International Journal of Pharmacy
Journal Homepage: http://www.pharmascholars.com

Research article **CODEN: IJPNL6**

RP-HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF EMTRICITABINE, RILPIVIRINE AND TENOFOVIR EMPLOYING RESPONSE SURFACE DESIGN

T.Sudha[1]*, P.Shanmugasundram[2]

[1]Department of Pharmaceutical Analysis, The Erode College of Pharmacy, Erode, India
[2]Department of pharmaceutical Analysis, School of pharmaceutical Sciences, Vels University, Chennai, India

***Corresponding author e-mail:** jvchrsty@yahoo.co.in

ABSTRACT

A high performance liquid chromatographic method has been developed and optimized for the simultaneous determination of emtricitabine, rilpivirine and tenofovir in bulk and in tablet dosage form. In this work, multiple response simultaneous optimization using Derringer's desirability function was employed for the development. The ranges of three experimental factors used for the optimization were acetonitrile concentration (50 -60%), buffer pH (2.5-3.5) and flow rate (0.8-1.2ml/min). The influence of these independent variables on the output responses such as capacity factor of the first peak (k_1), resolution ($Rs_{2,3}$) and retention time (Rt) were evaluated. The experimental responses were fitted into a second order polynomial equation. The optimized assay conditions were acetonitrile: phosphate buffer (69.7: 30.3%v/v) (pH 2.5) as the mobile phase and flow rate at 1.2 ml/min. While using this optimum condition, the total run time was less than 7 min were achieved. The optimized assay condition was validated according to ICH guidelines to confirm specificity, linearity accuracy and precision.

Keywords: Central composite design, Response surface design, Emtricitabine, Rilpivirine, Tenofovir, HPLC.

INTRODUCTION

In statistical experimental design, also known as design of experiments (DOE) is the methodology of how to conduct and plan of experiments in order to extract the maximum amount of information in the fewest number of analyses. The application of mathematical, statistical and logical principle of chemistry, i.e. chemometrics offers a sound alternative for optimization of chemical system and process. Hence it is applied to determine in an efficient way the set of condition that are required to obtain a product or process with desirable, often optimal characteristics [1]. Experimental design has been used for separation optimization [2-4] and for validation in RP-HPLC method [5-7]. The experimental design has shown utility in pharmaceutical development. Multivariate methods are based on the design of an experimental plan (i.e. a series of experiments in each of which the values for several parameters are changed at the same time). The results of these experiments are then evaluated using simple statistical methods like analysis of variance and regression analysis. The main experimental designs that are carried out include screening and optimization designs. With the first one it is possible to determine the parameters that have an effect, interaction effects among these parameters, influence of this interaction (positive or negative), and their significance. The most commonly used designs are fractional and full factorial designs at two levels for each parameter. In order to find the optimum, optimization designs such as Box-Behnken design or Central composite designs are used [8]. The central composite design is a widely used design for optimization of chromatographic system [9]. This design consists of a two –level factorial design and additional axial points. The

experiments will approximate the surface of a sphere. The factorial points will contribute in estimating the interaction terms and the axial points will contribute in estimating the quadratic terms. It is necessary to include at least one center point in the design. This design is often formed in this way and its result can be used to determine a quadratic response surface that has curvature and can be used to predict factor levels that produce maximum or minimum response values [10]. However the HPLC method intended to be applied for the pharmaceutical or industrial environment, the analysis time is usually optimized without losing resolution [11]. When one needs to optimize more than one response at a time the use of multicriteria decision making (MCDM) is the better choice. The different approaches of MCDM include the path of the steepest ascent, constrained optimization procedure, pareto-optimality, utility function and Derringer's desirability function. When there is a mix of linear and non- linear responses, or when all response models are of linear or non -linear, pareto-optimality, utility function or Derringer's desirability function can be used. There are many ways in which the individual desirabilities can be combined. If the combined criterion is a simple arithmetic average, it is called as utility function and if it is a geometric mean it is referred as Derringer's desirability function. The idea of combining desirabilities as geometric mean was first presented by Harrington [12] but it was put into a more general form by Derringer [13]. The advantage of the Derringer's desirability function is that if one of the criteria has an unacceptable value, then the overall product will also be unacceptable, while the utility function this is not the case. Further, Derringer's method offers the user flexibility in the definition of desirability function. Derringer's desirability function was introduced in the chromatography by Deming implementing resolution and analysis time as objective function to improve separation quality. The Derringer's desirability function was applied to explore the user flexibility of this technique in selecting optimum chromatographic condition for the determination of drugs in variety of sample matrices.

