

Analytical Quality by Design Enabled Development and Validation of RP-HPLC Method for Simultaneous Estimation of Pregabalin and Duloxetine HCL in its Pharmaceutical Dosage Form

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Abstract:

This study aims to develop and validate a Reverse Phase High Performance Liquid Chromatography method for simultaneous estimation of Pregabalin and Duloxetine from their pharmaceutical tablet dosage form using the Analytical Quality by Design Approach (AQbD). For quality reasons, it is crucial to estimate both drugs simultaneously in their fixed dose combination. The AQbD approach ensures an optimized, robust and well controlled analytical method in accordance with the regulatory guidelines. The optimized method was developed using a C18 Phenomenex column (250 × 4.6 mm, 5 μm). The Critical Method Parameters (CMPs) identified were mobile phase composition and flow rate. Critical Analytical Attributes (CAA) were Retention Time (RT) and Theoretical Plate Count. Central Composite Design was used for optimization. The optimized mobile phase composition consists of acetonitrile and buffer (0.1 M potassium dihydrogen orthophosphate and 0.05 M ammonium dihydrogen orthophosphate) in the ratio of 35:65 v/v at a 1.0 mL/min flow rate. The wavelength of detection was 237 nm. Validation was carried out as per ICH Q2 (R2) guidelines. RT observed for pregabalin and duloxetine were found to be 2.00 and 3.09 minutes, respectively. Pregabalin demonstrated linearity in the range of 45-105 μg/mL and 12-28 μg/mL by duloxetine. ANOVA helped to link the effect of CMPs with that of CAAs. For the developed method, accuracy, precision, and robustness were within tolerable levels. Thus, the developed HPLC method was found to be robust, simple and cost effective. AQbD helped in the systematic development of the method with utmost analytical control.

Keywords: RP-HPLC, Analytical Quality by Design, Pregabalin, Duloxetine HCL, Central Composite Design.

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Introduction

Neuropathic pain is experienced by a patient when the nervous system is malfunctioned or gets damaged. It is a chronic type of pain and exerts great deal of burden on the patients, healthcare system and society. The prevalence of neuropathic pain observed all over the globe is around 7-8 % of the total population and is estimated to increase rapidly in the coming years. The pain is different than nociceptive pain and hence different approach, different therapeutic agents are required to treat the condition. Thus, management of neuropathic pain is more critical and complicated than nociceptive pain [1]

Pregabalin (PREG) is a GABA-aminobutyric acid (GABA) derivative and exerts anticonvulsant effect [2]. Chemically it is N-methyl-3-naphthalen-1-yl oxy-3-thiophen-2 yl propan-1-amine having molecular weight of 159.23 g/mol [3]. It efficiently impedes calcium channels and serves the dual role of an anticonvulsant and an anti-anxiety agent. Just

like gabapentin, PREG exerts healing effects by binding to alpha 2-delta (α2δ) subunit of voltage-gated calcium channels [2,4,5]. It helps to manage seizures and anxiety-related complications. It also exhibits analgesic properties making it crucial in treating neuropathic pain. Duloxetine HCL (DULO) is majorly used to treat major depressive disorder as it exerts antidepressant effect. It also has been found to be effective in managing diabetic peripheral neuropathy [6]. Chemically, it is 3-(aminomethyl)-5-methylhexanoic acid [4] having molecular weight of about 333.38 g/mol [3,7]. It belongs to a newer generation of selective serotonin and norepinephrine reuptake inhibitor (SSNRI) used against major depressive disorders [8]. A combination of these two drugs is available in the market to treat the neuropathic pain.

Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) is a highly sensitive and sophisticated approach amongst most of the

frequently applied techniques in pharmaceutical analysis for the separation, identification, and quantification of components in complex pharmaceutical mixtures. It plays a key role in precise determination of active and inactive components from various formulations. Thus, it is extremely useful in both research and quality control studies. It has become an indispensable component of drug development and regulatory testing due to its versatility, high detection sensitivity, and ability to handle analytes with a wide range of polarities. The RP-HPLC, includes a hydrophobic stationary phase in combination with a relatively polar mobile phase. The difference in polarities allows the analytes to be resolved according to their own polarity and their interactions with the column packing material and mobile phase [9,10].

