Original Article

Development and Validation of an Analytical Method for Related Substances in N-acetyl–L-cysteine Effervescent Tablets by RP-HPLC

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ABSTRACT

Background: The reported chromatographic methods for N-acetyl cysteine [NAC] are reverse phase HPLC and ion pair chromatography [IPC] for related substances test in bulk and in formulations. No reported stability indicating methods for the estimation of related substances in NAC effervescent formulation was found in literature. **Objective:** The present work was aimed at developing a selective, sensitive and reproducible stability indicating high-performance liquid chromatographic method for the quantitative determination of known, unknown impurities, degradation impurities and process-related impurities of NAC effervescent formulation. **Method:** A reversed phase ion pair chromatographic method was developed employing Cadenza C18 column as the stationary phase and 0.01M octane sulphonate [pH 2.20], methanol and acetonitrile in the ratio 90:8:2 as the mobile phase. A gradient programme was followed with a run time of 55 minutes. 0.3 M hydrochloric acid was selected as the optimum diluent. The performance of the method was validated according to the ICHQ2R1 guidelines. **Results:** The method was found to be linear from 1.5 to 25μg/ml for impurities A, C and D and from 2.0 to 25 μg/ml for impurity B. The official impurities C and D were mapped in all stress conditions. Additionally, impurity B was also seen in acidic conditions. **Conclusion:** The results from the study demonstrate that the method is suitable for evaluating the stability of NAC effervescent tablet.

Key words: N-acetyl cysteine, Reverse phase HPLC, Effervescent formulation, Ion pair chromatography, Related substances,Validation.

Key message: An ion pair chromatographic method was developed for quantifying the related substances of N-acetyl cysteine effervescent Tablets. Selection of diluent was an important variable in the method development.

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INTRODUCTION

Acetyl cysteine also known as N-acetyl-L-cysteine [NAC] is derived from cysteine by attaching an acetyl group to the amino group. It's basically a prodrug that is converted to cysteine and absorbed in the intestine into the blood stream. Cysteine is an important constituent of glutathione and hence acetyl cysteine aids in replenishing glutathione stores. The chief use of the drug is as a mucolytic agent as it helps loosen mucus in the airways due to emphysema, bronchitis, pneumonia and cystic fibrosis. It acts as an antidote of paracetamol poisoning by replenishing the glutathione reserves in the body. Glutathione acts as an antioxidant by conjugating the toxic metabolites of paracetamol poisoning. Other uses include in the treatment of HIV, chronic obstructive pulmonary disease, renal impairment, mild to moderate traumatic brain injury, idiopathic interstitial pulmonary fibrosis, colon polyps, adjunct in the treatment of *Helicobacter pylori*, contrast induced nephropathy, prophylactic of gentamycin-induced hearing loss in

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patients on renal dialysis, treatment of infertility in patients with clomiphene-resistant polycystic ovary syndrome, neuropsychiatric and neurodegenerative disorders including cocaine, cannabis, smoking addictions, alzheimer's and parkinson's diseases, autism, compulsive and grooming disorders, schizophrenia, depression, and bipolar disorder. Recent studies have revealed that NAC inhibits muscle fatigue and can be used to enhance performance in exercise and endurance training.[1-3] Analytical techniques like colorimetry,[4,5] chemiluminiscence,[6,7] electrochemical detection,[8-15] flurimetry,[16,17] turbidimetry and nephlometry,[18] liquid chromatography tandem mass spectrometry,[19-21-44]gas chromatography mass spectrometry,[22,23] and capillary electrophoresis[24-26] have been employed in literature for the quantification of acetyl cysteine. Acetyl cysteine has also been simultaneously quantified along with other drugs like clomiphene citrate,[27] arginine,[28] and cefexime trihydrate.[29] Stability testing studies of drugs in API and formulation provide evidence on the intrinsic stability of the molecule in response to environmental stress factors like temperature, humidity and light. This in turn helps in establishing shelf life for the drug product and recommended storage conditions. Forced degradation studies assist in developing a stability indicating method, they also offer vast knowledge on the possible degradation pathways and degradation products of the drug in bulk and formulation.[30-33] The related substances [Figure 1] as described by the European pharmacopoeia and British pharmacopoeia are L-cystine [impurity A], L-cysteine [impurity B], N,N'-diacetylcystine [impurity C] and N,S diacetylcysteine [impurity D].[34,35]Among chromatographic methods literature reveals separation methods like reverse phase HPLC and ion pair chromatography for related substances test of NAC in bulk and drug products.[34-44] Literature also reports expensive and less widely available techniques like LC-UV-MS[44] and capillary electrophoresis-mass spectrometry[25] for quantifying the related substances of acetyl cysteine. According to our findings, none of the currently available analytical methods is stability indicating. Based on the literature review there are no reported methods for the estimation of related substances in effervescent formulation of NAC by HPLC. The literature survey reveals that no reference exists for the quantitative determination of impurities by a stability-indicating HPLC method.

