RESEARCH ARTICLE Am. J. PharmTech Res. 2015; 5(4) ISSN: 2249-3387

AMERICAN JOURNAL OF PHARMTECH RESEARCH

Journal home page: http://www.ajptr.com/

New Stability Indicating Analytical Method Development and Validation for the Simultaneous Estimation of Azithromycin and Ambroxol Hydrochloride in Bulk and Tablet Dosage Form Using RP-HPLC

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ABSTRACT

A simple, economic, rapid, high range and accurate stability indicating RP-HPLC method was development for simultaneous estimation of Azithromycin and Ambroxol Hydrochloride in their combined tablet dosage form. This method was carried out by using Isocratic peak HPLC instrument with kromasil C-18 Column (250 mm X 4.6mm,5um) with mobile phase consisting a mixture of Methanol: Acetonitrile: Phosphate buffer in the ratio of 70:20:10 (v/v), at a flow rate of 1.1 ml/min with UV detection at 221nm . The retention time for Azithromycin and Ambroxol Hydrochloride are 9.08 and 5.39min respectively. Suitability, specificity, linearity, accuracy, precision, stability, and sensitivity of this method for the quantitative determination of the drugs were proved by validation in accordance with the requirements laid down by International Conference on Harmonization (ICH) Q2 (R1) guidelines. To establish stability indicating nature of the LC method, forced degradation of drug substances was performed under stress conditions like thermal, oxidation, peroxide, UV light, acid and base hydrolysis) The limit of quantification (L.O.Q) for Azithromycin and Ambroxol Hydrochloride are found to be 0.70ug/ml 1.00ug/ml.Then the limit of detection (L.O.D) for Azithromycin and Ambroxol Hydrochloride are found to be 0.15ug/ml and 0.3ug/ml respectively. The results of the study showed that the proposed method is reliable and robust and can be used as quality control tool for the estimation of these drugs in combined pharmaceutical solid dosage forms.

Keywords: Azithromycin, Ambroxol Hydrochloride, RP-HPLC method, Validation.

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Received 27 July 2015, Accepted 01 August 2015

Please cite this article as: Subbareddy P *et al.*, New Stability Indicating Analytical Method Development and Validation for the Simultaneous Estimation of Azithromycin and Ambroxol Hydrochlorde in Bulk and Tablet Dosage Form Using RP-HPLC. American Journal of PharmTech Research 2015.

INTRODUCTION

Azithromycin ($C_{38}H_{72}N_2O_{12}$) is an Antibiotic, useful for the treatment of Bacterial infections. It is an Azilide,a sub-class of macrolide antibiotic[1]. It is derived from Erythromycin, with a methyl substituted nitrogen atom incorporated into the lactone ring. Chemically it is (2R,3S,4R,5R,8R,10R,11R,12S,13S,14S)-11-(2S,3R,4S,6R)-4-(dimethylamino)-3-hydroxy-6-methyl tetrahydro-2H-Pyran-2-yloxy)-2-ethyl-3,4,10-trihydroxo-13-((2S,4R,5S)-5-hydroxy-4-methoxy-4-tetrahydro-2H-pyran-2-yloxy)-3,5,6,8,10,12,14-hepatamethyl-1-oxa-6 cyclopentadecan -5-one. It is on the world health organization's list of essential medicines, a list of the most important medications needed in a basic health system[2]. Ambroxol hydro chloride ($C_{13}H_{18}Br_2N_2O.HCL$) is a 2-amino-3,5- dibromo-N-(trans- 4- Hydroxy cyclo hexyl) Benzyl amine[3]. It is a metabolite of bromhexine, used in the treatment of respiratory disorders with productive cough, surfactant stimulation, mucokinethc and secretagogue activity are the major pharmacodynamics activity of the substance[4]. The literaturesurvey revealed that few HPLC methods have been reported for the estimation of Azithromycin and Ambroxol Hydrochloride[5-9]. Hence we attempted to develop a simple, accurate and economical analytical RP-HPLC, method for simultaneous estimation of Azithromycin and Ambroxol Hydrochloride API and tablet dosage forms.