Traditional way for HPLC method optimization often followed one-factor-at-a-time (OFAT) approach, where individual parameters were varied at a time and its effect on the method was evaluated while all the other factors were held constant. This strategy proved to be very time-consuming and at times failed to reveal the interactions between critical independent variables. Recently the Quality by Design (QbD) framework has emerged as an effective and systematic approach for developing formulations and chromatographic methods, that enables a more comprehensive understanding of method parameters, their interactions and their influence on overall performance of the method [11–15]. It helped to gain deeper insights into the variables that affect resolution, identification of factors and their magnitude to impact the chosen responses, and exploring the multidimensional interactions that could occur among these factors.

The principles of Quality by Design in analytical method development helps to develop a method with significant robustness and improved method performance since, it helps in optimization of the method.

According to ICH Q8 guideline, QbD means an organised method for development that commences with proper understanding and defining the objectives and helps to increase product and process understanding and methods to control the process, based on sound scientific knowledge gained in the process and managing the quality risks. Similarly, AQbD approach helps to extend these principles of QbD to analytical processes, ensuring that analytical methods developed are scientifically understood, robust, and sensitive to perform throughout the product lifecycle [16–24].

Literature reports various analytical methods for alone or simultaneous estimation of PREG and DULO by RP-HPLC method [4,6]. But, these methods lack optimization and also reports high retention times as major limitations and scope for

further research. Thus, these limitations raises suspicions about method accuracy and specificity.

The primary objective of this study was to develop an optimized, precise, accurate, and sensitive RP-HPLC method for the simultaneous estimation of PREG and DULO with the help of Analytical Quality by Design (AQbD) framework. Critical factors influencing the method performance were identified based on series of preliminary experiments and a structured risk assessment. Central Composite Design (CCD) was applied to determine the interaction of critical factors with each other and how they affected the method performance. Thus, insights were gained about optimized range of parameters for optimal performance of the method [25]. The validation of the method was carried out as per the ICH Q2 (R2) guidelines [26]. Pregabalin and duloxetine HCl were also simultaneously evaluated in marketed capsules.

Experimental

Chemicals and Reagents: Pure API (Active Pharmaceutical Ingredient) PREG and DULO were obtained from Pramukh Pharmachem Pvt. Ltd., Gujrat., India. HPLC grade Acetonitrile was obtained from Alpha Chemika, India. PREABA-D (PREG 75 mg and DULO 20 mg) was purchased from the local pharmacy store.

Instrumentation and Optimized HPLC condition: Method development and analysis by isocratic separation was performed using HPLC (MAKE - WATERS SCIENTIFIC and MODEL – WATERS 1525) with solvent delivery pump, autosampler injector and a UV/Visible detector. Separation was carried out using Phenomenex column (C18, 250mm, 4.6 mmL). Before use, all the solvents were degassed using an ultra sonicator. The optimum mobile phase comprised of Acetonitrile and Buffer (0.1 M KH₂PO₄ & 0.05M NH₄H₂PO₄) in the proportion of 35:65 v/v; having flow rate of a 1 mL/min; injection volume of 20 µL and detection wavelength was 237 nm.

Software: The data was collected and processed using the Waters- Empower software. Design Expert trailed Version 13 software was used in experimental design for optimization of various chromatographic parameters as well as to create design space.

Sample Preparations

Preparation of Standard Solution: Accurately weighed and dissolved about 75 mg of Pregabalin and 20 mg of Duloxetine Hydrochloride in 100 mL of mobile phase and sonicated for 10 minutes. Standard solution having concentration as 750 µg/mL PREG & 200 µg/mL DULO were obtained.

Preparation of Sample Solution (Test Solution): For analysis of the marketed formulation, 20 capsules (PREGABA-D) having combination of

Pregabalin and Duloxetine HCL were weighed and crushed to get a fine powder using a mortar and pestle. Accurately weighed a quantity of the sample equivalent to 75 mg of Pregabalin and 20 mg of Duloxetine HCL and dissolved using sonicator in 100 mL mobile phase. It was then filtered using a 0.45 μ m membrane filter. From the above stock solution, 1 mL of solution was pipette out and was diluted to 10 mL using mobile phase to get 75 μ g/mL test solution of pregabalin and 20 μ g/mL of test solution of duloxetine HCL.