On screening the reported chromatographic methods for their suitability to the NAC effervescent formulation, the impurities L-cystine, L-cysteine and the placebo components were seen to elute at the same retention time. Hence, it was felt necessary to develop an accurate, selective and sensitive stability-indicating HPLC method

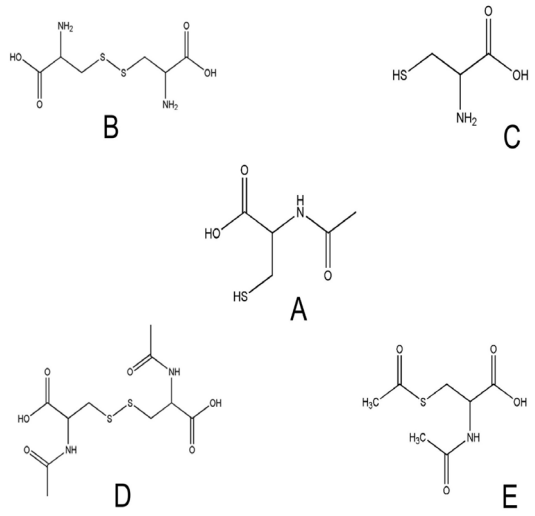

Figure 1: Chemical structures of[A] N-acetyl-L-cysteine, [B] L-cystine[Impurity A],[C]L-cysteine [Impurity B],[D] N,N'-diacetyl -L-cystine [Impurity C] [E] N,S-diacetyl -L-cysteine [Impurity D].

for the determination of NAC and its related compounds. This method was successfully validated according to the International Conference on Harmonization [ICH] guideline Q2R1.[45]

EXPERIMENTAL

Instrumentation

The liquid chromatography method development was carried out using Agilent 1260 infinity series, which consisted of a pumping system, a thermostat column compartment, UV-DAD detector and an auto sampler [Agilent, USA]. Data were collected on a PC equipped with the Open-LAB Chem- station version C. 01. 04 [35]. The method validation was carried out on Agilent 1260 and Shimadzu LC-20 prominence system. Shimadzu LC-20 prominence is equipped with a Shimadzu LC-20AD prominence pump, Shimadzu SPD-M10 diode array detector, Shimadzu SIL-20AC HT auto sampler and a Shimadzu CTO-10AS column compartment. The data were collected and analysed on a PC equipped with LC solutions version 1.25.

Materials

NAC[97%], L-cystine [98%], L-cysteine[97%] were purchased from Sigma Aldrich, [Bangalore, India], N-acetyl cysteine impurity C CRS [61.9%] and N-acetyl cysteine impurity D CRS were purchased as European reference standards. The in house HPLC water [Milli -Q] was used. Methanol [HPLC grade], acetonitrile [HPLC grade], octane-1-sulphonic acid sodium monohydrate

[HPLC grade], orthophosphoric acid [AR], 37% hydrochloric acid [AR] were purchased from Rankem [Mumbai, India]. The effervescent placebo was manufactured and supplied from the formulation facility of STEER Life India Pvt. Ltd. Bengaluru.

Preparation of Solutions

Mobile phase:

- Mobile phase A: 0.01M octane-1-sulphonic acid sodium monohydrate, pH 2.2 adjusted with dilute ortho phosphoric acid.
- Mobile phase B: A mixture of 200 ml of acetonitrile and 800 ml of methanol.

