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Original Article

SIMULTANEOUS ESTIMATION OF LAMIVUDINE, ABACAVIR AND DOLUTEGRAVIR BY UPLC METHOD

SOMSHANKAR DUBEY, MAHESH DUGGIRALA

Gandhi Institute of Technology and Management
Email: somshankarbhu@yahoo.co.in

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ABSTRACT

Objective: To develop a simple, rapid, sensitive and effective reverse phase ultra-performance liquid chromatographic method (RP-UPLC) for simultaneous quantification of lamivudine, abacavir and dolutegravir in pure and tablet dosage forms.

Methods: Chromatographic separation was performed by using Waters-ACQUITY UPLC system equipped with auto sampler, photodiode array (PDA) detector, zodiac sil RP C18 (4.6 mm × 250 mm, 3.0 µm) column, phosphate buffer (pH 3.0) and methanol in the ratio of 30:70 %v/v have been delivered at a flow rate of 0.25 ml/min and the detection was carried out using a Ultraviolet (UV) detector at a wavelength of 260 nm at ambient column temperature. The mobile phase was used as diluent.

Results: The retention time (Rt) for lamivudine, abacavir and dolutegravir were 1.763, 2.247 and 3.175 min respectively. A good linear response was obtained in the range of 15-75 µg/ml, 30-150 µg/ml and 2.5-12.5 µg/ml, respectively. The Limit of Detection (LOD) values were found to be 0.021, 0.330 and 0.038 µg/ml, respectively and the Limit of Quantitation (LOQ) values were 0.056, 1.320 and 0.095 µg/ml, respectively.

Conclusion: It was concluded that the developed RP-UPLC method was effective, suitable and conducive for analyzing lamivudine, abacavir and dolutegravir in pharmaceutical formulations. The method was quantitatively evaluated in terms of precision, linearity, accuracy (recovery), selectivity and robustness in accordance with standard guidelines.

Keywords: Reverse phase ultra-performance liquid chromatography, Lamivudine, Abacavir, Dolutegravir, Degradation

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INTRODUCTION

Antiretroviral therapy (ART) has evolved significantly over the last three decades since the development of the first nucleoside analogues NRTIs (nucleoside reverse transcriptase inhibitors). Since the arrival of triple therapy, the challenge of sustained and complete viral suppression has been solved for the majority of patients [1]. The major limiting factors for improving the long-term success of ART are tolerability and convenient pill burden [2]. The latest class of the antiretroviral drugs developed was integrase inhibitors (INI). Dolutegravir (fig. 1) is an integrase inhibitor, particularly focused on maintaining a favorable safety profile and a high-efficiency rate within a single-tablet regimen (STR). It improves resistance barrier and allowing co-formulation with an NRTI backbone. Dolutegravir has been compared against both other classes of Human Immunodeficiency Virus (HIV) antiretrovirals as well as other integrase nuclear strand inhibitors. In August 2013, Dolutegravir was approved by Food and Drug Administration (FDA) for its use in both patients who have never taken ART (ART-naïve) and patients who have taken ART (ART-experienced) [3-5]. It is predicted that very soon an STR containing dolutegravir, abacavir and lamivudine will become available.

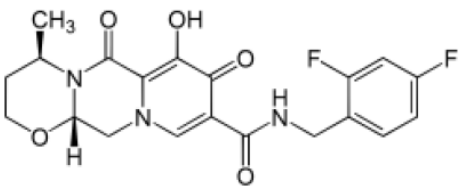

Fig. 1: Chemical structure of dolutegravir

Abacavir (fig. 2) [6] is a synthetic analogue of the naturally occurring purine nucleoside, guanine and it is a type of NRTI (nucleoside analog reverse transcriptase inhibitor) with the HIV antiretroviral activity agent. It differs from other reverse transcriptase inhibitors (didanosine, lamivudine, stavudine, zalcitabine and zidovudine) in structure. It belongs to the class of a carbocyclic nucleosides analogue rather than a dideoxynucleoside analogue. It is converted by intracellular enzymes to the carbovir triphosphate, active metabolites. Abacavir is vigorous *in vitro* against HIV-1 and HIV-2. It is a poor inhibitor of cellular Deoxyribonucleic acid (DNA) polymerases α, β and γ. After oral administration of abacavir sulphate is rapidly absorbed and it is distributed extensively. An absolute bioavailability of abacavir sulphate is ~83%, which is not affected by food. In December 1998, abacavir is approved by FDA.

