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ARTICLE

DEVELOPMENT & VALIDATION OF HIGH PRESSURE LIQUID CHROMATOGRAPHY ANALYTICAL METHOD FOR RELATED SUBSTANCE OF ACETYLCYSTEINE EFFERVESCENT TABLET

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Abstract

The HPLC method was developed specially for the related substances of n-acetyl-l-cysteine (NAC) effervescent tablet formulation where the issue of merging of impurities peaks was observed during the development of the method. So, development was triggered with an aim of separation of all the known possible impurities peaks from that of principal peak of acetylcysteine as well as each four known impurities. Finally the analytical method for related substance of acetylcysteine with a gradient program on HPLC was developed and validated with the mandatory experiments. The related substance method was efficient to separate the co-eluting peaks of impurities and its individual known impurities from the principal peaks specifically for the effervescent formulation. The method was specific, selective and reproducible. Recovery and linearity was also found within the acceptance criteria. Solution stability was also established and evaluated during the method validation. Method was successfully evaluated for robustness using various alterations in the method parameters for its functionalities.

Keywords: Acetylcysteine, N-Acetyl-L-Cysteine, NAC, Related Substance, HPLC

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1. Introduction

N-Acetylcysteine (acetylcysteine or n-acetyl-l-cysteine or NAC or (2R)-2-acetamido-3-sulfanylpropanoic acid) acts as a mucolytic agent and used to reduce the viscosity of pulmonary secretions in respiratory diseases. It is also used as an effective antidote in the treatmentof paracetamol poisoning [1-3].

The mucolytic action of NAC is probably due to its ability to decrease the viscosity of secretions by breaking the disulphide bonds of the protein network [4].

Additionally, NAC is acting as effective antioxidant and being studied for the treatment of various diseases such as nephropathy [5], liver failure [6], chronic obstructive pulmonary disease [7] and brain disorders [8]. It has also been used as a metal chelating agent [9]and radioprotective agent [10].

Liquid chromatography (LC) methods either as a conventional method or derivatization method have been extensivelyreported for the assay of N-acetylcysteine in pharmaceuticalformulations and biological samples [11-16]. Certain LC methodsfor quantitative analysis of NAC were based on various other detectors. However, the UV detector was found as universal detector [17-21]. Majority of Related Substance method were described, but the chromatographic issues were arised during the analysis of effervescent tablets formulation due to extensive merging of the impurities peaks, which were interfering with the principal and each other among known impurities. One literature was observed which represented the method for effervescent tablets [22], however, with this method during development, the chromatography was not acceptable with the test formulation.

So, the aim was to develop the specific and selective related substances method using the universal detector (UV) using HPLC method specifically for effervescent tablets formulations without the derivatization sample preparation.

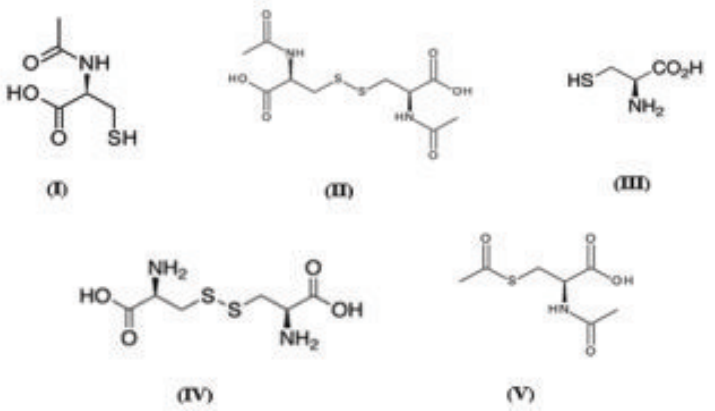

Figure-1 Chemical Structure of Acetylcysteine (I), Impurity-A (II - L-cystine), Impurity-B (III - L-cysteine), Impurity-C (IV - N,N-diacetyl-L-cystine), Impurity-D (V - N,S-diacetyl-L-cysteine)

Literature described the transformation of one polymorphic form to another dihydrate form, which is showing variable dissolution behavior of carbamazepine [11–13]. There have been several reports showing irregular dissolution [14–16], bioequivalence failures [17–19], and clinical failures of carbamazepine [20]. Certain literatures have been found which indicates the impact on polymers to make the drug release profile prolonged. But the optimization of polymer levels and combination of polymers plays a foremost role in achieving a successful extended release formulation which is correlating with the innovator formulation in each dissolution medium i.e. original medium as well as different biological pH.

