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A New Stability Indicating RP-HPLC Method for Simultaneous Estimation of Acebrophylline and N-Acetylcysteine in Tablet Dosage Form and Its Validation as Per ICH Guidelines

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ABSTRACT

A simple, accurate, precise and stability-indicating RP-HPLC method was developed for the simultaneous estimation of acebrophylline and N-acetylcysteine in tablet dosage form. Chromatogram was run through Kromasil $C_{18,}$ column (250 x 4.6 mm, 5µm) consisting of the mobile phase, 0.2M sodium hydrogen phosphate buffer (Na_2HPO_4): acetonitrile taken in the ratio of 50:50 v/v, pumped at a flow rate of 1.0 mL/min. Temperature was maintained at 30°C and the optimized wavelength selected for the present investigation was 260 nm. Retention time of acebrophylline and N-acetylcysteine were found to be 2.257 and 2.875 minutes, respectively. LOD, LOQ values obtained from regression equations of acebrophylline and N-acetylcysteine were 0.42, 1.27 and 0.23, 0.68 mcg/mL respectively. Hydrolysis, oxidation, photolysis and thermal degradation are evaluated by subjecting the drug substance to stress conditions. Good separation of the drugs and their degradation products were observed using this method. This validated method can be applied for the simultaneous estimation of acebrophylline and N-acetylcysteine in commercially available formulation sample.

INTRODUCTION

Acebrophylline[1] is a xanthine derivative (Figure 1) containing ambroxol and theophylline as main constituents, the drug is used in the treatment of asthma, COPD, bronchitis, lungs inflammation sinusitis, tightness of chest *etc.* Acebrophylline helps the patient to breathe easily by relaxing the smooth muscle present in lungs and airways. Theophylline acts as a bronchodilator by inhibiting the intercellular phosphodieterases which increases the adenosine monophosphate cyclic levels and it enhances the relaxation of bronchial muscles. Ambroxyl acts as a mucolytic and expectorant by increasing the serous gel phase. It simulates the cilia mobility and thus increases the mucociliary clearance Phospholipase A and phosphatidylcholine is inhibited by Acebrophylline thus producing pro-inflammatory substance like leukotriene and tumor necrosis factors. Acebrophylline reduces the inflammation of airway by inhibiting the synthesis of these inflammatory mediators. Acetylcysteine[2], (Figure 2) also known as N-Acetylcysteine is a medication that is used to treat paracetamol (acetaminophen) overdose, and to loosen thick mucus in individuals with cystic fibrosis or chronic obstructive pulmonary disease. It can be taken intravenously, by mouth, or inhaled as a mist.

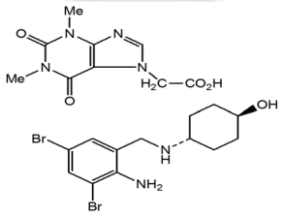

Figure No. 1: Chemical structure of acebrophylline

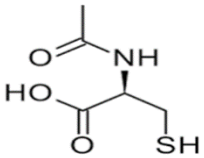

Figure No. 2: Chemical structure of N-acetylcysteine

There are few reported HPLC and other analytical methods[3-14] for the determination of acebrophylline and N-acetylcysteine in single and combination with other formulations. So far, to our knowledge, no stability-indicating assay method has been developed for the simultaneous estimation of acebrophylline and N-acetylcysteine in presence of their degradants using the ICH recommended guidelines. The focus of the present study was to

develop a simple, rapid, precise, and accurate isocratic reversed-phase stability-indicating HPLC method for the simultaneous estimation of acebrophylline and N-acetylcysteine in tablet dosage form.

