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ANALYTICAL METHOD DEVELOPMENT & VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF GLECAPREVIR & PIBRENTASVIR IN ITS BULK AND PHARMACEUTICAL DOSAGE FORM.

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Abstract:

A rapid and precise reverse phase High Performance Liquid Chromatographic method has been developed for the validation of Pibrentasvir and Glecaprevir, in its pure form as well as in tablet dosage form. Chromatography was carried out on a Phenomenex Gemini C18 (4.6×250mm) 5µ column using a mixture of Methanol: TEA Buffer (65:35 v/v) as the mobile phase at a flow rate of 1.0ml/min, the detection was carried out at 265nm. The retention time of the Pibrentasvir and Glecaprevir was 2.121, 3.643 ±0.02min respectively. The method produce linear responses in the concentration range of 5-25µg/ml of Pibrentasvir and 18.75-93.75µg/ml of Glecaprevir.

The method precision for the determination of assay was below 2.0%RSD. The method is useful in the quality control of bulk and pharmaceutical formulations.

Keywords: *Pibrentasvir, Glecaprevir, RP-HPLC, validation.*

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INTRODUCTION:

Analytical chemistry [1] is the branch of chemistry involved in separating, identifying and determining the relative amounts of the components making up a sample of matter. It is mainly involved in the qualitative identification or detection of compounds and the quantitative measurement of the substances present in bulk and pharmaceutical preparation.

Measurements of physical properties of analytes such as conductivity, electrode potential, light absorption or emission, mass to charge ratio, and fluorescence, began to be used for quantitative analysis of variety of inorganic and biochemical analytes. Highly efficient chromatographic and electrophoretic techniques began to replace distillation, extraction and precipitation for the separation of components of complex mixtures prior to their qualitative or quantitative determination. These newer methods for separating and determining chemical species are known collectively as instrumental methods of analysis.

Introduction to hplc:

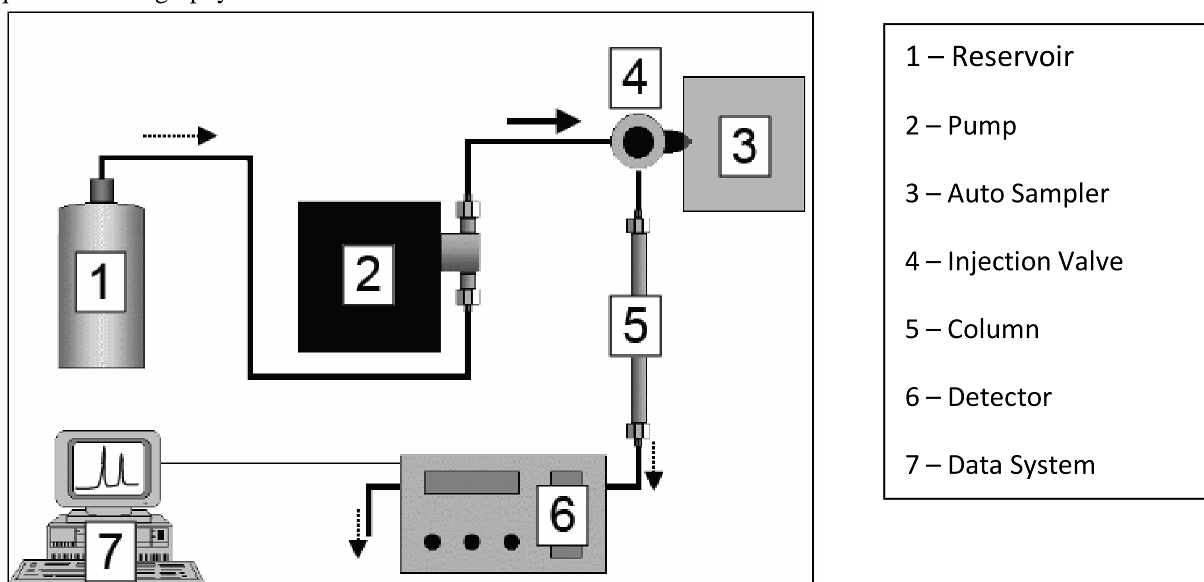
HPLC [3] is a type of liquid chromatography that employs a liquid mobile phase and a very finely divided stationary phase. In order to obtain satisfactory flow rate liquid must be pressurized to a few thousands of pounds per square inch.

