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Development and Validation of RP-HPLC Method for the Estimation of N-Acetylcysteine in Wet Cough Syrup

Shaikh Sana[a], Dr. Athawale Rajani[a*], Dr. Nadkar Sumedha[b], Phadtare Pravin[b] and Dr. Naik Shripad[b]

[a]C.U. Shah College of Pharmacy, S.N.D.T Women's University, Sir Vithaldas Vidya Vihar, Juhu road, Santacruz (West), Mumbai-400 049, India.

[b] Novartis Healthcare Pvt. Ltd., OTC Buisness Unit, R&D Lab, Tiffany, 6th Floor, Hiranandani Business Park, Off Ghodbunder Road, Patlipada, Thane (W)- 400 607, India.

Abstract

N-Acetylcysteine is an active pharmaceutical agent and nutritional supplement primarily used as a mucolytic agent and in the management of Paracetamol overdose. A simple, specific and precise reverse phase high performance liquid chromatographic (RP HPLC) method for the analysis of Acetylcysteine in wet cough syrup dosage form has been developed and validated. Sample was resolved on a Waters Symmetry, C18 (150mm X 4.6 mm i.d., particle size 3.5µ) column. The gradient system was used with mobile phase consisting of Acetonitrile and 0.05 M phosphate buffer (pH was adjusted to 3.0 ± 0.05 by using orthrophosphoric acid), at a flow rate of 0.8 ml/min at ambient temperature. Detection was carried out at 214nm. The retention time of about 4.6 minutes was recorded. Force degradation studies were carried out for acidic, alkaline, oxidative, reductive and photolytic exposure of the drug substance and drug product. The method was found to be specific for Acetylcysteine and was able to resolve the NAC peak from formulation excipients. The calibration curve was linear over the concentration range of 400-600 µg/ml (R=0.999). The proposed method was applicable to routine analysis of Acetylcysteine in wet cough syrup dosage form.

*Corresponding author, Mailing address:
Dr. Athawale Rajani
E-mail:rajani.athawale@gmail.com, sana304@gmail.com

Key words:
Acetylcysteine, RP-HPLC, Mucolytic Agent, Validation, Force Degradation, Excipients.

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1. INTRODUCTION

N-Acetylcysteine (NAC) (Figure 1) is mainly used as a mucolytic agent in bronchitis or pulmonary diseases.

It depolymerises mucopolysaccharides, reduces the viscosity of pulmonary secretions [1]. Besides mucolytic effect it also has anti-oxidant and anti-inflammatory effects and it is used as an antidote in Paracetamol poisoning [2].

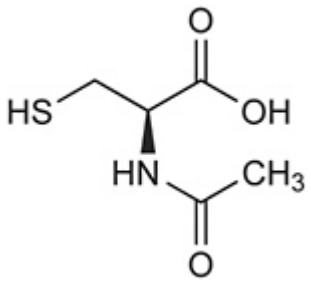

Figure 1 Structure of N-acetylcysteine

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 thus facilitates easy removal of the same and therefore improving conditions such as bronchitis and flu [3].

A number of spectrophotometric [4], colorimetric [5], chemiluminescence [6], HPLC (high performance liquid chromatography) with electrochemical [7-10], fluorimetric [2, 11], mass [12-13] and ultraviolet [14-16] detectors and GC [17] have been widely applied as the main methods of detection in biological samples and pharmaceuticals. 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 this compound.

Other published methods suffer from lack of selectivity and sensitivity or require expensive equipment. British Pharmacopoeia [18] and European Pharmacopoeia [19] describe an iodimetric method for the determination of NAC which lack accuracy and sensitivity. USP [20] mentions HPLC method with UV detector for assay. Forced degradation studies were carried out to identify likely degradation products, the possible degradation pathways and the intrinsic stability of the molecule and to evaluate the stability indicating power of the analytical procedure used. In forced degradation studies, samples were treated with acid, base, peroxide, sulfite, heat, and light and kept under extreme conditions.

The present report describes a simple, specific, and rapid HPLC method with UV detection to determine NAC and to perform the quality control of Wet Cough Syrup, a new oral formulation containing 20mg/ml of NAC. The developed method was also validated according to ICH guidelines [21].

