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SIMULTANEOUS ESTIMATION OF N-ACETYL CYSTEINE AND AMBROXOL HYDROCHLORIDE FROM TABLET DOSAGE FORM BY HPLC METHOD

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ABSTRACT

A simple, precise, accurate and rapid RP-HPLC method has been developed for simultaneous estimation of N-Acetylcysteine and Ambroxol Hydrochloride in tablets dosage form. The method was carried out on C18 column (25cm x4.6 mm x 5µm) with a mobile phase consisting of Phosphate buffer solution and Methanol in 70:30 ratio. The flow rate was adjusted to 1.0 ml/minute and detection was carried out at 230 nm. The retention time obtained for N-Acetylcysteine and Ambroxol Hydrochloride was 3.38 and 22.23 minutes respectively. The calibration areas were linear in the concentration range of 40-150 µg/ml for Ambroxol Hydrochloride and 40-150 µg/ml for N-Acetylcysteine. The developed method was svalidated in terms of accuracy, precision, linearity, limit of detection, limit of quantification, ruggedness and solution stability. The proposed method can be used for simultaneous estimation of these two drugs in tablet dosage form.

KEYWORDS: Ambroxol Hydrochloride, N-Acetylcysteine, RP-HPLC, Validation, Tablet dosage form.

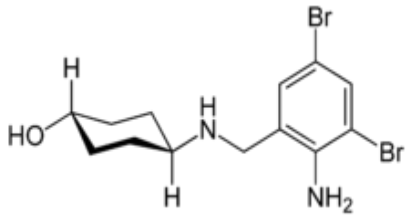
INTRODUCTION

Ambroxol Hydrochloride (Figure.1) is chemically 1({[2-4 Amino-3, 5 dibromophenyl] methyl} amino) cyclohexanol, monohydrochloride, which is a semi synthetic derivative of vasicine obtained from the Indian shrub “Adhatoda Vasica”. It is an expectorant and

mucolytic agent, which is used in the treatment of Bronchial Asthma and Chronic Bronchitis[1]. Ambroxol Hydrochloride also been reported to show cough suppressing and anti-inflammatory properties. Recently the inhibition of nitric oxide dependent activities of soluble guanylate cyclase was suggested as one of the molecular mechanisms of the therapeutic action of Ambroxol Hydrochloride, also used in pulmonary alveolar proteinosis in pulmonary distress and infant respiratory distress syndrome.[2] [3]

N-Acetylcysteine (Figure.2) is chemically ((2R)-2-(acetylamino)-3- sulfanylpropanoic acid) mainly used as a mucolytic agent in bronchitis or pulmonary diseases. It depolymerises mucopolysaccharides, reduces the viscosity of pulmonary secretions[4]. Besides mucolytic effect it also has anti-oxidant and anti-inflammatory effects and is used as an antidote in Paracetamol poisoning [5]. N-Acetylcysteine causes cleavage of disulfide bonds by converting them to two sulfhydryl groups. This action results in the breakup of mucoproteins in lung mucus,reducing their chain lengths and causes thinning of the mucus, facilitating easy removal of the same and therefore improving conditions such as Bronchitis & Flu[6] . A number of Spectrophotometric[7], Colorimetric[8], Chemiluminescence[9], HPLC[10–13], MassSpectroscopy[15][16], Fluorimetric[5][14] and Ultraviolet[17][18] and Gas Chromatography[19] methods have been widely applied as the main methods of detection in pharmaceutical samples. Several liquid chromatographic (LC) methods involve spectrophotometric detection with pre or post column derivatization, where time-consuming extraction or long derivatization steps hamper the studies of these compounds.

Literature survey showed that very few analytical methods have been reported for the estimation of Ambroxol Hydrochloride and N-Acetylcysteine in combination using UV-VIS spectrophotometer, LC-MS, RP-LC, HPLC with Potentiometric detection etc. Fixed combination containing N-Acetylcysteine (200mg) and Ambroxol Hydrochloride (30 mg) is available in the tablet dosage form and only one method was available for estimation but there was no method available for simultaneous estimation of these two actives. So efforts were taken to make available simultaneously evaluating, optimized, simple and cost effective HPLC method for estimation of N-Acetylcysteine and Ambroxol HCl in tablet dosage form as per guidelines laid down by International Conference on Harmonization (ICH)[20].