Emtricitabine and tenofovir drugs are nucleoside reverse transcriptase inhibitor. They are combined with a new antiretroviral drug rilpivirine. It is a non – nucleoside transcriptase inhibitor. Three drugs FDC comprising of Emtricitabine, tenofovir and rilpivirine form one of the first line regimens in HIV therapy. Emtricitabine (Fig 1) is an analogue of cytidine. The drug works by inhibiting reverse transcriptase, the enzyme that copies HIVRNA into new viral DNA. Tenofovir (Fig 1) inhibits the activity of HIV reverse transcriptase by competing with the natural substrate deoxyadenosine 5'-triphosphate and, after incorporation into DNA, by DNA chain termination. Rilpivirine (Fig 1) is a diarylpyrimidine. It inhibits HIV-1 replication by non-competitive inhibition of HIV-1 reverse transcriptase (RT). Rilpivirine does not inhibit the human cellular DNA polymerases α, β and γ. In literature survey simultaneous estimation of emtricitabine, tenofovir disoproxil fumarate and rilpivirine in bulk form by RP-HPLC and RP-UPLC methods were reported [14-15].Bioequivalence of the emtricitabine, rilpivirine and tenofovir disoproxil fumarate single tablet regimen and combination therapies, effectiveness, and adherence in patients with HIV infection: clinical utility of a single tablet of emtricitabine, rilpivirine and tenofovir were reported [16-17]. On the other hand, several methods have been cited in the literature for the estimation of tenofovir [18-22], emtricitabine [23-27] and rilpivirine [28-32] individually. In spite of that, an intensive search exposed to the best of our knowledge that there are only few works describing the method for the simultaneous determination of these drugs in pharmaceutical formulations. However, no method related to the chemometric optimization for the simultaneous determination of these drugs using HPLC in the commercial pharmaceutical mixture is available. Therefore, in the present study a HPLC method was developed, optimized and validated for the determination of EMF, RPV and TEN in bulk and in tablet formulation. In order to understand the sensitivity of the chromatographic factors on the separation analytes and to simultaneous optimization of resolution and analysis time, chemometric protocols of response surface design and Derringer's desirability function were successfully employed.

MATERIALS AND METHODS:

Chemicals and Reagents: Pure standards of Tenofovir and Emtricitabine were obtained from M/S Pharma Train, (Hyderabad, India). Rilpivirine pure sample was purchased from Yarrow Chem products, (Mumbai, India). Tablet formulation of Complera (300 mg of tenofovir, 25 mg of rilpivirine and 200 mg of emtricitabine) was purchased from local pharmacy. The reagents were purchased as follows: methanol HPLC grade was procured from Merck (Mumbai, India). Dipotassium hydrogen phosphate, potassium dihydrogen phosphate and phosphoric acid were obtained from SD fine Chemicals (Mumbai, India). The HPLC grade water was prepared by using Milli - Q Academic, Millipore (Bangalore, India).

Chromatographic conditions: High performance liquid chromatography method was performed with Shimadzu prominence equipment comprising LC20AD solvent delivery modules, SPD 20A UV-

visible detector, a Rheodyne model 7125 injection valve fitted with a 20µl loop, and SPD-20A detector. Compounds were separated on a 250 mm X 4.6 mm i.d., 5µm particle, phenomenex, Gemini C_{18} column and a personal computer. The equipment was situated in an air conditioned laboratory (20±2˚ C). The chromatographic software Autochro 3000 (Shimadzu) was used for data acquisition and treatment of chromatographic data.

Preparation of Standard solution: About 20 mg of Emtricitabine, 30 mg of tenofovir and 2.5 mg of rilpivirine were weighed accurately and transferred into a 100 ml volumetric flask. 10 ml of the mobile phase was added and sonicated for 15 min. and the volume was made up to 100 ml with the mobile phase. From the standard stock solution 10 ml of the solution was pipetted out and transferred into a 100 ml volumetric flask. Then it was made up to the volume with mobile phase to get a concentration of 20 µg/ml for emtricitabine, 30µg/ml for tenofovir and 2.5 µg/ml for rilpivirine.

