INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES
[ISSN: 0975-4725; CODEN(USA): IJPS00]
Journal Homepage: https://www.ijpsjournal.com

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Research Article

Development, Optimization and Validation of Analytical Procedures for The Simultaneous Determination of Selected Antiviral Drug Combinations

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ARTICLE INFO

Published: 20 Dec. 2024

Keywords:
Absorptivity Factor Method, RP-HPLC, Pibrentasvir, Glecaprevir, Lamivudine, Tenofovir.

DOI:
10.5281/zenodo.14536508

ABSTRACT

A novel, precise, and accurate methodology was developed for the quantification of Pibrentasvir (PBR) and Glecaprevir (GLE) in bulk dosage form using UV-Vis spectrophotometry, as well as for Lamivudine (LAM) and Tenofovir (TNF) in bulk drug formulation employing the RP-HPLC technique. The absorbance of GLE and PBR followed Beer's law within concentration ranges of 6.0 - 8.5 µg/ml and 2.0 - 4.5 µg/ml, respectively. For the simultaneous quantification of LAM and TNF in combination tablet formulations, chromatographic separation was achieved using a Phenomenex C18 column (250×4.6 mm, 5 µm) with a mobile phase consisting of phosphate buffer (pH 5) and acetonitrile (60:40 v/v) at a flow rate of 1 ml/min. The detection wavelength was set at 264 nm. The retention times for LAM and TNF were determined to be 4.4 minutes and 1.8 minutes, respectively. Multiple analytical performance metrics, such as linearity, precision, accuracy, limit of detection (LOD), and limit of quantification (LOQ) were assessed and found to comply with International Council for Harmonization (ICH) guidelines (Q2A and Q2B). The established methods demonstrated good precision, with a relative standard deviation below 2% for both drug combinations.

INTRODUCTION

The number of newly launched pharmaceuticals is escalating daily, making the development of appropriate analytical methods crucial for the qualitative and quantitative assessment of drugs in analytical chemistry. Analytical method development involves establishing a series of experimental conditions for performing analytical operations on chemical samples. Established analytical methods can evaluate the identification, potency, purity, and stability of drug substances, drug products, and other pharmaceutical

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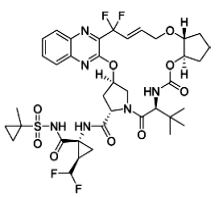
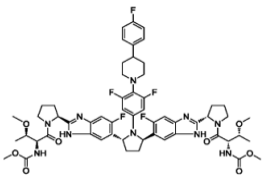
Relevant conflicts of interest/financial disclosures: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

constituents [1]. Viral infections are of paramount importance in today's healthcare landscape for several reasons. Viral illnesses such as influenza, hepatitis, and HIV/AIDS can lead to widespread outbreaks and pandemics, causing significant morbidity, mortality, and strain on healthcare systems. Consequently, the clinical relevance of antiviral agents used in treating HIV and hepatitis infections is substantial. Combination drug therapy offers a convenient once-daily dosage regimen to enhance adherence, effectively combat viral infections, and minimize side effects. The aim of this project is to develop simple, precise, sensitive, and accurate methods for the quantification of certain antiviral medications, specifically Pibrentasvir - Glecaprevir and Lamivudine-Tenofovir in multicomponent dosage forms [2,3]. An Absorptivity Factor approach was employed using a UV-Visible spectrophotometer for the simultaneous quantification of PBR and GLE, as no prior UV-Visible spectrophotometric analytical techniques were identified in the literature. Additionally, an RP-HPLC method was established for the simultaneous quantification of LAM and TNF in multicomponent formulations, aiming for enhanced accuracy and sensitivity compared to existing methodologies [4-19]. Validation of the analytical methods was based on ICH guidelines. These guidelines recommend key performance criteria such as accuracy, precision, linearity, Limit of Detection (LOD), Limit of Quantification (LOQ), robustness, ruggedness, and system suitability tests [20].

