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SIMULTANEOUS ESTIMATION OF HYDROCORTISONE AND KETOCONAZOLE IN PHARMACEUTICAL DOSAGE FORM BY RP-HPLC METHOD

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ABSTRACT: A simple, selective, rapid, precise and economical reverse phase high performance liquid chromatographic method has been developed for the simultaneous estimation of hydrocortisone and ketoconazole from pharmaceutical formulation. The method was carried out on a C18 (25 cm × 4.6 mm i.d., 5 µ) column with a mobile phase consisting of methanol: water (adjusted to pH 3.0 using triethylamine) in the ratio of 70:30 v/v. The retention time of hydricortusone and ketoconazole was 3.50 min and 6.00 min respectively with the flow rate of 1mL/ min. Eluents were detected at 221 nm. The linear regression analysis data for the linearity plot showed good linear relationship with correlation coefficient value for hydrocortisone and ketoconazole 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.

INTRODUCTION: Hydrocortisone (hydro) is chemically (1S, 2R 10S, 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 **Fig. 1** [1]. Ketoconazole (keto) is chemically 1-[4-(4-{2-(2, 4-dichlorophenyl)- 2- (1H-imidazol-1-ylmethyl)-1, 3-dioxolan-4-yl] methoxy] phenyl0piperazin- 1- yl] ethan-1-one. It is used as antifungal agents. Structure of keto was shown in **Fig. 2** [2]. 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.

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Several analytical methods have been reported for keto 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 ketoconazole by HPLC in pharmaceutical dosage forms. This present study reports for the first time simultaneous estimation of hydrocortisone and ketoconazole 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 keto was provided by Mylan laboratory, as gift samples. Analytical grade methanol was purchased from Merck & Co. Glass wares used in each procedure were soaked overnight 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.

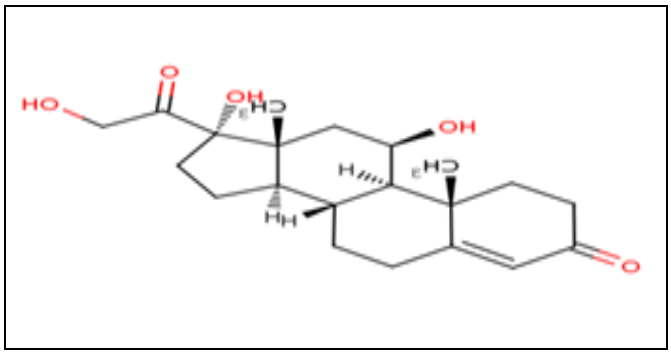

FIG. 1: HYDROCORTISONE

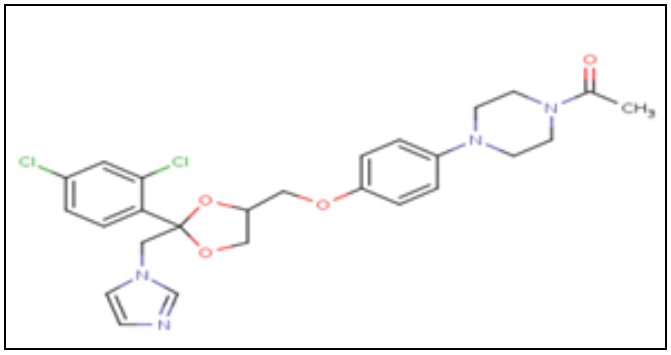

FIG. 2: KETOCCONAZOLE

Instrumentation: HPLC system (Agilent HPLC Model-1100 with ezchromelite software) containing C18 (Qualisil BDS, 250 × 4.6 mm, 5µ) column with UV- VWD detection. Lab India-3000 + UV-Visible double beam spectrophotometer with a fixed slit width 1 nm and 1cm matched quartz cells was used for all the spectral measurements.

RESULTS AND DISCUSSION:

Method:

Optimisation of the Chromatographic Conditions: The mobile phase consisted of methanol and water in ratio methanol: water (70:30), pH was adjusted to 3.0 with tea 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 221 nm. The results of the optimized chromatogram was shown in **Fig. 3** and **Table 1**.

