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DEVELOPMENT AND VALIDATION OF RPHPLC METHOD FOR QUANTIFICATION OF N-ACETYLCYSTIENE AND ACEBROPHYLLINE IN BULK AND ITS PHARMACEUTICAL FORMULATION

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ABSTRACT

In the present work, a simple, precise, accurate and cost effective extractive RP-HPLC method has been developed for the simultaneous estimation of the Acebrophylline and N-Acetylcysteine in Tablet dosage form. Chromatogram was run through Ascentis C18 150 x 4.6 mm, 5μ. Mobile phase containing Buffer OPA: Acetonitrile taken in the ratio 40:60 was pumped through column at a flow rate of 1.0ml/min. Buffer used in this method was OPA. Temperature was maintained at 30°C. Optimized wavelength selected was 225nm. Retention time of Acebrophylline and N-Acetylcysteine were found to be 2.855min and 2.280. %RSD of the Acebrophylline and N-Acetylcysteine were and found to be 0.7 respectively. %Recovery was obtained as 99.13% and 100.72% for Acebrophylline and N-Acetylcysteine respectively. LOD, LOQ values obtained from regression equations of Acebrophylline and N-Acetylcysteine were 0.10, 0.30 and 0.28, 0.86 respectively. Regression equation of Acebrophylline is $y = 45305x + 13941$ and $y = 33545x + 5956.8$ of N-Acetylcysteine. Retention times were decreased, so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.

Key Words: Acebrophylline, N-Acetylcysteine, RP-HPLC

INTRODUCTION:

A wide variety of challenges is encountered while developing the methods for different drugs depending on its nature and properties. Different physico- chemical methods (Sharma, 2007) are used to study the physical phenomenon that occurs as a result of chemical reactions. Among the physico-chemical methods, the most important are optical (refractometry, polarimetry, emission and fluorescence methods of analysis), photometry (photocolorimetry and spectrophotometry covering UV-Visible, IR Spectroscopy and nepheloturbidimetry) and chromatographic (column, paper, thin layer, gas liquid and high performance liquid chromatography) methods. Methods such as nuclear magnetic resonance (NMR) and Para magnetic resonance (PMR) are becoming more and more popular. The combination of mass spectroscopy (MS) with gas chromatography is one of the most powerful tools available. The chemical methods include the gravimetric and volumetric procedures which are based on complex formation; acid-base, precipitation and redox reactions. Titrations in non-aqueous media and complexometry have also been used in pharmaceutical analysis.

❖ The aim of present study was to develop and validate RP-HPLC method for quantification of N-Acetylcystiene and acebrophylline in bulk and its pharmaceutical formulation.

Method validation

Validation of an analytical method is the " A documented program ,which provide a high degree of assurance that a specific process will consistency produce, a product meeting its predetermined specification and quality attribute". Validation is concerned with assuring a measurement process produces valid measurement. Results from method validation can be used to judge the quality, reliability and consistency ofanalytical results.[9,10]

Aim and Objective of Present Work

AIM:

- To develop the RP-HPLC method for the combination of N-Acetylcysteine and acebrophylline.
- To validate the developed method and apply the same method for the marketed products.

Differences in HPLC instrumentation, laboratory equipment and reagent sources and variations in the skills and background of personnel may require specific features in the HPLC method. In addition, the development of different formulations of the same drug with varying strengths or physical forms may require flexibility in method procedures.

Method validation study include system suitability, linearity, precision, accuracy, specificity, robustness, limit of detection, limit of quantification and stability of samples, reagents, instruments.

OBJECTIVE:

- To develop a simple, economic, precise, accurate, and eco-friendly method of RP-HPLC for the combination of N-Acetylcysteine and acebrophylline.

E NEED TO PERFORM THIS ANALYTICAL METHOD INSTEAD OF EXISTING METHODS

- To provide alternative method is required for regulatory compliance.
- For providing sufficient reliability and sensitivity
- For reduction of cost and time

PLAN OF WORK:

The plan of work is designed by following some simple and effective steps. They are as follows,

1. Selection of the drugs
2. Selection of preliminary parameters for the chosen drugs
3. Selection of the stationary phase
4. Selection of the mobile phase
5. Selection of suitable wavelength
6. **Drug Profile:**

Introduction of N-Acetylcysteine

Description: Acetylcysteine. Acetylcysteine, also known as N-acetylcysteine (NAC), 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.

