



NEW RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF ANTIFUNGAL DRUG ITRACONAZOLE IN ITRACONAZOLE LOADED INVASOMES GEL

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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 (ITZ) in bulk and prepared in house invasomes gel formulation. Acetonitrile and Methanol was used as a mobile phase for chromatographic separation in the ratio of 20mM KH₂PO₄: Acetonitrile (pH adjusted to 4.0 with OPA) at flow rate of 1.0 ml/min and estimation on Thermo C18 (250 × 4.6 mm) in the ratio of 50:50 v/v at flow rate of 1.0 ml/min. The detection was carried out with UV detector set at 262nm. The retention time for itraconazole was found to be 2.258±0.095 minutes. The linearity range for itraconazole was found to be 5-25 µg/ml with coefficient of linear regression 0.998. The method was validated in accordance with the requirements of International Conference on Harmonization (ICHQ2 A & B) guidelines for specificity, linearity, accuracy, precision, LOD & LOQ.

Keywords: Method development, validation, ICH Guidelines, Itraconazole, Invasomes gel.

1. INTRODUCTION

Itraconazole is a synthetic triazole antifungal agent. Itraconazole is a 1:1:1:1 racemic mixture of four diastereomers (two enantiomeric pairs), each possessing three chiral centers. It may be represented by the following nomenclature: 4-[4-[4-[4-[[2-(2, 4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazin-1-yl]phenyl]-2-(1-methylpropyl)-2,4-dihydro-1,2,4-triazol-3-one (Fig. 1). It has a molecular formula is C₃₅H₃₈Cl₂N₈O₄ and a molecular weight is 705.64. It is a white to slightly yellowish powder. It is insoluble in

water, very slightly soluble in alcohols, and freely soluble in dichloromethane [1-4]. It is one of world health organization's lists of essential medicines⁵. Analytical methods such as UV spectrophotometric methods, Reverse Phase High Performance Liquid Chromatography, HPLC-FLD, Ultra Pressure Liquid Chromatography⁶⁻¹⁰, 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 prepared Invasomes gel.

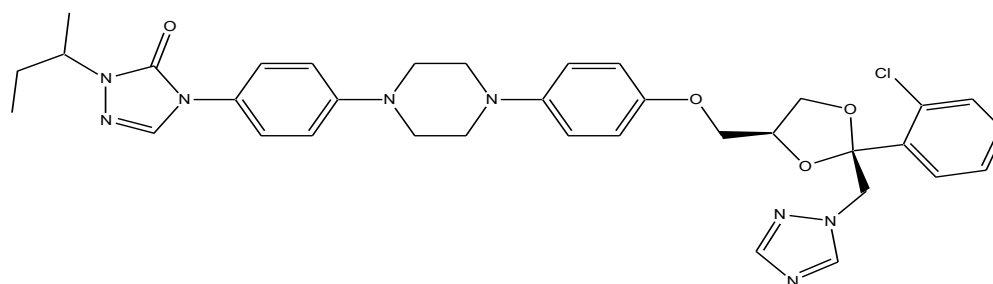


Fig. 1: Structure of Itraconazole

2. MATERIAL AND METHODS

Working standards of pharmaceutical grade itraconazole was purchased from Himedia Pvt. Ltd, India. The

formulation was prepared in house in sophisticated instrument lab, SRK University, Bhopal, HPLC grade methanol and acetonitrile was obtained from Merck

(India) limited. All other chemical used were of analytical grade. Triple distilled water was used for whole experiment was generated in house.

2.1. Methods

The isocratic mobile phase consisted 20mM KH_2PO_4 : Acetonitrile (pH adjusted to 4.0 with OPA), flowing through the column at a constant flow rate of 1.0 ml/min. The mobile phase was filtered through nylon 0.22 μm membrane filters and was degassed before use (30 min). A Thermo (C-18) Column (5 μm , 250mm x 4.60mm) was used as the stationary phase. By considering the chromatographic parameter, sensitivity and selectivity of method for drugs, 262 nm was selected as the detection wavelength for UV-Visible detector.

2.2. Selection of Diluent

Diluent used for preparation of sample were compatible with mobile phase and had no any significant affect on retention and resolution of analyte. After various trials, 7.4 pH phosphate buffers were used as diluents.