System suitability

Following solutions were freshly prepared in 0.3M hydrochloric acid

- Resolution solution: An equal proportion mixture of 3000 µg/ml NAC, 6 µg/ml impurity A, 6 µg/ml impurity B, 6 µg/ml impurity C and 6 µg/ml impurity D.
- Diluted standard solution: 10µg/ml NAC.
- Diluent: 0.3 M hydrochloric acid

Forced Degradation

- Acid degradation: API, placebo and placebo spiked with NAC were refluxed separately with 5ml of 1 M hydrochloric acid for 15 mins at 80 °C. The stressed samples (pH 1.30-1.87) were cooled, neutralized with 1M sodium hydroxide and diluted with diluent to a final concentration of 3.0mg/ml in case of API and placebo spiked with NAC
- Alkali degradation: API, placebo and placebo spiked with NAC were refluxed separately with 5ml of 1 M sodium hydroxide for 15 minutes at 80 °C. The stressed samples (pH 11.40-12.80) were cooled, neutralized with 1M hydrochloric acid and diluted with diluent for a final concentration of 3.0 mg/ml in case of API and placebo spiked with NAC.
- Peroxide degradation: API, placebo and placebo spiked with NAC were sonicated with 5 ml of 0.3% v/v hydrogen peroxide for 2 minutes. The stressed samples were cooled and diluted with diluent for a final concentration of 3.0 mg/ml in case of API and placebo spiked with NAC.
- Thermal degradation: API, placebo and placebo spiked with NAC were weighed separately in standard flasks, capped and kept in a hot air oven at 80°C for 2 hr. The stressed sample were cooled and dissolved with diluent for a final concentration of 3.0 mg/ml in case of API and placebo spiked with NAC.
- Photolytic degradation: API, placebo and placebo spiked with NAC were kept in sunlight for 5 days. The stressed sample was then dissolved with diluent for a final concentration of 3.0 mg/ml in case of API and placebo spiked with NAC.

Table 1: Optimised gradient programme

Time[minutes]	0.01M octane-1-sulphonic acid monohydrate sodium salt	Methanol : Acetonitrile [80:20 v/v]
0	90	10
17	90	10
20	70	30
32	70	30
35	90	10
55	90	10

RESULTS

Optimized chromatographic conditions

The chromatographic separation was performed on a Cadenza C_{18} column [150 mm X 4.6 mm, 3µ] from Almkat. The mobile phase consists of 0.01 M octane -1-sulphonic acid sodium of pH 2.2 and methanol: acetonitrile [80:20 v/v] in the organic phase. A gradient program was followed [Table 1] for 55 minutes. The flow rate was 1ml/minute and the sample injection volume was 10µl. Column temperature was maintained at ambient. The detection wavelength was set at 210nm. 0.3M hydrochloric acid was used as the diluent.

Forced degradation studies

Forced degradation studies were performed as per Q1 A(R2)[33] to assess the specificity and the stability indicating capacity of the method. Stressed drug substance, stressed placebo, and stressed placebo spiked with NAC were subjected to acid, alkali, peroxide [oxidative], thermal, photolytic [sunlight] and humidity with temperature conditions and injected into the HPLC. The specificity of the method, mass balance and the mapping of the official impurities in the stress conditions were carried out [Table 2 and Table 3]. There were no co-elution of impurities or placebo with the NAC peak and the official impurities peaks. The per cent degradation of NAC in the sample [placebo spiked with NAC] was seen to be in the range of 5-21% with the maximum degradation in photolytic condition. In comparison the degradation in API is from 12-22 % with the maximum degradation seen in thermal conditions. Investigating the difference

in the degradation pattern of API and formulation has been undertaken and the work is in progress.

Method validation

The method was validated to show compliance with regulatory requirements. The guideline as per the International Conference on harmonisation for validation of analytical procedures: text and methodology: Q2 [R1] was followed.[45]

System Suitability: System suitability test was carried out to verify that the analytical system was working as desired and can give precise and accurate results. Diluted standard and resolution solution were injected five times into the HPLC system. The results are displayed in Table 4. All the values were found to be within acceptable limits.

Specificity and forced degradation: The capability of the method to measure the analyte among excipients was evaluated by chromatographing the blank, placebo, resolution solution, and placebo spiked with resolution solution at specification level as per the optimized chromatographic conditions [Figure 2]. The peak purity of the NAC peak and its related substances were evaluated by the diode array detector and the peak was considered pure if the single point thre shold [SPT] was less than the peak purity index [PPI] [Figure 3]. The drug substance, placebo, and placebo spiked with NAC were exposed to forced degradation under acid, alkali, peroxide [oxidative], thermal, photolytic and humidity with temperature conditions. The resultant samples were

