Research Article

KETOCONAZOLE HPLC METHOD DEVELOPMENT AND VALIDATION: A NOVEL APPROACH

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ABSTRACT

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. Flow rates was maintained at 1ml/min throughout the process for mobile phase combinations. In this method ICH guidelines were used for validation studies. After analysis of different research and review articles it came to light that HPLC method development of ketoconazole has been done in different dosage forms but less work is done on solid dosage forms and hence still there is enormous potential for new methods to be developed in solid dosage form with different mobile phase combinations.

Keywords: ketoconazole, Linearity, ICH

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 embodying two nitrogen atoms. 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.

Ketoconazole cure fungal infections that has dissemination to distinctive fragments of the body through the blood such as the mouth, skin, urinary tract and blood and certain fungal infections that evolve on the skin, lungs and can transmit through the body. Fungal skin or nails fungal infections also treated with ketoconazole that cannot be treated with other

medications. Ketoconazole(cis1acetyl4[4[[2(2,4dichlorophenyl)2(1Himidazol1ylmethyl)1,3dioxolan4yl]methoxy]phenyl]piperazine) has complex structure act by slowing the growth of fungi¹⁻⁴.

There are only few relevant HPLC methods available for the estimation of ketoconazole in pharmaceutical solid dosage forms⁵⁻⁹. The objective of this research is to develop a novel, easy, precise, less time utilizing and reproducible method and validate it according to ICH guidelines.

MATERIALS AND METHODS

Wavelength Selection

Standard stock solution of ketoconazole was prepared by dissolving 20mg of ketoconazole standard into 100ml of methanol which gives 200µg/ml of standard stock solution. From this solution 1ml was taken and diluted with 10ml of methanol to give 20 µg /ml standard working solution which was sonicated for 15min to remove dissolved gases. UV spectra of above solution taken between the range of 200nm-400nm using methanol as a blank.

Mobile Phase Selection

Standard stock solution of ketoconazole was prepared by dissolving 20 mg of ketoconazole standard into 100ml of methanol which gives 200 µg /ml of standard stock solution. From this solution 1ml was taken and diluted it with 10 ml of mobile phase (mobile phase which used for trials) to give 20 µg /ml standard working solution. Above working standard preparation was injected for mobile phase selection. Different mobile phase combinations were used for the study until system suitability parameters was obtained.

Instrumentation and Developed Method Detail

Chromatographic Condition: The HPLC system consisted of Agilent 1220 Infinity consisting of gradient pump having a flow range from 0.2 up to 10 mL/min and Inertsil octa decyl silane (150X4.6X5 μ) C- 18 column was used. The HPLC system was equipped with the software EZChrom Software having double-beam photometer with light source deuterium lamp and wavelength limit of 190-600 nm having integrated degasser¹⁰. The Mobile phase was a composite of buffer (pH-4) and Methanol in the ratio of 30:70. Flow rate was maintained at 1ml/minute. Wavelength for the chromatographic separation used was 274 nm. Injection volume was maintained at 20 μ l. The column was maintained at ambient temperature. The mobile phase was purified by passing through 0.45 μ m micron filter prior to use.

Materials

Ketoconazole reference standard was furnished by the Yaksh Pharma Ghatkopar Mumbai, India. The pharmaceutical dosage form of ketoconazole, Ketovate tablets, was procured from local pharmacy with a 200mg label claim, manufactured by Bal Pharma, India. All chemicals used was of HPLC grade, Methanol and water both HPLC grade, was from Rankem, disodium hydrogen phosphate and potassium dihydrogen phosphate from Fisher scientific.

Procedure For Buffer Preparation (Phosphate Buffer, pH 4)- Disodium hydrogen phosphate 5.04 g was dissolved in 3.01 g of potassium dihydrogen phosphate in ample water to produce 1000 ml. pH of the solution was modified with glacial acetic acid¹¹.

U.V Spectra of Ketoconazole-U.V spectra was obtained from double beam UV-spectrophotometer having model UV-2700 .

U.V spectra of the ketoconazole standard is shown in Figure 1.