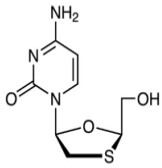
Structure of Glecaprevir



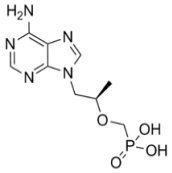
Structure of Pibrentasvir



Structure of Lamivudine



Structure of Tenofovir



Simultaneous Estimation Of Pibrentasvir And Glecaprevir In Combined Dosage Form Using Absorbtivity Factor Method

Chemicals and reagents

Pibrentasvir (PBR) and Glecaprevir (GLE) were obtained as gift samples from Mylan Pharmaceuticals Private Limited, Karnataka. Tablet formulation Mavyret (Intas Pharmaceuticals Ltd., India) labelled to contain 100 mg Glecaprevir and 40 mg Pibrentasvir was procured from a local pharmacy.

Instruments

UV-Visible Spectrophotometer (Shimadzu-1800, software version - UV probe 2.32), Digital pH meter (Systronics), Electronic weighing balance (Shimadzu AY220), Ultrasonicator RC system-MU700.

Preparation of standard stock solution

50 mg of the reference standard of PBR and GLE were accurately weighed and transferred into two separate 50 ml volumetric flasks. Both drugs were dissolved in acetonitrile and volume was made up

to mark with same to get 1000 µg/ml of standard solution. 1ml of the above standard solution was transferred separately into 10 ml volumetric flask and made up to the volume to get 100 µg/ml standard stock solution.

Selection of analytical wavelength

From the stock solutions 20 µg/ml of both drugs were prepared and scanned in the spectrum mode from 200 - 400 nm. PBR and GLE showed absorbance maxima at 252 nm and 246 nm respectively as shown in Fig 1.

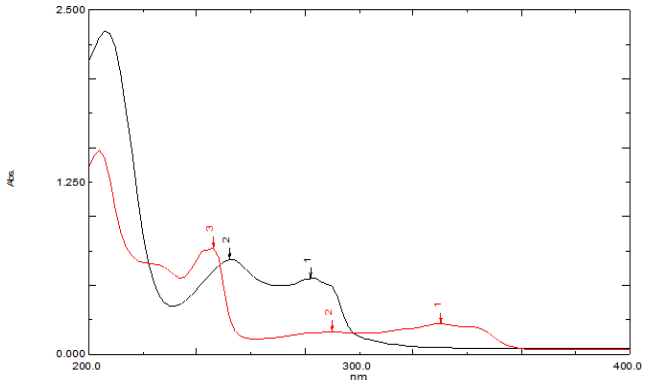


Fig 1: Overlain spectrum of PBR and GLE

Preparation of sample solution

Twenty tablets of Mavyret were accurately weighed and pulverized. A quantity of tablet powder equivalent to 100 mg of PBR was transferred into a 100 ml volumetric flask. To this, 50 ml of acetonitrile was added, and the mixture was sonicated to dissolve the contents. The volume was then made up to 100 ml with acetonitrile and filtered through a 0.45 µm membrane filter, discarding the initial few milliliters of the filtrate. From this stock solution, suitable aliquots were diluted with acetonitrile to achieve a final concentration of 20 µg/ml each for PBR and GLE, considering their amounts in the combined tablet formulation.

Absorptivity factor method

When it comes to the investigation of binary mixes, the absorptivity factor approach is applied, with the exception of circumstances in which there is a large variance in the absorptivity of both drugs. As a precautionary measure, this is taken to eliminate the possibility of an isoabsorptive point occurring. It is possible to employ the absorptivity factor approach in order to ascertain the concentration of drug x in a mixture that contains two medicines, x and y, where drug y can be determined by any of the well established spectrophotometric methods. The calculation of the absorptivity factor, which is the ratio between the two absorptivities (ax, ay) at the intersection point with the same absorbance value, is the foundation of this method [21]. Also known as the absorptivity factor, this particular point is referred to as the absorbtivity factor point (F), and calculation of it can be accomplished by utilizing the equation,

$A_m = a_y (Fc_x + c_y)$

A_m is absorbance of mixture

a_y is absorptivity of y at F

c_x and c_y are the concentrations of x and y respectively.

