Research Article

Implementation of Quality by Design Approach to Develop and Validate Analytical Method for Simultaneous Estimation of Duloxetine Hydrochloride and Methylcobalamin in Pharmaceutical Dosage form by RP-HPLC Method

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ABSTRACT

Quality by Design (QbD) is a philosophy that refines the level of knowledge associate with a product and process to deliver a product with the desired critical quality attributes. The objective of study is to develop and demonstrate multivariate Quality by Design approach and to quantify the constitute concentration of Duloxetine HCl and Methylcobalamin drugs in standard mixture and capsule dosage form by reversed phase high-performance liquid chromatography method. The developed method employed mobile phase 0.05 M Potassium dihydrogen phosphate buffer (pH 3.5±0.05): Methanol (70:30, v/v) and flow rate 1.0 mL/min which was optimized with help of design expert software. Method was developed using column Hypersil BDS C18 and detection wavelength at 215 nm. The retention time was 6.94±0.09 and 4.83±0.16 minutes for Duloxetine HCl and Methylcobalamin respectively. High linearity of the developed method was confirmed over concentration range of 30-90 µg/mL for Duloxetine HCl and 1.5-4.5 µg/mL for Methylcobalamin. Limit of detection (LOD) and limit of quantification (LOQ) were found to be 1.51 and 4.58 µg/mL for Duloxetine HCl as well as 0.09 and 0.28 µg/mL for Methylcobalamin, respectively. The method was validated for precision, accuracy, robustness, limit of detection and limit of quantification according to International Conference on Harmonization guidelines. The results show that the Quality by Design concept can be effectively applied to optimize method with fewer trials and error-free experimentation.

Keywords: Design expert software, Duloxetine hydrochloride (DUL), Methylcobalamin (MEC), Quality by design (QbD), RP-HPLC, Validation

Received 9 Jan 2016 Received in revised form 28 Jan 2016 Accepted 30 Jan 2016

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INTRODUCTION

Quality by design is a systematic approach to develop that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management [1]. Traditional chromatographic method development has always involved the time-consuming process of varying one system parameter at a time, examining its effect on the method, and system operation. This generally requires a large number of experimental runs and in most situations the developed method requires further development [2]. The objective of the QbD initiative is to demonstrate both understanding and control of pharmaceutical processes to deliver high quality pharmaceutical products while affording opportunities for continuous improvement. QbD delivers a better understanding of method capabilities and limitations and ensures a superior chance of successful downstream method validation and transfer. The QbD concept can be extended to analytical methods [3]. The analytical methods used for the analysis of active pharmaceutical ingredients (API) and drug products are an integral part of the QbD.

Duloxetine HCl (DUL) - (3*S*)-*N*-methyl-3-(naphthalen-1-yloxy)-3-(thiophen-2-yl)propan-1-amine hydrochloride has an empirical formula of $C_{18}H_{19}NOS.HCl$ and a molecular weight of 333.38 g/mol (**Fig. 1**) [4]. It is a potent inhibitor of serotonin and norepinephrine reuptake and thus it is used for major depressive disorders [5-7], anxiety disorder, and pain associated with diabetic peripheral neuropathy or fibromyalgia. Furthermore, it provides evidence of an effect on pain in the case of urinary incontinence [8, 9] independent of its effect on depression. Therefore, Duloxetine HCl is used in the treatment of the different symptoms of depression [10].

Methylcobalamin (**Fig. 2**) is MeCbl; Coα-[α-(5,6-dimethylbenz-1H-imidazolyl)]-Coβmethylcobamide and has an empirical formula $C_{63}H_{91}CoN_{13}O_{14}P$. It is a cobalamin and it is a form of Vitamin B_{12}. Vitamin B_{12} is used in the body in two forms such as methylcobalamin and 5-deoxyadenosyl cobalamin.

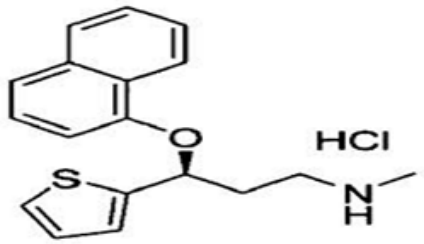

Figure 1: Chemical structure of Duloxetine Hydrochloride

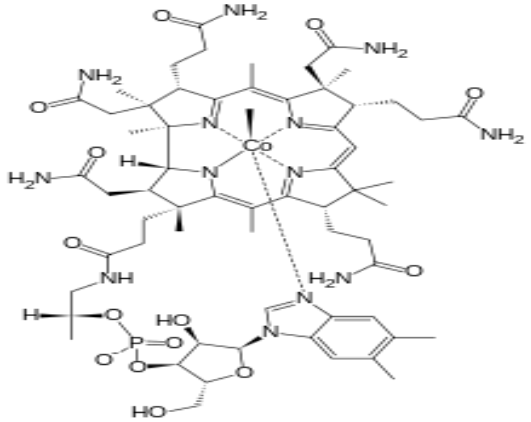

Figure 2: Chemical structure of Methylcobalamin

The methionine synthase is an enzyme responsible for conversion of the amino acid homocysteine into methionine and this enzyme requires Methylcobalamin as a cofactor. Methylcobalamin is also used in the treatment of peripheral neuropathy, diabetic neuropathy, hearing loss, Alzheimer's disease and as a preliminary treatment for amyotrophic lateral sclerosis [11].

