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RP-HPLC Method Development and Validation for Simultaneous Estimation of Hydrocortisone and Acyclovir in Pharmaceutical dosage forms.

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ABSTRACT

The Chromatographic condition were successfully developed for the separation of Acyclovir and Hydrocortisone by using Agilent C_8 column (25 cm x 4.6 mm i.d., 5 µ). A simple, selective, rapid, precise and economical reverse phase high performance liquid chromatographic method has been developed for the simultaneous estimation of Hydrocortisone and Acyclovir from pharmaceutical formulation. The method was carried out on a C_8 (25 cm x 4.6 mm i.d., 5 µ) column with a mobile phase consisting of Methanol: water (adjusted to pH 3.0 using o-phosphoric acid) in the ratio of 80:20 v/v. The retention time of Hydricortusone and Acyclovir was 3.50 min and 6.00 min respectively with the flow rate of 1mL/ min. Eluents were detected at 254 nm. The linear regression analysis data for the linearity plot showed good linear relationship with correlation coefficient value for Hydrocortisone and Acyclovir were R^2=0.9995 and R^2=0.9996 in the concentration range of 10-40µg. mL^{-1} , 20-80 µg. mL^{-1} respectively. The relative standard deviation for intra-day precision was lower than 2.0 %. The method was validated according to the ICH guidelines. The method was also found to be robust. The developed method was validated in terms of accuracy, precision, linearity, limit of detection, limit of quantification and solution stability.

Keywords: RP-HPLC, Hydrocortisone, Acyclovir, Agilent , Mobile Phase, Retention time.

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INTRODUCTION

Hydrocortisone(HYDRO) is chemically (1S,2R10S,11S,14R,15S,17S)-14,17-dihyroxy-14-(2-hydroxyacetyl)-2,15-dimethyl tetracyclo[8.7.0.0^{2,7}heptadec-6-en-5-one. HYDRO belongs to Anti-inflammatory Agents. Structure of HYDRO was shown in figure 1[1].

Acyclovir is chemically 2-amino- (hydroxyethoxy)methy6,9-dihydro-3H-purin-6-one. It is used as Antiviral agents .Structure of ACYCLO was shown in figure 2[2].

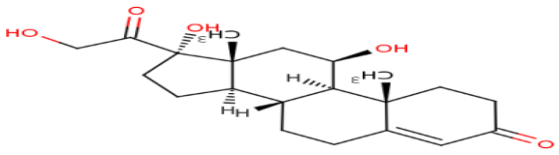

Figure 1: Hydrocortisone

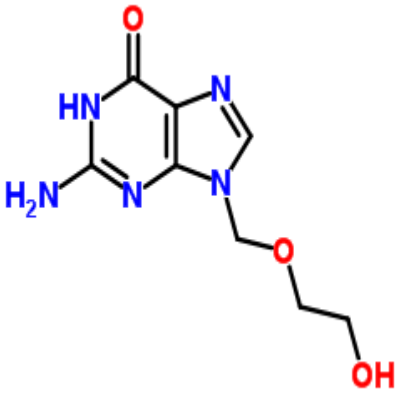

Figure 2: Acyclovir

The review of literature revealed that various analytical methods involving spectrophotometry TLC, HPLC, HPTLC have been reported for HYDRO in single form and in combination with other drugs. Several analytical methods have been reported for ACYCLO in single form and in combination with other drugs including spectrophotometry HPLC, HPTLC, LC – MS. To date, there have been no published reports about the simultaneous estimation of Hydrocortisone and Acyclovir by HPLC in pharmaceutical dosage forms. This present study reports for the first time simultaneous estimation of Hydrocortisone and Acyclovir by HPLC in pharmaceutical dosage form. The proposed method is validated as per ICH guidelines.

Experimental

MATERIALS AND REAGENTS

Analytically pure HYDRO was kindly provided by Hetero Laboratory, and ACYCLO was provided by Mylan Laboratory, as gift samples. Analytical grade methanol was purchased from Merck & Co. Glasswares used in each procedure were soaked over night in a mixture of chromic acid and sulphuric acid rinsed thoroughly with double distilled water and dried in hot air oven. Water (HPLC grade) were purchased from Merck, India. Triple distilled water is used for all purpose.

Instrumentation

HPLC system (Agilent HPLC Model-1100 with Ezchromelite Software) containing C_8 (Qualisil BDS, 25 x 4.6 mm, 5µ) column with UV- VWD detection. LABINDIA-3000$^+$ UV-Visible double beam spectrophotometer with a fixed slit width 1nm and 1cm matched quartz cells was used for all the spectral measurements.

