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Quality by Design (QBD) Approach Prior to The Validation for Simultaneous Estimation of Related Substances in Lopinavir- Ritonavir Soft Gelatin Capsules by High Performance Liquid Chromatography

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ABSTRACT

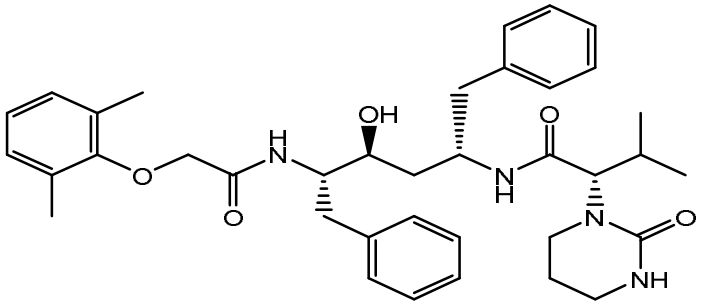
The present study focuses to determine the design space for a stability-indicating HPLC method prior to validation by systematic design of experiments (DoE) approach with the principle of quality by design (QbD). A simultaneous multivariate approach was carried out for mobile phase pH, flow rate, percentage of organic content and column temperature by employing DoE. Statistical analysis of the experimental data is not sufficient enough to cover all the significant chromatographic factors by performing one factor at a time instead of multi variant fractional factorial design. By analyzing the statistical experimental data for resolution to screen the chromatographic factors, flow and temperature displayed the most effective chromatographic factors. The inferences evaluated includes summary of fit, lack of fit, analysis of variance and parameter estimates. The chromatographic factors within the acceptable limits were displayed as a Contour plot defining the 'design space' of the method. A satisfactory QbD was deduced to finalize the method prior to validation from the range of operating conditions. The stability-indicating method is simple, rapid and robust for the related substances determination of lopinavir and ritonavir in lopi-rito soft gelatin capsules. The method was validated according to ICH guidelines for accuracy, precision, linearity, range, specificity, ruggedness and robustness (one factor varied at a time).The method has been successfully transferred to the quality control department for product analysis of manufactured batches and stability samples.

Keywords: Quality of design (QbD), Design of experiments (DoE), Stability indicating HPLC, lopi-rito soft gelatin capsules.

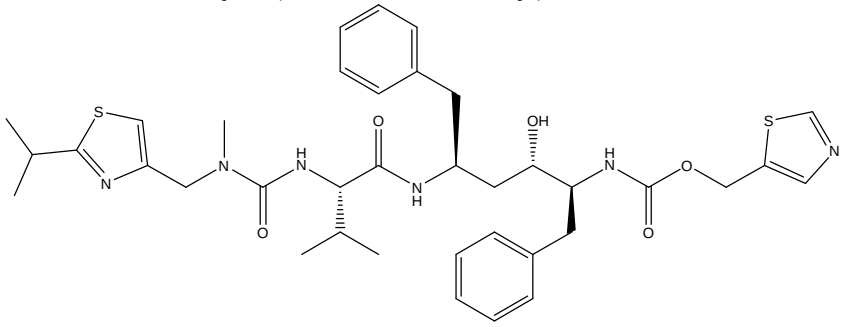
INTRODUCTION

Lopinavir and Ritonavir is a human immunodeficiency virus (HIV) protease inhibitor and chemically designated as (2S)-N-{(2S,4S,5S)-5-[2-(2,6 dimethylphenoxy)Acetamido)-4-hydroxy-1,6-diphenylhexane-2-yl}-3-methyl-2-(2- oxo-1,3-diazine-1-yl) butanamide and (5S,8S,10S,11S)-10-Hydroxy-2-methyl-5-(1-

