

Research Article



Development and Validation of Chromatographic Method for Related Substances of Raltegravir in Raltegravir Tablets by Using Quality by Design (Qbd) Approach

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ABSTRACT

This paper outlines the application of Quality by Design (QbD) concepts to the development of a stability indicating robust HPLC method for a complex molecule such as Raltegravir and its degradants in presence of inactive excipients. In the QbD based development approach we have considered, identification of Analytical Target Profile (ATP), risk assessment to identify the failure modes and to understand the interaction of method parameters on ATP. The above mentioned objectives and tools (viz., DOE, risk assessment) enable efficient experiment design to improve the understanding and robustness of the method. The method was optimized by using an Inertsil (C18 x 2.5 μ) reverse phase column by following DoE approach. By employing DoE, a multivariate approach was carried out for Flow rate, Column Temperature, Organic solvent ratio in mobile phase and Buffer PH. A two level full factorial design is employed and statistical analysis of the experimental data is used to determine significant influential chromatographic parameters. The experimental data for optimization of USP resolution is critical ATP. Organic phase is identified as critical parameter interacting with the Flow and pH to achieve the desired resolution of NLT 1.8. The method was validated according to ICH guidelines for Accuracy, Precision, Linearity, Range, Specificity, Ruggedness and Robustness (one factor varied at a time).

Keywords: Quality of design (QbD), Design of experiments (DoE), Stability indicating HPLC, Robustness, Raltegravir Tablets.

INTRODUCTION

Raltegravir (RAL), a hydroxypyrimidinone carboxamide derivative, is an integrase strand-transfer inhibitor (INSTI) used in the treatment and management of human immunodeficiency virus (HIV) infection¹. It was first approved by USFDA in 2007 for the treatment of HIV treatment-experienced patients². RAL is considered as the first generation INSTI that has demonstrated considerable efficacy in the treatment of naive as well as HIV treatment-experienced adult patients with viral resistance. It inhibits the catalytic activity of HIV-1 integrase enzyme, which is responsible for viral replication by blocking the viral DNA into the cellular genome by binding to the integrase-viral DNA complex³⁻⁵. RAL is approximately 83% plasma bound and gets rapidly absorbed from the gastrointestinal tract, with peak plasma concentration achieved within 0.5–1.3 h. It undergoes hepatic metabolism mainly by uridine diphosphate glucuronosyl transferase enzyme to give an inactive glucuronide metabolite, with only 9% of the administered dose excreted unchanged in the urine⁶.

Simple Spectrophotometric Method for Estimation of Raltegravir Potassium in Bulk, Pharmaceutical Formulations and in tablets⁷⁻⁸.

HPLC-MS-MS method for the determination of raltegravir in human plasma and rat plasma were reported in⁹⁻¹². Quantification of the HIV-integrase inhibitor raltegravir and detection of its main metabolite in human plasma using SPE, LLE methods and with fluorescence detector

were reported in¹³⁻¹⁷. Validated reverse phase HPLC method for determination of raltegravir in pharmaceutical preparation was reported in¹⁸. Identification and characterization of degradation products of raltegravir using LC, LC-MS/TOF was reported in¹⁹. Validated stability-indicating UPLC assay method and degradation behavior of Raltegravir was reported in²⁰. Development and validation of RP-HPLC method for determination of raltegravir and its impurities in bulk drug and dosage forms was reported in²¹. But to the best of our knowledge, according to the reported literature there is no Quality by design: approach prior to the validation of a stability-indicating RP-HPLC method for the related substances determination of Raltegravir in Raltegravir tablets available. QbD approach for pharmaceutical development by defining quality target Analytical 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)[22] was carried out. QbD approach enables building efficiency by using risk based evaluation of probable and critical parameters, and usage of structured experiment design approach using DOE to finalize the stability-indicating method, and validation.

A QbD approach determines the Design Space for a stability-indicating method for Raltegravir in Raltegravir Tablets has been established. The method is validated according to the ICH guidelines. Chemical structure of Raltegravir was shown in Figure 1.



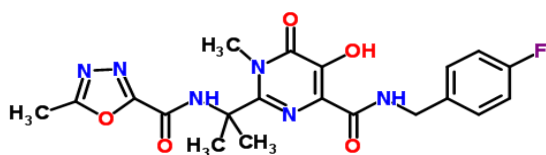


Figure-1: Chemical Structures of Raltaglavir

N-(4-Fluorobenzyl)-5-hydroxy-1-methyl-2-((5-methyl-1,3,4-oxadiazol-2-yl)carbonyl)amino-2-propanyl-6-oxo-1,6-dihydro-4-pyrimidinecarboxamide

MATERIALS AND METHODS

RAL active pharmaceutical ingredient (API), reference standards and its impurities, placebo and standards of impurities were supplied by Hetero drugs laboratories, Hyderabad, India. Methanol (HPLC grade, supplied by E-Merck), Triethyl amine (HPLC grade, supplied by E-Merck), ortho-Phosphoric acid (AR grade, supplied by E-Merck) were used as such supplied by the manufacturer. Water collected from Millipore system used for analysis.

