RESEARCH PAPER

QUALITY-BY-DESIGN ORIENTED DEVELOPMENT AND VALIDATION OF A ROBUST RP-HPLC METHOD FOR ANALYSIS OF A CYSTEAMINE HYDROCHLORIDE AND ITS FORCED DEGRADATION STUDY

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Abstract:

The present study focused on the development and validation of an AQbD-based stability-indicating RP-HPLC method for analysis of Cysteamine Hydrochloride used in cystinosis treatment. Central Composite Design (CCD) was applied to optimize chromatographic parameters such as mobile phase composition and flow rate, and their effects on retention time, peak area, and theoretical plates were evaluated using Design-Expert® Software.

Chromatographic separation was achieved on a C18 column using Acetonitrile and 0.1% OPA water as mobile phase with detection at 210 nm. The method was validated according to International Council for Harmonisation guidelines and showed excellent linearity, accuracy, precision, robustness, and sensitivity with low %RSD values. Forced degradation studies under acidic, basic, oxidative, neutral, and photolytic conditions confirmed the stability-indicating nature of the method by effectively separating degradation products from the drug peak. Oxidative degradation showed maximum degradation, whereas neutral and photolytic conditions showed comparatively less degradation.

Overall, the developed RP-HPLC method was simple, accurate, precise, robust, sensitive, and suitable for routine quality control and stability studies of Cysteamine Hydrochloride formulations.

Key word: Cysteamine Hydrochloride, Analytical Quality by Design (AQbD) ,Central Composite Design (CCD), Forced Degradation Study

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Introduction :

Analytical Quality-by-Design (AQbD) applies QbD principles to analytical methods to ensure consistent and reliable performance throughout the method li fecycle. Key elements include Analytical Target Profile (ATP), Critical Quality Attributes (CQAs), Critical Method Parameters (CMPs), risk assessment, Design of Experiments (DoE), and control strategy. AQbD enhances method understanding, robustness, and regulatory acceptance, especially for stability-indicating methods.[1]**Experimental Run Data : E**xperimental run data are an important part of AQbD-based RP-HPLC method development as they help in understanding the effect of critical analytical parameters on chromatographic performance. In the present study, Central Composite Design (CCD) was used to optimize the RP-HPLC method for analysis of Cysteamine Hydrochloride by varying factors such as mobile phase composition and flow rate. The obtained responses including retention time, peak area, theoretical plates, and tailing factor were statistically evaluated to determine optimized chromatographic conditions. The experimental run data also supported development of a robust and stability-indicating analytical method suitable for routine quality control analysis.[2]

Cysteamine as the Drug of Choice for Cystinosis : Cysteamine (β-mercaptoethylamine) is the only approved treatment for cystinosis. It reduces intracellular cystine by converting it into compounds that can exit lysosomes through alternative transporters. Analytical evaluation of cysteamine is challenging due to its:High polarity,Low molecular weight,Thiol functional group,Oxidative instability These properties complicate chromatographic analysis and necessitate stability and degradation studies during formulation and regulatory assessment.[3]

Literature Survey :Several researchers have reported the use of AQbD principles for development of robust and stability-indicating RP-HPLC methods.

Reid et al. emphasized the importance of Design of Experiments (DoE) in analytical method optimization. Rozet et al. highlighted the application of Quality-by-Design concepts for improving robustness and reliability of chromatographic methods. Peraman et al. reported successful the basis for analytical method validation and forced degradation studies.[4,5]

Instrumentation : The analysis of Cysteamine Hydrochloride was carried out using a Agilent technologies,1260 Infinity system equipped with a quaternary gradient HPLC pump and Photo Diode Array (PDA) detector using a reverse phase HPLC column. The chromatographic signal was monitored and integrated using ChromNAV chromatographic software.

Chemicals and reagents :

The chemicals and reagents used during the analytical study were of HPLC grade to ensure accuracy and reliability of the results. Methanol (HPLC grade), Ortho Phosphoric Acid (OPA, development of stability-indicating RP-HPLC methods using AQbD approaches, while Karmarkar et al. demonstrated the application of Central Composite Design (CCD) for optimization of chromatographic conditions. Regulatory guidelines such as ICH Q2(R1), Q8(R2), and Q1A(R2) provide

Material & method:

0.1%), Water (HPLC grade), and Acetonitrile were procured from Loba Chemie. All chemicals were used as received without any further purification and were suitable for chromatographic analysis. The active pharmaceutical ingredient (API) used in the study was Cysteamine Hydrochloride, which was obtained from Scienlogy Research Lab. The marketed formulation used for analysis was Cystagon capsules (50 mg), manufactured by Mylan Pharmaceuticals. Both the API and formulation were used for the development and validation of the analytical method.

Structure of Cysteamine HCL

Table No 1 : Experimental trial for choice of column

Column	Observation	Inference
C8	Poor Retention of Analyte	Broad and poor peak shape
C18	Improved Retention Of Analyte	Better peak shape

Table No 2 : Experimental trial for choice of mobile Phase

Mobile phase Composition	Observation	Inference
Water : Methanol	No precision in Retention time. Broad Peak with tailing	Use of buffer required and use of methanol to improve peak shape
Methanol : 0.1 % Orthophosphoric Acid	No precision in Retention time. Good Peak shape	Use of buffer and methanol required.

Determination of λmax of Cysteamine HCl by UV-Spectroscopy

An accurately weighed 10 mg of Cysteamine Hydrochloride was transferred into a 10 mL volumetric flask and dissolved in acetonitrile. The volume was made up to the mark with acetonitrile to obtain a stock solution of 1000 µg/mL. The solution was scanned in the UV region of 200–400 nm using acetonitrile as blank to determine the wavelength of maximum absorption (λmax). The obtained spectrum was used for further analytical studies.[6]

Selection of RP-HPLC Chromatographic Parameters by QbD Approach

A Central Composite Design (CCD) using Design-Expert® Software Version 13 was employed for optimization of the RP-HPLC method under Quality by Design (QbD) principles. The effects of mobile phase composition and flow rate on chromatographic responses such as retention time, peak area, and theoretical plates were evaluated through 13 experimental runs consisting of factorial, axial, and centre points. The CCD model enabled assessment of linear, interaction, and quadratic effects of variables and facilitated identification of robust chromatographic conditions with minimum experimental trials.[7,8]

Preparation of Standard Sample Solution from Marketed Formulation

Twenty capsules of Cystagon containing Cysteamine Hydrochloride were weighed, and powder equivalent to 10 mg of drug was transferred into a 100 mL volumetric flask. The drug was extracted with acetonitrile by sonication for 15 minutes, and the volume was made up with acetonitrile to obtain a stock solution of 100 µg/mL.