The combined dosage form of these drugs is used for the treatment of neuropathic pain associated with peripheral neuropathy especially diabetic neuropathy. Duloxetine HCl is not official in any pharmacopoeia. Methylcobalamin is official in Japanese Pharmacopoeia [12]. The combination of these two drugs is not official in any pharmacopoeia; hence no official method is available for the simultaneous estimation of Duloxetine HCl and Methylcobalamin in their combined dosage forms. A literature survey indicated few analytical methods like Spectrofluorimetric method [13], gas chromatography [14], MS, NMR spectrometry, and X-ray analysis [15, 16], HPLC and HPTLC methods [17-40], UV Spectrophotometric method [41-49] and capillary electrophoresis with laser-induced fluorescence detection method [50] which have been reported for the determination of DUL and MEC in individual or combination with other drugs in pharmaceutical dosage form and in biological samples. This paper aims to describe the development and validation of new, simple, economical and robust RP-HPLC method for the simultaneous determination of Duloxetine HCl and Methylcobalamin in standard and in its pharmaceutical dosage form by implementing the Quality by Design approach.

MATERIALS AND METHODS

Reagents and Materials:

Duloxetine HCl (DUL) and Methylcobalamin (MEC) were procured as gratis samples from Sunrise Pharma Pvt Ltd (Satej, Ahmedabad, India). Capsule formulation, Duzela® M 30 (Sun Pharma laboratories ltd., India), was obtained commercially with the labeled amounts of 30 mg of DUL and 1.5 mg of MEC. Analytical reagents potassium dihydrogen orthophosphate and ortho phosphoric acid were purchased from Merck, Mumbai, India. HPLC grade methanol was purchased from Finar Chemicals Ltd., Ahmedabad, India. HPLC grade water was purchased from Rankem, Ankleshwar, India.

Instrument and Chromatographic Conditions:

A HPLC system (SPD-20AT), equipped with pump (LC-20AT), system controller (SCL-20AT), on-line degasser (DGU-14A), low-pressure gradient flow control valve (FCV-20AL), solvent delivery module (LC-20AD), injector (Rheodyne injector, 20 µL capacity), column oven (CTO-20AT), UV/Vis detector (SPD-20AT), Diode Array Detector (SPD-M20A) and Spinchrom software (Shimadzu, Kyoto, Japan). A double beam UV–visible spectrophotometer (SHIMADZU, Japan Model: 1800), having a pair of 10 mm matched quartz cuvettes, was used to measure absorbance of the resulting solutions.

The chromatographic column Hypersil BDS C18 (250 mm x 4.6 mm, 5 µm particles) was used. A buffer 0.05 M Potassium dihydrogen phosphate was prepared and pH-adjusted to 3.5±0.05 using ortho-phosphoric acid. Mobile phase consisting of a phosphate buffer and methanol in the ratio of 70:30 (v/v) was used in isocratic reversed phase liquid chromatography method. The flow rate of the mobile phase was 1.0 mL/min, the column was maintained at 25°C and detection was at 215 nm. The injection volume was 20 µL and mobile phase was used as diluent.

Solution preparation:

Preparation of standard mixture solution:

For RP-HPLC, a standard stock solution was prepared in mobile phase containing 600 µg/mL of DUL and 30 µg/mL of MEC. Pipette out 1 mL from standard stock solution into a 10mL volumetric flask and make up with mobile phase to get the working standard solution containing 60 µg/mL of DUL and 3 µg/mL of MEC.

Preparation of Sample Solution:

Twenty capsules were opened and transferred the contents (each capsule containing 30 mg of DUL and 1.5 mg of MEC) equivalent to 60 mg of DUL and 3 mg of MEC into a 100 mL volumetric flask. The 60 mL of mobile phase was added into volumetric flask and sonicated for 30 minutes with intermediate shaking. The final volume was made up to 100 mL and prepared stock solution containing 600 µg/mL of DUL and 30 µg/mL of MEC. Aliquots of the stock solutions were appropriately diluted with mobile phase to obtain working sample solution containing 60 µg/mL of DUL and 3 µg/mL of MEC.

Method Development:

Based on sample solubility, pKa value and solvent polarity, various mobile phase compositions were tried to get a good separation. The standard solution containing mixture of DUL and MEC and as well as individual drugs were run in different mobile phases in order to find the best conditions for separating both the drugs simultaneously. The composition of mobile phase for separation was determined as phosphate buffer (pH 3.5): methanol. The column for RP-HPLC method was selected Hypersil BDS - C_{18} (5 µm, 250 mm x 4.6 mm i.d.). For quantitative analytical purposes, the wavelength was set at 215 nm as the optimum wavelength throughout the experiment for DUL and MEC.

