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Reverse Phase-High Performance Liquid Chromatography method development and validation for estimation of efavirenz by Quality by Design approach

Waghmare Santosh A [a], Kashid Arun M [b]

[a] JSPM's Charak College of Pharmacy and Research(Affiliated to Savitribai Phule Pune University), Wagholi, Pune- 412207, India

[b] Department of Pharmaceutical Chemistry, Sinhgad Institute of Pharmacy, Narhe, Pune 411041, Maharashtra, India

ABSTRACT

Quality by design (QbD) refers to the achievement of certain predictable quality with desired and predetermined specifications. A very useful component of the QbD is the understanding of factors and their interaction effects by a desired set of experiments by using software (design expert 8). The present study describes the development of a comprehensive science and risk based HPLC method which is given by design expert 8 and subsequent validation for the analysis of Efavirenz active pharmaceutical ingredient (API) using a quality by design approach. An efficient experimental design based on systematic scouting of all four key components of the RP-HPLC method (column, pH, mobile phase and flow rate) is presented. The described method was linear. $R^2 = 0.9998$. The precision, ruggedness and robustness values were also within the prescribed limits (<1% for system precision and <2% for other parameters). Chromatographic peak purity results indicated the absence of co-eluting peaks with the main peak of Efavirenz. The proposed method can be used for routine analysis of Efavirenz in quality control laboratories.

Keywords: Quality by design, HPLC, Efavirenz, design expert 8.

1. INTRODUCTION

1.1 Introduction to Drug Molecule

Chemically efavirenz is (4S)-6-chloro-4-(2-cyclopropylethynyl)-4-(trifluoromethyl)-2,4-dihydro-1H-3,1-benzoxazin-2-one. It has molecular formula of $C_{14}H_9ClF_3NO_2$. It is crystalline powder having white or slightly yellowish colour. The melting point of Efavirenz is ranging from 136.0^0C to 141.0^0C. Efavirenz is a non-nucleoside reverse transcriptase inhibitor (NNRTI). Efavirenz is directly connected to the enzyme and blocks the enzyme catalytic site. It is used as a part of highly active antiretroviral therapy for the treatment of human immunodeficiency virus. The drug is used in combination with other anti-retroviral agents for the treatment of HIV-1 infection in children's and adults. The usual dosage of efavirenz is 600mg per day. It is usually taken on empty stomach at bedtime to reduce neurological and psychiatric adverse effects. Structure of efavirenz is given in Figure no 1.[1]

1.2 Quality by Design (QbD) is a concept first outlined by Juran in various publications. Juran believed that quality could be planned and that most quality crises and problems relate to the way in which quality was planned in the first place. Experimental design (DoE), as a tool of QbD, can be defined as the strategy for setting up experiments in such a manner that the information required is obtained as efficiently and precisely as possible. Experimental design (DoE) is a powerful technique used for exploring new processes, gaining increased knowledge of the existing processes and optimizing these processes for achieving world-class performance. Experimentation is performed to determine the relationship between factors acting on the response.[2]
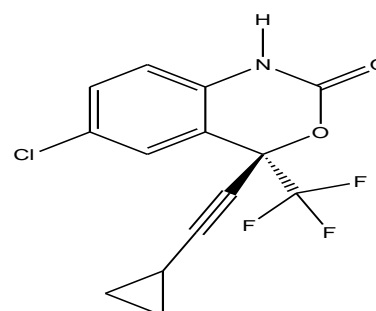

Figure 1: Chemical structure of efavirenz

The choice of DoE is very important for the success of any industrial experiment. The proper selection was based on the process knowledge and depended on the following factors:

- Number of factors and interactions to be studied

- Complexity of using each design

- Statistical validity and effectiveness of each design

- Ease of understanding and implementation

- Nature of the problem

- Cost and time constraints

  The potential benefits of using a DoE rather than using traditional "one factor at a time" experimentation are summarized below:

- Improved process yield and stability

- Improved profits

- Improved process capacity

- Reduced process variability

- Reduced manufacturing costs

- Reduced process design and developing time.[2]

1.3 Full three-level factorial designs by Design expert 8 Software.

Full three-level factorial design is an experimental matrix that has limited application in RSM when the factor number is higher than 2 because the number of experiments required for this design (calculated by expression N = 3k, where N is experiment number and k is factor number) is very large, thereby losing its efficiency in the modelling of quadratic functions. Because a complete three-level factorial design for more than two variables requires more experimental runs than can usually be accommodated in practice, designs that present a smaller number of experimental points, such as the Box–Behnken, central composite, and Doehlert designs, are more often used. However, for two variables, the efficiency is comparable with designs such as central composite. The majority of applications of three-level factorial designs are in the area of chromatography.[2,3,4,5,6.]

2. MATERIAL AND METHODS

2.1 Apparatus

A UV- vis Spectrophotometer (Jasco/ v-630), was used with quartz cells of 10mm path length; HPLC (Jasco 12000 series); Column Symmetry C-18, 4.6*250mm, 5μm particle size (Waters); analytical balance (Contech, CB-50 series); ultrasonicator cleaning bath (spectra lab / UCB-400); filter papers 0.45μm.

2.2 Materials

Efavirenz reference standard was obtained as gift sample from Lupin Ltd. Aurangabad, Maharashtra, India and formulation was procured from the market. Ammonium formate buffer of research grade was obtained from Fine chem. Industries Ltd. Mumbai. Acetonitrile of HPLC grade was used for research from Merck Pvt. Ltd. Mumbai.

Stock solution of Efavirenz 1000μg/ml was prepared by using 10mg of Efavirenz in 10ml of methanol.

