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NEW ANALYTICAL METHOD DEVELOPMENT & VALIDATION FOR THE DETERMINATION OF FOSTEMSAVIR & GANCICLOVIR IN BULK DRUG & FORMULATION BY RP-HPLC.

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Abstract:

High performance liquid chromatography (HPLC) is an essential analytical tool in assessing drug product. HPLC methods should be able to separate, detect, and quantify the various drugs and drug related degradants that can form on storage or manufacturing, detect and quantify any drugs and drug-related impurities that may be introduced during synthesis. A rapid and precise reverse phase high performance liquid chromatographic method has been developed for the validation of Fostemsavir and Ganciclovir, in its pure form as well as in tablet dosage form. Chromatography was carried out on a X-Terra C18 (4.6×150mm) 5µ column using a mixture of Methanol: Water (65:35%v/v) as the mobile phase at a flow rate of 1.0ml/min, the detection was carried out at 220nm. The retention time of the Fostemsavir and Ganciclovir was 2.9, 3.6 ±0.02min respectively. 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: *Ganciclovir, Fostemsavir, RP-HPLC, validation.*

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INTRODUCTION:

In the modern pharmaceutical industry, high-performance liquid chromatography (HPLC) is the major and integral analytical tool applied in all stages of drug discovery, development and production. It is ideal for the analysis of many drugs in both dosage forms and biological fluids due to its simplicity, high specificity and good sensitivity.

High Performance Liquid Chromatography (HPLC) is a technique that has arisen from the application to liquid chromatography the use of an instrumentation that was originally developed for gas chromatography. High Pressure Liquid Chromatography was developed in the mid-1970 and was improved with the development of column packing material and the additional convenience of on-line detectors. The various components of HPLC are pumps (solvent delivery system), mixing unit, gradient controller and solvent degasser, injector (manual or automatic), guard column, analytical columns, detectors, recorders and/or integrators. Recent models are equipped with computers and software for data acquisition and processing. The mobile phase in HPLC refers to the solvent being continuously applied to the column or stationary phase at a flow rate of 1-5 cm3/min. The mobile phase acts as a carrier for the sample solution. The chemical interactions of the mobile phase and sample with the column determine the degree of migration and separation of components contained in the sample. The mobile phase can be altered in order to manipulate the interactions of the sample and the stationary phase.

Types of Chromatogphy:

Normal-phase chromatography:

Mechanism: Retention by interaction with the polar surface of the stationary phase with polar parts of the sample molecules.
Stationary phase: SiO_2, Al_2O_3, $-NH_2$, -CN, -Diol, $-NO_2$, etc.
Mobile phase: Heptane, hexane, cyclohexane, $CHCl_3$, CH_2Cl_2, dioxane, methanol, etc.
Application: Separation of non-ionic, non-polar to medium polar substances. Disadvantage: Lack of reproducibility of retention times as water or protic organic solvents change the hydration state of the silica or alumina chromatographic media.

Reversed-phase chromatography:

Mechanism: Retention by interaction of the stationary phase's non-polar hydrocarbon chain with non-polar parts of the sample molecules.
Stationary phase: n-octadecyl (RP-18), n-octyl (RP-8), ethyl (RP-2), phenyl, $(CH_2)_n$-CN, $(CH_2)_n$-diol, etc.
Mobile phase: Methanol, acetonitrile, water, buffer (sometimes with additives of THF orDioxane), etc.

Application: Separation of non-ionic and ion forming non-polar to medium polar substances (carboxylic acids, hydrocarbons). If ion forming substances (as carboxylic acids) are to be separated, a pH control by buffers is necessary.

Reversed-phase ion-pair chromatography:

Mechanism: Ionic sample molecules are ionically bound to an ion-pair reagent. The ion- pair reagent contains an unpolar part suitable for interaction with the unpolar hydrocarbon chain of the stationary phase.
Stationary phase: Reversed phase materials (RP-18, RP-8, CN), etc.
Mobile phase: Methanol, acetonitrile, buffer with added ion-pair reagent in the concentration range of 0.001 to 0.01 M, etc.
Application: Ionic substances often show very poor retention in reversed phase chromatography. To overcome this difficulty an ion-pair reagent is added to the eluent.

Ion-exchange chromatography:

Mechanism: Retention of reversible ionic bonds on charged groups of the stationary phase.