Software aided method optimization:

Response surface methodology, such as the Box-Behnken or Central Composite Design (CCD), was used to optimize a response. Central Composite Design- statistical screening design was used to optimize the compositional parameters and to evaluate interaction effects and quadratic effects of the chromatographic conditions on the retention time for RP-HPLC method. A 2-factorial design is suitable for exploring quadratic response surfaces and constructing polynomial models with Design Expert_ (Version 7.0.0.1, Trial Version). The Central Composite design was specifically selected since it requires fewer runs than a Box-Behnken in cases of two variables. A design matrix comprising of 13 experimental runs was constructed. The non-linear computer generated quadratic model is given as

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_1 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \varepsilon$$

Where, ***Y*** is the measured response retention time associated with each factor level combinations; mobile phase composition **(X_1)** and flow rate of mobile phase **(X_2)**. The dependent and independent variables selected are shown in (**Table 1**) along with their low, medium and high levels, which were selected based on the results from preliminary experimentation.

The combination range of two factors used to prepare the 13 analytical trials and the respective observed responses of method are given in (**Table 2**).

Table 1: Variables selected in Central Composite Design

	Levels used		
Independent variables	**Low (-1)**	**Medium (0)**	**High (+1)**
X_1 = Mobile phase composition	65	70	75
X_2 = Flow rate(mL/min)	0.8	1.0	1.2
Dependant variables	Constraints		
Y_1 (retention time of DUL)	$5.243 \leq Y_1 \geq 9.013$		
Y_2 (retention time of MEC)	$3.810 \leq Y_2 \geq 6.277$		

Table 2: Observed responses in Central Composite Design for 13 analytical trials

Run	Independent Variable		Dependent Variable	
	Amount of Buffer (X_1) (mL)	**Flow rate (X_2) (mL/min)**	**Retention time (DUL) (Y_1) (min)**	**Retention time (MEC) (Y_2) (min)**
1	70.00	1.20	5.743	4.007
2	65.00	1.20	5.243	3.810
3	75.00	1.20	6.500	4.570
4	70.00	0.80	8.317	5.767
5	70.00	1.00	6.937	4.863
6	70.00	1.00	6.940	4.869
7	70.00	1.00	6.935	4.861
8	75.00	1.00	7.617	5.300
9	70.00	1.00	6.937	4.863
10	75.00	0.80	9.013	6.277
11	65.00	0.80	8.037	5.767
12	65.00	1.00	6.640	4.787
13	70.00	1.00	6.931	4.859

It was observed that the best-fitted model was the quadratic model. The comparative values of R^2, SD and predicted residual sum of square (PRESS) for the different proposed models are given in (**Table 3**) along with the regression equation generated for finally selected responses.

Furthermore, the model was validated by the application of analysis of variance (ANOVA) to examine the significance of the model which showed that the responses achieved significant differences in their values.

Table 3: Summary results of regression analysis for model and responses *(Y)*, Regression equations for the finally suggested Quadratic model

Response	Models	R^2	Adjusted R^2	Predicted R^2	SD	PRESS	%C.V.
Retention time for DUL (Y_1)	Quadratic	0.9993	0.9987	0.9926	0.036	0.090	0.51
	Regression equation	$Y_1 = 6.94 + 0.54X_1 - 1.31X_2 + 0.070X_1X_2 + 0.18X_1^2 + 0.082X_2^2$					
Retention time for MEC (Y_2)	Quadratic	0.9986	0.9976	0.9866	0.033	0.075	0.67
	Regression equation	$Y_2 = 4.86 + 0.30X_1 - 0.90X_2 + 0.063X_1X_2 + 0.20X_1^2 + 0.043X_2^2$					

The model was examined using a lack of fit test, which indicated an insignificant lack of fit value corresponding with a higher p-value as compared to the model F-value.

The results of ANOVA statistical analysis for responses (Y_1 and Y_2) for the finally suggested quadratic model are shown in (**Table 4**).

Table 4: Summary results of ANOVA statistical analysis for models and response (*Y*) for the finally suggested quadratic model

ANOVA Statistical Analysis for response variable retention time										
Source	**Sum of Squares**		**Degree of freedom**		**MS**		**F- Value**		**P- Value**	
	DUL	**MEC**	**DUL**	**MEC**	**DUL**	**MEC**	**DUL**	**MEC**	**DUL**	**MEC**
Model	12.25	5.60	5	5	2.45	1.12	1896.93	1009.96	<0.0001	<0.0001
Amount of Buffer	1.72	0.53	1	1	1.72	0.53	1329.36	477.49	<0.0001	<0.0001
Flow rate	10.35	4.90	1	1	10.35	4.90	8012.98	4418.76	<0.0001	<0.0001
Residual	0.00904	0.00776	7	7	0.00129	0.00111	---	---	---	---
Lack of fit	0.00899	0.00771	3	3	0.003	0.00257	272.70	183.61	<0.0001	<0.0001
Pure error	0.00004	0.00005	4	4	0.00001	0.00001	---	---	---	---

Model p-value of <0.0001 indicates that 0.01% chance for large "Model F-value" due to noise. Values of "Prob > F" (p-value) less than 0.05 indicate model terms are significant. Only statistically significant ($p<0.05$) coefficients are included in the equations. 3D response surfaces were also analyzed to visualize the effects of the parameters and their interactions on the responses. (**Fig. 3 and 4)** show the effect of interactions on response Y_1 and Y_2 respectively. In order to get the best chromatographic performance, the multicriteria methodology is employed by means of Derringer's desirability function. Individual desirability functions range from 0 (undesired response) to 1 (fully desired response). A value of D close to 1 indicates that combination of different criteria is globally optimal.

