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Research

Method Development and Validation by RP-HPLC For Estimation of Acyclovir and Hydrocortisone in Bulk and Pharmaceutical Dosage Form

Gajula Karishma Begum*, Dr. V. Jagadeesh Babu, Dr. D. Varun

Department of Pharmaceutical Analysis, Sri Indu Institute of Pharmacy, Sheriguda (V), Ibrahimpatnam, Telangana, 501510

*Author for Correspondence: Gajula Karishma Begum
Email: Gajulakarishmabegum@gmail.com

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Abstract

A rapid and reliable Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) method was developed and validated for the simultaneous estimation of Acyclovir and Hydrocortisone in bulk and pharmaceutical dosage forms. Chromatographic separation was achieved using a Waters HPLC system equipped with an auto sampler and PDA detector (Model 996), employing a Gemini C18 column (4.6 × 150 mm, 5 µm) maintained at 30°C. The mobile phase consisted of Acetonitrile and Water in a 50:50 v/v ratio, with a flow rate of 1.0 mL/min. Detection was carried out at 238 nm, with an injection volume of 10 µL and a total run time of 8 minutes. The method was validated as per ICH guidelines, demonstrating excellent linearity, precision, accuracy, specificity, and robustness. The retention times of both drugs were distinct and interference-free. This method is efficient, economical, and well-suited for routine quality control and analysis of Acyclovir and Hydrocortisone in pharmaceutical preparations.

Keywords: RP-HPLC, Acyclovir, Hydrocortisone, Gemini C18 column, simultaneous estimation, validation.

1. INTRODUCTION

Analysis may be defined as the science and art of determining the composition of materials in terms of the elements or compounds contained in them. In fact, analytical chemistry is the science of chemical identification and determination of the composition (atomic, molecular) of substances, materials and their chemical structure.

Chemical compounds and metallic ions are the basic building blocks of all biological structures and processes which are the basis of life. Some of these naturally occurring compounds and ions (endogenous species) are present only in very small amounts in specific regions of the body, while others such as peptides, proteins, carbohydrates, lipids and nucleic acids are found in all parts of the body. The main object of analytical chemistry is to develop scientifically substantiated methods that allow the qualitative and quantitative evaluation of materials with certain accuracy. Analytical chemistry derives its principles from various branches of science like chemistry, physics, microbiology, nuclear science and electronics. This method provides information about the relative amount of one or more of these components.[1]

Every country has legislation on bulk drugs and their pharmaceutical formulations that sets standards and obligatory quality indices for them. These regulations are presented in separate articles relating to individual drugs and are published in the form of book called "Pharmacopoeia" (e.g. IP, USP, and BP). Quantitative chemical analysis is an important tool to assure that the raw material used and the intermediate products meet the required specifications. Every year number of drugs is introduced into the market. Also quality is important in every product or service, but it is vital in medicines as it involves life.

There is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, report of new toxicities and development of patient resistance and introduction of better drugs by the competitors. Under these conditions standard and analytical procedures for these drugs may not be available in Pharmacopoeias. In instrumental analysis, a physical property of the substance is measured to determine its chemical composition. Pharmaceutical analysis comprises those procedures necessary to determine the identity, strength, quality and purity of substances of therapeutic importance. [2]

Pharmaceutical analysis deals not only with medicaments (drugs and their formulations) but also with their precursors i.e. with the raw material on which degree of purity and quality of medicament depends. The quality of the drug is determined after establishing its authenticity by testing its purity and the quality of pure substance in the drug and its formulations.

