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DEVELOPMENT AND VALIDATION OF REVERSE-PHASE HPLC METHOD FOR ESTIMATION OF HAMYCIN AND KETOCONAZOLE IN PHARMACEUTICAL CREAM

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ABSTRACT: A simple, accurate rapid and precise RP-HPLC method has been developed and validated for determination of Hamycin and Ketoconazole in Pharmaceutical Cream. The RP-HPLC separation was achieved on Thermosil C-18, (250 mm, 4.6 mm, 5µm) using mobile phase 0.4 % (v/v) diisopropylamine in methanol (v/v): 0.5% (w/v) Ammonium acetate in distilled water (90:10 % v/v) pH 6.5 adjusted with Glacial acetic acid at flow rate of 1.0 ml/min at ambient temperature. The retention times were 2.433 min. for Hamycin and 4.711 min. for Ketoconazole. Calibration plots were linear over the concentration range 50-250 µg/ml for Hamycin and 200-1000 µg/ml. Quantification was achieved with UV detection at 263 nm over the Beer-Lambert's range.The method was validated statistically and applied successfully for the determination of Hamycin and Ketoconazole. Validation studies revealed that method is specific, rapid, reliable, and reproducible. The high recovery and low relative standard deviation confirm the suitability of the method for the routine determination of Hamycin and Ketoconazole in pharmaceutical cream. The proposed method was validated as per the ICH and USP guidelines.

INTRODUCTION: Ketoconazole is Chemically Cis-1-acetyl-4-[4-[2-(2,4-dichlorophenyl)-2H-imidazolyl methyl)-1,3-dioxolan-4-yl] methoxy] phenyl]- piperazine is a topical as well as systemic antifungal agent (**figure 1**).

Ketoconazole is practically insoluble in water; sparingly soluble in strong acid, soluble in strong bases. It is an imidazole derivative with molecular weight 531.44 [1, 2].

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It inhibits cytochrome P450 dependent lanosterol C14 demethylase, which is responsible for production of ergosterol, a necessary component in fungal cell wall synthesis [3, 4]. Ketoconazole is a weak base with pKa values of 2.94 and 6.51 [5]. Ketoconazole is an antifungal drug approved by the US FDA in 1981. Only a few analytical methods for the determination of the drug in biological samples and in the presence of other drugs have been reported [6-10].

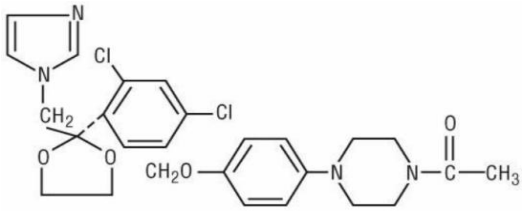

FIG. 1: STRUCTURE OF KETOCONAZOLE

Hamycin is a polyene antimycotic organic compound. It is a heptaene antifungal compound rather similar in chemical structure to amphotericin B except that it has an additional aromatic group bonded to the molecule (**figure 2**). Hamycin is obtained from a strain of Streptomyces bacteria growing in soil i.e., Streptomyces pimprina. This compound is being produced in India by Hindustan Antibiotics Limited, located at Pimpri, Pune, Maharashtra, India. It is useful as an antifungal antibiotic drug for topical as well as systemic mycoses.

It is Yellow amorphous powder, no definite M.P., decompose after 160^0 C. UV max (80% methanol): 383 nm (E1%1cm916). An amphoteric compd. Almost insoluble in water, benzene, chloroform, dry lower aliphatic alcohols, ether. Solution in basic solvents such as pyridine, and in aqueous lower alcohols. In conc. H_2SO_4 gives stable blue color, no coloration with ferric chloride or with HCl. Hamycin is a rigid, rod-shaped molecule that kills cells by disrupting the cell membrane, causing leakage of electrolytes and small molecules. Hamycin bind to ergosterol, the major membrane lipid in fungal cells.[7, 8]

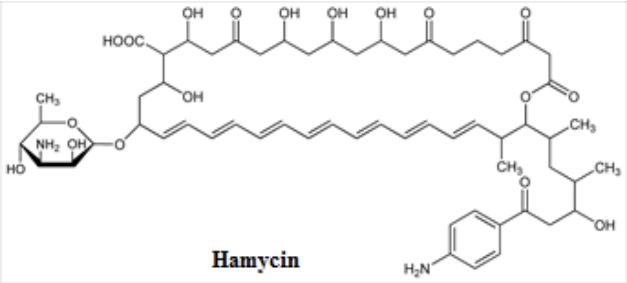

FIG. 2: STRUCTURE OF HAMYCIN

MATERIALS AND METHODS: All the reagents used were of HPLC grade and analytical grade from Rankem, India. Reference standard of Ketoconazole was supplied as gift sample from Genpharma International Pvt. Ltd., Pune and Hamycin was supplied as gift sample from Hindustan Antibiotic Limited, Pune.

