SIMULTANEOUS RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR ACYCLOVIR AND DEXAMETHASONE

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Abstract

A precise and robust method was developed for simultaneous estimation of Acyclovir [ACY] and Dexamethasone [DEX]by RP-HPLC technique. The Method used Agilent 1260 Infinity II model HPLC with DAD detector and column of Agilent Zorbax Bonus RP with dimension 250 x 4.6 mm, 5 µm. The Mobile phase employed was 0.1% Trifluoroacetic acid and Methanol using a gradient elution program. Flow rate at 1.0 ml/min and wavelength at 324 nm with run time of 10 minutes. The retention time of Acyclovir and Dexamethasone peakswas at2.34 minutes and 5.01 minutes, respectively. The method was validated as per ICH guidelines. The instrument precision, Method precision and Intermediate precision had %RSD of 0.09%, 0.11% and 0.10% respectively for ACY and 0.01%, 0.02% and 0.10% respectively for DEX. Method was linear for concentration range 0.025µg/ml to 60µg/ml with r^2 of 0.9998for ACY and 0.025 µg/ml to 60 µg/ml with r^2 of 0.9997 for DEX and accurate at 80%, 100% and 120% with % RSD for ACY of 0.07%, 0.09% and 0.07% and for DEX of 0.08%, 0.02% and 0.01%, respectively. Acyclovir and Dexamethasonewere studied for stress stability and found that ACY was susceptible to acid Hydrolysis with 11.71% degradation and Dexamethasone degraded in photolytic condition with 11.37% degradation. The established method can be used in commercial sense as it is very linear and the LOD and LOQ for ACY are very low as 0.41µg/ml and 1.25µg/ml and for DEX as low as 0.44 µg/ml and 1.33 µg/ml. The method was found to be robustness and precise.

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1. Introduction

Acyclovir is a drug that is used to treat infections caused by specific viruses. Cold sores around the mouth (caused by herpes simplex), shingles (produced by herpes zoster), and chickenpox are all treated with this medication. This medicine is also used to treat genital herpes outbreaks. Acyclovir is intended to help persons with recurrent outbreaks minimize the frequency of future episodes. Acyclovir is a kind of antiviral medication. It is not, however, a treatment for these illnesses. [1]

Acyclovir is a synthetic purine nucleoside analogue with in vitro and in vivo inhibitory activity against herpes simplex virus types 1 (HSV-1), 2 (HSV-2), and varicella-zoster virus (VZV). [2]

The chemical name (IUPAC) of Acyclovir is 2-amino-9-(2-hydroxyethoxymethyl)-1*H*-purin-6-one(Figure 1).

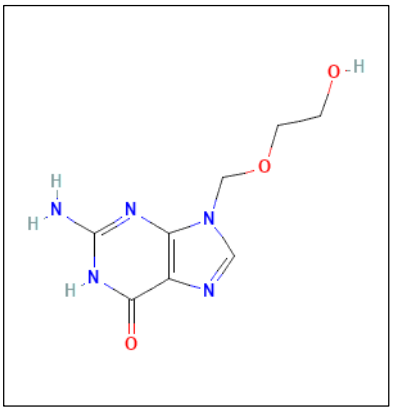


Figure 1: Chemical Structure of Acyclovir [3]

Dexamethasone belongs to the corticosteroid class of medicines. It reduces symptoms like edema and allergic responses by lowering your immune system's response to numerous infections. It is also used for treatment of Arthritis, blood/hormone issues, allergic responses, skin illnesses, eye difficulties, respiratory problems, intestinal disorders, cancer, and immune system disorders are all treated with dexamethasone. [4]

The chemical name (IUPAC) of Dexamethasone is (8S,9R,10S,11S,13S,14S,16R,17R)-9-fluoro-11,17-dihydroxy-17-(2-hydroxyacetyl)-10,13,16-trimethyl-6,7,8,11,12,14,15,16-octahydrocyclo-penta[a]phenanthren-3-one (Figure 2).

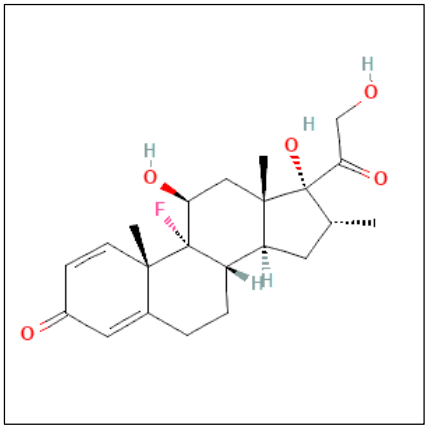


Figure 2: Chemical Structure of Dexamethasone [5]

According to the literature review [6-15], there are many Liquid Chromatography analyses for bulk and pharmaceutical dosage form has been reported for Acyclovir and Dexamethasone individually. But, current study was planned for development and validation of method developed for Acyclovir and Dexamethasone in combination was identified in a very short run time method.

Table No. 1: Quality Target Profile for Acyclovir and Dexamethasone for HPLC Method development.

Parameter	**Limits**
Theoretical Plates	Not less than 2000
Asymmetry	Not More than 2.0 (Fairly at 1.0)
Tailing Factor	Not More than 2.0 (Fairly at 1.0)
Run time	Not More than 20 minutes
Resolution	Not Less than 2.0

2. Material And Method

2.1. Chemicals and Reagents

Aadhaar Life Sciences Pvt. Ltd. provided a complimentary sample of Acyclovir (Purity ≥ 99.8%) and Dexamethasone (Purity ≥ 99.8%). Trifluoroacetic acid and methanol were purchased from Merck in India and was of HPLC grade. Internal Milli-Q system provided water was used. All weighing was done using calibrated NABL scales. Samples were produced using Type A glassware and the analytical balance.

2.2. Instrumentation

Agilent 1260 Infinity II with a DAD detector and quaternary pump was the tool utilized for development and validation. Agilent's openlabsEzChrom software was employed. The labmanultrasonicator and the Aczet analytical balance were used for wet chemistry.