System Suitability: Six standard solutions containing 75 μ g/mL PREG and 20 μ g/mL DULO were injected to determine the system suitability parameter. All Percent Relative Standard Deviations (% RSD) were calculated for resolution, Retention Time (RT), and theoretical plates.

Method Validation: The developed method for estimation of pregabalin and duloxetine HCL was validated as per ICH Q2(R2) guidelines. As per the guidelines, parameters assessed were accuracy, precision, linearity, limit of detection (LOD), limit of quantification (LOQ), specificity, and robustness.

Results

Method Development as Per Experimental Design: Based on literature search and preliminary trials, critical factors (independent variables) were identified and confirmed using the Ishikawa diagram. The independent factors identified for method development included percent (%) ratio of organic solvent in mobile phase and flow rate. Key four dependent factors identified were RT of PREG and DULO, TPC for PREG and DULO. CCD was employed to evaluate how the independent variables influences dependent variables. Independent variables were then evaluated at two concentrations, low (-1) and high (+1). The factor % organic phase used in mobile phase was assigned as X1 and was studied at two levels- 60 % and 70 % and flow rate was assigned as X2 at two levels- 0.8 and 1.2 mL/min. The retention time of PREG (Y1), RT of DULO (Y2), TPC of PREG (Y3) and TPC of DULO (Y4) were employed as responses in experimental design. The CCD gave a protocol for 13 runs. The 13 runs, using the conditions, were performed and then recording of responses was done as give in Table 1.

Table 1: Layout of 13 experimental trials by CCD

Std	Run	Factor 1 A= Mobile phase Compositon (% Buffer) (X1)	Factor 2 B= Flow rate (mL/min) (X2)	Response 1 Retention time of PREG (Y1)	Response 2 Retention time of DULO (Y2)	Response 3 TPC of PREG (Y3)	Response 4 TPC of DULO (Y4)
1	7	60	0.8	2.74	3.925	4582	6766
2	9	70	0.8	2.721	3.878	4705	6795
3	12	60	1.2	1.983	2.824	4418	6221
4	1	70	1.2	2.002	3.571	4305	5422
5	4	57.9289	1	2.169	3.152	4376	6454
6	8	72.0711	1	2.235	4.147	4240	2272
7	5	65	0.717157	2.474	3.437	4761	6645
8	10	65	1.28284	1.823	2.529	4534	6064
9	3	65	1	2	3.091	4449	5979
10	11	65	1	2.007	3.102	4602	5919
11	6	65	1	2	3.092	4564	6002
12	13	65	1	2.005	3.101	4578	5935
13	2	65	1	1.997	3.086	4502	6009

Risk Assessment: A risk evaluation was performed to identify potential sources of variability and the associated risk of such variation on the performance of the method. Risk assessment study was performed as per the tools described in ICH Q9 guidelines. Using these tools, Ishikawa diagram was studied for risk assessment as given in Figure 1 [24].

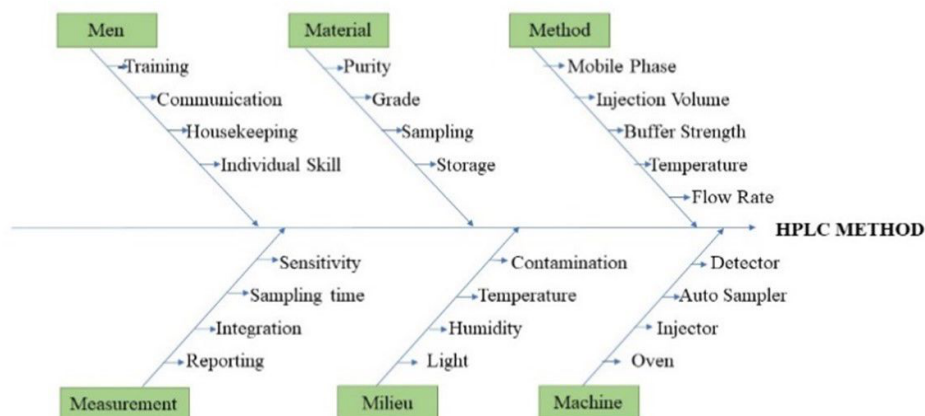


Figure 1: Ishikawa fishbone diagram

Analysis of variance (ANOVA) for the retention time of Pregabalin (Y1) and Duloxetine HCL (Y2): Once the data was obtained by running the 13 runs as per the CCD protocol, it was inserted in the software to evaluate its effects and impacts by statistical analysis.

