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RP-HPLC method development and validation for the estimation of Acetazolamide in bulk drug and formulations with forced degradation studies

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ABSTRACT

This work has been done with a motto to develop a simple, accurate, precise, reproducible and economic reverse phase HPLC method for Acetazolamide in bulk drug as well as in formulations. in the current developed method C8H column (250x4.6mm) was used as stationary phase and potassium dihydrogen phosphate buffer (pH 3), Acetonitrile & water in a ratio 30:20:50 as mobile phase. ICH guidelines were followed for method validation and different parameters studied include linearity range, system suitability, specificity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), Robustness and Solution Stability. Forced degradation studies were also done by exposing the drug to different stress conditions (Photolytic, thermal, acidic, alkaline, & Oxidative). The developed validated method was also utilized to quantify the Acetazolamide in the marketed formulation successfully. The method was found linear within the range of 20-120µg/mL with LOD of 37.60ng/mL & LOQ of 0.11396µg/mL whereas recovery was found within the range of 99.98% -100.77%. Method was found to be able to distinguish the parent drug from degraded products & quantify Acetazolamide in Dosage form (101.14%). A linear, robust & economic RP-HPLC method was developed with LOD & LOQ within the good range which can be adopted for the routine analysis of Acetazolamide in bulk drugs & formulations.

Key word: Acetazolamide, HPLC, validation, stability.

INTRODUCTION

Acetazolamide (ACZ), a carbonic anhydrase inhibitor (CAI), till now is used orally for the reduction of intraocular pressure (IOP) in patients suffering from glaucoma. It is used in the pre-operative management of closed angle glaucoma or as an adjunct therapy in the treatment of open angle glaucoma [1]

Literature shows few reported method for the estimation of Acetazolamide in bulk as well as in the dosage form. But still there is a need to develop a more precise, accurate & economic method. In the present study an economic method has been developed by keeping the organic solvent at the minimum possible level which will finally reduce the cost of the method.

MATERIALS AND METHODS

Chemicals:
All the chemicals used were of AR grade. Solvents used are of HPLC grade and purchased from MERCK. The water used was distilled and deionised by using Millipore (ELIX) system.

1. INSTRUMENTATION:
Shimadzu LC-2010C HT system was used which is equipped with quaternary pump, auto sampler unit, online degasser, column oven and PDA detector (SPD-M20A). All the data was processed and monitored at LC SOLUTION software provided by Shimadzu.

2. CHROMATOGRAPHIC CONDITIONS
C8 column (250x4.6mm & 5µm particles) (Spincotech Pvt. ltd) was used as stationary phase. The mobile phase composed of three different components in the ratio 30:20:50 which are 10mM potassium dihydrogen phosphate buffer (pH 3- adjusted with O-phosphoric acid), Acetonitrile and water. The flow rate was optimized at 0.8mL/minute and the injection volume was kept 20µl with a run time of 10 minutes. The chromatograms were recorded at 265nm and column temperature was maintained at 25^0C throughout the study period. Different samples prepared as well as mobile phase were filtered using 0.22µm filter and degassed by ultrasonication (Metrex) prior to use.

3. PREPARATION OF STANDARD STOCK SOLUTION
A stock solution of Acetazolamide of concentration 1000µg/mL was prepared by dissolving 10 mg of Acetazolamide in 10mL of Millipore water in a volumetric flask. Thereafter solutions of different concentration (20-120µg/mL) were prepared by diluting the stock solution.

4. METHOD VALIDATION
Different parameters i.e. linearity, range, system suitability, specificity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), Robustness and Solution Stability were studied for the validation of the developed method & method was validated as per the ICH guidelines. [2]

6.1. LINEARITY & RANGE
Stock solution of concentration 1000µg/mL was diluted to get the different concentrations 20 µg/mL, 40 µg/mL, 60 µg/mL, 80 µg/mL, 100 µg/mL and 120 µg/mL and 20µl of each concentration was then injected using auto sampler unit and the chromatograms were recorded. A graph of Area under curve vs. concentration was then plotted to get Calibration curve. The equation, slope, intercept and regression coefficient (R^2) were determined.

