DEVELOPMENT AND VALIDATION OF KETOCONAZOLE BY RP-HPLC

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ABSTRACT : The objective of the present research work was to develop a innovative, simple, and economic method for estimation of Ketoconazole in bulk and dosage form by RP-HPLC.The chromatographic conditions were performed on Phenomenex Luna C18, 100A, 5µm, 250mmx4.6mm i.d.as stationary phase and mobile phase was prepared with a mixture of Acetonitrile : 0.2% triethylamine (pH-6.5) = 70:30 (pH-6.5) flow 1.0 ml/min, with Injection Volume 10µl, at detection wavelength 243 nm and run time at 5.0 min.The analytical method is valid for estimation of Ketoconazole over a range of 10µg/ml–50 µg/ml. The results of system suitability test, linearity, precision and accuracy, robustness, specificity, LOD and LOQ and stabilities presented in this report are within the acceptance range.A specific, sensitive, economic method estimation of Ketoconazole has been developed based on ICH Guidelines with bulk and dosage forms.
Key Words: Ketoconazole , HPLC, Method Development, ICH, Validation, Accuracy, Precision.

I.INTRODUCTION

Ketoconazole is a lipophilic imidazole derivative appears as white to off white crystalline powder. The drug is not miscible in water, miscible in strong bases and soluble to a low extent in strong acid, having molecular weight of 531.44. It is a feeble base, having acid dissociation constant values of 6.51 and 2.94, it contains five-membered azole ring emboding two nitrogen atoms.[1-4] It is a chiral drug containing a racemic (1:1) mixture of enantiomers of the *cis* configuration. It is a expansive spectrum antifungal imidazole agent that has been shown to be efficient in the treatment of human superficial and systemic fungal infections, against *Candida* species, tinea cruris, tinea pedis, seborrheic dermatitis, possessing anti-inflammatory and minute antibacterial activities with topical together with systemic action. ketoconazole is formulated in a variety of dosage forms including tablets, topical creams, ointments, gels and antidandruff shampoo. Ketoconazole as an azole derivative act by inhibition of sterol 14-α-demethylase, a microsomal cytochrome P450 dependent enzyme system which converts lanosterol into ergosterol indispensable for fungal cell membrane organization. Ketoconazole obstruct the changeover of the lanosterol in ergosterol and disturb the virtue of membrane-bound enzymes and fungal cell membranes. This ketoconazole action increases membrane permeability and the leakage of small ions, amino acids and proteins from the fungi, leading to cell death. Ketoconazole spectrum of activity is broad as an antifungal drug.

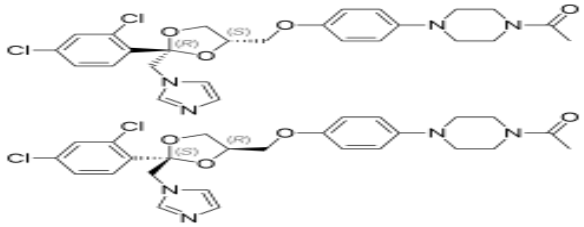


Fig-1: Structure of Ketoconazole

IUPAC Name : 1-[4-(4-{[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy}phenyl)piperazin-1-yl]ethan-1-one

There are only few relevant HPLC methods available for the estimation of ketoconazole in pharmaceutical solid dosage forms[5-9]. The objective of this research is to develop a novel, easy, precise, less time utilizing and reproducible method and validate it according to ICH guideline.[10-11]
A survey of literature reveals that good analytical methods are not available for Ketoconazole. The present research manuscript describes innovative, simple, economical, accurate, specific, robust, rugged and rapid RP-

HPLC method developed in selected solvent system (Mobile Phase) and validated in accordance with International Conference on Harmonization (ICH) Guidelines Q2 (R1), for the estimation of Ketoconazole in bulk drug and in its dosage forms.

II.EXPERIMENTAL

2.1 Materials and Methods:

Pharmaceutical grade working standard Ketoconazole were obtained from Syncorp Pvt. Laboratories, Hyderabad, India. All chemicals and reagents were HPLC grade and were purchased from S D Fine-Chem Limited & Loba Chemie Pvt Ltd, Mumbai, India.

2.2 Instrumentation:

The analysis was performed using HPLC (Waters-717 series) with PDA detector and data handling system EMPOWER2 software, UV-Visible double beam spectrophotometer (ELICO SL-159), analytical balance 0.1mg Sensitivity (SHIMADZU), pH meter (Labindia), ultra sonicator. The column used is Phenomenex Luna C_{18}, 100A, 5µm, 250mmx4.6mm i.d. (as Stationary phase) with the flow rate 1.0ml/min (isocratic).