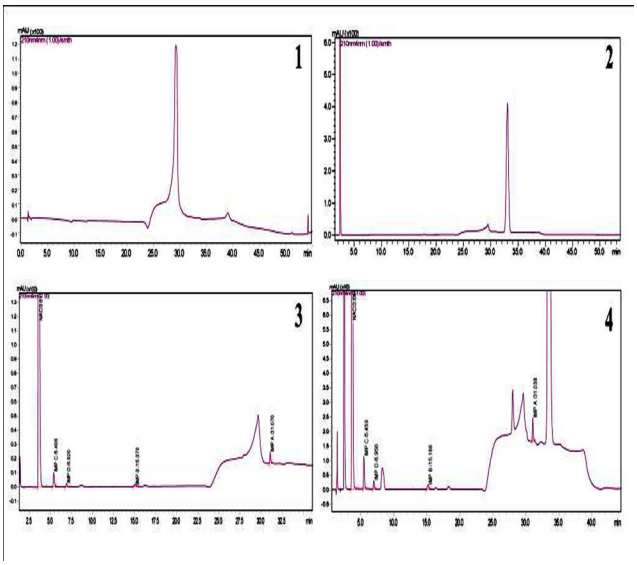

Figure 2: The chromatograms representing the specificity of the developed method: [1] Blank [2] Placebo solution [3] Resolution solution [4] Placebo spiked with NAC and known impurities.

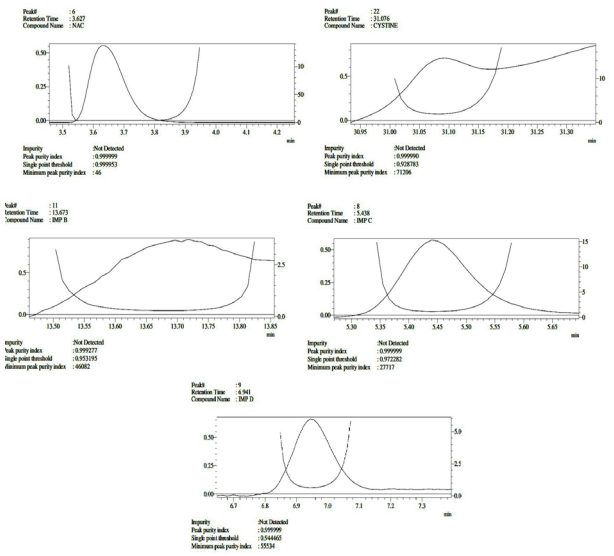

Figure 3: Peak purity curve of (A) N-acetyl-L-cysteine (B) L-cysteine [Impurity B] (C) L-cystine[Impurity A] (D) N,N'-diacetyl -L-cystine [Impurity C] (E) N,S-diacetyl -L-cysteine [Impurity D].

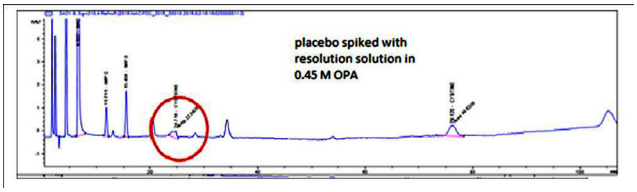

Figure 4: The chromatogram showing the additional peak co-eluting with L-cysteine when OPA was used as the diluent.

chromatographed on the HPLC after suitable treatment and dilution to establish the stability indicating power of the method [Figure 5]. The peak purity of the NAC peak was evaluated in all cases by the diode array detector and the peak was considered pure if the single point threshold[SPT]was less than the peak purity index [PPI] [Table 2 and Table 3].

Limit of detection [LOD] and limit of quantification [LOQ]**:** The LOQ and LOD were established by determining the signal to noise ratio. The experiment was executed by chromatographing separately samples of blank (diluent and placebo) and placebo spiked with impurities A, B, C, D at 0.75µg/ml, 1µg/ml, 1.5µg/ml and 2µg/ml. Detection and quantification limits for NAC and impurities A, C and D were found to be 0.75µg/ml and 1.5µg/ml respectively. For impurity B the acceptable LOD and LOQ results were obtained at 1µg/ml and 2µg/ml receptively.

*Linearity and range***:** The linearity was determined by the linear regression analysis. The linearity was obtained for NAC from LOQ to 150%w/w sample concentration i.e. 1 to 4000µg/ml and for impurities A, B, C and D from LOQ to 150% w/w of specification level, i.e. for A, C, D