The Analysis of Variance (ANOVA) was performed to identify significant and insignificant factors and interactions. The statistical results of ANOVA helped to identify the key factors having statistically significant impact on the response variable(s).

Factors having significant effects are considered critical for the method as they have potential to influence the responses. Thus, such types of key factors were identified which need to be controlled or optimized for method robustness. The term significant implies that the identified factor has critical impact on responses which in turn is dependent on p-value. For a factor to be significant, the p value should fall below 0.05. The results of ANOVA for the response 1 (Y1) are as shown in Table 2 and for response Y2 are shown in Table 3.

Table 2 :ANOVA table for retention time of Pregabalin

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	0.9410	5	0.1882	12.08	0.0025	significant
A-Mobile Phase composition (% Buffer)	0.0011	1	0.0011	0.0699	0.7991	
B-Flow rate	0.7180	1	0.7180	46.07	0.0003	
AB	0.0004	1	0.0004	0.0232	0.8833	
A ²	0.1496	1	0.1496	9.60	0.0174	
B ²	0.1000	1	0.1000	6.42	0.0390	

Table 3: ANOVA table for retention time of Duloxetine HCL

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2.42	5	0.4831	19.50	0.0006	significant
A-Mobile Phase Composition (% buffer)	0.5550	1	0.5550	22.40	0.0021	
B-Flow rate	0.9059	1	0.9059	36.57	0.0005	
AB	0.1576	1	0.1576	6.36	0.0397	
A ²	0.7847	1	0.7847	31.67	0.0008	
B ²	0.0000	1	0.0000	0.0019	0.9663	

The Model F-value of 12.08 for Y1 implies that the model is significant. There is only a 0.25 % chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are

significant. The Model F-value of 19.50 for Y2 implies that the model is significant. There is only a 0.06 % chance that an F-value this large could occur

due to noise. P-values less than 0.0500 indicate model terms are significant.

Response Surface Plots: Figure 2 below, graphically represents the type and magnitude of impact of independent variables on dependent variables.

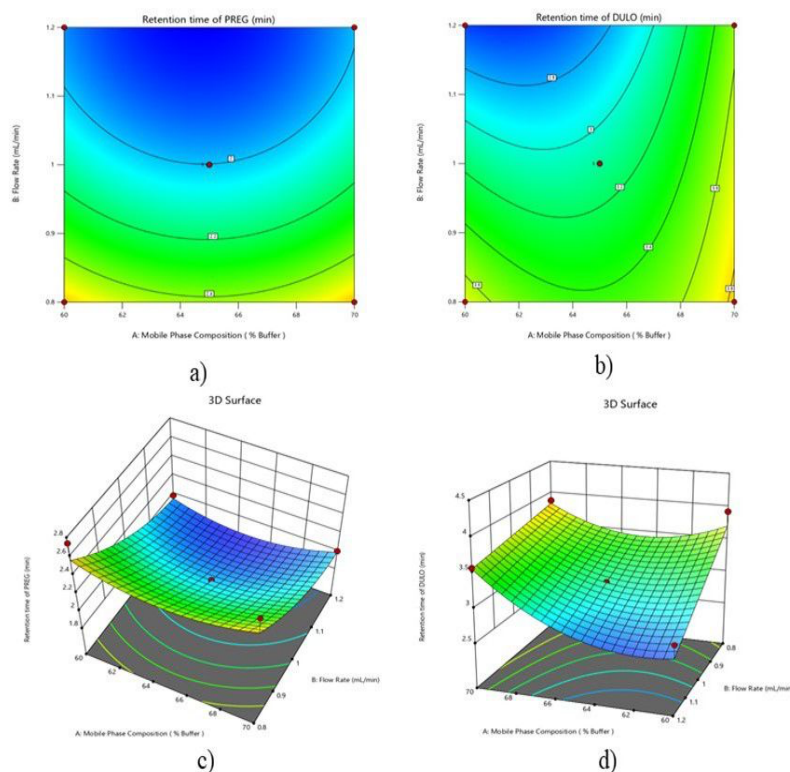


Figure 2: Contour plot (a-b) and 3-D response plot (c-d) for retention time (RT) of PREG and DULO

Contour plot (a) and 3D surface plot (c) graphically helps to correlate effect of independent variables on dependent variables. Here, it can be observed that as the flow rate increases, RT of Pregabalin decreases. This can be due to reduced time allowed for interaction between the column and the drug. Since high flow rate pushes the analyte faster through the column.

