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STABILITY INDICATING RP-HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF ASCARBIC ACID AND N-ACETYL CYSTENE IN BULK AND PHARMACEUTICAL FORMULATION

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ABSTRACT

The present study was designed to develop and validate a simple, sensitive, precise and accurate stability indicating RP-HPLC method for simultaneous estimation of Ascorbic acid and N-Acetylcysteine in bulk and tablet dosage form. The chromatographic separation was achieved on Denali C18 (4.6 x 250mm, 5µm) as stationary phase with a mobile phase of 0.1% OPA: acetonitrile (50:50 v/v) at a flow rate of 1 ml/min and PDA detection at 220 nm. The proposed method was validated for system suitability, specificity, linearity, accuracy, precision, LOD, LOQ and robustness as per ICH guidelines. The retention times of Ascorbic acid and N-Acetylcysteine were found to be 2.587 ± 0.06 and 3.254 ±0.05 min respectively. The calibration curves were linear in the concentration range of 25% to 150% of the working concentration (r^2=0.999) for both the drugs in binary mixture. The accuracy was found to be 99.45% and 99.31% for Ascorbic acid and N-Acetylcysteine respectively. The LOD was found to be 0.51 µg/ml and 0.24 µg/ml and LOQ was found to be 1.55 µg/ml and 0.73 µg/ml for Ascorbic acid and N-Acetylcysteine respectively. The percentage recoveries for both drugs were in the range of 99-100%. Hence the proposed stability indicating RP-HPLC ethod can be used in routine analysis of tablets containing Ascorbic acid and N-Acetylcysteine.

KEYWORDS: Ascorbic acid, N-Acetylcysteine, RP-HPLC, stability, Method development and Validation.

INTRODUCTION

Ascorbic acid is chemically known as (5R)-5-[(1S)-1,2-dihydroxyethyl]-3,4-dihydroxy-2,5-dihydrofuran-2-one (Fig. 1). It was used to treat vitamin C deficiency, scurvy, delayed wound and bone healing, urine acidification, and in general as an antioxidant. It has also been suggested to be an effective antiviral agent.

N-Acetyl cysteine is chemically known as (2R)-2-acetamido-3-sulfanylpropanoic acid (Fig. 2). It is a pharmaceutical drug and nutritional supplement used primarily as a mucolytic agent and in the management of Paracetamol (acetaminophen) overdose. Other uses include sulfate repletion in conditions, such as autism, where cysteine and related sulfur amino acids may be depleted.[8]

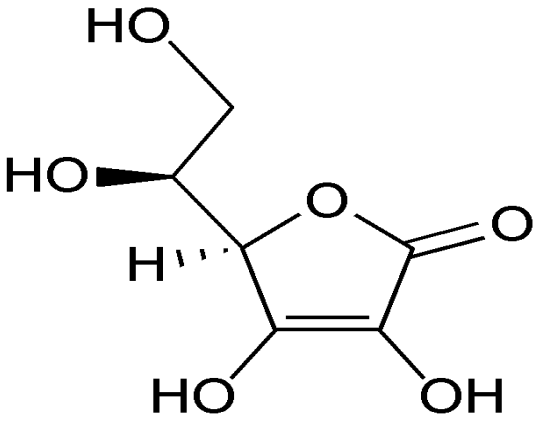

Fig. 1: Structure of Ascorbic acid.

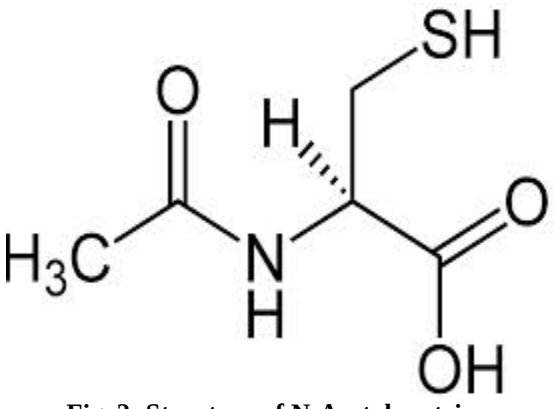

Fig. 2: Structure of N-Acetyl cysteine.

