DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF CHLORDIAZEPOXIDE AND AMITRIPTYLINE: APPLICATION OF QbD APPROACH

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ABSTRACT

With the objective of establishing a design space for the simultaneous assessment of chlordiazepoxide and amitriptyline by RP-HPLC, a methodical design of experiments was carried out. The method was developed using Symmetry ODS C18 (4.6mm 250mm, 5m) HPLC column, a mobile phase consisting of Methanol: Acetonitrile (35:65v/v), a flow rate of 1 mL per minute, and UV detection at 228 nm for both chlordiazepoxide and amitriptyline. The method was found to be linear in the range of 6.0-14.0 g mL1 and 30.0-70.0 g mL1, with a correlation coefficient of 0.9999 in each case. The recovery values ranged from 99.94% to 100.09% and 99.26% to 100.63% for chlordiazepoxide and amitriptyline, respectively. Statistical analysis of experimental data was carried out using Design-Expert software employing the response-surface methodology, central composite design, and quadratic model. In order to optimize the method, perturbation, contour, 3D, and design-space plots were taken into consideration along with response factors like resolution and retention time. Through the QbD approach, we established the design space and demonstrated the resilience of the method under chromatographic conditions. Furthermore, it has been established that this

method is stability-indicating and robust and can be used for standard analysis in quality control labs.

Keywords: RP-HPLC, Chlordiazepoxide, Amitriptyline, forced degradation studies, Quality by Design (QbD)

INTRODUCTION

7-chloro-2-(methylamino)-5-phenyl-3H-1,4-benzodiazepin-4-ium-4-olate is the chemical name for chlordiazepoxide. It is a benzodiazepine derivative that acts as an anticonvulsant and sedative. Additionally, it has been used to alleviate the symptoms of alcohol withdrawal. It is an odorless, white or slightly yellow crystalline powder that dissolves in water, alcohol, and ether but is almost completely insoluble in petroleum spirit, chloroform, and ether. Chlordiazepoxide is efficiently absorbed from the GI tract after oral administration; however, peak plasma levels do not appear for up to 4 hours[1]. The IUPAC name of amitriptyline hydrochloride, is dimethyl (3- tricyclo [9.4.0.03,8] pentadeca-1(15),3,5,7,11,13-hexaen-2-ylidene propyl) amine. This tricyclic antidepressant (TCA) is frequently used to treat depression and neuropathic pain[2]. Crescent Therapeutics Ltd markets chlordiazepoxide in combination with amitriptyline under the brand name Limbitrol. Few methods have been laid out for estimating these drugs in combination [8–13], however, no stability-indicating method for concurrently estimating this combination utilizing the QbD approach has been published. Therefore, we have aimed at the development of an RP-HPLC method for the simultaneous quantification of amitriptyline and chlordiazepoxide in pharmaceutical formulation.

MATERIALS AND METHODS

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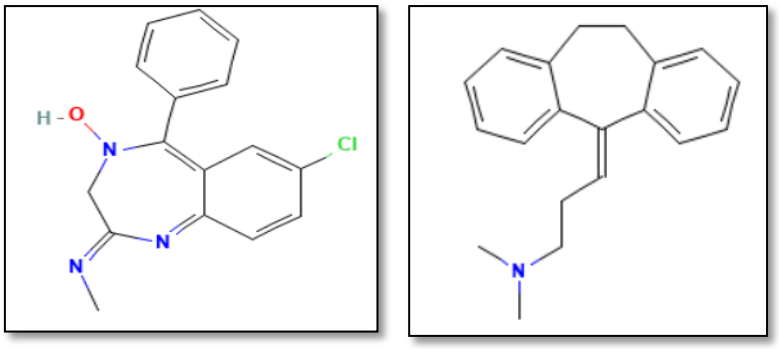
Chemicals and Reagents

Chlordiazepoxide and Amitriptyline HCl were procured from Aurobindo Pharma Hyderabad. The structure of the compounds is shown in Figure 1. Methanol and acetonitrile of HPLC grade were procured from Merck in India. A Millipore purification system was used to produce HPLC-grade water.