6.2. PRECISION
Three different concentrations were selected for Interday & intraday precision studies which are 20 µg/mL, 60 µg/mL and 100 µg/mL. Three responses of each concentration were recorded by injecting 20 µl of sample on a single day for intraday whereas for interday on three different days.

6.3 ACCURACY
In a prequantified solution of drug a known amount of drug was added & chromatogram was recorded as per the optimized chromatographic conditions. The percentage recovery was then calculated by fitting the area of the sample in the calibration curve equation. [4]

6.4. LIMIT OF DETECTION (LOD) & LIMIT OF QUANTIFICATION (LOQ)
As per ICH guidelines following equations were used to determine the LOD & LOQ
$LOD = 3.3 \times \sigma / S$
$LOQ = 10 \times \sigma / S$
Where σ is standard deviation of y intercepts & S is the slope regression line of calibration curve. [4]

6.5. ROBUSTNESS
Deliberate changes were made in the optimized working parameters of the developed method. Changes were made in temperature conditions, wavelength & instrument. Retention time, plate count and peak asymmetry parameters were observed. [5]

6.6. ANALYSIS OF MARKETED FORMULATION
A marketed formulation of Acetazolamide (Diamox) was procured from local pharmacy. The tablet was crushed & it was diluted to get a solution equivalent to 100µg/mL of Acetazolamide and was analyzed using the optimized chromatographic conditions.

The chromatogram was also observed for the unwanted peaks due to excipients present in the formulation, at the optimized RT, to confirm the specificity of the method

6.7. SYSTEM SUITABILITY
It was determined by giving the six injections of the same concentration (20µg/mL) applying the chromatographic conditions of the proposed method. Thereafter %RSD of retention time, peak area, peak asymmetry, and theoretical plate were calculated.

6.8. SOLUTION STABILITY
For solution stability chromatograms of the same concentration were recorded at different time intervals (3, 9, 12, and 24 hours) & changes in RT, peak area, peak asymmetry & theoretical plates were observed.

5. FORCE DEGRADATION STUDIES:
7.1. ALKALINE & ACIDIC DEGRADATION STUDIES: 1mL solution of drug (1mg/mL) was treated with 9mL of 0.1 N Sodium hydroxide/ 0.1N HCL & refluxed on a boiling water bath for 2 hours. The solution was then cooled to room temperature & neutralized to ph 7. This solution was then properly diluted to get a solution of 20µg/mL and analyzed by proposed method.

7.2. OXIDATIVE DEGRADATION STUDIES 1mL solution of drug (1mg/mL) was treated with 9mL of 30% Hydrogen peroxide & refluxed on a boiling water bath for 2 hours. This solution was then properly diluted to get a solution of 20µg/mL and analyzed by optimized method.

7.3. THERMAL DEGRADATION STUDIES: 10 mg of drug was stored at 55°C for 3 h in oven separately. The drug was then properly diluted to reach a final concentration of 20 µg/mL. The chromatograms were run by injecting the sample in the column.

7.4. PHOTOLYTIC DEGRADATION STUDIES: 10 mg of drug was dissolved in 10 mL of Millipore water. The solutions were kept in the sun light for 8 h. The drug was then properly diluted to reach a final concentration of 20 µg/mL & analyzed by the optimized HPLC method. [4]

RESULTS AND DISCUSSION

8.1 LINEARITY & RANGE
The proposed method was found linear in the range of 20µg/mL-120µg/mL with a regression coefficient of 1.0000 and the regression line was having a slope of 63,041.76 and y-intercept 43,862.87 (equation y = 63,041.76x + 43,862.87) [Table 1][Figure 1(i), 1(ii)]

Table 1 Linearity & Range parameters

S.No.	Parameter	Result
1	Linearity (range) (µg/mL)	20-120
2	Retention time (min)	6.8
3	Regression coefficient (r^2)	1.0000
4	Slope	63,041.76
5	Intercept	43,862.87
6	Equation	y = 63,041.76x + 43,862.87

