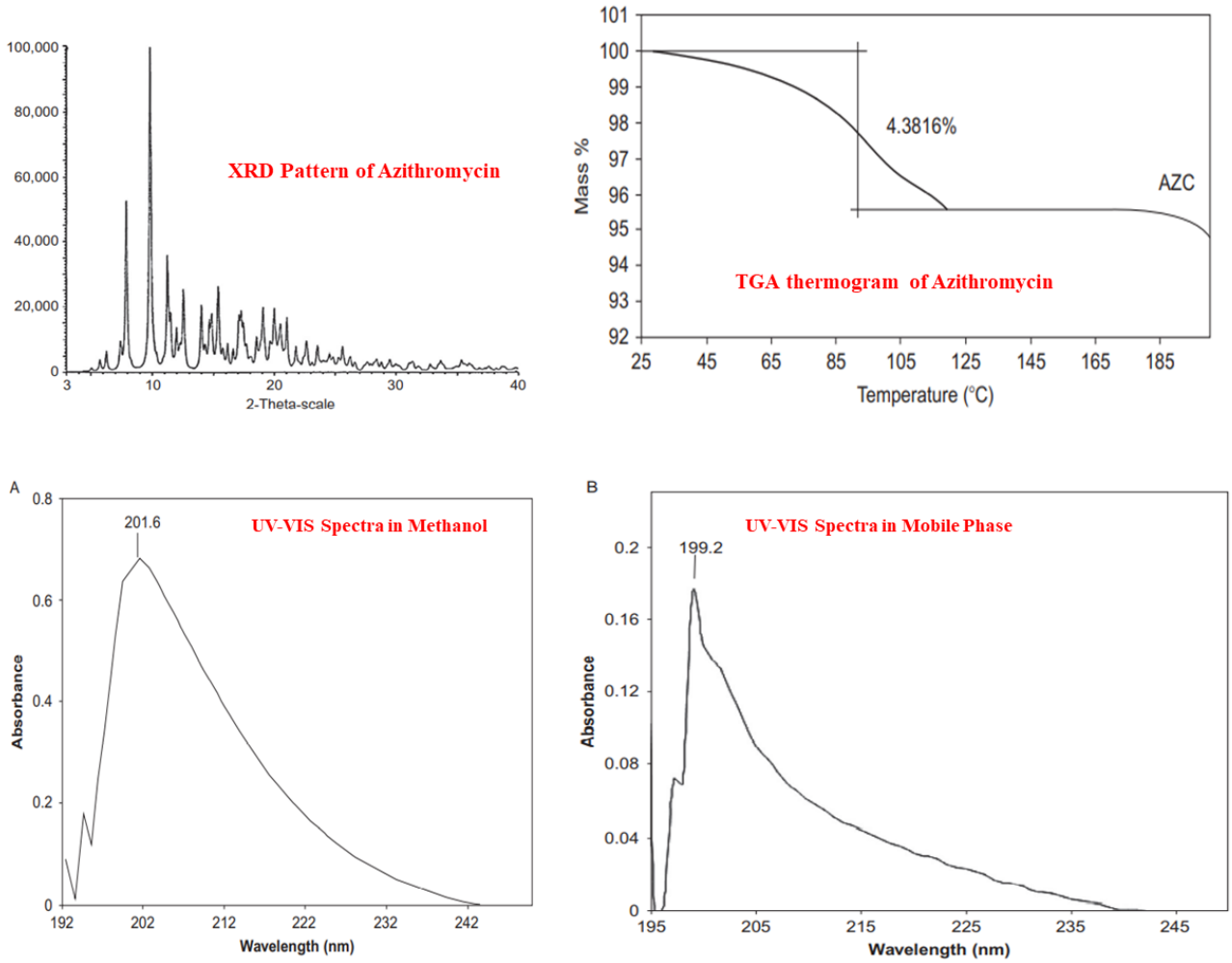


Fig 2. XRD Patterns, TGA Thermogram , UV-VIS Spectra of Azithromycin in Methanol and in Mobile phase

Equipment's used for the testing of Azithromycin

Different Instruments are required for the testing of Azithromycin in wet lab Analytical weighing balance. Test tubes. Volumetric flask. Pipette, Spatula.PH-meter, Karl Fischer Titrator Spectrophotometer, HPLC with the list of chemical like water, Ethanol, Methanol, Methylene Chloride, Dried Methanol, Karl Fischer reagent with specific amount.

