Validation of new sensitive stability indicating RP-HPLC method for the pharmacokinetical study of fluconazole in an animal model

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ABSTRACT

This study was aimed to develop a simple, sensitive RP-HPLC-UV method for the detection of fluconazole (FLZ) in rat plasma. The proposed method is selective, reproducible, reliable and highly sensitive. Chromatic separation of fluconazole and internal standard were achieved by carrying out on an octadecyl silane (ODS-3, www.glsciencesinc.com) Hypersil C18 column (250 mm × 4.6 mm × 5 µm) using water (pH 5.2, adjusted with orthophosphoric acid) and acetonitrile (80:20, v/v) as the mobile phase at a flow rate of 2.5 mL/min with detection at 260 nm. The drug sample was prepared by one-step liquid-liquid extraction (LLE) from rat plasma using sodium hydroxide and dichloromethane. Here methyl paraben (MP) (0.5 µg/mL) was used as internal standard (IS). The chromatographic mean retention times of drug and methyl paraben were less than 8 min and 14 minutes respectively. The calibration standards were prepared in the range of 0.1-20 µg/mL with an average coefficient of variation less than 0.05%. The accuracy was within a range of 97.41-99.33%. The lower limit of detection (LLOD) and quantification (LLOQ) was 0.0013 µg/mL and 0.013 µg/mL respectively where no interferences detected in the chromatograms. The stability study of forced and non forced degradation was also performed with plasma, drug and internal standard to demonstrate the stability-indicating power of the method. This HPLC method was applied successfully to the pharmacokinetic study in rat after iv bolus injection and monitoring in receiving the fluconazole therapy.

Keywords: Fluconazole (FLZ), Methyl paraben (MP), Reverse phase- high performance liquid chromatography (RP- HPLC), Plasma pharmacokinetics, Validation

INTRODUCTION

Fluconazole (FLZ) is a fluorine-substituted triazole agent which is one of the most commonly prescribed systemic and superficial fungal infections, following both oral and intravenous administration Fig. (1). It is a water soluble, low molecular weight compound with limited plasma binding (11-12%) and long elimination half life. The time to reach steady state with once daily dosage is approximately 7 days, with an elimination half-life of 30 h.¹,² that excreted predominantly unchanged (75±9%) in urine.³,⁴ Fluconazole is almost completely absorbed (>90%) from the gastrointestinal tract after oral administration. The recommended dosage of FLZ is generally 100-200 mg once daily, and the time to attain peak concentration in plasma is 1.7-4.3 h after single oral dose drug administration.

Literature survey has revealed various methods for estimation of FLZ in biological fluids and in pharmaceutical formulations, such as TLC-densitometry,⁵,⁶ electrochemically,⁷,⁸ pH indicator absorbance ratio method,⁹ titrimetry,¹⁰ supercritical fluid chromatography (SFC) with UV-detection,¹¹ IR Spectroscopic,¹²,¹³ UV-spectrophotometric,¹⁴,¹⁵,¹⁶ microbiological method,¹⁷,¹⁸ gas liquid chromatography (GLC)¹⁹-⁴³, HPLC for biological fluid,⁴⁴-⁵⁵ and high performance liquid chromatography for pharmaceutical dosage forms.⁵⁶-⁶⁰ The reported methods have some disadvantages. In the GC method, the column has to be pre-treated with benzoyl chloride to avoid fluconazole being adsorbed to the column and, additionally, a time consuming extraction procedure is employed. There was no facility in our laboratory for the reported bioassays. On the other hand, the cited HPLC method has the disadvantage that some assays used high buffer concentrations, high concentrations of IS or solid...
phase extraction, which resulted in high % RSD when applied in laboratory. The majority utilize ultraviolet detection (UV) and a reversed-phase octadecylsilyl (C18) column, however, existing methods have shortcomings in terms of pharmacokinetics studies, i.e., lengthy running times (>10.0 min), a narrow concentration range (LLOQ ≥ 0.2 µg/mL) and with the lack of an internal standard (IS). Therefore, a sensitive and a short chromatographic run-times of HPLC-UV analytical method is required to quantify FLZ levels in animal and human plasma to support pharmacokinetic and bioequivalence studies.