. HCL

Fig . 1 Ambroxol Hydrochloride

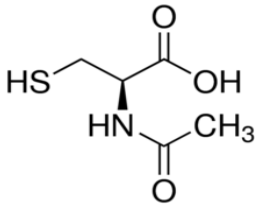


Fig.2 N-Acetylcysteine

EXPERIMENTAL

Materials and Method

Methanol, HPLC grade was procured from Merck (India) Limited. Potassium dihydrogen phosphate AR grade, Triethylamine AR grade, Phosphoric acid AR grade were procured from Fischer scientific, India. Water HPLC grade was obtained from Milli-Q-RO water purification system. Reference standard of Ambroxol Hydrochloride and N-Acetylcysteine were received as gift from Wallace Pharmaceuticals Pvt. Ltd, Goa, India.

Chromatographic separation was performed on HPLC system with following details

System : Thermo Fischer Ultimate 3000

Electronic Balance : LCGC

Sonicator : Spectra Physics

pH Meter: Digisun AS220/X

Injector: Auto sampler

Elution: Isocratic

Column: C18 (250 x 4.6 mm x 5 µm) Cosmosil

Column: Thermostat column compartment

Detector: DAD-300 Diode Array Detector

Software : Chromeleon

Column Temperature : 40°C

Chromatographic conditions

A Cosmosil C18 (250 mm X 4.6 mm X 5µm) column was used at 40°C temperature. Mixed 700 ml of Buffer solution, 300ml of Methanol to make 1000 ml of mobile phase. This was filtered through glass fiber filter (0.45 µ). This was degassed. Flow rate was maintained at 1.0 ml /minute. The elution was observed at 230 nm. Some trials were carried out w. r. t change in the ratio of constituents of the mobile phase like 50:50/40:60/ 30:70/60:40 etc. of buffer solution and Methanol. Injection volume and runtime were 20µl and 30 minutes respectively. In the ratio of 70:30 retention time for N-Acetylcysteine and Ambroxol Hydrochloride observed at about 3.4 minutes and 22.3 minutes respectively. The two peaks were well resolved with good, sharp shape and symmetry.

Preparation of Mobile Phase

Mixed 700 ml of buffer solution, 300ml of Methanol to make 1000 ml of mobile phase. This was filtered through glass fiber filter (0.45 µ) & degassed.

Buffer solution

Dissolved 1.36 g of potassium dihydrogen phosphate in 900 mL of water. Added 2 mL of triethylamine, adjusted to pH 3.0 ±0.05 with diluted phosphoric acid and then diluted to 1000 mL with water. Filtered the solution through 0.45 µ filter .

Preparation of standard stock solution

1. **Ambroxol Hydrochloride standard solution (Solution "A")**

(Concentration: 0.3 mg/mL of Ambroxol hydrochloride) Weighed accurately about 30mg of Ambroxol Hydrochloride working standard and transferred to a 100ml volumetric flask. Added 60ml of mobile phase and dissolved it completely. Made up volume with additional mobile phase. Mixed well.

2. **N-Acetylcysteine standard solution (Solution 'B')**

(Concentration: 1.0 mg/mL of N-Acetylcysteine): Weighed accurately about 50mg of N-Acetylcysteine. Made up volume with additional mobile phase and mixed well. Working standard transferred to a 50 ml volumetric flask. Added 30 ml of mobile phase and dissolved completely. Made up volume with additional mobile phase. Mixed well.

3. **Standard solution**

(Concn: 30 mcg/mL of Ambroxol HCl and 200 mcg/mL of N-Acetylcysteine)

Transferred 5 mL of standard solution (A) and 10 mL of standard solution (B) into a 50 mL volumetric flask. Diluted to volume with diluent and mixed.