MATERIALS AND METHODS

MATERIALS AND CHEMICALS

Authentic samples of acebrophylline and N-acetylcysteine were procured from Dr. Reddy's Laboratories Limited (Hyderabad, India). Pulmoclear tablets (containing acebrophylline (100mg) and N-acetylcysteine (600 mg) were manufactured by Fourrts India Laboratories Ltd, India) procured from local pharmacy. HPLC-grade chemicals were purchased from Spectrochem Pvt. Ltd. (Mumbai, India). Sodium dihydrogen phosphate (Rankem, Mumbai, India) and orthophosphoric acid (Qualigens Fine Chemicals, Mumbai, India) were analytical reagent grade.

Apparatus

Chromatography was performed on a Shimadzu (Shimadzu Corporation, Kyoto, Japan) chromatographic system equipped with an isocratic HPLC pump (Shimadzu LC-20AT) and a Photodiode array detector (Shimadzu SPD-20AV) with a Rheodyne syringe-loading sample fixed loop (20 µL) injector (7725). The LC separations were performed at ambient temperature on a Kromasil $C_{18,}$ column (250 x 4.6 mm, 5µm), (Torrance, CA). Data were acquired and processed by the use of Spinchrom (CFR version 2.4.1.93) software. Degassing of the mobile phase was done by sonication in an Ultrasonic bath (Ultrasonics Selec, Vetra, Italy). The standard substances were weighed on a Precisa (205 ASCS Swiss Quality, Switzerland) analytical balance. Photostability studies were carried out in a photostability (NEC-103R Newtronic, Mumbai, India) chamber, which was set at 25ºC ± 1ºC. Thermal stability study was carried out in a hot air oven (Sedko Laboratory, Equipments, Ahmedabad, India).

Chromatographic separations

HPLC studies were individually carried out for all the reaction solutions and in a mixture of the solutions in which decomposition was observed. The separation was carried out under isocratic elution with sodium dihydrogen phosphate buffer (adjusted pH with 0.5%

orthophosphoric acid)–acetonitrile (50:50 v/v) as the mobile phase. The mobile phase was filtered through a 0.45-µm nylon filter and degassed before use. The flow rate was 1 mL/min, and the detection wavelength was 260 nm.

Preparation of standard stock solutions: Accurately weighed 75mg of N-acetylcysteine, 12.5 mg of acebrophylline transferred to 25ml volumetric flask and 3/4th of diluents was added to these flasks and sonicated for 10 minutes. Flask were made up with diluents (Water: Acetonitrile, 50:50) and labeled as standard stock solution (3000µg/ml of N-acetylcysteine and 500 µg/ml of acebrophylline).

Preparation of standard working solutions: 1ml from each stock solution was pipetted out and taken into a 10 mL volumetric flask and made up with diluent to get the final concentration of 300µg/mL N-acetylcysteine and 50 µg/mL of acebrophylline.

Preparation of sample stock solutions: 20 tablets were weighed and the average weight of each tablet was calculated, then the weight equivalent to one tablet was transferred into a 100 mL volumetric flask, 50 mL of diluents was added and sonicated for 25 min, further the volume was made up with diluent and filtered by HPLC filters to get the concentration of (6000µg/ml of N-acetylcysteine and 1000µg/ml of acebrophylline).

Preparation of Sample working solutions: 0.5ml of filtered sample stock solution was transferred to 10 mL volumetric flask and made up with diluent so as to get the concentration of 300µg/ml of N-acetylcysteine and 50µg/mL of acebrophylline, respectively.

Method validation

The developed method was validated according to ICH guidelines[15-17] with respect to system suitability parameters, specificity, precision, linearity, accuracy, Limit of detection (LOD), Limit of quantitation (LOQ). The system suitability parameters were determined by preparing standard solutions of N-acetylcysteine (300 ppm) and acebrophylline (50 ppm) and the solutions were injected six times and the parameters like peak tailing, resolution and USP plate count were determined. The % RSD for the area of six standard injections results should not be more than 2%. The specificity of the method is found to be correct if the interfering peaks in blank and placebo at retention times of these drugs were not found. The linearity of the system was studied by preparing the standard stock solution by accurately weighing 75mg of N-acetylcysteine, 12.5mg of acebrophylline and transferring to 25ml volumetric flask and

3/4th of diluents was added and sonicated for 10 minutes. The final solution concentration was (3000µg/ml of N-acetylcysteine and 500µg/ml of acebrophylline). The standard solution were prepared across a range of 25%, 50%, 75%, 100%, 125% and 150% by pipetting 0.25ml, 0.5ml, 0.75ml, 1.0ml, 1.25ml, and 1.5ml from each of the standard stock solutions and made up the volume to 10ml respectively.

