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Research Article

Quality by Design Assisted Analytical Reverse-phase High Performance Liquid Chromatography Method Development and Validation for the estimation of Glasdegib

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ABSTRACT

The present work aims to develop and validate a simple, accurate and robust RP-HPLC method for the estimation of Glasdegib by using Analytical Quality by Design (AQbD) approach. Design of experiments was applied for multivariate optimization of RP-HPLC method. The critical method parameters were systematically optimized using Box-Behnken design (BBD). Design Expert® (11.1.0.1) modeling software (Stat-Ease Inc., Minneapolis, MN, USA) was used to generate 2D Contour and 3D Surface plots. Chromatographic separation was accomplished on Kromasil 100 C18 (250×4.6 mm, 5 µm) column at 30^0C. The optimized and predicted data from Design Expert software consisted of mobile phase phosphate buffer pH 4.4 and Acetonitrile (51.8:49.2 %v/v), pumped at a flow rate of 0.98mL /min gave the desirability function of 1. The UV detector was set at 225nm. The developed method was linear with a correlation coefficient of 0.9992. The optimized chromatographic method was validated as per ICH Q2 (R1) guidelines for system suitability, specificity, linearity, accuracy, precision, limit of detection (LoD), and limit of quantitation (LoQ). The drug's stability was examined under different stress conditions forcibly and significant degradation was found in 20% H_2O_2.

Introduction

Glasdegib, chemically known as 1-[(2*R*, 4*R*)-2-(1*H*-benzimidazol-2-yl)-1-methylpiperidin-4-yl]-3-(4-cyanophenyl) urea is used as an antineoplastic agent in the treatment of acute myeloid leukemia (AML).[1] This condition is characterized by abnormal production of myeloblasts, red cells, or platelets, most commonly seen in adults. It is a hedgehog signaling pathway inhibitor that binds to smoothened (SMO) receptors and blocks signal transduction.[2] It is used in combination with low dose Cytarabine for the treatment of newly diagnosed AML in adults more than 75 years of age. It is available under the brand name Daurismo, tablets containing 25mg/100mg of Glasdegib.[3] The chemical structure of Glasdegib was shown in Fig. 1.

An extensive literature survey has revealed that only stability-indicating RP-HPLC[4] method, pharmacological[5] and pharmacokinetic[6] studies were reported for the estimation of Glasdegib. There is no RP-HPLC method reported for the estimation of Glasdegib by using the

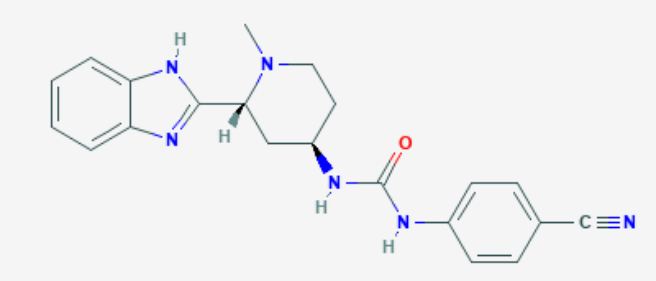

Fig.1: Structure of Glasdegib

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AQbD approach and its application to forced degradation studies. The traditional RP-HPLC method development consists of trial and error by varying only one factor simultaneously, which is time-consuming. But in the case of QbD there is an assessment of all factors that strongly influence the method's results.[7] In QbD, robustness and ruggedness are verified early in the method development stage to ensure method performance over the product's lifetime. Hence the present work is aimed to develop and validate simple, rapid, precise, robust RP-HPLC method for the estimation of Glasdegib assisted with DoE by using Box-Behnken design (BBD) followed by graphical interpretation of data by Response surface methodology (RSM).[8] The significance of the model was studied using Analysis of Variance (ANOVA). Optimization was done by applying the probability function.

Materials and Method

Chemicals

Acetonitrile, HPLC grade water, and potassium dihydrogen orthophosphate were purchased from Merck India Pvt. Ltd, Mumbai, India. API of Glasdegib was obtained as a gift sample from Sterling biologicals, Ahmedabad, India.

Equipment

The FTIR/ATR (BRUKER ALFA) spectrophotometer and UV-VIS spectrophotometer (Shimadzu -1800, Japan) were used to authenticate the drug sample. HPLC study was carried out on WATERS HPLC 2965 system with photodiode array (PDA) Detector.