In general, the enhanced dissolution pattern is depending upon either by dissolution medium pH change or by addition of the solubilizer, like surfactants and cyclodextrin derivatives in the preparation of dissolution medium [21–27]. SLS has been proven as the agent of choice because it is cost-effective and it holds good solubilizing capacity even at quite low concentrations. Already, several authors reported that SLS can be used to enhance dissolution of low water-soluble compounds [28]. Till date, many authors had published the articles on the addition and usage of SLS like sodium taurocholate or other surfactant for executing the dissolution of low soluble drug like carbamazepine using less dissolution media volume. Carbamazepine solubility was also distinctly increased in several nonionic surfactants [29]. But with the medium volume 1800mL according to USP method, very few articles were present which shows the surfactant assisted dissolution to get the higher release profile. Even the data of innovator formulation in original medium as well as in the acidic medium has not been described at a large extent.

But here, the aim of carrying out the in-vitro drug dissolution at various biological pH to partially evaluate the effect of pH on drug release and absorption during in-vivo conditions. And specifically to evaluate the effect of surfactant on drug release and to set the minimum possible optimum concentration of surfactant to achieve desired drug release with a minimum variability in the results of both – innovator as well as test formulations.

1. Experimental

2.1 Chemicals and materials

Acetylcysteine working standard (100.17%) was prepared from an API, against a standard procured from SimsonPharma, Mumbai, India.Impurities standard (Impurity – A, B, C, and D) were procured from TRC, Canada. Methanol (HPLC grade, Merck, India), Methanol (MS Grade, J. T. Baker, USA), Hydrochloric acid (GR Grade, Merck, India), Ammonium Sulfate (GR Grade, Merck, India), Sodium Pentane Sulfonate (HPLC Grade, Merck, India) were used during the development and validation experiments.

2.2 Instrumentation

A high pressure liquid chromatograph system (1260-Infinity-II, Agilent, Germany& LC-2010, Shimadzu, Japan)coupled with UV& PDA detector and quaternary pump, Micro analytical balance (MSA6-6S-000-AM, Sartorius, Japan), pH meter (Thermo Orion Star II, Thermo, USA), Membrane filter (nylon) (Pall corporations, USA), Syringe filter (Nylon, 0.22µ) (Millipore, USA), Ultrasonicator (Bioneeds scientific corporation, India), Water purification system (Milli-Q, Millipore, USA) and RO water systemwere used during method development and validation.

2.3 Chromatographic method parameters

For carrying out related substance experiment, HPLC column – XBridge C18(250 x 4.6mm, 5µ) (Waters, USA) was connected with the LC system and stabilized with the mobile phase at 30°C. Mobile phase was eluted at a 1.0mL/min follow rate with a gradient program. Gradient program was set for Mobile Phase-A as 0à3 (90%), 3à12 (10%), 12à25 (10%), 25à35 (90%). Standard and samples were injected with 100µL injection volume during analysis. The signal of eluted components will be monitored continuously using PDA detector and specifically at 205nm using UV detector using the respective chromatography softwares (LC Solution, Shimadzu, Japan and OpenLab CDS, Agilent, Germany).

2.4 Analytical Procedure

2.4.1 Mobile Phase preparation

Mobile Phase-A consists of 0.5%w/v solution of ammonium sulfate in 0.01M sodium pentane sulfonate in water. pH should be adjusted to pH 2.0 using 2M HCl.Mobile Phase-B consists of amixture of 100mL of methanol and 900mL of a 0.5%w/v solution of ammonium sulfate in 0.01M sodium pentane sulfonate in water. pH of the mixture was adjusted to pH 2.0 using 2M HCl.

2.4.2 Standard Solutions preparation

Impurity A: 5.0mg of Impurity-A standard was dissolved in 5mL of 0.1N HCl and diluted to 50mL with mobile phase-B. 3mL of the resulting solution was diluted up to 10mL with mobile phase-B.

Impurity B:5.0mg of Impurity-B standard was diluted to 50mL with mobile phase-B. 3mL of the resulting solution was diluted up to 10mL with mobile phase-B.

Impurity C: 5.0mg of Impurity-C standard was diluted to 50mL with mobile phase-B. 6mL of the resulting solution was diluted up to 10mL with mobile phase-B.

