Development and Validation of a Rapid Chemometric Assisted LC Assay with PDA Detection Method for the Simultaneous Estimation of HIV Tablet Containing Emtricitabine, Tenofovir Disoproxil Fumarate in Three Component Bulk and Pharmaceutical Dosage Forms Containing Rilpivirine

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Abstract: This report describes a rapid, precise, simple and accurate method was developed and optimized RP-HPLC method for the simultaneous determination of emtricitabine, tenofovir disoproxil fumarate with rilpivirine hydrochloride in bulk and commercial pharmaceutical preparations. The analysis has been performed by employing Phenomenex C18 analytical column (150 mm × 4.6 mm i.d. 5 μm) and the optimum conditions predicted were MeCN-potassium dihydrogen phosphate buffer (20 mM, pH 3.3)-triethylamine 58.72:41.23:0.05 (v/v) as mobile phase The detection was carried out at 270 nm. Emtricitabine, tenofovir and rilpivirine were eluted at 2.508, 3.402 and 4.297 respectively, at a mobile phase flow rate 1.7ml/min. The method was validated according to ICH guidelines. The method was validated for specificity, precision, linearity, accuracy, limits of detection, limits of quantitation and robustness. The linearity of emtricitabine, tenofovir and rilpivirine were in the range of 40-72, 60-108 & 5-9µg/ml respectively. This optimized method was validated and has been successively applied to bulk and Commercial formulation and no interference from the tablet excipients was found. Hence, it can be employed for the routine analysis in quality control laboratories.

Key words: Centralcompositedesign · Derringer’s Desirability Function · ICH · RP-HPLC · Emtricitabine · Tenofovir Disoproxil Fumarate · Rilpivirine

INTRODUCTION

Three drug FDC comprising of emtricitabine, tenofovir disoproxil fumarate, rilpivirine form one of the first line regimens in HIV-Therapy [1]. Emtricitabine (EMT) chemically it is 5-fluoro-1-(2R, 5S)-[2 - (Hydroxymethyl) -1,3 - oxathiolan - 5-y] cytosine which differs from other cytidine analogs, in that it has a fluorine in 5th position Fig. 1. Tenofovir disoproxil fumarate chemically it is 9 [(R) 2[bis [Isopropoxycarbonyl] oxy] Methoxy] phosphinyl] methoxy propyl] adenine fumarate. Both drugs block the enzyme reverse transcriptase, an essential enzyme that is required for the replication of viral DNA [2]. Rilpivirine chemically it is a-[4- (4-[(1E)-2-cyanoeth-1-en-1-yl]-2,6 dimethylphenyl] amino) pyrimidin-2-yl] amino] benzonitrile. It is next generation non nucleoside reverse transcriptase inhibitor (NNRTIs) for the treatment of HIV. NNRTIs block HIV replication by inhibiting HIV reverse transcriptase.

Literature indicates spectrophotometry [3-6], HPLC [7-9], HPTLC [10] and LC/MS/MS [11, 12] methods for determination of TEN individually and in combination with other drugs in pharmaceutical formulations, drug substance and biological matrices. Similarly for EMT individually and in combination with other drugs by UV [13], HPLC in pharmaceutical formulations, drug substance and biological matrices [14-17], LC/MS/MS [18] and Stability indicating liquid chromatographic methods [19] were reported. A detailed literature survey for RPV revealed that few analytical methods are available using Spectrophotometric [20], HPLC [21] & HPTLC [22], individually. However, an intensive literature search revealed to the best of our knowledge that only two methods are available for the simultaneous estimation of these analytes EMT, TDF and RPV in pharmaceutical mixtures [23, 24]. In the reported methods the analysis time is more than 10 minutes and these methods didn’t apply a systematic optimization procedure for the
separation and quantitation of these analytes. But these methods employed a time consuming trial and error approach resulting only in an apparent optimum and information concerning the sensitivity of the factors on the analytes separation and interaction between factors are not available.

Conversely this manuscript describes the development and validation of an isocratic RP-HPLC method for the routine quality control analysis of EMT, TDF & RPV in a pharmaceutical laboratory using design of experiment approach. Response Surface methodology from Central Composite was used to optimize the developed method and provide information on their interaction effects on the separation characteristics. In the commencement, the factorial design was employed to mark the importance of the curvature term for all the chromatographic responses \((k, R_s, R_t)\). Subsequently, the chromatographic factors that had the plentiful effects were optimized using a central composite design and response surface methodology [25,26].