The rate of distribution of drugs between Stationary and mobile phase is controlled by diffusion process. If diffusion is minimized faster and effective separation can be achieved. The techniques of high performance liquid chromatography are so called because of its improved performance when compared to classical column chromatography advances in column chromatography into high speed, efficient ,accurate and highly resolved method of separation.

For the recent study Clonazepam and Propranolol was selected for estimation of amount of analyte present in formulation and bulk drug. The HPLC method is selected in the field of analytical chemistry, since this method is specific, robust, linear, precise and accurate and the limit of detection is low and also it offers the following advantages

- Speed many analysis can be accomplished in 20min (or) less.
- Greater sensitivity (various detectors can be employed).
- Improved resolution (wide variety of stationary phases).
- Re usable columns (expensive columns but can be used for many analysis).
- Ideal for the substances of low viscosity.
- Easy sample recovery, handling and maintenance.
- Instrumentation leads itself to automation and quantification (less time and less labour).
- Precise and reproducible.
- Integrator itself does calculations.
- Suitable for preparative liquid chromatography on a much larger scale.

HPLC Basic Instrumentation:



1 – Reservoir
2 – Pump
3 – Auto Sampler
4 – Injection Valve
5 – Column
6 – Detector
7 – Data System

Fig-1: Schematic diagram of a basic HPLC system

HPLC components:
The essential components [4] of a complete HPLC system are solvent delivery system (Pump), detector, fixed volume injector loop or auto sampler, solvent reservoirs, packed column, data system and recorder. A schematic of a simplified HPLC system is shown in Figure 1.

Column:
The column is probably the heart of HPLC system. The development of this column technology leads to the evolution of the HPLC instrumentation systems used today. The conventionally used HPLC columns are particle packed columns. The key of column selection when previous separation is not available resides in knowing the chemistry of the sample. Columns should never be dry. A dry column will eventually have voids because the packing will shrink away from the wall, which would result in band broadening. Before running a sample in HPLC the column should be equilibrated. Usually column equilibrium is achieved after passage of 10 – 20 column volumes of the new mobile phase through the column. Insufficient column equilibrium usually leads to retention difference.

Pump:
The solvent delivery system or as it is commonly called the pump includes two major types, constant volume or flow and constant pressure. Constant volume pumps are mechanically driven systems, most commonly using screw driven syringes or reciprocating pistons. On the other hand, constant pressure pumps are driven or controlled by gas pressure.

Injector or Auto sampler:
Samples are usually introduced by syringe injection via a manual injector into the mobile phase stream or by the use of an auto sampler. The important aspects in sample introduction are precise and reproducible injections. This is especially important with quantitative analysis where the reproducibility of the peak response is dependent on the precision of the sample introduction. Direct syringe injection through a manual injector was the first popular method of sample introduction. As HPLC instrumentation evolved, many auto sampler techniques were applied so that sample introduction has become more precise and rapid.

Detector:
HPLC detectors include ultraviolet-visible, fluorescence, electrochemical, refractometer, mass spectrometer and others. The UV visible absorption detector is the most widely used detector in liquid chromatography, since most organic compounds show some useful absorption in the UV region. This detector is fairly universal in application, although sensitivity depends on how strongly the sample absorbs light at a particular wavelength.

Solvent reservoir:
Different containers are used as a solvent delivery system reservoir. The best material from which the containers are made is glass. Plastic containers are not recommended as it leads to plasticizer leaching. The container should be covered to prevent solvent evaporation. The tubing from the reservoir can be made of stainless steel or Teflon, and both are satisfactory.

Data handling and analysis:
Data handling in HPLC is as important to the success of any experiment or analysis as any other components in the system. It is part of good HPLC techniques to properly label and document the analytical results. The advanced computer softwares used now in data handling and analysis allow easy recording and storage of all chromatographic data.

Normal phase chromatography:
In normal phase mode the stationary base (eg; silica gel) is polar in nature and the mobile phase is non-polar. In this technique, non-polar compound travel faster and are eluted first. This is because less affinity between solute and stationary phase and take more time to elute.

Reverse phase chromatography:
The popularity of reversed phase liquid chromatography is easily explained by its unmatched simplicity, versatility and scope. Neutral and ionic analytes can be separated simultaneously. Retention in RPLC is believed to occur through nonspecific hydrophobic interaction of the solute with the stationary phase. The near universal application of RPLC stems from the fact that almost all organic compounds have hydrophobic regions in their structure and are capable of interacting with the stationary phase.