Figure 1: Structures of Chlordiazepoxide and Amitriptyline



Method development:

Selection of Wavelength:

Isobestic point of Chlordiazepoxide and Amitriptyline hydrochloride was determined by dissolving it in methanol and scanned between 200-400nm. The isobestic point was found to be 254nm.

Standard solution preparation:

Preparation of stock:

50 mg of chlordiazepoxide and 125 mg of amitriptyline hydrochloride were accurately weighed and transferred into a 50 ml volumetric flask. The sample was then thoroughly dissolved by adding 15 ml of mobile phase and sonicating it for 20 minutes. This solution was then filtered and used in the experiment.

Preparation of standard:

5mL of the stock solution was pipetted into a 50mL volumetric flask. It was then added with diluent to obtain a final concentration of 100g/mL of chlordiazepoxide and 250g/mL of amitriptyline hydrochloride, respectively.

Test Solution Preparation:

Preparation of stock:

Twenty tablets were weighed and finely ground. The tablet powder containing about 50mg of Chlordiazepoxide and 125mg of Amitriptyline HCl was then transferred to a 50mL volumetric flask. After that, 15mL of mobile phase was added and sonicated for 15 minutes. The required volume was then raised by adding a diluent.

Preparation of standard:

About 5mL of the supernatant liquid was pipetted out into a 50 mL volumetric flask. The diluent was then added and mixed well to get final concentration of about

100µg/mL of Chlordiazepoxide and 250 µg/mL of Amitriptyline hydrochloride respectively. The above solution was then filtered through a membrane filter.

Method Validation

According to the ICH (Q2) specifications, the proposed method was validated for specificity, accuracy, precision, linearity, limit of detection (LOD), the limit of quantification (LOQ), ruggedness, and robustness. [3-6].

RESULTS AND DISCUSSION

Method development and optimisation

The primary goal of method development is to achieve an optimal separation of chlordiazepoxide and amitriptyline. The challenge in developing selective and sensitive methods is to obtain symmetrical peaks as well as proper resolution between two compounds. A gradient elution is always preferred over an isocratic elution for achieving good peak symmetry and resolution; thus, gradient elution was used.

Figure 2: Optimized Chromatogram (Sample)

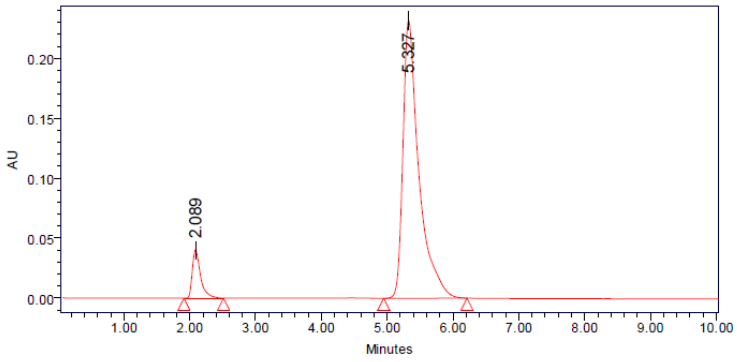


Table 1: Optimized method data

S. No.	Peak Name	R_t	Area	Height	USP Resolution	USP Tailing	USP plate count
1	Chlordiazepoxide	2.089	298698	3658		1.68	6859
2	Amitriptyline	5.327	4758695	29586	8.64	1.85	8789

According to the ICH (Q2) specifications, the proposed method was validated for specificity, accuracy, precision, linearity, limit of detection (LOD), limit of quantification (LOQ),

ruggedness, and robustness. It was found to be linear in the range of 30.0-70.0 g mL^{-1} for Chlordiazepoxide and 6.0-14.0 g mL^{-1} for Amitriptyline, with a correlation coefficient of 0.9999 in each case. The recovery values for Chlordiazepoxide and Amitriptyline were 99.94% to 100.09% and 99.26% to 100.63%, respectively.