2. EXPERIMENTAL

2.1. Chemicals and reagents

N-Acetylcysteine was received from Moehs (Barcelona, Spain); acetonitrile, methanol, ortho phosphoric acid and water (HPLC Grade), hydrogen peroxide 30% (v/v) and hydrochloric acid were obtained from Fischer Scientific (Mumbai, India). Analytical grade potassium phosphate, sodium metabisulfite, sodium hydroxide and sodium hydrogen sulfite were purchased from Merck (Mumbai, India).

2.2 Chromatographic Conditions

Analysis was performed on Waters alliance 2695 (MA, USA) HPLC system equipped with a binary pump, a vacuum degasser, a thermostated column compartment, an autosampler and Waters 2996 PDA (photo diode array) detector. Empower 2 software for data handling were used. Injection volume was kept constant 10 µL. Separation was achieved using various columns like Inertsil ODS 3V C18 (150 mm × 4.6 mm i.d. 5-µm particles), Zorbax Cyano (150 mm × 4.6 mm i.d. 3.5-µm particles) and Waters Symmetry C18 (150 mm × 4.6 mm i.d. 3.5-µm particles) under reversed-phase partition chromatographic conditions. HPLC method development process utilized isocratic and then gradient system with various mobile phase

compositions containing different ratio of acetonitrile, methanol and phosphate buffer (pH 3 ± 0.05 adjusted with orthophosphoric acid). The flow rate was varied from 0.8 to 1.5 ml/min and the analytes were monitored at 214 nm. The system was used in an air-conditioned HPLC laboratory (20 ± 2°C). Before analysis the mobile phase was degassed by use of a sonicator (Branson 8510, Danbury, USA) and filtered through a 0.45 µm filter (Millipore, Bangalore, India). Sample solutions were also filtered through a 0.45µm filter. The system was equilibrated before each injection.

2.3 Preparation of standard and sample solutions for HPLC analysis

2.3.1 Preparation of standard solutions for development trials

NAC standard solution for method development trials was prepared by dissolving approximately 25 mg of NAC in approximately 30 mL of Sodium metabisulphite (1g in 2000mL, used as diluent) by sonicating for 5 mins and diluting to 50 ml with diluents. Amber colour volumetric flasks were used as NAC is light sensitive.

2.3.2 Preparation of standard solutions for Linearity Response

The stock standard solution (1000 µg/ml) was prepared by dissolving 100 mg of NAC in 100 ml of diluent. Various standard solutions were then prepared by diluting the above stock solution with diluent to yield nominal concentrations over a range of 400–600 µg/mL. According to ICH guidelines, for the assay of a drug substance or a finished (drug) product normally from 80 to 120 percent of the test concentration should be considered and a minimum of 5 concentrations is recommended [21]. The test concentration was 500 µg/mL, thus range selected was 400–600 µg/mL and 7 concentrations were prepared.

2.3.3 Preparation of sample solution for development trials

A 5 ml of wet cough syrup was transferred to 200 ml of amber colour volumetric flask and diluted to volume with diluent. Solution was filtered through 0.45 µm (23 mm i.d) syringe filter (MDI, India).

***2.3.4 Preparation of sample solution for recovery** study*

A 5 ml of placebo was transferred to 200 ml of amber colour volumetric flask to which 12.5 ml, 25 ml and 37.5 ml of standard stock solution of NAC prepared for linearity was added and diluted to volume to get concentration 250, 500 and 750 ppm respectively. Solution was filtered through 0.45 µm syringe filter.

2.3.5 Preparation of sample solution for Force Degradation Studies

NAC was weighed equivalent to 50 mg and transferred to a series of 200.0 mL volumetric flasks. Similarly 5 ml of Wet Cough Syrup was transferred to a series of 200.0 mL volumetric flasks. The solutions were kept under the following different conditions:

- *Refluxed for 2 h after addition of 5 ml of 0.5M NaOH (Alkali)*
- *Refluxed for 2 h after addition of 5 ml of 0.5M HCl (Acid)*
- *Refluxed for 2 h after addition of 5 ml of 2% H_2O_2 (Oxidation)*
- *Refluxed for 2 h after addition of 5 ml of 5% Na_2SO_3 (Reduction)*
- *Exposed to 80°C for 24 h (Thermal)*
- *Exposure to UV light for 24 h at 254nm (Photolytic)*

After exposure to different stress conditions samples were diluted to volume with diluents and filtered. Solutions were injected separately and the content of NAC was calculated by comparing the peak area of the sample with that of standard.