Instrumentation

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 an Inertsil C18 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 used for degassing of the mobile phase. A Mettler-Toledo electronic balance was used for weighing the materials. The HPLC system was connected with Empower 2 Chromatographic Manager Software 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).

Preparation of Standard Solution

22 mg of Raltaglavir working standard was accurately weighed and transferred into a 100 mL volumetric flask. 60 mL of diluent was added into the flask and diluted to the mark with diluent. Filtered through 0.45 μ nylon filter. A 5 ml of the above solution was transferred into a 100 ml volumetric flask and diluted with diluents.

Preparation of Placebo

100 mg of placebo was accurately weighed and transferred into a 50 mL volumetric flask. To it 30 mL of

diluent was added and sonicated in cold water for 30 min. Diluted to the further volume with diluent and filtered through 0.45 μ nylon filter.

Preparation of Sample Solution

20 tablets were weighed and crushed into a fine powder. A sample equivalent to about 100 mg of Raltaglavir was accurately weighed and transferred into a 50 mL volumetric flask. 30 mL of diluent was added and sonicated in cold water for 30 min. Diluted to the mark with diluent. Filtered through 0.45 μ nylon filter.

Preparation of Mobile Phase A (pH 4.0)

1 mL of orthophosphoric acid was transferred into 1000 mL of Methanol. PH was adjusted to 4.0 \pm 0.04 with orthophosphoric acid. Prepared mobile phase was filtered through 0.45 μ or finer porosity membrane filter.

Preparation of Mobile Phase B

1 mL of orthophosphoric acid was transferred into 1000 mL of Methanol. Prepared mobile phase was filtered through 0.45 μ or finer porosity membrane filter.

Preparation of Buffer pH 7.5

1 mL of orthophosphoric acid was transferred into 1000 mL of Methanol. PH was adjusted to 7.5 \pm 0.04 with Triethyl amine. Filtered through 0.45 μ or finer porosity membrane filter.

Preparation of Diluent

500 mL of buffer solution with pH 7.5 and Methanol 500 mL was mixed well and degassed the mixture.

RESULTS AND DISCUSSION

Method Development Strategy and Optimization

QbD-based analytical method development commenced with method scouting. The structure of Raltaglavir contains amine and alcohol functional groups on the basis of this, HPLC development trials were initiated with a mobile phase containing basic pH buffer to retain the analyte in its unionized form. Medium polar solvent like Methanol was used as with a low UV cut-off which makes the stability-indicating method more sensitive. Development trials were performed to optimize the separation by varying the factors such as flow (from 0.8 to 1.2 mL min⁻¹) and various ratios of Methanol to OPA buffer (between pH 3.0 and 5.0). To obtain a desired resolution, less particle size HPLC columns (C-18 or C-8) were exercised.

Desired separation was achieved on the sample solution spiked with all impurities on an Intertsil C18 x 2.5 μ , and reverse phase column (250 mm x 4.6 mm) connected to a 2996 PDA detector with a mobile phase of pH 3.5buffer. Organic phase consists of a degassed gradient of Buffer and Methanol. Detector wave length was set at 300 nm and Injection volume is 20 μ L and column oven temperature is at 40°C. Mobile phase, flow rate, organic phase ratio buffer ratio (0 \rightarrow 5, 20:80, 5 \rightarrow 10, 60:40,

10→30, 55:45, 30→35, 55:45, 35→60, 30:70, 60→65, 80:20, 65→80, 20:80. Methanol was used as a sample preparation diluent. The screening phase of method development is based on early risk assessment test variables: mobile phase type, 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) of number of peaks, resolution, and peaks having peak tailing less than 1.2 was maximized, and the software modeled the contour plot for various columns. The pH, organic content and 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 4.0, temperature 40°C, solvent 80% and Gradient time is 80 min. At this point, the critical method parameters (CMPs) and critical method attributes/responses (CMAs) were determined.

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).

