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METHOD DEVELOPMENT AND VALIDATION OF ELBASVIR AND GRAZOPREVIR BY RP-HPLC

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ABSTRACT

A rapid, simple, and precise RP-HPLC method has been developed for the simultaneous estimation of the antiviral drugs Elbasvir and Grazoprevir in bulk and t dosage forms. The analysis was carried out using a Waters Symmetry Shield C18 column(4.6×250mm,5.0µm)with a mobilephase consisting of 0.1% ortho-phosphoric acid (pH 2.2) and acetonitrile in a 45:55 (v/v) ratio. The flow rate was maintained at 1 mL/min, and the column effluents were monitored using a PDA detector at 260 nm. The retention times for Grazoprevir and Elbasvir were found to be 2.124 minutes and 3.628 minutes, respectively, with correlation coefficients of 0.999 for both drugs. The performance of the developed method was validated according to ICH guidelines, assessing parameters such as accuracy, specificity, linearity, precision, and robustness. The recovery was within the acceptance range of 98–102%, and the detector response was linear over a concentration range of 12.5–150 µg/mL for both drugs .The proposed method is reliable and can be effectively employed for the quality control analysis of Elbasvir and Grazoprevir in pharmaceutical formulations.

INTRODUCTION

Pharmaceutical analysis is concerned not only with medicaments (medicaments and their formulations)but also with their precursors i.e. with raw material on which degree of purity and quality of medicament is dependent. Quality of a drug is ascertained after ascertaining its authenticity by ascertaining purity and quality of pure substance in the drug and its formulations. Quality is necessary in all product or service but it is crucial in medicine since it deals with life. Unlike common consumer commodities there can be no"second quality"in medicines. Quality control is a notion, which aim to deliver an ideal product by series of steps aimed at preventing and eradicating errors at various production stages.[1-5]The provision of timely,accurate,and consistent data is the core of the work of analytical chemists and particularly so in drug discovery, drug development, and drug manufacturing. Analytical information is utilized to screen prospective drug candidates, assist in drug synthesis development, facilitate formulation studies, and monitor bulk pharmaceutical and formulated product stability, and release testing of final products. The reliability of analytical data plays an important role in the success of a drug development program. The method development and validation process directly influences the reliability of thesed ata. Elbasvir, with the molecular formula $C_{49}H_{55}N_9O_7$ and a molecular weight of 882.02 g/mol, is a solid substance with a melting point of 242ºC. It is highly lipophilic (LogP=6.17)and has limited aqueous

solubility, being insoluble in water and hygroscopic. Elbasvir is mostly available in tablet form and is approved for the treatment of chronic HCV infection, genotypes 1 and 4, in adults. It acts as an antiviral by inhibiting the HCV replication complex and thus blocking viral replication. Elbasvir has extensive plasma protein binding in its pharmacokinetics, with greater than 99.9% bound in the bloodstream. It has pKa valuesof12.42foritsstrongestacidicgroupand 5.39 for its strongest basic group. Typical untoward effects from its administration include fatigue, headache, and nausea.

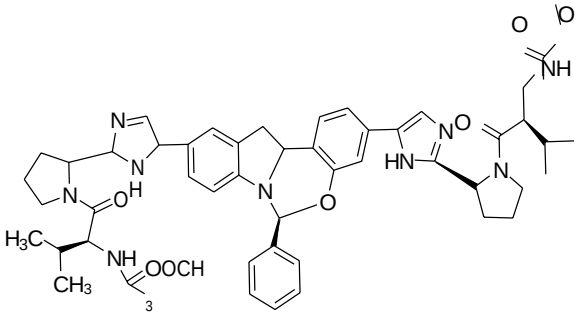

Figure1:StructureofElbasvir

Grazoprevir is a solid pharmaceutical compound with the chemical formula $C_{38}H_{50}N_6O_9$Sandamolecularweightof 766.90 g/mol. It appears as a solid and exhibits a melting point of 174ºC. The drug is hygroscopic andinsolubleinwater,withalog P value of 3.14, indicating moderate lipophilicity.Grazoprevir has pKa values of 5.31 (acidic) and 1.81 (basic), influencing its ionization and solubility under physiological conditions. It is formulated in tablet dosage form and primarily indicated for the treatment of chronic hepatitis C virus (HCV) infections, specifically genotypes 1 and 4 in adult patients. Grazoprevir acts as an inhibitor of HCV replication, thereby reducing viral load in infected individuals. Pharmacokinetically, the drug exhibit sa half-life of approximately 31 hours, supporting once-daily dosing. Despite its therapeutic benefits, common adverse effects include fatigue, headache, and nausea.

