



Received on 20 October, 2016; received in revised form, 14 December, 2016; accepted, 16 December, 2016; published 01 May, 2017

A NOVEL STABILITY INDICATING RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF N-ACETYLCYSTEINE AND AMBROXOL IN COMBINED TABLET DOSAGE FORM

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Keywords:

N-Acetyl-L-cysteine, Ambroxol Hydrochloride, RP-HPLC, Mucolytic

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ABSTRACT: A new, simple, sensitive, precise, accurate and stability indicating RP-HPLC method has been developed for the simultaneous determination of N-Acetyl-L-cysteine and Ambroxol Hydrochloride in combined tablet formulation. The combination is mainly used as mucolytic. The study aimed at developing and validating a method which can be used easily and effectively for determination of given drugs in various formulations. The analysis was carried out at 220 nm and the range was obtained as 3-18 µg/ml for AMB and 20-120 µg/ml for NAC. Recovery studies indicated that the drug recovery from the formulation was within acceptable limits. The developed method was statistically evaluated. The %RSD values reported were less than 2 all through the validation which indicates variations in the results are minimal. Thus the proposed method of analysis was found to be simple, sensitive, accurate, precise, rapid and also economical and can be applied successfully for simultaneous estimation of both N-Acetyl-L-cysteine and Ambroxol Hydrochloride in bulk and combined tablet formulation.

INTRODUCTION: N-Acetyl-L-cysteine (NAC), ((2R)-2-(acetylamino)-3- sulfanylpropanoic acid), (Fig. 1) is mainly used as a mucolytic for the treatment of bronchitis, pulmonary diseases and other respiratory diseases ¹. The viscosity of pulmonary secretions is reduced by depolymerizing the muco-polysaccharides. It is also used as an antidote in Paracetamol poisoning. NAC has been proven effective as anti-inflammatory agent ². Recent studies suggest that NAC is a powerful antioxidant and a potential therapeutic agent in the treatment of HIV infections, heavy metal toxicity, cancer, heart disease, and other diseases characterized by free radical and oxidant damage ³.

Various spectrophotometric, colorimetric, chemiluminescence, HPLC, fluorimetric, electrochemical, mass spectrometric and UV detectors and GC have been widely applied as the primary methods of detection in biological samples and pharmaceuticals ⁴.

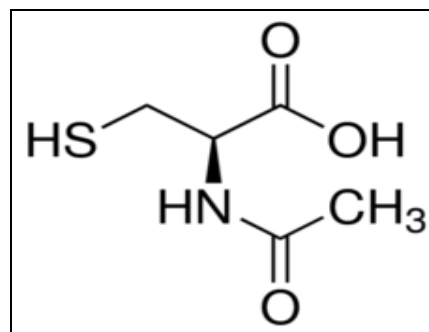


FIG. 1: CHEMICAL STRUCTURE OF N-ACETYL-L-CYSTEINE

Ambroxol hydrochloride (AMB) is chemically, trans-4-((2-amino-3, 5-dibromobenzyl) amino) cyclohexanol hydrochloride (Fig. 2).

<p>QUICK RESPONSE CODE</p>	<p>DOI: 10.13040/IJPSR.0975-8232.8(5).2161-67</p> <hr/> <p>Article can be accessed online on: www.ijpsr.com</p>
<p>DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.8(5).2161-67</p>	

AMB is effective in treating bronchial hyper-reactivity and acts as a mucolytic along with cough suppressant action⁵.

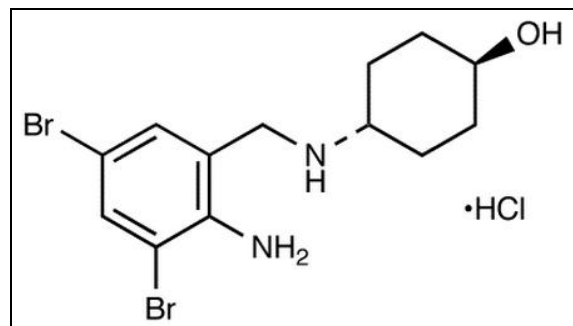


FIG. 2: CHEMICAL STRUCTURE OF AMBROXOL HYDROCHLORIDE

AMB is a metabolite of Bromhexine which primarily acts a mucolytic agent and expectorant for the treatment of acute and chronic disorders of excess or thick mucus. For the determination of AMB, there are various reported methods like HPLC, GC, LC-MS and Capillary electrophoresis.

