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Review

Analytical Method Validation for Related Substances of Carbocisteine Syrup 250mg/5ml BY HPLC

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Abstract

The objective of this study is to validate the HPLC method to be used for the determination of related substances of Carbocisteine syrup 250mg/5ml by HPLC.

Introduction: Carbocisteine syrup is used to treat respiratory conditions with thick mucus, brochitis, cystic fibrosis and COPD (Chronic Obstructive Pulmonary Disease).

Materials and Methods: Waters HPLC Model no. 2695, HPLC Column: Lichrosphere-100 NH2, 250 mm x 4.6 mm, 5-µm was used with Mobile phase Buffer and Acetonitrile in the ratio of 570:430%v/v.

Results and Discussion: System precision and System suitability, Specificity, Limit of Detection (DL) & Limit of Quantitation (QL), Precision at QL, Method precision, Linearity, Accuracy, Range, Intermediate Precision and Robustness were evaluated.

Conclusion: The HPLC method for Related substances of Carbocisteine Syrup 250mg/5ml has been validated. The test method was found to be specific, precise, linear and accurate in the range of QL (Limit of Quantitation) to 200% of Carbocisteine specification level and can be used for intended purpose.

Keywords: HPLC, Carbocisteine syrup, brochitis, cystic fibrosis and COPD (Chronic Obstructive Pulmonary Disease), Acetonitrile

Introduction:

IUPAC name of Cabocisteine is (2R)-2-amino-3-(carboxymethylsulfanyl) propanoic acid, molecular structure is given in Figure 1. Molecular weight is 179.20 g/mol. Carbocisteine is a white crystalline powder with very low water solubility, measured at approximately 0.16% w/v. It is practically insoluble in ethanol and ether. Its solubility is significantly improved in alkaline solutions (by forming salt with NaOH) or in diluted mineral acids. The melting point of carbocisteine is (208-213°C).

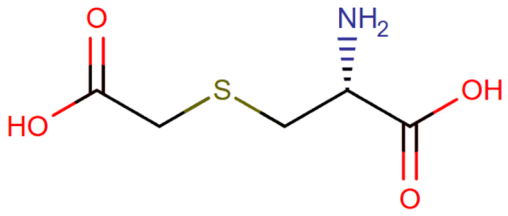

Figure.1.Carbocisteine

Carbocisteine impurities primarily consist of process-related compounds, including unreacted raw materials like cystine and chloroacetic acid, as well as synthetic by-products such as N,S-dicarboxymethyl-cysteine (a major synthesis by-product), carbocisteine lactam (a degradation or synthetic by product), and carbocisteine sulfoxides (oxidation products). These impurities arise from synthesis or degradation (oxidation/moisture)[1].

Materials and Methods:

Chemical and reagents include Potassium dihydrogen phosphate, Ortho phosphoric acid and Sodium hydroxide were purchased from Rankem, Acetonitrile, Hydrogen peroxide (30%) were purchased from Fisher scientific, Hydrochloric acid was purchased from Merck, 0.45 µm Nylon filter was purchased from Axiva, 0.45 µm PVDF filter was purchased from Simple pure.

Standards and Samples like Carbocisteine working standard, Placebo for Carbocisteine, Carbocisteine drug substance, Carbocisteine Lactum and Carbocisteine Sulfoxide were purchased from Pharma Zell. Carbocisteine drug product 250mg/5ml and Placebo were prepared in-house.

Waters HPLC Model no. 2695, HPLC Column: Lichrosphere-100 NH2, 250 mm x 4.6 mm, 5-µm was used with Mobile phase Buffer and Acetonitrile in the ratio of 570:430%v/v.

Analytical test method:

Mobile phase preparation: The composition of mobile phase is Buffer and Acetonitrile in the ratio of 570:430%v/v. Buffer was prepared by dissolving 6.8g of Potassiumdihydrogen phosphate anhydrous in 1000 ml of HPLC water (Milli Q Water) and pH was adjusted to 3.50 by using diluted phosphoric acid. The solution was filtered and degassed before usage.