The solution was filtered through a 0.45 µm membrane filter. Further, 3 mL of the stock solution was diluted to 10 mL with mobile phase to obtain a working solution of 30 µg/mL for RP-HPLC analysis.[9]

Chromatographic conditions:

The chromatographic analysis was carried out using an Agilent Technologies HPLC system (Model 1100) equipped with CHEMSTATION software for data acquisition and processing. Separation was achieved on a C18 column having dimensions of 4.6 × 250 mm with a particle size of 5 µm. The mobile phase consisted of Acetonitrile and 0.1% Orthophosphoric Acid (OPA) water in the ratio of 80.5:19.5 (v/v). The analysis was performed at a detection wavelength of 210 nm with a flow rate maintained at 0.97 mL/min. The column temperature was kept at 25°C throughout the analysis, and the sample injection volume was 20 µL.

Initial Method Development:

Choice of Column: In order to select the most suitable chromatographic column for the analysis of Cysteamine Hydrochloride, several preliminary experimental trials were carried out using different chromatographic conditions. Based on the observations obtained from the initial experimental runs and chromatographic peak characteristics, the Agilent C18 column was selected for further method development studies due to its satisfactory peak symmetry, retention behavior, and resolution.

software-Aided Method Development:

A new Reverse Phase-High Performance Liquid Chromatographic (RP-HPLC) method was developed for the estimation of Cysteamine Hydrochloride using a Quality-by-Design (QbD) approach. The analytical Quality-by-Design methodology combined with Design of Experiments (DoE) was applied to achieve a robust and optimized chromatographic method. The method development process mainly involved the following phases:

a)ScreeningPhase

b) Statistical Analysis and Final Optimization

Analytical Method Validation of Cysteamine Hydrochloride by RP-HPLC method

System Suitability:

System suitability testing is an essential part of analytical method validation and was performed to verify the performance of the developed RP-HPLC method for Cysteamine Hydrochloride. The test was carried out by injecting six replicate injections of the standard Cysteamine Hydrochloride solution under optimized chromatographic conditions. Various chromatographic parameters such as retention time, theoretical plates, peak area, and tailing factor were evaluated to ensure the adequacy, reproducibility, and suitability of the chromatographic system.

Limit of Detection (LOD) and Limit of Quantification (LOQ):

The Limit of Detection (LOD) and Limit of Quantification (LOQ) of the developed RP-HPLC method for Cysteamine Hydrochloride were determined by injecting progressively lower concentrations of the standard solution. The concentrations producing signal-to-noise ratios of approximately 3:1 and 10:1 were considered as the LOD and LOQ, respectively.

Linearity:

The linearity of the developed RP-HPLC method was evaluated over a suitable concentration range around the working concentration of Cysteamine Hydrochloride. Different concentrations of standard solutions were prepared and injected into the HPLC system. Calibration curves were constructed by plotting peak area versus concentration, and the correlation coefficient was calculated to assess the linear relationship of the method.

Precision and Accuracy:

The precision of the developed analytical method was evaluated in terms of intraday and interday precision and expressed as percentage Relative Standard Deviation (%RSD). Different concentration levels of Cysteamine Hydrochloride standard solutions were analyzed repeatedly on the same day and on different days to determine the precision of the method. Accuracy of the developed method was determined by recovery studies performed at different concentration levels, and the percentage recovery of Cysteamine Hydrochloride was calculated to confirm the accuracy and reliability of the RP-HPLC method.

Analysis of Marketed Formulation:

Twenty capsules of Cystagon containing Cysteamine Hydrochloride were accurately weighed and finely powdered. An amount of powder equivalent to 10 mg of Cysteamine Hydrochloride was transferred into a 10 mL volumetric flask and dissolved using the selected diluent. The solution was sonicated for 20 minutes to ensure complete extraction of the drug and then diluted up to the mark with the diluent. The resulting solution was filtered through Whatman filter paper to remove insoluble excipients. A suitable aliquot of the filtrate was further diluted to obtain the desired working concentration of Cysteamine Hydrochloride. Finally, 20 µL of the prepared sample solution was injected into the RP-HPLC system under optimized chromatographic conditions for analysis.

Stability Studies:

To establish the stability-indicating capability of the

developed RP-HPLC method, the standard stock solution of Cysteamine Hydrochloride was subjected to various stress conditions to induce degradation. The drug was exposed to acidic, alkaline, oxidative, photolytic, and thermal degradation conditions. Standard and stressed sample solutions were analyzed under optimized chromatographic conditions to evaluate the ability of the method to separate Cysteamine Hydrochloride from its degradation products.

Preparation of Standard Solution:

An accurately weighed quantity of 5 mg of Cysteamine Hydrochloride working standard was transferred into a 50 mL volumetric flask and dissolved in diluent to obtain a standard stock solution having a concentration of 100 µg/mL. This solution was used as the untreated standard solution for analysis.

Acidic Degradation:

For acidic degradation studies, an accurately weighed quantity of Cysteamine Hydrochloride was treated with 0.1 N hydrochloric acid (HCl) solution and kept at room temperature for 3 hours to promote acid hydrolysis. After the degradation period, the stressed solution was neutralized using an appropriate quantity of sodium hydroxide solution to stop further degradation. The solution was then diluted with the mobile phase to obtain the desired concentration, filtered through a 0.45 µm membrane filter, and analyzed using the developed RP-HPLC method. The chromatogram obtained was evaluated to determine the extent of acidic degradation and formation of degradation products.

Basic Degradation:

For alkaline degradation studies, an accurately weighed quantity of Cysteamine Hydrochloride was treated with 0.1 N sodium hydroxide (NaOH) solution and maintained at room temperature for 3 hours. This study was performed to evaluate the stability of the drug under alkaline conditions. After completion of the degradation period, the degraded solution was neutralized with hydrochloric acid solution. The final solution was diluted with the mobile phase, filtered through a 0.45 µm membrane filter, and injected into the RP-HPLC system for chromatographic analysis. The chromatogram obtained was used to evaluate the degradation behavior of Cysteamine Hydrochloride under basic conditions.