The red area in desirability plot indicates prediction at all points in this region is one. The yellow area in overlay plot indicates all the constraints are satisfied in this region. Desirability and overlay plot were obtained from the model for the selected responses, which is shown in (**Fig. 5 and 6**) respectively.

Calibration curve of Duloxetine HCl and Methylcobalamin:

A standard mixture stock solution was prepared in mobile phase containing 600 µg/mL of DUL and 30 µg/mL of MEC. Aliquots of stock solution (0.5, 0.75, 1.0, 1.25 and 1.50 mL) were transferred into series of 10 mL volumetric flasks and diluted up to mark with mobile phase. These yielded concentration of 30, 45, 60, 75 and 90 µg/mL of DUL and 1.5, 2.25, 3.0, 3.75 and 4.50 µg/mL of MEC respectively. An aliquot of 20 µL of each solution was injected under operating chromatographic condition. The data of peak areas plotted against the corresponding concentrations were treated by linear least-square regression analysis and also found out correlation co-efficient and regression line equation for DUL and MEC. Each response was an average of five determinations. The linear regression data for the calibration curves (n=5) is shown in (**Table 5**).

Method validation:

The analytical method was validated for specificity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ) and robustness in accordance with ICH guideline [51].

Specificity:

Specificity of analytical method is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Specificity of the method was evaluated by comparison between chromatogram of standard and test solutions. There should be absence of any interfering peak with the peak of analyte. The result of system suitability parameters with optimized chromatographic conditions are shown in (**Table 6**).

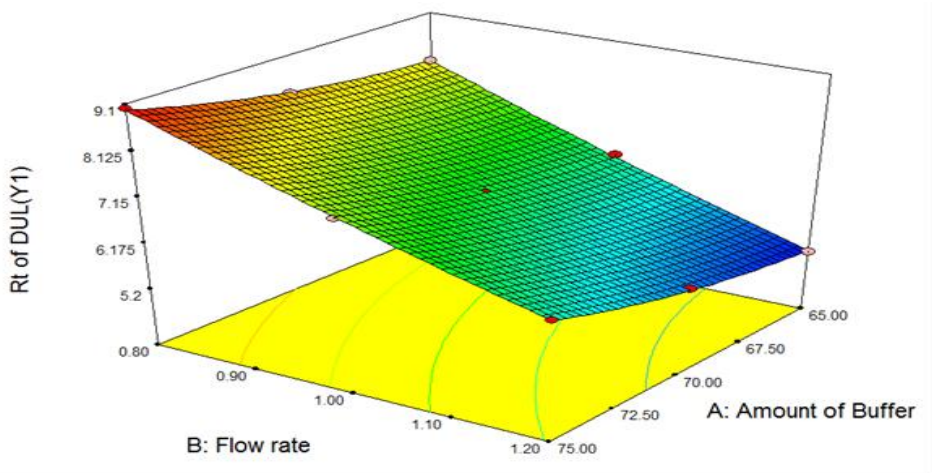

Figure 3: 3D Response surface plot showing the effect of mobile phase composition (amount of buffer) and flow rate on retention time of Duloxetine HCl

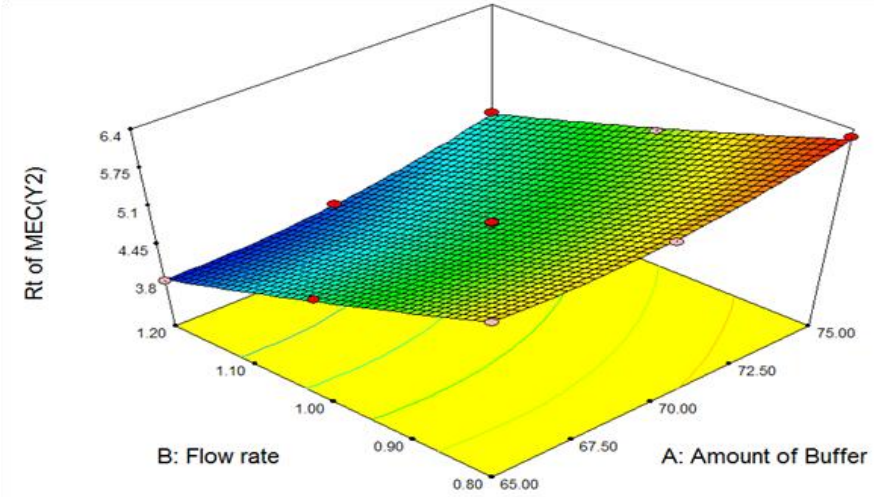

Figure 4: 3D Response surface plot showing the effect of mobile phase composition (amount of buffer) and flow rate on retention time of Methylcobalamin

