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Potential Use of a Simplified Method for Determination of Itraconazole Levels in Plasma and Esophageal Tissue by Using High-Performance Liquid Chromatography

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A simplified high-performance liquid chromatographic assay was developed for determination of itraconazole levels in plasma and esophageal tissue in four patients with AIDS who had been receiving daily oral doses of 100 mg of itraconazole in solution for at least 3 weeks for therapy of esophageal candidiasis. Itraconazole levels were about three times higher in esophageal tissue than in plasma (means ± standard errors of 0.69 ± 0.50 μg/g and 0.24 ± 0.16 μg/ml, respectively; $P = 0.04$). This method is quick (it requires only 1 h for completion) and sensitive (the limits of detectability for itraconazole in plasma and esophageal tissue are 0.005 μg/ml and 0.01 μg/g, respectively), and it can be reliably used in clinical and research settings (accuracy, >95%; absolute recovery from biological samples, 80 to 90%; coefficient of variation, 3.3 to 6.6%).

Esophageal candidiasis is a major cause of morbidity in immunocompromised subjects, such as neutropenic patients with malignancy (1) and patients with AIDS (16). In fact, *Candida* infection is the most common cause of esophageal disease in patients with AIDS, affecting 10 to 60% of this population (7, 13, 16). Because of their relative safety and ease of administration, the azoles have replaced amphotericin B in recent years as primary therapeutic agents for esophageal candidiasis in patients with AIDS (12). Of the various azole drugs that are currently in use, itraconazole is the most lipophilic and hence has the greatest affinity for body tissues (10, 15). Itraconazole concentrations in body tissues such as the lung, kidney, brain, and epidermis are severalfold higher than in plasma (5, 8, 15, 19). Studying the concentrations of itraconazole in esophageal tissue and plasma may provide important insight into the pharmacodynamic properties of itraconazole in human subjects with esophageal candidiasis.

A simplified high-performance liquid chromatographic (HPLC) method was developed and used to determine itraconazole levels in plasma and esophageal tissue of patients with AIDS who were participating in a double-blinded clinical trial that evaluates the efficacy of oral itraconazole solution (100 mg daily) versus that of fluconazole tablets (100 mg daily) for therapy of esophageal disease. A total of eight patients were studied. Because of the double-blinded nature of the clinical trial, patients' identities, randomization groups, and certain clinical information were unknown to the investigators. However, four of the eight patients had no detectable levels of itraconazole and their plasma samples yielded fluconazole on analysis; therefore, these patients were excluded from analysis in this study. None of the patients enrolled in the clinical trial were permitted to receive medications that may affect the gastrointestinal absorption or hepatic metabolism of azole drugs. Plasma specimens and biopsies of esophageal tissue were concurrently obtained from these eight patients on two occasions: (i) initially, when esophageal candidiasis was diagnosed by upper gastrointestinal endoscopy before institution of antifungal therapy, and (ii) at least 3 weeks later, when follow-up endoscopy was done 12 to 16 h after the last dose of antifungal medication. Coded clinical specimens were stored at −20°C until analyzed by HPLC; pilot trials in which weekly testing was performed had demonstrated that itraconazole levels (initial solution of 1 μg/ml) remained stable at −20°C for at least 8 weeks (range of calculated levels, 0.96 to 1.02 μg/ml).

One hundred milligrams of itraconazole (Janssen Research Foundation, Titusville, N.J.) was initially mixed with 100 μl of chloroform (Burdick and Jackson, Muskegon, Mich.) and then dissolved in 10 ml of dimethyl sulfoxide (Sigma Chemical Co., St. Louis, Mo.) to yield stock at 10 mg/ml. Various working solutions of itraconazole were prepared by dissolving the stock solution in methanol (Burdick and Jackson). The internal standard R51012 (Research Diagnostics Inc., Flanders, N.J.) was prepared in the same fashion as that used for itraconazole to yield a working solution of 1 mg/ml. Esophageal tissue specimens were weighed (range, 0.012 to 0.018 g; because of the difficulty in dissecting small pieces of esophageal tissue, the whole biopsied specimen from each patient was used for analysis), mixed with cold phosphate-buffered saline (pH 7.8) at a weight/volume ratio of 1:20, and homogenized by using a disposable tissue grinder (Baxter Scientific, McGaw Park, Ill.). One milliliter of acetonitrile (Burdick and Jackson) and 1 μl (1 μg) of the internal standard were added to the tubes containing 250-μl aliquots of esophageal tissue homogenate (or plasma), and the mixtures were agitated on a vortex mixer for 1 min and then centrifuged at 1,000 × *g* for 5 min. The supernatant fluid was decanted and dried in air for 30 min. The residues were redissolved in 100-μl aliquots of mobile phase, which were then injected into the HPLC apparatus.