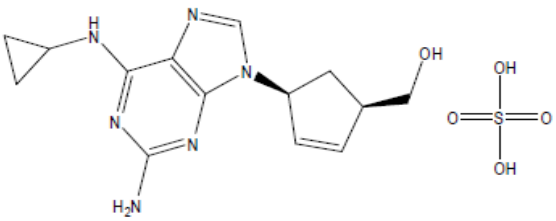

Fig. 2: Chemical structure of abacavir

Lamivudine (fig. 3) [7] is a drug in the same category of nucleoside reverse transcriptase inhibitors as abacavir. It is an analogue of cytidine and it can reduce both types of the reverse transcriptase of hepatitis B virus and HIV reverse transcriptase. Lamivudine administered orally, it is rapidly absorbed with a bioavailability of 80% to 87%. FDA approval granted in 1995 for lamivudine to use pediatric and adult based on increases in CD4 T-lymphocyte count [8] on a regimen of zidovudine and lamivudine compared with either drug alone or compared with a combination of zalcitabine and zidovudine was initial approval [9]. Lamivudine is in combinations with several triple nucleoside analogues has been shown to lead to high virologic failure in previously untreated individuals [10-13].

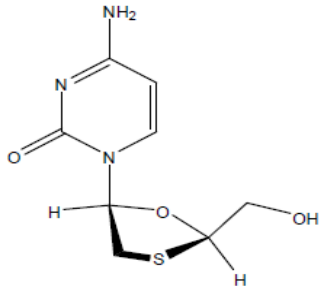

Fig. 3: Chemical structure of lamivudine

In the literature, numerous methods were described to determine separately or in a combination of lamivudine, abacavir and dolutegravir with other drugs in pharmaceutical formulation [14-23]. Still, very few methods were reported to determine these drugs simultaneously in biological matrices by using High-performance liquid chromatography (HPLC), titrimetry and UV-visible spectrophotometer [24-35]. Literature survey results, till now no UPLC analytical work on the determination of this combination.

Our aim was to develop a simple, accurate, sensitive method for simultaneous determination of lamivudine, abacavir and dolutegravir in combined pharmaceutical dosage form by UPLC with UV detection, where simple mobile phase composition was used for chromatographic separation without any ion-pairing agent. Total retention time for analysis was short with a good resolution between these components. All these reasons make this new method really lucrative. This method was also validated for linearity, sensitivity, precision, accuracy, selectivity and degradation studies according to the International Conference on Harmonization (ICH) guidelines.

MATERIALS AND METHODS

Chemicals and reagents

Lamivudine, abacavir and dolutegravir were obtained from Pharmatrain (Kukatpally, Hyderabad). The chemicals and solvents used in this study were of analytical grade and HPLC grade, respectively. Potassium dihydrogen phosphate (KH_2PO_4), di-potassium hydrogen phosphate (K_2HPO_4), orthophosphoric acid and acetonitrile were obtained from Merck (Mumbai, India). Milli-Q-Water purification system manufactured by Millipore (USA) generated water having a resistivity of 18.2 MΩ×cm.

Equipment

Waters-ACQUITY UPLC consisted of binary solvent manager with part number: 186015001, sample manager with part number: 186015005, single column manager with part number: 186015007 and PDA detector with part number: 186015026 with Waters Empower 2 PC workstation used for method development and validation.

