Development and Validation of Analytical Method for Fluconazole and Fluconazole Related Compounds (A, B, and C) in Capsule Formulations by HPLC with UV Detection

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Abstract
A simple and stability-indicating liquid chromatographic method was developed and validated for the analysis of Fluconazole and its related compound (A, B, and C) in capsule formulations. Liquid chromatography with a UV detector at a wavelength of 260 nm using a reversed-phase C18 column was employed in this study. Isocratic elution was employed using a mixture of methanol and water (40:60, v/v). This new method was validated in accordance with USP requirements for new methods for assay determination, which include accuracy, precision, specificity, linearity and range. The current method demonstrates good linearity over the range of 0.05-0.15 mg/ml of Fluconazole. The accuracy of the method is 99.3%. The precision of this method reflected by relative standard deviation of replicates is 0.61%. Validation of the same method for Fluconazole related compounds analysis was also performed according to USP requirements for quantitative determination of impurities which include accuracy, precision, linearity and range, selectivity, and Limit of quantitation (LOQ). Low LOQ of the related compounds using this method enables the detection and quantitation of these impurities at low concentration.

Key words: Fluconazole; Related compounds; Validation; Impurities; Pharmaceutical dosage forms.

Introduction
Fluconazole is a part of a family of triazole antifungal drugs, which exhibit a broad spectrum of activity [1]. It has desirable pharmacologic properties, including a relatively long half-life, the ability to be administered either orally or parenterally [1]. In USP, Fluconazole as raw material is described, while formulated Fluconazole does not appear in the USP or in BP. However, Fluconazole is formulated in different dosage forms. In USP, three potential impurities of Fluconazole is listed (Fluconazole related compounds A, B, and C). Figure 1 shows the structure of Fluconazole and its related compounds (A, B, and C). In this respect, a stability-indicating test method for the analysis of Fluconazole and Fluconazole related compounds is needed. Many methods are developed for the analysis of Fluconazole in pharmaceutical preparations [2] and in body fluids as plasma and serum [1, 3-6]. The stability of reconstituted Fluconazole oral suspension was also investigated by Dentinger et. al. using an HPLC

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method \(^7\). All of these methods, however, are not used for the analysis of Fluconazole related compounds (A, B, and C).

![Diagram of Fluconazole, Fluconazole related compound A, Fluconazole related compound B, and Fluconazole related compound C.](image)

**Figure 1**: Structure of Fluconazole, Fluconazole related compound A, Fluconazole related compound B, and Fluconazole related compound C.

The objective of the current work is, therefore, to develop a simple RP-HPLC, stability-indicating method for analysis of Fluconazole and Fluconazole related compounds in capsule formulations. Validation of the current method was conducted for both determination of Fluconazole in capsule formulations (assay), and for quantitative determination of its related compounds. Validation of the method for Fluconazole was performed according to the requirements of USP for assay determination which include accuracy, precision, specificity, linearity and range, while validation of the method for Fluconazole related compounds was performed according to the requirements of USP for quantitative determination of impurities which include accuracy, precision, specificity, linearity and range, and LOQ.

**Experimental**

**Chemicals**

Methanol HPLC grade was from J.T Baker (NJ, USA). Fluconazole and Fluconazole related compounds were received from USP (Rockville, MD, USA).
**Apparatus**

HPLC system (Merck Hitachi Lachrome Elite HPLC system, Japan) with an L-2130 pump, an L-2200 autosampler, L-2300 column oven, and L-2490 UV detector was employed. The Ezochrom Elite software was employed. The chromatographic analysis was performed on HX749288, LiChroCart, HPLC-cartage Purospher STAR RP-18 endcapped (5 µm), (150 mm length, 4.6 mm inner diameter) (Waters Corporation, Milford, Massachusetts, USA), as well as on RP-8 endcapped (5 µm), (150 mm length, 4.6 mm inner diameter) (Waters Corporation, Milford, Massachusetts, USA). The column was kept at room temperature.