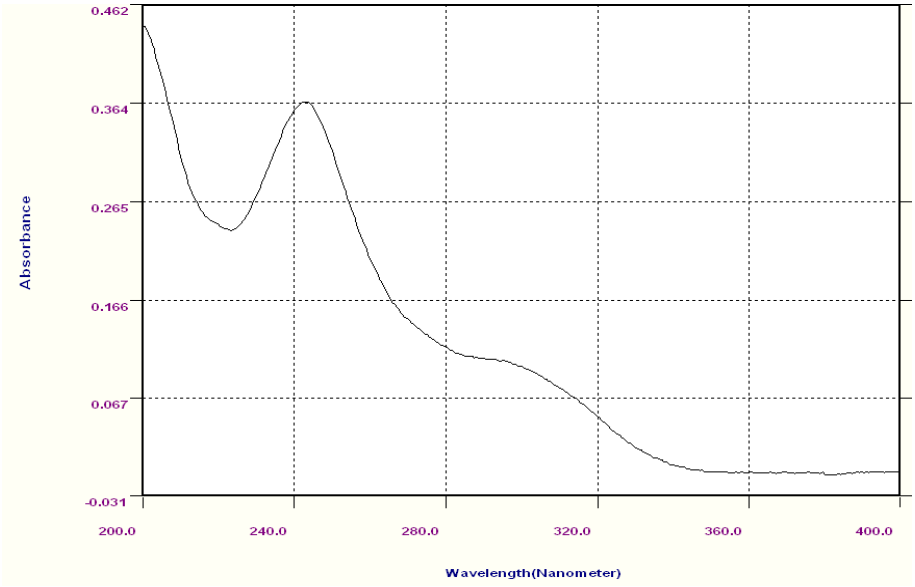
2.3 Sample & Standard Preparation for the Analysis

25 mg of Ketoconazole standard was transferred into 25 ml volumetric flask, dissolved & make up to volume with mobile phase. Further dilution was done by transferring 0.1ml of the above solution into a 10ml volumetric flask and make up to volume with mobile phase.

2.4 Selection of wavelength

The standard & sample stock solutions were prepared separately by dissolving standard & sample in a solvent in mobile phase diluting with the same solvent. (After optimization of all conditions) for UV analysis. It scanned in the UV spectrum in the range of 200 to 400nm. While scanning the Ketoconazole solution we observed the maxima at 243 nm.

Fig-2: UV Spectrum for Ketoconazole



2.5 Method Development

2.5.1 Preparation of 0.2% triethylamine (pH- 6.5)Solution:

Dissolve 7.0ml of triethylamine in 800ml of water. Adjust the pH to 6.5 +\- 0.1 with phosphoric acid and dilute with water to 1000ml and filtered through 0.45 µm filter under vacuum filtration.

2.5.2 Preparation of Mobile Phase:

The mobile phase used in this analysis consists of a mixture of ACN and 0.2% triethylamine with pH adjusted to 6.5 with ratio of 70:30.

700 ml of this Acetonitrile was added and properly mixed with 300 ml of 0.2% triethylamine with pH adjusted to 6.5 and a homogenous solution is achieved. This mobile phase was filled and sonicated for 15 minutes before using in the experiment.

2.5.3 Summary of Optimized Chromatographic Conditions:

The Optimum Chromatographic conditions obtained from experiments can be summarized as below:

Table-1: Summary of Optimised Chromatographic Conditions

Mobile phase	Acetonitrile : 0.2% triethylamine = 70:30 (pH-6.5)
Column	Phenomenex Luna C_{18}, 100A, 5µm, 250mmx4.6mm i.d.
Flow rate	1.0 ml/ min.
Wavelength	243nm
Sampling System	Automatic
Temp. of Auto sampler	Ambient
Volume of injection	10µl
Run time	05min.
Mode of Separation	Isocratic

