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DEVELOPMENT OF VALIDATED RP-HPLC METHOD FOR ESTIMATION OF CAPECITABINE BY QBD APPROACH

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Keywords:

Capecitabine, Quality by design approach, RP-HPLC, DOE, ICH guidelines validation

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ABSTRACT: It belongs to the group of medications called as anti-metabolites and is a fluoropyrimidine carbonate with anti-cancer properties. The chemotherapy drug capecitabine is taken orally and is used to treat metastatic colorectal and breast cancers. A review of the literature reveals several analytical techniques have been developed for Capecitabinein single and combined with other drugs. No QbD-assisted RP-HPLC method is available to estimate Capecitabine. So, present work describes the development and validation of QbD driven RP-HPLC method for Capecitabine. This method has been developed using Agilent 1100 series HPLC with ODS (water) column (150 × 4.5mm, 5 µm). Based on the RP-HPLC method development Mobile phase and Flow rate were selected as CAA and retention time, peak area, theoretical plates, tailing factor of drug were monitored using Design Expert 13.0.0.5. By applying CCD, 8 trials having 2 factors and 4 responses method had been selected for method development of capecitabine. The optimum method development was selected based on the criteria of Retention time, Peak area, Theoretical plates, Tailing factor. The retention time of Capecitabine is 2.721 min. The method was validated for specificity, linearity, accuracy, precision, limit of quantification, limit of detection, robustness in accordance with ICH guidelines. Limit of detection and limit of quantification for estimation of Capecitabine found to be 0.2371 µg/mL and 0.7185 µg/mL.

INTRODUCTION: Capecitabine CAP [N4-pentoxycarbonyl- 5- deoxy-5-fluorocytidine] is an anticancer prodrug of 5- fluorouracil (5-FU) that was designed to undergo preferential conversion to 5-FU within tumors [1]. 5-FU has also been widely used as an anticancer agent in the chemotherapy of solid tumors but its efficacy is limited by dihydropyrimidine dehydrogenase catalyzed formation of dihydro-5-fluorouracil [2].

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Since, it lacks selectivity toward tumour cells, 5-FU also exhibits significant toxicity [3]. The activation of Capecitabine follows a pathway with three enzymatic steps and two intermediary metabolites, 5'-deoxy-5- fluoro cytidine (5'-DFCR) and 5'-deoxy-5-fluorouridine (5'-DFUR), to form 5-fluorouracil [4].

Prodrug of 5-FU have been developed to improve efficacy and to reduce side effect and toxicity [5]. There is now some evidence to suggest that 5-FU is most active when given by prolonged intravenous infusion [6]. Capecitabine is an orally administered chemotherapeutic agent used in the treatment of metastatic breast and colorectal cancers [7]. Capecitabine reached peak blood levels in about 1.5 hours (Tmax) with peak 5-FU levels occuring

slightly later, at 2 hours [8]. Plasma protein binding of Capecitabine and its metabolites is less than 60% and is not concentration-dependent [9, 10, 11].

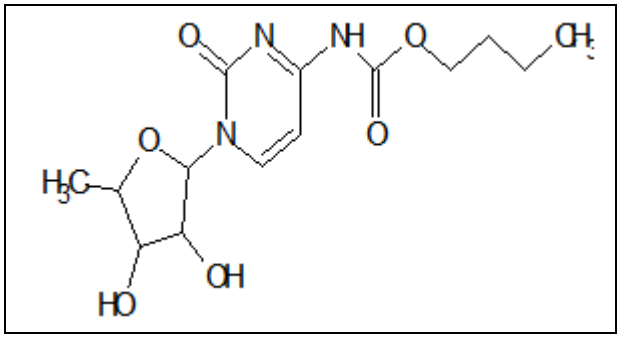

FIG. 1:

Literature survey reveals that various methods have been reported for capecitabine individually or in combination with other drugs those are UV [3, 6, 12, 15] HPLC [8, 14], stability indicating [2, 4, 15], stability by LC-MS [16], Stability UV by QbD [17, 18], but no one has developed RP-HPLC by Quality by Design method of these drug, which is fast, simple, and sensitive with less run time and good peak symmetry. Finally, the established method was validated with respect to specificity, linearity, precision, accuracy, robustness, LOD and LOQ according to ICH guidelines.