methylethyl)-1-[2-(1-methylethyl)-4-thiazolyl]-3,6-dioxo-8,11-bis(phenylmethyl)-2,4,7,12-etraazatri decan -13 -oic acid 5-thiazolyl methyl ester respectively. The chemical structure of lopinavir and ritonavir were given in (fig.1). This class of drugs inhibits the HIV protease preventing cleavage of the gag-pol poly protein, reducing the probability of viral particles reaching a mature infectious state. Administered alone, lopinavir has insufficient bioavailability. However, like several HIV protease inhibitors, its blood levels are greatly increased by low doses of ritonavir, a potent inhibitor of cytochrome P450 3A4 [2-3] and therefore lopinavir is co-administered with sub-therapeutic doses of ritonavir by oral route of administration. In literature, LPV and RTV have been reported to be quantified individually or in combination by Spectrophotometric methods [5–7] HPTLC method [8] HPLC methods [9-12] from bulk drug and dosage forms as well as RP-HPLC/MS methods [13–17] for simultaneous determination of LPV and RTV and in combination with other antiviral drugs in the biological matrices are reported. But as far as the literature survey as concerned there is a no Quality by design: approach prior to the validation of a stability-indicating RP-Hplc method for the related substances determination of lopinavir and ritonavir in lopi-rito soft gelatin capsules**.** QbD approach for pharmaceutical development by defining quality target test profile, critical method attribute (CMA), and critical method parameters (CMP) to assess risk, design space (DS) and acceptable ranges of the operating conditions are recommended in ICH guideline Q8 (R2) [19]. QbD approach is executed by only twenty experiments under a fractional factorial design (FFD) to finalize the robust and stability-indicating method prior to validation shows a new feature with valuable parameters. A QbD approach to determine the DS for a stability-indicating method for lopinavir and ritonavir in lopi-rito soft gelatin capsules has been established. The method is validated according to the ICH guidelines [20].



Lopinavir:(2S)-*N*-[(2S,4S,5S)-5-{[2-(2,6-dimethylphenoxy)acetyl]amino}-4-hydroxy-1,6-diphenyl-hexan-2-yl]-3-methyl-2-(2-oxo-1,3-diazinan-1-yl)butanamide.



Ritonavir: 1,3-thiazol-5-ylmethyl [3-hydroxy-5- [3-methyl-2-[methyl- [(2-propan-2-yl-1, 3-thiazol-4-yl) methyl] carbamoyl] amino- butanoyl] amino-1,6-diphenyl-hexan-2-yl] amino formate

Figure-1: Chemical structures of Lopinavir and Ritonavir

MATERIALS AND METHODS

Spectrum Pharma research solutions, Hyderabad provided all the reference standards, chemicals and laboratory manufactured finished drug product used in the experiments. Potassium dihydrogen orthophosphate (Merck, India), HPLC grade acetonitrile (J. T. Baker; USA), hexane sulphonic acid sodium salt (Sigma- Aldrich, USA), hydrochloric acid (Qualigens, India), potassium dihydrogen phosphate (E-Merck, USA), triethylamineHPLC grade (E-Merck. USA), ortho phosphoric acid 88 % w/w AR Grade (E-Merck, USA),hexane HPLC grade(E-Merck, India)sodium acetate trihydrate AR Grade (Merck, India), and HPLC grade methanol (Merck, India) were used as received. Amber colored glassware and HPLC vials were used for standard and sample preparation. Millipore purified water was further filtered through a 0.45- µ membrane filter (Durapore, Millipore) to provide Milli-Q water.