Chromatographic conditions:

Column	: Lichrosphere-100 NH2, 250 mm x 4.6 mm id., 5-µm.
Flow rate	: 1.0 mL /min.
Wavelength	: 205 nm
Column temperature	: 25°C
Injection Volume	: 20 µL
Run Time	: For Standard: 15 minutes, For Sample: 30 minutes.
Diluent	: HPLC Water

Standard and check standard stock Preparation: Stock solution was prepared by weighing 20 mg of Carbocisteine working standard and transferring into 100 ml volumetric flask. 1 ml of 1 N Sodium hydroxide solution was added, then 40 ml of diluent was added, the solution was Sonicated for 2-3 minutes till clear solution was obtained. 1 ml of 1N Hydrochloric acid solution was added, remaining volume was made up with diluent and mixed well.

Diluted standard and check standard preparation: 5 ml of the above solution was Pipetted into 50 ml volumetric flask and made up to mark with diluent.

Test preparation: Shake the bottle 10 sec then Weigh and Transfer accurately 2ml syrup solution into a 25 mL volumetric flask (Concentration: 4 mg / mL). Add 10mL of diluent and shake the flask for 2 minutes. Make up to mark with diluent. Filter the portion of

above solution throw 0.45 µm syringe filter and inject
in into HPLC.

Procedure: Sequence of injections is given in the following table (Table 1)

Table.1. HPLC Sequence of injections

S. No.	Sample ID	No of Injections
1	Blank	2
2	Standard preparation	6
3	Check Standard preparation	1
4	Test Placebo Preparation for RS	1
5	Test Preparation for RS	1
6	Bracketing standard	1

System suitability:
Standard preparation:

i) Tailing factor for Carbocisteine peak should be not more than 2.0
ii) Theoretical plates should not be less than 2000.
iii) %RSD of six replecates of standard solution should not be more than 5.0
iv) Bracketing standard %RSD should not be more than 5.0

Check standard preparation: % of the recovery should be 95.0 to 105. 0.

Elution order of impurities:

Table.2. Elution order of impurities

S.No	Impurity	RRT	RRF
1	Carbocisteine Lactum	About 0.6	5.89
2	Carbocisteine Sulfoxide	About 1.3	1.80

Calculations:

$$\text{Carbocisteine Lactum and Sulfoxide} = \frac{T}{S} \times \frac{Ws}{100} \times \frac{5}{50} \times \frac{25}{Wt} \times \frac{Density}{L} \times \frac{P}{100} \times CF \times 100$$

$$\text{Unknown impurity} = \frac{T}{S} \times \frac{Ws}{100} \times \frac{5}{50} \times \frac{25}{Wt} \times \frac{Dencity}{L} \times \frac{P}{100} \times CF \times 100$$

Where,
T = Area of impurity in test preparation.
S = Area of standard preparation.
Ws = Weight of standard taken, in mg, for standard preparation.
Wt = Weight of sample taken, in mg.
P = Potency of Carbocisteine standard calculated as Carbocisteine.
D = Dencity of Carbocisteine syrup solution .
L = Labeled amount of Carbocisteine mg/ml.
CF = Correction factor

RESULTS AND DISCUSSION

Method Validation Summary

System precision and system suitability: The standard, check standard and spiked standard solution (six times) with known impurities at specification level were prepared as per test method and injected into HPLC system. The system suitability parameters, % RSD for peak areas of Carbocisteine and known impurities and RRTs for Carbocisteine and known impurities were evaluated as per the test method and found to be within the acceptable limits. The results and of standard solution are given in Table 3 and Table 4. Typical chromatogram is given in Figure 2.

