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Application of Elements of Quality by Design to Development and Optimization of HPLC Method for Fingolimod

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Purpose: A HPLC method for Fingolimod was developed using a Quality by Design concept. QbD has gained importance in recent times due to regulatory requirements. Actual study was started after determination of target profile and qualification of instrument.
Methods: Separation was carried on a Grace C-8 column (4.6 x 250 mm, 5-µm particle size).The composition of mobile phase was methanol and 20 mM ammonium formate buffer of pH5.8 in gradient mode HPLC method development is affected by critical factors like pH, flow rate and mobile phase composition.
Results: To study the effects of these three factors on USP tailing, Box Behnken optimization model was applied. Desirability of the model was set at Tailing less than 1.2.Analysis of results was

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done using surface diagrams. Verification of Software generated results was done by taking six replicates of the run. Thus developed and optimised method was Finally validated as per ICH guideline.

Conclusion: A Quality by Design approach has been successfully utilised in method development of the Fingolimod in bulk. All key aspect of QbD were tried to be implemented in said study.

Systematic approach was utilized for method development which includes beginning with determination of target profile characteristics, instrument qualification, risk assessment, design of experiment and validation.

Three factors i.e. Ph, flow rate and methanol concentration were analysed for their effect on USP tailing as a responce. Interaction and quadratic effect of the factors were studied with least possible runs by using Box Behnken model. Response surface diagrams and contour plots were studied for coming to conclusion which factors are affecting response and their limits were recorded. Optimum run condition was obtained; Replicates of run having optimized condition were taken to confirm the predicted response with actual response.

Keywords: Fingolimod; Quality by design (QbD) application; development of HPLC method.

1. INTRODUCTION

Fingolimod [Trade name: Gilenya, Novartis (FTY720),; 2-amino-2-[2-(4-octylphenyl) ethyl]-1,3-propanediol] is a recently discovered molecule approved by the United States Food and Drug Administration (USFDA) for treating multiple sclerosis [1]. Fingolimod (FTY720) is a oral immunomodulating agent which preventslymphocyte recirculation from lymphoid organs [2]. Fingolimod primarily decrease the entry of T lymphocytes from secondary lymphoid organs, thereby inhibiting neuroinflammation [3]. A method for simultaneous quantification of FTY720using liquid chromatography–tandem mass spectrometry (LC–MS/MS) is reported [3]. To our knowledge HPLC method using simple UV detector which is developed using systematic approach of method development is not available. "QbD is systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control based on sound science and quality risk management" (ICH guidelines, 2012).QbD has gained importance in recent times due to regulatory requirements. USFDA has accelerated QbD drive to encourage the risk based approach and thorough understanding of processes which is ultimately going to help the regulatory bodies in review process. The main aspect in QbD is that quality is 'built inby understanding the effect of the various system parameters.Effects are analysed for their influence on quality of product that is desired which supports in establishing the design space which is defined as the "multidimensional combination and interaction of input variables that have been demonstrated to provide assurance of quality" [4].

Some of HPLC and UPLC method are reported for estimation of drug substance. In some reports statistical approach or experimental design are used [5,6,7,8,9,10,11].

HPLC method is extensively used as quality control test at various levels of drug development for API's and for formulations. Considering the potential, we have developed a HPLC method using the principles of QbD which helped to gain the knowledge from experimental design and risk assessment. For optimization of the said method a Box-Behnken design was used where in three factors were studied at three levels. Factors which were studied are pH, flow rate, and methanol concentration in mobile phase. Effect of these three parameters on tailing factor was studied.

2. EXPERIMENTAL

2.1 Materials

Reference standard of Fingolimod was obtained from Glenmark Pharmaceutical limited Mumbai. HPLC grade methanol, ammonium formate, acetic acid and triethyle amine (TEA) of Merk were used. Water used in the method is of HPLC grade and is generated from a water purification system by Millipore, USA.

2.2 Instrumentation and Chromato-graphic Conditions

RP-HPLC of Fingolimod was carried out using Jasco equipped with PU 2089 quaternary gradient pump and Grace C_8 column.