2.3. HPLC Method Development

2.3.1. The table 2 describes trials done during the development phase.

Table No. 2. Method development

Trail No.	**Mobile Phase**	**Ratio**	**Diluent**	**Column**	**Wavelength**
1	MeOH : 0.1% TFA	50-50	50 0.1% TFA : 50 MeOH	Agilent Zorbax Bonus RP (250 x 4.6mm, 5µ)	250
2	MeOH : 0.1% TFA	60-40	50 0.1% TFA : 50 MeOH	Agilent Zorbax Bonus RP (250 x 4.6mm, 5µ)	250
3	MeOH : 0.1% TFA	G1	50 0.1% TFA : 50 MeOH	Agilent Zorbax Bonus RP (250 x 4.6mm, 5µ)	242
4	MeOH : 0.1% TFA	G2	50 0.1% TFA : 50 MeOH	Agilent Zorbax Bonus RP (250 x 4.6mm, 5µ)	242
5	MeOH : 0.1% TFA	G3	50 0.1% TFA : 50 MeOH	Agilent Zorbax Bonus RP (250 x 4.6mm, 5µ)	242

6	MeOH : 0.1% TFA	G4	50 0.1% TFA : 50 MeOH	Agilent Zorbax Bonus RP (250 x 4.6mm, 5µ)	242
7	MeOH : 0.1% TFA	G4	50 0.1% TFA : 50 MeOH - STK, 100% MeOH -WS	Agilent Zorbax Bonus RP (250 x 4.6mm, 5µ)	242
8	MeOH : 0.1% TFA	G5	50 0.1% TFA : 50 MeOH - STK, 100% MeOH -WS	Agilent Zorbax Bonus RP (250 x 4.6mm, 5µ)	242
9	MeOH : 0.1% TFA	G6	50 0.1% TFA : 50 MeOH - STK, 100% MeOH -WS	Agilent Zorbax Bonus RP (250 x 4.6mm, 5µ)	242
10	MeOH : 0.1% TFA	G6	50 0.1% TFA : 50 MeOH	Agilent Zorbax Bonus RP (250 x 4.6mm, 5µ)	242
11	**MeOH : 0.1% TFA**	**G7**	**50 0.1% TFA : 50 MeOH**	**Agilent Zorbax Bonus RP (250 x 4.6mm, 5µ)**	**242**

Table 3: Gradient programs

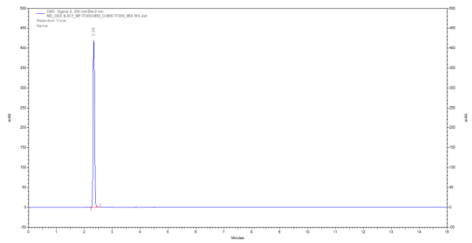
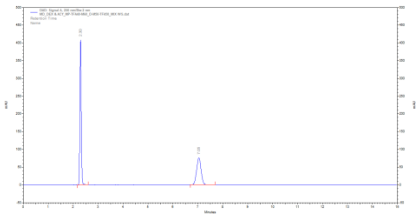
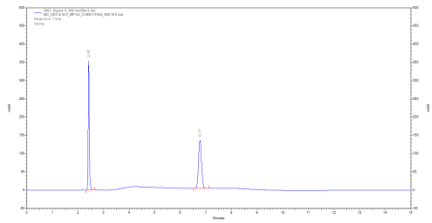
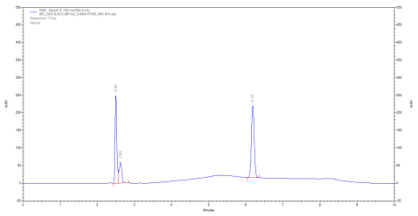
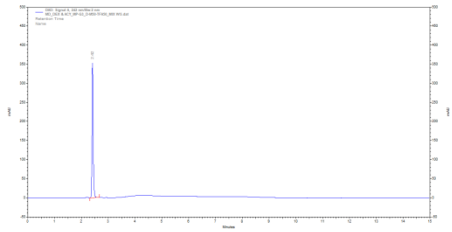
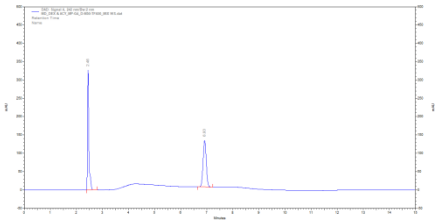
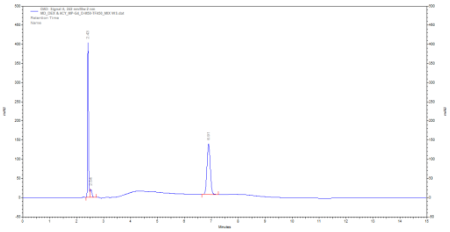
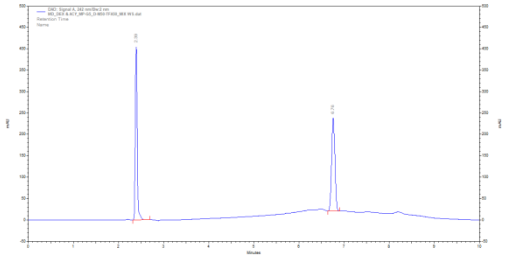
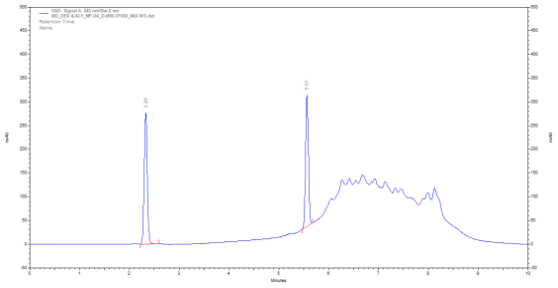
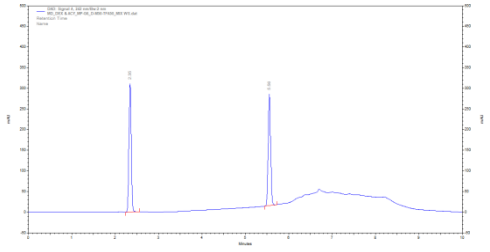
Gradient Trails	**Gradient Programs**		
G1	**Time (min.)**	**0.1% TFA (%)**	**Methanol (%)**
	0.00	60.0	40.0
	1.00	30.0	70.0
	5.00	30.0	70.0
	8.00	60.0	40.0
	15.00	60.0	40.0
G2	**Time (min.)**	**0.1% TFA (%)**	**Methanol (%)**
	0.00	70.0	30.0
	2.00	20.0	80.0
	5.00	20.0	80.0
	8.00	70.0	30.0
	15.00	70.0	30.0
G3	**Time (min.)**	**0.1% TFA (%)**	**Methanol (%)**
	0.00	60.0	40.0
	1.00	40.0	60.0
	5.00	40.0	60.0
	8.00	60.0	40.0
	15.00	60.0	40.0
G4	**Time (min.)**	**0.1% TFA (%)**	**Methanol (%)**
	0.00	65.0	35.0
	1.00	30.0	70.0
	5.00	30.0	70.0
	8.00	65.0	35.0
	15.00	65.0	35.0
G5	**Time (min.)**	**0.1% TFA (%)**	**Methanol (%)**
	0.00	60.0	40.0
	3.00	20.0	80.0

