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Research Article

Development and validation of a reversed-phase HPLC method for simultaneous estimation of azithromycin in tablet dosage form

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Abstract

An isocratic, precise and accurate reversed-phase liquid chromatographic method was developed for the quantitative determination of Azithromycin in tablet dosage form. The separation was carried out using a mobile phase consisting of buffer Potassium dihydrogen Phosphate: Acetonitrile (HPLC grade) (pH 7.5 adjusted with ortho phosphoric acid). The column used was Hypersil- keystone C18 (250 X 4.60 mm), 5µm column with flow rate of 1.2 ml/min using U.V. Visible detector. The detection was monitored at 215nm and the run time was 25min. The volume of injection loop was 10µl prior to injection of the drug solution the column was equilibrated for at least 15 min. The retention times of Azithromycin were found to be 9.761. Results of analysis were validated statistically and by recovery studies. Forced degradation method is used for detection of degraded impurities in body fluids shows better results than reported. The results of the study conclude that the proposed RP-HPLC method is a simple, specific, definite, precise, and less time consuming method which is useful for the routine determination of Azithromycin in its pharmaceutical dosage form.

Keywords: Azithromycin, RP-HPLC, buffer Potassium dihydrogen Phosphate, Acetonitrile

1. Introduction

Nowadays, purity testing and quantitative estimation of drugs in single or combined dosage forms is necessarily [1-3]. The determination of the concentration of the drug and it's metabolites in biological fluids and prepared samples of dosage form for treatment, is also an important task. The accurate determination and quantification analytical techniques are very helpful in to achieve that goal. The scope of developing and validating and analytical method is to ensure a suitable method for a particular analyte more specific, accurate and precise. The accurate determination and quantification analytical techniques are very helpful to achieve that goal. A review of literature reveals that good analytical methods are not available for the drug Azithromycin.[4-6] There are very few methods in the literature for the estimation of Azithromycin in dosage forms.[7-9] The development of analytical method for the determination of drugs in bulk, in dosage forms or in body fluids have received attention in recent years because of their importance in quality control, bioavailability and pharmacokinetic study etc. Developing, validating and analytical methods ensure a suitable method for a particular analyte and provide specific, accurate and precise result. The main objective for that is to improve the condition and Parameters, which should be followed in the development and validation.

The main objective of the stability indicating HPLC method is to estimate the related substances of Azithromycin in the presence of degraded impurities, employed by forced degradation method. [10-12]

- To optimize the method according to different parameters by taking different individual conditions to avoid error, unreliability and contamination.

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- To carry out the validation of method according to the International Conference on Harmonization (ICH) (Q1A, Q2B) guidelines..
- To develop a new, simple, economical, selective, precise, reproducible, and stability-indicating high-performance liquid chromatographic (HPLC) method with a wide linear range and good sensitivity for assay of Azithromycin in the bulk drug and in formulations using UV detection.[13]

2. Materials and Methods

Methanol (AR Grade) was used as solvent. Pure Standard gift sample of azithromycin dihydrate (AZI) was provided by Dr. Reddy's Laboratories Ltd., Hyderabad (INDIA). Tablets of "Zithium-O" of Alkem Laboratories Ltd. (azithromycin dihydrate- 500 mg) was purchased from local market.

2.1 Instruments

The HPLC system consisted of a Water's 600 controller pump, a U.V. Visible detector (wavelength tunable), a Hypersil- keystone C18 (250 X 4.60 mm), 5µm column and a Data ace software (Chromatography workstation). Volumetric glassware Class 'A' were used.

2.2 Determination Of Solubility:-

Solubility of Azithromycin was performed in different solvents and result was shown in (Table no.1).

2.3 Experimental Procedure:-

HPLC Conditions: The mobile phase consisting of buffer Potassium dihydrogen Phosphate: Acetonitrile (HPLC grade) (pH 7.5 adjusted with ortho phosphoric acid) were filtered through 0.45µ membrane filter before use, degassed and were pumped from the solvent reservoir in the ratio of 55:45v/v was pumped into the column at a flow rate of 1.2 ml/min. The column temperature was 30°C. The detection was monitored at 215nm and the run time was 25min. The volume of injection loop was 10µl prior to injection of the drug solution the column was equilibrated for at least 15 min. with the mobile phase.

2.3.1. Preparation of Standard Stock Solution:-

10 mg of Azithromycin was weighed accurately and transferred to a 10ml volumetric flask, and the volume was adjusted to the mark with the mobile phase, to give a stock solution of 1000ppm.

2.3.2. Preparation of Working Standard Solution:-

From stock solutions of Azithromycin 1 ml was taken and diluted up to 10 ml. from this solution 0.1, 0.2,0.3, 0.4,and 0.5 ml solutions were transferred to 10ml volumetric flasks and make up the volume up to 10 ml with mobile phase, gives standard drug solution of 10, 20, 30, 40 and 50 µg/ ml concentration.

2.3.3. Preparation of the Calibration Curves of the Drug:-

The standard drug solution was chromatographed and the mean area of drug was calculated (Table-5) and plotted against the concentration of the drug. The regression equation was found out by using this curve. Typical chromatograms (Fig-1 to 9) and the calibration curve (Fig- 10) were obtained (Table-6).

2.3.4. Preparation of Analysis of marketed Formulation:

Tablets equivalent to 10 mg Azithromycin was weighed and transferred to a 10 ml volumetric flask and volume was made up to 10 ml with Diluent to obtain concentration of 1000µg/ml. 1 ml of filtrate was taken in 10 ml volumetric flask and volume was made up to 10 ml with Diluent to obtain concentration of 100µg/ml. Further 1 ml of this solution was taken and diluted up to 10 ml obtain final concentration of 10µg/ml of Azithromycin.

3. Validation

3.1. System Suitability Studies

The column efficiency, resolution and peak asymmetry were calculated for the standard solutions (Table-3).

3.2. Specificity

Specificity of method was done by comparing the chromatogram of single drug with the chromatogram of drug mixture and blank (mobile phase) (Table-2, 4).

3.3. Selectivity

Various mobile phases had been trailed. The details of the same are presented in the (Table-3) for the selection of suitable solvent system, flow rate and ratio.

3.4. Linearity and Range

The calibration curve was constructed by plotting response factor against concentration of drugs. The slope and intercept value for calibration curve was y = 42349x - 21996 (R^2=0.997) for Azithromycin. The result shows that an excellent correlation exists between areas and concentration of drugs within the concentration range indicated above.

3.5. Assay for the marketed formulation

The marketed formulation "Zithium-O" of Alkem Laboratories Ltd. was taken for assay. The results obtained are presented in the (Table-8).

3.6. Robustness and Ruggedness

The effect of pH (9 and 9.4), temperature (25, 35 ^{0}C) and flow rate (1 and 1.3 ml/min.) had been studied to check the robustness of the method. The results are presented in the table. The ruggedness of the method was assessed by analyst to analyst precision. (Table-9 to 11)

3.7. Precision

Precision was calculated by relative standard deviation (%RSD) known as percentage coefficient of variance (%CV), using the formula: (Table-12, 13)

%RSD = Standard deviation / Mean × 100

3.8. Accuracy

The accuracy of the method was done by addition of standard drug solution to pre-analyzed sample solution at three different levels 80, 100 and 120 %. Mean percentage recovery was determined. % recovery was calculated by the given formula- (Table-14)

% Recovery =Amount recover / Total present amount × 100

3.9. Forced degradation study

Forced degradation studies of the drug sample were also performed using the following condition: Acid hydrolysis (1N HCl), basic hydrolysis (0.1 N NaOH), Heat (80^{0}C for 48hr), photolytic (UV), oxidation (30% hydogen peroxide) and reduction (10% sodium metabisulphite) was carried out for Azithromycin.