TABLE 1: OPTIMIZED CHROMATOGRAPHIC CONDITIONS OF HYDROCORTISONE AND KETO-CONAZOLE ON C18 COLUMN

S. no.	Parameters	Hydrocortisone	Ketoconazole
1	Mobile phase optimized	MeOH : H_2O (70 : 30)	MeOH : H_2O (70 : 30)
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

Preparation of Standard Stock Solutions: Accurately weighed 10 mg of hydro and keto 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 keto. From this solution 1 ml was transferred to volumetric flask of 100 ml capacity. Volume was made up to the mark to give a solution containing 100 µg/ml of hydro and 100 µg/ml keto.

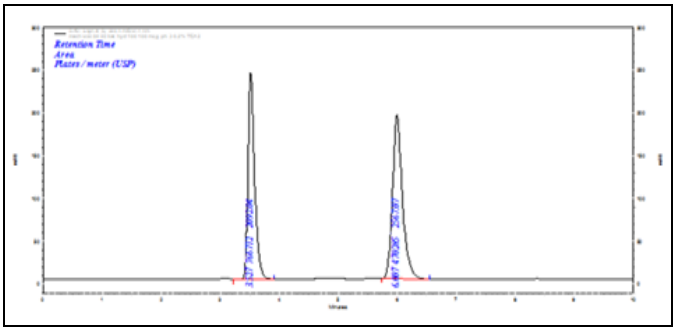

FIG. 3: OPTIMIZED CHROMATOGRAM OF HYDROCORTISONE AND KETOCONAZOLE

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

Determination of Hydrocortisone and Keto-conazole in their Combined Dosage:

Sample Preparation: A powder quantity equivalent to 10 mg hydro and 20 mg keto 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 keto. 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 4** and **Fig. 8**.

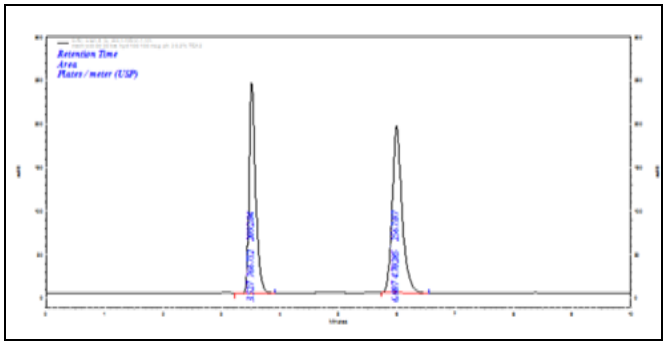

FIG. 8: ASSAY CHROMATOGRAM OF 10 HYDROCORTISONE 20 KETOCONAZOLE

TABLE 4: ASSAY REPORT OF FORMULATION

S. no.	Brand name	Content	Peak Area mean ± S.D.	Assay	%RSD
1	Ketocon + plus	10 µg/ml Hydrocortisone	760340 ± 6610	101.00%	1.785
		20 µg/ml Ketoconazole	476733 ± 5687	96.50%	1.145

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.

TABLE 2: SYSTEM SUITABILITY PARAMETERS FOR HYDROCORTISONE AND KETOCONAZOLE

Parameter	Values obtained (n = 6)		Acceptance Criteria
	Hydrocortisone	Ketoconazole	
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	----
Rt	3.50	6.00	-----

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 keto and for this, parameters like plate number (n), tailing factor, HETP, peak asymmetry of samples were measured, and shown in **Table 2**.

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 6.00 min, the proposed method was specific for the detection of hydro and keto 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 keto 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 keto. 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 keto respectively. The mean and correlation coefficient of standard curves (N = 3) were calculated. The represented data was shown in below **Fig. 4, 5, 6, 7** and **Table 3**.