3. RESULTS AND DISCUSSION

3.1. Chromatography

Initially USP method was tried using Inertsil ODS 3V C18 (150 mm × 4.6 mm i.d. 5-µm particles) column

and KH_2PO_4 (0.05M), pH 3 buffer as mobile phase with flow rate of 1.5 ml/min, NAC got eluted with t_R of 8.95 mins., when reviewed the chromatogram for checking the peak purity of NAC it was found that it was failing i.e; (purity angle > purity threshold) 0.284 > 0.272. It means other components were coeluting with active peak so organic phase was tried in combination with buffer to get good separation between active peak and excipients peaks. Buffer: ACN (80:20) and buffer: methanol (80:20) were tried. In comparison to methanol

(100 mins), acetonitrile causes elution of components faster with run time of 30 mins in isocratic program. To reduce run time gradient program system was tried. The gradient program mentioned in table 1 was set with flow rate of 1.2 ml/min and run time of 10 mins, NAC got eluted at t_R of 3.2. In this case also peak purity of NAC peak was failing (8.651 > 1.668). The gradient program table 2 was set with flow rate of 0.8 min and run time of 20 mins. Peak shape of NAC was not proper with peak tailing of 1.43, thus column was changed to Zorbax Cyano (150 mm × 4.6 mm i.d. 3.5-µm particles) but same problem was observed. Further Waters Symmetry C18 (150 mm × 4.6 mm i.d. 3.5-µm particles) column was used, NAC eluted at 4.6 mins with proper peak shape and peak purity passing i.e; (Purity angle < purity threshold) 0.721 < 3.934 [Figure 2 and Figure 3].

Table 1: Gradient program with run time of 10 mins.

Time (mins)	Mobile phase A	Mobile phase B
0	95	5
2	95	5
2.10	20	80
6	95	5
10	95	5

Table 2: Gradient program with run time of 20 mins.