Structure:

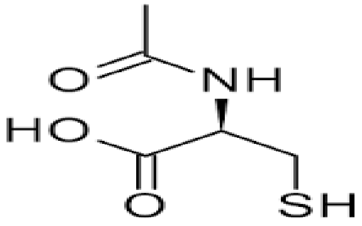

Fig. 1. Structure Of N-Acetylcysteine

Introduction of Acebrophylline

Description: Acebrophylline is an effective bronchodilator medicine used to treat blockage, swelling, and irritation associated with bronchial asthma and chronic obstructive pulmonary disease (COPD) in adults.

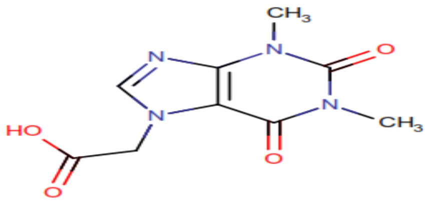

Fig. 2. Structure Of Acebrophylline

EXPERIMENT

Material and Methods-

N-Acetylcystiene API, Acebrophylline API, Pulmonuclear tablet combination of N-Acetylcystiene and acebrophylline from BMR chemicals private limited.

HPLC grade distilled water, Ortho phosphoric acid, Acetonitrile, Methanol-RANKEM CHEMICALS WITH GR GRADE.

Diluent -

The mobile phase was used as a diluent.

Preparation of Standard stock solutions: Accurately weighed 60mg of N-Acetylcysteine, 10mg of Acebrophylline and transferred to 50ml volumetric flask and3/4 th of diluents was added to these flasks and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution. (1200µg/ml of N-Acetylcysteine and200µg/ml of Acebrophylline)

Preparation of Standard working solutions (100% solution): 1ml from each stock solution was pipette out and taken into a 10ml volumetric flask and made up with diluent. (120µg/ml N-Acetylcysteine of and 20µg/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 tablet was transferred into a 500ml volumetric flask, 50ml of diluents was added and sonicated for 25 min, further the volumewas made up with diluent and filtered by HPLC filters (1200µg/ml of N-Acetylcysteine and 200µg/ml of Acebrophylline).

Preparation of Sample working solutions (100% solution):1ml of filtered sample stock solution was transferred to 10ml volumetric flask and made up with diluent. (120µg/ml of N-Acetylcysteine and20µg/ml of Acebrophylline)

Preparation of buffer:

0.1%OPA Buffer: 1ml of ortho phosphoric acid was diluted to 1000ml with HPLC grade water.

Selection of wavelength:

Wavelength was selected by scanning the standard solutions of both the drugs over 200nm to 400nm. Both the components show reasonably good response at 225nm. Therefore, wavelength 225nm was selected for further studies.

Selection of Diluent: Based up on the solubility of the drugs, diluent was selected, Acetonitrile and Water taken in the ratio of 50:50

METHOD VALIDATION

Validation Parameters:

1**. Linearity:**

The linearity of a method is a measure of how well a calibration plot of response vs. concentration approximates a straight line. Linearity can be assessed by performing single measurements at several analyte concentrations. The data is then processed using a linear least-squares regression. The resulting plot slope, intercept and correlation coefficient provide the desired information on linearity.

2. Precision:

Precision can be defined as "The degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogenous sample". A more comprehensive definition proposed by the International Conference on Harmonization (ICH) divides precision into three types:

Repeatability: Repeatability is the precision of a method under the same operating conditions over a short period of time.

Intermediate precision: Intermediate precision is the agreement of complete measurements (including standards) when the same method is applied many times within the same laboratory

Reproducibility: Reproducibility examines the precision between laboratories and is often determined in collaborative studies or method transfer experiments.

3. Accuracy:

The accuracy of a measurement is defined as the closeness of the measured value to the true value. In a method with high accuracy, a sample (whose "true value" is known) is analyzed and the measured value is identical to the true value. Typically, accuracy is represented and determined by recovery studies. There are three ways to determine accuracy:

- Comparison to a reference standard
- Recovery of the analyte spiked into blank matrix or
- Standard addition of the analyte.

It should be clear how the individual or total impurities are to be determined. For e.g., Weight / weight or area percent in all cases with respect to the major analyte.