4. Results and discussion

4.1 Results of solubility study

Table No. 1- Solubility of Azithromycin

S. No.	Solvent	Solubility
1	Ethanol	Freely soluble
2	0.1 N NaOH	Freely soluble
3	Hydrochloric acid	Slightly soluble
4	Methylene chloride	Freely soluble

4.2 Results of HPLC Method

4.2.1 Mobile Phase Selection:-

Table-2 Selection of Mobile phase, pH and flow rate

Mobile phase	Ratio	Flow rate ml/min	Conclusion
Methanol: Phosphate Buffer	90:10	1.5	Poor resolution
Buffer: Methanol	60:40	1.0	short retention time
Phosphate Buffer:Methanol	20:80	1.5	Very small retention, peak broadening of azithromycin
Buffer: Methanol: Acetonitrile	65:25:10	1.2	Poor peak shape
Acetonitrile: Buffer	50:50	1.0	Asymmetric peak, long retention time
Buffer: Acetonitrile	55:45	1.2	Symmetric peak, good retention time

Table-3 Description of System suitability

S.N.	Parameters	Azithromycin
1.	Resolution	6.6871
2.	Theoretical Plate	213883.5573
3.	HETP	0.1482
4.	Tailing Factor	1.113
5.	Retention time	9.761
6.	Asymmetry	1.414

During mobile phase optimization and considering the system suitability parameters like RT, Tailing factor, No. of theoretical plates and HETP, the mobile phase Buffer: Acetonitrile (55:45), 7.5 pH at λ_{max}-215nm was found satisfactory. After mobile phase selection, effect of pH and flow rate was seen. It was found that pH 7.5 and 1.2ml/min. is suitable for the drug.

4.2.2. Selection of Separation Variable:

Table-4 Specificity representation

S. No.	Peak name	Retention Time
1	**Diluent**	No peaks are obseved at retention time of main peak
2	**Placebo**	No peaks are observed at the retention time of main peak
4	**Main Peak Azithromycin**	9.761

The chromatogram of blank, single drug and mixture of drug are given below:

Chromatogram of Blank

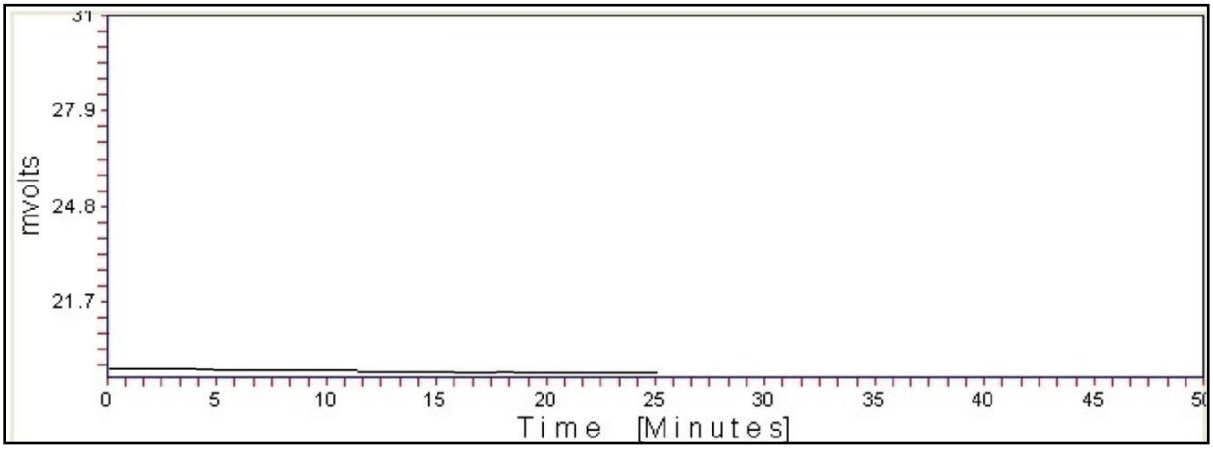

Fig. 1: Chromatogram of Blank

Chromatograms of Azithromycin

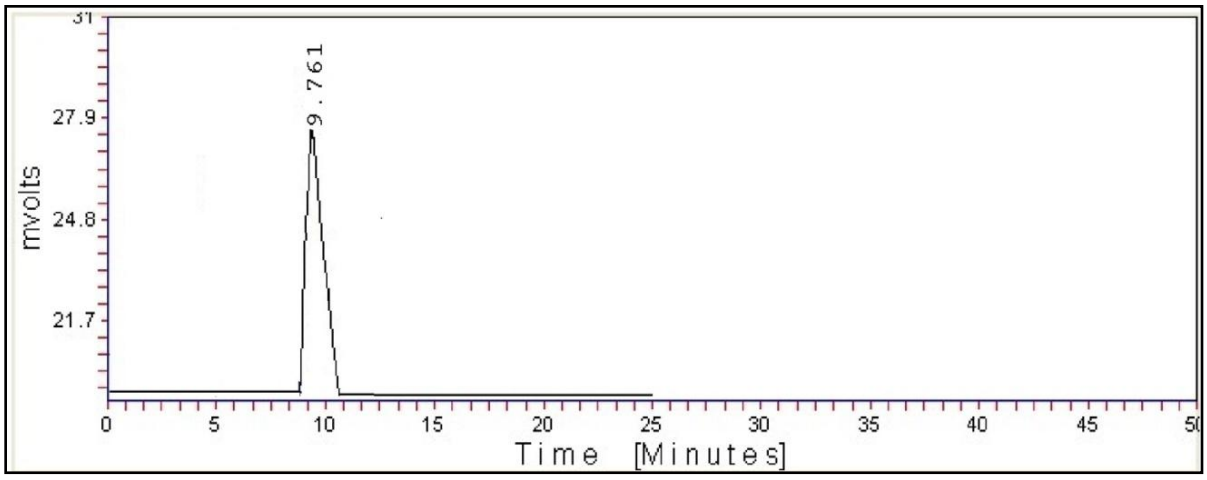

Fig. 2: Chromatogram of Azithromycin

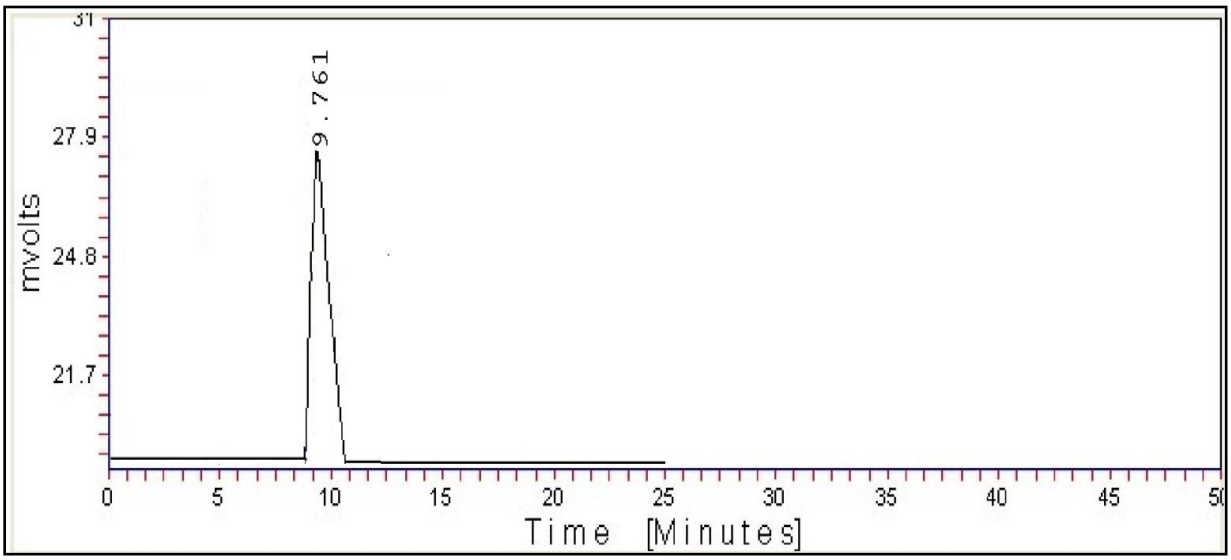

Fig. 3: Chromatogram of Azithromycin 10ppm

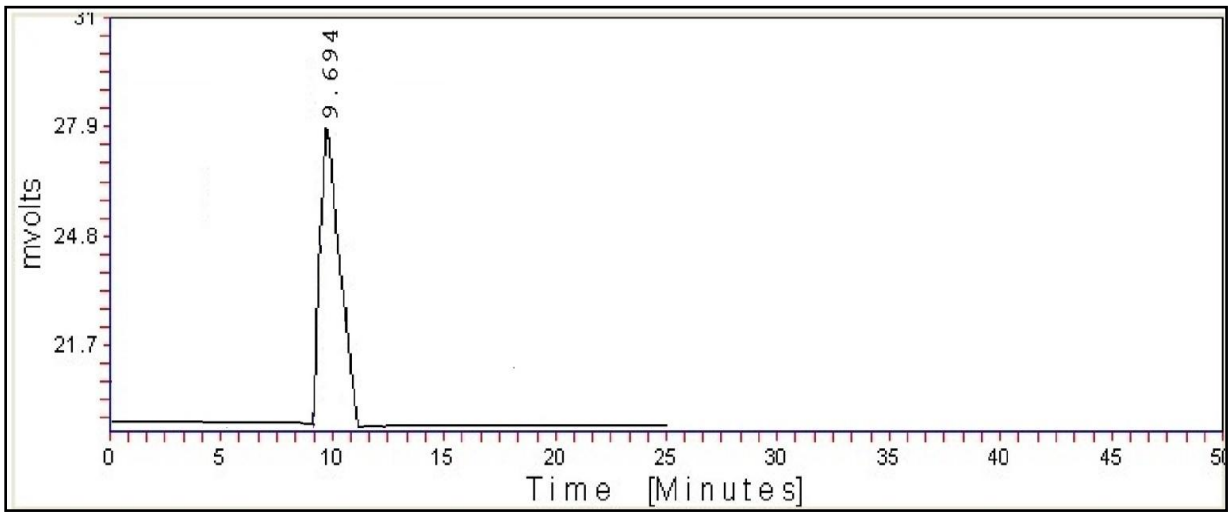

Fig. 4: Chromatogram of Azithromycin 20ppm

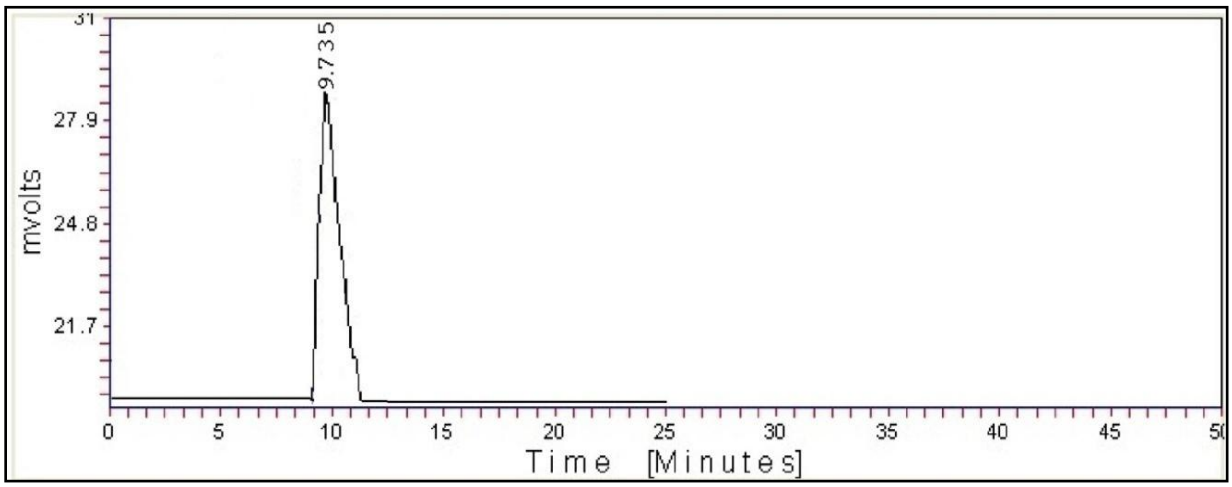

Fig. 5: Chromatogram of Azithromycin 30ppm

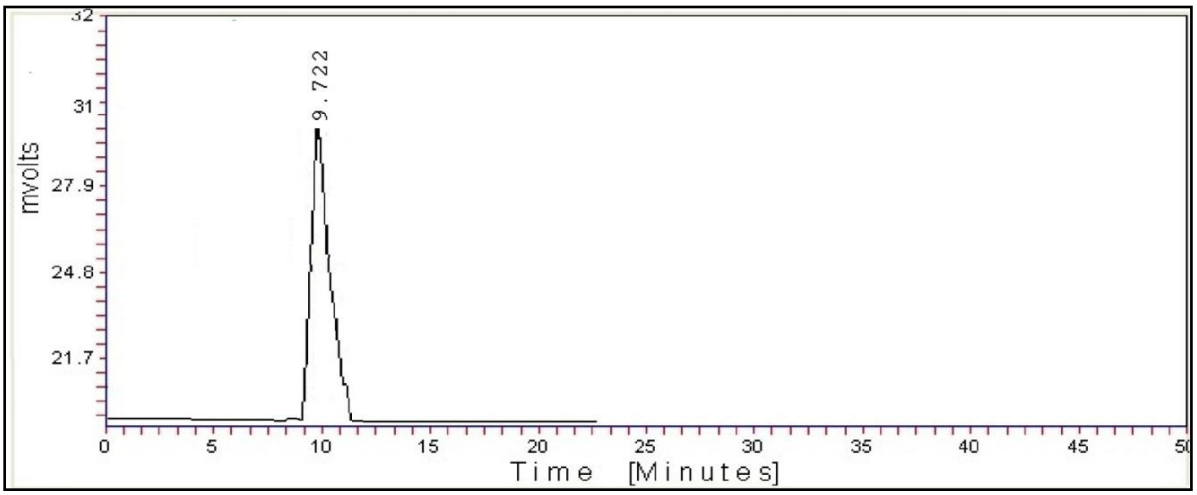

Fig. 6: Chromatogram of Azithromycin 40ppm

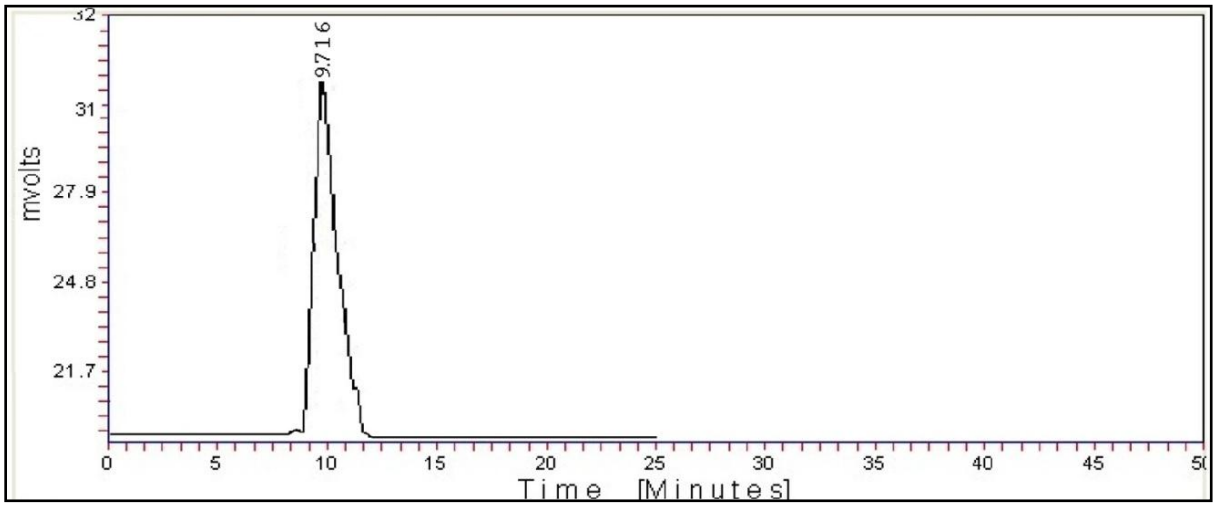

Fig. 7: Chromatogram of Azithromycin 50ppm

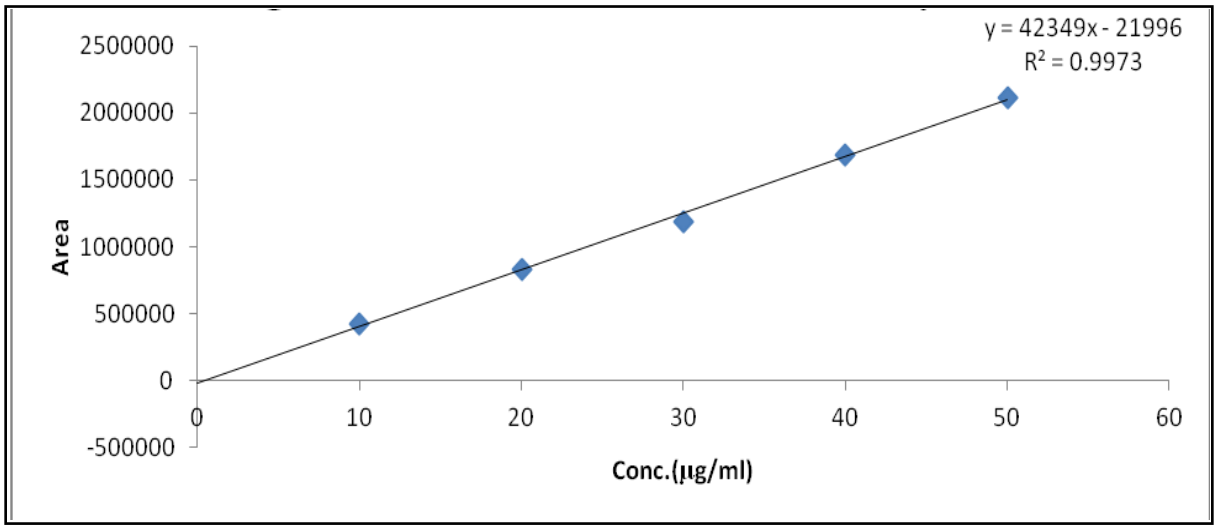

Fig. 8: Standard curve of Azithromycin

Table 5- Peak areas of Azithromycin

Conc. µg/mL	10	20	30	40	50	Mean
Rep.						
1	421490.2676	835975.247	1186498.755	1682949.213	2115443.832	
2	421487.4121	835970.1523	1186502.354	1682940.543	2115445.242	
3	421486.3864	835971.0114	1186501.111	1682951.448	2115448.546	
Mean	421488.022	835972.1369	1186500.74	1682947.068	2115445.873	
S.D.	1.642141359	2.727463054	1.828175334	5.760273302	2.419648959	
R.S.D%	0.000389606	0.000326262	0.000154081	0.000342273	0.00011438	
LOQ	0.000389419	0.000646793	0.000433535	0.001365997	0.000573798	**0.000682**
LOD	0.001056286	0.001754406	0.00117595	0.003705222	0.001556408	**0.00185**

Table 6- Standard Curve of Azithromycin