MATERIALS AND METHOD

Instrumentation

To develop a High Pressure Liquid Chromatographic method for simultaneous estimation of Azithromycin and Ambroxol isocratic PEAKHPLC instrument with kromasil C18 column (250 mm x 4.6 mm, 5µ) and Electronic balance-DENVER (SI234) was used. The instrument is equipped with a LC 20AT pump for solvent delivery and variable wavelength programmable LC – 7000 UV-detector. A 20µL Rheodyne inject port was used for injecting the samples. Data was analyzed by using PEAK software.

Preparation of standard solution

Standard stock solution of Azithromycin and Ambroxol pure drug (1mg/ml) was prepared by accurately weighing about 100 mg of each drug in 100 ml volumetric flask separately. Then the drugs were dissolved with 25ml of methanol, and sonicated to dissolve it completely and made up to the mark with the same solvent. The contents were mixed well and filtered through Ultipor N_{66} Nylon 6, 6 membrane sample filter paper. Appropriate volumes of these solutions were further diluted with mobile phase to get required concentrations for construction of calibration curve.

Procedure for pharmaceutical formulation

Sample solution was prepared by a composite of 20 Azithromycin and Ambroxol combination tablets (Aziriv-A: 500mg of Azithromycin and 60mg of Ambroxol) were grinded to a fine, uniform size powder. An amount of drug equivalent to10 mg of Candesartan was accurately weighted and quantitatively transferred into 100 ml volumetric flasks. Approximately 30 ml mobile phase was added and the solution was sonicated for 15 min. The flask was makeup to volume with mobile phase, and mixed well. Then the solution is filtered through 0.45µm nylon 6 6 membrane filter paper. Then an amount of the solution was diluted with mobile phase to a concentration of 100µg/ml of Azithromycin. Then based on the label claim of the Hydrochlorothiazide, a concentration of 12µg/ml of Ambroxol solution was obtained.

Method Development

To develop a simple and robust method for the simultaneous determination of Azithromycin and Ambroxol Hydrochloride in combined tablet dosage form using HPLC.The spectra of diluted solution of the Azithromycin and Ambroxol Hydrochloride in methanol are recorded separately on UV spectrophotometer. The peaks of maximum absorbance wavelengths are observed. The spectra of the both Azithromycin and Ambroxol Hydrochloride were showed that a balanced wavelength was found to be 221 nm. To effect ideal separation of the drug under isocratic conditions, mixture of solvents like Methanol, Acetonitrile and water with or without different buffers in the different combinations were tested has mobile phases on RP-C-18 stationary phase. Finally find that a mixture of Methanol, Acetonitrile & phosphate buffer in the ratio of 70:20:10(v/v) was proved to be the most suitable of all the combinations since the chromatographic peaks obtained werebetter defined & resolved and almost tree from tailing. Flow rates of the mobile phase were changed from 0.6-1.1ml/min for optimum separation. A minimum flow rate as well as minimum run time gives the maximum saving of the usage of solvents. It was found from the experiments that 1.1 ml/min flow rate was ideal for the successful elution of the analyte. No interference in blank and placebo solutions for both drug peaks in the trail injections with a runtime of 12min. The above optimized chromatographic conditions were followed for the simultaneous determination of Azithromycin and Ambroxol Hydrochloride in bulk samples and its combined tablet formulations.

Validation of the developed method

A new optimized method suitable for the simultaneous routine analysis of Azithromycin and Ambroxol Hydrochloride in bulk and pharmaceutical formulation samples was successfully developed. The developed method was validated as per ICH guidelines.

Linearity

Linearity was established by preparing six different concentrations of Azithromycin and Ambroxol Hydrochloride based on the label claim of the formulation dosages form. Azithromycin of 20-150 ug/ml & Ambroxol of 3-18 ug/ml solutions were mixed. The peak area vs concentration data were evaluated by linear regression analysis.

Precision

Precision of the method was determined by performing intraday precision by making six injections (concentration of Ambroxol 12.5 ug/ml & Azithromycin 100 ug/ml) on the same day. The studies also repeated on next day to determine interday precision. The % RSD was calculated in both intraday and interday precision.