4. Specificity / selectivity:

The terms selectivity and specificity are often used interchangeably. According to ICH, the term specific generally refers to a method that produces a response for a single analyte only while the term selective refers to a method which provides responses for a number of chemical entities that may or may not be distinguished from each other. If the response is distinguished from all other responses, the method is said to be selective. Since there are very few methods that respond to only one analyte, the term selectivity is usually more appropriate. The analyte should have no interference from other extraneous components and be well resolved from them. A representative chromatogram or profile should be generated and submitted to show that the extraneous peaks either by addition of known compounds or samples from stress testing are baseline resolved from the parent analyte.

5. Robustness:

The concept of robustness of an analytical procedure has been defined by the ICH as "a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters". A good practice is to vary important parameters in the method systematically and measure their effect on separation. The variable method parameters in HPLC technique may involves flow rate, column temperature, sample temperature, pH and mobile phase composition.

6. Limit of detection:

Limit of detection (LOD) is the lowest concentration of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. With UV detectors, it is difficult to assure the detection precision of low level compounds due to potential gradual loss of sensitivity of detector lamps with age or noise level variation by detector manufacturer. At low levels, assurance is needed that the LOD and LOQ limits are achievable with the test method each time. With no reference standard for a given impurity or means to assure detect ability, extraneous peak(s) could "disappear / appear." A crude method to evaluate the

feasibility of the extraneous peak detection is to use the percentage claimed for LOD from the area counts of the analyte. Several approaches for determining the LOD are possible, depending on whether the procedure is a non-instrumental or instrumental.

- Based on visual evaluation
- Based on signal-to-noise
- Based on the standard deviation of the response and the slope

The LOD may be expressed as:

$$LOD = 3.3\,\sigma / S$$

Where, σ = Standard deviation of Intercepts of calibration curves

S = Mean of slopes of the calibration curves. The slope S may be estimated from the calibration curve of the analyte.

7. Limit of quantitation:

Limit of quantitation (LOQ) is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. Several approaches for determining the LOQ are possible depending on whether the procedure is a non-instrumental or instrumental.

- Based on visual evaluation
- Based on signal-to-noise Approach
- Based on the standard deviation of the response and the slope

The LOQ may be expressed as:

$$LOQ = 10\,\sigma / S$$

Where,

σ = Standard deviation of Intercepts of calibration curves

S = Mean of slopes of the calibration curves.

Table.1. Characteristics to be validated in HPLC

Characteristics	Acceptance Criteria
Accuracy/trueness	Recovery98-102% (individual)
Precision	RSD <2%
Repeatability	RSD <2%
Intermediate Precision	RSD <2%
Specificity/ Selectivity	No interference
Detection Limit	S/N >2 or3
Quantitation Limit	S/N >10
Linearity	Correlation coefficient R^2>0.999
Range	80 –120 %

RESULT AND DISCUSSION

- A new RP-HPLC method was developed for the simultaneous estimation of N-Acetylcysteine and acebrophylline by trial and error method i.e., by changing column and mobile phase.

- UV overlain spectra of N-Acetylcysteine and acebrophylline shows that both the drugs absorbs appreciably at 225nm, so 225nm was selected as the detection wavelength in liquid chromatography.

- Optimization of mobile phase was performed based on resolution, asymmetric factor and peak area obtained.

- Different mobile phases were tried but, satisfactory separation, well resolved and good symmetrical peaks were obtained with the mobile phase OPA: Acetonitrile (40:60).

- The retention time of NAC was found to be 2.280 min and that of ACB was found to be 2.855 min respectively.

- Resolution between NAC and ACB was found to be 0.7% which indicate good separation of both the compounds.

- The asymmetric factor for NAC and ACB was found to be 1.428 and 1.423.

- The calibration curves for NAC and ACB was obtained by plotting the respective peak areas versus their concentration over the range of 50-180µg/ml and 5-30µg/ml with correlation coefficient(r^2)= 0.999 which indicates good correlation exist between conc. and response.

- Detection limit for NAC and ACB was 0.28 and 0.10µg/ml and quantitation limit was 0.86µg/ml and 0.3µg/ml, which suggest that a nano gram quantity of both the compounds can be estimated accurately, the low values indicates that the method is sensitive.

- The results for stability studies revealed that the retention time and peak area of NAC and ACB remained almost unchanged and no significant degradation was observed within the indicated period.