Advantages of HPLC[2]:

1) It provides specific, sensitive and precise method for analysis of the different complicated sample.
2) There is ease of sample preparation and sample introduction.
3) There is speed of analysis.
4) The analysis by HPLC is specific, accurate and precise.
5) It offers advantage over gas chromatography in analysis of many polar, ionic substances, high molecular weight substances, metabolic products and thermolabile as well as nonvolatile substances.

Applications of HPLC:

a) Natural Products: HPLC is an ideal method for the estimation of various components in plant extracts which resemble in structure and thus demand a specific and very sensitive method e.g., analysis of digitalis, cinchona, liquorice, and ergot extracts.
b) Stability studies: HPLC is now used for ascertaining the stability of various pharmaceuticals. With HPLC the analysis of the various degradation products can be done and thus stability indicating HPLC systems have been developed.

c) Bioassays and its complementation: Complex molecules as antibiotics and peptide hormones are mainly analysed by bioassay which suffer from high cost, necessity replicates, poor precision and length of time required. Also bioassay gives an overall estimate of potency and gives no guidance about the composition. Thus HPLC can be used to complement bioassays and give an activity profile. It has been used for analysis of chloramphenicol, penicillins, clotrimoxazole, sulfas and peptides hormones.
d) HPLC has also been used in the cosmetic industry for quality control of various cosmetics.

The basic components of HPLC are:

Pumping System: The HPLC pump is very important component of the system. It delivers the constant flow of the mobile phase or phases so that the separation of the components of the mixture occur in a reasonable time. Its performance directly affects retention time, reproducibility and detector sensitivity. Three main types of pumps are used in HPLC to propel the liquid mobile phase through the system are as under;

Isocratic: In this system, the things are kept constant throughout the run. In the case of pumping of mobile phase, the mobile phase composition is kept constant throughout the run. The nominal flow rate accuracy required is ±1% of the set flow

Gradient: There is some change purposely incorporated during the particular sample run to achieve a better or/and faster separation. In case of pumping mobile phase, the composition of mobile phase is continuously varied during the particular run. The gradient accuracy of ±1% of the step gradient composition is typical.

Sample Introducing Device

It is not possible to use direct syringe injection on column like GC, as the inlet pressure in LC is too high. Insertion of the sample onto the pressurized column must be as a narrow plug so that the peak broadening attributable to this step is negligible. The injection system itself should have no dead (void) volume. There are three important ways of introducing the sample into injection port.

Chromatographic Column

Column is a heart of chromatography. The column is usually made up of heavy glass or stainless steel tubing to withstand high pressure. The columns are usually 10-30 cm long and 4-10 mm inside diameter containing stationary phase at particle diameter of 25 µm or less. Columns with an internal diameter of 5 mm give good results because of compromise between efficiency, sample capacity, and the amount of packing and solvent required.

Column packing:

The packing used in modern HPLC consists of small, rigid particles having a narrow particle size distribution. There are three main types of column packing in HPLC.

Detector

The function of the detector in HPLC is to monitor the mobile phase as it merges from the column. There are several detectors available in the market. However UV Visible detector, photo diode array detector, fluorescence detector, conductometric and coulometric detector are more commonly used. The new ELSD detector is proving to be important detector, while the MS detector is outstanding. Detectors are usually of two types:

Data handling Device

Computer-based system that controls all components of HPLC instrument (eluent composition (mixing of different solvents); temperature, injection sequence, etc.) and acquires data from the detector and monitors system performance (continuous monitoring of the mobile phase composition, temperature, back pressure, etc.)

Methods for Quantitative Analysis

Normalized peak area
External standard calibration
Internal standard calculation method
Standard addition calculation method

Area normalization method

After integrating all significant peaks in a chromatogram, total peak area may be calculated. Area (%) of any individual peak is called normalized peak area. This technique is widely used particularly in preliminary method development.

External standard calibration

The external standard is the same substance as that being analysed in the sample. This method of calculation is most commonly used. The external standard must be pure or its composition known through prior analysis. Many times, the standard is costly or is in short supply so a secondary standard can be employed.

Internal standard calculation method

It is the addition of known quantity of a foreign substance (internal standard-IS) to the analyzed

sample, the response coefficient of which is known or arbitrarily fixed. Addition of internal standard is essential for the sample requiring significant pre-treatment such as derivatisation, extraction to reduce chances of error due to these steps as it is expected to mimic the behaviour of analyte in such re-treatment steps. A calibration curve is produced by analyzing different concentrations of the pure drug with constant amount of internal standard and from the chromatogram. Calculate the ratio (Rs) for each concentration of the analyte.

