Research Paper

Development and Validation of Ultra Performance Liquid Chromatographic Method for the Simultaneous Estimation of Lamivudine, Tenofovir Disoproxil Fumarate, Doravirine and Efavirenz in Bulk and Pharmaceutical Formulations

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A simple and rapid stability indicating reverse phase ultra-performance liquid chromatographic method has been established and validated for the simultaneous quantification of lamivudine, tenofovir disoproxil fumarate, doravirine and efavirenz in bulk and pharmaceutical formulations. The chromatographic separation was performed on Acquity Ethylene Bridged Hybrid Phenyl (50 mm×2.1 mm, 1.7 µm) column. The isocratic elution system of water and acetonitrile in the ratio 50:50 v/v pumped at a flow rate 0.4 ml/min in isocratic mode. The injection volume set was 1 µl and the detection wavelength was 238 nm. The column temperature was set at 30º. The retention times of lamivudine, tenofovir disoproxil fumarate, doravirine and efavirenz were found to be 1.012 min, 1.233 min, 1.428 min and 1.666 min respectively with a total run time of 3 min. The proposed method was validated according to International Council on Harmonisation Q2 (R1) guidelines. The percentage recoveries were found to be in the range of 99.56 -100.40 %. The relative standard deviation values obtained during precision studies were found to be less than 2. Linearity between concentration and response was found within the specified concentration range and the correlation coefficient was found to be 0.999 for all the drugs. Degradation studies were carried out under various stress conditions such as acid, base, oxidation, heat and light and found no interference of degraded impurity peaks at the retention time of analyte peaks. Hence, the proposed ultra-performance liquid chromatographic method can be utilized in the routine quality investigation of lamivudine, tenofovir disoproxil fumarate, doravirine and efavirenz either individually or simultaneously in bulk and co-formulated dosage forms.

Key words: Lamivudine, tenofovir disoproxil fumarate, doravirine, efavirenz, reverse phase ultra-performance liquid chromatographic method, method development and validation

Antiretroviral drugs are used for the treatment of retroviral infection, primarily human immune virus (HIV). Combinational therapy of antiretroviral drugs is preferred for highly active treatment. Antiretroviral drug molecules such as lamivudine, tenofovir disoproxil fumarate, doravirine and efavirenz were selected for the study, as these are the drugs among most commonly prescribed for HIV therapy. Lamivudine (fig. 1A) is chemically 4-amino-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]pyrimidin-2-one. It is formulated as tablets and oral solution. Tenofovir disoproxil fumarate (fig. 1B) is {[(2R)-1-(6-aminopurin-9-yl)propan-2-yl]oxymethyl-(propan-2-yloxycarbonyloxymethoxy) phosphoryl}oxymethylpropan-2-yl carbonate;(E)-but-2-enedioic acid. Doravirine (fig. 1C) is 3-chloro-5-({1-[(4-methyl-5-oxo-4,5-dihydro-1H-1,2,4-triazol-3-yl)methyl]-2-oxo-4-(trifluoromethyl)-1,2-dihydropyridin-3-yl}oxy)benzonitrile. Efavirenz (fig. 1D) is (4S)-6-chloro-4-(2-cyclopropylethynyl)-4-(trifluoromethyl)-2,4-dihydro-1H-3,1-benzoxazin-2-one. These are the drugs that belong to the class of reverse transcriptase inhibitors. They are available