Extensive literature survey revealed that there were few analytical methods for the estimation of specified drugs with other combinations.[1-7] There was no stability indicating reverse phase high performance liquid chromatography (RP-HPLC) method reported for the estimation of Ascorbic acid and N-Acetyl cysteine. Hence we planned to develop a simple stability indicating analytical method for simultaneous estimation of Ascorbic acid and N-Acetyl cysteine in bulk and pharmaceutical preparations.

MATERIALS AND METHODS

MATERIALS

Ascorbic acid and N-Acetylcysteine pure drugs (API), received from spectrum labs, Hyderabad as a gift sample. Combination Ascorbic Acid and N-Acetylcysteine tablets were obtained commercially. Distilled water, Acetonitrile, Ortho-phosphoric acid. All the above chemicals and solvents were procured from Rankem.

METHODS

Diluent

Water : acetonitrile has taken in the ratio 50:50% v/v.

Buffer 0.1%OPA

Accurately 1ml of OPA in a 1000ml of volumetric flask add about 900ml of milli-Q water added and degas to sonicate and finally make up the volume with water.

Preparation of Standard stock solutions

Accurately weighed 16.25mg of N-Acetylcysteine, 125mg of Ascorbic acid and transferred to 50ml volumetric flasks and 3/4 th of diluents was added to these flask and sonicated for 10 minutes. Flask was made up with diluents and labeled as Standard stock solution. (325µg/ml of N-Acetylcysteine and 2500µg/ml Ascorbic acid).

Preparation of Standard working solutions (100% solution)

1ml from stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (32.5µg/ml of N-Acetylcysteine and 250µg/ml of Ascorbic acid)

Preparation of Sample stock solutions

20 tablets were weighed and the average weight of each tablet was calculated, then the weight equivalent to 1 tablet was transferred into a 100 ml volumetric flask, 50ml of diluents was added and sonicated for 25 min, further the volume was made up with diluent and filtered by HPLC filters (325µg/ml of N-Acetylcysteine and 2500µg/ml of Ascorbic acid)

Preparation of Sample working solutions (100% solution)

1ml of filtered sample stock solution was transferred to 10ml volumetric flask and made up with diluent. (32.5µg/ml of N-Acetylcysteine and 2500 µg/ml of Ascorbic acid).

Chromatographic conditions

The chromatographic conditions were performed on Denali C18 (4.6 x 250mm, 5µm particle size) at 30^0C. The samples were eluted with 0.1% ortho phosphoric acid and acetonitrile (50:50 v/v) as the mobile phase. The measurements were carried out with an injection volume of 10 µl, the flow rate was set to 1 ml/min at a detection wavelength 220nm by using PDA detector.

RESULTS AND DISCUSSION

Method development

A series of trails was conducted with different columns with different mobile phase ratios to develop a suitable RP-HPLC method for estimation of Ascorbic acid and N-Acetylcysteine in bulk and tablet dosage form. Denali C_{18} column was found to be satisfactory for better separation and good resolution, analytes were checked with PDA detector at 220nm was considered satisfactory for detecting both the drugs with adequate sensitivity. A typical RP-HPLC chromatogram for simultaneous determination of Ascorbic acid and N-Acetylcysteine from standard preparation and from pharmaceutical formulation was shown in (Fig 3 and 4).