Chromatographic conditions

The chromatographic analysis was performed in an isocratic elution mode for 8 min run time at ambient column temperature. The mobile phase consists of phosphate buffer (2.95 g of potassium dihydrogen phosphate and 5.45 g of dipotassium hydrogen phosphate in 1 l Milli-Q-Water) and adjusted pH to 3.0 with orthophosphoric acid-methanol (30:70%v/v), the flow rate of pump was set to 0.25 ml/min, Zodiac SIL RP C18 column (length 250 mm × 4.6 mm inner diameter, 3 µm particle size), the chromatogram was monitored with UV detector at 260 nm and injection volume was 5 µl. The mobile phase was used as diluent.

Methodology

Preparation of standard solution

The standard stock solution was prepared by taking accurately weighted 15 mg, 30 mg and 2.5 mg of lamivudine, abacavir and dolutegravir working standards into 10 ml clean and dried volumetric flask, add diluent let it be dissolved completely and using the same diluent make volume up to the mark. Taken 1 ml of the above solution into 10 ml volumetric flask and add diluent to make it up to the mark. For the preparation of the standard solution, 1.6 ml of solution was taken from above stock solution into 10 ml volumetric flask and added diluent to make it up to the mark.

Preparation of sample solution

For the preparation of sample solution, 10 tablets were accurately weighed and crushed to obtain a fine powder. The quantity of powder equivalent to 15 mg of lamivudine, 30 mg of abacavir and 2.5 mg of dolutegravir was transferred to 10 ml volumetric flask and add diluent, let it be dissolved completely and using the same diluent make the volume up to the mark. Take 1 ml of the above solution into 10 ml volumetric flask and add diluent to make it up to the mark. The obtained solution was appropriately diluted with the mobile phase to get the final dilution of 0.024 mg/ml lamivudine, 0.048 mg/ml abacavir and 0.004 mg/ml dolutegravir.

RESULTS AND DISCUSSION

To optimize the RP-UPLC parameters, to reach a good resolution and peak tailing for lamivudine, abacavir and dolutegravir, many chromatographic parameters were tested. Several mobile phases of different ratios were analyzed to get good resolution, peak shape and to provide sufficient selectivity for the drugs. The phosphate buffer provided a higher sensitivity and selectivity than other buffers did. Using methanol and acetonitrile as organic components shown results of higher sensitivity, but varying the amounts of methanol and acetonitrile in the mobile phase affected the resolution, tailing factor, theoretical plates and run time. Varying the pH of the mobile phase resulted in poor peak shapes and poor resolution. So we introduced potassium dihydrogen phosphate and dipotassium hydrogen phosphate into the mobile phase to adjust the pH of the buffer to 3.0. The optimized mobile phase consisted of 2.95 g of potassium dihydrogen phosphate and 5.45 g of dipotassium hydrogen phosphate in 1 l Milli-Q-Water and adjusted pH to 3.0 with orthophosphoric acid-methanol (30:70 %v/v). The column elution was monitored at 260 nm and the injection volume was 5 µl. The column oven temperature was maintained at 25 °C (ambient). The Zodiac SIL RP C18 (4.6 mm × 250 mm with a particle size of 3 µm) was used with a constant flow rate of 0.25 ml/min in isocratic mode. Retention times (Rt) of lamivudine, abacavir and dolutegravir were approximately 1.763±0.06 min, 2.247±0.07 min and 3.175±0.06 min, respectively, in all the analytical runs. The standard and sample chromatograms were shown in fig. 4 and 5.

System suitability test

Before sample analysis, the chromatographic parameters used in this analysis must confirm the system suitability parameters within the limits. The retention time (Rt), tailing factor (T) and theoretical plate number (N) for the principal peak and its degradation product were evaluated for lamivudine, abacavir and dolutegravir. The tailing factors were 1.23, 1.25 and 1.29 for lamivudine, abacavir and dolutegravir, respectively. The theoretical plate numbers (N) were 2755, 2190 and 2693 respectively. The retention times (Rt) of the drugs were 1.763 min, 2.247 min and 3.175 min respectively. System suitability parameters (table 1) satisfied the USP guidelines and ICH guidelines.