**MATERIAL AND METHODS**

**Chemicals and Reagents:** Working standards of tenofovir, emtricitabine and rilpivirine were donated by Strides arco Ltd, Bangalore, India. Acetonitrile (MeCN) was of HPLC grade and potassium dihydrogen phosphate and triethylamine were of analytical reagent grade purchased from SD Fine Chemicals, Mumbai, India. The HPLC grade water was prepared by using Milli-Q Academic, Millipore and Bangalore, India. The tablet Complera was purchased from the local market.

**Standard Solutions:** Stock standard solutions of EMT, TDF and RPV were prepared in mobile phase. Working standard solutions were freshly obtained by diluting the stock standard solutions with mobile phase during the day of analysis. The standard solution prepared for the optimization procedure constituted EMT, TDF and RPV at 56.0, 84.0, 7.0μg/ml, respectively.

**Sample Preparation:** Twenty tablets were weighed and powdered. An amount of tablet powder equivalent to 40mg of EMT, 60 mg of TDF and 5 mg of RPV were accurately weighed and transferred into a 100 ml volumetric flask. This mixture was subjected to sonication for 10 min for complete extraction of drugs. From the above solution 7ml of solution was pipetted out into 50ml volumetric flask. The solution was made up to the mark with a mobile phase to obtain a concentrations of EMT, TDF, RPV as 56.0, 84.0, 7.0 μg/ml respectively.

**Chromatographic Conditions:** Chromatographic separations were carried out on a Phenomenex C\textsuperscript{18} analytical column (150 mm × 4.6 mm i.d. 5 μm). The mobile phase consisted of MeCN-potassium dihydrogen phosphate buffer (20mM, pH 3.3) - triethylamine. Wavelength of 270 nm was selected for detection. The injection volume of the sample was 20 μl. The HPLC system was used in an air conditioned laboratory atmosphere.

**Instrumentation:** Chromatographic analysis was performed on a Shimadzu HPLC which contains SPD M20A PDA detector, a rheodyne injector valve with a 20 μl loop volume and Shimadzu chromatographic software LC Solution assisted for data collections and processing. The mobile phase was degassed using Branson sonicator (Branson Ultrasonic’s Corporation, USA). The weighing was done on a Sartorius balance and all pH measurements were done on pH meter.

**Software:** Experimental design, data analysis and desirability function calculations were performed by using Design-Expert trial version 7.0.0. (Stat-Ease Inc. Minneapolis).

**Design of Experiments:** Preliminary experiments indicated that the variables, such as MeCNconcentration, buffer concentration and flow rate were the main factors that
affected the capacity factor of the first peak $k_1$ resolutions of the 2nd and 3rd peak and Retention time of the third peak $t_R$. Thus, a central composite rotatable design-response surface methodology (CCRD-RSM) was used to methodically examine the influence of these three critical variables on the responses above said. The details of the design are listed in Table 1. For each factor, the experimental range was selected on the basis of the results of preliminary experiments. The value range of the variables was MeCN concentration (A) of 55% to 60% V/V, buffer concentration (B) of 10 to 20 mM and flow rate (C) of 1.30 to 1.70 mL/min A total of 20 tests were conducted. All the formulations in these experiments were prepared in duplicate.

**Validation:** Validation studies were conducted using the optimized assay conditions based on the principles of validation described in the ICH guidelines “Text on Validation of Analytical Procedures” and “Q2B, Validation of Analytical Procedures: Methodology” [27]. Key analytical parameters including specificity, accuracy, precision, linearity, detection limit and quantification limit were evaluated. For specificity study, placebo containing lactose monohydrate, Croscarmellose sodium, Povidone, Microcrystalline Cellulose, titanium dioxide and lactose monohydrate, Croscarmellose sodium, Povidone, Microcrystalline Cellulose, titanium dioxide and magnesium stearate was used. Linearity were established over the concentration range of 40-72 µg/ml for EMT 60-108 µg/ml for TDF & 5-9 µg/ml for RPV. LOD & LOQ were calculated from the standard deviation ($\sigma$) of the response and the slope (S) of the calibration curve in accordance to the following equation: $3.3\sigma/S$ and $10\sigma/S$. Also, robustness of the proposed method was assessed with respect to small alterations in the MeCN concentration (58.72 ± 0.5%), the buffer concentration (20 ± 2.0 mm) and the flow rate (1.7± 2.0 ml/min).