Table 2: Forced degradation and mapping of official impurities in NAC API

Stress Condition	% assay	% degradation of NAC	% impurities	Mass balance	Peak purity index	Single point threshold	Peak purity Result	Remarks (impurity expressed as %w/w)
Control	95.86	-	3.50	99.36	0.9999	0.999936	Pass	C-2.47 D-1.02
Acid Hydrolysis	73.5	22.3	28.72	108.0	1.0000	0.999946	Pass	C-2.0 D-12.04 B4.82 UI-17.67
	UI: 2.53,13.05,23.04 and 26.2 mins							
Alkali hydrolysis	79.8	16	13.64	98.4	0.9999	0.999952	Pass	C-5.74 D-6.14 UI-5.02
	UI: 2.7and 3.2 mins							
Peroxide	83.8	12	15.82	98.6	0.9999	0.9952	Pass	C-10.15 D-1.06 UI-3.38
	UI: 13.29and 26.7mins							
Heat	65.4	30.4	31.83	97.3	1.0000	0.99999	Pass	C-7.85 D-6.3
	UI: 13.29, 14.77 and 26.77mins							
H/ T	83.8	12	17.21	100.7	0.999999	0.999952	Pass	C-10.64 D-20.79 UI-0.39
	UI: 13.92,26.4,27.6 and 28.21 mins							
Photolytic	83.2	12.6	18.43	101.7	1	0.999996	Pass	C-6.37 D-11.72 UI-0.34
	UI: 4.39,6.1,6.38,6.6,19.27,26.35 and 50.24mins							

B-impurity B, C-impurity C, D-impurity D, UI-Unknown impurities, H/T -humidity with temperature

Table 3: Forced degradation and mapping of official impurities in effervescent formulation

Stress Condition	% assay	% degradation of NAC	% Impurities	Mass balance	Peak purity index	Single point threshold	Peak purity result	Remarks [impurity expressed as %w/w]
Control	96.9	-	2.9	99.7	1.00000	0.99995	Pass	C-1.99 D-0.88
Acid	84.9	12	7.0	96.0	0.99998	0.99995	Pass	C-4.13,D-3.81 B-0.265U-3.19
	U I- 13.97, 14.53,26.29,53.29and 53.4mins							
Alkali hydrolysis	92.3	4.6	4.9	96.1	0.99999	0.99520	Pass	C-1.5D-1.06, U-1.07
	UI 4.2,11.37and 32.24mins							
Peroxide	91.4	5.5	11.2	102.6	0.99999	0.99991	Pass	C-2.22 D-0.11 U-0.88
	UI- 2.52,4.39,8.00,13.06,14.04,19.59and 26.11							
Heat	85.1	11.8	16.2	101.4	0.99998	0.99995	Pass	C-6.98,D-0.11 B-0.24, U-9.13
	UI: 2.9,4.27,8.03,13.18,15.49 and 26.7							
H/T	84.8	12.1	19.5	104.3	1.00000	0.99992	Pass	C-9.8,D-6.2 U-3.27
	UI: 6.92,13.75,23.2and 26.36mins							
Photolytic	75.6	21.3	22.9	98.5	0.99999	0.999953	Pass	C-14.35,D-4.60 U-3.89
	UI - 5.16,29.81,31.48,33.38,33.67,33.84,46.26 and 54.09 mins							

B-impurity B , C-impurity C, D-impurity D, UI-Unknown impurities, H/T humidity with temperature

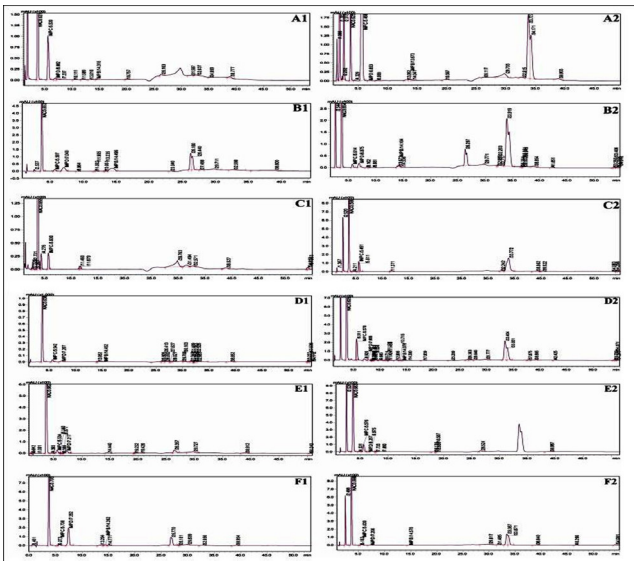

Figure 5: Forced degradation study:[A1] Peroxide degradation -API [NAC] [A2] Peroxide degradation-Effervescent tablet placebo spiked with NAC [B1] Acid Hydrolysis - API [NAC] [B2] Acid Hydrolysis – Effervescent tablet placebo spiked with NAC [C1]Alkali Hydrolysis - API [NAC] [C2] Alkali Hydrolysis – Effervescent tablet placebo spiked with NAC [D1] Humidity with temperature - API [NAC] [D2] Humidity with temperature – Effervescent tablet placebo spiked with NAC[E1] Photolytic [Sunlight] - API [NAC] [E2] Photolytic [Sunlight] – Effervescent tablet placebo spiked with NAC[F1] Thermal - API [NAC] [F2] Thermal – Effervescent tablet placebo spiked with NAC.