Accuracy- Recovery Studies

Recovery of method was determined by adding 50%, 100% & 150% of the test drive to previously analyzed sample of standard Azithromycin and Ambroxol Hydrochloride (API). For each concentration of levels three sets were prepared and injected in duplicate. The recovery of added drug was determined.

Robustness

To determine the robustness of the method, experimental conditions were purposely altered and chromatographic characters are evaluated. Influence of small changes in chromatographic conditions such as changes in flow rate (±0.25ml/min), % organic Phase (± 2%) and column temperature (±5^{o}c) were studied to determine the Robustness of the method

System Suitability

Six replicate injections of standard preparations were injected and asymmetries, theoretical plates, tailing factor and % relative standard deviation (%RSD) for peak area were determined.

Forced degradation studies

Forced degradation studies of both the drugs were carried out under conditions of hydrolysis, dry heat, oxidation, UV light and photolysis. 500mg of Azithromycin and 60mg of Ambroxol Hydrochloride were weighed and transferred into two 50 ml volumetric flasks and diluted up to the mark with methanol. These stock solutions were used for forced degradation studies. Forced degradation in basic media was performed by taking 10 ml of stock solution of Azithromycin and Ambroxol each in separate round bottom flasks. Then 10 ml of 5 N NaOH was added and these mixtures were heated for up to 8 h at 70^{0}C in dark, in order to exclude the possible degradative effect of light. Forced degradation in acidic media was performed by keeping the drug in contact with 1N HCl for up to 30 h at ambient temperature as well as heating for up to 8 h at 70 0 in dark. Degradation with hydrogen peroxide was performed by taking 10 ml of stock solution two different flasks and adding 10 ml of 30% (w/v) hydrogen peroxide in each of the flasks. These mixtures were kept for up to 4 days in the dark. To study neutral degradation, 10 ml of stock solution was taken in two different flasks, and then 10 ml of HPLC grade water was added in each flask, these mixtures were heated for 6 h at 700 in the dark. For dry heat degradation, solid drugs were kept in Petri dish in oven at 100^0C for 12 h. Thereafter, 10 mg each of Azithromycin and Ambroxol were weighed and transferred to two separate 10 ml volumetric flasks and diluted up to the mark with methanol. The photostability was also studied by exposing both the drugs to direct sunlight in for 5 h on a wooden plank. For UV degradation study, both the drugs were exposed to UV radiation of a wavelength of 256 nm and of 1.4 flux intensity for 12 h in UV chamber.

Analysis of tablet formulation

The prepared formulation solution was injected into HPLC systems and a chromatogram was recorded. The injections were repeated six times and peak areas were recorded. A representative chromatogram has been given in figure-1. The peak area of each of the drug was determined and the amount of each drug present per tablet was estimated from the respective calibration curves. The stability of the sample in mobile phase was analyzed after 24 hours; it was found no change in analytical parameters.

RESULTS AND DISCUSSION

The development of an analytical method for the determination of drugs by HPLC has received considerable attention in recent years because of their importance in quality control of drug products. The objective of this study was to develop a rapid and sensitive HPLC method for estimation of Azithromycin and Ambroxol Hydrochloride in tablet formulations using the most commonly employed RP C-18 column with UV detection. The mobile phase was optimized with Methanol: Acetonitrile: Phosphate buffer in 70:20:10 (v/v). From the overlain spectrum of Azithromycin and Ambroxol Hydrochloride, wavelength was selected, at 221nm, is absorptive point for both the drugs. Good resolution was carried out at 221nm and both drugs showed good absorbance at this wavelength. Optimized chromatographic conditions were shown in table 1. Results of formulation chromatograms were shown in figure- 1 all parameters of these proposed method was validated as per the ICH guidelines. A good linear relation was observed with the concentration range of 3-18 ug/ml for Ambroxol with regression equation $y=19042\ x-8584.3(r^2 =0.9987)$ and 20-150 ug/ml for Azithromycin with regression equation $y=7218.4\ x+183528(r^2$

