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Original Article

Application of Reversed-Phase HPLC Method for the Simultaneous Determination of Lenacapavir and Bictegravir in Tablets Dosage Form

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ABSTRACT

Background: Human Immunodeficiency Virus is a debilitating viral infection that compromises the immune system, rendering individuals vulnerable to opportunistic infections. While antiretroviral therapy can effectively manage the condition, the development of novel treatment strategies remains crucial. This study aims to create and validate a robust reversed-phase HPLC routine for the concurrent quantification of lenacapavir and bictegravir, two antiretroviral agents. **Materials and Methods:** A novel chromatographic separation method was developed utilizing a Hypersil ODS C_{18} column (4.6x250 mm, 5 µm particle size). The mobile phase, a gradient mixture of 0.1% OPA buffer and acetonitrile, was optimized for efficient analyte elution. Chromatographic detection was performed at 220 nm with a flow rate of 1.0 mL per minute, injecting 20 µL of sample volume for each analysis. **Results:** Linearity for lenacapavir was established across a concentration range of 72-216 µg/mL, while bictegravir demonstrated linearity from 24-72 µg/mL. The chromatographic retention times for lenacapavir and bictegravir were computed to be 2.17 and 11.46 min, respectively. Recovery studies indicated that both analytes could be accurately quantified, with recovery percentages falling within the 98-102% range. **Conclusion:** In accordance with ICH requirements, the novel approach was successfully validated. The devised method is dependable and cost-effective for routine analysis with respect to all parameters that have been evaluated.

Keywords: Lenacapavir, Bictegravir, Simultaneous estimation, Liquid chromatography, Validation.

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INTRODUCTION

Acquired Immunodeficiency Syndrome (AIDS) is a condition resulting from infection with the "Human Immunodeficiency Virus (HIV)", which dwindles the immune system, making the body prone to serious infections and illnesses. The primary means of transmission is via heterosexual intercourse. On each day, more than 5,000 individuals contract the infection, with 500 of them being children. However it is projected to decrease to 8.5 by the year 2040. Based on the global health sector HIV plan by the World Health Organisation (WHO), the global number of HIV infections will decrease from 1.5 million in 2020 to 335,000 by 2030.[1-3] The incidence of individuals with HIV is on the rise as a result of breakthroughs in Antiretroviral Therapy (ART), which has significantly decreased HIV-related illness and death rates. However, several patients still struggle to maintain adequate viral suppression, leading to the progress of viral mutations and drug resistance. It is imperative to develop initiatives to address the increasing prevalence of HIV. Despite extensive endeavours to create efficacious drugs and a vaccine, a comprehensive therapy, remedy, or vaccination for AIDS is still some years in the future. Continuing to increase efforts to avert the propagation of HIV by solving behaviours that lead to transmission is of utmost importance.[4]

The HIV treatment guidelines suggests using a "combination of two nucleoside reverse transcriptase inhibitors along with either an integrase strand transfer inhibitor, a non-nucleoside reverse transcriptase inhibitor, or a boosted protease inhibitor" as the primary treatment for the majority of patients having HIV. The aforementioned blend of anti-retroviral effectively reduces viral load to undetectable levels, boosts $CD4^+$T cells, inhibits transmission and prolongs lives in a significant number of patients.[5-7]

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Bictegravir (BIC; GS-9883) is a new and powerful inhibitor of HIV-1 Integrase (IN) that is taken once a day without any requirement of any booster dose. It selectively targets the activity of IN in transferring strands, with an IC_{50} (Inhibitory Concentration) of 7.5±0.3 nM. Additionally, it effectively inhibits the integration of HIV-1 in the human cells. The BIC demonstrated selective and high *in vitro* activity in both primary human T-lymphocytes and cell lines. The concentration required for 50% effectiveness ranges from 1.5 to 2.4 nM and the selectivity indices can reach up to 8,700 compared to its toxicity.[8] BIC demonstrates synergistic antiviral effects *in vitro* when combined with tenofovir alafenamide, emtricitabine, or darunavir. It also retains strong antiviral efficacy against HIV-1 strains that are resistant to other types of antiretroviral drugs.[9]