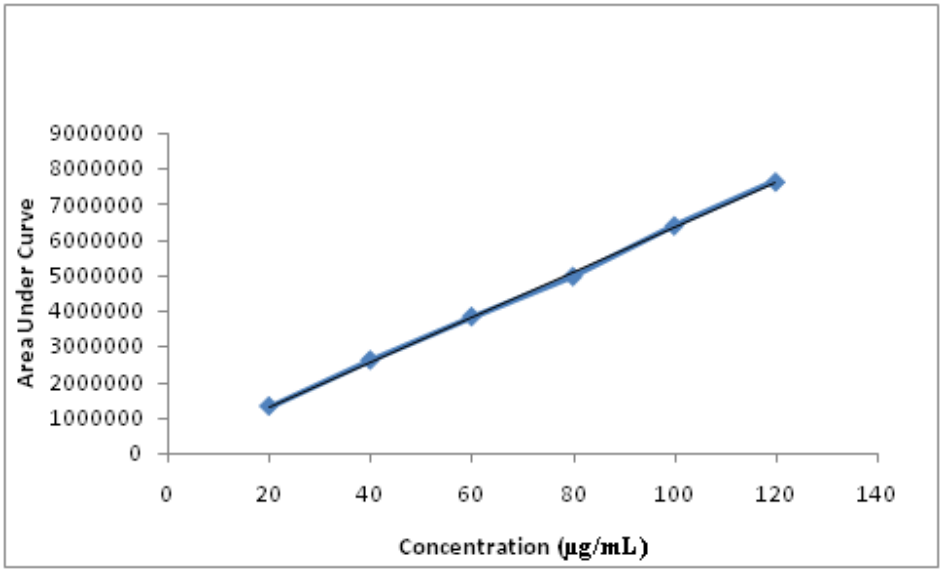

Figure 1(i) standard plot of Acetazolamide

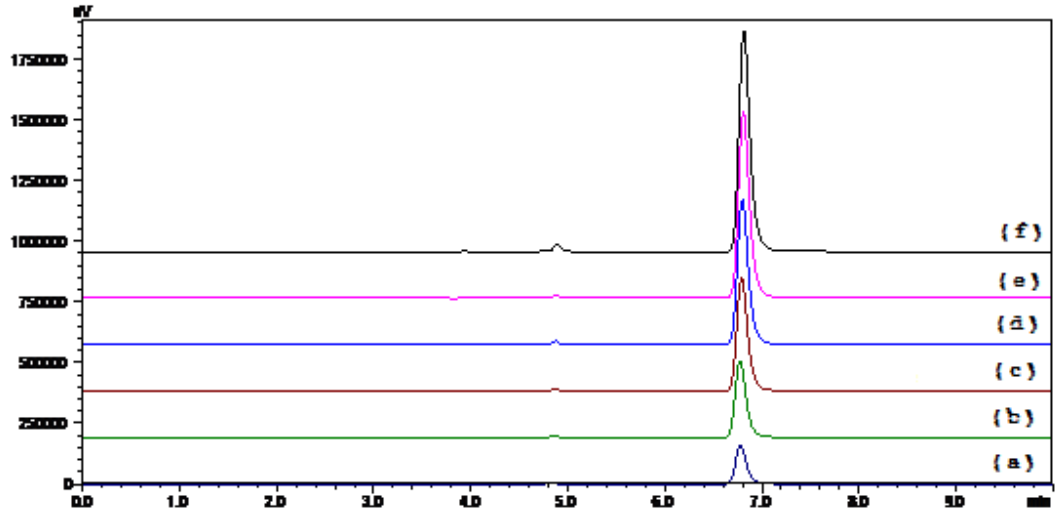

Figure 1 (ii) Overlay chromatogram of Acetazolamide [(a) 20µg/mL (b) 40 µg/mL (c) 60 µg/mL (d) 80µg/mL (e) 100µg/mL (f)120 µg/mL]