Table 3: System suitability data for Carbocisteine

System suitability parameters for Carbocisteine	Method Precision	Intermediate precision	Accuracy	Linearity	Acceptance criteria
%RSD	1.4	1.0	1.9	2.3	Not more than 5.0
Tailing factor	1.1	1.1	1.1	1.1	Not more than 5.0
Theoretical plates	8242	8486	7811	8208	Not less than 2000
Check standard recovery (%)	97.9	103.1	102.1	102.9	Between 95.0 to 105.0

Table 4: System precision data for Carbocisteine

S. No.	Type of compound	% RSD	Acceptance criteria
1.	Carbocisteine	1.4	Not more than 5.0

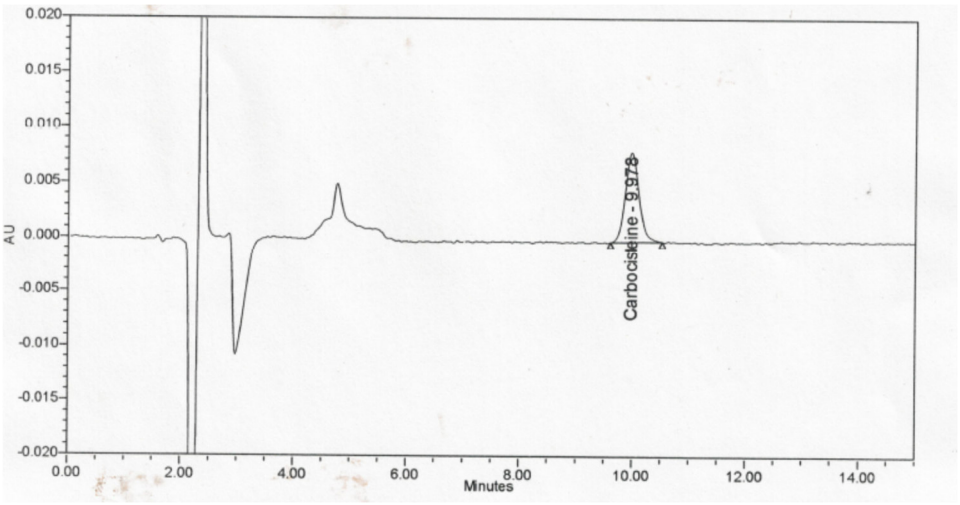

Figure.2. Typical chromatogram of standard solution

Specificity:

Blank interference: Blank solution was prepared in and injected as per test method. It was observed that no blank peaks were eluting at the retention time of Carbocisteine and known impurities peaks.

Placebo interference: Placebo solution was prepared in duplicate and injected as per test method. It was observed that no placebo peaks were interfering at the retention time of Carbocisteine and known impurities peaks.

Impurity interference: Individual impurities solutions were prepared at specification level of test concentration and analyzed as per test method. It was observed that impurities are not co eluting with each other and with Carbocisteine peak. Spiked standard and sample solutions were prepared in single by spiking Carbocisteine and all known impurities at

specification level of test concentration and analyzed as per test method. Peak purity of the Carbocisteine peak in the spiked sample solution was found to be within the acceptable limit. Retention times of known impurities in the individual preparations are comparable with the retention times of respective impurities in the spiked test preparation.

Forced degradation: Performed the forced degradation studies on the drug substance, placebo and the drug product for Acid, Alkali, Peroxide, Water, Thermal and Humidity degradations and injected as per test method. Percentage net degradation was calculated and evaluated the Carbocisteine peak purity in each stressed condition using Empower software. The peak purity of Carbocisteine peak in the entire stressed sample was found to be within the acceptable limit. The results are given in Table 5 to Table 8.

Table.5. Blank and Placebo interference

Sample No.	Peak found at RT of Carbocisteine peak (Yes / No)	
	Blank	Placebo
1	No	No
2	No	No

Acceptance criteria: Chromatogram of blank and placebo should not show any peak at the retention time of Carbocisteine peak and known impurities peaks.

Table.6.Impurity interference

Peak Name	RT of impurities & main analyte		
	Spiked sample RT	Individual impurities	Acceptance criteria
Carbocisteine	9.8min	N/A	Known impurities should not co-elute with each other and with Carbocisteine peak.
Carbocisteine Lactum	6.03	6.04	
Carbocisteine Sulfoxide	13.12	13.16	

Table.7.Peak purity table

Sample Name	Peak Name	Purity angle	Purity threshold	Total Peak purity	Acceptance criteria
Sample (Un spiked)	Carbocisteine	0.223	0.409	Pass	Passed

Acceptance criteria:

1. The peak purity of Carbocisteine peak in stressed sample should pass
2. Purity angle should be lessthan purity threshold in waters empower soft ware

Table.8.Peak purity table

S. No.	Condition	Procedure	Purity angle	Purity threshold	Peak Purity
1.	Sample Acid degradation	1N HCl at 60°C for 30 mts	0.114	0.277	Pass
2.	Sample Alkali degradation	1N NaOH at 60°C for 30 mts	0.080	0.259	Pass
3.	Sample Peroxide degradation	1% H_2O_2 at 60°C for 30 mts	0.096	0.263	Pass
4.	Sample Water degradation	Water at 60°C for 30 mts	0.122	0.289	Pass
5.	Sample Thermal degradation	Sample kept at 60°C for 2 Hrs	0.153	0.296	Pass

6.	Sample Humidity degradation	Sample kept in humidity chamber 1 day	0.140	0.301	Pass
7.	API Alkali degradation	1N NaOH at 60°C for 30 mts	0.076	0.256	Pass
8.	API Peroxide degradation	1% H_2O_2 at 60°C for 30 mts	0.091	0.261	Pass
9.	API Water degradation	60°C at 60°C for 30 mts	0.079	0.261	Pass
10.	API Acid degraded	1N HCl at 60°C for 30 mts	0.078	0.258	Pass
11.	API Humidity degradation	Sample kept in humidity chamber 1 day	0.085	0.259	Pass
12.	API Thermal degradation	Sample kept at 60°C for 2 Hrs	0.089	0.263	Pass

Acceptance criteria:

1. The peak purity of Carbocisteine peak in stressed sample should pass
2. Purity angle should be lessthan purity threshold in waters empower soft water