MATERIALS AND METHOD:

Materials: Capecitabine, pure drug was gifted by Shree Industrial Training Center and Research Laboratory, Jalgaon, Maharashtra. The solvent used in this experiment were of HPLC grade.

Methods: The analysis was performed using High Performance Liquid Chromatography (Agilent tech gradient system) with PDA detector having Chemstation software, UV-Visible spectro-photometer (model 2080), pH meter (VSI 1-B), electronic balance (WENSAR High Resolution Balance), Ultra sonicator. The column used is Symmetry C18 Column, 150 mm x 4.6 mm and 5µm (as Stationary phase) with the flow rate 0.8 ml/min.

Standard Stock Preparation for the Analysis: Accurately weighed 10 mg of Capecitabine and transferred to 50 mL volumetric flasks, 3/4th of diluents was added and sonicated for 10 minutes. Flasks were made up with diluents and labeled as Standard stock solution (1000µg/mL of Capecitabine). 1mL from above solution was pipetted out and taken into a 10mL volumetric flask and made up with diluent. (100 µg/mL of Capecitabine).

Sample Stock Preparation for the Analysis: 10 tablets were weighed and the average weight of each tablet was calculated, then the weight equivalent to tablet was transferred into a 10 mL volumetric flask, 5mL of diluents was added and sonicated for 25 min, further the volume was made up with diluent and filtered by HPLC filters (1000 µg/mL of Capecitabine). 1mL of filtered sample stock solution was transferred to 10mL volumetric flask and made up with diluent. (100 µg/mL of Capecitabine).

Determination of Detection Wavelength: Between 200 to 400 nm, the standard solution was scanned as shown in the **Fig. 2**, the wavelength of maximum absorption for drug was determined to be 240 nm.

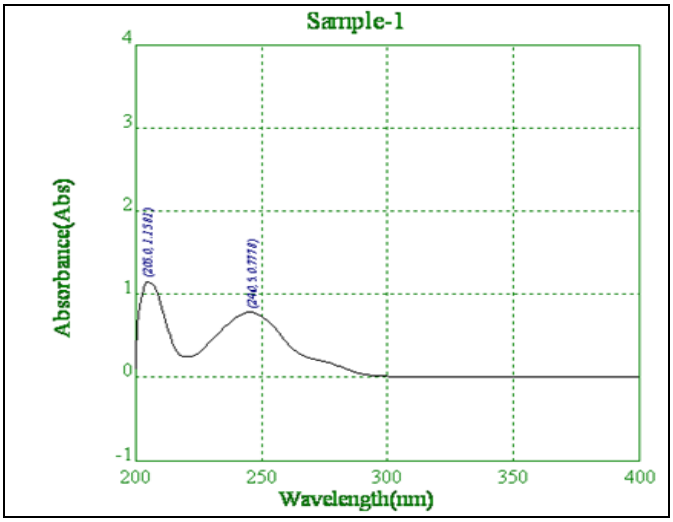

FIG. 2: UV SPECTRUM OF CAPECITABINE

Chromatographic Condition: The column C-18 (150 mm x 4.6 mm having 5µm particle size equilibrated with mobile phase consisting of 0.1% OPA: Methanol taken in a ratio of 49:51 V/V) was used. The flow rate was kept at 0.8mL/min, and column temperature were at 25°C. Eluents were supervised using a PDA detector at 240 nm.