Apparatus and Chromatographic Conditions:

Equipment: High performance liquid chromatography (Shimadzu LC 2010) equipped with Auto Sampler and UV detector. HPLC Column: Thermo Scientific C_{18} column (5 µm, 250 X 4.6 mm ID), Column temperature: Ambient temperature, Mobile Phase: 4 % (v/v) diisopropyl-amine in methanol (v/v): 0.5%, (w/v) Ammonium acetate in distilled water (90:10 % v/v), pH 6.5 adjusted with Glacial acetic acid. UV detection: 263 nm Injection volume: 20 µL, Run time: 7 mins.

Preparation of Mobile phase: The mobile phase is prepared by mixing 0.4 %(v/v) diisopropylamine in methanol (v/v): 0.5% (w/v) Ammonium acetate in distilled water (90:10 % v/v) pH 6.5 adjusted with Glacial acetic acid. Filtered and degas it.

Diluent Preparation: The Mobile phase was used as diluent.

Preparation of standard solution: 10 mg of Hamycin and 40 mg Ketoconazole weighed and transferred to 100 ml volumetric flasks respectively. It was dissolved in the mobile phase consisting of 0.4% (v/v) diisopropylamine in methanol (v/v): 0.5% (w/v) Ammonium acetate in distilled water (90:10 % v/v) and the solutions were made up to mark with same mobile phase to obtain stock solutions of concentration 100 µg mL^{-1} of Hamycin and 400 µg mL^{-1} Ketoconazole each.

Preparation of sample solution: The sample solution was prepared by weighing accurately and transferred 2 gm of cream formulation which contain 10 mg of Hamycin and 40 mg of Ketoconazole into a 100mL clean dry volumetric flask. About 70mL of diluent was added to this and sonicated to dissolve it completely. Finally the volume was made to the mark with the same solvent.

Injection of Standards and Samples into the Chromatographic system: 20 µL of each standard and sample solution was injected into the chromatographic system and measured the areas of Hamycin and Ketoconazole peaks. %Assay of both the drug was calculated using the appropriate formulae.

System Suitability Results: To ascertain resolution and reproducibility of proposed chromatographic system for estimation of Hamycin and Ketoconazole in formulation system suitability parameters were studied.

System suitability parameters were calculated and are given in **Table 2**.

TABLE 1: ASSAY OF CREAM FORMULATION

Sr. No.	Label claim (mg/2 gm)		Amount Found (mg/2 gm)		% Amount Found	
	Hamycin	Ketoconazole	Hamycin	Ketoconazole	Hamycin	Ketoconazole
1.	10	40	9.93	38.62	99.33	96.54
2.	10	40	9.85	39.62	98.49	99.05
3.	10	40	9.75	39.29	97.47	98.22
4.	10	40	10.02	40.07	100.16	100.18
5.	10	40	9.91	39.34	99.10	98.36
6.	10	40	9.86	39.61	98.58	99.03

TABLE 2: SYSTEM SUITABILITY PARAMETERS

Parameter with Std values	Hamycin	Ketoconazole
Resolution NLT 2		**10.43**
Tailing factor NMT 2	1.26	1.06
Number of Theoretical plates	**9299**	**9689**

