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Development And Validation Of RP-HPLC Method For Estimation Of Some Antiviral Drugs Using Aqbd Approach

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ABSTRACT
A precise and robust method was developed method for the estimation of some antiviral drugs i.e. Acyclovir (ACY), Abacavir (ABA), Sofosbuvir (SOF) and Lopinavir (LPV) in bulk and pharmaceutical dosage form. The Method used Agilent 1260 Infinity II model HPLC with DAD detector and Phenomenex Kinetex XB-C8 (150 mm × 4.6 mm, 5µm). The Mobile phase combination used was 0.2% Perchloric acid and Acetonitrile [50:50]. Flow rate at 0.5ml/min and wavelength at 210 nm with run time of 10 minutes. The retention time of Acyclovir, Abacavir, Sofosbuvir and Lopinavir peaks was at 2.9minutes, 3.29 minutes, 4.49 minutes and 14.19minutes, respectively. The developed method was validated according to ICH Q2 (R1) guidelines. % RSD for Instrument precision, Method Precision and Intermediate precision was found to be below 2.0% for the antiviral drugs. The Intra& Inter-day precision for Acyclovir, Abacavir, Sofosbuvir and Lopinavir had a %RSD of less than 2.0% for all the APIs. Method was linear and accurate for concentration range of 40-60 µg/ml for all four APIs. For accuracy, maximum % RSD of ACY at 80% which was 0.21%, for ABA at 100% which was 0.12%, for SOF and LPV at 120% which was 0.39% and 0.34% respectively. The method was found to be robust for simultaneous estimation of Acyclovir, Abacavir, Sofosbuvir and Lopinavir.

Keywords: Acyclovir, Abacavir, Sofosbuvir, Lopinavir, RP-HPLC, Robustness, AQbD, Precision

Introduction

Since the beginning of human civilization, infectious diseases have been well recognized to the general public. A variety of microorganisms, including bacteria, viruses, and fungus, are responsible for the transmission of infectious diseases [1]. Viruses are considered to be obligatory intracellular pathogens because they replicate by utilizing the cellular machinery of their host. [2].The genetic material of viruses can be either DNA or RNA, and they are known to cause a wide range of diseases in humans, animals, and plants. [3].In light of the fact that viral infections have been responsible for the deaths of millions of people all over the world throughout the course of human civilization, the development of dynamic antiviral drugs is an urgent necessity. [4]. There is a category of medications known as antiviral drugs, which are specifically employed for the treatment of viral illnesses. As a result, it is challenging to identify therapeutic targets that will interfere with the virus without causing damage to the cells of the host**.** Hence, with variety of antiviral drugs the major challenge is quantification of these drugs. So, current studies focus on estimation of some antiviral drugs using a single method which can used for simultaneous as well for individual quantification.

Acyclovir is an antiviral agent [5] primarily utilized for treating herpes simplex virus infections, chickenpox, and shingles [6]. Additional applications encompass the prophylaxis of cytomegalovirus infections post-transplant and the management of severe complications associated with Epstein–Barr virus infection [6, 7]. It can be administered orally, topically as a cream, or via injection [6, 8].

Abacavir is a nucleoside reverse transcriptase inhibitor, specifically a carbocyclic 2'-deoxyguanosine, recognized as a guanosine analog. Ingesting abacavir with food does not significantly impact drug exposure and can be consumed without consideration of meals. The antiviral efficacy of abacavir arises from its intracellular metabolite, carbovir-triphosphate, which disrupts HIV RNA-dependent DNA polymerase (reverse transcriptase), resulting in the suppression of viral replication [9] This intracellular anabolite exhibits a prolonged elimination half-life exceeding 20 hours, permitting once-daily administration [10,11].

Sofosbuvir was identified in 2007 and received approval for medical application in the United States in 2013 [12] It is included in the World Health Organization's List of Essential Medicines [13].Sofosbuvir is an authorized pharmaceutical agent with robust antiviral efficacy against various genotypes of the hepatitis C virus (HCV) [14]. The acid dissociation constant (pKa) of sofosbuvir is around 9.3, and being a class 3 chemical, it exhibits good solubility and low permeability

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[15]. The phosphorylation of sofosbuvir within the host cell (hepatocyte) transforms it into the active form, nucleoside triphosphate, which halts RNA replication in the emerging viral genome by competing with the virus's nucleotides. Sofosbuvir demonstrates robust antiviral efficacy, exceeding 90%, even in cases of liver cirrhosis and previous non-responsiveness to ribavirin and interferon [16, 17].
Lopinavir is a protease inhibitor classified as an antiretroviral medication. It is utilized for HIV infections as a fixed-dose combination with another protease inhibitor, ritonavir (lopinavir/ritonavir) [18]. It received patent protection in 1995 and obtained medical approval in 2000 [20] Regarded as a second-line therapy in Western countries, it remains prescribed in low- and middle-income countries, particularly for children with HIV. Lopinavir with ritonavir may be administered as a tablet or an oral solution, the latter being the preferable choice for youngsters. During the initial phases of the COVID-19 pandemic, lopinavir was repurposed to target the SARS-CoV-2 virus with the aim of inhibiting its protease activity [19].

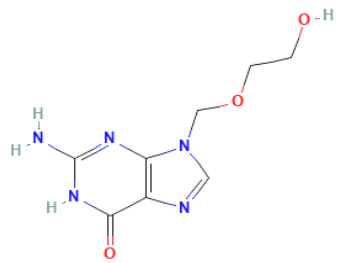

Figure 1. Structure of Acyclovir [7]

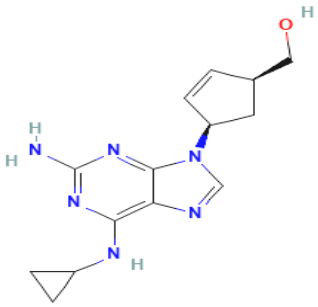

Figure 2. Structure of Abacavir [11]

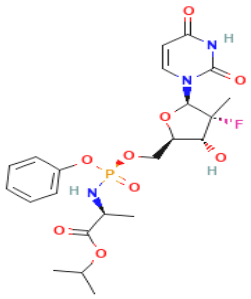

Figure 3. Structure of Sofosbuvir [20]

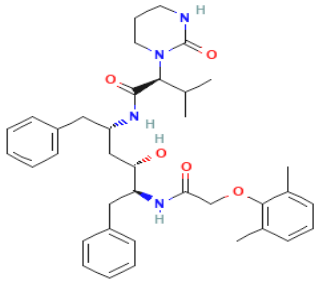

Figure 4. Structure of Lopinavir [24]

Literature Review

Based on a comprehensive study of the literature, numerous published reverse phase (RP)-HPLC procedures have been developed for quantifying Acyclovir, Abacavir, Sofosbuvir, and Lopinavir separately in pharmaceutical formulations [20–29]. The goal of this work was to provide an authentic, quick, accurate, and dependable analytical technique using RP-HPLC that could measure Acyclovir, Abacavir, Sofosbuvir, and Lopinavir in pharmaceutical formulations via RP-HPLC, validated in accordance with ICH requirements. A validated technique for measuring Niraparib in bulk and pharmaceutical formulations was effectively executed.