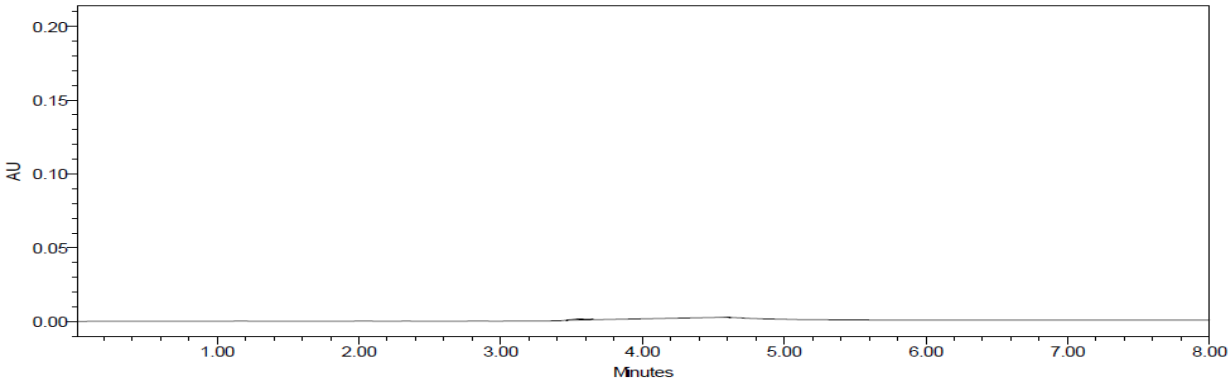
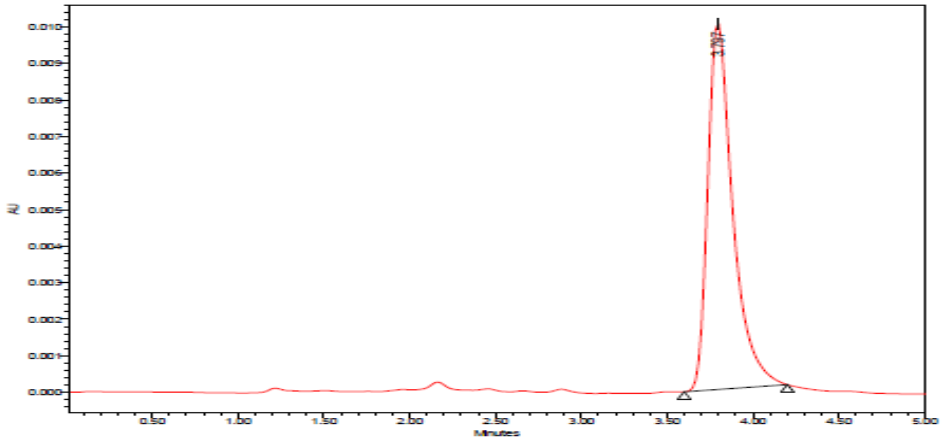


Fig-3: Chromatogram for Blank Preparation



Fig–4: Chromatogram of Ketoconazole in Optimized Condition

2.6 Method validation:

2.6.1 Linearity & Range:

Calibration standards at five levels were prepared by appropriately mixed and further diluted standard stock solutions in the concentration ranges from 10-50µg/mL for Ketoconazole. Samples in triple injections were made for each prepared concentration. Peak areas were plotted against the corresponding concentration to obtain the linearity graphs. Chromatograms of each solution were recorded.

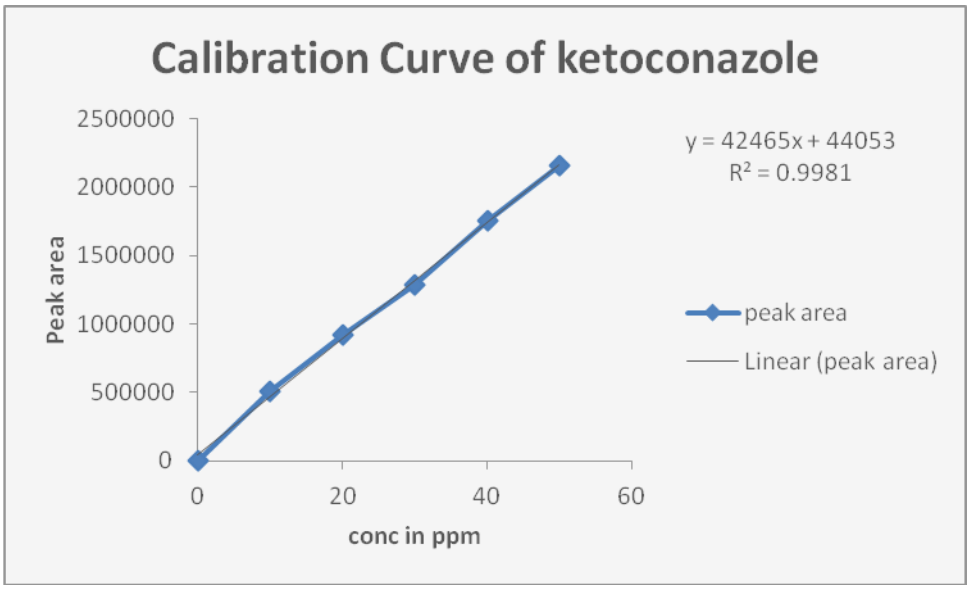


Fig-5: Calibration Curve of Ketoconazole(API)