Objective of Study: Both the drugs are official in IP and USP. Extensive literature survey revealed that there are RP-HPLC and UV spectrophotometric methods available for determination of NAC and AMB individually, or in combination with other drugs, but no method is reported for simultaneous estimation of NAC and AMB in combined dosage form using RP-HPLC method. The study was thus performed with an aim to develop a simple, economic, sensitive, rapid, accurate and precise method for the determination of NAC and AMB in combined tablet dosage form.

Formulation details: NAC and AMB containing AMBRONAC tablets, manufactured by Tablets (India) Ltd. were used. The content of the tablets were 200mg of NAC (USP) and 30mg of AMB (IP) in each tablet.

MATERIALS AND METHODS:

Instrumentation: The experimental part of this research was carried out on Shimadzu HPLC using the software 'Spinchrom'. All the glasswares used were of 'A' grade.

Reagents and chemicals: The active pharmaceutical ingredients in highly pure form were obtained as a gift sample from Kores (India) Pharmaceutical Division Pvt. Ltd., Roha, Raigad

(AMB) and Pallav Chemicals, Mumbai (NAC). Tablet dosage form consisting NAC (200mg) and AMB (30mg) named AMBRONAC, manufactured and marketed by Tablets (India) Ltd. was obtained. All other chemicals required of analytical grade and solvents for mobile phase of HPLC grade, were procured from S.D. Fine Chemicals, Mumbai for the research.

TABLE 1: INSTRUMENTS USED

Sr. No.	Name of the instrument	Company
1	HPLC	Shimadzu
2	Weighing balance	CA 123
3	pH meter	PCI analytics
4	Sonicator	Expo Hi-Tech

Preparation of Potassium dihydrogen orthophosphate (pH 3.5): Accurately weighed 2.72 g of Potassium dihydrogen orthophosphate, dissolved in 1000 ml of water and adjusted the pH with o-phosphoric acid to 3.5 ± 0.05 . The solution was filtered using 0.45 μ membrane filter.

Preparation of Mobile Phase: Phosphate buffer was prepared using Potassium dihydrogen orthophosphate (pH to 3.5) with orthophosphoric acid. It was then sonicated and filtered using 0.45 μ membrane filter. Mixture of phosphate buffer (pH 3.5): acetonitrile (70:30 v/v) used as mobile phase.

Preparation of Diluent: Mobile phase was used as a diluent.

Preparation of standard stock solution (NAC 1000 μ g/ml and AMB 150 μ g/ml): About 100 mg of NAC and 15 mg of AMB were accurately weighed and transferred into 100 ml volumetric flask respectively. The solution was then sonicated to dissolve and diluted up to the volume with the diluent and filtered through 0.45 μ membrane filter.

Preparation of standard solution (NAC 100 μ g/ml and AMB 15 μ g/ml): About 1 ml of standard stock solution of NAC and AMB was transferred into 100 ml volumetric flask and diluted up to the volume with diluent and then filtered through 0.45 μ m membrane filter.

Preparation of sample solution (NAC 100 μ g/ml and AMB 15 μ g/ml): Average weight of 10 tablets was determined and tablets were transferred into a 200 ml volumetric flask. About 100 ml diluent was

added and solution was sonicated with intermittent shaking for 20 minutes. The volume was made up using the diluent. Accurately 1 ml of the above solution was transferred into 100 ml volumetric flask and diluted up to the volume with the diluent.