Hence, the RT of PREG is lowered. The concentration of organic phase (% volume of buffer) too affects the RT. As buffer concentration increases, the retention time increases slightly but not significantly. This is because in RP-HPLC, the polarity of stationary phase and the mobile phase is different. Buffer in polar mobile phase, have lower elution strength than organic solvents like acetonitrile. Hence Pregabalin interacts for slightly longer time with the column which results in increased RT of PREG. The p-value of 0.7991 from ANOVA Table 2, suggests that the mobile phase alone has minimal impact on RT of PREG. However,

the quadratic term (A^2) is significant with p value of 0.0174 indicates that very high or very low buffer % volume can increase retention time significantly. Contour plot (b) and 3D surface plot (d) indicates that as concentration of organic phase i.e. % volume of buffer increases, RT of Duloxetine HCL increases. This can be attributed to the relatively more lipophilic (non-polar) nature of duloxetine HCL and its preference for the non-polar mobile phase resulting in reduced interaction with the non-polar C18 stationary phase causing increased RT of DULO. Also, as flow rate increases, RT of Duloxetine HCL decreases because with higher flow rates the analytes get pushed faster through the column, reducing interaction time with the stationary phase. Hence, the RT of DULO decreases with increase in flow rate.

Analysis of variance (ANOVA) for the TPC of Pregabalin (Response 3) and Duloxetine HCL (Response 4)

Table 4: ANOVA table for TPC of Pregabalin

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	2.386+05	5	47717.79	11.40	0.0029	significant
A-Mobile Phase Composition (% buffer)	4155.67	1	4155.67	0.9926	0.3523	
B-Flow rate	97908.88	1	97908.98	23.39	0.0019	
AB	13924.00	1	13924.00	3.33	0.1110	
A ²	83125.03	1	83125.03	19.86	0.0030	
B ²	25410.03	1	25410.03	6.07	0.0432	

Table 5: ANOVA table for TPC of Duloxetine HCL

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	6.523E+06	2	3.262E+06	3.46	0.0723	significant
A-Mobile Phase Composition (% buffer)	5.585E+06	1	5.585E+06	5.92	0.0353	
B-Flow rate	9.382E+05	1	9.382E+05	0.9942	0.3422	

Table 4 and 5 represents the statistical results of ANOVA for TPC for PREG and DULO respectively.

The Model F-value of 11.40 for TPC of PREG implies that the model is significant. There is only a 0.29 % chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. The Model F-value of 3.46 for TPC of DULO implies that the model is

significant and there is a 7.23 % chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant.

Response surface plots: Figure 3 graphically represents the type and magnitude of impact of independent variables on dependent variables.

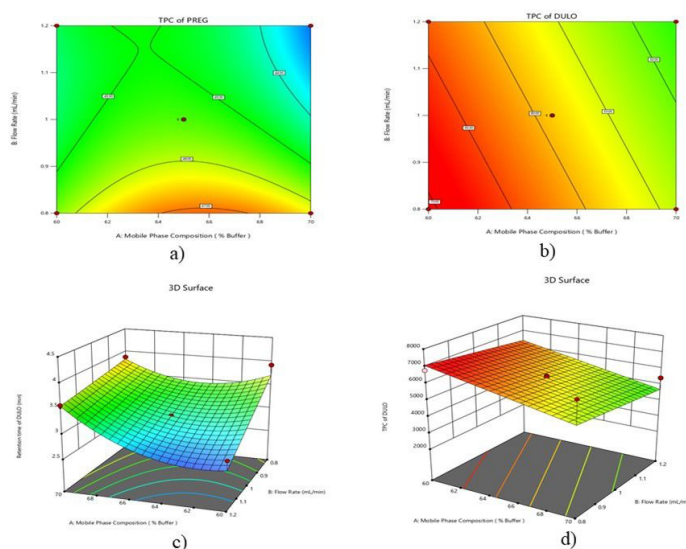


Figure 3: Contour plot (a-b) and 3-D response plot (c-d) for theoretical plate count (TPC) of PREG and DULO

Contour plot (a) and 3D surface plot (c) shows that as the flow rate increases, TPC of PREG decreases. This can be attributed to the fact that at higher flow rate the analytes are forced to move faster through the column resulting in reduced interaction of the mobile phase with the column.