Preparation of sample solution: Ten tablets were accurately weighed and crushed into fine powder. The powder equivalent to one tablet (200 mg of Emtricitabine, 300 mg of Tenofovir and 25 mg of rilpivirine) was taken in a 100 ml volumetric flask. About 50 ml of diluent was added, shaken for 5 minutes on a rotatory shaker and then sonicated for 20 minutes with intermediate shaking. After that the volume was finally made up to the mark with 100 ml. A sample solution was centrifuged at 500 rpm for 5 minutes to get a clear solution. Then 10 ml of supernatant solution was diluted and made up to the volume with 100 ml diluent. 1 ml of the above solution was pipetted out and transferred into a 10 ml volumetric flask and made up to the volume with the same. Then it was filtered through a 0.45µ membrane filter. So the final concentrations were 20 µg / ml for emtricitabine, 30 µg / ml for tenofovir and 2.5 µg / ml for rilpivirine.

RESULTS AND DISCUSSION

Column chemistry (C_{18}), solvent type (MeCN or MeOH), solvent strength and flow rate were then varied to determine the best chromatographic conditions that give quality separation. The mobile phase conditions were optimized such that the first eluting component does not interfere with the peaks of solvent and excipient. Other criteria like analysis time, appropriate *k* range ($1<k<10$) for eluted peaks, tailing factor, assay sensitivity and noise were also considered. The analytes emtricitabine, tenofovir and rilpivirine were predominantly polar and have low molecular mass. Therefore a phenomenex Gemini C_{18} column (150 mm X 4.6mm i.d., 5 µm) and mobile phase consisted of MeCN: phosphate buffer (pH 3.0) were tried to examine initial separation conditions. Different ratios of mobile phases MeCN: phosphate buffer (pH 3.0) (50:50, 60:40, 40:60, 55:45%v/v) were tried. Among these, the mobile phase composition ratio 55:45% v/v resulted in a quality separation in terms of peak symmetry, optimum resolution and reasonable run time.

Before starting on optimization procedure it is important to investigate the curvature term using factorial design with center points. ANOVA generated 2^K factorial design showed that curvature was significant for all the responses (k_1, $Rs_{2,3}$, tR_3) since p value was less than 0.05. This implied that quadratic model should be considered to model the separation process. In order to obtain the second order predictive model, central composite design (CCD) - a design type under response methodology - was employed. CCD was chosen due to its flexibility and it could be applied to optimize an HPLC separation by gaining better understanding of factor's main and interaction effects. The selection of factors for optimization was based on preliminary experiment and prior knowledge from literature as well as certain instrumental limitations. From preliminary experiments a Gemini C_{18} column stationary phase and mobile phase consisted of MeCN: phosphate buffer (pH 3.0) was employed. The volume of phosphate buffer in the mobile phase was fixed at (45%) and only MeCN content was varied. The mobile phase flow rate could also moderately influence selectivity in HPLC analysis. Therefore the key factors selected for optimization process were MeCN concentration (A), Buffer pH (B) and flow rate (C). Table 1 showed the levels of each factor studied for finding out the optimum values and responses. In table 1 the ranges of each factor used were MeCN concentration (50-60%v/v), buffer pH (2.5-3.5) and flow rate (0.8- 1.2 ml / min). As response variables, the capacity factor for the first eluted peak Tenofovir (k_1), the resolution between two peaks tenofovir and emtricitabine ($Rs_{2,3}$), the retention time of the last peak rilpivirine (tR_3) were selected. For an experimental design with the three factors, including linear, quadratic and cross terms, the model can be expressed as $Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2$

where Y is the response to be modeled, β is the regression coefficient and X_1, X_2 and X_3 represent factors A, B and C respectively. Statistical parameters obtained from ANOVA for the reduced models were given in table 2. The insignificant terms

($p > 0.05$) were eliminated from the model through backward elimination process to obtain a simple and realistic model. Since R^2 [33] always decreases when a regressor variable is eliminated from a regression model, in statistical modeling the adjusted R^2 which takes the number of regressor variables into account, is usually selected.

