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Stability Indicating RP-HPLC Method for Simultaneous Estimation of N-Acetylcysteine and Ascorbic Acid

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ABSTRACT

A precise and robust RP-HPLC has been developed and validated for simultaneous estimation of N-Acetylcysteine and Ascorbic acid. The chromatographic separation was carried out on Kromasil C18 (4.6 x 250mm, 5µm) with a mixture of 0.01N potassium dihydrogen phosphate buffer PH 3.0: acetonitrile (55:45,% v/v) as mobile phase at 220 nm. The retention times were 2.836 min and 3.675 min for N-Acetyl cysteine and Ascorbic acid, respectively. Calibration plots were linear about 60-400 µg/ml and 8-50µg/ml for N-Acetylcysteine and Ascorbic acid, respectively. The proposed method was validated according to ICH guidelines for the parameters like linearity, accuracy, precision, percent recovery, robustness, ruggedness, the limit of detection, and the limit of quantitation. The % RSD is found to be less than 2 % and the tailing factor for both the drugs are found to be less than 2. The number of theoretical plates for N-Acetylcysteine and Ascorbic acid was found to be more than 2000. All validation parameters were within the acceptable range. The developed method was successfully applied for the estimation of N-Acetylcysteine and Ascorbic acid in bulk and pharmaceutical dosage forms.

INTRODUCTION

Acetylcysteine, also known as N- Acetylcysteine (NAC), is a medication that is used for the treatment of Paracetamol overdose and to loosen thick mucus in individuals with Cystic fibrosis. Chemically it is (2R)-2- acetylamino-3- sulfanyl propanoic acid. Its molecular formula is $C_5H_9NO_3S$ and 163.1g/ml is the molecular weight.

Ascorbic acid is chemically (5R)-5- [(1S)- 1,2- dihydroxy ethyl]-3,4- dihydroxy-2,5-dihydrofuran-2-one. It is six carbon compound related to glucose. It is found naturally in citrus fruits. It is used to treat Vitamin C deficiency, Scurvy, delayed wounds and bone healing, urine acidification, and in general as an anti-oxidant. It has also been suggested to be an effective anti-viral agent.

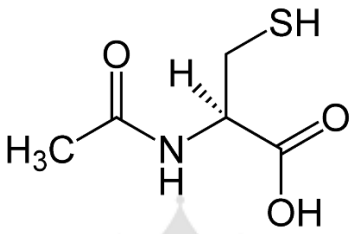

Figure No.1: Chemical Structure of N-Acetyl cysteine

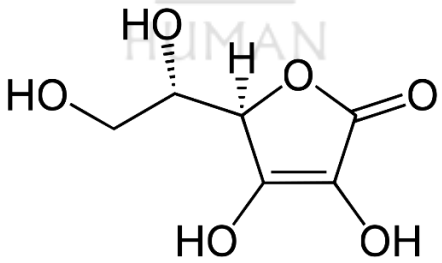

Figure No.2: Chemical Structure of Ascorbic acid

Materials and Reagents: Acetylcysteine and Ascorbic acid pure drugs were kindly gifted from Spectrum labs Hyderabad. HPLC grade Potassium dihydrogen phosphate (KH_2PO_4), acetonitrile procured from Merck, India. High pure water was prepared by using Millipore Milli Q plus a purification system.

Chromatographic Conditions: Analysis was done by using water HPLC 2695 SYSTEM equipped with quaternary pumps, Photo Diode Array detector, and Autosampler integrated with Empower 2 Software. The purity determination was performed on a stainless steel column 250 mm long,4.6 mm internal diameter filled with Octadecyl silane chemically

bonded to porous silica particles of 5µm diameter (Kromasil C18 (4.6 x 250mm, 5µm)with the mobile phase containing phosphate buffer and Acetonitrile in the ratio of 55:45 (%v/v) at 30^0C temperature. The flow rate was kept at 1 ml/min and the elution was monitored at 220 nm.

Selection and Preparation of Mobile Phase: 0.01N KH_2PO_4: Acetonitrile (55:45, %v/v)

Preparation of buffer:

0.01N KH2PO4 Buffer: Accurately weighed 1.36gm of Potassium dihydrogen phosphate transferred into a 1000 ml Volumetric flask add about 900ml of milli-Q water and degas to sonicate and finally make up the volume with water then PH adjusted to 3.0 with dil. Orthophosphoric acid solution.

Preparation of Standard Stock Solution: Accurately weighed and transferred 62.5mg of N-Acetylcysteine,8.125mg of Ascorbic acid working standards into a 25ml clean dry volumetric flasks, added 15 ml diluents, sonicated for 10 minutes, and made up to the final volume with diluents and labeled as Standard stock solution. (2500µg/ml of Acetylcysteine and 325µg/ml of Ascorbic acid).