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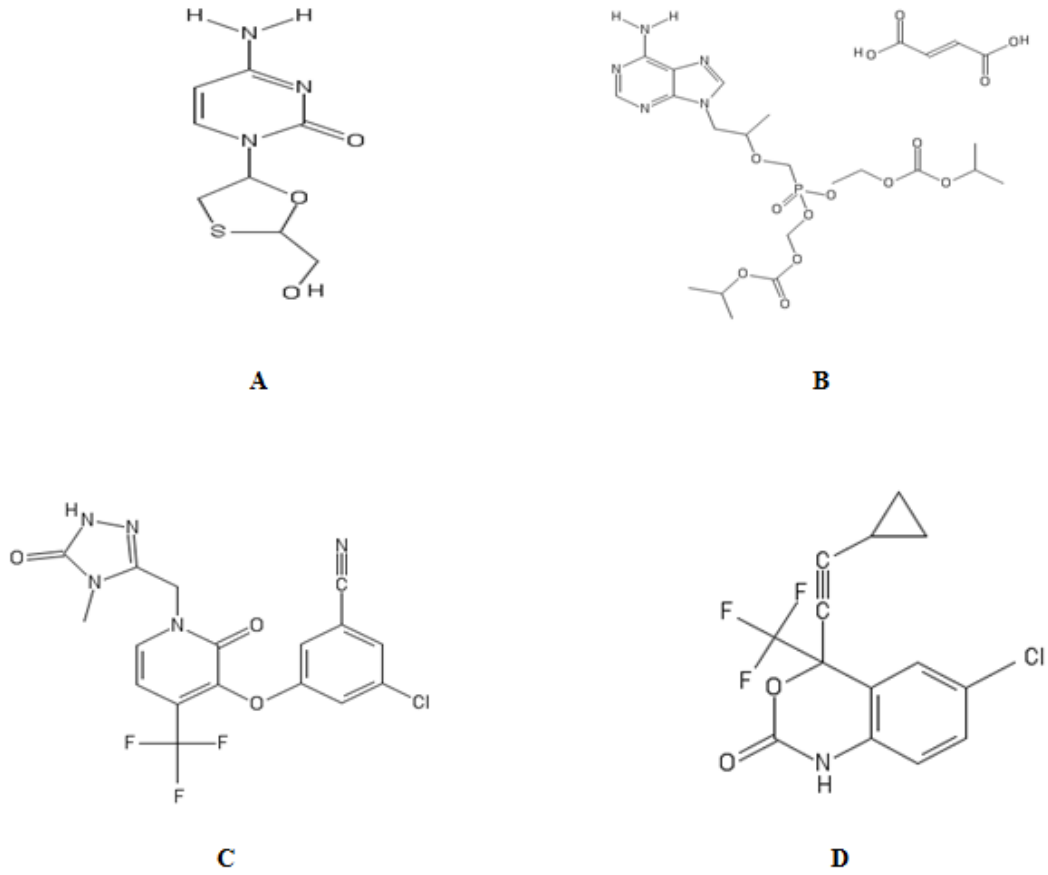

Fig. 1: Structure of analytes. Structures of A. lamivudine, B. tenofovir disoproxil fumarate, C. doravirine and D. efavirenz

as single drug formulations with varied strengths and also as co-formulated dosage forms with other HIV drugs used in the effective management of retro viral infections and Hepatitis B. A combination of lamivudine, tenofovir disoproxil fumarate and efavirenz tablets are available in the market with different trade names such as Trioday, Vonaday and Telura by various manufacturers. Lamivudine and tenofovir disoproxil fumarate with doravirine combinational tablets are available with the trade name Delstrigo. An extensive literature survey revealed analytical methods for the estimation of specified drugs individually[1,2] and also in combination with other drugs[3-5]. An ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method was reported for the impurities characterization of doravirine[6]. Few liquid chromatographic methods were reported for the simultaneous estimation of efavirenz along with impurities[7,8]. There were simultaneous Reverse-phase high performance liquid chromatography (RP-HPLC) methods proposed for efavirenz in combination with lamivudine and tenofovir disoproxil fumarate[9-12], but in those methods efavirenz has longer retention time (RT) and the run time of the studies was high leading to longer analysis time. In addition to these, there was no ultra-performance liquid chromatography (UPLC) method yet reported for the simultaneous estimation of the proposed lamivudine, tenofovir disoproxil fumarate, doravirine and efavirenz. Henceforth, there is a scope for the development of a simple and fast method that can analyze all the four drugs either in single or combination formulations as well as in bulk. The present investigation depicts the development and validation of accurate and isocratic Reverse-phase ultra-performance liquid chromatography (RP-UPLC) method with excellent resolution for the assay of lamivudine, tenofovir disoproxil fumarate, doravirine and efavirenz. Therefore, this method can be used for the routine quality control of bulk and marketed formulations of selected drugs, utilizing a simple mobile phase in short time.

MATERIALS AND METHODS

Combinational tablets containing 300 mg of lamivudine, 300 mg of tenofovir disoproxil fumarate, 100 mg of doravirine (formulation A) and the reference standards

were procured from Spectrum Pharma Research Solutions, Hyderabad. Trioday tablets manufactured by Cipla (formulation B) were purchased from the local market. Acetonitrile and HPLC grade water of analytical grade from Merck Ltd., Mumbai. The diluent used was water and acetonitrile in the ratio of 50:50 v/v.