Figure 1 U.V spectra of standard ketoconazole

METHOD DEVELOPMENT

Trials obtained during method development- Different trials was performed during method development some important trials are given from figure 2 to figure 8

Figure 2: Trial 1- This trial was performed in mobile phase combination of water-methanol 50-50.

Figure 3: Trial 2- This trial was performed in mobile phase combination of water-methanol 40-60.

Figure 4: Trial 3- This trial was performed in mobile phase combination of water acetonitrile 40-60.

Figure 5: Trial 4- This trial was performed in mobile phase combination of phosphate buffer pH 5.0-methanol 40-60.

Figure 6: Trial 5- This trial was performed in mobile phase combination of phosphate buffer pH 5.5-methanol 40-60.

Figure 7: Trial 6- This trial was performed in mobile phase combination of phosphate buffer pH 4.5-Methanol 40-60.

Figure 8: Final trial 7- This trial was performed in mobile phase combination of phosphate buffer pH 4.0-Methanol 30-70.

METHOD VALIDATION

(1) **Linearity and Range-** Standard stock solution of ketoconazole was prepared by dissolving 20mg of ketoconazole standard into 100ml of methanol which gives 200µg/ml of standard stock solution. Method linearity was displayed over the concentration range of 10 µg /ml to 30 µg /ml. Concentration range of 10 µg /ml to 30 µg /ml was obtained by dissolving 0.5ml, 0.75ml, 1ml, 1.25ml, 1.5ml of ketoconazole standard stock solution with mobile phase diluted with 10ml to obtain 10 µg /ml, 15 µg/ml, 20 µg/ml, 25 µg/ml, 30 µg/ml of final concentration of ketoconazole for linearity determination¹².

(2) **Precision Studies of Ketoconazole-** Precision studies was carried out in three different ways as intraday precision, interday precision and repeatability. Intraday precision studies was conducted for three different concentrations at three times each for single day and interday precision studies was conducted for three different concentrations at three times at alternate days respectively and repeatability for one concentration at six times.

Intraday Precision

Intraday precision was performed for three concentrations at lower concentration KTC-10µg/ml ,middle concentration KTC-20µg/ml and higher concentration KTC-30µg/ml .

Interday Precision

Interday precision was performed for three different concentrations respectively at lower concentration KTC-10µg/ml ,middle concentration KTC-20µg/ml and higher concentration KTC-30µg/ml for day-1, day-2 and day-3.

Repeatability Studies

Repeatability studies was carried out for one concentration six times for the middle concentration at KTC 20 µg/ml.

(3) **RECOVERY/ACCURACY STUDIES-** Recovery studies were performed by spiking the sample with the standard and then finding out the recovery at 80,100,120 percent. In this a chunk of standard drug was mixed to the sample preparation and find out recovered amount from sample preparation as % recovery.

Procedure: Standard Stock Solution of Ketoconazole- Standard stock solution of Ketoconazole was made by dissolving 20mg of standard drug in 100 ml of Methanol to make 200 µg/ml solution.

Standard Working Solution for Recovery (KTC-20µg/ml): 1ml of standard stock solution of ketoconazole was taken and diluted with 10ml of mobile phase.

Sample Stock Solution: Weigh and powdered 20 tablets. Take tablet powder equivalent to 20mg of ketoconazole in to a 100ml

volumetric flask. Add 60 ml Methanol. Shake for 15 minutes and sonicate for 10 minutes. Make up volume with Methanol. Filter this solution through 0.45 µm micron filter prior to use to give KTC-200µg/ml)

Sample Solution for Recovery (KTC-20 µg/ml):

Take 1ml from sample stock solution and then diluted with 10 ml of mobile phase.

Recovery Solution Preparation: Recovery solution was prepared for standard by dissolving 0.8ml, 1ml and 1.2 of standard ketoconazole and then volume make upto 10ml with the diluent. Recovery solution of sample was prepared by dissolving 1ml from sample stock solution and then diluted with 10ml of mobile phase to give 20µg/ml. After that Inject standard solution for recovery, sample solution for recovery and recovery solutions of 80%, 100% and 120% and then calculate % recovery.