Results of UV Method:

Oxidative Degradation:

For oxidative degradation studies, an accurately weighed quantity of Cysteamine Hydrochloride was treated with 3% hydrogen peroxide (H_2O_2) solution and kept at room temperature for 3 hours. Oxidative stress conditions were applied to study the susceptibility of the drug towards oxidation. After completion of the degradation period, the solution was diluted appropriately with the mobile phase without neutralization. The prepared sample was filtered through a 0.45 µm membrane filter and analyzed using the developed RP-HPLC method. The chromatographic results were evaluated for oxidative degradation products and percentage degradation of the drug.

Photolytic Degradation:

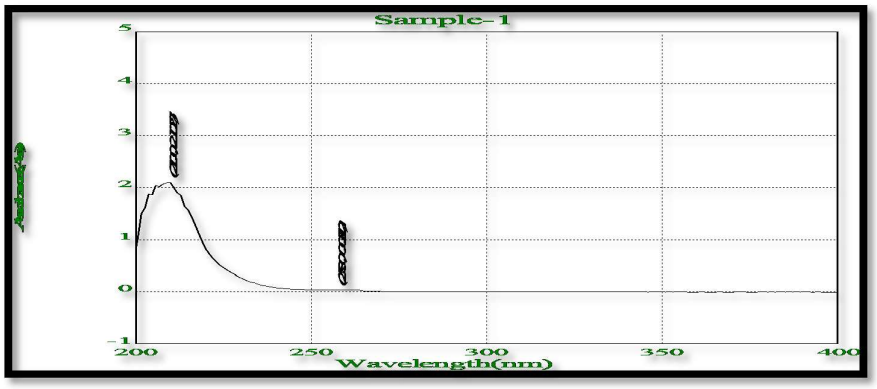
For photolytic degradation studies, the prepared solution of Cysteamine Hydrochloride was exposed to UV/light conditions for 24 hours to investigate the effect of light on drug stability. After exposure, the solution was diluted with the mobile phase to obtain the required concentration for analysis. The sample solution was filtered through a 0.45 µm membrane filter before injection into the HPLC system. The chromatogram obtained after analysis was compared with that of the untreated standard solution to determine the extent of photolytic degradation and to confirm the stability-indicating nature of the developed RP-HPLC method.

Results and discussion:

Choice of column:

Cysteamine Hydrochloride possesses polar characteristics due to the presence of amino and thiol functional groups, making reverse-phase chromatography suitable for its analysis under optimized chromatographic conditions. Different reverse-phase columns were evaluated during method development, and the C18 column was selected because it provided better retention behavior, improved peak symmetry, and satisfactory chromatographic performance. Acetonitrile and 0.1% Orthophosphoric Acid (OPA) water were selected as the mobile phase components to achieve good resolution, proper peak shape, and reproducible chromatographic results. The optimized chromatographic conditions using the Agilent C18 column enabled efficient separation and accurate analysis of Cysteamine Hydrochloride along with its degradation products during forced degradation studies.

Fig No 1 : UV Spectra of



Cysteamine HCl at 210 nm

The sample was scanned between 200-400 nm wavelength and sample showed its maximum wavelength at 210 nm as shown in fig no 1.

Results of QbD:

A Central Composite Design (CCD) using Design-Expert® version 13 software was applied for optimization of the RP-HPLC method for Cysteamine Hydrochloride by evaluating two critical method parameters: mobile phase composition (A) and flow rate (B). The experimental design consisted of 13 trial runs including 4 factorial points, 4 axial points, and 5 center points. The factorial points were used to evaluate the main effects of the selected variables, whereas the axial points were used to study the quadratic effects of the method parameters. The center points were repeated five times to determine the repeatability of the method and to estimate experimental error. Different combinations of mobile phase composition consisting of Acetonitrile and 0.1% Orthophosphoric Acid (OPA) water along with varying flow rates were investigated during the optimization process. Based on desirability function and chromatographic responses such as retention time, peak area, tailing factor, and theoretical plates, the optimized chromatographic condition was obtained at the selected mobile phase composition and flow rate, which provided satisfactory chromatographic performance for Cysteamine Hydrochloride analysis. Among all experimental runs, the optimized run demonstrated consistent peak symmetry, acceptable retention time, and improved chromatographic efficiency. Thus, the Central Composite Design successfully established a reliable quadratic model and robust optimized chromatographic conditions for the analysis of Cysteamine Hydrochloride, as shown in Table No. 1.

	Independent Variable		Dependent Variable		
	Factor 1	Factor 2	Response 1	Response 2	Response 3
Run	A:MOBILE PHASE	B:FLOW RATE	R1(RT)	R2(AREA)	R3(TP)
	%	ML/MIN	MIN	AUC	TP
1	80	0.9	3.242	678.504	7603
2	80	0.9	3.243	678.959	7631
3	85	1	2.654	643.662	7806
4	80	0.758579	3.884	818.75	8882
5	**87.0711**	**0.9**	**2.848**	**691.559**	**9356**
6	85	0.8	3.3	794.263	9700
7	80	0.9	3.244	678.512	7613
8	80	1.04142	2.812	585.135	6835
9	75	1	3.419	612.22	6462
10	80	0.9	3.246	679.869	7632
11	80	0.9	3.249	679.148	7643

12	75	0.8	4.261	768.447	7438
13	72.9289	0.9	4.116	682.664	6692

Table No 3 : Qbd runs

DESIGN EXPERT STUDIES:

Response 1: R1(RT)

Factor coding was coded and sum of squares was Type III–Partial. The model showed a significant F-value of 5016.89, indicating that the developed model was highly significant with only a 0.01% chance of occurring due to noise. Model terms A, B, AB, A^2, and B^2 were found to be significant ($p < 0.0500$). The Lack of Fit F-value of 32.36 was significant, suggesting that the model did not adequately fit the experimental data.