S.No.	Conc.(µg/ml)	Area	Height
1	10	421488.022	7760.0687
2	20	835972.1369	14880.0421
3	30	1186500.74	22300.3312
4	40	1682947.068	31051.175
5	50	2115445.873	38799.847

The calibration curve was constructed by plotting response factor against concentration of drugs. The slope and intercept value for calibration curve was y = 42349x - 21996 (R^2=0.997) for Azithromycin. The result shows that an excellent correlation exists between areas and concentration of drugs within the concentration range indicated above. The results for calibration curves are given in Fig 7.

4.2.3. Assay of Formulation:-

Table-7: Assay in Tablet Formulation

Brand Name	Azithromycin	
	Label Claim (mg)	% Assay
Zithium-O	500	99.7
	500	99.8
	500	99.8
	500	99.6
	500	99.8
Mean		99.74
SD		0.089442719
%RSD		0.089675876

Robustness:

Table-8: Effect of pH

Individual Drug

pH	Azithromycin			
	Rt	Area	Tailing	Plate count
9	9.752	421461.02	1.122	213880.47
9.4	9.664	421455.41	1.142	213879.52
Mean	9.708	421458.215	1.132	213879.995
S.D.	0.062225397	3.966869042	0.014142136	0.671751442
%RSD	0.6409703	0.000941225	1.249305267	0.000314079

Formulation

pH	Azithromycin			
	Rt	Area	Tailing	Plate count
9	9.747	421448.24	1.127	213879.87
9.4	9.672	421453.79	1.145	213883.46
Mean	9.7095	421451.015	1.136	213881.665
S.D.	0.053033009	3.924442636	0.012727922	2.538513344
%RSD	0.546197112	0.000931174	1.120415674	0.001186877

