Validated HPLC method for the determination of fluconazole in human plasma

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Received 24 February 2005; revised 17 May 2005; accepted 19 May 2005

ABSTRACT: A high-performance liquid chromatographic assay with UV detection was developed for the determination of fluconazole in human plasma. The method utilized solid-phase extraction for sample clean-up. The separation was performed on a C_{18} column by isocratic elution with a mobile phase of 10 mM acetate buffer at pH 5.0 and methanol and UV detection at 210 nm. Validation was performed according to the current recommendations of the USFDA bioanalytical method validation guidance. The method proved to be specific, accurate, precise and linear between 200 and 10,000 ng/mL with correlation coefficients greater than 0.999. The coefficient of variation was within 11% and relative deviation was less than 10%. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: fluconazole; HPLC; UV detection

INTRODUCTION

Fluconazole is an orally bistriazole antifungal agent used in the treatment of superficial and systemic candidiasis in patients with acquired immunodeficiency syndrome (AIDS). Several methods have been reported for quantitative analysis of fluconazole in human plasma and biological samples. Bioassay is time-consuming (Troke et al., 1985; Van’t Wout et al., 1988; Rex et al., 1991) and is not very reliable since medium conditions are known to influence the in vitro activity of fluconazole (Marchetti et al., 2001). Gas chromatography is very sensitive, but the sample preparation is sometimes laborious (Wood and Tarbit, 1986) and the validated concentration range is too narrow (0.01–0.15 µg/mL) for use in pharmacokinetics and bioavailability studies (Debruyne et al., 1988). Because of these disadvantages, high-pressure liquid chromatography (HPLC) is the method of choice for fluconazole determination in biological samples (Hosotsubo et al., 1990; Inagaki et al., 1992; Koks et al., 1996; Cociglio et al., 1996; Mathy et al., 2003). The purpose of the present study was to develop a validated HPLC method for the determination of fluconazole in human plasma which proved to meet the currently accepted USFDA (2001) bioanalytical method validation guidance.

EXPERIMENTAL

Chemicals. Fluconazole was obtained from Stancross S. A. (Montevideo, Uruguay), while phenacetin, internal standard (IS), was obtained from BDH Chemicals (Poole, UK). Sodium acetate (Fluka, Neu-Ulm, Germany), sodium dihydrogen phosphate (Carlo Erba, Milano, Italy) and glacial acetic acid (Merck, Darmstadt, Germany) were analytical grade. HPLC-grade methanol was obtained from Mallinckrodt (KY, USA). Drug-free human plasma was purchased from the Thai Red Cross Society (Bangkok, Thailand).

Instrumentation and chromatographic conditions. The HPLC system (Dionex, Germering, Germany) consisted of a pump with integrated degasser (P680A LPG), an autosampler (ASI-100), a column oven (TCC-100) and a UV–vis diode array detector (UVD 340U). The separation was performed at 35°C on a Luna C_{18}(2) column (150 × 4.6 mm, i.d.) protected by a C_{18} guard column (4.0 × 3.0 mm) (both Phenomenex, USA). The mobile phase was a mixture of 10 mM sodium acetate buffer (adjusted to pH 5.0 with glacial acetic acid) and methanol (65:35) at a flow rate of 1 mL/min. Detection was monitored at 210 nm.

Preparation of calibration curves and quality controls. Stock solutions (1000 µg/mL) of fluconazole and IS were separately prepared in methanol. A series of fluconazole working solutions were prepared by diluting stock solution with methanol–water (50:50). Calibration samples were prepared by spiking 450 µL blank plasma with 50 µL fluconazole working solutions to produce final concentrations of 200, 500, 1000, 2000, 5000, 7500 and 10,000 ng/mL. Quality control

samples (QCs) of fluconazole in plasma were prepared at low (QCL), medium (QCM) and high (QCH) concentrations of 500, 3000 and 9000 ng/mL, respectively.

**Sample extraction.** Calibration samples and QCs were extracted using solid-phase extraction. A 50 µL aliquot of IS solution (25 µg/mL) and 550 µL 0.1 M phosphate buffer pH 6.0 were added to 500 µL plasma sample and vortexed for 15 s. A 990 µL aliquot of sample solution was loaded onto a solid-phase extraction cartridge (Waters Oasis™) which was preconditioned with 2 mL methanol and 2 mL 0.1 M phosphate buffer pH 6.0. After loading, each column was washed with 1 mL 0.1 M phosphate buffer pH 6.0, followed by 1 mL mixture of methanol and 0.1 M phosphate buffer pH 6.0 (15:85), and eluted with 2 mL methanol. The eluate was evaporated to dryness under vacuum at 40°C. The residue was reconstituted with 500 µL of mobile phase and 40 µL were injected into the HPLC.

