RESEARCH ARTICLE

Development, Optimization, and Validation of a RP-HPLC Method with PDA Detection for the Concurrent Quantification of Emtricitabine, Tenofovir Alafenamide, and Dolutegravir in Both Bulk and Pharmaceutical Dosage Forms

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ABSTRACT

A simple, quick, precise and accurate HPLC (reverse phase) approach has been designed and optimized using a chemometric tool to estimate dolutegravir, emtricitabine and tenofovir alafenamide, respectively. A design of central composite was used to optimize the response surface. As a result of trial and error, the parameters such as rate of solvent flow, buffer pH, and methanol concentration in the solvent system were determined. System responsiveness was estimated using the optimization process's capacity factor, resolution, and retention time. The separation was achieved by using a solvent system containing 58.90% methanol and 41.10 triethylamine buffer (4.56 pH) at the rate of flow of 0.8 mL per minute on a phenomenex carbon (18) column (150.4 4.6 mm; I.D., 5) under optimal conditions. The duration of retention for emtricitabine was 2.91 minutes, for tenofovir alafenamide it was 6.114 minutes; and for dolutegravir it was 8.824 minutes. A correlation coefficient of 0.9998, 0.9997, and 0.9999 was determined for emtricitabine's calibration curves from 40 to 200 g/mL, tenofovir alafenamide's from 5 to 25 g/mL, and emtricitabine's from 10 to 50 g/mL, respectively. This approach was effective in estimating the simultaneous doses of medications in commercial combination forms.

Keywords: Central composite design, Optimum conditions, RP-HPLC, Validation, Emtricitabine, Tenofovir alafenamide, Dolutegravir.

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INTRODUCTION

By competing with deoxycytidine 5'-triphosphate, emtricitabine 5'-triphosphate inhibits HIV-1 reverse transcription in addition to incorporating into a nascent viral DNA chain, causing it to terminate. Emtricitabine 5'-triphosphate has a moderate inhibitory effect on mammalian DNA polymerase 1, 2, and 3. Chemically, called (5) Fluro (1) (2R,5S) (2-[hydroxy-methyl] (1) (3) oxathiolan (5) yl) cytosine. Emtricitabine 5'-triphosphate is formed by pyro phosphorylation of emtricitabine, which is an analog of cytidine in nucleoside form.[1, 2]

A prodrug for tenofovir known as tenofovir alafenamide is available in the oral form (Figure 1b). The intracellular concentration of tenofovir-diphosphate (TDP) of tenofovir alafenamide fumarate (TAF) is greater than that of TDP because TAF circulates 90% less in the blood. There are chemical names for tenofovir alafenamide (TA). One of those is propan-2-yl (2S)-2-[(S)-((2R)-1-(6-amino-9H-purin-9-yl) propan-2-yl] oxy-methyl) (phenoxy) phosphoryl] amino propanoate.[3,4]

As shown in Figure 1, dolutegravir (Figure 1c) is a monocarboxylic acid amide derived from (4R,12aS) by conventional condensation of its carboxyl group with its amino group, which is 2,4-difluorobenzylamine-7-hydroxy-4-methyl-6-8-dioxo-3-4-6-8-12-12a-hexa-hydro-2H-pyrido- [1',2':4,5] pyrazino[2,1-b] [1,3] oxazine (9) carboxylic acid. Salts of this drug are used to treat HIV-1 (as their sodium salts). HIV-1

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integration is inhibited by this compound. As an organic heterotricyclic molecule derived from a mono-carboxylic acid and an organo-fluoride chemical, it is called monocarboxylic acid. It is a conjugate of dolutegravir.[5]

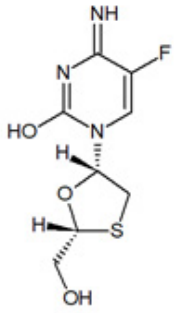
Various analytical techniques have been reported for determining these selected drugs, including dolutegravir, tenofovir, and emtricitabine. These include UV-spectroscopy[6], HPTLC[7], HPLC[8], UPLC[9], as well as LC/MS[10] in biological samples. RP-HPLC was used to simultaneously quantitate EMTR, TENO ALA, and DOLU, but no publication discusses the chemometrics methodology used.