Accuracy of the system was studied by preparing the standard stock solution, accurately weighed 75mg of N-acetylcysteine, 12.5mg of acebrophylline and transferred to 25ml volumetric flask and 3/4th of diluents was added to these flasks and sonicated for 10 minutes. Flask were made up with diluents and labeled as standard stock solution. (3000µg/ml of N-acetylcysteine and 500 µg/ml of acebrophylline). Accuracy was studied with a concentration range of 50%, 100% and 150% by pipetting 0.5 ml, 1.0 ml and 1.5 ml of sample stock solution to 10 ml volumetric flask and added 1ml from each standard stock solution and made up to the mark respectively. Precision was carried out by inter and intraday analysis. Robustness of the method was carried out by small deliberate changes in method like flow rate, mobile phase ratio, and temperature but no recognized change in the method would be found. Robustness conditions like Flow minus (0.9 ml/min), Flow plus (1.1ml/min), mobile phase minus (65Buffer: 35Acetonitrile), mobile phase plus (55Buffer:45Acetonitrile), temperature minus (25°C) and temperature plus (35°C) was maintained and samples were injected in duplicate and %RSD was calculated. The LOD and LOQ value can be obtained by the calibration curve and the assay was carried out in triplicate (*n*= 3) at three different concentration levels for N-acetylcysteine and acebrophylline**,** respectively.

Forced Degradation studies

Oxidation degradation:

To 1 ml of stock solution of N-acetylcysteine and acebrophylline, 1 mL of 20% hydrogen peroxide (H_2O_2) was added separately. The solutions were kept for 30 min. For HPLC study, the resultant solution was diluted to obtain 300µg/ml & 50µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Acid degradation:

To 1 ml of stocks solution N-acetylcysteine and acebrophylline, 1mL of 2N Hydrochloric acid was added and refluxed for 30 mins. The resultant solution was diluted to obtain 300 µg/mL & 50µg/mL solution and 10µl solutions were injected into the system and the chromatograms were recorded to assess the stability of sample.

Alkali degradation:

To 1 ml of stock solution N-acetylcysteine and acebrophylline, 1 ml of 2N sodium hydroxide was added and refluxed for 30 mins. The resultant solution was diluted to obtain 300µg/ml & 50µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Dry Heat degradation (Thermal):

The standard drug solution was placed in oven at105°C for 1hour to study dry heat degradation. For HPLC study, the resultant solution was diluted to 300 µg/ml & 50µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of the sample.

Photolytic degradation:

The photochemical stability of the drug was also studied by exposing the 3000µg/ml & 500µg/ml to UV Light by keeping the beaker in UV Chamber for 1day or 4000 Watt hours/m^2 in photostability chamber. For HPLC study, the resultant solution was diluted to obtain 300 µg/ml & 50µg/ml solutions and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Neutral Degradation:

Stress testing under neutral conditions was studied by refluxing the drug in water for 1 hour at a temperature of 60ºC. For HPLC study, the resultant solution was diluted to 300µg/ml & 50µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of the sample.