Standard addition calculation method

The principle involved is that, the analytical signal is proportional to concentration, the initial analyte content is determined through measurements of this signal before and after the addition of known amount of the analyte to the analyzed sample. The method of standard addition, also denoted as “spiking,” is used when an analyte is to be quantified inside the matrix, the effect of which are likely to affect the chromatographic peak behavior.

ATERIALS AND METHODS:

Fostemsavir -Procured by Glenmark, provided from Sura labs, Ganciclovir-Procured by Glenmark, provided from Sura 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 Fostemsavir and Ganciclovir 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 2.25ml of the above Fostemsavir and Ganciclovir and 0.45ml of the Clonidine 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, Acetonitrile: Water with varying proportions. Finally, the mobile phase was optimized to Methanol and water in proportion 65:35 v/v respectively.

Optimization of Column:

The method was performed with various columns like C18 column, X- bridge column, Phenomenex Luna. Xterra C18 (4.6 x 150mm, 5µm) was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

Validation

Preparation of mobile phase:

Preparation of mobile phase:

Accurately measured 650ml (65%) of Methanol and 350ml of Water (35%) 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:

Trail 4 (Optimized chromatogram):

Column : Xterra C18 (4.6×250mm) 5µ
Column temperature : 40˚C
Wavelength : 220nm
Mobile phase ratio : Methanol: Water (65:35 v/v)
Flow rate : 1ml/min
Injection volume : 10µl
Run time : 10minutes

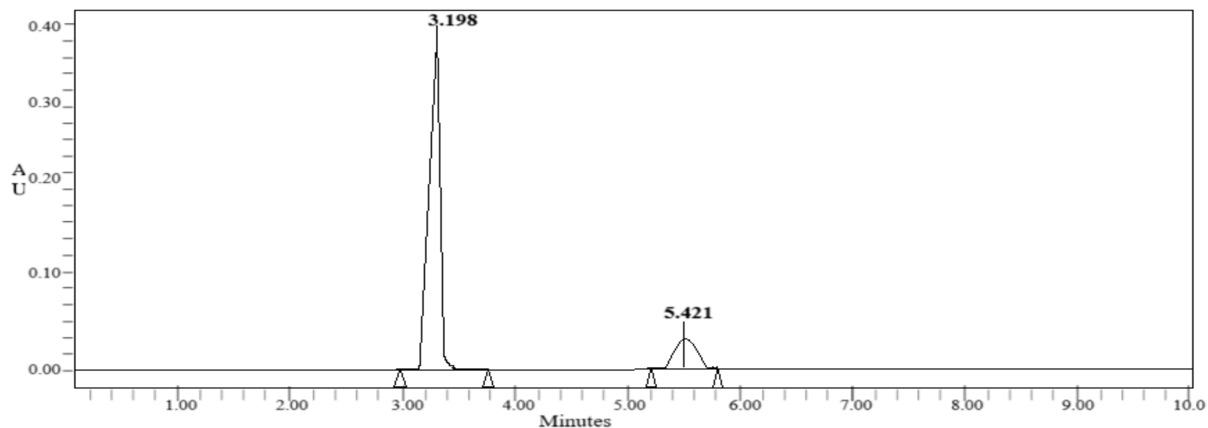


Figure : Optimized Chromatogram (Standard)

Table : Optimized Chromatogram (Standard)

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	USP Resolution
1	Fostemsavir	3.202	2391746	39726	1.2	9028	
2	Ganciclovir	5.463	194627	8497	1.1	7398	7.4