Creating and improving HPLC procedures[11] might be a clever process that requires careful consideration of a number of aspects. It has taken decades to refine high-performance liquid chromatography (HPLC) procedures, but this has only provided an apparent optimum without knowing the sensitivity of the parameters to separation and interactions between the analytes. Therefore, any chemometric techniques, such as the overlapped resolution maps, the factorial design12, and the response surface approach,[13,14] may be helpful. To simultaneously quantify EMTR, TENO ALA, and DOLU, a fast, easy, and reliable RP-HPLC technique was developed and validated using the DoE technique. An optimization method based on the central composite design is employed in response surface methodology. Using the chemometric technique, we can establish parameters for analysis by identifying the variables that influence the chromatographic behavior of the compounds we are studying.

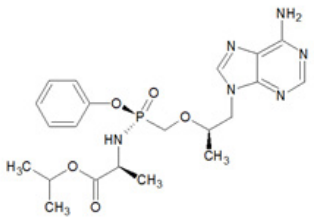
METHODOLOGY

Materials Used

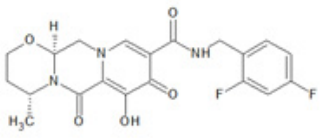
EMTR, TENO ALA, and DOLU working standards were gifts from Emcure Pharmaceuticals Ltd. in Jammu, Pune, India. Qualigens Fine Chemical Pvt. Ltd., Mumbai, provided Orthophosphoric acid, triethyl amine (AL grade) and methanol (HPLC grade). Milli-Q grade water provided by Qualigens was collected. The tablet formulation Spegra contains 200 mg of emtricitabine, 25 mg of tenofovir alafenamide, and 50 mg of dolutegravir were purchased from a local pharmacy.



(A) Emtricitabine



(B) Tenofovir Alafenamide



(C) Dolutegravir

Figure 1: Structure for Analytes

Condition Criteria (instrumentation)

A Shimadzu HPLC system is made up of the following components: Pump (LC 20AD) for solvent delivery, 20 micro litre capacity of heodyne loop injector, a Photodiode array detector (SPD M20A), data collecting and processing unit (R & LC Solution). A Phenomenex Luna C-18 column (150 mm i.d., 5-micron size of the particle) employed methanol-orthophosphoric acid buffer (pH 4.5) (60:40v/v) at a flow rate of 1.0 mL per minute was employed as the stationary phase. Detection at 265 nm was performed with PDA detectors. A 0.45 micron membrane filter was used to filter solvent system before use.

Solvent System Preparation

A mixture of 0.6-liter methanol and 0.4-liter ortho phosphoric acid buffer (with a pH 4.5 adjusted with triethyl amine) was placed in an ultra-sonic water bath for 5 minutes to degas them. After filtration with a 0.45 pore filter under vacuum, the mixture was transferred to a 1 litre volumetric flask.

Standard Working Stock Solution Preparation

Precisely measured quantities of 40 mg emtricitabine, 5 mg tenofovir alafenamide, and 10 mg dolutegravir were introduced to 100 mL volumetric flask. The mixture was mixed and sonicated for 15 minutes, followed by the addition of 10 mL of mobile phase. In 3 mL was pipetted and transferred from the standard stock solution to 10 mL volumetric flask. To achieve concentrations of 30 µg per mL for emtricitabine, 15 µg per mL for tenofovir alafenamide, and 120 µg per mL for dolutegravir, the flask was subsequently filled with the mobile phase solution.

Sample Solution Preparation

After accurately weighing ten tablets, a fine powder was made by pulverizing the tablets. In a volumetric flask, 200 mg of emtricitabine, 25 mg of tenofovir alafenamide, and 50 mg of dolutegravir were combined into the powder equivalent of one Spegra tablet. An amount of 50 mL of solvent was introduced into a rotary shaker, agitated for 5 minutes, and then subjected to a sonication period of 20 minutes, intermittently shaken. It was eventually possible to adjust the volume to 100 mL. Five minutes of centrifugation at 500 rpm were required to achieve a clear solution from the sample mixture. The supernatant solution was diluted with a diluent of 10 mL (emtricitabine, tenofovir, and dolutegravir) after the supernatant was diluted with 2.0 mL of diluent. A pipette was used to draw 3.0 mL of the previously mentioned solution, transfer it, and then supplement it in a 10 mL volumetric flask with the same solution. After that, a membrane filter of 0.45 was used for filtration. The ultimate concentrations of emtricitabine were 120 µg per mL, tenofovir were 15 µg per mL, and dolutegravir were 30 µg per mL.