Chromatographic separation was achieved on a Grace C-8 column (4.6 x 250 mm, 5-µm particle size). Analysis of the data was done using ChromPass version 1.8.6.1 software. The mobile phase used was consisting of methanol and 20mM ammonium formate buffer of pH 6.3. Acetic acid and triethylamine were used for adjustment of mobile phase pH of 5.8 and 6.8 respectively. The buffer solution was then filtered and degassed.

2.3 Methods

2.3.1 Fingolimod sample preparation

By weighing 10 mg of Fingolimod and dissolving in 100 ml methanol stock solution of Fingolimod was prepared. Further serial dilutions were madeto get a final concentration of 100 µg/ml Fingolimod. From above stock solution 10µg/ml sample was prepared for analysis.

2.3.2 Analytical target profile

"QbD is a systematic approach to product, process design and development," [12]. Therefore, QbDstarts with determination of method goal. In QbD more focus is given on process understanding [13]. Here method intent was, The HPLC method of Fingolimod should be accurate, precise and robust with tailing less than 1.2, theoretical plates as per standard, short analysis time i.e. less than 10 min. InQbDusing design space a robust method must be developed.

2.3.3 Instrument qualification

High formalities of validation procedures in the field of pharmaceutical analyses is due to the importance to demonstrate the suitability of these procedures for the intended use. Therefore, it is very much essential to see that whether the equipment and/or the analyticaltesting system is properly tested, designed, maintained, and calibrated.Different phases for Qualification of analytical instrumentsneeds to be performed.

Since HPLC are “off the shelf” equipment, we can ignore Design Qualification. To see wheather instrument is received and installed properly or not, Installation Qualification is needed. As far as practical experimentation is considered only operational qualification and performance qualification combine parameters were done as reported by [14].

Precision of injection volume

With fixed 20µl injection, peak areas obtained helps in determining precision of injection volume. Calibrated dosage loop tolerance limit set was <1% RSD.

Injection carryover: By measuring the absorption of a blank injected after analysis, Injection carryover was determined. There should not be any peak from previous analysis.

Flow rate accuracy: By setting a flow rate of 1.0 ml/min, 2.0 ml/min and 2.5 ml/min for 10 min, 10 min, and 10 min respectively and by measuring the volumetric flow rate of mobile phase through the column, Flow rate accuracy was determined. RSD should be<1% or tolerance limit is $\pm$ 3%.

Flow rate precision: By recording the RSD% of retention times a flow rate precision was determined. Limit set was <1.0% RSD.

Wavelength accuracy: It was done by scanning a compound with known specific maxima.Tolerance limit is Specific maxima ±2nm.

Linearity of detector: Linearity of detectorwas determined by injecting increasing concentrations of test substance and Tolerance limit set was $R^2 \geq 0.999$.

2.3.4 Risk assessment

Risk assessment is needed to enhance quality of method. In addition, risk assessment determines the how input variables affect performance of method. Among the Different tools for risk assessment [15] here Ishikawa or fishbone diagram is studied (Fig. 3).

2.3.5 Method design

It is the multidimensional combination and interaction of input variables (e.g., material attributes) and process parameters that have been demonstrated to provide assurance of quality. Optimization was done by response surface methodology, applying a three level Box–Behnken design with three centre points (Table 1). Three factors selected were pH, flow rate and methanol concentration in mobile phase. Evaluation of main factors, their interaction and quadratic effects on peak USP tailing factor were done. Injection volumes of 20µl, column oven temperature were kept constant as their effect on

tailing was less significant. Experiments were conducted by making injections of the standard Fingolimod solution and the average of USP tailing was analysed using Design Expert 8 software (Table 2). Application of multivariate regression analysis resulted in a fitted full quadratic model for the average responses for peak USP tailing, given by the equation: 1.

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3$$

Where Y is the response, β_0 is the arithmetic mean response.β_1, β_2 and β_3 are regression coefficients of the factors X_1, X_2 and X_3, respectively [16,17].

Critical Quality Attributes (CQA)

Factors that influence the quality of the product are recognized, and their effect on development of method is studied [16].

From risk assessment by fishbone diagram critical factors that significantly affects the tailing was determined. Among them critical factors were methanol concentration in mobile phase flow rate and pH. Selection of stationary phase was also critical parameter. The nature of the drug is more retentive on C18 than C8.Butfor HPLC method to be effective it should have lesser retention time.