Table-9 Effect of temperature

Individual Drug

Temp. °C	Azithromycin			
	Rt	Area	Tailing	Plate count
25°C	9.709	421450.243	1.113	213880.22
35°C	9.743	421473.472	1.112	213885.34
Mean	9.726	421461.8575	1.1125	213882.78
S.D.	0.024041631	16.42538342	0.000707107	3.62038672
%RSD	0.247189292	0.003897241	0.06356016	0.001692697

Formulation

Temp. °C	Azithromycin			
	Rt	Rt	Rt	Rt
25°C	9.711	421502.121	1.201	213788.86
35°C	9.732	421434.224	1.126	213889.54
Mean	9.7215	421468.1725	1.1635	213839.2
S.D.	0.014849242	48.01042952	0.053033009	71.19151063
%RSD	0.152746412	0.011391235	4.558058323	0.033292077

Table-10 Effect of flow rate

Individual Drugs

Flow rate ml/min	Azithromycin			
	Rt	Rt	Rt	Rt
1	9.698	421471.23	1.211	213881.44
1.3	9.763	421453.46	1.127	213875.26
Mean	9.7305	421462.345	1.169	213878.35
S.D.	0.0459619	12.5652875	0.05939697	4.369919908
%RSD	0.4723492	0.002981355	5.081006811	0.00204318

Formulation

Flow rate ml/min	Azithromycin			
	Rt	Rt	Rt	Rt
1	9.688	421470.44	1.184	213873.986
1.3	9.767	421482.24	1.178	21388.454
Mean	9.7275	421476.34	1.181	117631.22
S.D.	0.0558614	8.343860018	0.004242641	136107.825
%RSD	0.5742630	0.001979675	0.359241379	115.7072289

Ruggedness:

Table-11 Result of Analyst to Analyst Precision Azithromycin

	Label claim Analyst	Amount found* mg/tab	Label claim (%)
1	500	498.94	99.788
2	500	499.91	99.982
3	500	497.58	99.516
Mean	500	498.81	99.762
SD	0	1.170427272	0.234085454
% RSD	0	0.234643907	0.234643907

4.2.5. Precision:

Precision was calculated by relative standard deviation (%RSD) known as percentage coefficient of variance (%CV):

Table-12 Intra-day and Inter-day precision Azithromycin

Intra-day Precision		Inter-day Precision	
	% Label Claim		**% Label Claim**
	Azithromycin		**Azithromycin**
After 1hr	99.6	First day	99.5
After2hr	99.3	Second day	98.9
After3hr	98.9	Third day	98.9
After4hr	98.6		
After5hr	99.7		
Mean	99.26666667	**Mean**	99.1
SD	0.43204938	**SD**	0.346410162
% RSD	0.435241148	**% RSD**	0.349556167

4.2.6. Accuracy:

The accuracy of the method was done by addition of standard drug solution to pre-analyzed sample solution at three different levels 80, 100 and 120 %. Mean percentage recovery was determined. % recovery was calculated.

Table-13 Recovery Studies and Statistical Validation for Accuracy of Tablets Formulation

Statistical Validation of Recovery Studies	**80**	**100**	**120**
Recovery (%)	**AZI**	**AZI**	**AZI**
Amount Present (mg)	500	500	500
	500	500	500
	500	500	500
Amount of Std. Added (mg)	400	500	600
	400	500	600
	400	500	600
Amount Recovered (mg)	498.69	499.71	499.64
	499.83	498.92	499.58
	498.98	499.85	498.75
% Recovery	99.738	99.942	99.928
	99.966	99.784	99.916
	99.796	99.97	99.75
Mean Recovery	99.83333333	99.89866667	99.86466667
SD	0.118496132	0.100286257	0.099485342
%RSD	0.118693955	0.100387983	0.099620162

4.2.7. Degradation of Azithromycin

Chromatograms of degradation study of Azithromycin at different parameter is shown-

Blank for Hydrochloric acid

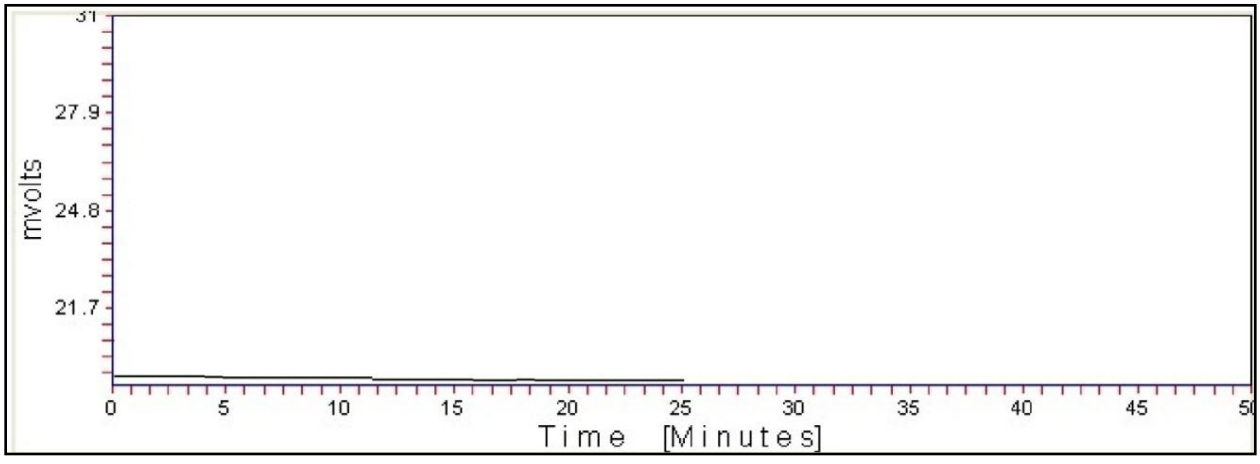

Fig. 9: Chromatogram of Hydrochloric acid

Azithromycin Acid Degradation

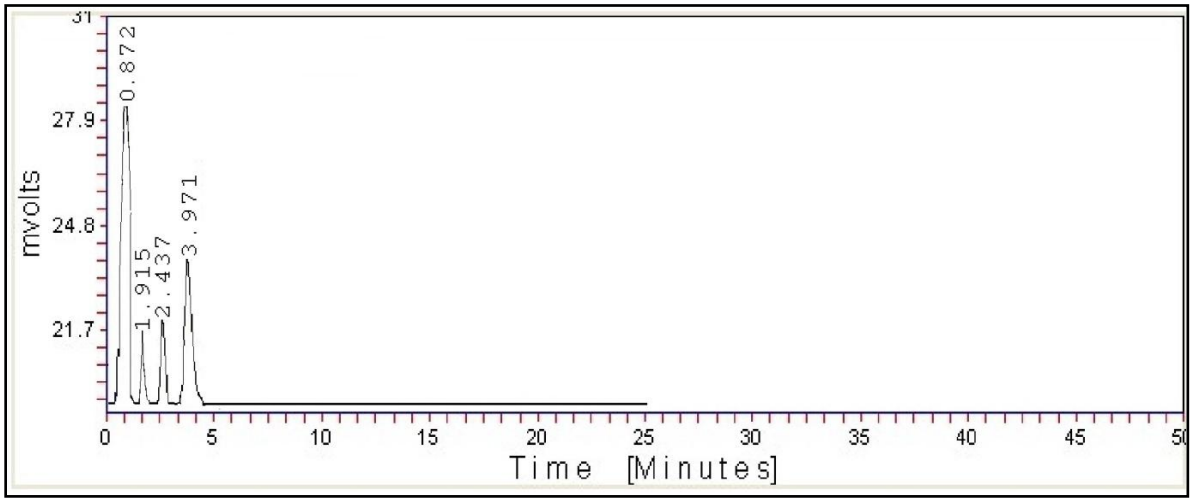

Fig. 10: Chromatogram of Azithromycin Acid Degradation

Degradant Peak	Retention time	Area	Peak Purity	Height
1	0.872	290342.411	999	4275.234
2	1.915	22451.236	999	232.986
3	2.437	28604.412	999	527.652
4	3.971	79678.335	999	2854.298

Azithromycin get completely degraded and form degradants at 0.872, 1.915, 2.437 and 3.971. The degradants was compared with the previous reporting in literature and the degradant on 3.971 may be correlated to azithromycin.