Initial Method Development by QbD Approach: A Quality by Design with Design of Experiments approach to the development of an analytical method mainly involves two phases as follows:

a) Screening Phase

b) Statistical Analysis and Final Optimization

Screening Phase: The experimental design was constructed using design expert software version 13 (13.0.0.5) for the study of different variables (% organic phase, flow rate) and to verify method performances. The levels of these variables are as given in **Table 2**. The retention time, peak area, theoretical plates, tailing factor were used as a response in experimental design as controlling response, which is expected to affect and control method responses.

A 2^4 factorial design consisting of two factors at four responses were considered for the experimental plan. Initially and after confirming that the process is a non-linear, Central Composite Design was used. The experimental observations along with Design (DOE) plan are shown in **Table 3.**

TABLE 1: FACTORS AND LEVELS OF INDEPENDENT VARIABLES

CMPs	Unit	Type	Subtype	Min	Max
% Org ratio	%	Numeric	Continuous	50	52
Flow rate	mL/min	Numeric	Continuous	0.7	0.9

TABLE 2: CENTRAL COMPOSITE DESIGN AND RESPONSES

		Factor 1	Factor 2	Response 1	Response 2	Response 3	Response 4
Std	Run	A: Methanol %	B: Flow rate ml/min	RT	PA	TP	TF
8	1	51	0.9	2.439	4532.87	4672	0.96
1	2	50	0.7	3.177	5809.89	5591	0.94
4	3	52	0.9	2.416	4519.05	4583	0.97
7	4	51	0.6	3.667	6706.97	5939	0.93
6	5	52.4	0.8	2.737	4999.33	5387	0.98
3	6	50	0.9	2.439	4514.01	4256	0.97
5	7	49.5	0.8	2.792	5009.9	4436	0.96
2	8	52	0.7	3.138	5806.34	5760	0.98

Statistical Analysis and Final Optimization: The responses obtained after carrying out the above trial runs were fed back to Design Expert software and plots like 3D-response surface plots and Graph plots were plotted. These graphs demonstrated how important procedure factors affected the chosen quality criteria. The analysis of these plots was used to estimate as to which method parameter gave the most acceptable responses. Thus, based on these observations, the final critical method parameters of the method were determined and the optimized chromatographic conditions were finalized. Additionally, the significance of each method parameter chosen for the study was determined using a statistical analysis tool like ANOVA for each individual response using the p value (probability).

Validation of the Optimized Method: Validation of analytical procedures was performed for Capecitabine using the following parameters.

Specificity: To demonstrate the method's precision, the following solutions will be prepared and injected (double-checked the peak purity).

1. Blank (methanol 100% as a diluent)
2. Standard solution
3. Sample solution
4. Placebo treatments

Linearity: The linearity of the method was studied over six different concentrations of Capecitabine in between 10-50µg/mL. Peak area was plotted against concentration on the x axis to create the calibration curve. Values for the correlation coefficient and regression line equation were calculated.

Accuracy (% Recovery): Accuracy of the method was confirmed by a recovery study from marketed formulation at 3-level of standard addition. Percentage recovery of capecitabine was found out.

Precision: The precision is reported in terms of Relative standard deviation (RSD). There are three levels of precision: repeatability, reproducibility and intermediate precision. It takes place using a sample API.

- Repeatability (Intraday precision)
- Intermediate precision (Interday precision)

Limits of Detection and Quantitation: Limits of detection (LOD) and limit of quantitation (LOQ) were determined from the signal-to-noise ratio. The detection limit was referred to as the lowest level of concentration resulting in a peak area of three times the baseline noise. The quantitation limit was referred to as the lowest possible concentration that provided a peak area with a signal-to-noise ratio higher than ten.

$$LOD = 3.3 \times \delta/S$$

$$LOQ = 10\times \delta/S$$

Robustness: For robustness studies 100 µg/mL of capecitabine was used. In order to demonstrate the robustness of the procedure, the following optimized conditions were slightly varied.