Final method:

Reagents:

1. Potassium dihydrogen orthophosphate (AR grade)
2. Orthophosphoric acid (GR grade)
3. Water (HPLC grade or equivalent)
4. Acetonitrile (HPLC grade)

TABLE 2: CHROMATOGRAPHIC CONDITIONS

Column	Hypersil (C18)
Wavelength	220 nm
Flow Rate	1.0 ml/min
Run time	8 minutes
Injection Volume	20 μ l
Retention time of NAC	About 2.9 minutes
Retention time of AMB	About 5.6 minutes
Mobile Phase	Phosphate buffer (pH 3.5): Acetonitrile (70:30 v/v)

Method Validation:⁶

Specificity: To ensure the absence of interference from blank those are likely to be present in NAC and AMB drug product.

Acceptance criteria: There should be no interference from blank and placebo at the retention time of NAC and AMB.

Accuracy: To evaluate the recovery at each level. Working standards of the drugs were added at three different concentrations (levels) 80 %, 100 % and 120 %. The measurements were done in triplicates.

Acceptance criteria: Individual % recovery of NAC and AMB at each level should be between 98 % - 102% and % RSD should not be more than 2.0 at each level.

Precision: System precision: Prepared and injected six replicates of standard solutions as per the proposed method of analysis into HPLC system. Calculated the mean, SD and % RSD for peak areas of NAC and AMB.

Method precision: Six samples of a single batch of same strength (40/25 mg) of NAC and AMB were analyzed as per the test method. The % assay and % RSD for NAC and AMB in six samples was calculated.

Intermediate precision or Ruggedness: Ruggedness of the method was verified by analyzing six samples of the same batch used for method precision as per proposed method by different analyst, different column serial number on different day and injected into different HPLC system. The % assay, % RSD and cumulative %RSD of twelve samples (six samples of method precision and six samples of intermediate precision) of NAC and AMB in tablets was determined.

Acceptance criteria: % RSD and cumulative % RSD should not be more than 2.0 and % assay should be within the specification limit.

Linearity: Linearity involves selection of minimum 5 concentrations in the order of linearity. Acceptance criteria: Correlation coefficient (R²) should be not less than 0.999.

Range: For range, data shall be considered from linearity and accuracy.

Acceptance criteria: Correlation coefficient (R²) should be not less than 0.999 and individual % recovery of NAC and AMB at each level should be between 98 % - 102 %.

Robustness: Robustness of the method was evaluated by changing the flow rate by ± 10 %, by changing the column temperature by $\pm 5^{\circ}\text{C}$ and by changing the pH of buffer by ± 0.1 units. Acceptance criteria: % RSD should be not more than 2.0.

RESULT AND DISCUSSION: The analytical method validation parameters were determined according to the ICH guidelines^{6, 7}. Before validation of the developed method, analysis of blank solution (only mobile phase), standard solution (mixed standard) and sample solution (tablet mixture) was carried out.

Specificity:

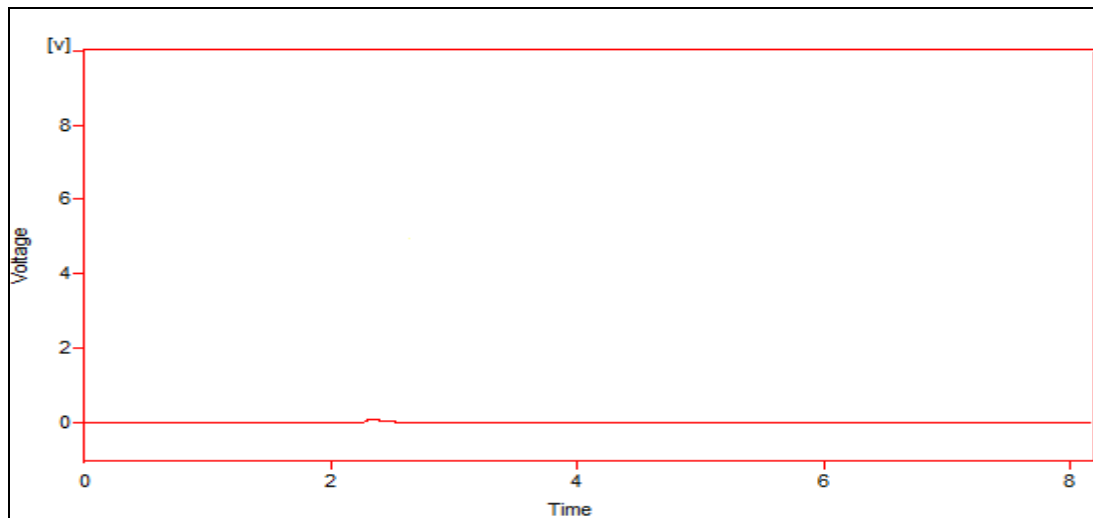


FIG. 3: CHROMATOGRAM FOR BLANK SOLUTION

No peak was observed at the retention times of both the drugs in blank solution and thus the method is specific.

