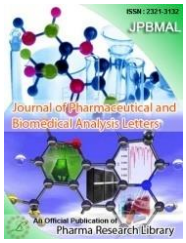

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RP-HPLC Method Development and Validation of a Stability Indicating Cidofovir in Its Pure and Dosage Form

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A b s t r a c t
High performance liquid chromatography is at present one of the most sophisticated tool of the analysis. The estimation of Cidofovir was done by waters HPLC with auto sampler and PDA detector. The buffer 1ml TFA in 1000ml water. Adjust this solution to pH 3 by using acid / base based on the PH of the resulted solution and Mix a mixture of above ACN 650ml (65%), 350 ml TFA (35%) and degas in ultrasonic water bath for 5 minutes. Filter through 0.45 µ filter under vacuum filtration. Inspire (150X4.6mm 5µm) or equivalent chemically bonded to porous silica particles was used as stationary phase. The detection was carried out using UV detector at 230nm. The solutions were chromatographed at a constant flow rate of 1ml/min and Injection volume 20µl, the run time 10min, the linearity range was found to lie from 20µg/ml to 50µg/ml of Cidofovir. The results obtained on the validation parameters met ICH and USP requirements. It inferred the method found to be simple, accurate, precise and linear. The method was found to be having suitable application in routine laboratory analysis
Keywords: TFA, ICH, USP, Cidofovir, CAN, HPLC, PDA

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1. Introduction

HPLC is an analytical technique in which solutes are resolved by differential rates of elution as they pass through a chromatographic column. The method of separation by this instrument is governed by distribution between the mobile phase and stationary phase. The instrumentation is made-up of eight basic components, mobile phase reservoir, solvent delivery system, sample introduction device, column, detector, waste reservoir, connective tubing and computer, integrator or recorder. The successful use of HPLC for the possible problem requires the right combination of variety of operating conditions such as the type of column packing and mobile phase, column length and diameter, mobile phase flow rate , column temperature and sample size [1]. Now a day reversed-phase chromatography is the most commonly used separation technique in HPLC due to its broad application range. It is estimated that over 65% (possibly up to 90%) of all HPLC separations are carried out in the reversed phase mode. The reasons for this include the simplicity, versatility and scope

of the reversed-phase method as it is able to handle compounds of a diverse polarity and molecular mass [2-4].

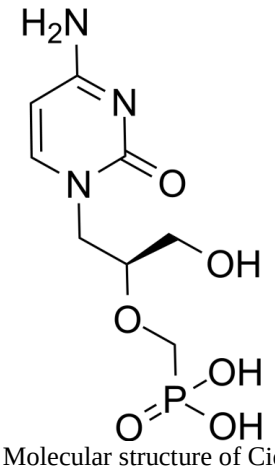

Fig.1: Molecular structure of Cidofovir

Molecular Formula: $C_8H_{14}N_3O_6P1$
Molecular Weight : 279.19 g/mol2
IUPAC Name : {[(S)-1-(4-amino-2-oxo-1,2-dihydropyrimidin-1-yl)-3-hydroxypropan-2-yl]oxy}methyl)phosphonic acid3
Drug Category : Antiviral medication
Indications : Used to treat cytomegalovirus (CMV) retinitis in individuals with AIDS (acquired immunodeficiency syndrome)12. - Also investigated for progressive multifocal leukoencephalopathy and anti-smallpox efficacy.
Pharmacology : Classified as a nucleotide analogue. Suppresses CMV replication by selective inhibition of viral DNA synthesis.
Contraindications: Allergic to cidofovir. Moderate to severe kidney disease. History of severe allergic reaction to probenecid or sulfa drugs1.
Adverse Effects: Vision changes, kidney problems, low blood cell counts, and pancreatitis. - Common side effects include nausea, vomiting, diarrhea, pain, weakness, rash, headache, and hair loss.

2. Materials and Methods

Table 1: Instruments used

SN	Instrument	Model
1	HPLC	WATERS, software: Empower, 2695 separation module.2487 UV detector.
2	UV/VIS spectrophotometer	LABINDIA UV 3000$^+$
3	pH meter	Adwa – AD 1020
4	Weighing machine	Afcoset ER-200A
5	Pipettes and Burettes	Borosil
6	Beakers	Borosil

Table 2: Chemicals used

S.No	Chemical	Brand
1	CIDOFOVIR	Supplied by MSN LAB
2	KH_2PO_4	FINAR chemical LTD
3	Water and Methanol for HPLC	Standard solutions Ltd
4	Acetonitrile for HPLC	Standard solutions Ltd
5	HCl, H_2O_2, NaOH	MERCK

Optimized chromatographic conditions:
Instrument used : High performance liquid chromatography Equipped with Auto Sampler and PDA detector
Temperature : Ambient
Column : INSPIRE (150X4.6mm 5µm)
Mobile phase : (65: 35) ACN: Trifluoroacetic acid
Flow rate : 1ml/min
Wavelength : 230nm
Injection volume : 20 µl
Run time : 10 min.

