ORIGINAL ARTICLE

Simultaneous Quantification of Anti-retroviral Drugs Atazanavir, Lamivudine, Tenofovir, and Ritonavir in Bulk and Pharmaceutical Formulation by RP-HPLC

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Abstract

Objective: Quality is important in every product or service, but it is vital in medicine as it involves life. Therefore, analytical methods which are a measure of quality of the drugs play a very comprehensive role in drug development and follow-up activities. As like any drug, the perfect estimation of the quality and quantity of the anti-viral drugs with respect to their potency, safety, and dosage etc., is the need of the hour so that these drugs will serve the actual purpose for which they are intended. **Materials and Methods:** The chromatographic separation was achieved using Waters C8 (100 × 4.6 mm ID) 1.7 µm column, and isocratic mobile phase consists potassium phosphate buffer pH3.5:acetonitrile (55:45) %v/v with a flow rate of 1.0 mL/min. The detection was carried out at 280 nm. The current method was validated according to the ICH guidelines for accuracy, precision, linearity, specificity, robustness, and ruggedness. **Results:** The retention times obtained for atazanavir (ATZ), lamivudine (3tc), tenofovir, and ritonavir (RTV) were 2.05, 3.33, 4.56, and 6.97 min, respectively. The calibration curves of peak area versus concentration were linear from 15 to 45 µg/mL for Atazanavir (ATV), 3TC, tenofovir disoproxil fumarate, and 5–15 µg/mL and had regression coefficient (r^2) 0.999. Limit of detection was found to be 1.2, 0.9, 0.5, 0.2 µg/mL and limit of quantification was found to be 3.6, 2.8, 1.8, 0.8 µg/mL, respectively, for ATZ, 3TC, tenofovir, and RTV. The % assay of the marketed dosage form was found to be 99.8, 99.5, 100.2, and 99.4 for ATZ, 3TC, tenofovir, and RTV. **Conclusion**: The experimental study results revealed the suitability of proposed method that can be used for simultaneous estimation of ATZ, 3TC, tenofovir, and RTV in bulk and their pharmaceutical formulations for routine quality control analysis.

Key words: Antiretroviral, ICH, quality control, RP-HPLC, validation

INTRODUCTION

Humans have been fighting viruses since the species has existed. Emerging viral infections continue to pose a major threat to global public health. For some viral diseases, vaccines and antiviral drugs have prevented widespread infection or helped people recover. Some viruses pose a bigger threat than others. Powerful antiviral drugs have made it possible for people to live for years with human immunodeficiency virus (HIV).[1,2]

AIDS is a chronic, potentially life-threatening condition caused by the HIV. By damaging immune system, HIV interferes with body's ability to fight the organisms that cause diseases. There is no cure for HIV/AIDS, but there are medications that can dramatically slow the progression of the disease. These medicinal products have reduced AIDS deaths in many developed nations.

In general, the term "antiretroviral drug" is used for anti-HIV drugs. Since the discovery of HIV, there has been a desperate

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need to develop easy and convenient methods to evaluate antiretroviral drugs.[3]

The primary goal of antiretroviral therapy for HIV infection is suppression of viral replication. Evidence indicates that the optimal way to achieve this goal is by initiating combination therapy with two or more antiretroviral agents.[4] Introduction of HIV protease inhibitors (PIs) within antiretroviral therapy, in association with nucleoside reverse transcriptase inhibitors (NRTIs), started a new era in the battle against HIV and enabled the construction of highly active antiretroviral therapy, which dramatically decreased mortality in HIV-infected populations in developed countries.[5] Because of the potential for viral resistance, non-NRTIs s and PIs should only be used in combination therapy.

High-performance liquid chromatography (HPLC) is the dominant separation technique in modern pharmaceutical and biomedical analysis because it results in highly efficient separations and in most cases provides high detection sensitivity. Most of the drugs in multicomponent dosage forms can be analyzed by HPLC method because of the several advantages such as rapidity, specificity, accuracy, precision, and ease of automation in this method.[6] Reversed-phase chromatography is the most commonly used HPLC separation mode.

Atazanavir (ATZ) is an azapeptide HIV-1 PIs. The compound selectively inhibits the virus-specific processing of viral Gag and Gag-Pol polyproteins in HIV-1-infected cells, thus preventing formation of mature virions. ATZ has an important role in the treatment of both antiretroviral-naive and antiretroviral-experienced individuals.[7] It is a white-to-pale yellow crystalline powder with a molecular formula of $C_{38}H_{52}N_6O_7$ and a molecular weight of 704.9 g/mol. Its chemical structure is shown in Figure 1. Lamivudine (3TC) is a nucleoside analog reverse transcriptase inhibitor that has been widely used against HIV infection which also has antiviral effects against hepatitis B.[8] 3TC is the (–) enantiomer of a cytidine analog with sulfur substituted for the 3′ carbon atom in the furanose ring [(–) 2′,3′-dideoxy, 3′-thiacytidine].[9] It is a white to beige powder with a molecular formula of $C_8H_{11}N_3O_3S$ and a molecular weight of 229.26 g/mol. Its chemical structure is shown in Figure 2. Tenofovir disoproxil fumarate (TDF) is a nucleotide analog of adenosine monophosphate and it is the first nucleotide reverse transcriptase inhibitor approved for the treatment of HIV infection. TDF is converted to tenofovir by serum esterases. Tenofovir is then activated by phosphorylation by cellular kinases. It is a white to off-white crystalline powder with a molecular formula of $C_{23}H_{34}N_5O_{14}P$ and molecular weight of 635.52 g/mol. Its chemical structure is shown in Figure 3. Ritonavir (RTV) is an inhibitor of HIV protease and a potent inhibitor of CYP3A4 and CYP2D6. RTV inhibits the HIV viral proteinase enzyme that normally cleaves the structural and replicative proteins that arise from major HIV genes, such as gag and pol. RTV prevents the cleavage of the gag-polpolyprotein, which results in non-infectious, immature viral particles.[10] It is a white-to-tan powder with a molecular weight of 720.9 g/mol and molecular formula of $C_{37}H_{48}N_6O_5S_2$. Its chemical structure is shown in Figure 4.