Table 2 showed that the adjusted R^2 values were well within the acceptable limits of $R^2 \geq 0.80$ [34], which revealed that the experimental data showed a good fit with second order polynomial equations. For all the reduced models *p* value < 0.05 was obtained, implying these models were significant. The adequate precision value is a measure of the signal (response) to noise (deviation) ratio. A ratio greater than 4 is desirable [35]. The ratio was found to be in the range from 12.00 to 18.22 which indicated an adequate signal and therefore the model was significant for the separation process. The coefficient of variation (C.V) is a measure of reproducibility of the model and as a general rule a model can be considered reasonably reproducible if it is less than 10%.

In table 2 the interaction terms with the largest term coefficient among the fitted model was BC (+ 0.54) of $Rs_{2,3}$ model. The positive interaction between B and C was statistically significant (< 0.0001) for $Rs_{2,3}$. The existence of such interactions emphasized the necessity to carry out active multifactor experiments for the optimization of chromatographic separation. In order to gain a better understanding of the results the predicted models were presented in the form of perturbation plot (Fig 2a, 2b, 2c) and 3D response surface plot (Fig 3a, 3b, 3c)**.** Variables giving quadratic and interaction terms with the largest absolute coefficients in the fitted models were chosen for the axes of the response surface plots. Consequently, factors A and C were selected for the response plots of $k_{1,}$ $Rs_{2,3}$ and tR_3 with factor B held constant usually at central value of phosphate buffer pH 3.00. All these three dimensional plots were beneficial to gain an overall understanding of the influence of phosphate buffer pH and flow rate on analysis time ($Rs_{2,3}$). Perturbation plots provide silhouette views of the response surface plots, where it shows how the response changes as each factor moves from a chosen reference point, with all other factors held constant at the reference value.

A steepest slope or curvature indicates the sensitiveness of the response to a specific factor. Figure 2b showed that phosphate buffer pH (factor B) had most the important effect on resolution between emtricitabine and rilpivirine $Rs_{2,3}$ followed by factor C and then factor A. The rest of the factors (MeCN concentration and flow rate) had significant effect on tR_3 and k_1. When k_1 and tR_3 values were increased, the level of MeCN concentration (factor A) increased and when k_1 and tR_3 values decreased, the level of flow rate (factor C) increased. Analysis of the perturbation plot and response surface plot of optimization models revealed that factor B and C had the significant effect on separation of analytes, whereas the factor A, MeCN concentration was of little significance. Derringer's desirability function was employed for global optimization of three responses and to select different optimal conditions for the analysis of formulation. The criteria for the optimization of each individual response were shown in table 3.

From the above table it could be seen under the column criteria that the response of tR_3 was minimized in order to shorten the analysis time and the response of $Rs_{2,3}$ was minimized to allow the base line separation of emtricitabine and rilpivirine. In order to separate the first eluting peak of tenofovir from the solvent front, $k_1$was maximized. Importance could range from 1 to 5 which gave emphasis to a target value. Following the conditions and restrictions above, the optimization procedure was carried out. The response surface obtained for the global desirability function was presented in figure 4. From the figure it could be concluded that there was a set of coordinates producing high desirability value (D = 0.701), MeCN concentration 69.7%, buffer pH 2.5, and flow rate of 1.2 ml/min. The optimized formulation assay conditions were using Gemini C_{18} column with MeCN: phosphate buffer pH 2.5 (69.7:30.3%v/v) as mobile phase at a flow rate of 1.2 ml/min and UV detection at 280 nm. The predicted response values corresponding to the latter value of D were $K_1 = 0.95$, $Rs_{2,3} = 3.50$, $tR_3 = 6.60$ minutes. The agreement between experimental and predicted responses under optimal conditions was shown in table 4 and the optimized chromatogram was shown in figure 5.

Method Validation: The proposed method was validated as per ICH guidelines [36-37]

Linearity: The linearity study was conducted for standard stock solutions of tenofovir, emtricitabine and Rilpivirine. For the construction of calibration curves, five calibration standard solutions were prepared over the concentration range of 10-50µg/ml for tenofovir, emtricitabine and 4-12µg/ml for rilpivirine. The results were summarized in table 5. It showed good correlation between analytes peak area and concentration with $r^2 > 0.9998$ (n = 6).