Table 1: System suitability parameters

Parameters	Lamivudine	Abacavir	Dolutegravir
Retention time	1.763	2.247	3.175
USP plate count	2755	2190	2693
USP tailing	1.23	1.25	1.29
Standard area	935905	195063	27100

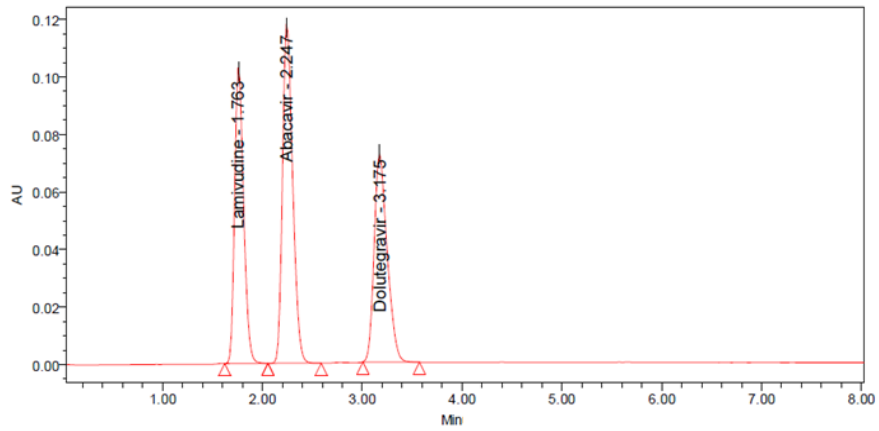

Fig. 4: Standard chromatogram

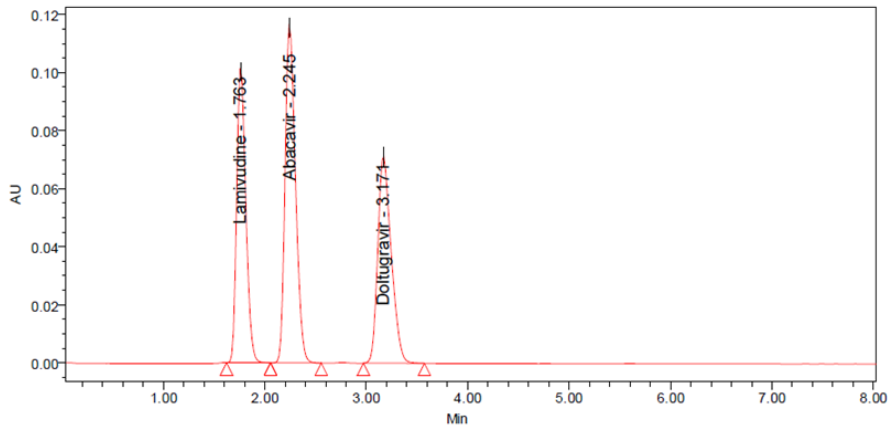

Fig. 5: Sample chromatogram

Table 2: Assay results from pharmaceutical formulation of lamivudine, abacavir and dolutegavir

Drug name	Label claim (mg)	% assay
Lamivudine	300	99.31
Abacavir	600	99.22
Dolutegravir	50	99.75

Data of n= 2 replicates

Assay of pharmaceutical formulation

The proposed method was effectively applied to find lamivudine, abacavir and dolutegravir in their tablet dosage form. The results obtained (table 2) were comparable with the corresponding labelled amounts.

Method validation

According to the international conference on harmonization (ICH) guideline, ICH Q2(R1) [33], this method was validated.

Linearity

Calibration plots for the analytes were prepared with standard stock solutions to yield the concentration ranges of 15-75 µg/ml for lamivudine, 30-150 µg/ml for abacavir, 2.5-12.5 µg/ml for dolutegravir into the UPLC system. In between the ranges given above, five concentrations were taken and triplicate injection of each concentration was performed.