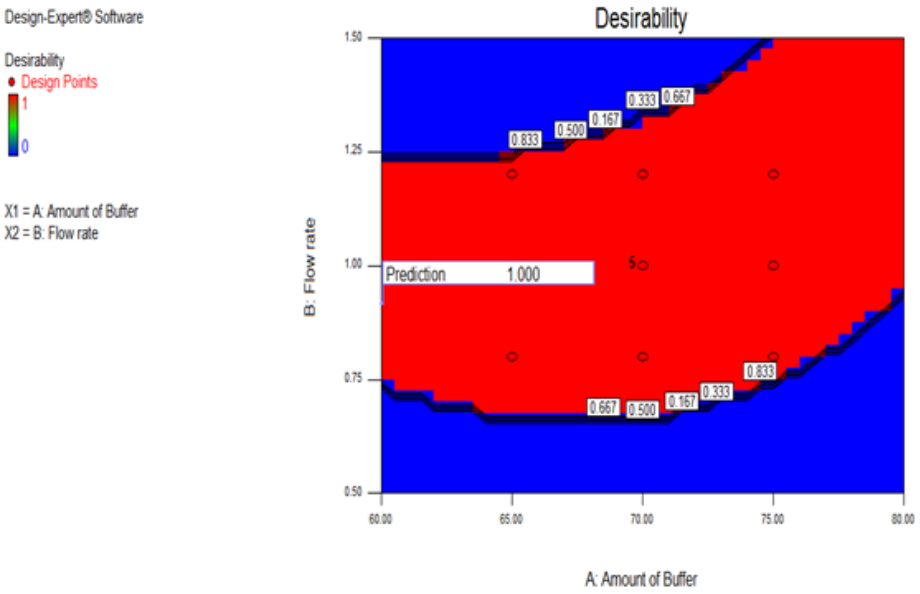

Figure 5: Desirability showing the effect of mobile phase composition (amount of buffer) and flow rate on retention time of Duloxetine HCl and Methylcobalamin

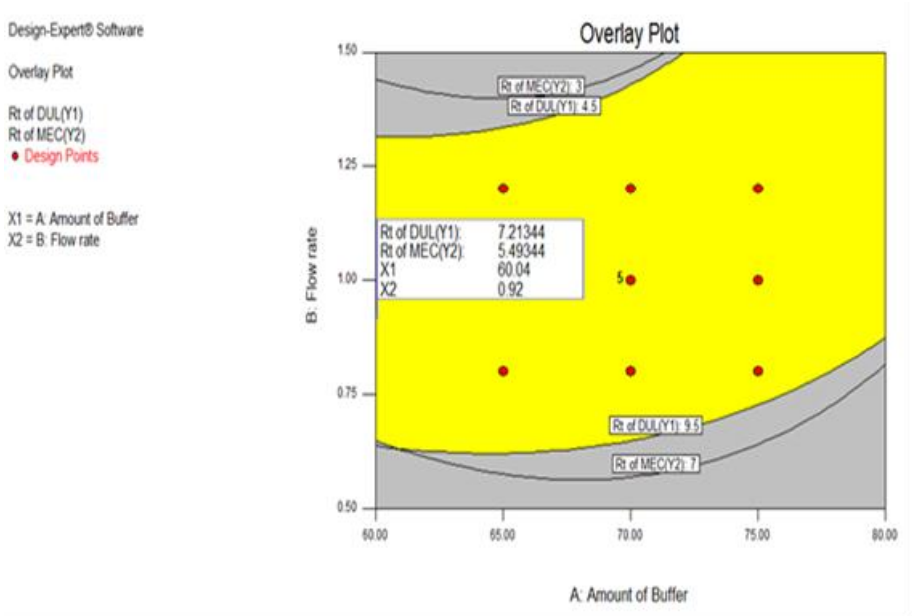

Figure 6: Overlay plot showing the effect of mobile phase composition (amount of buffer) and flow rate on retention time of Duloxetine HCl and Methylcobalamin

Table 5: Linear regression data for calibration curve (n=5)

Parameter	DUL	MEC
Linearity range (µg/mL)	30-90 µg/mL	1.5-4.5 µg/mL
Regression equation	Y = 121.4x + 43.76	Y = 57.43x + 0.649
Co- relation co- efficient	0.999	0.999
Slope±SD	121.40±0.25	57.43±0.49
Intercept±SD	43.76±2.67	0.649±1.58
LOD	1.51	0.09
LOQ	4.58	0.28

Table 6: Optimized chromatographic conditions and system suitability parameters of RP-HPLC method