RESULTS AND DISCUSSION

HPLC method development and optimization

Different parameters like buffer, organic modifier ratio, and pH were optimized to achieve good separation between N-acetylcysteine and acebrophylline, and the degradation products formed under various conditions. Initial studies on individual reaction solutions were carried out using buffer–methanol (50:50 v/v) as the mobile phase. Several studies were carried out by decreasing the percentage of methanol from 50% to 35% until satisfactory resolution was obtained. Another attempt was made by substituting HPLC water with methanol. The advantages observed were smoothening of baseline, but peaks were not well resolved. After several attempts, it was found that good resolution was obtained with 50% buffer (0.2M Sodium hydrogen phosphate), and 50% acetonitrile gave sufficient separation as well as symmetrical peak shape. So, finally, the above mobile phase was selected for both validation as well as for assay. It was then applied to a mixture of those stressed samples in which there was recognizable different degradation products were formed. The method worked well with the mixture of degradation solutions and was even applicable to formulations.

Validation of the method

The method was validated with respect to following parameters.

Linearity: Linear calibration plots of each drug for the previously mentioned method were obtained over the calibration ranges 12.5-75 µg/mL and 75-450 µg/mL N-acetylcysteine and acebrophylline, respectively; the correlation coefficient obtained was greater than 0.999 for both drugs (Table 2). The results show that good correlation existed between the peak area and concentration of the analyte.

LOD and LOQ: The LOD values for N-acetylcysteine and acebrophylline were shown in (Table 5 and Figure 4).

Precision: Data obtained from analysis of the samples on the same day (n = 3) and on consecutive days (n = 3) are given in Table 3. As evident, the % RSD values of the data obtained were well below 2%.

Accuracy: Percentage recovery was calculated from differences between the peak areas obtained for fortified and unfortified solutions. As shown from the data in Table 4, good recoveries were made at each added concentration, confirming that the method was accurate.

Degradation studies: Good resolution was obtained between the drugs and the degradation products formed under the various stress conditions, indicating the specificity of the method. The resolution factor (Rs) from acidic, alkaline, neutral, oxidative, and thermal degradation products was always ≥ 1.8, which ensured complete separation of acebrophylline and N-acetylcysteine from their degradation products. The drugs underwent degradation in acidic, basic and oxidative degradation, while in thermal, neutral and photolytic degradation the drugs underwent only little degradation (Table 7 & Figure 5). Studies performed to determine the purity of acebrophylline and N-acetylcysteine peaks using a PDA detector showed purity angle (PA) values of 0.074 and 0.063 and purity threshold (TH) values of 0.256 and 0.272 for acebrophylline and N-acetylcysteine, respectively. The PA value was found to be less than the TH value, indicating that the acebrophylline and N-acetylcysteine were free from any co-eluting peak.

Robustness: The results presented in Table 6 indicate that the selected factors remained unaffected by slight variation of these parameters. It was also found that acetonitrile from the different manufacturers does not have significant influence on the determination. Insignificant differences in peak areas and less variability in retention times were observed.

System suitability: The results (Table 1) obtained from system suitability tests are in agreement with the United States Pharmacopoeia requirements[18]. The variation in retention times among six replicate injections of acebrophylline and N-acetylcysteine standard solutions was very low, rendering RSD of less than 2 %, respectively.

Applicability of the developed method to marketed formulation

The developed method (Figure 3a) was successfully applied to analyze N-acetylcysteine and acebrophylline, in marketed formulation (Figure 3b). A clear separation of the drugs and degradation products was achieved in the tablet with no interference from excipients (Table 8).

CONCLUSION

In this study, acebrophylline and N-acetylcysteine were subjected to stress studies under various ICH-recommended conditions. The additional findings in this study show that the drugs underwent nearly 5-6% degradation in acidic, basic and oxidative degradation, while mild degradations were observed in thermal, photolytic and neutral degradations. The method was validated for parameters like linearity, accuracy, specificity, robustness, and system suitability. Application of this method for the analysis of acebrophylline and N-acetylcysteine in tablet dosage form shows that there is no interference of excipients or degradation products in the analytical determination. Thus, the proposed method could be regarded as the stability-indicating method for the simultaneous estimation of acebrophylline and N-acetylcysteine either in bulk drug or in pharmaceutical formulations.