=0.9988). Linearity results were shown table -2& graphs were shown in figure-2. Accuracy results were expressed as percent recoveries of the particular components in the samples. The overall results of percent recoveries (mean ±%RSD) of drug-matrix solutions are indicating good accuracy of the proposed RP-HPLC method. The % RSD recovery was found to be 0.48, 0.27, and 0.12 for Azithromycin (table 3), 0.43, 1.05, 1.29 for Ambroxol (table 3) for 50%, 100% and 150% respectively. During the forced degradation study, in all the degradation conditions, additional degradation peaks were observed but the retention time of standard was not changed. The assay also found to be very high and the method can successfully separate the degradation products also. The degradation results were given in table 9 and chromatograms were given in figure 3

Table 1: Optimized chromatographic conditions for Azithromycin and Ambroxol

S. No	Parameter	Results
1	MP	Methanol: Acetonitrile: Phosphate Buffer 70:20:10 v/v
2	Wavelength	221nm
3	Stationary Phase	RP- C18 Column
4	pH of MP	4.1
5	Flow Rate	1.1ml/min
6	Pump Mode	Isocratic
7	Pump Pressure	10.5±5MPa
8	Api Concentration	Azithromycin – 100µg/ml Ambroxol– 12.5µg/ml
9	RT	Ambroxol – 5.39min Azithromycin – 9.08min
10	Resolution	Ambroxol – Azithromycin – 12.08
11	Area	Azithromycin – 724792 Ambroxol -161385
12	Theoretical Plates	Ambroxol – 6163 Azithromycin – 11823
13	Tailing Factor	Ambroxol – 1.77 Azithromycin – 0.83

Table 2:- Linearity results for Ambroxol and Azithromycin

S. No	Ambroxol		Azithromycin	
	Concentration in µg/ml	Peak Area	Concentration in µg/ml	Peak Area
1	3	52734	20	344230
2	6	100977	50	527121
3	9	161385	75	724792
4	12	222886	100	896551
5	15	273135	125	1088169
6	18	337020	150	1273865
	Slope	19042	Slope	7218.4
	Intercept	- 8584.3	Intercept	183528
	r^2	0.9987	r^2	0.9988

Table 3: Recovery Results of Azithromycin

% of Recovery	Azithromycin					
	Target Conc., (µg/ml)	Spiked conc., (µg/ml)	Final Conc., (µg/ml)	Conc., Obtained	% of Assay	%RSD of recovery
50%	50	25	75	74.37	99.15	0.48
	50	25	75	73.70	98.27	
	50	25	75	74.25	99.00	
100%	50	50	100	98.95	98.95	0.27
	50	50	100	99.19	99.20	
	50	50	100	98.67	98.67	
150%	50	75	125	124.38	99.50	0.12
	50	75	125	124.65	99.72	
	50	75	125	124.60	99.68	

Table 4: Recovery Results of Ambroxol

% of Recovery	Ambroxol					
	Target Conc., (µg/ml)	Spiked conc., (µg/ml)	Final Conc., (µg/ml)	Conc., Obtained	% of Assay	%RSD of recovery
50%	6	3	9	8.98	99.83	0.43
	6	3	9	8.92	99.09	
	6	3	9	8.92	99.10	
100%	6	6	12	12.03	100.26	1.05
	6	6	12	11.86	98.85	
	6	6	12	11.79	98.23	
150%	6	9	15	14.71	98.10	1.29
	6	9	15	14.80	98.68	
	6	9	15	15.08	100.55	

Table 5: Ruggedness of Ambroxol & Azithromycin

S.NO	Ambroxol at 12.5µg/ml	Azithromycin at 100µg/ml
	Peak Area	Peak Area
1	159291	716970
2	161724	711323
3	160570	725445
4	160278	727969
5	163520	716574
6	162589	728197
	0.975	**0.980**