Blank for Sodium hydroxide alkali

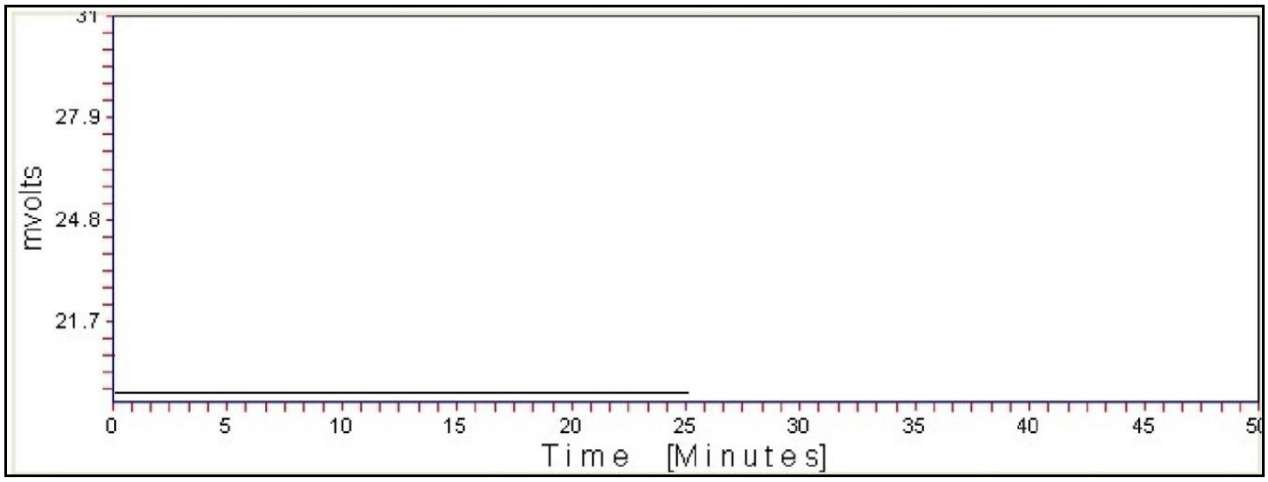

Fig. 11: Chromatogram of Sodium hydroxide alkali

Azithromycin with NaOH

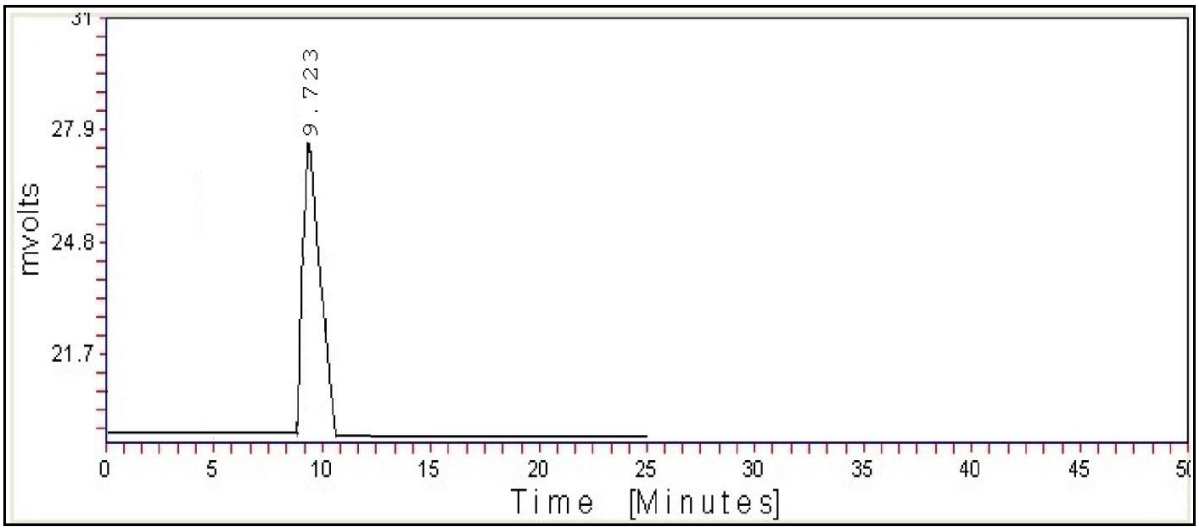

Fig. 12: Chromatogram of Azithromycin with NaOH

Peak	Retention time	Area	Peak Purity	Height
Azithromycin	9.723	421130.541	999	7765.0589

No degradation was observed when basic hydrolysis was performed.