- The %Recovery was obtained as 99.13% and 100.72% for ACB and NAC respectively. The % RSD was found to be less than 2%, which shows that the method is precise.
- The proposed liquid chromatographic method was applied for the determination of NAC and ACB in tablet formulations and the assay values for both the drugs were comparable with the corresponding labeled amount.
- All the validation parameters results for the combination of N-Acetylcystiene and acebrophylline are mentioned in the table.

Table. 2. Validation Parameters data

Parameters	Acebrophylline	N-Acetylcysteine	LIMIT
Linearity Range(µg/ml)	5-30 µg/ml	30-180 µg/ml	R< 1
Regression coefficient	0.999	0.999	
Slope(m)	45305	4223	
Intercept(c)	13941	33545	
Regression equation (Y=$mx + c$)	y =45305.x +13941	y = 33545x + 5956.3	
Assay (% mean assay)	99.27%	99.57%	90-110%
Specificity	Specific	Specific	No interference of any peak
System precision %RSD	0.5	0.6	NMT 2.0%
Method precision %RSD	0.7	0.7	NMT 2.0%
Accuracy %recovery	99.13%	100.72%	98-102%

LOD		0.10	0.28	NMT 3
LOQ		0.30	0.86	NMT 10
Robustness	FM	0.6	0.4	%RSD NMT 2.0
	FP	0.2	0.4	
	MM	0.8	1.3	
	MP	0.9	0.7	
	TM	0.9	0.8	
	TP	0.9	0.7	

Table.3. Instrumentation

INSTRUMENT USED	COMPANY
HPLC 2695 system	WATERS
UV spectrophotometer	PG T60
Digital electronic balance	DENVER ELECTRONICS
Ultra sonicator	BVK ENTERPRISES
pH meter	

Chromatographic conditions

Mobile phase : OPA: Acetonitrile (40:60)
Flow rate : 1.0ml/min
Column : Ascentis C_{18} (4.6 x 150mm, 5µm)
Detector wave length : 225nm
Column temperature : 30°C
Injection volume : 10µL
Run time : 10 min
Diluent : Water and Acetonitrile in the ratio 50:50

Table. 4. Accuracy of N-Acetylcysteine

% LEVEL	AMOUNT TAKEN µg/mL	AMOUNT SPIKED	AMOUNT RECOVERED	% RECOVERY	MEAN %RECOVERY
50%	120	60	183.36	101.87	100.34%
	120	60	181.327	100.74	
	120	60	177.146	98.41	
100%	120	120	238.569	99.4	100.5%
	120	120	241.707	100.71	
	120	120	243.366	101.4	
150%	120	180	305.927	101.98	101.32%
	120	180	302.273	100.76	
	120	180	303.702	101.23	

Table.5. Accuracy of Acebrophylline

% LEVEL	AMOUNT TAKEN µg/mL	AMOUNT SPIKED	AMOUNT RECOVERED	% RECOVERY	MEAN %RECOVERY
50%	20	10	30.111	101.37	101.03%
	20	10	30.456	101.52	
	20	10	30.063	100.21	
100%	20	20	40.188	100.47	100.12%
	20	20	39.604	99.04	
	20	20	40.34	100.85	
150%	20	30	50.463	100.94	100.06%
	20	30	49.563	99.13	
	20	30	50.066	100.13	

Table. 6. System precision of Acebrophylline and N-Acetylcysteine

S. No	Area of Acebrophylline	Area of N-Acetylcysteine
1.	918686	4043221
2.	918284	4087891
3.	916869	4058297
4.	924941	4029594
5.	909503	4061933
6.	919243	4087812
Mean	917921	4061458
S.D	4974.2	23457.9
%RSD	0.5	0.6

Table. 7. Repeatability of Acebrophylline and N-Acetylcysteine

S. No	Area of Acebrophylline	Area of N-Acetylcysteine
1.	911247	4063209
2.	908454	4053333
3.	915791	4098738
4.	921275	4017469
5.	903916	4088506
6.	917554	4040395
Mean	913040	4060275
S.D	6375.8	30201.9
%RSD	0.7	0.7

Table.8. Intermediate precision of Acebrophylline and N-Acetylcysteine

S. No	Area of Acebrophylline	Area of N-Acetylcysteine
1.	918686	4043221
2.	918284	4087891
3.	916869	4058297
4.	924941	4029594
5.	909503	4061933
6.	919243	4087812
Mean	917921	4061458
S.D	4974.2	23457.9
%RSD	0.5	0.6