This paper describes an stability indicating RP HPLC-UV method to determine the plasma FLZ concentrations. It utilizes liquid-liquid extraction and can analyze over the concentration range 0.10- 20.00 µg/mL by using methyl paraben as internal standard (IS). The purpose of this study was to develop a rapid, sensitive and selective method for the determination of fluconazole in plasma.

Table 1: System Suitability Parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>API (FLZ)</td>
<td>0.796</td>
</tr>
<tr>
<td>MP (IS)</td>
<td>0.923</td>
</tr>
</tbody>
</table>

Table 2: Results of Regression Analysis of Linearity Data of Fluconazole in Plasma*

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression equation</td>
<td>Y= 0.0030X + 0.0004</td>
</tr>
<tr>
<td>Linearity (µg/mL)</td>
<td>0.1-20</td>
</tr>
<tr>
<td>Slope</td>
<td>0.003</td>
</tr>
<tr>
<td>Intercept</td>
<td>0.0004</td>
</tr>
<tr>
<td>Stand. error of line</td>
<td>0.0002</td>
</tr>
<tr>
<td>Stand. error of slope</td>
<td>0.000023471</td>
</tr>
<tr>
<td>Correlation coefficient (r²)</td>
<td>0.9995</td>
</tr>
<tr>
<td>LLOD (µg/mL), % RSD (n=3)</td>
<td>0.00134, 0.93</td>
</tr>
<tr>
<td>LLOQ (µg/mL), % RSD (n=3)</td>
<td>0.01343, 0.87</td>
</tr>
</tbody>
</table>

*Linearity data is based on peak area ratio of fluconazole (API analyte) and methyl paraben (IS) LLOD- Lower limit of detection LLOQ- Lower limit of quantification

Table 3: Range (Known Concentration)

<table>
<thead>
<tr>
<th>Concentration level (µg/mL)</th>
<th>Recovery Quantity (mg)</th>
<th>Mean ± SD (n=3)</th>
<th>Mean percentage ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.00</td>
<td>1.96</td>
<td>1.96 ± 0.01</td>
<td>97.41 ± 0.54</td>
</tr>
<tr>
<td>2.50</td>
<td>2.41</td>
<td>± 0.15</td>
<td></td>
</tr>
<tr>
<td>3.50</td>
<td>3.61</td>
<td>3.53 ± 0.04</td>
<td>101.93 ± 0.26</td>
</tr>
<tr>
<td>5.50</td>
<td>5.59</td>
<td>5.62 ± 0.06</td>
<td>101.93 ± 0.26</td>
</tr>
</tbody>
</table>

SD- Standard deviation

Table 4: Accuracy Analysis by Recovery of the Drug

<table>
<thead>
<tr>
<th>Concentration level (µg/mL)</th>
<th>Recovery Quantity (mg)</th>
<th>Mean ± SD (n=3)</th>
<th>Mean % ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.50</td>
<td>2.41</td>
<td>± 0.15</td>
<td>96.29</td>
</tr>
<tr>
<td>3.50</td>
<td>3.61</td>
<td>3.58 ± 0.04</td>
<td>102.21</td>
</tr>
<tr>
<td>5.50</td>
<td>5.59</td>
<td>5.61 ± 0.06</td>
<td>101.93</td>
</tr>
</tbody>
</table>

SD- Standard deviation
MATERIALS AND METHODS

Materials

Fluconazole powder was obtained as gift sample from Mankind Pharmaceutical Ltd, New Delhi, India. The purity of drug according to the manufacturer’s recommendations was found to be 99.99 % (n=6). Methyl paraben (MP), sodium hydroxide, hydrochloric acid, phosphoric acid, hydrogen peroxide of highest analytical grade were purchased from Himedia laboratories, Mumbai, India. Acetonitrile and water of HPLC grade were purchased from Fisher Scientific Ltd, Mumbai and Rankem RFCL, India respectively. In house double distilled water was used other than HPLC method development. Nylon membrane filter (0.22µm porosity) was purchased from Pall Corporation, Mumbai, India.