Validation of Azithromycin by Using HPLC method:

SOLVENT/MOBILE PHASE

- Phosphoric acid (Analytical grade)
- Acetonitrile
- Distilled water
- Buffers / Solution preparations

Mobile Phase Preparations

Buffer: Dissolve 4.6 g/l of monobasic potassium phosphate (KH2PO4) in 900 ml water. Adjust with 1N NaOH solution to a pH of 7.5 and dilute with water to 1L.

Mobile phase: Mix the following solvents in a given ratio,

Acetonitrile	:	Buffer
65%	:	35%

Filter through 0.45µm filter paper and degas the mobile phase in an Ultra sonic bath for 5-10 minutes.

Diluents: Mobile Phase

STANDARD PREPARATION

- Weigh accurately 100 mg of USP Azithromycin (……...mg) or working standard, and transfer to 100ml volumetric flask and dissolve it in diluents to the volume and mix for 2-5 minutes (final concentration 1.0mg/ml).

(**Note**: Sonicate if required)

SAMPLE PREPARATION

- Take NLT 20 tablets of sample of Azithromycin 250/500 mg tablet and grind to powder and take powder equivalent to 1 tablet (250/500) and transfer to 100 ml volumetric flask and add about 80 ml diluents, sonicate for 2-5 minutes, cool and dilute to volume with diluents and mix for 2-3 minutes.
- Take 10/5 ml (250/500 mg tablet) of above solution in a 25 ml volumetric flask and dilute to volume with diluent for the final concentration 1.0mg/ml.

CHROMATOGRAPHIC CONDITIONS:

The following conditions have been found suitable

Dimensions	:	25cm x 4.6mm
Packing	: Packing L1	
Temperature	:	50 ± 1°C
Flow rate	:	2.0 ml/min
Detector	:	UV at 210 nm.
Injection volume	:	100 µl

System suitability:
Sample: Standard solution
Tailing factor: NMT 2.0
RSD: NMT 2.0%

II. RESULTS

1.1 Linearity of Azithromycin:

Linearity experiments were conducted to identify the range over which Azithromycin exhibit linear response. The stock solution of Azithromycin was prepared by dissolving equivalent to 200 mg (Azithromycin) of finely powder homogeneous sample of Azithromycin 500 mg tablet into 100 ml of diluents for final concentration of 2.0 mg/ ml of Azithromycin.

The stock solution was gravimetrically diluted in diluents to concentrations of 100ppm (0.1 mg/ml) ,200 ppm (0.2 mg/ml) ,500 ppm (0.5 mg/ml) ,1000 ppm (1.0 mg/ml) ,1500 ppm (1.5 mg/ml) and 2000 ppm (2.0 mg/ml) respectively.

The calibration graph is shown below, indicates linear relationship observed between the concentration and absorbance of the solutions. The R^2 of calibration data point was calculated to 0.9999. This indicates that the test procedure obeys Beer's law.

Sr. No.	Concentration (mg/ml)	injections	Retention time	Peak areas	Average peak areas of test solution
1	0.10	1	7.447	138203	138203
2	0.20	1	7.409	275010	275010
3	0.50	1	7.396	680860	680860
4	1.00	1	7.614	1407801	1407801
5	1.50	1	7.817	2125592	2125592
6	2.00	1	7.911	2879616	2879616