4. **Sample solution**

(Concentration 30 mcg/mL of Ambroxol HCl and 200 mcg/mL of N-Acetylcysteine) Weighed and transferred 20 tablets to mortar and pestle, ground and mixed to form uniform powder.Transferred an accurately weighed portion of the tablet powder containing 30 mg (about 300 mg) of Ambroxol hydrochloride into a 100 mL volumetric flask. Added about 60 mL of diluent and sonicated to dissolve for about 15 minutes. Allowed to cool to room temperature. Diluted to volume with diluent and mixed. Filtered the solution through 0.45 µ filter, discarding the first few mL of the filtrate. Diluted 5 mL of the filtrate to 50 mL with diluent and mixed.

Analysis of Marketed product

To determine the content of Ambroxol hydrochloride and N-Acetylcysteine in conventional tablet (Brand name: Pulmobreathe/Marketed product), Label claim: 30 mg Ambroxol Hydrochloride and 200 mg N-Acetylcysteine per tablet. Twenty tablets were weighed. Their mean weight determined and finely powdered. The weight of the tablet triturate equivalent to 30 mg of Ambroxol Hydrochloride and 200 mg of N-Acetylcysteine were transferred into a 100 ml volumetric flask containing 70 ml diluent, sonicated for 30 minutes and diluted up to 100 ml with diluents. Allowed to cool to room temperature. Diluted to volume with diluents and mixed. Filtered the solution through 0.45 µ filter, discarding the first few mL of the filtrate. Diluted 5 mL of the filtrate to 50 mL with diluent and mixed.

Order of injections: Injected 20 µL of the blank (diluent), standard solution (5 replicates), blank (diluents), and sample solutions into the equilibrated system.

RESULTS AND DISCUSSION

Method Development

1) **Solubility:** To arrive at right choice of Mobile Phase and diluents solubility of each compound was checked in all HPLC compatible solvents. Since target of Method Development was to estimate two compounds simultaneously, it was necessary to find a common solvent or diluents in which all compounds will have satisfactory solubility. Additionally the selected diluents should be capable to extract both compounds from tablets dosage form. A detailed and thorough suitability study narrowed down to Phosphate buffer with Methanol in the ratio of 70:30.
2) **Selection of UV detection wavelength:** A detailed review of UV spectrum of two compounds suggested that 230 nm was the most suitable wavelength, which could be employed for detecting all these components.
3) **Selection of working pH range for mobile phase**: Since two active compounds are present in sample matrix, pH of mobile phase plays very vital role in separation. pKa values of all these two compounds were studied to select proper pH of mobile phase. From the study, conclusion was acidic pH would be better choice for separation of two actives. pH of 3.0 ±0.05 was finalized.
4) **Selection of Column:** In a reverse phase chromatographic method development, selection of proper column was one of the key factors of Method Development. In reverse phase chromatographic separation wide range of columns like C8,C18,Cyano, Phenyl etc.

of different make were available, which could be used for separation. Extensive literature survey revealed that in general 150 or 250 mm columns having diameter 4.6 mm and particle size 5 µm had been used for method development. Trials were taken on various columns and came to the conclusion for C18 column with 4.6 mm diameter with 5µm particle size was finalized.

Final Method optimization

Final optimization was done to fix the remaining method parameters like flow rate of mobile phase, column oven temperature, concentration of each compound in standard and sample preparation. Effect of each individual parameter on separation was studied. Typical chromatogram showing separations between two compounds are shown in Fig.5.

Method Validation

The developed method was validated as per ICH (International Conference on Harmonization) guidelines with respect to System suitability, Precision, Specificity, Linearity, Accuracy, Limit of Detection, Limit of Quantification, Ruggedness and Robustness.

Specificity

Specificity is the ability of Analytical Method to indentify and quantify the compounds of interest, without any interface in the presence of impurities or degradants which are likely to be present. Interferences may be either from blank or from placebo with the retention times of Ambroxol Hydrochloride and N-Acetylcysteine . Identification of Ambroxol Hydrochloride, N-Acetylcysteine from sample solution was done by comparing retention time of standard solution of individual components. Peak purity of both actives in sample solution were checked to confirm uniformity of all these peaks using Photo Diode Array Detector (PDA). Compliance of the method of the requirement for blank interference, identification and peak purity tests indicate that method is specific.