Material and Methods

Chemicals and Reagents

Aadhaar Life Sciences Pvt. Ltd. provided the complimentary samples of Acyclovir, Abacavir, Sofosbuvir and Lopinavir. HPLC-grade Acetonitrile was procured from Qualigens, India. Analytical grade of Perchloric acid was acquired from Molychem, Mumbai. A HPLC grade water was utilized via the internal Milli-Q system. All of the weighing was carried out on scales that had been calibrated by NABL lab. The preparation of samples was carried out with the use of the analytical balance and Type A glassware.The instrument named Agilent 1260 Infinity II equipped with a quaternary pump and DAD detector was used for HPLC Method development and validation. Software from Agilent Open lab CDS Ezchrom edition was used. Labman ultrasonicator and the Aczet analytical balance was used for wet chemistry purposes.

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Preparation of Mobile Phase and Diluent
Mobile Phase: 50%- 0.2% Perchloricacid: 50% Acetonitrile
Mix separately measured 500 ml of 0.2% Perchloric acid and 500 ml of Acetonitrile into a suitable container. Filter the mobile phase through 0.45 µm nylon membrane filter. Briefly sonicate to degas.

Preparation of Standard Solution
A. Working Standard:
1. Acyclovir Standard Stock Solution-I (SSS-I):
Initially Prepare a Standard Stock Solution (SSS-I) of by adding 5 mg of Acyclovir in 10 ml volumetric flask & add 5 ml diluent, mix for 2 minutes and make the volume to 10 ml with diluent.(Conc. of Acyclovir = 500 µg/ml).
2. Abacavir Standard Stock Solution-II (SSS-II):
To prepare a Standard Stock Solution (SSS-II) of add 5 mg of Abacavir in 10 ml volumetric flask & add 5 ml diluent, mix for 2 minutes and make the volume to 10 ml with diluent. (Conc. of Abacavir = 500 µg/ml).
3. Sofosbuvir Standard Stock Solution (SSS-III)
To prepare a Standard Stock Solution (SSS-III) of add 5 mg of Sofosbuvir in 10 ml volumetric flask & add 5 ml diluent, mix for 2 minutes and make the volume to 10 ml with diluent. (Conc. of Sofosbuvir = 500 µg/ml).
4. Lopinavir Standard Stock Solution (SSS-IV)
To prepare a Standard Stock Solution (SSS-IV) of add 5 mg of Lopinavir in 10 ml volumetric flask & add 5 ml diluent, mix for 2 minutes and make the volume to 10 ml with diluent. (Conc. of Lopinavir = 500 µg/ml).
5. Then add 1.0 ml of each SSS-I, SSS-II, SSS-III&SSS-IV in 10 ml volumetric flask and add 4 ml diluent and vortex and make up the volume with diluent. (Conc. of Acyclovir, Abacavir, Sofosbuvir &Lopinavir = 50 µg/ml).

Methodology
Development and Optimization of Method using AQbD Design:
1. Analytical Target Profile (ATP)
This approach allows for the quantification of substances in pharmaceutical formulations. The procedure ensures a high degree of accuracy and precision. A detailed overview of the performance attributes, outlining the intended function and expected performance standards of analytical measurement. It highlights performance attributes such as specificity, precision, accuracy, and linearity.
2. Critical Analytical Attributes (CAA)
The CAA has a crucial function in determining the factors that influence the parameters of the CAA. Attributes of the analytical method must be maintained within suitable limits to attain the desired ATP. Examples include peak area, retention time, plate count, tailing factor, resolution, and relative retention times.
3. Critical Method Parameters (CMP)
Independent analytical variables affecting CAA include the composition of the mobile phase, pH levels, buffer strength, flow rate, and injection volume.
4. Critical Method Material Attributes (CMMA)
A Critical Method Material Attributes (CMMA) represents a distinct category of risk factors linked to manufacturing or preparation variables. CMMAs can be directly manipulated by experimenters, exerting a considerable influence on CAAs. Therefore, the reagents utilized in the procedure must be of high purity and analytical reagent grade, while the glassware should conform to Type A standards. The identification of these parameters represents a crucial phase in Quality by Design, as it establishes the input variables necessary for controlling Critical Analytical Attributes and ensuring they remain within the defined range for quality and safety purposes.

Selection of Wavelength:
The selection of wavelength was carried out by using PDA option from Diode Array Detector. The solution injected was allowed to scan from 190 to 400 nm and based on the appropriate intensity of the all four drugs wavelength was selected.

HPLC Method Development
Multiple trials were done during the development phase with the results and observations were optimized using AQbD design.

Method validation
1. Specificity
Acyclovir, Abacavir, Sofosbuvir & Lopinavir were injected at 50µg/ml doses, respectively, and peaks were found by Retention Time analysis. Blank injection ensured that the blank peak would not interfere with the major analyte peaks.
2. System Suitability
System Suitability Testing limits are acceptance criteria that must be met prior to sample analysis. The theoretical plate count, tailing factor, and resolution should meet ICH guidelines.

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3. Accuracy
The assessment of accuracy involves introducing a known concentration of analyte standard into the sample matrix of interest and subsequently analysing the sample through the method under validation. The methodology and computation for accuracy, expressed as percentage recovery. Three replicate injections were performed at each level to calculate drug concentration, recovery percentage, and standard deviation.

4. Precision
Instrument Precision is quantified through the injection of a sequence of standards or by analysing a series of samples obtained from multiple samplings of a homogeneous lot. Precision is determined by calculating the relative standard deviation (% RSD) from the measured standard deviation (SD) and mean values.