TABLE 3: CALIBRATION OF HYDROCORTISON AND KETOCONAZOLE

Concentration of hydrocortisone (µg/ml)	Peak Area mean ± SD (n=3) of Hydrocortisone	Concentration of ketoconazole (µg/ml)	Peak Area mean ± SD (n=3) of Ketoconazole	% RSD	
				Hydro	Keto
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

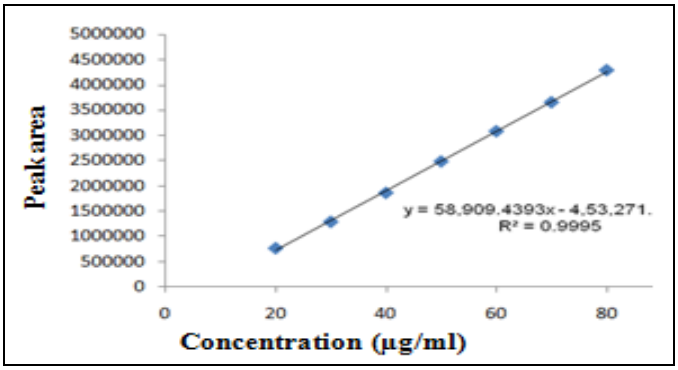

FIG. 4: LINEARITY OF KETOCONAZOLE AT 221nm

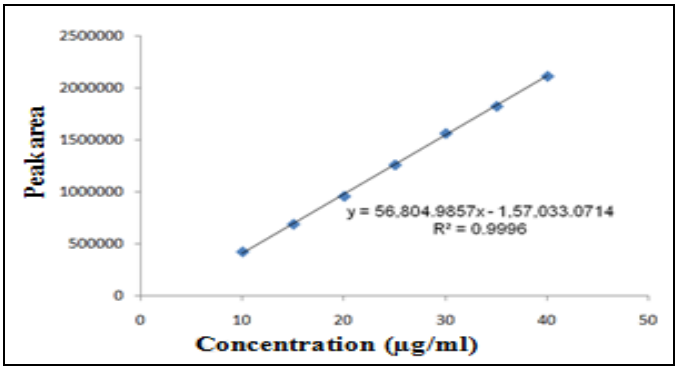

FIG. 5: LINEARITY OF HYDROCORTISONE AT 221 nm

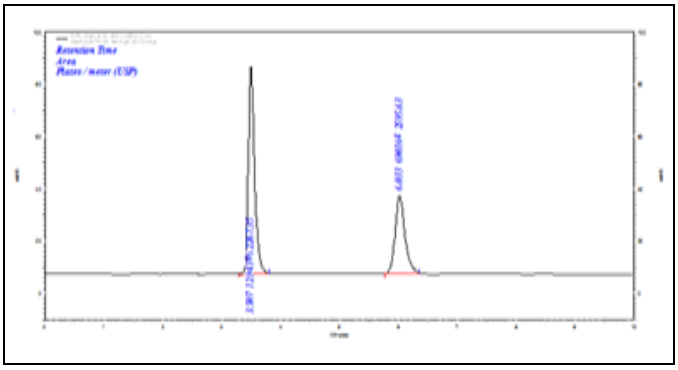

FIG. 6: LINEARITY CHROMATOGRAM OF 15 HYDROCORTISONE 30 KETOCONAZOLE

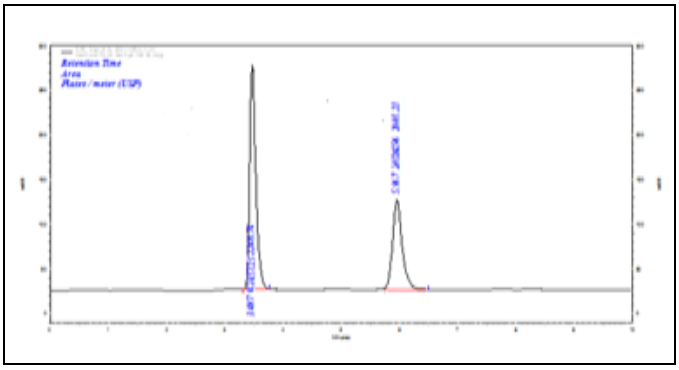

FIG. 7: LINEARITY CHROMATOGRAM OF 40 HYDROCORTISONE 80 KETOCONAZOLE

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 5** and **Fig. 9, 10, 11**.