Software

The software used is Empower 2 for HPLC method development and validation. Design Expert® (11.1.0.1) modeling software (Stat-Ease Inc., Minneapolis, MN, USA) was used to generate contour plots and 3D surface plots.

Authentication and Identification of Sample

By UV-VIS Spectra

A total of 10 µg/mL concentration of Glasdegib was dissolved in the methanol and UV spectrum. The absorption maxima were found to be 225 nm shown in the Fig. 2.

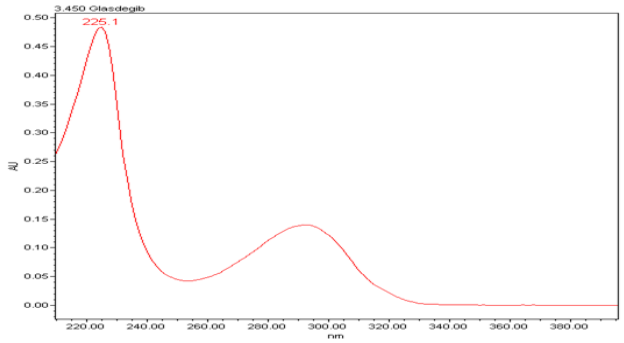

Fig. 2: UV Spectra of Glasdegib

By IR Spectra

Glasdegib was scanned in FTIR spectrometer (Bruker-ALFA) from 4000 to 400 cm^{-1} and characteristic peaks of functional groups were identified at 3308, 2247, 1746, 1494,1190 cm^{-1} shown in the Fig. 2.

Preparation of Mobile Phase

The mobile phase was prepared by using HPLC grade Acetonitrile (ACN) and Phosphate buffer (HPLC grade) in a 50:50 ratio.

Preparation of buffer

Accurately weighed 1.36 gm of potassium dihydrogen Orthophosphate was dissolved in 1000ml of Volumetric flask add about 900ml of milli-Q water, sonicate and finally make up the volume with water. The pH of the solution was observed as 4.8. Further pH was adjusted using orthophosphoric acid and triethylamine solutions.

Preparation of Standard Stock Solution

Accurately weighed 6.25 mg of Glasdegib was transferred to a 25ml volumetric flask, 3/4th of final volume was filled with mobile phase and sonicated to dissolve completely. The final volume was made upto 25 mL with mobile phase and labeled as a standard stock solution (250 µg/mL of Glasdegib). A 1-mL of the above stock solution of Glasdegib was pipetted out and taken into 10 mL volumetric flask and made up to volume with the mobile phase. (25 µg/mL of Glasdegib).

Preparation of Synthetic Mixture

Laboratory synthetic mixture was prepared using suitable excipients, which are mentioned in FDA label. In a motor and pestle, take accurately weighed 25 mg of Glasdegib, 75 mg of microcrystalline cellulose (MCC), 46.5 mg of sodium starch glycolate (SSG), 2.5 mg of Magnesium stearate, 1 mg of Dibasic calcium phosphate anhydrous. The contents were thoroughly mixed.

Preparation of Sample Solution

The above-prepared synthetic mixture was transferred into a 100 mL clean dry volumetric flask, add mobile phase to dissolve the drug and sonicate it for 30 minutes. After dissolving, make up the volume up to the mark with mobile

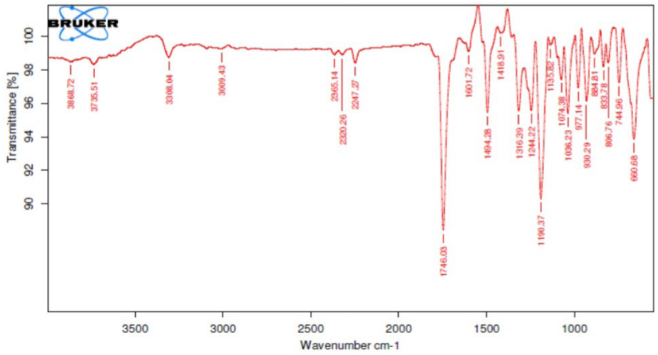

Fig. 3: IR Spectra of Glasdegib

phase. It is the stock solution having a concentration of 250 µg/mL of Glasdegib. Then it is filtered through 0.45 µm membrane filter. Further take 1-mL of above solution into 10 mL volumetric flask and dilute up to the mark with mobile phase (25 µg/mL of Glasdegib).