Equipment and Chromatographic Conditions: HPLC Method development and its Quantitative estimation were performed using a waters 2996 PDA HPLC instrument for the analysis. The instrument was provided with 2695 separation module, the analysis was carried out on a Kromasil C18, 5 µ, and reverse phase column (250 mm x 4.6 mm) connected to a 2996 PDA detector. For sample injection an auto injector was employed. A spectra lab model UCB 50-ultrasonic cleaning bath was used for degassing of the mobile phase. A Metler-Toledo electronic balance was used for weighing the materials. The HPLC system was connected with Empower 2 Chromatographic Manager Soft Ware for its automatic operation, recording and integrating and analysis of the results. A Thermo Orion pH meter (3 Star Plus) was used to measure the pH of the mobile phase. The mobile phase and sample preparation used a Sonic 420 (LUC-420) sonicator for the preparation of the solutions. Hydrolytic degradation studies involved water baths equipped with an MV controller (Julabo, Seelbach, Germany) and thermal stability was performed in an air oven (MACK Pharmatech, Hyderabad, India). The photo stability study of the finished drug product dosage form was carried out in a photo stability chamber (Sanyo, Leicestershire, UK).

Methodology

Preparation of standard solution: Accurately weigh and transfer about 133 mg of Lopinavir working standard and 34 mg of Ritonavir working standard into a 100 mL volumetric flask. Add about 50 mL of diluent and sonicate to dissolve. Dilute to mark with diluent. Dilute 2 mL to 100 mL with diluent. Filter through 0.45µ nylon filter.

Preparation of sample solution: Weigh 20 capsules. Collect the contents of 20 capsules in a dry beaker by incising with the help of a sharp blade. Wash the empty capsule shells with hexane and let the shells dry in air. Weigh empty shells and calculate average fill weight. Accurately weigh and transfer content of sample equivalent to about 133.3 mg of Lopinavir into a 25 mL volumetric flask. Add about 15 mL of diluent and sonicate in cold water for 5 min. Dilute to mark with diluent. Filter through 0.45µ nylon filter.

RESULTS AND DISCUSSION

Method Development Strategy and Optimization: QbD-based analytical method development commenced with method scouting. The structures of Lopinavir and ritonavir contains amine and hydroxyl functional groups and on the basis of this, HPLC development trials were initiated with a mobile phase containing acidic pH buffer to retain the analyte in its unionized form. A high polar solvent like acetonitrile was used as with a low UV cut-off which makes the stability-indicating method more sensitive. A number of development trials were performed to optimize the separation by varying the factors such as flow (from 1.0 to 2.0 mL min^{-1}) and various ratios of acetonitrile to 0.05 M potassium phosphate buffer (between pH 2.5 and 4.5). To obtain a rapid method, short length HPLC columns (C-18 or C-8) were exercised. Desired separation was achieved on the sample solution spiked with all impurities on a Kromasil C18, 5 µ, and reverse phase column (250 mm x 4.6 mm) connected to a 2996 PDA detector with a mobile phase of pH 3.3buffer. Organic phase consists of a degassed mixture of acetonitrile and methanol

in the ratio of 90: 10 (v/v). Detector wave length was set at 215 nm and Injection volume is 10 µL and column oven temperature is at 30^0c. Mobile phase, flow rate, organic phase ratio buffer ratio were represented in **table 1**. Methanol was used as a sample preparation diluent. The screening phase of method development is based on early risk assessment test variables: mobile phase ratio, pH, column chemistry, and run time. The statistical design of experiments using full factorial design or other default designs can be used. The critical method attributes (CMA) are resolution, and peaks having peak tailing less than 1.2 was maximized, and the software modeled the contour plot for various columns. The Flow rate, pH, organic content and oven temperature were considered for designing of the experiments. The method was further optimized by studying the gradient endpoint percent strong solvent in combination with narrow pH and temperature ranges around the best values identified from the screening experiments. This stage optimized mean method performance, with the analysis modeling and Best Overall Answer feature identifying the best conditions as pH 3.3, temperature 30 °C are represented in **table-2.** At this point, the critical method parameters (CMPs) and critical method attributes/responses (CMAs) were determined.

