Analysis of Fluconazole in Human Urine Sample by High Performance Liquid Chromatography Method

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Abstract. A method for determination of fluconazole, antifungal drug in human urine by using reversed-phased high performance liquid chromatography (RP-HPLC) with ultraviolet (UV) detector was developed. Optimization HPLC conditions were carried out by changing the flow rate and composition of mobile phase. The optimum separation conditions at a flow rate 0.85 mL/min with a composition of mobile phase containing methanol:water (70:30, v/v) with UV detection at a wavelength 254 nm was able to analyze fluconazole within 3 min. The excellent linearity was obtained in the range of concentration 1 to 10 µg/mL with $r^2 = 0.998$. The limit of detection (LOD) and limit of quantitation (LOQ) were 0.39 µg/mL and 1.28 µg/mL, respectively. Solid phase extraction (SPE) method using octadecylsilane (C18) as a sorbent was used to clean-up and pre-concentrated of the urine sample prior to HPLC analysis. The average recoveries of fluconazole in spiked urine sample was 72.4% with RSD of 3.21% (n=3).

Keywords: Antifungal drug, Fluconazole, Human urine, RP-HPLC, SPE

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1. Introduction

An antifungal drug had widely used as a medication to treat fungal infection such as a ringworm, candidiasis, foot fungus and many others. Until 1970, antifungal agents had many problems included toxicity, narrow activity spectrum or low pharmacological activity. But, the patients who acquire immunodeficiency syndrome (AIDS), undergoing anti-cancer chemotherapy or having suffered an organ transplant are necessary to take effective drug for the bacterial and fungal disease treatment. It is because the immune system of the cancer patients who is receiving chemotherapy usually at high risk of developing invasive fungal infections. Hence, this has resulting in increasing clinical demand for newer and safer antifungal drugs which arrived infection focus, efficient and lower toxicity [1].

Fluconazole is a former synthetic triazole antifungal agents which available for over two decades that safe and effective in cancer patients. According to the clinical pharmacokinetics of fluconazole, it has been indicated effective at wide range of body sites because of its large distribution volume, long half life and also rapid absorption after oral administration. In the pharmaceutical analysis, high performance liquid chromatography (HPLC) is the most extensively technique used [2-5]. The application of modern HPLC toward quantification of various compounds is very applicable because it can perform a separation, identification and also purification. The analysis using HPLC also perform in a shorter time and the most important thing that HPLC is characterized by high resolution, selectivity, sensitivity, precision and accuracy. In this report, solid phase extraction (SPE) coupled with HPLC was successfully applied to the determination of fluconazole in human urine sample.

2. Material and Method

2.1. Chemical and Reagents
Fluconazole (99.1% purity) was purchased from Sigma (St Louis, MO, USA). Methanol (HPLC) grade was purchased from J. T. Baker, USA. Purified water was collected from a Millipore Simplicity (Simpak®2). All solvents are filtered through 0.45 µm membrane prior to usage. Human urine samples were collected from healthy drug-free volunteers.

2.2. Chromatographic Instrumentation
The HPLC system was an Agilent 1100 Series (Germany) and consisted of a Model Agilent 1100 pump, an on-line solvent vacuum degasser, an auto sampler with 20 µL injection loop, and an Agilent 1100 Series UV detector. The analytical column LichroCART® C18 (150 mm x 4.6 mm x 5 µm particles) was obtained from Germany. The Agilent Chem Station software was used to record chromatograms and calculate peak area. The mobile phase consisted of a mixture of methanol and water (70:30 v/v). The HPLC system was operated isocratically at a flow rate of 0.85 mL/min and the effluent of the column was monitored at a wavelength of 254 nm. An Agilent 1100 Series UV Spectrophotometer (Germany) was used to record the absorption maxima of analyte.

2.3. Preparation of Standards
Stock solution (2000 µg/mL) of fluconazole was prepared by dissolving ten mg accurately weighed fluconazole in methanol as a solvent. For the quantitation of fluconazole, an appropriate standard working solution 100, 50, 20 and 10 µg/mL were prepared by diluting a stock solution in methanol. Serial dilutions from 10 µg/mL of standard working solution were made to obtain four different concentrations in the range of 0.5 to 10 µg/mL using methanol. Three replicates for each concentration were used for calibration standards to determine the limit of detection, linearity range and correlation coefficients. The stock and standard working solutions were sealed and stored in the refrigerator at temperature 5ºC prior to use.
2.4. Sample Preparation
The sample preparation involved spiking the human urine sample with standard fluconazole solutions. Sample was spiked with 5 ppm of standard fluconazole after the blank sample was confirmed that no fluconazole was detected in the sample using HPLC-UV. For the sample pre-treatment, the human urine was filtered through a Buchner funnel prior extraction with solid phase extraction and the spiked sample was added with 1.0 M sodium hydroxide to maintain the basic condition of drug. After that, the spiked sample human urine was introduced to the C18 extraction cartridge. The eluent was reconstituted with 500 µL of methanol and 20 µL was injected into HPLC-UV for analysis.