Parameter	Chromatographic conditions and system suitability
Column	Hypersil BDS - C_{18} (5 µm, 250 mm x 4.6 mm i.d.)
Mobile phase	0.05 M Potassium dihydrogen phosphate buffer-Methanol (pH 3.5; 70:30, v/v)
Flow rate	1 mL/min
Detection wave length	By UV at 215 nm.
Run time	10 minutes
Temperature	25°C
Volume of injection loop	20 (µL)
Retention time (min)	6.94 (DUL) and 4.83 (MEC)
Theoretical plates [th.pl] (Efficiency)	7384 (DUL) and 8167 (MEC)
Capacity factor	1.39 (DUL) and 0.66 (MEC)
Peak asymmetry	1.37 (DUL) and 1.30 (MEC)
% RSD of peak area	0.29 (DUL) and 0.37 (MEC)
Resolution factor	7.63 (DUL)

Precision:

Intraday and Interday precision study of DUL and MEC was carried out by estimating corresponding responses 3 concentrations and 6 replicates on the same day and on 3 different days for the concentration

covering the specified range of Duloxetine HCl (30, 60 and 90 µg/mL) and Methylcobalamin (1.5, 3 and 4.5 µg/mL). The results of intraday and Interday precision are shown in (**Table 7**).

Table 7: Intra-day and Inter-day precision of RP-HPLC method (n=6)

Drug	Amount (µg/mL)	Intra-day Precision		Inter-day Precision	
		% Assay*±SD	%RSD	% Assay*±SD	%RSD
DUL	30	99.78±0.23	0.23	99.83±0.38	0.38
	60	99.09±0.24	0.25	98.88±0.24	0.25
	90	99.78±0.88	0.88	99.34±0.62	0.63
MEC	1.5	99.08±1.12	1.13	99.18±0.58	0.59
	3.0	99.62±0.36	0.36	99.41±0.36	0.36
	4.5	99.26±1.03	1.03	98.94±1.31	1.32

*n = 6 replicate analysis for each interval.

Limits of detection (LOD) and quantification (LOQ):

Limit of Detection (LOD) is the lowest concentration in a sample that can be detected, but not necessarily quantified under the stated experimental conditions. The limit of quantitation (LOQ) is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy. The LOD and LOQ were calculated using the equations as per ICH guideline.

LOD = 3.3 SD/S and LOQ = 10 SD/S

Where, SD is standard deviation of the peak area (n=5), taken as measure of the noise and S is the slope of the corresponding calibration curve. Results are shown in (**Table 5**).

Accuracy, as recovery:

Accuracy was evaluated in triplicate, at three different concentrations equivalent by spiking to 80, 100, and 120% of the active ingredient, by adding a known amount of DUL and MEC standard to a sample of known concentration and calculating the recovery of DUL and MEC and % RSD for each concentration. The previously analyzed samples (30 µg for DUL and 1.5 µg for MEC) were spiked with extra concentration levels of 24, 30 and 36 µg for DUL and 1.2, 1.5 and 1.8 µg for MEC and the mixtures were reanalyzed by the developed Method. The recovery studies were carried out in triplicates each level. (**Table 8**)

Table 8: Results of Recovery Studies by Standard Addition Method

	Amount taken	Amount added	Total amount recovered† (Mean±SD)	% Recovery	% RSD
For DUL	30.0 µg/mL	24.0 µg/mL	53.92±0.137	99.85	0.25
	30.0 µg/mL	30.0 µg/mL	59.94±0.157	99.90	0.26
	30.0 µg/mL	36.0 µg/mL	66.21±0.245	100.31	0.37
For MEC	1.5 µg/mL	1.2 µg/mL	2.67±0.011	99.07	0.40
	1.5 µg/mL	1.5 µg/mL	3.05±0.004	101.62	0.12
	1.5 µg/mL	1.8 µg/mL	3.30±0.010	99.95	0.31

†Three independent analyses at each level.

Robustness:

The robustness of the method was investigated by making small deliberate changes in the chromatographic conditions at two different levels. The chromatographic conditions selected for deliberate changes were different compositions of the mobile phase e.g. Phosphate buffer: methanol (65:35, 70:30 and 75:25 v/v) and flow rate of mobile phase (0.8, 1.0 and 1.2 mL). The results are shown in (**Table 9**).

Table 9: Results from Robustness Experiments for HPLC Method

Condition	Value	% Recovery		% RSD		Retention time (R_t)	
		DUL	MEC	DUL	MEC	DUL	MEC
Flow rate of mobile phase (± 20% absolute)	0.8 mL/min	101.76	101.34	0.27	0.67	7.03±0.008	4.94±0.011
	1.2 mL/min	100.25	99.87	0.45	0.36	6.88±0.010	4.83±0.012
Mobile phase ratio (± 2% absolute)	Buffer: Methanol (68:32)	100.87	100.62	0.90	0.94	7.01±0.025	4.91±0.008
	Buffer: Methanol (72:28)	99.55	99.50	0.17	0.37	6.86±0.007	4.81±0.007
pH (± 0.2 absolute)	3.3	101.64	100.99	0.38	0.57	7.03±0.006	4.93±0.007
	3.7	101.21	100.83	0.26	0.41	6.98±0.006	4.88±0.007