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CONFLICTS OF INTEREST: Nil

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Table No. 1: System suitability parameters for acebrophylline and N-acetylcysteine

Sr. No.	Acebrophylline			N-Acetylcysteine			
Inj	RT(min)	USP Plate Count	Tailing	RT(min)	USP Plate Count	Tailing	Resolution
1	2.255	3081	1.14	2.876	10604	1.39	4.6
2	2.261	3983	1.23	2.883	11372	1.48	4.8
3	2.262	3764	1.23	2.884	11187	1.48	4.7
4	2.262	3869	1.22	2.885	11050	1.48	4.7
5	2.263	3615	1.15	2.885	10439	1.48	4.6
6	2.264	3737	1.14	2.885	10571	1.48	4.7

Table No. 2: Linearity table of acebrophylline and N-acetylcysteine.

Acebrophylline		N-Acetylcysteine	
Conc (µg/mL)	**Peak area**	**Conc (µg/mL)**	**Peak area**
0	0	0	0
12.5	14932	75	315596
25	29436	150	635244
37.5	43066	225	961915
50	57193	300	1274645
62.5	72517	375	1582450
75	86607	450	1898400

Table No. 3: System precision table of acebrophylline and N-acetylcysteine

Sr. No.	Area of Acebrophylline	Area of N-Acetylcysteine
1.	56777	1280666
2.	56887	1284229
3.	57428	1276856
4.	57570	1277965
5.	56981	1274966
6.	57010	1272319
Mean	57109	1277834
S.D	316.2	4207.9
% RSD	0.6	0.3

Table No. 4: Accuracy study of acebrophylline and acetylcysteine

% level	Amount Spiked (µg/ml)	Amount recovered (µg/ml)	% recovery	Mean % Recovery	Amount Spiked (µg/ml)	Amount recovered (µg/ml)	% recovery	Mean % Recovery
50%	25	24.84	99.37	99.84%	150	149.1	99.4	99.58%
	25	24.93	99.72		150	149.6	99.7	
	25	25.24	100.97		150	150.1	100.0	
100%	50	49.75	99.50		300	299.1	99.7	
	50	50.00	100.00		300	297.7	99.2	
	50	49.64	99.29		300	298.6	99.5	
150%	75	74.86	99.82		450	449.3	99.8	
	75	74.91	99.88		450	447.7	99.5	
	75	75.00	100.00		450	446.6	99.3	

Table No. 5: LOD and LOQ value of acebrophylline and N-acetylcysteine

Molecule	LOD(mcg/ml)	LOQ(mcg/ml)
Acebrophylline	0.42	0.23
N-Acetylcysteine	1.27	0.68

Table No. 6: Robustness data for acebrophylline and N-acetylcysteine.

Sr. No.	Condition	%RSD of Acebrophylline	%RSD of N-Acetylcysteine
1	Flow rate (-) 0.9ml/min	0.5	0.3
2	Flow rate (+) 1.1ml/min	0.6	0.2
3	Mobile phase (-) 65B:35A	0.2	0.9
4	Mobile phase (+) 55B:45A	0.1	0.3
5	Temperature (-) 25°C	0.3	0.6
6	Temperature (+) 35°C	0.4	0.5

B: Buffer and A: Acetonitrile

Table No. 7: Degradation data of acebrophylline and N-acetylcysteine

Type of degradation	Acebrophylline			N-Acetylcysteine		
	Peak area	% of drug recovered	% of drug degraded	Peak area	% of drug recovered	% of drug degraded
Acid	53991	94.16	5.84	1214176	94.83	5.17
Base	54232	94.58	5.42	1223675	95.57	4.43
Peroxide	54320	94.74	5.26	1232246	96.24	3.76
Thermal	56300	98.19	1.81	1250355	97.65	2.35
UV	56621	98.75	1.25	1253217	97.88	2.12
Neutral	56954	98.75	1.25	1270132	99.20	0.80