it is 1.5 to 25µg/ml and for impurity B it is 2.0 to 25µg/ml. In all the cases the regression coefficient was found to be not less than 0.99.The details of the calibration graph i.e. slope; regression coefficient and Y-intercept are depicted Table 4.

Accuracy and Precision: Accuracy, method precision and intermediate precision were evaluated. The method precision was evaluated by spiking the effervescent placebo with impurities at LOQ level and at specification level [Table 4] and the %RSD of six replicate injections were calculated. The intermediate precision was [Table 4] determined by spiking the placebo with impurities at specification level on different days and on different instruments and the %RSD of five injections were calculated. In all the experiments the % RSD was found to be less than 10%. Accuracy was performed as a single experiment at three different levels using three separate solutions by spiking the effervescent placebo at LOQ level, at specification level [0.5% w/w] and at 150 %w/w of specification level and the percent accuracy were calculated at all levels. (Table 4).

Robustness: The solution at specification level was used to evaluate the robustness of the method by making small but deliberate changes in flow rate [±0.1ml/min], dwell

Table 4: Validation data

Validation parameter	Parameter	Levels	NAC	Imp A	Imp B	Imp C	Imp D
System suitability	R_t RRT T_f for NAC	-	3.6 - 1.17	31.07 8.63 -	15.07 4.18 -	5.41 1.50 -	6.92 1.92 -
	Rs between impurity C and D	-	-	-	6.57	-	-
	% RSD of area response for triplicate injection of NAC in diluted standard.	-	0.70	-	-	-	-
Linearity	Slope Intercept Regression coefficient	- - -	3319.2 89766 0.999	3826.1 115.6 0.999	1569.4 238.39 0.999	4585 622.66 1.000	6771.90 264.13 0.9998
Method precision (n=6)	% RSD	LOQ Specification level Intermediate precision	-	2. 5 2. 3 3. 71	4. 2 0. 02 3. 12	2. 1 0. 50 2. 12	4. 4 0. 50 3. 63
Accuracy (n=3)	Mean % recovered (%RSD)	LOQ Specification level 150% w/w of specification	-	104.73 (2.2) 100.22 (2.4) 92.9 (1.3)	106.63 (1.8) 100.06 (3.9) 102.54 (1.8)	99.96 (7.0) 93.47 (1.0) 108.98 (0.5)	104.73 (2.2) 100.22 (2.4) 92.90 (1.3)

Table 5: Robustness data

Robustness parameter		Parameter	NAC	Imp A	Imp B	Imp C	Imp D	Cal t value	Table t value
Change in Flow rate (ml/min)	0.9	Elution volume (ml)	3.60	29.18	15.28	5.42	8.16	0.045	2.355
	1		3.62	30.96	15.56	5.35	6.87	-	-
	1.1		3.69	33.44	16.61	5.55	8.23	0.156	2.015
Change in column temperature	20◦C	Retention time (mins)	3.74	33.12	17.09	5.77	7.18	0.98	2.35
	25◦C		3.62	30.96	15.96	5.35	6.87	-	-
	30◦C		3.5	31.20	14.94	5.02	6.52	0.84	2.35
Dwell volume	Agilent 1260	Retention time (mins)	3.56	28.46	15.80	5.61	7.18	0.4123	0.7078
	Shimadzu LC-20AD		3.62	30.96	14.76	5.35	6.87	0.4123	0.7078
pH	2.0	Tailing factor	1.14	1.01	1.00	1.14	1.02	-	-
	2.2		1.48	1.13	1.05	1.32	1.07	2.000	2.353
	2.4		1.41	1.01	1.03	0.95	1.00	0.400	2.353
Change in organic	80:20	Tailing factor	1.14	1.01	1.00	1.14	1.02	-	-
	88:12		1.43	1.3	0.97	1.24	2.00	0.19	0.090

volume[Agilent 1260 infinity and Shimadzu LC-20AD prominence], column oven temperature[±5°C], pH of the mobile phase[±0.2] and per cent change in organic phase[88:12;methanol: ACN]. The student's t-test was used as the statistical tool to determine the statistical significance and in all the conditions there was no significant difference from the optimum conditions. The results are as displayed in Table 5.