**Standard Solutions Preparation**

Nominal standard solution of Fluconazole was prepared by dissolving 10 mg of Fluconazole in 100 ml of mobile phase to obtain a solution having a known concentration of 0.1 mg/ml. Stock solution of Fluconazole related compounds was prepared by dissolving 10 mg of each (A, B, C) in 100 ml mobile phase. Standard solution of Fluconazole related compounds was prepared by diluting 5 ml of Stock Solution to 100 ml mobile phase to obtain a solution having a known concentration of 0.005 mg per ml of each.

Resolution solution was prepared by dissolving 50.0 mg of Fluconazole and 5.0 ml from each of Fluconazole related compounds A, B, and C stock solution in 100 ml.

Samples of formulated Fluconazole (capsules) were prepared by dissolving a quantity of the powdered product equivalent to 250.0 mg of Fluconazole in 25.0 ml mobile phase to get a concentration of 10.0 mg/ml Fluconazole.

**Results and Discussion**

**Method Development**

As fluconazole and its related compounds are hydrophobic and neutral compounds, reversed-phase separation mode (i.e. hydrophobic stationary phase and polar mobile phase) was employed for this separation. I have started with a mixture of methanol and water (buffer was not used as fluconazole and its related compounds are not charged), on a C8 column. By injecting every compound individually, good peaks for all compounds were obtained. However, for the separation of the mixture of fluconazole and its related compounds, C8 showed no adequate resolution between fluconazole related compounds A and B, as well as between fluconazole and fluconazole related compound C, using mobile phase of methanol and water with different volume fractions (20% to 80% of methanol). Afterwards, C18 column was tested (as it is more hydrophobic than C8 and it gives stronger retention of neutral compounds), and fortunately, good separation with good resolution of the four compounds was obtained (Figure 2) using mobile phase of methanol and water with optimum composition of 40% methanol and 60% water. Different flow rates (1.0, 1.5, and 2.0 ml/min) were tested and optimum of 1.5 ml/min was chosen. UV detection was
performed at 260 nm (as it gives good response for the four compounds), and the injection volume of 20 µL was found to be suitable for this analysis.

![Chromatogram of Fluconazole related compound A (1), Fluconazole related compound B (2), Fluconazole (3), and Fluconazole related compound C (4). Mobile phase: water, methanol (60:40, v/v), flow rate 1.5 mL/min, injection volume 20 µL. Column: reversed phase C18, 5 µm, 15 cm length, 4.6 mm inner diameter, UV detection: 260 nm.](image)

**Figure 2:** Chromatogram of Fluconazole related compound A (1), Fluconazole related compound B (2), Fluconazole (3), and Fluconazole related compound C (4). Mobile phase: water, methanol (60:40, v/v), flow rate 1.5 mL/min, injection volume 20 µL. Column: reversed phase C18, 5 µm, 15 cm length, 4.6 mm inner diameter, UV detection: 260 nm.

**Method Validation**

**Validation of Fluconazole Method of Analysis**

After method development, the validation of the current test method for Fluconazole was performed in accordance with USP requirements for assay determination (Category-I: Analytical methods for quantitation of active ingredients in finished pharmaceutical products) which include accuracy, precision, specificity, linearity and range.

**Linearity and Range**

Linearity is the ability of a method to elicit test results that are directly proportional to analyte concentration within a given range. Range is the interval between the upper and lower levels of analyte that have been demonstrated to be determined with precision, accuracy, and linearity using the method as written. The accepted criteria for linearity is that the correlation coefficient ($R^2$) is not less than 0.990 for the least squares method of analysis of the line [8].

Standard solutions covering the range between 50-150% of the nominal standard concentration (0.1 mg/mL) were prepared by diluting specific volume of the
stock standard (1.0 mg/ml of fluconazole) to get several concentrations (0.05, 0.075, 0.1, 0.125, 0.15 mg/mL). The peak area was recorded and plotted versus standard concentrations. Results have shown that the method is linear (straight line equation is $y = 1745.2x - 4.52$, where $y$ is the peak area, and $x$ is the concentration of fluconazole in mg/ml) over the specified range with $R^2$ of 0.992. These findings demonstrate linearity of this method over the specified range.