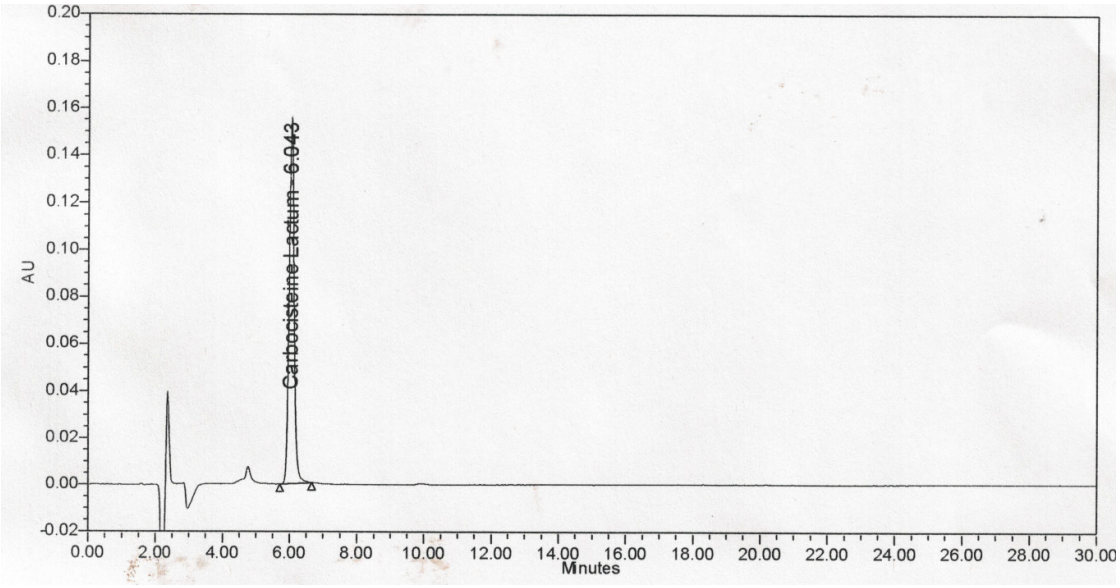

Figure.3.Typical chromatogram of Carbocisteine Lactum

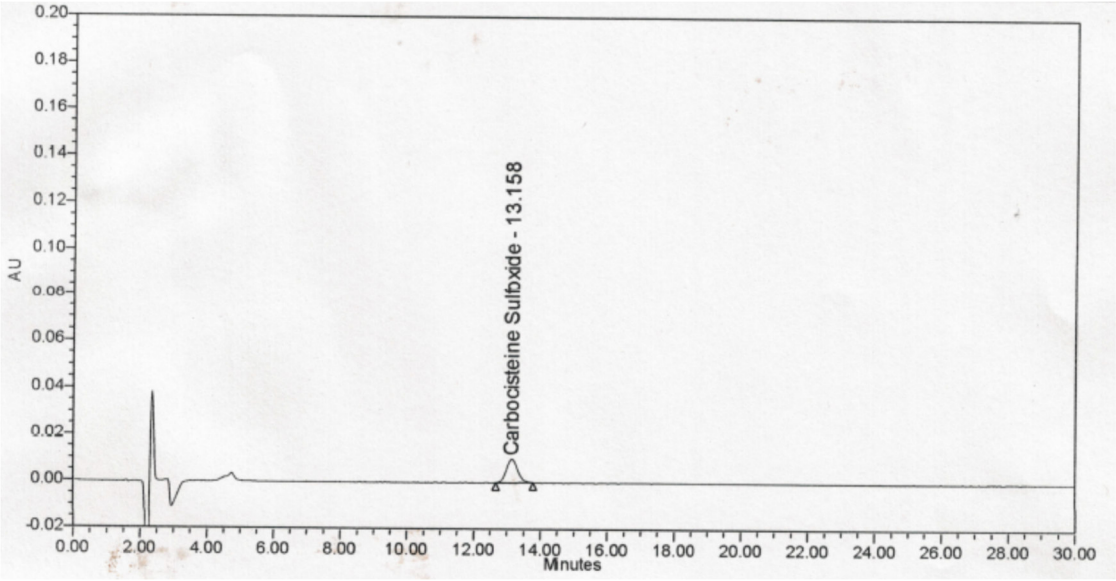

Figure.4.Typical chromatogram of Carbocisteine Sulfoxide

DL, QL establishment and Precision at QL: To establish the DL and QL for Carbocisteine peak, appropriate concentrations of the Carbocisteine and known impurities were prepared and injected as per test method. Signal to noise ratio for DL of impurities carbocisteine Lactum,Carbocisteine sulfoxide and Carbocisteine were found to be 3,3 and 2 respectively and for QL were found to be 10,12 and 9 respectively. Carbocisteine and known impurities solutions were spiked on the placebo at about QL concentration and injected six times in to the HPLC system. % RSD for individual known impurity and Carbocisteine of six samples were calculated and found to be within the acceptance criteria. The results are summarized in Table.9. and Table.10.

Table.9.QL and DL data of Carbocisteine with S/N ratio

Sample Name	DL Concentration(%w/w)	S/N Ratio	QL Concentration (%w/w)	S/N Ratio
Carbocisteine	0.01	2	0.03	9
Carbocisteine Lactm	0.02	3	0.03	10
Carbocisteine Sulfoxide	0.02	3	0.05	12

Acceptance criteria: For DL signal to noise ration should be about 3. For QL signal to noise ration should be about 10.

Table.10.Precision at QL data for Carbocisteine

No. of Injection	Content at QL		
	Carbocisteine	**Carbocisteine Lactum**	**Carbocisteine Sulfoxide**
1	0.01	0.05	0.04
2	0.01	0.05	0.04
3	0.01	0.05	0.04
4	0.01	0.04	0.04
5	0.01	0.04	0.04
6	0.01	0.04	0.04
Mean	0.01	0.05	0.04

% RSD	0.0	12.17	0.0

Acceptance criteria: % RSD for % of individual known impurity and Carbocisteine should be no more than 15.0

Method precision: To evaluate the precision for RS method, six samples were prepared by spiking the individual known impurities at specification (0.5% and 1% m/m) level and analyzed as per test method. The % RSD of six samples for each individual known impurity and the % of total impurities were calculated and found to be within the acceptance criteria. The results are summarized in Table.11.