Table 2: System suitability data of Chlordiazepoxide

S.No.	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Chlordiazepoxide	2.090	289854	3526	8659	1.82
2	Chlordiazepoxide	2.090	285745	3541	8642	1.83
3	Chlordiazepoxide	2.089	289587	3612	8674	1.82
4	Chlordiazepoxide	2.089	285466	3584	8692	1.83
5	Chlordiazepoxide	2.085	285987	3572	8654	1.82
Mean			**287327.8**			
Std. Dev			**2194.024**			
% RSD			**0.763596**			

Table 3: System suitability data of Amitriptyline

S.No.	Drug	Rt	Area	Height	USP plate count	USP Tailing
1	Amitriptyline	5.289	4658745	28564	8659	1.82
2	Amitriptyline	5.289	4652587	28457	8647	1.83
3	Amitriptyline	5.338	4674833	28952	8632	1.82
4	Amitriptyline	5.327	4685825	28754	8645	1.83
5	Amitriptyline	5.262	4652145	28964	8694	1.82
Mean			4664827			

Std. Dev	14905.35
% RSD	0.319526

The chromatographic parameters were optimized as part of the development of a stability-indicating method for better compound separation and quantification. Stress tests were carried out to determine the stability-indicating property and specificity of the proposed method. Intentional degradation was carried out under stress conditions of UV radiation (254 nm), acid (0.5N HCl), base (0.5N NaOH), and oxidation (3.0% H2O2). The study time for light studies was 10 days, however, the study period for heat, acid, base, and oxidation was 24 hours.

Table 4: Summary data of forced degradation studies

S.No.	Sample name	Assay of chlordiazepoxide	% Degradation	Assay of amitriptyline	% Degradation
1	Acid	76.2	16.7	64.9	31.1
2	Base	75.9	15.2	64.3	32.7
3	Peroxide	72.1	21.8	71.4	24.6
4	Heat	73.4	20.5	72.7	26.3
5	UV light	90.9	08.0	78.4	21.2

Application of QbD Approach

A design of experiments (DoE) research is a series of experiments in which purposeful modifications to input factors are carried out in order to identify the reasons for substantial changes in output responses. The study was carried out using a QbD approach with the aim of generating a robust design space for operational chromatographic conditions. Three critical quality attributes (CQA) that could potentially influence the separation were identified to be flow rate, column temperature, and perchloric acid concentration.

For this study, Design Expert 12.0 version software7 was used and a response surface methodology was chosen. Responses (Res1, Res2, Res3) were collected from the resolution between chlordiazepoxide and amitriptyline. For this study, flow rates of 0.6-1.0 mL/min,

column temperatures of 55-65 °C, and perchloric acid concentrations of 0.05-0.15% were suggested.

To examine the impact of selected variables on resolution, a response surface methodology, three-level factorial design was used. Twenty-three trials were carried out randomly, with each run including a spiked sample comprising chlordiazepoxide and amitriptyline.