- (49 %) 0.1% OPA: Methanol (51 %) ratio of mobile phase
- 0.80 mL/min of flow rate
- 25℃ of temperature

RESULTS AND DISCUSSION:

Statistical Analysis of Experimental Data by Design-expert Software: Analysis of variance (ANOVA) was applied to study the significance of the model generated for the five responses shown in the **Tables 3-6**. 2D Contour and 3D Surface plots were analyzed to visualize the effect of factors and their interactions on the Design Expert® software's responses. The regions shaded in dark blue represent lower values, and shaded in dark red represents higher values. The regions shaded in light blue, green and yellow represents intermediate values.

TABLE 3: ANOVA TABLE FOR RETENTION TIME USING CCD

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.40	5	0.2799	1549.87	0.0006	significant
A-Methanol	0.0024	1	0.0024	13.03	0.0689	
B-Flow rate	0.5346	1	0.5346	2960.41	0.0003	
AB	0.0001	1	0.0001	0.3544	0.6120	
A^2	0.0000	1	0.0000	0.1913	0.7045	
B^2	0.0048	1	0.0048	26.57	0.0356	
Residual	0.0004	2	0.0002			
Cor Total	1.40	7				

The Model F-value of 1549.87 implies the model is significant. There is only a 0.06% chance that an F-value this large could occur due to noise. P-values Model terms are significant when the value is less than 0.0500. In this case B, B^2 are significant model terms.

TABLE 4: ANOVA TABLE FOR PEAK AREA USING CCD

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	4.453E+06	5	8.907E+05	1005.37	0.0010	significant
A-Methanol	133.37	1	133.37	0.1505	0.7354	
B-Flow rate	1.679E+06	1	1.679E+06	1895.03	0.0005	
AB	18.45	1	18.45	0.0208	0.8985	
A^2	3370.25	1	3370.25	3.80	0.1904	
B^2	12467.08	1	12467.08	14.07	0.0643	
Residual	1771.85	2	885.92			
Cor Total	4.455E+06	7				

The Model F-value of 1005.37 implies the model is significant. There is only a 0.10% 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 B is a significant model term.

TABLE 5: ANOVA TABLE FOR THEORETICAL PLATES USING CCD

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2.859E+06	2	1.429E+06	35.03	0.0011	significant
A-Methanol	4.329E+05	1	4.329E+05	10.61	0.0225	
B-Flow rate	2.423E+06	1	2.423E+06	59.37	0.0006	

Residual	2.040E+05	5	40808.93
Cor Total	3.063E+06	7	

The Model F-value of 35.03 implies the model is significant. There is only a 0.11% 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 are significant model terms.

TABLE 6: ANOVA TABLE FOR TAILING FACTOR USING CCD

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.0022	4	0.0005	18.48	0.0188	significant
A-Methanol	0.0006	1	0.0006	19.56	0.0215	
B-Flow rate	0.0001	1	0.0001	2.54	0.2094	
AB	0.0004	1	0.0004	13.45	0.0351	
B^2	0.0005	1	0.0005	16.38	0.0272	
Residual	0.0001	3	0.0000			
Cor Total	0.0023	7				

The Model F-value of 18.48 implies the model is significant. There is only a 1.88% chance that an F-value this large could occur due to noise. Model terms are considered significant when the P-value is less than 0.0500. A, AB, and B2 are important model terms in this instance.