Quality control is a concept which strives to produce a perfect product by series of measures designed to prevent and eliminate errors at different stages of production. The decision to release or reject a product is based on one or more type of control action. With the growth of pharmaceutical industry during last several years, there has been rapid progress in the field of pharmaceutical analysis involving complex instrumentation. Providing simple analytical procedure for complex formulation is a matter of most importance. So, it becomes necessary to develop new analytical methods for such drugs. In brief the reasons for the development of newer methods of drugs analysis are:

1. The drug or drug combination may not be official in any pharmacopoeias.
2. A proper analytical procedure for the drug may not be available in the literature due to Patent regulations.
3. Analytical methods for a drug in combination with other drugs may not be available.
4. Analytical methods for the quantitation of the drug in biological fluids may not be available.
5. The existing analytical procedures may require expensive reagents and solvents. It may also involve cumbersome extraction and separation procedures and these may not be reliable. [1, 2]

1.2 INTRODUCTION TO HPLC

HPLC is also called as high pressure liquid chromatography since high pressure is used to increase the flow rate and efficient separation by forcing the mobile phase through at much higher rate. The pressure is applied using a pumping system. The development of HPLC from classical column chromatography can be attributed to the development of smaller particle sizes. Smaller particle size is important since they offer more surface area over the conventional large particle sizes. The HPLC is the method of choice in the field of analytical chemistry, since this method is specific, robust, linear, precise and accurate and the limit of detection is low and also it offers the following advantages.

1. Improved resolution of separated substances
2. column packing with very small (3,5 and 10 µm) particles
3. Faster separation times (minutes)
4. Sensitivity
5. Reproducibility
6. continuous flow detectors capable of handling small flow rates
7. Easy sample recovery, handling and maintenance. [6]

1.3 INSTRUMENTATION OF HPLC

The basic liquid chromatograph consists of six basic units. The mobile phase supply system, the pump and programmer, the sample valve, the column, the detector and finally a means of presenting and processing the results.

1.3.1 Mobile phase (solvent) reservoirs and solvent degassing

The mobile phase supply system consists of number of reservoirs (200 mL to 1,000 mL in capacity). They are usually constructed of glass or stainless steel materials which are chemically resistant to mobile phase**.**

Mobile phase

Mobile phases in HPLC are usually mixtures of two or more individual solvents. The usual approach is to choose what appears to be the most appropriate column, and then to design a mobile phase that will optimize the retention and selectivity of the system. The two most critical parameters for nonionic mobile phases are strength and selectivity. [8,24]

Mobile phase preparation

Mobile phases must be prepared from high purity solvents, including water that must be highly purified. Mobile phases must be filtered through ≤1 μm pore size filters and be degassed before use.

Degassing of solvents

Many solvents and solvent mixtures (particularly aqueous mixtures) contain significant amounts of dissolved nitrogen and oxygen from the air. These gasses can form bubbles in the chromatographic system that cause both serious detector noise and loss of column efficiency. These dissolved gases in solvent can be removed by the process of degassing. Every solvent must be degassed before introduction into pump as it alter the resolution of column and interfere with monitoring of the column effluent.

Degassing is done in many ways:

- By warming the solvents
- By stirring vigorously with a magnetic stirrer
- By subjecting to vaccum filtration
- By ultra sonication (using ultrasonicator)
- By bubbling He gas through the solvent reservoir. [8]

EXPERIMENTAL METHODS

INSTRUMENTS USED

HPLC	WATERS Alliance 2695 separation module, Software: Empower 2, 996 PDA detector.
pH meter	Lab India
Weighing machine	Sartorius
Volumetric flasks	Borosil
Pipettes and Burettes	Borosil
Beakers	Borosil

CHEMICALS USED

Acyclovir	Provided by Sura Pharma labs
Hydrocortisone	Provided by Sura Pharma labs
Water and Methanol for HPLC	LICHROSOLV (MERCK)
Acetonitrile for HPLC	Merck

HPLC METHOD DEVELOPMENT

TRAILS

Preparation of standard solution

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

Further pipette 0.2ml of the Acyclovir and o.4ml of the Hydrocortisone 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.