Table 5: Stability Summary of Fluconazole in Forced and Non Forced Condition in Plasma

<table>
<thead>
<tr>
<th>Storage condition</th>
<th>Time</th>
<th>Mean percentage recovery of FLZ ± SD (n=3)</th>
<th>Remark: Significant degradation found: Yes/No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid hydrolysis</td>
<td>Same day</td>
<td>99.39 ± 0.67</td>
<td>No</td>
</tr>
<tr>
<td>(1 N HCl, at 5 °C), Forced</td>
<td>Inter day</td>
<td>99.10 ± 0.78</td>
<td>No</td>
</tr>
<tr>
<td>Base hydrolysis</td>
<td>Same day</td>
<td>99.41 ± 0.17</td>
<td>No</td>
</tr>
<tr>
<td>(1 N NaOH, at 5 °C), Forced</td>
<td>Inter day</td>
<td>98.34 ± 0.78</td>
<td>No</td>
</tr>
<tr>
<td>Oxidation</td>
<td>Same day</td>
<td>98.39 ± 0.69</td>
<td>No</td>
</tr>
<tr>
<td>(35% H2O2), Forced</td>
<td>Inter day</td>
<td>97.91 ± 0.78</td>
<td>No</td>
</tr>
<tr>
<td>Thermal</td>
<td>Same day</td>
<td>99.89 ± 0.67</td>
<td>No</td>
</tr>
<tr>
<td>(5°C), Forced†</td>
<td>Inter day</td>
<td>99.72 ± 0.73</td>
<td>No</td>
</tr>
<tr>
<td>Room temperature</td>
<td>Same day</td>
<td>99.12 ± 0.77</td>
<td>No</td>
</tr>
<tr>
<td>(25 °C), Non-forced†</td>
<td>Inter day</td>
<td>99.08 ± 0.23</td>
<td>No</td>
</tr>
</tbody>
</table>

Same day means less than ≤ 4 hrs Interday studies means ≥ 12 hrs †Thermal forced at (5 °C) and room temperature (25 °C) non forced degradation study were in distilled water media

Apparatus

Reverse-phase high performance liquid chromatography (RP-HPLC) determinations were performed with Agilent Technologies (model 1120, compact) G4288A- Gradient, low pressure binary gradient pump consisting of vacuum in built degasser unit, non-PDA, VWD-UV detector, column oven, manual injector equipped with 50 µL injector loop, having analytical syringe (50 µL, Germany) and EZChrom Elite (3.0.1) with LMD software controlled. Ultrasonic bath degasser and cleaner were used (Lansany, Chandigarh, India). All weights were taken on electronic balance (Vibra, DJ- 150S-5, Shinko Denshi, Japan).

Analytical technique

Chromatographic conditions

Chromatic separation was carried out at 45°C on a Hypersil ODS-3, C18 column (250 mm × 4.6 mm × 5 µm particle sizes, GL Sciences, Inc. USA, www.glsciencesinc.com). The fluconazole was separated gradiently with a mobile phase consisting of water (pH = 5.2) and acetonitrile (80:20 v/v). The pH of inorganic (water) was adjusted with orthophosphoric acid. The mobile phase was filtered and degassed for five minutes in a bath sonicator prior to use. To reach equilibrium the analysis was usually started after the passage of 60-70 mL of mobile phase. The flow rate was 2.5 mL/min. The injection volume was 50 µL, and the eluted analytes for drug was traced by UV-detection at 260 nm. Different system suitability test parameters were studied. The system precision and intermediate precision was calculated in term of percentage RSD. The others system suitability test parameters like retention time, peak area, peak width (5%), tailing factor (USP), peak width USP (min), peak width (10% cm), peak asymmetry factor at 10% height, capacity factor , plates/meter (USP), theoretical plate and resolution (USP) were calculated for API and internal standard separately that were given in Table 1.
Calibration standard and quality control

Fluconazole standard stock solution of 100 µg/mL was prepared by dissolving accurately 10 mg of drug in 100 mL of distilled water and passed through 0.22 µm membrane filter. Working solutions of FLZ were prepared from stock solution by serial dilution with mobile phase to concentration of 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 15.0 and 20.0 µg/mL. The accurate weighed 10 mg of methyl paraben (IS) was also dissolved (prior having 0.5 ml methanol) in water to prepare a stock solution 100 µg/mL and this solution was further diluted with mobile phase to provide a working IS solution of concentration 0.5 µg/mL. All the stock and working solutions were stored at 5°C. Calibration standard were prepared by adding 100 µL plasma, 1 mL of IS equivalent to 0.5 µg and standard FLZ dilutions equivalent to 0.1-20.0 µg/mL, respectively. Different concentrations (0.5, 5, 20 µg/mL) other than calibration curve were used with blood plasma for quality control.