Table No. 8: Analysis of tablets containing acebrophylline and N-acetylcysteine in combination (n = 3)

Tablet	Drug (mg/tab)	% Drug obtained ± SD	Std error of estimation
Pulmoclear tablets	Acebrophylline (100mg) N-acetylcysteine (600 mg)	99.38 ± 0.15 98.31 ± 0.16	0.109 0.114

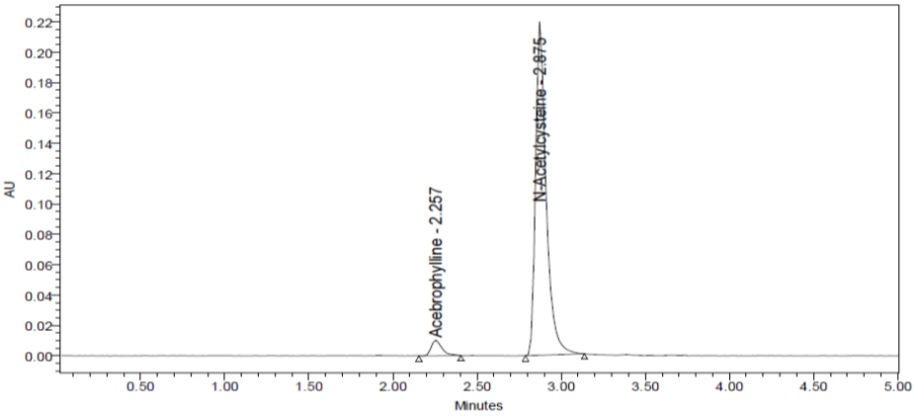

Figure No. 3 a: Standard Chromatogram of acebrophylline and acetylcysteine (showing retention times at 2.2 and 2.8 minutes, respectively)

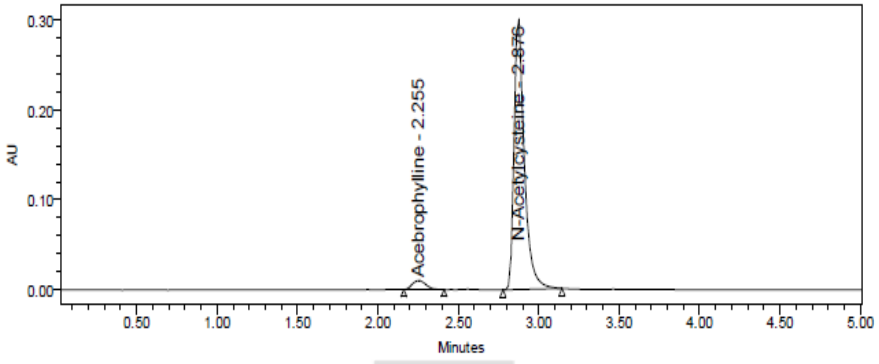

Figure No. 3 b: Sample Chromatogram of acebrophylline and acetylcysteine (showing retention times at 2.2 and 2.8 minutes, respectively)