Method Development

Optimized Chromatographic Conditions

The initial trials are needed to optimize the final method. Chromatographic separation was accomplished on Kromasil 100 C18 (250×4.6 mm, 5 µm) column at 30^0C. A mixture of Phosphate buffer and acetonitrile (50:50 %v/v) was used a mobile phase pumped at a 1-mL /min flow rate. The UV detector was set at 225 nm.

Experimental Design

The method was optimized using Box- Behnken Design (BBD).[9] Total three factors viz; % Organic composition, flow rate and pH of the buffer were optimized. So BBD was used to optimize these parameters varied over three levels (high, mid, and low). Different ranges of three parameters 40-60% acetonitrile, pH 4.3- 5.3, and flow rate of 0.9-1.1mL/min were taken as shown in Table 1.

A 3-factor 3-level BBD design was established. This study design of 17 experimental runs was generated and analyzed by Design-Expert software, as shown in Table 2.

Method Validation

The final optimized chromatographic analytical method was validated as per the International Conference on Harmonization (ICH) Q2(R1) guidelines for system suitability, specificity, linearity, accuracy, precision, limit of detection, limit of quantitation and robustness.[10]

Linearity

Standard calibration curve was generated with six different concentrations over the range of 6.25-37.5µg/mL. A linear calibration curve was generated between peak area and drug concentration. The linearity was examined

Table 1: Design summary of BBD

Design summary					
File version: DX 11.0.0 Study Type: Response surface Design Type: Box-Behnken design			CQA: Retention time, Theoretical plates and asymmetry Runs: 17		
CMPs	*Unit*	*Type*	*Subtype*	*Min.*	*Max.*
pH	-	Numeric	Continuous	4.3	5.3
Flow rate	ml/min	Numeric	Continuous	0.9	1.1
%Org ratio	-	Numeric	Continuous	40	60

CMP: Critical method parameters, CQA: Critical quality attributes

Table 2: Box-Behnken experimental design matrix with responses

Trail no	*S.No*	*Flow rate (FR) (ml)*	*% Organic modifier (MP)*	*pH of buffer*	*Retention Time (RT) (min)*	*USP theoretical plates (TP)*	*Asymmetry factor (SY F)*
14	1	1	50	4.8	3.58	4809.7	1.5
16	2	1	50	4.8	3.55	5397.8	1.5
6	3	1.1	50	4.3	2.94	5091.3	1.5
8	4	1.1	50	5.3	3.80	3364.8	1.4
15	5	1	50	4.8	3.54	5202.4	1.5
9	6	1	40	4.3	2.92	5907.2	1.5
10	7	1	60	4.3	4.00	4474.7	1.6
17	8	1	50	4.8	3.61	4808.7	1.5
1	9	0.9	40	4.8	3.57	5466.7	1.5
5	10	0.9	50	4.3	3.69	4301.0	1.4
4	11	1.1	60	4.8	3.70	2548.4	1.4
2	12	1.1	40	4.8	2.83	4971.7	1.6
11	13	1	40	5.3	3.35	5865.7	1.5
13	14	1	50	4.8	3.44	5358.0	1.5
7	15	0.9	50	5.3	3.68	5691.4	1.5
3	16	0.9	60	4.8	4.77	3996.2	1.7
12	17	1	60	5.3	5.13	2000.8	1.1

using linear regression, which was calculated by the least square regression method.

Accuracy

Accuracy was carried out by adding a known amount of standard to the sample solution at 50, 100, and 150% in triplicate, and samples were analyzed using the optimized method. Percentage recovery was calculated.

Precision

The precision of the optimized method was determined by studying the intermediate precision and repeatability. Six working sample solutions of 20 µg/mL are injected on the same day and next day of the preparation of samples and the % RSD of the peak area was calculated.

Limits of detection and Quantitation

LoD and LoQ were evaluated using the standard deviation method. LoD was defined as 3.3σ/S and LoQ as 10σ/S based on the standard deviation of the response(S) and slope of the calibration curve(S).

Robustness

Small deliberate changes in the method were made like flow rate (0.9-1.1 mL/min), proportion of organic composition in the mobile phase (40-60%) and temperature of the column (25-35°C). %RSD of the above conditions was calculated.

System suitability

The system suitability was determined by taking six replicates of the drug at the same concentration of 20 µg/mL. The acceptance criteria was ± 2% for the percent coefficient of variation (% CV) for the peak area, retention time of drug, USP plate count, and asymmetry.

