

## Assay of Fluconazole by Megabore Capillary Gas-Liquid Chromatography with Nitrogen-Selective Detection

STEVEN C. HARRIS,<sup>1,2†\*</sup> JACK E. WALLACE,<sup>2</sup> GEORGE FOULDS,<sup>3</sup> AND MICHAEL G. RINALDI<sup>1,2</sup>

*Laboratory Service, Audie L. Murphy Memorial Veterans' Hospital,<sup>1</sup> and Department of Pathology, University of Texas Health Science Center at San Antonio,<sup>2</sup> San Antonio, Texas 78284, and Drug Metabolism Department, Pfizer Central Research, Groton, Connecticut 06340<sup>3</sup>*

Received 29 September 1988/Accepted 16 February 1989

**A megabore column gas-liquid chromatographic method which uses nitrogen-phosphorus detection was developed for the analysis of fluconazole in plasma, serum, cerebrospinal fluid, or urine. The assay was linear from 0.2 to 200 µg/ml and had an average coefficient of variation of 7%. The suitability of the assay for pharmacokinetic studies was demonstrated.**

Fluconazole [UK-49,858; 2-(2,4-difluorophenyl)-1,3-bis(1*H*-1,2,4-triazol-1-yl)-2-propanol; Fig. 1] is a triazole compound with potent antifungal activity which is currently being evaluated in clinical trials (5). It is well absorbed after oral administration (4) and shows good penetration into cerebrospinal fluid (1, 3). Early analytical methods for assay of this compound in biological fluids, designed to support preliminary animal and human studies, were effective but rather complex. Both gas-liquid chromatographic and high-performance liquid chromatographic (HPLC) approaches were used (2, 3, 6). However, with this compound now in clinical trials, a simpler and more rapid and reliable analytical method was desirable for therapeutic drug monitoring. We therefore developed the following method, which uses megabore column gas-liquid chromatography with nitrogen-selective detection.

### MATERIALS AND METHODS

**Instrumentation.** A Varian 6000 (Varian Instruments, Walnut Creek, Calif.) gas chromatograph with a nitrogen-phosphorus detector operating at an applied voltage of 5 to 10 mV was employed. A fused silica DB-5 megabore column (45 m long, inner diameter of 0.55 mm; J. W. Scientific, Folsom, Calif.) with a 1-µm-thick bonded liquid phase was used. The instrument conditions were as follows: injector temperature, 225°C; detector temperature, 275°C; column temperature, 190°C.

**Reagents.** Fluconazole and internal standard UK-54,373 [1-fluoro-2-(2,4-difluorophenyl)-1,3-bis(1*H*-1,2,4-triazol-1-yl)-2-propanol; Fig. 1] were supplied by Pfizer Central Research, Groton, Conn. Other reagents used and their suppliers were as follows: methylene chloride (HPLC grade; Burdick & Jackson, Muskegon, Mich.); methanol (HPLC grade; Mallinckrodt, Paris, Ky.); filter paper (9 cm; 610 grade; VWR Scientific Inc., San Francisco, Calif.); sodium bicarbonate (certified A.C.S.; Fisher Scientific Co., Pittsburgh, Pa.); sodium carbonate (Sigma Chemical Co., St. Louis, Mo.).

**Standards and controls.** Plasma standards were prepared by adding appropriate amounts of fluconazole in methanol to tubes that had been washed in a chromic acid bath and then sonicated in water purified by ion exchange, carbon filter, and 0.45-µm filter. The methanol was removed either by blowing nitrogen or air into the tubes at room temperature or by vacuum. For plasma, serum, and cerebrospinal fluid, standards fortified to 0.0, 0.2, 0.5, 1.0, 2.0, 2.5, 5.0, 10.0, 15.0, 20.0, and 40.0 µg/ml were used in each analysis. For urine, standards fortified to 0.0, 5.0, 10.0, 20.0, 40.0, 60.0, 80.0, 100.0, and 200.0 µg/ml were employed in each group of urine specimens analyzed. Controls of 2 and 7 µg of fluconazole per ml, prepared in the same manner as were the standards, were also included in each group of serum or urine specimens assayed.