The literature survey reveals that no spectroscopic and liquid chromatographic procedures have been reported for the simultaneous determination of ATZ, 3TC, tenofovir,

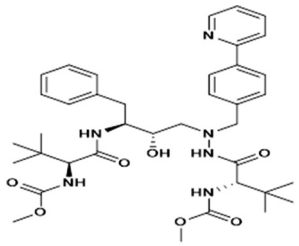

Figure 1: Chemical structure of atazanavir

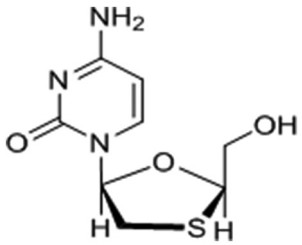

Figure 2: Chemical structure of lamivudine (3TC)

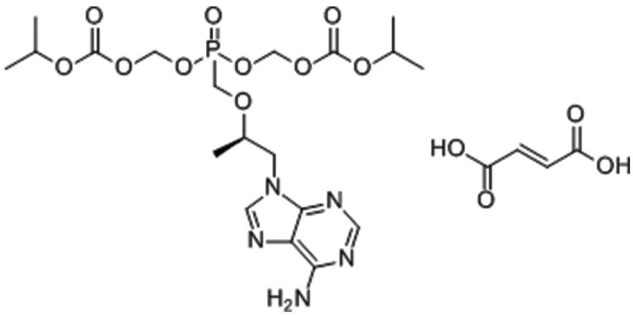

Figure 3: Chemical structure of tenofovir disoproxil fumarate

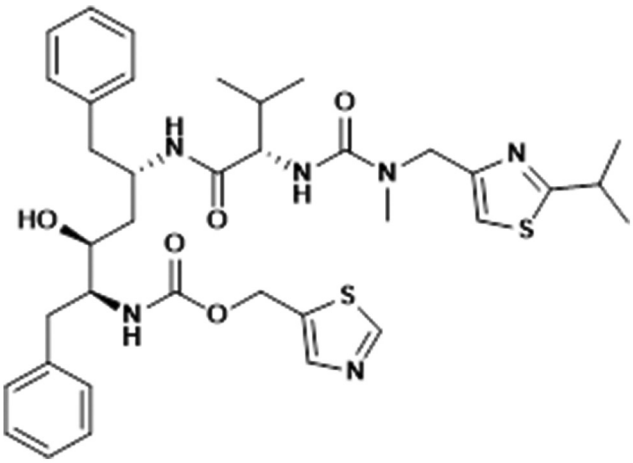

Figure 4: Chemical structure of ritonavir

and RTV. As there is a no HPLC method for simultaneous estimation of ATZ, 3TC, tenofovir, and RTV in bulk and pharmaceutical dosage form, it is felt worthwhile to develop a rapid, sensitive HPLC method for their estimation in bulk and pharmaceutical dosage.

The present study involves the development of HPLC method for the simultaneous estimation of ATZ, 3TC, tenofovir TDF, and RTV in bulk and combined tablet dosage form, which is fast and sensitive with better resolution and peak symmetry. Finally, the developed method was validated as per ICH guidelines.

MATERIALS AND METHODS

Chemicals and reagents

The reference samples of ATZ, 3TC, TDF, and RTV were provided as a gift sample from Chandra labs, Hyderabad. VIRO4® kit (Emcure) tablets labeled to contain ATZ 300 mg, 3TC 300 mg, TDF 300 mg, and RTV 100 mg were purchased from the local pharmacy store. HPLC-grade acetonitrile was purchased from Merck, Hyderabad. Potassium Phosphate Buffer AR Grade was purchased from Rankem, Mumbai, India. HPLC-grade water was used throughout the process, which was prepared using Millipore MilliQ water purification system.

HPLC method development

Instrumentation

The instruments used in the study were electronic balance (Sartorius), ultra-sonicator (Phoenix), and digital pH meter (Thermo). Agilent HPLC system model 1290 equipped with PDA detector, autosampler, and column Waters C8 (100 × 4.6 mm ID) 1.7 µm, respectively. The output signal was monitored and integrated using Lab solution software.

Chromatographic conditions

The present assay was carried out on Agilent HPLC system model 1290 equipped with PDA detector, auto sample injector, and column Waters C8 (100 × 4.6 mm ID) 1.7 µm, respectively. The output signal was monitored and integrated using Lab solution software. The isocratic mobile phase consisted of potassium phosphate buffer pH 3.5:acetonitrile (55:45) %v/v, flowing through the Waters C8 (100 × 4.6 mm ID) 1.7 µm column at a constant flow rate of 1.0 mL/min at ambient temperature. The mobile phase was pumped through the column at a flow rate of 1.0 mL/min with a sample injection volume of 20 µL. Detection of the analytes was carried out at a wavelength of 280 nm.