		5.00	20.0	80.0	
		8.00	60.0	40.0	
		15.00	60.0	40.0	
G6		**Time (min.)**	**0.1% TFA (%)**	**Methanol (%)**	
		0.00	50.0	50.0	
		3.00	0.0	100.0	
		5.00	0.0	100.0	
		8.00	50.0	50.0	
		15.00	50.0	50.0	
G7		**Time (min.)**	**0.1% TFA (%)**	**Methanol (%)**	
		0.00	50.0	50.0	
		2.00	0.0	100.0	
		4.00	0.0	100.0	
		4.01	50.0	50.0	
		10.00	50.0	50.0	

Table 4: Results for Method Development

Trail No.	**Acyclovir**					**Dexamethasone**				
	RT	**TP**	**Aysmmetry**	**Resolution**	**Peak Purity**	**RT**	**TP**	**Aysmmetry**	**Resolution**	**Peak Purity**
1	2.34	9050	1.10	0.00	1.00	-	-	-	-	-
2	2.30	7879	1.08	0.00	0.98	7.05	7211	1.02	21.79	1.00
3	2.42	13985	1.20	0.00	1.00	6.77	15483	1.01	29.06	0.55
4	Peak Split					6.19	31549	1.06	24.71	0.62
5	2.42	13888	1.21	0.00	0.98	-	-	-	-	-
6	Peak Split					6.93	14828	1.01	29.44	0.57
7	Peak Split					6.91	15052	1.00	21.40	0.53
8	2.39	15955	1.21	0.00	0.87	6.76	39766	1.07	41.31	0.73
9	2.33	6062	1.00	0.00	0.92	5.57	34470	1.03	28.68	1.00
10	2.35	9017	1.04	0.00	1.00	5.56	34657	1.00	31.49	1.00
11	**2.34**	**9176**	**1.10**	**0.00**	**1.00**	**5.01**	**41154**	**0.99**	**27.20**	**1.00**

For all the above trials, wavelength was kept constant at 242 nm, as this was predetermined using HPLC DAD detector. Diluent was as 50-50 0.1% Trifluoroacetic acid-Methanol for all trails. Column used for all trails was Agilent Zorbax Bonus RP (250 x 4.6 mm, 5 micron). Based in the predetermined quality target profile for development work, the condition for trial 11 was finalized.

Trail 1 	Trial 2 
Trial 3 	Trial 4 
Trial 5 	Trial 6 
Trial 7 	Trial 8 
Trial 9 	Trial 10 

Trial 11

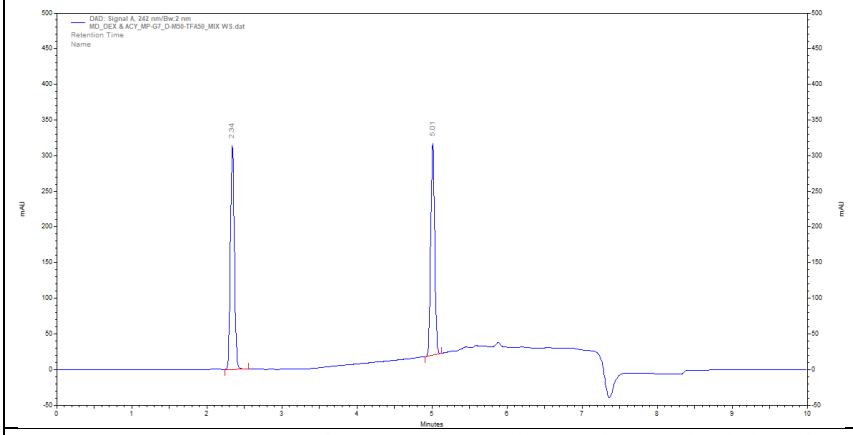


Figure No 3: Chromatogram shows the method development for all Trials

2.3.2. Final Chromatographic Conditions:

Table No. 5: Final Chromatographic Condition

Parameter	**Condition**
HPLC Instrument	Agilent 1260 Infinity II
Column	Agilent Zorbax Bonus RP, 5µ, 250 x 4.60 mm (Part #880668-901)
Wavelength	242 nm
Mobile Phase	0.1% Trifluoroacetic acid and Methanol
Elution Type	Gradient
Gradient Program	**Time (min.)** / **0.1% TFA (%)** / **Methanol (%)**: 0.00 / 50.0 / 50.0; 2.00 / 0.0 / 100.0; 4.00 / 0.0 / 100.0; 4.01 / 50.0 / 50.0; 10.00 / 50.0 / 50.0
Diluent	0.1% Trifluoroacetic acid and Methanol (50:50) v/v
Run time	10 minutes
Injection Volume	10 micro liters
Flow Rate	1.0 ml/min
Column oven Temperature	30°C

2.3.3. Preparation of 0.1% Trifluoroacetic acid:

Measure 1000 ml of purified water and add 1.0 ml of with Trifluoroacetic Acid. Mix and filter through 0.45 micron filter and sonicate to degas.