RESULTS AND DISCUSSION

Method

Optimization of the chromatographic conditions

The mobile phase consisted of methanol and water in ratio Methanol: Water (80:20), pH was adjusted to3.0 with o-phosphoric acid to water. The contents of the mobile phase were filtered before use through a 0.45µ membrane and degassed for 10 min. The mobile phase was pumped from the solvent reservoir to the column at a flow rate of 1.0 ml/min and the injection volume was 20 µL. The column temperature was maintained at ambient temperature. The eluents were monitored at 254 nm. The results of the optimized chromatogram was shown in Figure and Table1.

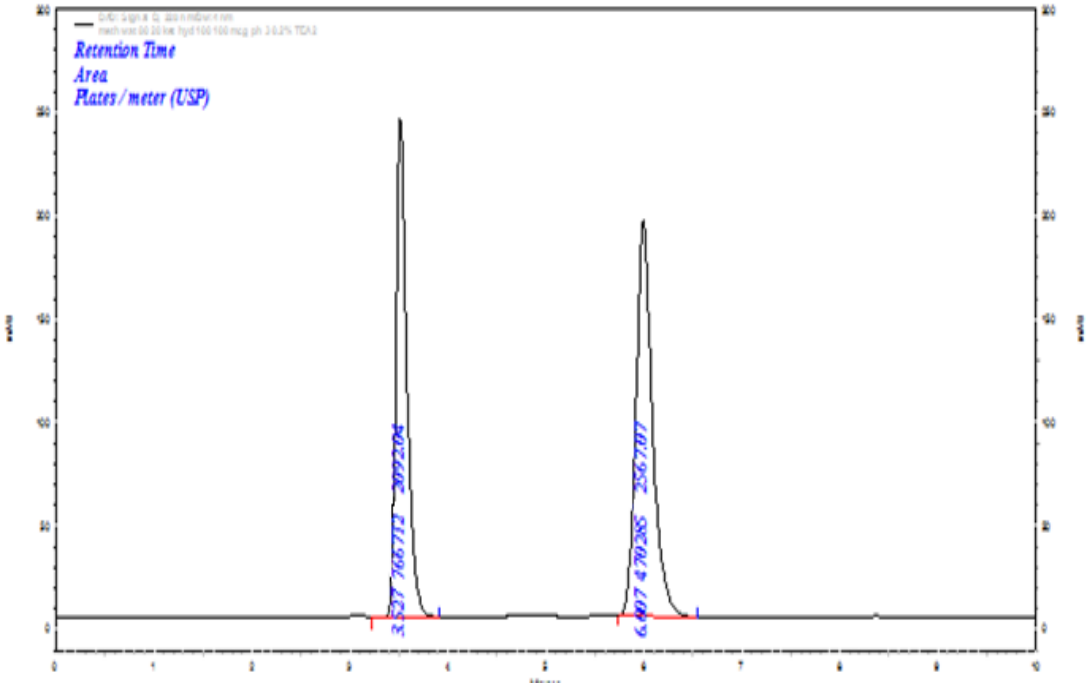

Figure 3: optimized chromatogram of Hydrocortisone and Acyclovir

Preparation of standard stock solutions

Accurately weighed 10 mg of HYDRO and ACYCLO standard were transferred to separate 10 ml volumetric flask and dissolved in 10 ml methanol. The flasks were shaken and volume was made up to the mark with methanol to give solutions containing 1000 µg/ml HYDRO and 1000 µg/ml ACYCLO. From this solution 1ml was transferred to volumetric flask of 100 ml capacity. Volume was made up to the mark to give a solution containing 100µg/ml of HYDRO and 100µg/ml ACYCLO.

Calibration of standards

The standard calibration curve was constructed for Hydrocortisone and Acyclovir. Different volumes of stock solutions of each were accurately transferred in to 10mL volumetric flasks and diluted to mark to yield a concentration range of 10-40 µg/ml solutions of Hydrocortisone and 20-80µg/ml solutions of Acyclovir. The calibration line was obtained by plotting the peak area against concentration of drug.

Determination of Hydrocortisone and Acyclovir in their Combined Dosage

Sample preparation

A powder quantity equivalent to 10 mg HYDRO and 20 mg ACYCLO was accurately weighed and transferred to volumetric flask of 100 ml capacity, methanol was transferred to this volumetric flask and sonicated for 15 min. The flask was shaken and volume was made up to the

mark with methanol. The above solution was filtered through Whatmann filter paper (0.45µ). From this solution 2 ml was transferred to volumetric flask of 100 ml capacity. Volume was made up to the mark to give a solution containing 10µg/ml of HYDRO and 20µg/ml of ACYCLO. The resulting solution was analyzed by proposed method. The quantification was carried out by keeping these values to the straight line equation of calibration curve. The results were shown in Table IV and Figure.