Preparation of TFA pH 3:
To prepare TFA solution, by adding 1ml TFA in 1000ml water. Adjust this solution to pH 3 by using acid / base based on the ph of the resulted solution.

Preparation of mobile phase:
Mix a mixture of above ACN 650ml (65%), 350 ml TFA (35%) and degas in ultrasonic water bath for 5 minutes. Filter through 0.45 µ filter under vacuum filtration.

Diluent Preparation:
Acetonitrile: TFA (65:35) ratio.

System Suitability: Tailing factor for the peaks due to Cidofovir in Standard solution should not be more than 2.0
Theoretical plates for the Cidofovir peaks in Standard solution should not be less than 2000
Calculation: (For Cidofovir)

$$\%\ Assay = \frac{AT}{AS} * \frac{WS}{DS} * \frac{DT}{WT} * \frac{Average\ weight}{Label\ Claim} * \frac{P}{100} * 100$$

Where,
AT= average area counts of sample preparation.
AS= average area counts of standard preparation.
WS= Weight of working standard taken in mg.
P= Percentage purity of working standard
LC= Label Claim mg/ml.

System Suitability Results:

- Tailing factor Obtained from the standard injection is 1.16
- Theoretical Plates Obtained from the standard injection is 3338

Validation parameters:

1. Assay:
Standard Solution Preparation:
Accurately weigh and transfer 25 mg of Cidofovir working standard into a 25 ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.

Sample Solution Preparation:
Accurately weigh and transfer equivalent to 25 mg of Cidofovir equivalent weight of the sample into a 25 ml clean dry volumetric flask add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.
Procedure: Inject 10 µL of the standard, sample into the chromatographic system and measure the areas for the Cidofovir peaks and calculate the % Assay by using the formulae.
2. Linearity:
Preparation of stock solution:
Accurately weigh and transfer 25 mg of Cidofovir working standard into a 25 ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Preparation of Level – I (10ppm of Cidofovir):
0.1ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with Diluents.
Preparation of Level – II (20ppm of Cidofovir):
0.2ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with Diluents.
Preparation of Level – III (30ppm of Cidofovir):
0.3ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with Diluents.
Preparation of Level – IV (40ppm of Cidofovir):
0.4ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with Diluents.
Preparation of Level – V (50ppm of Cidofovir):
0.5ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with Diluents.
3. Precision:
Preparation of stock Solution:
Accurately weigh and transfer 25 mg of Cidofovir working standard into a 25 ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.
Procedure:
The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.
4. Intermediate precision/ruggedness:
To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day within the laboratory.
Preparation of stock solution:
Accurately weigh and transfer 25 mg of Cidofovir working standard into a 25 ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.
Procedure:
The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.
5. Accuracy:
For accuracy determination, three different concentrations were prepared separately i.e. 50%, 100% and 150% for the analyte and chromatograms are recorded for the same.
Preparation of Standard stock solution:
Accurately weigh and transfer 25 mg of Cidofovir working standard into a 25 ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.
Preparation Sample solutions:
For preparation of 50% solution (With respect to target Assay concentration): Accurately weigh and transfer 12.5mg of Cidofovir working standard into a 25 ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.
For preparation of 100% solution (With respect to target Assay concentration): Accurately weigh and transfer 25 mg of Cidofovir working standard into a 25 ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.
For preparation of 150% solution (With respect to target Assay concentration): Accurately weigh and transfer 37.5 mg of Cidofovir working standard into a 25ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.
Procedure:
Inject the standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions. Calculate the Amount found and Amount added for Cidofovir and calculate the individual recovery and mean recovery values.
5. Limit of Detection:
Preparation of Cidofovir solution:
Preparation of 0.5µg/ml solution: Accurately weigh and transfer 25 mg of Cidofovir working standard into a 25 ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 1ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents. Further pipette 1ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents. Further pipette 0.5 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluent.

6. Limit of Quantification:

Preparation of Cidofovir solution:

Preparation of 1.9µg/ml solution: Accurately weigh and transfer 25 mg of Cidofovir working standard into a 25 ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 1ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents. Further pipette 1ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents. Further pipette 1.9ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluent.