Table.11.Method Precision data

Sample No.	Carbocisteine Lactum content(%w/w)	Carbocisteine Sulfoxide content(%w/w)	Total impurities (%w/w)
1	1.01	0.5	1.51
2	1.04	0.52	1.56
3	1.03	0.5	1.53
4	1.03	0.51	1.54
5	1.05	0.53	1.58
6	1.01	0.51	1.52
Mean	**1.03**	**0.51**	**1.54**
% RSD	**1.6**	**2.3**	**1.7**

Acceptance criteria: The %RSD for each individual known impurity and % of total impurities should not be more than 15.0 for replicate preparations.

Linearity

A series of Carbocisteine and known impurities (Carbocisteine Lactum,Carbocisteine sulfoxide) solutions were prepared in the concentration ranging from QL to 200% of specification level and injected into the HPLC system as per the test method. Linearity of detector response was established by plotting a graph between concentration and response of Carbocisteine peak and impurity peaks. The detector response was found to be linear from about QL to 200% of specification level. The square of correlation coefficient, intercept and residual sum of squares were calculated and found to be within the acceptable limit. The linearity graphs are given in figure 5 to figure 6.

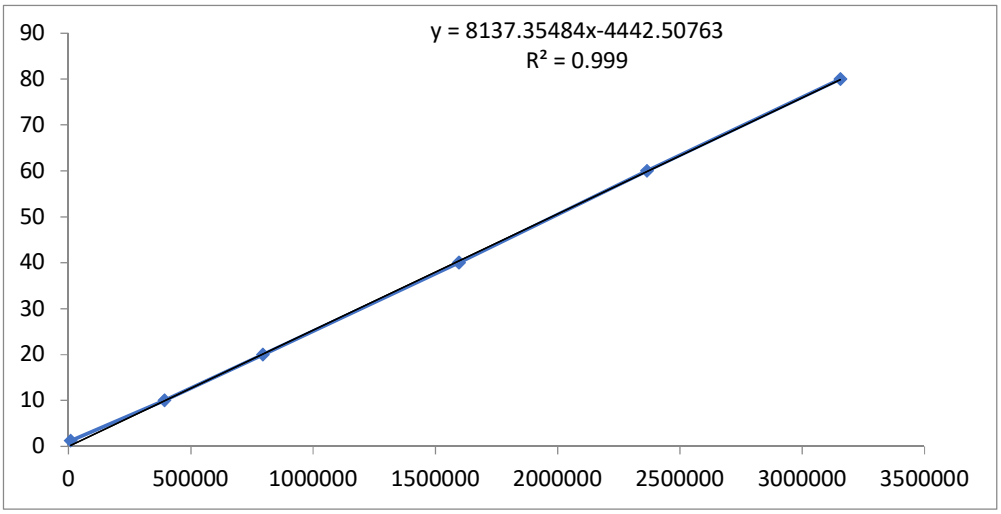

Figure.5.Linearity of detector response graph for Carbocisteine

Acceptance criteria: Square of Correlation coefficient should be not less than 0.99.

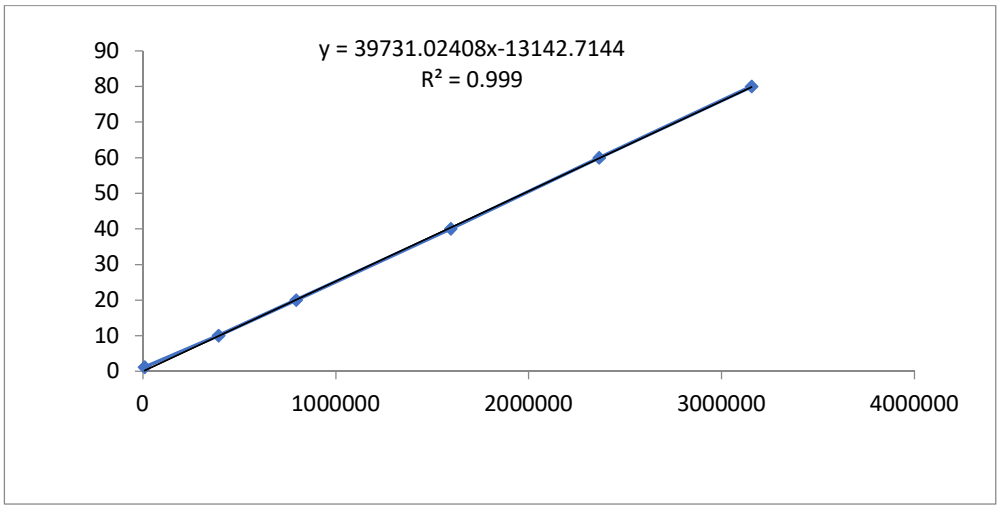

Figure.6.Linearity of detector response graph for Carbocisteine Lactum

Acceptance criteria:

1. Square of Correlation coefficient should be not less than 0.99.

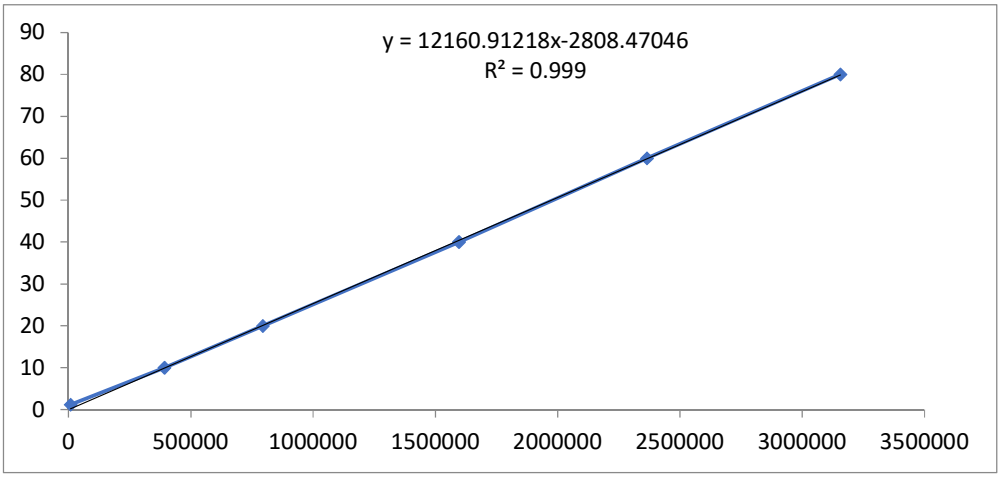

Figure.7.Linearity of detector response graph for Carbocisteine Sulfoxide

Acceptance criteria: Square of Correlation coefficient should be not less than 0.99.

Accuracy

A series of solutions were prepared in triplicate by spiking the test preparation with known impurities at the specification limit in the range of about QL to 200% of test concentration and injected into HPLC system and analyzed as per the test method. Individual % recovery, mean % recovery, % RSD and linearity of the test method are calculated at each level and the results were found to be within the acceptable limits. The accuracy results are summarized in Table 12 and Table 13.

Table.12.Accuracy data of Carbocisteine Lactum

S. No.	% spike level	Amount added (%w/w)	Amount recovered (%w/w)	% Recovery	% Mean recovery	% RSD
1.	LQ	0.12	0.118	98.69	100.75	3.44
2.		0.12	0.115	96.11		
3.		0.12	0.124	103.09		
4.		0.12	0.118	98.67		

5.		0.12	0.123	102.63		
6.		0.12	0.126	105.32		
1.	100	4	4.03	100.78	102.26	1.48
2.		4	4.12	103.05		
3.		4	4.08	102.09		
4.		4	4.08	101.87		
5.		4	4.19	104.83		
6.		4	4.04	100.91		
1.	200	8	7.64	95.45	96.26	0.7
2.		8	7.80	97.43		
3.		8	7.71	96.36		
4.		8	7.70	96.27		
5.		8	7.66	95.79		
6.		8	7.70	96.27		