Table No 4 : Anova of Retention time

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2.79	5	0.5579	5016.89	< 0.0001	significant
A-MOBILE PHASE	1.55	1	1.55	13921.52	< 0.0001	
B-FLOW RATE	1.13	1	1.13	10143.87	< 0.0001	
AB	0.0096	1	0.0096	86.36	< 0.0001	
A^2	0.0952	1	0.0952	855.98	< 0.0001	
B^2	0.0174	1	0.0174	156.24	< 0.0001	
Residual	0.0008	7	0.0001			
Lack of Fit	0.0007	3	0.0002	32.36	0.0029	significant
Pure Error	0.0000	4	7.700E-06			
Cor Total	2.79	12				

Fit Statistics for Response 1 : The Predicted R^2 value of 0.9981 was in close agreement with the Adjusted R^2 value of 0.9995, indicating good model predictability. The Adeq Precision value of 227.637, which is greater than 4, demonstrated an adequate signal-to-noise ratio, confirming that the model was suitable for navigating the design space.

$$\mathbf{R1\ (RT) = 3.24 - 0.4399A - 0.3755\ B + 0.0490\ AB + 0.1170A^2 + 0.0500\ B^2}$$

The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.

Fig no 2 : Normal plot of Residuals

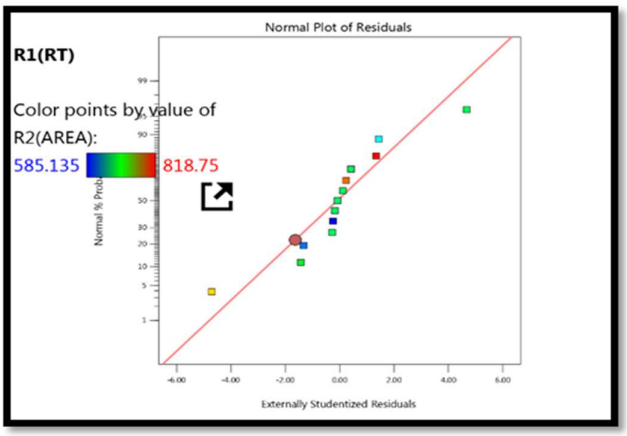


Fig No 3 : Residual vs.Run

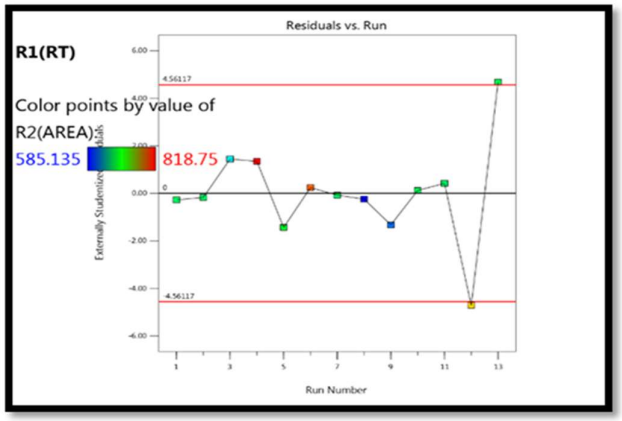


Fig No 4 : Predicted vs. Actual

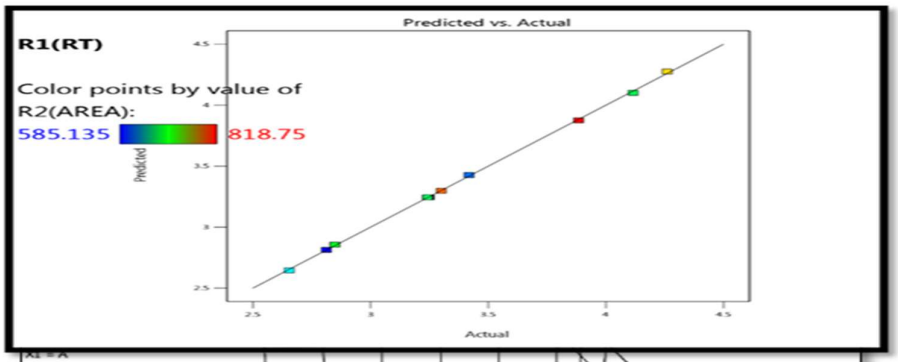
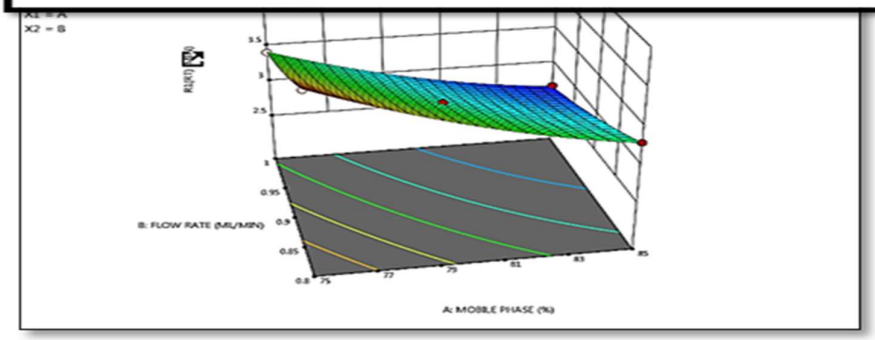


Fig No 5: Counter Plot and 3D Plot for response 1: Retention Time



Response 2: R2(AREA) :

Factor coding is **Coded**.

Sum of squares is **Type III - Partial**

The **Model F-value** of 141.06 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, B, B^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The **Lack of Fit F-value** of 552.47 implies the Lack of Fit is significant. There is only a 0.01% chance that a Lack of Fit F-value this large could occur due to noise. Significant lack of fit is bad -- we want the model to fit

Table 6 : ANOVA for response 2

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	52896.22	5	10579.24	141.06	< 0.0001	significant
A-MOBILE PHASE	609.61	1	609.61	8.13	0.0247	
B-FLOW RATE	50754.39	1	50754.39	676.75	< 0.0001	

AB	7.91	1	7.91	0.1055	0.7548	
A^2	301.83	1	301.83	4.02	0.0849	
B^2	1363.91	1	1363.91	18.19	0.0037	
Residual	524.98	7	75.00			
Lack of Fit	523.72	3	174.57	552.47	< 0.0001	significant
Pure Error	1.26	4	0.3160			
Cor Total	53421.20	12				

The **Predicted R^2** of 0.9302 is in reasonable agreement with the **Adjusted R^2** of 0.9832; i.e. the difference is less than 0.2.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 38.292 indicates an adequate signal. This model can be used to navigate the design space.

Final Equation in Terms of Coded Factors

$$\mathbf{R2(Area)= 679.00+8.73A-79.65B+1.41\ AB+6.59\ A^2+14.00\ B^2}$$

The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.

