**Fluconazole: Stability and analysis in human plasma by simple high performance liquid chromatography**

**Summary**

A simple reversed phase high performance liquid chromatography assay to measure fluconazole level in human plasma was developed and validated, and fluconazole stability was studied. Fluconazole and acetophenetidin (internal standard) were detected spectrophotometrically at 260 nm after separation on Nova-Pak C18 cartridge at room temperature (retention time 5.7 and 11.8 minutes, respectively). The mobile phase, a mixture of 0.01 M phosphate buffer (pH 7.0) and acetonitrile (75: 25, v: v), was delivered at a flow rate of 1.0 ml/minute. Plasma samples were deproteinized by Amicon Centrifree system. No interference in blank plasma or by eleven commonly used drugs was observed. The limit of detection was 0.1 µg/ml. Calibration curves were linear (R^2 ≥ 0.9987) over the range of 0.2 (lower limit of quantitation) to 12 µg/ml, and intra and inter-run coefficients of variation and bias were ≤ 8.6% and ≤ 9.2%, and < 16.3% and ≤11.5%, respectively. Mean extraction recovery of fluconazole and acetophenetidin were 90% and 83%, respectively. Fluconazole was stable in plasma for 5 hours at room temperature (≥ 94%), 10 weeks at −20°C (103%), and after 3 cycles of freeze at −20°C and thaw at room temperature (≥ 87%). In deproteinized samples, fluconazole was stable for 16 hours at room temperature (≥ 100%) and 48 hours at −20°C (≥ 96%). Fluconazole stock solution (1 mg/ml in methanol / phosphate buffer 10:90) was stable for 6 hours at room temperature (≥ 99 %) and 3 weeks at −20°C (≥ 90%).

**Key Words:** Fluconazole, Stability, Acetophenetidin, HPLC.

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**INTRODUCTION**

Fluconazole, 2-(2, 4-difluorophenyl)-1, 3-bis (1H-1, 2, 4-triazol-1-yl)-2-propanol (Fig. 1), is a triazole drug that is administered orally or intravenously for the treatment of systemic and localized fungal infections. It is also used in patients with the acquired immunodeficiency syndrome as suppressive therapy for cryptococcal meningitis. Fluconazole circulates...
in plasma as 11-12% protein-bound. (1) Its peak plasma level after the oral administration of a single therapeutic dose of 200 mg is around 6-7 µg/ml, (2) and concentrations of 8 to 9 µg/ml are considered therapeutic for candidasis. (3-4)

Several methods to determine fluconazole level in human plasma have been reported. They were based on high-performance liquid chromatography (HPLC), (2-8, 20) gas chromatography, (9-12) liquid chromatography/tandem mass spectrometry (LC-MS-MS), (13-15) bioassay, (16-17) and micellar electrokinetic capillary chromatography (MECC) (18) These assays involved time-consuming liquid-liquid or column extraction steps, (3, 5, 9-12) had high level of detection, (2, 7, 20) used equipment that might not be available in clinical laboratory settings, (13-15) or did not incorporate an internal standard. (3, 6, 7)

The aims of the present study were to develop and fully validate a simple and reliable method for the measurement of therapeutic fluconazole levels in human plasma and to determine the stability of fluconazole under various conditions encountered in the clinical laboratory. We propose a single deproteinization step using commercially available filters followed by an HPLC with UV detection with a run time of 13 minutes per injection.

**EXPERIMENT**

**Apparatus**

The liquid chromatography system consisted of Waters Alliance 2690 Separations Module and Waters 996 photodiode array detector set at 260 nm (Waters Corporation, Milford, MA, USA). Data were collected and processed with a Pentium III computer using Millennium® Chromatography Manager Software (Waters Corporation, Milford, MA, USA).

**Chemicals and reagents**

Analytical reference standards of fluconazole and acetophenetidin (internal standard) were obtained from Janssen Biotech N.V., Hill Road, NJ, USA and Sigma Chemical Co., St. Louis, MO, USA, respectively. Acetonitrile, methanol, 85% phosphoric acid, and disodium hydrogen phosphate were all HPLC grade and obtained from Fisher Scientific, Fair Lawn, NJ, USA. Water for the HPLC was generated by reverse-osmosis and further purified by passing through Milli-Q system obtained from Millipore Co. (Bedford, MA, USA).