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

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

Preparation of Sample solutions: 1ml of filtered sample stock solution was transferred to a 10ml volumetric flask and made up with diluent. (250µg/ml of Acetylcysteine and 32.5 µg/ml of Ascorbic acid).

Method Validation

According to ICH guidelines method was validated. The validation includes system suitability, linearity, precision, accuracy, specificity, robustness, the limit of detection (LOD), and the limit of quantification (LOQ).

System suitability test

System suitability tests were performed following USP 24/NF 19 to confirm that the reproducibility of the equipment was adequate for the analysis. The test was performed before the analysis of each batch of samples to ensure the reproducibility of the chromatographic system. All the system suitability parameters were within the range and the results were tabulated in Table-1.

Linearity and Range: The linearity of the method was evaluated by analyzing six (n=6) calibration standards of each Acetylcysteine and Ascorbic acid for a concentration range of 60- 400µg/ml&8-50µg/mL The calibration curve was constructed by plotting peak area against the concentration of standard drugs. The straight-line equation was determined. The regression equations were obtained as follows: y =6906.5x+2098.1(R^2 =0.9995) for Acetylcysteine(Fig No.5) and y= 24210x+ 4352.5(R^2=0.9999) for Ascorbic acid(Fig No.6), where y = peak area, x = concentration of solution; R^2= the square of determined correlation coefficient. The results implied that the method developed was linear over the specified range (Table-2).

Precision. The Repeatability studies were carried out by estimating the response of six different concentrations of Acetylcysteine and Ascorbic acid, results are reported in terms of relative standard deviation (% RSD)(Table No.3).

Accuracy: Recovery studies were performed by standard addition method at three levels i.e., 50%, 100%, and 150%. Known amounts of standard NAC and AA were added to pre-analyzed samples and they were subjected to the proposed HPLC method. Results of recovery studies are shown in Table No.4.

Robustness: The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. The robustness of the method was investigated under a variety of conditions including changes in the composition of buffer in

the mobile phase and flow rate. % RSD of the assay was calculated for each condition. The degree of reproducibility of the results obtained as a result of small deliberate variations in the method parameters has proven that the method is robust (Table-6).

Sensitivity and selectivity: LOD and LOQ decide about the sensitivity of the method.LOD and LOQ were calculated by the equations given in the ICH guidelines. The LOD with a signal/noise ratio of 3:1 was found to be 0.18 µg/ml for Acetylcysteine and 0.31 µg/ml for Ascorbic acid. The LOQ with a signal/noise ratio of 10:1were found to be 0.55 µg/ml for Acetylcysteine and 0.95µg/ml for Ascorbic acid. The results are given in Table 5.

Assay: Onika Organics, bearing the label claim Acetylcysteine 250mg, Ascorbic acid 32.5mg. An assay was performed with the above formulation. The average % Assay for Acetylcysteine and Ascorbic acid obtained was 100.39 and 100.13% respectively and results were shown in Table-7&8.

Forced Degradation Studies:

Oxidative degradtion: To 1 ml of stock solution of Acetylcysteine and Ascorbic acid, 1 ml of 20% hydrogen peroxide (H_2O_2) was added separately. The solutions were kept for 30 min at 60^0c.For HPLC study,the resultant solution was diluted to obtain 250 ppm(NAC) & 32.5 ppm (AA) solution and 10µl were injected into the system, and the chromatograms were recorded to assess the stability of sample (Fig.9).

Acid Degradation: To 1 ml of stock solution of Acetylcysteine and Ascorbic acid, 1 ml of 2N Hydrochloricacid was added and refluxed for 30mins at 60^0c.The resultant solution was diluted to obtain 250ppm (NAC)&32.5ppm(AA)solution and10µl solutions were injected into the system and the chromatograms were recorded to assess the stability of sample (Fig.7).

Alkali Degradation Studies: To 1 ml of stock solution of Acetylcysteine and Ascorbic acid, 1 ml of 2N sodium hydroxide was added and refluxed for 30mins at 60^0c. The resultant solution was diluted to obtain 250ppm (NAC) &32.5ppm (AA) solution and 10µl was injected into the system and the chromatograms were recorded to assess the stability of sample (Fig.8).

Thermal Degradation Studies: The standard drug solutions were placed in an oven at105°C for1h to study dry heat degradation. For the HPLC study, the resultant solution

was diluted to 250 ppm (NAC) &32.5ppm (AA) solution and10µl were injected into the system and the chromatograms were recorded to assess the stability of the sample (Fig.10).

Photo Degradation: The photochemical stability of the drug was also studied by exposing the Acetyl cysteine2500 ppm &Ascorbic acid 325 ppmsolution to UV Light by keeping the beaker in UV Chamber for 1day or 200 Watt-hours/m^2 in a photostability chamber for the HPLC study, the resultant solutions were diluted to obtain 250ppm(NAC))&32.5ppm(AA)solutions and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample(Fig.11).