Forced Degradation Studies[11]

Acid Hydrolysis

To 1 ml of stock solution, 1ml of 2N HCl solution was added. The degradation sample was placed for reflux in Radley apparatus (Veego) with continuous stirring at 70° C for 60 minutes. The sample was neutralized with 2N NaOH, diluted upto 10 mL with mobile phase and analyzed using HPLC system.

Base hydrolysis

To 1-mL of stock solution, 1-mL of 2N NaOH solution was added. The degradation sample was placed for reflux in Radley apparatus (Veego) with continuous stirring at 70°C for 60min. The sample was neutralized with 2N HCl, diluted upto 10 mL with mobile phase.

Neutral hydrolysis

A 1-mL of stock solution was diluted to 10ml with HPLC grade water. The degradation sample was placed for reflux in Radley apparatus with continuous stirring at 70° C for 4 hours.

Oxidative study

To 1 ml of stock solution, 1ml of 20% H_2O_2 solution was added. The degradation sample was kept in the dark area without disturbance at room temperature for 4 hours. The sample was diluted upto 10 mL with the mobile phase.

Thermal degradation

A 25 mg Glasdegib was taken in a Petri dish and placed in a hot air oven at 70°C for 60 minutes. The sample was diluted with mobile phase and analyzed using the HPLC system.

Photo Degradation

A 25 mg Glasdegib was uniformly spread in a Petri dish and was exposed to direct sunlight for 24 hours. The sample was diluted with mobile phase and analyzed by the HPLC system.

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 three responses shown in the Tables 3-5.[12]

2D Contour and 3D Surface plots[13] 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 BBD

ANOVA for response surface linear model						
Analysis of variance table [Partial sum of squares - Type III]						
Source	*Sum of squares*	*d f*	*Mean square*	*F Value*	*p-value Prob> F*	*Inference*
Model	4.49	3	1.50	20.01	< 0.0001	Significant
A-FR	0.7308	1	0.7308	9.78	0.0080	Significant
B-MP	3.03	1	3.03	40.55	< 0.0001	Significant
C-pH	0.7248	1	0.7248	9.70	0.0082	Significant
Residual	0.9717	13	0.0747			

The Model F-value of 20.01 implies the model is significant. There is only a 0.01% 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, C are significant model terms.

Table 4: ANOVA table for theoretical plates using BBD

ANOVA for Response Surface 2F1 model						
Analysis of variance table [Partial sum of squares - Type III]						
Source	*Sum of Squares*	*d f*	*Mean Square*	*F Value*	*p-value Prob> F*	*Inference*
Model	1.687E + 07	6	2.812E + 06	8.94	0.0015	Significant
A-FR	1.380E + 06	1	1.380E + 06	4.39	0.0626	
B-MP	1.071E + 07	1	1.071E + 07	34.06	0.0002	Significant
C-pH	7.963E + 05	1	7.963E + 05	2.53	0.1426	
AB	2.321E + 05	1	2.321E + 05	0.7383	0.4103	
AC	2.184E + 06	1	2.184E + 06	6.95	0.0249	Significant
BC	1.570E + 06	1	1.570E + 06	4.99	0.0495	Significant
Residual	3.144E + 06	10	3.144E + 05			

The Model F-value of 8.94 implies the model is significant. There is only a 0.15% 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, AC, BC are significant model terms.