Lenacapavir (LEN), commonly referred to as GS-6207, is a novel HIV-1 capsid inhibitor that is used alongside other antiretroviral drugs to treat HIV-1 infection that is resistant to multiple drugs.[10,11] Additionally, it shows promise as a preventive measure for HIV infection, known as pre-exposure prophylaxis. LEN is used in combination due to its strong synergistic effects and lack of resistance with other authorised antiretroviral drugs. Additionally, it has antiviral action at very low concentrations. Extended-release formulations of LEN are given every 26 weeks (or six months) as a subcutaneous injection after an initial time of taking the medication orally. On December 22, 2022, the FDA approved lenacapavir for the treatment of adults with a history of severe treatment failure resulting from resistance, intolerance, or other safety concerns with their existing antiretroviral therapy regimen.[12]

Gilead Sciences is currently working on an oral two-drug regimen, bictegravir+lenacapavir, inspired by the achievements of other successful two-drug regimens. A clinical trial (NCT 05502341) is now being conducted in multiple countries to evaluate the effectiveness of this combination in individuals with HIV.[13] The trial features an intricate structure, but, following a two-day initial dose of lenacapavir, the majority of participants will receive a combination of bictegravir at a daily dosage of 75 mg together with lenacapavir administered at either 25 mg or 50 mg per day. If the bictegravir+lenacapavir combination proves to be efficacious, it is quite anticipated that it will receive approval for therapeutic use.[14] The combination may be considered as a viable choice for individuals with multidrug-resistant HIV or for patients whose physicians recommend a nucleoside-free treatment plan.

The IUPAC name for lenacapavir is "N-[(1S)-1-[3-[4-chloro-3-(methanesulfonamido)-1-(2,2,2-trifluoroethyl)indazol-7-yl]-6-(3-methyl-3-methylsulfonylbut-1-ynyl)pyridin-2-yl]-2-(3,5-difluoro phenyl)ethyl]-2-[(2S,4R)-5,5-difluoro-9-(trifluoro methyl)-7,8-diazatricyclo[4.3.0.02,4]nona-1(6),8-dien-7-yl]acetamide"andfor bictegravir is "(1S,11R,13R)-3,6-dioxo-7-[(2,4,6-trifluorophenyl)methylcarbamoyl]-12-oxa-2,9-diazatetracyclo[11.2.1.02,11.04,9]hexadeca-4,7-dien-5-olate". The chemical structure of LEN and BIC were shown in Figure 1. A good peak separation between the drug and its degradants is recommended for any HPLC technology developed for active drugs in recent years.[15-18] The extensive literature review revealed no analytical approach has been described for the simultaneous analysis of LEN and BIC in any application; the methods mentioned in literature are for the investigation of bictegravir with combination of other anti-retroviral drugs.[19,20] As a result, this technique has been developed to use a UV detector coupled High-Performance Liquid Chromatography (HPLC) to analyse LEN and BIC simultaneously in tablet dosage form. The LEN/BIC in tablet dosage formulation was effectively analysed using the established approach.

MATERIALS AND METHODS

Instruments

A Shimadzu HPLC (model no. LC-2030C) with a UV detector (model SPD-M20A) was the instrument employed in the investigation. Data collection was managed using Empower software, version 2. Separations were achieved using a "Hypersil ODS C_{18} column (4.6×250 mm, 5 µm)". A Rheodyne injection valve equipped with a 20 microlitre sample loop was used to introduce the sample. All weighing measurements were performed on a Mettler Toledo analytical balance.

Chemicals and materials

High-purity HPLC reagents and solvents were employed in this study. Acetonitrile (CH_3CN) and orthophosphoric acid (H_3PO_4) were procured from SD Fine Chem Ltd., Mumbai, India. The reference standards for LEN and BIC were supplied by Ascentyo Biosciences, Hyderabad, India.

Chromatographic conditions

A gradient elution method was used, combining a 0.1% orthophosphoric acid buffer with acetonitrile (specific composition outlined in Table 1) as mobile phases A and B. The chromatographic analysis was performed at ambient temperature (~25ºC) with a continuous flow rate (~1.0 mL/min). Twenty microliters of each sample were injected into the HPLC system. Detection of LEN and BIC was achieved at a wavelength of 220 nanometers using a UV detector. A diluent was prepared by combining 50:50 v/v acetonitrile with water.

Preparation of standard solution

An accurate weighing scale was used to transfer 180 mg of LEN and 60 mg of BIC working standards into a 100 mL Volumetric Flask (VF) in order to prepare the stock solution. An appropriate diluent was used to dissolve the solids entirely. The same diluent was then used to raise the solution to the final volume. Next, 5 mL of the stock solution was added to a 50 mL VF and mixed

to make a working solution. Linearity studies were conducted for both LEN (72 to 216 mg/mL) and BIC (24 to 72 mg/mL) to establish calibration curves.