5. Linearity
The assessment of linearity involves the introduction of a sequence of standards at least five distinct concentrations spanning from different concentrations of the working range. The linearity graph will be constructed depicting the relationship between concentration and peak area response.

6. LOD and LOQ
Limits of detection (LOD) and quantification (LOQ) represent the method's capacity to detect and quantify the smallest analyte amounts. The following formulae determined the standard deviation and regression line slope needed to calculate LOD and LOQ.

$$LOD = \frac{3.3 \times Std.ErrorofIntercept}{CoefficientsofXVariable\ 1}$$

$$LOQ = \frac{10 \times Std.ErrorofIntercept}{CoefficientsofXVariable\ 1}$$

7. Robustness
The Robustness study involved altering the column temperature by a range of ± 2˚C and the Change in strength of Mobile phase A ± 2%.

8. Inter-day & Intraday Precision:
In the morning and evening, the prepared working standard underwent evaluation, and the %RSD was computed to assess the stability of the solution concerning intraday precision. On the second day, the identical solution was administered, and the intraday precision along with the %RSD was evaluated against the morning data.

Results and Discussion
Wavelength Selection
The maximum absorption of Acyclovir, Abacavir, Sofosbuvir and Lopinavir was found to be at 210 nm.

Method Development
Following all of the trials (Table 1), it was discovered that the peak of highest absorption occurred at a wavelength of 210 nm. The diluent was maintained at a constant ratio of 50-50 0.2% Perchloric acid & Acetonitrile throughout all of the trials. Throughout all of the tests, Phenomenex Kinetex XB C8 (150 x 4.6 mm, 5 micron) was used because of it particle size and silica bed.

Table 1.Method Development for Acyclovir, Abacavir, Sofosbuvir and Lopinavir HPLC

Trial No.	Mobile Phase	Ratio	Flow rate (ml/min)	Wavelength (nm)	Observation
1	0.1% PA: ACN	50-50	1	250	3 peaks eluted early and 4th peak eluted at about 9 min. but 4th peaks intensity was too small so wavelength changed to 210 nm and increased 0.1% PA by 10% for separation of first 3 peaks
2	0.1% PA: ACN	60-40	1	210	For 2nd peak split was observed, and 4th peak did not eluted. So ACN was increased from 40 to 60%
3	0.1% PA: ACN	40-60	1	210	1st and 2nd peaks were merged whereas 3rd and 4th peak eluted early so to separate first 2 peaks flow rate needs to be decreased by half
4	0.1% PA: ACN	40-60	0.5	210	First two peaks still merged, but intensity increased compared to Trial 3 and slight

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					separation in peak 1 and 2 so strength of perchloric acid reduced to 0.05% from 0.1%
5	0.05% PA: ACN	40-60	0.5	210	First two peaks got completely merged, so 0.05% perchloric acid increased by 10%
6	0.05% PA: ACN	50-50	0.5	210	1st and 2nd peaks separated by 4th eluted very late at around 20 minutes so higher strength of perchloric acid is required for separation of first 2 peaks
7	0.2% PA: ACN	40-60	0.5	210	First two still merged, need to decrease ACN by 5 %
8	0.2% PA: ACN	45-55	0.5	210	First two still merged, need to decrease ACN by 5 %
9	0.2% PA: ACN	50-50	0.5	210	First two still slightly merged, 4th peak eluted very late so by taking trial 8 need to decrease Sample concentration from 50 ug/ml to 5 ug/ml for each drug
10	0.2% PA: ACN	45-55	0.5	210	First 2 peaks merged
11	0.2% PA: ACN: THF	35-55-10	0.5	210	First 2 peaks merged due to THF
12	0.2% PA: ACN: Water	40-50-10	0.5	210	Due to water in mobile phase at 10%, abacavir and acyclovir merged completely
13	0.2% PA: ACN	55-45	0.5	210	First 2 peaks merged
14	0.2% PA: ACN	50-50	0.5	210	Separation achieved between 1 and 2, for more resolution increase 0.2% PA by 2%
15	0.2% PA: ACN	52-48	0.5	210	Separation achieved between 1 and 2, but resolution decreased so for more resolution increase ACN by 2%
16	0.2% PA: ACN	48-52	0.5	210	Separation achieved between 1 and 2, so should go with trial 14 only

NOTE: sequence of peaks: Peak 1- Acyclovir, Peak 2- Abacavir, Peak 3: Sofosbuvir and Peak 4: Lopinavir

In accordance with the Analytical target profile that had been established in advance for the development work AQbD design was utilized to optimize the results. After the optimization, it was found that, the mobile phase having 0.2% Perchloric acid: Acetonitrile in ratio of 50: 50 and Flow rate 0.45 ml/min and Injection volume 10µl will be the final method for simultaneous estimation of Acyclovir, Abacavir, Sofosbuvir and Lopinavir.

Table 2. Optimization of Method development using AQbD design

Run	0.2% PA	Flow rate	Acyclovir		Abacavir		Sofosbuvir		Lopinavir	
			TP	RT	TP	RES	TP	RES	RT	RES
1	48	0.45	6741	2.88	8145	2.22	9897	5.38	9.93	23.15
2	52	0.5	6208	2.63	7256	2.76	10221	9	17.32	34.17
3	52	0.55	6165	2.39	7479	2.69	9717	7.75	14.09	31.18
4	50	0.5	6253	2.6	7676	2.56	9654	6.87	12.74	28.61
5	**50**	**0.45**	**6601**	**2.91**	**7913**	**2.7**	**10207**	**7.8**	**15.35**	**30.87**
6	48	0.55	6159	2.36	7434	2.1	9474	5.3	8.25	23.6
7	52	0.45	6573	2.91	7885	2.69	10067	8.06	17.43	31.22
8	48	0.5	6458	2.6	7749	2.1	9775	5.35	8.98	23.23
9	50	0.55	6187	2.37	7391	2.5	9813	7.23	12.14	29.38

RT – Retention Time, TP – Theoretical Plates, RES - Resolution

ANOVA spectrums for Acyclovir from Design expert software

Response 1: Theoretical Plates

From the 3D surface plot (Figure 5), the grey area with respect to the two axes, A: Mobile phase A % and B: Flow rate, with balanced mobile phase A concentration and low flow rate, the theoretical plates will be more than 6000. This is in accordance with the contour plot.

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Response 2: Retention Time

From the 3D surface plot (Figure 6), it can be incurred that with decrease in flow rate, the retention time increases for Acyclovir. There is no significant effect of mobile phase A concentration change of 2% on the retention time.