Table.9. Linearity Data of Acebrophylline and N-Acetylcysteine

Acebrophylline		N-Acetylcysteine	
Conc (μg/mL)	Peak area	Conc (μg/mL)	Peak area
0	0	0	0
5	242381	30	1061194
10	492293	60	1994923
15	685116	90	2956215
20	920941	120	4087434
25	1142880	150	5017766
30	1371032	180	6057273

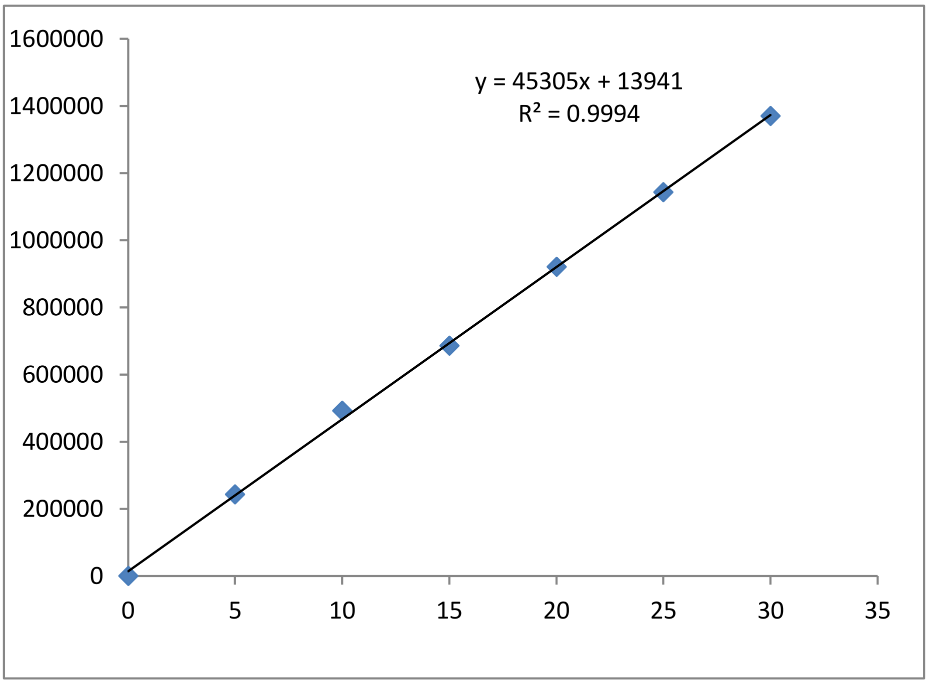

Fig.3. Observations of Standard Calibration Curve of Acebrophylline

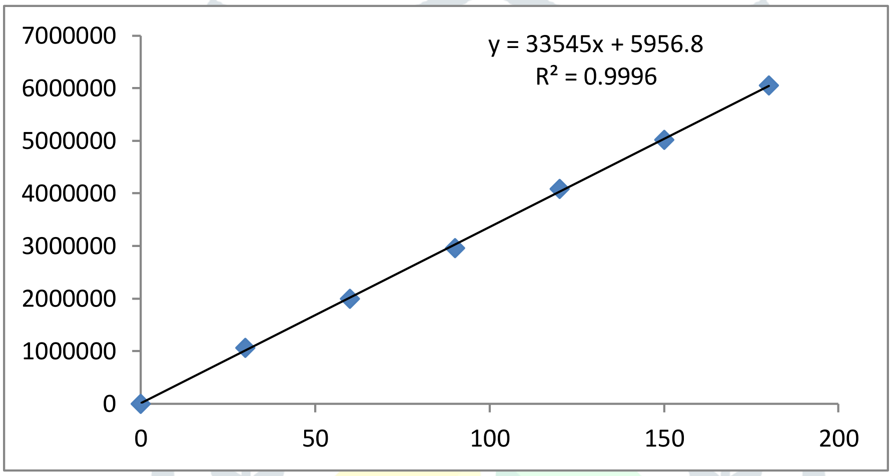

Fig .4. Observations of Standard Calibration Curve of N-Acetylcysteine

Table.10. Robustness data of Acebrophylline and N-Acetylcysteine

S.no	Condition	%RSD of Acebrophylline	%RSD of N-Acetylcysteine
1	Flow rate (-) 0.9ml/min	0.6	0.4
2	Flow rate (+) 1.1ml/min	0.2	0.4
3	Mobile phase (-) 65B:35A	0.8	1.3
4	Mobile phase (+) 55B:45A	0.9	0.7
5	Temperature (-) 25°C	0.9	0.8
6	Temperature (+) 35°C	0.9	0.7