A decrease in the polarity of the mobile phase leads to a decrease in retention. It is also generally observed in RPLC that branched chain compounds are retained to a lesser extent than their straight chain analogues and that unsaturated compounds are eluted before their fully saturated analogs. A wide variety of RP-HPLC columns are available. Most columns are silica based. Silica offers good mechanical stability. A typical

stationary phase is formed by chemically bonding a long-chain hydrocarbon group to porous silica. Typical ligands are n-octadecyl (C18), n-octayl (C8), n-butyl (C4), diphenyl (C2), and cyano propyl.

Parameters affecting separation [6]:
Separation in reversed phase chromatography is affected by stationary phase type and column length. It is also affected by organic solvent type and percentage in the mobile phase and by mobile phase pH. Flow rate could also affect separation in reversed phase chromatography; however it is usually limited by the developed backpressure. Moreover temperature of the column also has an effect on separation.

MATERIALS AND METHODS:

Pibrentasvir-Procured from Mylon, provided by Sura Pharma labs, Glecaprevir-Procured from Mylon, provided by Sura Pharma labs, Water and Methanol for HPLC-LICHROSOLV (MERCK), Acetonitrile for HPLC -Merck.

HPLC METHOD DEVELOPMENT:

TRAILS

Preparation of standard solution:
Accurately weigh and transfer 10 mg of Pibrentasvir and Glecaprevir working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol.

Further pipette 0.15ml of Pibrentasvir and 0.56ml of Glecaprevir from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure: Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines.

Mobile Phase Optimization:
Initially the mobile phase tried was methanol: Water, Methanol: Phosphate buffer and ACN: Water with varying proportions. Finally, the mobile phase was optimized to TEA buffer (pH 4.0), Methanol in proportion 65:35 v/v respectively.

Optimization of Column:
The method was performed with various C18columns like Symmetry, X terra and ODS columns. Phenomenex Gemini C18 (4.6×250mm) 5µ was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

Optimized chromatographic conditions:
Instrument used : Waters Alliance 2695 HPLC with PDA Detector 996 model.
Temperature : 45ºC
Column : Phenomenex Gemini C18 (4.6×250mm) 5µ
Mobile phase : Methanol: TEA Buffer (65:35 v/v)
Flow rate : 1.1ml/min
Wavelength : 265nm
Injection volume : 10µl
Run time : 6 minutes

Validation

Preparation of buffer and mobile phase:

Preparation of Triethylamine buffer (pH-4.0):

Take 6.0ml of Triethylamine in to 750ml of HPLC water in a 1000ml volumetric flask and mix well. Make up the volume up to mark with water and adjust the pH to 4.0 by using Orthophosphoric acid, filter and sonicate.

Preparation of mobile phase:
Accurately measured 350 ml (35%) of TEA buffer and 650 ml of HPLC Methanol (65%) were mixed and degassed in a digital ultrasonicater for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation: The Mobile phase was used as the diluent.

RESULTS AND DISCUSSION:

Optimized Chromatogram (Standard)
Mobile phase ratio : Methanol: TEA Buffer (65:35 v/v)
Column : Phenomenex Gemini C18 (4.6×250mm) 5µ
Column temperature : 45ºC
Wavelength : 265 nm
Flow rate : 1.1ml/min
Injection volume : 10µl
Run time : 6 minutes