8.2 PRECISION

In precision studies the area values were obtained for both intraday & interday precision. The % RSD of three concentrations viz. 20 µg/mL, 60 µg/mL & 100 µg/mL, for intraday were 0.030006, 0.001195 & 0.018035while it was 0.761414, 1.494905 & 0.068324 for interday studies. All the three concentrations were found with no significant change as the values of % RSD was within the limit. (<2%) (Table 2)

Table 2 intraday & Interday Precision parameters

Intraday				Interday		
Conc. (µg/mL)	**Mean Area**	**SD**	**%RSD**	**Mean Area**	**SD**	**%RSD**
20	1306528	392.0387	0.030006	1306867	9950.67	0.761414
60	3835340	45.82576	0.001195	3800968	56820.88	1.494905
100	6392927	1152.968	0.018035	6395097	4369.384	0.068324

8.3 ACCURACY

In this study 70%, 100% & 130% of 10 µg/mL solution was added to the 10 µg/mL & analyzed by the proposed method & the % recoveries for these three added concentrations, found were 100.77%, 100.16% and 99.981% respectively.

The method was found accurate as good recoveries (99.98% -100.77%) were obtained for various added concentrations (table 3)

Table 3 Accuracy parameter of validation for three different concentrations

Percentage	Amount added (µg/mL)	Total drug (µg/mL)	Drug found (µg/mL)	Amount recovered (µg/mL)	% Recovery	SD	%RSD
70%	7	17	17.05425	7.054255	100.7751	6938.328	0.620049
100%	10	20	20.01682	10.01682	100.1682	3514.777	0.269174
130%	13	23	22.99754	12.99754	99.98109	25067.95	1.678274

8.4 LIMIT OF DETECTION (LOD) & LIMIT OF QUANTIFICATION (LOQ)

The limit of detection was found to be 37.60ng/mL while the value obtained for limit of quantification was found to be 0.11396µg/mL.

The limit of detection was found 37.60ng/mL which shows that quantities in nanograms can also be determined by the proposed method whereas limit of quantification was found to be 00.11396µg/mL. (Table 4)

Table 4 Limit of Detection (LOD) & Limit Of Quantification (LOQ)

LOD	37.60ng/mL
LOQ	0.11396µg/mL

8.5 ROBUSTNESS

8.5.1. Wavelength (± 2nm): Deliberate changes were made in wavelength (± 2nm) & sample was analyzed at 263nm & 267nm [Figure 2(i) & figure 2(ii)]. The values obtained for sample analyzed at 263nm for retention time (RT), Plate count (PC) & peak asymmetry (PA) were found 6.869, 13726.83, 1.376 respectively while the results for sample analyzed at 267nm were found 6.869, 13721.04 & 1.377 respectively.