**Assay procedure.** To assay plasma, serum, or cerebrospinal fluid, 0.5 ml of specimen was pipetted into a tube fitted with a Teflon-lined cap. One level spoonful of a Coors 01 spatula filled with NaHCO<sub>3</sub>-Na<sub>2</sub>CO<sub>3</sub> (2:1) was added to the specimen to adjust the pH to 9.0 and to provide a salting-out effect. A 10-ml sample of extracting solvent (methylene chloride containing 0.1875 µg of the UK-54,373 internal standard per ml) was added to the tube, and the mixture was mechanically shaken for 15 min. The tube was subsequently centrifuged in a floor model centrifuge (model CU-5000; International Equipment Co., Needham Heights, Mass.) for 5 min at 2,000 rpm. The upper aqueous layer was aspirated to waste, and the organic layer was filtered through paper to remove residual water deposits and particulate matter. The organic layer was then evaporated to near dryness under vacuum and redissolved in 50 µl of methanol. Next, 2 to 4 µl of the methanolic extract was injected into the gas chromatograph. Time for the chromatographic analysis was generally less than 10 min.

The procedure for assay of fluconazole in urine was identical to that described for plasma, serum, or cerebrospinal fluid, except that the urine was either diluted 1/10 before using a 0.5-ml specimen or was utilized without dilution in combination with a different concentration range of spiked urine standards. For the undiluted urine specimens, the extracting solvent contained 1.875 µg of the internal standard per ml, a 10-fold increase over the amount used for plasma or serum.

\* Corresponding author.

† Present address: Veterans Administration Medical Center, Battle Creek, MI 49016.

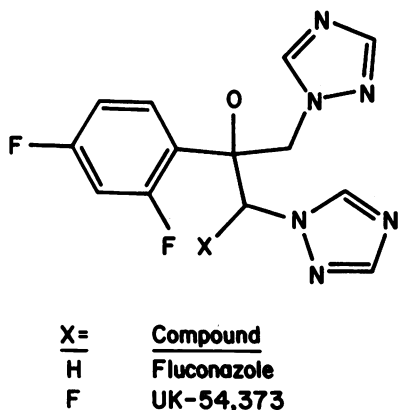


FIG. 1. Structures of fluconazole and internal standard UK-54,373.

### RESULTS

A representative chromatogram is presented in Fig. 2. Fluconazole was well separated from the internal standard, and endogenous peaks did not interfere. The lower limit of detection, defined as the mean of the serum blank plus 3 standard deviations was 0.04  $\mu\text{g/ml}$ ,  $n = 20$ .

A typical standard curve is shown in Fig. 3. Linearity extended from 0.2 to 200  $\mu\text{g/ml}$ . The assay had within-run and run-to-run coefficients of variation of 8.1% ( $n = 10$ ) and 9.9% ( $n = 10$ ), respectively, at a concentration of 2.0  $\mu\text{g/ml}$  and of 6.3% ( $n = 10$ ) and 4.5% ( $n = 10$ ), respectively, at a concentration of 7.0  $\mu\text{g/ml}$ .

A study was performed to examine recovery of fluconazole from fortified plasma samples. Recovery was calculated by determining the relative chromatographic response of drug extracted from plasma and drug dissolved directly in methanol. Recoveries were 86% at 1.0  $\mu\text{g/ml}$  and 93% at 20  $\mu\text{g/ml}$ .

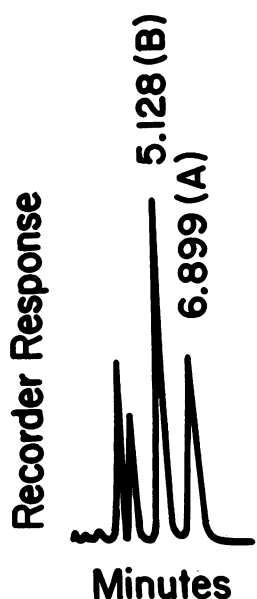


FIG. 2. Typical chromatographic separation of fluconazole (peak A; 2.4  $\mu\text{g/ml}$ ) and internal standard UK-54,373 (peak B; 3.75  $\mu\text{g/ml}$ ) from a patient plasma sample.