Fig No 6 : Normal plot of Residuals

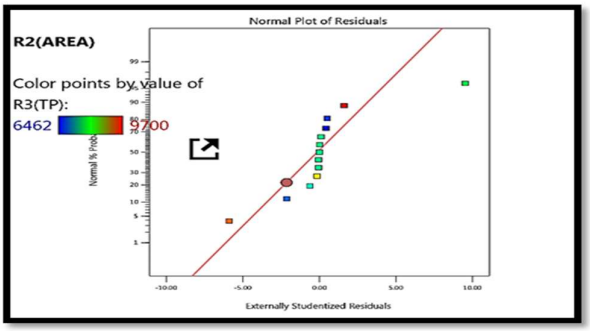


Fig No 7 : Residual vs. Run

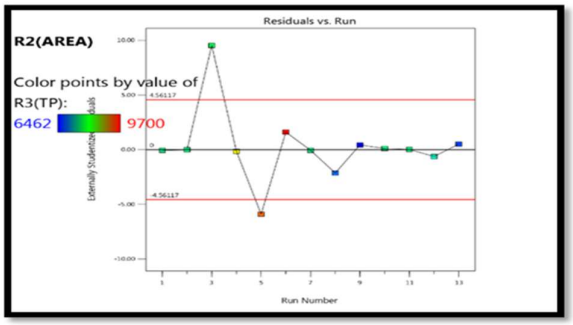


Fig No 8 : Predicted vs. Actual

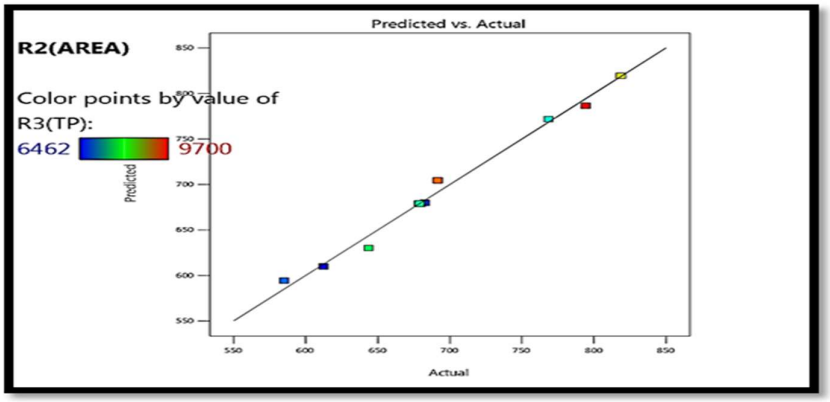
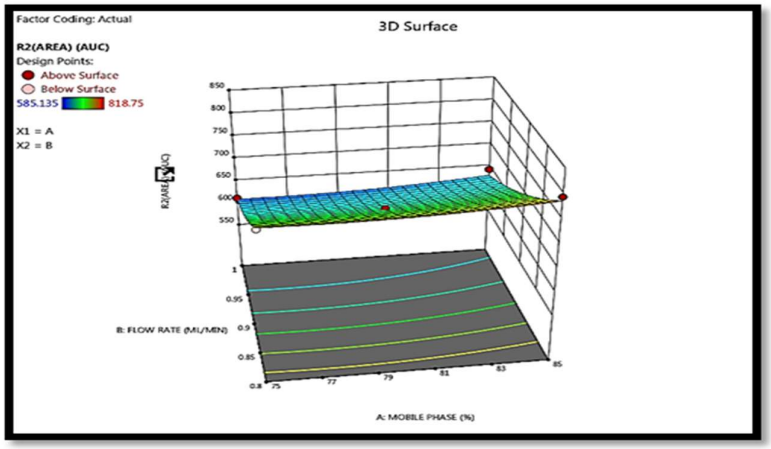


Fig No 9 : Counter and 3D Plot for response 2: Area



Response 3: R3(TP)
ANOVA for response 3
Factor coding is Coded**.**
Sum of squares is **Type III – Partial**
The **Model F-value** of 780.32 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.
P-values less than 0.0500 indicate model terms are significant. In this case A, B, AB, A^2, B^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.
The **Lack of Fit F-value** of 25.05 implies the Lack of Fit is significant. There is only a 0.47% chance that a Lack of Fit F-value this large could occur due to noise. Significant lack of fit is bad – we want the model to fit.

Table No 7 : ANOVA for response 3

Source	**Sum of Squares**	**Df**	**Mean Square**	**F-value**	**p-value**	
Model	1.142E+07	5	2.283E+06	780.32	< 0.0001	significant
A-MOBILE PHASE	6.796E+06	1	6.796E+06	2322.66	< 0.0001	
B-FLOW RATE	4.154E+06	1	4.154E+06	1419.80	< 0.0001	
AB	2.107E+05	1	2.107E+05	72.00	< 0.0001	
A^2	2.188E+05	1	2.188E+05	74.79	< 0.0001	
B^2	62271.48	1	62271.48	21.28	0.0024	
Residual	20481.66	7	2925.95			
Lack of Fit	19446.46	3	6482.15	25.05	0.0047	significant
Pure Error	1035.20	4	258.80			
Cor Total	1.144E+07	12				

Fit statistics for response 3
The **Predicted R^2** of 0.9878 is in reasonable agreement with the **Adjusted R^2** of 0.9969; i.e. the difference is less than 0.2.
Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 89.381 indicates an adequate signal. This model can be used to navigate the design space.
Final Equation in Terms of Coded Factors

$$R3(TP)= 7624.40+921.68A-720.61B-229.50\ AB+177.36\ A^2+94.61\ B^2$$

The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.