Table 1: Central composite arrangement and responses

Run	*Space type*	*Factor-1 methanol concentration*	*Factor-2 buffer pH*	*Factor 3 flow rate (mL/min)*	*Response 1 capacity factor (k_1)*	*Response 2 resolution ($rs_{2,3}$)*	*Response 3 retention time (rt_2)*
7	Center	60	4.5	1	0.963	7.41	4.438
10	Center	60	4.5	1.0	0.963	7.41	4.438
12	Center	60	4.5	1.0	0.963	7.41	4.438
17	Center	60	4.5	1.0	0.963	7.41	4.438
18	Center	60	4.5	1.0	0.963	7.41	4.438
19	Center	60	4.5	1.0	0.963	7.41	4.438
2	Axial	68.409	4.5	1	0.964	7.722	5.836
4	Axial	60	4.83	1	1.007	7.843	7.214
13	Axial	60	4.5	0.66	1.164	7.298	3.656
15	Axial	60	4.16	1	0.942	7.437	5.369
16	Axial	51.591	4.5	1	1.129	8.43	15.015
20	Axial	60	4.5	1.33	1.061	7.682	6.8
1	Factorial	55	4.3	1.2	0.964	7.722	5.836
3	Factorial	55	4.7	1.2	0.972	7.595	5.101
5	Factorial	65	4.7	1.2	0.967	7.232	4.165
6	Factorial	55	4.3	0.8	1.045	7.678	7.652
8	Factorial	65	4.3	1.2	1.07	7.738	8.735

Analytical Software

Stat-Ease Inc., headquartered in Minneapolis, provided the trial version 12.0.0 of Design-Expert for use in the experiment design, data analysis, and computation of the desirability function.

Experimental Design

Trial and error was utilized to choose the variables, such as Buffer, pH, the rate of flow, and the proportion of methanol. These variables were assessed in order of importance: retention time, resolution, and capacity. An in-depth analysis of these three variables, including flow rate, pH buffer, and methanol concentration in the mobile phase, was conducted using a CCD-RSM. Table 1 is a list of the design's specifics. The experimental range for each element was chosen based on the findings of trial studies. The variables' ranges were 50–70% by volume of methanol in the mobile phase (A), 0.8–1 mL/min for flow rate, and 4.3–4.7 for buffer pH. 20 experiments in total were run in a random sequence.

RESULTS AND DISCUSSION

The CCD -RSM is an alternative methodology since it allows for the investigation of a various parameters at various degree using a small number of tests. Table 1 displays the factors that were examined for capacity factor, resolution, and retention time and the experimental findings related to those variables. These variables were selected after considering the outcomes of our first studies. Response surface regression study utilizing Design-Expert software produced a mathematical link between variables and responses.

It is possible to express an experimental design incorporating linear, quadratic, and cross terms as follows:

$$y=\beta0+ \beta1\ X1 + \beta2\ X2 + \beta3\ X3 + \beta12\ X1\ X2 + \beta13\ X1X3 + \beta23\ X2X3 + \beta11\ X12 + \beta22\ X22 + \beta33\ X32$$

Where,

X1, X2, and X3 = components A, B, and C, and *y*= represents the reaction that has to be modeled.

Table 2 displays the statistical parameters from the condensed models calculated using ANOVA. The model was backward eliminated to remove an unimportant variable ($p > 0.05$). A regression model's adjusted R^2 is often used since it always drops when a regressor variable is removed from the equation.[15]

Experimental results showed a satisfactory fit with polynomial equations of second order, as the modified R^2 values fell within the permissible limits of $R^2 \geq 0.80$.[16] The *p-values* of all of the reduced models were below 0.05, indicating they were all significant. As a result of the acceptable precision value, the signal to noise (response) to deviation ratio is calculated. Ideally, there should be a ratio of at least 4.[17] Using the model, a sufficient signal was discovered within the range of 13.314 to 19.417, which was the crucial process of separation. A model's coefficient of variation, or CV, should be less than 10%. This parameter measures the repeatability of a model. Table 2 shows that among the models that were fitted, the interaction term with the highest coefficient was AB (+0.2966) of the $Rs_{2,3}$ model. Statistically significant interactions between A and B were observed in $Rs_{2,3}$ (<0.0001).