Neutral Degradation Studies: Stress testing under neutral conditions was studied by refluxing the drug in water for 1hr at a temperature of 60º. For the HPLC study, the resultant solutions were diluted to 250ppm (NAC) &32.5ppm (AA) solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of the sample (Fig.10)**.**

RESULTS AND DISCUSSION

In this study, separation of NAC and AA were done on the C18 column. Optimization of mobile phase composition was performed based on resolution among drugs and degradation products, asymmetric factor, and theoretical plates. The mobile phase consisted of 0.01N KH_2PO_4 and acetonitrile in a ratio of 55:45 % v/v was selected to give sharp and well-resolved peaks for NAC and AA. The retention times for NAC and AA were 2.836±0.2 and 3.765±0.3 min, respectively. UV overlaid spectra of NAC and AA showed that both the drugs absorbed appreciably at 220 nm, so the same wavelength was selected as the detection wavelength during degradation studies. The optimized method was validated as per ICH Q2 guidelines. The method was specific in presence of degradation products as shown in figs. 7 to 12. The linearity was proven by calibration curves and it was found to be linear over the range of 60-400 µg/ml for NAC and 8-50 µg/ml for AA. The data of correlation coefficient (R^2 value) for NAC and AA were 0.9995 and 0.9993, respectively. The intermediate precision was tested by interday precision and was found to be less than 1.5% against the acceptance limit of 2%. The low RSD (%) value indicated that the method is more precise. The recovery studies for accuracy were tested at three different levels (50, 100, and 150%) and their results were between 98-102%, thus the accuracy of the method was proven. The detection limits (LOD) for NAC and AA were 0.18 µg/ml and 0.31µg/ml, respectively, while quantitation limits (LOQ) were 0.55 and 0.95 µg/ml respectively. The lowest LOD value

indicated the method is more sensitive. The robustness of the method was studied by changing the flow rate of the mobile phase from 1 ml/min to 0.9 ml/min and 1.1 ml/min and pH change of ±0.1 units. The mobile phase composition was changed to 45:65, by increasing the percentage of acetonitrile, the retention time for NAC and AA were observed to be 2.634 min and 3.285 min, respectively. The corresponding assay value for all robust conditions was between 98-102% against the Assay value of optimized conditions. Solution stability study was done as a part neutral degradation study and the results revealed that NAC and were stable in solution up to 24 h.

The method has fulfilled all validation parameters as per ICH guidelines. A forced degradation study was carried out by subjecting both drugs to acid and alkali hydrolysis, chemical oxidation, and photolytic and thermal degradations.

CONCLUSION

A simple, Accurate, precise method was developed for the simultaneous estimation of the Acetylcysteine and Ascorbic acid in the Tablet dosage form. The results of stress testing undertaken according to the ICH guidelines reveal that the method is specific and stability-indicating. The proposed method can separate these drugs from their degradation products in tablet dosage forms and hence can be applied to the analysis of routine quality control samples.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this paper.

ACKNOWLEDGMENTS

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ABBREVIATIONS

NAC: N-Acetylcysteine

AA: Ascorbic acid

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Table No.1: System suitability parameters for N-Acetylcysteine and Ascorbic acid

S no	N-Acetylcysteine			Ascorbic acid		
Inj	RT(min)	USP Plate Count	Tailing	RT(min)	USP Plate Count	Tailing
1	2.835	6382	1.58	3.675	7399	1.47
2	2.836	6327	1.58	3.677	7775	1.50
3	2.836	6596	1.57	3.678	7455	1.48
4	2.836	6492	1.58	3.678	7336	1.48
5	2.836	6733	1.57	3.678	7437	1.48
6	2.838	6705	1.56	3.678	7644	1.49

Table No.2: Linearity table for N-Acetylcysteine and Ascorbic acid.

Acetylcysteine		Ascorbic acid	
Conc (µg/mL)	**Peak area**	**Conc (µg/mL)**	**Peak area**
0	0	0	0
62.5	457286	8.125	212067
125	854923	16.25	378852
187.5	1270656	24.4	611215
250	1748864	32.5	788198
312.5	2142821	40.625	992094
375	2608884	48.75	1179413

Table No.3: System precision of N-Acetylcysteine and Ascorbic acid

S. No	Area of Acetylcysteine	Area of Ascorbic acid
1.	1748735	783315
2.	1716992	782358
3.	1706486	788962
4.	1723659	789698
5.	1712329	789269
6.	1705050	789169
Mean	1718875	787129
S.D	16158.7	3346.9
%RSD	0.9	0.4