Limit of detection and Limit of Quantification (LOD and LOQ): Limit of detection and limit of quantification were calculated from the linearity

studies. Linearity study was performed three times and the value of slope and intercept were calculated from the calibration curve. It was used to calculate the LOD and LOQ values. LOD and LOQ were calculated by using the following formulas
LOD = 3.3 x std. dev / slope, LOQ = 10 x std. dev / slope
Limit of detection was found to be 0.0085µg/ml, 0.23µg/ml and 0.26 µg / ml for tenofovir, emtricitabine and rilpivirine respectively. Limit of quantification was found to be 0.025 µg / ml, 0.7041µg/ml and 0.8137 µg / ml for tenofovir, emtricitabine and rilpivirine respectively. The report was shown in table 5.

Precision: The precision of the method was confirmed by intraday analysis. The analyses of standards were carried out five times in the same day. The percentage RSD value of the intraday analysis of analytes was found to be 0.99 for tenofovir, 1.35 for emtricitabine and 0.46 for rilpivirine. The %RSD value was found to be less than 2%. This indicated that the developed method had good precision with repeatability.

Accuracy: To evaluate the accuracy of the method, known amount of pure drugs were added to the previously analysed solution of formulation and the mixture was analysed by the proposed method. The amount of drug recoveries was calculated. The percentage recovery was found to be in the range of 99.25 % - 99.84%. The % RSD values were found to be less than 2%. This indicated that there was no interference due to excipients used. Hence the accuracy of the method was confirmed.

Ruggedness: It refers to the precision of a lab over multiple days which may include multiple analysts, multiple instruments and different sources of the reagents. The developed method was validated for ruggedness. It was confirmed by using different analysts. The percentage RSD values were found to be less than 2% for three analytes. Hence the precision was further confirmed. The results were shown in table 5.

CONCLUSION

The analytes Emtricitabine, Rilpivirine and Tenofovir had been simultaneously analysed in pharmaceutical formulations by using HPLC. Time of analysis, resolution and quality of the peaks were simultaneously optimized by applying useful tools of chemometrics: response surface design and Derringer's desirability function. The results of the study demonstrated the benefit of applying this approach in selecting optimum conditions for the determinations of drugs in pharmaceutical formulations. This method reduced overall assay development time and provided essential information regarding the sensitivity of various chromatographic variables on separation attributes. The validation study supported the selection of the assay conditions by confirming that the assay was accurate, linear, precise and robust.