**Chromatographic conditions**

The separation of compounds was achieved on a 4-µm (particle-size), 8 x 100-mm Nova-Pak C₁₈ radial compression cartridge in conjunction with an RCM-100 radial compression module, and a Nova-Pak C₁₈ 4-µm insert in conjunction with Guard Pak pre-column module. The mobile phase consisted of 0.01 M disodium hydrogen phosphate (pH adjusted to 7.0 with phosphoric acid) and acetonitrile (75: 25 v: v) and was delivered at a flow rate of 1.0 ml/min at room temperature. The mobile phase was filtered through a 0.45 µm membrane filter (Millipore Co., Bedford, MA, USA) and degassed before use.

**Stock and working standard solutions**

Fluconazole stock solution (1 mg/ml) was prepared in methanol/0.01 M disodium hydrogen phosphate buffer (pH 7) (10: 90, v: v) and filtered through 0.2 µm Gelman filter (Gelman Sciences, Ann Arbor, MI, USA). Acetophenetidin (IS) stock solution (1 mg/ml) was prepared in methanol/0.01 M disodium hydrogen phosphate buffer (pH 7) (50 : 50, v : v). Aliquots from these stock solutions were transferred into 4.0 ml amber vials and stored at −20°C until used. Fluconazole working solution (100 µg/ml) was prepared daily by diluting fluconazole stock solution with blank human plasma (leftover plasma obtained from the blood bank of King Faisal Specialist Hospital and Research Centre, with the Research
Ethics Committee approval (RAC 2031081). The IS working solution (100 µg/ml) was prepared daily by diluting the IS stock solution with 0.01 M disodium hydrogen phosphate buffer (pH 7).

Calibration standards and quality control (QC) samples
Nine calibration standards were prepared by mixing blank human plasma with the appropriate volumes of fluconazole working solution to produce final concentrations in the range of 0.2 – 12.0 µg/ml. Quality control samples were prepared by mixing four different volumes of fluconazole working solution with human blank plasma to produce QC samples with a final concentrations of 0.2, 0.5, 5.0, and 10.0 µg/ml. Samples were vortex-mixed for 10 seconds then 0.5 ml aliquots were transferred into 1.5 ml microcentrifuge tubes and stored at −20°C until used.

Sample preparation
Aliquots of 0.5 ml of plasma samples, calibration standards, or QC samples in 1.5 ml microcentrifuge tubes were allowed to equilibrate at room temperature prior to analysis. To each tube 10 µl of the IS working solution (100 µg/ml) and 200 µl of 0.01 M of disodium hydrogen phosphate buffer (pH adjusted to 7.0) were added. The samples were vortexed for 30 seconds, sonicated for 1 minute, transferred to an Amicon Centrifree micropartition system (Millipore Co., Bedford, MA, USA), and centrifuged at 3000 rpm for 15 minutes. 200 µl of the ultra-filtrate was transferred to an autosampler vial and 100 µl was injected into the HPLC system.

Stability studies
Plasma samples: The stability of fluconazole in human plasma was assessed by analyzing QC samples at two concentrations, 0.5 and 10.0 µg/ml. Five aliquots of each QC sample were freshly prepared, deproteinized, and immediately analyzed (baseline). Counter and long term stability were assessed by placing five aliquots of each QC sample on the bench-top at ambient temperature for 5 hours or at −20°C for 10 weeks before being processed and analyzed. Further, five aliquots of each QC sample were processed and stored at ambient temperature for 16 hours or at −20°C for 48 hours before analysis (auto-sampler stability). Finally, freeze and thaw stability was estimated by analyzing fifteen aliquots of each QC sample stored at −20°C for 24 hours, and then left to completely thaw unassisted at ambient temperature (up to three cycles) before analysis.