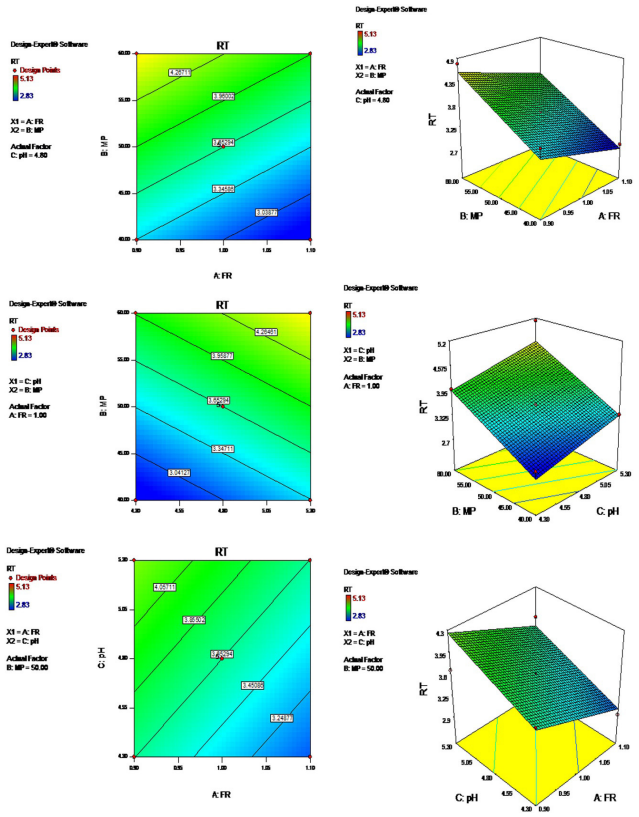

Fig. 4: 2D Contour & 3D Surface plots of retention time as a function of pH, flow rate and organic phase composition

From the above 2D Contour and 3D Surface plots of retention time shown in the Fig. 4, it was found that at a higher flow rate, lower organic phase composition and lower pH, the value of retention time is less.

From the above 2D Contour and 3D Surface plots of theoretical plates shown in the Fig. 5, it was found that at the flow rate does not have significant impact on theoretical plates. Lower organic phase composition, and higher pH, the value of theoretical plates is more.

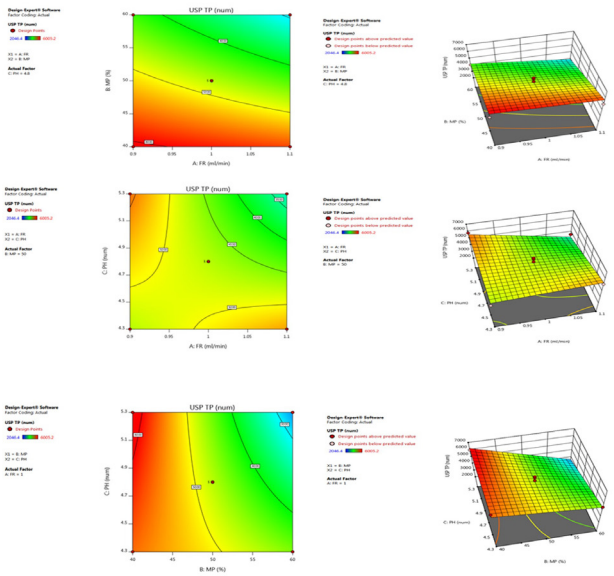

Fig. 5: 2D Contour and 3D Surface plots of theoretical plates as a function of pH, flow rate and organic phase composition

From the above 2D Counter and 3D Surface plots of asymmetry shown in the Fig. 6, it was found that at the flow rate does not have significant impact on asymmetry. higher organic phase composition, and higher pH, the value of asymmetry is less.

Design Validation

From the actual versus predicted plots[14] (Fig. 7) for the three responses, it was observed that the selected models for the respective responses were suitable for the selected design as these plots indicated the uniform distribution of the data points around 45° line. It was further evidenced from the ANOVA Tables 3-5 that the selected models were significant with $p < 0.05$. Hence the selected models were suitable for the design employed in this work.

Table 5: ANOVA table for asymmetry using BBD

ANOVA for response surface quadratic model						
Analysis of variance table [Partial sum of squares - Type III]						
Source	*Sum of Squares*	*d f*	*Mean Square*	*F Value*	*p-value Prob> F*	*Inference*
Model	0.2473	9	0.0275	4.53	0.0295	Significant
A-FR	0.0120	1	0.0120	1.98	0.2021	
B-MP	0.0210	1	0.0210	3.47	0.1050	
C-pH	0.0512	1	0.0512	8.44	0.0228	Significant
AB	0.0272	1	0.0272	4.49	0.0718	
AC	0.0100	1	0.0100	1.65	0.2399	
BC	0.0729	1	0.0729	12.02	0.0104	Significant
A^2	0.0040	1	0.0040	0.6566	0.4444	
B^2	0.0016	1	0.0016	0.2573	0.6276	
C^2	0.0480	1	0.0480	7.91	0.0260	Significant
Residual	0.0424	7	0.0061			

The Model F-value of 4.53 implies the model is significant. There is only a 2.95% 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, C, BC, C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant.