**RESULTS AND DISCUSSION**

**Optimization of Formula:** The central composite rotatable design-response surface methodology (CCRD-RSM) constitutes an alternative approach because it offers the possibility of investigating a high number of variables at different levels with only a limited number of experiments. The variables in Table 1 were chosen taking into account our preliminary experiments. Table 4 showed the experimental results concerning the tested variables on the capacity factor of the first peak $k_1$, resolutions of the 2nd and 3rd peak and Retention time of the third peak $t_R$. The three dependent values ranged from 1.19 to 2.26, 2.08 to 8.97 and 3.41 to 12.61. A mathematical relationship between factors and parameters was generated by response surface regression analysis using Design-Expert® 7.0 software.

It is momentous to scrutinize the curvature term utilizing CCD with centre points before starting the optimization procedure. ANNOVA generated for CCD exhibited the curvature is significant for all three responses. Since p value less than 0.05, quadratic model should be used. The mathematical equation of quadratic model of the three independent factors is given in equation 1.

$$Y= \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_1X_2 + \beta_5X_1X_3 + \beta_6X_2X_3 + \beta_7X_1^2 + \beta_8X_2^2 + \beta_9X_3^2$$

(1)

The statistical analysis of the results generated the following polynomial equations:

- Capacity factor of the first peak $k_1 = +1.52-0.32xA+0.11xA^2$
- Resolutions of the 2nd and 3rd peak $R_{s3} = +5.26-1.93xA-0.074xB-0.53xB^2$
- Retention time of the third peak $t_R = +6.86 - 2.25xA - 0.033xB - 1.90xC + 0.44xAxC + 0.48xA^2C + 0.37xBB^2 + 0.21xC^2$

Where A, B and C represent the coded values of the MeCN concentration, buffer concentration and flow rate respectively.

Statistical parameters obtained from ANOVA for the reduced models are 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. The adjusted $R^2$ values were well within the acceptable limits of $R^2 = 0.80$ [28] revealing that the experimental data fits the second-order polynomial equation. $P$ value of <0.05 is obtained for all the reduced models, implying the significance. The adequate precision was found to be in the range of 32.59-42.04 indicating an adequate signal and the model is significant for the separation process [29]. The coefficient of variation (C.V) a measure of reproducibility of the model was found to less than 10% and could be considered reasonably reproducible.

The perturbation plots and three-dimensional (3D) response surface graphs for the most statistical significant variables on the evaluated parameters are shown in Fig. 2 & 3.

The response surface diagrams showed that changing the fraction of MeCN from low to high results in a rapid decline in the retention time of RPV both at the low
Table 1: Experimental responses and central composite rotatable design measures

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<tr>
<th>Design Points</th>
<th>MeCN (%v/v)</th>
<th>Buffer conc.</th>
<th>Flow rate (ml/min)</th>
<th>K₁</th>
<th>Rₛ₂₃</th>
<th>tᵣ³</th>
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<td>6.84</td>
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Table 2: Statistical parameters obtained from ANNOVA for CCD

<table>
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<tr>
<th>Responses</th>
<th>Regression model</th>
<th>Adjusted R²</th>
<th>Model P value</th>
<th>% C.V</th>
<th>Adequate precision</th>
</tr>
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<td>K₁</td>
<td>A1.52 - 0.32 x A + 0.11 x A²</td>
<td>0.9201</td>
<td>&lt;0.0001</td>
<td>5.04</td>
<td>32.599</td>
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<td>Rₛ₂₃</td>
<td>A + 5.26 - 1.93 x A - 0.074 x B - 0.53 x B²</td>
<td>0.9602</td>
<td>&lt;0.0001</td>
<td>7.05</td>
<td>42.049</td>
</tr>
<tr>
<td>tᵣ³</td>
<td>A + 6.86 - 2.25 x A - 0.033 x B - 1.90 x C + 0.44 x AxC + 0.48 x A² x B² + 0.21 x C²</td>
<td>0.9791</td>
<td>&lt;0.0001</td>
<td>5.31</td>
<td>36.060</td>
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</table>

Fig. 2: Perturbation plots showing the effect of each of the independent variables on (a) K₁, (b) Rₛ₂₃, and (c) tᵣ³. Where A is the concentration of acetonitrile, B the buffer molarity and C the mobile phase flow rate.
Fig. 3: Response surfaces related to percentage acetonitrile concentration and Flow rate: (a) Capacity factor of the first peak \( k_1 \), (b) resolution of the second and third peak \( R_{S_{2,3}} \), (c) retention time of \( t_{R_3} \).