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 keto was carried out by estimating different concentrations of hydro (10, 25, 40 µg/ml) and keto (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 6**.

TABLE 5: RECOVERY REPORT OF HYDROCORTISONE AND KETOCONAZOLE

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
Keto	20	80%	16	16.15 ± 0.168	0.234	100.93
		100%	20	20.22 ± 0.46	0.902	101.00
		120%	24	23.96 ± 0.24	0.262	99.83

Precision Intra-Day and Inter-Day Precision:

TABLE 6A: INTRA-DAY PRECISION DATA FOR HYDROCORTISONE AND KETOCONAZOLE

S. no.	Conc. (µg/ml) of Hydro	Peak Area mean ± S.D (n=3) of Hydro	Conc. (µg/ml) of Keto	Peak Area mean ± S.D (n=3) of Keto	% RSD	
					Hydro	Keto
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

TABLE 6B: INTER-DAY PRECISION DATA OF HYDROCORTISONE AND KETOCONAZOLE

S. no.	Conc. (µg/ml) of Hydro	Peak Area mean ± S.D. (n=3) of Hydro	Conc. (µg/ml) of Keto	Peak Area mean ± S.D. (n=3) of Keto	%RSD	
					Hydro	Keto
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

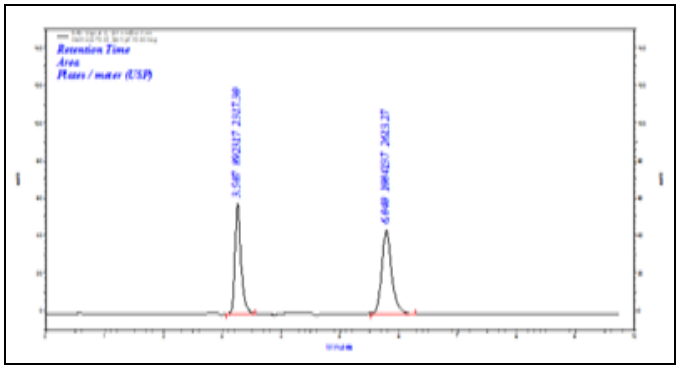

FIG. 9: RECOVERY 80% CHROMATOGRAM

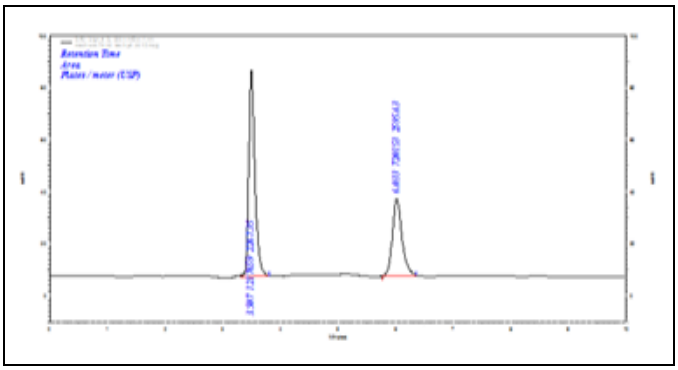

FIG. 10: RECOVERY 100% CHROMATOGRAM

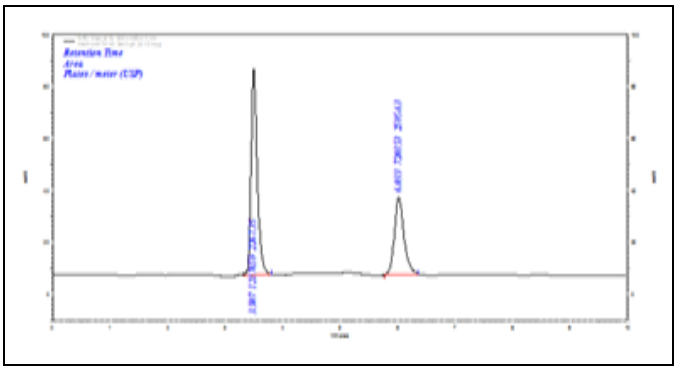

FIG. 11: RECOVERY 120% CHROMATOGRAM

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 (±0.1 ml) and organic solvent content (±2 ml) 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 7** and **Fig. 12, 13**.