2.3.4. Preparation of Diluent

Mix separately measured 50 mL of Methanol with 50 mL of 0.1% Trifluoroacetic acid into a suitable container and mix well. Mixture is to be filtered through 0.45 µm nylon membrane filter. Briefly sonicate to degas.

2.3.5. Preparation of Standard Stock Solution

a. Preparation of Acyclovir Standard Stock Solution (SSS-I)

Weigh 5 mg of Acyclovir into 10 ml of volumetric flask dilute with diluent, vortex for 1 min. Sonicate for 5 minutes. Concentration of Acyclovir standard stock solution 500 µg/ml.

b. Preparation of Dexamethasone Standard Stock Solution (SSS-II)

Weigh 5 mg of Dexamethasone into 10 ml of volumetric flask dilute with diluent, vortex for 1 min. Sonicate for 5 minutes. Concentration of Dexamethasone standard stock solution 500 µg/ml.

c. Preparation of Working Standard

Transfer 1.0 mL of SSS-I and 1.0 mL of SSS-II using a pipette/micro pipette directly into a 10 mL volumetric flask. Make up to the volume with diluent. Vortex the flask with cap on for 1 minute to mix the contents. (Conc. Acyclovir & Dexamethasone be 50 µg/mL).

2.4. Method validation

2.4.1. Specificity

The test is termed specific if the analyte can be evaluated without interference any specific components like impurities, degradants, or excipients). It was validated by comparing the ACY & DEX chromatograms to a blank chromatogram.

2.4.2. System Suitability

Using a series of tests, the suitability and performance of the system were examined. Theoretical Plate count, tailing factor, and peak purity are all found to be within allowed ranges for the ICH guideline system.

2.4.3. Accuracy

To determine the accuracy of a technique, one must examine how closely its test findings correspond to the actual value. In the recovery studies, three distinct concentration levers were evaluated. At each level, three replicate injections were performed and the amount of drug present, the percentage of recovery, and the related standard deviation were calculated.

2.4.4. Precision

Analytical precision is determined by the degree of concordance between individual test results. Multiple samples of a uniform sample were examined. The repeatability, intra-day, and inter-day variations were utilized to assess the precision of the current method. This parameter was validated by analyzing samples collected at various times of day and on many days. Precision was performed as Instrument precision (how good the instrument preform back to back replicate injection of same concentration), Method Precision (one analyst inject 6 different samples with sample drug concentration and confirm the %RD), Intermediate precision (second analyst injects 6 samples with sample drug concentration and % RSD of total 12 injections is confirmed).

2.4.5. Linearity

Methodological linearity is the capacity of an analytical method to yield results proportionate to analyte concentrations within a given range. There were five sets of standard solutions used to determine linearity. On the calibration curve, the peak area against concentration of the standard solution was plotted, and the regression equation was developed. The least-squares method was utilized to determine the slope, intercept, and correlation coefficient.

2.4.6. Forced Degradation

Forced degradation studies were carried out to know the potential impurities in the drug under highly stressed condition described in table 6.

Table No. 6. Stress stability studies conditions.

Degradation Condition	**Strength and process**
Acid	1 N Hydrochloric Acid, 1.0 mL, for 10 minutes
Base	0.1 N Sodium Hydroxide, 1.0 ml, for 10 minutes
Peroxide	30% H2O2, 1.0 mL, for 60 minutes

UV	254 nm for 16 hour.
Dry heat	Thermal (110°C for 8 hours in oven)

2.4.7. LOD and LOQ

The LOD and LOQ are denoting ability of the method to detect and quantify smallest amount of analyte, respectively. The LOD and LOQ were calculated by using standard deviation and slope of regression line by using following equations.

2.4.8. Robustness

To check the method was robust enough in different condition, changes were made in column temperature and Mobile phase composition as below: Column temperature Decreased and Increase to 33ºC and 37ºC and its effects on the main peaks were studied. Flowrate by 20% and its effect on the main peaks were studied.

2.4.9. Solution Stability

The solution stability can be demonstrated by periodic analysis of the same working standard and shall be evaluated as per the procedure given. Perform System suitability according to the analytical method at 0 hours (the area of working standard injection from the system suitability is considered as stability T0). Store working standard solution in the original volumetric flask, parafilmed at room temperature to check solution stability at different time intervals. Inject the stored solution as a part of system suitability in a new sequence. To determine the working standard solution stability at different time interval, use peak area of working standard injected from initial time to the end of stability study and determine cumulative % RSD of working standard peak area.

3. Results And Discussion

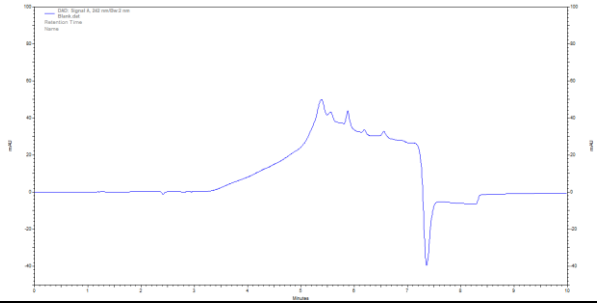
3.1. Specificity

The identification solutions were prepared as specified the protocol and injected. The retention time for each peak are shown in Table 7. The peaks due to Acyclovir & Dexamethasone are well separated from each other as well as any peaks due to diluent.There is no significant interference due to diluent at the RT of and Acyclovir & Dexamethasone peak.