Linearity

Linearity shows proportionate response of analyte against concentration of analyte. Linearity of the method was estimated by using five concentrations of each compound within the 50% to 150% range of working concentration. For linearity experiments 49.91 mg to 149.30 mg for Ambroxol Hydrochloride and 49.81 mg to 147.11 mg for N-Acetylcysteine were used. Linearity curves for Ambroxol Hydrochloride and N-Acetylcysteine are as shown in Fig. 3 & Fig.4 respectively.

Linearity- N-Acetylcysteine	
50%	49.81 **µg** /ml
100%	98.35 **µg** /ml
150%	147.11 **µg** /ml

Linearity- Ambroxol HCl	
50%	49.91 **µg** /ml
100%	98.74 **µg** /ml
150%	149.30 **µg** /ml

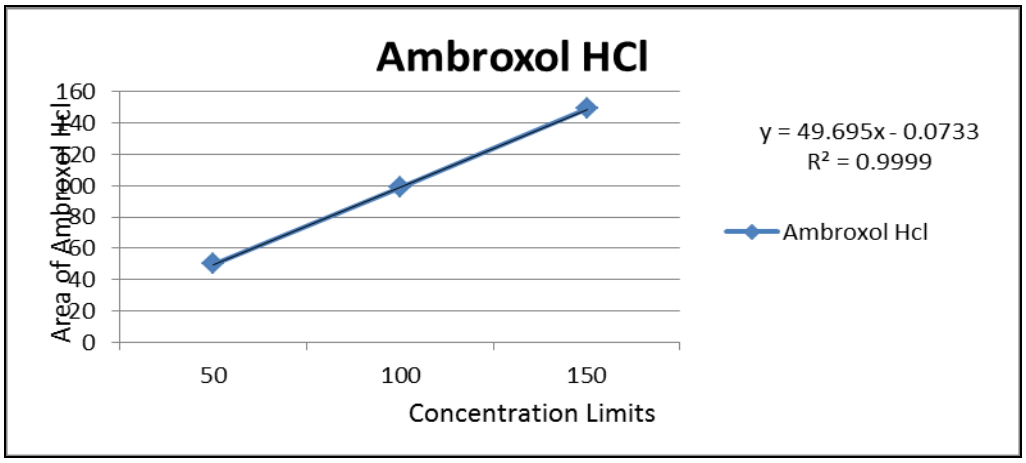


Fig .3 . Linearity curve for Ambroxol HCl

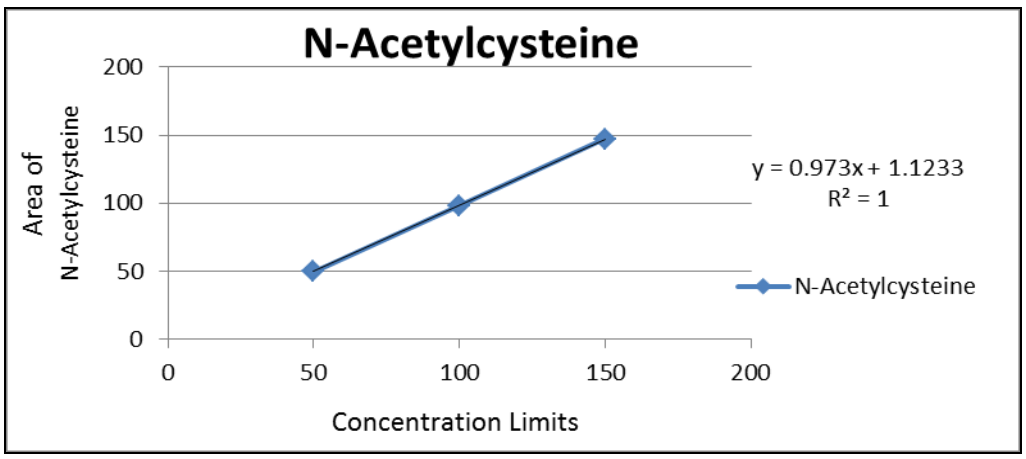


Fig .4 . Linearity curve for N-Acetylcysteine

Accuracy

Accuracy of a method is the closeness of observed values obtained using the method to the true value. Estimation of recovery by standard addition is a sound approach to demonstrate accuracy of the method. During recovery experiment, known amount of reference standard of each compound were spiked into the placebo of the sample at three different levels i.e.50%, 100% and 150% of sample concentration and prepared three samples of each level. These spiked samples along with one control sample were analyzed. The experimental value of each compound obtained for each level was calculated and compared with actual added amount of respective component. Mean accuracy in percentage was calculated for all the three levels.

Precision

Precision of the method was demonstrated by repeatability (Intra- assay Precision) and intermediate Precision (Inter- assay). Six different sample solutions of same concentration were prepared from same uniform sample and analysed against working standard solution. Assay values for each component were calculated and relative standard deviation (RSD) of assay values were evaluated. Very low RSD values indicate closeness of the results. Percent RSD of assay values from six samples were less than 1.0% for each compound indicates that the method is precise or repeatable.