Table 7: LOD and LOQ

Test	Ambroxol	Azithromycin
LOD	0.30µg/ml	0.15µg/ml
LOQ	1.00µg/ml	0.70µg/ml

Table 6: Robustness

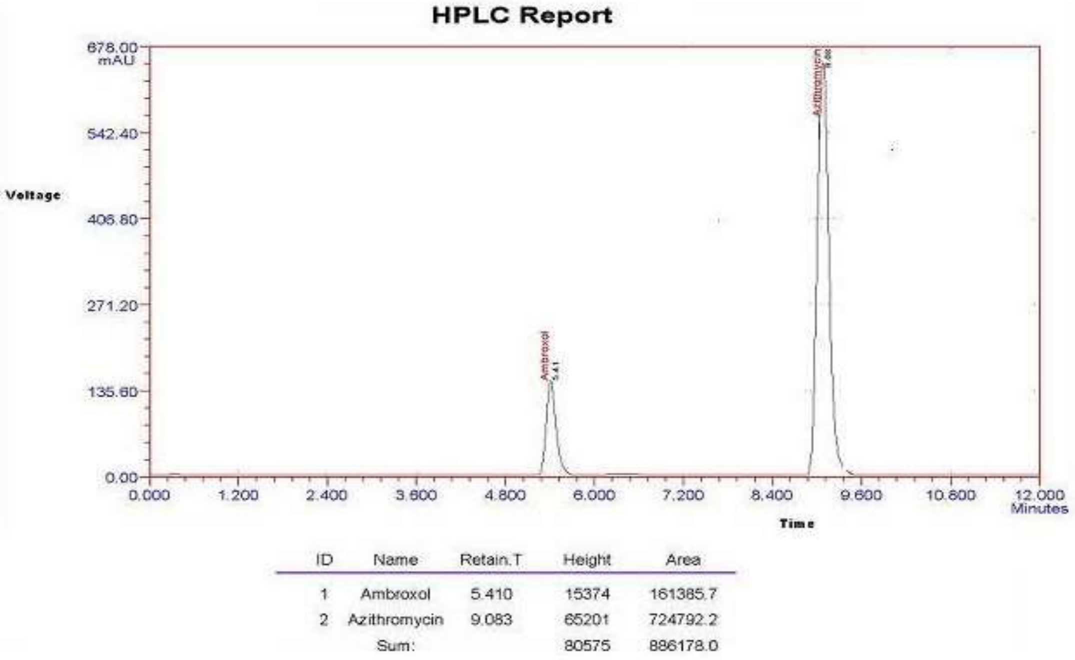
Drug Condition	Azithromycin		Ambroxol	
	Mean area	% Difference	Mean area	% Difference
Standard	724792		161385	
Mp Changes 1	711460	1.839	161562	0.110
Mp Changes 2	713223	1.596	162035	0.403
WL Changes 1	724729	0.0086	159950	0.890
WL Changes 2	712790	1.656	159092	1.421
pH Change 1	714335	1.443	159496	1.170
pH Change 2	722464	0.321	160215	0.725

Table 8: Analysis of a marketed formulation

S.NO	Drug	Brand	Dosage	Amount Prepared	Amount Found	%Assay
1	Ambroxol	AZIRIV-A	60mg	12µg/ml	11.92µg/ml	99.333
2	Azithromycin	AZIRIV-A	500mg	100µg/ml	99.17µg/ml	99.17

Table 9: Forced degradation results

S.NO	Condition	Number of additional peaks observed
1	Acidic	3
2	Base	3
3	Aqueous	0
4	Light	2
5	Peroxide	3
6	Thermal	2
7	UV Light	3



ID	Name	Retain.T	Height	Area
1	Ambroxol	5.410	15374	161385.7
2	Azithromycin	9.083	65201	724792.2
	Sum:		80575	886178.0

