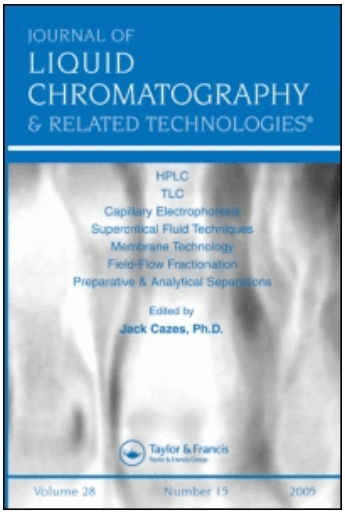

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A Simple and Rapid Method for Determination of Fluconazole in Human Plasma Samples by High-Performance Liquid Chromatography

F. J. Flores-Murrieta[a]; V. Granados-Soto[a]; E. Hong[a]
[a] Sección de Terapéutica Experimental Departamento de Farmacología y Toxicología, Centro de Investigacíon y de Estudios Avanzados del Instituto Politécnico Nacional, México, D.F., Mexico

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A SIMPLE AND RAPID METHOD FOR DETERMINATION OF FLUCONAZOLE IN HUMAN PLASMA SAMPLES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

F. J. FLORES-MURRIETA, V. GRANADOS-SOTO, AND E. HONG

Sección de Terapéutica Experimental
Departamento de Farmacología y Toxicología
Centro de Investigación y de Estudios Avanzados
del Instituto Politécnico Nacional
Apartado Postal 22026
14000 México, D.F., Mexico

ABSTRACT

A rapid and simple method for determination of fluconazole in plasma samples by high performance liquid chromatography was developed. The method includes a single extraction of alkalinized plasma with ethyl acetate. Plasma extracts were analyzed on a reverse-phase column eluted with a mixture of acetonitrile and 0.05 M sodium monohydrogen phosphate (pH 4) and detected by absorbance at 210 nm. Retention times for fluconazole and the internal standard were 7 and 13 min respectively. The method was linear in the range of 0.1 to 5 μg/ml and the detection limit of the method was 20 ng/ml. This method is suitable for determination of fluconazole after administration of the drug at therapeutic doses, and it could be used in pharmacokinetic studies of the drug in humans.

INTRODUCTION

Fluconazole, [2-(2,4-difluorophenyl)-1,3-bis-(1-(1-1,2,4-triazol-1-yl)-propan-2-ol], is an agent with an important antifungic activity (1,2). That compound has been shown to be effective in the treatment of peripheral and systemic mycoses (3,4). To our knowledge, only two methods for its quantitative determination in body fluids are available, one by gas chromatography (GC) with electron capture detection and one by high performance liquid chromatography (HPLC) (5,6). The methods reported have some disadvantages. In the GC method the column has to be pre-treated with benzoyl chloride to avoid that fluconazole is adsorbed to the column and, additionally, a time consuming extraction procedure is employed. On the other hand, the HPLC method has the disadvantage of employing a detection wavelength where sensitivity is very poor and the compound has to be detected at 0.002 absorbance units full scale (AUFS) and consequently, an important noise is present. An additional disadvantage is that a time consuming extraction procedure was performed. In this paper, a simple and rapid method for determination of fluconazole in plasma samples by HPLC is described with enough sensitivity for realization of pharmacokinetic studies after administration of therapeutic doses of the drug.

MATERIAL AND METHODS

Reagents and solutions

Fluconazole was provided by Laboratorios Senosiain, S.A. de C.V. (Mexico City). TR3318, 5-phenyl-5-(2-pyridyl)hydantoin (fig. 1), the internal standard, was

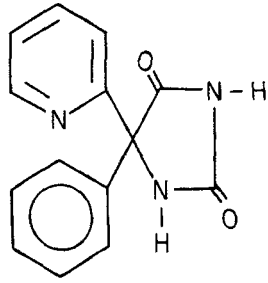

Fig. 1. Chemical structure of the internal standard used in the determination of fluconazole.

provided by the Sección de Terapéutica Experimental del Centro de Investigación y de Estudios Avanzados del I.P.N. (Mexico City). Acetonitrile chromatographic grade was purchased from Merck (Darmstadt, Germany). Deionized water was obtained through a Milli Q system (Continental Water Systems, El Paso, TX, USA). All other reagents were of analytical grade.