Preparation of sample stock solution

Ten tablets, which were manufactured in-house, were finely ground to a powder. Each tablet contained 300 mg of LEN and 50 mg of BIC. A precisely measured quantity of this powder, corresponding to 180 mg of LEN and 60 mg of BIC, was put to a 100 mL VF and dissolved in 50 mL of diluent. The solution was filled up to the desired volume using the diluent, resulting in a stock solution. A VF was filled with 5 mL of the stock solution, which was then diluted to 50 mL in order to achieve the target concentrations of 60 µg/mL for BIC and 180 µg/mL for LEN. The amounts of LEN and BIC in the samples were quantified by analyzing the corresponding peak areas in the chromatograms.

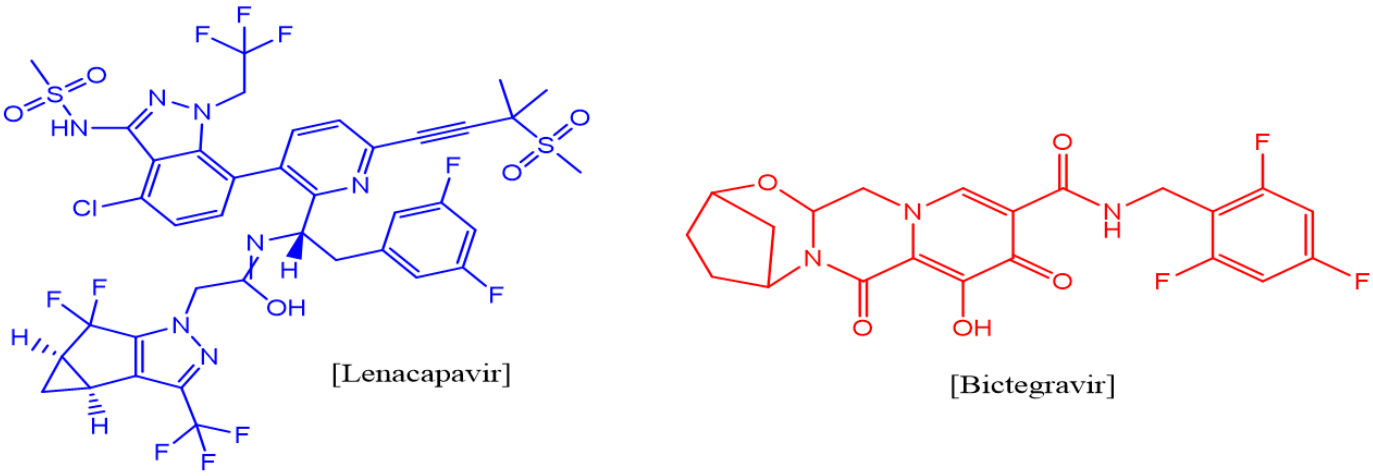

Figure 1: Chemical structures of drugs.

