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Application of QbD in development and validation of a rapid RP-HPLC method for determination of Atazanavir in bulk drug

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Abstract

The present study describes a simple, accurate, precise and cost effective reverse phase high-performance liquid chromatographic (RP-HPLC) method for determination of Atazanavir in bulk.The systematic approach of QbD was use for the analytical method development. Chromatographic separation was carried out with C18 column; different mobile phases were tried starting with methanol and water. The separation was carried on Grace C-18 column (4.6×250 mm, 5-µm particle size) with mobile phase of acetonitrile: water (60:40). Peak was obtained at retention time of 5.56 min flow rate of 1 ml/min. Detection was done using UV detector at 252 nm. Optimization was done by response surface methodology, applying a three level Box Behnken design with three centre points. Three factors selected were injection volume, column oven temperature and acetonitrile concentration in mobile phase. The optimized chromatographic method was validated according to the International Conference on Harmonization (ICH) Q2 (R1) guidelines for linearity, range, accuracy, precision and robustness.

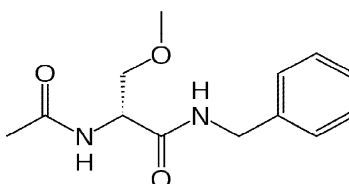
Key words: RP-HPLC; QbD; Acetonitrile (ACN); Atazanavir.

Introduction

Quality by Design (QbD) is, "a 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." QbD has become an important concept for the pharmaceutical industry that is further defined in the International Conference on

Harmonisation (ICH) guidance on pharmaceutical development. This approach ensures a very high likelihood of method success during the product lifecycle. The scientific understanding gained during the method development process can be used to devise method control elements and to manage the risks identified. Thus, the validation which is usually performed after method development will serve the purpose of confirming method performance as opposed to identifying potential problem areas. [1]

Atazanavir [7, 11]



Atazanavir is Chemically N^2-acetyl-N-benzyl-D-homoserinamide. Atazanavir is an anticonvulsant compound used for treatment of partial onset seizures and neuropathic pain..

Trospium chloride is a white crystalline powder having melting point 250.29^0 C. It is soluble in Acetonitrile and water.

The aim of this work is to use QbD as an approach for development of an analytical RP-HPLC method for estimation of Atazanavir in bulk and also validate the method as per ICH guidelines.

Material and method for Atazanavir

Materials

Reagent and chemical used

Reference standard of Atazanavir was obtained from Glenmark pharmaceutical limited Mumbai. HPLC grade methanol, acetonitrile, acetic acid and of merc were used. All aqueous solutions were prepared with HPLC grade ready water obtained in-house, Milli-Q water purification system (Millipore, USA).

Instrumentation

- HPLC analysis was carried out using Jasco HPLC 2080 model chromatograph equipped with a PU 2080 isocratic pump, UV-2075 plus detector, analytical column was a Grace RP C-18 column (4.6 × 250 mm, 5 µm particle size). Data analysis was carried out using JASCO BORWIN software.

- Milli-Q water purification system (Millipore, USA).
- UV visible spectrometer (Double Beam), JASCO 630V and wavelength range of 200 to 400nm.

a) Atazanavir sample preparation

Atazanavir stock solution for optimization of experiments was prepared by accurately weighing 10mg of Atazanavir and dissolving in 10ml Methanol to yield a final concentration of 1000µg/ml Atazanavir. From above stock solution 100µg/ml sample was prepared for analysis.

b) Mobile phase preparation

Mobile phase was prepared by mixing acetonitrile: water (60:40) the mobile phase was filtered through 0.45µm and degassed before use.

c) Wave selection for analysis

Appropriate dilutions of Atazanavir were prepared and samples were scanned using UV spectrometer in the range of 200nm to 400nm. An absorbance maximum was obtained at 252nm.

d) Analytical target profile

"QbD is systematic approach to product, process design and development."[2]. Hence it began with determination of goal or method intent. In emphasis given on the product and process understanding [43]. Here method intent was to develop HPLC method of Atazanavir which is robust, accurate, precise and USP tailing less than 1.2, number of theoretical period as per requirement and short analysis time i.e. less than 10 min. as per QbD norms a robust method should be developed with help of visualized a design space.

e) Instrument Qualification[47]