Impurity D: 5.0mg of Impurity-D standard was diluted to 50mL with mobile phase-B. 3mL of the resulting solution was diluted up to 10mL with mobile phase-B.

Acetylcysteine:5.0mg of Acetylcysteinestandard was diluted to 50mL with mobile phase-B. 6mL of the

resulting solution was dilutedup to 10mL with mobile phase-B.

Standard Solution: 1.0mL of each standard stock solution (Acetylcysteine and Impurities A, B, C, D) were transferred in to the 10mL volumetric flask and diluted with mobile phase-B.

2.4.3 Sample (Test) Solution Preparation

10 effervescent tablets were transferred in to 1000mL volumetric flask. To this, 5mL 1M HCl was added to dissolve and then diluted it with mobile phase-B. 5mL of this solution was transferred in 50mL of volumetric flask and diluted with mobile phase-B. Finally the sample was filtered through 0.22µ nylon syringe filter.

2.5 Method Development & Optimization

Initially during the development of the formulation product related substance (RS) tests, the RS method for raw material from European Pharmacopoeia (EP) was adopted. However, using this method, the issues were raised in the chromatography of the sample solution, where the excipients peaks were observed at the retention time of known impurities and principal peak with a greater intensity. Also the method was not so much efficient to separate out the impurities with a reasonable resolution.

So, ultimately the desire is to separate those known impurities with the enough resolution and the overall chromatography should be free from effervescent tablet excipients peaks. However, to develop the effervescent tablet, focus was also established for the similarity in the behavior of effervescence, appearance of the solution and taste of solution between the test formulation and innovator formulation.

To attain this goal, multiple trials were taken for the usage of mobile phase buffer, mobile phase, mobile phase pH, mobile phase ratio, mobile phase elution program, diluent, stationary phase (LC column) and various grades and types of excipients in the formulations.

Mobile Phase Buffer: Water, ammonium sulfate, Phosphate buffer, triethylamine, ammonium acetate, ammonium formate, tetrabutyl ammonium hydroxide

Mobile Phase: Acetonitrile: Buffer, Methanol: Buffer, Acetonitrile: Methanol: Buffer

Ion Pair Reagent: Pentane sulfonate, Octane sulfonate

Mobile Phase Elution: Isocratic, Gradient

Mobile Phase pH: pH 2.0, 2.5, 3.0, 4.0, 7.5

Grade of Solvent in Mobile Phase: HPLC and MS Grade

Stationary Phase (Column): GL Science Inertsil ODS C18 (3µ), Thermo Hypersil ODS (3µ), AgelaVenusil XBP Polar Phenyl (5µ), Waters X-bridge (3.5µ), Phenomenex Gemini C18 (5µ), PhenomenexSynergi Polar-RP (4µ)

In majority of above multiple combination chromatographic trials, the desired separation was not achieved, except limited number of trials.The chromatography was not powerful with respect to higher resolution and elimination of placebo interference.