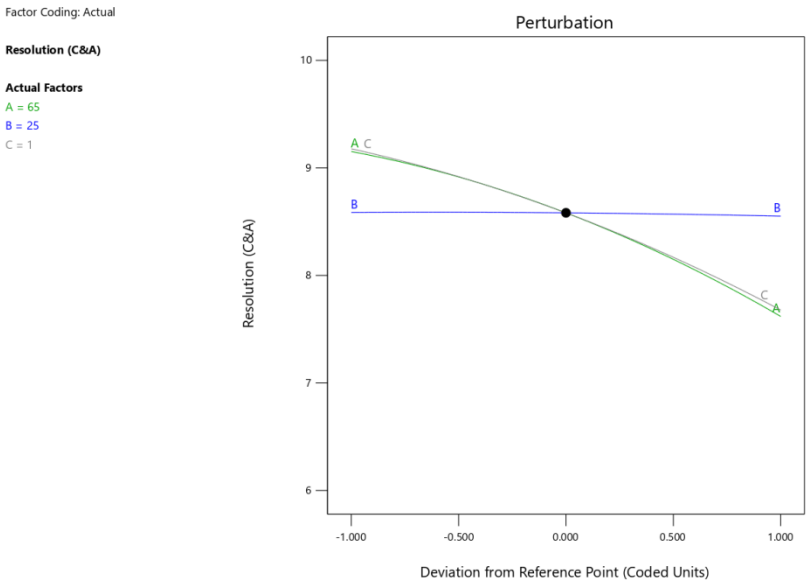
Table 5: Design summary layout

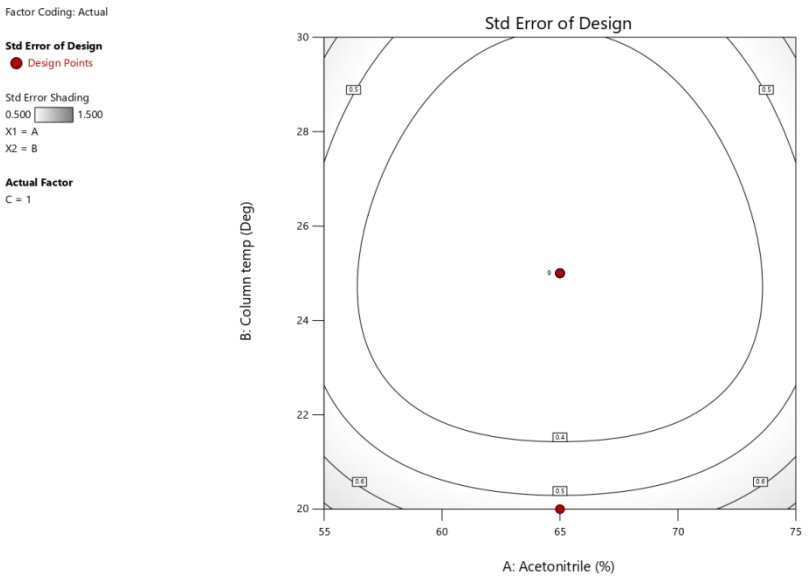
Std	Run	Factor-1 A: Acetonitrile %	Factor-2 B: Column temp Deg	Factor-3 C: Flow mL	Response 1 Resolution (C&A)
4	1	75	30	0.7	8.8
17	2	65	25	1	8.5
3	3	55	30	0.7	8.9
1	4	55	20	0.7	9.6
7	5	55	30	1.3	8.9
16	6	65	25	1	8.6
12	7	65	35	1	8.5
19	8	65	25	1	8.5
10	9	80	25	1	7
20	10	65	25	1	8.6
8	11	75	30	1.3	7
5	12	55	20	1.3	8.1
14	13	65	25	1.3	7.7
9	14	50	25	1	9.3
15	15	65	25	1	8.5
13	16	65	25	0.7	9.2
11	17	65	20	1	8.6
18	18	65	25	1	8.6
6	19	75	20	1.3	6.3
2	20	75	20	0.7	8.1
21	21	65	25	1	8.6
22	22	65	25	1	8.6
23	23	65	25	1	8.7

The effect of CQA on response variables was described using perturbation plots, contour plots, and 3D response surface plots.

Figure 3: Perturbation and contour plots

a. Perturbation plot
b. Contour plot for Res 1
c. Contour plot for Res 2
d. Contour plot for Res 3
e. 3D response surface plot for Res 1
f. 3D response surface plot for Res 2
g. 3D response surface plot for Res 3