DISCUSSION

The major objective of method development was to achieve separation between NAC and its related compounds. The hurdle was to obtain sufficient selectivity and resolution among structurally similar impurities, degradants and placebo components within a reasonable run time. For selecting the wavelength the UV absorption spectra of NAC and related compounds were studied and an absorption maximum was observed at 210 nm. This wavelength was seen to be of high sensitivity for all related substances and a minimal difference in response factors was observed. For choosing the column, literature was scanned and the C_{18} column was chosen as the stationary phase. NAC and its impurities are highly polar in nature and for their optimum retention a column with a greater non polarity is required.[46] Unsatisfactory results were observed on chromatographing the placebo and the resolution solution in the literature reported conditions owed to the impurities L-cysteine, L-cystine and the placebo components eluting near the void volume. The pKa of NAC are 3.24 [carboxylic acid moiety] and 9.52 [-SH group].[1,2] As per Henderson-Hesselbach equation above their pka acid moieties are known to exist in their ionised forms and elute early from the column, hence trials were performed at pKa -1 i.e.at pH 2.2.[47] The impurities L-cysteine and L-cystine are polar in nature with their Log P being -2.5 and -5.08 respectively.[1,2] For retaining such compounds on non-polar stationary phase mobile phase modifiers like ion pair reagents needs to be employed. 0.01M octane-1-sulphonic acid was employed to shift the impurities to a longer retention time. The anionic part [sulphate] of the ion pair reagent binds to the amino group of the impurities and the non-polar part binds to the non-polar chain on the column and hence increases the retention of the impurities.[47] Literature was reviewed and methanol was seen to be the organic phase of choice. To further reduce the run time and maintain selectivity among structurally similar impurities, degradants and placebo components, 20 parts of acetonitrile was included as part of the organic phase. Acetonitrile is known to have greater elution strength than methanol.[47] Ambient column temperature was maintained and a flow rate of 1ml/minute was used in all the method development trials. The isocratic mode of solvent delivery was followed initially with 90 parts of buffer and 10 parts of methanol, a run time of 75 minutes was observed. To further reduce the run time a gradient solvent delivery was seen imperative. Various gradients were tried and a final gradient [Table 1] with a run time of 55 minutes was optimized. During the course of the method development an interesting observation was the absence of L-cystine

[impurity A] when the placebo spiked with the resolution solution was chromatographed. Further investigations revealed that the pH of the above solution was 3.31 which differ from mobile phase pH of 2.2. The effervescent couple was suspected to cause this change in pH. Effervescent couple is a mixture of citric acid, sodium bicarbonate and sodium carbonate, which in presence of moisture instantaneously reacts and forms carbon dioxide and water. The evolved carbon dioxide dissolves in water to form carbonic acid which keeps the pH of the solution acidic. On storage the solution slowly loses its carbon dioxide and becomes alkaline. At this stage it became imperative to choose an appropriate diluent to recover the impurity L-cystine from the solution. Mineral acids like hydrochloric acid and orthophosphoric acid were screened to choose an optimum diluent. Various strengths of hydrochloric acid [from 0.1M to 0.3M] and orthophosphoric acid [1%v/v to 5% v/v] were tested. The pH of the initial solution, pH immediately after the addition of the placebo and at 3 hours, 6 hours and 24 hours were measured. 0.3 M Hydrochloric acid and 0.45 M orthophosphoric acid solutions were the lowest molar concentration acid solutions which maintain the pH of the placebo solution less than or equal to 2.20 which corresponds to the mobile phase pH. To choose the better diluent among 0.3M hydrochloric acid and 0.45M OPA, placebo spiked with the resolution solution were prepared in both the diluents and chromatographed in the optimized conditions. When 0.45M OPA was used as the diluent an additional peak was seen to co elute at the retention time of L-cysteine. 0.3M hydrochloric acid was selected to be the appropriate diluent as the resolution between the impurities were good and there was no interference from the placebo peaks. [Figure 2 and Figure 4]

Forced degradation studies provide knowledge on the possible degradation pathway and degradation products in API and effervescent formulation of NAC. NAC undergoes various transformations to form its known impurities and unknown impurities in different stress conditions [Table 2 and Table 3].The main degradant in NAC are impurity C and impurity D which are formed in all the stress conditions are due to the high susceptibility of the thiol moiety to oxidize and form disulphide. This impurity is also seen to form during storage of NAC. In addition to the impurity C and D in the acidic condition, impurity B is also seen to be formed this is due to the breaking of the N-C bond in acidic conditions.