Blank for H_2O_2 degradation

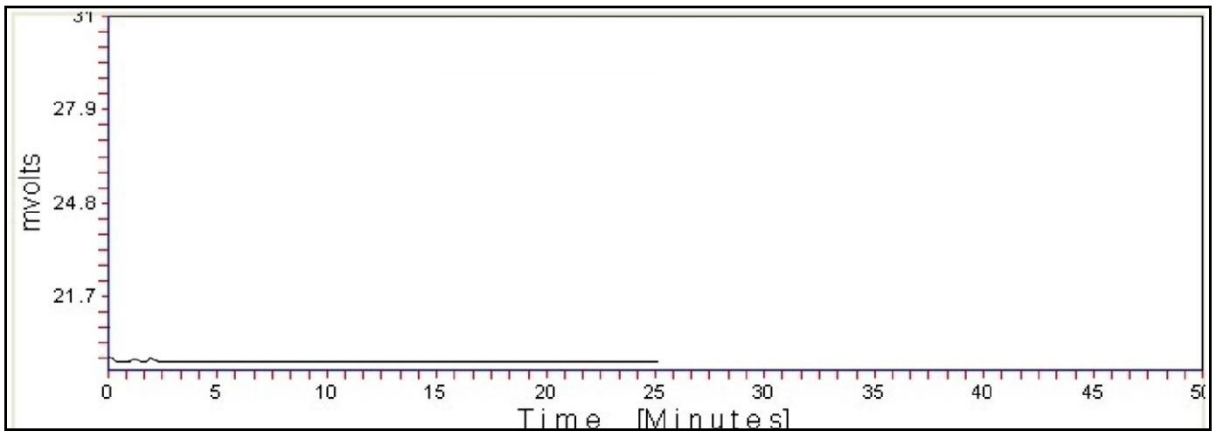

Fig. 13: Chromatogram of Blank for H2O2 degradation

Azithromycin with H_2O_2

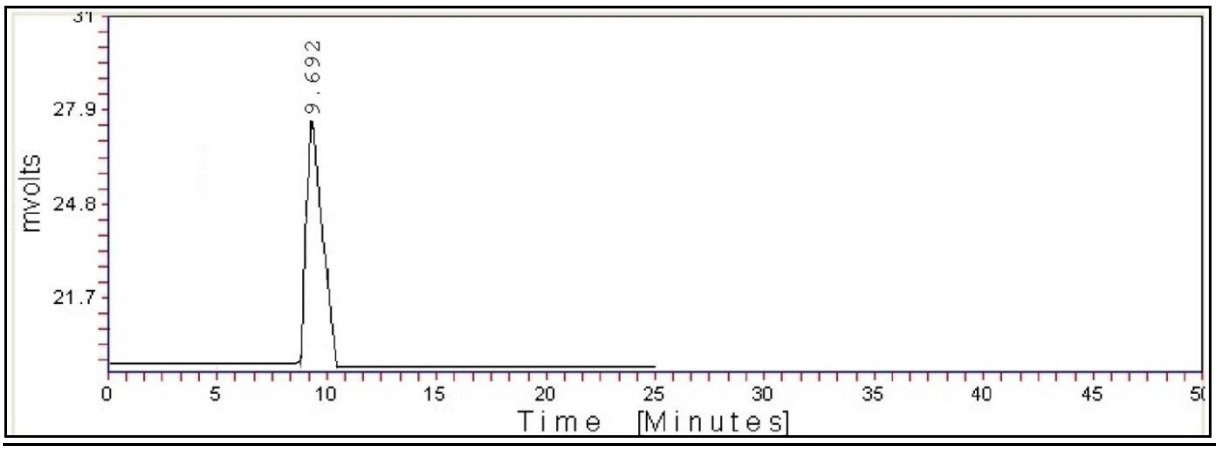

Fig. 14: Chromatogram of Azithromycin with H_2O_2

Peak	Retention time	Area	Peak Purity	Height
Azithromycin	9.692	420986.214	999	7740.5247

No degradation was observed when treated with hydrogen peroxide (oxidation) was performed.

Blank for Sodium Bi Sulphite degradation

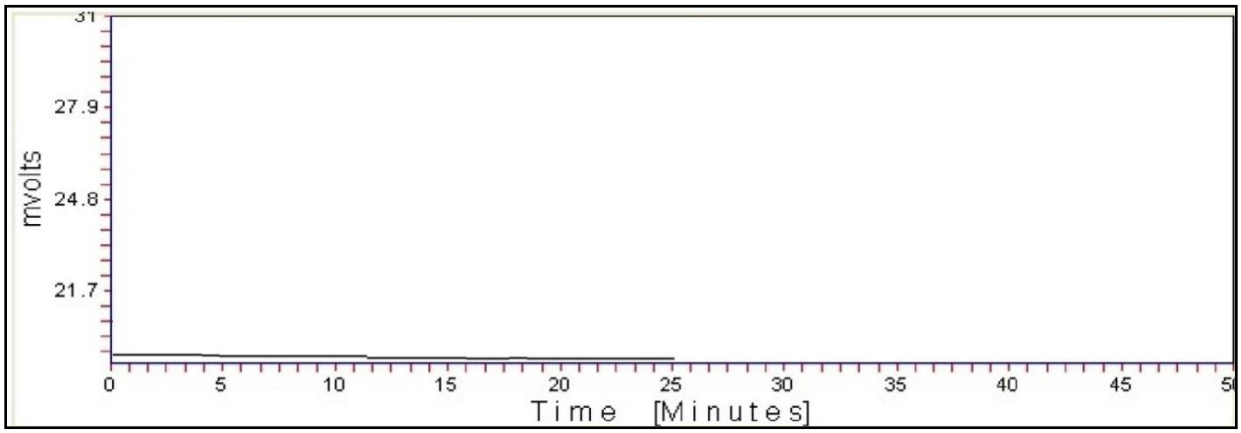

Fig. 15: Chromatogram of Blank for Sodium Bi Sulphite degradation

Azithromycin with Sodium bi sulphite

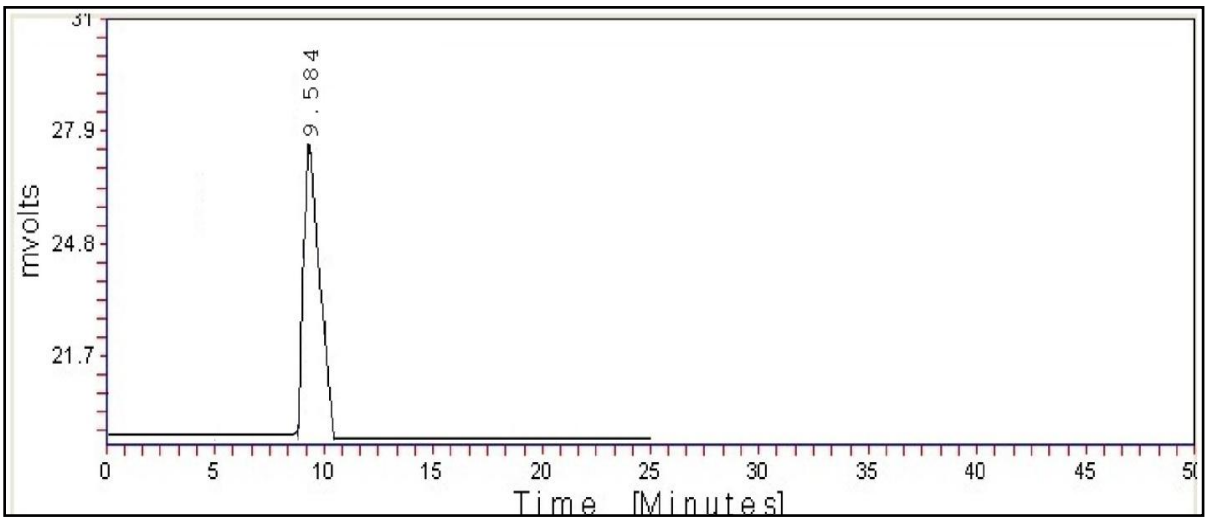

Fig. 16: Chromatogram of Azithromycin with Sodium bi sulphite

Peak	Retention time	Area	Peak Purity	Height
Azithromycin	9.584	420748.116	999	7732.4982

No degradation was observed when treatment with sodium bi sulphite was performed.