2.3 Chromatographic System

Analysis was carried on Symmetry C-18 4.6*250mm, 5μm particle size column at 292 nm. The samples were introduced through a Rheodyne injector valve with 20-μl sample loop. The mobile phase consisted of acetonitrile and ammonium formate (72:28, v/v), filtered through a membrane filter (0.45μ), degassed in ultrasonic bath, and pumped at a flow rate of 1.0 ml/min.

3. RESULTS AND DISCUSSION

3.1 Design of Experiment

Two Columns have used

✓ Column C8

✓ Column C18

3.1.1 3 level Factorial design (Miscellaneous Factorial design)

Selected Factorial design was miscellaneous Factorial design due to it has flexibility to change/add/delete any parameter at any time when our experiment is going on.it provide facility to give standard run at one time at only one mobile phase.[6]

3.1.2 There are selected four mobile phase

✓ Acetonitrile : Water

✓ Acetonitrile: Ammonium format buffer

✓ Methanol : Water

✓ Methanol : Ammonium format buffer.[3,4,5,6]

3.1.3 Miscellaneous Factorial design can pick up one mobile phase. So selected each mobile phase one by one.

✓ Acetonitrile : Water

✓ Change pH Range: 3-5

✓ Change Mobile phase proportion Range: 60-80% (Consider Acetonitrile)

✓ Change flow rate range: 0.8-1.2

When all above ranges put in Miscellaneous Factorial design. It gave 27 run at different pH, Mobile phase proportion and flow rate.

Followed same procedure for each mobile phase. That is for column C-8 has four mobile Phase and column C-18 has four mobile Phases with 27 run for each mobile phase. Each column has its 108 run. After completion of all trails software give one optimize best value for each column. Optimization means finding an alternative with the most cost effective or highest achievable performance under the given constraints, by maximizing desired factors and minimizing undesired ones. In comparison, maximization means trying to attain the highest or maximum result or outcome without regard to cost or expense.

Miscellaneous factor design was utilised for method development in order to evaluate the effects of amount of buffer, buffer pH and flow rate on responses. Total 27 runs were suggested by the software. Factors and responses considered for study were shown in table 1. Ranges considered were based upon previous univariate chromatographic separation studies. For amount of organic range was 60 to 80 % v/v, for pH of buffer 3 to 5 and for flow rate 0.8 to 1.2 mL min^{-1}.[7,8.]

3.2 Optimization

3.2.1 Screening design for suitable chromatographic condition:

Determination of suitable column and solvent system based on peak parameters. Methanol: water/ methanol: buffer (Amonium formate)/ ACN: water and ACN: buffer, these four mobile phases were selected for screening study on C8 and

C18 columns at pH 3.0, 4.0 and 5.0. These mobile phases were screened by varying the organic phase composition from 60 to 80 % v/v and flow rate from 0.8 to 1.2 mL/ min flow rate consider to be standard 1 ml/min because result are not satisfactory with 0.8 and 1.2 ml/min.

3.2.2 Results of various trials, having organic phase composition 60 % v/v are shown in following tables.

Table 1: Trials performed on C8 column at mobile phase (60:40 v/v) with aqueous phase pH 3

Sr.no.	Composition	Observation	Remarks
1	Methanol: buffer (60:40 v/v)	No peak appearance	Not satisfactory
2	Methanol: water (60:40 v/v)	No peak appearance	Not satisfactory
3	ACN: buffer (60:40 v/v)	Greater peak asymmetry and lower theoretical plates	Not satisfactory
4	ACN: water (60:40 v/v)	Greater peak asymmetry and lower theoretical plates	Not satisfactory

Table 2: Trials performed on C18 column at mobile phase (60:40 v/v) with aqueous phase pH 3

Sr. no.	Composition	Observation	Remarks
1	Methanol: buffer (60:40 v/v)	Greater peak Asymmetry and lower theoretical plates	Not satisfactory
2	Methanol: water (60:40 v/v)	No peak appearance	Not satisfactory
3	ACN: buffer (60:40 v/v)	Less peak asymmetry but less theoretical plates	Not satisfactory
4	ACN: water (60:40 v/v)	Less peak asymmetry with more theoretical plates	Satisfactory

Table 3: Trials performed on C8 column at mobile phase (60:40 v/v) with aqueous phase pH 4

Sr.no.	Composition	Observation	Remarks
1	Methanol: buffer (60:40 v/v)	No peak appearance	Not satisfactory
2	Methanol: water (60:40 v/v)	No peak appearance	Not satisfactory
3	ACN: buffer (60:40 v/v)	More retention time	Not satisfactory
4	ACN: water (60:40 v/v)	More retention time	Not satisfactory

Table 4: Trials performed on C18 column at mobile phase (60:40 v/v) with aqueous phase pH 4

Sr.no.	Composition	Observation	Remarks
1	Methanol: buffer (60:40 v/v)	No peak appearance	Not satisfactory
2	Methanol: water (60:40 v/v)	No peak appearance	Not satisfactory
3	ACN: buffer (60:40 v/v)	Good peak properties	Satisfactory
4	ACN: water (60:40 v/v)	More retention time	Not satisfactory

Table 5: Trials performed on C8 column at mobile phase (60:40 v/v) with aqueous phase pH 5

Sr. no.	Composition	Observation	Remarks
1	Methanol: buffer (60:40 v/v)	No peak observed	Not satisfactory
2	Methanol: water (60:40 v/v)	No peak appearance	Not satisfactory
3	ACN: buffer (60:40 v/v)	Greater peak asymmetry with less theoretical plates	Not satisfactory
4	ACN: water (60:40 v/v)	More retention time	Not satisfactory

