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# Note

# Rapid determination of serum levels of a new antifungal agent, fluconazole, by high-performance liquid chromatography

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Fluconazole [UK-49 858; 2-(2,4-difluorophenyl)-1,3-bis(1*H*-1,2,4-triazol-1-yl)propan-2-ol] (Fig. 1) is a new triazole with broad-spectrum antifungal activity, which is currently used in clinical trials and seems to have potential in the management of fungal infections without major side-effects [1]. This drug has a long half-life and is excreted predominantly unchanged from kidney [2]. Since the plasma concentration can easily increase in patients with renal dysfunction, and patients with severe infection often have accompanying multiple organ failure, it is necessary to have a simple and accurate method to determine the drug in biological fluids.

The currently available assays for fluconazole are a bioassay, a gas chromatographic (GC) assay connected to a nitrogen-selective detector and a high-performance liquid chromatographic (HPLC) method [3–5]. Since several antifungal imidazoles and most triazoles show a discrepancy between in vivo

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and in vitro antifungal activity and medium conditions are known to influence the in vitro activity of fluconazole [6], the bioassay of fluconazole is not very reliable. From the viewpoint of time required, the bioassay is not a practical way to monitor drug levels in therapeutic use. Although the GC assay is highly sensitive, it is too complicated for bedside drug monitoring and is not satisfactorily reproducible. The HPLC method also has several drawbacks: the extraction procedure is complicated; the recovery and accuracy are poor; and the quantitative range is too narrow for use with patients receiving the drug. Therefore, no simple and specific method for fluconazole is available.

This paper describes a reversed-phase HPLC method for fluconazole determination in serum; it has been rigorously tested for possible interferences from other drugs and plasma factors.

## EXPERIMENTAL

### *Reagents and standards*

HPLC-grade acetonitrile was purchased from Wako (Osaka, Japan). Fluconazole and the internal standard [UK-47 265; 2-(2,4-dichlorophenyl)-1,3-bis(1*H*-1,2,4-triazol-1-yl)propan-2-ol] (Fig. 1) was kindly provided by Pfizer Pharmaceutical (Tokyo, Japan).

### *Apparatus*

The HPLC system consisted of a Shimadzu (Kyoto, Japan) Model LC-6A HPLC pump, a Model SPD-6A variable-wavelength UV detector, a Model SIL-1A sample injector and a Model C-R4A data module. The column was an AM-312 ODS analytical column (15 cm×0.6 cm I.D. particle size 5 $\mu$m), from Yamamura Laboratory (Kyoto, Japan).

### *Chromatographic conditions*

The mobile phase was acetonitrite–distilled water (15:85, v/v). It was filtered and degassed by vacuum and sonication. The chromatograph was operated at room temperature (22–25°C). The mobile phase flow-rate was 1.5 ml/

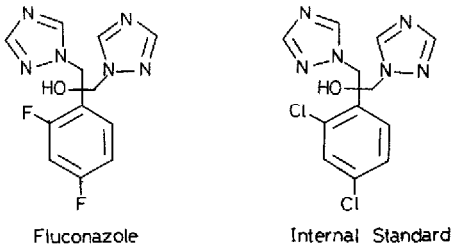


Fig. 1. Structures of fluconazole and the internal standard.

min, and the column pressure was ca. 70 bars (1000 p.s.i.). The effluent from the column was monitored at a wavelength of 210 nm.

*Standard and sample preparation*

A stock standard solution was prepared at a concentration of 1 mg/ml in ethanol and stored at −70°C. Six reference samples containing 0.5, 1.0, 5.0, 10.0, 25.0 and 50.0 μgl fluconazole were prepared by diluting this stock solution with drug-free pooled human serum. These samples were used to derive the calibration curve and to assess the accuracy and the precision of the assay. For routine use in the assay, drug-free pooled human serum containing 10 μg/ml fluconazole was prepared as a single-point standard and stored in 1-ml aliquots at −70°C. Plasma samples from patients receiving fluconazole were stored at −70°C until analysis. A 100-μl volume of serum standard or sample was pipetted into a 2-ml micro-centrifugal tube, and proteins were precipitated by adding an equal volume of acetonitrile. The mixture was vortex-mixed for 30 s, kept standing for 5 min at room temperature and then centrifuged at 12 000 *g* for 2 min. Finally, 25 μl of the supernatant were injected into the HPLC system.