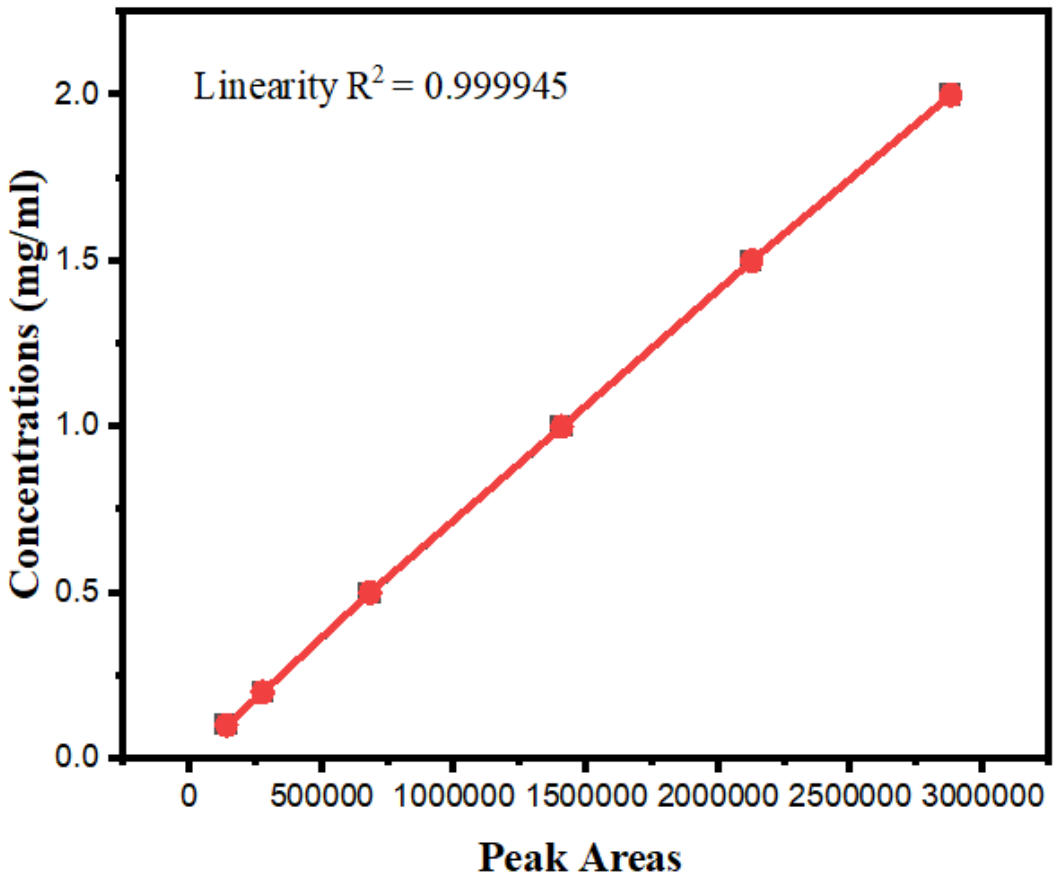


Fig 3. Representation of Linearity at different concentrations in mg/ml from the stock solution

The calibration graph showing linear relationship between the absorbance and concentration of analyte in the solution is shown below. The calibration graph is showing linear relationship between the concentration and absorbance of the solutions. There by indicating that the test procedure follows Beer's law.

1.2 **Precision:**

Verification of tests for assay includes an investigation of precision. This is tested by repeatability and reproducibility if the test method evaluated as repeatability and reproducibility.

1.3 **Repeatability (Azithromycin)**

It was calculated from the results of six consecutive determinations. To check repeatability, six samples were drawn and test solutions were prepared and tested according to the test procedure.

Weight of Reference Standard = 103 mg Purity 95.22%

Peak areas of Reference Standard = 1- 1426955 2- 1413563 3- 1404738

4- 1404387 5- 1411127

Average peak area of reference standard = 1412154

Average weight /tablet = 384 mg/tab (Azithromycin 250 mg tablet)

Sr. No.	Concentration (mg/ml)	Injections	Retention time	Peak areas	Average peak areas of test solution	%age Results of LC	Variation from theoretical Results

1	1.0	1	7.558	1414111	1408744	99.83%	-0.17%
		2	7.406	1408707			
		3	7.349	1403415			
2	1.0	1	7.330	1369415	1370301	98.13%	-1.87%
		2	7.321	1366980			
		3	7.460	1374509			
3	1.0	1	7.540	1394766	1401177	99.29%	-0.71%
		2	7.629	1410600			
		3	7.454	1398165			
4	1.0	1	7.526	1434449	1418406	100.51%	0.51%
		2	7.369	1418113			
		3	7.266	1402656			
5	1.0	1	7.249	1408425	1414598	100.25%	0.25%
		2	7.412	1409379			
		3	7.520	1425991			
6	1.0	1	7.582	1399084	1378112	98.69%	-1.31%
		2	7.259	1370587			
		3	7.228	1364666			

Range: (98.13 --- 100.51%)
Mean : 99.45%
Standard Deviation = 0.9223448%
Relative Standard Deviation = 0.927445% ± 2.00 %

1.4 **Reproducibility of (Azithromycin)**

To check the repeatability; 2 sets of five samples equivalent to 100 % of label claim were prepared and assayed by two analysts individually.