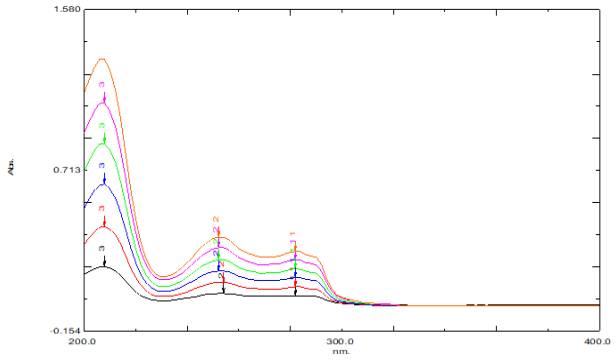


Fig 2: Calibration curve of PBR (2- 12 µg/ml)

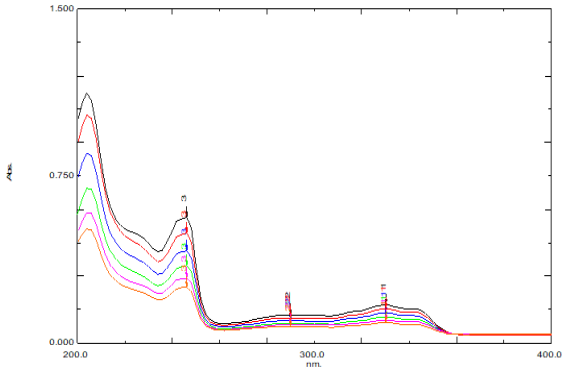


Fig 3: Calibration curve of GLE (6 -16 µg/ml)

Validation of proposed methods

After the development of UV-spectrophotometric methods for the estimation of drugs in a combined dosage form, validation of the method was performed. Performance characteristics are expressed in terms of analytical parameters [20].

Simultaneous Estimation Of Lamivudine And Tenofovir In Combined Dosage Form Using RP-HPLC

Chemicals and reagents

Lamivudine (LAM) and Tenofovir (TNF) were obtained as gift sample from Mylan Pharmaceuticals Private Limited, Karnataka. Tenolam tablets labelled to contain Lamivudine 300 mg and Tenofovir 300 mg (Hetero Drugs Ltd., India) was procured from local pharmacy.

Instruments

Liquid chromatography (Shimadzu LC-20AD, UV-detector (Shimadzu SPD-20A), Analytical column (Phenomenex C18 (250×4.6 mm & 5 µm) Data processor (LC solution software (Schimadzu, Japan)), Injector (Rheodyne-7725 (Capacity loop of 20 µl)), Syringe (Hamilton, 25 µl), Pump (Shimadzu LC 20 AD), Electronic weighing balance (Shimadzu AY220).

Selection of analytical wavelength

The standard solutions of LAM (5 µg/ml) and TNF (5 µg/m) in acetonitrile were scanned in UV region and an overlain spectrum as shown in Fig 4 was recorded. It was observed that both drugs show absorbance at 264 nm. Therefore 264 nm was considered as optimum due to less interference and appreciable absorbance.

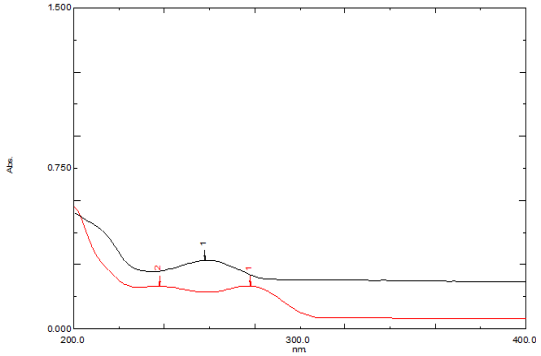


Fig 4: Overlain spectra of LAM and TNF (5 µg/ml)

Preparation of standard stock solution and calibration curve

10 mg of reference standards of both LAM and TNF were accurately weighed and transferred into two separate 10 ml volumetric flasks. Both drugs were dissolved in acetonitrile and volume was made up to mark with same to get 1000 µg/ml of standard solution. 1 ml of the above standard solution was transferred separately into 10 ml volumetric flasks and made up to the volume to get 100 µg/ml standard stock solutions. Standards containing 2.0 - 12 µg/ml of LAM and 0.5 – 3.0 µg/ml TNF were prepared by transferring appropriate aliquots from the above solutions. The drugs were injected into HPLC system by using mobile phase consisting of phosphate buffer (pH 5.0) and acetonitrile in the ratio 60:40 and their chromatograms were recorded as in Fig 5, Fig 6 & Fig 7. Peak areas were recorded both for LAM and TNF.