Stock solutions of fluconazole corresponding to 1 mg/ml were prepared in the mobile phase (see under), and of the internal standard corresponding to the same concentration was prepared in a mixture of methanol-water (60:40, v/v). Standard solutions were prepared by diluting the stock solutions ranging from 0.1 to 100 μg/ml in mobile phase. A standard solution of the internal standard was prepared at a fixed concentration of 10 μg/ml in mobile phase. Sodium borate (0.025 M), providing a pH of 9.0, was prepared in deionized water.

Extraction procedure

Plasma samples (1 ml) were pipetted into 15 ml conical glass tubes and 1 μg of the internal standard were added. Plasma was alkalinized by addition of 0.5 ml

of 0.025 M sodium borate (pH 9). 5 ml of ethyl acetate were added and samples were extracted by agitation in vortex at maximum speed for 1 min. To separate the layers, samples were centrifuged at 4000 g for 10 min and the organic layer was transferred to another conical glass tube. The organic layer was evaporated to dryness in a water bath at 50°C under nitrogen stream. Dry residue was redissolved in 200 μl of mobile phase (see under) and aliquots of 80 μl were injected into the chromatographic system.

Chromatographic system

The chromatographic system was manufactured by Waters (Waters Assoc., Milford, MA, USA) and was formed by a model 510 solvent delivery system, an U6K injector, a model 490 detector. Chromatograms were recorded in a model 4270 integrator (Varian, Palo Alto, CA, USA). Separation of the compounds was performed on a C_8 Novapak column of 150 mm X 3.9 mm I.D. of 4 μm particle size eluted by a mixture of 0.02 M sodium monobasic phosphate adjusted to pH 4 with *o*-phosphoric acid with acetonitrile (88:12, v/v). To evaluate the optimum wavelength for detection of fluconazole, an ultraviolet absorbance spectrum was obtained (fig. 2). As a good absorbance was obtained at 210 nm, an evaluation of the signal to noise ratio was performing comparing against 260 nm and it was found that the best signal to noise ratio was at 210 nm. Therefore, detection was performed at that wavelength using a sensitivity of 0.05 AUFS.

Calibration

The method was calibrated by addition of known amounts of fluconazole and the internal standard to drug-free plasma samples (1 ml). Calibration curves were established for fluconazole concentrations ranging from

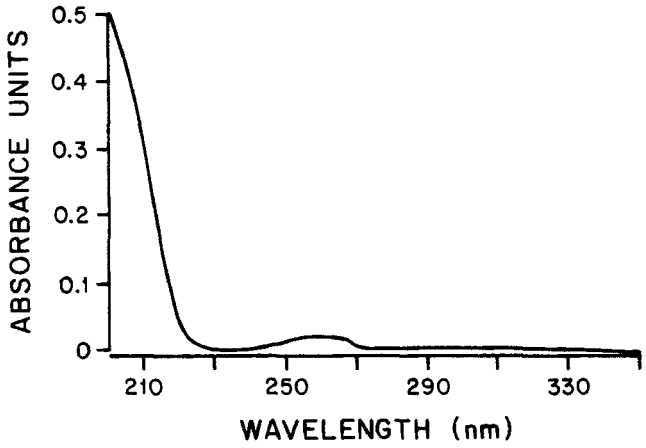

Fig. 2. Ultraviolet absorbance spectrum obtained with a concentration of 111 μg/ml of fluconazole.

0.1 to 5 μg/ml. The internal standard was used at a fixed concentration of 1 μg/ml. The actual sample concentration of fluconazole was calculated by determination of the peak-height ratios of fluconazole to the internal standard.

RESULTS

Typical chromatograms obtained after injection of plasma extracts into the chromatographic system are shown in fig. 3. Retention times for fluconazole and the internal standard were 7 and 13 min respectively. No interfering peaks occurred at these times. Any endogenous contaminants remaining in the extracts were eluted before the fluconazole, and samples could be injected immediately after elution of the internal standard. A linear relationship was obtained when height ratios of

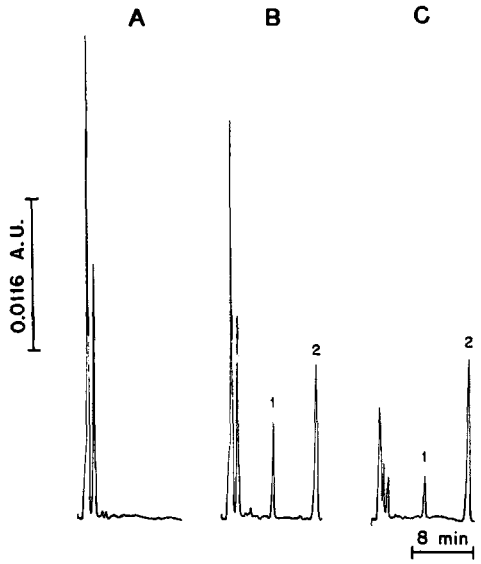

Fig. 3. Typical chromatograms resulting of the injection of plasma extracts into the chromatographic system. A) Drug-free plasma, B) plasma spiked with 1 μg/ml of fluconazole (1) and 1 μg/ml of the internal standard (2), and C) plasma obtained from a subject 72 h after the administration of a capsule of 100 mg fluconazole.

fluconazole to the internal standard were plotted against fluconazole concentration ranging between 0.1 and 5 μg/ml (r = 0.9998), as shown in fig. 4.