(4) **LIMIT OF DETECTION (D.L)-** There are different ways to find the limit of detection according to the ICH guidelines. In one method calibration curve construct standard deviation of y-intercepts of regression lines is used as the standard deviation. The values of standard deviation and slope are put in the equation to get LOD.

$$D.L = 3.3\sigma/S$$

(5) **LIMIT OF QUANTITATION (Q.L)-** There are different ways to find the limit of detection according to the ICH guidelines. In one method calibration curve construct standard deviation of y-intercepts of regression lines is used as the standard deviation. The values of standard deviation and slope are put in the equation to get LOD.

$$Q.L = 10\sigma/S$$

(6) ASSAY STUDIES

Materials

Ketoconazole reference standard was furnished by the Yaksh Pharma Ghatkopar ,mumbai, India. The pharmaceutical dosage form of ketoconazole, Ketovate tablets, was procured from local pharmacy with a 200 mg label claim, manufactured by Bal Pharma, India. All chemicals used was of HPLC grade, Methanol and water both HPLC grade, was from Rankem, disodium hydrogen phosphate and potassium dihydrogen phosphate from Fisher scientific.

Assay: Standard stock solution of ketoconazole was prepared by dissolving 20 mg of ketoconazole standard into 100ml of methanol which gives 200 µg /ml of standard stock solution.

Working Standard Preparation: From this solution take 1ml and dilute it with 10ml of mobile phase to give 20 µg /ml standard working solution which was sonicated for 15min to remove dissolved gases.

Assay preparation (Marketed Formulation)

Sample Stock Solution

Weigh and powder 20 tablets. Take tablet powder equivalent to 20 mg Ketoconazole in to a 100 ml volumetric flask. Add 60 ml Methanol. Shake for 15 minutes and sonicate for 10 minutes. Make up volume with Methanol.

Working Sample Preparation

Take 1ml from sample stock solution and dilute it with 10 ml of mobile phase. Inject working standard preparation and working sample preparation for assay analysis.

(7) **Specificity**-To analyse the specificity of the method different chromatograms was obtained for sample, blank and standard to ascertain that the method is specific or not or is there any interference from any impurities or exipients.

(8) **Robustness Study** – Robustness studies was performed by injecting working standard preparation for different flow rate, different pH and different mobile phase compositions. Flow rate was changed from +0.2ml/minute to -0.2ml/minute, Buffer pH from +0.2 pH to -0.2pH and solvent percentage in mobile phase from +2% solvent to -2% solvent in mobile phase

RESULT AND DISCUSSION

Mobile Phase Selection

The final mobile phase combination selected which shows system suitability parameter was Phosphate buffer pH 4.0-Methanol in the ratio of 30-70 in standard ketoconazole. System suitability parameters are shown in table 1 for trial 7

Table 1 System suitability parameters for final selected trial

Retention time[min]	Area	Height	Assymetry	Theoretical plate
3.303	2263.226	387.261	1.318	6940

Linearity

The linearity graph and linearity chromatogram are shown in figure 9 and figure 10 and table 2 represents linearity data concentration vs time. The method was found to show linearity in the domain of 10 µg/ml to 30 µg/ml respectively.

Figure 9 Linearity graph of ketoconazole

Figure 10 Linearity chromatogram of ketoconazole

Table 2 Linearity data- concentration versus time

S.No	Concentration(µg/ml)	Area
1	10	1136.791
2	15	1667.695
3	20	2276.669
4	25	2812.87
5	30	3401.921

Precision studies of ketoconazole-Precision studies was carried out and the method was found to be precise for intraday, interday and on repeatable determinations. Table 3-5 represents intraday precision figure. The % R.S.D was found within the limits.

Intraday Precision

Table 3,4,5 show the area, average area, standard deviation and %RSD at 10 µg/ml, 20 µg/ml and 30 µg/ml respectively for intraday precision.