Analytical procedures in pharmaceutical analysis are subjected to highly formalized validation procedures in order to demonstrate that they are suitable for the intended use. As a consequence, prior to method validation it is necessary to assure that the equipment or analytical test system itself is adequately design, maintained, calibrated and tested. These tests are called as analytical instrument qualification (AIQ). Qualification phases for analytical instrument are,

- Design qualification
- Installation qualification
- Operational qualification
- Performance qualification

Here in HPLC system are "of the shelf" equipment, design qualification may be disregarded here. Installation qualification establishes that the instrument is received as designed and that it is properly installed. As far as practical experimentation is considered only operational qualification and performance qualification combine parameters were done as reported by L.Kaminski et al.[47]

Precision of injection volume

It was determined by comparing peak area received with fixed 20µl injection and calibrated dosage loop tolerance limit set was <1%RSD.

Injection carryover

Injection carry over was determined by running a blank test directly after an analysis and measuring possible absorption there should not be any peak from previous analysis.

Flow rate accuracy

It was determine by measuring the volumetric flow rate of mobile phase through the column over a previously set period of time 1.0ml/min for 10 min, 2.0 ml/min for 5 min, 2.5 ml/min for 10 min. RSD should be <1% or tolerance limit is ±3%.

Flow rate precision

A flow rate precision was determined by measuring the RSD of retention times. Limit set was <1% RSD.

Wavelength accuracy

It was done by scanning the compound with known specific maxima. Tolerance limit is specific maxima ±2nm.

Linearity of detector

Linearity of detector was determined by injecting increasing concentration of test substance and tolerance limit set was $R^2 \geq 0.999$.

f) Risk assessment

It is commonly understood that risk is defined as the combination of probability of occurrence of harm and severity of that harm. Risk assessment help to increase quality of method or process. Also it is determine for effect of input variable on method or process. From risk assessment one can recognise critical attributes that are going to affect final quality of product. A risk assessment is helpful for effective communication between FDA and industry, research/development, and manufacturing and among multiple manufacturing sites within company. Various tools for risk assessment are [9],

- Ishkawa or fishbone diagram,
- Failure mode effect analysis(FMEA),
- Pareto analysis.

Risk assessment helps to increase quality of method or process. Also it is determinant for effect of input variables on method or process. From risk assessment one can recognise critical attribute that are going to affect final quality of product. Ishkawa or fishbone diagram and Pareto analysis are studied for said method by software generated reports.

g) **Initial Chromatographic condition**

Chromatographic separation was carried out with C18 column; different mobile phases were tried starting with methanol and water, then with methanol and 0.05M KH_2po_4 of pH 4.6. Then separation was carried on Grace C-18 column (4.6×250 mm, 5-µm particle size) with mobile phase of Acetonitrile: water having pH value of 4.5 (60:40 v/v) filtered through 0.45µ membrane filter and degassed in a sonicator for 10 min before use. Peak was obtained at retention time of 5.56 min flow rate of 1 ml/min, prior to the injection of drug solution: column was equilibrated with mobile phase flowing through the system. Detection was done using UV detector at 252nm. Further changes were done according to optimization model. pH was change by using acetic acid.

h) **Method design**

Optimization was done by response surface methodology, applying a three level Box Behnken design with three centre points (Table 8). Three factor selected were

injection volume, column oven temperature and acetonitrile concentration in mobile phase. Evaluation of main factor, their interaction and quadric effect on peak USP tailing factor were done. Injection flow rate and wave length were kept constant as their effect on tailing was less significant. Experiments were conducted by making triplicate injections (total 51 runs) of standard Atazanavir solution and the average of USP tailing was analysed using Design Expert 8 software.(table 9) Application of multivariate regression analysis resulted in a fitted full quadrate model for the average responses for peak USP tailing given by the equation 1

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

Where Y is the response, β_0 is the arithmetic mean response. B_1 β_2 and β_3 are regression coefficients of the factor X_1, X_2 and X_3 respectively. B_{11}, β_{22} β_{33} are squared coefficients β_{12}, $\beta_{13}$and β_{23} are interaction coefficients. [73, 74]

Table 1: Chromatographic factors and response variable for Box Behnken experimental design.