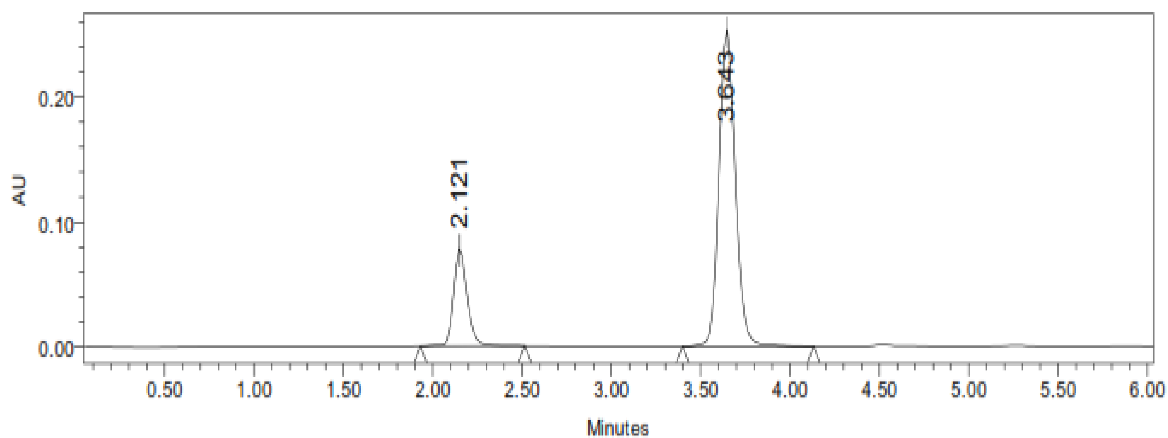


Figure: Optimized Chromatogram (Standard)

Table: Optimized Chromatogram (Standard)

S.no	Name	RT	Area	Height	USP Tailing	USP Plate Count	Resolution
1	Pibrentasvir	2.121	406433	77644	1.2	4009	
2	Glecaprevir	3.643	1592811	251532	1.1	7849	9.8

Optimized Chromatogram

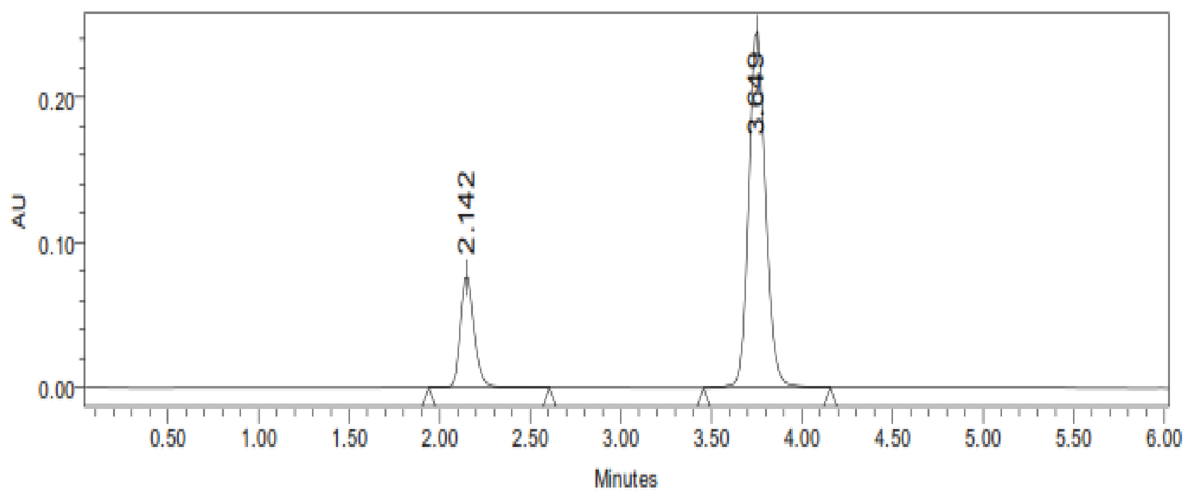


Figure: Optimized Chromatogram (Sample)

Table: Optimized Chromatogram (Sample)

S.no	Name	Rt	Area	Height	USP Tailing	USP Plate Count	Resolution
1	Pibrentasvir	2.142	403871	77464	1.2	4136	
2	Glecaprevir	3.649	1573821	259361	1.1	7812	10.3

Acceptance criteria:

- Resolution between two drugs must be not less than 2
- Theoretical plates must be not less than 2000
- Tailing factor must be not more than 2.
- It was found from above data that all the system suitability parameters for developed method were within the limit.

System Suitability:

Table: Results of system suitability for Pibrentasvir

S.No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Pibrentasvir	2.152	382726	70725	5271	1.2
2	Pibrentasvir	2.157	382621	70625	5928	1.2
3	Pibrentasvir	2.141	389172	70617	5283	1.2
4	Pibrentasvir	2.133	384152	70718	5763	1.2
5	Pibrentasvir	2.166	389721	70172	6222	1.2
Mean			385678.4			
Std. Dev.			3497.932			
% RSD			0.906956			

Acceptance criteria:

- %RSD of five different sample solutions should not more than 2
- The %RSD obtained is within the limit, hence the method is suitable.