Table.11. Assay Data of Acebrophylline

S.no	Standard Area	Sample area	% Assay
1	918686	911247	99.07
2	918284	908454	98.77
3	916869	915791	99.57
4	924941	921275	100.16
5	909503	903916	98.28
6	919243	917554	99.76
Avg	917921	913040	99.27
Std± **N=6**	4974.2	6375.8	0.7
%RSD	0.5	0.7	0.7

Table.12. Assay Data of N-Acetylcysteine

S.no	Standard Area	Sample area	% Assay
1	4043221	4063209	99.64
2	4087891	4053333	99.40
3	4058297	4098738	100.51
4	4029594	4017469	98.52
5	4061933	4088506	100.26
6	4087812	4040395	99.08
Avg	4061458	4060275	99.57
Std± **N=6**	23457.9	30201.9	0.74
%RSD	0.6	0.7	0.7

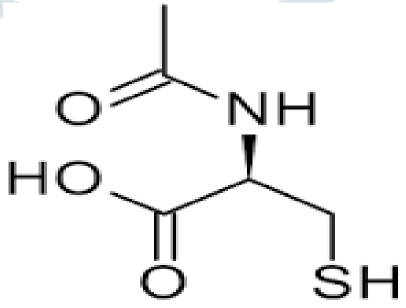

Fig.5. Structure of Acebrophylline

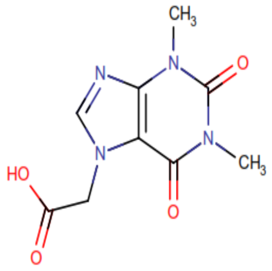

Fig.6. Structure of N-Acetylcysteine

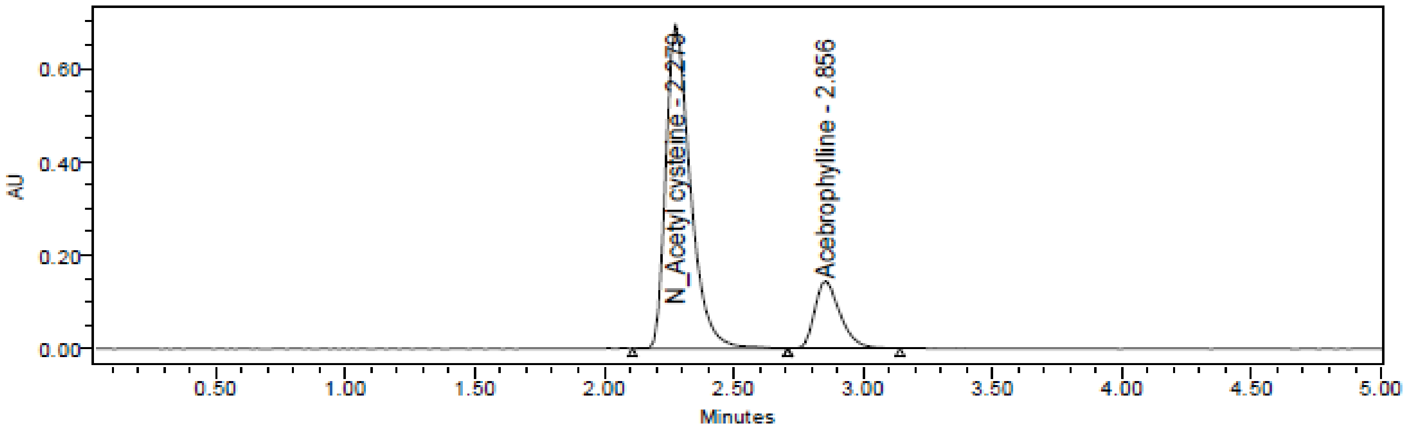

Fig.7. Chromatogram obtained by tablet formulation of Acebrophylline and N-Acetylcysteine

Discussion

Analysis of bulk and tablet formulation was done and the results obtained within acceptable limits. The results obtained for validation study were within the limit specified by the ICH guidelines and hence the method was found to be linear, precise.

The results of recovery study were within ICH limits, thusindicating the accuracy of method.

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