Time (mins)	Mobile phase A	Mobile phase B
0	95	5
5	95	5
10	20	80
15	95	5
20	95	5

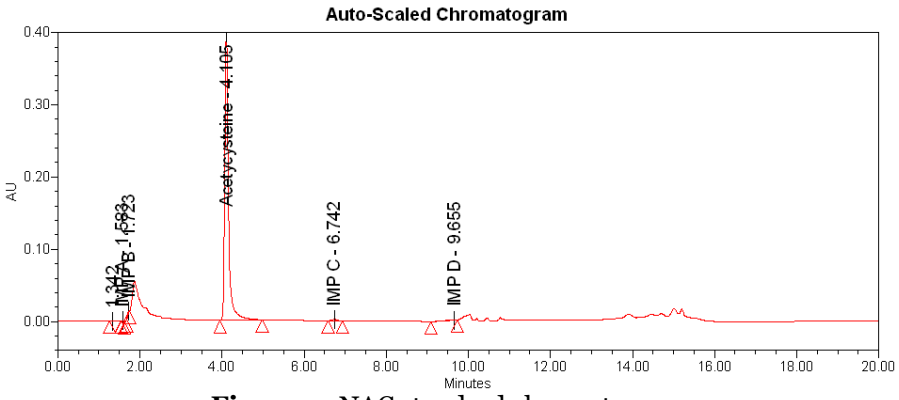

Figure 2: NAC standard chromatogram

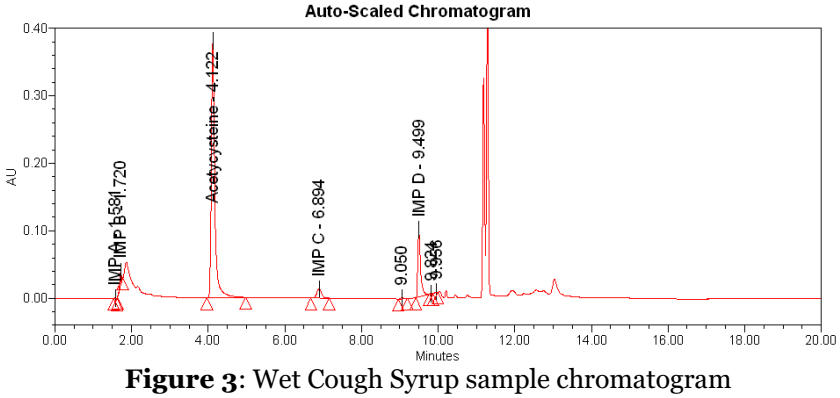

Figure 3: Wet Cough Syrup sample chromatogram

3.1.1 System Suitability

System suitability was evaluated by preparing NAC standard solution of 500 µg/ ml and injecting 5 injections (n=5). The RSD (%) of peak area was within 2%, indicating the suitability of the system. The number of theoretical plates and the USP tailing factor were within the acceptance criteria of >2000 and ≤ 2 respectively, indicating good column efficiency and optimum mobile phase composition (Table 3).

Table 3: System Suitability Parameters

Parameters	NAC
Tailing Factor	1.2
Theoretical Plates	5865
Relative Standard Deviation	0.11

3.2. Establishing stability indicating aspect of the developed method

Following the degradation period, all samples were prepared for analysis as previously described and chromatographed (Figure 4-9). Results are shown in table 4. The results indicate that the drug was found to be susceptible to degradation in all most all stress conditions of acid, alkali, reduction, oxidation, heat and light. Decrease in the absolute peak area of the parent peak and appearance of any extra peaks due to possible degradation products were monitored. This method was able to identify many more additional peaks, possibly due to the degradation products along with the parent peak. USP Pharmacopoeia specifies four impurities; these impurities were identified and marked as impurities A, B, C and D. Other unknown impurities were also seen in the chromatogram. Impurity was not generated under above mentioned stress conditions. Impurities B, C and D appeared at retention time of approximately 1.8, 6.8 and 8.8 respectively. NAC was found to be more prone to oxidative degradation and leads to formation of impurity B. All of the above forced degradation studies have clearly indicated that the HPLC method developed for NAC is indeed stability indicating. The peak of NAC was a single component sharp peak and none of the degradation products co-eluted with parent drug peak.

Table 4: Force Degradation Results

Sample (Treated)	%Un-degraded
Reflux for 2 h with 0.5 M HCl	80
Reflux for 2 h with 0.5M NaOH	81.5
Reflux for 2 h with 2% H_2O_2	9.2
Reflux for 2 h with 5% Na_2SO_3	76
Exposed 80°C for 24 hrs	91
Exposure to UV Light for 24 hrs at 254.0 nm	91

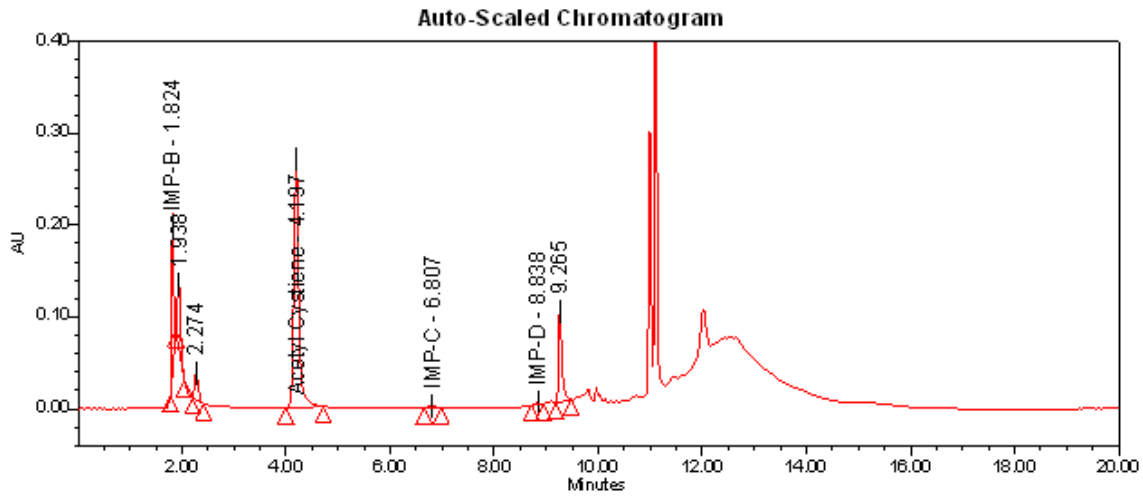

Figure 4: Chromatogram of Wet Cough Syrup formulation in 0.5 N HCl (reflux 2h)