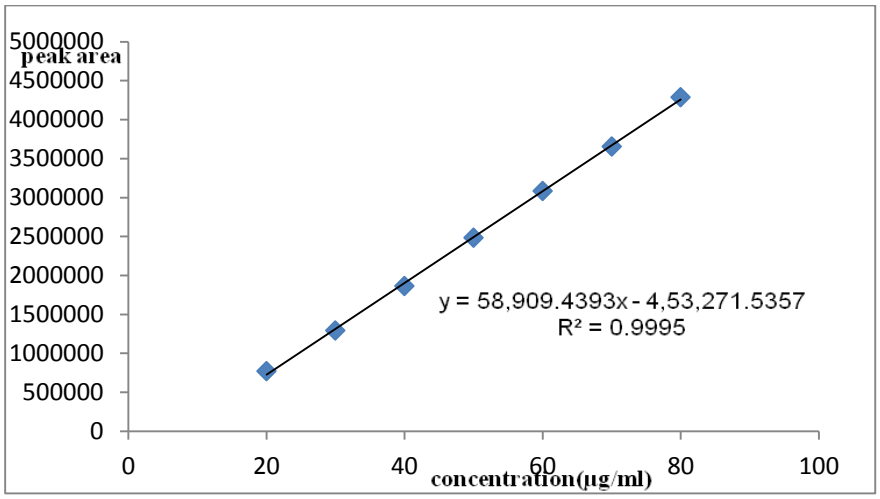

Figure 4: Linearity of Acyclovir at 254nm

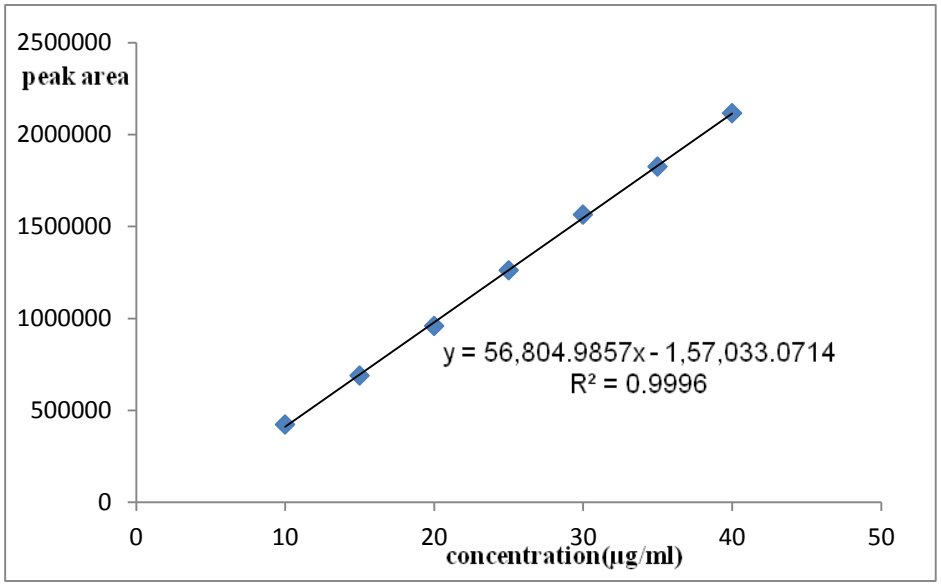

Figure 5: Linearity of Hydrocortisone at 254nm

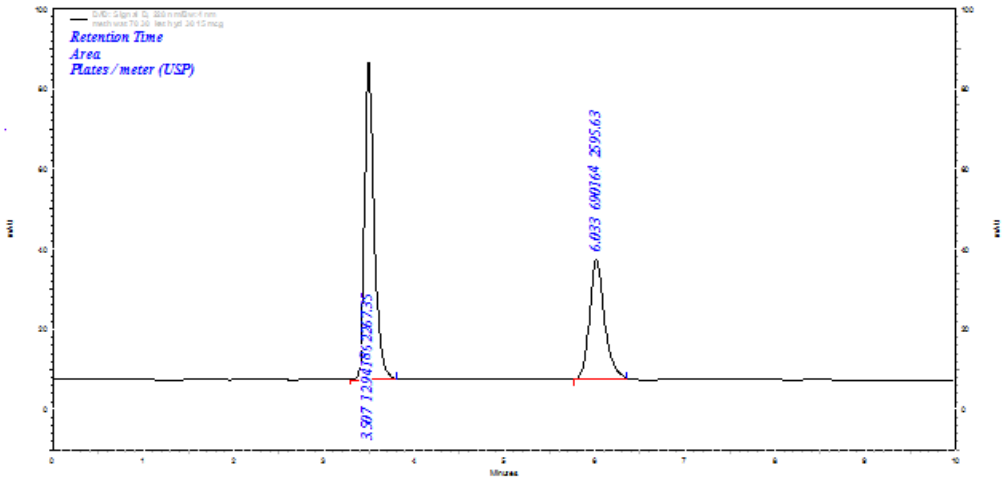

Figure 6: Linearity chromatogram of 15 Hydrocortisone 30 Acyclovir

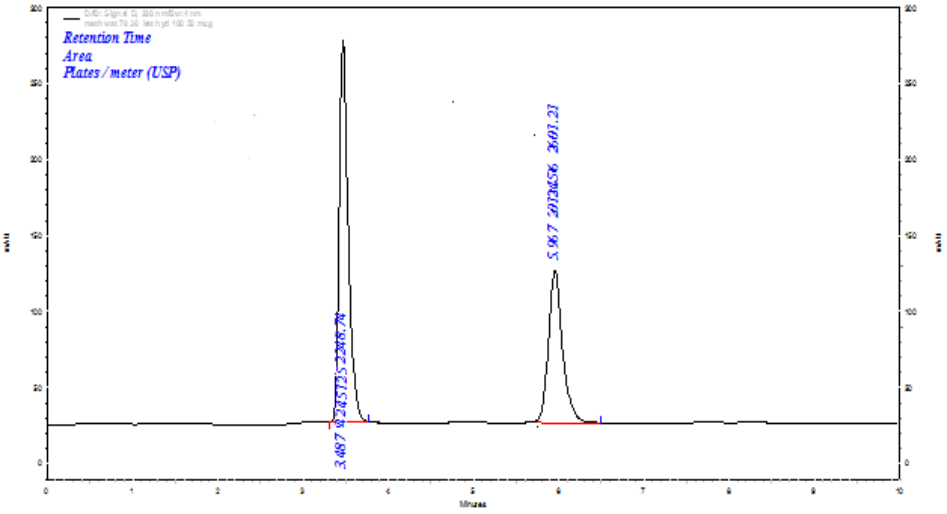

Figure 7: Linearity chromatogram of 40 Hydrocortisone 80 Acyclovir

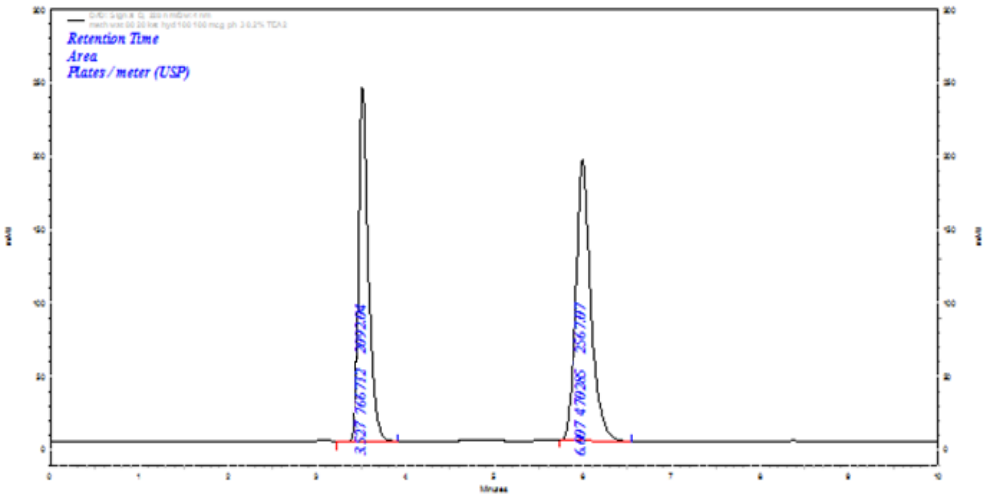

Figure8: Assay chromatogram of 10 Hydrocortisone 50 Acyclovir

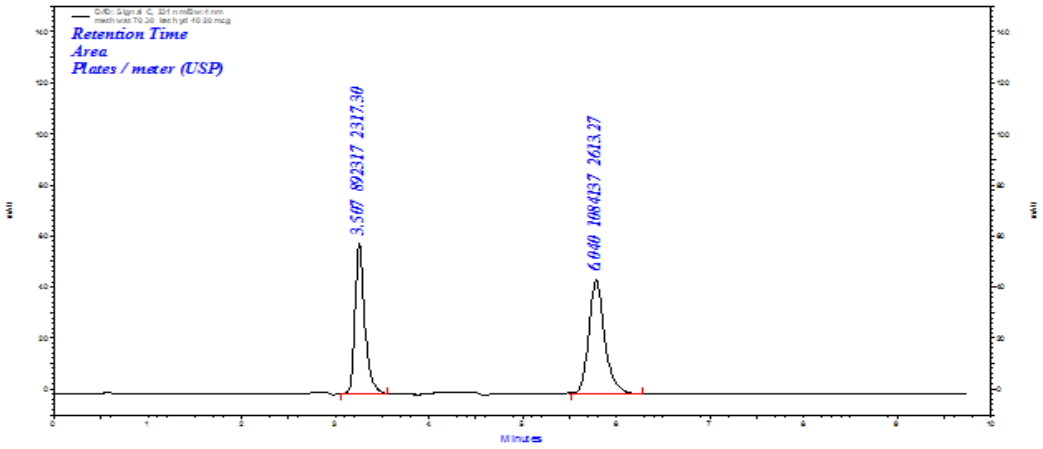

Figure 9: Recovery 80% chromatogram

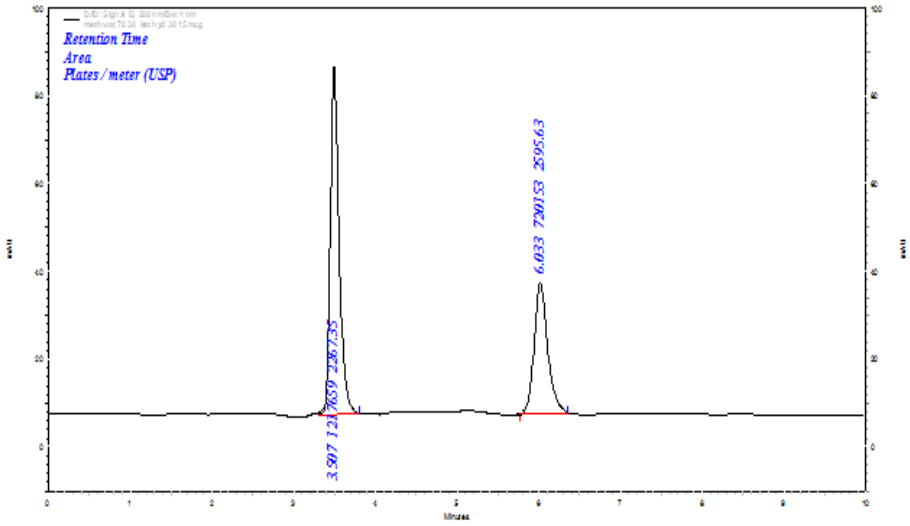