Table 6: Trials performed on C18 column at mobile phase (60:40 v/v) with aqueous phase pH 5

Sr. no.	Composition	Observation	Remarks
1	Methanol: buffer(60:40 v/v)	No peak observed	Not satisfactory
2	Methanol: water (60:40 v/v)	No peak observed	Not satisfactory
3	ACN: buffer (60:40 v/v)	More retention time	Not satisfactory
4	ACN: water (60:40 v/v)	More retention time	Not satisfactory

3.2.3 Results of various trials, having organic phase composition 70 % v/v are shown in following tables.

Table 7: Trials performed on C8 column at mobile phase (70:30 v/v) with aqueous phase pH 3

Sr. no.	Composition	Observation	Remarks
1	Methanol: buffer (70:30 v/v)	Greater peak asymmetry and lower theoretical plates	Not satisfactory
2	Methanol: water (70:30 v/v)	More retention time	Not satisfactory
3	ACN: buffer (70:30 v/v)	Greater peak asymmetry and lower theoretical plates	Not satisfactory
4	ACN: water (70:30 v/v)	Greater peak asymmetry	Not satisfactory

Table 8: Trials performed on C18 column at mobile phase (70:30 v/v) with aqueous phase pH 3

Sr. no.	Composition	Observation	Remarks
1	Methanol: buffer (70:30 v/v)	More retention time	Not satisfactory
2	Methanol: water (70:30 v/v)	More retention time	Not satisfactory
3	ACN: buffer (70:30 v/v)	Less peak asymmetry with more theoretical plates and good retention time	More satisfactory
4	ACN: water (70:30 v/v)	Less peak asymmetry with more theoretical plates and good retention time	satisfactory

Table 9: Trials performed on C8 column at mobile phase (70:30 v/v) with aqueous phase pH 4

Sr.no.	Composition	Observation	Remarks
1	Methanol: buffer (70:30 v/v)	Greater peak asymmetry with lower theoretical plates	Not satisfactory
2	Methanol: water (70:30 v/v)	No peak appearance	Not satisfactory
3	ACN: buffer (70:30 v/v)	Less peak asymmetry with more theoretical plates and good retention time	satisfactory
4	ACN: water (70:30 v/v)	Greater peak asymmetry with more theoretical plates	Not satisfactory

Table 10: Trials performed on C18 column at mobile phase (70:30 v/v) with aqueous phase pH 4

Sr.no.	Composition	Observation	Remarks
1	Methanol: buffer (70:30 v/v)	More retention time	Not satisfactory
2	Methanol: water (70:30 v/v)	More retention time	Not satisfactory
3	ACN: buffer (70:30 v/v)	Less peak asymmetry with more theoretical plates and good retention time	Satisfactory
4	ACN: water (70:30 v/v)	Less peak asymmetry with more theoretical plates and good retention time	Satisfactory

Table 11: Trials performed on C8 column at mobile phase (70:30 v/v) with aqueous phase pH 5

Sr. no.	Composition	Observation	Remarks
1	Methanol: buffer (70:30 v/v)	More retention time	Not satisfactory
2	Methanol: water (70:30 v/v)	Greater peak asymmetry	Not satisfactory
3	ACN: buffer (70:30 v/v)	Greater peak asymmetry with less theoretical plates	Not satisfactory
4	ACN: water (70:30 v/v)	Greater peak asymmetry with less theoretical plates	Not satisfactory

Table 12: Trials performed on C18 column at mobile phase (70:30 v/v) with aqueous phase pH 5

Sr. no.	Composition	Observation	Remarks
1	Methanol: buffer (70:30 v/v)	More retention time	Not satisfactory
2	Methanol: water (70:30 v/v)	More retention time	Not satisfactory
3	ACN: buffer (70:30 v/v)	Less peak asymmetry with more theoretical plates and good retention time	More satisfactory
4	ACN: water (70:30 v/v)	Less peak asymmetry with more theoretical plates and good retention time	satisfactory

3.2.4 Results of various trials, having organic phase composition 80 % v/v are shown in following tables.

Table 13: Trials performed on C8 column at mobile phase (80:20 v/v) with aqueous phase pH 3

Sr. no.	Composition	Observation	Remarks
1	Methanol: buffer (80:20 v/v)	Greater peak asymmetry and More retention time	Not satisfactory
2	Methanol: water (80:20 v/v)	Greater peak asymmetry	Not satisfactory
3	ACN: buffer (80:20 v/v)	Greater peak asymmetry	Not satisfactory
4	ACN: water (80:20 v/v)	Greater peak asymmetry and lower theoretical plates	Not satisfactory

Table 14: Trials performed on C18 column at mobile phase (80:20 v/v) with aqueous phase pH 3

Sr. no.	Composition	Observation	Remarks
1	Methanol: buffer (80:20 v/v)	Lower theoretical plates	Not satisfactory
2	Methanol: water (80:20 v/v)	Lower theoretical plates	Not satisfactory
3	ACN: buffer (80:20 v/v)	Less retention time	Not satisfactory
4	ACN: water (80:20 v/v)	Lower theoretical plates	satisfactory

Table 15: Trials performed on C8 column at mobile phase (80:20 v/v) with aqueous phase pH 4

Sr.no.	Composition	Observation	Remarks
1	Methanol: buffer (80:20 v/v)	Greater peak asymmetry and lower theoretical plates with more retention time	Not satisfactory
2	Methanol: water (80:20 v/v)	Greater peak asymmetry	Not satisfactory
3	ACN: buffer (80:20 v/v)	Greater peak asymmetry and lower theoretical plates	Not satisfactory
4	ACN: water (80:20 v/v)	Greater peak asymmetry and lower theoretical plates	Not satisfactory