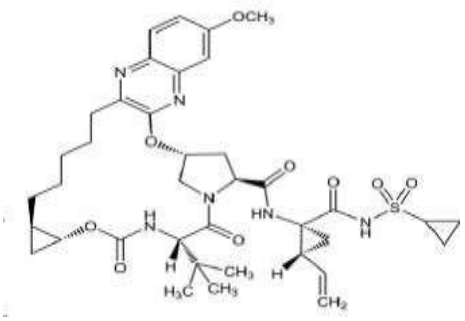

Figure2:StructureofGrazoprevir

Several analytical methods have been reported for the estimation of, including UV spectrophotometry , HPLC, GC, and LC-MS UV spectrophotometric methods often require derivatization to enhance TA's absorbance. Although existing HPLC methods offer good sensitivity, they frequently involve complex mobile phases, labor-intensive sample preparation, or lack adequate stability-indicating properties. LC-MS methods, while highly sensitive, are costly and require sophisticated instrumentation.

In light of these challenges, there is a clear need for a simple, rapid, and validated RP-HPLC method for the routine quantification of Elbasvir and Grazoprevir in pharmaceutical formulations. This study focuses on developing and validating an RP-HPLC method that is accurate, precise, sensitive, and capable of indicating stability, in compliance with ICH guidelines . The developed method will be evaluated for system suitability, specificity, precision, accuracy, linearity, robustness, and reproducibility to confirm its suitability for pharmaceutical quality control applications.

MATERIALSANDMETHODS

Analytical work was conducted with a series of calibrated equipment and high-purity chemicals. An HPLC setup (ALLIANCE Waters e2695) with Empower 2.0 softwarewas utilized for chromatographic analysis. pH was measured using an Eutech digital pH meter, and sample weights were measured precisely using a Sartorius analytical balance. Borosil glassware in the form of pipettes, beakers, and burettes was used to prepare solutions. Sonication was done using an Ultra

Sonicator (Model UCA701) by Unichrome, and the mobile phase was delivered using an isocratic pump. The chemicals employed in the HPLC procedure were HPLC-grade acetonitrile and ortho phosphoric acid (Rankem), and Milli-Q water generated in-house. Elbasvir and Grazoprevir, the active pharmaceutical ingredients (APIs), were sourced from Sinfachem Limited. All solvents and reagents employed were of HPLC or analytical grade to provide reproducible results and high accuracy.

METHODDEVELOPMENT

Selection and preparation of mobile phase: Several mobile phases containing orthophosphoric acid and acetonitrile in different ratios were tried by different columns, flowrates. Good peak symmetry, resolution and retention time was observed with mobile phase comprised of0.1% Ortho Phosphoric acid: Acetonitrile, (45:55, v/v) premixed. Further sonication was done for 30 min and filtered.

Preparation of standard stock solution: Accurately weighed 5 mg of Elbasvir& 10mg of Grazoprevir standards were taken in a 10mL clean dry volumetric flask respectively and 5mL of diluent was added andsonicated for 30 minutes. The final volume is made upto the markwith diluents to get a concentrationof 100 µg/mL ofGrazoprevir and 50µg/mL of Elbasvir. From the above two stock solutions, 1mL was diluted to 10mL using diluent.