Fig No 10 :Normal plot of Residuals

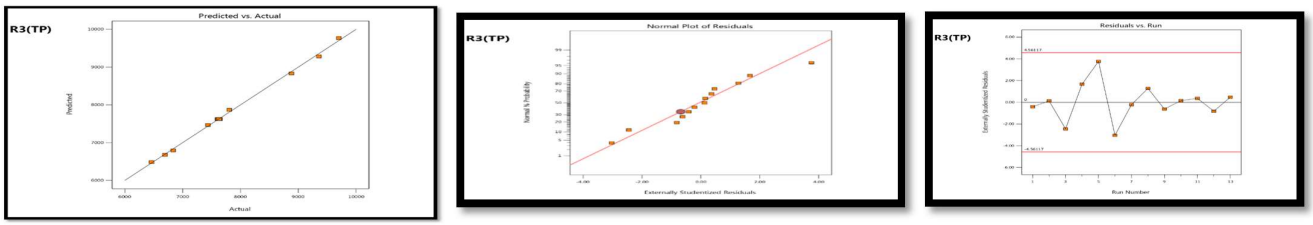


Fig No 11 ;Residual vs. Run **Fig No 12 Predicted vs Actual**

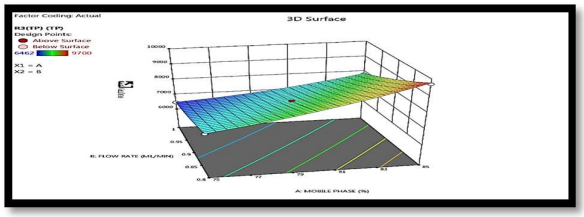


Fig No 13 : Counter and 3D Plot for response 3 : Theoritical Plates

Factors :

Table No 8 : Optimized conditions

Factor	Name	Level	Low Level	High Level	Std. Dev.	Coding
A	**Mobile phase**	87.07%	75.00	85.00	0.0000	Actual
B	**Flow rate**	0.9766 ml/min	0.8000	1.0000	0.0000	Actual

Confirmation Location

MOBILE PHASE	FLOW RATE
87.07 %	0.976567 ml/min

Result: The Central Composite Design successfully optimized the chromatographic conditions. Among the 13 runs, Run 5 (87.07% mobile phase and 0.9 mL/min flow rate) showed optimum performance with suitable retention time, good peak area, and high theoretical plates, and was selected for further RP-HPLC method development.

Results of RP-HPLC Method Development:

System Suitability:

A sample was prepared and introduced and checked for system suitability parameters including

Retention Time: In HPLC (High-Performance Liquid Chromatography), retention time (tR) is the time it takes for a specific compound to travel through the column and elute at the detector.
Theoretical plates: Theoretical plate number (N) is an index that indicates column efficiency. It describes the number of plates as defined according to plate theory, and can be used to determine column efficiency based on calculation in which the larger the theoretical plate number the sharper the peaks.
Asymmetry (Tailing factor): In High-Performance Liquid Chromatography (HPLC), peak asymmetry refers to the non-symmetrical shape of a chromatographic peak, where the peak's leading and trailing edges are not identical. Instead of a bell-shaped curve, the peak may be elongated or broadened on one side (fronting or tailing).
Limit of Detection (LOD) and Limit of Quantitation (LOQ) :
The method exhibits good sensitivity, as indicated by the low LOD and LOQ values. This confirms that the method is capable of detecting and quantifying the analyte at very low concentrations with acceptable accuracy and precision. As shown in table no 9 and 10

LOD=	3.3 X SD/ Slope
	3.2670247
	0.1048804

LOQ=	10X SD/Slope
	9.9000748
	0.3178194

Linearity :
The linearity of the developed RP-HPLC method for Cysteamine Hydrochloride was evaluated over the concentration range of 10–50 µg/mL. A linear relationship between concentration and peak area was observed under optimized chromatographic conditions. The mean peak areas obtained for concentrations of 10, 20, 30, 40, and 50 µg/mL were 315.79, 623.06, 934.47, 1228.03, and 1570.88 respectively. The %RSD values were found within acceptable limits (0.03–0.56%), indicating good precision and reproducibility of the method. The average standard deviation obtained was 0.99, confirming the reliability of the developed RP-HPLC method for quantitative analysis of Cysteamine Hydrochloride As shown in table no 11.

Table No 11 : Linearity

Conce	Area I	II	III	IV	V	VI	Mean	SD	%RSD
10	314.5435	317.03873	0.099	31.15	**315.89**	10.14	315.79	1.76	0.56
20	623.2924	622.83398	0.099	31.15	**623.16**	20.01	623.06	0.32	0.05
30	935.7508	933.18518	0.099	31.15	**934.57**	30.00	934.47	1.81	0.19
40	1228.447	1227.6204	0.099	31.15	**1228.13**	39.43	1228.03	0.58	0.05
50	1570.557	1571.2114	0.099	31.15	**1570.98**	50.43	1570.88	0.46	0.03
							Avg SD	0.99	

Acceptance Criteria:
Correlation coefficient (R^2) should be 0.999.
The linearity of the system was illustrated graphically and found to be linear.

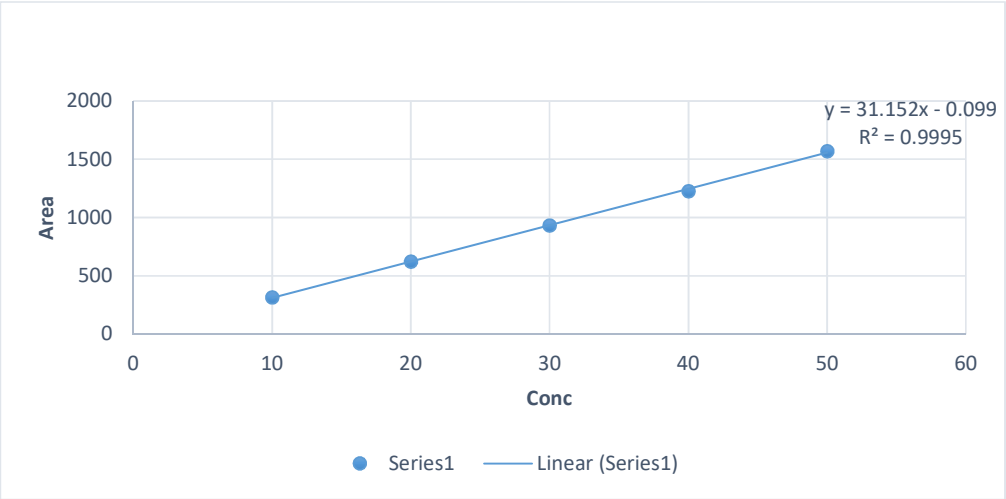


Fig. No 14 :

Calibration curve of Cysteamine HCl

Precision:

Precision of the developed RP-HPLC method for Cysteamine Hydrochloride was expressed in terms of Relative Standard Deviation (%RSD) for both intraday and interday studies. Intraday precision was evaluated by analyzing replicate injections of standard Cysteamine Hydrochloride solutions within the same day, whereas interday precision was determined by analyzing the solutions on different days. The obtained %RSD values for both studies were found to be within the acceptable limit of less than 2.0%, indicating good precision and reproducibility of the developed analytical method.