RESULTS AND DISCUSSIONS

Optimized Chromatogram (Standard)

Mobile phase ratio	:	Acetonitrile: Water (50:50v/v)
Temperature	:	30ºC
Column	:	Gemini C18 (4.6×150mm, 5μ)

Flow rate	:	1ml/min
Wavelength	:	238 nm
Injection volume	:	10 µl
Run time	:	8 min

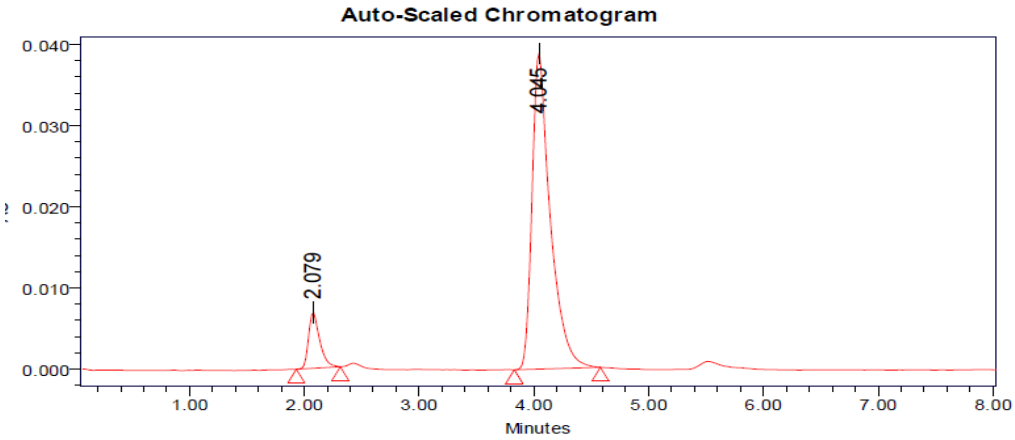

Fig 1: Optimized Chromatogram (Standard)

Table 1: Optimized Chromatogram (Standard)

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Acyclovir	2.079	46168	6841	1.33	4251
2	Hydrocortisone	4.045	429069	38885	1.59	5224

Optimized Chromatogram (Sample)

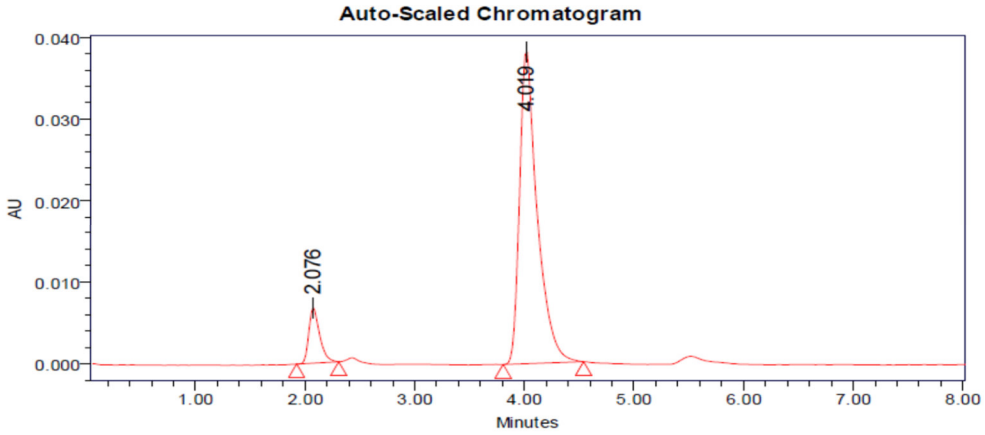

Fig 2: Optimized Chromatogram (Sample)

Table 2: Optimized Chromatogram (Sample)

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Acyclovir	2.076	46150	6766	1.36	5152
2	Hydrocortisone	4.019	427826	38246	1.58	6071

Acceptance Criteria

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

VALIDATION

Blank

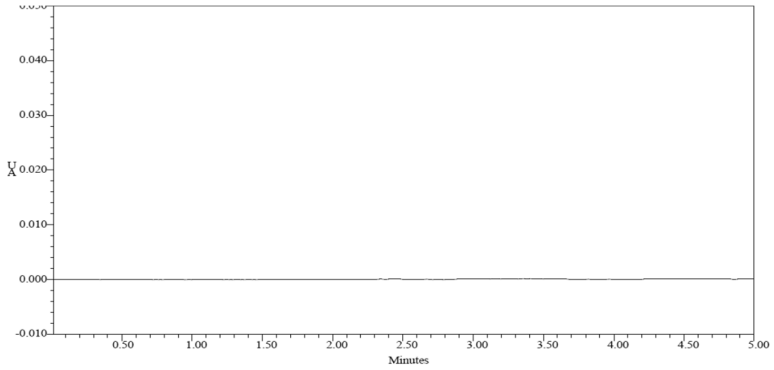

Fig 3: Chromatogram showing blank (mobile phase preparation)

SPECIFICITY

The ICH documents define specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components.