Thus, resulting in lower TPC. Just like mobile phase i.e. % volume of buffer alone does not have significant impact, but exerts effect at extremes, flow rate can reduce TPC because PREG is polar and it elutes quickly. Whereas, change in % volume

of buffer didn't greatly influence peak sharpness. But if buffer concentration is too high or too low (extreme) then the ionization in the mobile phase may slightly disturb peak shape which reduces TPC.

Contour plot (b) and 3D surface plot (d) shows that as the concentration of organic phase i.e. % volume of buffer increases, the TPC of DULO increases. This might be because, DULO is more lipophilic (non-polar) and at higher buffer concentration i.e. having more polar mobile phase, DULO can have better interaction with the stationary phase (non-

polar) and also helps in stabilizing ionization of DULO, which results in sharper peaks and higher TPC. But changes in flow rate does not significantly impact TPC for DULO. This might be because DULO due to its non-polar nature elutes later than PREG. So, even if flow rate changed, it got enough time to equilibrate with the stationary phase even at slightly higher flow rate.

Method Operable Design Region- Design Space:

The Graphical optimization performed using Design Expert software provided with a design space as shown in Figure 4. A design space is a graphical representation of the region whose boundaries constitute the limits of optimal conditions for CPMs or specific criteria to achieve a certain goal of the experiment. Within this region, variations of CPMs will always result in outcomes that will satisfy the objective of the experiment.

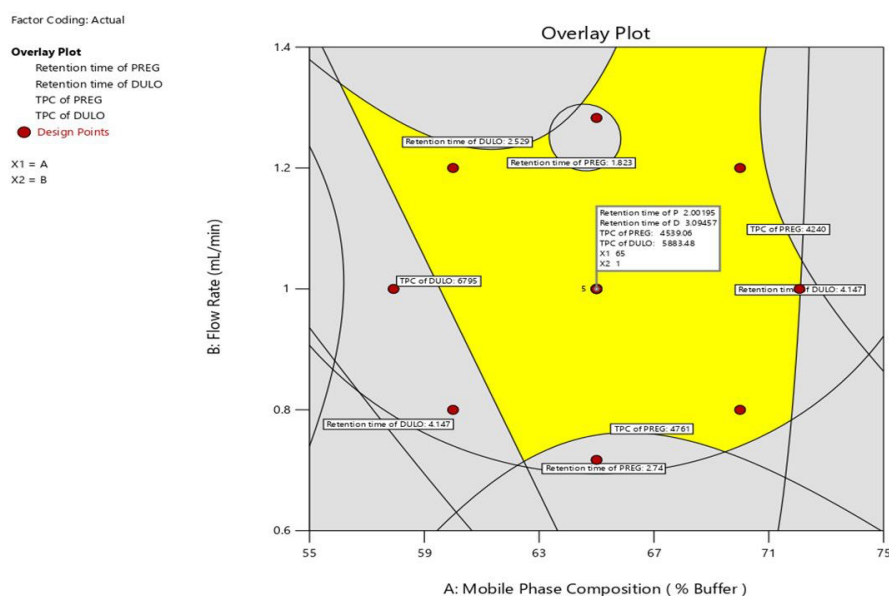


Figure 4: Overlay plot showing the optimal analytical design space

Method Optimization: Figure 5 shows chromatogram for optimized conditions of CPMs. The optimized mobile phase composed of Acetonitrile to Buffer ratio of 35:65 and flow rate of a 1 mL/min which resulted in retention time of PREG (Y1) as 2.0 minutes and retention time of DULO (Y2) as 3.092 minutes.