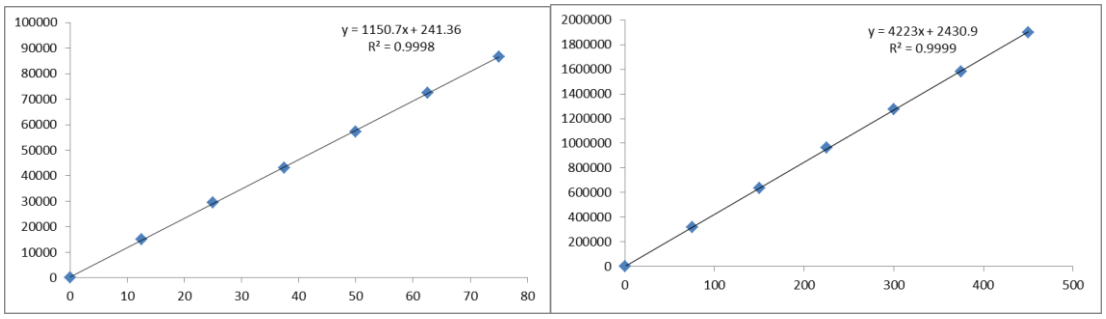

Figure No. 4: Calibration curves of acebrophylline and acetylcysteine

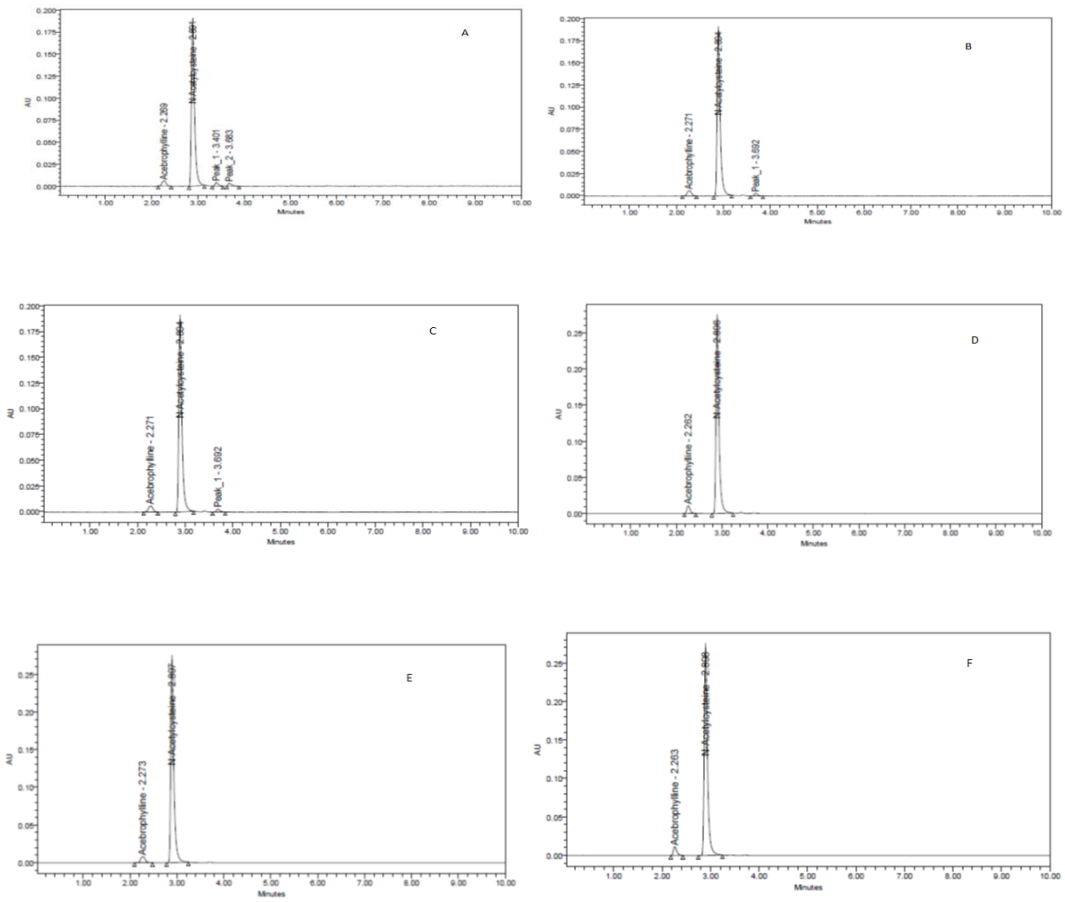

Figure No. 5: Forced degradation chromatograms of acebrophylline and acetylcysteine (A: Acid degradation, B: Base degradation, C: Peroxide degradation, D: Thermal degradation, E: UV degradation, F: Neutral degradation)