Blank for UV Light degradation

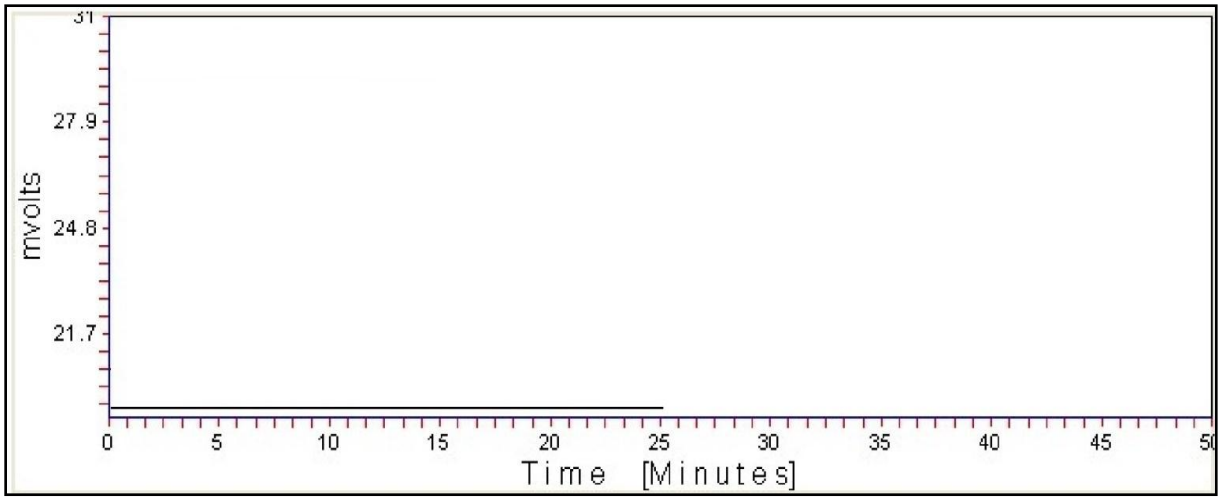

Fig. 17: Chromatogram of Blank for UV Light degradation

Azithromycin with UV light

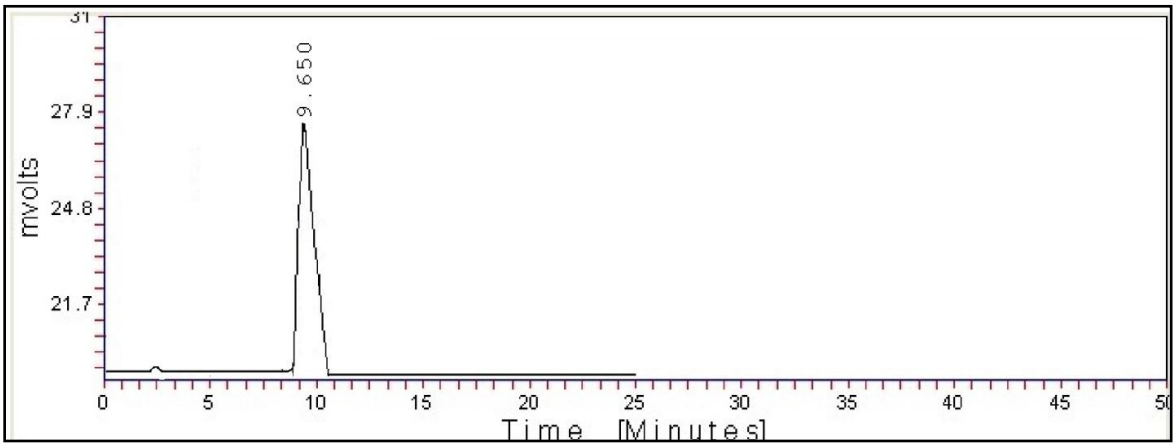

Fig. 18: Chromatogram of Azithromycin with UV light

Peak	Retention time	Area	Peak Purity	Height
Azithromycin	9.65	420751.421	999	7733.1754

No degradation was observed when treatment with UV light was performed.

Blank for temperature degradation

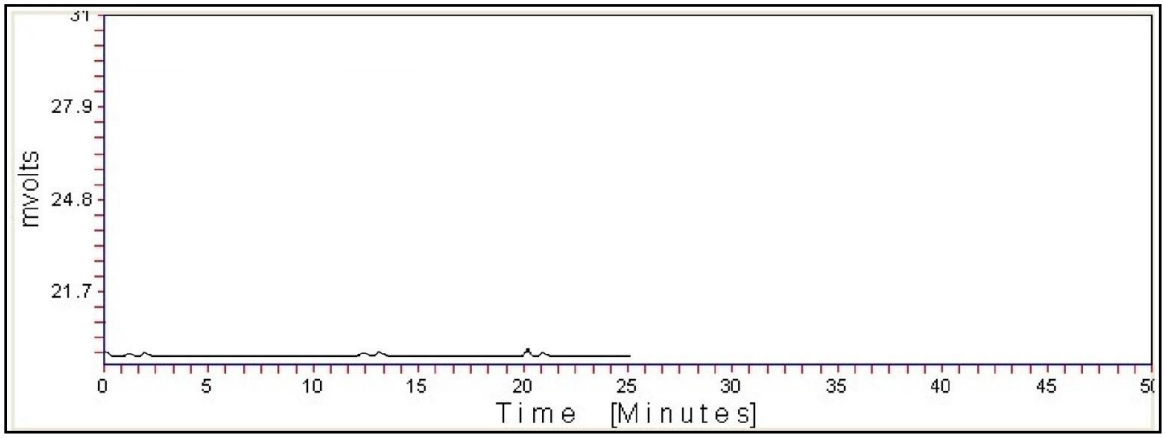

Fig. 19: Chromatogram of Blank for temperature degradation

Azithromycin with temperature

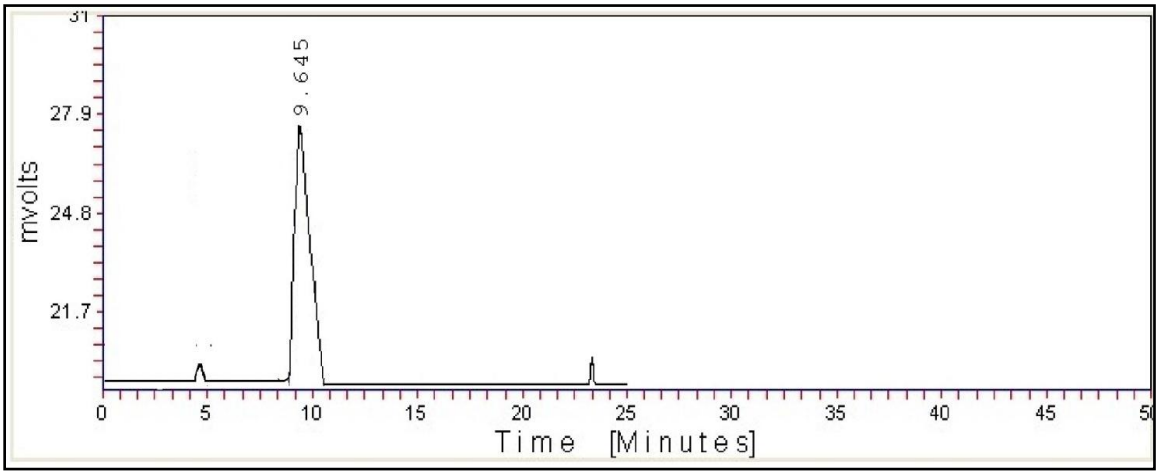

Fig. 20: Chromatogram of Azithromycin with temperature

Peak	Retention time	Area	Peak Purity	Height
Azithromycin	9.645	420883.207	999	7741.2243

No degradation was observed when treatment with dry heat was performed.

5. Conclusion

We can conclude that the simultaneous spectrophotometric methods for quantitative estimation of pharmaceuticals are fast, less time consuming, reproducible and highly sensitive even microgram of compound can be measured. Performing a through method validation can be a tedious process, but the quality of data generated with the method is directly linked to the quality of this process. Time constraints often do not allow for sufficient method validations. Many researchers have experienced the consequences of invalid methods and realized that the amount of time and resources required to solve problems discovered later exceeds what would have been expended initially if the validation studies had been performed properly.

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