Ruggedness

The intermediate precision was evaluated by preparing six different sample solution of same concentration as prepared in method precision and anlaysed on different days. Percent cumulative RSD of assay results for twelve samples were done. Six samples for method precision and six for intermediate precision were calculated. Percent RSD of assay values of each compound from twelve samples were less than 1.0%. The closeness of assay results and percent RSD values demonstrated that the method is rugged.

Robustness

Robustness is a validation parameter, which shows ability of analytical method to remain unaffected by slight but deliberate changes in method parameters. Robustness was demonstrated by making slight changes in parameters like flow rate (±5%), column temperature (± 2°C) and mobile phase composition (± 5%). Robustness study demonstrates that by making slight but deliberate changes in method parameters, method remains unchanged and gives consistent results. Results of original conditions and altered conditions are comparable.

Solution Stability

The Solution Stability of sample solution was evaluated by comparison of assay value of freshly prepared samples at room temperature for 24 hours. Standard solution and sample solution were prepared as mentioned in chromatographic conditions. Sample solution was analyzed and assay value was calculated against standard solution. Both the solutions were kept at room temperature for 24 hours were reanalyzed against freshly prepared standard solution and assay values were compared. Assay value of stored samples were compared with initial assay value. Difference between these two assays was less than 2.0% for both actives. Study demonstrated that sample solutions were stable up to 24 Hours.