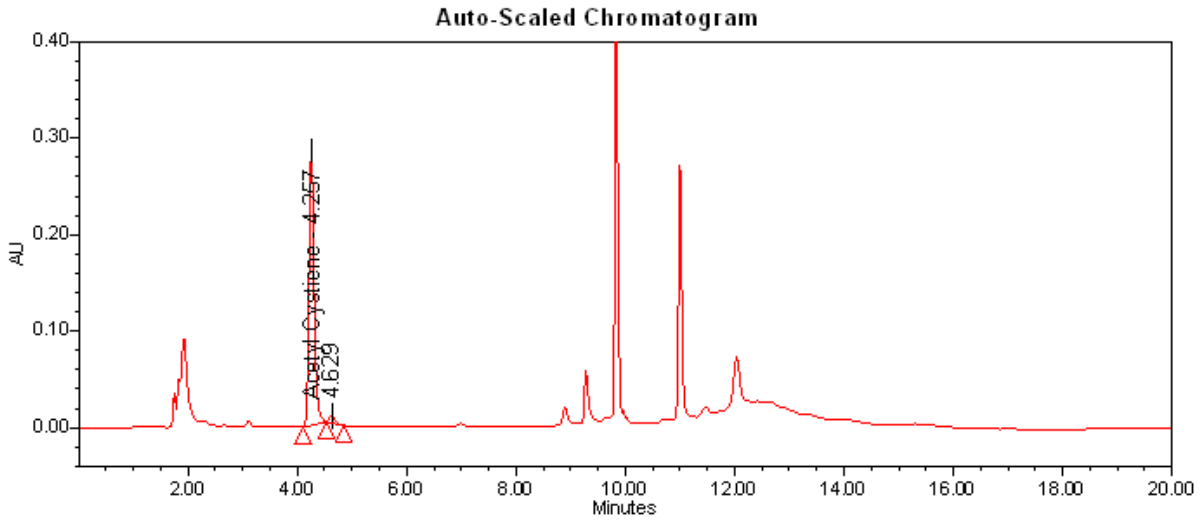

Figure 5: Chromatogram of Wet Cough Syrup formulation in 0.5 N NaOH (reflux 2h)

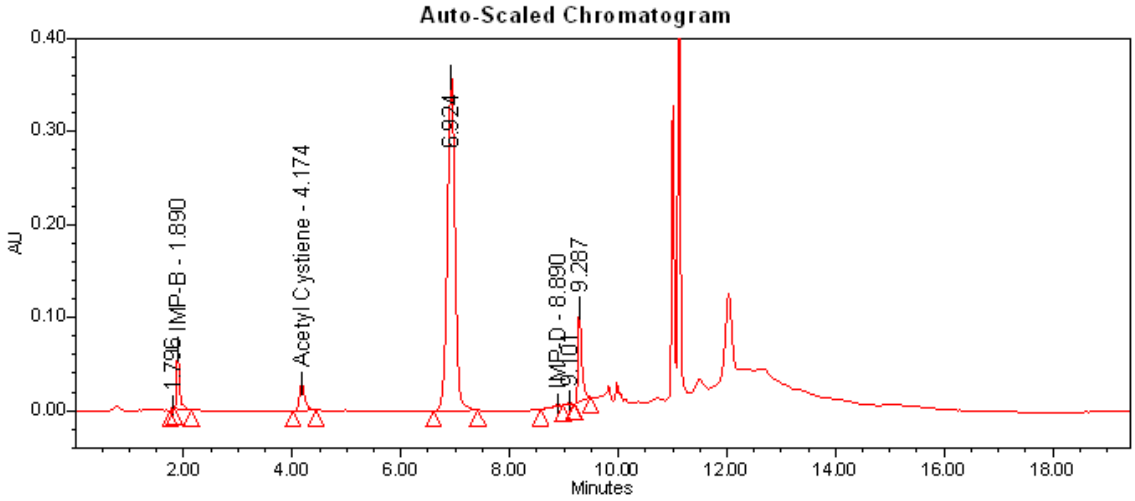

Figure 6: Chromatogram of Wet Cough Syrup formulation in 2% H_2O_2 (reflux 2h)