**Validation of the method.** The specificity of the method was investigated using six different sources of human plasma. The calibration curve was obtained by plotting peak area ratios (y) of drug to internal standard and drug concentrations (x). Linear regression was performed with a weighting factor of 1/x^2. The intra-day and inter-day precision and accuracy were determined using five replicates of QCL, QCM, QCH and at LLOQ. Accuracy expressed as relative deviation and precision expressed as coefficient of variation should be within 15% except at LLOQ, where it should be within 20%. Stability of fluconazole in plasma was determined for short- and long-term storage, after three freeze–thaw cycles, and stability of the extracts (post preparative stability), stock solutions of fluconazole and internal standard.

**RESULTS AND DISCUSSION**

**Specificity**

Under the described conditions, the retention times of fluconazole and internal standard were 7 and 11 min, respectively. No peak interferences were found in six different sources of human plasma, as shown in Fig. 1.

**Linearity, accuracy and precision**

The method exhibited a good linearity over a concentration range 200–10,000 ng/mL. A representative regression line was \( y = 0.0003x - 0.0053 \) with the correlation coefficient always greater than 0.999 as determined on five different days. Results of intra- and inter-day accuracy and precision were within the acceptance criteria as shown in Table 1.

**Recovery**

Recovery of fluconazole from human plasma was calculated by comparing peak areas of extracted samples with unextracted standard solutions at three QC levels. The average percentages of recovery of fluconazole

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**Table 1. Intra- and inter-day accuracy and precision for fluconazole determination in human plasma**

<table>
<thead>
<tr>
<th>Fluconazole calculated concentration (ng/mL)*</th>
<th>LLOQ (200 ng/mL)</th>
<th>QCL (500 ng/mL)</th>
<th>QCM (3000 ng/mL)</th>
<th>QCH (9000 ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intra-day (n = 5)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Accuracy as RD (%)</td>
<td>2.5</td>
<td>7.3</td>
<td>−2.1</td>
<td>1.6</td>
</tr>
<tr>
<td>Precision as CV (%)</td>
<td>6.5</td>
<td>0.6</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td>Day 2</td>
<td>7.1</td>
<td>6.4</td>
<td>4.3</td>
<td>3.0</td>
</tr>
<tr>
<td>Accuracy as RD (%)</td>
<td>10.9</td>
<td>6.2</td>
<td>1.0</td>
<td>1.5</td>
</tr>
<tr>
<td>Precision as CV (%)</td>
<td>199.6 ± 17.3</td>
<td>486.3 ± 20.6</td>
<td>2716.2 ± 85.9</td>
<td>8436.6 ± 108.2</td>
</tr>
<tr>
<td>Accuracy as RD (%)</td>
<td>−0.2</td>
<td>−2.7</td>
<td>−9.5</td>
<td>−6.3</td>
</tr>
<tr>
<td>Precision as CV (%)</td>
<td>8.7</td>
<td>4.2</td>
<td>3.2</td>
<td>1.3</td>
</tr>
<tr>
<td><strong>Inter-day (n = 15)</strong></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Accuracy as RD (%)</td>
<td>3.2</td>
<td>3.6</td>
<td>−2.4</td>
<td>−0.6</td>
</tr>
<tr>
<td>Precision as CV (%)</td>
<td>8.6</td>
<td>7.0</td>
<td>6.2</td>
<td>5.0</td>
</tr>
</tbody>
</table>

* Values are reported as mean ± SD.
Determination of fluconazole in plasma

The extraction efficiency of the analytical method was consistent and reproducible.

**Stability**

The stability of fluconazole in human plasma was tested using three replicates of two QC levels (QCL and QCH) under tested conditions compared with freshly prepared QCs. The results indicated that fluconazole in human plasma was stable for three cycles of freeze and thaw, short-term (6 h), long-term (stored at −20°C for 2 months) and post-preparative (22 h in autosampler) with mean values in the range 98.6–104.1 and 95.5–99.8% for QCL and QCH, respectively.

Stock solutions of fluconazole and phenacetin kept at room temperature were stable for at least 24 h with mean values of 100.6 and 100.8%, respectively and those stored at 4°C were stable for at least 2 months with recoveries of 99.6 and 97.4%, respectively.

**CONCLUSION**

An HPLC-UV method for the determination of fluconazole in human plasma was developed and fully validated with a lower limit of quantification of 200 ng/mL. No interference of the analyte was observed. It was shown that the method is accurate, precise and linear over the concentration range 200–10,000 ng/mL. The proposed method is sufficiently sensitive and reproducible for pharmacokinetic and bioequivalence studies.

**Acknowledgement**

This work was supported in part by the Government Pharmaceutical Organization, Bangkok, Thailand.

**REFERENCES**