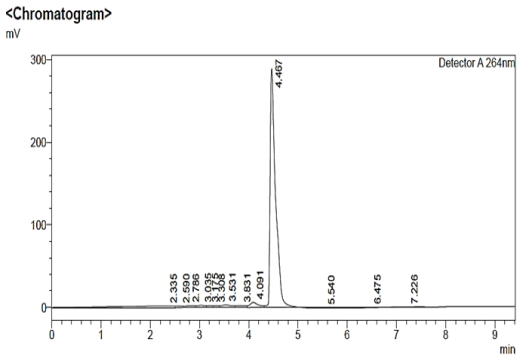


Fig 5: Chromatogram of LAM

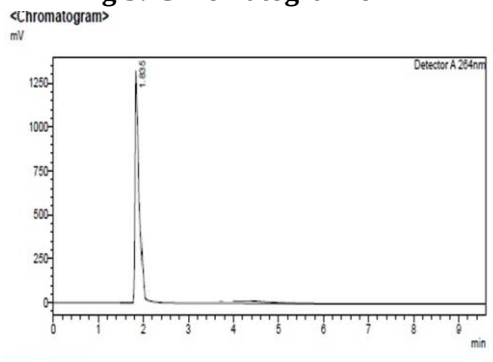


Fig 6: Chromatogram of TNF

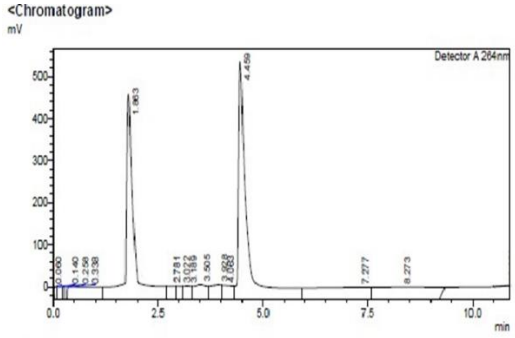


Fig 7: Chromatogram of drug combination

Preparation of sample stock solutions

Ten tablets of sample were accurately weighed, their average weight was calculated and ground to fine powder. An amount equivalent to 100 mg LAM was accurately weighed and transferred to 100 ml volumetric flask, add acetonitrile and then sonicated for 15 minutes. Adjust the volume with acetonitrile and filtered through 0.45 µm nylon filters. From this solution 1 ml was taken and made up to 10 ml with mobile phase. From the above solution 1 ml was taken in to 10 ml volumetric flask and made to final volume with acetonitrile. The solution injected in to HPLC system and chromatograms were recorded.

Validation of proposed method

The developed analytical method for LAM, TNF was validated as per ICH guideline in the terms of linearity, precision, accuracy, specificity, sensitivity, system suitability, robustness which are required to justify the purpose of the developed method [20].

RESULTS AND DISCUSSION

UV- Vis spectrophotometric method for Pibrentasvir and Glecaprevir

Linearity

The calibration plot of absorbance versus concentration was found to be linear over the concentration range selected as shown in Table 1. It indicate that test results were directly proportional to the concentration of the analyte in the sample as in Fig 8 and Fig 9.

Table 1: Linearity table

Glecaprevir		Pibrentasvir	
Concentration (µg/ml)	**Absorbance**	**Concentration (µg/ml)**	**Absorbance**
6.0	0.223	2.0	0.128
6.5	0.292	2.5	0.189
7.0	0.346	3.0	0.268
7.5	0.415	3.5	0.335
8.0	0.490	4.0	0.394
8.5	0.562	4.5	0.465

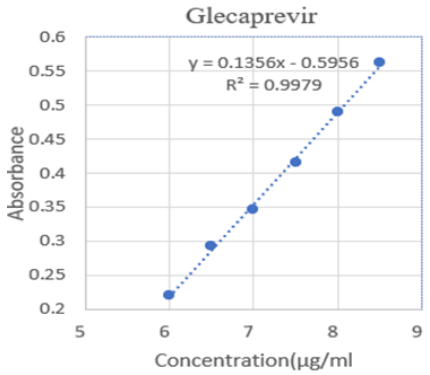


Fig 8: Linearity graph (6.0 - 8.5 µg/ml)

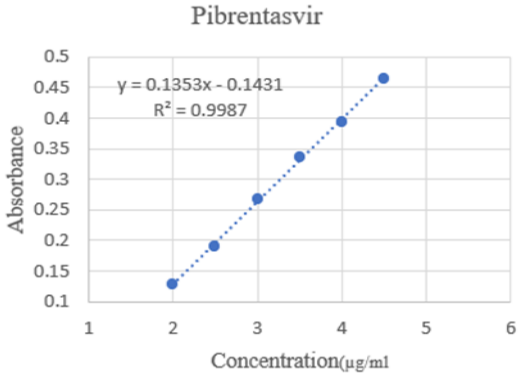


Fig 9: Linearity graph (2.0 - 4.5 µg/ml)

Accuracy and Precision

Recovery and precision studies were done to validate the accuracy and reproducibility of the developed method as shown in Table 2 and 3. The % recovery was found to be within 98.16 % - 102.50 % which indicate the accuracy of the developed method.