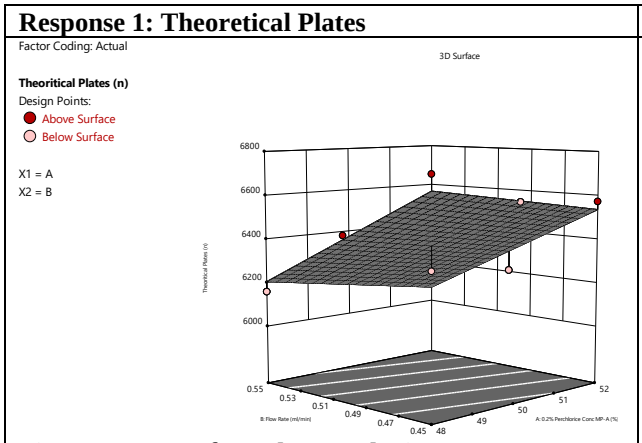

Figure 5. 3D Surface Plot Acyclovir

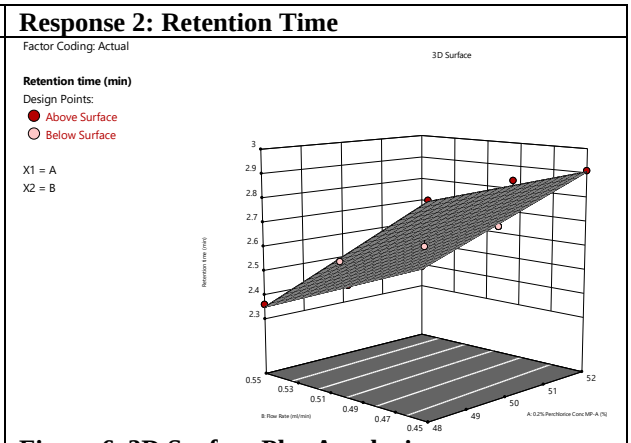

Figure 6. 3D Surface Plot Acyclovir

ANOVA spectrums for Abacavir from Design expert software

Response 1: Theoretical Plates

From the 3D surface plot (Figure 7), the grey area with respect to the two axes, A: Mobile phase A % and B: Flow rate, with balanced mobile phase A concentration and low flow rate, the theoretical plates will be more than 8000. This is in accordance with the contour plot.

Response 2: Resolution

From the 3D surface plot (Figure 8), it can be incurred that with decrease in flow rate, the Resolution increases for Abacavir. There is no significant effect of mobile phase A concentration change of 2% on the Resolution.

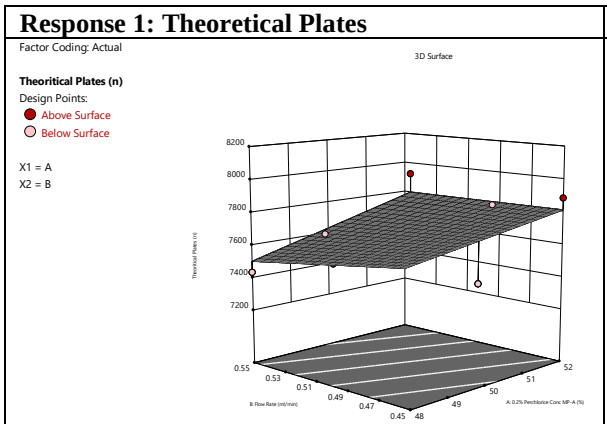

Figure 7. 3D Surface Plot Abacavir

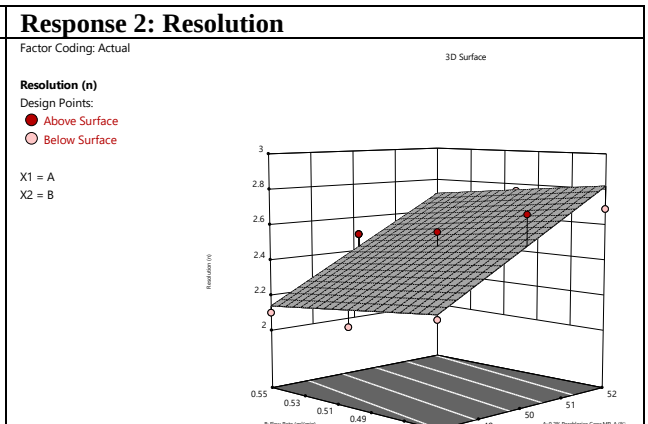

Figure 8. 3D Surface Plot Abacavir

ANOVA spectrums for Sofosbuvir from Design expert software

Response 1: Theoretical Plates

From the 3D surface plot (Figure 9), the grey area with respect to the two axes, A: Mobile phase A % and B: Flow rate, with balanced mobile phase A concentration and low flow rate, the theoretical plates will be more than 10000. This is in accordance with the contour plot.

Response 2: Resolution

From the 3D surface plot (Figure 10), it can be incurred that with decrease in flow rate, the Resolution increases for Sofosbuvir. There is no significant effect of mobile phase A concentration change of 2% on the Resolution.

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Response 1: Theoretical Plates