Figure 10: Recovery 100% chromatogram

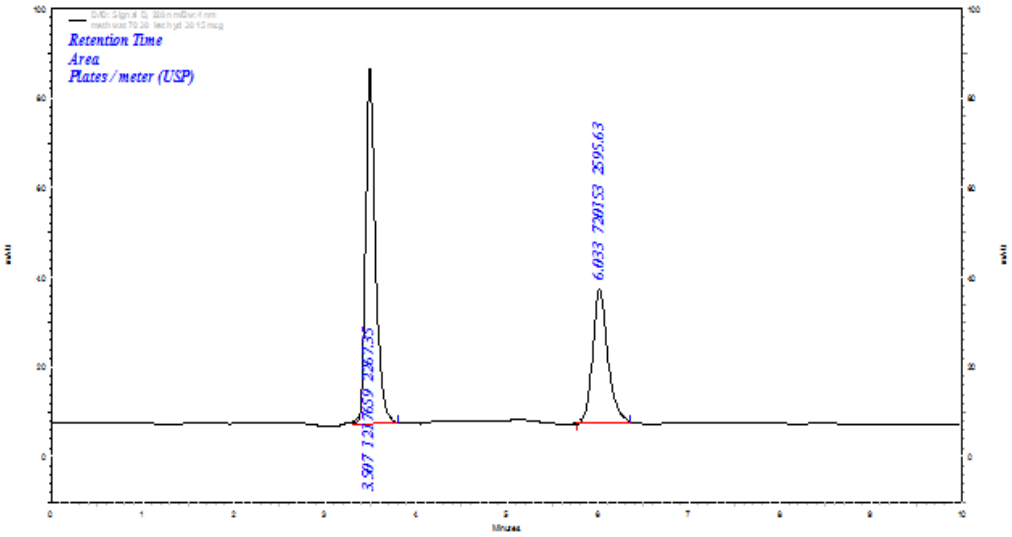

Figure 11: Recovery 120% chromatogram

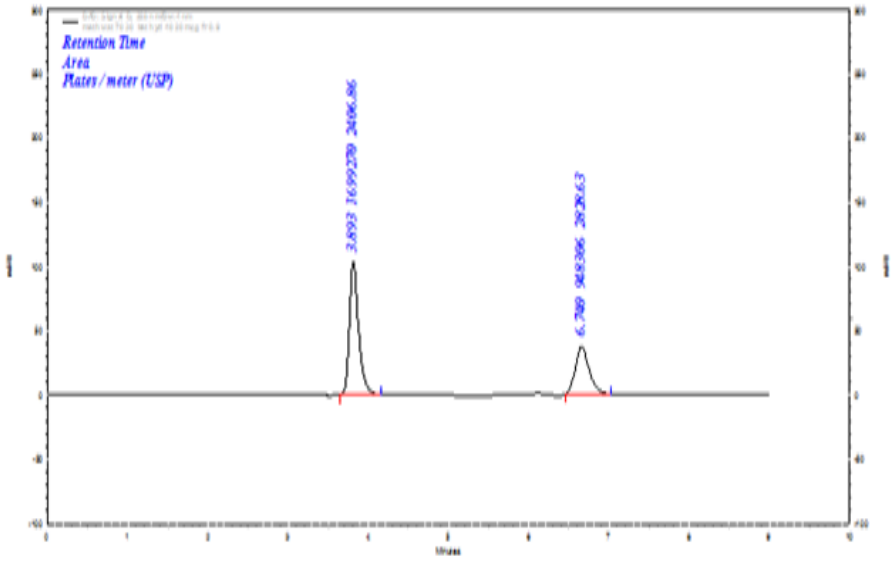

Figure 12: Robust chromatogram of Flow rate 0.9ml/min

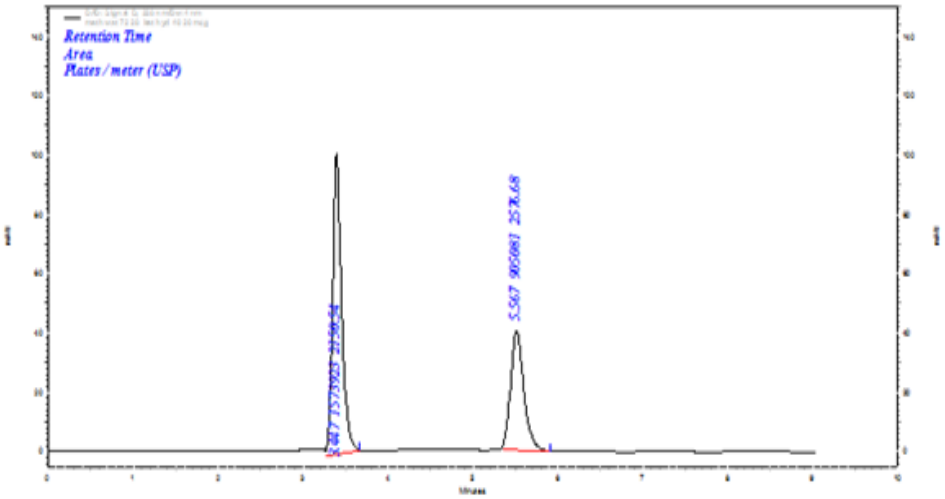

Figure 13: Robust chromatogram of mobile phase composition of Methanol: Water (82:18)

Table I: Optimized Chromatographic conditions of Hydrocortisone and Acyclovir on C_8 Column

S.No	Parameters	Hydrocortisone	Acyclovir
1	Mobile Phase Optimized	MeoH : H_2O (80 : 20)	MeoH : H_2O (80 : 20)
2	Flow Rate (ml/min)	1	1
3	Run Time (min)	10	10
4	Column Temperature ^OC	23	23
5	Volume of Injection (µl)	20	20
6	Detection Wavelength (nm)	221	221
7	Retention time Rt	3.50	6.00

Method validation

The proposed method has been extensively validated in terms of specificity, linearity, accuracy, precision, limits of detection (LOD) and quantification (LOQ), robustness and reproducibility. The accuracy was expressed in terms of percent recovery of the known amount of the standard drugs added to the known amount of the pharmaceutical dosage forms. The precision (Coefficient of Variation - C.V.) was expressed with respect to the repeatability, intra-day and inter-day variation in the expected drug concentrations. After validation, the developed methods have been applied to pharmaceutical dosage form.

System Suitability Criteria

It is defined as tests to measure the method that can generate result of acceptable accuracy and precision. The system suitability was carried out after the method development and validation have been completed. The system suitability was assessed by five replicate analyses of the drugs at concentrations of 20 µg mL^{-1} of HYDRO and 10 µg mL^{-1} of ACYCLO and for this, parameters like plate number (n), tailing factor, HETP, peak asymmetry of samples were measured, and shown in Table II.