Analyst 01
Weight of Reference Standard = 105 mg Purity 95.22%

Peak areas of Reference Standard = 1- 2136483 2- 2134658 3- 2137504

4- 2141810 5- 2139665

Average peak area of reference standard = 2138024

Average weight /tablet = 781 mg/tab (Azithromycin 500 mg tablet)

Sr. No.	Concentration (mg/ml)	Injections	Retention time	Peak areas	Average peak areas of test solution	% age Results of LC	Variation from theoretical Results
1	1.0	1	9.978	2102128	2102222	98.52%	-1.48
		2	9.971	2102500			
		3	9.964	2102040			
2	1.0	1	9.968	2095304	2094602	98.16%	-1.84%
		2	9.979	2093763			
		3	9.986	2094739			
3	1.0	1	9.978	2147060	2145911	100.57%	0.57%
		2	9.967	2147047			
		3	9.971	2143626			
4	1.0	1	9.982	2091365	2093937	98.13%	-1.87%
		2	9.987	2093514			
		3	9.974	2096933			
5	1.0	1	9.966	2148469	2145849	100.44%	0.44
		2	9.971	2145322			
		3	9.979	2143756			

Range: (98.13 – 100.57%)
Mean : 99.16%
Standard Deviation = 1.2345971%
Relative Standard Deviation = 1.24506 % ± 2.00 %

1.5 Analyst 02

Weight of Reference Standard = 105.6 mg Purity 95.22%

Peak areas of Reference Standard = 1- 2150885 2- 2148886 3- 2142951

4- 2146782 5- 2146351

Average peak area of reference standard = 2147171

Average weight /tablet = 781 mg/tab (Azithromycin 500 mg tablet)

Sr. No.	Concentration (mg/ml)	injections	Retention time	Peak areas	Average peak areas of test solution	% age Results of LC	Variation from theoretical Results
1	1.0	1	9.933	2141379	2140063	100.87%	0.87%
		2	9.937	2139688			

		3	9.954	2139124			
2	1.0	1	9.952	2088660	2093386	98.41%	-1.59%
		2	9.953	2095486			
		3	9.938	2096012			
3	1.0	1	9.937	2142922	2143014	100.48%	0.48%
		2	9.943	2142177			
		3	9.950	2143944			
4	1.0	1	9.945	2088870	2086008	98.06%	-1.94%
		2	9.937	2085821			
		3	9.941	2083333			
5	1.0	1	9.946	2085252	2084844	98.16%	-1.84%
		2	9.951	2084155			
		3	9.956	2081126			

Range: (98.06 – 100.87%)
Mean : 99.19%
Standard Deviation = 1.3631324%
Relative Standard Deviation = 1.3742639% ± 2.00 %

1.6 **Accuracy (Azithromycin)**

The accuracy of test method is determined by spiked Placebo method.

Spiked Placebo Method:

Three samples of 500 gm each were prepared in the lab according the manufacturing procedure of the product and quantities equivalent to 80 %, 100 % and 120 % of the labeled amount of analyte were added to each Placebo. Test solutions were prepared of each concentration (i.e. 80%, 100% and 120%) assayed in duplicate and tested according to the test procedure of the product Results are tabulated below:

Weight of Reference Standard = 54.3 mg, Purity 95.22%

Peak areas of Reference Standard = 1- 2189123 2- 2187705 3- 2190355

4- 2192317 5- 2187988

Average peak area of reference standard = 218949

Different concentrations are according to 250 mg (Azithromycin 250 mg) tablet

Contents of Active Added in Placebo (% of Label Claim)	80 % (200mg/tab)	100 % (250mg/tab)	120 % (300 mg/tablet)
Weight of Placebo for each tablet		126mg/tablet	

Standard Compression weights for each sample	336 mg/Tab	336 mg/Tab	390 mg/Tab	390 mg/Tab	442 mg/Tab	442 mg/Tab
Weights of each concentration Samples taken for analysis eq. to 250 mg Azithromycin	420 mg	420 mg	400 mg	400 mg	368mg	370 mg
Peak area of test solution	2102070	2107479	2193388	2175532	2107604	2170711
Recovery for each concentration	198.55 mg/tab	199.07 mg/tab	252.5 mg/tab	250.45 mg/tab	298.89 mg/tab	306.17 mg/tab
% age of label claim	99.27%	99.53%	101.00%	100.18%	99.63%	102.05%
Variation from Theoretical Results or Difference	-0.73%	-0.47%	1.00%	0.18%	-0.37	2.05%
Average results of each concentration	99.40%		100.59%		100.84%	
Standard Deviation	-0.60%		0.59%		0.84%	

The results of assay show contents of the analyst's equivalent to the theoretical contents in Placebo. The deviation observed is within the limits.