Stock solution: The stability of fluconazole and IS (1 mg/ml in a mixture of methanol/ phosphate buffer) were evaluated for up to 6 hours at room temperature and up to 3 weeks at −20°C. After completion of the storage time, the solutions were diluted in phosphate buffer to 100 µg/ml or 20 µg/ml for fluconazole and the IS, respectively. 100 µl was injected into the HPLC system in five replicates. Stability was assessed by comparing the peak height of each compound with that of the freshly prepared solutions.

Calculations
Precision was calculated as coefficient of variation (standard deviation divided by mean measured concentration × 100) and bias (inaccuracy) as mean measured concentration – nominal concentration / nominal concentration × 100.

RESULTS AND DISCUSSION
Optimization of the chromatographic conditions
A simple high performance liquid chromatographic assay for the quantification of fluconazole levels in human plasma was developed. The assay was performed at room temperature on a 4-µm (particle-size), 8 × 100-mm Nova-Pak C₁₈ radial compression cartridge in conjunction with an RCM-100 radial compression module, a Nova-Pak C₄ 4-µm insert in conjunction with Guard Pak pre-column module and Waters 996 photodiode array detector set at 260 nm. The chromatographic conditions were optimized by examining various ratios and pH of the mobile phase. Mobile phase selection was based on peak parameters (symmetry, tailing), run time, and ease of preparation. The best mobile phase consisted of 0.01 M disodium hydrogen phosphate (pH adjusted to 7.0 with phosphoric acid) and acetonitrile (75: 25, v: v) with a flow rate of 1 ml/minute, and a run time of 13 minutes. Under the described conditions, fluconazole and the IS were well resolved with a retention time of 5.7 and 11.8 minutes, respectively.
Chromatograms of blank human plasma spiked with fluconazole at a final concentration of 0.0, 0.5, 5.0, 10.0 µg/ml and 2.0 µg/ml of IS are shown in Figure 2.

**Assay Validation**
The analytical method was fully validated with respect to the parameters described in the US Food and Drug Administration (FDA) bioanalytical method validation guidelines. (19)

**Specificity**
To evaluate specificity, plasma samples from six different drug-free plasma were screened for the presence of endogenous components, which might co-elute with fluconazole or the IS. Specificity was also checked for possible interferences by eleven frequently used medications, namely: nizatidine, domperidone, gatifloxacine, aspirin, acetaminophen, ibuprofen, ranitidine, nicotinamide, ascorbic acid, caffeine, and omeprazole. All blank human plasma samples were free from co-eluting peaks, and none of the drugs interfered with fluconazole or the IS.

**Linearity**
Linearity of the method was evaluated using nine concentrations over the range of 0.2-12.0 µg/ml. Calibration curves were linear with an $R^2 = 0.9987$. The mean concentrations, back calculated from peak height ratios using individual regression equation are given in Table 1 with their respective mean, standard deviation, biases and precisions. Mean (SD) of slope, 

**Table 1: Back-Calculated Fluconazole Concentrations of Six Calibration Curve**

<table>
<thead>
<tr>
<th>Nominal Concentration (µg/ml)</th>
<th>0.2</th>
<th>0.4</th>
<th>0.6</th>
<th>0.8</th>
<th>1.0</th>
<th>2.0</th>
<th>4.0</th>
<th>8.0</th>
<th>12.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0.23</td>
<td>0.39</td>
<td>0.64</td>
<td>0.79</td>
<td>1.02</td>
<td>1.95</td>
<td>3.93</td>
<td>8.14</td>
<td>11.92</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.04</td>
<td>0.05</td>
<td>0.06</td>
<td>0.04</td>
<td>0.02</td>
<td>0.06</td>
<td>0.25</td>
<td>0.27</td>
<td>0.19</td>
</tr>
<tr>
<td>*Precision (% CV)</td>
<td>19.1</td>
<td>12.2</td>
<td>8.7</td>
<td>5.2</td>
<td>2.0</td>
<td>2.9</td>
<td>6.2</td>
<td>3.3</td>
<td>1.6</td>
</tr>
<tr>
<td>†Bias</td>
<td>12.6</td>
<td>−1.7</td>
<td>6.1</td>
<td>−1.5</td>
<td>1.6</td>
<td>−2.4</td>
<td>−1.7</td>
<td>1.7</td>
<td>−0.7</td>
</tr>
</tbody>
</table>

* Coefficient of variation (CV, %) = Standard Deviation (SD) divided by mean measured concentration $\times 100$.
† Bias = (mean measured concentration – nominal concentration divided by nominal concentration) $\times 100$. 