Calibration curves were plotted between analyte concentrations versus that analyte area. Linearity regression analysis of the data gave correlation coefficient value, slope and intercept. For concentration between 15 µg/ml and 150 µg/ml, the calibration curves were linear. By the values of the correlation coefficients (R^2), the linearity of the calibration curves was validated. The correlation coefficient was 0.999 for these three drugs. The results of the linearity experiment were listed in table 3. Linearity graphs were shown in fig. 6, 7 and 8.

Table 3: Linearity results of lamivudine, abacavir and dolutegravir

Parameters	Lamivudine	Abacavir	Dolutegravir
Concentration range(µg/ml)	15-75	30-150	2.5-12.5
Correlation coefficient	0.999	0.999	0.999
Intercept	1397	21826	10988
Slope	14378	6610	59803

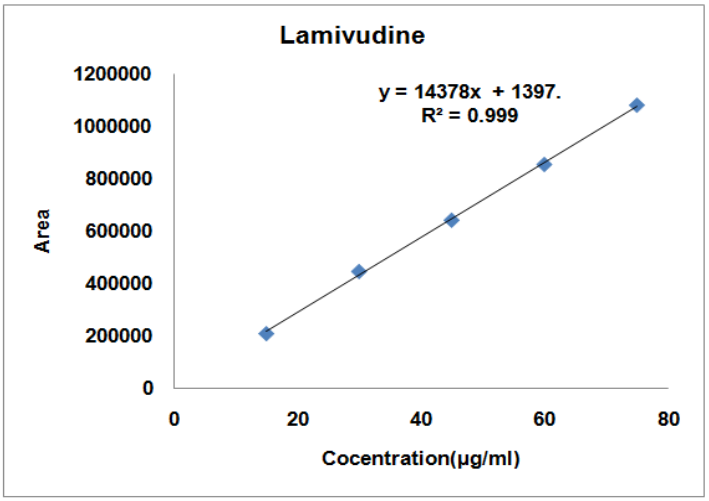

Fig. 6: Linearity graph of lamivudine

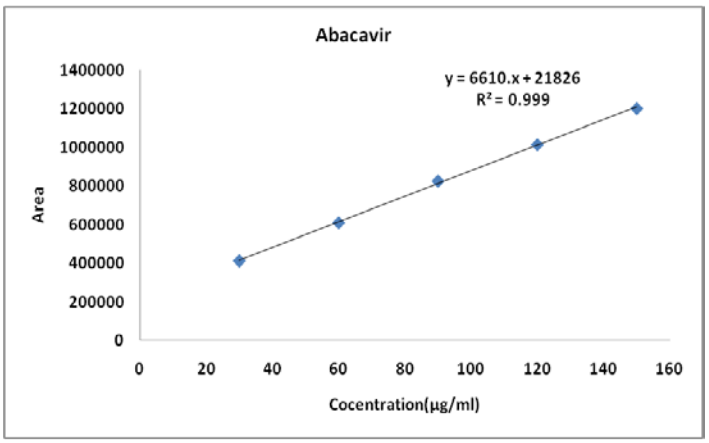

Fig. 7: Linearity graph of abacavir

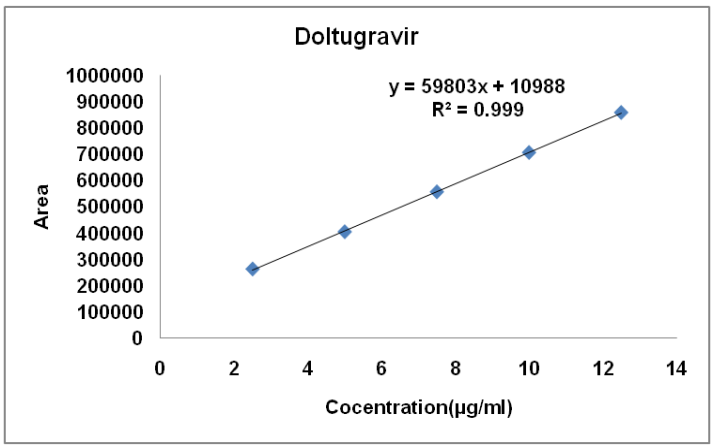

Fig. 8: Linearity graph of dolutegravir

Accuracy/Recovery

Accuracy was performed using a standard addition technique by recovery studies. The pre-analyzed samples were spiked with extra 50%, 100%, and 150% of each standard lamivudine, abacavir and dolutegravir and by using the proposed method mixtures were analyzed. The recovery studies were conducted in triplicate. The proposed method afforded a recovery of 98.60–101.69% after the additional standard drug solution was spiked with the presciently analyzed test solutions. The recovery percentages were in the ranges from 98.96 to 100.92 %, from 99.80 to 101.69 % and from 99.60 to 100.34% respectively. The values of the recovery (%) were shown in table 4, which indicates the accuracy of the proposed method.

Table 4: Accuracy results of lamivudine, abacavir and dolutegravir

Drug name	% concentration	Area	Amount added (mg)	Amount found (mg)	% recovery	mean recovery
Lamivudine	50%	573733	7.5	7.42	98.96	99.9
	100%	1158357	15	14.98	99.9	
	150%	1755375	22.5	22.70	100.92	
Abacavir	50%	732134	15	15.2	101.52	101.00
	100%	1557348	30	29.94	99.8	
	150%	2380289	45	45.76	101.69	
Dolutegravir	50%	541198	1.25	1.25	100.34	99.98
	100%	1074405	2.5	2.49	99.6	
	150%	1618551	3.75	3.75	100.02	

Data of n=3 replicates

Precision