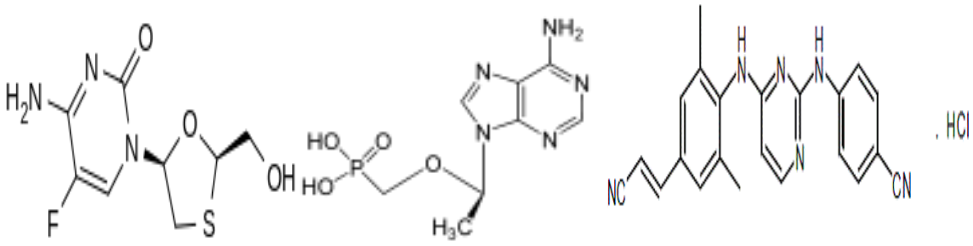

Figure1:The chemical structures of analytes

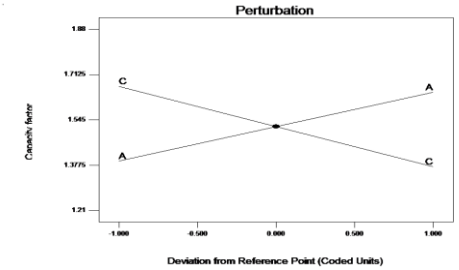

Figure 2(a): capacity factor

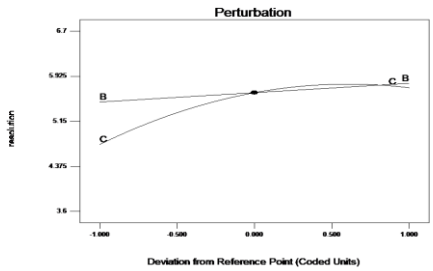

Figure 2(b): Resolution

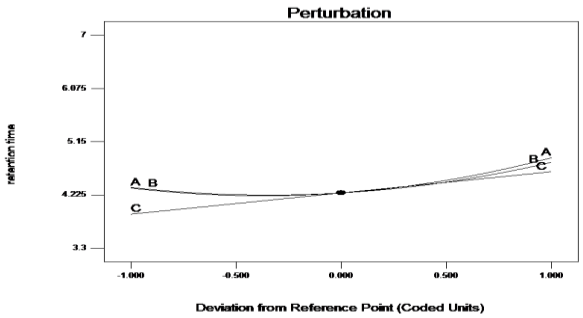

Figure 2(c): Retention time

Figure 2: Perturbation plots for responses

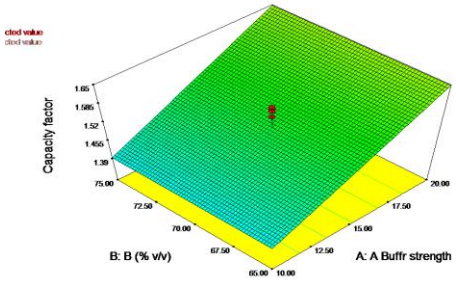

Figure 3(a): Capacity factor

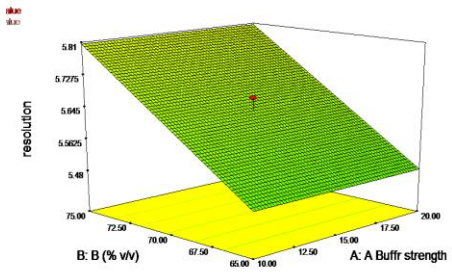

Figure 3(b): Resolution

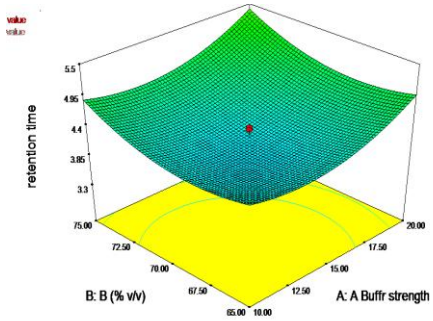

Figure 3(c): Retention time

Figure 3: Response surface plots for responses

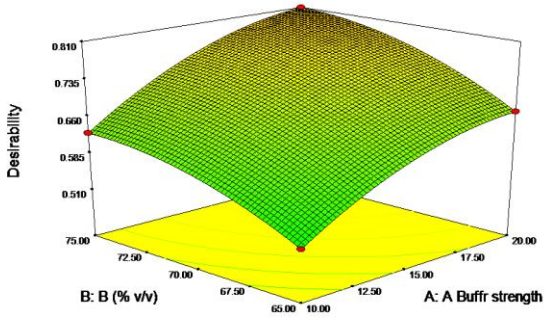

Figure 4: Graphical representation of overall desirability function

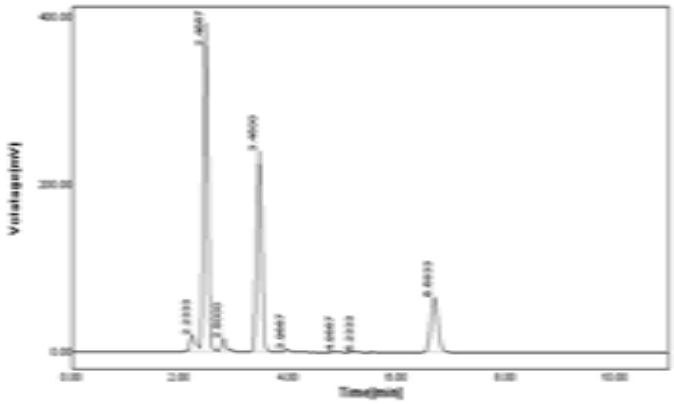

Figure 5: Chromatogram for optimized assay method

Table 1: Central composite arrangement and responses

Run	Type	ACN (%v/v)	pH	Flow rate ml/min	Capacity factor	resolution	Retention time
19	Fact	50.00	2.50	0.80	1.00	5.26	6.11
7	Fact	60.00	2.50	0.80	0.95	4.75	6.00
13	Fact	50.00	3.50	0.80	1.21	9.15	21.00
12	Fact	60.00	3.50	0.80	0.94	5.87	11.96
5	Fact	50.00	2.50	1.20	1.00	4.15	4.10
18	Fact	60.00	2.50	1.20	0.87	3.57	3.96
16	Fact	50.00	3.50	1.20	1.25	9.05	14.28
14	Fact	60.00	3.50	1.20	0.92	5.71	8.20
20	Axial	46.59	3.00	1.00	0.83	6.21	11.56
10	Axial	63.41	3.00	1.00	0.91	3.54	6.68
9	Axial	55.00	2.16	1.00	1.10	2.26	4.58
8	Axial	55.00	3.84	1.00	0.91	5.00	7.50
11	Axial	55.00	3.0	0.66	1.14	5.57	14.7
17	Axial	55.00	3.0	1.34	1.00	1.84	4.36
6	Center	55.00	3.0	1.00	0.91	3.82	9.30
15	Center	55.00	3.0	1.00	0.93	3.83	9.32