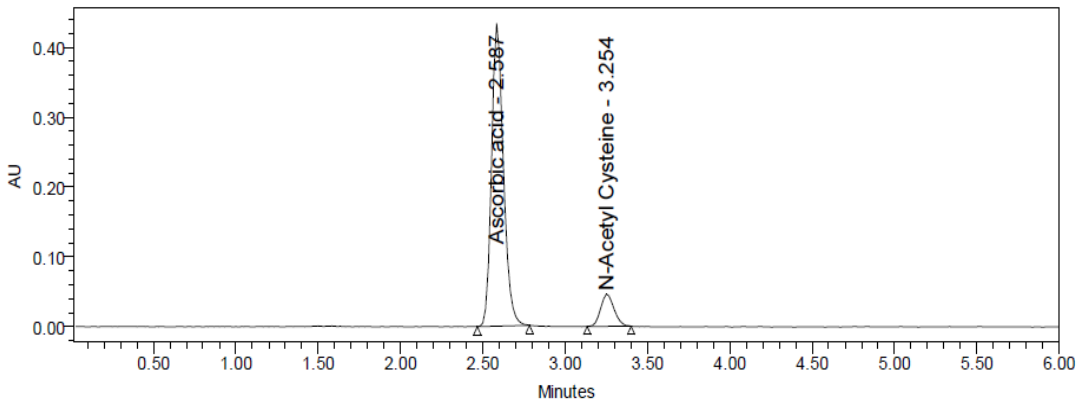

Fig. 3: Chromatogram of Ascorbic acid and N-Acetylcysteine in standard preparation.

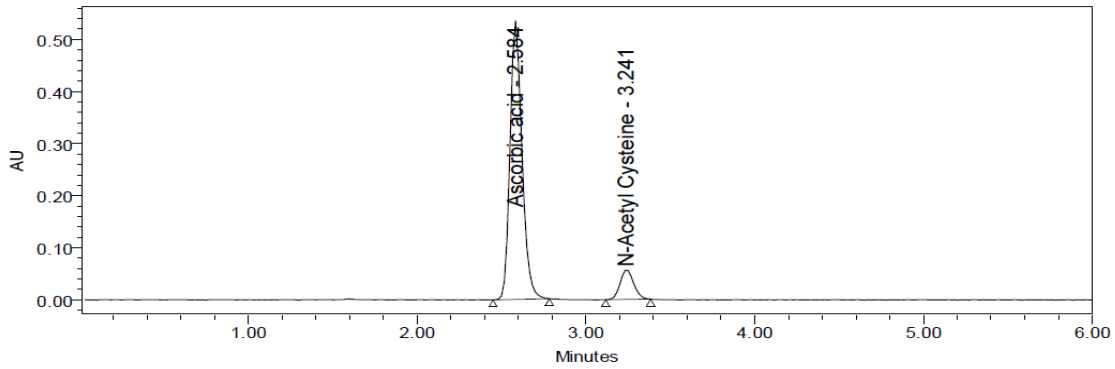

Fig. 4: Chromatogram of Ascorbic acid and N-Acetylcysteine in sample preparation.

Method validation

The objective of the method validation is to demonstrate that the method is suitable for its intended purpose as it is stated in ICH guidelines.[9] The developed RP-HPLC method was validated for parameters like system suitability, specificity, linearity, accuracy, precision, LOD, LOQ and robustness.

System suitability

To ensure the system suitability parameters, the standard solutions were prepared as per the test method and injected into the chromatographic system. The parameters such as theoretical plates, resolution and asymmetric factor were evaluated. The system suitability parameters were tabulated in Table 1. All the parameters were found to be within the limits.

Table 1: Results of system suitability.

Analytes	Retention times	Resolution	Theoretical Plates	Tailing Factor
Ascorbic acid	2.581± 0.03 min	-	6024	1.18
N-Acetylcysteine	3.077± 0.05 min	5.9	7447	1.10

Specificity

To check the specificity of the developed analytical method, blank and placebo injections were prepared as per the test method and injected into the chromatographic system. The chromatograms of blank and sample was shown in (Fig. 5 and 6). From the results it was found that there were no interfering peaks at retention times of analytes. Hence the results manifest that the developed method was said to be specific for the estimation for the estimation of Ascorbic acid and N-Acetylcysteine.