Chromatographic Condition	Level used		
	Low	Centre	High
Injection volume (X_1)	15	20	25
Column oven temp (X_2)	18	20	22
ACN Conc. (X_2)	50	60	70

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Table 2: Box Behnken method used for Atazanavir optimization

(Where '+' indicate the high value, '-'indicates lower value and '0' is the centre)

Run	Coded (X_1,X_2, X_3)	Injection volume (X_1)	Column oven temp (X_2)	ACN Conc. (X_2)
1	+0+	25	20	70
2	-0+	15	20	70
3	000	20	20	60
4	000	20	20	60
5	++0	25	22	60
6	0++	20	22	70
7	-0-	15	20	50
8	+0-	25	20	50
9	0+-	20	22	50
10	+-0	25	18	60
11	000	20	20	60
12	-+0	15	22	60
13	0-+	20	18	70
14	000	20	20	60
15	--0	15	18	60
16	0--	20	18	50
17	000	20	20	60

i) Critical Quality Attribute (CQA)

From software generated data the critical factor which affect the tailing factor and capacity factor were determined. Factor such as injection volume, column oven temp and acetonitrile conc were found to be critical. Selection of stationary phase was also critical

parameter. The nature of the drug is more retentive on C18 than C8. But for HPLC method to be effective it should have lesser retention time.

j) Method validation

The optimized chromatographic method was validated according to the International Conference on Harmonization (ICH) Q2 (R1) [75] guidelines for linearity, range, accuracy, precision and robustness. For system suitability, standard solution of 100µg/ml of Atazanavir was prepared by diluting and mixing drug with methanol. Five replicate injection of the system standard solution were analysed before sample analysis. The acceptance criteria for Atazanavir were less than 2% relative standard deviation (RSD)for peak area, retention time, symmetry USP tailing factor less than 1.2 and number of theoretical plates greater than 2000 for all peaks.

Linearity

As per ICH guidelines the linearity of analytical procedure is its ability (within in a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in sample. Standard calibration curves were prepared with five different concentrations by making serial volume to volume dilution of stock solution with methanol, over the range of 20, 40, 60, 80 and 100µg/ml. Three replicate injections of each concentration were made to determine the linearity of Atazanavir over the concentration range. Linear concentration curves of peak area versus drug concentration were plotted using linear least squares regression and evaluated for linearity.

Accuracy and precision

According to ICH Q2 guidelines accuracy of analytical procedure is the closeness of agreement between a reference or true value and value obtained while precision is usually reported as the per cent relative standard concentration standard deviation of a set of responses [75]. Accuracy and precision of the method were evaluated for Atazanavir drug substance by analysing standard samples prepared daily from stock solution. Three replicate of each low (20µg/ml), intermediate (60µg/ml), high (100µg/ml) standard were analysed daily over three days as a part of validation and quality control. Accuracy and

precision were determined by analysing the mean, standard deviation and relative standard deviation of the peak areas and their resultant concentrations. An acceptance criterion for precision is that the RSD of the standards should not be more than 2.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate change in method parameter and provide an indication of its reliability during normal usage [41]. There should be reliability of an analysis with respect to deliberate variation in method parameter such as flow rate (±0.1ml/min), pH (±0.1 units), and mobile phase proportion.

Result and discussion

Preliminary studies

Chemically, Atazanavir Sulfate [1] is (3S,8S,9S,12S)-3,12-Bis(1,1-dimethylethyl)-8-hydroxy-4,11-dioxo-9-(phenylmethyl)-6-[[4-(2-pyridinyl) phenyl] methyl]-2,5,6,10,13 pentaazatetradeanedioic acid dimethyl ester;1-[4-(pyridine-2-yl)phenyl]-5S,2,5-bis[[N (methoxy carbonyl)-L-tert- leucinyl]amino]-4S hydroxyl-6-phenyl-2-azahexane.It contain amino group in its structure hence it may be more retained on C18 column hence flow rate has to be increased in order to carry drug substance with mobile phase also retention time has to be consider while optimization. Different mobile phases were tried starting with methanol and water, then with methanol and 0.05M KH_2po_4 of pH 4.6. Then separation was carried on Grace C-18 column (4.6×250 mm, 5-µm particle size) with mobile phase of Acetonitrile: water having pH value of 4.5 (60:40 v/v). Peak was obtained at retention time of 5.56 min flow rate of 1 ml/min, column oven temperature of 20^0C. Further optimization was done by carrying runs as by Box Behnken design.