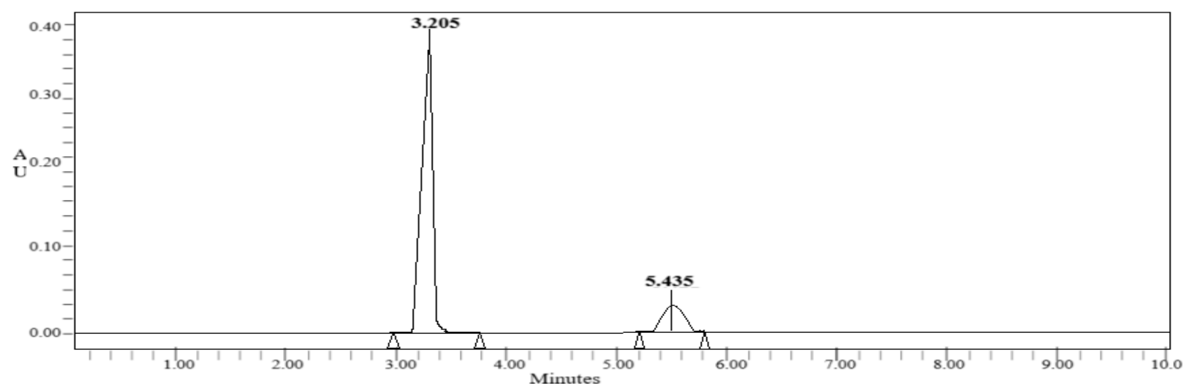


Table : Optimized Chromatogram (Sample)

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	USP Resolution
1	Fostemsavir	3.213	2381649	391846	1.2	9472	
2	Ganciclovir	5.478	191057	8104	1.1	8936	7.5

Acceptance criteria:

- Resolution between two drugs must be not less than 2
- Theoretical plates must be not less than 2000

System suitability:

Table : Results of system suitability for Fostemsavir

S.No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Fostemsavir	3.202	2391745	394171	8952	1.2
2	Fostemsavir	3.215	2391646	381946	9561	1.2
3	Fostemsavir	3.225	2381645	391746	6572	1.2
4	Fostemsavir	3.205	2385630	386562	6452	1.2
5	Fostemsavir	3.198	2385634	389164	7452	1.2
Mean			2387275			
Std. Dev.			4363.768			
% RSD			0.182798			

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 Ganciclovir

S.No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Ganciclovir	5.431	198363	7917	5272	1.1
2	Ganciclovir	5.425	197487	7486	6291	1.1
3	Ganciclovir	5.420	198355	7859	6184	1.1
4	Ganciclovir	5.435	197353	7926	7145	1.1
5	Ganciclovir	5.421	198454	7946	6946	1.1
Mean			198015.4			
Std. Dev.			535.1778			
% RSD			0.27032			

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: Peak results for assay standard

Fostemsavir

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Fostemsavir	3.202	2391745	397161	1.2	9473
2	Fostemsavir	3.215	2391646	389173	1.2	9746
3	Fostemsavir	3.225	2381645	391723	1.2	8916

Ganciclovir

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Resolution
1	Ganciclovir	5.431	198363	7811	1.1	8492	7.49
2	Ganciclovir	5.425	197487	8193	1.1	8916	7.52
3	Ganciclovir	5.420	198355	7972	1.1	9372	7.44

Assay (Sample):
Fostemsavir

S. No	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Fostemsavir	3.202	2391745	381612	1.2	9472
2	Fostemsavir	3.215	2391646	391746	1.2	8927
3	Fostemsavir	3.225	2381645	381634	1.2	9017

Ganciclovir

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Resolution
1	Ganciclovir	5.431	198363	8174	1.1	9284	7.18
2	Ganciclovir	5.425	197487	8942	1.1	8974	7.44
3	Ganciclovir	5.420	198355	7294	1.1	9017	7.38