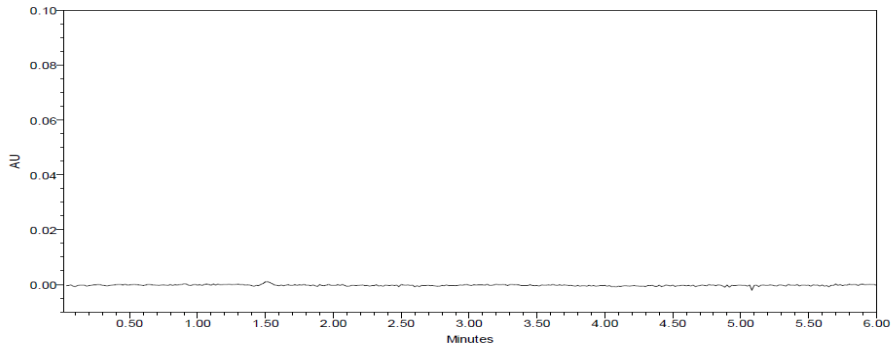

Fig. 5: Chromatogram of blank.

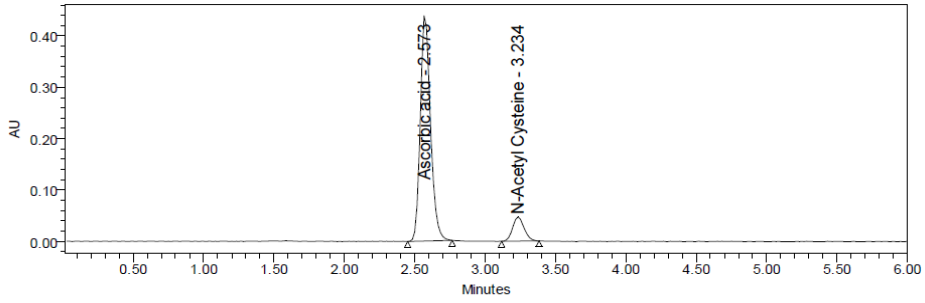

Fig. 6: Chromatogram of sample.

Precision

To ensure the precision of the analytical method by method precision studies. The sample solution was prepared at six working concentration level and analysis was carried out at replicates. The sample solutions of Ascorbic acid and N-Acetylcysteine were prepared as per the test method and injected six times into the column. The results of precision were tabulated in Table 2. RSD values was calculated and reported. The obtained RSD values are found within the limits, indicating the developed method was said to be precise.

Table 2: Results of precision

N*	Ascorbic acid		N- Acetylcysteine	
	Rt (min)	Peak area	Rt (min)	Peak area
1	2.587	2184562	3.254	257832
2	2.584	2210490	3.243	265091
3	2.573	2182022	3.234	260671
4	2.596	2187403	3.258	262119
5	2.570	2170328	3.232	259753
6	2.584	2188379	3.247	262033
Mean		2187197		261250
SD		13132.3		2465.0
RSD (%)		0.6		0.9

* number of replicates=6, SD= standard deviation and RSD= relative standard deviation.

Linearity

To ensure the linearity of the test solutions for the assay method was prepared from Ascorbic acid and N-Acetylcysteine standard stock solutions at five concentration levels from 50% to 150% of assay concentrations. The peak area versus concentration data was treated by least-square linear regression analysis. The calibration curves were plotted for Ascorbic acid and N-Acetylcysteine was shown in (Fig. 7 and 8). The results were tabulated in Table 3 have shown an excellent correlation between peak areas and concentration range of 62.25-375 µg/ml for Ascorbic acid and 8.125 – 48.75 µg/ml for N-Acetylcysteine. The correlation coefficients were found to be 0.999 for both the drugs, which meet the method validation acceptance criteria and hence the method was said to be linear at the specified concentration range for the mentioned drugs.