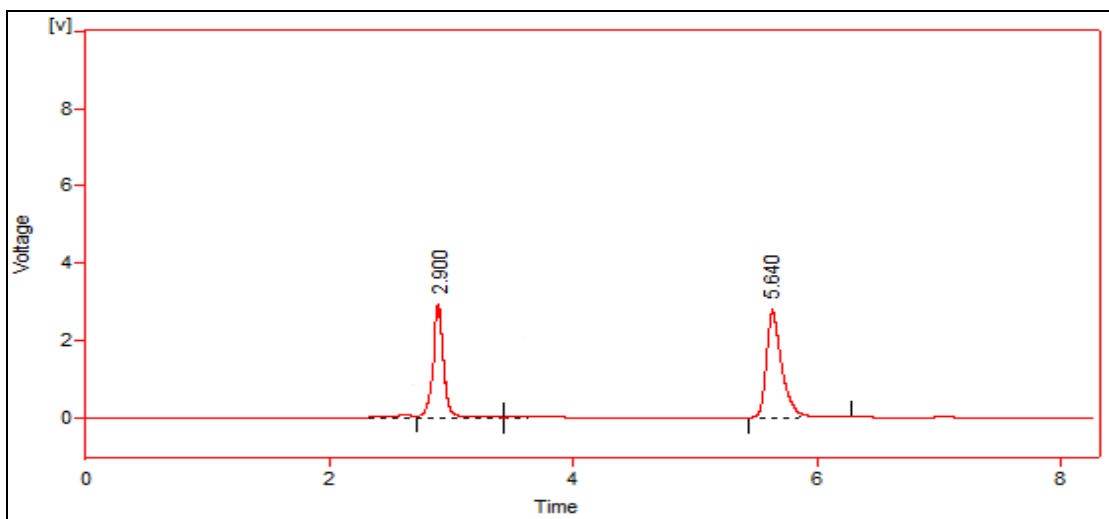


FIG. 4: CHROMATOGRAM FOR STANDARD SOLUTION

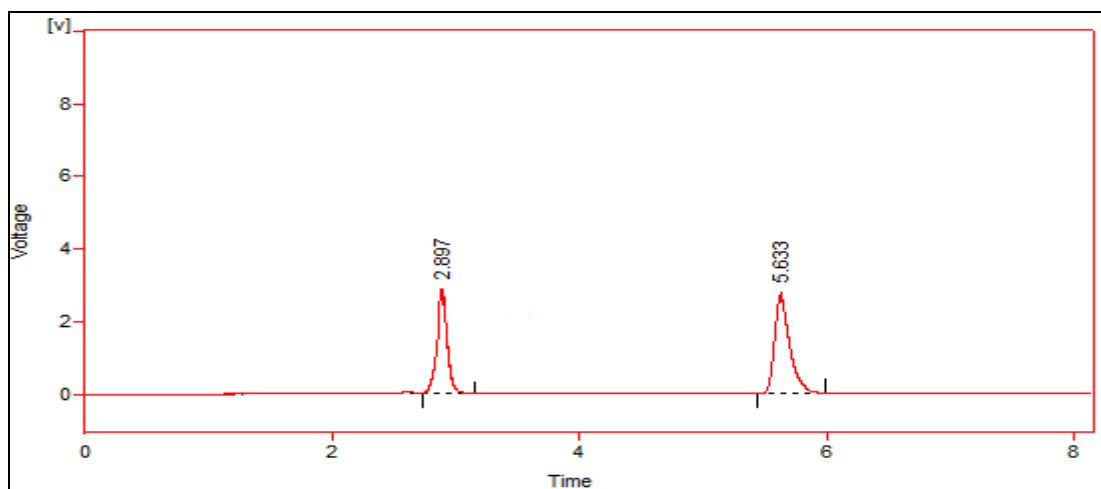


FIG. 5: CHROMATOGRAM FOR SAMPLE SOLUTION

Accuracy: Initially a standard solution was analyzed and then accuracy was determined at three concentrations 80 %, 100 % and 120 %. From the % RSD values of NAC and AMB at all 3 concentrations were considered for the calculations and amount found was compared with the amount added for preparing these concentrations.