7. 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.

- The flow rate was varied at 0.81 ml/min to 1.2 ml/min.
- Standard solution 30 µg/ml of Cidofovir prepared and analyzed using the varied flow rates along with method flow rate.
- The Organic composition in the Mobile phase was varied from 40% to 60%

Standard solution 30 µg/ml of Cidofovir was prepared and analyzed using the varied Mobile phase composition along with the actual mobile phase composition in the method.

3. Results and Discussion

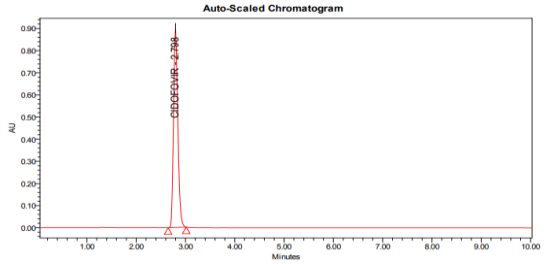

Figure 3: Chromatogram for system suitability

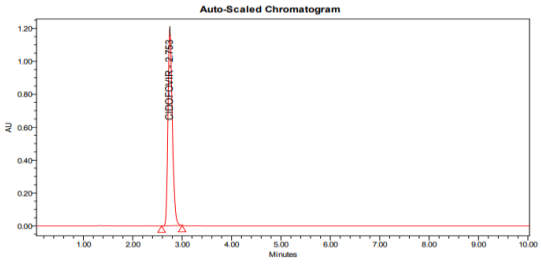

Figure 4: Chromatogram for Standard

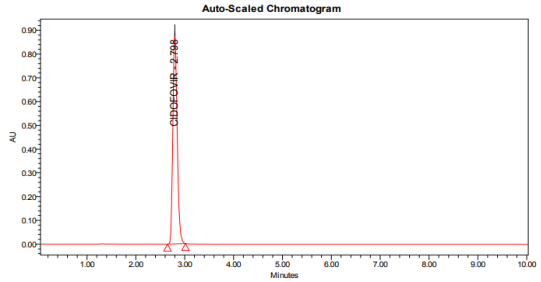

Figure 5: Chromatogram for Sample

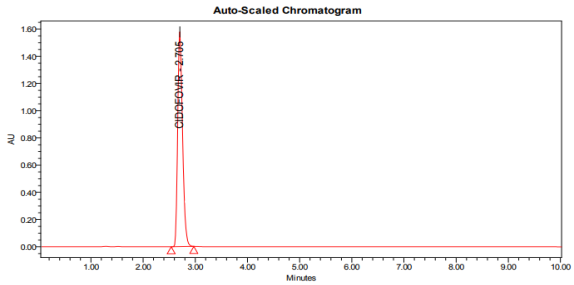

Figure 6: Chromatogram for linearity

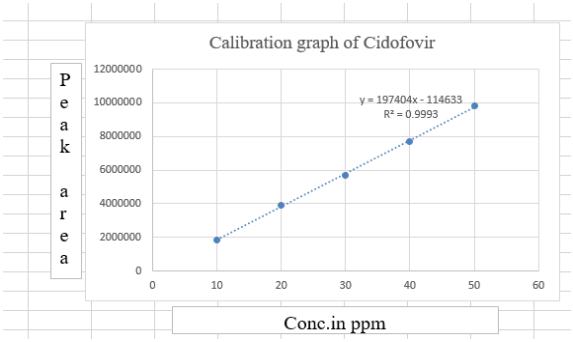

Figure 7: Calibration graph for Cidofovir

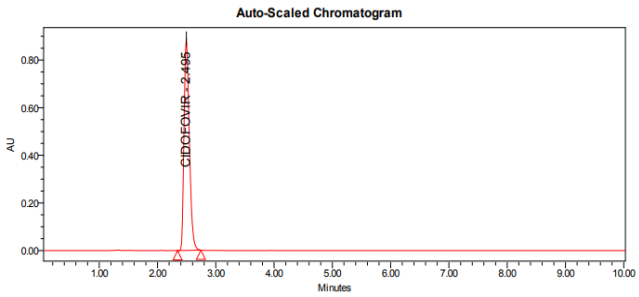

Figure 8: Chromatogram for Precision

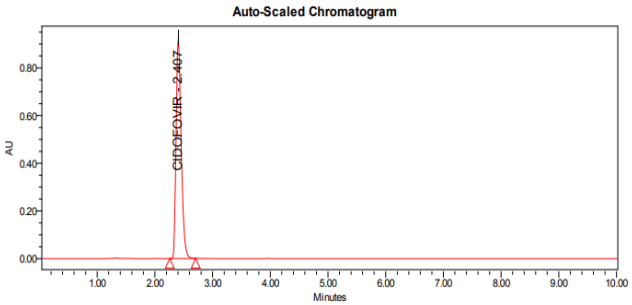

Figure 9: Chromatogram for ID Precision

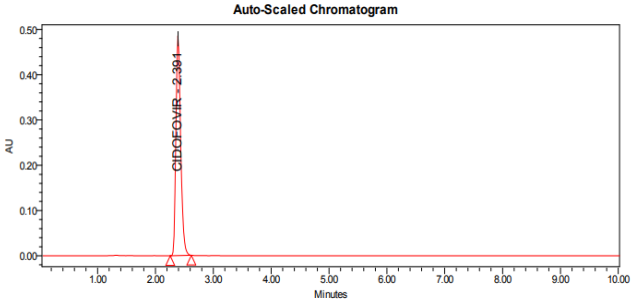

Figure 10: Chromatogram for Accuracy 50%

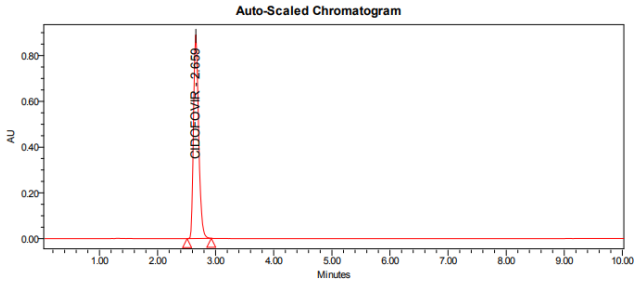

Figure 11: Chromatogram for Accuracy 100%

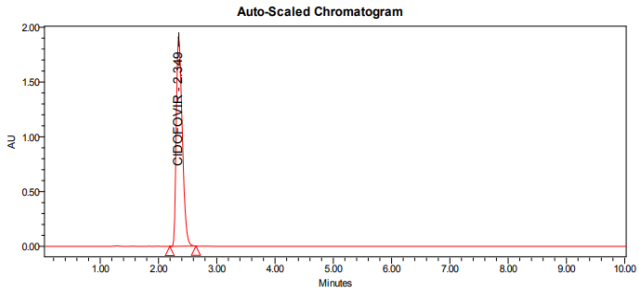

Figure 12: Chromatogram for Accuracy 150%-3

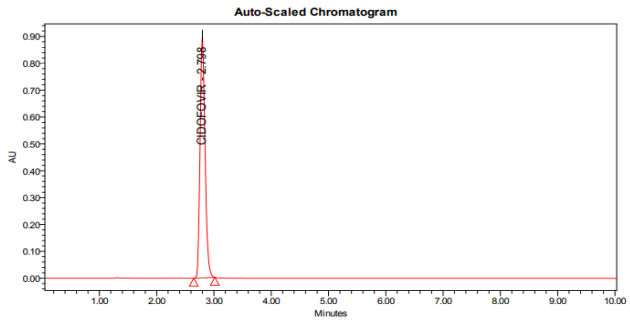

Figure 13: Chromatogram of Cidofovir showing LOD

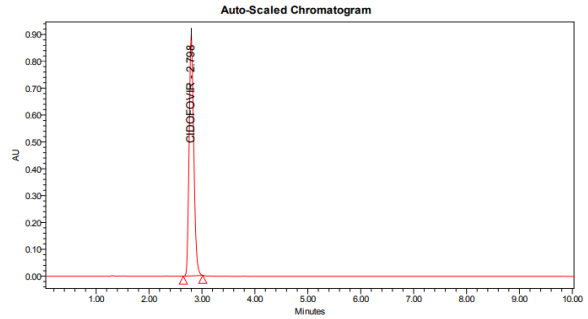

Figure 14: Chromatogram of Cidofovir showing LOQ

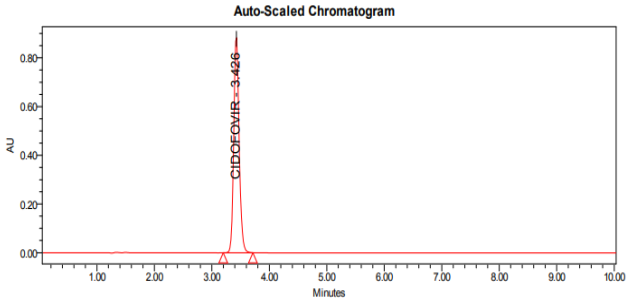

Figure 15: Chromatogram showing less flow

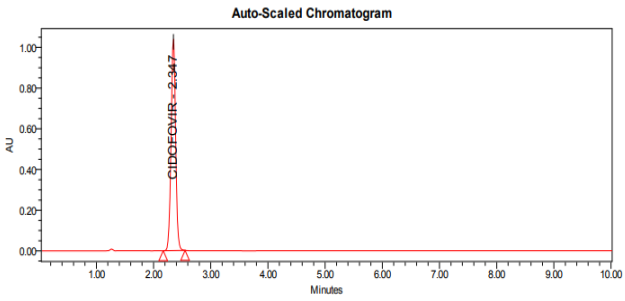