2.3. Standard preparation

Accurately weighed 10 mg API of ITZ was transferred into 10 ml volumetric flask separately and added 5ml of 7.4 pH phosphate buffers as diluents, sonicated for 20 minutes and volume was made up to 10ml with 7.4 pH phosphate buffers to get concentration of solution 1000 $\mu\text{g}/\text{ml}$ (Stock-A)

A 5 ml of solution was taken from stock-A of both the drugs and transferred into 50ml volumetric flask separately and diluted up to 50 ml with diluent (7.4 pH phosphate buffers) to give concentration of 100 $\mu\text{g}/\text{ml}$ of ITZ respectively (Stock-B). 0.5ml, 1.0ml, 1.5ml, 2.0ml and 2.5ml of stock-B were taken separately in 10 ml volumetric flask and volume was made up to 10ml with (7.4 pH phosphate buffers). This gave the solutions of 5 $\mu\text{g}/\text{ml}$, 10 $\mu\text{g}/\text{ml}$, 15 $\mu\text{g}/\text{ml}$, 20 $\mu\text{g}/\text{ml}$ and 25 $\mu\text{g}/\text{ml}$, for ITZ.

2.4. Preparation of invasomes

Invasomes were prepared by Mechanical dispersion technique by previously reported technique with minor alteration. Invasomes were formulated with the concentrations of soya lecithin and ethanol. Drug and Terpenes in varying concentrations were dissolved in ethanolic phospholipid solution. To obtain a clear solution, mixture obtained was vortexed and sonicated for 5min. Phosphate buffer saline (PBS) (pH: 6.8) was

added to the solution by a syringe under constant vortexing. The vortexing was continued for an additional 5 min. The most suitable preparation was used for further studies¹¹.

2.5. Preparation of invasomes loaded gel

The incorporation of the itraconazole loaded invasomes (equivalent to 1 %) into separate 10gm gels was achieved by slow mechanical mixing at 25 rpm for 10 minutes. The optimized formulation was incorporated into carbapol gel concentration 1.5 % w/w.

2.6. Assay of prepared invasomes loaded gel

The amount equal to 5mg of itraconazole invasomes was taken in 10ml volumetric flask. The volume was made up to the mark by diluent and filtered by whatmann filter paper (no. 41) and the filtrate was used to prepare samples of appropriate concentration.

2.7. Method validation

As per ICH guidelines, the method was validated and following parameters were evaluated [12-13]

2.7.1. Linearity

Linearity of itraconazole was established by response ratios of drug. The response ratios (response factor) were calculated by dividing the AUC with respective concentration. The curve was plotted between response ratios and concentration which shows the good linearity of drugs in the concentration ranging from 1-5 $\mu\text{g}/\text{ml}$.

2.7.2. Specificity

Specificity of the method was carried out to assess unequivocally the analyte presence of the components that might be expected to be present, such as impurities, degradation products and matrix components.

2.7.3. Precision

Precision was determined by repeatability, Intermediate precision and reproducibility of all three drugs.

2.7.4. Repeatability

The repeatability was performed for five replicate at five concentrations in linearity range for itraconazole that indicates the precision under the same operating condition over short interval time.

2.7.5. Intermediate Precision

2.7.5.1. Day to day precision

Intermediate precision was also performed within laboratory variation on different days for all three drugs simultaneously in five replicate at five concentrations.

2.7.5.2. Analyst- to- analyst precision

Analyst to analyst variation was performed by different analyst in five replicates at five concentrations.

2.7.6. Reproducibility

The reproducibility was performed by chemical to chemical (use of Rankem chemicals in place of Merck chemicals) variation in five replicate at five concentrations.

2.7.7. Accuracy (% recovery)

This study was carried out using pre analyzed tablet solution. A definite concentration of pure drug was added (80 %, 100 % and 120 % level) and then recovery was studied. A pre analyzed tablet solution containing 5mg of itraconazole were taken in 10 ml volumetric flasks and known concentrations of pure drug solution was added to them, which were prepared from standard stock solution of itraconazole. It was repeated at 5 concentrations and 3 replicate levels. Calculation was done from the label claim and the average weight of the final product.