For the precision, repeatability expressed the same chromate-graphic parameters within the short time interval. For the intermediate precision, repeatability expressed in a different day under same chromatographic parameters. The same concentration sample was injected in six replicates for intraday (precision) and six replicates for interday (intermediate precision). The peak areas for injections recorded and then calculated % Relative Standard Deviation (%RSD) for intraday (precision) and interday (intermediate precision). The intra-day precision of the method ranged from 0.17 to 0.20 % RSD and inter-day precision of the method was found from 0.11 to 0.17 % RSD for lamivudine, abacavir and dolutegravir, which signify that the developed method was precise (table 5). The lowest values of the RSD (%) specify that the chosen method is repeatable.

Table 5: Precision and inter-day precision results for lamivudine, abacavir and dolutegravir

Injection	Precision			Inter-day precision		
	Lamivudine	Abacavir	Dolutegravir	Lamivudine	Abacavir	Dolutegravir
Injection 1	641219	831356	654221	637987	828667	652517
Injection 2	641645	831763	654574	638983	829544	654557
Injection 3	642197	832877	655600	639198	829935	654622
Injection 4	643020	833975	656731	639852	830731	654726
Injection 5	644273	835545	657468	639951	830995	655234
Injection 6	642460	833403	656718	640553	831033	655761
Average	642469.0	833153.2	655885.3	639420.6	830151.0	654569.7
Standard deviation	1083.8	1529.5	1302.6	899.8	942.9	1104.5
% RSD	0.17	0.18	0.20	0.14	0.11	0.17

Data of n= 6 replicates

Limit of detection (LOD) and limit of quantification (LOQ)

The lowest amount of analyte in the drug, which can be detected, but not necessarily quantified, indicates the limit of detection (LOD). The lowest amount of analyte in the drug, which can be quantitatively determined with suitable precision and accuracy indicates the limit of quantification (LOQ). The limit of quantification (LOQ) and limit of detection (LOD) were determined based on the slope and the standard deviation of the response using the signal-to-noise ratio (S/N) as per ICH guidelines Q2(R1) 2005. The LODs for lamivudine, abacavir and dolutegravir were found to be 0.021, 0.330 and 0.038 µg/ml and the LOQs were 0.056, 1.320 and 0.095 µg/ml, respectively (table 6).