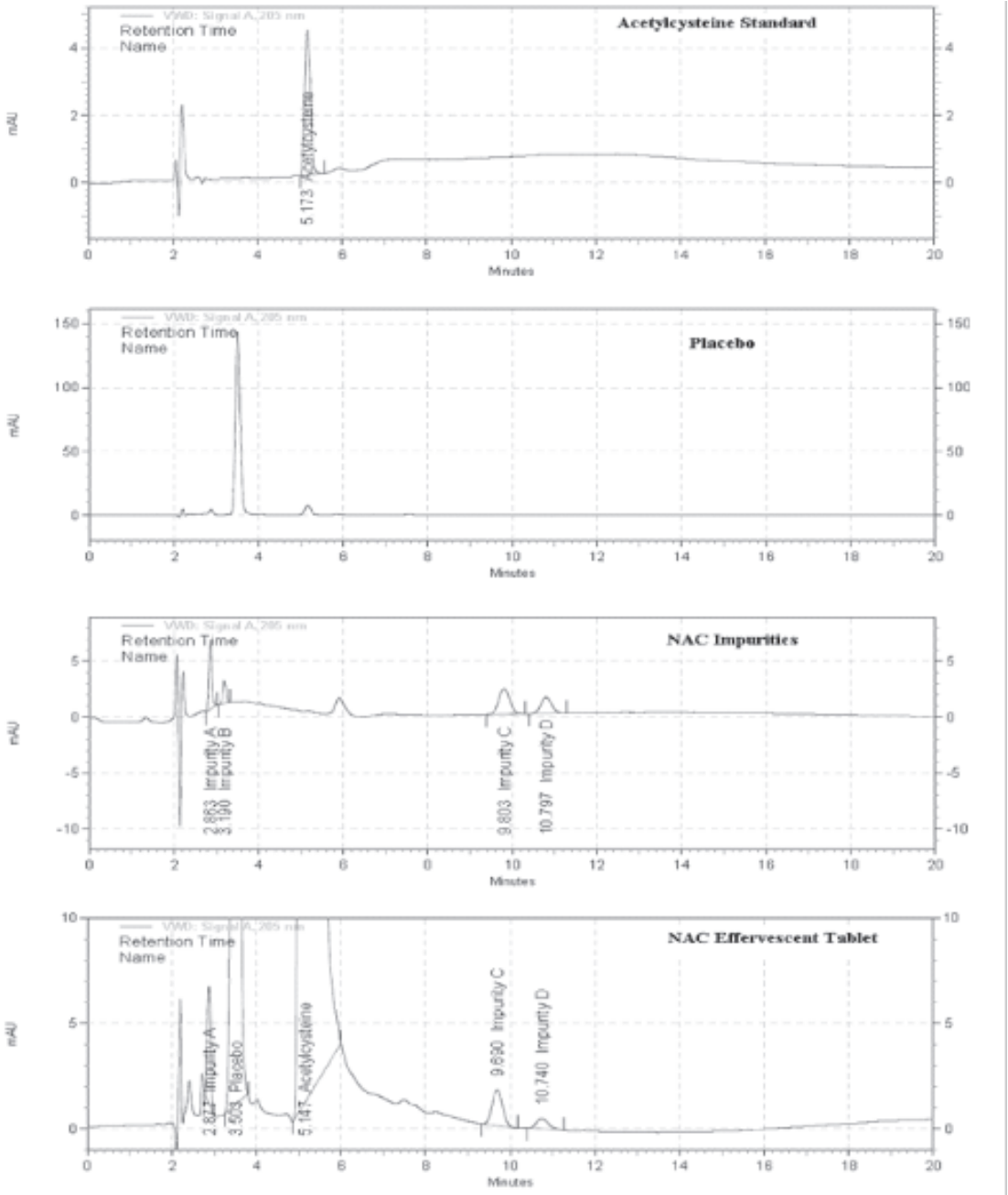

Figure-2 Excipient (Placebo) Interference and Merged Impurity Peaks

Impurity A and B are very fast eluting in almost all the condition and very difficult to separate with good resolution. Also the principal analyte is eluting just after the both impurity A and B. If the isocratic modewas selected, then impurity C and D would be eluting very late and still the difficulties in separation of Impurity A, B and principal peak. So, trials were arranged in such manner to effectively separate both the impurities A and B from each other as well as from excipient peak and principal peaks and to elute the impurities C and D to be eluted earlier after elution of principal analyte. So, overall analysis time and cost for one run can be reduced.

During development, one method was also applied using fluorescence detector to detect the impurity-A & B by derivatization method using fluorenyl-methyl-oxy-carbonyl chloride (FMOC-Cl), but still the method specificity was not achieved with respect to formulation samples. And also we could not able to analyze impurities C and D with this method. So, in that case, separate methods were needed to be developed, which was not suitable.