**Accuracy and precision**

Accuracy of a method is expressed as percentage of analyte recovered by spiking samples in placebo of the drug formulation [8]. To document accuracy, a minimum of nine determinations over a minimum of three concentration levels covering the specified range (for example, three concentrations, three replicates for each) were collected. It was performed at 50, 100, and 150% levels of label claim. The RSD of the replicates provides the analysis variation and gives an indication of the precision of the test method. Moreover, the mean of the replicates, expressed as % of label claim, indicates the accuracy of the test method. The mean recovery of the assay should be within 100 ± 2.0% at each concentration over the range of 50-150% of nominal concentration [8].

To prepare accuracy standard solutions, placebo of the drug formulation has to be prepared according to the formulation procedure. To the required quantity of placebo, a known quantity of Fluconazole with the same proportion as in the drug formulation was added to get three concentrations (0.05, 0.10 (nominal concentration), and 0.15 mg/ml). Results have shown that the mean recovery of the assay is within 100 ± 2.0%, and the RSD is lower than 1.0%, (Table 1).

**Table 1**: Percentage recovery of Fluconazole in capsule formulation at three concentration levels.

<table>
<thead>
<tr>
<th>Fluconazole Concentration (mg/ml)</th>
<th>% recovery</th>
<th>RSD for 3 replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>98.7</td>
<td>0.3%</td>
</tr>
<tr>
<td>0.10</td>
<td>99.1</td>
<td>0.8%</td>
</tr>
<tr>
<td>0.15</td>
<td>100.1</td>
<td>0.7%</td>
</tr>
</tbody>
</table>

Precision is the measure of the degree of repeatability of an analytical method under normal operation and is normally expressed as the RSD for a statistically significant number of samples. RSD for the peak areas of the samples should not be greater than 1.5% [8].

In this study, precision is determined from six different samples of the formulated fluconazole (capsule formulation). Results have shown that the RSD of the peak areas of the six samples (3 replicate injections for each sample) is 0.75%. These results show that the current method for Fluconazole analysis is repeatable.
Specificity (Stability Indicating Evaluation)

Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components. It is a measure of the degree of interferences from such components, ensuring that a peak response is due to a single component only. Specificity is measured and documented in a separation by the resolution, plate count (efficiency), and tailing factor.

Specificity of the current method was demonstrated by good separation of Fluconazole from its related compounds (A, B, and C) with adequate resolution, (Figure 2). Table 2 shows the chromatographic parameters of the separated peaks in figure 2.

Table 2: Chromatographic parameters of Fluconazole and Fluconazole related compounds peaks in figure 1.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Theoretical plates</th>
<th>Asymmetry</th>
<th>Resolution</th>
<th>Relative retention time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluconazole related compound A</td>
<td>1100</td>
<td>1.36</td>
<td>2.1</td>
<td>0.60</td>
</tr>
<tr>
<td>Fluconazole related compound B</td>
<td>1250</td>
<td>1.22</td>
<td>2.3</td>
<td>0.77</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>1530</td>
<td>1.36</td>
<td>4.1</td>
<td>1.0</td>
</tr>
<tr>
<td>Fluconazole related compound C</td>
<td>1270</td>
<td>1.42</td>
<td></td>
<td>1.70</td>
</tr>
</tbody>
</table>

Validation of the Method of Analysis of Fluconazole Related Compounds

Validation of the method for Fluconazole related compounds analysis was performed according to USP requirements for quantitative determination of impurities (Category II) which include accuracy, precision, specificity, linearity and range, and LOQ.

Limit of Detection and Limit of Quantitation

Limit of detection (LOD) is the lowest concentration of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. LOD can be determined by preparing a solution that is expected to produce a response that is approximately 3 to 10 times of the baseline noise. The solution is injected three times, and the signal and the noise for each injection are recorded. Each signal to noise ratio (S/N) is then calculated, and averaged. The concentration of the solution is used for determination of the detection limit if the average S/N ratio is between 3 and 10. If it is not between 3 and 10, the solution concentration is modified as necessary and the experiment is repeated. LOD may be expressed as:

\[
LOD = 3.3 \sigma/S
\]

Where \( \sigma \) is the standard deviation of the response, and S is the slope of the calibration curve.