Preparation of sample solutions of elbasvir and grazoprevir Stock solution: Accurately weighed 10 tablets crush in mortor, transferred equivalent to 10 mg of elbasvir and 20 mg grazoprevir sample into a 10 ml clean, dry volumetric flask addedabout 7 ml of diluent and sonicated it up to 30 min to dissolve it completely and made volume up to the mark with the same solvent. Then it was filtered through 0.45µ membrane filter (Stock solution). Further pipetted out 0.3 ml of elbasvir and grazoprevir from the above stock solution into a 10ml volumetric flask and diluted upto the mark with diluent.(50ppm ELBA and 100 ppm of GRAZO). 0.25 □L of the standard, sample injected into the chromatographic system and measures the areas for elbasvir and grazoprevir

DeterminationofWorkingWavelength (λ_{max}):

In simultaneous estimation of twodrugs isobestic wavelength was used. Isobestic point is the wavelength where the molar absorptivity is the same for two substancest hat are interconvertible. So this wavelength was Used in simultaneous estimation to estimate Two drugs accurately.Thewavelength of maximum absorption of the solution of the drugs in mixture of 0.1% Ortho Phosphoric acid: Acetonitrile, (55:45, v/v) were scanned using PDA Detector within the wavelength region of 200–400 nm .The absorption curve shows isobestic point at 260 nm. Thus 260 nm was selected as detector wavelength for the HPLC chromatographic method.

Selection of wavelength: Good response for both the drugs was detected from UV spectraas 260 nm . Hence detection was executed at 260 nm. **4.1.5. 0.1% Ortho Phosphoric acid Preparation:** To prepare a 0.1% Ortho Phosphoric acid solution with a pH of 2.2, concentrated (85%) phosphoric acid was diluted with deionized water. The pH was then carefully adjusted to 2.2 using a pH meter and small additions of either concentrated phosphoric acid or a dilute sodium hydroxide solution, as necessary.

Preparation of Mobile Phase: Mobile phase was prepared by mixing Acetonitrile and 0.1% Ortho Phosphoric acid (pH 2.2): Acetonitrile, (45:55, v/v) premixed .It was filtered through 0.45µmembranefiltertoremovetheimpurities which may interfere in the final chromatogram.

Chromatographic conditions: uring the selection of chromatographic conditions, numbers of trails were carried out and the best trail was selected for optimized method.

Sample Preparation: Label Claim: **100**mg of Grazoprevir+ 50mg of Elbasvir**Preparationof Diluent:**MobilePhaseused as diluents.

Chromatographic Conditions: The optimized chromatographic conditions for the method include the use of a mobile phase consisting of 0.1% ortho phosphoric acid (pH 2.2) and acetonitrile in a 45:55 (v/v) ratio, premixed prior to use. Separation was carried out on a Waters Symmetry Shield C18 column with dimensions of 4.6 × 250 mm and aparticle size of 5.0 µm. The flow rate was maintained at 0.9 mL/min, and the column temperature was set at 30°C to ensure consistent performance. An injection volumeof 10 µL was used, with a total run time of 6 minutes. Detection was performed using a Photodiode Array(PDA) detector. The method was found to be suitable for validation basedon these optimized conditions. The Elbasvir peak was observed at 2.124 and Grazoprevir peak was observed at 3.628 min. Thistrial was optimized.

ANALYTICAL METHOD VALIDATION (HPLC)

The method was validated for its linearity range, accuracy, precision, and specificity.

Method validation was carried out as per ICH guidelines.

SPECIFICITY: From Specificity test it was evident that there is no interference of placebo at the retention time of Elbasvir and Grazoprevir. Interference from blank and placebo was not observed at any peak of interest.

Discussion: Retention times of Elbasvir and Grazoprevir was found to be 2.124 min and 3.628minrespectively.Wedidnotfoundand interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific.

LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTIFICATION (LOQ):

The limit of detection (LOD) limit of quantification (LOQ) of the drug carry was calculated using the following equation as per international conference harmonization(ICH) guidelines. The lower detection limit and lower quantitation limit was necessarily determined and calculated from the signal-to-noise ratio using 100 µg/ml of Grazoprevir and50µg/mLofElbasvir.10µLofthesewere injected and the chromatograms were recorded. The peak areas were observed.