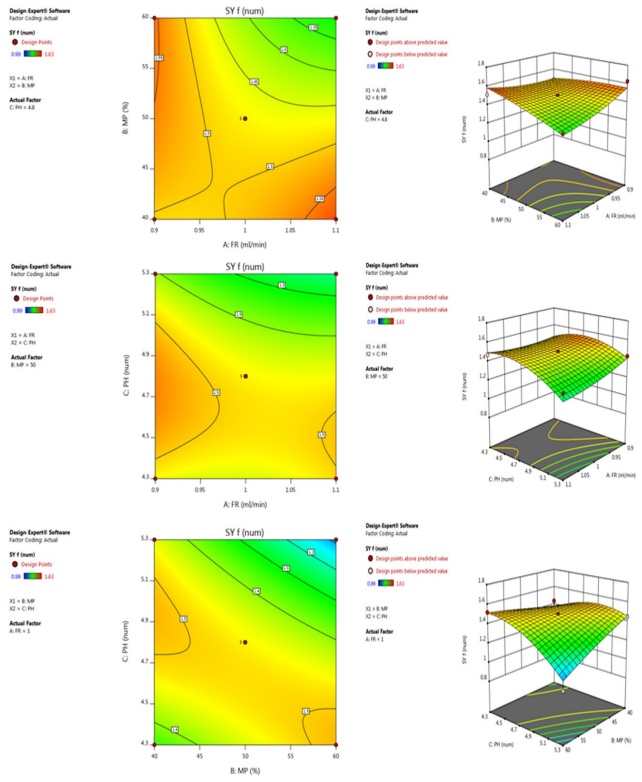

Fig. 6: 2D Contour and 3D Surface plots of asymmetry as a function of pH, flow rate and organic phase composition

Optimization by Desirability Function

A composite desirability was applied to get an optimum set of conditions based on each response's specified goals and boundaries.[15] This desirability function depends on a scale of desirability function ranges between d = 0 for a completely undesirable response to d = 1 for a fully desirable response. Based on the specified goals and boundaries for the retention time (minimum), theoretical plates (maximum) and asymmetry (minimum) composite

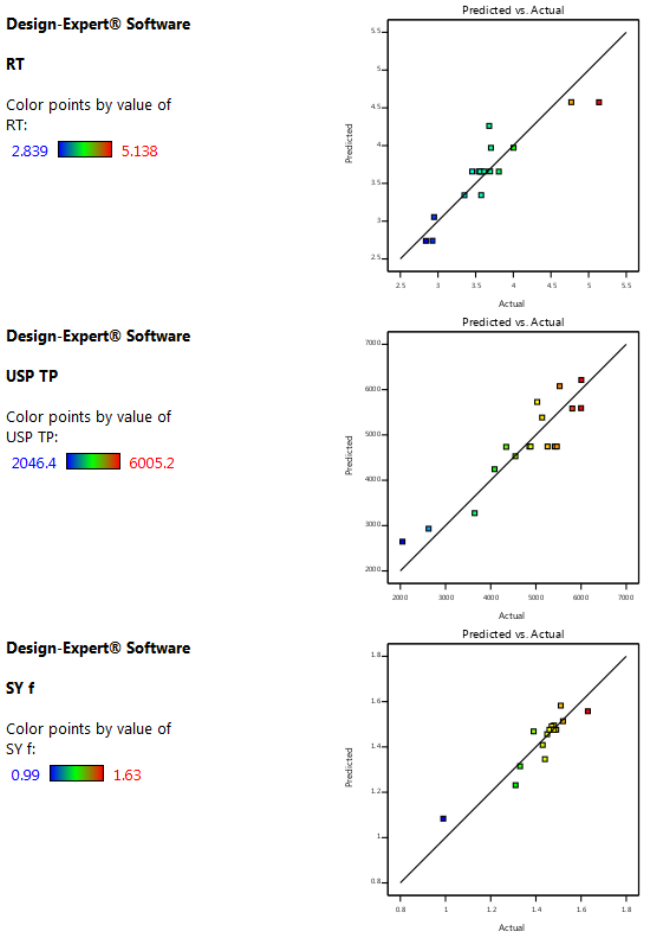

Fig. 7: Actual versus predicted plots for retention time, theoretical plates, and asymmetry

desirability (D) of 1 was obtained as shown in Fig. 8. To confirm these optimum set of conditions, three replicate injections of 25 µg/mL Glasdegib were analyzed to determine if their observed retention time, asymmetry and theoretical plates were within the predicted ranges shown in the Table 7 and the corresponding optimized chromatogram of standard and sample was shown in the Fig. 10 & 11.