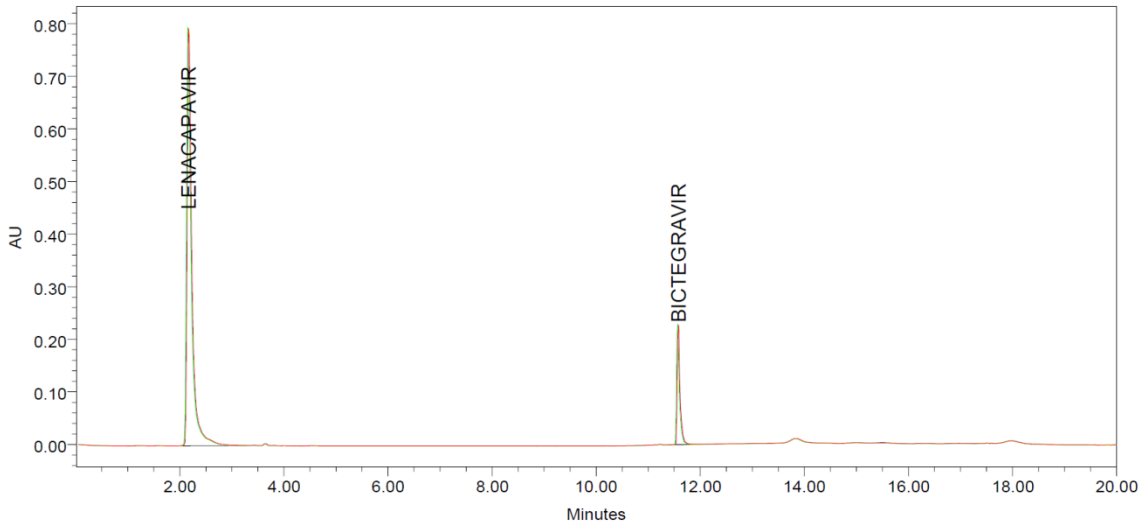

Figure 2: Chromatogram of standard solution of LEN and BIC.

Table 1: Time programming of gradient of elution.

Time (min)	Mobile phase A (% v/v) (0.1% OPA buffer)	Mobile phase B (% v/v) (CH_3CN)
0	80	20
6	80	20
10	30	70
15	30	70
17	80	20
20	80	20

Table 2: System suitability parameters.

Sl. No.	Parameters	Lenacapavir	Bictegravir	Limits
1.	Relative retention time (min)*	2.17	11.46	-
2.	% RSD of retention time	0.38	1.39	Not more than 2
3.	Peak area*	6359815	1059821	-
4.	% RSD of peak area	0.3	0.3	Not more than 2
5.	Theoretical plates	35867	28563	More than 2000
6.	Tailing factor	1.79	1.54	Less than 2
7.	Resolution	16.3	11.21	More than 2

*Mean of six determinations.

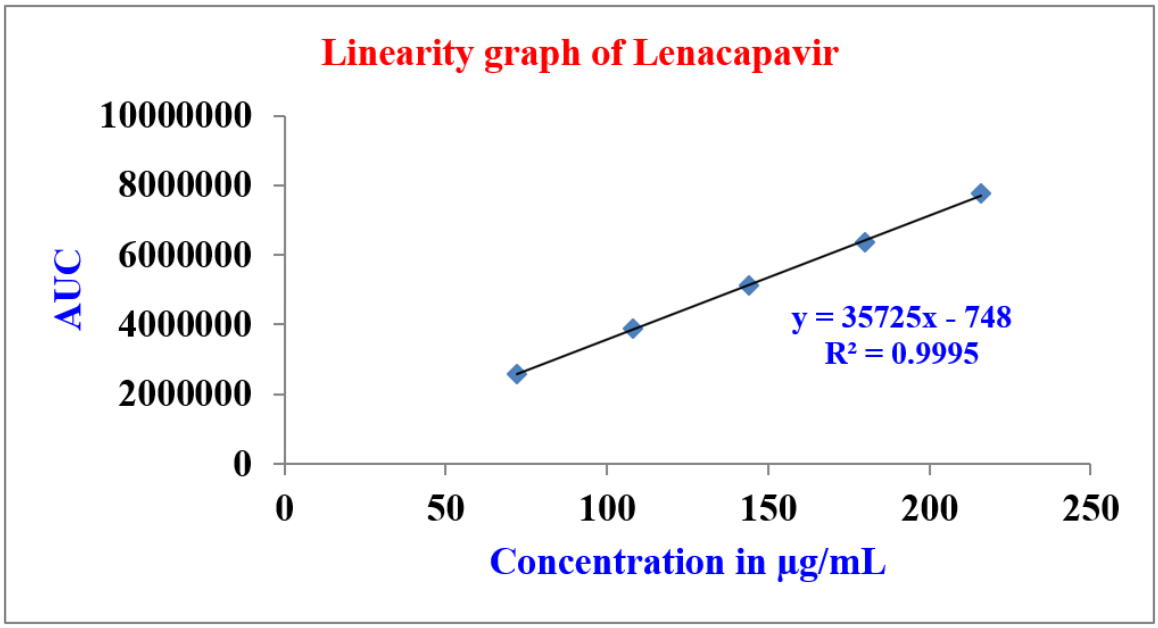

Figure 3: Linearity graph of Lenacapavir.