Assay (Standard)

Table 3: Peak results for assay standard of Acyclovir

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Acyclovir	2.078	49569	6811	6945	1.51
2	Acyclovir	2.080	49649	6999	6149	1.57
3	Acyclovir	2.078	49731	6972	6473	1.49
4	Acyclovir	2.079	49479	6971	6190	1.49
5	Acyclovir	2.082	49684	6841	6294	1.49
Mean			49607			
Std. Dev.			107.963			
% RSD			0.217637			

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 4: Peak results for assay standard of Hydrocortisone

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Hydrocortisone	4.041	423328	44147	7672	1.35
2	Hydrocortisone	4.033	423805	44538	7786	1.13
3	Hydrocortisone	4.050	423229	44964	5772	1.34
4	Hydrocortisone	4.045	423876	44959	5191	1.35
5	Hydrocortisone	4.032	423575	38885	5137	1.35
Mean			423559.5			
Std. Dev.			328.2606			
% RSD			0.0775			

Acceptance Criteria:

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

Assay (Sample)

Table 5: Peak results for Assay sample of Acyclovir

S.No.	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Acyclovir	2.078	46684	6918	1.34	5217	1
2	Acyclovir	2.079	46168	6841	1.33	5251	2
3	Acyclovir	2.077	46088	6851	1.37	7127	3

Table 6: Peak results for Assay sample of Hydrocortisone

S.No.	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Hydrocortisone	4.050	430575	39127	1.60	6197
2	Hydrocortisone	4.045	429069	38885	1.59	6224
3	Hydrocortisone	4.037	429543	38892	1.58	8203

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

The % purity of Acyclovir and Hydrocortisone in pharmaceutical dosage form was found to be 98.2%

LINEARITY

Table 7: Chromatographic Data For Linearity Study For Acyclovir

Concentration	Average
20	15065
40	31009
60	46166
80	60569
100	76862

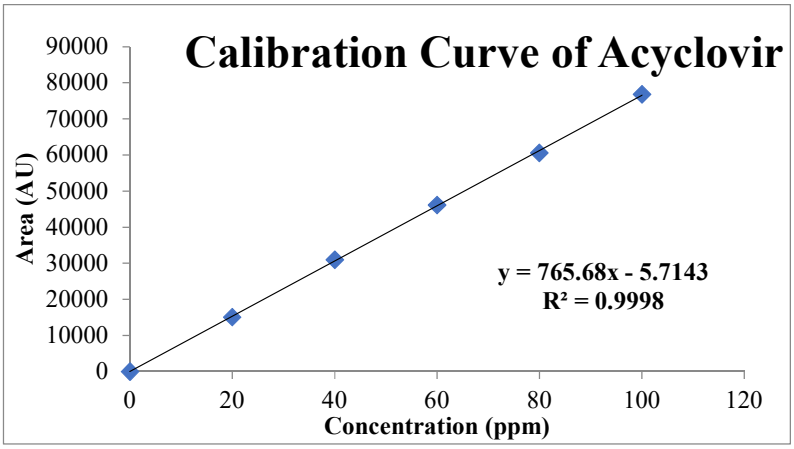

Fig 4: Chromatogram showing linearity level

LINEARITY PLOT

CONCLUSION: Correlation Coefficient (r) is 0.99, and the intercept is 5.7143. These values meet the validation criteria.