Overlay Plot

The overlay contour plot shows the QbD design space where the method meets the mean performance goals and robustness criteria. The flag represents an optimized combination of the three selected independent factors, which gives the selected desirability of minimum retention time, maximum theoretical plates, and minimum asymmetry values shown in the Fig. 9.

Method validation

The developed method was linear over the concentration range with 5-30 µg/mL with a correlation coefficient of 0.999. For the accuracy studies at 50,100 and 150% levels, the % recovery of the drug was to be within 98-102%. Intermediate precision and repeatability were carried out, and the % RSD values were found to be less than 2%. LoD and LoQ values were found to be 0.007 and 0.02 µg/mL. The robustness of the developed method was checked by making minor changes in the experimental conditions like flow rate, % organic composition and temperature, and %RSD values for the peak area were found to be less than 2%. From the system suitability tests, the number of theoretical plates was found to be more than 5000 and the tailing factor was less than 2. The summary of the method validation parameters was shown in Table 8.

Forced Degradation Studies

Forced degradation studies of Glasdegib in various conditions like acidic, basic, peroxide, thermal, photolytic, and hydrolytic was observed. The drug showed significant degradation in peroxide condition represented in Fig. 13.

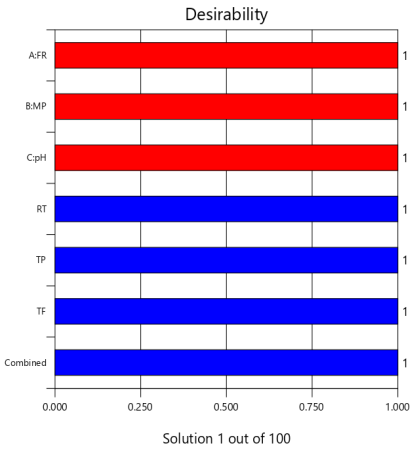

Fig. 8: Overall desirability of the optimized method

Table 6: Final optimized HPLC chromatographic conditions

Chromatographic condition	*Value*
Mobile phase	(51.8%) 0.01N KH_2PO_4: Acetonitrile (49.2%)
pH	4.4
Flow rate	0.98 mL/min

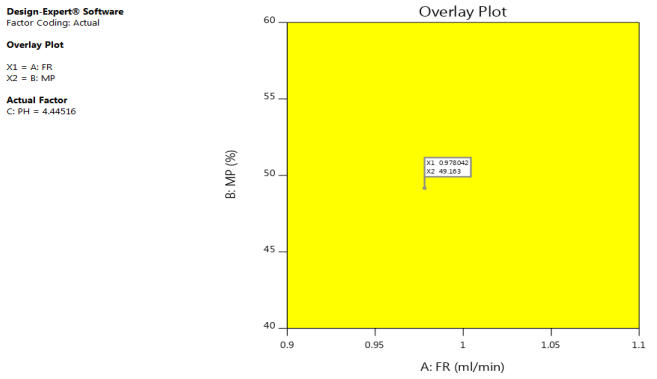

Fig. 9: Overlay contour plot for design space

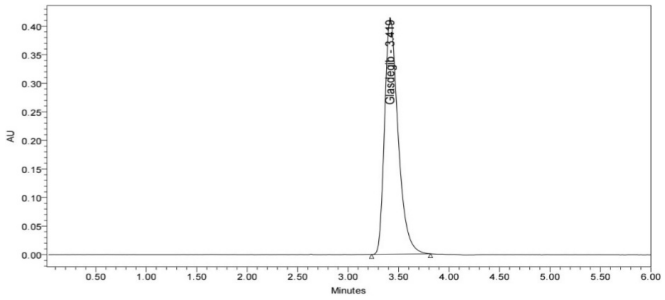

Fig.10: Chromatogram of the optimized method (Standard chromatogram)

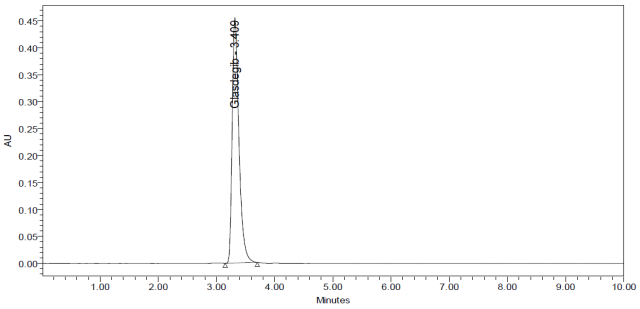

Fig. 11: Chromatogram of sample

Table 7: Responses of the optimized method

S.No.	*Response variables*	*Predicted value*	*Actual value*	*Desirable Range*
1	Retention time(min)	3.458	3.419	2.83–4.08
2	Theoretical plates	5000.31	5358.0	3668.86–6331.76
3	Asymmetry	1.47	1.5	1.27–1.67