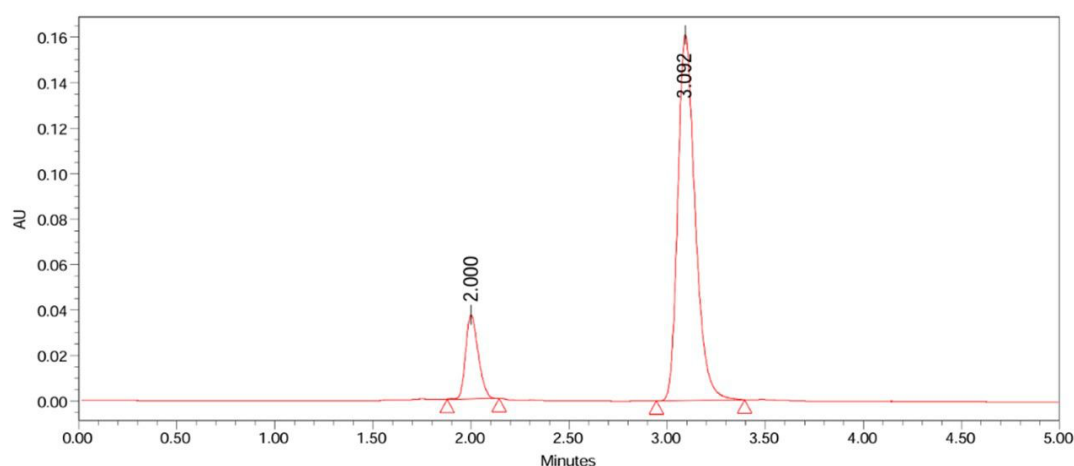


Figure 5: Chromatogram of Optimized Condition

System suitability: Results of the system suitability test are represented in Table 6. The % RSD obtained for all the parameters was less than 2 %. Thus, it passes the test for suitability.

Table 6: System suitability results for the developed HPLC method

Parameters	Average $\pm$ SD	% RSD
Retention time (RT1)	0.003507136	0.175664417
Retention time (RT2)	0.003507136	0.113591497
Tailing Factor of PREG	0.001437	0.113611853
Tailing Factor of DULO	0.006324555	0.486509053
TPC of PREG	64.12305878	1.426610686
TPC of DULO	12.57643299	0.209671667

Method validation

Linearity, LOD and LOQ: Concentration of PREG and DULO and corresponding peak areas of the chromatograms demonstrated a significant linear relationship over the concentration ranges of 45-105 $\mu\text{g/mL}$ and 12-28 $\mu\text{g/mL}$ respectively. The experiments were carried out using optimized chromatographic conditions that resulted in correlation coefficient of $R^2 = 0.999$ for PREG and $R^2 = 0.9994$ for DULO. The analytical outcomes for

linearity with slope and intercept are depicted in Table 7. The Standard Deviation (SD) of the response and regression line slope values were used to calculate the values of LOD and LOQ, used to demonstrate the high sensitivity of the proposed method.

The values for LOD and LOQ were found to be 4.70, 14.24 for PREG and 0.255, 0.774 for DULO respectively.

Table 7: The results of Linearity parameters for PREG and DULO

Parameters	PREG	DULO
Concentration ($\mu\text{g/mL}$)	45-105 $\mu\text{g/mL}$	10-30 $\mu\text{g/mL}$
Regression equation	$2200.3x + 2647.2$	$55209x - 87212$
Correlation coefficient (R^2)	0.999	0.9994
LOD ($\mu\text{g/mL}$)	4.70	0.255
LOQ ($\mu\text{g/mL}$)	14.24	0.774