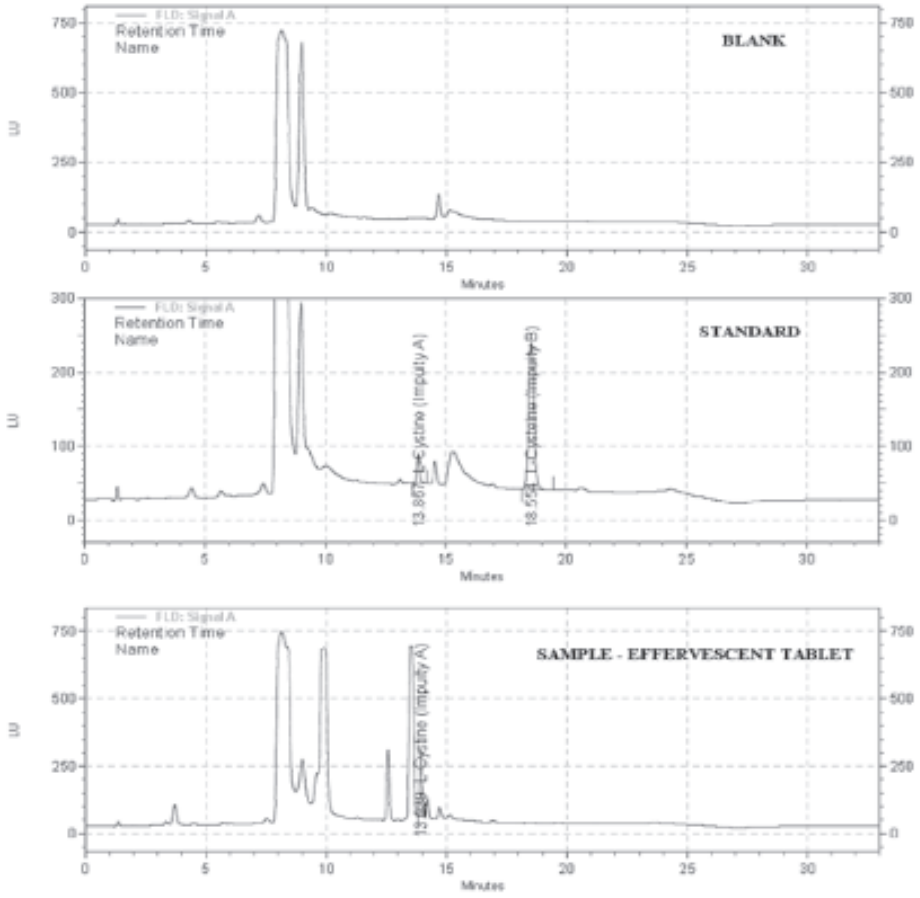

Figure-3-Chromatography with fluorescence detector after derivatization for Impurity A and B

Reasonably better chromatography was achieved with Waters X-bridge column with lower particle size and using ion pair reagent in the mobile phase with low pH and the gradient elution of mobile phase at 30°C column temperature and by using superior grade of methanol (MS grade, J.T.Baker). With this chromatography, the interference of impurities with each other was resolved. Only certain excipients within the effervescent formulation were creating a minor problem in terms of specificity compared to major issues with previous methods. So, finally to resolve the same, grade as well as replacement in the excipients were done in the effervescent formulation manufacturing process to achieve the completely smooth chromatography which can be efficient to analyze the related substances of the acetylcysteine within the product. Lastly, the formulation was developed as per the goal and evaluated for its outcome. The related substance test for this formulation was performed using as such sample solution, impurity spiked sample solution with the final optimized parameters as described above. The chromatography was well accepted with respect to routine chromatographic system suitability parameters (Resolution, Signal-to-Noise Ratio, Theoretical Plates, Tailing Factor etc.). The method specificity was also evaluated using the diluent, standard solution, placebo solution and sample solution.