Figure 16: Chromatogram showing more flow

Table 3: Results of system suitability parameters

S.No	Name	RT(min)	Area (µVsec)	Height (µV)	USP tailing	USPplatecount
1	Cidofovir	2.79	5415431	8932	1.22	4932.41

Figure 4: Results of standard and sample of Cidofovir

S.No	Name	RT(min)	Area (µVsec)	Height (µV)	USP tailing	USP plate count
1	Cidofovir Std	2.75	5415434	8933	1.32	4934.41
2.	Cidofovir sample	2.79	5415431	8932	1.22	4932.41

Table 5: Results of Assay for Cidofovir

	Label Claim (mg)	% Assay
Cidofovir	670 mg	98.7206

Table 6: Area of different concentration of Cidofovir

S. No	Cidofovir	
	Concentration (µg/ml)	Area
1	10	1842517
2	20	3936132
3	30	5715431
4	40	7725126
5	50	9818216

Table 7: Results of Precision for Cidofovir

Injection	Area
Injection-1	5572689
Injection-2	5545558
Injection-3	5520838
Injection-4	5517424
Injection-5	5503044
Injection-6	5457925
Average	5519580
Standard Deviation	38926.68
%RSD	0.7

Table 8: Results of LOD

Drug name	Baseline noise(µV)	Signal obtained (µV)	S/N ratio	CONC
Cidofovir	59	173	2.93	0.59µg/ml

Table 9: Results of LOQ

Drug name	Baseline noise(µV)	Signal obtained(µV)	S/N ratio	CONC
Cidofovir	59	585	9.91	1.9µg/ml

Signal to noise ratio shall be 10 for LOQ solution

Table 10: Results for variation in flow for Cidofovir

S. No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.8	4922.20	1.12
2	1	4932.41	1.22
3	1.2	4933.41	1.19

*****Results for actual flow (0.8 ml/min) have been considered from Assay standard.

Table 11: Results for variation in mobile phase composition for Cidofovir

S.No	Change in Organic Composition in theMobile Phase	System Suitability Results	
