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Development and Validation of a Simple and Rapid HPLC Method for Determination of Itraconazole in Bulk and Marketed Formulation

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ABSTRACT

A new simple, accurate, rapid, selective and robust high pressure liquid chromatography (HPLC) method was developed and validated for estimation of itraconazole in bulk and marketed formulation. Acetonitrile and double distilled water was used as a mobile phase for chromatographic separation and estimation on HiQSil C18- HS (250 × 4.6 mm) in the ratio of 90:10 v/v at flow rate of 1.0 ml/min. The detection was carried out with UV detector set at 263 nm. The retention time for itraconazole was found to be 7.75 minutes. The linearity range for itraconazole was found to be 5-60 µg/ml with coefficient of linear regression 0.991. The method was validated in accordance with the requirements of International Conference on Harmonization (ICHQ2 (R1) 2005) guidelines for accuracy, precision, LOD & LOQ, linearity and robustness.

Keywords: Simple, HPLC, Chromatographic separation, Itraconazole, ICH

INTRODUCTION

Itraconazole (ITZ) belongs to triazole class of antifungal agents with molecular weight 706 g/mol and chemical structure shown in Figure 1 [1]. Itraconazole is an orally active antifungal agent, which displays broad spectrum activity against a number of fungal infections [2]. The antifungal action of itraconazole is due to its binding of fungal cytochrome P-450 which leads to inhibition of ergosterol synthesis. Ergosterol is an essential element of the cell membrane which plays an important role in the

growth of fungal and yeast colonies alongwith perturbation of membrane bound enzyme function and membrane permeability [3]. Itraconazole is metabolized [4-6] by means of CYP3A4 enzymatic system to form three active metabolites viz. hydroxy itraconazole, keto-itraconazole and N-desalkylitraconazole. Itraconazole and its metabolites are potent inhibitors of CYP3A4. Analytical methods such as UV spectrophotometric methods [7,8], Visible spectrophotometric method [9], Reverse Phase High Performance Liquid Chromatography [10-15], LCMS [16-18], Ultra Pressure Liquid Chromatography [19], HPTLC [20] methods have been reported for the analysis of Itraconazole. The objective of the present study was to develop simple, accurate, specific and precise HPLC method for the determination of Itraconazole in bulk and pharmaceutical dosage form.

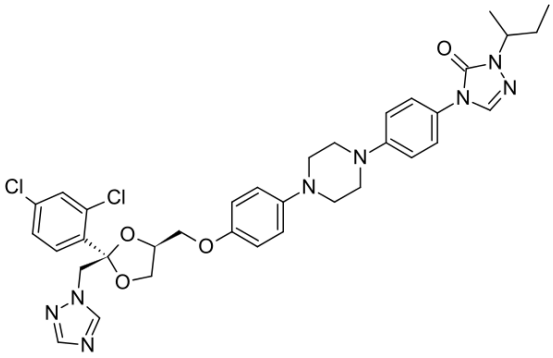

Figure 1: Chemical structure of Itraconazole.

MATERIALS AND METHODS

Pharmaceutical grade ITZ was obtained as generous gift from Amoli organics. Fixed dose capsules (Brand name: Itrasys 100) containing 100 mg of ITZ was purchased. Methanol, acetonitrile (ACN) was purchased from SD Fine Chemicals, Mumbai.

Instruments

HPLC analysis was performed by using Agilent 1200 series which is provided with variable wavelength detector. EZChrom software was used to record the chromatogram.

Experimental procedure

Analytical method development

For preparation of standard stock solution 100 mg ITZ was accurately weighed and transferred into 100 ml volumetric flask and volume was made up to 100 ml with methanol. Working solution was prepared from standard solution. 1ml from stock solution was pipetted out and transferred to 10 ml volumetric flask and volume was made up with the mobile phase.

Preparation of sample solution for estimation from marketed formulation

Marketed ITZ capsules (20 Capsules) were accurately weighed, emptied and crushed into a fine powder. The weight of powder equivalent to 500 mg of ITZ was transferred into 100 ml volumetric flask and dissolved in methanol. The mixture was subjected

to sonication to dissolve drug and then volume was made up to the mark. The solution was filtered through 0.45 µm filter paper. Further, dilutions were made with mobile phase to yield extract.

Selection of detection wavelength

UV absorption spectrum for 10 ppm solution of ITZ was obtained by scanning over the range of 200-400 nm.

Optimization of chromatographic conditions

Many preliminary trials were carried out for selection and optimization of mobile phase, flow rate, volume of sample to be injected and column temperature.