Table No.4: Accuracy study of N-Acetyl cysteine and Ascorbic acid

% level	Amount Spiked(µg/ml)	Amount recovered(µg/ml)	% recovery	Mean % Recovery	Amount Spiked(µg/ml)	Amount recovered(µg/ml)	% recovery	Mean % Recovery
50%	125	124.83	99.86\	99.68%	16.25	16.03	98.65	99.26%
	125	124.92	99.94		16.25	16.16	99.44	
	125	125.14	100.11		16.25	15.98	98.34	
100%	250	247.35	98.94		32.5	32.21	99.10	
	250	249.08	99.63		32.5	32.35	99.54	
	250	249.64	99.86		32.5	32.55	100.15	
150%	375	372.86	99.42		48.75	48.56	99.61	
	375	372.91	99.44		48.75	47.97	98.4	
	375	375.28	100.00		48.75	48.82	100.14	

Table No.5: LOD and LOQ values of N-Acetyl cysteine and Ascorbic acid

Drug Name	LOD	LOQ
N-Acetyl Cysteine	0.18	0.55
Ascorbic acid	0.31	0.95

Table No.6: Robustness data for N-Acetylcysteine and Ascorbic acid

S.no	**Condition**	**%RSD of Acetylcysteine**	**%RSD of Ascorbic acid**
1	Flow rate (-) 1.1ml/min	0.5	0.7
2	Flow rate (+) 1.3ml/min	0.6	0.4
3	Mobile phase (-) 35B:65A	0.6	0.7
4	Mobile phase (+) 45B:55A	0.3	0.6
5	Temperature (-) 25°C	0.8	0.7
6	Temperature (+) 35°C	0.3	0.2

A: Acetonitrile, B: Buffer

Table No.7.Assay Data of Acetylcysteine

S.no	**Standard Area**	**Sample area**	**% Assay**
1	1748735	1707526	99.14
2	1716992	1717278	99.71
3	1706486	1721123	99.93
4	1723659	1711035	99.34
5	1712329	1728736	100.37
6	1705050	1733161	100.63

Table No.8. Assay Data of Ascorbic acid

S.no	Standard Area	Sample area	% Assay
1	783315	783159	99.30
2	782358	783192	99.30
3	788962	780405	98.95
4	789698	789221	100.07
5	789269	783710	99.37
6	789169	784933	99.52
Avg	787129	784103	99.42
Stdev	3346.9	2913.2	0.4
%RSD	0.4	0.4	0.4

Table No.9: Degradation data of N-Acetyl cysteine and Ascorbic acid

Drug Name	Parameters	% Drug degraded	Purity angle	Purity Threshold
N-Acetyl Cysteine	Oxidation	7.24	0.173	0.386
	Acid	4.69	0.398	0.574
	Alkali	4.54	0.372	0.556
	Thermal	3.62	0.426	0.686
	UV	1.26	0.334	0.569
	Water	0.87	0.146	0.353
Ascorbic acid	Oxidation	7.28	0.184	0.388
	Acid	4.67	0.244	0.382
	Alkali	4.60	0.235	0.357
	Thermal	3.52	0.122	0.297
	UV	1.58	0.158	0.336
	Water	1.26	0.166	0.386