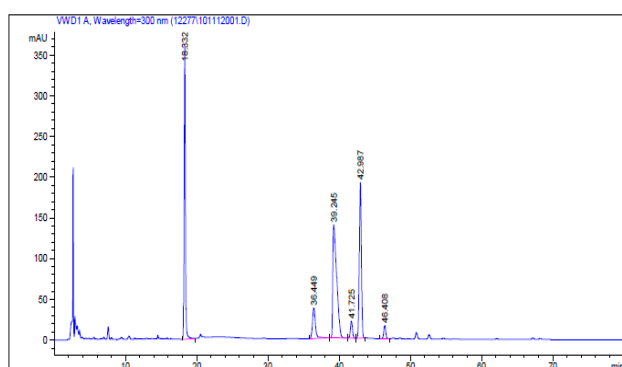


Figure-2: HPLC Chromatogram of the Raltaglavir spiked with Impurities

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 every run was repeated in duplicate. Under DoE trials, various levels of the factors are shown in Table 3. 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. HPLC chromatogram of the Raltaglavir 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.

Statistical Analysis and Inferences

The two responses for the chromatographic factors namely Flow and resolution were discussed in the statistical approach to determine the design space, where the values of factors and responses were considered as continuous. Null hypothesis (H0) was defined at a significant level of $p \geq 0.05$ 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. Responses for the multivariant factors for the designed experiments were reported in Table 1.

The Summary of Fit

The 'summary of fit' report, has shown that the mean response for twenty observations, is 2.01 for the flow of the mobile phase R^2 is high at 0.9086 denoting that 90.86 % of the observed variation can be explained by the grouping variable.

Analysis of Variance (ANOVA)

Probability value denoted by (prob> F). Here the "p value" is significant at alpha (α) = 0.05 with a value less than < 0.0001 . The p value obtained was enough for rejecting the null hypothesis with all the parameter estimates equal to zero. Anova for factorial model were represented in **Table 2**.

Lack of Fit

The "lack of fit" test reminds that anything was missing out of the model. The model was a good fit if the "p value for lack of fit" should not be above 0.05. Here it was 0.0913, which was not significant. Hence, the model was a good fit.

Risk Assessment

"Parameter estimates" designed the model, which represented the main effect and the other factors affecting the variability. If the p value associated with the factor was smaller than 0.05, then it can be concluded that the true value of the slope was significantly different from zero.

The observed p value of 0.0064 was the lowest for the organic content of the mobile phase. Out of all the given other factors, Flow was the most influential chromatographic factor that can explain the most variability in Resolution.

Table 1: Data Table Responses of Multivariant Designed Experiments

		Factor 1	Factor 2	Factor 3	Factor 4	Peak Purity	Response 1
Std	Run	A:Flow	B:C.Temp	C:organic	D:pH		Resolution
16	1	1.20	35.00	35.00	4.50	Yes	2.2
14	2	1.20	35.00	25.00	4.20	Yes	2.3
6	3	0.80	45.00	25.00	4.50	Yes	1.5
11	4	1.00	40.00	30.00	4.00	Yes	2
12	5	1.00	40.00	30.00	4.00	Yes	2.1
1	6	0.80	35.00	25.00	4.20	Yes	1.8
9	7	1.00	35.00	35.00	4.20	Yes	2
7	8	0.80	45.00	35.00	4.20	Yes	1.9
10	9	1.00	40.00	30.00	4.00	Yes	2.1
15	10	1.20	35.00	25.00	4.50	Yes	2.1
2	11	0.80	35.00	25.00	4.50	Yes	1.6
4	12	0.80	35.00	35.00	4.50	Yes	1.9
3	13	0.80	35.00	35.00	4.20	Yes	2.1
8	14	0.80	45.00	35.00	4.50	Yes	2.1
5	15	0.80	45.00	25.00	4.20	Yes	1.8
13	16	1.00	40.00	30.00	4.00	Yes	2.1
17	17	1.20	45.00	25.00	4.20	Yes	2.1
18	18	1.20	45.00	25.00	4.20	Yes	2.1
19	19	1.20	45.00	35.00	4.20	Yes	2.1
20	20	1.20	45.00	35.00	4.50	Yes	2.2

Table 2: Anova for Selected Factorial Model

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	0.7	7	0.1	17.04	< 0.0001	significant
A-Flow	3.29E-04	1	3.29E-04	0.056	0.8167	
B-C. Temp	4.08E-03	1	4.08E-03	0.7	0.4204	
C-organic	0.064	1	0.064	10.88	0.0064	
D-pH	0.029	1	0.029	4.94	0.0462	
AC	0.064	1	0.064	10.93	0.0063	
BC	0.017	1	0.017	2.92	0.1133	
CD	0.062	1	0.062	10.54	0.007	

Pareto Plot

The 'Pareto plot' is a plot of scaled estimates. The most important factor with the longest horizontal bar appeared on the top among all the factors.