3	Center	55.00	3.0	1.00	0.94	3.80	9.30
1	Center	55.00	3.0	1.00	0.92	3.82	9.33
2	Center	55.00	3.0	1.00	0.91	3.84	9.34
4	Center	55.00	3.0	1.00	0.92	3.80	9.30

Table 2: Reduced response surfaces models and statistical parameters obtained from ANOVA

Responses Regression model Adjusted R^2	Model p value	% C.V	Adequate Precision
K_1 1.52+0.13A-0.15C0.8117	<0.0001	5.19	18.049
$Rs_{2,3}$ +5.65+0.16B+0.49C 0.8212+0.54BC-0.40C^2	<0.0001	5.60	18.229
tR_3+4.26+0.26A+0.22B+0.37C0.8388+0.50AC+0.42BC+0.35A^2 +0.31B^2	<0.0001	8.53	12.008

Table 3: Criteria for the Optimization of the Individual Responses

Response	Lower limit	Upper limit	Criteria/Goal
k_1	0.83	1.25	Maximize
$Rs_{2,3}$	3.0	5.0	Minimize
tR_2	3.96	21	Minimize

Table 4: The comparison of experimental and predictive values of different objective functions under optimal conditions

Optimum conditions	Acetonitrile (%v/v)	Buffer (pH)	Flow rate (ml/min)	K_1	$Rs_{2,3}$	tR_3
Predictive	69.7	2.52	1.2	0.95	3.50	6.60
Experimental	69.7	2.52	1.2	0.91	3.54	6.68
Average error				4.21	1.14	1.2
Desirability value= 0.701						

Table 5: Reports for validation parameters

Parameters	Tenofovir	Rilpivirine	Emtricitabine
Range(µgmL-1)	10-50	4-12	10-50
Y=mx + c	Y=40652.2X+221309	Y=376839X+4698107	Y=398106X+423656
Regression coefficient	0.9998	0.9995	0.9996
Slope (m)	40652.2	376839	398106
Intercept (c)	221309	4698107	423656
LOD (µgmL-1)	0.0082	0.2685	0.2320
LOQ(µgmL-1)	0.0250	0.8137	0.7041
Accuracy (%RSD)	0.37	0.49	0.52
Precision(%RSD)	0.99	0.46	1.35
Ruggedness			
Analyst I(%RSD)	1.250	1.232	1.417
Analyst II(%RSD)	1.313	0.950	0.733