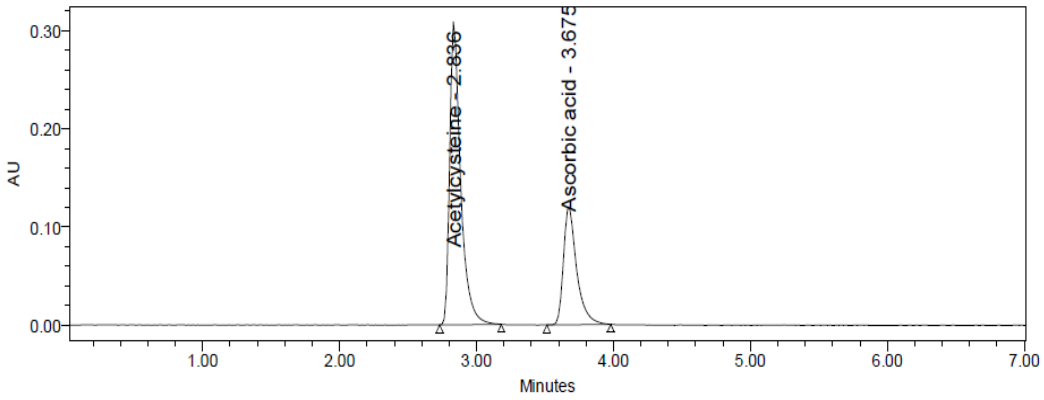

Figure No.3: Standard Chromatograms of N-Acetyl cysteine and Ascorbic acid

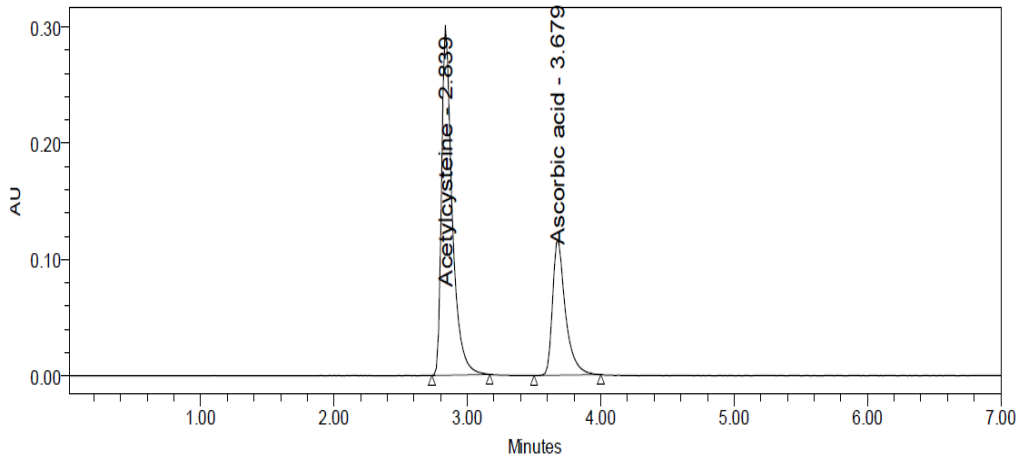

Figure No.4: Sample chromatograms of N-Acetyl cysteine and Ascorbic acid

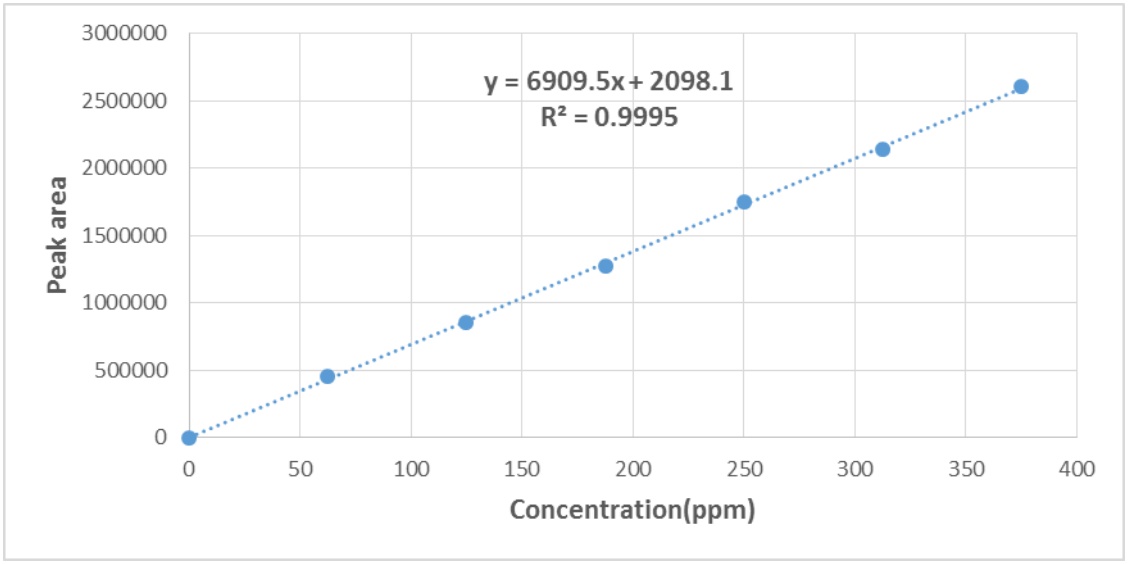

Figure No.5: Calibration plot of N-Acteyl cysteine