Table 16: Trials performed on C18 column at mobile phase (80:20 v/v) with aqueous phase pH 4

Sr.no.	Composition	Observation	Remarks
1	Methanol: buffer (80:20 v/v)	No peak appearance	Not satisfactory
2	Methanol: water (80:20 v/v)	No peak appearance	Not satisfactory
3	ACN: buffer (80:20 v/v)	Less retention time	Satisfactory
4	ACN: water (80:20 v/v)	Good peak properties	satisfactory

Table 17: Trials performed on C8 column at mobile phase (80:20 v/v) with aqueous phase pH 5

Sr. no.	Composition	Observation	Remarks
1	Methanol: buffer (80:20 v/v)	Greater peak asymmetry	Not satisfactory
2	Methanol: water (80:20 v/v)	less theoretical plates	Not satisfactory
3	ACN: buffer (80:20 v/v)	Greater peak asymmetry with less theoretical plates	Not satisfactory
4	ACN: water (80:20 v/v)	Greater peak asymmetry with less theoretical plates	Not satisfactory

Table 18: Trials performed on C18 column at mobile phase (80:20 v/v) with aqueous phase pH 5

Sr. no.	Composition	Observation	Remarks
1	Methanol: buffer (80:20 v/v)	lower theoretical plates	Not satisfactory
2	Methanol: water (80:20 v/v)	lower theoretical plates	Not satisfactory
3	ACN: buffer (80:20 v/v)	Good peak properties	satisfactory
4	ACN: water (80:20 v/v)	Lower retention time	Not satisfactory

3.3 Optimized trials suggested by software based on desirability value

This methodology is initially based on constructing a desirability function for each individual response. The scale of individual desirability function ranges between i= 0, for completely undesirable response and i =1, for fully desired response. Selection of trial was based on maximum desirability value. Therefore, first trial which was having desirability one (i=1) selected for method optimization.[8, 9,10.]

Table 19: Optimized trials suggested by software based on desirability value

Sr. no.	Amount of CAN	pH of buffer	Flow rate	Retention time	Tailing factor	Theoretical plates	Desirability
1	**72.03**	**5.0**	**1.0**	**5.6**	**0.96**	9084.48	**1.0**
2	72.00	5.0	1.0	5.9	1.01	9124.84	0.9
3	72.56	5.0	1.1	5.7	1.03	9025.81	1.0
4	70.02	5.0	1.0	5.76	0.99	8997.3	0.96

3.3.1 Optimized chromatographic conditions

Mobile phase: Ammonium format buffer: ACN (28:72 v/v), pH of buffer: 5, Analytical column: C_{18} column Waters XBridge (4.6× 250mm id. particle size 5μm), UV detection: 247 nm, Injection volume: 20 μL, Flow rate: 1.00 mL min^{-1}, Temperature: Ambient, Run time: 10 min

3.4 Effect of independent variables

3.4.1 Effect of independent variables on retention time (Y_1):

The equation for response surface quadratic model is as follows

$Y_1 = +5.56 -2.58 X_1+ 0.50X_2 -1.52 X_3 + 0.14 X_1X_2 +0.79X_1X_3+ -0.099 X_2X_3 + 1.45X_1^2 + 0.46X_2^2 + 0.96 X_3^2$ (1)

Where, X_1= A, X_2 = B and X_3= C

a graphical representation of amount of ACN (A) and flow rate (C), while pH of buffer (B) is maintained constant at its optimum of 5. An increase in flow rate resulted in decrease in retention time (Y_1), while increase in % v/v of buffer (or amount of buffer) resulted in increase in retention time (Y_1). Combination of amount of buffer and flow rate showed decrease in response.
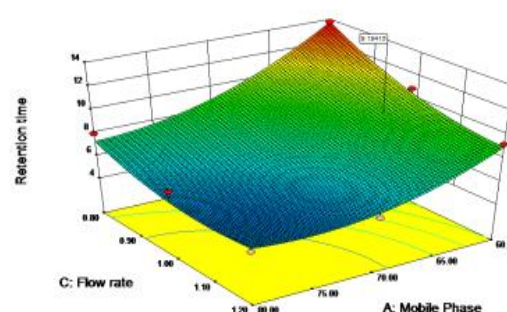

Figure 2(a): Three-dimensional plot for retention time as a function of flow rate and amount of ACN. Constant factor (pH of buffer- 5.0)

Fig.3 (b) shows a graphical representation of pH of buffer (B) and amount of buffer (A), while flow rate (C) is maintained constant at its optimum of 1 mL min^{-1}. Increase in pH of buffer showed increase in retention time (Y_1), also increase in amount of buffer showed synergistic effect on Y_1. Combination of amount of buffer and pH of buffer showed antagonistic effect on response.
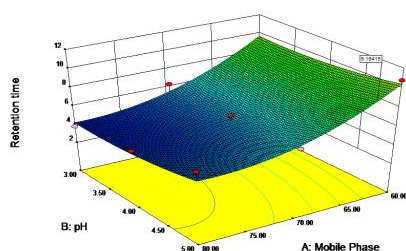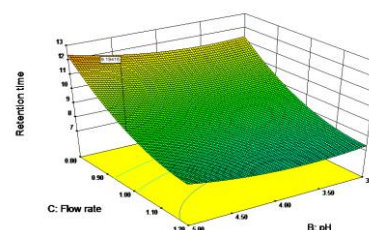
Figure 3(b): Three-dimensional plot for retention time as a function of pH of buffer and amount of buffer. Constant factor (flow rate- 1mL min^{-1})

Fig.4.(c) shows a graphical representation of pH of buffer (B) and flow rate (C), while amount of buffer (A) is maintained constant at its optimum of 40 % v/v. An increase in flow rate showed antagonistic effect on response (Y_1), while increase in pH of buffer showed synergistic effect on the response. pH of buffer and flow rate combination found for having synergistic effect on response.