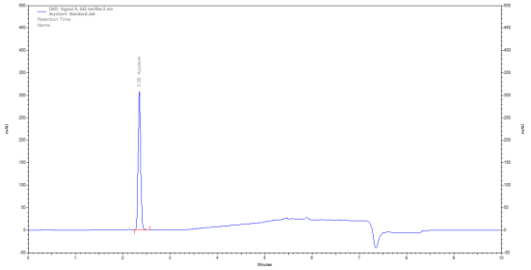
Table no. 7: Specificity and ID of API and its related substances

Solution	Retention Time (min) RT Acyclovir	Retention Time (min) RT Dexamethasone
Diluent		
ID_Acyclovir	2.34	
ID_Dexamethsone		5.01
Working Standard	2.34	5.01

a. Diluent



b. Acyclovir ID



c. Dexamethasone ID

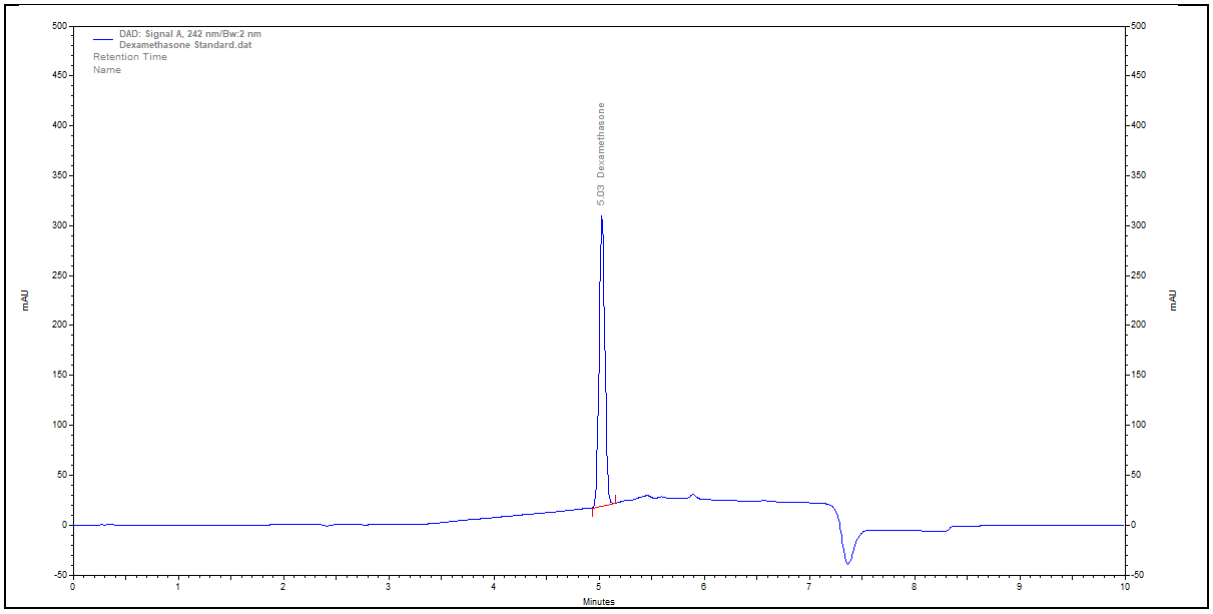


Figure No.4: Chromatogram ID. a]Diluent, b] Acyclovir ID, c] Dexamethasone ID

3.2. Instrument Precision, Method Precision, Intermediate Precision and System suitability

The maximum tailing of peak among all standard injections is 1.06 for Acyclovir and 1.10 for Dexamethasone.The minimum number of theoretical plates among all working standard injections for Acyclovir is 8754 & Dexamethasone peak is 38841. %RSD for the peak area is 0.09% for Acyclovir & 0.01% for Dexamethasone peak from 6 injections of working standard. The data demonstrate that the instrument precision is established. The relative standard deviation for Method Precision of Area of six sample preparations is 0.11% for Acyclovir & 0.02% for Dexamethasone. The relative standard deviation for intermediate precision of Area from six sample preparations is 0.10% for Acyclovir and 0.10% for Dexamethasone.The absolute difference of % RSD obtained in method precision and intermediate precision is 0.01% for Acyclovir & 0.08% for Dexamethasone.

Table 8: Instrument Precision, Method Precision, Intermediate Precision and System suitability for Acyclovir

System Suitability - Acyclovir				**Peak Area**		
Reps	**RT**	**Theoretical Plates**	**Asymmetry**	**Instrument Precision**	**Method Precision**	**Intermediate Precision**
Rep 1	2.35	8861	1.06	2415631	2401475	2417741
Rep 2	2.35	8754	1.05	2415874	2408741	2418147
Rep 3	2.35	8867	1.01	2412147	2408774	2413674
Rep 4	2.35	8891	1.03	2415576	2405747	2412144
Rep 5	2.35	9105	1.05	2417789	2405687	2413598

Rep 6	2.35	8845	1.05	2418746	2405671	2413247
Average	2.35			2415961	2406016	2414759
%RSD	0.00			0.09	0.11	0.10

Table 9: Instrument Precision, Method Precision, Intermediate Precision and System suitability for Dexamethasone

System Suitability - Acyclovir					**Peak Area**		
Reps	**RT**	**Theoretical Plates**	**Asymmetry**	**Resolution**	**Instrument Precision**	**Method Precision**	**Intermediate Precision**
Rep 1	5.03	38846	1.05	26.57	2299076	22890357	2290478
Rep 2	5.03	38872	1.06	26.57	2299874	22899741	2294741
Rep 3	5.03	38841	1.10	26.57	2299476	22892547	2295582
Rep 4	5.03	38876	1.09	26.57	2299987	22897416	2292268
Rep 5	5.03	38844	1.05	26.57	2299876	22891477	2296789
Rep 6	5.03	38856	1.01	26.57	2299774	22895741	2292441
Average	5.03				2299677	22894547	2293717
%RSD	0.00				0.01	0.02	0.10

3.3. Linearity of Acyclovir and Dexamethasone

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 is shown in table 10 and 11and graphs in figure 5 and 6 for Acyclovir and Dexamethasone respectively.