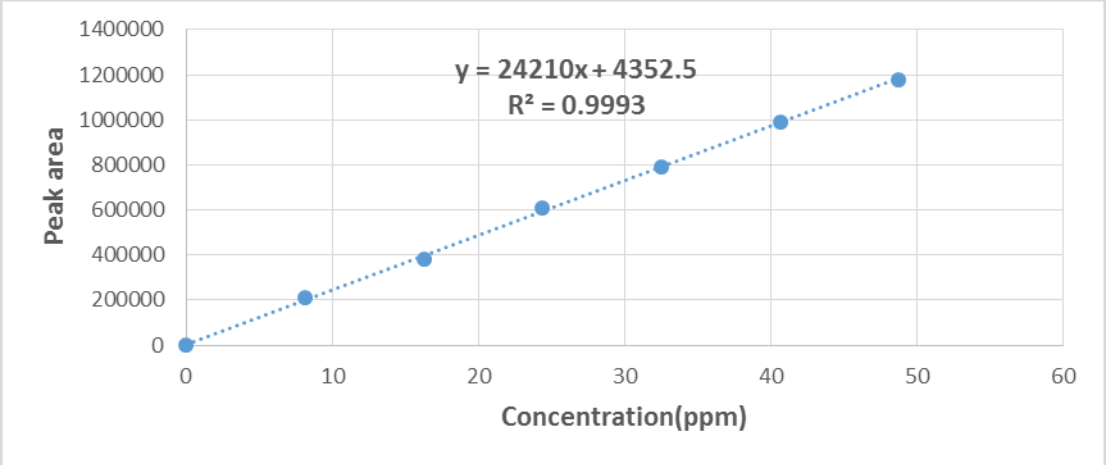

Figure No.6: Calibration plot of Ascorbic acid

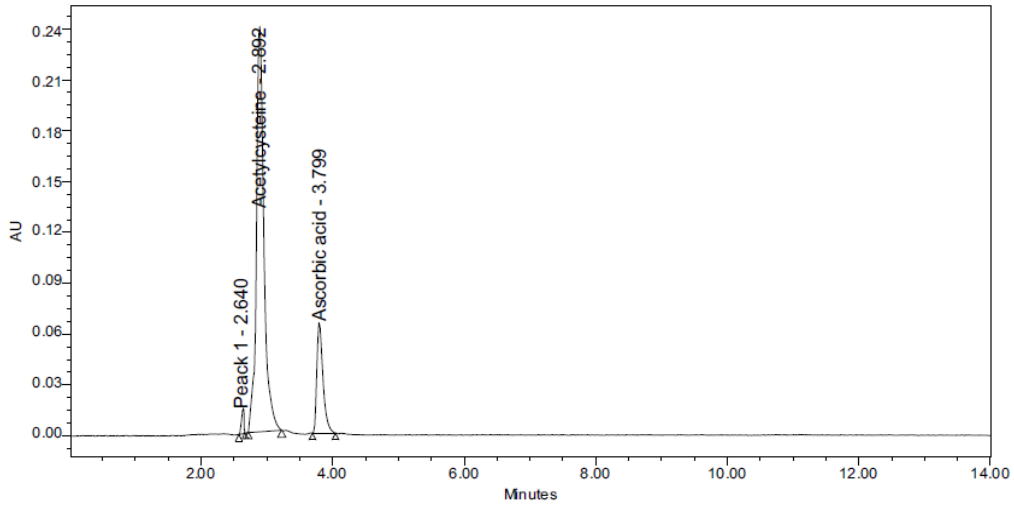

Figure No.7: Acid degradation chromatograms of N-Acetyl cysteine and Ascorbic acid

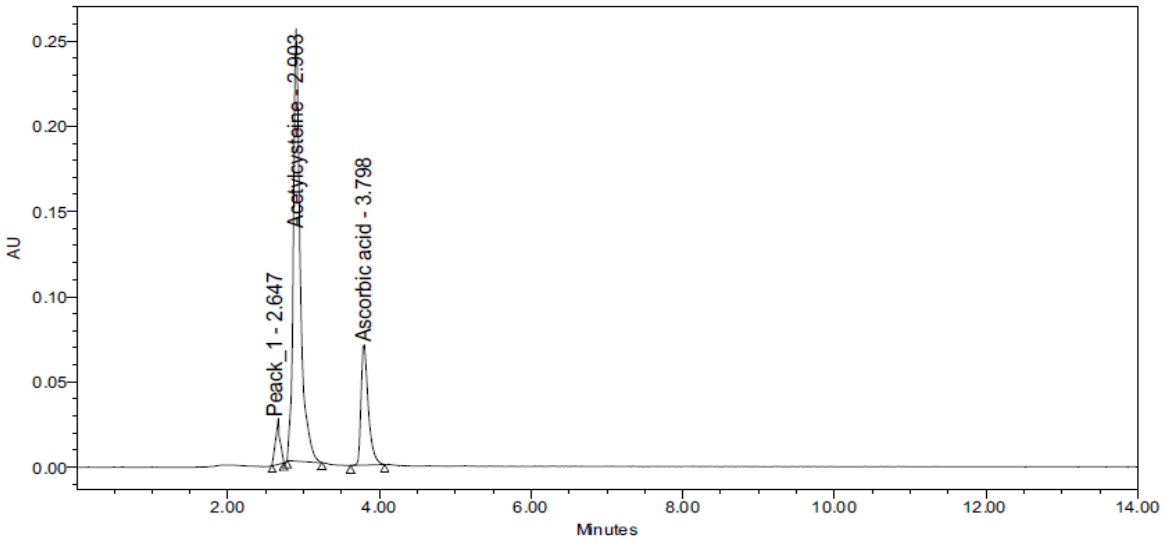

Figure No.8: Base degradation chromatograms of N-Acetylcysteine and Ascorbic acid

