ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 1992, p. 603–606
0066-4804/92/030603-04$02.00/0

Vol. 36, No. 3

Assay of Fluconazole by High-Performance Liquid Chromatography with a Mixed-Phase Column

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Received 24 June 1991/Accepted 27 December 1991

A mixed-phase liquid chromatographic column was used to assay fluconazole in plasma, serum, and cerebrospinal fluid. The assay was linear from 0.2 to 20 μg/ml, with an average coefficient of variation of less than 5%. The partitioning of the drug between serum and cerebrospinal fluid was determined for 34 patients. The method was demonstrated to be suitable for both pharmacokinetic studies and monitoring of patients receiving treatment with this antifungal agent.

Fluconazole is part of a growing family of triazole antifungal drugs which exhibit a broad spectrum of activity. It has desirable pharmacologic properties, including a relatively long half-life, the ability to be administered either orally or parenterally, and good penetration into cerebrospinal fluid (CSF) (1, 3, 5). With respect to side effects, it is superior to imidazole antifungal drugs introduced earlier (8). It has already been approved by the U.S. Food and Drug Administration for the treatment of certain systemic fungal infections and is currently in clinical use in the United States and Europe as well as in clinical trials for other applications. A continuing increase in systemic fungal infections concomitant with growing immunosuppressed population established the relevance of fluconazole in the drug treatment arena.

Several assays are currently in use for determining fluconazole concentrations in biologic specimens. Both gas-liquid chromatographic (2, 4) and high-performance liquid chromatographic (HPLC) (7) assays have been reported. Bioassay techniques are also available (6).

The assay described in this report is an HPLC method that is done with a mixed-phase column and UV spectrophotometric detection. It has been developed for determining drug concentrations in serum, plasma, and cerebrospinal fluid (CSF).

MATERIALS AND METHODS

Instrumentation. A model 5020 (Varian Instruments, Walnut Creek, Calif.) liquid chromatograph with a variable-wavelength UV detector (Varian UV-50 or equivalent) was used. A Varian mixed-phase PTHAA-5 liquid chromatographic column (15 cm long; 4-mm inner diameter) was used.

Reagents. Fluconazole and internal standard UK-54,373 [2-(2,4-difluorophenyl)-1,3-bis(1H-1,2,4-triazol-1-yl)-2-propanol; Fig. 1] were kindly supplied by Pfizer Central Research, Groton, Conn. The other reagents used and their suppliers were as follows: methylene chloride (HPLC grade; Burdick & Jackson, Muskegon, Mich.), methanol (HPLC grade; Mallinckrodt, Paris, Ky.), and sodium bicarbonate (American Chemical Society certified; Fisher Scientific Co., Pittsburgh, Pa.). Sodium carbonate, potassium phosphate (monobasic), phosphoric acid (85%), and acetonitrile were purchased from Sigma Chemical Co., St. Louis, Mo.

Chromatographic conditions. The mobile phase was prepared by mixing acetonitrile with filtered 0.051 M monobasic phosphate buffer adjusted to pH of 3.0 at 15:85 (vol/vol). The flow rate was 0.9 ml/min. The UV detector was set at a wavelength of 210 nm. The HPLC apparatus was operated at room temperature (25 to 28°C).

Standards and controls. Stock standards at concentrations of 1 and 100 μg/ml in methanol were prepared by placing appropriate amounts of fluconazole in polypropylene tubes with screw-cap tops and then adding 0.10 ml of methanol. Working serum standards with fluconazole concentrations of 0.0, 0.2, 0.5, 1.0, 2.0, 2.5, 5.0, 10.0, 15.0, 20.0, 40.0, 50.0, and 100 μg/ml were prepared by placing various amounts of the two stock standard solutions in 15-ml glass tubes, taking the methanol to near dryness, and resuspending the fluconazole residue in drug-free serum. Controls with concentrations of 2.0, 7.5, and 33.5 μg/ml were similarly prepared. All of the working standards and the controls were included with each batch of specimens analyzed.

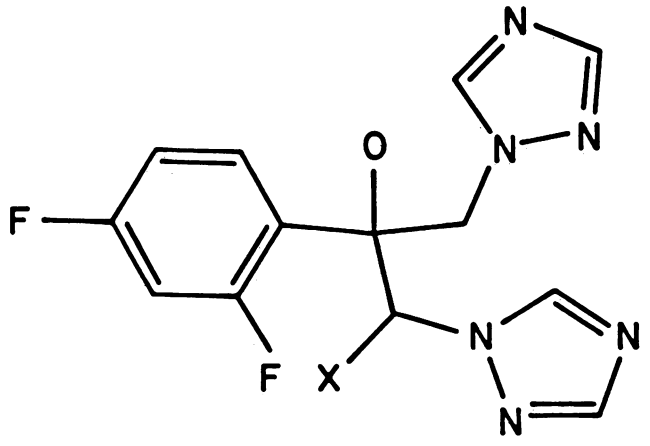

FIG. 1. Chemical structures of fluconazole (Flu) and the internal standard (I.S.), UK-54,373. X is H in fluconazole and F in UK-54,373.