		USP Plate Count	USP Tailing
1	10% less	4930.92	1.20
2	*Actual	4932.41	1.22
3	10% more	4932.08	1.16

*****Results for actual Mobile phase composition have been considered from Accuracy standard.

4. Conclusion

The estimation of Cidofovir was done by waters HPLC with auto sampler and PDA detector. The buffer 1ml TFA in 1000ml water. Adjust this solution to pH 3 by using acid / base based on the PH of the resulted solution and Mix a mixture of above ACN 650ml (65%), 350 ml TFA (35%) and degas in ultrasonic water bath for 5 minutes. Filter through 0.45 µ filter under vacuum filtration. Inspire (150X4.6mm 5µm) or equivalent chemically bonded to porous silica particles was used as stationary phase. The detection was carried out using UV detector at 230nm. The solutions were chromatographed at a constant flow rate of 1ml/min and Injection volume 20µl, the run time 10min, the linearity range was found to lie from 20µg/ml to 50µg/ml of Cidofovir. The correlation coefficient obtained was 0.999 which is in the acceptance limit. The % RSD values of Cidofovir are found to be 0.7 indicating less than 2% precision of the method and Intermediate precision for Cidofovir found to be 0.5. The percentage recovery varies from 98-102% of Cidofovir found to be 99.31. LOD and LOQ were found to be within limit. The results obtained on the validation parameters met ICH and USP requirements. It inferred the method found to be simple, accurate, precise and linear. The method was found to be having suitable application in routine laboratory analysis with high degree of accuracy and precision.

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