2.3.6 Method validation

Validation of the optimized chromatographic method was done as per (ICH) Q2 (R1) guidelines. The validation was done for linearity, range, precision, robustness and accuracy [18]. The acceptance criteria for Fingolimod were less than 2.0 USP tailing factor, relative standard deviation (RSD) for retention time and peak area less than 2% and theoretical plate count greater than 2000.

Linearity: By diluting a stock solution with methanol, in the range of 5, 7.5, 10, 12.5,15, 20µg/ml, standard calibration curves were prepared. The linearity of Fingolimod was determined by three replicate injections of each concentration. Evaluation of linearity was done by plotting Linear calibration curves.

Table 1. Three levels for Box Behnken of three factors

Chromatographic conditions	Level used		
	Low (X_1)	Centre (X_2)	High(X_3)
pH	5.8	6.3	6.8
Flow rate	1.1	1.3	1.5
Methanol concentration	65%	70%	75%

Table 2. Box Behnken design

Run	Coded (X_1, X_2, X_3)	pH	Flow rate	Methanol concentration
1	+0+	6.8	1.3	75
2	-0+	5.8	1.3	75
3	000	6.3	1.3	70
4	000	6.3	1.3	70
5	++0	6.8	1.5	70
6	0++	6.3	1.5	75
7	-0-	5.8	1.3	65
8	+0-	6.8	1.3	65
9	0+-	6.3	1.5	65
10	+-0	6.8	1.1	70
11	000	6.3	1.3	70
12	-+0	5.8	1.5	70
13	0-+	6.3	1.1	75
14	000	6.3	1.3	70
15	- - 0	5.8	1.1	70
16	0--	6.3	1.1	65
17	000	6.3	1.3	70

Accuracy and precision: According to ICH Q2 guidelines accuracy reflects proximity between a true value and obtained value whereas precision is evaluated as the % RSD of response [14]. Analysis of standard samples which were prepared from stock solution helps in, accuracy and precision evaluation. Three replicates of each low (5 µg/ml), intermediate (10 µg/ml), high (20 µg/ml) standards were analyzed daily for three days. Precision study is acceptable if % RSD of the standards should be less than 2.

Robustness

In robustness study, we must get reliable results with intentional variations in parameters of method like flow rate by (± 0.2 ml/min), pH by (+1units), mobile phase proportion by ± 2% of the optimized conditions.

3. RESULT AND DISCUSSION

3.1 Preliminary Studies

Fingolimod contains amino group in its structure hence it may be more retained on C18 column hence flow rate has to be increase in order to carry drug substance with mobile phase also retention time has to be considered while optimization. Different mobile phases were tried starting with methanol and water, then with methanol and 0.05 M KH_2PO_4 of pH 4.6. Then separations were carried on C8 column using a mobile phase of methanol: 20mM ammonium formate having pH value of 6.3(70:30 v/v). With a flow rate of 1.3ml/min and column oven temperature of 30^{o}C, Peak was obtained at retention time of 5.25 min. Runs as suggested by Box-Behnken model were recorded for further optimization.

3.2 Instrument Qualification

Instrument qualification was done by considering combine parameters for OQ and PQ as it is mentioned in methods section, results are given in Table 3.

3.3 Method Design

For the USP tailing factor of peak, Multivariate regression analysis was applied and fitted full quadratic model was obtained.

p-values and Regression analysis obtained from software generated report are presented in Table 4.

Table 3. Instrument qualification -combine parameters for OQ and PQ

Module	Parameter	Findings	Limits
Injector	Precision of injection volume	RSD :0.6	<1% RSD
	Injection carryover	No carryover	No carryover
Solvent delivery system	Flow rate accuracy	Expected volume ± 0.8 %	Expected volume ±3%
	Flow rate precision	RSD:0.8	<1% RSD
Detector	Wavelength accuracy	Specific maxima +1nm	Specific maxima ± 2nm
	Linearity of detector response	R^2>=0.999	R^2> 0.999

Table 4. Regression coefficients and associated probability values (p-value) for USP tailing