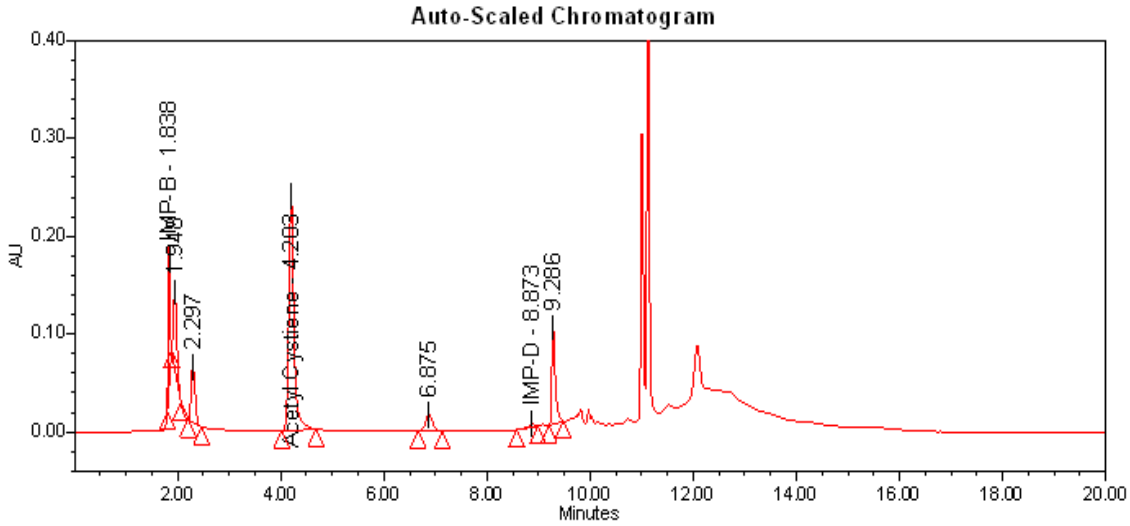

Figure 7: Chromatogram of Wet Cough Syrup formulation in 5% Na_2SO_3 (reflux 2h)

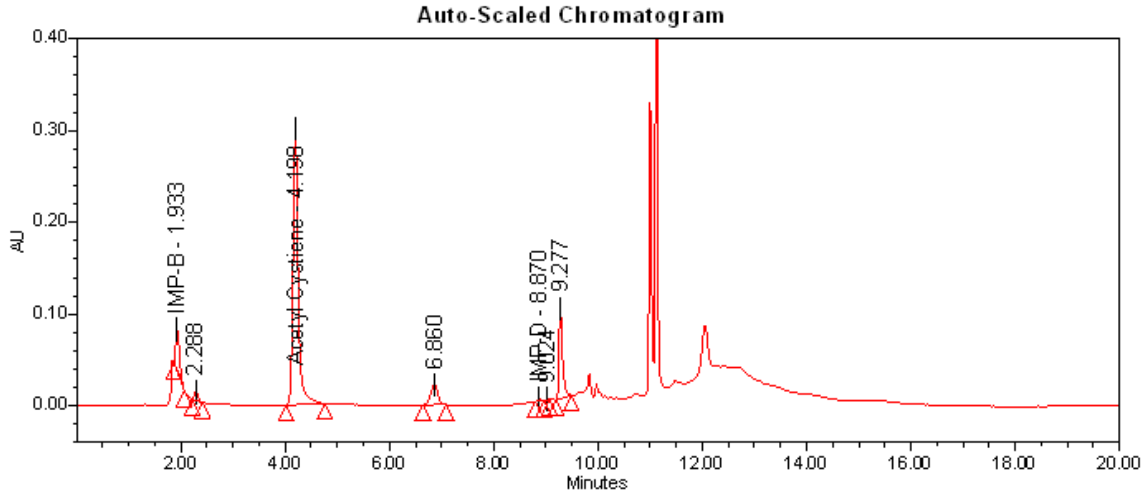

Figure 8: Chromatogram of Wet Cough Syrup formulation exposed to 80°C (24h)