Limit of quantitation can be determined in the same manner but using the formula

\[
10 \sigma/S.
\]
Results showed that LOD and LOQ using this method is 0.001 and 0.005 mg/ml, respectively, for Fluconazole related compound A, 0.002 and 0.005 mg/ml for Fluconazole related compound B, and 0.001 and 0.004 mg/ml, For Fluconazole related compound C. Low LOD and LOQ permits the detection of Fluconazole related compounds at low concentrations. The working concentration of these related compounds was chosen to be 0.005 mg/ml (near the LOQ) so that it can be detected and quantitated in formulated Fluconazole capsules at low concentration levels.

**Linearity and Range**

Linearity of the current method was established using five concentrations: 50%, 75%, 100%, 125%, and 150% of the working concentration (0.005 mg/ml) of Fluconazole related compounds. Results have shown that this method is linear over the range of 50-150% with $R^2$ of 0.997, 0.990, 0.992, for Fluconazole related compounds A, B, and C, respectively.

**Accuracy and precision**

Accuracy of the method for Fluconazole related compounds analysis was demonstrated by spiking samples of Fluconazole capsules with known amounts of Fluconazole related compounds A, B, and C. Accordingly, three solutions were prepared for this study having a concentration of 0.1 mg/ml of Fluconazole and three different concentrations of Fluconazole related compounds: 0.0025 mg/ml (50%), 0.0050 mg/ml (100%), and 0.0075 mg/ml (150%). Low concentration of Fluconazole related compounds relative to Fluconazole was employed to check if this low concentration of Fluconazole related compounds can be recovered in the presence of high concentration of Fluconazole. Percentage recovery of Fluconazole related compounds at these levels was found consistent as can be seen in Table 3. The chromatogram (Figure 3) of Fluconazole related compounds (0.005 mg/ml of each) and Fluconazole (0.10 mg/ml) shows that these related compounds can be recovered at this low concentration.

**Table 3:** Percentage recovery of Fluconazole related compounds at three concentration levels.

<table>
<thead>
<tr>
<th>Fluconazole related compound concentration</th>
<th>% recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fluconazole related compound A</td>
</tr>
<tr>
<td>0.0025</td>
<td>98.1</td>
</tr>
<tr>
<td>0.0050</td>
<td>99.6</td>
</tr>
<tr>
<td>0.0075</td>
<td>98.8</td>
</tr>
</tbody>
</table>

Precision of the method for Fluconazole related compounds analysis was demonstrated by analyzing 6 replicates of the working concentration of the related compounds (0.005 mg/ml) and calculating the RSD for the peak responses (Area).
Results have shown that the RSD for these 6 replicates is 0.82%, 0.76%, and 0.6% for Fluconazole related compounds A, B, and C, respectively.

Figure 3: Chromatogram of Fluconazole (0.1 mg/ml) and Fluconazole related compounds A, B, and C (0.005 mg/ml each). For other experimental conditions, see fig. 2.

Specificity

Specificity of the current method for Fluconazole related compounds analysis was demonstrated by separation of these related compounds from Fluconazole with adequate resolution; see figure 2 and table 2.

Conclusion

A simple, accurate and precise stability-indicating HPLC analytical method was developed and validated for the analysis of Fluconazole in capsule formulations. The current method has the ability to separate Fluconazole from its related compounds. LOD and LOQ for these related compounds using this method are low, which enables the detection and quantitation of these related compounds at low concentration.

Acknowledgments

I would like to thank gratefully Birzeit Pharmaceuticals for their support and providing us with the necessary instruments/apparatus to perform this study.
Abbreviations
LOD: Limit of detection
LOQ: Limit of quantitation
UV: Ultra Violet
HPLC: High Performance Liquid Chromatography
RP: Reversed Phase
USP: United States Pharmacopeia
RSD: Relative Standard Deviation

References