Figure 1: Chromatogram of Ambroxol and Azithromycin Formulation HPLC Report

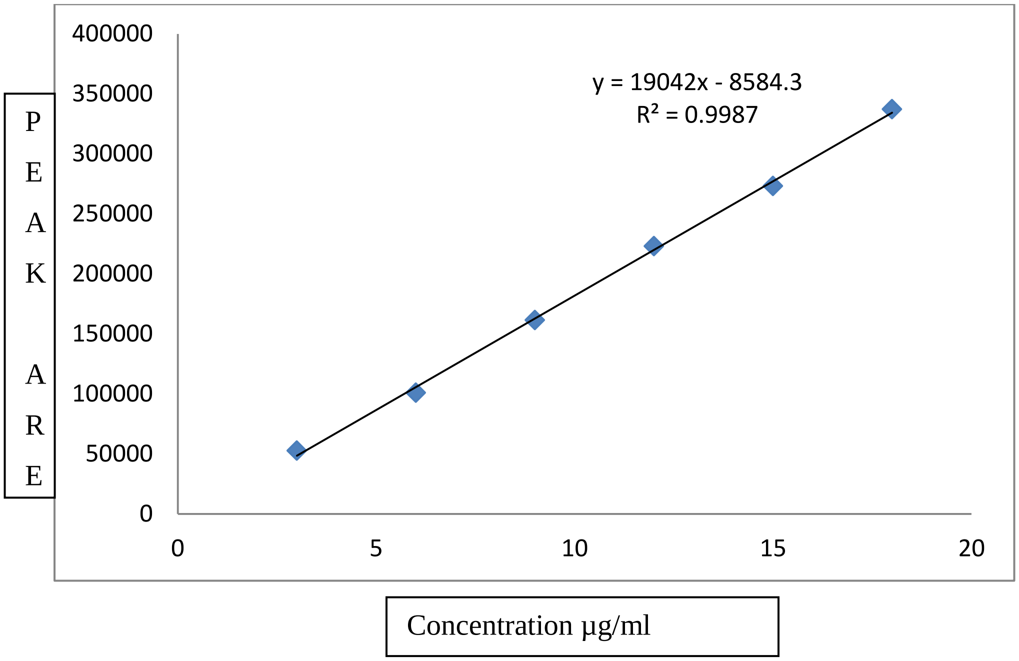
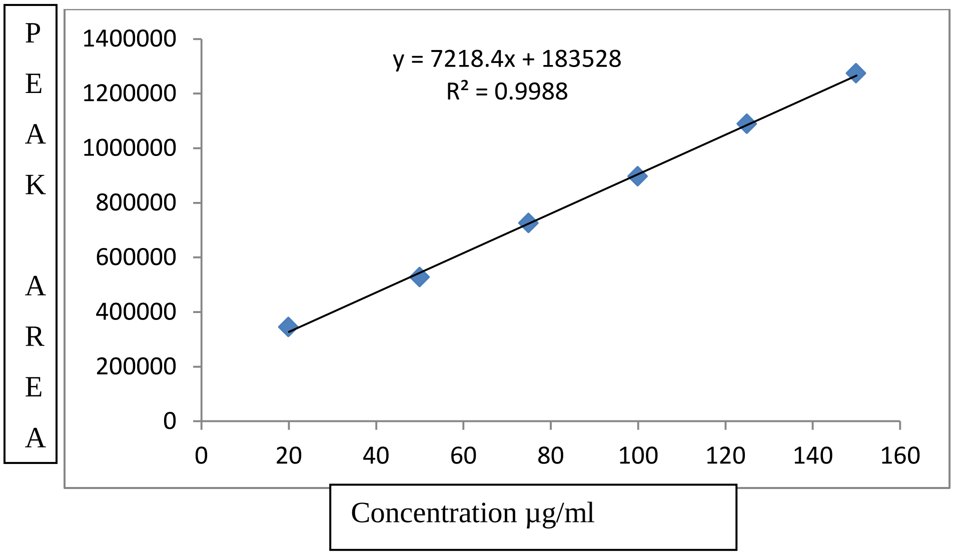


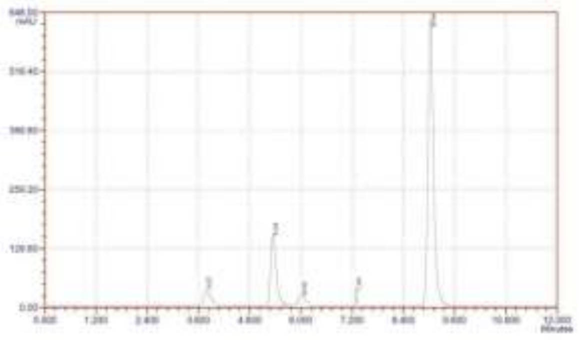
Figure2: Calibration Curves for Ambroxol and Azithromycin