Table-2: Concentration of Ketoconazole

CONC.(µg/ml)	MEAN AUC (n=6)
0	0
10	512458
20	924587
30	1287954
40	1754261
50	2154783

2.6.2. Accuracy:

To determine the accuracy of the proposed method, recovery studies were carried out by adding different amounts (80%, 100%, and 120%) of pure drug of Ketoconazole were taken and added to the pre-analyzed formulation of concentration 10µg/ml. From that percentage recovery values were calculated. The results were shown in table-3.

Table-3: Accuracy Readings of Ketoconazole

S. No.	Conc amount added	Peak Area	Conc. Amount Found	% Recovery of Pure drug	Statistical analysis
S_1 : 80 %	8	379492	7.899188	98.73984	Mean= 98.470% S.D. = 0.25366581 R.S.D.= 0.25%
S_2 : 80 %	8	377781	7.858896	98.23619	
S_3 : 80 %	8	378457	7.874815	98.43518	
S_4 : 100 %	10	464589	9.90312	99.0312	Mean= 100.382%

S_5 : 100 %	10	469845	10.02689	100.2689	S.D. = 1.41142 R.S.D.= 1.400086%
S_6 : 100 %	10	476547	10.18472	101.8472	
S_7 : 120 %	12	549458	11.90168	99.1807	Mean= 100.465% S.D. = 1.54521 R.S.D. = 1.5380%
S_8 : 120 %	12	562547	12.20991	101.7493	
S_9 : 120 %	12	563586	12.23438	101.9532	

2.6.3. Precision:

2.6.3.1. Repeatability

The precision of each method was ascertained separately from the peak areas & retention times obtained by actual determination of six replicates of a fixed amount of drug. Ketoconazole (API). The percent relative standard deviation was calculated for Ketoconazole are presented in the Table-4.

Table-4: Repeatability Readings of Ketoconazole

HPLC Injection Replicates of Ketoconazole	Retention Time	Area
Replicate – 1	3.797	10652428
Replicate – 2	3.799	10578247
Replicate – 3	3.801	10353404
Replicate – 4	3.802	10576244
Replicate – 5	3.805	10176758
Replicate – 6	3.803	10325646
Average	3.801167	10443787.89
Standard Deviation	0.002858	185788.2
% RSD	0.075181	1.778935%

2.6.3.2. Intermediate precision:

The intra & inter day variation of the method was carried out & the high values of mean assay & low values of standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Ketoconazole revealed that the proposed method is precise.

Table-5: Results of Intra-Assay & Inter-Assay

Conc. Of Ketoconazole (API) (µg/ml)	Observed Conc. Of Ketoconazole (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
8	08.36	0.85	08.32	0.99
10	10.29	0.44	10.55	0.25
12	12.41	0.15	12.62	0.34

2.6.4. Method Robustness:

Influence of little changes in optimized chromatographic conditions like changes in flow rate (± 0.1ml/min), mobile phase ratio (±2%), Wavelength of detection (±2nm) and Acetonitrile content in mobile phase (±2%) studied to measure the robustness of the method are also in favour of (Table-36, % RSD < 2%) the developed RP-HPLC method for the analysis of Ketoconazole (API).

Table-6: Results of Method Robustness Test

Change in parameter	% RSD
Flow (1.1 ml/min)	0.40
Flow (0.9 ml/min)	0.42
Temperature (27^0C)	0.08
Temperature (23^0C)	0.06
Wavelength of Detection (241 nm)	0.32
Wavelength of detection (245 nm)	0.38

2.6.5. LOD & LOQ:

The detection limit (LOD) and quantitation limit (LOQ) may be expressed as:

L.O.D. = 3.3(SD/S).

L.O.Q. = 10(SD/S)

Where, SD = Standard deviation of the response

S = Slope of the calibration curve

2.6.6. System Suitability Parameter

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. Following system suitability test parameters were established. The data are shown in Table-7.