Instrument Qualification

Instrument qualification was done by considering combine parameter for operational qualification and performance qualification as it is mentioned in method section, result are given in **(table3)**

Table3: Result of instrument qualification in term of OQ & PQ

Module	Parameter	Findings	Limits
Injector	Precision of injector volume Injection carryover	RSD : 0.6 No carryover	<1% RSD No carryover
Solvent delivery system	Flow rate accuracy Flow rate precision	Expected volume +/-8% RSD: 0.8	Expected volume +/- 3% <1%RSD
Detector	Wavelength accuracy Linearity of detector response	Specific maxima +1nm R^2=0.999	Specific maxima +/-2nm $R^2 \geq 0.999$

Method design- Box Behnken

Multivariate regression analysis was applied and fitted full quadratic model was obtained for the USP tailing factor of peak. Factor considered here are injection volume, column oven temperature and acetonitrile concentration. Regression analysis and p-values obtained from software generated report are given in **(table4)**

Table4: Regression coefficients and associated probability values (p-values) for USP tailing of Atazanavir

Term	Coefficient	p-value
Intercept	1.36	< 0.0001
Injection volume	-0.011	0.0193
Column oven temperature	-2.500E-003	0.5231
Acetonitrile concentration	0.094	< 0.0001
Injection volume x Column oven temperature	0.000	1.0000
Injection volume x Acetonitrile concentration	7.500E-003	0.1970
Column oven temperature x Acetonitrile concentration	5.000E-003	0.3736
Injection volume x Injection volume	-0.051	< 0.0001
Column oven temperature x Column oven temperature	-0.034	0.0003
Acetonitrile concentration x Acetonitrile concentration	0.069	< 0.0001

Analysis of variance (ANOVA) was perform to study the significance of the factors and interaction terms on the response i.e. USP tailing of the peak, p-value simply provide the cut-off beyond which we assert that the findings are 'statistically significant' by convention, it is $p<0.05$[13].

A value of Probe > F was found to be less than 0.05, hence model was found to be significant for prediction of response. Entire model was fitted well for optimization. Also a lack fit was significant. Significant factors found were injection volume (p-value0.0193), acetonitrile concentration (p-value <0.0001), interaction of injection volume x injection volume (p-value, 0.0001), interaction of column oven temperature x column oven temperature (p-value 0.0003), and acetonitrile concentration x acetonitrile concentration (p-value <0.0001)

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Three of the factors were found to affect the peak response from their respective coefficients. Injection volume, column oven temperature, interaction of injection volume x injection volume and interaction of column oven temperature x column oven temperature is showing inverse relationship with tailing. Flow rate also has shown effect on response.

Response surface and contour plot were studied to visualize effect of factor and their interaction so as to develop design space for robust method 3D graph are given below in **Fig.1 (A.B.C)**

From the graph some facts about effect of the factors and their interaction on the response can be found. Curvatures in the contour plot show nonlinear relationship between factor. From**(Fig.1A)** showing effect of injection volume and column temp(where acetonitrile concentration is constant at 60%, it can be observed that between injection volume of 22µl tailing was found to be more than 1.36, tailing was in specified limit between injection volume 19-20µl.

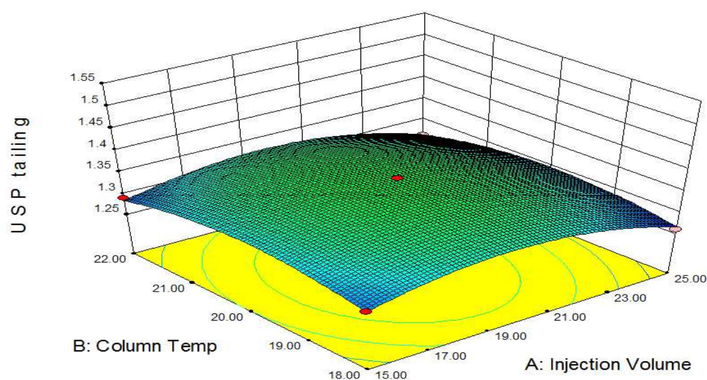
When column oven temperature was kept constant at 20° C **(Fig. 1B)** and effectof acetonitrile concentrationand injection volume was observed.it was found that at injection volume show no significant effect acetonitrile concentration in between 43-50% tailing factor exceeded the limit. But at injection volume 20µl it was within the limit. Hence at lower injection volume response was optimum though acetonitrile percentage is varied and at higher injection volumeit was out of the specified limit of 1.36.