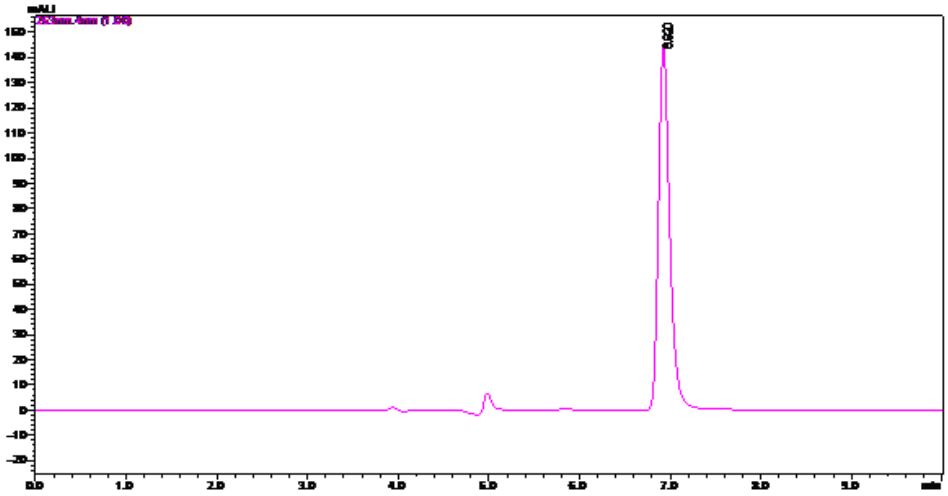

Figure 2(i) Chromatogram at 263nm

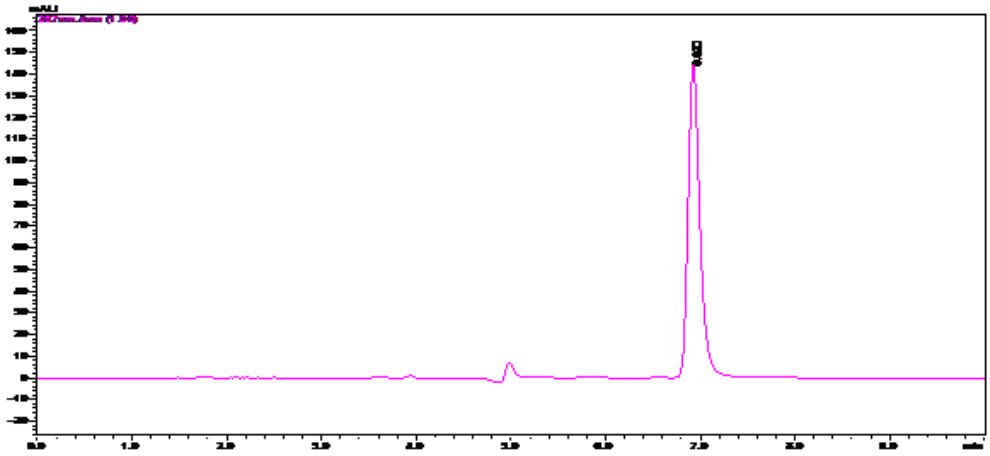

Figure 2(ii) Chromatogram at 267nm

8.5.2. Temperature (± 1 °C): The current method was optimized at 25°C and to study temperature robustness samples were analyzed at 24 °C & 26 °C and results obtained for both temperatures for RT, PC & PA were found to be 6.722, 15429.12, 1.449 & 6.769, 15562.5, 1.446 respectively.

8.5.3. Instrument alteration: The chromatographic conditions optimized at HPLC system Shimadzu LC2010HT (auto sampler unit) were reproduced on the Shimadzu LC20AD (manual sampling unit) with PDA (SPD M20A) & Fluorescence (RF 20A) detector & the results for RT, PC, PA were 6.752, 14150.78 & 1.393 respectively. Deliberate changes were made in wavelength (± 2nm), temperature (± 1 °C) & instrument and it was found that all the parameters were within the prescribed limit i.e. plate count was found >4000 and tailing factor was found <2. (Table 5)

Table 5 Wavelength, Temperature & Instrument Parameters for Robustness Studies

Parameter	Optimized	Used	Retention Time (RT), Min	Plate Count	Peak Asymmetry
Wavelength (± 2nm)	265nm	263	6.869	13726.83	1.376
		267	6.869	13721.04	1.377
Temperature (± 1 °C)	25°C	24 °C	6.722	15429.12	1.449
		26 °C	6.769	15562.5	1.446
Instrument	LC2010HT	LC20AD	6.752	14150.78	1.393

8.6 SOLUTION STABILITY

100µg/mL concentration solution was used for solution stability studies & the mean area was found to be 6382675 with % RSD 0.284309.

100µg/mL concentration solution was used for solution stability studies and the solution was found stable as no significant change was observed in the peak area (RSD <2%) (Table 6)

Table 6 Solution Stability parameter

Conc. Used(µg/mL)	Mean area	SD	%RSD
100	6382675	18146.5	0.284309

8.7 SYSTEM SUITABILITY:

For system suitability six injections of 20µg/mL concentration were injected and responses were observed for Rt, peak area, peak asymmetry & theoretical plates, the values for which are 6.8165, 1305975, 1.32, 15500.67 respectively & %RSD for all these parameters were found to be 1.766408, 0.055015, 11.742095, 1.286925 respectively.