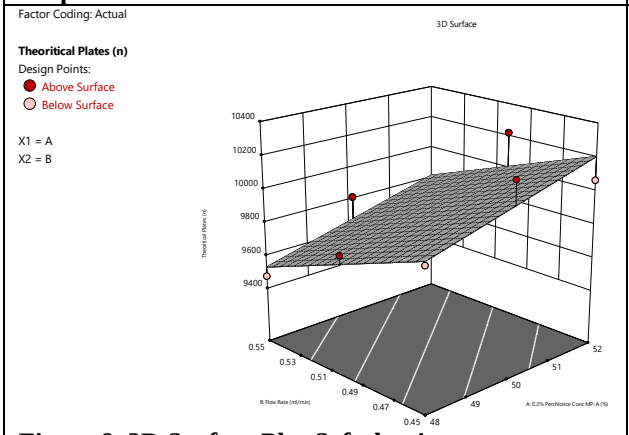

Figure 9. 3D Surface Plot Sofosbuvir

Response 2: Retention Time

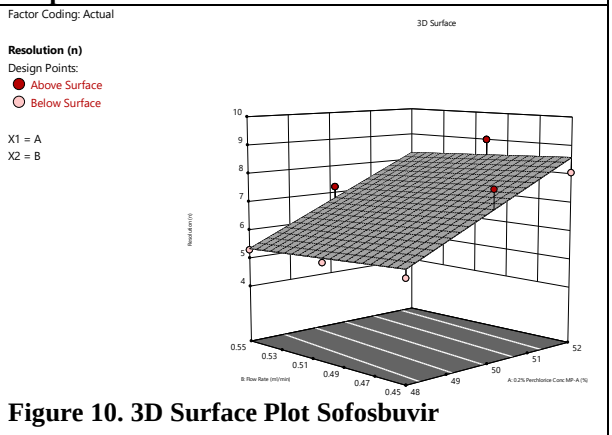

Figure 10. 3D Surface Plot Sofosbuvir

ANOVA spectrums for Lopinavir from Design expert software

Response 1: Retention time

From the 3D surface plot (Figure 11), it can be incurred that with decrease in flow rate, the retention time increases for Lopinavir. There is no significant effect of mobile phase A concentration change of 2% on the retention time.

Response 2: Resolution

From the 3D surface plot (Figure 12), it can be incurred that with decrease in flow rate, the Resolutionincreases for Lopinavir. There is no significant effect of mobile phase A concentration change of 2% on the retention time.

Response 1: Retention Time

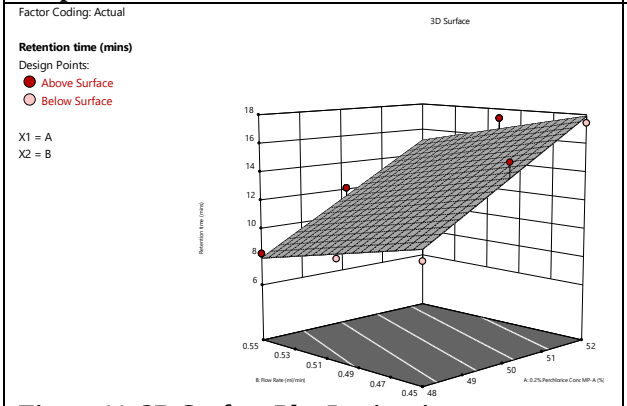

Figure 11. 3D Surface Plot Lopinavir

Response 2: Resolution

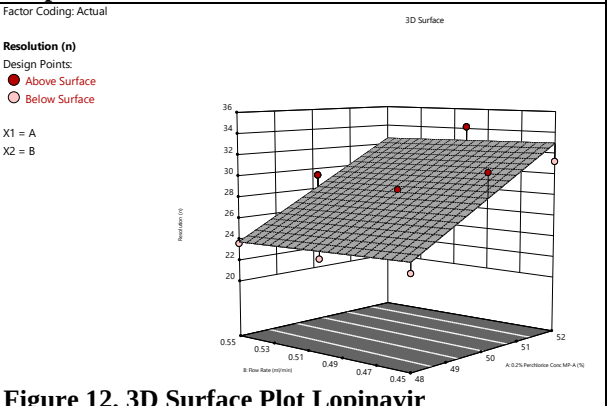

Figure 12. 3D Surface Plot Lopinavir

Final Chromatographic Conditions:

Table 3.Final Chromatographic Condition

Parameter	**Condition**
HPLC Instrument	Agilent 1260 Infinity II
Column	Phenomenex Kinetex XB-C8 (150 mm x 4.60 mm,5µm)
Wavelength	210 nm
Mobile Phase	Mobile Phase A –0.1% Perchloric acid-50 % Mobile Phase B – Acetonitrile- 50%
Diluent	0.1% Perchloric acid : Acetonitrile (50:50) v/v
Run time	20 minutes
Injection Volume	10 micro liters
Flow Rate	0.45 ml/min
Column oven Temperature	30°C (± 2°C allowed by Robustness)

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Method Validation

1. Specificity

Specificity was performed to check if there was any interaction between the peaks from blank or the APIs. Based on specificity data (Table 4), it was found that the Retention time of APIs and Drug product peak was same.

Table 4.Specificity results of Acyclovir, Abacavir, Sofosbuvir and Lopinavir

Sample ID	Acyclovir		Abacavir		Sofosbuvir		Lopinavir	
	RT	%Assay	RT	%Assay	RT	%Assay	RT	%Assay
Blank	-		-		-		-	
Working Standard	2.9	98.54%	3.29	99.73%	4.42	99.97%	14.19	99.92%
Drug Product	2.9		3.29		4.42		14.19	

For Acyclovir, Abacavir, Sofosbuvir and Lopinavir it is 2.9 minutes, 3.29 minutes, 4.42 minutes and 14.19 minutes respectively. There is no interference of blank with main peak, confirming the identification of Acyclovir, Abacavir, Sofosbuvir and Lopinavir in APIs and DPs. The marketed product assay for Acyclovir, Abacavir, Sofosbuvir and Lopinavir were found to be 98.54%, 99.73%, 99.97% and 99.92% respectively.

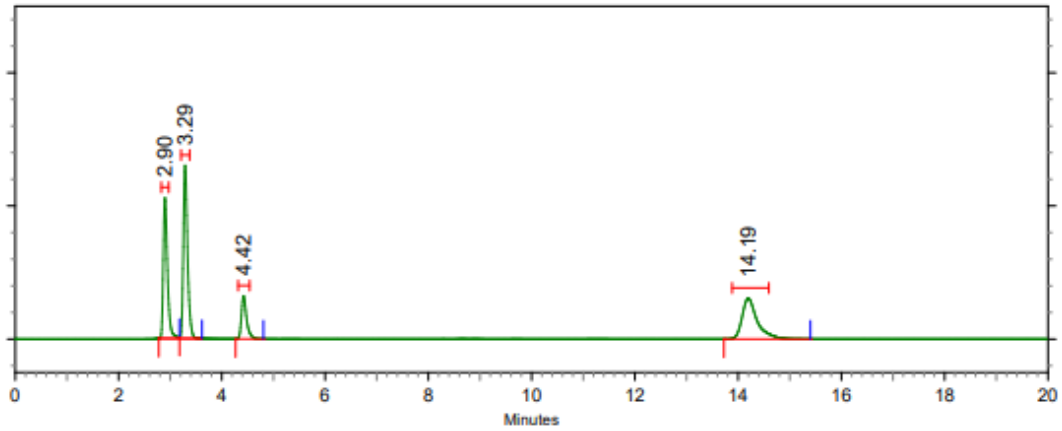

Figure13. Working Standard Chromatogram

Instrument Precision, Method Precision, Intermediate Precision and System suitability

The data given in Table 5 to 8 shows Instrument precision, method precision, and Intermediate precision for Acyclovir, Abacavir, Sofosbuvir and Lopinavir, respectively. This %RSD demonstrates that the approach is highly accurate and reliable when used by various analysts and for multiple sample preparations with the same concentration.