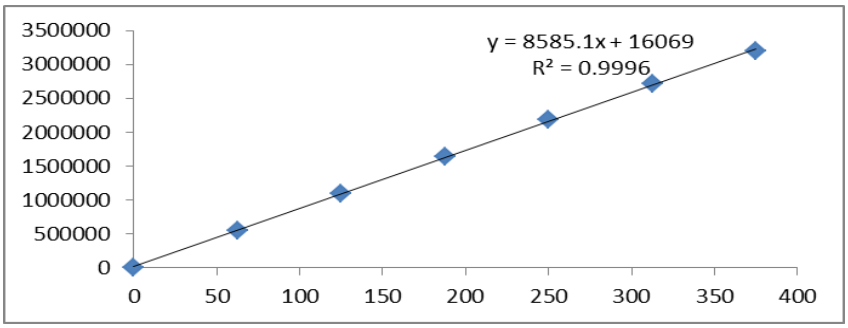

Fig. 7: Linearity chart of Ascorbic acid

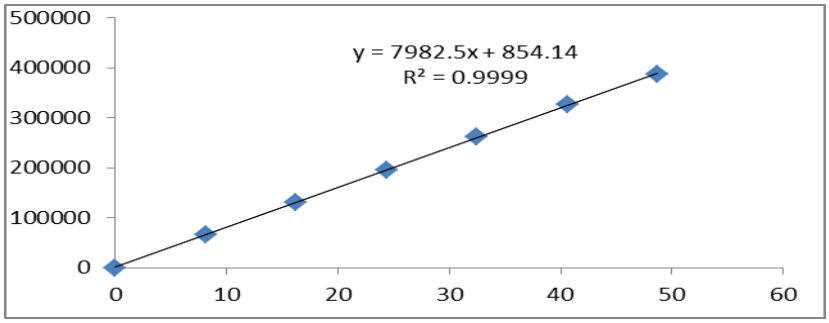

Fig. 8: Linearity chart of N-Acetylcysteine.

Table 3: Results of linearity.

Analytes	Correlation Coefficients (r^2)
Ascorbic acid	0.999
N-Acetylcysteine	0.999

Accuracy

To ensure the inerrancy and accuracy of the method recovery studies were carried out by standard addition method. A known quantity of pure drug corresponding to the specified level of label claim was added to the pre-analysing sample and the contents were reanalyzed by the proposed method and the percentage recovery was reported. The results were given in Table 4 and 5. The recoveries of the drugs were found to be within the specified range of 98-101%, hence concluding that the method was accurate for the determination of Ascorbic acid and N-Acetylcysteine.

Table 4: Results of accuracy of Ascorbic acid.

% Level	Amount Spiked (µg/ml)	Amount Recovered (µg/ml)	% Recovery	% Mean Recovery
50	125	123.45	98.76	
100	250	249.60	99.84	99.45
150	375	374.04	99.73	

Table 5: Results of accuracy of N-Acetyl Cysteine.

% Level	Amount Spiked (µg/ml)	Amount Recovered (µg/ml)	% Recovery	% Mean Recovery
50	16.25	16.15	99.40	
100	32.5	32.41	99.71	99.31
150	48.75	48.17	98.81	

Limit of detection and limit of quantitation

To determine the lowest amount of analyte in the sample, limit of detection (LOD) and limit of quantitation (LOQ) were established at signal-to-noise ratio of 3:1 and 10:1 respectively. The LOD and LOQ of Ascorbic acid and N-Acetyl Cysteine were experimentally determined by injecting six injections of each drug and results were given in Table 6. The LOD and LOQ of Ascorbic acid and N-Acetyl Cysteine were found to be 0.51 µg/ml, 0.24 µg/ml and 1.55, 0.73 µg/ml respectively. From the results of LOD and LOQ it was concluded that the developed method has better sensitivity.

Table 6: Results of LOD and LOQ.