Chromatographic conditions:

The instrument used was Acquity UPLC H-Class system of Waters, Milford, USA. It consists of binary solvent manager, auto sampler and Ultraviolet (UV) detector. The output signal was monitored and processed using Empower software. Chromatographic separation was performed on Acquity Ethylene Bridged Hybrid (BEH) Phenyl column (50 mm×2.1 mm, 1.7 µm) with UV detection at 238 nm. The mobile phase consisting of water and acetonitrile in the ratio 50:50 v/v was pumped at a flow rate 0.4 ml/min in isocratic mode with an injection volume of 1 µl and the column temperature was set at 30º.

Preparation of standard solution:

Accurately 7.5 mg of lamivudine, 7.5 mg of tenofovir disoproxil fumarate, 2.5 mg of doravirine and 15 mg of efavirenz were weighed, then transferred into a clean and dry 25 ml volumetric flask. To this, 15 ml of diluent was added, sonicated for 10 min and made up to the final volume with diluent. This was considered as the primary stock solution. From the primary stock, 1 ml was taken into a 10 ml volumetric flask and made up to the volume with diluent. This was considered as working standard solution with final concentrations of 30 µg/ml of lamivudine, 30 µg/ml of tenofovir disoproxil fumarate, 10 µg/ml of doravirine and 60 µg/ml of efavirenz.

Sample solution preparation (Formulation A):

Combinational tablets containing 300 mg of lamivudine, 300 mg of tenofovir disoproxil fumarate, 100 mg of doravirine were taken for the sample solution preparation. Twenty tablets were grinded into fine powder in a mortar. From this, the weight of powder equivalent to 7.5 mg of lamivudine, 7.5 mg of tenofovir disoproxil fumarate, 2.5 mg of doravirine was taken and transferred into 25 ml clean dry volumetric flask, 15 ml of diluent was added, sonicated for 15 min, filtered and made up to the final volume with diluent. From the above solution, 1 ml was taken into a 10 ml volumetric flask and made up to the volume with diluent.

Sample solution preparation (Formulation B):

Trioday tablets were taken for the sample solution preparation. Twenty tablets were grinded into fine powder in a mortar. From this, the weight of powder equivalent to 7.5 mg of lamivudine, 7.5 mg of tenofovir disoproxil fumarate, 15 mg of efavirenz was taken and transferred into 25 ml clean dry volumetric flask, 15 ml of diluent was added, sonicated for 15 min, filtered and made up to the final volume with diluent. From the above solution, 1 ml was taken into a 10 ml volumetric flask and made up to the volume with diluent.

Preparation of forced degradation samples:

As a part of forced degradation studies, the sample of acid degradation was prepared by taking 1 ml of standard stock solution and 1 ml of 1 Normal (1 N) hydrochloric acid and refluxed for 30 min at 60º. After refluxing, the resultant solution was neutralized by adding 1 N sodium hydroxide solution and was diluted up to 10 ml with diluent. The base degradation sample was prepared by taking 1 ml of standard stock and 1 ml of 1 N sodium hydroxide and refluxed for 30 min at 60º. After refluxing, the resultant solution was neutralized by adding 1 N hydrochloric acid solution and was diluted up to 10 ml with diluent. During oxidative degradation, to 1 ml of standard stock, 1 ml of of 10 % hydrogen peroxide was added and the resultant solution was kept undisturbed for 30 min at 60º, then the volume was made up to 10 ml with diluent. During sample preparation of thermal degradation, the standard stock solution was kept in the oven at 60º for 6 h. From the resultant stock solution, 1 ml was taken into 10 ml volumetric flask and made up to the volume with diluent. As a part of the photo stability study, 10 ml of stock solution was kept under UV light for 3 d. From the resultant stock solution, 1 ml was taken into a 10 ml volumetric flask and made up to the volume with diluent[13].