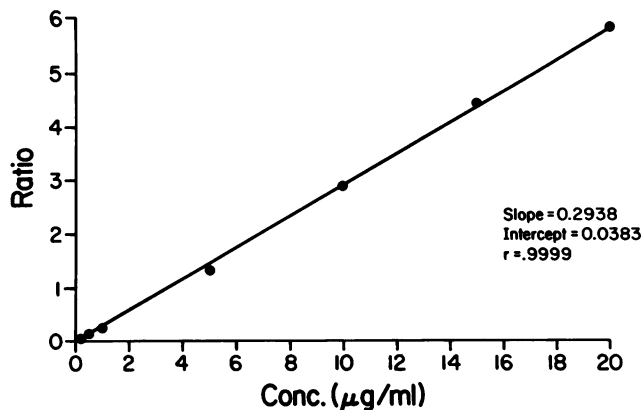


FIG. 3. Typical dose-response curve for gas chromatographic assay. Points of curve include: 0.0, 0.2, 0.5, 1.0, 5.0, 10.0, 15.0, and 20.0  $\mu\text{g/ml}$ .

To validate the analytical procedure, 26 samples of fresh human serum were fortified with fluconazole concentrations between 0.0 and 6.0  $\mu\text{g/ml}$  in another laboratory, randomized, and assayed blindly. A similar procedure was followed to produce 26 fresh human urine samples with fluconazole concentrations of 0.0 to 130  $\mu\text{g/ml}$ . These concentrations were chosen because they are representative of those previously seen in phase I/II studies of fluconazole in humans. The assayed concentrations are presented in Fig. 4a plotted against the nominal concentrations. The correlation coefficients were 0.9942 and 0.9979 for serum and urine, respectively.

To further examine the accuracy of the assay at those lower concentrations expected from low-dose studies in humans, 23 additional samples of fluconazole in fresh human serum were similarly prepared and randomized. The results of assays for fluconazole correlated very well ( $r = 0.999$ ) with the nominal concentrations (Fig. 4b). At three concentrations (0.5, 1.2, and 4.0  $\mu\text{g/ml}$ ) for which quadruplicate samples were provided, the mean measured concentrations were 86, 96, and 101% of the nominal concentrations and the coefficients of variation were 9, 5, and 1%.

A typical patient pharmacokinetic profile is presented in Fig. 5. The subject received 100 mg of fluconazole orally every morning for 9 days to achieve steady-state conditions. Then, on day 10, a blood sample was obtained before the dose, a final 100-mg oral dose was given, and blood samples were then obtained periodically over the next seven days.

### DISCUSSION

The assay, as presented, is suitable for use in both clinical and research laboratories. It is simple and reproducible enough for clinical application and yet has sufficient precision and linearity over the concentration range evaluated for pharmacokinetic studies. Furthermore, the robustness of this approach has been validated by our experience of over 3,000 assays to date on the same column without significant column maintenance problems.

When first developing this assay, we evaluated both gas-liquid chromatographic and HPLC approaches. While reasonable results were achieved with HPLC, the ease of use, sensitivity, and ability to automate the gas-liquid chromatographic assay made it, from our point of view, the