Preparation of plasma sample

The blood samples were received in EDTA treated anticoagulants tubes and centrifuged at 3000 rpm for 10 minutes, in cooling centrifuge and separated plasma was stored at -20°C in cryovials until analysis. After thawing at room temperature an aliquate of each plasma sample (100 µL) was pipette into a glass tube and 0.1 mL of IS working solution (50 µg/mL) was added. After vortexing briefly 20 µL of 5 N NaOH and 5 mL of dichloromethane were added to each sample shaken for 10 min and then centrifuged at 3000 rpm for 10 min at 4°C. The upper aqueous layer was then removed and remaining of the organic layer was evaporated to dryness using evaporation.

### Table 6: Chromatographic Conditions and Range Investigated During Robustness Testing

<table>
<thead>
<tr>
<th>Chromatographic changes</th>
<th>Mean retention time ± SD, (min), (n=3), %RSD</th>
<th>Mean peak area ± SD, (n=3), %RSD</th>
<th>Mean theoretical plates count (USP) ± SD, (n=3), %RSD</th>
<th>Mean plates/meter count (USP) ± SD, (n=3), %RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>†Condition I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flow rate (mL/min)</td>
<td>2.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>45</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UV-Wavelength (nm)</td>
<td>260</td>
<td>7.83±0.01, 0.12</td>
<td>358970.66±1072.00, 0.29</td>
<td>9385.33±38.60, 0.41</td>
</tr>
<tr>
<td>pH of mobile phase</td>
<td>5.2</td>
<td>0.12</td>
<td>1072.00, 0.29</td>
<td>38.60, 0.41</td>
</tr>
<tr>
<td>Mobile phase comp.</td>
<td>(Acetonitrile: Water)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Condition II</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flow rate (mL/min)</td>
<td>2.3</td>
<td>8.49±0.01, 0.11</td>
<td>399080±6398.19, 1.60</td>
<td>10547.67±13.28, 0.12</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td></td>
<td>8.29±0.01, 0.12</td>
<td>394008±3714.65, 0.95</td>
<td>10593.34±51.07, 0.48</td>
</tr>
<tr>
<td>Condition III</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>40</td>
<td>8.05±0.03, 0.39</td>
<td>371897±3695.49, 0.99</td>
<td>9705.00±220.03, 2.26</td>
</tr>
<tr>
<td>Condition IV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UV- Wavelength</td>
<td>258</td>
<td>7.83±0.01, 0.12</td>
<td>362708.34±2672.28, 0.73</td>
<td>9920.00±88.64, 0.89</td>
</tr>
<tr>
<td>Condition V</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change in mobile phase pH</td>
<td>5.0</td>
<td>7.87±0.01, 0.12</td>
<td>324380.34±3018.32, 0.83</td>
<td>9791.34±66.29, 0.39</td>
</tr>
<tr>
<td>Condition VI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mobile phase comp.</td>
<td>(Acetonitrile: Water)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>†</td>
<td>Optimized parameters, SD- Standard deviation , %RSD- Percentage related standard deviation</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
system at 50°C. The residue obtained was dissolved in 1 mL of mobile phase and vortexing for 10 min. After filtering by 0.22 µm membrane filter, samples were injected into the system.

The in vivo study, on animal, was based on "Breeding of and Experiments on Animals (Control and Supervision) Rules 1998" as amended by Government of India, Ministry of Environment and Forests (CPCSEA), with permission number 1149/ac/07/CPCSEA by local ethical committee.

**Preparation of plasma samples for stability**

Stability study of the drug FLZ and methyl paraben (IS) was investigated for one day in plasma as forced and non-forced degradation using the conditions: acid hydrolysis (1 N HCl), base hydrolysis (1 N NaOH), thermal (50°C) and oxidation (35% hydrogen peroxide). A total area peak purity test was carried out for the FLZ and MP by using a non-PDA detector in stress and non-stress samples.

**Validation of the analytical method**

**Specificity (selectivity)**

Blank samples obtained from pooled plasma, which were collected under controlled conditions, were subjected to the preparation procedure described above and evaluated to determine the extent to which endogenous plasma components may interfere with the chromatograph determinates of FLZ and MP (IS) levels.