Table 8: Results of the validation parameters

S.No.	*Parameter*		*Results*
1	Linearity	Linearity Range(µg/mL)	5-30
		Correlation Coefficient	0.9992
		Regression equation	y = 19574x + 9258.2
2	Accuracy (% recovery)	50, 100, and 150% levels	Between 99.21-100.05
3	Precision (% RSD of peak area)	Intermediate precision	0.8
		Repeatability	0.7
4	Sensitivity	LOD(µg/mL)	0.007
		LOQ(µg/mL)	0.02
5	Robustness (% RSD of peak area)	Flow rate (± 0.1mL/min)	0.5
		Organic phase (± 10%)	0.7
		Temperature(± 5ºC)	0.6
6	System suitability	Retention time (min)	3.459
		Tailing factor	1.395
		Plate count	5073.66

Table 9: Results of forced degradation studies

S.No.	*Stress condition*	*% Drug recovered*	*% drug degraded*
1	Acidic (2N HCl, 70^0, 60 min)	91.77	8.23
2	Alkali (2N NaOH,70^0, 60 min)	90.28	9.72
3	Neutral (H_2O, 70^0, 4 hrs)	97.31	2.69
4	Oxidative (20% H_2O_2, 4 hrs)	82.49	17.51
5	UV light (24 hrs)	97.31	2.69
6	Thermal (70^0, 60 min)	95.98	4.02

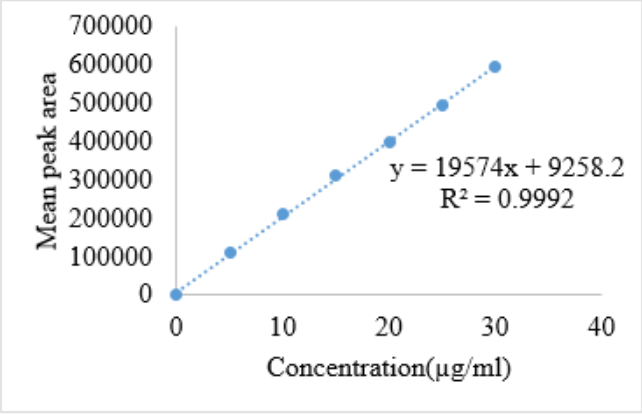

Fig. 12: Linearity curve of Glasdegib

Results of forced degradation studies were presented in Table 9.

A simple, accurate and robust RP-HPLC method was developed to estimate Glasdegib by using AQbD approach. The critical method parameters selected were % of organic mobile phase, flow rate and aqueous phase pH. The critical quality attributes are retention time, theoretical plates and asymmetry. The critical method parameters were systematically optimized using Box-Behnken design (BBD). Optimized chromatographic conditions consists of mobile phase phosphate buffer pH 4.4 and acetonitrile

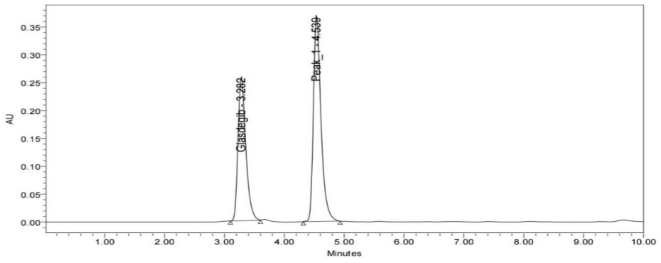

Fig. 13: Chromatogram of peroxide degradation

(51.8:49.2 %v/v), pumped at a flow rate of 0.92mL/min. The retention time of the drug was found to be 3.41 minutes. Theoretical plates and asymmetry were found to be within limits. The developed method was validated as per ICH Q2 (R1) guidelines. The utilization of response surface methodology provides better insight for method development and robustness testing. Degradation studies were performed in various stress conditions and the drug was found to be degraded more in peroxide condition.

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