Table 10: Linearity Results (Acyclovir)

% Level	Conc (ug/ml)	Area
0.05	0.025	2805
0.1	0.05	5034
0.5	0.25	21304
1	0.5	43085
2.5	1.25	98205
5	2.5	177257
10	5	242359
20	10	484489
40	20	966708
60	30	1447890
80	40	1934624
100	50	2415631
120	60	2892076

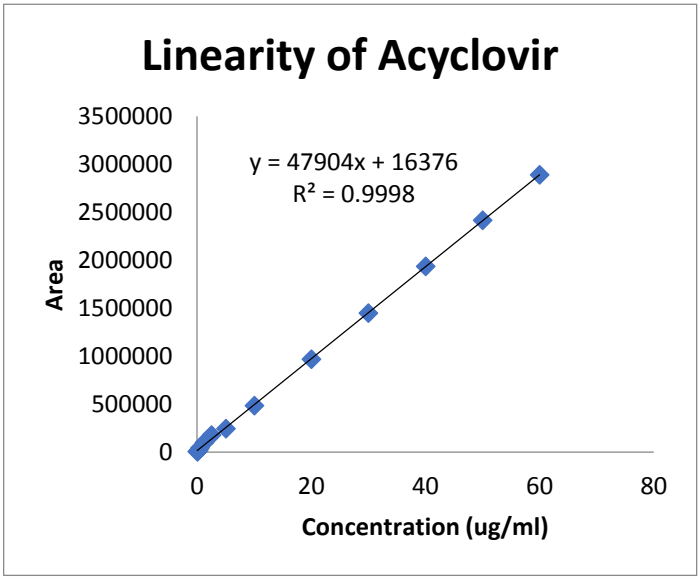


Figure No. 5 Linearity of Acyclovir

Table 11: Linearity Results (Dexamethasone)

% Level	Conc (ug/ml)	Area
0.05	0.025	1034
0.1	0.05	3227
0.5	0.25	14103
1	0.5	33262
2.5	1.25	83962
5	2.5	169462
10	5	223722
20	10	455379
40	20	915335
60	30	1374109
80	40	1837430
100	50	2299076
120	60	2760763

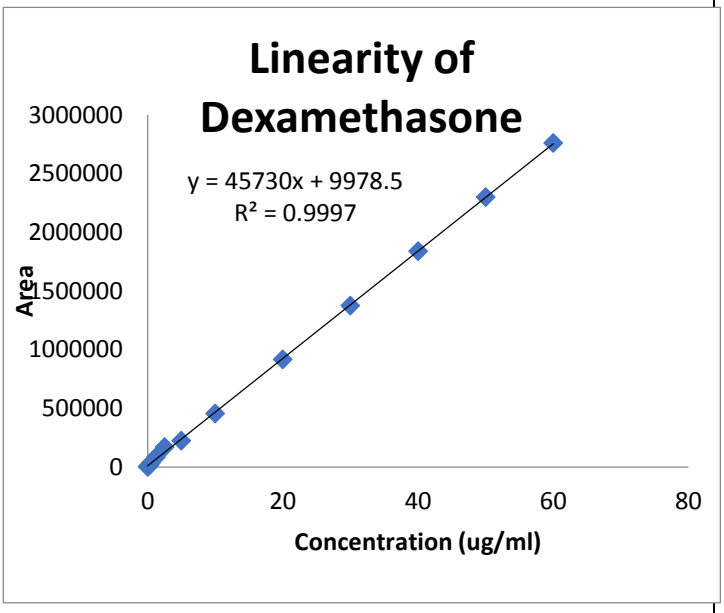


Figure No. 6.: Linearity of Dexamethasone

3.4. LOD and LOQ for Acyclovir and Dexamethasone

The Limit of Detection (LOD) and Limit of Quantitation (LOQ) were determined for ACY & DEX. The results of analysis are shown in table 12.

Table No. 12. LOD and LOQ for Acyclovir and Dexamethasone

	Acyclovir		**Dexamethasone**	
LOD	0.41	ug/ml	0.44	ug/ml
LOQ	1.25	ug/ml	1.33	ug/ml

The data demonstrate that the Accuracy, Linearity, LOD and LOQ are established for Acyclovir & Dexamethasone. The method is linear and accurate in above ranges.

3.4. Accuracy

Accuracy was performed in triplicates and it was observed that the method was accurate for the range 80%, 100% and 120% for ACY and DEX. The relative standard deviation for 80%, 100% and 120% were 0.07%, 0.09% and 0.07% respectively for ACY. The relative standard deviation for 80%, 100% and 120% were 0.08%, 0.02% and 0.01% respectively for DEX. The accuracy determined the methods ability to analyses different concentration of drug in solution accurately. The accuracy data is shown in table 13 and 14.

Table 13: Accuracy results (Acyclovir)

Sample ID	**Reps**	**Spiked Conc (ug/ml)**	**Area**	**Amount Recovered (ug/ml)**	**% Recovery**	**RSD**
80%	Rep 1	40.00	1934624	40.04	100.10	0.07
	Rep 2	40.00	1937451	40.10	100.24	
	Rep 3	40.00	1935715	40.06	100.15	
100%	Rep 1	50.00	2415631	49.99	99.99	0.09
	Rep 2	50.00	2415874	50.00	100.00	
	Rep 3	50.00	2412147	49.92	99.84	
120%	Rep 1	60.00	2892076	59.85	99.76	0.07
	Rep 2	60.00	2891774	59.85	99.75	
	Rep 3	60.00	2895563	59.93	99.88	

Table 14: Accuracy results (Dexamethasone)

Sample ID	**Reps**	**Spiked Conc (ug/ml)**	**Area**	**Amount Recovered (ug/ml)**	**% Recovery**	**RSD**
80%	Rep 1	40.00	1837430	39.95	99.87	0.08
	Rep 2	40.00	1839570	40.00	99.99	
	Rep 3	40.00	1840133	40.01	100.02	
100%	Rep 1	50.00	2299076	49.99	99.97	0.02
	Rep 2	50.00	2299874	50.00	100.01	
	Rep 3	50.00	2299476	50.00	99.99	
120%	Rep 1	60.00	2760763	60.03	100.04	0.01
	Rep 2	60.00	2760541	60.02	100.03	
	Rep 3	60.00	2760258	60.01	100.02	

3.5. Solution Stability

To know the long the prepared solution is stable, the working standard were analyzed at different time point and it was confirmed that it was stable for upto 2 days. This was proved by analyzing the same at 5 different time points. Stability data is shown in table 15.