Table 2: Result of recovery study

Drugs	Amount taken (µg/ml)	Amount added (µg/ml)	Amount recovered (µg/ml)	Percentage recovery
GLE	4	2 4 6	5.89 8.20 9.89	98.16 102.50 98.90
PBR	10	8 10 12	18.51 20.25 21.84	102.83 101.25 99.31

Table 3: Results of precision study

	Absorbance Of PBR						Absorbance Of GLE					
Wavelength (Λ)	246 nm			252 nm			246 nm			252 nm		
Concentration (µg/Ml)	2	4	6	2	4	6	6	8	10	6	8	10
DAY 1												
Absorbance	0.079 0.081 0.080	0.115 0.113 0.110	0.192 0.191 0.192	0.128 0.126 0.129	0.189 0.188 0.186	0.268 0.265 0.269	0.250 0.252 0.249	0.292 0.290 0.289	0.346 0.345 0.343	0.092 0.091 0.090	0.110 0.111 0.111	0.159 0.158 0.157
Mean	0.080	0.112	0.192	0.127	0.187	0.267	0.250	0.290	0.344	0.091	0.110	0.588
Sd	0.001	0.003	0.005	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.005	0.001
%Rsd	1.250	1.074	0.260	0.787	0.534	0.749	0.400	0.344	0.290	1.098	0.454	0.170

DAY 2												
Absorbance	0.078 0.078 0.076	0.113 0.113 0.115	0.192 0.191 0.192	0.126 0.124 0.126	0.187 0.185 0.185	0.267 0.268 0.264	0.249 0.248 0.248	0.290 0.290 0.288	0.343 0.343 0.342	0.091 0.090 0.092	0.111 0.110 0.110	0.157 0.158 0.154
Mean	0.077	0.113	0.191	0.125	0.185	0.266	0.248	0.289	0.342	0.091	0.110	0.156
SD	0.001	0.001	0.005	0.001	0.001	0.002	0.005	0.001	0.0005	0.001	0.005	0.002
%RSD	1.298	0.884	0.260	0.88	0.540	0.751	0.201	0.346	0.1461	1.098	0.454	1.282
DAY 3												
Absorbance	0.076 0.075 0.074	0.110 0.109 0.109	0.191 0.190 0.191	0.125 0.126 0.123	0.184 0.184 0.182	0.265 0.265 0.263	0.246 0.247 0.248	0.290 0.290 0.288	0.342 0.341 0.340	0.088 0.090 0.088	0.110 0.109 0.109	0.156 0.154 0.154
Mean	0.075	0.109	0.190	0.124	0.183	0.264	0.247	0.289	0.341	0.088	0.109	0.154
SD	0.001	0.001	0.005	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.005	0.001
%RSD	1.333	0.917	0.263	0.806	0.546	0.761	0.404	0.346	0.293	1.136	0.458	0.649

Limit of detection and quantitation

By using calibration curve, the standard deviation of the response (SD) and slope of the calibration curve (S) was determined. From this LOD and LOQ was calculated as shown in Table 4.

Table 4: Limit of detection and quantification

DRUGS	LOD (µg/ml)	LOQ (µg/ml)
Glecaprevir	1.103	3.405
Pibrentasvir	1.088	2.360

Result of sample analysis

The concentration of PBR estimated by simultaneous equation method is found to be 19.05 µg/ml. By using the above concentration, the concentration of GLE was determined by using absorptivity factor method was found to be 18.21 µg/ml. The percentage purity was reported in Table 5.