Analytical method validation

Performance characteristics of analytical HPLC method were validated statistically in accordance to the ICH guidelines for analytical method validation [21]. The details are mentioned in Table 1.

Table 1: Analytical method validation parameters and their determination.

<table>
<tr><th>Parameter</th><th colspan="2">Method / procedure followed</th></tr>
<tr><td>Specificity</td><td colspan="2">As per ICH guidelines, specificity should be carried out to ensure identity of an analyte. To determine specificity chromatograms were generated for blank and ITZ.</td></tr>
<tr><td>Accuracy</td><td colspan="2">As per ICH guidelines, accuracy should be evaluated by using a minimum of 9 determinations over a minimum of three concentration levels covering the specified range i.e., 3 concentration levels in triplicate. (e.g., 3 concentrations/ 3 replicate each). Accuracy of the method is described as percent recovery of known added amount of analyte in sample. The percent recovery was calculated by performing recovery studies in triplicates of three concentration levels viz. 80%, 100%, 120% of 10 ppm solution of ITZ. Recovery studies were also carried out on marketed preparation containing ITZ</td></tr>
<tr><td rowspan="3">Precision</td><td colspan="2">Precision was carried out at two levels.</td></tr>
<tr><td>Repeatability</td><td>Intermediate precision</td></tr>
<tr><td>Repeatability was evaluated by using minimum of 9 determinations covering the specified range for the procedure (e.g., 3 concentrations/ 3 replicates each)</td><td>Intermediate precision was established to study the effects of random events i.e., days, on the precision of the analytical procedure. Intraday and interday precision studies were carried out by taking 9 determinations of 3 concentrations/3 replicates each, at 3 times in a same day and on 3 different days, respectively</td></tr>
<tr><td colspan="2">Precision is described as standard deviation and relative standard deviation (coefficient of variation) for each type of precision</td></tr>
<tr><td>Detection limit and quantification limit</td><td colspan="2">Detection limit (DL) and quantification limit (QL) is calculated based on the standard deviation of the response and the slope
LOD(DL)=3.3σ/S LOQ(QL)=10 σ/S
σ = Standard deviation of response estimated based on the calibration curve. S = Slope of the calibration curve.</td></tr>
<tr><td>Linearity</td><td colspan="2">A linear relationship was assessed across the range of 5 to 60 mg for ITZ. According to ICH guidelines, for the establishment of linearity, a minimum of 5 concentrations are recommended. Linearity is reported by the value of the correlation coefficient, y-intercept, and slope of the regression line along with a plot of the data.</td></tr>
<tr><td>Robustness</td><td colspan="2">Robustness was assessed for proving the reliability of an analytical method with respect to deliberate variations in method parameters. To establish robustness of analytical method following factors were studied:
1. Influence of variations of Wavelength
2. Influence of variations in mobile phase composition
3. Influence of variations in flow rate</td></tr>
</table>

Analytical method development

Selection of wavelength

For detecting UV absorption spectrum 10 ppm solution of ITZ was scanned between 200-400 nm and 263 nm (an isosbestic wavelength) was selected as a detection wavelength for chromatographic determination of ITZ as shown in Figure 2.

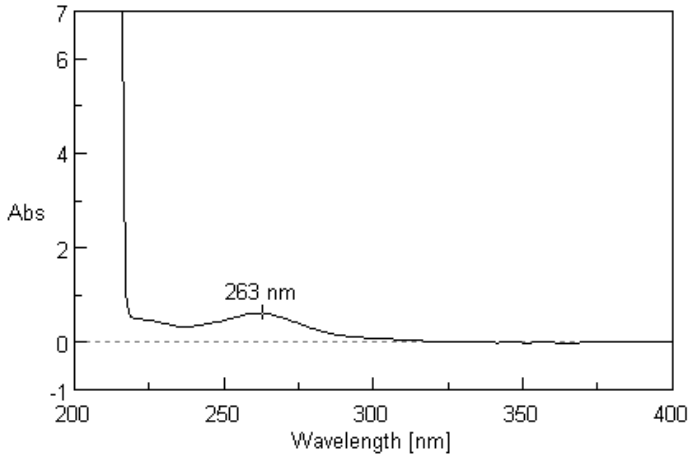

Figure 2: UV spectra of ITZ.

Optimization of chromatographic conditions

C18 column was used for chromatographic estimation of ITZ. Many preliminary trials were carried out for selection of mobile phase; as given in Table 2.

Table 2: Optimization trials for mobile phase composition.