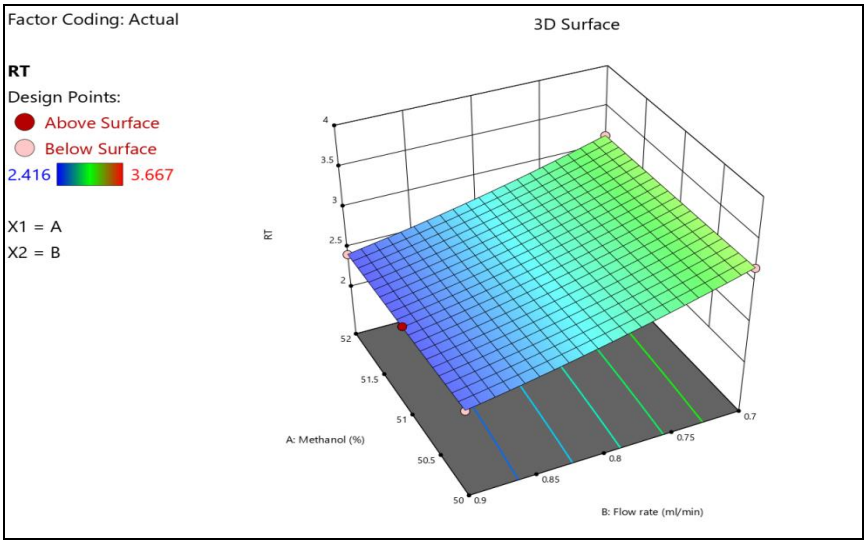

FIG. 3: CONTOUR PLOT FOR RT OF CAPECITABINE AGAINST MOBILE PHASE AND FLOW RATE

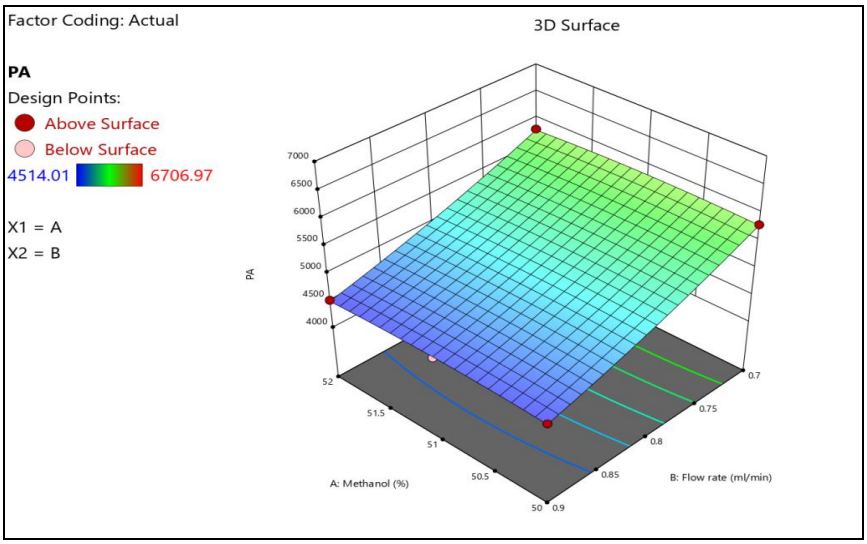

FIG. 4: CONTOUR PLOT FOR PEAK AREA OF CAPECITABINE AGAINST MOBILE PHASE AND FLOW RATE

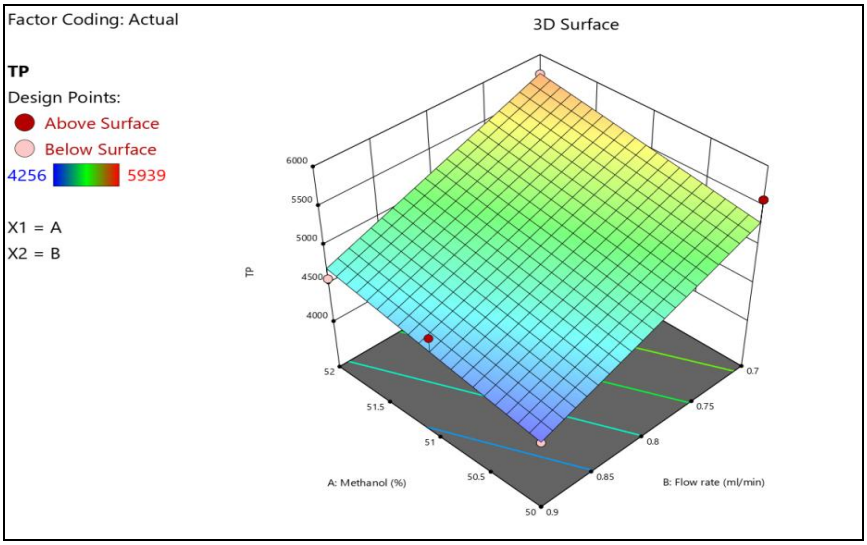

FIG. 5: CONTOUR PLOT FOR THEORETICAL PLATES OF CAPECITABINE AGAINST MOBILE PHASE AND FLOW RATE

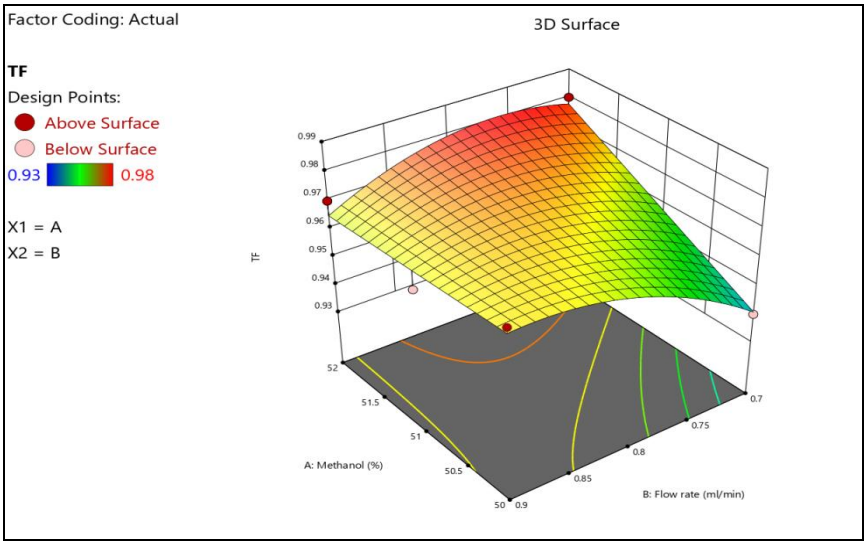

FIG. 6: CONTOUR PLOT FOR TELLING FACTORS OF CAPECITABINE AGAINST MOBILE PHASE AND FLOW RATE

From the above 2D Contour and 3D Surface plots of retention time, peak area, theoretical plates and tailing factors, shown in the above Figures. It shows the two-dimensional contour plot as a function of Organic ratio, Flow rate. Based on the color code, the working region can be easily identified. Retention time maps represent the value of the retention time, with warm "red" colors indicating larger retention time, cold "blue" colors lower and light green to yellow color represent intermediate retention time.

Design Validation: From the actual versus predicted plots for the four responses, it was observed that the selected models for the respective responses were suitable for the selected design. It was further evidenced from the ANOVA **Tables 3-6** that the selected models were significant with $p < 0.05$. Hence the selected models were suitable for the design employed in this work.