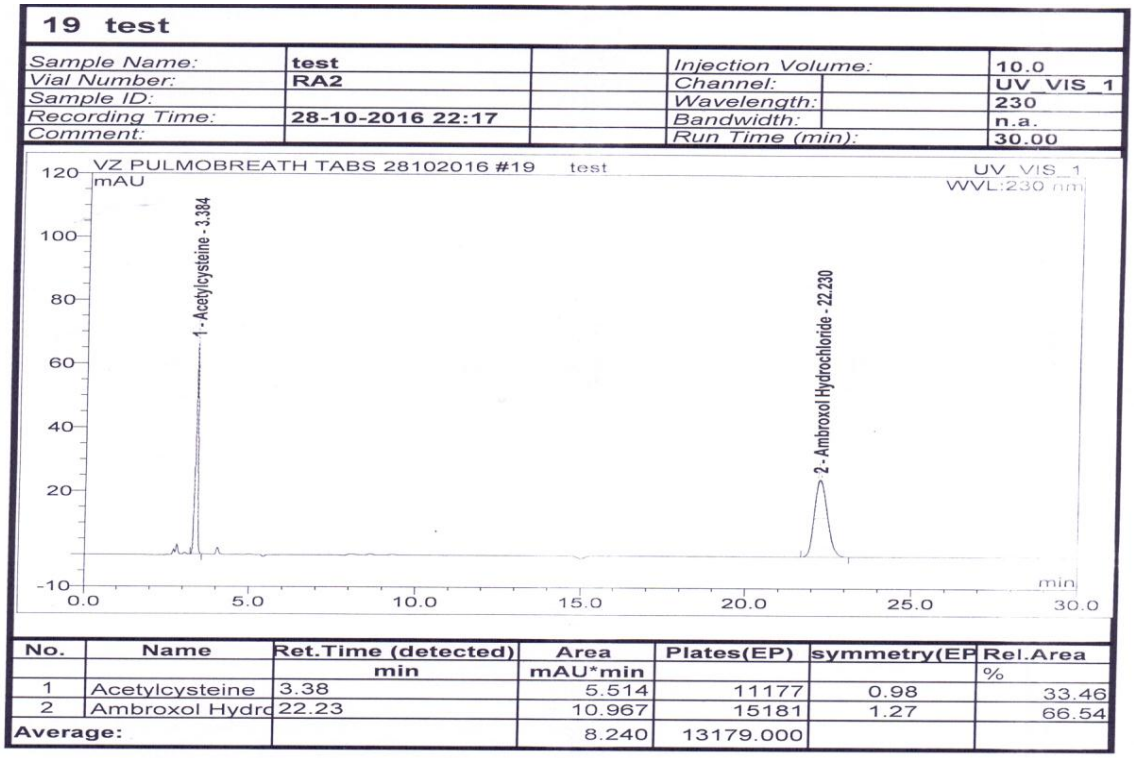


Fig.5 HPLC Chromatogram showing retention time of N-Acetylcysteine and Ambroxol HCl

No	Name	Ret.Time (Minutes)	Asymmetry (EP)	Relative Area	Theoretical Plates(EP)
1	N-Acetylcysteine	3.38	0.98	40.17	11177
2	Ambroxol HCl	22.23	1.27	N.A	15181

Table No.1. Precision of Ambroxol HCl and N-Acetylcysteine

Sr. No.	Ambroxol Hydrochloride standard solution	Ambroxol Hydrochloride test solution	N-Acetylcysteine Standard solution	N-Acetylcysteine Test solution
1	10.9873	11.0542	4.9995	5.5131
2	10.9822	11.0258	5.0100	5.5094
3	10.9765	11.0249	5.0160	5.5200
4	10.9618	10.9931	5.0497	5.5169
5	10.9689	11.0102	5.0673	5.5089
Average	**10.9753**	**11.0216**	**5.0285**	**5.5137**
RSD in %	0.0929	0.2046	0.5707	0.0869

Table No.2. Accuracy of Ambroxol HCl and N-Acetylcysteine

Sr. No	Conc. in %	% w/w Recovery Ambroxol HCl	% w/w Recovery Acetylcysteine	Average Recovery % Ambroxol HCl	Average Recovery % Acetylcysteine	Mean Recovery in % Ambroxol HCl	Mean Recovery in % Acetylcysteine
1	50 %	49.91	49.81	99.83	99.80	99.37 %	98.68 %
2	100 %	98.74	98.35	98.74	98.35		
3	150 %	149.30	147.11	99.54	98.07		

Table No.3. Ruggedness of Ambroxol HCl and N-Acetylcysteine

Chemist A				
Sr. No.	**Standard area of Ambroxol HCl**	**Standard area of N-Acetylcysteine**	**Test area of Ambroxol HCl**	**Test area of N-Acetylcysteine**
1	10.9873	4.9995	11.0014	5.5006
2	10.9822	5.0100	10.9940	5.5056
3	10.9765	5.0160	10.9889	5.5084
4	10.9618	5.0497	10.9840	5.4981
5	10.9689	5.0673	10.9971	5.4899
Average	**10.9753**	**5.0285**	**10.9931**	**5.5035**
RSD %	**0.0929**	**0.5707**	**0.0600**	**0.1300**
Assay: Ambroxol Hydrochloride: 29.11 mg **,N-Acetylcysteine:** 206.39 mg				
Chemist B				
Sr. No.	**Standard area of Ambroxol HCl**	**Standard area of N-Acetylcysteine**	**Test area of Ambroxol HCl**	**Test area of N-Acetylcysteine**
1	10.9893	4.9875	11.0014	5.5006
2	10.9872	5.0112	10.9940	5.5056
3	10.9787	5.0158	10.9889	5.5084
4	10.9629	5.0537	10.9901	5.4997
5	10.9799	5.0668	11.0112	5.5031
Average	**10.9796**	**5.0270**	**10.9971**	**5.5035**
RSD %	**0.0900**	**0.6500**	**0.0800**	**0.0700**
Assay: Ambroxol Hydrochloride: 29.57 mg **,N-Acetylcysteine:** 205.95 mg				