Table II: System suitability parameters for Hydrocortisone and Acyclovir

Parameter	Values obtained (n = 6)		Acceptance Criteria
	Hydrocortisone	Acyclovir	
Plate Count	2092 ± 62	2567± 87	>2000
Tailing Factor	1.100 ± 0.032	1.097 ± 0.054	≤ 2.0
Capacity factor	0.3	1.44	< 2
HETP	0.03789	0.03210	----
R_t	3.50	6.00	-----

Validation parameters

Method was validated as per ICH (Q2) guidelines with respect to linearity, accuracy, precision, specificity, robustness, limit of detection and limit of quantification.

Specificity

Commonly used excipients (starch, microcrystalline cellulose and magnesium stearate) were spiked into a pre weighed quantity of drugs. Specificity of the method was shown by quantifying the analyte of interest in the presence of matrix and other components. Blank injections have shown no peaks at retention time of 3.50 min and 6.00min, the proposed method was specific for the detection of HYDRO and ACYCLO respectively. The selectivity of the method was performed by injecting the solution after the degradation. The degradants formed during solution

stability study were well separated from the analyte peak after 20 hrs of sample preparations. Thus the method can be applied to evaluate the stability of the solution.

Linearity

Appropriate volume of aliquot from HYDRO and ACYCLO standard stock solution was transferred to volumetric flask of 10 ml capacity. The volume was adjusted to the mark with methanol to give a solutions containing 10-40 µg/ml HYDRO and 20-80 µg/ml ACYCLO. The slope, Y-intercept and correlation coefficient were calculated. The regression line relating standard concentrations of drug using regression analysis, the calibration curves were linear in the studied range and equations of the regression analysis were obtained: Y=56804.9857x+157033.0714; R^2=0.9995 for HYDRO and Y=58909.4393x+453271.5356; R^2=0.9996 for ACYCLO respectively. The mean and correlation coefficient of standard curves (N=3) were calculated. The represented data was shown in below figure 4,5,6,7 and Table III.

Table III: Calibration of Hydrocortisone and Acyclovir

Concentration of Hydrocortisone (µg/ml)	Peak Area mean± SD (n=3) of Hydrocortisone	Concentration of Acyclovir (µg/ml)	Peak Area mean± SD (n=3) of Acyclovir	% RSD	
				Hydro	Acyclo
10	766712± 6621	20	470286± 1056	0.86	0.25
15	1294165 ± 21330	30	690164± 4385	1.70	0.64
20	16672234 ± 10838	40	434455 ± 4677	0.60	1.06
25	2345254± 11178	50	1134567± 51295	0.45	0.46
30	3145342 ± 51089	60	1456980 ± 51026	1.67	0.36
35	3312467 ± 10021	70	1723673 ± 49568	0.28	0.29
40	4245125±11582	80	2012456±18089	0.27	0.90

Table IV: Assay report of formulation

S. No.	Brand name	Content	Peak Area mean ± S.D	Assay	%RSD
1	Xerese	10 µg/ml Hydrocortisone	760340 ± 6610	101.00%	1.785
		50 µg/ml -Acyclovir	476733± 5687	96.50%	1.145

Accuracy

Accuracy was assessed by determination of the recovery of the method by addition of standard drug to the pre-quantified placebo preparation at 3 different concentration levels 80, 100 and 120 %, taking into consideration percentage purity of added bulk drug samples. Each concentration was analyzed 3 times and average recoveries were measured. Results of assay and recovery were presented in the Table V and Figure 9,10,11.

Table V: Recovery Report of Hydrocortisone and Acyclovir

Drug	Amount taken (µg/ml)	Recovery Level	Amount of Drug Added	Amount of Drug Found (µg/ml) Mean± S.D	% RSD	% Recovery
Hydro	10	80%	8	8.06 ± 0.22	0.225	100.05
		100%	10	10 .00 ± 0.18	0.814	100.00
		120%	12	12.17 ± 0.12	0.373	101..41
Acyclo	50	80%	16	46.15 ± 0.168	0.234	100.93
		100%	20	50.22 ± 0.46	0.902	101.00
		120%	24	53.96± 0.24	0.262	99.83

Precision

The repeatability was evaluated by assaying 6 times of sample solution prepared for assay determination. The intraday and interday precision study of HYDRO and ACYCLO was carried out by estimating different concentrations of HYDRO (10, 25, 40 µg/ml) and ACYCLO (20, 50, 80 µg/ml), 3 times on the same day and on 3 different days (first, second, third) and the results are reported in terms of C.V. The results are shown in Table VI.