Term	Coefficient	p-value
pH	-5.01560	*0.6253*
Flow rate	0.84500	0.6253
Methanol %	-0.09110	0.0262
pH× flow rate	0.10000	0.4936
pH× methanol%	0.0020	0.7287
Flow rate× methanol%	-0.0300	0.0670
pH× pH	-0.26000	0.0016
Flow rate× Flow rate	0.25000	0.5720
Methanol % × methanol%	0.00080	0.2241

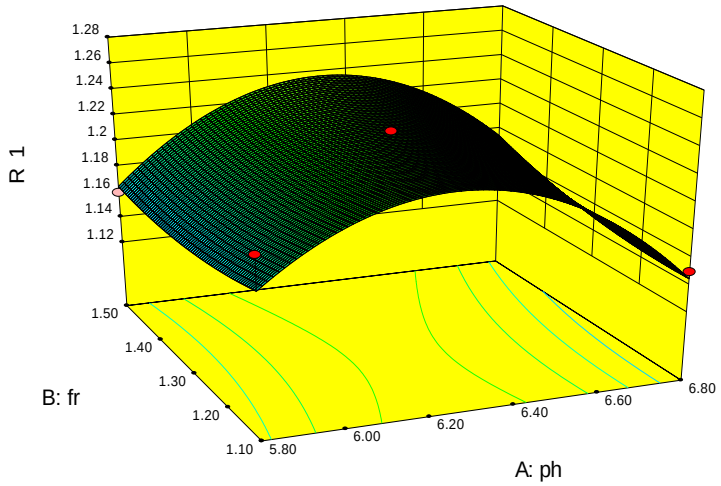
To analyse the effect of the different factors on USP tailing, Analysis of variance (ANOVA) was performed. p-values helps to prove that the findings are 'statistically significant' it is $p<0.05$ [19].

A value of Prob > F was found to be less than 0.05, hence model was observed to be significant for prediction of response. p-value more than the 0.05 indiactes that model is not fit for the prediction of response. Entire model fitted well for optimization. A lack of fit was not found to be statistical significant and prob>F = 0. 01052. Model used was accurate with R^2 of 0.968.

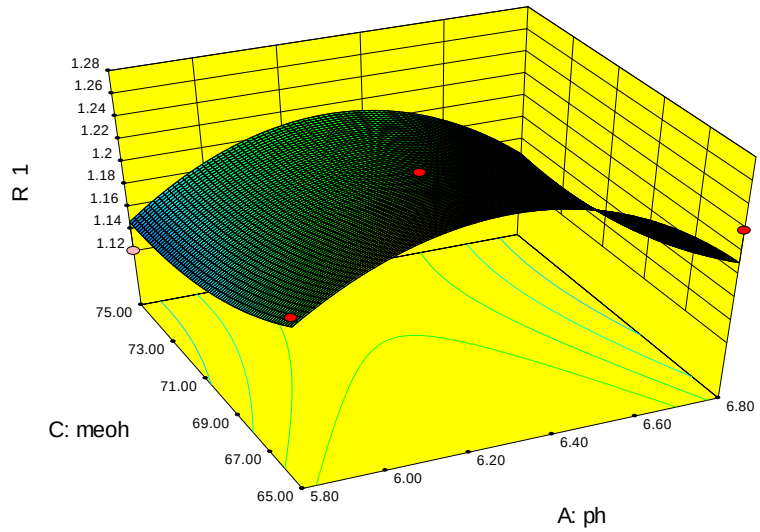
Significant factors found were Methanol (p-value 0.0262) and interaction of pH× pH (p- value 0.0016).

Three of the factors were found to affect the peak response from their respective coefficients. pH,methanol and interaction of Flow rate× methanol is showing inverse relationship with tailing. Flow rate also has shown effect on response.

Response surface and contour plot were analysed to study effect of different factors. This will help to develop design space for robust method. 3-D graph are presented in Fig. 1 (A, B, C).