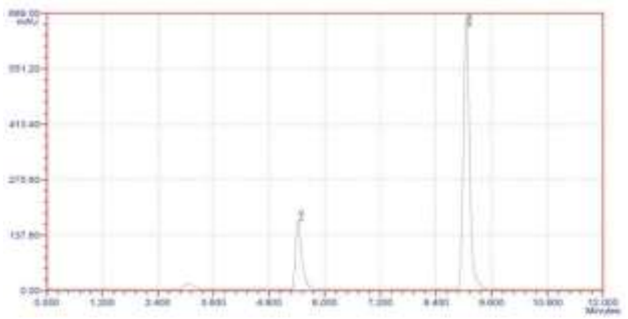
Calibration curve of Ambroxol



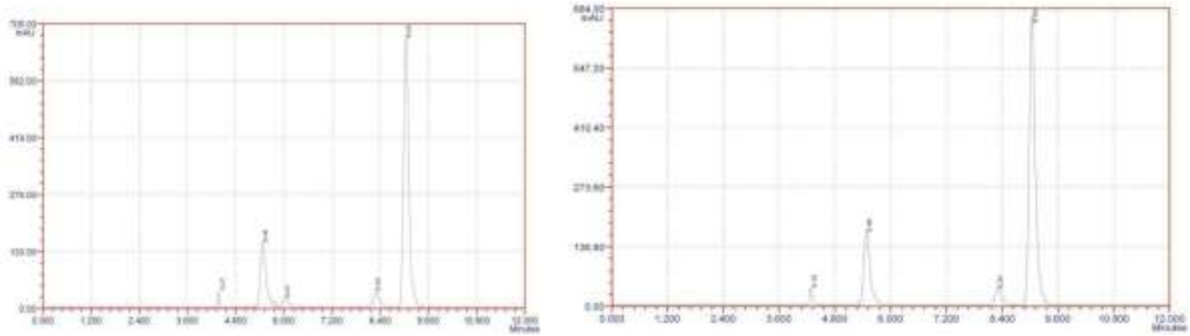
Calibration curve of Azithromycin

Acidic Aqueous

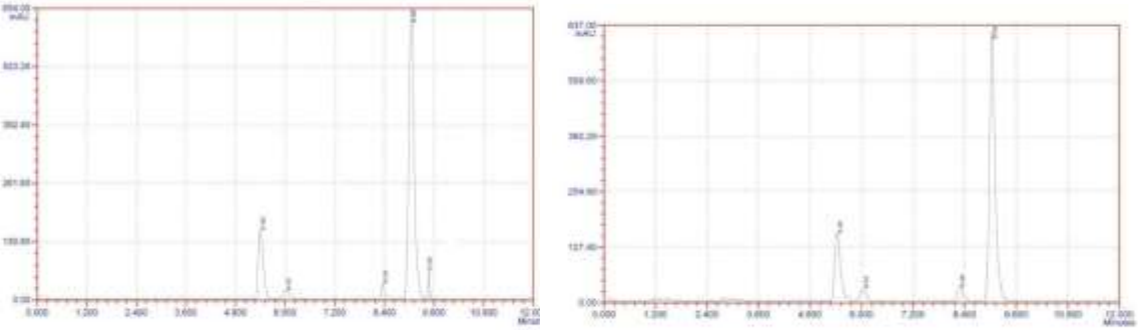




Base Light



Peroxide **Thermal**



UV

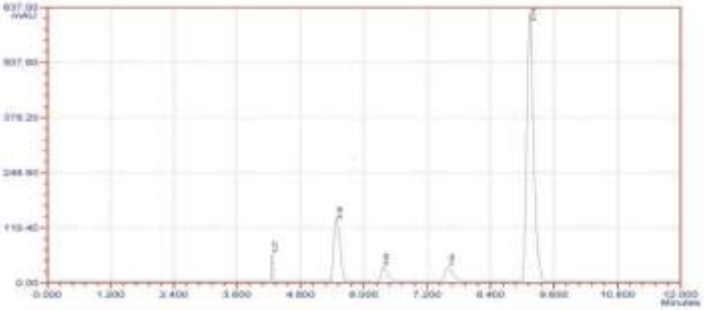


Figure 3: Degradation chromatograms of Ambroxol and Azithromycin

CONCLUSION

A Validated stability indication RP-HPLC method for determination of Azithromycin & Ambroxol HCL in marketed formulation was developed. The developed method is simple, accurate, precise and specific. It is suggested for routine analysis of Azithromycin & Ambroxol in its marketed formulation.

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