Figure 4(c): Three-dimensional plot for retention time as a function of flow rate and pH of buffer. Constant factor (% v/v of buffer- 40)

Fit summary: Quadratic model was suggested by the software.

ANOVA: ANOVA of developed Full three level factorial model for retention time (Y_1).

Values of "Prob > F" (p- value) less than 0.0500 indicate model terms are significant.
In this case A, B, BC, A^2 and C^2 are significant model terms.

Table 20: Significance of *p* value on model terms of retention time

Model terms	*p* value	Effect of factor	Remarks
A (X_1)	**0.0001**	-2.58	Significant
B (X_2)	**0.0115**	+0.50	Significant
C (X_3)	**0.0001**	-1.52	Significant
AB (X_1X_2)	0.5381	+0.14	Insignificant
AC (X_1X_3)	**0.0021**	+0.79	Significant
BC (X_2X_3)	0.6555	-0.099	Insignificant
A^2 (X^2_1)	**0.0002**	+1.45	Significant
B^2 (X^2_2)	0.1565	+0.46	Insignificant
C^2 (X^2_3)	**0.0061**	+0.96	Significant
Overall model	**0.0029**	-	**Significant**

3.4.2 Effect of independent variables on tailing factor (Y_2):

After applying experimental design, suggested Response Surface Linear Model was found to be significant with model F value of 23.79, *p* value less than 0.005 and R^2 value of 0.7563. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of % C.V. and adjusted R^2 were 3.78 and 0.7245 respectively. The model for response Y_2 (tailing factor) is as follows:

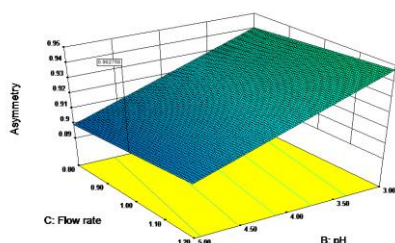$$Y_2 = +0.98 +0.071X_1-0.021X_2+3.333 \text{ E-003}X_3$$

Figure 5(a): Three-dimentional plot for tailing factor as a function of flow rate and amount of buffer. Constant factor (pH of buffer- 5)

Fig.6.(b) shows a graphical representation of pH of buffer (B) and amount of buffer (A), while flow rate (C) is maintained constant at its optimum of 1.0 mL min^{-1}. An increase in pH of buffer had antagonistic effect on response (Y_2) while increase in amount of ACN showed synergistic effect on it. Amount and pH of buffer combination showed antagonistic effect on response.
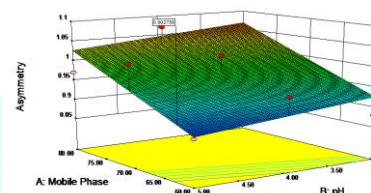

Figure 6(b): Three-dimentional plot for tailing factor as a function of pH of buffer and % v/v of buffer. Constant factor (flow rate- 1mL min^{-1})

Fig.7.(c) shows a graphical representation of pH of buffer (B) and flow rate (C), while amount of buffer (A) is maintained constant at its optimum 40 % v/v. An increase in pH of buffer and flow rate showed antagonistic effect on response (Y_2). Combination of flow rate and pH of buffer showed synergistic effect.
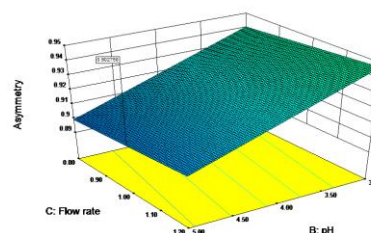

Figure 7(c): Three-dimentional plot for tailing factor as a function of flow rate and pH of buffer. Constant factor (amount of buffer)

Fit summary: Response Surface Linear Model was suggested by the software.

ANOVA: ANOVA of developed CCD model for tailing factor (Y_2).

Values of "Prob > F" (p- value) less than 0.0500 indicate model terms are significant.
In this case A, B are significant model terms.

Table 21: Significance of *p* value on model terms of tailing factor

Model terms	*p* value	Effect of factor	Remarks
A (X_1)	**0.090**	+0.071	Significant
B (X_2)	**0.0271**	-0.021	Significant
C (X_3)	0.7054	+3.333	Insignificant
Overall model	0.044	-	**Significant**

For response Y_2, factor X_1 and X_3 was having synergistic effect with *p* value 0.090 and 0.7054. Therefore we can conclude that increment in amount of ACN and flow rate was responsible for decrease in tailing factor and thus showed the direct relationship between them. Terms X_2 were responsible for significant increase in tailing factor with *p* values -0.0271. Therefore we can conclude that combination of factors showed inverse relationship with response. Factors amount of ACN and combination of flow rate and amount of buffer differs from zero with a great margin therefore they were having more significant effect than other factors.