Table 5: Analysis of sample

Drugs	Method	Estimated concentration (µg/ml)	Percentage purity(% w/w)

Pibrentasvir	Simultaneous equation method	19.05	99.51
Glecaprevir	Absorptivity factor method	18.21	98.87

RP- HPLC method for lamivudine and tenofovir

Accuracy and precision

Accuracy and precision studies were carried out and results were satisfactory. Accuracy determined by standard addition method shows % recoveries from 98.12 % - 100.87 % for LAM and 98.20 % - 101.98 % for TNF. Percentage RSD for the peak area of LAM and TNF for 3 replicate injections of standard solution was within the limit of < 2%. The results were shown in Table 6, Table 7 and Table 8.

Table 6: Accuracy table for lamivudine

Percentage level	Amount spiked (µg/ml)	Amount recovered (µg/ml)	Percentage recovery
50%	30 30 30	60 60 60	100.87 99.45 100.58
100%	60 60 60	60 60 60	100.68 99.84 98.12
150%	90 90 90	60 60 60	98.51 99.33 100.10

Table 7: Accuracy table for tenofovir

Percentage level	Amountspiked (µg/ml)	Amount recovered (µg/ml)	Percentage recovery
50%	30 30 30	60 60 60	98.54 99.56 98.65
100%	60 60 60	60 60 60	101.98
150%	90 90 90	60 60 60	98.20 99.95 98.48

Table 8: Results of precision study

Sl. No.	Area of lamivudine	Area of tenofovir
1	2151689	16433158
2	2158746	16249653
3	2257456	16333452
Mean	2189297	16399922
S.D	59132.79	57564.1
%RSD	1.8	0.357

Linearity

The linearity of the drug response on the basis of variable concentration has been found to be within the range of 2.0 - 12.0 µg/ml for LAM & 0.5 - 3.0 µg/ml for TNF as shown in Table 9. The graph of peak response versus concentration shows the linear graded response within the same range of concentrations as shown in Fig 10 & Fig 11.

Table 9: Linearity table for concentration v/s peak area

Lamivudine	Tenofovir	Lamivudine	Tenofovir
Concentration (µg/ml)	Peak Area	Concentration (µg/ml)	Peak Area
2	554678	2	554678
4	1345789	4	1345789
6	2151683	6	2151683
8	3024568	8	3024568
10	3754899	10	3754899
12	4578912	12	4578912

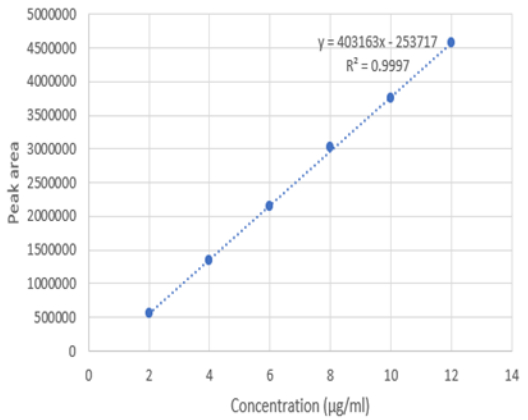


Fig 10: Linearity graph for lamivudine

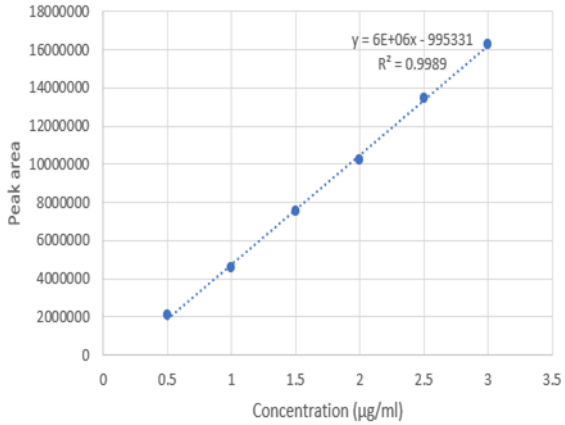


Fig 11: Linearity graph for tenofovir

Limit of detection and quantitation

By using calibration curve, the standard deviation of the response (SD) and slope of the calibration curve (S) was determined. From this LOD and LOQ was calculated as shown in Table 10.

Table 10: LOD and LOQ

Drugs	LOD (µg/ml)	LOQ (µg/ml)
Lamivudine	1.30	2.50
Tenofovir	0.25	0.75

System suitability study

The system suitability parameters observed by using these optimized conditions were reported. The results of the system suitability test assure the adequacy of the proposed HPLC method for routine analysis of LAM and TNF alone or in combination discussed in Table 12.