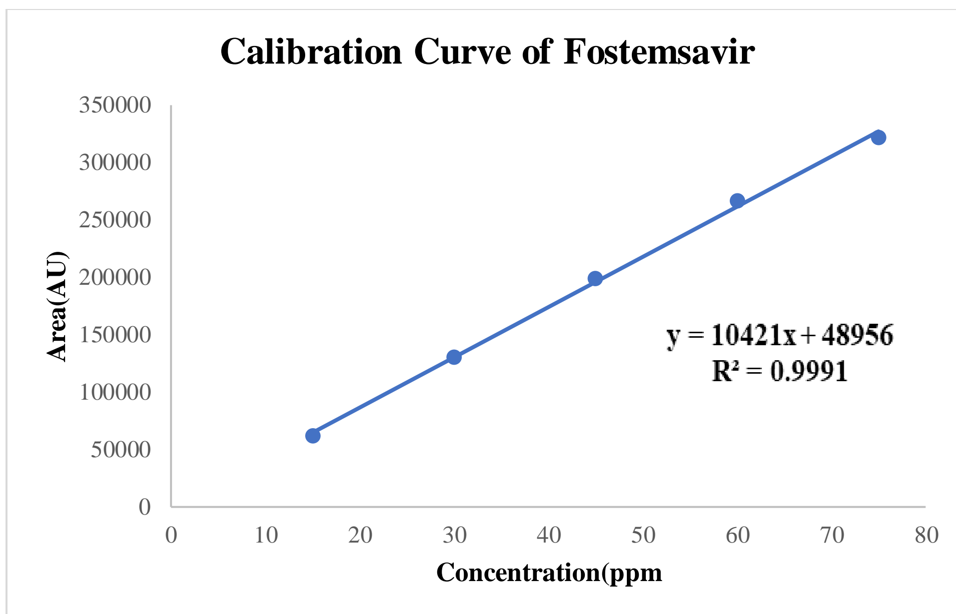
%ASSAY =

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

The % purity of Fostemsavir and Ganciclovir in pharmaceutical dosage form was found to be 99.2%.

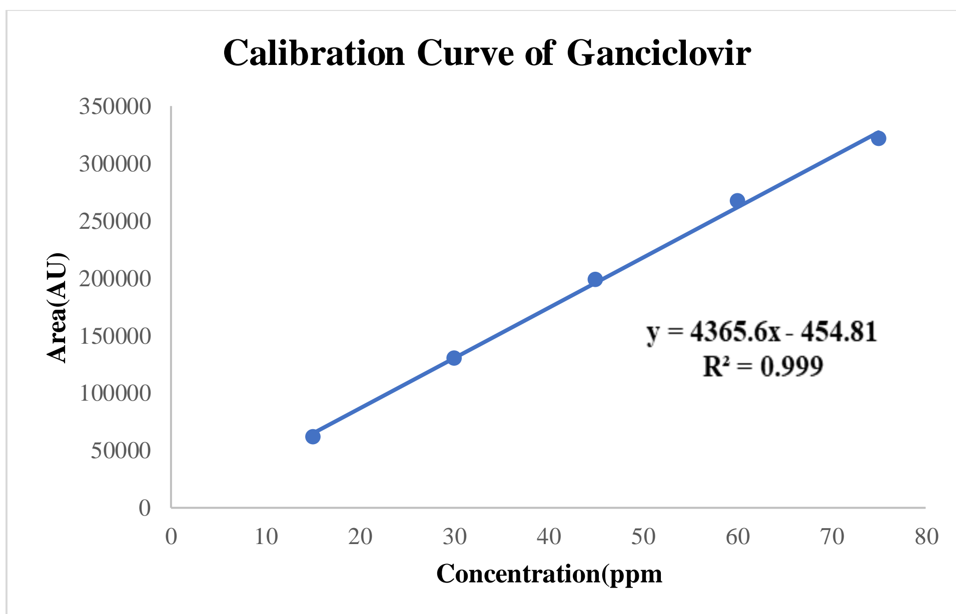
Linearity
Chromatographic data for linearity study:
Fostemsavir

Concentration µg/ml	Average Peak Area
75	909890
150	1583642
225	2395381
300	3185090
375	3943728



Ganciclovir

Concentration µg/ml	Average Peak Area
15	61956
30	130215
45	198698
60	267013
75	321662



Repeatability:

Table 7: Results of repeatability for Fostemsavir:

S. No	Peak name	Retention time	Area(µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Fostemsavir	3.220	2397163	381741	8155	1.2
2	Fostemsavir	3.215	2391742	371742	9174	1.2
3	Fostemsavir	3.225	2371845	391746	7154	1.2
4	Fostemsavir	3.205	2361749	391847	9917	1.2
5	Fostemsavir	3.198	2371645	384622	9247	1.2
Mean			2378836			
Std.dev			14959			
%RSD			0.628798			

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 7.12: Results of repeatability for Ganciclovir:

S. No	Peak name	Retention time	Area(µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Ganciclovir	5.431	198465	7291	6274	1.1
2	Ganciclovir	5.425	193642	7219	6592	1.1
3	Ganciclovir	5.420	196463	7194	6028	1.1
4	Ganciclovir	5.435	194645	8174	6927	1.1
5	Ganciclovir	5.421	198466	8653	5920	1.1
Mean			196334			
Std.dev			2190.193			
%RSD			1.115534			

Intermediate precision:

Day 1:

Table: Results of Intermediate precision for Fostemsavir

S.No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Fostemsavir	3.220	2397163	395275	9375	1.2
2	Fostemsavir	3.215	2391742	392175	9275	1.2
3	Fostemsavir	3.225	2371845	312947	8265	1.2
4	Fostemsavir	3.205	2361749	310585	6254	1.2
5	Fostemsavir	3.198	2371645	310694	9028	1.2
6	Fostemsavir	3.205	2378836	358373	8928	1.2
Mean			14959			
Std. Dev.			0.628798			
% RSD			2397163			