(A)



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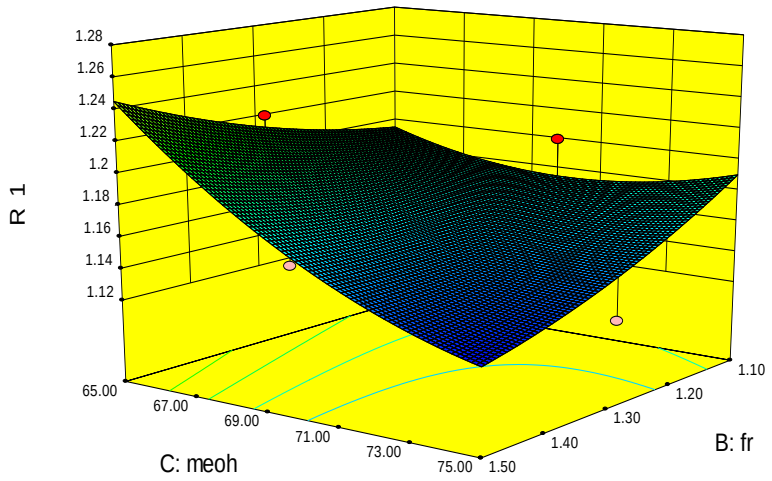

(C)

Fig. 1. Response surface (3D) and contour plots showing the effects of pH, flow rate and methanol (meoh) concentration on USP tailing factor of Fingolimod. A) Effect of pH and flow rate B) effect of pH and methanol (meoh). C) Effect of flow rate and methanol (meoh)