Table No. 15. Solution stability of ACY & DEX

Days	Sample ID	Acyclovir				Dexamethasone			
		Area	% Assay	% RSD	Cumm % RSD	Area	% Assay	% RSD	Cumm % RSD
Control	WS	2415961	100.00	-	-	2299677	100.00	-	-
Day 0	WS	2410569	99.78	0.16	-	2298471	99.95	0.04	-
Day 1	WS	2418741	100.12	0.24	0.40	2259713	98.26	1.20	1.24
Day 2	WS	2399488	99.32	0.57	0.96	2248697	97.78	0.35	1.59
Day 3	WS	2356703	97.55	1.27	2.23	2187740	95.13	1.94	3.53
Day 4	WS	2347811	97.18	0.27	2.50	2157411	93.81	0.99	4.52

The cumulative RSD after 2 days keep the volumetric flask at room temperature and dry place away from light was 0.96% for ACY and 1.59 for DEX. The specification limits are of 2% and therefore, the working standard is stable for atleast2days.

3.6. Robustness

Robustness is done to check how deviating the method is with respect to 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 column temperature and Mobile phase as shown in table 13. All the runs were done in triplicates for working standard and system suitability data is shown in table 13.

Table 16: Change in Flowrate Acyclovir

Flowrate					
Condition	**Area**	**RT**	**TP**	**Asymmetry**	**Resolution**
Increase (1.2 ml/min)	2006205	1.95	7352	1.07	0.00
Normal (1 ml/min)	2415631	2.35	8861	1.06	0.00
Decrease (0.8 ml/min)	3027579	2.95	11442	1.04	0.00

Table 17: Change in Column Oven Temperature Acyclovir

Column Oven Temperature					
Condition	**Area**	**RT**	**RP**	**Asymmetry**	**Resolution**
Increase (32℃)	2417884	2.35	8547	1.03	0.00
Normal (30℃)	2415631	2.35	8861	1.06	0.00
Decrease (28℃)	2426874	2.35	8541	1.04	0.00

Table 18: Change in Flowrate Dexamethasone

Flowrate					
Condition	**Area**	**RT**	**TP**	**Asymmetry**	**Resolution**
Increase (1.2 ml/min)	1826771	4.36	35861	1.07	26.27
Normal (1 ml/min)	2299076	5.03	38846	1.05	26.57
Decrease (0.8 ml/min)	2825966	5.99	34889	1.07	25.54

Table 19: Change in Column Oven Temperature Dexamethasone

Column Oven Temperature					
Condition	**Area**	**RT**	**RP**	**Asymmetry**	**Resolution**
Increase (32℃)	2265742	5.03	34785	1.05	26.57
Normal (30℃)	2299076	5.03	38846	1.05	26.57
Decrease (28℃)	2293374	5.03	37540	1.02	26.57

There was no change in theoretical plate, asymmetry and peak purity with respect to change in column temperature and Flow rate.

3.8. Forced degradation

Stress degradation carried out showed very high degradation in photolytic condition with upto 11.37% for DEX and 39.43% in acidic condition for ACY. There was no significant degradant peak observed in the current method.

Table No. 20: Forced Degradation Assay results for Acyclovir

Sample Name	**Degradation Condition**	**% Assay**	**Peak Purity**	**% Degradation**
Control	NA	100.00	1.000	NA
Acid	1 N Hydrochloric Acid, 1.0 mL, @ Room Temperature for 10 minutes	60.57	0.998	11.71
Base	1 N Sodium Hydroxide, 1.0 mL, @ Room Temperature for 10 minutes	98.08	0.996	1.92
Peroxide	30% H_2O_2, 1.0 mL, @ Room Temperature for 60 minutes	99.89	0.992	0.11
UV	254 nm for 16 hours	99.84	0.990	0.16
Dry Heat	Thermal (110°C for 8 hours in an oven)	99.49	1.000	0.51

Table No. 21: Forced Degradation Assay results for Dexamethasone

Sample Name	**Degradation Condition**	**% Assay**	**Peak Purity**	**% Degradation**
Control	NA	100.00	1.000	NA
Acid	1 N Hydrochloric Acid, 1.0 mL, @ Room Temperature for 10 minutes	97.56	0.998	2.44
Base	1 N Sodium Hydroxide, 1.0 mL, @ Room Temperature for 10 minutes	94.15	0.996	5.85
Peroxide	30% H_2O_2, 1.0 mL, @ Room Temperature for 60 minutes	98.13	0.992	1.87
UV	254 nm for 16 hours	88.63	0.990	11.37
Dry Heat	Thermal (110°C for 8 hours in an oven)	99.43	1.000	0.57