The fact that these interactions occur highlights the need for active multifactor research to optimize chromatographic separation. Figures 2 and 3 provide the projected models' perturbation plots and 3D responses surface plots, respectively, to help the reader better comprehend the findings. The response

Table 2: Analysis of variance using reduced response surface models

Retaliation	*Regression model*	R^2 *(adjucted)*	*p-value*	*% C. V*	*Adequate precision*
K_1	+0.9635-0.0369A+0.0144B-0.0359C-0.0459AB+0.0536AC-0.0346BC+0.0260A^2+0.0006B^2+0.0494C^2	0.9242	<0.0001	2.29	19.417
$Rs_{2,3}$	+7.43+0.0552A-0.1686B+0.2221C+0.2966AB-0.3299AC+0.2149BC+0.1388A^2-0.0203B^2-0.0734C^2	0.9303	<0.0001	8.02	14.815
tR_2	+4.47-0.9468A-0.5074B+0.0790C-0.6821AB+0.1774AC-0.0721BC+1.91A^2+0.4434B^2+0.0674C^2	0.8091	<0.0001	7.38	13.314

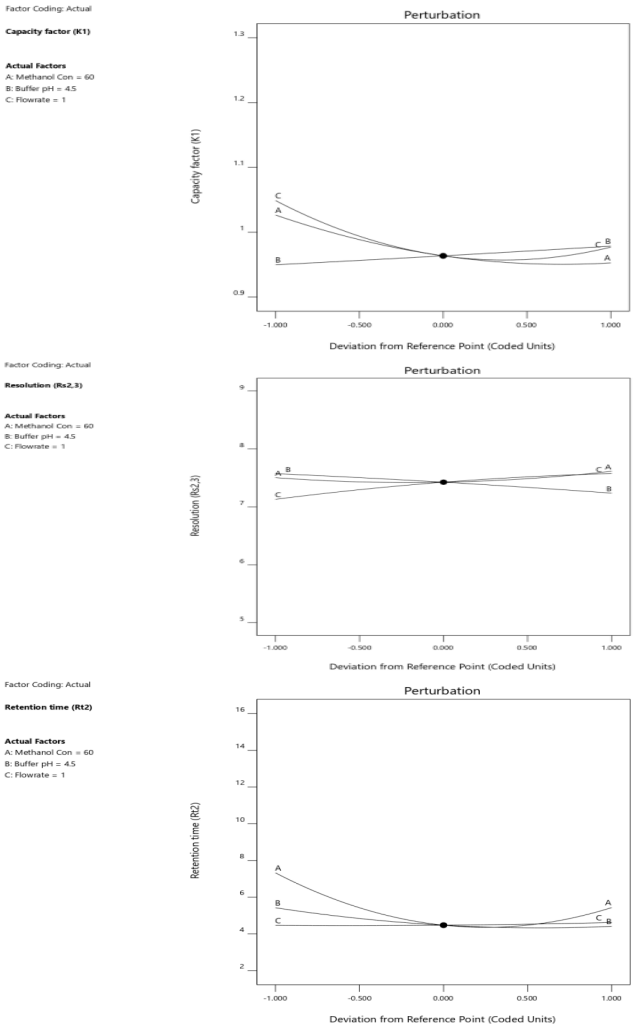


Figure 2: Perturbation plots for Responses (K_1, $Rs_{2,3}$, tR_2)