Table 3: Intraday precision at 10 µg/ml

STANDARD	AREA
1	1124.4
2	1135.658
3	1128.802
Average	1129.62
Standard deviation	5.673401449
%RSD	0.502239819

Table 4: Intraday precision at 20 µg/ml

STANDARD	AREA
1	2267.69
2	2256.368
3	2260.882
Average	2261.646667
Standard deviation	5.699601506
%RSD	0.252011138

Table 5: Intraday precision at 30 µg/ml

STANDARD	AREA
1	3390.793
2	3366.973
3	3383.848
Average	3380.538
Standard deviation	12.25010918
%RSD	0.362371587

Interday Precision

Table 6,7,8 show the area, average area, standard deviation and % RSD at 10 µg/ml, 20 µg/ml and 30 µg/ml respectively for interday precision.

Table 6: Interday precision at 10 µg/ml

STANDARD	AREA
1	1117.623
2	1095.277
3	1106.229
Average	1106.376
Standard deviation	11.17373
%RSD	1.009939

Table 7: Interday precision at 20 µg/ml

STANDARD	AREA
1	2276.815
2	2297.355
3	2274.376
Average	2282.849
Standard deviation	12.6219
%RSD	0.552901

Table 8: Interday precision at 30 µg/ml

STANDARD	AREA
1	3385.015
2	3408.77
3	3378.073
Average	3390.619
Standard deviation	16.0976
%RSD	0.474769

Repeatability Studies- Table 9 show the area, average area, standard deviation and % RSD at 20 µg/ml. The method was found to be precise with the repeatability studies.

Table 9: Repeatability study at 20 µg/ml

STANDARD	AREA
1	2269.981
2	2283.65

3	2295.139
4	2285.97
5	2308.867
6	2290.403
Average	2289.001667
Standard deviation	12.91417178
%RSD	0.56418359

Recovery Studies

Results obtained from recovery studies are given in table 10 and table 11 with average recovery found out to be 101.112, 99.862 and 98.573 respectively, standard deviation was 0.9340987, 0.9819543 and 0.9852619 and %R.S.D was 0.9238260, 0.9833150 and 0.9995260. Percentage recovery was found to be within limits. The representative chromatograms obtained during recovery studies are given in figure 11 and figure 12 for sample for recovery and standard for recovery respectively.

Table 10: Area of sample and standard

Spiking of standard	Area of sample spike with standard	Area of sample	Net area of standard	Area of standard
80%	2056.222	1133.024	923.198	1144.716
	2052.145	1133.024	919.121	1144.716
	2068.573	1133.024	935.549	1144.716
100%	2283.841	1133.024	1150.817	1144.716
	2263.255	1133.024	1130.231	1144.716
	2281.372	1133.024	1148.348	1144.716
120%	2499.043	1133.024	1366.019	1144.716
	2489.807	1133.024	1356.783	1144.716
	2472.39	1133.024	1339.366	1144.716

Table 11: Percentage recovery, standard deviation and % R.S.D

Sample amount (mcg/ml)	Standard Amount added(mcg/ml)	Standard Amount recovered(mcg/ml)	%Recovery	Average	Standard Deviation	%R.S.D
10	8	8.064864997	100.8108125	101.112	0.9340987	0.9238260
10	8	8.029249176	100.3656147			
10	8	8.172760755	102.1595094			
10	10	10.05329706	100.5329706	99.862	0.9819543	0.9833150
10	10	9.873462064	98.73462064			
10	10	10.03172839	100.3172839			
10	12	11.93325681	99.44380673	98.573	0.9852619	0.9995260
10	12	11.85257304	98.771442			
10	12	11.70042176	97.5035147

Figure 11: Chromatogram obtained for the sample for recovery **Figure 12: Chromatogram obtained for the standard for recovery**

(4) LIMIT OF DETECTION (D.L)- The limit of detection was found out to be 0.809077855.

(5) LIMIT OF QUANTITATION (Q.L)- The limit of quantitation was found out to be 2.451751077.

(6) ASSAY STUDIES –Assay results are given in table 12 which shows the area of standard, area of samples, % assay, average assay, standard deviation and %R.S.D of assay and

table 13 which shows label claim, result (actual) found, assay, average of assay, standard deviation and %R.S.D of ketoconazole and figure 13-15 contains chromatograms obtained for the sample for first, second and third determination and figure 16 contains chromatogram of the standard.