2.5. Extraction Procedure
The isolation of fluconazole from human urine was conducted by using solid phase extraction (SPE). The SPE C18 cartridge with a capacity of 3 mL was placed in a 12-port of SPE vacuum manifold system. The vacuum pressure was adjusted to -0.5 Hg/bar and each column was activated by preconditioning with 5 mL of methanol followed by 5 mL of deionized water. The approximately 1.0 mL of spiked sample was introduced into the cartridge under a vacuum at a flow rate 0.5 mL/min. The solid phase in the cartridge was not allowed to become dry at any moment. Afterwards, analyte was eluted with 3 mL methanol. The eluent was concentrated under a stream of nitrogen to dryness until all the solvent evaporated. Finally, the analyte residue was dissolved in 500 µL methanol and aliquot (200 µL) was injected into HPLC-UV. Analysis of a blank sample and spiked sample were performed for a comparison purpose.

3. Results and Discussions

3.1 Effect of Mobile Phase
Addition of organic modifier increase the strength of eluting, varying it composition will affect the elution time for analyte. The results shows that retention time will be longer when the percentage of water higher than organic modifier or in other word, retention time of analyte will be shorter with increasing of organic modifier. Fluconazole was a polar compound will have a strong interaction with the high percentage of organic solvent which was methanol. Methanol lowering the polarity of mobile phase cause the analyte less retained in mobile phase. Analyte will be eluted faster and resulting in the shorter retention time.

A column with a high number of theoretical plates will have a narrower peak at a given retention time than a column with a lower N number. The effect of organic modifier (methanol) on some basic compound was gave a significantly better peak shape [6]. This result may become significance in this study because the properties of fluconazole which was also a basic compound. The higher percentage of organic modifier in mobile phase may results a narrow peak. The sharp peak, which present in the density profiles of water, result from solvent molecules directly hydrogen bonding with surface silanols. The result obtained, the peak shape of analyte will become narrower due to high value of N when the percentage of water was increased. This result was different from the literature stated above and it was due to a different protonation of analyte in mobile phase which gave a possible influential factor of different in a peak shape.

3.2 Effect of Flow Rate
Flow rates of mobile phase have important effect on the analyte retention time. Higher flow rates lead to a shorter retention time and vice versa. The result shows that the retention time was decrease as the mobile phase flow rate was increased. A further improvement in separation may be possible by varying column condition such as flow rate to improve the column plate number, N. An increase in N leads to an increase in resolution and usually longer run time. Conversely, a decrease in N can provide
a shorter run time. The result shows that the high flow rate was decreased the value of \( N \) which means that the resolution was not good and gave a slightly broad peak.

### 3.3 Linearity and Recovery

The HPLC method assay for determination of fluconazole was linear from 1 to 10 µg/mL and show a good correlation coefficients, \( r^2 = 0.998 \). The limit of detection and limit of quantification of fluconazole was 0.39 µg/mL and 1.28 µg/mL respectively. The low limit of detection showed the method developed is sensitive and sufficient to determine fluconazole in urine samples for routine analysis.

The absolute recovery of fluconazole from human urine sample was estimated by comparing the peak height obtained from chromatogram of standards with those obtained by the spiked urine sample with known concentration of fluconazole (figure 1). The recovery of fluconazole obtained in urine sample was 72.4% with RSD 3.27% (\( n = 3 \)).

![Chromatogram of 10 ppm fluconazole standard (a) and spiked urine sample after SPE procedure (b). HPLC conditions: methanol:water (70:30,v/v) as mobile phase, flow rate of 0.85 mL/min and UV detection at 254 nm. Column: LichroCART® C₁₈ (150 mm x 4.6 mm x 5 µm particles).](image)

**Figure 1.** Chromatogram of 10 ppm fluconazole standard (a) and spiked urine sample after SPE procedure (b). HPLC conditions: methanol:water (70:30,v/v) as mobile phase, flow rate of 0.85 mL/min and UV detection at 254 nm. Column: LichroCART® C₁₈ (150 mm x 4.6 mm x 5 µm particles).

### 4. Conclusions

In this study, high performance liquid chromatography (HPLC) method for analysis of fluconazole in human urine has been developed. The HPLC system was performed with C₁₈ (150 mm x 4.6 mm x 5 µm particles) column using methanol:water (70:30,v/v) as a optimum mobile phase. The calibration graph was obtained in the range of 1 to 10 ppm. The correlation coefficient from the graph is 0.998. The limit of detection (LOD) for fluconazole is 0.39 ppm while limit of quantifying (LOQ) is 1.28 ppm. Solid phase extraction (SPE) using octadecylsilane (C₁₈) was used for sample clean-up and pre-concentration method. The SPE coupled with HPLC-UV method has been successfully applied to determination of fluconazole in urine sample with percentage of recovery = 72.4% (RSD = 3.27%) with relatively shorter analysis time (3 min) compared to the previous method (10 min) [7].
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