REFERENCES

1. Massart DL, Vandeginste BGM, Buydens LMC, De Jong S, Lewi PJ, Smeyers-Verbeke J. Handbook of Chemometrics and Qualimetrics, Part A., Amsterdam; Elsevier: 1997, pp. 643.
2. Araujo P. Trends anal Chem, 2000; 19(9): 524-529.
3. Gennaro MC, Marengo E, Gianotti V, Angioi S. J Chromatogr A, 2002; 945(1-2):287.
4. Chen J, Glancy K, Chen X, Alasandro M. J Chromatogr A, 2001; 917(1-2): 63-73.
5. Christine Ye,June Liu,Feiyan Ren,Ngozi Okafo. J Pharm Biomed. Anal, 2000; 23 (2-3): 581-589.
6. Ficarra R, Ficarra P, Tommasini S, Melardi S, Calabro ML, Fluranetto S, Semreen M. J Pharm Biomed Anal, 2000; 23 (1): 169-174.
7. Ficarra R, Calabro ML, Cutroneo P, Tommasini S, Melardi S, Semreen M. Fluranetto S, Ficarra P, AltavillaG. J Pharm Biomed Anal, 2002; 29(6): 1097 -1103.
8. Ana B. Baranda, Nestor Etxebarria, Rosa M. Jimenez, Rosa M. J Chromatogr Sci, 2005; 43:505-512.
9. Ferreira SLC, Bruns RE, Da Silva EGP, Dos Santos WNL, Quintella CM, David JM, De Andrade JB, Breitkreitz MC, Jardim ICSF, Neto BB. J Chromatogr A, 2007; 1158: 2–14.
10. Myers RH, Montgomery DC, Anderson Cook CM. Response surface Methodology, Process and product Optimization Using Designed Experiments. New York; John Wiley and Sons: 1995.
11. Deming SN. J Chromatogr, 1991; 550:15-25.
12. Harrington EC. Ind Qual cont, 1965; 21(10): 494-498.
13. Derringer G, Suich R. J Qual techn, 1980; 12(4): 214-218.
14. Pranitha D, Vanitha C, Prince Francis, M. Alagar Raja, P. Vishnu vardan, Surendar M, David Banji. J Pharm Res, 2012; 5(8): 4600-4602.
15. Kavitha KY, Geetha G, Hariprasad R, Venkatnarayana R, Subramanian G. Int J Compre Pharma,2013;4(1):1-6.
16. Mathias A, Menning M, Wiser L, Wei X, Dave A, Chuck S, Kearney BP. J Bioequivalence & Bioavailability, 2012; 4(7):100.
17. Weinberg, Mark A. Research & Palliative Care, 2013; 5:41.
18. Kandagal PB, Manjunatha DH, Seetharamappa J, Kalanur S. Analytical Letters, 2008; 41(4):561-570.
19. Gnanarajan G, Gupta AK , Juyal V, Kumar P, Yadav PK, Kailash P. Pharma methods, 2009; 1(4): 351-353.
20. Choi SO, Rezk N, Kim JS, Kashuba ADM. J Chromato Sci, 2010; 48(3): 219–223.
21. Yadav M, Mishra T, Singhal P, Goswami S, Shrivastav PS. J Chromato Sci, 2009; 47(2): 140–148.
22. Shirkhedkar Atul A, Bhirud Charushila H, Surana Sanjay. J Res Chem Env, 2008; 12(1): 49–50.
23. Peepliwal A, Bonde CG. J Pharma Res, 2010; 3(8): 1712–1715.
24. Droste JAH, Aarnoutse RE, Burger DM. J. Liq. Chromatogr. Related Techn, 2007; 30(18): 2769–2778.
25. Pendela M, Mamade DA, Hoogmartens J, Van Schepdael A, Adams E.Talanta,2010; 82(1):125–128.
26. Ashenafi D, Verbeek A, Hoogmartens J, Adams E. J. Separation Science, 2009; 32(11): 1823–1830.
27. Seshachalam U, Haribabu B, Chandrasekhar KB. J. Separation Science, 2007; 30(7): 999–1004.
28. Sudha T, Shanmugasundram P. World. J Pharma Res, 2012; 1(4): 1183-1196.
29. Masthanamma SK, Alekhya Gottumukkala. Int J Pharma Sci Res, 2014; 5(2): 483-489.
30. Addepalli Raju V, Appala Raju Nemala. Asian J Biomed Pharma. Sci,2013; 3(21): 23-29,
31. Venkata Reddiah CH, Prama Devi K, Mukkanti K. Int J Pharma Pharmac Sci, 2012; 4 (3):595-599,
32. Mohanareddy Chilukuri, Hussian Reddy K, Narayanareddy P, Venkataramana M. Int J Clin Pharmacology and toxicology, 2012; 1(1): 1-13,
33. Parajo JC, Alonso JL, Lage MA, Vazquez D. Bioprocess Engineering,1992; 8: 129-136,
34. Lundstedt T, Seifert E, Abramo L, Thelin B, Nystrom A, Pettersen J, Bergman R. Chemom Intell Lab System, 1998; 42(1-2):3–40.
35. Beg Q, Sahai V, Gupta R. Process Biochem, 2003; 39(2): 203-209.
36. International conference on Harmonization guidance for Industry In: Q2A Text on validation of Analytical methods. Switzerland; IFPMIA: 1994, pp. 1-4.
37. International conference on Harmonization guidance for Industry In: Q2B Text on validation of Analytical methods. Switzerland; IFPMIA: 1996, pp. 1-8.