LOD=3.3Xσ/SLOQ=10Xσ/S

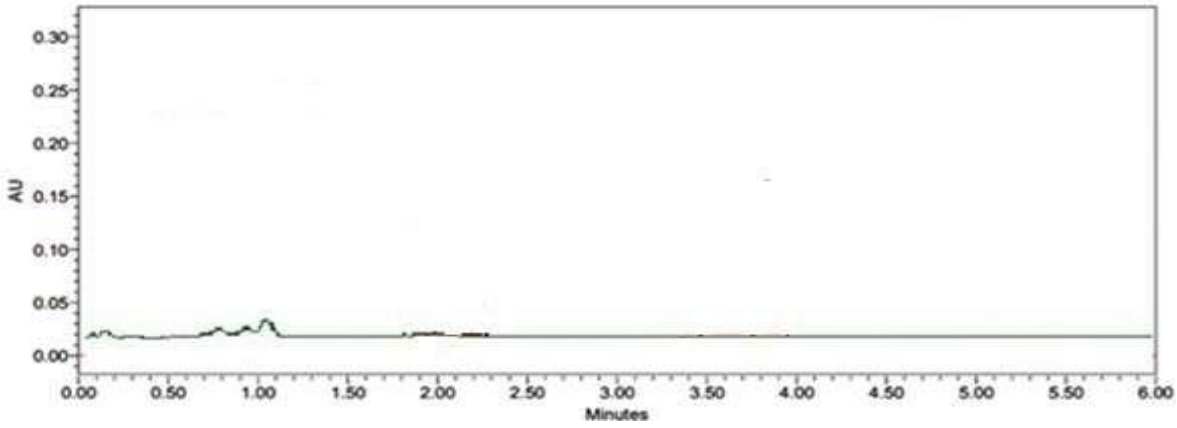

Figure3:Chromatogramof Placebo

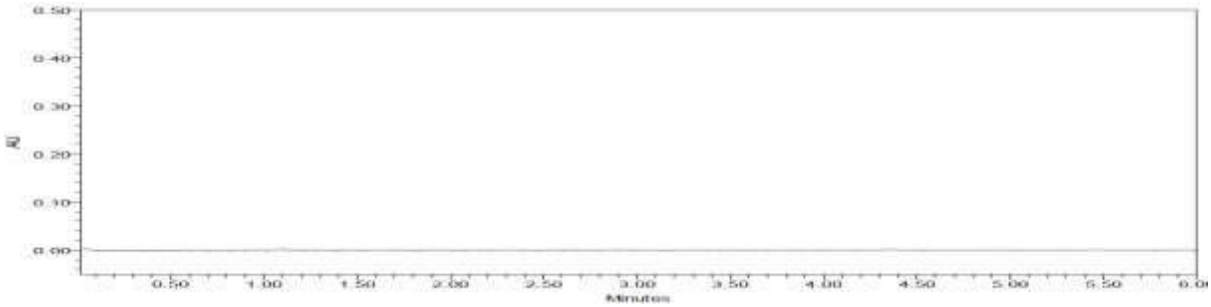

Figure4:ChromatogramofBlank

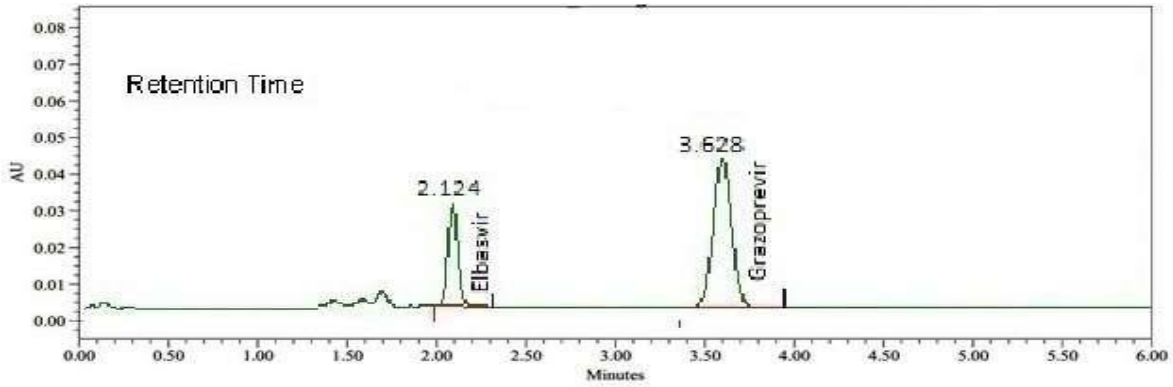

Figure5:Optimized chromatogram

PRECISION-SystemPrecision:[11-16]

In method precision, a homogenous sample of single batch is analyzed 6 times. This indicates whether a method is giving constant results for a single batch .From a single volumetric flask of working standard solution six injections were given and the obtained areas were mentioned above. Average area, standard deviation and % RSD were calculated for two drugs. %RSD obtained as 1.71 % and 1.98% respectively for Elbasvir and Grazoprevir. As the limit of Precision was less than "2" the system precision was passed in this method.