Table 5. Precision and system suitability for Acyclovir

Sample ID	Acyclovir System Suitability					Peak Area		
	RT	TP	Asymmetry	Peak Purity	Resolution	Instrument Precision	Method Precision	Intermediate Precision
Rep 1	2.90	6847	1.21	1.00	0.00	6193961	6194251	6186547
Rep 2	2.90	6754	1.22	1.00	0.00	6195478	6173524	6193648
Rep 3	2.90	6862	1.21	1.00	0.00	6196374	6186954	6263147
Rep 4	2.90	6965	1.22	1.00	0.00	6186358	6203251	6176487
Rep 5	2.90	6584	1.22	1.00	0.00	6186369	6203144	6188547
Rep 6	2.90	6931	1.23	1.00	0.00	6194287	6181241	6174214
Average	2.90					6192137.83	6190394.167	6197098.333
STDEV	0.00					4554.79	12024.55	33188.27
%RSD	0.00					0.07	0.19	0.54

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Table 6. Precision and system suitability for Abacavir

Sample ID	AbacavirSystem Suitability					Peak Area		
	RT	TP	Asymmetry	Peak Purity	Resolution	Instrument Precision	Method Precision	Intermediate Precision
Rep 1	3.29	8283	1.11	1.00	2.72	7734878	7731214	7743698
Rep 2	3.29	8168	1.10	1.00	2.71	7736984	7710321	7756142
Rep 3	3.29	8265	1.11	1.00	2.72	7726958	7723251	7712474
Rep 4	3.29	8214	1.13	1.00	2.72	7731584	7736954	7736214
Rep 5	3.29	8174	1.12	1.00	2.73	7739625	7647254	7689257
Rep 6	3.29	8298	1.09	1.00	2.71	7728657	7658474	7688001
Average	3.29					7733114.33	7701244.67	7720964.333
STDEV	0.00					4910.88	38689.27	28812.71
%RSD	0.00					0.06	0.50	0.37

Table 7.Precision and system suitability for Sofosbuvir

Sample ID	Sofosbuvir System Suitability					Peak Area		
	RT	TP	Asymmetry	Peak Purity	Resolution	Instrument Precision	Method Precision	Intermediate Precision
Rep 1	4.42	10274	1.05	1.00	7.11	2337632	2334815	2336547
Rep 2	4.42	10123	1.07	1.00	7.1	2336512	2313254	2336954
Rep 3	4.42	10254	1.06	1.00	7.11	2335478	2356924	2341254
Rep 4	4.42	10554	1.25	1.00	7.11	2331543	2274164	2347214
Rep 5	4.42	10568	1.05	1.00	7.1	2339845	2296587	2331254
Rep 6	4.42	10748	1.08	1.00	7.11	2333521	2326254	2331121
Average	4.42					2335755.17	2316999.667	2337390.67
STDEV	0.00					2954.07	29197.89	6152.94
%RSD	0.00					0.13	1.26	0.26

Table 8.Precision and system suitability for Lopinavir

Sample ID	Lopinavir System Suitability					Peak Area		
	RT	TP	Asymmetry	Peak Purity	Resolution	Instrument Precision	Method Precision	Intermediate Precision
Rep 1	14.19	12904	1.44	1.00	28.99	6498900	6497652	6483514
Rep 2	14.19	12658	1.41	1.00	29.00	6497524	6413574	6493274
Rep 3	14.19	12874	1.42	1.00	28.99	6492645	6378364	6412547
Rep 4	14.19	12896	1.45	1.00	28.99	6497826	6521471	6423574
Rep 5	14.19	12987	1.42	1.00	29.00	6497845	6376512	6436578
Rep 6	14.19	12857	1.44	1.00	29.01	6493652	6486254	6475241
Average	14.19					6496398.67	6445637.833	6454121.333
STDEV	0.00					2580.17	63934.43	34094.67
%RSD	0.00					0.04	0.99	0.53

Linearity

Linearity was performed at different levels. The graph plotted between peak area and concentration showed linearity with correlation coefficient as shown in table below. The linearity data in shown in table 9 and graph in figure 14.

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Table 9. Linearity data of Acyclovir, Abacavir, Sofosbuvir and Lopinavir

% Level	Conc (ug/ml)	Acyclovir Peak Area	Abacavir Peak Area	Sofosbuvir Peak Area	Lopinavir Peak Area
80	**40**	4915697	6181854	1865944	5192589
90	**45**	5547325	6955165	2100877	5846656
100	**50**	6193961	7734878	2337632	6498900
110	**55**	6825784	8516182	2572249	7149579
120	**60**	7467704	9290714	2808051	7797798
R^2		1	1	1	1