Table 6: LOD and LOQ values of lamivudine, abacavir and dolutegravir

Drug	LOD concentration (µg/ml)	LOQ concentration (µg/ml)
Lamivudine	0.021	0.056
Abacavir	0.330	1.32
Doltugravir	0.038	0.095

Robustness

Robustness of the method was performed by making slight deliberate changes in the analytical methodology like flow rate and solvent ratio. It was observed that this method did not significantly affect in system suitability parameters like USP tailing factor, theoretical plates and resolution, which confirmed that the developed UPLC method is robust (table 7).

Table 7: Robustness study for the UPLC method

Drug	Parameter	Retention time	Peak area	USP plate count	USP tailing
Lamivudine	Flow1	1.950	712143	2504	1.26
	Flow2	1.607	731317	2563	1.25
	Low Organic	1.666	582337	2537	1.24
	High Organic	1.550	712143	2504	1.26
Abacavir	Flow1	2.475	928580	2698	1.32
	Flow2	2.039	757879	2904	1.29
	Low Organic	2.485	761420	2229	1.33
	High Organic	2.375	928580	2698	1.32
Dolutegravir	Flow1	3.488	731317	2809	1.38
	Flow2	2.877	596086	2421	1.34
	Low Organic	4.705	595173	3060	1.44
	High Organic	3.988	731317	2809	1.38

Degradation studies

According to stability testing of new drug substances and products, a guideline of ICH desires that to clarify the inherent stability characteristics of the active component stress testing was implemented. The aim of this work was to carry out the stress degradation studies on the lamivudine, abacavir and dolutegravir using the proposed method.

Formulation drug products were exposed to thermal stress, oxidative stress, photolytic, hydrolytic stress under acidic medium and basic medium. An ideal stability indicating method, quantifies the standard drug alone and also resolves its degradation products. So described different types of stress used were thermal, oxidative, photolytic, acidic and basic hydrolysis. Some unknown degradant peaks were observed in the acidic, basic, peroxide, photolytic and thermal studies. But based on the peak purity, no degradant peaks were reported at the retention time (Rt) of lamivudine, abacavir and dolutegravir. Therefore, the drugs were stable up to the specified period of 12 h when the proposed method is used, or they are susceptible to acids, alkali, hydrogen peroxide, photolytic and thermal.

Hydrolytic degradation under acidic conditions

Pipette 3 ml from standard stock solution containing 0.15 mg/ml, 0.3 mg/ml and 0.025 mg/ml of lamivudine, abacavir and dolutegravir into a 10 ml flask and added 1.0 ml of 0.1N HCl. Then, the volumetric flask was kept at room temperature (RT) for 6 h and then neutralized with 0.1 N NaOH and filled with diluents up to the mark. By using 0.45-micron syringe filters, filtered the solution and placed in vials. The results showed multiple peaks for the degradation products. The degradations percentage of the drugs observed were 5.00%, 8.03% and 20.69% (table 8), here no degradant peaks were observed at a retention time (Rt) of lamivudine, abacavir and dolutegravir.

Hydrolytic degradation under alkali conditions

Pipette 3 ml from a standard stock solution containing 0.15 mg/ml, 0.3 mg/ml and 0.025 mg/ml of lamivudine, abacavir and dolutegravir into a 10 ml flask and added 1 ml of 0.1N NaOH. Then, the volumetric flask was kept at room temperature (RT) for 6 h and then neutralized with 0.1N HCl and filled with diluents up to the mark. By using 0.45-micron syringe filters, filtered the solution and placed in vials. The results showed multiple peaks for the degradation products. The degradations percentage of the drug observed were 14.39%, 13.73% and 16.66% (table 8), here no degradant peaks were observed, at the retention time (Rt) of lamivudine, abacavir and dolutegravir.

Thermal-induced degradation

The sample treated with the thermal condition at 105 °C for 48 h. Then the sample was taken and diluted with diluent to prepare 45 µg/ml, 90 µg/ml and 7.5 µg/ml of lamivudine, abacavir and dolutegravir respectively were injected into UPLC and it was analyzed. No degradant peaks were observed, at the retention time (Rt) of these drugs. The degradation percentages were found to be 15.99, 4.91 and 17.11% (table 8).