TableNo1:Resultsfor specificity

Nameof thesolution	Retentiontimein minutes
Blank	0min(Absence of peaks)
Elbasvir	2.124min
Grazoprevir	3.628 min

TableNo.2:SystemprecisionofElbasvirand Grazoprevir

Determination	Areaof Analyte
50	15022
50	15545
50	15473
50	15675
50	15096
50	15239
Mean	15341
% RSD	1.71

Determination	Areaof Analyte
50	24747
50	25662
50	25618
50	26292
50	25376
50	25763
Mean	25576
% RSD	1.98

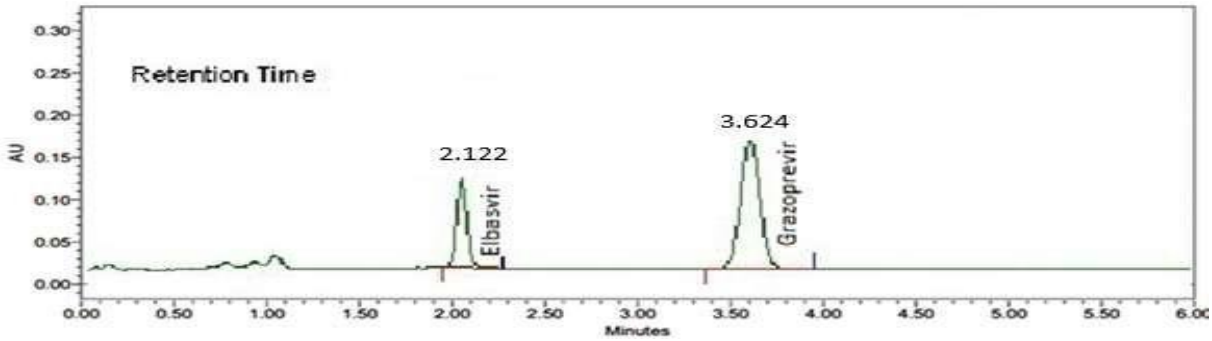

Figure6:System precision chromatogram-1

LINEARITY:[18-22]

Accuratelyweighandtransfer10mgof Elbasvir into 10 ml volumetric flask, 10mg of Grazoprevir working standard into another 10 ml clean dry volumetric flask add Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Preparation of Level –I (12.5 ppm of Elbasvir, 25 ppm of Grazoprevir): 0.25ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute upto the mark with diluent.

Preparation of Level–II (25 ppm of Elbasvir , 50 ppm of Grazoprevir): 0.5 ml of above stock solutions has taken indifferent 10 ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level–III (37.5 ppm of Elbasvir ,75 ppm of Grazoprevir): 0.75ml of above stock solutions has taken indifferent 50ml of volumetric flasks, dilute upto the mark with dilue.

Preparation of Level–IV (50 ppm of Elbasvir ,100 ppm of Grazoprevir) 1.0ml of above stock solutions has taken in different50mlofvolumetricflasks,dilute upto the mark with diluent**.**

Preparation of Level–V (62.5 ppm of Elbasvir, 125ppm of Grazoprevir) 1.25ml of above stock solutions has taken indifferent 50ml of volumetric flasks, dilute upto the mark with diluent**.**

Preparation of Level–VI(75 ppm of Elbasvir, 150 ppm of Grazoprevir) 1.5 ml of above stock solutions has taken in different 50ml of volumetric flasks, dilute upto the mark with diluent**. Procedure:** Inject each level into the chromatographic system and measure the peak area**.** Plot a graph of peak area versus concentration (on X-axis concentration and onY-axis Peak area) and calculate the correlation coefficient.

Range:[23-25]The Range of an analytical method is the interval between the upper and lower levels of analyte (including these levels)that have been demonstrated with precision, accuracy and linearity **Acceptance Criteria:**

Correlation coefficient should be not less than 0.999.