Table-1: Isocartic conditions

Time (min.)	Flow rate ($mLmin^{-1}$.)	Organic phase	Buffer (pH 3.3)
0	1.0	22	78
7	1.5	22	78
30	1.5	45	55
35	1.0	55	45
50	1.0	55	45
70	1.5	72	28
71	1.5	85	15
90	1.5	85	15
91	1.0	22	78
105	1.0	22	78

Table 2: Best overall Optimization conditions

Gradient time	15 min
Final % strong solvent	50%
pH	3.3
Oven temperature	30 °C

Design of Experiments (DoE): A four factor simultaneous multi-variant approach adopted under DoE is called multi-variation at a time (MVAT). An orthogonal and balanced FFD was employed to determine the main effects by the above experiments. The number of experimental points is expressed as 2^n-k in FFD, "2" denoting that each tested factor has two levels, where as 'n' indicating the total number of factors (n = 5) and 'k' is showing the number of the fraction of the full factorial to be used (k = 1). Combining the four experiments at the centre points of the factors (nominal values) with the total number of experiments as per required FFD design gives (2^5-1 = 2^4 = 16) + 4=20.Hence, twenty experiments are conducted and mean run was repeated in triplicate. Under DoE trials, various levels of the factors are shown in table 1. Multi chromatographic factors were varied simultaneously by this approach. The main purpose of the study was to identify the significant influential factors and their interaction impact on the response. Twenty experiments were performed under FFD as explained earlier. **Table 3** provides the values of main response

from experimental design. **Table-4** denotes the responses of Multi variant designed experiments for each experimental design. HPLC chromatogram of the lopinavir and ritonavir spiked with impurities is shown in **figure 2.** By screening the data of all responses the most influential factors for all the responses, are identified. QbD and statistical analysis are explained in detail below.

Table-3: Multi varaiation factors under DOE trails

Level	Level	Flow	Column Temperature	pH	% organic Ratio
	(0)center	1.3	25	3.1	50
	- Low level	1.5	30	3.3	55
Level	+ High level	1.7	35	3.5	60

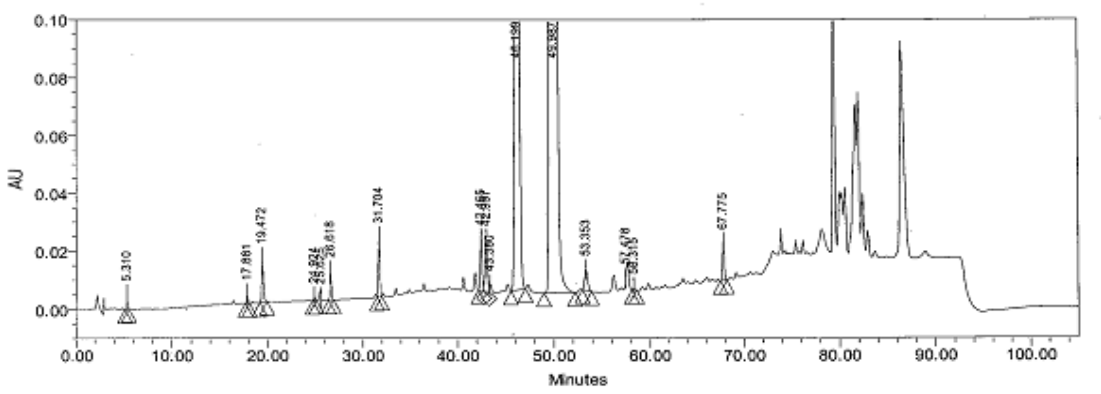


Figure-2: HPLC chromatogram of the lopinavir and ritonavir spiked with impurities

Statistical Analysis and Inferences: The two responses for the chromatographic factor namely resolution discussed in the systematic statistical approach to determine the design space, where the values of factors and responses are considered as continuous. Null hypothesis (H0) was defined at a significant level of $p \geq 0.005$ for the factor of influence to receive the required range of the response as per the requirement. The statistical analysis tools such as parameter estimates, prediction expression, and summary of fit, lack of fit, actual vs. predicted plot, prediction profiler, Pareto plot and Contour plot for each individual response are estimated to find out the most influential chromatographic factors design space.