Table No.4. Method validation of assay results of Ambroxol HCl and N-Acetylcysteine

<table>
<tr><th>Validation Parameters</th><th>Acceptance Criteria</th><th colspan="4">Observation</th></tr>
<tr><td rowspan="3">Specificity</td><td rowspan="3">Analyte Ambroxol Hydrochloride and N-Acetylcysteine chromatographic peak should be specific, pure and distinct from each other</td><td>Content</td><td>N-Acetylcysteine</td><td colspan="2">Ambroxol Hydrochloride</td></tr>
<tr><td>Retention time</td><td>About 3.4 min</td><td colspan="2">About 22.3 min</td></tr>
<tr><td colspan="4">No apparent interference observed between the peaks, Ambroxol Hydrochloride and N-Acetylcysteine peaks were selectively separated from formulation matrix.</td></tr>
<tr><td rowspan="4">Precision</td><td rowspan="4">The relative standard deviation should not be more than 2% for test solution and standard solution.</td><td colspan="2">Content</td><td colspan="2">RSD</td></tr>
<tr><td colspan="2">Ambroxol Hydrochloride</td><td colspan="2">Std =0.09%Test =0.20%</td></tr>
<tr><td colspan="2">N-Acetylcysteine</td><td colspan="2">Std =0.57%Test =0.09%</td></tr>
<tr><td colspan="4">It is observed that in precision the five replicate injections of homogeneous test of assay for Ambroxol Hydrochloride and N-Acetylcysteine and its standard solution the result are within specified RSD limit.</td></tr>
<tr><td rowspan="3">Accuracy</td><td rowspan="3">Recovery should be 98% to 102% with respect to the added percentage</td><td colspan="2">Content</td><td colspan="2">Average Mean recovery</td></tr>
<tr><td colspan="2">Ambroxol Hydrochloride</td><td colspan="2">99.37%</td></tr>
<tr><td colspan="2">N-Acetylcysteine</td><td colspan="2">98.68 %</td></tr>
<tr><td rowspan="3">Linearity</td><td rowspan="3">The test results with respect to test concentration should be linear and co- relation coefficient should not be less than 0.998.</td><td>Content</td><td colspan="2">Correlation coefficient</td><td>Slope</td></tr>
<tr><td>Ambroxol Hydrochloride</td><td colspan="2">0.999</td><td>1.0060</td></tr>
<tr><td>N-Acetylcysteine</td><td colspan="2">1.000</td><td>1.027</td></tr>
<tr><td rowspan="3">Ruggedness</td><td rowspan="3">The analytical result should be reproducible</td><td>Content</td><td colspan="2">Chemist A</td><td>Chemist B</td></tr>
<tr><td>Ambroxol Hydrochloride</td><td colspan="2">29.11 mg</td><td>29.57 mg</td></tr>
<tr><td>N-Acetylcysteine</td><td colspan="2">206.39 mg</td><td>205.95 mg</td></tr>
</table>

CONCLUSION

The developed method was accurate, simple, rapid and selective for the simultaneous estimation of Ambroxol Hydrochloride and N-Acetylcysteine in tablet dosage form. The sample preparation was simple, analysis time was short and elution by isocratic method.The retention time of N-Acetylcysteine and Ambroxol HCl were found to be 3.38 & 22.23 minutes respectively. The excipients of the commercial sample ,which was analyzed did not interfere in the analysis, which proved excellent specificity of the method finalized for these drugs analysis. Hence the proposed method can be conveniently adopted for the routine quality control analysis for this combination in tablet form.

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