Mobile phase components	Compositions	Retention time
ACN : Water	50:50	>15 min
ACN : Water	70:30	>11min
ACN : Water	90:10	<8min
ACN: Methanol : Water	50:40:10	>15min
ACN: Methanol : Water	60:30:10	>15min
ACN: Methanol : Water	70:20:10	>15min

The flow rate of the mobile phase was varied in the range of 0.5 to 1.2 ml/min and different injection volumes in the range of 20 µl to 50 µl were tried. Optimized mobile phase selected comprised of acetonitrile (ACN): water (90:10). Optimized chromatographic conditions are tabulated in Table 3.

Table 3: Optimized chromatographic conditions.

Mobile Phase	Acetonitrile: Water (90:10)
Stationary Phase	HiQSil C18- HS (250 × 4.6 mm)
Flow rate	1.0 ml/ min
Detection wavelength	263 nm
Injection volume	20 µl

Chromatogram obtained using these optimized chromatographic conditions showed that drug was well resolved and retained at 7.75 minutes. Representative chromatogram is shown in Figure 3.

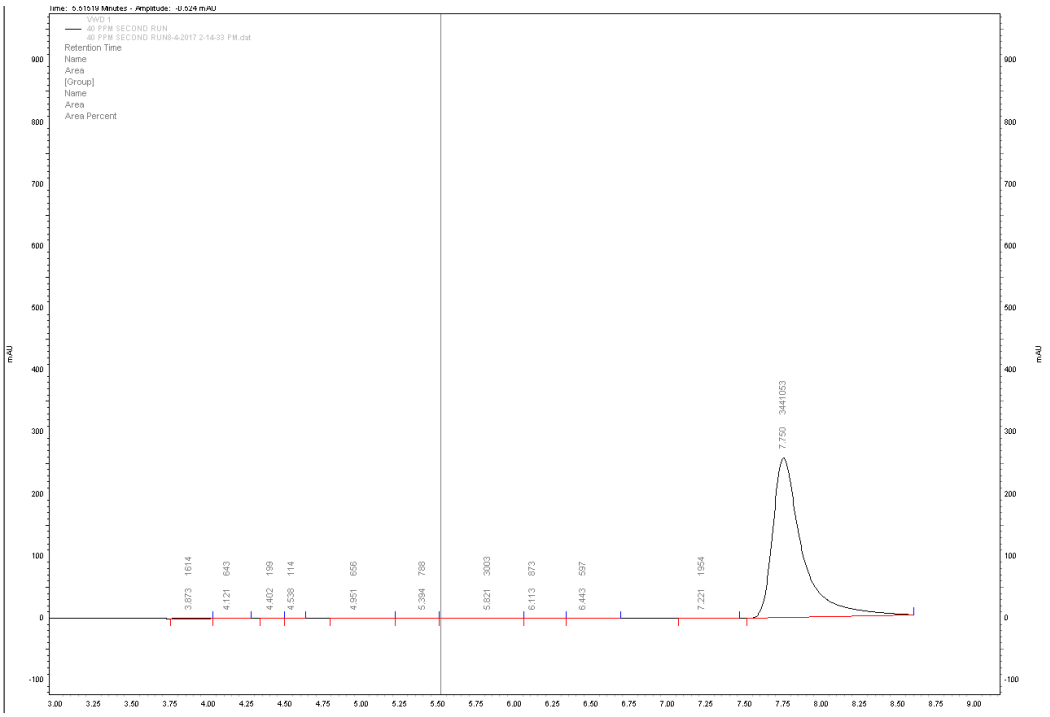

Figure 3: Representative chromatogram of ITZ.

Analytical method validation

Specificity

Chromatograms for blank and ITZ were generated individually to ensure the identity of analyte under study.

Linearity

Serial dilutions of ITZ were prepared making use of standard stock solution and dilutions were made with mobile phase. Responses were recorded as peak area. The peak areas were plotted against concentrations (PPM) to obtain the calibration curve. ITZ was found linear in the range of 5-60 ppm. The linearity plot of ITZ is given in Figure 4. The values of correlation coefficient, y- intercept and slope of regression line was observed to be 0.991, 21274 and 93046 respectively.