RESULTS

Analytical method development

In order to ensure accuracy, a dependable HPLC method was developed to analyse LEN and BIC simultaneously. This involved optimising several experimental parameters while keeping the circumstances consistent. Mobile phase composition, column type and flow rate were carefully adjusted to achieve optimal peak separation. In order to guarantee method reproducibility and facilitate validation, the study maintained constant values for parameters including elution mode, column temperature (25±2°C), injection volume (20 µL) and detector type. Spectral data was collected for each chromatographic run at the designated detection wavelength. Additional factors considered during method development included peak shape, system backpressure, analytical accuracy, peak resolution, analysis time and solvent consumption.

Validation

Once satisfactory chromatographic conditions were achieved, the analytical routine was validated according to the guiding principles outlined in ICH Q2 (R1).[21] Additionally, the method's solvent and reagent stability was assessed.

Evaluation of system suitability

To assess the HPLC system's performance, system suitability tests were conducted. Six replicate injections of a standard solution were analyzed. Column efficiency, peak count and peak shape (tailing factor) were determined from these injections. The system's consistency was assessed by calculating the RSD (Relative Standard Deviation) of the peak areas, which was expected to be less than two percent. Additionally, the column efficiency was required to be at least 2000 theoretical plates and the tailing factor for both analytes had to be less than 2.0. The results are shown in Table 2.

Table 3: **Results of precision study.**

Parameter	Lenacapavir			Bictegravir		
	Mean	SD	%RSD	Mean	SD	%RSD
System Precision	6372376.2	37616.6	0.6	1062872.3	7011.9	0.7
Method Precision	6363737	16586.5	0.3	1063164	8525.9	0.8
Intermediate Precision	6347716.57	27524.93	0.43	1061187.2	8624.6	0.8

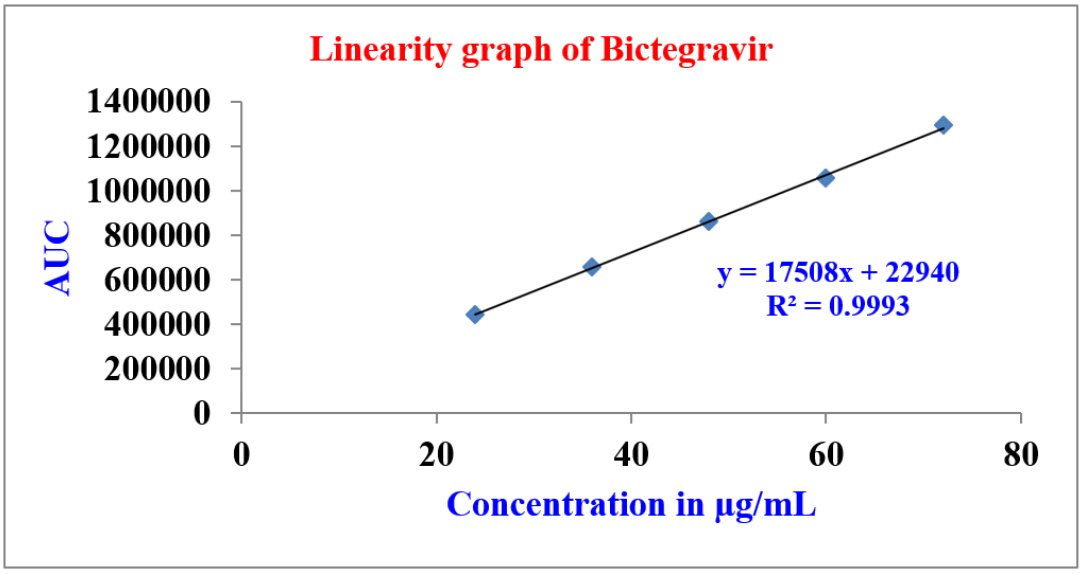

Figure 4: Linearity graph of Bictegravir.

Specificity and selectivity

To assess the method's ability to specifically detect the target drugs without interference, a two-step injection process was employed. Initially, a blank solution (free of any drug) was injected to establish a baseline and identify any potential system or mobile phase peaks. Subsequently, a drug solution containing both LEN and BIC was injected. By comparing the resulting chromatograms, the effectiveness of the chromatographic conditions in separating the target drugs from potential impurities was evaluated.

Linearity

A series of standard solutions were prepared to evaluate the linearity of the method, with concentration ranges of 72 to 216 µg/mL for LEN and 24 to 72 µg/mL for BIC. The peak areas of these solutions were recorded after they were analysed using HPLC. The peak area was plotted against concentration to construct calibration curves. The method's linearity was assessed by investigating the linearity of the calibration curves.