3.4.3 Effect of independent variables on theoretical plates (Y_3):

After applying experimental design, suggested Response Surface Quadratic Model was found to be significant with model F value of 4.38, *p* value less than 0.005 and R^2 value of 0.6988. There is only a 0.43% chance that a "Model F-Value" this large could occur due to noise. Values of % C.V. and adjusted R^2 were 18.43 and 0.5394 respectively. The model for response Y_3 (theoretical plates) is as follows:

$$Y_3 = +6409.99 -1700.54X_1 +283.43X_2 -322.85X_3 -602.20X_1X_2 + 445.98X_1X_3 -323.61X_2X_3 +243.15 X_1^2 +510.74X_2^2 +2176.65X_3^2$$ (3)

Fig.8 .(a) shows a graphical representation of amount of ACN (A) and flow rate (C), while pH of buffer (B) is varying from 3-5.

An increase in flow rate and amount of ACN showed antagonistic effect on response (Y_3) individually, while their combination showed synergistic effect on response.[1,10].
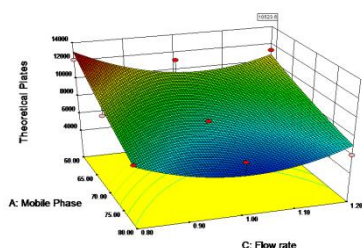

Figure 8(a): Three-dimensional plot for theoretical plates as a function of flow rate and amount of ACN. Constant factor pH-5

Fig.9.(b) shows a graphical representation of amount of ACN (A) and pH of buffer (B), while flow rate (C) is

maintained constant at its optimum value 1mL min^{-1}. An increase in pH of buffer showed increase in number of theoretical plates (Y_3), while increase in amount of ACN showed antagonistic effect on response. Combination of amount of buffer and pH of buffer showed antagonistic effect on it.
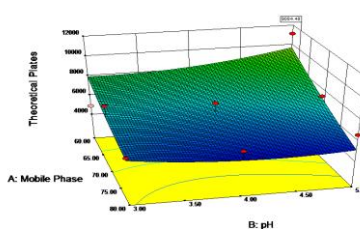

Figure 9(b): Three-dimensional plot for theoretical plates as a function of pH of buffer and % v/v of buffer. Constant factor (flow rate- 1 mL min^{-1})

Fig.10.(c) shows a graphical representation of flow rate (C) and pH of buffer (B), while amount of ACN (A) is maintained constant at its optimum value 70 % v/v. An increase in pH of buffer showed increase in number of theoretical plates (Y_3), while, increase in flow rate showed antagonistic effect on response. Combination of flow rate and pH of buffer showed antagonistic effect on response.
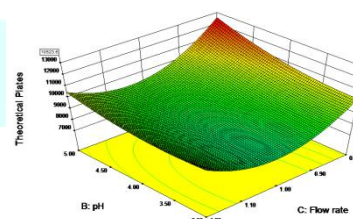

Figure 10(c): Three-dimensional plot for theoretical plates as a function of pH of buffer and flow rate. Constant factor (amount of buffer)

Fit summary: Quadratic model was suggested by the software

ANOVA: ANOVA of developed CCD model for theoretical plates (Y_3).

Values of "Prob > F" (p- value) less than 0.0500 indicate model terms are significant. In this case B, AC, B^2 and C^2 are significant model terms.

Table 22: Significance of *p* value on model terms of theoretical plates

Model terms	*p* value	Effect of factor	Remarks
A (X_1)	**0.0043**	-388.5	Significant
B (X_2)	0.4460	+240.4	Insignificant
C (X_3)	0.3866	-211	Insignificant
AB (X_1X_2)	0.1936	-419.2	Insignificant
AC (X_1X_3)	0.3302	+0.50	Insignificant
BC (X_2X_3)	0.4769	-78.1	Insignificant
A^2 (X^2_1)	0.7040	-210.2	Insignificant
B^2 (X^2_2)	0.4282	+387.2	Insignificant
C^2 (X^2_3)	**0.0030**	+73.2	Significant
Overall model	**0.0134**	**-**	**Significant**

For response Y_3, factor pH of buffer was having synergistic effect with *p* value 0.4460. Therefore we can conclude that increment in pH of buffer was responsible for increment in theoretical plates and thus showed the direct relationship between them.

Combination of amount of ACN and flow rate was responsible for significant increase in theoretical plates with significant *p* value of 0.0043. Therefore direct relationship between this combination and response is observed. Exponential terms (X^2_2) and (X^2_3) also showed direct relationship with response having significant *p* values 0.4282 and 0.0030 respectively. Factor pH of buffer and its exponential term differs from zero with a great margin therefore they were having more significant effect than other factors. Therefore it is concluded that pH of buffer and its exponential term was responsible for significant change in response i.e. nos. of theoretical plates. [10, 11,12,13,14]

3.5 Analytical Method Development

Different mobile phases were investigated to develop the suitable HPLC method for the analysis of Efavirenz in formulations. For the selection of media the criteria employed was sensitivity of the method, ease of sample preparation, miscibility of the drug, cost of solvents and applicability of method to various purposes. Retention time and peak area of EFV in the selected medium at respective wavelengths were determined and compared with the reference standards and formulation also.[9,10]
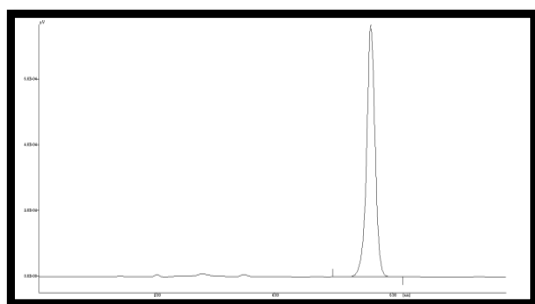
Chromatogram of Efavirenz

Figure 11: A typical chromatogram of Efavirenz

The applied chromatographic conditions permitted a good separation of Efavirenz in a short retention time of 5.642min. (Fig.11), no drug decomposition was observed during the analysis. The LC method was validated for the parameters reported below.