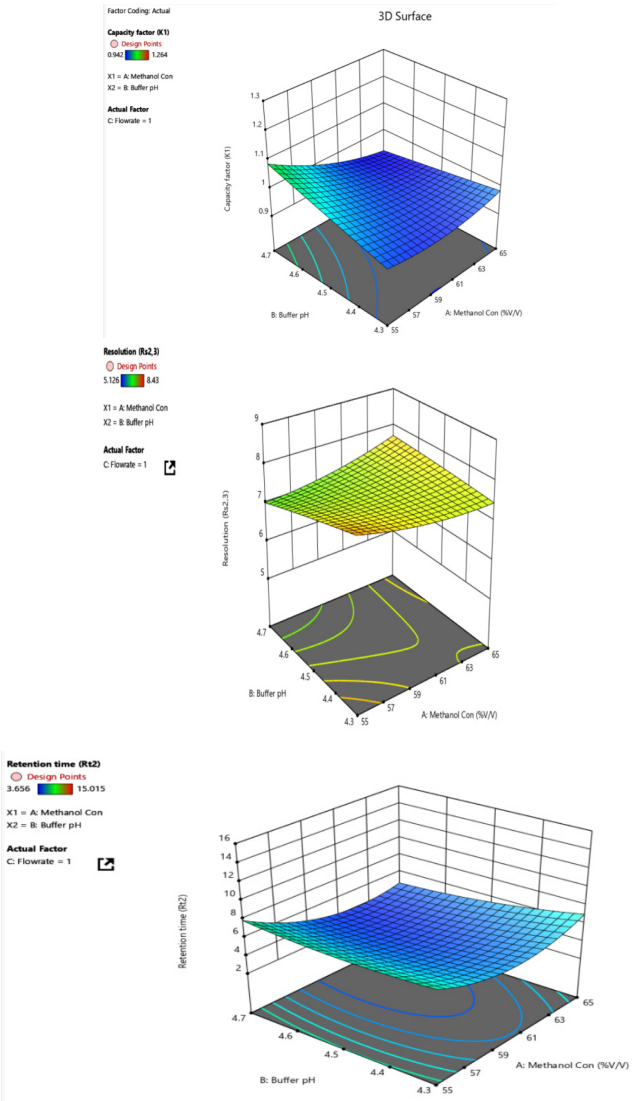


Figure 3: Response surface plots for Responses (K_1, $Rs_{2,3}$, tR_2)

surface plots were plotted using the variables with the largest absolute coefficients in the fitted models. For the response plots of k1, Rs2, 3, and tR2, factors A and C were selected for constant factor B at phosphate buffer pH 4.5. By comparing these three-dimensional maps, researchers can assess how pH and flow rate affect analysis time ($Rs_{2,3}$). Shadow plots demonstrate the responses of surface plots, which vary when elements deviate from selected reference points while keeping all other variables fixed.

The sharpest slope or curve shows the response's sensitivity to a particular element. Figure 2c demonstrated that factor B (phosphate buffer pH), followed by factors C and A, had the greatest impact on the retention period for tenofovir tR2. The other variables, including methanol content and flow rate, significantly impacted Rs2,.3, and k1. As k1 and Rs2,.3 values decreased, flow rate (factor C) increased, while

Table 3: Optimum individual response criteria

Retaliation	*Lower limit*	*Upper limit*	*Outcome*
k_1	0.94	1.26	Maximize
$Rs_{2,3}$	5.126	8.43	Minimize
tR_2	3.656	15.015	Minimize

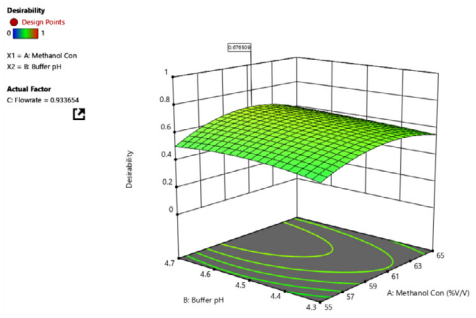


Figure 4: Visual diagram of overall desirability

methanol concentration (factor A) increased. Factor A and C of optimization models are determined by the analysis of perturbation plots and response surfaces, in contrast to factor B, phosphate buffer pH, significantly impacted the separation of analytes. Table 3 displays the standards for each individual response's optimization.

The table above shows the column criteria for separating emtricitabine, tenofovir, and dolutegravir. The responses of tR2 were minimized in order to save time during analysis. K1 was increased in order to best isolate emtricitabine's initial eluting peak from the solvent front. The importance is scaled from 1 to 5, emphasizing a goal value. The optimization process was completed while adhering to the aforementioned requirements and limitations. Figure 4 shows the response surface that was developed for the global desirability function.

This picture showed a high degree of desirability (D = 0.673), 58.90% methanol concentration, pH 4.5 buffer, and flow rate of 0.8 mL per minute. Formulation assay conditions were optimized with a C-18 column with methanol concentration, PO_4^{3-} buffer-pH 4.5 (58.90:41.10 v/v) as the mobile phase, and 0.8 mL per minute flow rate. By examining the projected response values based on D, we find K1 = 1.0, Rs2,3 = 7.03, and tR2 = 6.22 minutes. Based on the experimental and anticipated results under ideal circumstances, both Table 4 and Figure 5 demonstrate agreement between the relevant chromatograms.

Method Validation

The newly created and improved procedure has been validated as per ICH criteria.[18] This method has been analyzed in terms

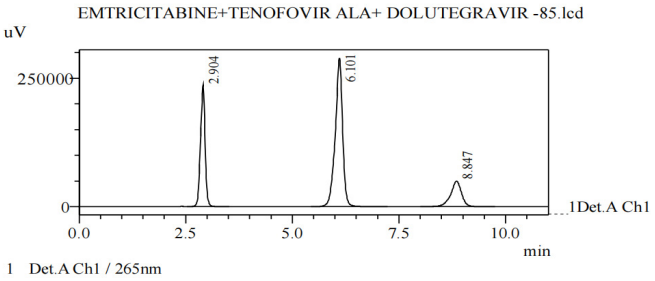


Figure 5: Optimized chromatogram

of linearity, precision, specificity, system appropriateness, accuracy, robustness, limit of detection, and limit of quantification. The method's specificity was confirmed by injecting the placebo and blank (synthetic mixes). Since the placebo and blank did not interact with the primary peaks, the procedure was unique to these two medications.