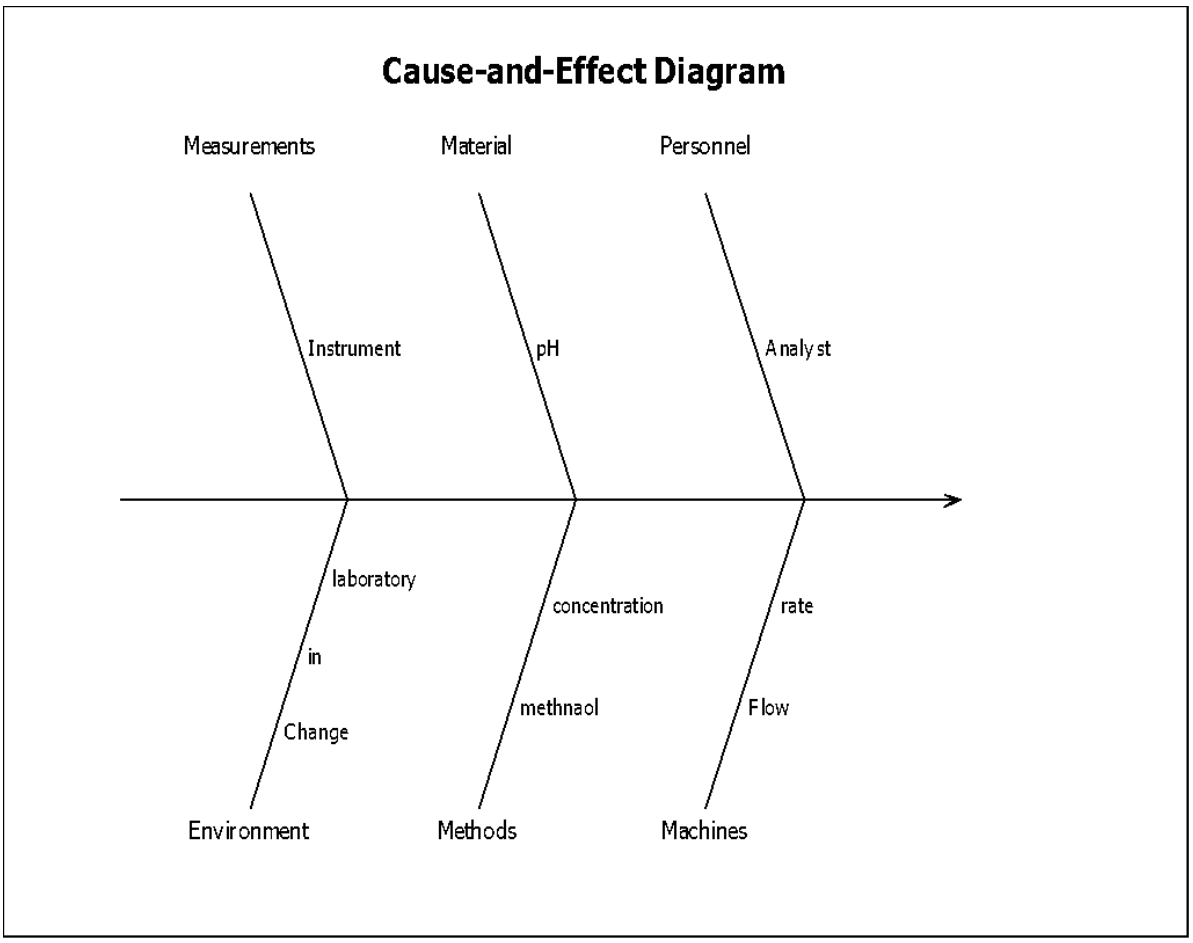

Fig. 2. Cause and effect diagram for risk assessment

Graphs clearly shows effect of the different factors and their interaction on the response. Nonlinear relationship between different factors is clearly understood by Curvatures present in the contour plot. From Fig. 1(A) showing effect of pH and flow rate (where methanol concentration is constant at 70%), it is observed that between pH of 6-6.9 tailing was found to be more than 1.2, tailing was in specified limit between lower pH of 5.8-5.9.

Flow rate is not showing much effect but when flow rate was increased throughout the pH range tailing was increased with increase in flow rate. If flow rate was kept constant at 1.3effect of methanol concentration and pH was observed which is given in (Fig. 1, B). It was found that at pH of around 6.6 and methanol concentration in between 67-68 tailing factor exceeded the limit. But at pH of 5.8 it was within the limit. Hence at lower pH response was optimum though methanol percentage is varied and at higher pH it was out of the specified limit of 1.2.

When pH was kept constant and flow rate and method phase concentration was studied, methanol concentration is not showing much effect but when flow rate was at lower limit peak tailing was increased (Fig. 1 C). From the three of diagrams conclusion can be drawn that pH either at lower side 5.8 -5.9 or more than 6.6 and, flow rate at high level between 1.4-1.5 should be maintained Methanol concentration has lesser effect on tailing but at higher concentration tailing was found to be lesser as well as higher concentration is desired for this particular drug as it is more retentive on stationary phase. Optimum condition chosen from obtained runs i.e. pH at 5.8, flow rate of 1.5 and methanol concentration of 75% (Fig. 3).

Set of conditions were analysed to compare predicted response with actual response six replicates of 5 µg/ml of solution at above specified conditions were taken difference in the response was not more than 3%.

Using the optimised method percentage purity of fingolimod was determined and found to be 99.87.

3.4 Method Validation

Validation of the method was performed as per ICH guidelines Q2. All the results were within the limit. Method was precise, robust accurate, and. Results of validation are given in (Tables 5, 6, 7) (Fig. 4).

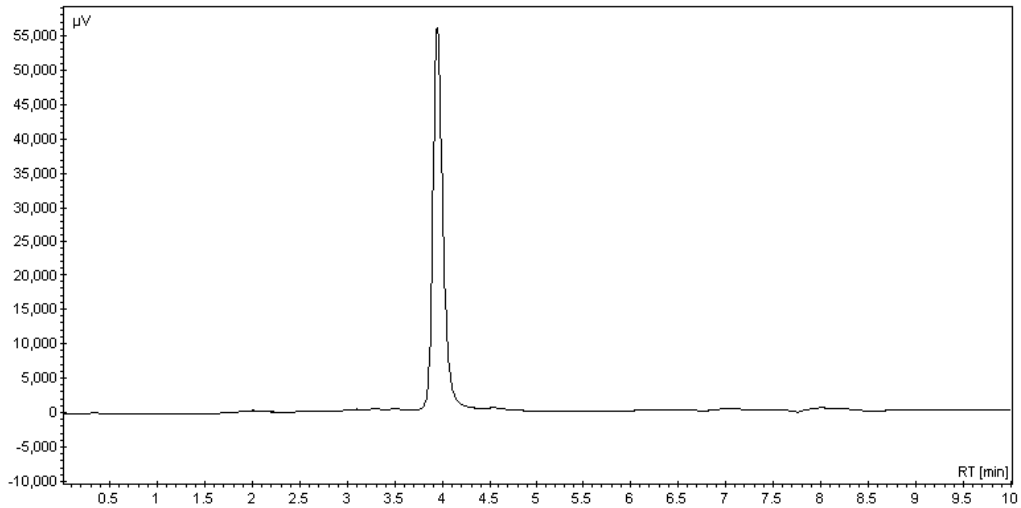

Fig. 3. Chromatogram of Fingolimod

Table 5. Validation of method in terms of accuracy and precision

Level	Intra-day		Inter-day	
	Average area	%RSD	Average area	%RSD
5 µg/ml	2587.05	0.112	2584.69	0.326
10 µg/ml	5159.58	0.029	5161.55	0.053
20 µg/ml	10455.77	0.053	10447.85	0.076