Determination of working wavelength (λ_{max})

In the simultaneous estimation of drugs, isosbestic wavelength is used. It is wavelength at which two (or more) chemical species have the same absorptivity. Hence, this wavelength is used in simultaneous estimation, to estimate the drugs accurately. 280 nm was selected based on the λmax of the four drugs.

Preparation of mobile phase

10 Mm buffer preparation: Accurately weighed and transferred an amount of 1.3600 g of potassium phosphate into a 1000 mL volumetric flask, this 500 mL of Milli-Q water was added and sonicated to dissolve and made up the volume with Milli-Q water, and pH was adjusted to 3.5 with dilute orthophosphoric acid.

Mobile phase: 450 mL of HPLC-grade acetonitrile was added to 550 mL of buffer solution and sonicated for 10 min (55:45% v/v).

Preparation of Standard stock solution for Ultraviolet (UV)

About 10 mg each of ATV, 3TC, TDF, and RTV pure samples were weighed and transferred into four different 50 mL volumetric flask. To each flask, 20 mL of mobile phase was added, sonicated and the volume was made up to mark with the mobile phase so the concentration of ATV, 3TC, TDF, and RTV would be 200 µg/mL each.

Dilutions

Necessary dilutions (2.5 mL in 50 mL) were made from standard stock solutions to get the concentration of 10 µg/mL each of ATV, 3TC, TDF, and RTV. The wavelength of maximum absorption (λ_{max}) of solution of the drugs in mobile phase was scanned using UV-Visible spectrophotometer within the wavelength region of 200–400 nm against mobile phase as blank. The absorption curve shows characteristic absorption maxima at 280 nm for ATZ [Figure 5], 272 nm for 3TC [Figure 6], 260 nm for TDF [Figure 7], and 300 nm for RTV [Figure 8]. 280 nm was selected as detector wavelength at which four drugs have absorbance for the HPLC chromatographic method.

Preparation of standard stock solutions

300 mg of ATV, 300 mg of 3TC, 300 mg of TDF, and 100 mg of RTV working standards were weighed and

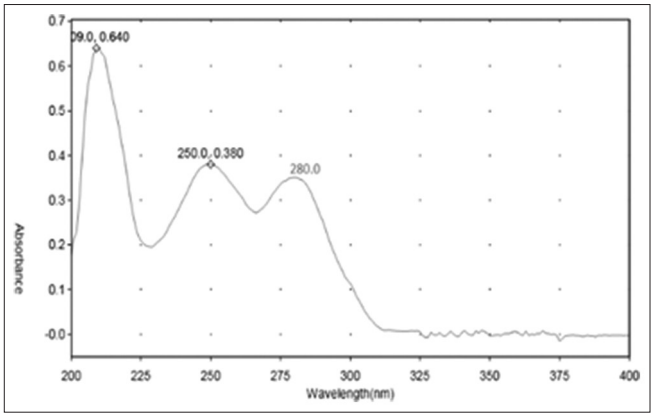

Figure 5: UV-VIS spectrum of ATV

transferred into a 100 mL clean dry volumetric flask, and then, 70 mL of mobile phase was added and sonicated for 30 min and made up the final volume with mobile phase and labeled as standard stock solution-I containing 3 mg/mL of ATV, 3 mg/mL of 3TC, 3 mg/mL of TDF, and 1 mg/mL of RTV (Standard stock solution I). 5 mL of the standard stock-I pipetted into 50 mL volumetric flask and made-up volume with mobile phase (Standard solution II). 5 mL of the standard stock-II pipetted into 50 mL volumetric flask and made-up volume with mobile phase (Working Standard solution). The final concentration of Working Standard solution would be 30 µg/mL of ATV, 30 µg/mL of 3TC, 30 µg/mL of TDF, and 10 µg/mL of RTV. The resulting solution is used to record the chromatogram [Figure 9].