Accuracy of the method was evaluated by recovery studies at different concentration levels using the solutions prepared for precision analysis. The percentage mean recovery of Cysteamine Hydrochloride was found within the acceptance criteria of 98.0%–102.0%, demonstrating the accuracy, reliability, and suitability of the developed RP-HPLC method for quantitative analysis. As shown in table no 12.

Intraday Precision:

Table no 12 : Intraday Precision

Conc.	Area I	II	III	Mean	Amt Found	AM	% Amt Fnd	SD	%RSD
10	310.9733	310.47830	310.82	310.73	9.98	0.997832	99.78	0.35	0.11
30	934.7319	933.83960	934.38	934.29	30.00	0.999877	99.99	0.63	0.07
50	1570.126	1567.48670	1568.91	1568.81	50.37	1.007323	100.73	1.87	0.12

Interday Precision:

Table no 13 : Interday Precision

Conc.	Area :I	II	III	Mean	Amt Found	AM	% Amt Fnd	SD	%RSD

10	315.0677	316.80563	316.04	315.94	10.15	1.014561	101.46	1.23	0.39
30	950.3685	944.28522	947.43	947.33	30.41	1.013832	101.38	4.30	0.45
50	1559.48	1556.0248	1557.85	1557.75	50.01	1.000226	100.02	2.44	0.16

Accuracy:

Accuracy of 80% :

Accuracy study of the developed RP-HPLC method for Cysteamine Hydrochloride at the 80% recovery level. The accuracy was evaluated using the standard addition method by adding 16 µg/mL of standard drug solution to a pre-analyzed sample solution containing 20 µg/mL of Cysteamine Hydrochloride.The obtained peak areas were 1117.21 and 1117.36365, corresponding to the amounts found of 35.86 µg/mL and 35.87 µg/mL respectively. The percentage recovery values obtained were 99.14% and 99.17%, with a mean percentage recovery of 99.16%. The standard deviation values for amount found, amount recovered, and percentage recovery were found to be very low, indicating minimal variation among replicate measurements. The %RSD value for percentage recovery was found to be 0.02%, which is well within the acceptable limit.These results indicate that the developed RP-HPLC method is accurate, reliable, and suitable for quantitative estimation of Cysteamine Hydrochloride. As shown in table no 14.

Table no 14 : Accuracy of 80% level

Sr no.	µgm/ml	Amt added	Area	Amt found	Amt recvd	% Recovery
1	20	16	1117.21	35.86	15.86	99.14
2	20	16	1117.36365	35.87	15.87	99.17
			Mean	35.86	15.86	99.16
			SD	0.003	0.003	0.02
			%RSD	0.010	0.022	0.02

Accuracy of 100% :

The accuracy study of the developed RP-HPLC method for Cysteamine Hydrochloride at the 100% recovery level was performed using the standard addition method. In this study, 20 µg/mL of standard drug solution was added to the pre-analyzed sample solution containing 20 µg/mL of Cysteamine Hydrochloride and analyzed under optimized chromatographic conditions.The peak areas obtained were 1240.50281 and 1243.52161, corresponding to the amounts found of 39.82 µg/mL and 39.91 µg/mL respectively. The percentage recovery values obtained were 99.10% and 99.59%, with a mean percentage recovery of 99.34%. The standard deviation values for amount found, amount recovered, and percentage recovery were found to be low, indicating good consistency of the analytical method. The %RSD value for percentage recovery was observed to be 0.34%, which is within the acceptable limit.These results confirmed that the developed RP-HPLC method is accurate, precise, and suitable for the quantitative estimation of Cysteamine Hydrochloride. As shown in table no 15.

Table no 15 : Accuracy of 100% level

Sr no.	µgm/ml	Amt added	Area	Amt found	Amt recvd	% Recv
1	20	20	1240.50281	39.82034703	19.82035	99.10
2	20	20	1243.52161	39.91725875	19.91726	99.59

			Mean	39.87	19.87	99.34
			SD	0.069	0.069	0.34
			%RSD	0.172	0.345	0.34

Accuracy of 120% :

The accuracy study of the developed RP-HPLC method for Cysteamine Hydrochloride at the 120% recovery level was carried out using the standard addition method. In this study, 24 µg/mL of standard drug solution was added to the pre-analyzed sample solution containing 20 µg/mL of Cysteamine Hydrochloride and analyzed under optimized chromatographic conditions.

The peak areas obtained were 1370.739 and 1359.70093, corresponding to the amounts found of 44.00 µg/mL and 43.64 µg/mL respectively. The percentage recovery values obtained were 100.01% and 98.53%, with a mean percentage recovery of 99.27%. The standard deviation values for amount found, amount recovered, and percentage recovery were found to be low, indicating good consistency and reliability of the analytical method. The %RSD value for percentage recovery was observed to be 1.05%, which is within the acceptable limit.

These results demonstrated that the developed RP-HPLC method is accurate, precise, and suitable for quantitative estimation of Cysteamine Hydrochloride at the 120% recovery level**.** As shown in table no 16.

Table no 16 : Accuracy of 120% level

Sr no.	µgm/ml	Amt added	Area	Amt found	Amt recvd	% Recv
1	20	24	1370.739	44.00127287	24.00127	100.01
2	20	24	1359.70093	43.64693194	23.64693	98.53
			Mean	43.82	23.82	99.27
			SD	0.251	0.251	1.04
			%RSD	0.572	1.052	1.05

Acceptance criteria:

The % Drug Recovery of drug at every spiked level should be within 98 – 102%.

Result: The recovery study represents that test method has an acceptable level % drug recovery that's why the method is accurate. As shown in table no 16.

Repeatability:

Repeatability of the developed RP-HPLC method for Cysteamine Hydrochloride was evaluated by analyzing three replicate injections of 50 µg/mL standard solution under optimized chromatographic conditions. The peak areas obtained were 1581.610, 1577.009, and 1579.41 with a mean peak area of 1579.31. The amount found was 50.70 µg/mL corresponding to 101.41% assay of the drug. The standard deviation and percentage Relative Standard Deviation (%RSD) were found to be 3.25 and 0.21% respectively.According to the acceptance criteria, the %RSD should not be more than 2.0%. Since the obtained %RSD value was less than 2.0%, the developed RP-HPLC method was found to be precise and repeatable for the analysis of Cysteamine Hydrochloride. As shown in table no 17.