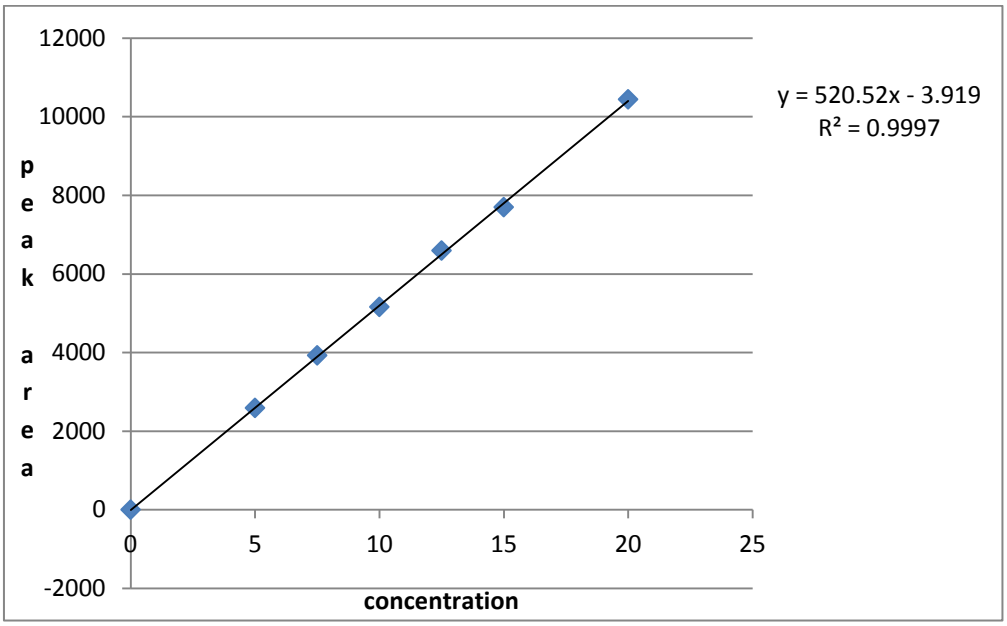

Fig. 4. Linearity of Fingolimod

Table 6. Linearity of FNG

Standard Concentration (µg/ml)	Peak area of FNG
5	2588.8
7.5	3926.6
10	5159.4
12.5	6594.1
15	7698.2
20	10442.2
Regression equation	y = 520.5x - 3.919
Regression coefficient	0.999

Table 7. Validation in terms of robustness

Sr. No.	variables		Retention time	Number of therotical plates
1	**pH unit**	+1	3.638	2375
		0	3.875	2378
		-1	3.852	2382
		Average	3.865	2377.3
		% RSD	0.3433	0.1699
2	**Flow rate**	+0.2	3.863	2403
		0	3.875	2378
		-0.2	3.894	2389
		Average	3.8813	2381.6
		% RSD	0.2826	0.2666
3	**Methanol concentration**	+2%	3.796	2384
		0	3.875	2378
		-2%	3.879	2380
		Average	3.8763	2378.66
		% RSD	0.0595	0.0485

4. CONCLUSION

A Quality by Design approach has been successfully utilised in method development of the Fingolimod in bulk. All key aspect of QbD were tried to be implemented in said study.

Systematic approach was utilized for method development which includes beginning with determination of target profile characteristics, instrument qualification, risk assessment, design of experiment and validation.

Three factors were analysed for their effect on response i.e. USP tailing factor. Interaction and quadratic effect of the factors were studied with least possible runs by using Box Behnken model. Response surface diagrams and contour plots were studied for coming to conclusion which factors are affecting response and their limits were recorded. Optimum run condition was obtained; Replicates of run having optimized condition were taken to confirm the predicted response with actual response.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

We conducted our research after obtaining proper IEC approval.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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