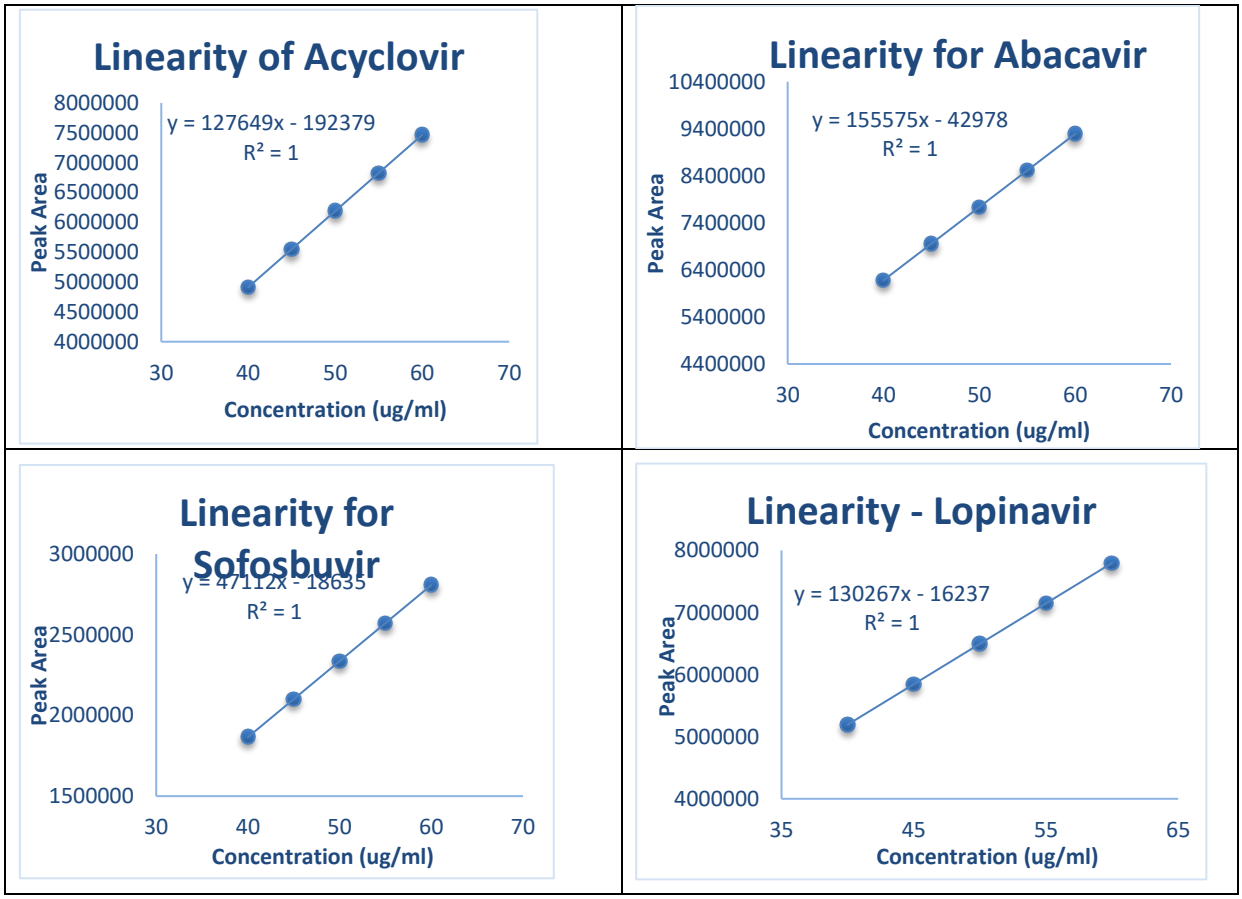
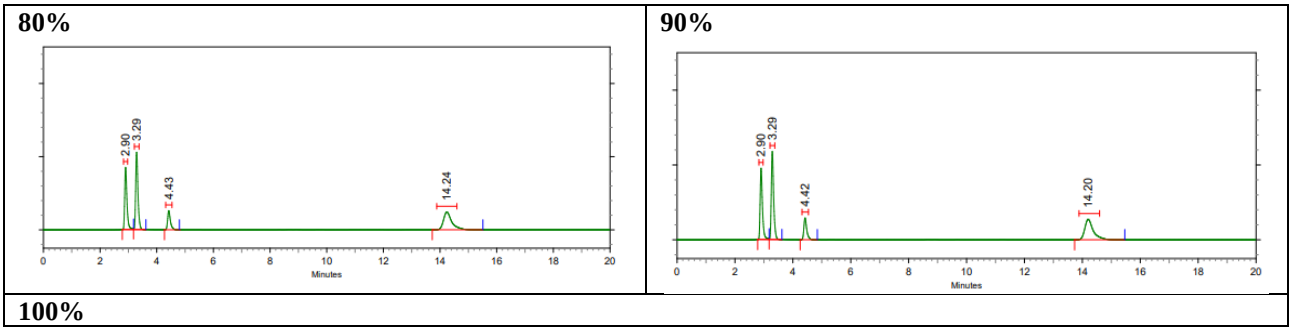


Figure 14: Linearity graph of Acyclovir, Abacavir, Sofosbuvir and Lopinavir

Linearity was performed for Acyclovir, Abacavir, Sofosbuvir and Lopinavir with concentration ranging from 40-60µg/ml, and the Correlation Coefficient was found to be 1 for all four APIs. The chromatograms for linearity are given in Figure 14.



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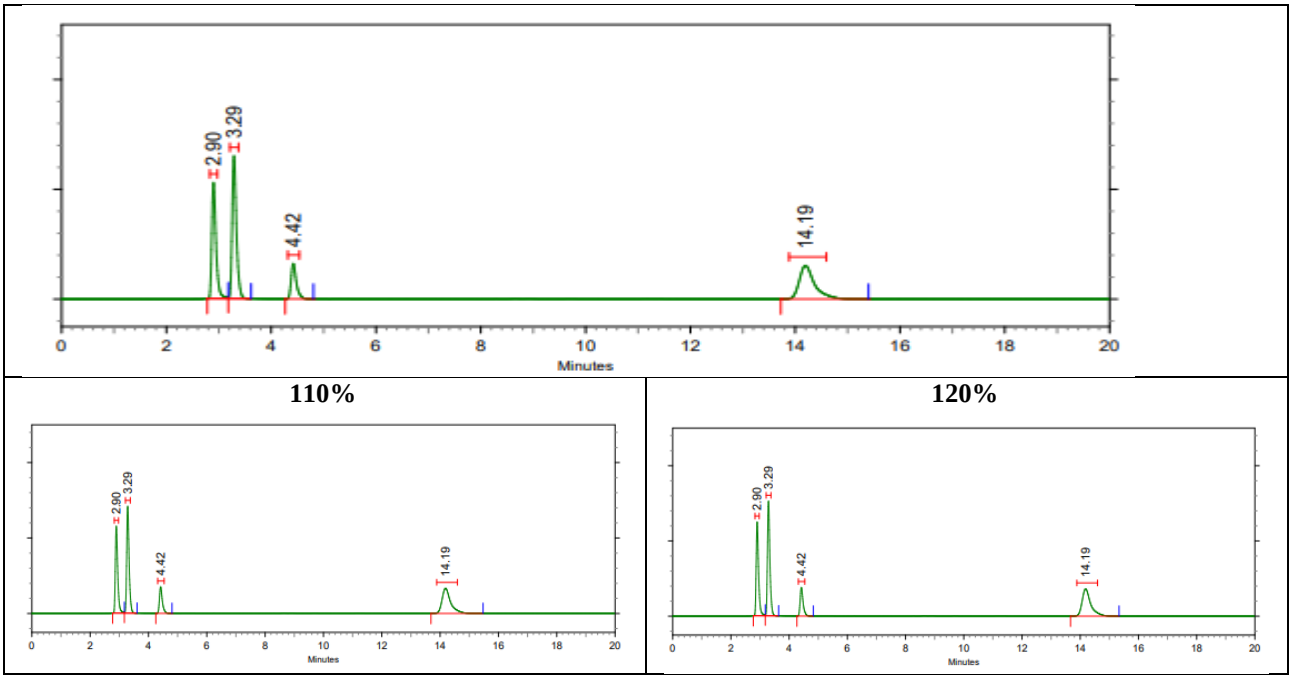

Figure 15.Linearity chromatograms of ACY, ABA, SOF & LPV

LOD and LOQ for ACY, ABA, SOF & LPV

The Limit of Detection (LOD) and Limit of Quantitation (LOQ) were determined for ACY, ABA, SOF & LPV. The results of analysis are shown in table 10.