**Calibration curve and range**

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### Table 7: Pharmacokinetic Characteristics after Single iv Bolus Administration of 2.5 mg/Kg Fluconazole in Six-Healthy Rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Mean ± SD (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC(_{0-n}) h (µg/h/mL)</td>
<td>163.7 ± 42.20</td>
</tr>
<tr>
<td>AUC(_{0-∞})h (µg/h/mL)</td>
<td>358.3 ± 160.30</td>
</tr>
<tr>
<td>C(_{max}) (g/h/mL)</td>
<td>2.5 ± 0.40</td>
</tr>
<tr>
<td>T(_{max}) (h)</td>
<td>5.0 ± 1.0</td>
</tr>
<tr>
<td>Cls (mL/kg, h)</td>
<td>8.2 ± 2.40</td>
</tr>
<tr>
<td>T(_{1/2}) (h)</td>
<td>29.60 ± 38.3</td>
</tr>
</tbody>
</table>

SD- Standard deviation, AUC\(_{0-n}\) - Area under the plasma concentration - time curve from 0 to n hours, AUC\(_{0-∞}\) - Area under the plasma concentration - time curve from 0 to ∞ hours, C\(_{max}\) - Maximum plasma concentration, T\(_{max}\) - Time required reaching maximum plasma concentration, Cls- Clearance, T\(_{1/2}\) - Half life of the drug.
Figure 3: Chromatogram of blank plasma

Figure 4: Chromatogram of fluconazole and methyl paraben, spiked with plasma
were obtained using blank plasma sample and nine prepared calibration samples covering the FLZ range (0.1-20.0 µg/mL) by determining the peak area ratios of FLZ verses IS. The linearity of the calibration curve was also examined on the basis of correlation coefficient (r²).

**Accuracy and precision**

The accuracy of the methods was determined by selecting three known plasma drug samples in triplicate (n=3) in the linearity range. The percentage recovery of above three known analyte was calculated. Precision study of the method was by calculating % RSD of repeating six-determinates (n=6) at 100 % concentration (5 µg/mL). The intermediate precision was determined by selecting six different samples by another analyst in same manner as precision.

**Stability**

Stability studies in stress and non-stress conditions of the active pharmaceutical ingredients were utilized for the possible degradation products and for stability indicating analytical procedures. It is the ability of the analytical method to measure the analyte response in the presence of its degradation products.

**Sensitivity**

The lower limit of detection (LLOD) and lower limit of quantification (LLOQ) was based on the standard deviation of the response and the slope of the calibration curve. The estimation of standard deviation of the response was by calculating the standard deviation of y-intercept of regression line and the slope was estimated from the calibration curve.

**Robustness**

To prove the selectivity and reliability of the analytical method during normal usage, some small but deliberate changes were made in the analytical method (e.g. flow rate ± 0.2 mL, column temperature ± 5°C, UV-detection wavelength ± 2 nm and pH ± 0.2). Changes in the chromatographic parameters i.e. area, retention time, plates/meter (USP) and theoretical plates (USP) were evaluated for the studies.

**Pharmacokinetics data and statistical analysis**

The following pharmacokinetic values were determined for the period 0-120 h, the area under the plasma concentration - time curves from time 0 to 120 h (AUC_0-120 h), the area under the plasma concentration - time curves from time 0 to ∞ (AUC_0-∞), the maximum plasma concentration (C_{max}), the time required to reach maximum plasma concentration (T_{max}), the clearances (Cl) and the half life (T_{1/2}). The values were calculated by non-compartment model method.

**RESULTS**

The method was validated according to ICH guidelines (ICH Topic Q 2B, 1996). The purpose of this study was to investigate and compare the feasibility of utilizing simple and selective chromatographic method, for the determination of the FLZ in animal, human and in others form of the drug.

**Chromatogram development**

Under the experimental condition described above, FLZ and MP (IS) were well separated from endogenous materials. Representative chromatograms of drug in non-biological fluid (Fig. 2), blank plasma sample (Fig. 3) and a plasma sample spiked with FLZ and MP were shown in Fig. 4, where no interference was found in chromatograms of randomly selected rat plasma samples. The retention time of FLZ and MP were 7.75 and 13.91 min respectively i.e. total running time for each sample was less than 15 min. Although other analytical methods using short (< 15 cm) column have been reported the peaks of FLZ or IS were not well resolved against the endogenous peaks from plasma, resulting in a long chromatographic run time for separation. This method reduced the analysis time compared to the existing FLZ method using MP (> 14 min).