Table VI: Precision

Intra-day and Inter-day Precision

Intra-day Precision Data for Hydrocortisone and Acyclovir

S. No	Conc. (µg/ml) of Hydro	Peak Area mean ± S.D (n=3) of Hydro	Conc. (µg/ml) of Acyclo	Peak Area mean ± S.D (n=3) of Acyclo	%RSD Hydro	Acyclo
1	10	754892± 6523	20	443468 ± 1034	0.86	0.25
2	25	2345254± 11032	50	1261647 ±51340	0.47	0.30
3	40	4234568± 11257	80	2015712 ±17045	0.29	0.80
				Avg. of %RSD	1.62	1.35

Inter-day Precision Data of Hydrocortisone and Acyclovir

S. No	Conc. (µg/ml) of Hydro	Peak Area mean ± S.D (n=3) of Hydro	Conc. (µg/ml) of Acyclo	Peak Area mean ± S.D (n=3) of Acyclo	%RSD Hydro	Acyclo
1	10	758345 ± 6530	20	4424453 ± 1123	0.90	0.30
2	20	2346537 ± 11212	50	1234567 ± 51433	0.50	0.89
3	80	4222567 ± 11421	80	2014589± 17312	0.35	0.30
Avg. of %RSD					1.75	1.49

Robustness

The robustness of the method was evaluated by analyzing the system suitability standards and evaluating system suitability parameter data after varying the HPLC pump flow rate (±0.1ml) and organic solvent content (±2ml) and pH (±0.2). None of the alterations caused a significant change in peak area R.S.D (%), USP tailing factor and theoretical plates. Although the changes

in retention times were more significant, and quantification was still possible. Results of robustness studies are shown in Table VII and Figure 12,13.

Table VII: Robustness studies of Hydrocortisone and Acyclovir

S. No	Parameter	Modification	Retention time		Tailing Factor	
			Hydro	Acyclo	Hydro	Acyclo
1	Flow rate	0.9 ml/min	3.55 min	6.76min	1.152	1.140
		1.0 ml/min	3.50min	6.00 min	1.100	1.097
		1.1 ml/min	2.88min	5.50 min	1.165	1.393
2	Mobile phase	73 : 27	3.19 min	6.75 min	1.152	1.146
	Composition	80 : 20	3.50min	6.00min	1.100	1.097
	(MeOH:H2O)	82 : 18	3.10min	5.58min	1.167	1.314
3	pH	3.0	3.50 min	6.00 min	1.100	1.097
		3.2	3.180min	6.12 min	1.149	1.110
4	Wavelength	251	3.18min	6.12 min	1.139	1.107
		254	3.50min	6.00min	1.100	1.097
		256	3.18min	6.1 2min	1.164	1.104

LOD and LOQ

LOD and LOQ were calculated from the formula 3.3 x (σ/S) and 10 x (σ/S), respectively where, σ is standard deviation of intercept and S is the mean of slope. The LOD and LOQ can also be determined by S/N. The value for LOD should be 3-5 whilst for LOQ 10-15. The results are presented in Table VIII.

Table VIII: LOD and LOQ data

S.No.	Parameter	Hydrocortisone	Acyclovir
1	LOD	1.294 µg/ml	0.241 µg/ml
2	LOQ	3.922 µg/ml	0.733 µg/ml

Solution stability and Mobile phase stability

The stability of HYDRO and ACYCLO in solution was determined by leaving test solutions of the sample and reference standard in tightly capped volumetric flasks at room temperature for 3 days during which they were assayed at 12 h intervals. Stability of mobile phase was determined by analysis of freshly prepared sample solutions at 12 h intervals for 48 h and comparing the results with those obtained from freshly prepared reference standard solutions. The mobile phase was prepared at the beginning of the study period and not changed during the experiment. The % assay of the results was calculated for both the mobile phase and solution-stability experiments.

CONCLUSION

The present study represents an accurate, precise and specific HPLC method for routine analysis of Hydrocortisone and Acyclovir combination in Cream(tablet) dosage form. In addition to

assay it may be used to detect related substance or other impurities which are formed during storage conditions and the analyte of interest could be estimated without any interferences. The use of C_8 column in the present work has shown better elution of analytes with good resolution, improved plate count, capacity factor, reduced tailing. So the C_{18} column can be used to achieve high specificity in shorter time of analysis Hydrocortisone to Acyclovir in tablet as per ICH Q2 (R2) guidelines.

The developed UV-spectrophotometric method for simultaneous determination of Hydrocortisone and Acyclovir in combined pharmaceutical dosage form is simple and reliable. From the study of validation parameters namely accuracy, precision (SD and RSD), (interday, intraday and different analyst), specificity, linearity and range, it was observed that the method is specific, accurate, precise and reproducible. Hence the method can be employed for routine analysis of dosage form.

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