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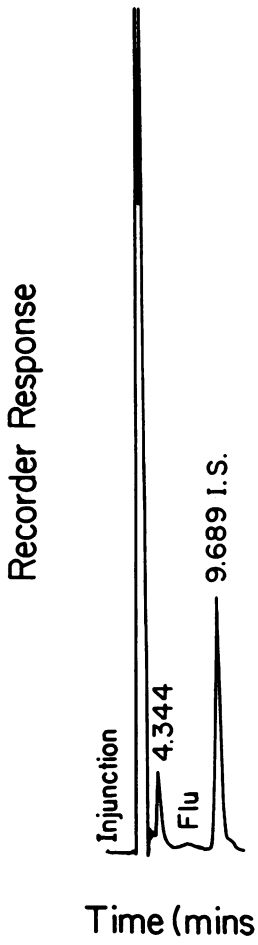

FIG. 2. Chromatogram of a spiked-serum assay with the internal standard (I.S.) but no fluconazole (Flu).

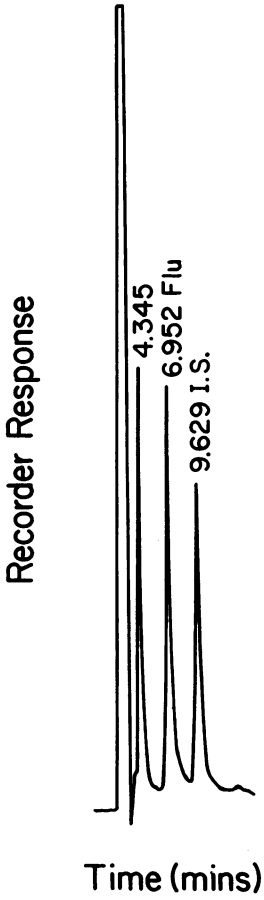

FIG. 3. Chromatogram of a spiked-serum assay with the internal standard (I.S.) and 10.0 μg of fluconazole (Flu) per ml.

Assay procedure. A 0.5-ml aliquot of plasma, serum, or CSF was pipetted into a tube with a Teflon-lined cap. Approximately 0.5 g of $NaHCO_3$-Na_2CO_3 (2:1) was added to the specimen to adjust the pH to 9.0 and to provide a salting-out effect. Extracting solvent (3.0 ml) was added to the tube. The extracting mixture was methylene chloride containing 1.0 μg of the internal standard, UK-54,373, per ml. The mixture was vortexed for 30 s and then frozen either by means of acetone-dry ice or in a freezer at −80°C for 15 min. While still frozen, the tubes were immediately centrifuged (model CU-5000 centrifuge; International Equipment Co., Needham Heights, Mass.) at 3,000 rpm for 15 min. After centrifugation, the organic layer was removed to a clean tube, and the aqueous layer was left behind. To remove small residual amounts of water containing residual amounts of serum, plasma, or urine constituents, we refroze the tube contents at −80°C. While the aqueous layer was still frozen, the organic layer was poured into a separate tube, and any additional frozen water droplets were left behind. The organic layer was evaporated to near dryness by passing a low stream of nitrogen or air over the surface. The residue was reconstituted in 100 μl of the mobile phase, of which approximately 50 μl was injected into the liquid chromatograph.

RESULTS

A chromatogram of a serum sample containing no fluconazole but spiked with an appropriate amount of the internal standard is shown in Fig. 2. A chromatogram of a serum sample spiked with 10 μg of fluconazole per ml and the internal standard is shown in Fig. 3. A chromatogram of a patient serum sample containing 17.9 μg of fluconazole per ml is shown in Fig. 4. Fluconazole was well separated from the internal standard and endogenous serum peaks. Figure 5 shows the results for a patient extract analyzed by a previously described gas-liquid chromatographic procedure (4).

Standard curves were constructed by plotting the peak area ratios, which were calculated by dividing the peak area of fluconazole by the peak area of the internal standard, against the concentrations of the standards. The assay was found to be linear from 0.2 to 20 μg of fluconazole per ml in serum.