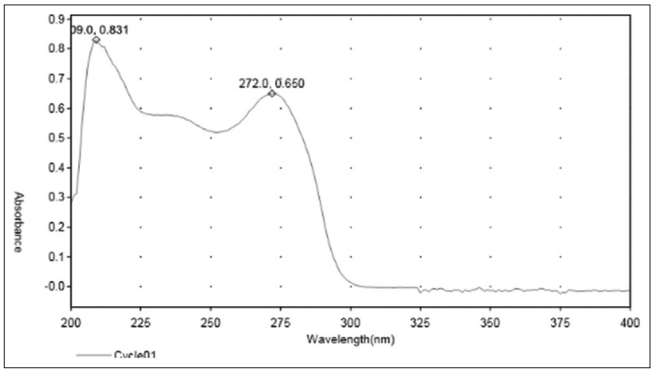

Figure 6: UV-VIS spectrum of 3TC

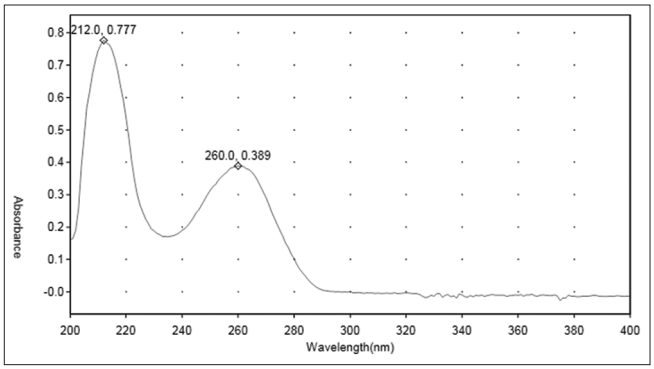

Figure 7: UV-VIS spectrum of TDF

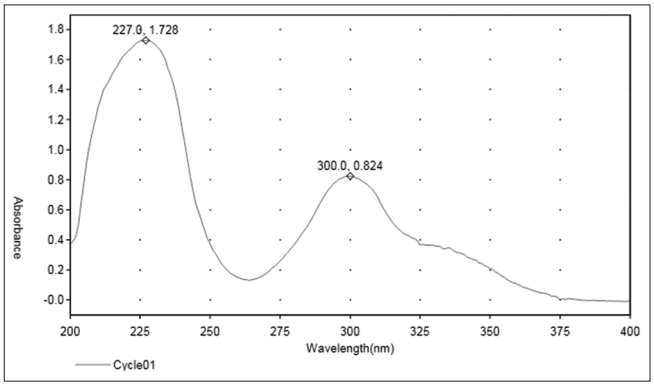

Figure 8: UV-VIS spectrum of RTV

Preparation of sample solution

Twenty tablets of VIRO4®kit were weighed and calculated average weight, and then, tablets were crushed into fine powder with mortar and pestle. The powder equivalent to 300 mg of ATV, 300 mg of 3TC, 300 mg of TDF, and 100 mg of RTV was transferred into 100 mL volumetric flask and added 70 mL of mobile phase then sonicated for 30 min with intermittent shaking, after 30 min made up the volume with mobile phase and labeled as Sample Stock solution-I, containing 3 mg/mL of ATV, 3 mg/mL of 3TC, 3 mg/mL of TDF, and 1 mg/mL of RTV, then centrifuged the sample at 10000RPM for 10 min. Pipetted 5 mL upper clear Sample Stock solution-I into 50 mL volumetric flask and made up volume with mobile phase and labeled as Sample Stock solution-II, containing 0.3 mg/mL of ATV, 0.3 mg/mL of 3TC, 0.3 mg/mL of TDF, and 0.1 mg/mL of RTV.

Pipetted 5 mL of the Sample Stock solution-II into 50 mL volumetric flask and made-up volume with mobile phase and filtered the solution through 0.45 µm filter paper (Working Sample solution). The final concentration of Working Sample solution would be 30 µg/mL ATV, 30 µg/mL 3TC, 30 µg/mL TDF, and 10 µg/mL of RTV. The resulting solution is used to record the chromatogram [Figure 10].

Optimized chromatographic conditions

After systematic and detailed study of the various parameters involved in the method, the following were found to be optimized conditions and employed for further studies [Table 1].

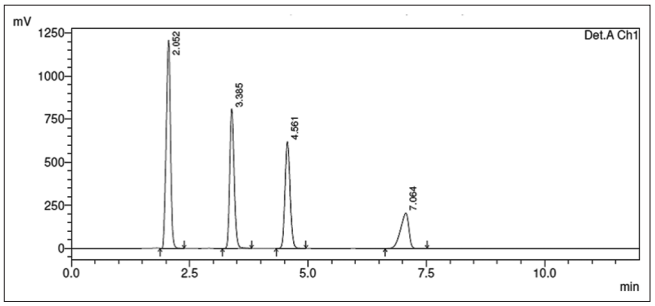

Figure 9: Typical optimized chromatogram of atazanavir, lamivudine, tenofovir, and ritonavir (standard)

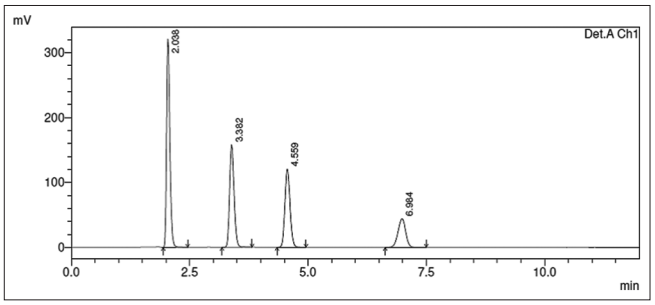

Figure 10: Typical sample chromatogram of atazanavir, lamivudine, tenofovir, and ritonavir (formulation)

Method validation

The developed method for ATV, 3TC, TDF, and RTV was validated for parameters such as system suitability, linearity and range, precision, accuracy, robustness, limit of detection (LOD), limit of quantification (LOQ), filter compatibility, and solution stability as per the ICH guidelines.

System suitability

To ensure the validity of the analytical procedure, a system suitability test was established. The following parameters such as asymmetry factor, theoretical plate number (N), resolution (Rs), and retention time (t_R) were analyzed using 20 µL of the working standard solution containing 30 µg/mL ATV, 30 µg/mL 3TC, 30 µg/mL of TDF, and 10 µg/mL of RTV were injected 6 times into HPLC system and the chromatograms were recorded for the same. The system suitability parameters are given in Table 2.