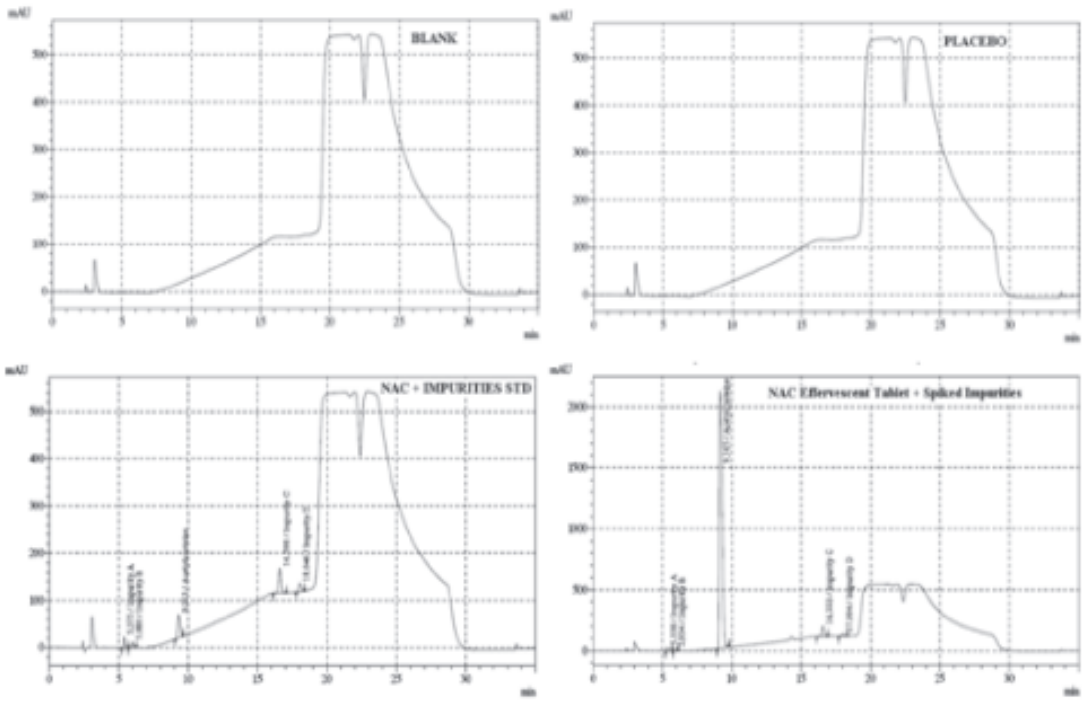

Figure-4-Final Optimized Method Chromatography

2.1 Method Validation

The related substance method for effervescent formulation was developed and optimized as in above section, was also undergone through complete validation by evaluating all the validation parameters. Specificity, accuracy, injection reproducibility, intra-assay, ruggedness using different equipment, linearity, range, solution stability at two different conditions – refrigerated and ambient temperature, robustness by change in mobile phase ratio, column oven temperature & mobile phase pH and forced degradation experiments using all degradation conditions were performed using the final method for validation. The finished product of effervescent tablets with final optimized formula and its placebo were used for the method validation purpose.Forced degradation was carried out by keeping the standard and sample at stressed condition for 24 hours. For oxidative, base and acid degradation, respectively 30% peroxide, 1N NaOH and 1N HCl solutions were used. For thermal hydrolysis, standard and sample were kept at 50°C and 80% RH conditions.

1. Results and discussion

The analytical method for RS of effervescent formulation was completely validated by evaluating all the mandatory parameters. Limit of Detection (LOD) and Limit of Quantitation (LOQ) were also found within the acceptance criteria with respect to signal to noise as well as %RSD and correlation co-efficient. Linearity and Range were accepted throughout the lowest LOD range. The results arerepresented in the following tables.The method was completely validated and it was observed that Impurity-C is more degradant via oxidative pathway and critical in terms of stability. This impurity is increased within the standard, upon storage for more time at room temperature. So, it's mandatory to prepare and inject the standard and samples solution freshly and within the short duration of time to get the exact values of impurities.

Table-1 Specificity

Sample Name	Sample ID	RT	Area	Resolution	TP	TF	Single Point Threshold
Blank	Diluent	No Additional Peak Observed					
Placebo	Placebo	No Additional Peak Observed					
Impurity A	Standard	5.4	156720	-	9602	1.4	0.988
Impurity B	Standard	6.0	90611	-	13915	1.3	0.981
Impurity C	Standard	16.6	659045	-	42949	1.1	0.998
Impurity D	Standard	18.1	237704	-	38842	1.2	0.996
Acetylcysteine+ Impurity A,B,C,D	Impurity A	5.4	184766	-	9135	1.4	0.990
	Impurity B	6.0	81194	2.8	13981	1.3	0.993
	Acetylcysteine	9.3	590698	12.5	12650	1.2	0.998
	Impurity C	16.6	619776	22.5	44244	1.2	0.998
	Impurity D	18.0	228378	4.3	39841	1.2	0.996
Acetylcysteine Tablet + Impurity A,B,C,D	Impurity A	5.3	208755	-	9825	1.4	0.993
	Impurity B	5.9	66052	2.9	14382	1.3	0.994
	Acetylcysteine	9.2	33829601	10.2	7041	1.7	1.000
	Impurity C	16.6	971725	19.6	43280	1.2	0.999
	Impurity D	18.0	225449	4.2	39258	1.2	0.995