Analysis of the marketed formulation:

To determine the DUL and MEC content of capsule formulation, twenty Duzela® M 30 capsules (label claim 30 mg DUL and 1.5 mg MEC) were weighed to determine the average weight of the capsules without shell and then crushed and prepared fine powder using a mortar and pestle. A portion of powder equivalent to the weight of 60 mg of DUL and 3 mg of MEC was accurately dissolved in mobile phase. Each solution was sonicated for 20 min to achieve complete dissolution of DUL and MEC and then dilution was done to get a stock solution of 600 µg/mL of DUL and 30 µg/mL of MEC. The resulting solution was filtered through whatman filter no 42. Aliquot of above stock solution was diluted with mobile phase to get solution of 60 µg/mL of DUL and 3 µg/mL of MEC. An aliquot of 20 µL from sample solution was injected under chromatographic condition. Peak area was measured and % assay was calculated. Response was an average of six determinations. The results of assay are shown in (**Table 10**). The chromatograms of standard and sample solutions are shown in (**Fig. 7 and 8**) respectively.

Table 10: Results Obtained by the Proposed Methods for the Assay of Drugs in Pharmaceutical Preparations (n=6)

Statistical value	DUL	MEC
Mean Peak Area*	7265.14	173.77
% Recovery	99.10	99.51
% RSD	0.76	0.97

* Six independent analyses; Duzela® M 30 capsules (label claim 30 mg DUL and 1.5 mg MEC)

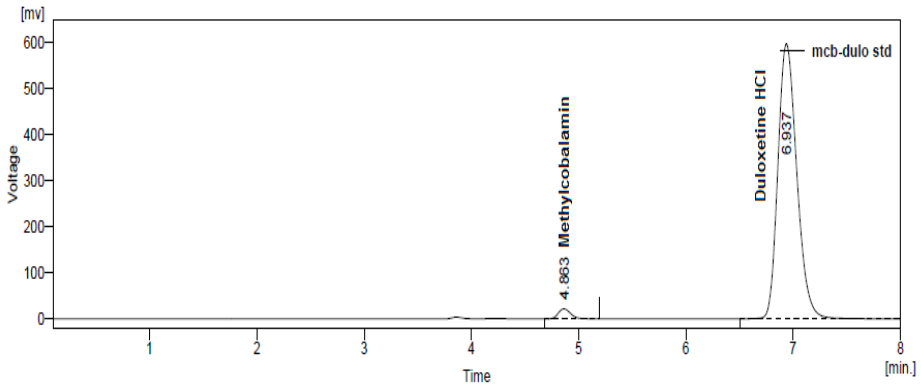

Figure 7: Chromatogram of Duloxetine HCl (60 µg/mL; Retention time: 6.937 min.) and Methylcobalamin (3 µg/mL; Retention time: 4.863 min) in Standard laboratory mixture

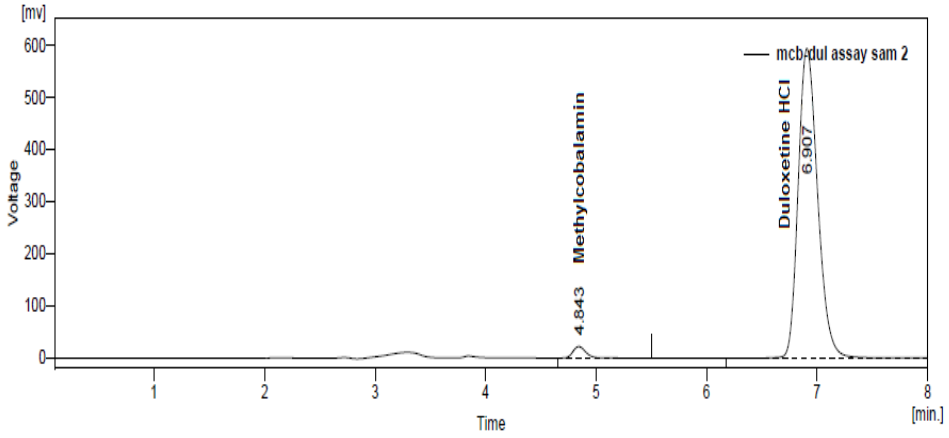

Figure 8: Chromatogram of Duloxetine HCl (60 µg/mL; Retention time: 6.907 min.) and Methylcobalamin (3 µg/mL; Retention time: 4.843 min) in marketed formulation