Table 1: Optimized chromatographic conditions

Instrument	Agilent HPLC system model 1290
Mobile phase	Potassium phosphate buffer pH 3.5:acetonitrile (55:45) %v/v
Column	Waters C8 (100×4.6mm ID) 1.7µm
Flow rate	1.0 mL/min
Column temperature	25°C
Sample temperature	25°C
Wavelength	280 nm
Injection volume	20 µL
Run time	12 min
Retention time	2.05, 3.33, 4.56 and 6.97 (ATV, 3TC, TDF and RTV)

TDF: Tenofovir disoproxil fumarate, 3TC: Lamivudine, RTV: Ritonavir

Specificity

It is the ability to unequivocally assess the analyte in the presence of components that may be expected to be present. Typically, these might include impurities, degradants, or matrix.

Blank solution was injected, and the chromatogram was recorded [Figure 11]. Placebo solution was prepared, it was injected, and the chromatogram was recorded [Figure 12].

Linearity and range

For establishing linearity, 2.5, 3.75, 5.0, 6.25, and 7.5 mL of standard stock-II solution containing 300 µg/mL of ATV, 300 µg/mL of 3TC, 300 µg/mL of TDF, and 100 µg/mL of RTV were pipetted out and transferred into a separate 50 mL volumetric flasks and diluted up to mark with mobile phase. Hence, the final concentrations were in the range of 15–45 µg/mL ATV, 15–45 µg/mL 3TC, 15–45 µg/mL TDF, and 5–15 µg/mL RTV. These standard solutions containing ATV, 3TC, TDF, and RTV were injected using a 20 µL injector, and the chromatograms were recorded at 280 nm and calibration curve was constructed by plotting the peak area versus drug concentration.

LOD and LOQ

A study to establish the LOD and LOQ for ATV, 3TC, TDF, and RTV was conducted. Series of very dilute LOD and LOQ solutions were prepared as per the test method and injected

Table 2: System suitability test parameters

S. No.	Peak name	Retention time (Minutes)	Peak area	Theoretical plates	Tailing factor	Resolution	%RSD
1.	Atazanavir	2.05	4549023	13896	1.0	-	0.2
2.	Lamivudine	3.39	3071198	37452	1.1	2.6	0.2
3.	Tenofovir	4.56	2685284	52044	1.0	2.8	0.3
4.	Ritonavir	5.20	1456064	47521	0.9	4.5	0.7

Table 3: Linearity data results

Atazanavir		Lamivudine		Tenofovir		Ritonavir	
Concentration (µg/mL)	Peak area	Concentration (µg/mL)	Peak area	Concentration (µg/mL)	Peak area	Concentration (µg/mL)	Peak area
15.0	2262511	15.0	1408736	15.0	1335486	5.0	729811
22.5	3358505	22.5	2145514	22.5	2001542	7.50	1072361
30.0	4405361	30.0	2958551	30.0	2658664	10.00	1442510
37.5	5636667	37.5	3689514	37.5	3356631	12.50	1807691
45.0	6775889	45.0	4523611	45.0	3986641	15.00	2163621
Regression equation y=15073x−34181		Regression equation y=10365x−16431		Regression equation y=88765x+4833		Regression equation y=14411x+2018	
Square of correlation coefficient (R2)=0.999		Square of correlation coefficient (R2)=0.999		Square of correlation coefficient (R2=0.999)		Square of correlation coefficient (R2=0.999)	

triplicate into the HPLC system. The LOD and LOQ were established based on signal-to-noise ratio. LOD was established by identifying the concentration which showed signal-to-noise ratio of 3, whereas LOQ was established by identifying the concentration which gave signal-to-noise ratio of about 10.

Accuracy

Accuracy of the method was determined by recovery studies. A known amount of ATV, 3TC, TDF, and RTV at each three concentration levels 50%, 100%, and 150% was added to a pre-analyzed sample solution (formulation) and injected in triplicate at each level into the HPLC system. The percentage recovery and mean percentage recovery of ATV, 3TC, TDF, and RTV at each level were calculated.

Method precision

Method precision was determined by injecting six different Working Sample Solutions of ATV (30 µg/mL), 3TC (30 µg/mL), TDF(30 µg/mL), and RTV(10 µg/mL) into HPLC system and chromatograms were obtained. The %RSD of the assay result of six preparations was calculated. The results for LOD and LOQ were computed and are presented in Table 4, while the precision at the LOQ is presented in Table 5.

Intermediate precision (Ruggedness)/interday precision

Intermediate precision (also called within-laboratory or within-device in different days, different analysts) is a measure of precision under a defined set of conditions: Same measurement procedure, same measuring system, same location, and replicate measurements on the same or similar objects over an extended period of time. The Intermediate Precision (ruggedness) was determined by injecting six different working sample solutions of ATV (30 µg/mL), 3TC (30µg/mL), TDF (30µg/mL), and RTV (10µg/mL) into HPLC system by different analysts on 2 different days and chromatograms were obtained. The %RSD of the assay results of six preparations was calculated.