Table 12: System suitability data

Drug	Retention time (min)	Area	Tailing factor (T)	Theoretical plates (N)	Resolution (R)
Tenofovir	1.835	16249653	0.74	5369	1.2
Lamivudine	4.457	2151683	0.83	4987	0.99

Robustness

Deliberated changes in chromatographic conditions like increasing or decreasing flow rate, pH, temperature of column, and wavelength. The data on the robustness of results were examined and found within the limit shown in Table 13.

Table 13: Results of robustness study

Paramete	Rt		Resolution	%RSD	
	TNF	LAM		TNF	LAM
Change in pH of mobile phase					
pH 5.5	1.835	4.457	0.74	5369	1.2
pH 4.8	1.863	4.467	0.83	4987	0.99
pH 5.0	1.839	4.477	0.74	5369	1.2
Change in temperature					
20ºC	1.834	4.456	1.33	0.48	1.33
25ºC	1.848	4.459	1.55	0.99	1.89
30ºC	1.854	4.463	1.66	0.88	0.87
Change in flow rate					

0.8mL/mi	1.825	4.456	1.60	0.56	1.38
1.0mL/mi	1.823	4.457	1.77	0.89	1.88
1.2mL/mi	1.824	4.459	1.86	0.97	1.96
Change in wavelength					
264nm	1.835	4.457	1.88	0.99	1.47
258nm	1.872	4.574	1.60	0.78	1.55

Analysis of sample preparation

The proposed method can be used for estimation of both drugs in dosage forms. The results as depicted in Table 14, Table 15, Table 16 indicate that each drug corresponds to requirements of label claim. The low % RSD values (< 2%) the suitability of method for routine analysis of LAM and TNF in pharmaceutical dosage forms.

Table 14: Assay data of lamivudine

Sl.no	Standard area	Sample area
1	2151689	92357646
2	2157346	92437542
3	2154442	92246437
Avg	2154492.33	92347208.33
S D	2828.84	96004.91

Table 15: Assay data of tenofovir

Sl No	Standard area	Sample area
1	16433156	145026496
2	16432252	145018774
3	16434145	145034218
Avg	16433184.33	145026496
S D	947.08	7721.68
%RSD	0.00576	0.00532

Table 16: Assay outcome

Drugs	Label claim (mg)	%Assay
Lamivudine	300	99.99
Tenofovir	300	99.80

CONCLUSION

This work has established a UV-Visible spectrophotometric approach for the concurrent determination of PBR and GLE, as well as an RP-HPLC method for the simultaneous quantification of LAM and TNF. We devised an absorptivity factor approach utilizing a UV-Visible spectrophotometer for the simultaneous assessment of PBR and GLE. The suggested method does not necessitate any complex mathematical analysis for the absorbance data. Due to its simplicity, this method is advantageous compared to two more complex alternatives: the derivative ratio method, which requires an additional step for generating the derivative curve, and the ratio subtraction method, which entails a series of division, constant subtraction, and multiplication steps. Secondly, an RP-HPLC method was devised for the simultaneous quantification of LAM and TNF in mixed dose forms, with the objective of enhancing accuracy and sensitivity. The proposed method offers advantages in operational simplicity and measurement sensitivity compared to previously published techniques for the analyzed mixtures. The methods are validated for accuracy, precision, and sensitivity, demonstrating satisfactory results for all validation parameters in accordance with ICH guidelines. Validated methods conserve time and resources by ensuring the use of reliable techniques for routine testing, thus eliminating the necessity for repeated troubleshooting or re-validation.

ACKNOWLEDGEMENT

The authors express gratitude to the Management and Principal of Ezhuthachan College of Pharmaceutical Sciences, Thiruvananthapuram, Kerala, for their continual assistance, encouragement, and support. Additionally, appreciation is extended to Mylan

Pharmaceuticals Private Limited, Karnataka, for supplying drug samples of glecaprevir, pibrentasvir, lamivudine, and tenofovir.

Conflicts Of Interest

The authors have no conflict of interest.

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HOW TO CITE: Anju A., Aparna R. V., Gouriga V. N., S. M. Sandhya*, Shaiju S. Dharan, Development, Optimization and Validation of Analytical Procedures for The Simultaneous Determination of Selected Antiviral Drug Combinations, Int. J. of Pharm. Sci., 2024, Vol 2, Issue 12, 2597-2608. https://doi.org/10.5281/zenodo.14536508