Method development:

Varied chromatographic conditions were tried to get sharp peaks with good resolution and for rapid elution. Different combinations of solvents in different ratios as mobile phase were examined and found that the suitable mobile phase for the separation was water and acetonitrile in the ratio of 50:50 v/v. It was pumped at a flow rate of 0.4 ml/min in an isocratic mode found quite well for the detection as well as simultaneous estimation of four drugs. Acquity BEH Phenyl column

(50 mm×2.1 mm, 1.7 µm) with UV detection at 238 nm was considered best for the separation. The injection volume of 1 µl and a column temperature of 30º were optimized for the study. Standard solutions were prepared and injected six times into the chromatographic system. The evaluated system suitability parameters were tabulated in Table 1. The resolution between two peaks must be greater than 2. The number of theoretical plates must be more than 2000 and the tailing factor must be less than 2. From the results, it was found that all the parameters were in compliance with the acceptance limits. Hence, these chromatographic conditions were optimized for the estimation of lamivudine, tenofovir disoproxil fumarate, doravirine and efavirenz. A typical UPLC chromatogram from standard preparation was shown in fig. 2.

RESULTS AND DISCUSSION

The developed reverse phase UPLC method was validated for parameters like specificity, accuracy, linearity, precision, Limit of detection (LOD), limit of quantitation (LOQ) and robustness according to ICH guidelines[14].

The specificity of the developed method was performed to analyze the interference components which were expected to be present include excipients, degradants and impurities with the analyte peaks during determination of analytes. To establish specificity, sample, blank and the impurities generated by forced degradation were injected into the chromatographic system and observed for any interfering peaks at the RT of analytes. The degradation was carried out intentionally by exposing the samples into various stress conditions. The sample chromatograms were shown in fig. 3. After the injection of sample, blank and degradation samples into the chromatographic system, no interfering peaks were observed at the RT of analytes. Hence, the method was found to be specific for the estimation of drugs.

The precision of the method expresses the closeness of agreement between a series of measurements. It was examined using pure samples. The working standard solutions were prepared and injected six times into the system. The results were tabulated in Table 2. The mean and standard deviation (SD) of peak areas were considered; percentage relative standard deviation (RSD) for peak areas was calculated and reported. RSD values were found within the limits, inferring good repeatability of the developed method.

The linearity was developed between concentration of standard solutions and their responses. The test solutions were prepared from standard solution at six concentration levels from 25 % to 150 % of assay concentration. The obtained peak area versus concentration was treated by the least squares linear regression analysis. The calibration plots were shown

TABLE 1: SYSTEM SUITABILITY

Analytes	RT (min)*	Resolution*	Theoretical plates*	Tailing Factor*
Lamivudine	1.013±0.002	-	3968.7±251.6	1.11±0.06
Tenofovir disoproxil fumarate	1.236±0.002	3.45±0.10	5977.8±341.4	1.20±0.03
Doravirine	1.430±0.002	3.00±0.09	7854.0±739.2	1.22±0.06
Efavirenz	1.671±0.003	3.62±0.12	9716.7±556.7	1.27±0.01