For system suitability six injections of 20µg/mL concentration were injected and responses were observed for RT, peak area & peak asymmetry and all were found within the limit. (RSD <2%) (Table 7)

Table 7 System Suitability parameters

Parameter	MEAN (n=6)	SD	% RSD
Rt	6.8165	0.120407	1.766408
Peak area	1305975	718.4769	0.055015
Peak asymmetry	1.32	0.022996	1.742095
Theoretical Plates	15500.67	199.4819	1.286925

8.8 ANALYSIS OF MARKETED FORMULATION

Solution of marketed formulation (concentration 100µg/mL) was injected at the optimized chromatographic conditions and 101.1428% recovery was obtained. (Table 8)(Figure 3)

Table 8 Analysis of Marketed Formulation

Conc.(µg/mL)	Area (n=3)	Conc. Recovered (µg/mL)	% Recovery	SD	%RSD
100	6420110	101.1428	101.1428	100000	1.557606

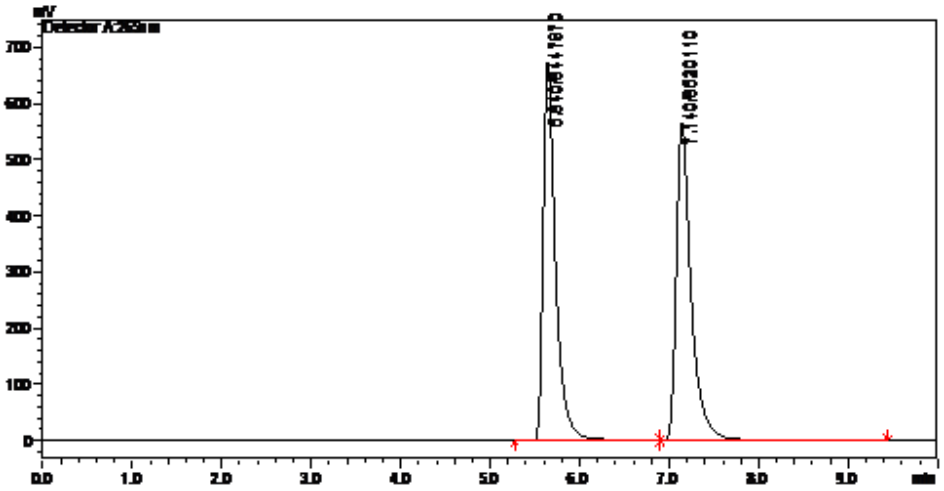

Figure 3 Chromatogram of marketed formulation with optimized chromatographic conditions

8.9 FORCED DEGARADATION STUDIES: An ideal stability indicating method is the one which is able to differentiate the degradation products & at the same time must be able to quantify the drug. In the present study the drug was forced to degrade under oxidative, acidic, alkaline, photolytic & thermal stress condition. After the forced degradation when drug was analyzed by the optimized method it was observed that the there was no degradation during thermal & photolytic stress conditions. On the other hand in case of Acidic & alkaline stress conditions peaks of 7 degradation products at different retention times along with the peak of API at 6.8 were found. Also under oxidative stress conditions there was 3 degradation products peaks & the chromatogram showed the extra peak of the hydrogen peroxide along with the peak of parent drug. (Figure no. 4)