When injection volume was kept constant and column oven temp and acetonitrile concentration was studied column oven temp is not showing much effect but when column temp was at higher limit peak tailing was decreased **(Fig.1C)**. from the three of diagram conclusion can be drawn that acetonitrile concentration at higher side tailing is above the specified limit and injection at high and lower level show tailing within the limit column oven temp has lesser effect on tailing but at higher value tailing was found to be lesser as well as higher value was desired for this particular drug as it is more retentive on stationary phase.

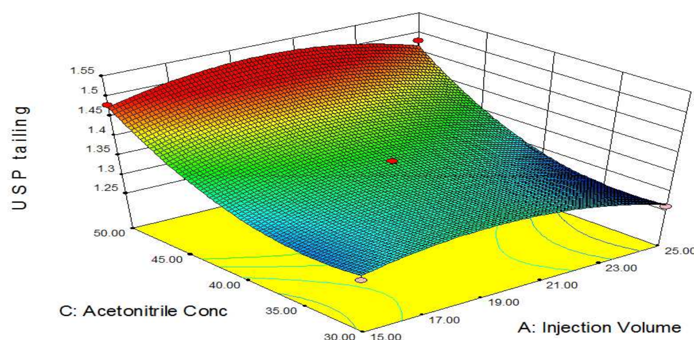
To get optimum set of condition to achieve desire goal composite desirability parameters were applied. Response was set to minimum tailing below target value of 1.36 as it is critical goal importance of 5+ was assigned.

Optimum condition having higher desirability was chosen from obtained runs i.e. injection volume of 20µl, column oven temp of 20° C and acetonitrile concentration of 40% **(fig.9)**.Set

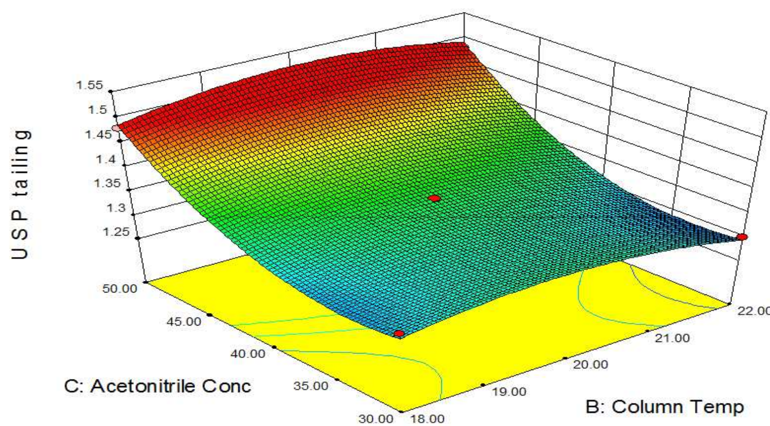
of condition were analyse to compare predicted response with actual response. Five replicates of 60μg/ml of solution at above specified condition were taken. Difference in the response was not more than 3%.



(A)



(B)



(C)

Figure1: Response surface (3D) and contour plots showing the effect of injection volume, acetonitrile concentration and column oven temperature on USP tailing factor of Atazanavir.

A) Effect of injection volume and column oven temperature. B) Effect of injection volume and acetonitrile concentration. C) effect of column oven temperature and acetonitrile concentration