*Each value is a mean of 6 observations±standard deviation

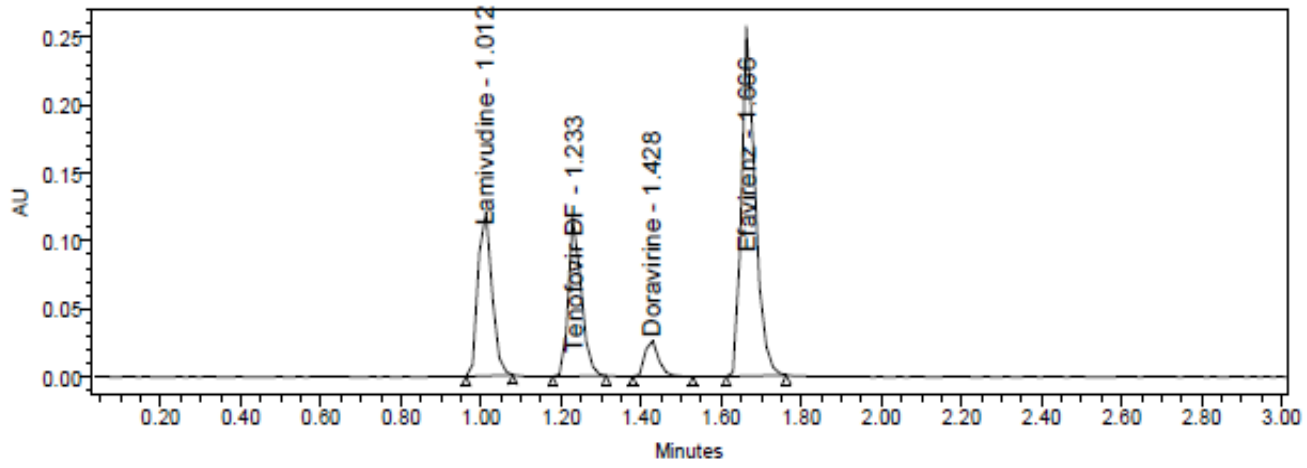

Fig. 2: Chromatogram of standard preparation

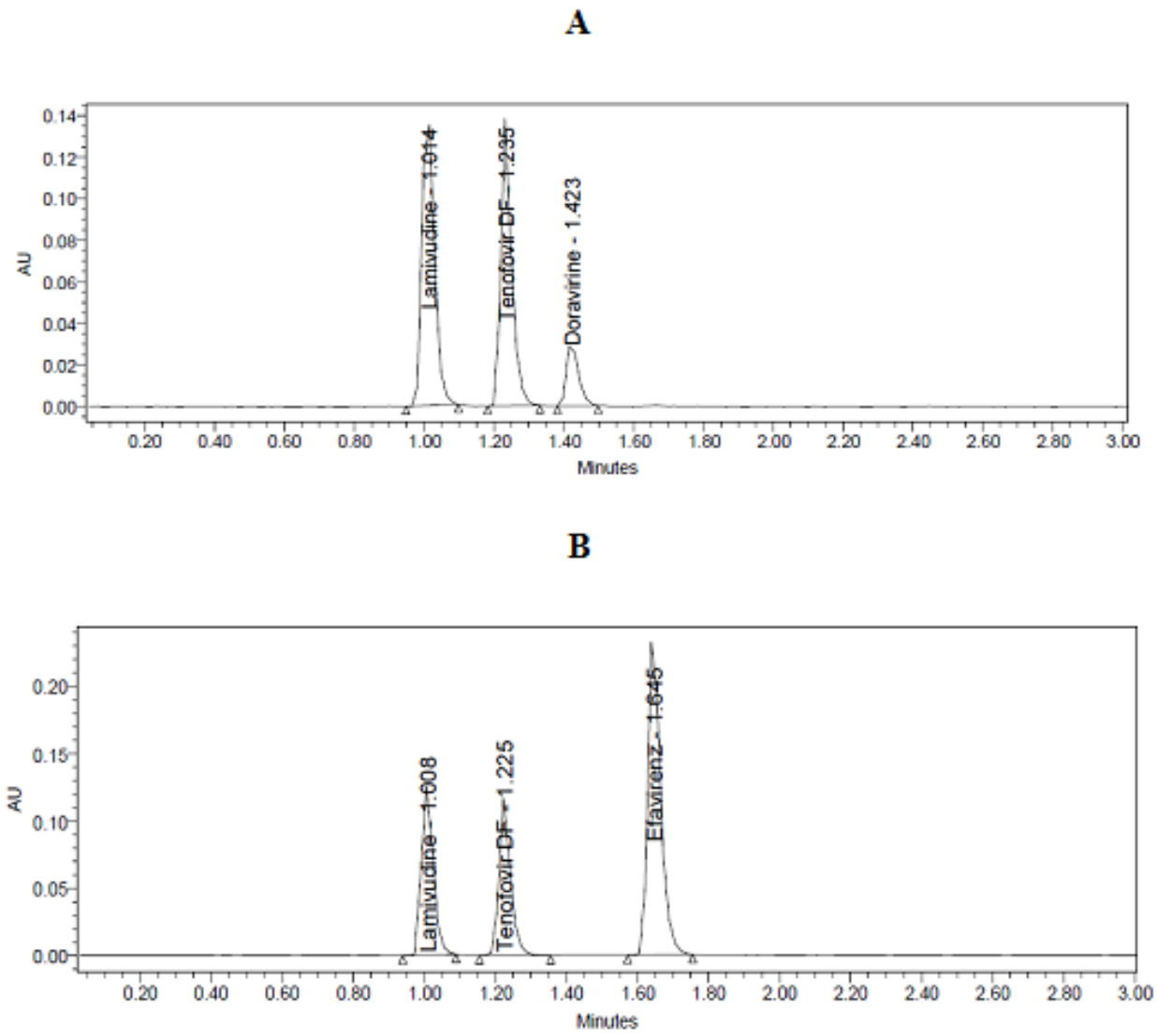

Fig. 3: Chromatograms of sample preparations. Chromatograms of A. Formulation A and B. Formulation B