Table-2 Recovery

Conc. Level (%)	Impurity-A		Impurity-B		Impurity-D		Impurity-C	
	Rec* (%)	RSD* (%)	Rec* (%)	RSD* (%)	Rec* (%)	RSD* (%)	Rec* (%)	RSD* (%)
10%	97.94	13.32	95.92	11.44	89.84	13.37	87.16	13.16
20%	100.64	7.15	98.82	9.48	98.88	3.97	97.39	8.43
50%	102.07	8.87	98.81	6.85	104.98	4.97	98.32	9.31
80%	101.43	8.81	98.29	7.78	103.92	6.48	96.65	9.22
100%#	99.79	9.24	97.71	7.05	103.86	5.69	109.9	6.15
150%	99.26	8.66	95.37	8.28	103.04	5.74	103.59	8.15

Values of Recovery and RSD were derived from triplicate preparation, *Rec -Recovery

#Conc. of Impurity-A, B, and D: 3ppm, Conc. of Impurity-C: 6ppm

Table-3 Reinjection Reproducibility

	Area		RT	
Standard	**Average**	**RSD (%)**	**Average**	**RSD (%)**
Impurity A	188.120	0.14	5.040	0.03
Impurity B	66.660	0.19	5.620	0.03
Acetylcysteine	543.560	0.07	8.990	0.02
Impurity C	592.420	0.20	16.490	0.01
Impurity D	188.310	0.63	17.880	0.01

Values were derived from 6 replicates injections

Table-4 Intra-Assay

	Area		RT	
	Average	**RSD (%)**	**Average**	**RSD (%)**
Standard				
Impurity A	188.12	0.14	5.04	0.03
Impurity B	66.66	0.19	5.62	0.03
Acetylcysteine	543.56	0.07	8.99	0.02
Impurity C	592.42	0.20	16.49	0.01
Impurity D	188.31	0.63	17.88	0.01
Sample + Spiked Imp.*				
Impurity A	175.119	0.42	5.05	0.05
Impurity B	76.384	0.62	5.62	0.06
Unknown 1	59.013	0.89	5.93	0.08
Unknown 2	92.16	3.85	7.81	0.03
Acetylcysteine	39667.747	0.19	8.89	0.02
Unknown 3	254.432	0.23	13.95	0.02
Impurity C	1413.205	5.81	16.48	0.03
Unknown 4	113.339	2.23	17.05	0.02
Impurity D	190.916	0.58	17.87	0.02

****Values of Average of Area & RT and RSD were derived from 6 sets of sample preparation***

Table-5 Ruggedness

	Experiment-I		Experiment-II		Experiment-III	
	Area		Area		Area	
	STANDARD					
	Average	RSD (%)	Average	RSD (%)	Average	RSD (%)
Impurity A	188.12	0.14	190074	0.24	205156	0.44
Impurity B	66.66	0.19	78520	1.25	65904	0.76
Acetylcysteine	543.56	0.07	589144	0.26	536781	0.28
Impurity C	592.42	0.20	648290	0.24	676710	0.20
Impurity D	188.31	0.63	202915	1.05	166509	0.83
	SAMPLE + Spiked Imp.*					
	Average	RSD (%)	Average	RSD (%)	Average	RSD (%)
Impurity A	175.738	0.62	187853	0.10	212299	0.22
Impurity B	76.549	0.96	83040	0.27	72916	0.29
Acetylcysteine	39740.994	0.20	38210250	0.02	37797758	0.05
Impurity C	1346.974	3.16	1153293	1.06	1161226	0.47
Impurity D	191.548	1.11	204794.5	0.30	216174.5	0.55

Values of Average of Area & RT and RSD were derived from 2 sets of sample preparation

Table-6 Linearity & Range

	Linearity			Range		
	Conc. (ppm)		Correlation coefficient (r^2)	Conc. (ppm)		Correlation coefficient (r^2)
	10%	150%		10%	150%	
NAC	0.611	9.160	0.9994	0.618	9.270	0.9991
Impurity A	0.260	3.902	0.9997	0.282	4.230	0.9996
Impurity B	0.365	5.471	0.9992	0.339	5.085	0.9987
Impurity C	0.543	8.150	0.9996	0.586	8.790	0.9992
Impurity D	0.301	4.521	0.9999	0.298	4.470	0.9995