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and intercept of the six curves were 17.17 (1.26), 0.004 (0.040), and 0.9987 (0.001), respectively (Table 2).

**Limits of detection and quantification**
The limit of detection, defined as three times the baseline noise, was 0.1 µg/ml. The limit quantification, defined as the lowest measured concentration with a precision and bias of < 20.0 %, was 0.2 µg/ml.

**Precision and bias (inaccuracy)**
The intra-day and inter-day precision and bias of the method were evaluated by analyzing four different concentrations of fluconazole over three different days. Intra-day precision and bias (n = 10) ranged from 3.6 % to 8.6 % and from −2.6 % to 16.5 %, respectively. The inter-day precision and bias (n = 20) ranged from 3.2 % to 9.2 % and from −2.4 % to 11.5 %, respectively (Table 3).

**Recovery**
The extraction recovery of fluconazole and the IS from plasma was calculated by comparing the mean peak areas of five replicates of four QC samples and five replicates of the IS (100 µg/ml) that were prepared in plasma (as described under sample preparation above), with the mean peak height of five replicates of equivalent concentrations prepared in the mobile phase. The recovery of fluconazole and the IS was ≥78% (mean 90%) and 83%, respectively (Table 4).

**Stability**
Stability studies were investigated at two concentrations (0.5 and 10.0 µg/ml) in plasma, and deproteinized samples as well as in stock solutions. The results revealed no significant decrease in measured concentrations or change in chromatographic behavior of fluconazole after storage of plasma for 5 hours at room temperature or 10 weeks at −20°C, or after three freeze-thaw cycles. Stock solutions of fluconazole and IS (1 mg/ml in methanol/ phosphate solution) were stable for at least 6 hours at room temperature (99% and 100%, respectively) and for at least 3 weeks at −20°C (100% and 90%, respectively). Finally, fluconazole was

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**Table 2: Summary of Regression Analysis of Six Calibration Curves of Fluconazole Assay**

<table>
<thead>
<tr>
<th>Linearity Range µg/ml</th>
<th>Slope Mean (SD)</th>
<th>Intercept Mean (SD)</th>
<th>R² Mean (SD)</th>
<th>LLD µg/ml</th>
<th>LLQ µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 – 12.0</td>
<td>17.17 (1.26)</td>
<td>0.004 (0.040)</td>
<td>0.9987 (0.001)</td>
<td>0.1</td>
<td>0.2</td>
</tr>
</tbody>
</table>

LLD, lower limit of detection. LLQ, lower limit of quantification.

**Table 3: Intra-day and inter-day precision and bias of fluconazole assay**

<table>
<thead>
<tr>
<th>Nominal Concentration (µg/ml)</th>
<th>Found Concentration (µg/ml)</th>
<th>Intra-Day (10)</th>
<th>Inter-day (20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>S.D</td>
<td>†Precision (%CV)</td>
<td>†Bias %</td>
</tr>
<tr>
<td>0.2</td>
<td>0.020</td>
<td>8.6</td>
<td>16.5</td>
</tr>
<tr>
<td>0.5</td>
<td>0.019</td>
<td>3.9</td>
<td>2.6</td>
</tr>
<tr>
<td>5.0</td>
<td>0.197</td>
<td>3.9</td>
<td>1.1</td>
</tr>
<tr>
<td>10.0</td>
<td>0.358</td>
<td>3.6</td>
<td>−1.2</td>
</tr>
</tbody>
</table>

* Coefficient of variation (CV, %) = Standard Deviation (SD) divided by mean measured concentration × 100.
† Bias = (mean measured concentration – nominal concentration divided by nominal concentration) × 100.
stable in deproteinized samples for at least 16 hours at room temperature or 48 hours at $-20^\circ$C (Table 5).