Fig. 4: Response surface Bar graph showing for the global desirability function and high level of buffer molarity. An increase in the buffer molarity results in a marginal decrease in \( t_{R_3} \) at a low level of factor A. This may be due to reduced silanol effects as a result of higher buffer molarity used. Setting the MeCN concentration at its lowest level, the buffer concentration has to be at its highest level to shorten \( t_{R_3} \). Especially this interaction is synergistic, as it led to a decrease in run time. The existence of such interactions explains the need to carry out active multifactor experiments for optimization of chromatographic separations.

Derringer’s desirability function was used to optimize three responses with distinct targets [30]. In this study the known criteria for the optimization were: capacity factor of the first peak, resolution between the critical peaks \( R_{S_{2,3}} \), and elution time of third peak \( t_{R_3} \). The criteria for the optimization of each individual response are shown in Table 3.

Criteria have been anticipated for selecting an optimum experimental condition for analyzing routine quality control samples. In order to separate the first eluting peak from the solvent front, \( K_1 \) was targeted at 1.4.
**Fig. 5:** Chromatograms from bottom to top corresponding to (a) a placebo solution (b) Synthetic mixture of EMT, TDF and RPV (56, 84 & 7 µg/ml respectively) (c) Real sample of Complera tablets containing EMT, TDF and RPV under optimum assay conditions for formulation.

The responses \( tR_3 \) was minimized, in order to shorten the analysis time while \( Rs_{2,3} \) was minimized to allow baseline separation of TDF and RPV. Importance can range from 1 to 5, which gives prominence to a target value. The optimization procedure was carried out with the above said conditions and restrictions.

The response surface plots obtained for the global desirability function Fig. 4 it can be concluded that there was a set of coordinates producing high desirability value (\( D = 0.923 \)) were MeCN concentration of 58.72 % w/v, 20mM buffer concentration and flow rate of 1.70 ml/min. The predicted response values corresponding to the latter value of D were: \( K1 = 1.4 \), \( Rs_{2,3} = 3.869 \) and \( tR_3 = 4.170 \) min.

The experiments could be performed under the optimal condition and the prediction efficiency of the model would be confirmed. The corresponding chromatogram is given in Fig. 5.

To investigate the predictability of the proposed model, the agreements between experimental and predicted responses for the predicted optimums are shown in Table 4. The Percentage of prediction error was calculated by Eq. (2). The average errors for \( K1 \), \( Rs_{2,3} \) and \( Rt_3 \) were 4.71, 3.94 and 4.40% respectively, indicating good correlation between the experimental and the predicted responses.

\[
\text{Percentage Error} = \frac{\text{Experimental} - \text{Predicted}}{\text{Predicted}} \times 100 \quad (2)
\]

**Assay Method Validation:** The present study was to check method’s validation for specificity, linearity, accuracy, intra/inter-day precision and robustness. The optimized HPLC method was specific in relation to the placebo used in this study. All placebo chromatograms showed no interference peaks. An excellent linearity was established at five concentration levels in the range of 40-72 µg/ml for EMT, 60-108 µg/ml for TDF and 5-9 µg/ml for RPV with \( R^2 \) of more than 0.999 for all the analytes. The slope and intercept of the calibration curve were 28963 and 8148.6 for EMT, 38969 and +3862.6 for TDF and 48818 and +5257.2 for RPV respectively. The LODs were 1.72, 2.11 and 2.76 ng/mL and the LOQs were 0.26, 0.42 and 0.182 ng/mL for EMT, TDF and RPV respectively. Accuracy (\( n = 9 \)) was in the range of 98 -102%; the values of standard deviation and% R.S.D. were found to be <2% shows the high accuracy of the method. The intra and inter-assay precision (\( n = 6 \)) was confirmed since, the %RSD were well within the target criterion of \( = 2\%\). Robustness study reveals that small changes did not alter the retention times, retention factor and resolutions and therefore it would be concluded that the method conditions are robust.

**CONCLUSION**

The selective isocratic RP-HPLC method developed, optimized and validated is very effective and sensitive. While using this optimum condition, baseline separation with minimum resolution of 2.0 and an analysis time of less than 5.0 min compared with the reported methods. Analysis time, resolution and quality of the peaks were simultaneously optimized by employing Central composite rotatable design. The validation study confirms the assay
was specific, accurate, linear, precise and robust. Therefore, this HPLC method can be used in routine quality control analysis in pharmaceutical industry.

REFERENCES