NMT – Not more than. **NLT** – Not less than

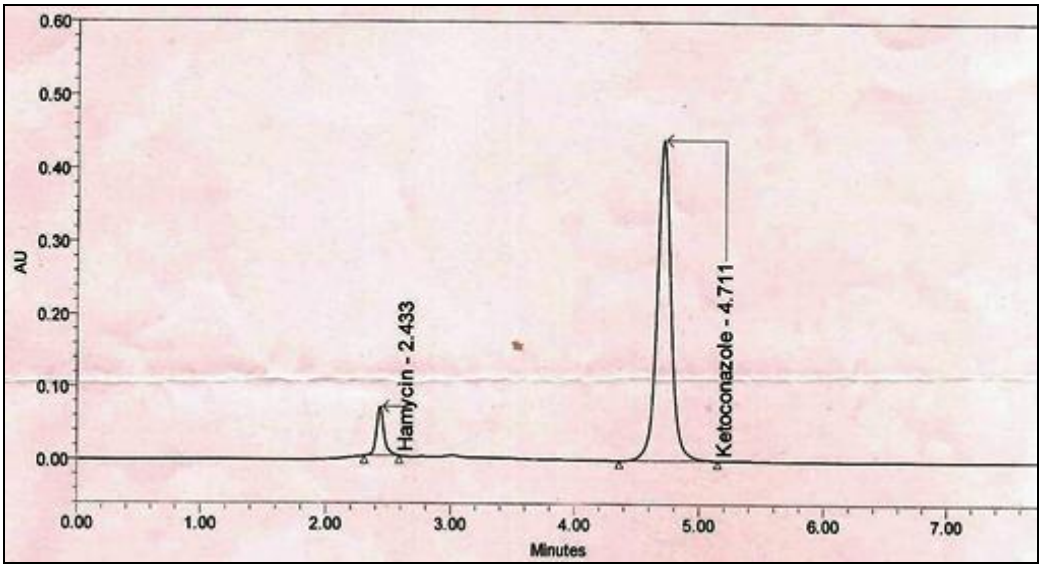

FIG 3: CHROMATOGRAM OF STANDARD MIXTURE OF HAMYCIN AND KETOCONAZOLE IN RATIO OF (1:4)

RESULTS:

RP-HPLC Validation:

Linearity: The calibration curves were found to be linear and in adherence to Beer's law over the concentration range of 50-250 µg mL^{-1} for Hamycin and 200-1000 µg mL^{-1} for Ketoconazole. The linearity was validated by the high values of the correlation coefficient. The results of the linearity studies are given in **Table 3**.

TABLE 3: LINEAR REGRESSION DATA FOR CALIBRATION CURVES OF HAMYCIN AND KETOCONAZOLE FOR RP–HPLC METHOD

Drugs	Linearity range* (µg ml^{-1})	Slope* ± S.D	y-intercept* ±S.D	Regression coefficient* (r^2)
Hamycin	50-250	2984 ±15	2291±943.11	0.999
Ketoconazole	200-1000	8842±21.50	52696±14053	0.999

*Average of six determinations

Precision:

a) **Repeatability:** The repeatability of sample application and measurement of peak area were expressed in terms of % R.S.D and was found to be less than 2 %. The results of the same are given in Table No.4.

TABLE 4: STATISTICAL VALIDATION OF REPEATABILITY FOR RP – HPLC METHOD

Drugs	Mean Content (%)*	S.D.*	% R.S.D.*
Hamycin	99.06	0.6319	0.637
Ketoconazole	96.46	1.6332	1.69

*Average of six determination

b) Intermediate precision: The intermediate precision of sample application and measurement of peak areas were expressed in terms of % Relative Standard Deviation (% R.S.D.) and were found to be less than 2% for Hamycin and Ketoconazole indicating that the proposed method provides acceptable intra-day and inter-day precision. The results of the same are given in **Table 5**.

TABLE 5: INTRA-DAY AND INTER-DAY PRECISION OF RP – HPLC METHOD

Drugs	Intra-day Precision*			Inter-day Precision*		
	Mean % content	S.D.	% R.S.D.	Mean % content	S.D.	% R.S.D.
Hamycin	98.95	0.9593	0.9695	98.52	0.6911	0.7012
Ketoconazole	99.19	0.6559	0.6613	98.56	0.3969	0.4028

*Average of three determinations

Robustness of the method: To evaluate the robustness of the method, the optimized method parameters were varied at different levels. The results presented in **Table 6** indicated that the selected factors (retention time t_R, peak area, and % content) were unaffected by small variations in the selected method parameters.