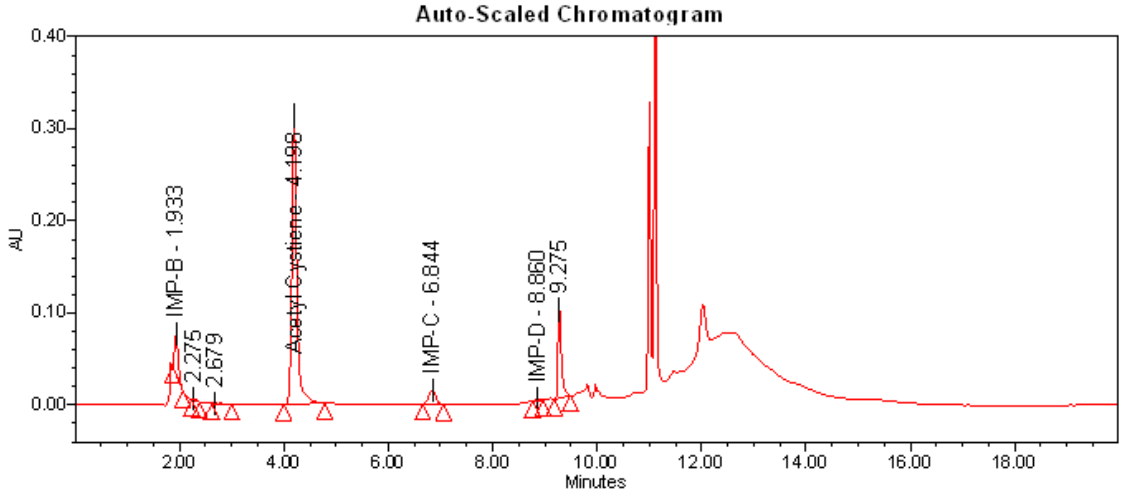

Figure 9: Chromatogram of Wet Cough Syrup formulation under UV light exposure (24h)

3.3. Assay validation

3.3.1. Linearity

Standard curves were constructed by plotting peak area versus concentration of the drug.

Standard curve for NAC was linear over the concentration range of 400–600 µg/ml. The equation of the standard curve correlating the peak area (Y) to the drug concentration (*X* in µg/ml) in this range was Y= 4745*X* - 2955, *R* > 0.999.

3.3.2. Precision

Precision of estimation of NAC by proposed method was ascertained by replicate analysis of six samples prepared from homogeneous sample of Wet Cough Syrup. Assay precision was expressed as the relative standard deviation (RSD, %), found to be ≤ 2.0% for both the drugs. The variability in the peak area of each injection is presented in table 5. Assay amount is calculated as follows:

$$\% \text{ Amount} = \frac{\text{Aspl} \times \text{Wmg} \times 1 \times \text{P} \times 200}{\text{Astd} \times 50 \times 20 \times 100 \times 5} \times 100$$

Aspl = Area of the NAC peak in the sample chromatogram

Astd = Area of the NAC peak in the standard chromatogram

P = Potency of the standard (100%)

W = Standard Weight (25 mg)

Table 5: Results of Precision Study

Injection	Peak area	Assay
1	2413808	103.14
2	2412155	103.07
3	2413606	103.13
4	2432896	103.95
5	2418573	103.34
6	2421999	103.49
%RSD		0.321057

3.3.3. Accuracy

Accuracy of the proposed method was ascertained on the basis of recovery studies performed by comparing the theoretical and measured concentrations of placebo formulation spiked with standard solutions of NAC at 50%, 100% and 150% of working level (500 ppm). It was determined by performing three repeated analysis. Results are shown in table 6. %RSD was found to be < 2.0%. % Recovery is calculated as follows:

$$\text{Amount Added} = \frac{\text{Wmg x ml of standard added}}{\text{100 ml x 50 ml}} \text{ x } 1000$$

$$\text{Amount Found} = \frac{\text{Aspl x Wmg}}{\text{Astd x 50 ml}} \text{ x } 1000$$

$$\% \text{ Recovery} = \frac{\text{Amount Found}}{\text{Amount Added}} \text{ x } 100$$