2.7.8. Robustness

As per ICH norms, small, but deliberate variations in concentration of the mobile phase were made to check

the method's capacity to remain unaffected. The ratio of mobile phase was changed from, acetonitrile: methanol (50:50 % v/v), to (45:55 % v/v).

2.7.9. LOD and LOQ

The LOD and LOQ of developed method were calculated based on the standard deviation of response and slope of the linearity curve.

3. RESULTS AND DISCUSSION

3.1. Chromatography

The mobile phase was chosen after several trials with methanol, acetonitrile, water and buffer solutions in various proportions and at different pH values. A mobile phase consisting of 20mM KH_2PO_4 : Acetonitrile (pH adjusted to 4.0 with OPA) was selected to achieve maximum separation and sensitivity. Flow rates between 0.5 and 1.5 min were studied. A flow rate of 1 ml/min gave an optimal signal-to-noise ratio with a reasonable separation time. Using a reversed-phase C_{18} column, the retention times for itraconazole was observed to be 2.258 ± 0.095 . Total time of analysis was less than 8 min. The maximum absorption of itraconazole was detected at 262nm and this wavelength was chosen for the analysis (Fig. 2).

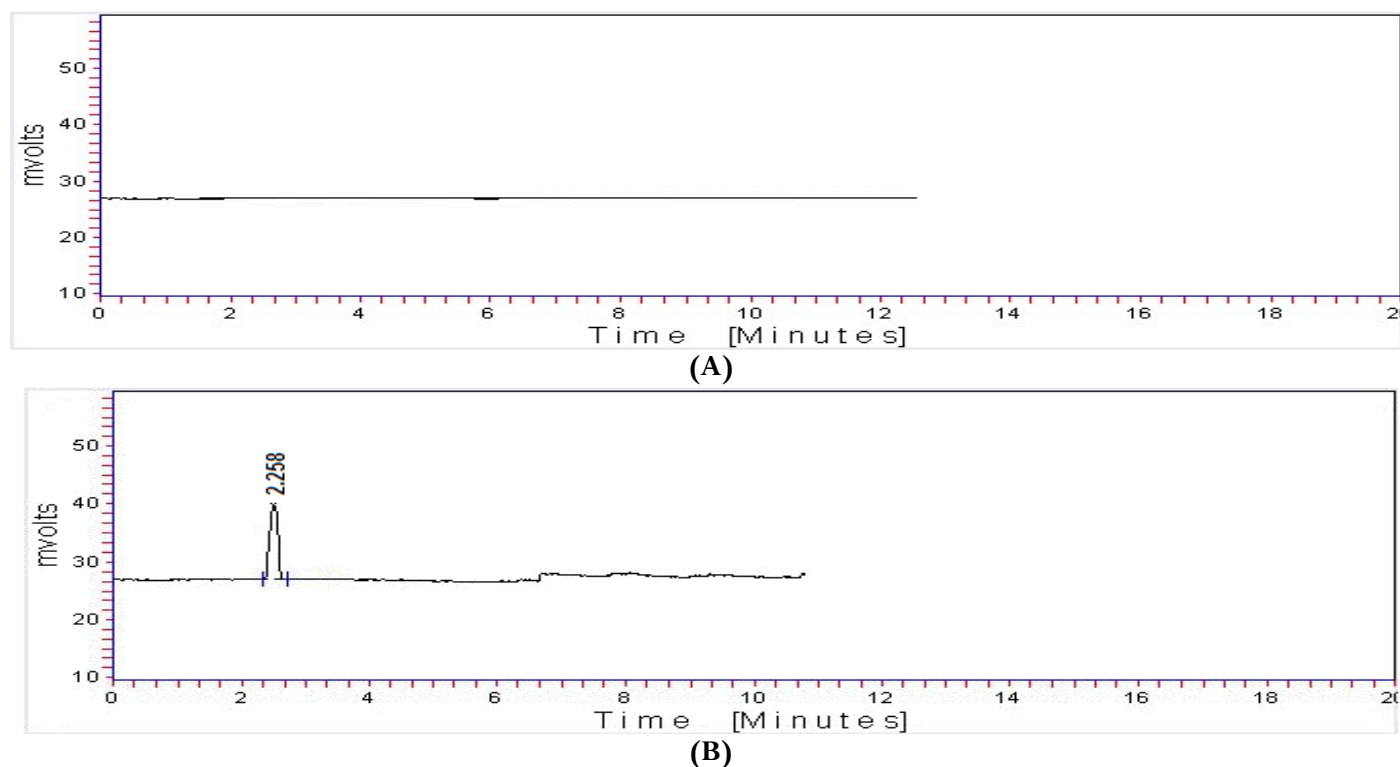


Fig. 2: Chromatograms of (A) Blank mobile phase (B) ITZ (10µg/ml) as standard

3.2. System suitability

System suitability parameters such as number of theoretical plates, HETP and peak tailing were determined. The results obtained are shown in table 1. The number of theoretical plates for itraconazole was 2558.333 and tailing Factor was found to be 1.238.

Table 1: Results of system suitability parameters

Parameters	Itraconazole
No. of Theoretical Plates	2558.333
Tailing Factor*	1.238
Retention time*	2.258±0.095
Calibration range (µg/ml)	5-25

*Each value is the mean ± SD of six determinations

3.2.1. Linearity

The calibration curve was linear over the concentration range of 5-25µg/ml for ITZ. The linearity was represented by a linear regression equation as follows:

$$Y (\text{ITZ}) = 49.82\text{conc} + 3.984 \quad (r^2 = 0.998)$$

3.2.2. Accuracy

Recovery studies were performed to calculate the accuracy of developed method to preanalysed sample solution, a definite concentration of standard drug (80%, 100%, and 120%) was added and then its recovery was analyzed. The value of percentage RSD was found less than 2 (0.433, 0.267 and 0.598) show good recovery at all three level 80, 100 and 120% respectively. Each level was made in triplicate (Table 2).