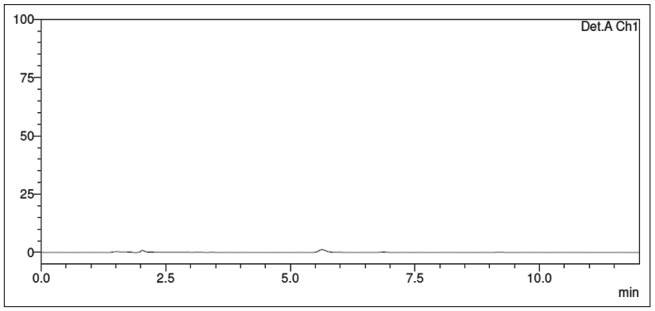

Figure 11: A typical chromatogram of ATV, 3TC, TDF, and RTV blank

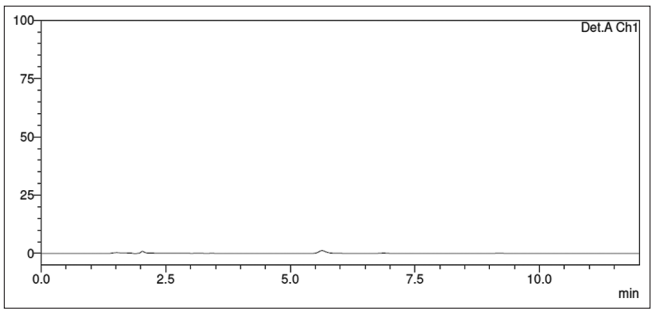

Figure 12: A typical chromatogram of placebo

Robustness

Working Standard solution was injected into the HPLC system at variable conditions such as column temperature ± 5°C and wavelength by ± 5 nm.

Assay

The commercial tablets kit (VIRO 4® KIT) was analyzed by injecting 6 replicates of Working Sample solutions

Table 4: Limit of detection and limit of quantification results

S. No.	Parameter	Measured values (µg/mL)			
		ATV	3TC	TDF	RTV
1.	Limit of detection	1.2	0.7	0.4	0.2
2.	Limit of quantification	3.7	2.0	1.1	0.5

TDF: Tenofovir disoproxil fumarate, 3TC: Lamivudine, RTV: Ritonavir

Table 5: Precision at LOQ

Injection	ATV	3TC	TDF	RTV
	Area	Area	Area	Area
1.	537524	197541	97484	48084
2.	536251	196854	97695	48077
3.	536842	198625	97854	47685
4.	536361	197004	97855	47896
5.	536444	197633	96854	47232
6.	535571	197511	97054	47869
AVG	536499	197258	97466	47807
% RSD	0.1	0.3	0.4	0.7

TDF: Tenofovir disoproxil fumarate, 3TC: Lamivudine, RTV: Ritonavir

Table 6: % recovery results of ATV

Spiked level	Amount spiked (µg/mL)	Amount recovery (µg/mL)	% recovery	Mean % recovery
50%	15	14.9	99.5	99.4
	15	14.9	99.4	
	15	14.9	99.2	
100%	30	29.9	98.8	99.6
	30	29.9	99.6	
	30	29.8	99.4	
150%	45	44.7	99.3	99.4
	45	44.8	99.5	
	45	44.7	99.3	

into the HPLC system and chromatograms were recorded. The amount of each drug present in marketed tablets was calculated by comparing the peak area of standard and sample. The % assay of ATV, 3TC, TDF, and RTV were found to be 98–102%. The results of assay are shown in Table 14.

$$\%\,Assay = \frac{AT}{AS}\times\frac{WS}{DS}\times\frac{5}{50}\times\frac{5}{50}\times\frac{DT}{WT}\times\frac{50}{5}\times\frac{50}{5}\times\frac{P}{100}\times\frac{AW}{LC}\times 100$$

Where,
AS: Average peak area due to standard preparation
AT: Peak area due to assay preparation (sample)
WS: Standard Weight of ATV/3TC/TDF/RTV in mg
WT: Weight of sample in assay preparation
DT: Dilution of assay preparation
DS: Dilution of standard preparation
P: Purity of ATV/3TC/TDF/RTV
AV: Average weight of tablets in milligrams
LC: Labeled claim of ATV/3TC/TDF/RTV in mg

Filter compatibility

Filter compatibility was determined by injecting unfiltered and filtered working sample solution through 0.45 µm PVDF (polyvinylidene fluoride) and 0.45 µm Nylon individually into HPLC system by discarding the 2 mL of filtrate. The difference between unfiltered sample % assay and filtered sample % assay should not be more than 2.0%. Filter compatibility results are shown in Table 15.

Solution stability of the standard and sample

Solution stability of the standard and sample was determined by injecting working standard solution and working sample solution into HPLC system at 12 h and 24 h of time intervals. Results of solution stability are shown in Tables 16 and 17.

RESULTS AND DISCUSSION

System suitability

The column efficiency for ATV, 3TC, TDF, and RTV peaks was identified from the theoretical plate count of more than 3000 and the tailing factor was <2.0. %RSD for peak areas from six replicate injections was found to be <2.0%. The results of other system suitability parameters, such as resolution, peak tailing, and theoretical plates, are presented in Table 2. All system-suitable parameters were found to be satisfactory.

Linearity

Linearity was evaluated by analyzing different concentrations. The correlation coefficient obtained was >0.999 for all the components. The slope and y-intercept values are also provided in Table 3, which confirmed good linearity between peak areas and concentration. The linearity graphs of ATV, 3TC, TDF, and RTV are shown in Figures 13-16, respectively.