A study was performed to determine the accuracy and precision of the assay. Known amounts of fluconazole were added to five serum samples. Ten extractions were performed with each fortified sample, and the concentrations were measured. The results are shown in Table 1. The excellent agreement between the measured concentrations and the spiked concentrations reflected the accuracy of the assay. The precision was demonstrated by the relatively small coefficients of variation at each concentration. Similar results were obtained for plasma and CSF.

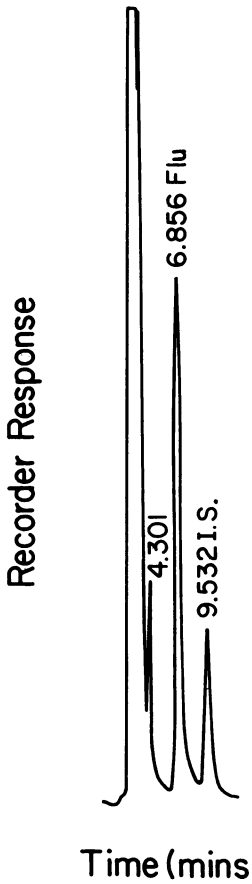

FIG. 4. Chromatogram of a patient serum assay with the internal standard (I.S.) and 17.9 μg of fluconazole (Flu) per ml.

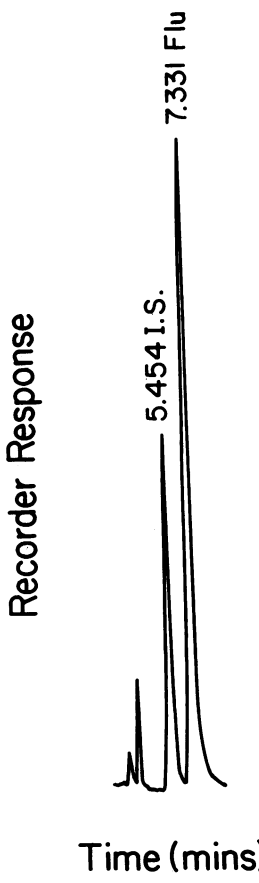

FIG. 5. Chromatogram of the same patient extract as that shown in Fig. 4 but assayed by a previously reported gas-liquid chromatographic procedure (4).

The assay had within-run or run-to-run coefficients of variation of 5.1% (n = 10) and 5.9% (n = 10), respectively, at a concentration of 5.0 μg/ml and of 3.3% (n = 10) and 3.9% (n = 10), respectively, at a concentration of 20.0 μg/ml. In practice, the linearity of the method was observed at concentrations of up to 200 μg/ml. Absolute recoveries of fluconazole were close to 100% (Table 1).

A comparison was also made between the proposed assay and an established gas-liquid chromatographic method for assaying fluconazole (4) (Fig. 6). Fifty-eight samples from actual patients receiving fluconazole therapy and containing fluconazole concentrations ranging from 0.0 to 34 μg/ml were assayed by both the HPLC method presented here and the reference method. Regression analysis on the resulting data demonstrated a high degree of correlation (r = 0.9978) between the two methods.

Another study was performed to determine the relative distributions of fluconazole in serum and CSF. The concentrations of fluconazole were determined in CSF and serum samples drawn simultaneously from patients participating in clinical trials of fluconazole efficiency. The samples were drawn at different time intervals postfluconazole dosing. The ratio of the concentration of fluconazole in serum to that in CSF was found to be constant for the time interval allowed between the administration of the drug and the drawing of the samples. The mean ratio was 1.21, and the coefficient of variation was 11.1% (n = 40).

DISCUSSION

The assay described here was done with a mixed stationary phase in the analytical column. This adaption serves as an improvement over the use of either a reverse-phase (C-18) or a normal-phase (CN) column. The combination of stationary phases effectively separates the drug from the internal standard and removes both from endogenous biologic materials that may be coextracted with the drug. Other, published techniques do not provide as clean a biologic extract as does the proposed method described in this report.

The extraction procedure is a single-step operation, and the alkaline conditions used provide for the selective removal of fluconazole from neutral and acidic analytes that may be given concomitantly to patients. Injection of a cleaner extract permits greater reproducibility between

TABLE 1. Recovery of fluconazole from serum spiked with fluconazole

Target concn (μg/ml)	Measured concn (mean ± SD μg/ml)	Coefficient of variation (%)
1.0	1.08 ± 0.09	8.24
5.0	5.09 ± 0.13	2.55
10.0	9.69 ± 0.44	4.57
15.0	14.74 ± 0.55	3.76
20.0	20.31 ± 0.66	3.27