Acceptance criteria:

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

Table : Results of Intermediate precision for Ganciclovir

S.No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Ganciclovir	5.431	198465	7194	8264	1.2
2	Ganciclovir	5.425	193642	7294	9174	1.2
3	Ganciclovir	5.420	196463	7147	9164	1.2
4	Ganciclovir	5.435	194645	7927	9733	1.2
5	Ganciclovir	5.421	198466	8238	9194	1.2
6	Ganciclovir	5.435	196334	7638	8973	1.2
Mean			2190.193			
Std. Dev.			1.115534			
% RSD			198465			

Acceptance criteria:

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

Table : Results of Intermediate precision Day 2 for Fostemsavir

S.No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Fostemsavir	3.220	2397163	391741	9264	1.2
2	Fostemsavir	3.215	2391742	391047	9746	1.2
3	Fostemsavir	3.225	2371845	391748	9816	1.2
4	Fostemsavir	3.205	2361749	391746	9917	1.2
5	Fostemsavir	3.198	2371645	381641	9742	1.2
6	Fostemsavir	3.205	2378836	381645	9017	1.2
Mean			14959			
Std. Dev.			0.628798			
% RSD			2397163			

Acceptance criteria:

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

Table: Results of Intermediate precision Day 2 for Ganciclovir

S.No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Ganciclovir	5.431	198465	7582	6272	1.1
2	Ganciclovir	5.425	193642	7184	6174	1.1
3	Ganciclovir	5.420	196463	7456	5184	1.1
4	Ganciclovir	5.435	194645	7814	6194	1.1
5	Ganciclovir	5.421	198466	7194	6292	1.1
6	Ganciclovir	5.435	196334	7745	6191	1.1
Mean			2190.193			
Std. Dev.			1.115534			
% RSD			198465			

Acceptance criteria:

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

Accuracy

The accuracy results for Fostemsavir

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	1217218	112.5	112.4	99.5	99.5
100%	2397141	225	225	100	
150%	3514547	337.5	332.5	98.4	

Acceptance Criteria:

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

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

The accuracy results for Ganciclovir

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	98598.67	22.5	22.4	99.9	99.4
100%	198359.7	45	45	100	
150%	291512.3	67.5	66.8	99	

Acceptance Criteria:

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

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

Robustness:

Table : Results for Robustness

Fostemsavir

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0mL/min	2397163	3.220	9028	1.2
Less Flow rate of 0.9mL/min	2391742	3.215	7381	1.2
More Flow rate of 1.1mL/min	2371845	3.225	9311	1.1
Less organic phase (about 5 % decrease in organic phase)	2361749	3.205	7462	1.2
More organic phase (about 5 % Increase in organic phase)	2371645	3.198	6817	1.1

Acceptance criteria:

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

Table: Results for Robustness

Ganciclovir:

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.1mL/min	198465	5.431	7398	1.1
Less Flow rate of 0.9mL/min	193642	5.425	6883	1.1
More Flow rate of 0.8mL/min	196463	5.420	9917	1.2
Less organic phase (about 5 % decrease in organic phase)	194645	5.435	8372	1.1
More organic phase (about 5 % Increase in organic phase)	198466	5.421	7716	1.2

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 analysis of Fostemsavir and Ganciclovir. This method is applicable for both pure forms and tablet dosage forms of these drugs. Chromatography was conducted using a X-Terra C18 column with a mobile phase consisting of Methanol and Water (65:35% v/v) at a flow rate of 1.0 ml/min. Detection was achieved at 220 nm, and the retention times for Fostemsavir and Ganciclovir were found to be 2.9 and 3.6 minutes, respectively, with a precision below 2.0% RSD for assay determination. The method proves effective for monitoring drug purity and quantification in bulk and pharmaceutical formulations.

The developed HPLC method offers a robust analytical tool for assessing the quality and purity of Fostemsavir and Ganciclovir in both raw materials and tablet formulations. Its precision and rapidity make it suitable for routine quality control applications in pharmaceutical manufacturing. By reliably separating, detecting, and quantifying these drugs and their potential degradants or impurities, the method ensures compliance with regulatory standards and maintains product efficacy and safety. This underscores its significance in pharmaceutical analysis and quality assurance processes.

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