Impurity A is not seen in any of the stress conditions thereby confirming it to be a process impurity only and not a degradant. Heat is seen to cause maximum degradation of NAC and photolytic conditions in placebo spiked with NAC. The mass balance was found to be in the range of 96.5 % to 103.5% in all stressed conditions of formulation stressed samples, thus proving the stability –indicating power of the method. Literature reports the formation of impurities B, C and D on subjecting the aqueous solution, and cough syrup to various forced degradation conditions.[39,40] From the forced degradation study conducted in our lab it is clear that known impurities B, C and D are degradation impurities which need to be strictly monitored during stability studies.

CONCLUSION

In the present work a sensitive, specific and reproducible stability indicating HPLC method was established for the quantification of the degradants and process-related impurities of NAC effervescent formulation. The need for the development of an analytical method was identified because of the inadequate capacity of the reported HPLC methods in resolving among the known impurities and placebo peaks. The developed method shows good separation and resolution between the known impurity, degradation impurities and process-related impurities of NAC effervescent formulation. The pH was observed to be a crucial component in the method as the effervescent couple alters the pH of the diluent. The diluent 0.3M hydrochloric acid has proved to be efficient in arresting the pH change within 10% from the mobile phase pH and thus providing appropriate recovery for the impurities. The method has been validated as per ICH guidelines for specificity, linearity, accuracy, and precision, limit of quantitation and limit of detection. The results demonstrate that the method is suitable for evaluating the stability of NAC effervescent Tablet.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ABBREVIATION USED

NAC: N-acetyl cysteine; **IPC:** Ion pair chromatography; **ICH:** International Conference on Harmonization; **SPT:** Single point threshold; **PPI:** Peak purity index; **LOD:** Limit of detection; **LOQ:** Limit of quantification; **RSD:** Relative standard deviation; **ACN:** Acetonitrile; **UV-** Ultraviolet; **OPA:** Orthophosphoric acid; **AR:** Analytical reagent; **HPLC:** High performance liquid chromatography; **CRS:** Certified refrence standard; **LC-UV-MS:** Liquid chromatography-Ultaviolet spectroscopy-mass spectrometry; **HIV:** Human immune deficiency virus; **API:** Active pharmaceutical ingredient.

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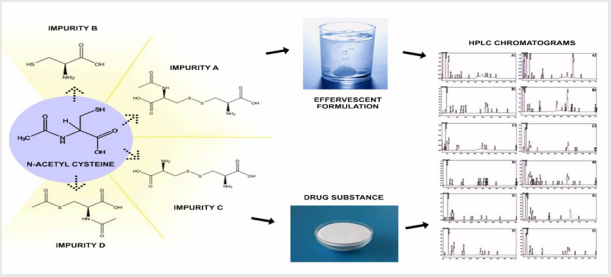
PICTORIAL ABSTRACT



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SUMMARY

- In the present work a stability indicating high-performance liquid chromatographic method was developed for the quantification of the related substances in N-acetyl cysteine [NAC] effervescent formulation. The analytical method was developed because of the insufficient capacity of the existing NAC related substances method in resolving among the known impurities and placebo peaks. The developed method employs a Cadenza C18 column as the stationary phase and 0.01M octane sulphonate [pH 2.20], methanol and acetonitrile in the ratio 90:8:2 as the mobile phase. A gradient programme of run time 55 minutes was followed. The pH of the effervescent couple was an important variable in method development. The method results in good separation between the official impurities and placebo as well as good resolution among the official impurities. The performance of the method was validated as per ICH Q2R1 for specificity, linearity, accuracy, and precision, limit of quantitation and limit of detection. The results demonstrate that the method is suitable for its intended use. Forced degradation studies were performed in stress conditions like acid, alkali, peroxide, heat, light and humidity. Per cent degradation of 5-21% and 12-22% was observed in sample and API. Heat is seen to cause maximum degradation of NAC and photolytic conditions in sample (placebo spiked with NAC). The mass balance was found to be in the range of 96.5 % to 103.5% in all stressed conditions. From the forced degradation study conducted in our lab it is clear that known impurities B, C and D are degradation impurities which need to be strictly monitored during stability studies

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