TABLE 3: ACCURACY STUDIES

Sr.No.	Level addition (%)	% Recovery*	
		NAC	AMB
1	80	79.43	78.8
2	100	99.44	99.80
3	120	118.03	118.6

*n=3

Precision: Precision was performed in three different levels as system precision, method precision and intermediate precision i.e. ruggedness.

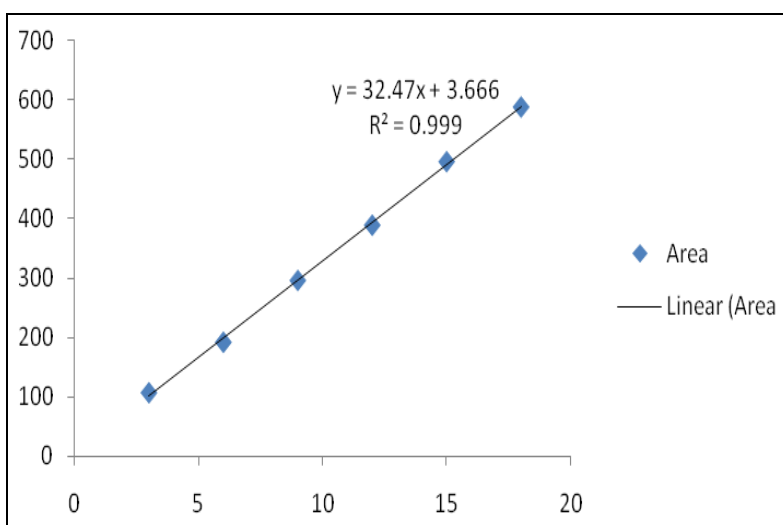
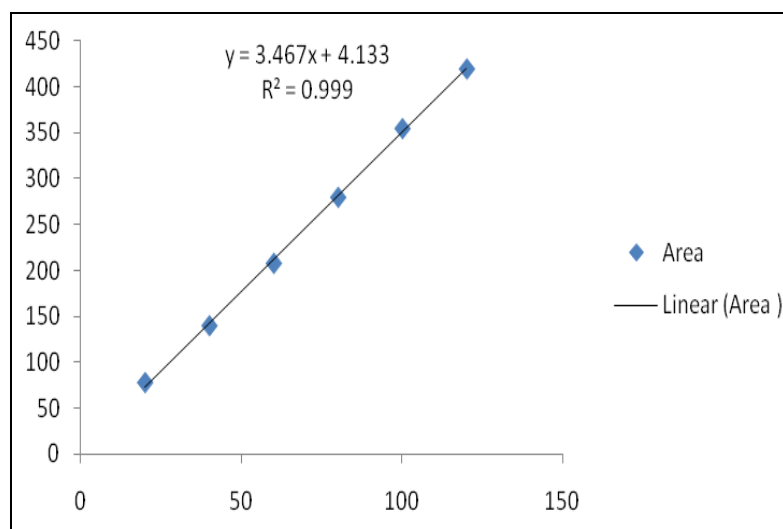
TABLE 4: PRECISION STUDIES

Sr. No.	Precision	NAC (% RSD)	AMB (% RSD)
1	Intraday*	0.12	0.21
2	Interday*	0.61	0.58
3	System*	0.09	0.16
4	Method*	0.1	0.42

*n=6

The % RSD values for all types of precision were found to be within the specified limit. This indicated that the method is rugged and can be used to analyze the formulations containing NAC and AMB effectively.