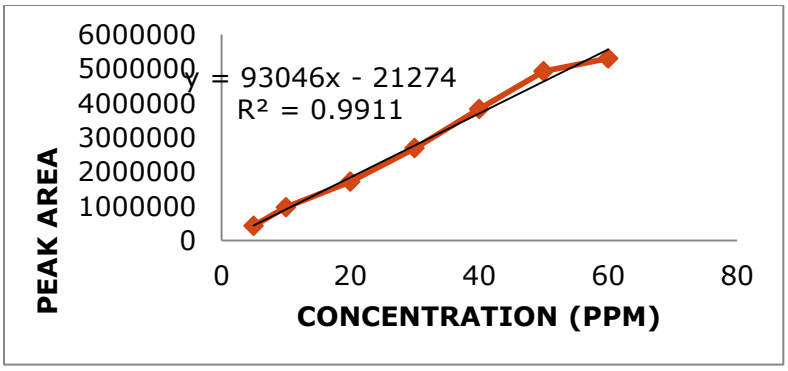

Figure 4: Linearity plot

Limit of detection and limit of quantitation

Values for detection limit and quantification limit calculated based on the standard deviation of the response and the slope of regression line. The calculated values of limit of detection (LOD) and limit of quantitation (LOQ) for ITZ were found to be 0.3356 µg/ml and 1.1657 µg/ml respectively.

Accuracy

Accuracy of the method is reported as percent recovery of known added amount of analyte in sample. The percent recovery was calculated by carrying studies in triplicates of three concentration levels viz. 80%, 100%, 120% of 10 ppm solution of ITZ. Results are tabulated in Table 4.

Table 4: Accuracy data.

	Observations					Inference
Drug	**% Level**	**Concentration before spiking (µg/ml)**	**Total Concentration after spiking (µg/ml)**	**Amount Recovered**	**% Recovery**	
ITZ (bulk)	80	10	18	18.25	101.43	Acceptable recovery hence accurate
	100	10	20	20.48	102.40	
	120	10	22	21.58	98.10	
ITZ (marketed)	80	100	40	39.332	98.33	
	100	100	50	51.105	102.21	
	120	100	60	58.458	97.43	

Precision

The results of interday and intraday precision studies are tabulated in Tables 5 and 6 respectively. Percent RSD values for intraday and interday precision were found within acceptable limit.

Table 5: Data for interday precision.

	Observation			Inference
Level	**LQC**	**MQC**	**HQC**	
Amount	20	40	50	Acceptable % RSD, hence precise
Peak area 1	171463.3	382984.5	4926687	
2	1523333.6	2980201.6	3638252.3	
3	1596485	3493588	4253147	
Avg. peak area	1511483.867	3434544.867	4272695.4	
S.D.	96527.66	427887.9	644439.8	
%RSD	0.374532	0.154391	0.273088	

Table 6: Data for intraday precision.

		Observation			Inference
Level		**LQC**	**MQC**	**HQC**	
Amount		20	40	50	
Peak area	1	1507672	2869451	3500742.6	Acceptable % RSD, hence precise
	2	1523333	2902283.6	3584101.3	
	3	1502327.6	2980201.6	3638252.3	
Avg. peak area		1511111.067	2917312.067	3574365.4	
S.D.		10916.78	56884.22	69269.91	
%RSD		0.599	0.947651	0.985184	

Robustness

To determine robustness of devised analytical HPLC method changes observed in retention time and response were recorded. Method was found to be reliable and robust as retention time and response are not much affected by deliberate variations in mobile phase composition, flow rate and changes in wavelength. The results obtained are tabulated in Table 7.

Table 7: Robustness.

Parameters and variations	**Level of variations**	**% RSD**	**Change in retention time (minutes)**
Proportion of organic phase in mobile phase 90:10(±2)	+2	0.507581	0.365
	-2	0.51.53	0.776
Flow Rate (1.0± 0.2)	+0.2	0.47246	0.128
	-0.2	0.1834	0.115
Wave length	+2	0.44286	0.175
	-2	0.85105	0.123

CONCLUSION

Most of the mobile phases reported for the HPLC separation of itraconazole were ternary or quaternary. Few reported mobile phases were binary that made use of phosphate buffer as one of the component, which can irreversibly damage the column. Therefore to extend column life, along with column friendly acetonitrile, we tried ultrapure water instead of phosphate buffer, the solvent that has lowest density. This binary mixture of ACN: water in the ratio 90:10 gave the best results with retention time of 7.75 minutes.

Significance

1. Itraconazole is an antifungal drug which is used in many conditions of infections and its method development for its detection from bulk and marketed preparations would help greatly for its rapid separation, testing and detection.

2. Acetonitrile (ACN) as one component of mobile phase performs dual functions of separation as well as column preservation.

3. Water as second component is comparatively much better than phosphate buffer for column life.

4. The retention time less than 8 minutes results in less solvent usage.

5. The use of 90 parts of ACN in mobile phase will yield better column life, results in lesser expenses and overall ultimately profit.

6. The sensitivity of proposed method can be proved by lowest values of LOD and LOQ as obtained by the method.

7. The percentage RSD for precision is <2 which confirms that the method is sufficiently precise.

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