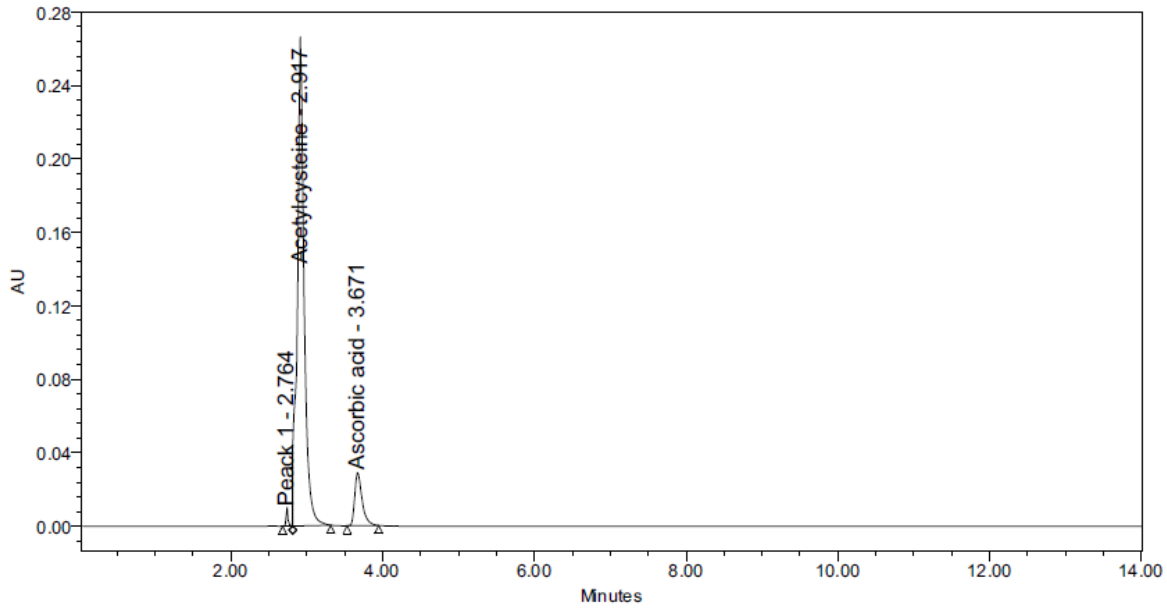

Figure No.9: Peroxide degradation chromatograms of Acetylcysteine and Ascorbic acid

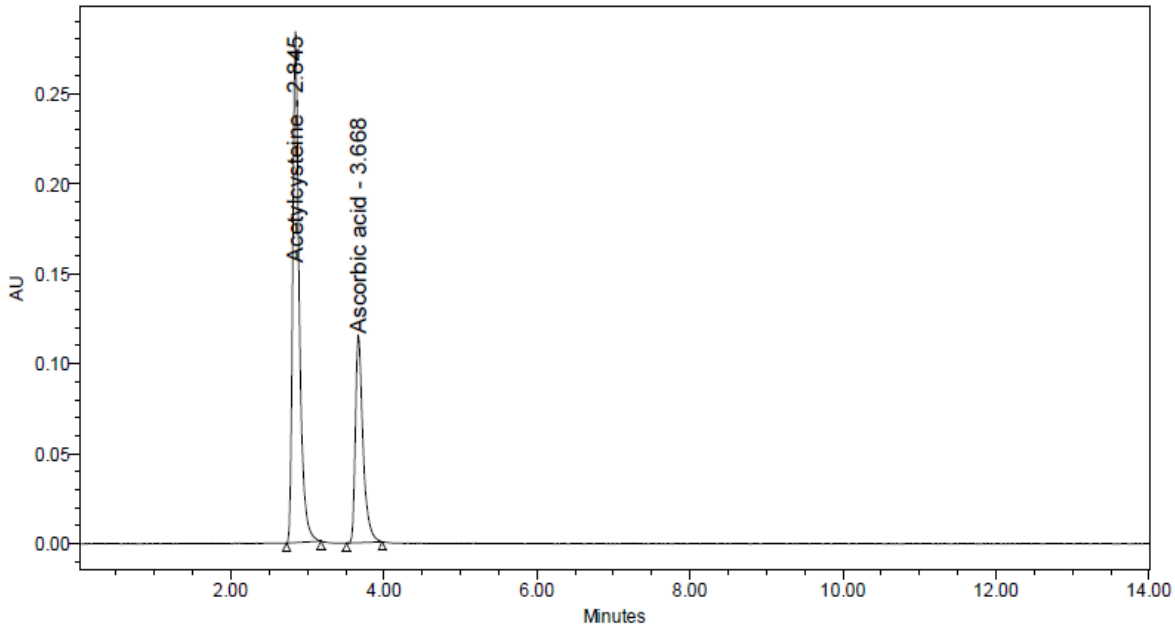

Figure No.10: Thermal degradation chromatograms of Acetylcysteine and Ascorbic acid