Drug	LOD (µg/ml)	LOQ (µg/ml)
Ascorbic acid	0.51	1.55
N-Acetyl Cysteine	0.24	0.73

Robustness

To ensure the inerrancy of the method by altering the chromatographic conditions like column temperature, mobile phase composition, flow rate etc can be reported. Minute changes in the operational conditions were allowed and the extent to which the method was robust was determined. A deviation of ± 2^0 c in the column temperature and ± 0.2 ml/min in the flow rate, were tried individually. A solution of 100% test concentration with the specified changes in the operational conditions was injected to the instrument in duplicate. The results were reported in the Table 7. From the results it was found that there was no significance difference was observed in system suitability parameters and found to be within the limits. Hence the results conclude that the method was unaffected and found to be robust.

Table 7: Results of robustness.

Analytes	Flow rate (ml)	% RSD	Column Temperature (^{0}C)	% RSD	Mobile phase	% RSD
Ascorbic acid	0.9	0.3	25	0.7	55:45	0.3
	1.1	0.7	35	0.7	45:55	0.6
N-Acetyl Cysteine	0.9	1.4	25	0.5	55:45	0.5
	1.1	0.2	35	0.5	45:55	0.4

*:Average of two determination.

Assay

Onika Organics, bearing the label claim Ascorbic acid 250mg, N-Acetyl Cysteine 32.5mg. Assay was performed with the above formulation. Average % Assay for Ascorbic acid and N-Acetyl Cysteineobtained was 99.94 and 99.29% respectively. Results were shown in Table 8 and 9. Hence the developed method was successfully applied for quality control of the formulation.

Table 8: Assay Data of Ascorbic acid.

S. No	Standard Area	Sample Area	% Assay
1	2184562	2180157	99.58
2	2210490	2183439	99.73
3	2182022	2194664	100.24
4	2187403	2202967	100.62
5	2170328	2172432	99.23
6	2188379	2194220	100.22
Average	2187197	2187980	99.94
Std Dev	13132.3	11240.9	0.51
% RSD	0.6	0.5	0.5

Table 9: Assay Data of N-Acetyl Cysteine.

S. No	Standard Area	Sample Area	% Assay
1	257832	260009	99.43
2	265091	260841	99.74
3	260671	258259	98.76
4	262119	259540	99.25
5	259753	258711	98.93
6	262033	260635	99.66
Average	260458	259666	99.29
Std Dev	2465.0	1033.9	0.40
% RSD	0.9	0.4	0.4

Forced Degradation Studies

The assay method was used to test the drug stability by conducting forced degradation studies for the drug substances under various stress conditions. Stress degradation studies were carried out of acid hydrolysis (2N HCl heated for 30 min at 60^0C), alkali hydrolysis (2N NaOH heated for 30 min at 60^0C), thermal degradation (samples placed in an oven at 105^0C for 1 hr), neutral degradation (drug in water heated for 1 hr at 600C), oxidative degradation (20% H_2O_2 heated for 30 min at 60^0C) and for photolytic stress studies, samples were exposed to UV light by keeping them in a UV chamber for 1 day. The chromatograms were shown in Fig. 9-14. Results were shown in Table 10.

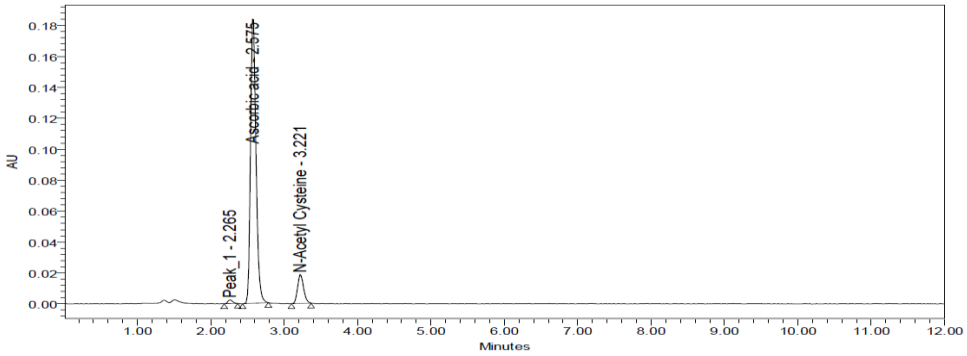

Fig. 9: Acid chromatogram of Ascorbic acid and N-Acetyl Cysteine.