Table-7: Data of System Suitability Parameter

S.No.	Parameter	Limit	Result
1	Resolution	Rs > 2	8.05
2	Asymmetry	T ≤ 2	Ketoconazole=0.14
3	Theoretical plate	N > 2000	Ketoconazole =2864

2.6.7 Estimation of Ketoconazole in Tablet Dosage Form

Twenty tablets were taken and the I.P. method was followed to calculate the average weight. Above weighed tablets were finally powdered and triturated well. Some quantity of powder which is equivalent to 25 mg of drug was transferred to a clean and dry 25 ml volumetric flask, make and solution was sonicated for fifteen minutes. Then the volume was made up to 25 ml with the same Mobile Phase. Then 10 ml of the prepared above solution was diluted to 100 ml with the help of mobile phase. The resulted solution was filtered through a membrane filter (0.45 µm) and sonicated to degas. The final solution prepared was injected in 5 replicates into the HPLC system and the s are record the observations. Two duplicate injections of the standard solution were also injected into the HPLC system and the peak areas were recorded. The data are shown in Table-8.

Table-8: Assay of KETOCONAZOLE Tablets

Brand name of Tablets	Labelled amount of Drug (mg)	Mean (±SD) amount (mg) found by the proposed method (n=6)	Assay + % RSD
KETOWELL 200	200	199.52(±0.06)	99.76 (±0.48)

III.RESULTS

The optimized chromatographic conditions were Phenomenex Luna C18, 100A, 5µm, 250mmx4.6mm i.d.as stationary phase and mobile phase was prepared with a mixture of Acetonitrile : 0.2% Triethylamine (pH-6.5) = 70:30 (pH-6.5) flow 1.0 ml/min, with Injection Volume 10µl, at detection wavelength 243 nm and run time at 5.0 min. In these chromatographic conditions the peak was pure, sharp, symmetric and found a greater number of theoretical plates.

The results obtained in method validation were

Linearity & Range: The calibration curve showed good linearity in the range of 10-50 µg/ml, for Ketoconazole (API) with correlation coefficient (r^2) of 0.999 (Fig-5). A typical calibration curve has the regression equation of y = 42465x + 44053 for Ketoconazole .

Accuracy: The mean recoveries were found to be 98.470, 100.382 and 100.465 for Ketoconazole . The limit for mean % recovery is 98-102% and as both the values are within the limit, hence it can be said that the proposed method was accurate.
Repeatability: The repeatability study which was conducted on the solution having the concentration of about 10 µg/ml for Ketoconazole (n =6) showed %RSD of 1.778935%. It was concluded that the analytical technique showed good repeatability.
LOD & LOQ: The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 0.02 & 0.08 µg/ml respectively.
Assay: The assay in KETOWELL 200 tablets containing Ketoconazole was found to be 99.76 %.

IV.DISCUSSION

To develop a precise, linear, specific RP-HPLC method for analysis of Ketoconazole , different chromatographic conditions were applied & the results observed were compared with the methods available in literatures.
Verma Vikrant*[12] et al.,The present research article describes the method development and validation of ketoconazole by an innovative HPLC method in dosage form that are solid in nature i.e tablets. Inertsil ODS-C18 column (150mm,4.6mm,5µm) was used for the study and mobile phase consists of buffer (pH-4) and Methanol in the mixture of 30:70. Wavelength for the chromatographic separation used was 274nm. Injection volume was maintained at 20µl. A wide variety of mobile phase combinations used for the study and system contains integrated degasser. **Prakash Vamanrao Diwan*[13] et al.,** Docetaxel has significant single agent activity in prostate cancer and ketoconazole also has activity as a second line hormonal agent. In vitro, ketoconazole is synergistic with some chemotherapy agents by enhancing the intracellular retention of the cytotoxic agent. A potential drug-drug interaction exists though between docetaxel and ketoconazole because both agents are metabolized hepatically by the cytochrome P-450 system. Hence, a nanoparticulate system was formulated by loading both drugs for tumor targeting. Assay and in vitro release of the formulation were conducted by developing simple, precise, accurate, and validated analytical method for simulataneous determination docetaxel and ketoconazole using reversed-phase high-performance liquid chromatography (RP-HPLC). The RP-HPLC method was developed using Waters Symmetry C18 column (25 cm × 4.5 mm, 5 µm) with a mobile phase consisting of acetonitrile and 0.2% triethylamine pH adjusted to 6.4 (48:52, v/v) at flow rate of 1 mL/min. **Ramavath Mohanbabu Naik*[14] et al.,** 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. **Sunil Kumar*[15] et al.,** 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.

V.CONCLUSION

A sensitive & selective stability indicting RP-HPLC method has been developed & validated for the analysis of Ketoconazole API. Based on peak purity results, obtained from the analysis of samples using described method, it can be concluded that the absence of co-eluting peak along with the main peak of Ketoconazole indicated that the developed method is specific for the estimation of Ketoconazole . Further the proposed RP-HPLC method has excellent sensitivity, precision and reproducibility.

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