TABLE 2: RESULTS OF PRECISION STUDIES

No. of injections	Lamivudine		Tenofovir disoproxil fumarate		Doravirine		Efavirenz	
	RT (min)	Peak area	RT (min)	Peak area	RT (min)	Peak area	RT (min)	Peak area
1	1.009	318566	1.233	301462	1.427	101594	1.666	642993
2	1.012	312155	1.235	299482	1.428	103694	1.670	645839
3	1.012	316678	1.236	300968	1.430	102241	1.671	642575
4	1.013	313755	1.236	298624	1.431	101216	1.672	644333
5	1.014	315265	1.238	298743	1.432	102155	1.674	645871
6	1.015	314625	1.238	300474	1.433	101927	1.675	642637
Mean		315174		299959		102138		644041
SD		2245.2		1185.8		851.2		1541.8
RSD (%)		0.7		0.4		0.8		0.2

Acceptance criterion is that RSD must be less than 2 %

in fig. 4. The results were tabulated in Table 3, which have shown excellent linearity between peak areas and concentration within the specified concentration range. The correlation coefficients were found to be 0.999 for all the four drugs, which met the acceptance criteria and hence the method was said to be linear within the specified concentration range.

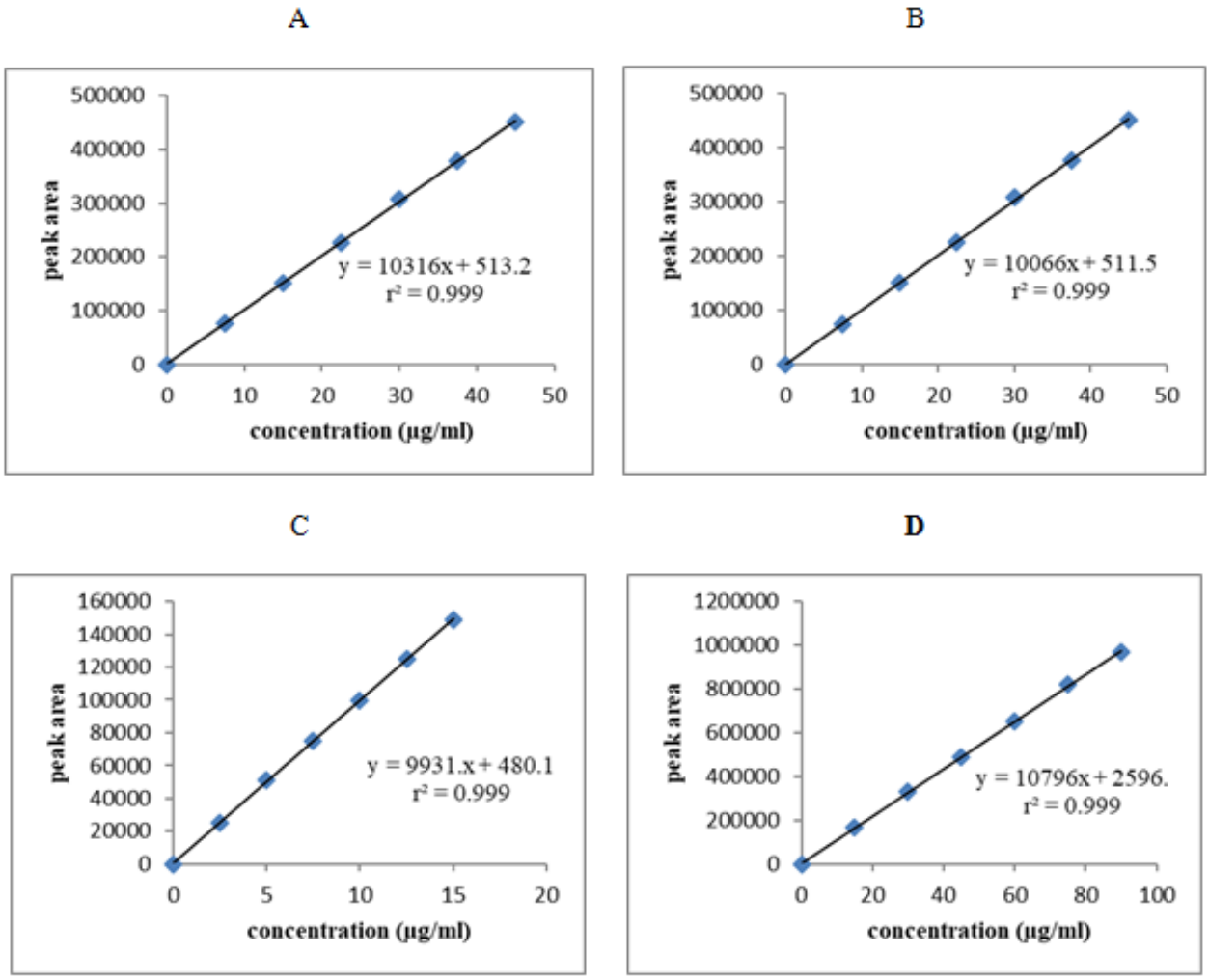

Fig. 4: Calibration plots of analytes. **Calibration plots of A. lamivudine, B. tenofovir disoproxil fumarate, C. doravirine and D. efavirenz**

TABLE 3: RESULTS OF LINEARITY

Percentage level	Lamivudine		Tenofovir disoproxil fumarate		Doravirine		Efavirenz	
	Concentration (µg/ml)	Peak area	Concentration (µg/ml)	Peak area	Concentration (µg/ml)	Peak area	Concentration (µg/ml)	Peak area
25	7.5	84266	7.5	75499	2.5	25201	15	164858
50	15	154822	15	150629	5	50845	30	328328
75	22.5	223455	22.5	226368	7.5	74935	45	488277
100	30	311473	30	308325	10	99965	60	650628
125	37.5	384776	37.5	377326	12.5	124983	75	818475
150	45	469557	45	450843	15	148844	90	968192
Correlation coefficient (r^2)	0.999		0.999		0.999		0.999	

Acceptance criterion is r^2 =0.99-1

The accuracy, also called as trueness, was determined by carrying out recovery studies where known quantities of the drug substance have been added to drug product. The developed analytical procedure has been applied so as to determine the added amount of drug substance. The percent recoveries were calculated and reported. The results of accuracy using formulation A and B were presented in Table 4 and Table 5. The results were found within the acceptance criteria which must be in the range of 98-102 %. Hence, the developed method was found to be accurate for the estimation of the mentioned drugs.