TableNo.3:Results of linearity for Elbasvir and Grazoprevir

S.No.	Elbasvir		Grazoprevir	
	Conc.(µg/ml)	Peakarea	Conc.(µg/ml)	Peakarea
1	12.5	3687	25	6454
2	25	7394	50	12908
3	37.5	12065	75	19462
4	50	15780	100	25816
5	62.5	18435	125	32280
6	75	22142	150	38724
Regressionequation	y=301.3x		y=258.03x	
Slope	301.3		258.08	
R^2	0.993		0.999	

TableNo.4:Recovery results of Elbasvir by RP-HPLC method

Analyte Level	Analyte Peak Area	Nominal Concentration	Actual Concentration	Individual %Recovery	Mean% Recovery	% RSD
Level1	7394	25	24.54	98.16	98.51	0.65
	7348	25	24.81	99.24		
	7399	25	24.53	98.12		
Level2	15780	50	49.99	99.99	100.06	0.41
	15768	50	50.21	100.42		
	15744	50	49.86	99.60		
Level3	22142	75	75.68	100.90	100.10	0.70
	22146	75	74.63	99.82		
	22148	75	75.85	101.13		

TableNo.5:Recovery results of Grazoprevir by RP-HPLC method

Analyte Level	Analyte PeakArea	Nominal Concentration	Actual Concentration	Individual% Recovery	Mean% Recovery	% RSD
Level1	12908	50	49.49	98.98	99.28	0.29
	12938	50	49.77	99.54		
	12982	50	49.65	99.31		
Level2	25816	100	100. 77	100.77	100.09	0.62
	25873	100	99. 57	99.57		
	25896	100	99. 92	99.92		
Level3	38724	150	149.23	99.48	99.45	0.44
	38726	150	149.81	99.87		
	38728	150	148.49	98.99		

ROBUSTNESS: As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method. Robustness of the developed method was reviewed by small variations in the three important factors which influence dramatically chromatographic separation which include flow rate (mL/min, ± 1) and organic phase composition (±5 %). The flow rate was varied at 0.9 ml/min to 1.1ml/min. On evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly.

TableNo.6:Robustness results of Elbasvir by RP-HPLC

Parameter	Condition	Retentiontime(min)	Peakarea
Flowrate Change	Lessflow(0.9ml)	2.128	15768
	High(1.1ml)	2.102	15986
MobilePhase composition	LessOrg(60:40)	2.318	15886
	Highorg(40:60)	2.120	15868

TableNo.7.Robustness results of Grazoprevir by RP-HPLC

Parameter	Condition	Retentiontime(min)	Peakarea
Flowrate Change	Lessflow(0.9ml)	3.772	25816
	High(1.1ml)	3.708	25873
MobilePhase composition	LessOrg(60:40)	3.968	27565
	Highorg(40:60)	3.689	25817

ASSAY

TableNo.8:Assay of Elbasvir and Grazoprevir

Drug	Avgsample area(n=2)	Std.Conc. (µg/ml)	Sample Conc. (µg/ml)	Label amount (mg)	Stdpurity	Amount found (µg/ml)	% assay
Elbasvir	15780	50	50	50	99.9	49.9	99.8
Grazoprevir	25816	100	100	100	99.8	98.5	99.6

Hence it indicates that the method is robust even by change in the flow rate ±10%.The variation of Organic Phase ratio. Standard solution of Elbasvir and grazoprevir was prepared and analysed using the variations in mobile phase ratio.

CONCLUSION:

The developed HPLC method for estimating the selected drugs is simple, rapid, accurate, precise, robust, and cost-effective. The mobile phase and solvents are easy to prepare, economical, reliable, sensitive, and time-efficient. The sample recoveries aligned well with their respective label claims, indicating no interference from formulation excipients during estimation. This makes the method suitable for routine analysis of the selected drugs in laboratory settings. The validation results confirm that the method is linear over a range of 25% to 150%, with recovery results falling within the acceptable rangeof98%to102%.Thelow%RSD values further demonstrate the method's accuracyand precision. Robustness was established by varying flow rate, mobile phase composition, and temperature.

As the system suitability parameters for the HPLC method used in estimating the selected drugs in their pure form produced satisfactory, accurate, and reproducible results without any interference from excipients, the proposed method is deemed simple and efficient for analytical purposes.

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