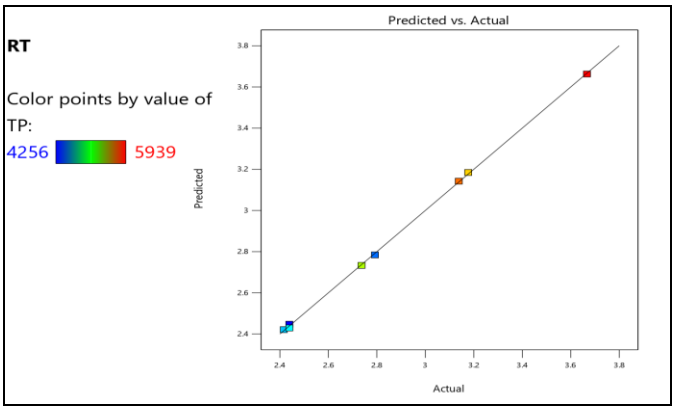

FIG. 7: COLOUR POINT BY VALUE OF RETENTION TIME PREDICTED VS ACTUAL

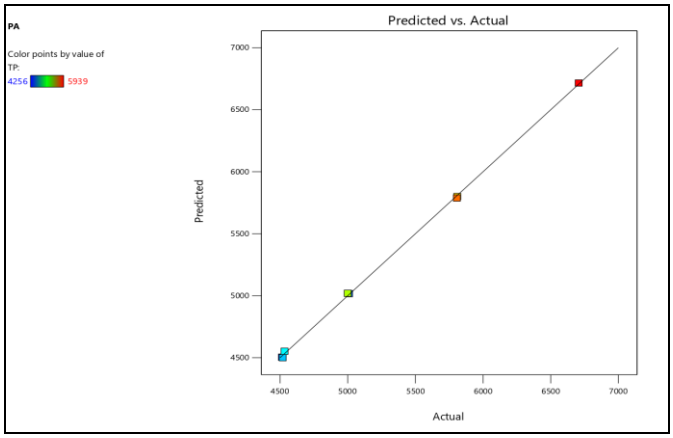

FIG. 8: COLOUR POINT BY VALUE OF PEAK AREA PREDICTED VS ACTUAL

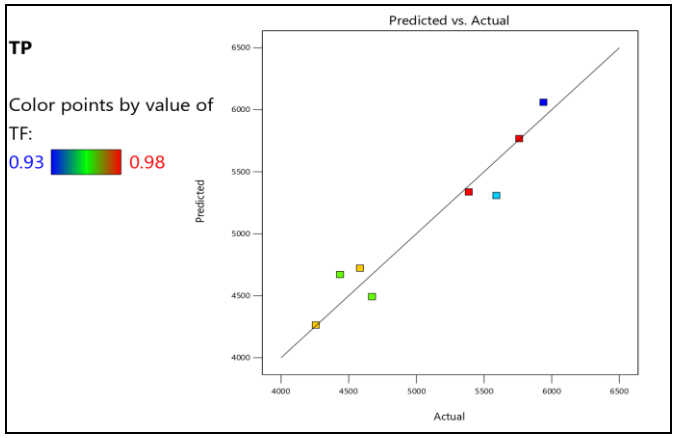

FIG. 9: COLOUR POINT BY VALUE OF THEORETICAL PLATES PREDICTED VS ACTUAL

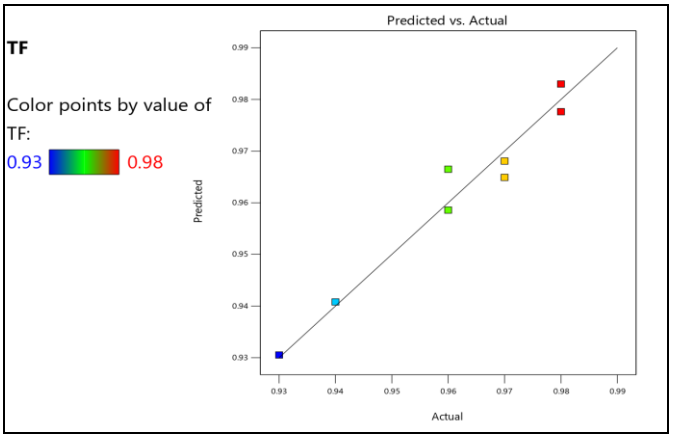

FIG. 10: COLOUR POINT BY VALUE OF TAILING FACTORS PREDICTED VS ACTUAL

TABLE 7: FINAL OPTIMIZED HPLC CHROMATOGRAPHY

Property	Value
Mobile phase	Methanol (51%): 0.1%OPA (49%)
Flow Rate	0.8 mL/min

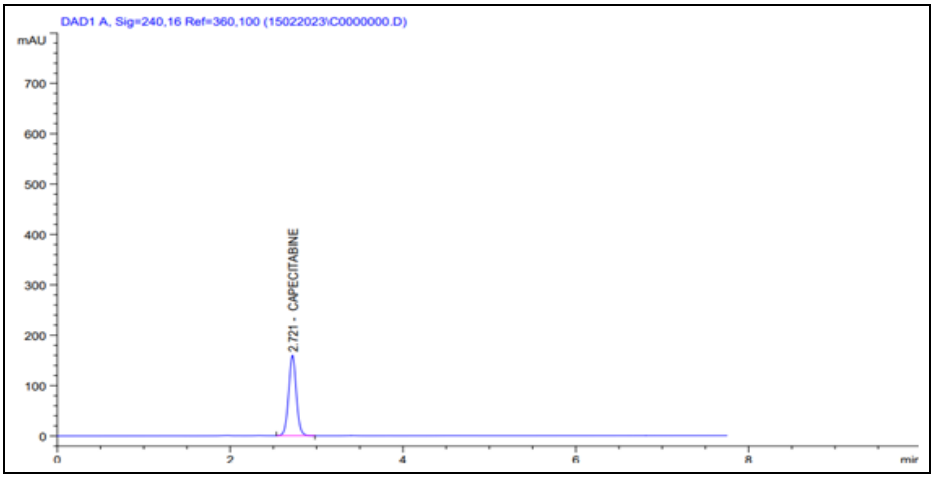

FIG. 11: CHROMATOGRAM OF FINAL OPTIMIZED METHOD

Method Validation: The proposed method was linear over the concentration range with 10-50 µg/ml with a correlation coefficient (R^2) 0.9993 respectively. For the accuracy studies at 80,100 and 120% levels, the drug recovery percentage was to be within 98-102%. Intermediate precision, reproducibility and repeatability were carried out and the % RSD values were found to be less than 2 %. LOD value was found to be 0.23 µg/mL of capecitabine. LOQ value was found to be 0.71

µg/mL of capecitabine. The robustness of the proposed method was checked by making minor changes in the experimental conditions like flow rate, % organic composition and wave length, and % RSD values for the peak area were found to be less than 2%. The summary of the method validation parameters was shown in **Table 8.**

TABLE 8: RESULTS OF THE VALIDATION PARAMETERS

Parameter		Capecitabine	Limit
Linearity Range (µg/ml)		10-50 µg/ml	
Regression coefficient		0.9993	
Slope (m)		102.96	