TABLE 6: **ROBUSTNESS TESTING FOR RP – HPLC METHOD**

Flow Rate (ml/min)	Chromatographic changes					
	Retention time*		Tailing Factor*		% Content*	
	Hamycin	Ketoconazole	Hamycin	Ketoconazole	Hamycin	Ketoconazole
0.8	3.0	5.9	1.21	1.23	98.95	99.19
1.0	2.4	4.7	1.1	1.25	99.16	99.34
1.2	2.0	3.9	1.14	1.11	99.2	98.59
Mean ± S.D.	2.46±0.5033	4.83±1.006	1.15±0.0556	1.19±0.0757	99.10±0.134	99.04±0.3968
% of Diisopropylamine in methanol in the mobile phase (v/v)						
92	2.41	4.18	1.25	1.2	99.1	97.92
90	2.4	4.71	1.22	1.12	99.23	99.41
88	2.54	5.13	1.21	1.23	98.17	98.13
Mean ± S.D.	2.45±0.0781	4.67±0.4760	1.23±0.0208	1.18±0.0568	98.83±0.5781	98.49±0.8064
pH of Mobile Phase						
6.3	2.4	4.63	1.23	1.11	98.87	99.09
6.5	2.4	4.71	1.13	1.15	99.13	99.29
6.7	2.61	5.01	1.21	1.12	99	98.49
Mean ± S.D.	2.47±0.1212	4.78±0.2003	1.19±0.0529	1.13±0.0208	99±0.13	98.96±0.4163

*Average of three determinations

Accuracy: The accuracy study was performed at three different levels (80%, 100% and 120% of the test concentration). The mean % recoveries were found to be between 98–102% as required by ICH guidelines. The results of the recovery studies and its statistical validation data are given in **Table 7 and Table 8** respectively.

TABLE 7: RECOVERY STUDIES FOR RP – HPLC METHOD

Level of % Recovery	Amount of drug present (mg/20gm)		Amount of standard added (mg)		Total amount recovered (mg)		% Recovery	
	HAM	KETO	HAM	KETO	HAM	KETO	HAM	KETO
80	100	400	80	320	177.90	716.16	98.83	**99.46**
	100	400	80	320	178.19	714.71	98.99	**99.26**
	100	400	80	320	178.90	715.40	99.38	**99.36**
100	100	400	100	400	198.51	790.18	99.25	**98.77**
	100	400	100	400	198.28	791.91	99.14	**98.99**
	100	400	100	400	198.83	791.66	99.42	**98.96**
120	100	400	120	480	216.51	874.18	98.41	**99.34**
	100	400	120	480	216.09	873.34	98.22	**99.24**
	100	**400**	120	**480**	215.80	**875.70**	98.10	**99.51**

TABLE 8: STATISTICAL VALIDATION OF RECOVERY DATA FOR RP – HPLC METHOD

Level of % Recovery	% Mean Recovery*		S. D. *		% R.S.D.*	
	HAM	KETO	HAM	KETO	HAM	KETO
80	99.06	99.36	0.2829	0.1	0.2855	**0.101**
100	99.27	98.91	0.1411	0.1193	0.1421	**0.1206**
120	**98.24**	**99.36**	**0.1563**	**0.1365**	**0.1591**	**0.1373**

*Average of three determinations

Limit of Detection and Limit of Quantitation: The limit of detection and limit of quantitation was calculated on the basis of standard deviation of the response and slope (**table 9**).

TABLE 9: LOD AND LOQ VALUES FOR RP – HPLC METHOD

Drugs	LOD (µg ml^{-1})	LOQ (µg ml^{-1})
Hamycin	1.048	3.176
Ketoconazole	5.244	15.893

DISCUSSION: A simple, specific, accurate, reproducible, precise and robust reverse phase high performance liquid chromatography method with UV detection at 263 nm has been developed for the simultaneous determination of Hamycin and Ketoconazole in formulation. Shimadzu LC2010 HPLC system was used for the analysis. Mobile phase consisted of a mixture of 0.4 %(v/v) diisopropylamine in methanol (v/v): 0.5% (w/v) Ammonium acetate in distilled water (90:10 % v/v) pH 6.5 with Glacial acetic was used.

The retention time for Hamycin and Ketoconazole were 2.4 and 4.7 min respectively. The system suitability parameters were calculated and are found within limits. Linear relationships were obtained between response and amount of drug with high correlation coefficients (r^2) in the range 50-250 µg mL^{-1} for Hamycin (r^2= 0.999) and 200-1000 µg mL^{-1} for Ketoconazole (r^2= 0.999). The LOD and LOQ were 1.048 and 3.176 µg mL^{-1} for Hamycin, 5.244 and 15.893 µg mL^{-1} for Ketoconazole respectively.

The results of formulation analysis and recovery studies were statistically validated as per ICH guidelines indicating high degree of accuracy. The low % R.S.D. value of intraday and interday precision studies revealed high degree of precision of the proposed method. The low % R.S.D. value for robustness study suggests that the developed RP-HPLC method is unaffected by small changes in process parameters.

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