Linearity: Linearity studies were performed at six different concentrations. The linearity data was calculated on individual drug basis and checked for the R² value.

**FIG. 6: LINEARITY PLOT FOR AMB****FIG. 7: LINEARITY PLOT FOR NAC**

The correlation coefficient (R^2) values for NAC and AMB were found to be 0.999 which were within acceptable limits. This indicated that the method is linear between the concentrations 3 μ g/ml and 18 μ g/ml for AMB and 20 μ g/ml and 120 μ g/ml for NAC.

Range: It was inferred from the data of accuracy and linearity that effective range for the estimation

of NAC and AMB was between the concentration 20 μ g/ml and 120 μ g/ml for NAC and 3 μ g/ml and 18 μ g/ml for AMB.

Robustness: Deliberate variations were made in the method and the % RSD was calculated. Plus flow rate, minus flow rate, plus pH, minus pH, plus temperature and minus temperature were the variations that were analyzed.

TABLE 5: ROBUSTNESS STUDIES (CHANGE IN FLOW RATE)

Sr. No.	Change in flow rate	NAC			AMB		
		Retention time	Area	% RSD	Retention time	Area	% RSD
1	0.9	3.0	356	0.11	5.8	497	0.13
2	1.0	2.9	353	0.09	5.6	501	0.20
3	1.1	2.8	353	0.14	5.6	499	0.21

TABLE 6: ROBUSTNESS STUDIES (CHANGE IN WAVELENGTH)

Sr. No.	Change in wavelength	NAC			AMB		
		Retention time	Area	% RSD	Retention time	Area	% RSD
1	215	2.9	350	0.09	5.8	494	0.22
2	220	2.9	355	0.05	5.9	502	0.22
3	225	2.8	340	0.18	5.9	490	0.15

Small but deliberate variations were made in the method parameters and it was found that % RSD values for all the variations were in acceptable limits. This indicated that the method is robust and

that it can be used within small variations of flow rate and wavelength without having a major effect on the results of % assay values.

System Suitability Test:

TABLE 7: SYSTEM SUITABILITY TEST

Sr. No.	Parameters	NAC	AMB
1	Retention time	2.9	5.6
2	Tailing factor	1.1	1.5
3	Theoretical plate number	5752	10165

Forced Degradation Studies:

TABLE 8: FORCED DEGRADATION STUDY RESULTS

Condition	% Assay	% Degradation w.r.t. control of NAC	% Assay	% Degradation w.r.t. control of AMB
Untreated Sample	99.44	—	99.70	—
Heat-Treated Sample	99.33	0.11	100.92	- 1.22
Acid-Treated Sample	50.6	48.84	99.34	0.36
Base-Treated Sample	61.88	37.56	98.68	1.02
Peroxide Treated Sample	136.01	-36.57	98.45	1.1
UV-Treated Sample	99.00	0.44	99.52	0.18

CONCLUSION: The method was developed and validated which concluded that the method is simple, economic, accurate, robust, precise and rugged. AMB is stable in all the conditions of acid, base, oxidation, heat and photolysis. NAC being an amino acid is not stable and shows some amount of

degradation in acid, base and peroxide. Thus, the proposed method is stability indicating and can be effectively applied for analysis of NAC and AMB in bulk dosage forms as well as in combined tablet dosage form.

ACKNOWLEDGEMENT: The authors are thankful to Oriental College of Pharmacy, Sanpada, Navi Mumbai for providing all the necessary facilities required for the research work. The authors are also thankful to all the teaching and non-teaching staff and the colleagues for their constant support. Hearty thanks to our parents. Also authors are very thankful to Kores India Pharmaceutical Division, Roha and Pallav Chemicals Pvt. Ltd. Mumbai, for providing gift samples.

CONFLICT OF INTEREST: None.

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How to cite this article:

Kinjawadekar V, Boddu S and Ghadge O: A novel stability indicating RP-HPLC method for the simultaneous estimation of n-acetylcysteine and ambroxol in combined tablet dosage form. *Int J Pharm Sci Res* 2017; 8(5): 2161-67. doi: 10.13040/IJPSR.0975-8232.8(5).2161-67.

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