Table-4: Data table of responses of Multi variant designed experiments

Experiment No	Flow rate	Column oven temp	Buffer	Buffer pH 4.0	Main peaks purity	Resolution B/w ritonavir peak and Alaninie N-acetyl impurity
1	1.3	35	50	3.1	Yes	1.9
2	1.3	35	50	3.5	Yes	1.9
3	1.3	35	60	3.1	Yes	2
4	1.3	35	60	3.5	Yes	1.9
5	1.3	25	50	3.1	Yes	1.9
6	1.3	25	50	3.5	Yes	1.9
7	1.3	25	60	3.1	Yes	1.8
8	1.3	25	60	3.5	Yes	1.9

9	1.5	30	55	3.1	Yes	1.8
10	1.5	30	55	3.3	Yes	1.9
11	1.5	30	55	3.3	Yes	1.8
12	1.5	30	55	3.3	Yes	1.7
13	1.5	30	55	3.3	Yes	1.8
14	1.7	35	50	3.1	Yes	1.6
15	1.7	35	50	3.5	Yes	1.5
16	1.7	35	60	3.5	Yes	1.9
17	1.7	25	50	3.1	Yes	1.7
18	1.7	25	50	3.1	Yes	1.6
19	1.7	25	60	3.1	Yes	1.5
20	1.7	25	60	3.5	Yes	1.7

The Summary of Fit: The 'summary of fit' report, has shown that the mean response for twenty observations, is 1.985 for the resolution with minimum 1.7 and Maximum 2.2.

Analysis of Variance (ANOVA): Probability value denoted by (prob> F).Here the "p value" is significant at alpha (a) = 0.05 with a value less than 0.002201.The p value obtained is enough for rejecting the null hypothesis with all the parameter estimates equal to zero. Anova for factorial model are represented in **table-5.**

Lack of Fit: The "lack of fit" test reminds that anything is missing out of the model. The model is a good fit if the" p value for lack of fit" should not be above 0.05. Here it is 0.922788, which is not significant. Hence, the model is a good fit.

Risk Assessment: "Parameter estimates" designs the model, which represents the main effect and the other factors affecting the variability. If the p value associated with the factor is smaller than 0.05, then it can be concluded that the true value of the slope is significantly different from zero. The observed p value of 0.004457 is the lowest for the Flow of the mobile phase. Out of all the given other factors, Flow and temperature are the most influential chromatographic factors that can explain the most variability in Resolution. Interaction effects are significant and hence in order to maintain statistical significance, the individual factors are also included in the model to maintain the statistical hierarchy (viz., p-value for factor "temperature" is 0.37, however then interaction of temperature and flow is found to be significant with p-value of 0.012)

Table-5: ANOVA for selected factorial model

Analysis of variance table [Partial sum of squares - Type III]						
	Sum of		Mean	F	p-value	
Source	Squares	df	Square	Value	Prob > F	
Model	0.375365	11	0.034124	9.059003	0.002201	significant
A-Flow	0.0577	1	0.0577	15.31771	0.004457	
B-C.Temp	0.003348	1	0.003348	0.888902	0.373372	
C-organic	0.006984	1	0.006984	1.85417	0.210406	
D-pH	0.000379	1	0.000379	0.100579	0.759254	
AB	0.016153	1	0.016153	4.288153	0.072141	
AC	0.03926	1	0.03926	10.42237	0.012086	
BC	0.011765	1	0.011765	3.123163	0.115168	
BD	0.001488	1	0.001488	0.394934	0.547236	