Oxidative degradation

Pipette 3 ml from a standard stock solution containing 0.15 mg/ml, 0.3 mg/ml and 0.025 mg/ml of lamivudine, abacavir and dolutegravir into a 10 ml flask and treated with 1 ml of 3% w/v of hydrogen peroxide as oxidation agent filled with diluents up to the mark. That volumetric flask was then kept at room temperature (RT) for 30 min. By using 0.45-micron syringe filters, filtered the solution and placed in vials. The observed degradation percentages were 9.97, 17.68 and 5.94%, respectively (table 8). No degradant peaks were observed at the retention time (Rt) of these drugs.

Photolytic degradation

The drug substances were exposed to the energy of a 1.2 million lux hr fluorescent light and to 200 W/Sq. the meter of UV light for approximately 7 d. Prepared the sample solution and then analysed the sample, here no degradant peaks were observed at the retention time (Rt) of lamivudine, abacavir and doutegravir. The degradation percentages were found to be 11.04, 16.65 and 18.24% respectively (table 8).

Table 8: Degradation results of lamivudine, abacavir and dolutegravir

Type of degradation	Lamivudine			Abacavir			Dolutegravir		
	Sample area	% recovered	%of degradation	Sample area	% recovered	%of degradation	Sample area	% recovered	%of degradation
Acid	889116	95.00	5.00	179391	91.97	8.03	214934	79.31	20.69
Alkali	801257	85.61	14.39	168274	86.27	13.73	225846	83.34	16.66
Thermal	786258	84.01	15.99	185487	95.09	4.91	254892	82.89	17.11
Oxidative	842575	90.03	9.97	160578	82.32	17.68	224635	94.06	5.94
Photolytic	832547	88.96	11.04	162587	83.35	16.65	221578	81.76	18.24

This method is specific for the determination of lamivudine, abacavir and dolutegravir with no interference and with good linearity, accuracy and precision. We achieved good separation for selected drugs. In addition, this separation technique uses simple, low cost and short runtime. The chromatographic conditions of this method were optimized for a short 8 min run time in RP-UPLC. It is an excellent method for the quantification of lamivudine, abacavir and dolutegravir in their pharmaceutical dosage forms.

At present, only HPLC methods were available in this combination. No UPLC methods were found till date.

CONCLUSION

The proposed RP-UPLC method for determination of lamivudine, abacavir and dolutegravir was developed and validated in pharmaceutical formulations. The prescribed method adapted the use of an economical and easily available mobile phase, stationary phase, convenient and easy extraction procedures. The method was sensitive enough to detect low concentration of 0.021 µg/ml, 0.330 µg/ml and 0.038 µg/ml for lamivudine, abacavir and dolutegravir respectively. Recovery of selected drugs from spiked control samples were>99% by using this method. A stability-indicating RP-UPLC method for the estimation of selected drugs in their solid dosage forms was established and validated in accordance with the ICH guidelines.

ABBREVIATION

ART: Antiretroviral theraphy; INI: Integrase inhibitors; STR: Single tablet regimen; NRTIs: Nucleoside Reverse Transcriptase Inhibitors; UV Detector: Ultraviolet Detector; RSD: Relative standard deviation; HPLC: High-Performance Liquid Chromatography; ICH: International Conference on Harmonization; SD: Standard deviation; PDA: Photo diode array; LOD: Limit of detection; LOQ: Limit of quantitation; DNA: Deoxyribonucleic acid; RNA: Ribonucleic acid; HIV: Human Immunodeficiency Virus; UPLC: Ultra Performance Liquid Chromatography; USP: United States Pharmacopeia; Rt: Retention time; RT: Room temperature.

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AUTHOR CONTRIBUTION

Corresponding author and first author proposed the design of the study. The first author drafted the manuscript and carried out the all

experimental works and performed the statistical analysis. Both authors read and approved the final manuscript.

AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest

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