**Degradation behavior (stability)**

The results obtained from the forced and non-forced degradation studies were summarized in Table 5. Forced acid hydrolysis study (1 N HCl, at 5°C) was found no remarked degradation (0.61% intraday and 0.90% interday). Degradation forced base hydrolysis (1 N NaOH, at 5°C) study results 0.59% in intraday and 1.66% in interday. Forced oxidative degradation (35% H₂O₂, at 5°C) study resulted 1.1% in same day and 2.09% in another day. The forced thermal degradation study resulted 0.11% in intraday and 0.92% in interday. Where nonforced degradation study of drug in distilled water at 25°C gives 0.88 % in same day and 0.92% in next day.

**Method validation**

**Specificity (selectivity)**

When the blank plasma with FLZ and MP (IS) were injected then eluted drug and internal
standard peak appears of good shape (Fig. 4) where no endogenous substance were found interfere with peaks. The FLZ in non biological sample and in blank plasma were separately injected that were reported in Figure 2 and 3.

**Calibration curve and range**

The linearity of ratio peak areas versus different concentrations was evaluated for FLZ and its related substances. The linear regression data for all tested components was presented in Table 2. The calibration curve was linear over the range of 0.10 – 20.00 µg/mL and showed good correlation coefficient i.e. $r^2 = 0.9995$ with regression intercepts not statistically significant different from zero. In addition to the range of known concentration calculation, samples 2.0 and 5.0 µg/mL was analyzed for their recovery and that was found between 97 to 100% (Table 3).

**Accuracy and precision**

The study of accuracy by percentage recovery of the method within the day of three different concentrations i.e. 2.50, 3.50 and 5.50 (total n=9) were computed in terms of standard deviation and found between 97 to 102 % respectively, showed great back-calculated value that were between range (80-120% according to ICH guidelines) (Table 4).

The precision of the method were determined from six replicate analysis (n=6) of one (5 µg/mL) spiked standard FLZ-plasma test sample at concentration within the linear range of FLZ. The percentage RSD of reproducibility was found to 0.796 for API (FLZ) and 0.923 for MP (IS) which was found less than one that proves highest precision and sensitivity of the method (Table 1). The intermediate precision was also studied and found % RSD as 0.831 and 0.897 for API and IS (Table 1).

**Sensitivity**

The lower limit of detection (LLOD) concentration was found to be 0.00134 µg/mL with % RSD 0.93 for n=3. Where the lower limit of quantification (LLOQ) was found to be 0.01343 µg/mL with 0.87 of % RSD (n=3), at an injection volume of 50 µL.

**Robustness**

The method was studied by changing the chromatographic conditions slightly and the results were presented in the Table 6. The change in chromatographic conditions (± 2%) resulted to change in the value of retention time, peak area, theoretical plates (USP) and plates per meter (USP) of API. There were no significantly changes occurs in different chromatographic parameters (Table 6).

**Pharmacokinetics study of FLZ**

Pharmacokinetic characteristics after single iv bolus administration of 2.5 mg/Kg FLZ in six-healthy rats results different pharmacokinetical parameters i.e. $AUC_{0-\infty}$, $AUC_{0-t}$, $C_{max}$, $T_{max}$, $CL_t$ and $T_{1/2}$ of the drug obtained were depicted in Table 7. So this method can be used to calculate the different pharmacokinetic parameters.

**DISCUSSION**

**Optimization of the chromatographic conditions**

In preliminary experiments FLZ subjected to separation by reversed phase HPLC using water and acetonitrile as mobile phase. Generally, the selection of the column depends on the characteristics of the compounds to be separated. Depending on the solubility of FLZ a C$_{18}$ column is more preferred. The HPLC conditions were optimized to study the effects of concentrations of organic solvent, pH of inorganic phase and gradient program on the separation of drug and internal standard. A different pH ranging from 4 to 6 was studied and found that pH 5.2 shows better peak shape and resolution behavior for the drug and internal standard. The effect of different ratio of organic and inorganic mobile phase was studies and selected 20: 80 ratios for further processing. Finally separation was conducted on an ODS-3, C$_{18}$ column maintained at 45 °C with gradient elution mode.