Table 7: % recovery results of 3TC

Spiked level	Amount spiked (µg/mL)	Amount recovery (µg/mL)	% recovery	Mean % recovery
50%	15	15.2	101.3	101.1
	15	15.2	101.4	
	15	15.1	100.5	
100%	30	29.9	99.5	99.4
	30	29.7	99.2	
	30	29.8	99.4	
150%	45	44.7	99.4	99.4
	45	44.8	99.6	
	45	44.7	99.3	

Table 8: % recovery results of TDF

Spiked level	Amount spiked (µg/ml)	Amount recovery (µg/mL)	% recovery	Mean % recovery
50%	15	15.1	101.0	100.5
	15	15.1	100.5	
	15	15.0	100.2	
100%	30	30.2	100.8	100.5
	30	30.0	100.2	
	30	30.2	100.5	
150%	45	44.9	99.7	99.5
	45	44.7	99.3	
	45	44.8	99.6	

Table 9: % recovery results of RTV

Spiked level	Amount spiked (µg/mL)	Amount recovery (µg/mL)	% recovery	Mean % recovery
50%	5	5.0	99.7	99.0
	5	4.9	98.5	
	5	4.9	98.8	
100%	10	9.9	99.3	99.4
	10	9.9	99.3	
	10	10.0	99.8	
150%	15	14.9	99.1	99.6
	15	14.9	99.6	
	15	15.0	100.1	

Table 10: Method precision results

Injection No.	% Assay of drugs			
	ATV	3TC	TDF	RTV
1.	99.86	99.63	100.14	99.50
2.	99.77	99.92	100.17	99.52
3.	99.93	99.91	100.32	99.53
4.	99.83	99.56	100.34	99.22
5.	99.85	99.49	100.35	99.46
6.	99.90	99.43	100.52	99.65
Average	99.9	99.5	100.4	99.6
% RSD	0.04	0.04	0.12	0.14

TDF: Tenofovir disoproxil fumarate, 3TC: Lamivudine, RTV: Ritonavir

Table 11: Intermediate precision results

Parameter	% Assay of drugs			
	ATV	3TC	TDF	RTV
Analyst-1	99.9	99.7	100.3	99.5
Analyst-2	99.7	99.3	100.1	99.4
Average	99.8	99.5	100.2	99.4
% RSD	0.11	0.25	0.15	0.06

TDF: Tenofovir disoproxil fumarate, 3TC: Lamivudine, RTV: Ritonavir

Table 12: Robustness results of ATV

Parameter	Setting	Theoretical plates	Tailing factor	Resolution
Column Temperature (±5°C)	20°C	13854	1.0	-
	30°C	14621	1.0	-
Wavelength (±5 nm)	275 nm	13351	1.0	-
	285 nm	13831	1.0	-

Table 13: Robustness results of 3TC

Parameter	Setting	Theoretical Plates	Tailing factor	Resolution
Column temperature (±5°C)	20°C	37963	1.0	2.6
	30°C	38541	1.1	2.6
Wavelength (±5 nm)	275 nm	38251	1.0	2.6
	285 nm	38211	1.0	2.6

Table 14: Robustness results of TDF

Parameter	Setting	Theoretical plates	Tailing factor	Resolution
Column temperature (±5°C)	20°C	52269	1.0	2.8
	30°C	52452	1.0	2.8
Wavelength (±5 nm)	275 nm	52622	1.0	2.8
	285 nm	52596	1.0	2.8

Table 15: Robustness results of RTV

Parameter	Setting	Theoretical Plates	Tailing factor	Resolution
Column temperature (±5°C)	20°C	47362	0.9	4.5
	30°C	45231	0.9	4.5
Wavelength (±5 nm)	275nm	45895	1.0	4.5
	285nm	45951	1.0	4.5

Table 16: Assay results (VIRO KIT)

Drug	ATV	3TC	TDF	RTV
Label claim (mg)	300	300	300	100
Amount found (mg)	299.4	298.5	300.6	99.4
% Assay	99.8	99.5	100.2	99.4