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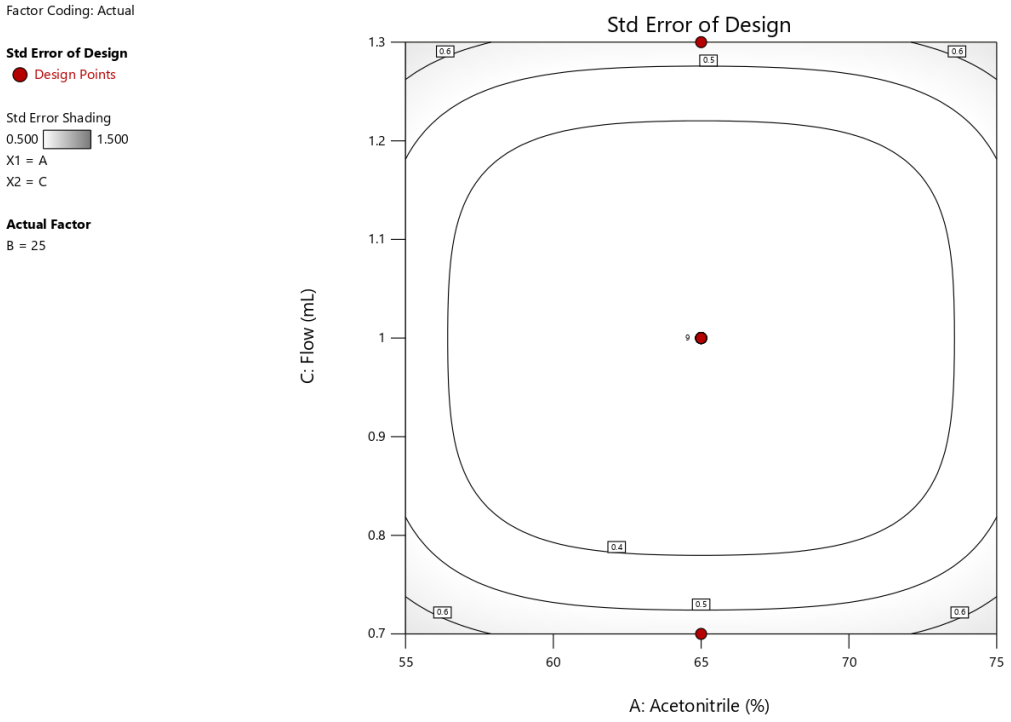
Std Error of Design
Design Points
Std Error Shading
0.500
1.500
X1 = A
X2 = B
Actual Factor
C = 1
Std Error of Design
B: Column temp (Deg)
A: Acetonitrile (%)
30
28
26
24
22
20
55
60
65
70
75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Std Error of Design
Design Points
Std Error Shading
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1.500
X1 = A
X2 = C
Actual Factor
B = 25
Std Error of Design
C: Flow (mL)
A: Acetonitrile (%)
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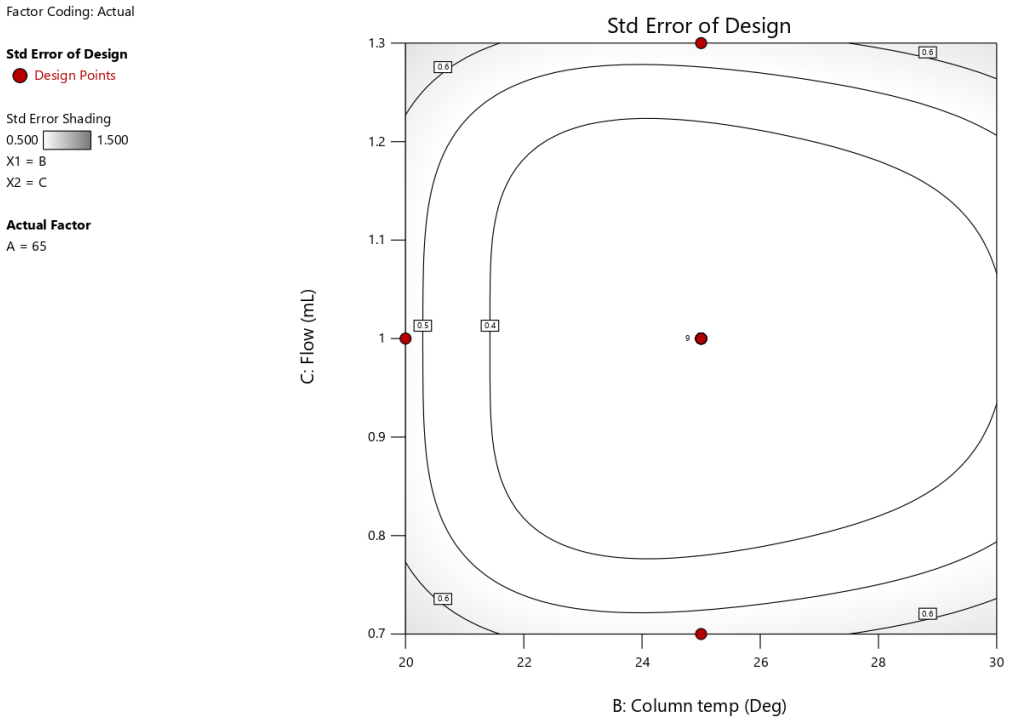
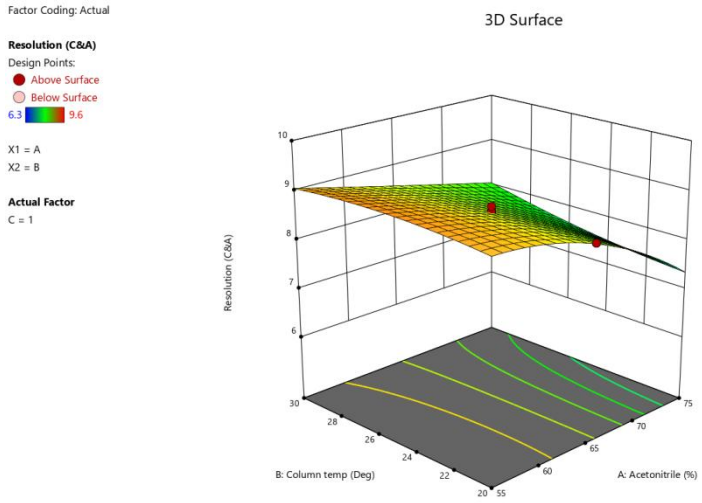
1.2
1.1
1
0.9
0.8
0.7
55
60
65
70
75