System performance was determined using retention duration, theoretical plates, asymmetry factor, and resolution. It can be concluded that the system is performing satisfactorily when the RSD is less than 2%. For Emtricitabine, tenofovir alafenamide, and dolutegravir, respectively, the calibration curves show linearity as they increase from 40 to 200 µg/mL, 5 to 25 µg/mL, and 10 to 50 µg/mL, with correlation coefficients of 0.9998, 0.9997, and 0.9999, respectively.

There was a significant difference between LoD and LoQ for emtricitabine, tenofovir alafenamide, and dolutegravir: 0.2642, 0.3352, 0.2269 and 0.8008, 1.0158, and 0.6573 µg per mL. Method [19] reported a higher LoQ and LoD value. This led to a more accurate devised procedure.

The preanalyzed formulation was formulated with emtricitabine, tenofovir alafenamide, and dolutegravir each at 50, 100, and 150% of their known concentrations. It was at these levels that recovery experiments were conducted. There was a mean percentage recovery of 99.97, 100.31, and 100.22% for emtricitabine, tenofovir alafenamide, and dolutegravir, respectively (Table 5). RSD was found to be less than 2%. In this case, the excipient did not interfere with the experiment. In this way, the correctness of the method was confirmed.

Precision investigation involved at least six injections of standard solution. Calculations were made for standard deviation and %relative standard deviation. Emtricitabine, tenofovir alafenamide, and dolutegravir had %RSD values of 0.56, 0.22, and 1.19 (Table 5). The approach was accurate as shown by the low %RSD figure.

The ruggedness of the developed approach was verified. It was verified utilizing several analyzers. A percentage RSD

Table 4: Predictions for different functions under optimal conditions compared to experimental values

Condition criteria	*Solvent used (methanol %v/v)*	*BUFFER (pH)*	*Flow rate (mL/min)*	K_1	$Rs_{2,3}$	tR_2
Predictive	58.90	4.5	0.8	1.0	7.03	6.22
Experimental	58.90	4.5	0.8	0.96	6.95	6.11
Average error				4.16	1.15	1.76
Desirability value= 0.673						

Table 5: Validation parameters

Parameters	*Emtricitabine*	*Tenofovir*	*Dolutegravir*
Range (µg/mL)	40–200	5–25	10–50
Y=mx + c	Y=16521X+945686	Y=287498X +9988576	Y=36376X + 656425
Regression coefficient	0.9998	0.9997	0.9999
Slope (m)	16521	287498	36376
Intercept (c)	945686	9988576	656425
Limit of detection (µg per mL)	0.2642	0.3352	0.2169
Limit of quantitation (µg per mL)	0.8008	1.0158	0.6573
Precision (%RSD)	0.5685	0.2276	1.1930
Accuracy (%)	99.97	100.31	100.22
Assay (%)	100.36	100.38	100.37

value of less than 2% was found for three analytes. In this way, accuracy was further supported.

The created approach was used with the sold dose types of tablets. Without altering the parameters of the established technique, the test was carried out on marketing pill dosage forms that were administered into HPLC. Emtricitabine contains 200.72 mg, tenofovir alafenamide contains 25.09 mg, and dolutegravir contains 50.18 mg per tablet (Table 5).

Using all validation parameters data (Table 5), the proposed and optimized approach was appropriate, linear, exact, accurate, and robust for estimating emtricitabine, tenofovir alafenamide, and dolutegravir simultaneously.

CONCLUSION

The suggested way to tackle the issue of looking for ideal RP-HPLC settings is effective and simple to implement. The derived quadratic model shows that changes in buffer pH have a large impact on retention duration and resolution, whereas methanol concentration and flow rate have a less dramatic but still significant impact. The experiment also showed that chromatographic procedures paired with chemometric instruments are an effective analytical tool since they may give important information about separation and elution time. This RP-HPLC technology can be used for regular quality control analyses in a pharmaceutical context because the created and optimized approach is specific, appropriate, linear, accurate, precise, and robust.

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