Table.13.Accuracy data of Carbocisteine sulfoxide

S. No.	% spike level	Amount added (%w/w)	Amount recovered (%w/w)	% Recovery	% Mean recovery	% RSD
1.	LQ	0.2	0.183	91.14	102.87	11
2.		0.2	0.243	120.66		
3.		0.2	0.192	95.41		
4.		0.2	0.189	94.04		
5.		0.2	0.218	108.29		
6.		0.2	0.216	107.65		
1.	100	2.01	1.98	98.67	100.56	1.77
2.		2.01	2.04	101.65		
3.		2.01	1.98	98.48		
4.		2.01	2.02	100.58		
5.		2.01	2.07	103.16		
6.		2.01	2.03	100.81		
1.	200	4.02	3.895	96.89	97.6	1.17
2.		4.02	3.975	98.89		
3.		4.02	3.888	96.71		
4.		4.02	3.971	98.77		
5.		4.02	3.867	96.2		
6.		4.02	3.945	98.13		

Acceptance criteria:

1. Individual % recovery and mean % recovery of the each known impurity should be between 70.0 to 130.0 for QL and 85.0 to 115.0 for Other Level.
2. % RSD at each level for replicate test preparations should be not more than 15.0

Range

Based on Method precision, Linearity and Accuracy data it can be concluded that the related substances method is precise, linear and accurate in the range of QL-200% of specification level.

Intermediate precision

To evaluate intermediate precision for RS method, six samples were prepared by spiking the individual known impurities at specification level and analyzed as per test method by using different column, by different analyst on different day. Percentage RSD for each known individual impurity and the % of total impurities for intermediate precision were calculated and found to be within the acceptable limits.

The overall % RSD of six samples in method precision, intermediate precision (n=6 and n=12) for each individual known impurity and % RSD for each individual known impurity and the % of total known

impurities were calculated and found to be within the acceptable limits.

Robustness

Filter Validation A study was conducted to evaluate the filter suitability by using two different types of filters namely 0.45 µm PVDF and 0.45 µm Nylon filters. Standard solution was prepared in single and test solution was prepared in duplicate by spiking the known impurities at specification level in as per the test method. Portion of standard and test solutions were filtered through 0.45 µm PVDF, 0.45 µm nylon filter and some portion of standard and sample solutions were centrifuged and analyzed as per test method. Similarity factors were calculated for the filtered standards against unfiltered standard (Centrifuged) and found to be within the specified limit. The difference in the percentage of individual known impurities and the percentage total impurities between unfiltered (centrifuged) and filtered samples were calculated and were found to be not meeting the acceptance limit. Both PVDF and Nylon filters were not suitable for the intended purpose.

Flow rate variation: A study was conducted to determine the effect of variation in flow rate. Blank, Standard, and spiked sample with known impurities at specification level were prepared as per the test method and injected into HPLC system with flow rates of 1.1mL/minute and 0.9mL/minute. System suitability parameters and RRTs of known impurities in spiked sample were evaluated and found to be within the specified limits as per test method.

Column oven Temperature variation: A study was conducted to determine the effect of variation in Column oven Temperature. Standard and spiked test preparations with known impurities at specification level were prepared as per the test method and injected into HPLC system with column oven temperature of 30°c. System suitability parameters and RRTs of known impurities in spiked sample were evaluated and found to be within the specified limits as per test method.

Effect of variation in mobile phase composition: A study was conducted to determine the effect of variation in mobile phase composition. Two different mobile phases of Buffer and Acetonitrile were prepared in the ratio 550:450v/v and 590:410%v/v as per the test method. Standard and spiked test preparations with known impurities at specification level were prepared as per the test method and injected into HPLC system System suitability parameters and RRTs of known impurities in spiked sample were evaluated and found to be within the specified limits as per test method.

Effect of pH variation in mobile phase: A study was conducted to determine the effect of variation in pH in mobile phase. Two mobile phases of pH 3.7 and 3.5 were prepared as per the test method. Blank, Standard and spiked test preparations with known impurities at specification level were prepared as per the test method and injected into HPLC. System suitability parameters and RRTs of known impurities in spiked sample were evaluated and found to be within the specified limits as per test method.

Conclusion

The HPLC method for Related substances of Carbocisteine Syrup 250mg/5ml has been validated. The test method was found to be specific, precise, linear and accurate in the range of QL (Limit of Quantitation) to 200% of Carbocisteine specification level and can be used for intended purpose.

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