CD	0.003213	1	0.003213	0.853083	0.382695	
ABC	0.023306	1	0.023306	6.187031	0.037676	
BCD	0.006795	1	0.006795	1.803867	0.216101	
Residual	0.030135	8	0.003767			
Lack of Fit	0.005135	4	0.001284	0.205399	0.922788	NotSignificant
Pure Error	0.025	4	0.00625			
Cor Total	0.4055	19				
Std. Dev.	0.061375		RSquared	0.925684		
Mean	1.985		Adj RSquared	0.823501		
C.V. %	3.091931		Pred Squared	0.701643		
PRESS	0.120984		AdeqPrecision	10.03566		

Pareto Plot: The 'Pareto plot' is a plot of scaled estimates. The most important factor with the longest horizontal bar appears on the top among all the factors. For this model, fig. 3 shows that Flow is the most influential factor for resolution. The prediction expression is valid for the range of levels covered by the factors.

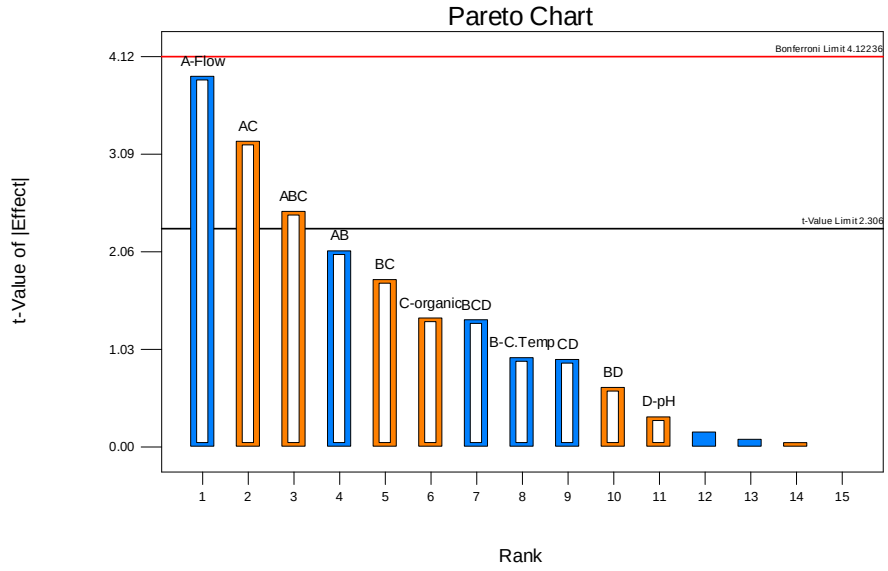


Figure-3: Pareto graph to show the influence of variables

Prediction Profiler: A plot of level of variables where one factor affecting the other can be observed in the "prediction profiler". Figure 4a depicts that the resolution is the steepest factor, which also indicates that the resolution is the most significant influential factor.

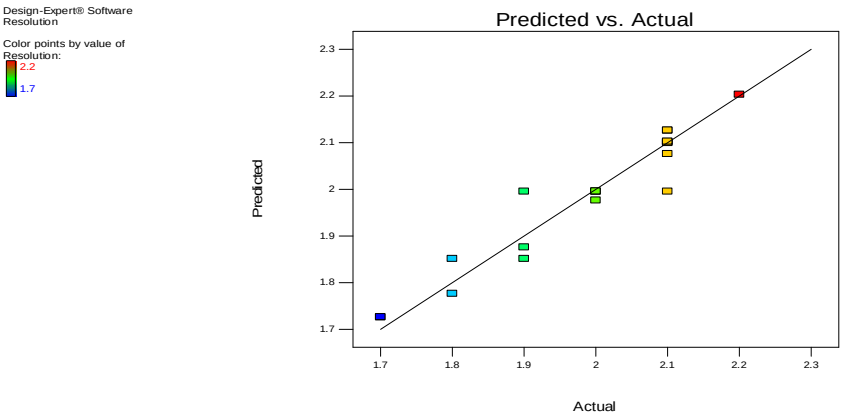


Figure-4a: Actual vs. Predicted plot for Resolution

Risk Reduction: DS. Vertical y-axis observed from the top view of a 3-D plot in the 2-D Contour profiler plot, shows the response. The three most highly influencing factors are on plane axis—resolution, flow and temperature. The nearby 3-D box reflects the shape of the response surface, Fig. 5c. The Contour plot depicts the most influential factors with respect to the allowed and forbidden regions of the response. This reflects the good agreement within the acceptance criteria.