For this model, Fig 2 shows that Flow was the most influential factor for resolution. The prediction expression was valid for the range of levels covered by the factors.



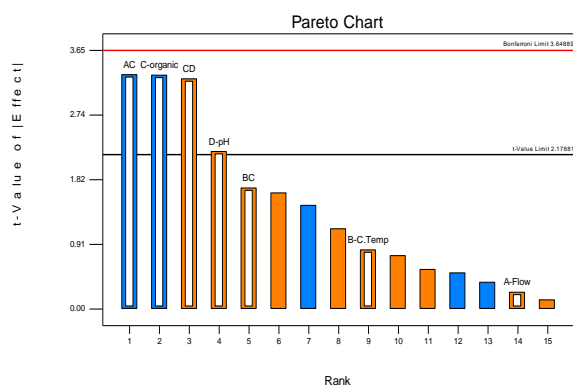


Figure 2: Pareto Graph to show the Influence of Variables.

Prediction Profiler

A plot of level of variables where one factor affecting the other can be observed in the “prediction profiler”. Figure 2a depicts that the resolution was the steepest factor, which also indicates that the resolution was the most significant influential factor. The errors (residuals) were not variable and almost constant across the range of resolutions without any outliers and exceptions.

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 4a. 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 raltagravir peak. The explanation about statistical analyses for “resolution” is below.

Risk Assessment

The design space yields a minimum resolution of 1.8 for the selected ranges of the method parameters. As the flow was increased, the resolution was improved; gave the organic and pH were constant.

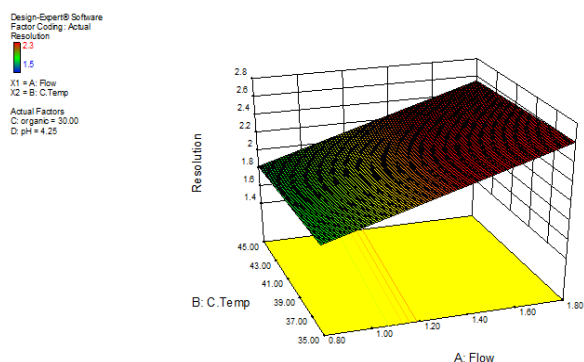


Figure 3a: Design space with respect to flow and temperature for resolution

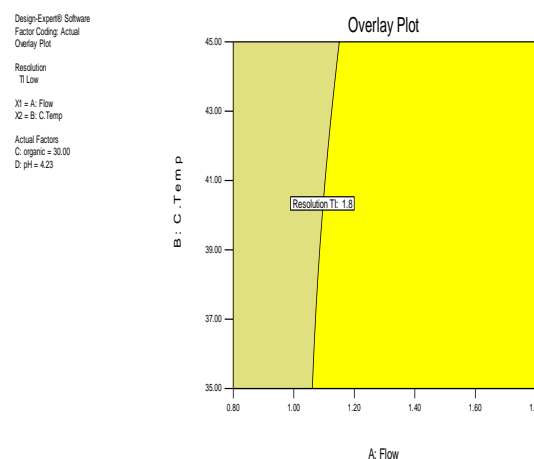


Figure 3b: Design space for the overlay plot for with respect to flow and temperature for resolution

Risk Reduction

The “Contour plot” (Fig 3b) depicts the influential Chromatographic factors with respect to the response of the allowed region. The Contour profiler (Fig 3a) 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 was 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 Raltagravir and their known related substances are determined in the desired range 0.249 µg/mL to 2.486 µg/mL for Impurity A, 0.241 µg/mL to 2.412 µg/mL for Impurity C, 0.243 µg/mL to 2.433 µg/mL for Impurity D, 0.247 µg/mL to 2.475 µg/mL for Impurity F, 0.249 µg/mL to 2.486 µg/mL for Impurity I, 0.118 µg/mL to 1.178 µg/mL for Impurity B, 0.089 µg/mL to 0.888 µg/mL for Impurity E, 0.082 µg/mL to 0.824 µg/mL for Impurity G, 0.086 µg/mL to 0.857 µg/mL for Impurity H and 0.051 µg/mL to 12.126 µg/mL for Raltagravir.

Data indicates that the method was linear. Acceptance criterion was its correlation coefficient should not be less than 0.98. Acceptance criterion for method precision was RSD should not be more than 5.0 % and for system precision RSD should not be more than 10.0 %. Data obtained indicates that the method had 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.

Application

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

CONCLUSION

QbD approach realized a simple, quick and new robust stability-indicating method analytical method which may be applied in routine quality control to determine the related substances for Raltegravir tablets. The factors influencing the responses were determined by performing simultaneous variation of factors under the multivariate 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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