Factor Coding: Actual
Std Error of Design
Design Points
Std Error Shading
0.500
1.500
X1 = B
X2 = C
Actual Factor
A = 65
Std Error of Design
C: Flow (mL)
B: Column temp (Deg)
1.3
1.2
1.1
1
0.9
0.8
0.7
20
22
24
26
28
30
0.6
0.5
0.4
9


Factor Coding: Actual
Resolution (C&A)
Design Points:
Above Surface
Below Surface
6.3
9.6
X1 = A
X2 = B
Actual Factor
C = 1
3D Surface
Resolution (C&A)
B: Column temp (Deg)
A: Acetonitrile (%)
10
9
8
7
6
30
28
26
24
22
20
55
60
65
70
75

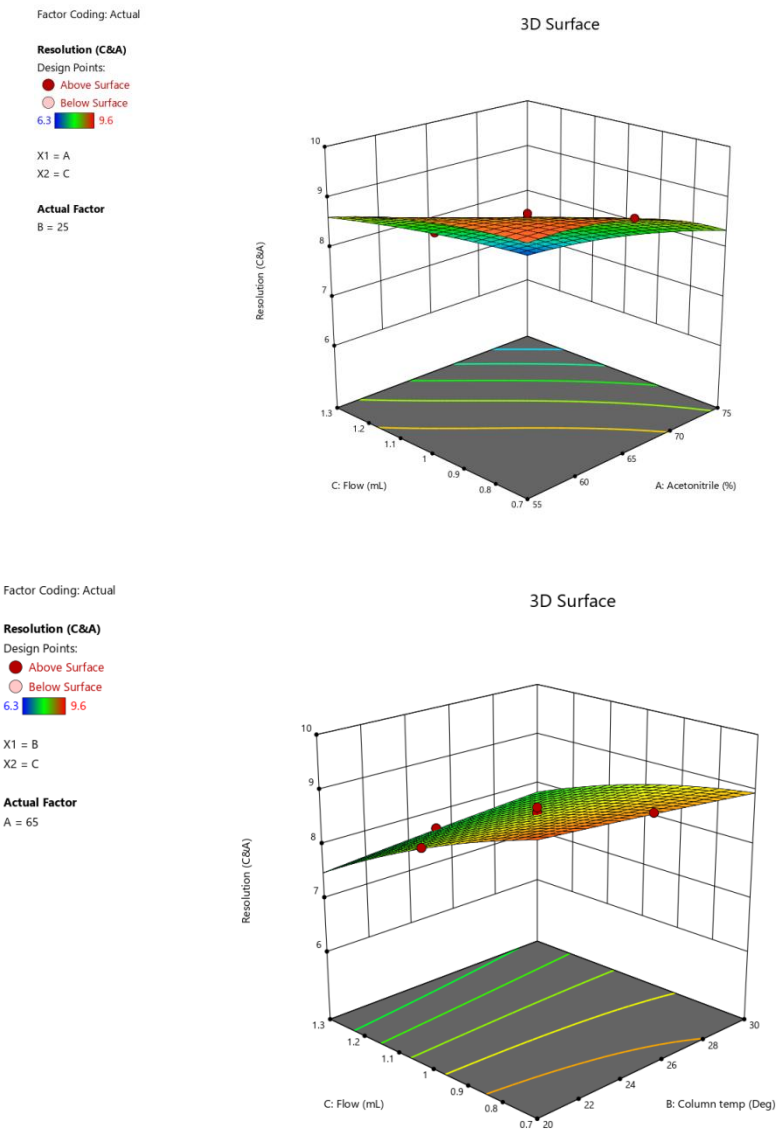
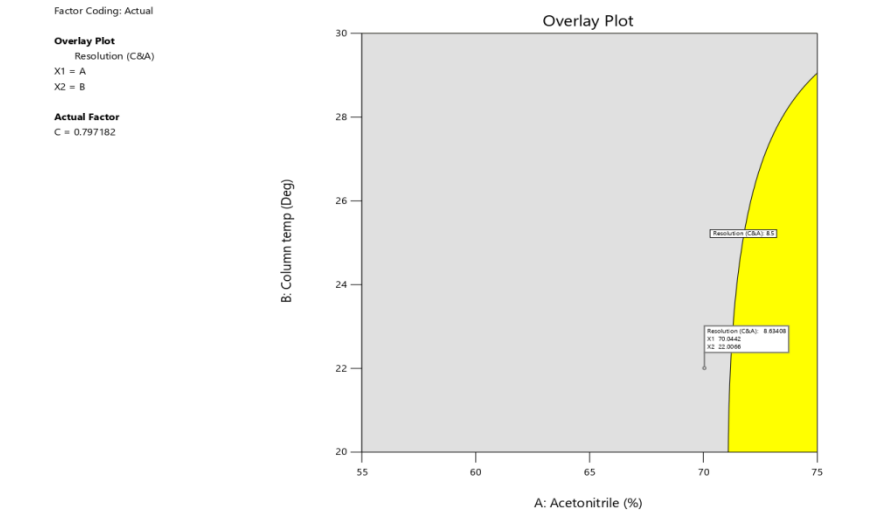
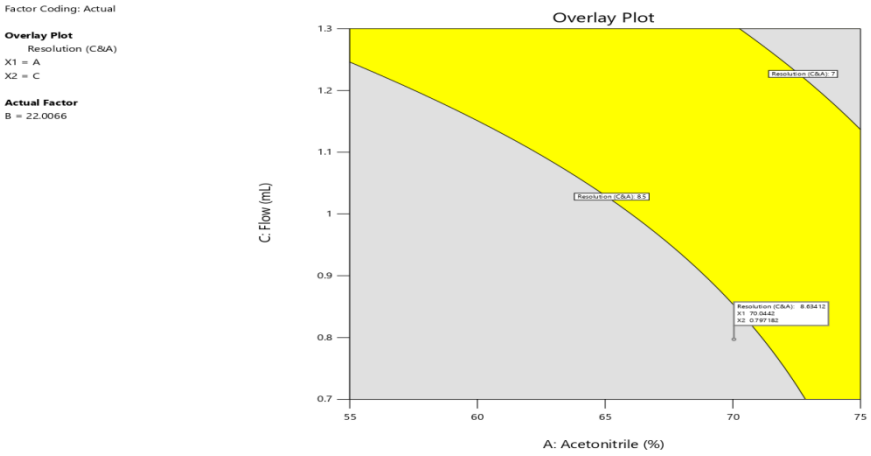
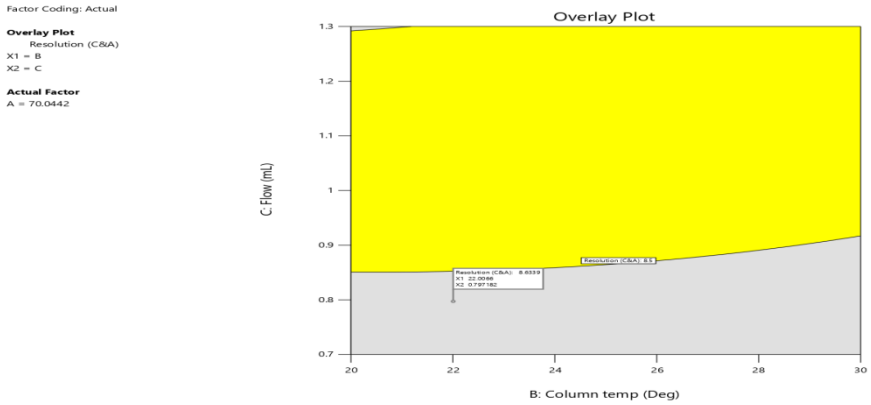