TABLE 4: RESULTS OF ACCURACY (FORMULATION A)

Analyte	Accuracy level (%)	*Amount spiked (µg/ml)	*Amount found (µg/ml)	#Mean percentage recovery
Lamivudine	50	15	15.06	
	100	30	30.21	100.40
	150	45	45.04	
Tenofovir disoproxil fumarate	50	15	14.93	
	100	30	30.26	100.19
	150	45	45.07	
Doravirine	50	5	5.03	
	100	10	10.00	100.23
	150	15	15.02	

*Each value is the mean of 3 observations, #Each value is the mean of 9 observations

TABLE 5: RESULTS OF ACCURACY (FORMULATION B)

Analyte	Accuracy level (%)	*Amount spiked (µg/ml)	*Amount found (µg/ml)	#Mean percentage recovery
Lamivudine	50	15	14.85	
	100	30	30.18	99.91
	150	45	45.06	
Tenofovir disoproxil fumarate	50	15	14.97	
	100	30	30.01	100.01
	150	45	45.08	
Efavirenz	50	30	30.16	
	100	60	59.35	99.56
	150	90	89.28	

*Each value is the mean of 3 observations, #Each value is the mean of 9 observations

The LOD and LOQ specify the lowest amount of analytes that can be detected and necessarily quantified respectively. Several methods were available for determining these limits. Among which, formula method has been applied to determine the limits. The values were calculated using the formulae. LOD=3.3 σ/S and LOQ=10 σ/S, Where, σ is the SD of the y-intercept and S is the slope of the calibration curves. The detection and quantitation limits were presented in Table 6. The itemized results showed the sensitivity of the developed method.

Robustness of the analytical method indicates the reliability of the method upon normal usage. It was examined by intentionally altering the chromatographic conditions so as to prove the capability of the method remained unaffected by those variations in chromatographic parameters. The parameters such as flow rate, column temperature, and mobile phase composition were altered to prove the robustness of the developed method. A deviation of ±0.1 ml/min in the flow rate, ±5º in the column temperature and 5 % variation in mobile phase ratio were tried individually. A standard solution at test concentration with the specified changes in the operational conditions was injected into the chromatographic system for six times. The results of robustness were presented in Table 7. The results infer that all the parameters were found to be within the limits even after slight variations in the chromatographic conditions. Hence, the method was found to be robust.