Resolution: For closely eluting impurity, the resolution was evaluated to determine the challenging chromatographic factors for spectral purity of the ritonavir peak. The explanation about statistical analyses for "resolution" is below.

Risk Assessment: p value lower than 0.05 are significant for the important chromatographic factors influencing the separation are .The actual vs. predicted plot, fig. 4a, depicts that there is enough evidence to reject the null hypothesis, assuming that all the parameter estimates are equal to zero. R^2 is high at 0.99988 for lopinavir and 0.99958 for ritonavir denoting that 99.73 % of the observed variation can be explained by the grouping variable. The mean of the resolution for twenty observations is 1.785. The null hypothesis evident that variation of all the chromatographic factors is the same and it has no impact on the "resolution".

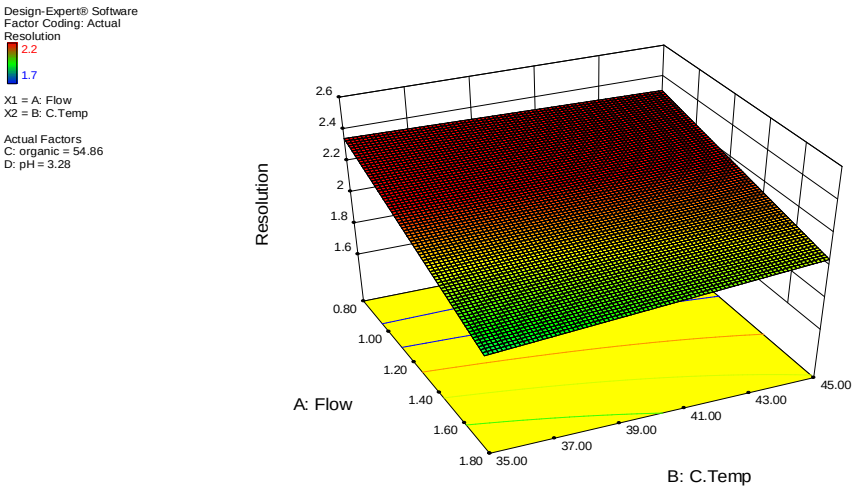


Figure-4a: Design space with respect to flow and temperature for resolution

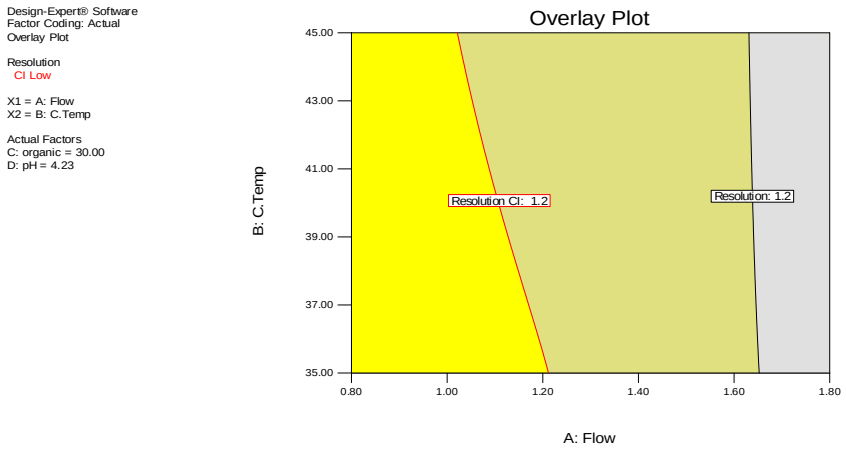


Figure-5c: Design space for the overlay plot of resolution with respect to temperature and flow

If the "p value" is lower than 0.05, and then it causes rejection of the null hypothesis. ANOVA, results shows the p value for the resolution model is significant (0.002201) is sufficient enough to reject the null hypothesis. Contradictorily, if the p value for "lack of fit" is above 0.05, is sufficient enough to reject the null hypothesis. Thus, the model is a significant fit to explain the variation effectively. 'Parameter estimates' has shown that the two chromatographic factors with a significant influence on resolution are the flow and the column temperature.