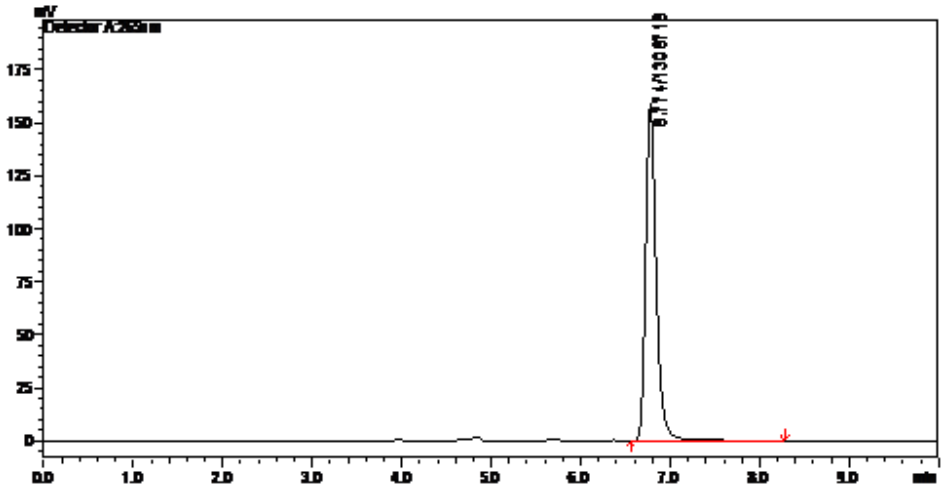

Figure 4 (i) Chromatogram after thermal degradation

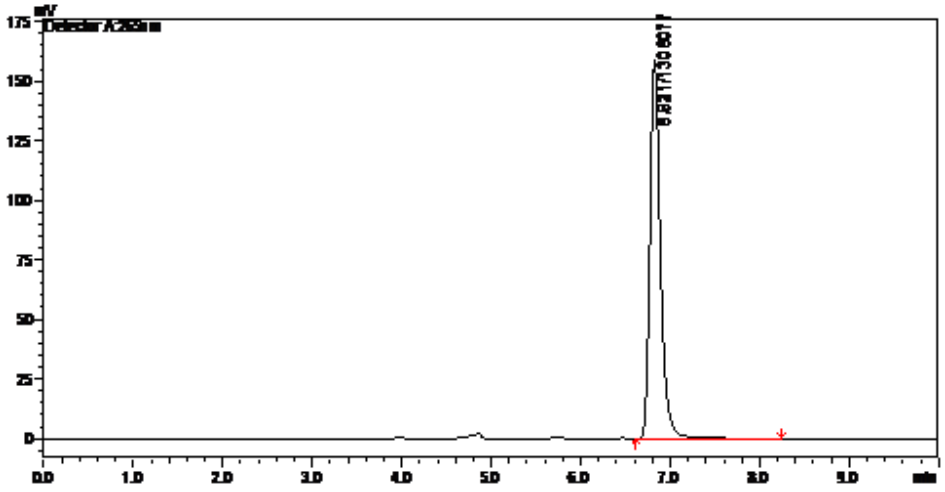

Figure 4 (ii) Chromatogram after photolytic degradation

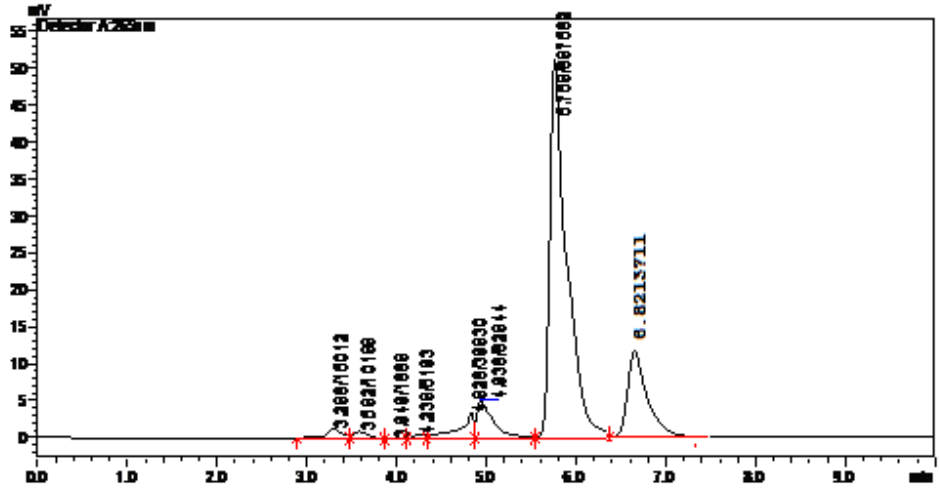

Figure 4 (iii) Chromatogram after acidic degradation

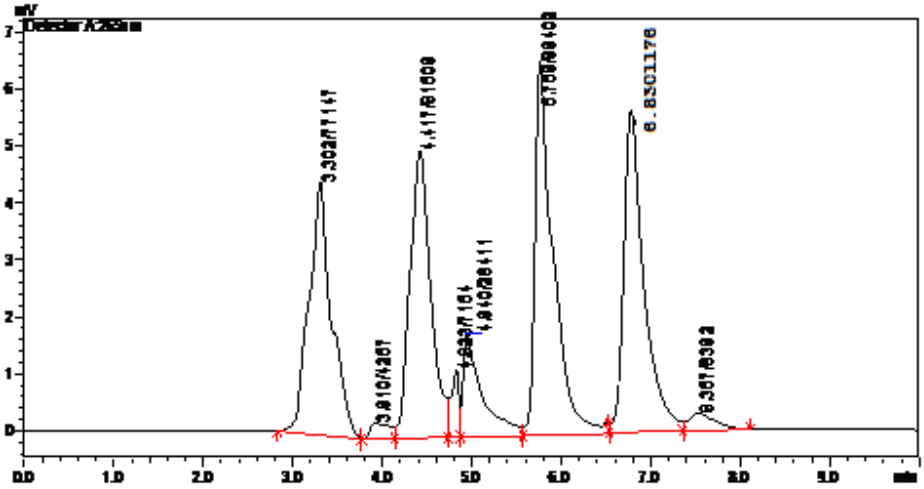

Figure 4 (iv) Chromatogram after alkaline degradation

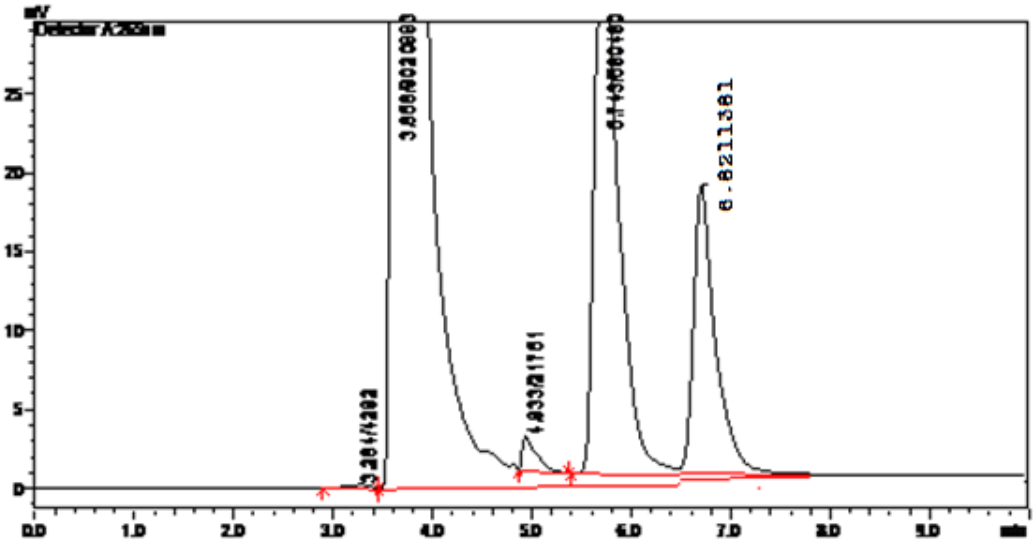

Figure 4 (v) Chromatogram after oxidative degradation

CONCLUSION

In the present study a RP-HPLC method has been described for the quantitative determination of Acetazolamide in bulk drug as well as in the formulation. The method has been validated for different parameters like linearity range, system suitability, specificity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), Robustness and Solution Stability and the results obtained were found statistically significant. Also when the drug was exposed to stress conditions (photolytic, thermal, acidic, alkaline & oxidative) no significant changes were observed in the chromatograms of the drug except the additional peaks of the degradation products. This method is very economic due to very less usage of organic content in the mobile phase and hence recommended for the routine quantitative analysis in the pharmaceutical industries.

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