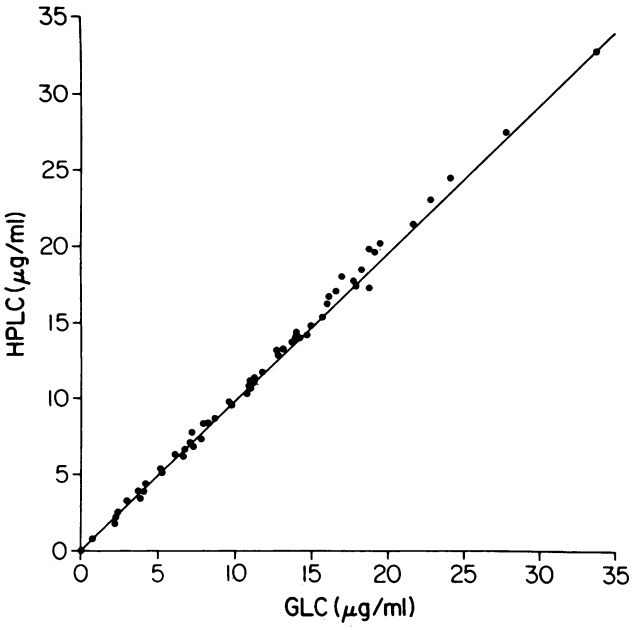

FIG. 6. Correlation (r^2 = 0.9978) of the previously reported gas-liquid chromatographic assay (GLC) of fluconazole (4) with the proposed HPLC assay.

chromatographic assays and provides for a longer life of the analytical column, both of which are highly desirable situations for clinical laboratory environments.

Several other triazole analogs of fluconazole were evaluated for possible inference with this assay. None of them yielded retention times near that of fluconazole or its internal standard.

The linearity range achieved for this assay (0.2 to 20 μg/ml) effectively covers the therapeutic range for fluconazole. The assay routinely takes less than 90 min to perform, including chromatography time, providing a turnaround time appropriate for clinical laboratory use.

In summary, the proposed fluconazole procedure meets the sensitivity, specificity, and time requirements for both therapeutic drug monitoring and pharmacokinetic studies.

ACKNOWLEDGMENTS

The excellent technical assistance of Asha Modak, Deanna A. McGough, Annette W. Fothergill, and Michael Wallace is deeply appreciated. The typing support of Rosyln Hickey is also acknowledged. Both fluconazole and the analog used as an internal standard in this study were graciously provided by Pfizer Central Research, Groton, Conn.

REFERENCES

1. **Arndt, C. A. S., T. J. Walsh, C. L. McCully, F. M. Bodis, P. A. Pizzo, and D. G. Poplack.** 1988. Fluconazole penetration into cerebrospinal fluid: implications for treating fungal infections of the central nervous system. J. Infect. Dis. **137**:178–180.
2. **Debruyne, D., J. P. Ryckelynck, M. C. Rigot, and M. Moulin.** 1988. Determination of fluconazole in biologic fluids by capillary column gas chromatography with a nitrogen detector. J. Pharm. Sci. **77**:534–535.
3. **Foulds, G., D. R. Brennan, C. Wajszczuk, A. Catanzaro, D. C. Garg, W. Knopf, M. Rinaldi, and D. J. Weidler.** 1988. Fluconazole penetration into cerebrospinal fluid in humans. J. Clin. Pharmacol. **28**:363–366.
4. **Harris, S. C., J. E. Wallace, G. Foulds, and M. G. Rinaldi.** 1989. Assay of fluconazole by megabore capillary gas-liquid chromatography with nitrogen-selective detection. Antimicrob. Agents Chemother. **33**:714–716.
5. **Humphrey, M. J., S. Jevons, and M. H. Tarbit.** 1985. Pharmacokinetic evaluation of UK-49,858, a metabolically stable triazole antifungal drug, in animals and humans. Antimicrob. Agents Chemother. **28**:648–653.
6. **McGinnis, M. R., and M. G. Rinaldi.** 1991. Antifungal drugs: mechanisms of action, drug resistance, suspectibility testing, and assays of activity in biological fluids, p. 198–257. *In* V. Lorian (ed.), Antibiotics in laboratory medicine, 3rd ed. The Williams & Wilkins Co., Baltimore.
7. **Nishihima, M. K., T. Okuda, N. Taenaka, and I. Yoshiya.** 1990. Rapid determination of serum levels of a new antifungal agent, fluconazole, by high-performance liquid chromatography. J. Chromatogr. **529**:223–228.
8. **Washton, H.** 1989. Review of fluconazole: a new triazole antifungal agent. Diagn. Microbiol. Infect. Dis. **12**:2295–2335.