The recoveries of fluconazole and the internal standard from plasma samples were similar and ranged between 90 and 100%, by comparison of peak heights from plasma extracts with those from standard solutions. The accuracy and precision of the method were evaluated by adding known amounts of fluconazole to drug-free plasma and analyzing the samples. An accuracy of 100.89 ± 2.42 was obtained for concentrations ranging between 0.1 to 10 μg/ml and the intra-assay and inter-assay coefficient of variation of the method ranged between 14% for 0.1 μg/ml and 4.5% for 0.5 μg/ml.

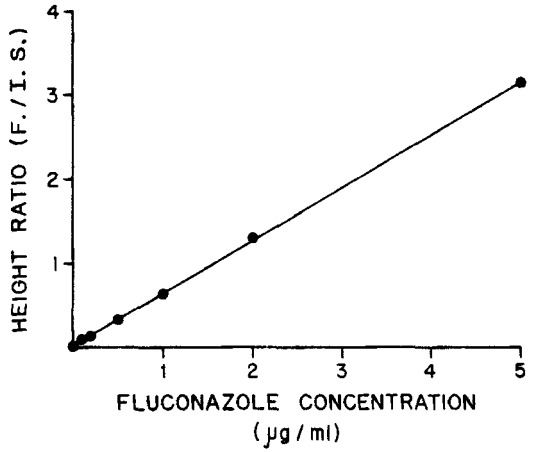

Fig. 4. Calibration curves of fluconazole in human plasma established in the range of 0.1 to 5 μg/ml. Data are expressed as mean of 6 determinations. In all cases the s.e.m. did not exceeded the symbol size.

To evaluate the usefulness of the method for determination of fluconazole after therapeutic doses of the drug, a human volunteer received an capsule of 100 mg of fluconazole p.o. and plasma samples were obtained at selected times. Temporal course of fluconazole concentrations are shown in the fig. 5. Pharmacokinetic parameters obtained in that subject were maximal concentration (C_{max}) of 2.49 μg/ml, time to reach the maximal concentration (t_{max}) of 3 h, area under the plasma level against time curve (AUC) of 112.77 μg·h/ml and half-life ($t_{1/2}$) of 27.15 h.

DISCUSSION

A new method for determination of fluconazole in plasma samples has been developed. This method is more

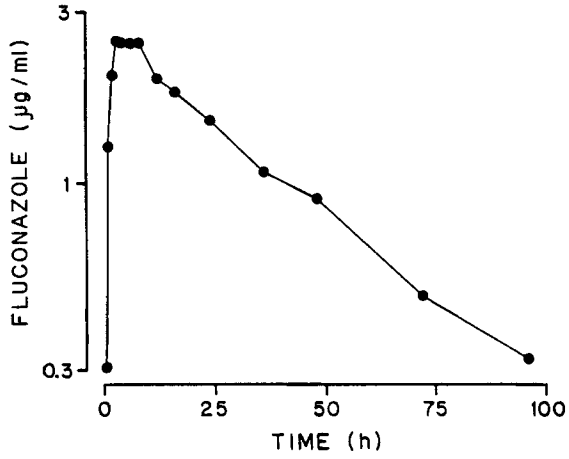

Fig. 5. Temporal course of fluconazole plasma levels in one male volunteer that received a single capsule of 100 mg of fluconazole.

rapid and simple than those previously reported by GC (3) and HPLC (4), since a single extraction is used. Additionally, it is more sensitive than that reported by HPLC, since a wavelength where the drug has a better signal to noise ratio was used. It is important to note that solvents used in the chromatographic system here reported have to be of a good quality, because the wavelength used in this method is very low and some interferences could appear if solvents of poor quality are used. As the method is rapid and simple, over 30 samples can be analyzed by one analyst in a working day. Results of this study allow us to conclude that the method here reported is suitable to carry out pharmacokinetic or bioavailability studies after administration of therapeutic doses of the drug.

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