Precision

Precision is a crucial quality of a method, indicative of the degree of agreement between independent test findings that are acquired through repeated use of the same analytical technique on the same sample. In order to thoroughly assess precision, this study followed the ICH validation guidelines. System, method and intermediate precisions (repeatability) were all included in the investigation (Table 3).

Method precision (Repeatability)

Method precision was assessed by preparing six replicate samples from a single batch. The percentage assay for each replicate was calculated. The method's precision was assessed by computing the RSD of the assay values.

Intermediate Precision (IP)

Intermediate precision evaluates a method's robustness under varying laboratory conditions. This includes factors like different analysts, days, equipment and columns. To assess intermediate precision, six samples were prepared from the same batch on different days by different analysts. The percentage assay of each sample was determined. The method's robustness has been assessed by determining the %RSD of the results.

Accuracy or recovery studies

Method accuracy was evaluated by comparing obtained results to accepted reference values. Standard solutions were added to placebo samples at concentrations equivalent to 80%, 100% and 120% of the desired concentration. The spiked samples were analyzed using the developed method. In compliance with the ICH guidelines, the average recovery for each analyte at each

Table 4: Results of accuracy study.

Drug	Level	Concentration (µg/mL)	Amount Recovered (µg/mL)	% Recovery
Lenacapavir	80	144	143.91	99.95
		144	143.93	
		144	143.96	
	100	180	180.03	99.99
		180	179.96	
		180	179.97	
	120	216	215.15	99.87
		216	215.11	
		216	216.91	
Bictegravir	80	48	47.72	99.58
		48	47.56	
		48	48.12	
	100	60	59.44	99.36
		60	60.13	
		60	59.29	
	120	72	72.16	99.49
		72	71.7	
		72	71.04	

concentration level should fall within 98-102%. Additionally, the Relative Standard Deviation (RSD) of recoveries at each level should not exceed 2% (Table 4).

Sensitivity

The sensitivity of the technique was tested by finding the Limit of Detection (LOD) and the Limit of measurement (LOQ). LOD stands for the lowermost concentration of analyte that can be safely distinguished from background noise. LOQ, on the other hand, stands for the lowest concentration that can be accurately measured. The values used to figure out these amounts were based on Signal-to-Noise (S/N) ratios. A typical S/N of 3 indicates LOD, while an S/N of 10 signifies LOQ in HPLC. Specific concentrations and corresponding peak areas were used in these calculations.

Robustness

Method robustness was assessed by intentionally introducing small variations to critical chromatographic parameters. To be more precise, the temperature of the column was changed by±5°C, the flow rate by±0.1 mL/min and the content of the mobile phase by±5%. The impact of these changes on the analytical results was evaluated to determine the method's resilience to minor fluctuations.

Ruggedness

Method ruggedness was evaluated by having multiple analysts perform the analysis on different days using different equipment. This assessed the method's reliability under varying experimental conditions.

Application of the developed method

The method's accuracy was assessed by performing consecutive injections of standard and sample solutions, each containing approximately 180 µg/mL of LEN and 60 µg/mL of BIC. The materials and methods section already addressed the production of these solutions in extensively. The following formula was used to compute the percentage assay:

$$\% \text{ Assay} = \text{TPA}/\text{SPA} * \text{MSG}/\text{DS} * \text{DT}/\text{SMG} * \text{PP}/100 * \text{AWT}/\text{Label Claim}$$

where TPA is the test sample (tablet) peak area, SPA is standard solution peak area, standard Substance Weight in mg (MSG), Sample powder weight in mg (SMG), Dilution factors of Standard and Test solutions (DS and DT, respectively), standard Percentage Purity (PP) and Average tablet Weight in mg (AWT) are all variables in this equation.

Mobile phase stability

Mobile phase stability was evaluated by preparing a 1:1 mixture of LEN and BIC. The stability of this mixture was assessed at 6, 12, 24 and 48 hr to determine any changes in composition over time.