1.7 **Robustness (Azithromycin)**

It is the measure how stable the test procedure is under slight variation in test procedure. The following changes were made deliberately in testing procedure. The test solution prepared according to the test procedure and kept at 15°C and 35°C for 24 hours and assayed according to the test procedure. The results are compound with initial results and tabulated below.

Weight of Reference Standard = 105.6 mg, Purity 95.22%

Peak areas of Reference Standard = 1- 2219609 2- 2218183 3- 2221598

4- 2219276 5- 2220025

Average peak area of reference standard = 2219738

Average weight /tablet = 781 mg/tab (Azithromycin 500 mg tablet)

Storage Condition	15°C		25°C		35°C	
	Sample I	Sample II	Sample I	Sample II	Sample I	Sample II
Weight of Samples	781 mg	781 mg	781 mg	781 mg	781 mg	781 mg
Peak area of test solution	2191216	2213546	2119053	2121011	2118688	2189230
% of Label Claim	99.38%	100.40%	96.11%	96.20%	96.34%	99.29%
Average	99.89%		96.16%		97.82%	
Standard Deviation	-0.11%		-3.84%		-2.18%	

The test samples are stable for up to twenty four hours kept under the temperature as low as 15°C and as high as 35°C. Based on linearity results test range of analytical control procedure for assay of Azithromycin is conformed as 0.1 to – 2.0 mg/ml of target.

Specificity:

Specificity of the analytical control procedure of assay of Azithromycin 500 mg tablet is determined by taken peak area of the placebo sample. The peak area obtained is 0.00.

III. Conclusion

A new, specific, and validated method for analyzing AZI was developed employing HPLC equipped with UV detection at 210 nm. This approach is exact, accurate, sensitive, and linear. This method can be used to analyze AZI at varied concentrations and in various medication formulations as well as raw materials.

Conflict of Interest:

The authors have no conflict regarding the data for the manuscript.

References

[1] Turchetta, S., P. Massardo, and P. Casellato, Process for preparing high purity azithromycin. 2003, WO2003102009A1.

[2] Bakheit, A.H., B.M. Al-Hadiya, and A.A. Abd-Elgalil, Azithromycin. Profiles of drug substances, excipients and related methodology, 2014. 39: p. 1-40.

[3] Lode, H., et al., Azithromycin—review of key chemical, pharmacokinetic and microbiological features. Journal of Antimicrobial Chemotherapy, 1996. 37(suppl_C): p. 1-8.

[4] Bright, G.M., et al., Synthesis, in vitro and in vivo activity of novel 9-deoxo-9a-AZA-9a-homoerythromycin A derivatives; a new class of macrolide antibiotics, the azalides. The Journal of Antibiotics, 1988. 41(8): p. 1029-1047.

[5] Fiese, E. and S. Steffen, Comparison of the acid stability of azithromycin and erythromycin A. Journal of Antimicrobial Chemotherapy, 1990. 25(suppl_A): p. 39-47.

[6] Cars, O., Pharmacokinetics of antibiotics in tissues and tissue fluids: a review. Scand J Infect Dis, 1991. 74(suppl): p. 23-33.

[7] Gladue, R. and M. Snider, Intracellular accumulation of azithromycin by cultured human fibroblasts. Antimicrobial Agents and Chemotherapy, 1990. 34(6): p. 1056-1060.

[8] Chang, H.R. and J. Pechere, In vitro effects of four macrolides (roxithromycin, spiramycin, azithromycin [CP-62,993], and A-56268) on Toxoplasma gondii. Antimicrobial Agents and Chemotherapy, 1988. 32(4): p. 524-529.

[9] Bayod-Jasanada, M., R.J. Carbajo, and F. López-Ortiz, Synthesis of 9-deoxo-9a-aza-9a-homoerythromycin A 11, 12-hydrogen borate and azithromycin 11, 12-hydrogen borate. A new procedure to obtain azithromycin dihydrate. The Journal of Organic Chemistry, 1997. 62(21): p. 7479-7481.

[10] Yang, B.V., M. Goldsmith, and J.P. Rizzi, A novel product from Beckmann rearrangement of erythromycin A 9 (E)-oxime. Tetrahedron letters, 1994. 35(19): p. 3025-3028.

[11] Pine, S.H. and B.L. Sanchez, Formic acid-formaldehyde methylation of amines. The Journal of Organic Chemistry, 1971. 36(6): p. 829-832.

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