3.6 System suitability study

According to USP, system suitability tests are integral part of liquid chromatographic methods. Retention time, peak area and number of theoretical plates were calculated for standard solutions. The obtained data was found to be within acceptable limits.

Table 23: System suitability parameters

Sr. No.	Parameters	Observation
1	Retention time	5.6
2	Peak area	764985.5
3	Theoretical plates	8182.42

3.7 Analytical Validation

3.7.1 Preparation of Calibration Curve

Stock solution was prepared using 10mg of Efavirenz in 10ml of methanol and further dilutions were done with mobile phase. Solutions were sonicated for 5 min in Ultrasonic clean bath and manually 20µl was injected through the Rheodyne injector. Five concentrations were taken 5-25µg/ml for the calibration curve at 292nm. Linearity graph for Efavirenz is shown in Figure no 12 and Overly spectra of Efavirenz Linearity shown in figure no 13.[6]
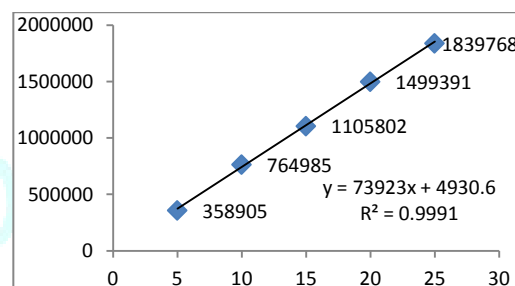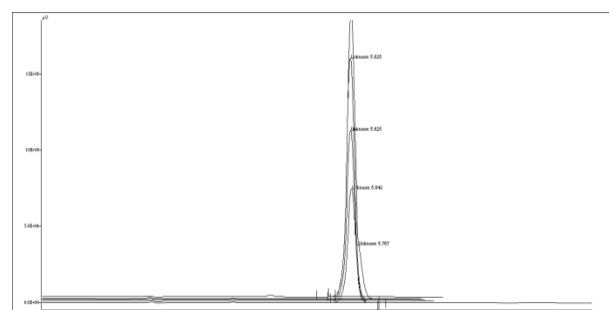

Figure 12: Linearity graph for Efavirenz

Figure 13: Overly spectra of Efavirenz Linearity

3.7.2 Selectivity

10 tab of EFV formulation were triturated in mortar pestle and 35mg (10 mg of pure drug) of tab including other excipients was transferred in 10 ml volumetric flask and volume was adjusted with methanol. Peak area and retention time of formulation was compared with the API of the drug. Typical peak of Efavirenz is shown in figure no 14.[7]
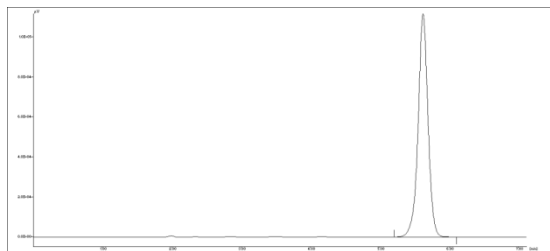
Figure 14: A typical chromatogram of Efavirenz [Concentration 15ug/ml]

3.7.3 Sensitivity

The sensitivity of measurement of EFV by use of the proposed method was estimated in terms of the limit of detection (LOD) and the limit of quantification (LOQ). The LOD and LOQ were calculated by the use of signal to noise

ratio. In order to estimate the LOD and LOQ values, the blank sample was injected six times and the peak area of this blank was calculated as noise level. The LOD was calculated as three times the noise level, while ten times the noise value gave the LOQ. LOD and LOQ were found to be 0.126 and 0.420.[9]

3.7.4 Accuracy

Accuracy of analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness. Accuracy should be established across the specified range of 98-102 %.

For accuracy three concentrations of five replicates were prepared and injected to get the peak area. Results obtained are shown in table 101.[11]

Table 24: Accuracy results of EFV by RP-HPLC

Concentration (ug/ml)	Peak area	Concentration Found (µg/ml)	Accuracy
7	276510	7.034880	100.498296
17	671362.4	17.25204161	101.4825
22	854793	21.99847332	99.9930

3.7.5 Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between the series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed

conditions. Method of precision was accessed on two levels, intra and intermediate precision. Solutions of known concentration were prepared in replicates and were injected to get the peak area. Results obtained are shown in table 99.[12]

Table 25: System Precision results for EFV by RP-HPLC

Sr. No.	Concentration (µg/ml)	Intraday precision (Area)	Interday precision (Area)
1	20	1504212	1512836
2	20	1504696	1516408
3	20	1525934	1538275
4	20	1499974	1502956
5	20	1506282	1519144
6	20	1485784	1495784
Mean		1504480.333	1514233.833
Std. Dev.		12911.11344	14672.11126
%RSD		0.858177615	0.968946205

3.7.6 Specificity

Chromatogram of EFV showed peak at a retention time of 5.018 min. The mobile phase designed for the method resolved the drug very efficiently; The Retention time of Efavirenz was 5.020 ± 0.0078min. The wavelength 292

nm was selected for detection because; it resulted in better detection sensitivity for the drug. The peak for Efavirenz from the tablet formulations was identified by comparing its retention time and Area of peak with those of standard Efavirenz. Recovery was found to be 99.28 ± 0.004. The result was shown in table 107.[13]

Table 26: Recovery results of EFV by RP-HPLC

Sample	Label Claim mg	Amount found* (mg)	Recovery ± SD* (%)	Retention time
Tab	600	599.96	99.28 ± 0.004	5.6 ± 0.0078 min