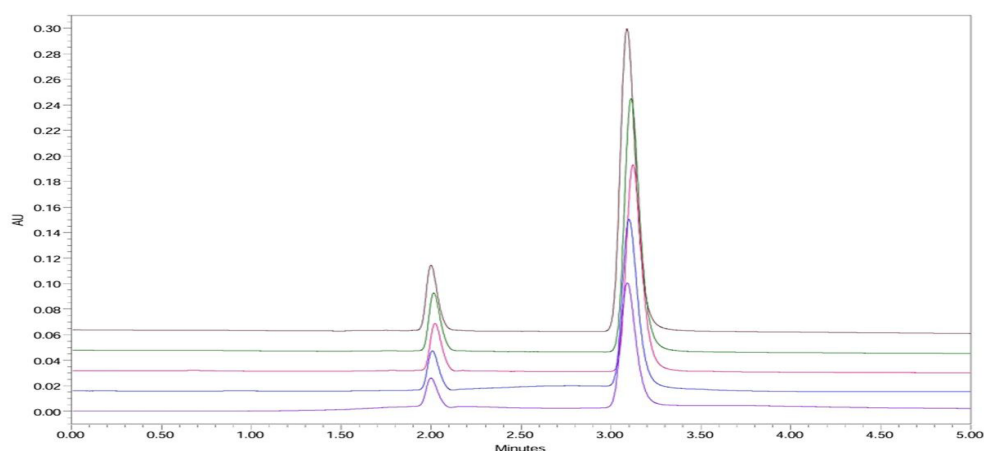
**Figure 6: Overlay Chromatogram of linearity**

Figure 6 represents an overlay chromatogram of PREG and DULO over the concentration range of their respective linearity ranges.

The retention times across all runs were found to be consistent, indicating excellent method reproducibility.

The progressive increase in peak area corresponding to increasing concentration indicated strong linearity, that was further supported by correlation coefficients of 0.999 and 0.9994 for PREG and DULO, respectively. Also, the consistent peak

shapes confirmed the robustness and suitability of the method for quantitative analysis.

Precision and accuracy: Outcome of the precision studies conducted are given in Table 8. The results indicated that the method is precise since no parameter showed % RSD more than 2 %. Percent recovery for accuracy testing of the developed method showed optimum results, representing sound accuracy of the method. The results of accuracy are depicted in Table 9.

Table 8: Precision results for PREG and DULO

PREG					DULO				
Conc.	Intraday		Interday		Conc.	Intraday		Interday	
	SD	% RSD	SD	% RSD		SD	% RSD	SD	% RSD
60	0.00047	0.02	0.00163	0.081	16	0.00094	0.03	0.00216	0.06
75	0.0017	0.08	0.007	0.35	20	0.00094	0.03	0.01228	0.39
90	0.001	0.06	0.014	0.72	24	0.02994	0.94	0.01461	0.47

Table 9: Accuracy results for PREG and DULO

Level of addition	% Mean recovery	SD	% RSD
PREG			
80%	99.70	1.3266	1.32
100%	100.87	1.7048	1.70
120%	99.66	1.3356	1.33
DULO			
80%	99.67	0.4018	0.40
100%	99.46	0.4681	0.46
120%	100.12	0.2641	0.26

Discussion

Use of AQbD framework helped in the successful development of a robust, validated RP-HPLC method for the simultaneous estimation of Pregabalin and Duloxetine HCl. Critical Method Parameters (CMPs) were systematically optimized using the CCD. Also, it helped in identifying a design space within the boundaries of which the results ensured consistent method performance. This developed method offers improved resolution, shorter retention times, and better peak symmetry with significant robustness than the previously reported methods. Excellent analytical performance is guaranteed by the high R^2 values (> 0.999) and low % RSD values ($< 2\%$) in linearity and precision studies. Furthermore, accuracy results help to validate the method's reliability for quantifying both analytes in pharmaceutical dosage forms.

Adopting the AQbD principles in method development ensures method reliability and facilitate the regulatory compliance in accordance with ICH Q2(R2) guidelines. This scientifically sound approach enhances method understanding and reduces the risk of failure in real-world industrial settings.

Conclusion

Simultaneous estimation of pregabalin and duloxetine HCl was performed using a method developed based on the Analytical Quality by Design (AQbD) strategy. The developed RP-HPLC method was robust and efficient for simultaneous estimation of the drugs in marketed capsule dosage form. Central Composite Design (CCD) was applied for optimization of the CMPs by evaluating both the interaction and quadratic effects of key independent factors on selected dependent responses. The optimization models were found to be statistically significant. Thus, it confirmed the reliability of the method predictions. Method validation was performed in

accordance with ICH Q2(R2) guidelines. It demonstrated the simplicity, preciseness, accuracy, and robustness of the developed method. The developed method exhibited high selectivity and is applicability for routine analysis of pregabalin and duloxetine HCl in pharmaceutical capsules.

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