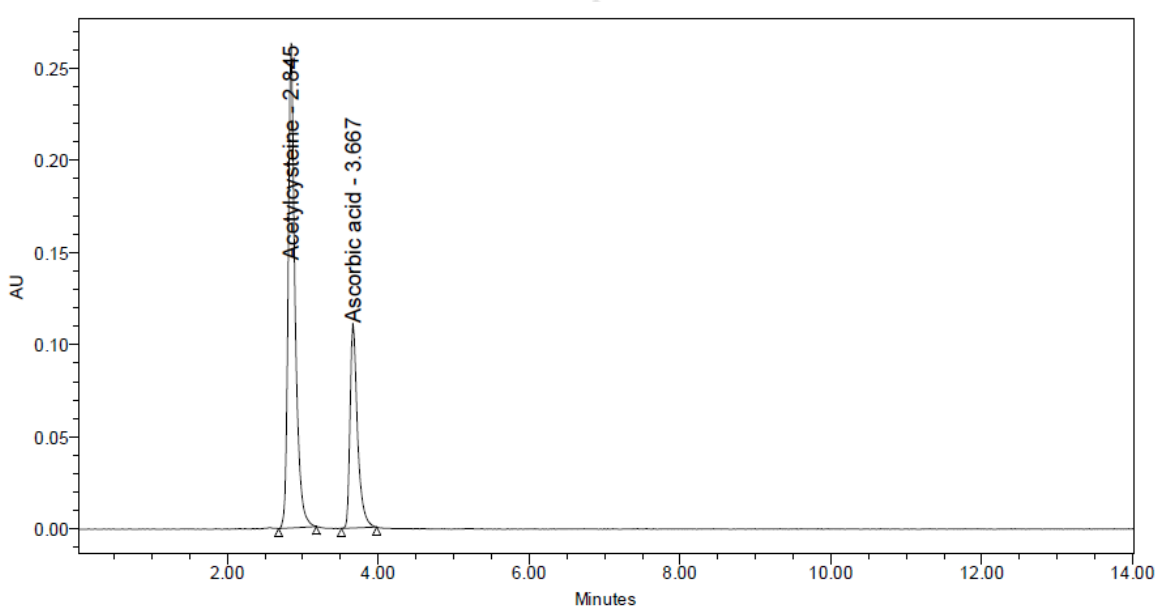

Figure No.11: UV degradation chromatograms of Acetylcysteine and Ascorbic acid

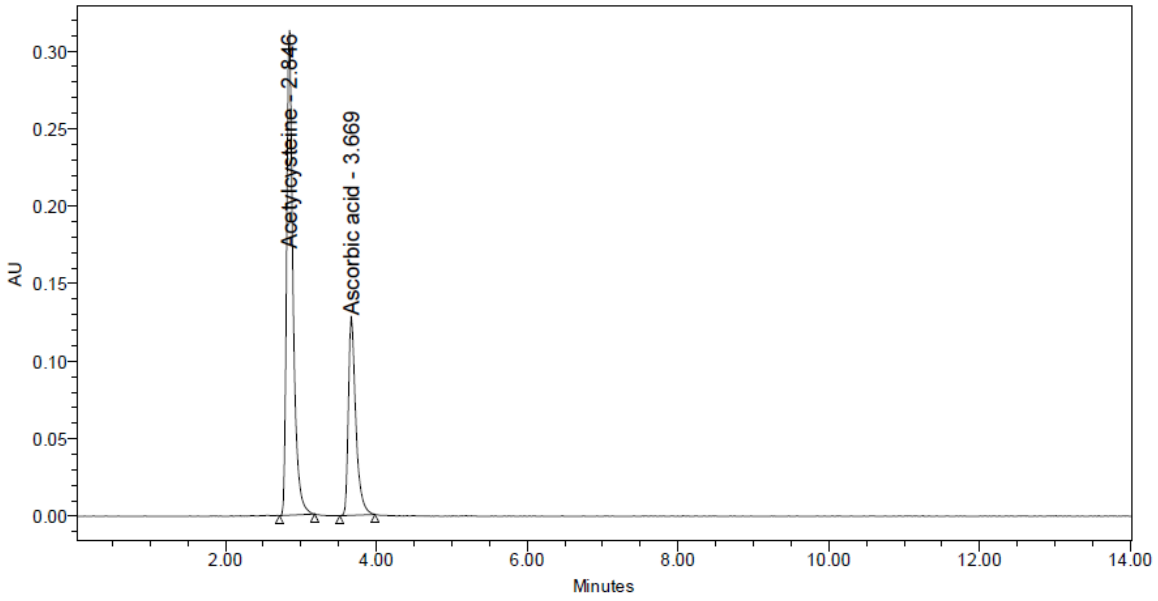

Figure No.12: Neutral degradation chromatogramsof Acetylcysteine and Ascorbic acid

 *Image* *Author -1*	***Author Name – Dr Vamseekrishna G*** *Professor* *Department of Pharmaceutical analysis,Nri college of Pharmacy,Pothavarappadu,Agiripalli,Andhra Pradesh.*
 *Image* *Author -2*	***G Sanjeev*** *IV B.Pharmacy student* *Nri college of pharmacy, Pothavarappadu,Agiripalli,Andhra Pradesh.*
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