Table 10.LOD and LOQ for Acyclovir, Abacavir, Sofosbuvir and Lopinavir

Name	LOD (µg/ml)	LOQ (µg/ml)
Acyclovir	0.33	1.00
Abacavir	0.17	0.51
Sofosbuvir	0.12	0.35
Lopinavir	0.17	0.50

The limits of detection (LOD) and quantification (LOQ) were markedly low, indicating that the approach is highly effective for quantifying low concentrations of the drug.

Accuracy

Accuracy for APIs were performed in triplicates and it was observed that the method was accurate for the range 80%, 100% and 120%. The accuracy determined is the methods ability to analyses different concentration of drug in solution accurately. The accuracy data is shown in table 11.

Table 11. Accuracy for Acyclovir, Abacavir, Sofosbuvir and Lopinavir

% Level	Reps	Acyclovir		Abacavir		Sofosbuvir		Lopinavir	
		% Recovery	%RSD	% Recovery	%RSD	% Recovery	%RSD	% Recovery	%RSD
80%	Rep 1	99.23	0.21	99.85	0.11	99.78	0.34	99.87	0.05
	Rep 2	99.57		100.04		100.21		99.81	
	Rep 3	99.19		100.03		99.53		99.77	
100%	Rep 1	100.03	0.04	99.95	0.12	99.92	0.31	100.15	0.09
	Rep 2	99.97		100.13		100.47		100.18	
	Rep 3	99.96		99.91		99.96		100.31	
120%	Rep 1	100.50	0.05	100.04	0.07	100.10	0.39	99.91	0.34
	Rep 2	100.39		100.01		100.52		100.05	
	Rep 3	100.45		100.14		100.88		100.57	

The average % recoveries of Acyclovir at 80%, 100% and 120% were found to be 99.33%, 99.99% and 100.45% respectively. The average % recoveries of Abacavir at 80%, 100% and 120% were found to be 99.97%, 100.00% and 100.06% respectively. The average % recoveries of Sofosbuvir at 80%, 100% and 120% were found to be 99.84%, 100.12% and 100.50% respectively and the average % recoveries of Lopinavir at 80%, 100% and 120% were found to be 99.82%, 100.21% and 100.18% respectively.

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Inter and Intraday Precision

Intra and inter day precision study was performed % RSD change in peak area of the APIs at different time points were recorded. The acceptance criteria is to have %RSD of peak area <2%. The Results are given in Table 12.

Table 12: Intra & Interday Precision of Acyclovir, Abacavir, Sofosbuvir and Lopinavir

Inter and Intraday precision			**Acyclovir**	**Abacavir**	**Sofosbuvir**	**Lopinavir**
Precision	**Interval**	**Sample ID**	**Peak Area**	**Peak Area**	**Peak Area**	**Peak Area**
Intraday	Morning	WS	6193648	7756142	2336954	6493274
	Evening	WS	6128298	7643920	2289109	6362928
Interday	Day 2	WS	6019827	7582981	2251829	6301928
Intraday - %RSD			**0.75**	**1.03**	**1.46**	**1.43**
Interday - %RSD			**1.44**	**1.15**	**1.86**	**1.53**

Robustness

Robustness is assessed to determine the extent to which the method deviates from its critical parameters. All over the world, the equipment is calibrated before use, but to know if the method is robust, changes were done in as shown in table 13 and 14.

Table 13: Robustness study - Change in Column oven temperature

Condition/ Column Temperature Change	**Sample ID**	**Acyclovir**	**Abacavir**	**Sofosbuvir**	**Lopinavir**
		Retention Time (min.)			
28℃	WS	2.90	3.29	4.42	14.19
30℃	WS	2.90	3.29	4.42	14.19
32℃	WS	2.90	3.29	4.42	14.19

Table 14: Robustness study – Change in Mobile phase A Strength

Condition/ Mobile Phase A Variation by 2%	**Acyclovir**		**Abacavir**		**Sofosbuvir**		**Lopinavir**			
	TP	**RT**	**TP**	**RES**	**TP**	**RES**	**TP**	**RES**	**ASY**	**RT**
MP A – Decrease	6741	2.88	8145	2.22	9897	5.38	12826	23.15	1.60	9.93
MP A – Normal	6601	2.91	7913	2.70	10207	7.80	14178	30.87	1.58	15.35
MP A - Increase	6573	2.91	7885	2.69	10067	8.06	12125	31.22	1.56	17.43

RT- Retention Time, TP – Theoretical Plates, RES – Resolution, ASY - Asymmetry

There is no significant effect found on Retention time of APIs with change in column oven temperature upto±2˚C.There is no significant effect found on Retention time and theoretical plates of APIs with change in strength of Mobile phase A upto ±2%. Hence, the method was found to be robust with a small change in column oven temperature and change in strength of Mobile phase A.

Conclusion

In this research article, a precise, accurate and eco-friendly method was developed for estimation of Acyclovir, Abacavir, Sofosbuvir and Lopinavir in bulk drugs and formulation by RP-HPLC technique. The developed method was successfully applied to marketed pharmaceutical dosage forms.

The developed method was optimized using AQbd approach and validated for accuracy, precision and robustness. The proposed methods were found to be appropriate due to its simplicity, reliability, sensitivity, rapidness and selectivity for detection at very low concentrations. Validation data demonstrates that, these methods are accurate, precise, simple and economic and can be used in the routine analysis of Acyclovir, Abacavir, Sofosbuvir and Lopinavir in various formulations.

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