Table: Results of system suitability for Glecaprevir

S.No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing	Resolution
1	Glecaprevir	3.674	1562821	227365	5827	1.1	10.1
2	Glecaprevir	3.631	1562726	226748	6183	1.1	10.1
3	Glecaprevir	3.625	1567361	227163	5029	1.1	10.1
4	Glecaprevir	3.692	1562811	226948	4920	1.1	10.1
5	Glecaprevir	3.629	1563816	226452	5183	1.1	10.1
Mean			1563907				
Std. Dev.			1982.03				
% RSD			0.126736				

Acceptance criteria:

- %RSD of five different sample solutions should not more than 2

- The %RSD obtained is within the limit, hence the method is suitable.

Assay (Standard):

Table: Peak results for assay standard of Pibrentasvir

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Pibrentasvir	2.152	409538	77074	1.2	4009	1
2	Pibrentasvir	2.198	409975	76001	1.2	4136	2
3	Pibrentasvir	2.179	409283	76048	1.2	5263	3

Table: Peak results for assay standard of Glecaprevir

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Glecaprevir	3.646	1609924	251956	1.1	7849	1
2	Glecaprevir	3.604	1601840	246020	1.1	7819	2
3	Glecaprevir	3.610	1602832	248287	1.1	7826	3

Assay (Sample):

Table: Peak results for Assay sample of Pibrentasvir

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Pibrentasvir	2.152	409538	77074	1.2	4009	1
2	Pibrentasvir	2.150	409975	76001	1.2	4136	2
3	Pibrentasvir	2.187	409911	77823	1.2	5173	3

Table: Peak results for Assay sample of Glecaprevir

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Glecaprevir	3.646	1609924	251956	1.1	7849	1
2	Glecaprevir	3.651	1601840	246020	1.1	7819	2
3	Glecaprevir	3.601	1603821	240291	1.1	6812	3

%ASSAY =

$$\frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

=1605195 /1604865 ×10/56.25×56.25/0.0102×99.5/100×0.1955/190×100

= 100.3%

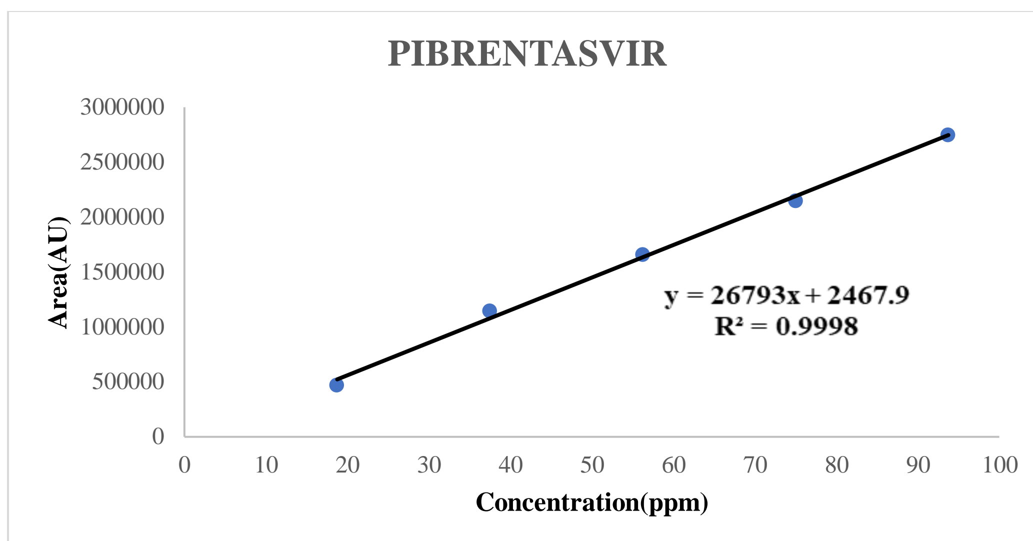
The % purity of Pibrentasvir and Glecaprevir in pharmaceutical dosage form was found to be 100.3 %

Linearity

Chromatographic data for linearity study:

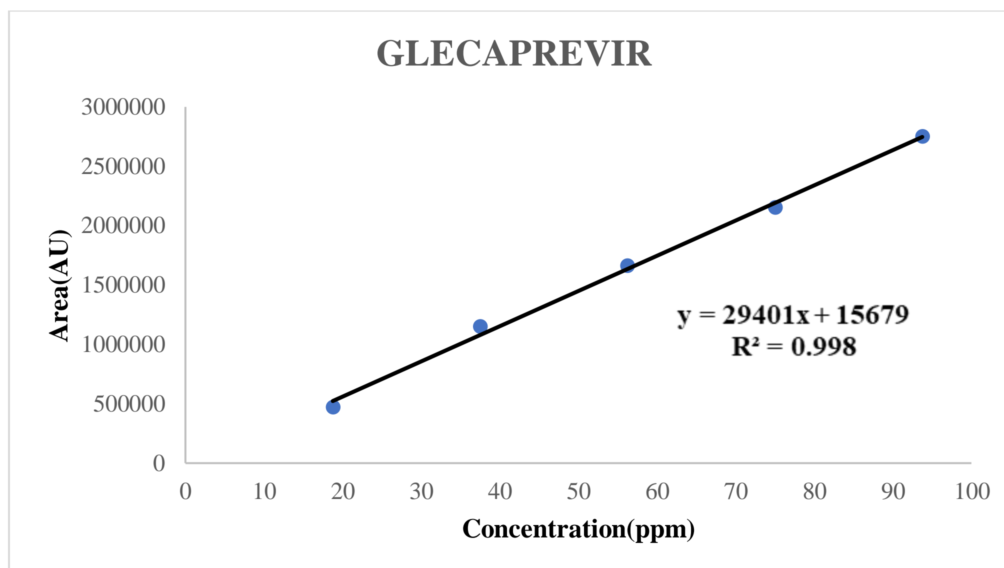
Chromatographic data for linearity study of pibrentasvir:

Concentration µg/ml	Average Peak Area
5	135005
10	277120
15	405128
20	534643
25	672357



Chromatographic data for linearity study of glecaprevir:

Concentration µg/ml	Average Peak Area
18.75	469094
37.5	1149397
56.25	1657592
75	2150412
93.75	2748444



Repeatability:

Table: Results of repeatability for Pibrentasvir:

S. No	Peak name	Retention time	Area(µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Pibrentasvir	2.157	400459	70717	1.2	4987
2	Pibrentasvir	2.159	402118	71819	1.2	5019
3	Pibrentasvir	2.186	405412	73930	1.2	5126
4	Pibrentasvir	2.160	406506	73333	1.3	4999
5	Pibrentasvir	2.170	407673	72623	1.2	5214
Mean			404433.6			
Std.dev			2716.809			
%RSD			0.671757			

Acceptance criteria:

- %RSD for sample should be NMT 2
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

Table: Results of repeatability for Glecaprevir:

S. No	Peak name	Retention time	Area(µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Glecaprevir	3.603	1617864	226985	1.1	7045
2	Glecaprevir	3.608	1618493	234764	1.1	7399
3	Glecaprevir	3.600	1628262	227712	1.2	7159
4	Glecaprevir	3.696	1615796	235459	1.1	7896
5	Glecaprevir	3.629	1619626	242158	1.1	7965
Mean			1620008			
Std.dev			4310.623			
%RSD			0.266086			

Intermediate precision:

Table: Results of Intermediate precision for Pibrentasvir

S.No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Pibrentasvir	2.198	405262	70572	5672	1.2
2	Pibrentasvir	2.196	405637	70516	5639	1.2
3	Pibrentasvir	2.160	405628	70572	6183	1.2
4	Pibrentasvir	2.160	405647	70372	5923	1.2
5	Pibrentasvir	2.160	405948	70592	6739	1.2
6	Pibrentasvir	2.186	408732	70526	5837	1.2
Mean			406142.3			
Std. Dev.			1287.197			
% RSD			0.316933			

Acceptance criteria:

- %RSD of six different sample solutions should not more than 2

Table: Results of Intermediate precision for Glecaprevir

S.No	Peak Name	Rt	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing	Resolution
1	Glecaprevir	3.623	1608292	235473	5372	1.1	10.1
2	Glecaprevir	3.611	1609283	235938	5927	1.1	10.1
3	Glecaprevir	3.696	1617836	235738	6129	1.1	10.1
4	Glecaprevir	3.696	1619743	235963	5284	1.1	10.1
5	Glecaprevir	3.696	1614262	231938	5284	1.1	10.1
6	Glecaprevir	3.642	1608471	235948	6347	1.1	10.1
Mean			1611315				
Std. Dev.			6077.093				
% RSD			0.377151				