Table 2: Results of recovery study

% Level	% Mean±SD*
80%	99.49±0.433
100%	99.77±0.267
120%	99.62±0.595

* Value of three replicate and three concentrations

3.2.3. Precision

3.2.3.1. Repeatability

Five dilutions in three replicates were analyzed in the same day for repeatability and results were found within acceptable limits (RSD < 2) as shown in table 3.

3.2.3.2. Intermediate precision

Five dilutions in three replicates were analyzed on two different days and by two analysts for day-to-day and analyst-to-analyst variations and results were found within acceptable limits (RSD < 2) as shown in table 3.

Table 3: Statistical data for precision

Statistical parameter	Itraconazole		
	Mean*	S.D*	R.S.D*
Repeatability	99.990	0.092	0.093
Intermediate Precision (I) (A day to day)	99.096	0.082	0.083
(II) Analyst to Analyst	99.033	0.081	0.081

*Mean of 15 determinations (three replicates at five concentration level)

3.2.4. Detection Limit and Quantitation Limit

The LOD and LOQ of developed method were calculated based on the standard deviation of response and slope of the linearity curve (Table 4).

Table 4: LOD and LOQ

Name	LOD (µg/ml)	LOQ (µg/ml)
Itraconazole	0.15	0.45

3.2.5. Analysis of (In-house) transfersomes formulation

The assay value of drugs was close to 100, SD and % RSD are less than 2 indicates no interference of excipients in the estimation of drug (Table 5).

Table 5: Analysis of Prepared Invasomes gel

S. No.	Parameter	Itraconazole
1.	Mean	99.85
2.	S. D.	0.104
3.	% RSD	0.105

4. CONCLUSION

The proposed method was successfully applied to the determination of Itraconazole from bulk and pharmaceutical (In-house) Invasomes gel formulation. The proposed HPLC method was validated as per the International Conference on Harmonisation (ICH) Q2B Guidelines, and was found to be applicable for routine quantitative analysis of Itraconazole by HPLC in pharmaceutical dosage form. The results of linearity, precision, accuracy and specificity, were proved to be within the limits. The method provides selective quantification of Itraconazole with no interference from other formulation excipients. The proposed method was highly reproducible, reliable, rapid, robust and specific. Therefore, a high percentage of recovery and the run time of less than seven minutes allow its application for the routine determination of Acyclovir in the pharmaceutical dosage form.

Conflict of interest

None declared

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