*Quantitation*

The calibration curve was derived by plotting the peak-height ratios of the drug and the internal standard (10 μg/ml) in standard samples against the concentration of the drug, using linear regression analysis. Quantitation of fluconazole in a sample was accomplished by interpolating the observed peak-height ratio into the calibration curve.

## RESULTS

*Chromatography of fluconazole*

Fig. 2 shows a typical chromatogram of fluconazole, which was found to have a retention time of 9.25 min and was well separated from the other detectable components in human serum at this wavelength. None of the drugs tested interfered with the fluconazole peak; they all had shorter retention times than fluconazole.

The least-squares linear regression analysis revealed that the relationship was linear ($r > 0.999$) up to 50 μg/ml.

*Recovery rate*

Known amounts of fluconazole were added to five serum samples. After protein precipitation, chromatographic measurement were performed in ten replicates. The recovery rate was calculated as the amount of drug measured divided by the amount of drug added, multiplied by 100. The resulting values were 92–101% (Table I).

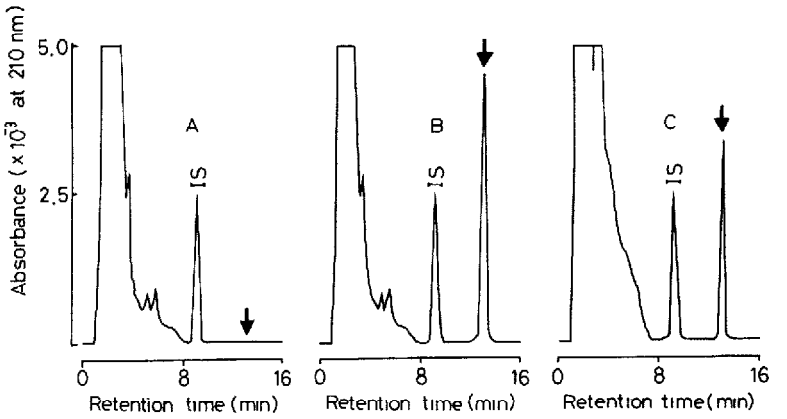


Fig. 2. HPLC profiles of fluconazole and the internal standard (IS). (A) Drug-free human serum; (B) human serum containing 10 μg/ml fluconazole; (C) serum collected from a patient following intravenous infusion of 400 mg of fluconazole. The calculated fluconazole concentration was 7.5 μg/ml.

TABLE I

RECOVERY OF FLUCONAZOLE FROM SERUM ($n=10$)

| Fluconazole added (μg/ml) | Fluconazole measured (mean ± S.D.) (μg/ml) | Recovery (mean ± S.D.) (%) |
|---|---|---|
| 0.5 | 0.46 ± 0.02 | 92.0 ± 3.82 |
| 1.0 | 0.98 ± 0.03 | 98.1 ± 2.75 |
| 5.0 | 4.87 ± 0.06 | 97.4 ± 1.10 |
| 10.0 | 10.03 ± 0.08 | 100.2 ± 0.74 |
| 25.0 | 25.13 ± 0.35 | 100.5 ± 0.63 |

*Precision*

The precision of the HPLC method was calculated for three concentrations of fluconazole in serum. Five replicate samples with fluconazole concentrations of 5.0, 10.0 and 25.0 μg/ml were injected into the system after pretreatment. Table II demonstrates that the method had a high degree of precision over this concentration range.