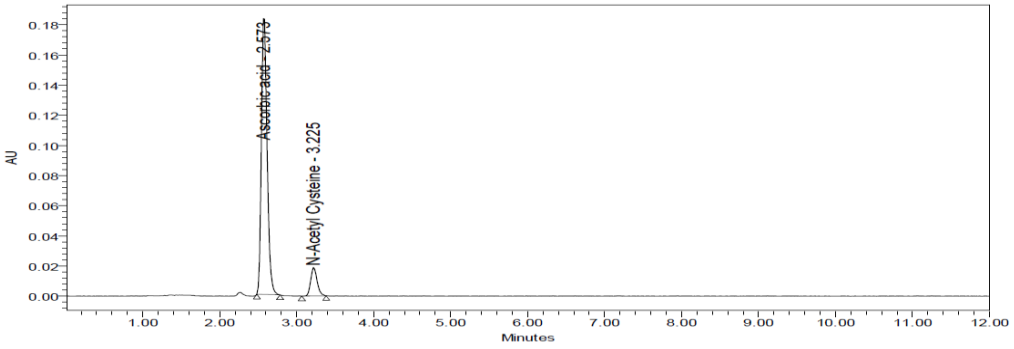

Fig. 10: Base chromatogram of Ascorbic acid and N-Acetyl Cysteine.

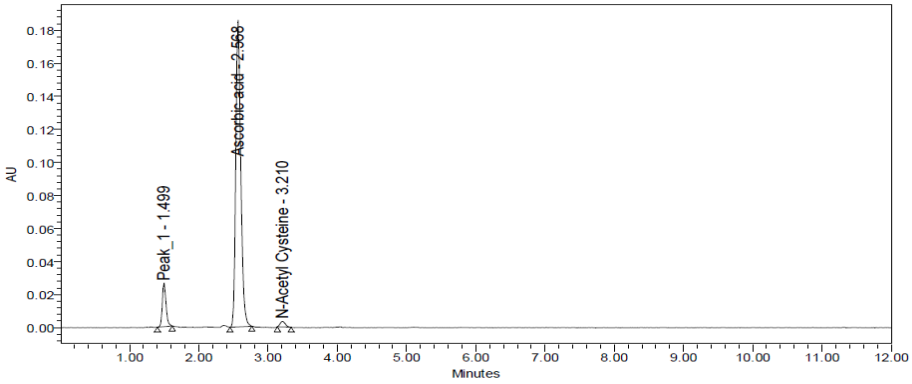

Fig. 11: Peroxide chromatogram of Ascorbic acid and N-Acetyl Cysteine.

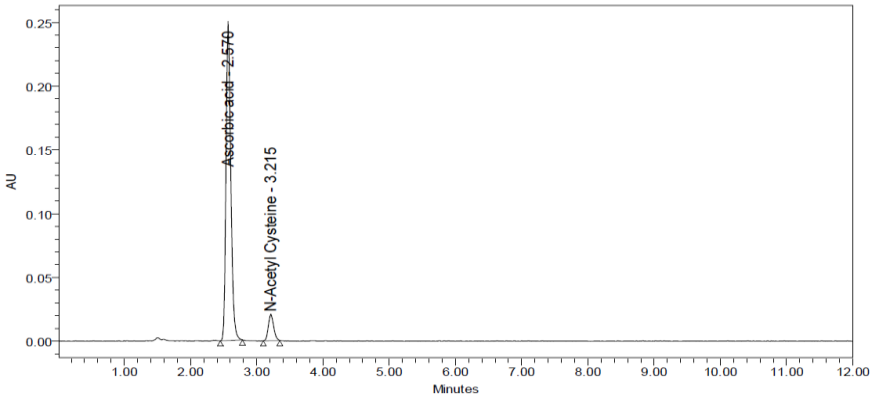

Fig. 12: Thermal chromatogram of Ascorbic acid and N-Acetyl Cysteine.