Table-7 Solution Stability

	Initial (0 hr)		Refrigerator Condition (10 hrs)			Ambient Condition (10 hrs)		
Standard								
	Area	**% Imp.**	**Area**	**% Imp.**	**% RSD**	**Area**	**% Imp.**	**% RSD**
Impurity A	208195	0.50	206564	0.50	0.07	205415	0.50	0.47
Impurity B	66923	0.51	67260	0.51	0.29	70751	0.54	3.29
Impurity C	679661	1.01	747556	1.10	6.40	832359	1.23	13.96
Impurity D	171738	0.52	176344	0.53	1.41	175651	0.53	1.13
Sample + Spiked Imp.								
	Area	**% Imp.**	**Area**	**% Imp.**	**% RSD**	**Area**	**% Imp.**	**% RSD**
Impurity A	211529	0.51	211942	0.52	0.62	208988	0.51	0.37
Impurity B	67504	0.52	68035	0.52	0.09	72144	0.55	4.05
Impurity C	788413	1.17	879191	1.30	7.40	851114	1.26	5.11
Impurity D	171123	0.52	182789	0.55	4.20	178082	0.53	2.35

Values of Average of Area & RT and RSD were derived from 2 sets of sample preparation

Table-8 Robustness

Mobile Phase Ratio			
Condition-1: MP-B Ratio_105 ml Methanol + 895 ml Buffer			
Condition-2: MP-B Ratio_95 ml Methanol + 905 ml Buffer			
Sample + Spiked Imp.	**%Assay**		**RSD (%)**
	Condition-1	**Condition-2**	
Impurity A	107.0	97.5	6.57
Impurity B	103.9	103.7	0.14
Impurity C	89.3	92.6	2.57
Impurity D	113.4	108.2	3.32
Column Oven Temperature			
Condition-2: Column temperature 32°C			
Sample + Spiked Imp.	**%Assay**		**RSD (%)**
	Condition-1	**Condition-2**	
Impurity A	95.2	98.1	2.12
Impurity B	98.0	98.3	0.22
Impurity C	106.3	101.7	3.13
Impurity D	94.6	97.4	2.06
Mobile Phase pH			
Condition-1: Mobile Phase pH 1.8			
Condition-2: Mobile Phase pH 2.2			
Sample + Spiked Imp.	**%Assay**		**RSD (%)**
	Condition-1	**Condition-2**	
Impurity A	101.2	97.1	2.92
Impurity B	105.9	99.1	4.69
Impurity C	86.0	90.0	3.21
Impurity D	104.7	98.4	4.39

Values were derived from 2 sets of sample preparation in each experiment

Table-9 Forced Degradation

% Degradation (For 24 Hrs)					
Known and Unknown Impurity	**Oxidative**	**Base**	**Acid**	**Thermal Hydrolysis**	**Photo**
Acetylcysteine Standard					
Unknown Impurities (%)	**0.21**	**0.12**	**0.33**	**1.68**	**0.19**
Impurity A	-	-	-	-	-
Impurity B	-	-	0.48	3.85	0.09
Impurity C	5.21	2.53	0.81	3.54	2.03
Impurity D	-	-	0.18	6.57	0.11
Total Degradation (%)	**5.42**	**2.65**	**1.80**	**15.64**	**2.42**
Acetylcysteine Sample					
Unknown Impurities (%)	**0.1**	**0.12**	**0.17**	**0.23**	**0.14**
Impurity A	-	-	-	-	-
Impurity B	-	-	0.08	0.89	0.70
Impurity C	24.64	2.63	1.14	3.51	1.06
Impurity D	0.04	0.04	0.73	5.40	2.69
Total Degradation (%)	**24.78**	**2.79**	**2.12**	**10.03**	**4.59**

Table-10 LOD-LOQ

	Impurity A	**Impurity B**	**Acetylcysteine**	**Impurity C**	**Impurity D**
LOD*					
% RSD	1.44	1.22	4.83	1.35	3.36
LOQ#					
% RSD	0.39	0.05	0.13	0.29	0.25

***Values were derived from 3 replicates injections, #Values were derived from 6 replicates injections**

4. Conclusions

The principal aim of the development of this related substance method specifically for the effervescent formulation was to separate out the excipient interference from that of known impurities and principal peaks as well as to separate out impurities from individual peaks as well as principal peak using the universal UV detector of liquid chromatography and without any derivatization method. So, in the development phase, more focus was given on the analytical method aspects as well as on the excipients grade and type of excipients used which may affect the overall chromatography, as the principal analyte and impurity have the wavelength maxima at lower side (205nm), so chances of poor chromatography is more higher. Finally the optimization was done using the theoretical approach for all the chromatographic parameters to develop the robust and accurate method for acetylcysteine related substance analysis.

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