*Profile in serum*

Fig. 3 shows a typical profile of the serum concentration of fluconazole in a patient with normal renal function following the intravenous infusion of 200 mg per day for five days. A plateau is reached four days after the start of administration. The half-life of fluconazole in serum was 1.5 days (36 h) following the end of the infusion. In four patients on continuous veno-venous haemodi-

TABLE II

WITHIN-DAY PRECISION FOR FLUCONAZOLE IN SERUM ($n=5$)

| Nominal concentration ($\mu$g/ml) | Concentration found (mean ± S.D.) ($\mu$g/ml) | Coefficient of variation (%) |
|---|---|---|
| 1.0 | 1.18 ± 0.046 | 3.92 |
| 5.0 | 5.01 ± 0.053 | 1.05 |
| 10.0 | 9.86 ± 0.056 | 0.57 |
| 25.0 | 24.76 ± 0.220 | 0.89 |
| 50.0 | 48.96 ± 0.260 | 0.53 |

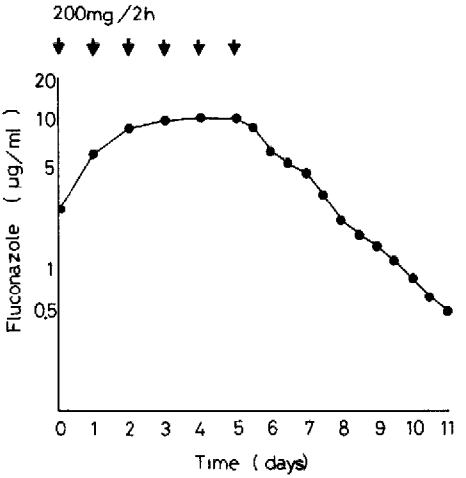


Fig. 3. Serum concentrations of fluconazole in a patient following intravenous administration of 200 mg per day for five days.

alysis for acute renal failure the serum fluconazole elimination rate was 0.17 ± 0.03 mg/h.

## DISCUSSION

The reversed-phase HPLC method reported here overcomes the problems of complicated procedures of the previously reported methods [3–5]. The required pretreatment is simply the precipitation of serum proteins with acetonitrile, and thereafter the supernatant is immediately injected into the HPLC apparatus. Although the GC method is superior to the present assay from the viewpoint of sensitivity, the range of the drug concentration, 0.5–50.0 mg/l, used in this assay is thought to cover the therapeutic range, based on the clinical observation of the serum levels of the patients treated with fluconazole against fungal infections [3,7].

The required sample volume of 100 $\mu$l is convenient and small enough for measurements in paediatric patients. Chromatographic separation was achieved on a reversed-phase ODS column. The mobile phase chosen for the analysis of fluconazole has also been used in laboratories for the determination of several other drugs, thus indicating a need for additional procedures for differentiation. The peak absorbance of fluconazole, at 260 nm, is too weak for therapeutic concentrations, therefore we adopted the wavelength of 210 nm. Although there are many substances that show non-specific absorption below 215 nm, the sensitivity was 100-fold higher at 210 nm than at 260 nm. Under the present HPLC conditions, fluconazole and the internal standard were well separated without any interference from other substances, and the absorbance showed a linear relationship with drug concentration. The assay is free from interference from other plasma substances and drugs, including amphotericin B and miconazole, and is suitable for use in clinical therapeutic drug monitoring.

In summary, we have developed an HPLC assay to determine serum fluconazole levels, which is simple and specific and requires only a half hour to produce results. It is helpful for pharmacokinetic studies and also therapeutic monitoring to maintain accurate drug levels in patients.

REFERENCES

1 H.S. Purba and D.J. Back, Br. J. Clin. Pharmacol., 21 (1986) 603.
2 M.J. Humphreys, S. Jevons and M.H. Tarbit, Antimicrob. Agents Chemother., 28 (1985) 648.
3 E.P. Fernandez, M.M. Patino, J.R. Graybill and M.H. Tarbit, J. Antimicrob. Chemother., 18 (1986) 261.
4 D. Debruyne, J.P. Ryckelynck, M.C. Bigot and M. Moulin, J. Pharm. Sci., 77 (1988) 534.
5 E.J. Kim, H.S. Lee, O.P. Zee and S.T. Lee, Arch. Pharm. Res., 11 (1988) 250.
6 K. Richardson, R.J. Andrews, M.S. Marriott, M.H. Tarbit and P.F. Troke, in K. Iwata and H. van den Bossche (Editors), In Vitro and In Vivo Evaluation of Antifungal Agents, Elsevier, Amsterdam, 1986, p. 147.
7 F.C. Odds, S.L. Cheesman and A B. Abbott, J. Antimicrob. Chemother., 18 (1986) 473.