TABLE 7: ROBUSTNESS STUDIES OF HYDROCORTISONE AND KETOCONAZOLE

S. no.	Parameter	Modification	Retention time		Tailing Factor	
			Hydro	Keto	Hydro	Keto
1	Flow rate	0.9 ml/min	3.55 min	6.76 min	1.152	1.140
		1.0 ml/min	3.50 min	6.00 min	1.100	1.097
		1.1 ml/min	2.88 min	5.50 min	1.165	1.393
2	Mobile phase	68 : 32	3.19 min	6.75 min	1.152	1.146
	Composition	70 : 30	3.50 min	6.00 min	1.100	1.097
	(MeOH:H_2O)	72 : 28	3.10 min	5.58 min	1.167	1.314
3	pH	3.0	3.50 min	6.00 min	1.100	1.097
		3.2	3.180 min	6.12 min	1.149	1.110
4	Wavelength	218	3.18 min	6.12 min	1.139	1.107
		221	3.50 min	6.00 min	1.100	1.097
		224	3.18 min	6.1 2 min	1.164	1.104

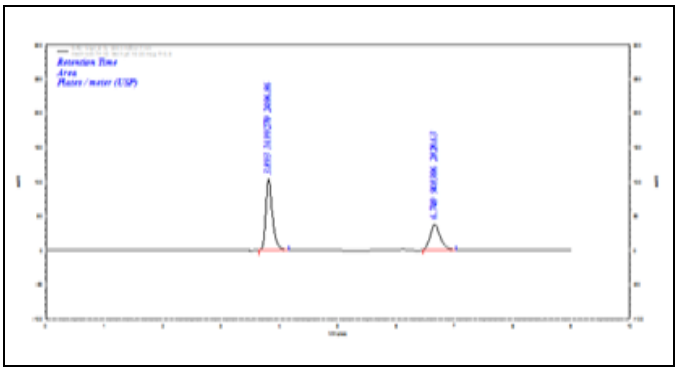

FIG. 12: ROBUST CHROMATOGRAM OF FLOW RATE 0.9 ml/min

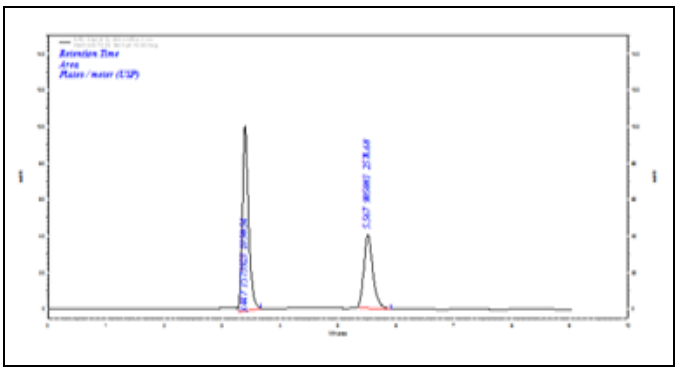

FIG. 13: ROBUST CHROMATOGRAM OF MOBILE PHASE COMPOSITION OF METHANOL: WATER (72:28)

LOD and LOQ: LOD and LOQ were calculated from the formula 3.3 × (σ/S) and 10 × (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 8**.

TABLE 8: LOD AND LOQ DATA

S. no.	Parameter	Hydrocortisone	Ketoconazole
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 keto 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 keto-conazole combination in 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 C18 column in the present work has shown better elution of analytes with good resolution, improved plate count, capacity factor, reduced tailing. So the C18 column can be used to achieve high specificity in shorter time of analysis hydrocortisone to ketoconazole in tablet as per ICH Q2 (R^2) guidelines.

The developed UV-spectrophotometric method for simultaneous determination of hydrocortisone and ketoconazole 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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CONFLICT OF INTEREST: Nil

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