Figure 4: Overlay plot showing the optimal analytical design space; yellow region indicates design space and gray region indicates responses below desired level.

a. Overlay plot for Res 1.
b. Overlay plot for Res 2.
c. Overlay plot for Res 3.







The analysis of variance (ANOVA) model was used to evaluate the statistically significant ($p>0.05$) and insignificant terms.

Table 6: ANOVA for reduced cubic model

Source	**Sum of squares**	**df**	**Mean Square**	**F-value**	**p-value**	
Model	12.88	13	0.9910	238.63	<0.0001	significant
A-Acetonitrile	2.65	1	2.65	636.94	<0.0001	
B-Column temp	0.0007	1	0.0007	0.1602	0.6983	
C-Flow	1.12	1	1.12	270.91	<0.0001	
AB	0.2113	1	0.2113	50.87	<0.0001	
AC	0.5512	1	0.5512	132.75	<0.0001	
BC	0.2813	1	0.2813	67.73	<0.0001	
A^2	0.3909	1	0.3909	94.13	<0.0001	
B^2	0.0015	1	0.0015	0.3606	0.5630	
C^2	0.1030	1	0.1030	24.79	0.0008	
ABC	0.2813	1	0.2813	67.73	<0.0001	
A^2B	0.0745	1	0.0745	17.94	0.0022	
A^2C	0.0202	1	0.0202	4.88	0.0546	
AB^2	0.0312	1	0.0312	7.53	0.0227	
Residual	0.0374	9	0.0042			
Lack of Fit	0.0018	1	0.0018	0.4092	0.5403	not significant
Pure Error	0.0356	8	0.0044			
Cor Total	12.92	22				

Factor coding is **closed**

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

We established a design space based on the design of experiments, and the appropriate chromatographic conditions will be chosen from within the design space.

CONCLUSION

For the simultaneous measurement of chlordiazepoxide and amitriptyline, a new straightforward and quick RP-HPLC method was developed. Due to its effective separation, and affordable analysis, the suggested approach is appropriate for the estimation of chlordiazepoxide and amitriptyline in combined solid oral dosage form and is suitable for analysis in quality control laboratories. Using the QbD method, we defined the design space for chromatographic parameters. According to ICH requirements, the devised method is validated and determined to be linear, accurate, robust, precise, and specific. This method has demonstrated stability-indicating power; as a result, it satisfies the desired regulatory requirement and may be used in quality control laboratories to test pharmaceutical formulations.

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