Acceptance criteria:

- %RSD of six different sample solutions should not more than 2

Table: Results of Intermediate precision Day 2 for Pibrentasvir

S.No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing	%Assay
1	Pibrentasvir	2.198	405423	70572	5672	1.2	100%
2	Pibrentasvir	2.196	405927	70516	5639	1.2	100%
3	Pibrentasvir	2.178	405029	70572	6183	1.2	100%
4	Pibrentasvir	2.142	405432	70372	5923	1.2	100%
5	Pibrentasvir	2.177	405062	70592	6739	1.2	100%
6	Pibrentasvir	2.177	408417	70526	5837	1.2	101%
Mean			405881.7				
Std. Dev.			1283.857				
% RSD			0.316313				

Acceptance criteria:

- %RSD of six different sample solutions should not more than 2

Table: Results of Intermediate precision Day 2 for Glecaprevir

S.No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing	Resolution
1	Glecaprevir	3.611	1638732	244384	5363	1.1	10.1
2	Glecaprevir	3.623	1637438	235827	6282	1.1	10.1
3	Glecaprevir	3.684	1638474	236382	5938	1.1	10.1
4	Glecaprevir	3.697	1634273	239183	6194	1.1	10.1
5	Glecaprevir	3.684	1636372	231931	5402	1.1	10.1
6	Glecaprevir	3.684	1639283	234356	5837	1.1	10.1
Mean			1637429				
Std. Dev.			1860.366				
% RSD			0.113615				

Acceptance criteria:

- %RSD of six different sample solutions should not more than 2

Accuracy:

The accuracy results for Pibrentasvir

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	201472.3	7.5	7.4	98.6	99.7%
100%	406193	15	15	100.3	
150%	607144	22.5	22.5	100.2	

Acceptance Criteria:

- The percentage recovery was found to be within the limit (98-102%).

The accuracy results for Glecaprevir

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	826527.7	28.12	28.6	101.6	99.6%
100%	1622241	56.25	55.7	99	
150%	2422702	84.37	82.9	98.2	

The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate.

Robustness

Pibrentasvir

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.1 mL/min	406433	2.121	4009	1.2
Less Flow rate of 1.0 mL/min	398841	2.210	3800.8	0.9
More Flow rate of 1.2 mL/min	389947	2.184	4800.8	1.1
Less organic phase	413898	2.200	4890.8	0.9
More Organic phase	389578	2.172	4190.8	0.7

Acceptance criteria:

The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

Glecaprevir

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.1 mL/min	1592811	3.643	7849	1.1
Less Flow rate of 1.0 mL/min	1613422	4.498	3312.2	0.9
More Flow rate of 1.2 mL/min	1619138	3.505	4312.2	0.8
Less organic phase	1616104	4.504	4392.2	0.9
More organic phase	1623185	3.512	4292.2	0.9

Acceptance criteria:

The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

CONCLUSION:

A rapid and precise reverse phase High Performance Liquid Chromatographic (HPLC) method has been developed and validated for the quantification of Pibrentasvir and Glecaprevir, both in their pure forms and in tablet dosage forms. The chromatographic separation was achieved using a Phenomenex Gemini C18 column with dimensions of 4.6×250mm and a particle size of 5µm. The mobile phase consisted of Methanol and TEA Buffer in a ratio of 65:35 (v/v), flowing at a rate of 1.0 ml/min. Detection was performed at a wavelength of 265nm. The retention times for Pibrentasvir and Glecaprevir were found to be 2.121 ± 0.02 min and 3.643 ± 0.02 min, respectively.

The method demonstrated linear responses within the concentration ranges of 5-25µg/ml for Pibrentasvir and 18.75-93.75µg/ml for Glecaprevir.

The developed HPLC method utilizing a Phenomenex Gemini C18 column with Methanol and TEA Buffer as the mobile phase has proven to be robust for the quantitative analysis of Pibrentasvir and Glecaprevir. The method offers several advantages, including rapid analysis and high precision in determining the concentrations of these compounds in both pure and tablet forms. The observed retention times were consistent and reproducible, indicative of the method's reliability. The linear responses obtained over the specified concentration ranges demonstrate the suitability of this method for routine quality control analysis of Pibrentasvir and Glecaprevir formulations.

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