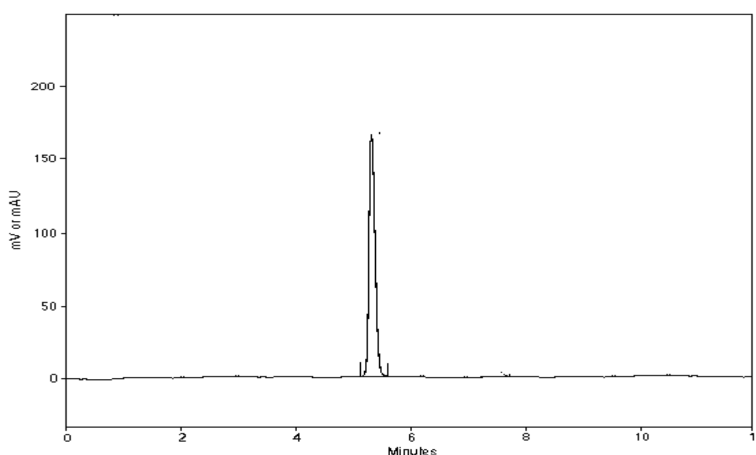


Figure 2: Chromatogram of Atazanavir

Method Validation

Method validation was done according to the ICH guideline Q2 [15]. Results were within the specified limit. Method was found to be accurate, precise and robust. Validation results are given below in **(table 5)**

Table 5: Validation of method in term of linearity, precision and accuracy of Atazanavir

Validation parameter	Result	Acceptance criteria
Linearity	Coefficient of	Coefficient of
(5-20 µg/ml)	Correlation-0.999	Correlation0>0.999
Accuracy	Recovery-99.5%	Recovery 98-102%
Precision		
Repeatability	RSD: 0.052%	RSD less than 2%

Linearity

A set of six solution of Atazanavir at concentration ranging from 20-100 ug/ml were prepared. Each sample was analysed in triplicate, calibration curve was constructed by plotting the peak area verses the concentration using linear regression analysis. The correlation coefficient was found to be 0.9975(**Table 6) (Fig.3)**

Table6: Linearity of Atazanavir

Standard Concentration(µg/ml)	Peak area of Atazanavir
20	3610
40	6345
60	9645
80	12540
100	16264
Regression equation	y=157.52x+229.9
Regression coefficient	0.9975

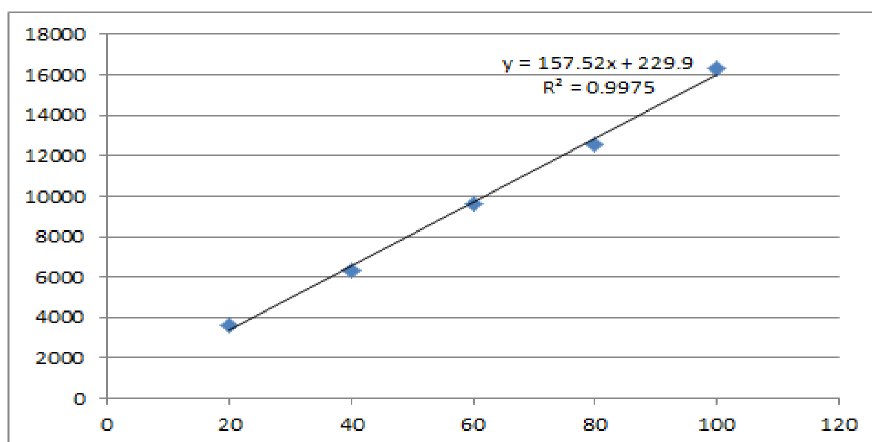


Figure 3: Linearity plot of Atazanavir

Repeatability

Repeatability was determined by running six replicates of samples and evaluating the average and %RSD for sample by comparing peak area.

Table7: repeatability

Sr.no	Concentration (µg/ml)	Peak Area
1	60	9639
2	60	9642
3	60	9645
4	60	9643
5	60	9645
Average		9642.8
%RSD		0.0258

Conclusion

The Quality by Design approach has been successfully used to develop HPLC method for Atazanavir API. Systematic approach was utilized to develop an efficient and robust method which includes beginning with determination of target profile characteristics, instrument qualification, risk assessment, design of experiment and validation.

Three factors that were determined to significantly affect the peaks were then analysed to determine their interactions and quadratic effects with the least possible runs by using Box-Behnken model in conjunction with response surface methodology.

Response surface diagrams and contour plots were studied for coming to conclusion which factor are affecting response and their limits were recorded.

A desirability function was applied to determine the optimum conditions. Optimum conditions were obtained; the one with higher desirability was selected. Replicates of run having optimized condition were taken to confirm the predicted response with actual response.

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