RESULTS AND DISCUSSION

A two-factorial, Central Composite Design-statistical experimental design was performed using 13 experimental trial runs for the optimization process of method. All trials and related observation data are shown in (**Table 2**). The values of R^2, SD and predicted residual sum of square (PRESS) for the quadratic proposed model are given in (**Table 3**). The model having low SD, higher R-square value and lower PRESS value were selected. The higher value of correlation coefficient signified an excellent correlation between the independent variables. All the above considerations indicate an excellent adequacy of the regression model. From the results of ANOVA (**Table 4**), response Y_1 and Y_2 showed that the predicted values for factors like amount of buffer in mobile phase (X_1) and flow rate (X_2) were under the satisfactory value with the predicted model F-value of 1896.93 (DUL) and 1009.96 (MEC). The model further suggested that the predicted values for both of the responses were closer to the actual values, indicating higher accuracy as well as precision for the obtained responses. The p-value less than 0.05 indicates model terms are significant. A positive value represents an effect that favours the optimization, while a negative value indicates an inverse relationship between the factor and the response. It was clear from the equation that the factor percentage of mobile phase composition (X_1) had a positive effect and flow rate (X_2) had a negative effect on the response retention time of DUL (Y_1) and retention time of MEC (Y_2). The relationship between responses and factors is not always linear. When we use different levels in an analysis or more than one factor is changed simultaneously, a factor can produce different degrees of response. Interaction of X_1 and X_2 produced positive impact on the response retention time Y_1 and Y_2 respectively. But the result in case of the square root of different factors did not repeat history as shown in its individual performance. In case of square root of factors X_1^2 and X_2^2 showed positive impact on the response retention time Y_1 and Y_2 respectively. Finally, the model was subjected to further analysis by the optimization module in the Design-Expert software which showed that optimized values for factors X_1 and X_2 were identical with the observed values. The selected optimized condition was 0.05 M Potassium dihydrogen phosphate buffer (pH 3.5): Methanol (70:30, v/v) as mobile phase and flow rate 1.0 mL/min for the final RP-HPLC analysis which showed good peak symmetry along with all other system suitability parameters and retention time 4.83±0.16 and 6.94±0.09 minutes for MEC and DUL respectively (**Table 6**). Chromatograms for standard and sample solution were obtained for RP-HPLC method. (**Fig. 7 and 8**)

The linear regression data for the calibration curves (n=5) as shown in (**Table 5**) showed a good linear relationship over

the concentration range of 30-90 µg/mL for DUL and 1.5-4.5 µg/mL for MEC with respect to peak areas. No significant difference was observed in the slopes of standard curves. The repeatability of sample application and measurement of peak area were expressed in terms of relative standard deviation (% RSD) and was found to be very low. The results depicted in (**Table 7**) were revealed intra-day and inter-day variation covering the specified range of Duloxetine HCl (30, 60 and 90 µg/mL) and Methylcobalamin (1.5, 3 and 4.5 µg/mL). Intraday and inter-day assay of samples were performed in the same laboratory. The low value of the RSD was indicative of the repeatability of the method. The limit of detection (LOD) and limit of quantification (LOQ) were estimated using standard deviation (SD) of the lowest response and slope of the calibration curve. With the help of it, exact value of LOD and LOQ were determined. Limits of detection (LOD) and quantification (LOQ) were found to be 1.51 and 4.58 µg/mL for DUL and 0.09 and 0.28 µg/mL for MEC, respectively. The recovery of the method was found to be varied from 99.85 to 100.31% for DUL and from 99.07 to 101.62% for MEC. Values of % recovery and % RSD were listed in (**Table 8**); less than 2 % RSD value indicated that the method was accurate. By introducing small changes in the mobile phase composition, flow rate and pH of mobile phase in chromatographic conditions, respective results were examined. There were very slight changes in the peak area, retention time, tailing factor and resolution. The lower value of SD and % RSD indicated the robustness of method as shown in (**Table 9**). The validated method was used to estimate the Duloxetine HCl and Methylcobalamin content of commercially available brands of the capsule containing 30 mg of DUL and 1.5 mg of MEC. Satisfactory results were obtained. Recovery of DUL and MEC from capsule formulation was 99.10% (RSD 0.76%) and 99.51% (RSD 0.97%) respectively. The amounts measured were in good agreement with the label claims. The results of the assay indicated the method was selective for analysis of DUL and MEC without interference from the excipients. (**Table 10; Fig. 8**)

CONCLUSION

A simple, rapid, accurate, and precise RP-HPLC analytical method with UV detection has been developed for the determination of Duloxetine HCl and Methylcobalamin in active pharmaceutical ingredients and in marketed capsule dosage form. A Quality by Design approach has been successfully applied for optimization of RP-HPLC method for estimation of Duloxetine HCl and Methylcobalamin. First, the method goals are clarified based on process understanding. The experimental design describes the scouting of the key components including mobile phase composition and flow rate. Their interrelationship is studied and optimized condition is obtained. A better understanding of the factors influencing chromatographic separation and greater confidence in the ability of the method to meet their intended purpose is done. It gives symmetric peak shape, good resolution and reasonable retention for it. The method was validated accordance to ICH guidelines. The method seems to be suitable for the quality control in the pharmaceutical industry because of its high sensitivity, simplicity and selectivity.

ACKNOWLEDGEMENT

The authors are thankful to Sunrise Pharma Pvt Ltd (Satej, Ahmedabad, India) for providing gift samples of DUL and MEC active pharmaceutical ingredients for research.

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