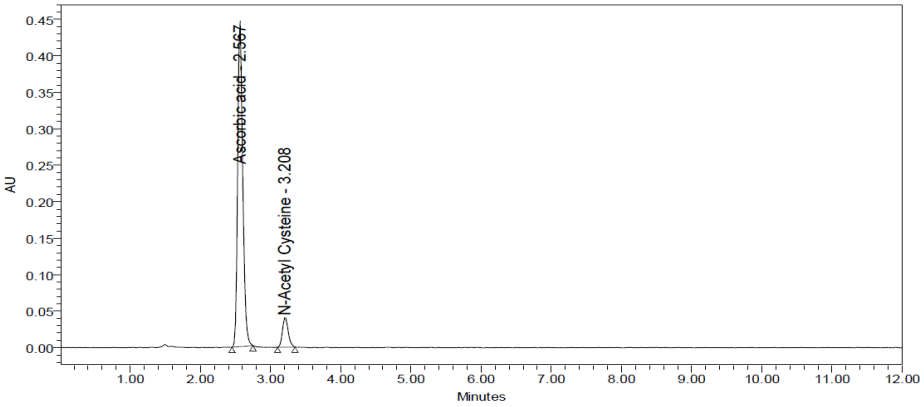

Fig. 13: UV chromatogram of Ascorbic acid and N-Acetyl Cysteine.

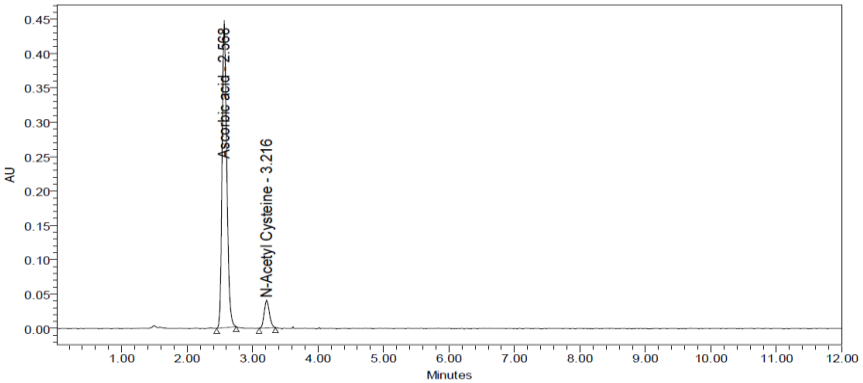

Fig. 14: Water chromatogram of Ascorbic acid and N-Acetyl Cysteine.

Table 10: Degradation data of Ascorbic acid and N-Acetyl Cysteine.

Analytes	Sample Name	% Drug Degraded	Purity Angle	Purity Threshold
	Acid	6.57	0.253	0.442
	Alkali	4.61	0.246	0.427
Ascorbic acid	Oxidation	8.02	0.141	0.446
	Thermal	2.65	0.355	0.427
	UV	1.38	0.230	0.437
	Water	1.38	0.250	0.435
	Acid	6.91	0.588	0.772
	Alkali	5.01	0.645	0.827
N-Acetyl Cysteine	Oxidation	8.04	0.125	1.578
	Thermal	4.36	0.653	0.841
	UV	1.14	0.661	0.842
	Water	0.92	0.123	0.842

CONCLUSION

The proposed stability indicating RP-HPLC method was found to be simple, accurate, precise, robust, rapid and economical. This method gives good resolution between two compounds with a short analysis time and can be used for routine quality control analysis in quality control departments for the determination of Ascorbic acid and N-Acetyl Cysteine in tablet dosage form.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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