During the forced degradation study, the standard and degraded samples were injected into the system with a run time of 10 min and the results were tabulated in Table 8. The percentage of drug degraded in the solution was calculated. The significant degradation was found in the presence of acid and base. Drugs were slightly degraded upon oxidation, under light and heat. The degradation products produced as a result of stress studies did not interfere with the RT of analytes. Therefore, the assay was considered as stability-indicating for the simultaneous estimation of lamivudine, tenofovir disoproxil fumarate, doravirine and efavirenz.

The applicability of the proposed method was confirmed by assaying formulations A and B. The standard and sample solutions were prepared and injected into the chromatographic system. The amount of drug present in the formulation was calculated and the results were tabulated in Table 9 and Table 10. The results were in

TABLE 6: RESULTS OF LOD AND LOQ

Parameter	Lamivudine	Tenofovir disoproxil fumarate	Doravirine	Efavirenz
LOD (µg/ml)	0.15	0.10	0.04	0.32
LOQ (µg/ml)	0.44	0.29	0.11	0.97

LOD is limit of detection and LOQ is limit of quantitation

TABLE 7: RESULTS OF ROBUSTNESS

Parameter	Lamivudine		Tenofovir disoproxil fumarate		Doravirine		Efavirenz	
	Tailing*	Plate count*	Tailing*	Plate count*	Tailing*	Plate count*	Tailing*	Plate count*
Low flow rate (0.3 ml/min)	1.16	3430.7	1.20	6147.3	1.21	8193.2	1.25	10002.2
High flow rate (0.5 ml/min)	1.23	4014.0	1.17	5833.5	1.22	7280.7	1.26	8786.7
Low column temperature (25°)	1.16	3684.8	1.17	5451.5	1.22	7491.8	1.26	9392.0
High column temperature (35°)	1.21	3586.5	1.24	5530.7	1.18	7577.5	1.29	8995.1
Low organic phase (55:45)	1.19	3995.5	1.21	5553.2	1.18	8271.2	1.27	10152.8
High organic phase (45:55)	1.22	3623.3	1.18	5864.0	1.22	7468.2	1.28	8765.0

*Each value is a mean of 6 observations, Acceptance criteria are tailing: <2, plate count: >2000

TABLE 8: FORCED DEGRADATION STUDIES

Stress conditions	Lamivudine	Tenofovir disoproxil fumarate	Doravirine	Efavirenz
	Percentage Degraded			
Acid	17.37	13.54	9.55	8.31
Base	12.76	15.01	3.34	6.82
Peroxide	3.81	6.93	8.52	8.51
Thermal	2.71	1.72	2.27	3.12
Photolysis	5.01	2.09	2.37	1.41

TABLE 9: ASSAY RESULTS OF FORMULATION A

S. No.	Lamivudine	Tenofovir disoproxil fumarate	Doravirine
	Percentage Assay		
1	99.40	99.96	99.58
2	99.28	100.25	101.33
3	99.14	99.54	100.00
4	99.63	99.43	100.47
5	99.65	100.24	99.52
6	100.09	99.54	101.29
Mean	99.53	99.83	100.36

Acceptance criterion is Percentage assay must be 98-102

TABLE 10: ASSAY RESULTS OF FORMULATION B

S. No.	Lamivudine	Tenofovir disoproxil fumarate	Efavirenz
	Percentage Assay		
1	99.26	99.92	100.28
2	100.32	99.20	100.07
3	99.28	100.70	99.12
4	99.23	100.29	99.10
5	100.25	100.29	98.97
6	100.30	99.98	99.99
Mean	99.77	100.06	99.59

Acceptance criterion is percentage assay must be 98-102

conformity with the label claim and hence the developed method can be effectively applied for quality analysis of formulations.

The proposed stability indicating UPLC method was found to be simple, accurate, precise, robust, quick and economic. All the four drugs were eluted within 3 min with good resolution utilizing a simple mobile phase, thereby drastically lessening the analysis time. This method can be used for the assay of drugs in bulk as well as in their dosage forms either individually or in combinations. Thus, the developed method can be employed for routine assay of bulk and tablets containing lamivudine, tenofovir disoproxil fumarate, doravirine and efavirenz in quality control divisions.

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Conflict of Interests:

The authors declared no conflict of interest.

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