3.7.7 Repeatability

Demonstration of precision was done under two categories. The injection repeatability (System Precision) was assessed by using six injections of the standard solution of EFV

and the % RSD of the replicate injections was calculated. Results obtained are shown in table 100.[14]

Table 27: Repeatability results for EFV by RP-HPLC

Sr. no	Concentration µg/ml	Peak Area
1	20	1490913
2	20	1497975
3	20	1499391
4	20	1516768
5	20	1499974
6	20	1516768
Mean		1503631.5
Std. Dev		10679.4255
% RSD		0.710242204

3.7.8 Robustness

Robustness is a measure of capacity of a method to remain unaffected by small, but deliberate variations in the method conditions, and is indications of the reliability of the method. A method is robust, if it is unaffected by small changes in operating conditions. To determine the robustness of this method, the experimental conditions were deliberately altered at three different levels and retention time and chromatographic response were evaluated. One factor at a time was changed to study the effect. Variation of mobile phase composition (72:28 v/v), flow rate by 1 ml/min (0.9 and 1.1 ml/min), Variation of wavelength by 247 nm (245nm and 247nm), Variations in pH by 5 (4.8 and 5.2) had no significant effect on the retention time and chromatographic response of 10 µg/ml solution, indicating that the method was robust, The result was shown in table 102, 103,104 and 105.[6,7.]

Mobile Phase

Table 28: Results of Robustness (Mobile Phase) for EFV by RP-HPLC

Buffer	Acetonitrile	Retention time
30	70	5.9
28	72	5.6
26	74	5.3
	%RSD	1.8

Wavelength

Table 29: Results of Robustness (Wavelength) for EFV by RP-HPLC

Wavelength	Peak area
245	742144.500
247	735436.709
249	724684.750
%RSD	1.2

Flow rate

Table 30: Results of Robustness (Flow rate) for EFV by RP-HPLC

Flow rate	Retention time
0.9 ml/min	6.1
1 ml/min	5.6
1.1 ml/min	5.1
%RSD	1.7

pH of mobile phase

Table 31: Results of Robustness (pH) for EFV by RP-HPLC

Sr. No	pH	Peak area
1	4.8	742144.500
2	5	765436.709
3	5.2	784684.750
	%RSD	1.2

3.7.9 Recovery

The recovery of the method was determined by use of standard additions at three different levels, i.e. multiple-level recovery studies. Preanalysed samples of EFV were spiked with extra 80, 100 and 120 % of the standard EFV and the mixtures were reanalysed by the proposed method and the % recovery was determined. Values were found to be within the limits and are presented in table 106.[2,3.]

Table 32: Recovery results of EFV by RP-HPLC

Sr. No	Amount of sample (µg/ml)	Amount of drug added (µg/ml)	Amount Recovered* (µg/ml)	% Recovery ± SD*
1	10	8	7.96	98.88 ± 0.31
2	10	10	9.88	99.01 ± 0.58
3	10	12	11.72	98.46 ± 0.41

4. SUMMARY AND CONCLUSION

7.1 Determination of Efavirenz by HPLC.

In this project, as per our objective RP-HPLC method was developed by implementing QbD methodology (RSM and 3 level factorial design) on analytical column- reversed phase Waters XBridge C18 (250mm×4.6mm×5µm) with mobile phase Amonium format: Acetonitrile (28:72 v/v). The flow rate used was 1 mL /min and UV detection was carried out at 247 nm. The retention time for Efavirenz was found to be 5.6 min. Before method optimization, screening studies were carried out on different mobile phases, varying composition and pH. Based on the results obtained from these studies, suitable mobile phase with appropriate composition and pH

was selected and utilized for method development using QbD methodology.

The RP-HPLC method developed for estimation of Efavirenz was validated as per ICH guidelines using various parameters.

Linearity for the drug by the proposed method was determined to study its ability to elicit test results which are directly proportional to the concentration of analyte in the sample response and was found to be in concentration range 5 to 25 µg/mL (R2 = 0.998).

The limit of detection (LOD) and limit of quantitation (LOQ) was established at a signal-to-noise ratio. LOD and LOQ were calculated as $3.3 \times \delta/S$ and $10 \times \delta/S$ respectively as per ICH

guidelines, where δ is the standard deviation of the response (y-intercept) and S is the slope of the calibration plot. LOD was found to be 0.126µg/ mL and LOQ was found to 0.420 µg/ mL

System suitability test ensures that the analytical system is working properly and can give accurate and precise results. System suitability tests includes resolution, tailing factor, number of theoretical plates, capacity factor etc. The results of all system suitability parameters were acceptable in their limits defined by official guidelines.

The proposed high-performance liquid chromatographic method has also been evaluated over the accuracy, precision and robustness and proved to be convenient and effective for the quality control of Efavirenz. Developed method was found simple and cost effective for the quality control of Efavirenz.

Moreover, the lower solvent consumption along with the short analytical run time of 10.0 min leads to a cost effective and environmentally friendly chromatographic procedure. Thus, the proposed methodology is rapid, selective, requires a simple sample preparation procedure, and represents a good procedure for Efavirenz.

Table 33: Results of the quantitative determination of Efavirenz.

Sr. no	Parameters	HPLC observations
1	Concentration range (µg mL-1)	5-25
2	Regression equation	y = 73923x + 4930.6
3	Correlation coefficient	0.998
4	LOD (µg mL-1)	0.126
5	LOQ (µg mL-1)	0.420

5. FUTURE SCOPE

QbD methodology can be applied for Bioanalytical method development and its validation for estimation of EFV.

QbD methodology can be applied for stability indicating studies of EFV.

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