Risk Reduction: The "Contour plot" depicts the influential Chromatographic factors with respect to the response of the allowed region .The Contour profiler is a two dimensional plot, in the top view it is a three dimensional plot. Here, "resolution" is depicted at the vertical axis, and on the horizontal perpendicular axis are the significant influential factors, i.e., column temperature and Flow. The resolution is found in an acceptable range with respect to its minimum and maximum values obtained from the experiments.

Risk Acceptance (Control Strategy): By employing a DoE approach, defined responses with an allowed designed responses is obtained. Hence, the employment of the method is defined at nominal values of all chromatographic factors.

Method Validation and Transfer: The analytical method was validated as per ICH guidelines. The evaluated parameters were precision, accuracy, linearity, range, LOD, LOQ, specificity and robustness. The method was found to be linear from the linearity of response for Lopinavir, Ritonavir and their known related substances are determined in the desired range (1.28 μg mL^{-1} to 12.82 μg mL^{-1} for pyrimidine acetic acid, 0.27 μg mL^{-1} to 3.2 μg mL^{-1} for thiazolyl methoxy carbonyl hexane hydrochloride, 0.43 μg mL^{-1} to 12.8 μg mL^{-1} for phenoxy acetic acid, 1.08 μg mL^{-1} to 3.22 μg mL^{-1} for L-valine thiazolyl methyl carbamate, 0.429 μg mL^{-1} to 12.87 μg mL^{-1} for amino amide, 0.69 μg $^{-1}$ mL to 8.25 μg mL^{-1} for ritonavir, 1.07 μg mL^{-1} to 32.09 μg mL^{-1} for lopinavir and 0.855 μg mL^{-1} to 12.827 μg mL^{-1} for D-valine analogue of lopinavir. Data indicates the method is linear. Acceptance criterion is its correlation coefficient should not be less than 0.98. Acceptance criterion for method precision is RSD should not be more than 10.0 % and for system precision RSD should not be more than 5.0 %. Data obtained indicates that the method has an acceptable level of accuracy with an acceptance criterion for recovery should be in the range of 80-120 %. Standard and test solutions were found to be stable up to 21 h on the bench top by determining the Cumulative RSD should not be more than 10 %. Method validation for robustness parameter, for column temperature, pH, wavelength, flow rate, and % organic content of mobile phase, varying only factor at a time, was found to be sufficient. The method can be successfully transferred to the QC and was employed for routine and stability sample analysis.

APPLICATIONS

The established QbD-based method development including stability indicating analytical method can be successfully transferred to the QC department in pharmaceutical industries for the routine and stability sample analysis.

CONCLUSIONS

QbD approach realized a simple, quick and new robust stability-indicating analytical method which may be applied in routine quality control to determine the related substances for lopinavir and ritonavir in Lopi-rito soft gel capsule formulation. The factors influencing the responses were determined by performing simultaneous variation of factors under the multivariant DoE approach. Significant experimental factors by employing statistical analysis are used to construct the acceptable design space for responses .Influential critical process parameters are identified by Using a QbD oriented, multivariate approach which is not possible under a conventional method validation's robustness approach. Allowed design space for the response was identified by using inferences from the data, obtained under risk management by evaluating, reducing and regulating the risk. The method validation results have proved that the flow Variation method is selective, precise, accurate, linear and robust.

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