Table 6: Results of Accuracy/Recovery Study

Concentration Level (%)	Area*	Amount Added (ppm)	Amount Found (ppm)	% Recovery
50	1219880	250.28	250.21	99.97
100	2321805	500.55	504.96	100.88
150	3500736	750.83	761.36	101.40
%RSD				0.7184

* n=3

3.3.4 Specificity

Specificity was performed by injecting solutions of sample as such, placebo, stressed sample (heat stressed) and impurities A, B, C, and D. No interference is observed with active peak and which is confirmed by checking peak purity of active peak which was passing as mentioned in table 7. Peak purity is said to be passed when purity angle is less than purity threshold. Figure 10 shows overlay chromatogram of sample as such, placebo, stressed sample and impurities.

Table 7: Results of peak purity

Sample	Purity angle	Purity threshold
Unstressed sample	0.057	0.263
Stressed sample	0.239	0.296

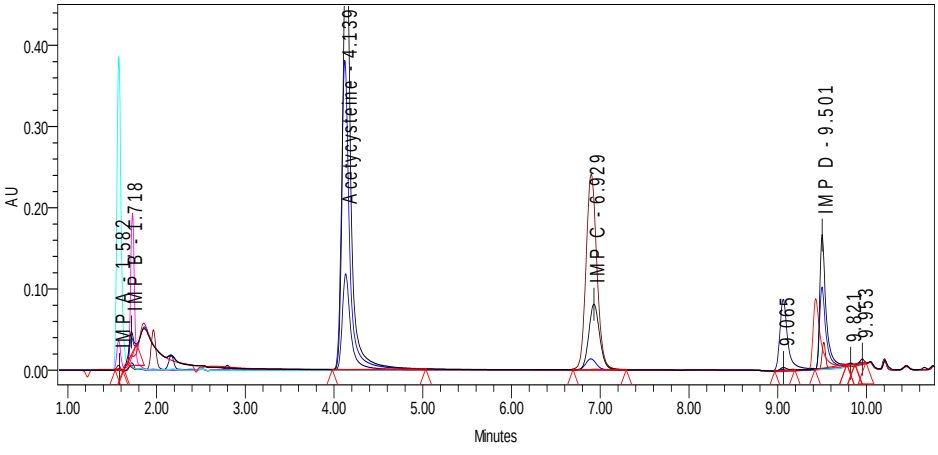

Figure 10: Overlay chromatogram of sample as such, placebo, stressed sample and impurities.

3.3.5 Robustness

Robustness of the proposed method was ascertained by deliberately changing the mobile phase pH, detection wavelength and flow rate of the mobile phase. Equal concentration of working standard solution and sample solution were injected separately (n=3), and the chromatograms were recorded. The content of NAC was calculated by comparing the peak area of sample with that of standard. % RSD of three robustness sample injection and % RSD of nine injections (sum of six injections of precision and three injections of robustness) were calculated. The RSD must be not more than ±2%. The results are recorded in table 8.

Table 8: Results of Robustness Study

Deliberate changes	Average % Assay*	% RSD
Change in Wavelength (211.0 nm)	102.2	1.24
Change in Wavelength (217.0 nm)	101.8	1.25
Flow rate (0.7 ml/min)	101.4	1.39
Flow rate (0.9 ml/min)	103.2	1.31
pH 2.9	102.9	1.21
pH 3.1	102.7	1.26

4. CONCLUSION

A simple, rapid, specific, and reproducible HPLC method for the quantitative determination of *N*-Acetylcysteine in Wet Cough Syrup prepared at 200mg/mL has been developed using C18 Waters symmetry column and validated. This method was found to be stability indicating. It is currently being employed in our laboratory for carrying out proof of concept study of Wet Cough Syrup containing NAC. It can be useful for routine monitoring in quality control laboratories and for investigating the stability of the drug.

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