**Conclusion**

A number of high performance liquid chromatography (HPLC) assays for the determination of fluconazole in plasma have been reported (2-8, 20). The drawbacks of the reported methods include lack of internal standard (3, 6, 7), high level of detection (2, 7, 20), and the use of time-consuming liquid-liquid, column extraction, or evaporation of organic extract under nitrogen steam (2, 5, 7). A simple method used acetonitrile as a protein precipitating solvent, however, it had a relatively high level of detection and low recovery of 0.625 µg/ml and 86.6%, respectively (20). Direct injection into the chromatographic system of small volumes of diluted plasma or extracts obtained from plasma protein precipitation can reduce the lifetime of the analytical column.

Therefore, this report described a single deproteinization step, using a commercially available filter that yielded a clear sample and high and reproducible recovery. The

**Table 4: Extraction recovery of Fluconazole and Acetophenetidin (IS)**

<table>
<thead>
<tr>
<th>Nominal Concentration (µg/ml)</th>
<th>* Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean †  SD Bias</td>
</tr>
<tr>
<td>Fluconazole</td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>78  4.822</td>
</tr>
<tr>
<td>0.5</td>
<td>87  3.813</td>
</tr>
<tr>
<td>5.0</td>
<td>96  1.64</td>
</tr>
<tr>
<td>10.0</td>
<td>99  1.31</td>
</tr>
<tr>
<td>IS</td>
<td>83  3.417</td>
</tr>
</tbody>
</table>

* Peak height of spiked plasma sample divided by peak height of spiked mobile phase sample × 100.
† Mean of 5 replicates. SD, Standard Deviation.

**Table 5: Stability of Fluconazole in Plasma Samples and Stock Solution**

<table>
<thead>
<tr>
<th>Nominal Concentration (µg/ml)</th>
<th>Stability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Undeproteinized</td>
</tr>
<tr>
<td>------------------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>0.5</td>
<td>97  104</td>
</tr>
<tr>
<td>10.0</td>
<td>94  103</td>
</tr>
</tbody>
</table>

Stability (%) = mean measured concentration (n=5) at the indicated time divided by mean measured concentration (n=5) at baseline x 100.

*Spiked plasma samples were filtered through Amicon Centrifree micropartition system and analyzed immediately (baseline, data not shown), after 5 hours at room temperature (5 hrs RT), after freezing at $-20^\circ$C for 10 weeks (10 weeks $-20^\circ$C), or after 1 to 3 cycles of freezing at $-20^\circ$C and thawing at room temperature (freeze-thaw); or filtered (deproteinized) and analyzed after leaving the ultrafiltrate 16 hours at room temperature (16 hrs RT) or 48 hours at $-20^\circ$C (48 hrs $-20^\circ$C).
†1 mg/ml in methanol: phosphate buffer (10 : 90, v : v).
proposed assay was sensitive, rapid, precise, specific, suitable for pharmacokinetic and bioequivalence studies, and was implemented successfully in the studies on fluconazole stability. Calibration curves were linear (R^2 ≥ 0.9987) over the range of 0.2 to 12 µg/ml, and intra and inter-run coefficients of variation and bias were ≤ 8.6% and ≤ 9.2%, and < 16.5% and 11.5%, respectively. Mean extraction recovery of fluconazole and acetophenetidin were 90% and 83%, respectively. Fluconazole was stable in plasma for 5 hours at room temperature (≥ 94%), 10 weeks at −20°C (103%), and after 3 cycles of freeze at −20°C and thaw at room temperature (≥ 87%). In deproteinized samples, fluconazole was stable for 16 hours at room temperature (≥100%) and 48 hours at −20°C (≥96%). Finally, fluconazole stock solution was stable for 6 hours at room temperature (≥ 99 %) and 3 weeks at −20°C (≥90%).

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REFERENCES
17. Rex JH, Hanson LH, Amantea MA, Stevens DA, Bennett JE. Standardization of a