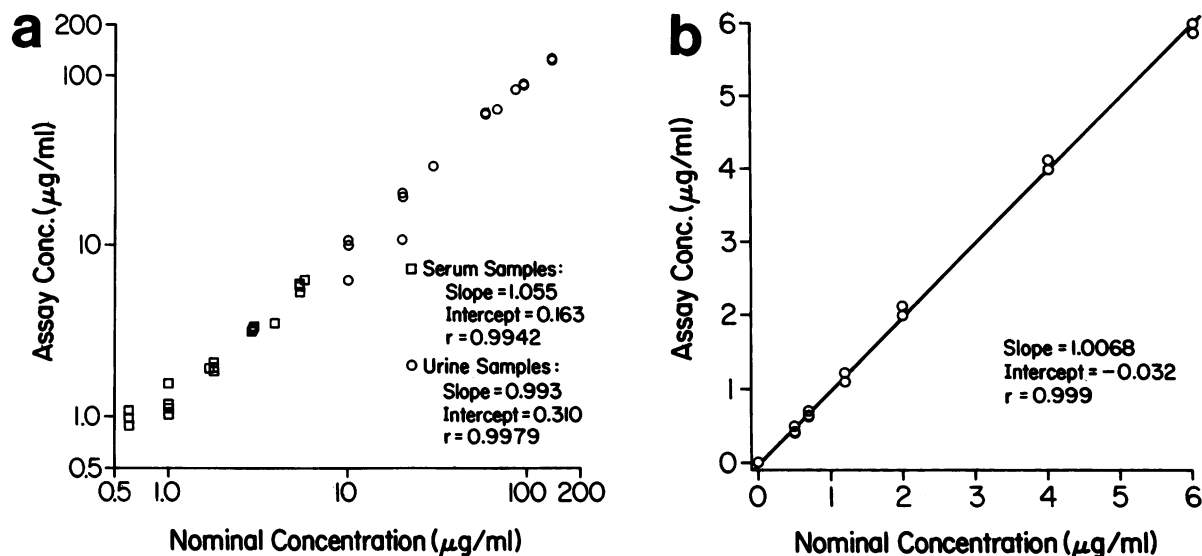


FIG. 4. Comparison of assayed concentrations and nominal concentrations of fluconazole in human serum and urine at usual therapeutic concentrations (a) and in human serum at subtherapeutic concentrations (b).

preferable one. Additionally, the described assay exhibits a 20-fold increase in sensitivity compared with the gas-liquid chromatographic assay of Wood and Tarbit (6). Although a bioassay is useful in many clinical settings, its inherently poorer precision and lower sensitivity make it a less desirable approach for evaluating pharmacokinetic profiles or in monitoring patients on low doses of fluconazole.

For an internal standard, a fluoro rather than the bulkier chloro analog was selected to minimize chromatographic time between the two peaks. This approach also allowed for excellent base-line separation between the solvent front and the first chromatographic peak, which was not achieved by Debruyne et al. (2). Also, we used a megabore rather than a small-bore capillary column, eliminating the inconveniences associated with the latter.

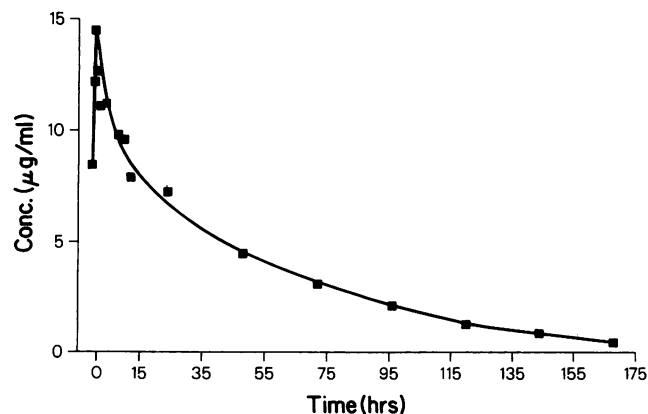


FIG. 5. Plasma fluconazole bioavailability profile on a single patient at steady state who received a final 100-mg oral dose of fluconazole at  $t = 0$ .

The assay presented herein is reliable, sensitive, rapid, and practical for the evaluation of parent drug concentrations in patients receiving fluconazole and for the performance of pharmacokinetic studies. The assay could be easily automated for situations requiring rapid processing of large numbers of specimens.

#### ACKNOWLEDGMENTS

We thank Asha Modak and Deanna A. McGough for excellent technical assistance and Ginny Wolfe and Sharyl Salazar for fine secretarial support.

This study was supported by the Veterans Administration and Pfizer Central Research, Groton, Conn.

#### LITERATURE CITED

- 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.
- Debruyne, D., J. P. Ryckelynck, M. C. Bigot, and M. Moulin. 1988. Determination of fluconazole in biological fluids by capillary column gas chromatography with a nitrogen detector. *J. Pharm. Sci.* **77**:534-535.
- 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.
- 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.
- Saag, M. S., and W. E. Dismukes. 1988. Azole antifungal agents: emphasis on new triazoles. *Antimicrob. Agents Chemother.* **32**:1-8.
- Wood, P. R., and M. H. Tarbit. 1986. Gas chromatographic method for the determination of fluconazole, a novel antifungal agent in human plasma and urine. *J. Chromatogr.* **383**:179-186.