Degradation chromatograms as below

Acid Degradation	**Photolytic degradation**

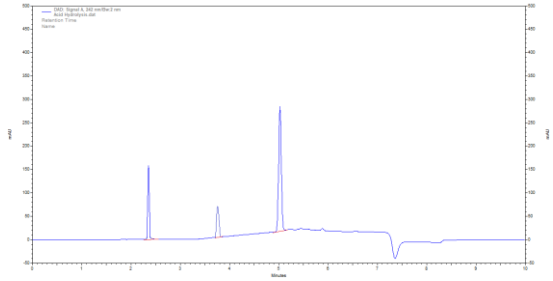
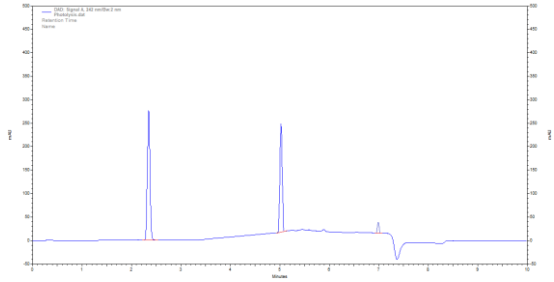

	

Figure No. 7.: Acid Stress Stability Chromatogram	**Figure No. 8: Photolytic Stress Stability Chromatogram**

4. Conclusion

In this research article, a precise and accurate method was developed based on method developed technique for Simultaneous estimation of Acyclovir and Dexamethasone in by RP-HPLC technique. The developed method was validated for accuracy, precision and robustness. Forced degradation data reveled that Acyclovir is significantly susceptible to acid hydrolysis and Dexamethasone is susceptible to photolytic degradation. There was more than 12% degradation found for 2 conditions i.e. Acid and Photolytic condition. Therefore, it is necessary to keep the bulk drug and formulation away from light and at room temperature. The current research more is much better than published articles with respect to detection limit, quantification limit and analysis time.

5. References

1. https://dailymed.nlm.nih.gov/dailymed/drugInfo.cfm?setid=d13b8cdd-59fd-472b-8125-a19f42ef5402
2. O'Brien JJ, Campoli-Richards DM: Acyclovir. An updated review of its antiviral activity, pharmacokinetic properties and therapeutic efficacy. Drugs. 1989 Mar;37(3):233-309.
3. National Center for Biotechnology Information (2023). PubChem Compound Summary for CID 135398513, Acyclovir. Retrieved May 28, 2023 from https://pubchem.ncbi.nlm.nih.gov/compound/Acyclovir.
4. https://dailymed.nlm.nih.gov/dailymed/drugInfo.cfm?setid=0277cc0a-2fd4-4605-a310-b613be84ee26##
5. National Center for Biotechnology Information (2023). PubChem Compound Summary for CID 5743, Dexamethasone. Retrieved May 28, 2023 from https://pubchem.ncbi.nlm.nih.gov/compound/Dexamethasone.
6. Zendelovska D, Simeska S, Atanasovska E, Georgievska K, Kikerkov I, Labachevski N, Jakovski K, Balkanov T. Determination of Acyclovir in Human Plasma Samples by HPLC Method with UV Detection: Application to Single-Dose Pharmacokinetic Study. Open Access Maced J Med Sci. 2015 Mar 15;3(1):32-6. doi: 10.3889/oamjms.2015.011.
7. Suchita V. Ghumre ,Varsha M. Jadhav and Vilasrao J. Kadam (2016). High Pressure Liquid Chromatographic Method Development and Validation for Estimation of Acyclovir In Bulk and Marketed Formulation.*International Journal of Pharmaceutical Sciences and Research.* Vol. 7(5): 2194-2200.
8. Ravi Sankar, A. Niharika, S. Siressha and OsKoushik (2015). Development and validation of RP-HPLC method for quantitative estimation of acyclovir in bulk drug and tablets. *Journal of*

Chemical and Pharmaceutical Sciences. 88(1):73-80

9. GunasekarManoharan and Ramadan Abdel Wahed Mohamed(2019).Development and Validation of a Stability-Indicating RP-HPLC Method for the Estimation of Aciclovir, in Bulk and Ointment Dosage Form. *International Journal of Pharmaceutical and PhytopharmacologicalResearch.*9(1):11-18.
10. Ch. VENKATA KUMAR, D. ANANTHAKUMAR and J. V. L. N. SESHAGIRI RAO. Development And Validation of RP- HPLC Method for the Determination of Acyclovir In Human Plasma. Int. J. Chem. Sci.: 8(1), 2010, 475-482.
11. Heda AA, Kathiriya JM, Gadade DD, Puranik PK. Development and Validation of RP-HPLC Method for Simultaneous Determination of Granisetron and Dexamethasone. Indian J Pharm Sci. 2011 Nov;73(6):696-9. doi: 10.4103/0250-474X.100255. PMID: 23112409; PMCID: PMC3480760.
12. AlAani H, Alnukkary Y. Stability-Indicating HPLC Method for Simultaneous Determination of Chloramphenicol, Dexamethasone Sodium Phosphate and Tetrahydrozoline Hydrochloride in Ophthalmic Solution. Adv Pharm Bull. 2016 Mar;6(1):137-41. doi: 10.15171/apb.2016.020. Epub 2016 Mar 17. PMID: 27123429; PMCID: PMC4845542.
13. Maria Cristina CocenzaUrban ,Rubiana Mara Mainardes, Maria Palmira DaflonGremiao(2009). Development and validation of HPLC method for analysis of dexamethasone acetate in microemulsions. *Brazilian Journal of Pharmaceutical Sciences 45(1).*
14. Fu-chaoChen,Lin-hai Wang,Jun Guo,Xiao-ya Shi and Bao-xia Fang (2017).Simultaneous Determination of Dexamethasone, Ondansetron, Granisetron, Tropisetron, and Azasetron in Infusion Samples by HPLC with DAD Detection. *Journal of Analytical Methods in Chemistry*. Article ID 6749087, 7 pages https://doi.org/10.1155/2017/6749087.
15. Iqbal, M.S., Shad, M.A., Ashraf, M.W. *et al.* Development and Validation of an HPLC Method for the Determination of Dexamethasone, Dexamethasone Sodium Phosphate and Chloramphenicol in Presence of Each Other in Pharmaceutical Preparations. *Chroma* **64**, 219–222 (2006). https://doi.org/10.1365/s10337-006-0019-3.