Intercept (c)		13.372	R< 1
Regression equation (y=mx+c)		y = 102.96x + 13.372	
Assay (% mean assay)		99.71%	90-110%
Specificity		Specific	No interference of any peak
System precision % RSD			
Interday:		0.11	
Intraday:		0.1	
Accuracy % recovery		99.72%	98-102%
LOD		0.2371 µg/ml	NMT 3
LOQ		0.7185 µg/ml	NMT 10
Robustness	Flow (-)	0.12	%RSD NMT 2.0
	Flow (+)	0.08	
	Mobile phase (-)	1.07	
	Mobile phase (+)	0.07	
	Wavelength (-)	0.31	
	Wavelength (+)	0.72	

CONCLUSION: Quality by design is an approach that aims to ensure the quality of medicine by employing statistical, analytical and risk management methodology in the design, development and manufacturing of medicines. Development of validated RP-HPLC method by QbD approach has been described. The QbD approach to method development has helped to better understand the method variables hence leading to less chance of failure during method validation. Optimized chromatographic conditions were performed such as composition of the mobile phase by several trials.

This have been done to achieve good resolution and the symmetric peak shapes of analyte. All the validated parameters were found within the acceptance criteria. Method has been confirmed by validation parameters as per the guidelines of ICH. The developed RP-HPLC method was found to be simple, linear, precise, and robust for determination of Capecitabine so the method developed was simple and robust that can be adopted in regular Quality control test in Laboratories & Industries.

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CONFLICTS OF INTEREST: Nil

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