**Degradation behavior (stability)**

Results obtained from the forced and non-forced degradation studies were summarized in Table 5. During the forced degradation study, the mean percentage recovery (n=3) of FLZ in same and inter day was found to be high pure and no considerable degradation of drug substance was observed in acidic, alkaline, oxidative, thermal and non-forced conditions. During analysis, a negligible amount of degradation had occurred, which was not considered relevant. The chromatographs were checked for the appearance of any extra or overlapping peaks. The purity of the principal and other chromatographic peaks was found to be satisfactory. This study confirmed the stability indicating power of the RP-HPLC method.

**Method validation**

**Specificity (selectivity)**
The data on selectivity studies revealed that the separated drug peak was satisfactorily separated from the mixture. Thus, optioned selected RP-HPLC method was found to be specific for the FLZ, as none of the excipients interfered with the calculation of FLZ and MP (IS). Therefore presented chromatographic condition was suitable for the bioassay as plasma analysis and plasma quantification of the drug.

**Calibration curve and range**

The calibration curve was linear over the high range (0.10 – 20.00 μg/mL) that was good because of producing high correlation coefficient value i.e. $r^2 = 0.9995$. The linear regression data for tested components was listed in Table 2, where relative retention factor for FLZ and MP confirms linearity and detector response at 260 nm to be linear over the studied range for all analytes.

**Accuracy and precision**

The accuracy by % drug recovery of three different selected concentrations was found to be between the predefined acceptances criteria. The precision of the method was studies for repeatability and intermediate precision. The obtained percentage relative standard deviation (% RSD) for repeatability that was less than one proves high precision of the method. The intermediate precision of the method was determined on six separate sample solution prepared from the same lot by spiking the drug and IS in plasma at specific level by a different analyst using a different mobile phase and on different day with a same brand of column. The overall RSD was evaluated and found to be 4.39 % for drug and 2.81 % for IS, which was within the acceptance criterion of not more than10.0%.

**Sensitivity**

The lower limit of detection and limit of quantification were determined for FLZ. The calculated LLOD and LLOQ levels of the components were verified for their precision. Percentage relative standard deviations (% RSD) were less than one reveals high value of detection and quantification of FLZ. The lower LLOQ values with higher precision indicate better sensitivity of the RP-HPLC.

**Robustness**

To evaluate the robustness of the method, the influence of small and predetermined alterations of analytical parameters on the quantization of the drug and selectivity was studied. In this sequence one parameter was selected and changed as reducing and increasing respectively. The SD and % RSD for change in mean retention time, mean peak area, mean theoretical plate and mean plates/meter were evaluated and it was found less than 2% for retention time and peak area that was excellent. The other parameters were also good because of showing near 2% RSD, except few of them who showed maximum 2.26 %. The studies indicated no effect on the determination of related drug and in the selectivity. The results obtained were within the acceptable limit and it was observed that there were no marked changes in the area of the chromatograms which demonstrated that the RP-HPLC method developed was robust.

**CONCLUSION**

In conclusion, this analytical procedure was validated in terms of specificity, linearity, range, precision, accuracy in terms of recovery, sensitivity in terms of LLOQ and robustness. The limit of quantification and recovery were more than adequate for use in pharmacokinetic studies. Our results indicate that this RP-HPLC procedure was a reproducible method that provides consistent quantification of FLZ in blood plasma samples. This method has been used successfully in pharmacokinetic study of FLZ.

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List of Abbreviations:

RP-HPLC UV- Reverse phase-high performance liquid chromatography ultra-violet
ODS- Octadecyl silane
LLE- Liquid-liquid extraction
IS- Internal standard
ICH- International conference on harmonisation
LLOD- Lower limit of detection
LLOQ- Lower limit of quantification
US-FDA- United state-food and drug administration
FLZ- Fluconazole
TLC- Thin layer chromatography
SFC- Supercritical fluid chromatography
GLC- Gas liquid chromatography
GC- Gas chromatography
VWD- Variable wavelength detection
EDTA- Ethylene diamine tetra acetic acid
MP- Methyl paraben
API- Active pharmaceutical ingredient
AUC sub 0 to n sup - Area under the plasma concentration - time curve from 0 to n hours
AUC sub 0 to infinity sup - Area under the plasma concentration - time curve from 0 to infinity hours
C max - Maximum plasma concentration
T sub max sup - Time required reaching maximum plasma concentration
Clsub s- Clearance
T sub 1/2 sup - Half life of the drug
µg- Microgram
mL -Milliliter
nm – Nanometer
RT- Retention time