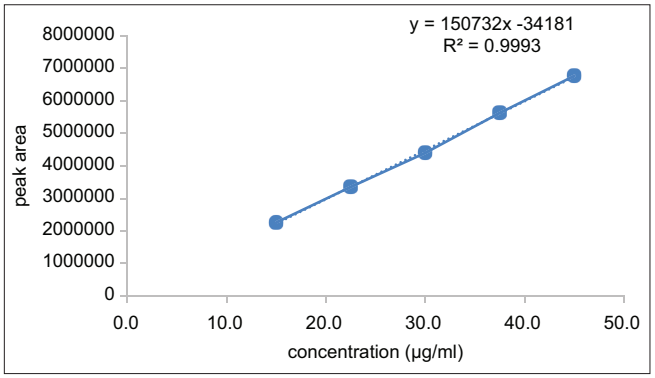

Figure 13: Standard calibration curve of ATV

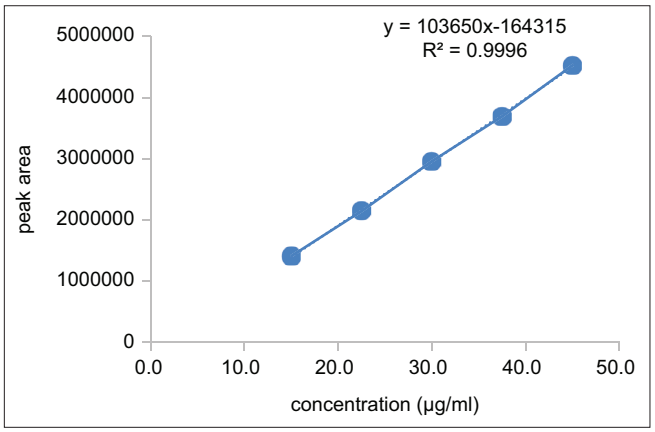

Figure 14: Standard calibration curve of 3TC

LoD and LoQ

The LOD and LOQ of ATV, 3TC, TDF, and RTV were calculated using the following equations (ICH, Q2 (R1)). The LoD and LoQ values are reported in Table 4.

These LoD=3.3×σ/S and LoQ=10×σ/S

Where σ = the standard deviation of the response and S = slope of the calibration curve.

Table 17: Filter compatibility results

Analyte	Initial % Assay	PVDF 0.45 μm %Assay	% Difference	NYLON 0.45 μm % Assay	%Difference
ATV	99.9	99.6	0.2	100.1	0.4
3TC	99.7	99.4	0.2	100.2	0.7
TDF	100.3	99.9	0.3	101.2	0.6
RTV	99.5	100.4	0.7	100.3	1.4

Table 18: Results of solution stability of standard

Analyte	Initial area	12 h Area	% Difference	24 h Area	% Difference
ATV	4550449	4585211	0.1	4567541	1.0
3TC	3071559	3075142	0.1	3075112	0.1
TDF	2697078	2698701	0.6	2673652	0.8
RTV	1457601	145362	0.4	144251	0.6

Table 19: Results of solution stability of sample

Analyte	Initial/Area	12 h/Area	% Difference	24 h/Area	% Difference
ATV	4542791	4585411	0.5	4582512	0.5
3TC	3059710	3055281	0.3	3056221	0.2
TDF	2688931	2687551	0.3	2684740	0.2
RTV	1448795	145212	0.3	145031	0.4

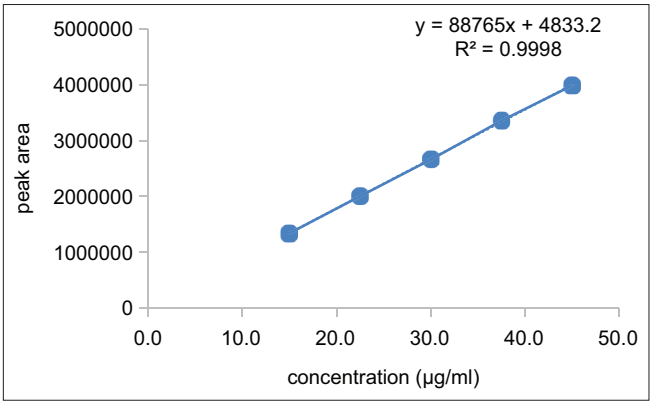

Figure 15: Standard calibration curve of TDF

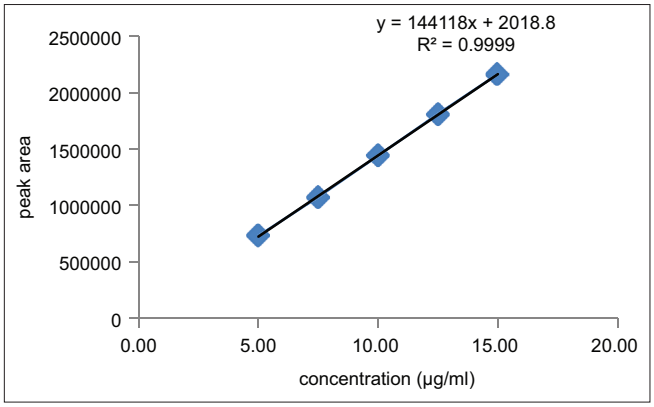

Figure 16: Standard calibration curve of RTV

Accuracy

The %recovery for ATV, 3TC, TDF, and RTV was within the range of 98–102%. The %RSD for ATV, 3TC, TDF, and RTV was found to be within 2%. Hence, the proposed method was accurate, and the results are summarized in Tables 6-9.

Method precision

%Assay for ATV, 3TC, TDF, and RTV was in the range of 98–102%. The %RSD for ATV, 3TC, TDF, and RTV was found to be within 2%. Hence, the method is precise, reproducible, and rugged for 48 h study and the results are summarized in Table 10.

Robustness

The system suitability parameters such as resolution, tailing factor, and theoretical plates of ATV, 3TC, TDF, and RTV remained unaffected by deliberate changes. The results are presented in Tables 12-15. Thus, the method was found to be robust concerning variability in applied conditions.

CONCLUSION

A convenient, rapid, accurate, and precise HPLC method was developed for the simultaneous determination of ATZ, 3TC, TDF, and RTV in pharmaceutical formulations. The assay provides a linear response across a wide range

of concentrations (15–45 µg/mL and 5–15 µg/mL). This method can be said to be economical with less consumption of solvents as the retention times were <7 min. The method is suitable for the determination of these drugs in tablets and hence can be used for routine quality control of ATZ, 3TC, tenofovir DF, and RTV in the tablet dosage form.

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