Table 8: Chromatographic Data for Linearity Study for Hydrocortisone

Concentration	Average
40	131289
80	284775
120	427559
160	555861
200	712514

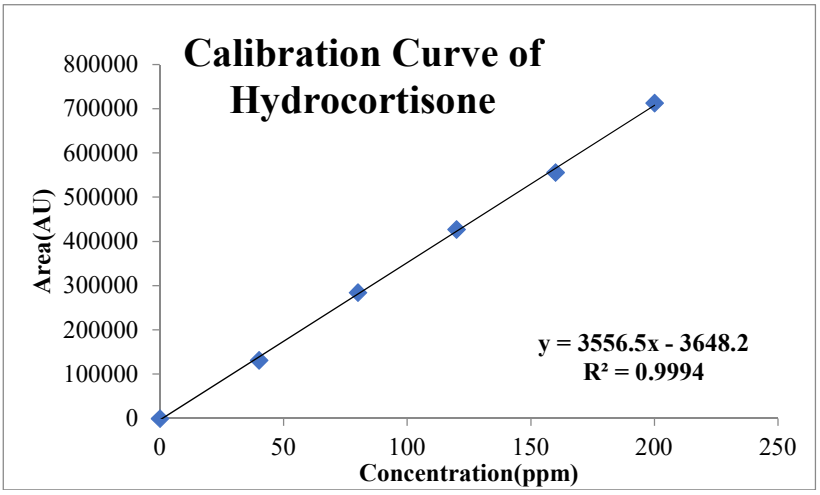

Fig 5: Chromatogram showing linearity level

LINEARITY PLOT

CONCLUSION: Correlation Coefficient (r) is 0.99, and the intercept is 3648. These values meet the validation criteria.

Precision: The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

REPEATABILITY

Obtained Five (5) replicates of 100% accuracy solution as per experimental conditions. Recorded the peak areas and calculated % RSD.

Table 9: Results of repeatability for Acyclovir

S. No.	Peak name	Retention time	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Acyclovir	2.077	46054	6784	4208	1.32
2	Acyclovir	2.076	46803	6867	6088	1.34
3	Acyclovir	2.076	46150	6766	4152	1.36
4	Acyclovir	2.077	46056	6715	4184	1.32
5	Acyclovir	2.074	46247	6746	4065	1.33
Mean			46262			
Std.dev			312.7099			
%RSD			0.675954			

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 10: Results of repeatability for Hydrocortisone

S. No	Peak name	Retention time	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Hydrocortisone	4.031	427962	38634	5158	1.57
2	Hydrocortisone	4.024	429623	38673	5092	1.58
3	Hydrocortisone	4.019	427826	38246	5071	1.58
4	Hydrocortisone	4.016	427829	38310	5046	1.58
5	Hydrocortisone	4.014	429559	38181	5036	1.58
Mean			428559.8			
Std.dev			943.2246			
%RSD			0.220092			

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.

Intermediate precision
Day 1:

Table 11: Results of Intermediate precision day1 for Acyclovir

S.No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Acyclovir	2.075	46204	6673	5117	1.33
2	Acyclovir	2.074	46300	6735	5043	1.36
3	Acyclovir	2.075	46259	6652	5087	1.28
4	Acyclovir	2.075	46223	6667	5134	1.31
5	Acyclovir	2.075	46205	6674	5151	1.32
6	Acyclovir	2.074	46189	6703	5157	1.33
Mean			46230			
Std. Dev.			41.88556			
% RSD			0.090603			

Acceptance criteria:
%RSD of Six different sample solutions should not more than 2.

Table 12: Results of Intermediate precision day1 for Hydrocortisone

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Hydrocortisone	4.013	428922	38004	7038	1.58
2	Hydrocortisone	4.011	428524	37935	7999	1.57
3	Hydrocortisone	4.010	427239	37850	7003	1.57
4	Hydrocortisone	4.008	427667	37780	7982	1.57
5	Hydrocortisone	4.006	427826	37824	7983	1.57
6	Hydrocortisone	4.006	427093	37970	7042	1.58
Mean			427878.5			
Std. Dev.			718.1952			
% RSD			0.16785			

Acceptance criteria:

- %RSD of Six different sample solutions should not more than

Day 2:

Table 13: Results of Intermediate precision Day 2 for Acyclovir

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Acyclovir	2.076	46803	6867	5149	1.57
2	Acyclovir	2.076	46056	6715	5190	1.13
3	Acyclovir	2.077	46252	6652	6088	1.58
4	Acyclovir	2.075	46205	6674	5184	1.58
5	Acyclovir	2.075	46940	7249	5087	1.57
6	Acyclovir	2.072	46727	6983	5151	1.57
Mean			46497.17			
Std. Dev.			369.4739			
% RSD			0.794616			

Acceptance criteria:
%RSD of Six different sample solutions should not more than 2 Table:

Table 14: Results of Intermediate precision Day 2 for Hydrocortisone

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Hydrocortisone	4.024	429623	38673	6789	1.49
2	Hydrocortisone	4.024	427829	38310	5772	1.34
3	Hydrocortisone	4.016	427263	37850	5092	1.32
4	Hydrocortisone	4.010	427826	37824	6046	1.28
5	Hydrocortisone	4.006	421284	40752	6003	1.32
6	Hydrocortisone	4.008	421832	40281	6983	1.33
Mean			425942.8			
Std. Dev.			3492.681			
% RSD			0.819988			

Acceptance criteria:

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

ACCURACY
Accuracy at different concentrations (50%, 100%, and 150%) was prepared and the % recovery was calculated.

Table 15: The accuracy results for Acyclovir

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	22938.33	30	29.9655	99.88	100.166
100%	45426	60	59.33511	98.89	
150%	70096.67	90	91.55572	101.7285	

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.

Table 16: The accuracy results for Hydrocortisone

%Concentration	Area	Amount	Amount	% Recovery	Mean
50%	209357	60	59.8	99%	99%
100%	420697.7	120	119.8	99%	
150%	631550.7	180	179.8	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. Hence method is accurate.

LIMIT OF DETECTION FOR ACYCLOVIR AND HYDROCORTISONE

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

LOD= 3.3 × σ / s

where

σ = Standard deviation of the response

S = Slope of the calibration curve

Result:

Acyclovir

=2.2µg/ml

Hydrocortisone

=7.3µg/ml

Quantitation limit

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined.

LOQ=10×σ/S

Where

σ = Standard deviation of the response

S = Slope of the calibration curve

Result:

Acyclovir

=6.8µg/ml

Hydrocortisone

=22.2µg/ml

Robustness

Table 17: Results for Robustness -Acyclovir

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 0.9mL/min	46168	2.079	4251	1.33
Less Flow rate of 0.8mL/min	51177	2.29	5269	1.38
More Flow rate of 1.0mL/min	42190	1.890	5126	1.32
Less organic phase	42402	1.885	5126	1.19
More organic phase	42112	1.908	5854	1.36

Acceptance criteria:

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

Table 18: Results for Robustness-Hydrocortisone

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 0.9mL/min	429069	4.045	5224	1.59
Less Flow rate of 0.8mL/min	472673	4.450	6328	1.58

More Flow rate of 1.0mL/min	392497	3.660	6217	1.54
Less organic phase	391379	4.251	6996	1.61
More organic phase	391703	3.239	6120	1.50

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

SUMMARY AND CONCLUSION

A simple, precise, and reliable Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) method was developed and validated for the simultaneous estimation of Acyclovir and Hydrocortisone in bulk and pharmaceutical dosage forms. The method employed a Waters HPLC system with an auto sampler and PDA detector (model 996), using a Gemini C18 column (4.6 × 150 mm, 5 µm) maintained at 30°C. The mobile phase consisted of Acetonitrile and Water in a 50:50 v/v ratio, delivered at a flow rate of 1.0 mL/min. Detection was carried out at 238 nm with an injection volume of 10 µL, and the total run time was 8 minutes. The method was validated according to ICH guidelines and demonstrated excellent linearity, accuracy, precision, specificity, and robustness. The retention times for both Acyclovir and Hydrocortisone were well-resolved, with no interference from excipients or degradation products. In conclusion, the developed RP-HPLC method is accurate, reproducible, and efficient, making it highly suitable for routine quality control and analysis in pharmaceutical formulations.

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