Table 12: Area of standard, area of samples average assay, standard deviation and % R.S.D of assay

S.No	Area of standard	
1.	2276.718	
S.No	Area Of Samples	% Assay

1.	2132.55	93.66772696
2.	2153.959	94.60807179
3.	2110.886	92.7161818
Average assay		93.66399352
Standard deviation		0.945950522
% RSD of assay		1.009940412

Table 13: Label claim, result (actual) found, assay, average of assay, standard deviation and %R.S.D of ketoconazole

Serial no	Label claim(w/w)	Result(w/w)	% Assay	Average % assay	Standard deviation	%RSD
1	200	187.3354539	93.66772696	93.66399352	0.945951	1.00994
2	200	189.2161436	94.60807179			
3	200	185.4323636	92.7161818

Figure 13: Chromatogram obtained for the sample preparation at first determination

Figure 16: The chromatogram obtained for the standard

Figure 14: Chromatogram obtained for the sample preparation at second determination

Figure 15: Chromatogram obtained for the sample preparation at third determination

(7) **SPECIFICITY**-To judge the specificity of the method following chromatograms was obtained for sample, blank and standard given in figure 17-19 and it was observed that no intervention was found from impurities and exipients in developed method.

Figure 17: Method specificity chromatogram obtained for sample

Figure 18: Method specificity chromatogram obtained for blank

Figure 19: Method specificity chromatogram obtained for standard

(8) ROBUSTNESS STUDIES OF KETOCONAZOLE –The robustness study was carried out and the method was found to be robust. Flow rate data are given in table 14 and table 15 for the variation in the flow rate by increasing or decreasing by 0.2 at 20 µg/ml. Table 16 and table 17 shows effect of change in mobile phase composition by 2% variation at 20 µg/ml and table 18 and table 19 shows the effect of change in pH by increasing or decreasing by 0.2 at 20 µg/ml.

Table 14: Effect of increase in flow rate

AT FLOW RATE +0.2	
S.NO	AREA
1	2234.003
2	2251.865
3	2233.858
Average	2239.909
Standard deviation	10.35474
%RSD	0.462284

Table 15: Effect of decrease in flow rate

AT FLOW RATE -0.2	
S.NO	AREA
1	2276.739
2	2299.579
3	2283.494
Average	2286.604
Standard deviation	11.73331
%RSD	0.513132

Table 16: Effect of change in mobile phase

AT MOBILE PHASE +2.0	
S.NO	AREA
1	2209.068
2	2222.375
3	2233.491
Average	2221.645
Standard deviation	12.22787
%RSD	0.550397

Table 17: Effect of change in mobile phase

AT MOBILE PHASE -2.0	
S.NO	AREA
1	2299.125
2	2322.16
3	2336.091
Average	2319.125
Standard deviation	18.66891
%RSD	0.804998

Table 18: Effect of increase in pH by 0.2

AT pH +0.2	
S.NO	AREA
1	2274.519
2	2290.516
3	2283.64
Average	2282.892
Standard deviation	8.024712
%RSD	0.351515

Table 19: Effect of decrease in pH by 0.2

AT pH -0.2	
S.NO	AREA
1	2245.274
2	2227.228
3	2238.356
Average	2236.953
Standard deviation	9.104479
%RSD	0.407004

CONCLUSION

A simple, rapid, less time consuming method was developed for ketoconazole in solid dosage form i.e. tablet the method was observed to be precise, specific, accurate and reproducible. This new innovative method save time and is suitable for use in pharma industries. In this method system suitability yardstick was met at combination of pH4 buffer and methanol at 30:70 ratio. Different trials by various solvent combinations was performed to attain the system suitability yardstick. This method was observed to be specific as there is no intervention of excipients or degradation products, % R.S.D was found to be within limits. Slight variation in mobile phase constitution, pH and flow rate variation does not effect the method performance and hence the method was found out to be robust.

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