Table no 17 : Repeatability

Conc	Area: I	II	III	Mean	Amt. Found	AM	% Amt Fnd	SD	%RSD
50	1581.610	1577.009	1579.41	1579.31	50.70	1.014066	101.41	3.25	0.21

Acceptance Criteria;

The % RSD should not be more than 2%.

The % RSD of drug is calculated and it is less than 2%, so the method is found to be precise.

Robustness: Robustness of the developed RP-HPLC method for Cysteamine Hydrochloride was evaluated by introducing small deliberate changes in mobile phase composition and detection wavelength. Slight variations in the mobile phase composition (79.5:20.5 and 81.5:18.5 Acetonitrile water) and wavelength (289 nm and 291 nm) did not significantly affect the chromatographic performance. The %RSD values obtained in all cases were below 2.0%, indicating that the method remained unaffected by minor changes in analytical conditions. The low LOD and LOQ values further confirmed the good sensitivity of the developed method. As shown in table no 18,19.

Table no 18 : Change in Mobile phase:

MP: 79.5 Acetonitrile +20.5 OPA			**MP: 81.5 Acetonitrile+18.5 OPA**		
Sr No.	Conc. µgm/ml	Area	Sr No.	Conc. µgm/ml	Area
1	30	934.8663	1	30	938.2513
2	30	939.987	2	30	939.9398
	Mean	937.43		Mean	939.096
	SD	3.621		SD	1.19
	%RSD	0.386		%RSD	0.1271

Table no 19 :Change in Wavelength

WAVE LENGTH CHANGE :289 nm			**WAVE LENGTH CHANGE :291 nm**		
Sr No.	µgm/ml	Area	Sr No.	µgm/ml	Area
1	30	913.7065	1	30	957.75183
2	30	918.0828	2	30	959.1783
	Mean	915.895		Mean	958.47
	SD	3.095		SD	1.01
	%RSD	0.3379		%RSD	0.11

Ruggedness:

Ruggedness of the method was evaluated under varied analytical conditions, and the obtained %RSD value was found to be 0.038%, which is well within the acceptable limit. The results demonstrated that the developed RP-HPLC method is rugged, reproducible, and capable of providing consistent and reliable results for the analysis of Cysteamine Hydrochloride. As shown in table no 20.

Table no 20 : Ruggedness

	Area						
Conc	**I**	**C (y-intercept)**	**M (Slope)**	**CM**	**Amt Found**	**LC**	**% Label Claim**
30.00	937.511	0.099	31.15	937.41	30.09348	1.003116	100.31
30.00	938.016	0.099	31.15	937.92	30.10969	1.0036563	100.37
Mean	937.76				30.10		100.34
SD	0.357				0.011		0.038
%RSD	0.038				0.038		0.038

Application on Marketed Formulation :

The developed stability-indicating RP-HPLC method was successfully applied for the estimation of Cysteamine Hydrochloride in the marketed formulation Cystagon capsules. The analysis revealed that the formulation contained drug content within acceptable limits of the labeled claim, indicating good agreement with the declared amount. The obtained results confirmed the suitability, accuracy, and reliability of the developed RP-HPLC method for routine quality control analysis of Cysteamine Hydrochloride in pharmaceutical dosage forms.

Assay:

Table No 21 : Assay Study

Conc.	Area	C (y-intercept)	M (Slope)	CM	Amt Found	LC (Linearity Coefficient)	% Label Claim
30.00	925.976	0.099	31.15	926.08	29.72954	0.9909847	99.10
30.00	928.437	0.099	31.15	928.54	29.80854	0.9936181	99.36
Mean	**927.21**				29.77		99.23
SD	**1.740**				0.056		0.186
%RSD	**0.188**				0.188		0.188

Acceptance criteria: The % labelled claim of drug at every spiked level should be within 98 – 102%.
Result: The % labelled claim of drug represents that test method has an acceptable level of % labelled claim. As shown in table no 21.

Results of Forced Degradation Studies:

Table No 22 : degradation after 2 hrs

	AFTER 2 HR: 30 MCG CYSTEAMINE HCL				
Sr. NO.	Degradation	Area of Standard	Area of degraded Sample	Degraded upto %	Actual % degradation
1	Acid Degradation	935.75079	919.84741	98.30	1.70
2	Basic Degradation	935.75079	916.14551	97.90	2.10
3	H_2O_2 Degradation	935.75079	891.09622	95.23	4.77
4	Neutral	935.75079	930.51703	99.44	0.56

After 2 hours, Cysteamine HCl shows minimal degradation under all stress conditions. The highest degradation is observed in oxidative (H_2O_2) condition (4.77%), indicating slight sensitivity to oxidation. Acidic and basic conditions show low degradation, while the drug remains highly stable in neutral condition. As shown in table no 22.

Table no 23 : Degradation study after 24 hrs:

AFTER 24 HR: 30 MCG CYSTEAMINE HCL					
	Degradation	Areaof Standard	Area of degraded Sample	Degraded upto %	Actual % degradation
	Acid Degradation	935.75079	902.83636	96.48	3.52
	Basic Degradation	935.75079	879.27814	93.96	6.04
	H_2O_2 Degradation	935.75079	775.08298	82.83	17.17
	Neutral	935.75079	930.45673	99.43	0.57
	Photo	935.75079	932.24869	99.63	0.37

After 24 hours, degradation increases significantly, especially under oxidative conditions (17.17%), confirming that the drug is highly susceptible to oxidation over time. Basic degradation is moderate, while acid degradation remains relatively low. The drug continues to be stable in neutral conditions, showing negligible degradation. As shown in table no 23.

Conclusion :

The present study successfully developed and validated an AQbD-based stability-indicating RP-HPLC method for estimation of Cysteamine Hydrochloride in Cystagon. Central Composite Design (CCD) was effectively used to optimize chromatographic conditions, providing satisfactory retention time, peak area, and theoretical plates. The method was validated as per International Council for Harmonisation guidelines and showed excellent linearity, accuracy, precision, robustness, sensitivity, and reproducibility. Forced degradation studies confirmed the stability-indicating nature of the method by effectively separating degradation products from the drug peak. Overall, the developed RP-HPLC method was found to be simple, accurate, precise, robust, sensitive, and suitable for routine quality control and stability studies of Cysteamine Hydrochloride formulations.

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