DISCUSSION

Lenacapavir, the first HIV treatment drug targeting the virus's capsid, is recommended for patients with drug resistance. It is used in combination with bictegravir, providing a safe, effective single-pill regimen. Suitable for limited treatment options, not newly diagnosed patients.[13] Based on the literature study, there is currently no established method for quantifying the dosage of mixed tablet forms in the Pharmacopoeias. There are only two approaches to analysis, specifically Reversed Phase High-Performance Liquid Chromatography (RP-HPLC), that can be used to estimate the amount of Bictegravir (BIC) in combination with Emtricitabine (EMT) and Tenofovir (TEN) drugs.[19,20] An extensive investigation has been conducted to develop a reliable RP-HPLC technique for the simultaneous determination of LEN and BIC. The drugs' solubility was evaluated in several solvents, such as water, methanol and acetonitrile, using different ratios. In the end, it was concluded that the active pharmaceutical ingredients can dissolve in a combination consisting of equal parts acetonitrile and water. The isosbestic point for LEN and BIC was decided on depending on the UV-absorption spectra intersected at a common point of maximum absorbance at 220 nm. In order to achieve the detection of both LEN and BIC on the same chromatogram, different mobile phases and elution procedures were initially experimented. The gradient elution with different mobile phase ratios in the optimized method were determined based on sensitivity, selectivity and appropriate chromatographic parameters of the developed peaks, including peak shape, peak purity, peak sharpness, tailing factor and resolution between the peaks. The initial approach involved utilizing an isocratic elution method with several mobile phases, such as acetonitrile and formic acid (0.1%), OPA buffer with methanol and acetonitrile. These compositions were used at a flow rate of 1 mL/min to separate the two drugs that have an absorption peak at 220 nm. Regrettably, the resolution of LEN and BIC was inadequate and the time analysis, specifically 30 min, was very lengthy. The resolution, retention period and tailing factor did not meet the desired standards. Finally, a mobile phase consisting of 0.1% OPA and acetonitrile was used, with a flow rate of 1 mL/min. After evaluating all of these variables, the Hypersil ODS C_{18} Column (4.6x250 mm, 5 µm) was selected as the best stationary phase among other tested ones. The C_{18} column we utilized is well favored because to its exceptional hydrophobic separation capabilities and extensive surface area coverage.[15] We have chosen to examine the utilization of an elution gradient (Table 1) in order to enhance the accuracy and efficiency of our investigation. In addition, the theoretical plate values for LEN and BIC in this approach were 35,867 and 28,563, respectively, both exceeding 2000. The tailing factor for both medications had values below 2 due to the presence of sharp peaks without any tailing or fronting. The strategy was approved in accordance with the ICH criteria Q2R1.[21] Analysis of a 100% concentration solution demonstrated that the parameters for system suitability met the acceptance criteria and précised in Table 2. The retention time of the LEN peak was 2.17 min and for BIC was 11.76 min as shown in Figure 2. Specifically, the RSD was less than or equal to 2%, the tailing factor was less than or equal to 2 and the plate count exceeded 2000. The linearity graph exhibited a strong correlation between the concentrations and peak area when measured within the range of 72 to 216 µg/mL for LEN and 24 to 72 µg/mL for BIC. The high correlation coefficients (R^2>0.999) for both LEN and BIC, as shown in Figures 3 and 4, provide strong indication of a precise linear association between the concentration of the analyte and the response of the detector. There were no peaks identified that co-eluted with the target compound, indicating a high level of specificity. The peak shape was sharp and symmetrical, further confirming the method's specificity. We found the LOD and LOQ for LEN and BIC to be 1.8, 0.06, 5.4 and 0.18 g/mL, respectively, indicating the sensitivity of the method. A quantitative analysis of a pharmaceutical tablet formulation containing LEN and BIC revealed respective contents of 99.72% and 99.85%. The mobile phase remained clear and free of particles throughout the stability study. System suitability tests, including precision and accuracy checks, were consistently met over the two-day observation period. These results indicate that the mobile phase was stable when stored at room temperature. The developed method is appropriate for conducting quality control studies on the formulations.

CONCLUSION

A novel RP-HPLC/UV routine was successfully established for the simultaneous quantification of LEN and BIC in tablet formulations. The method proved efficient and reliable for routine analysis. Comprehensive validation studies as per ICH guidelines demonstrated that all method parameters met acceptance criteria, confirming its sensitivity, accuracy, robustness and cost-effectiveness. This validated HPLC method offers a reliable approach for the simultaneous quantification of LEN and BIC in the dosage forms, with potential applications in future research.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

AIDS: Acquired immunodeficiency sydrome; **BIC:** Bictegravir; **ICH:** International conference on harmonization; **LEN:** Lenacapavir; **%RSD:** Percentage relative standard deviation; **RP-HPLC:** Reversed-phase high-performance liquid chromatography.

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