A Waters liquid chromatograph (Millipore Corp., Millford, Mass.) equipped with a model 510 solvent delivery system, a model 700 satellite WISP injector, a Waters Nova-pak C_{18} column (15 cm by 3.9 mm [inner diameter]), a model 486 UV detector, and a Waters Maxima 820 workstation for analysis of data was used. The UV detector was set at 263 nm with an absorbance unit full scale of 0.05. The mobile phase consisted

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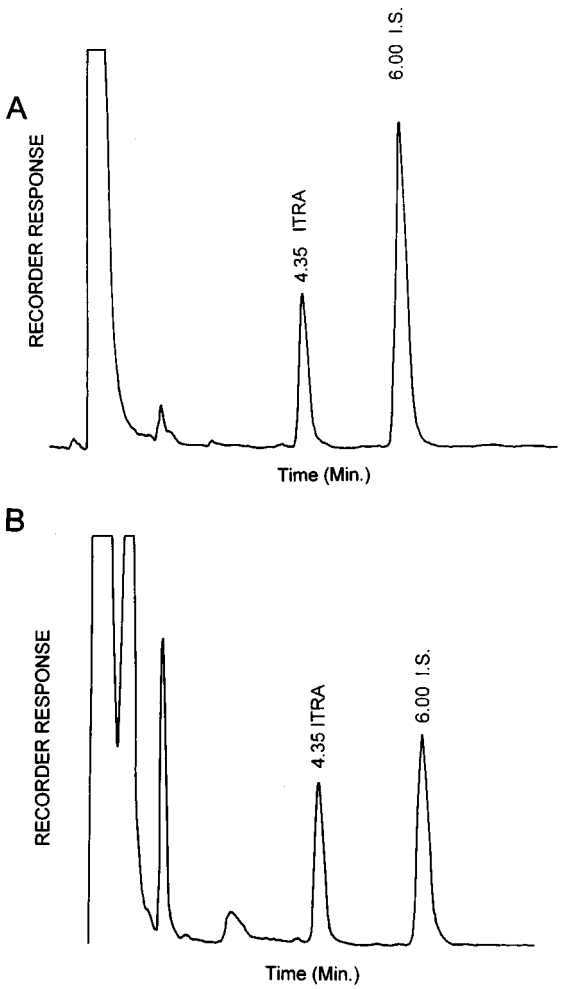

FIG. 1. (A) Chromatogram of drug-free plasma spiked with itraconazole (ITRA) at a concentration of 0.5 μg/ml and with the internal standard (I.S.) at a concentration of 4 μg/ml. (B) Chromatogram of drug-free esophageal tissue spiked with itraconazole (ITRA) at a concentration of 1.0 μg/g and with the internal standard (I.S.) at a concentration of 4 μg/g.

of a 60:40 (vol/vol) solution of acetonitrile and 10 mM K_2HPO_4 (Sigma), with the final pH adjusted to 7.8 with 85% orthophosphoric acid (Sigma). The flow rate was 1.3 ml/min, and the HPLC apparatus operated at ambient temperature (25 to 28°C). Itraconazole eluted at 4.35 min, followed by elution of the internal standard at 6.00 min. The separation of itraconazole and internal standard was complete without interference from endogenous substances in plasma and esophageal tissue (Fig. 1). No interference with this assay was observed when we injected a variety of drugs, including vancomycin, clindamycin, norfloxacin, tetracycline, minocycline, ampicillin, nafcillin, cefuroxime, cefazolin, cefoperazone, ceftriaxone, fluconazole, rifampin, zidovudine, zalcitabine, and folic acid.

In order to construct standard curves, drug-free samples of both plasma and esophageal tissue from patients who had undergone esophageal biopsy for conditions other than candidiasis were each spiked with the internal standard at a concentration of 4 μg/ml (or 4 μg/g) and with itraconazole at a concentration of 0.005, 0.01, 0.02, 0.05, 0.1, 0.5, 1.0, 2.0, 5.0, 10.0, or 20.0 μg/ml (or μg/g). The limits of detectability for itraconazole in plasma and esophageal tissue were 0.005 μg/ml and 0.01 μg/g, respectively. A linear relationship was established between the itraconazole-to-internal-standard peak area ratio and the itraconazole concentration between 0.005 and 20 μg/ml of plasma and between 0.01 and 20 μg/g of esophageal tissue. The correlation coefficient was 0.99. After construction of the standard curves, drug controls were measured with a consistent accuracy of >95%. The absolute recovery of itraconazole was determined by adding known quantities of the drug to blank clinical specimens and subjecting them to the extraction procedure outlined above. Experiments were done with different concentrations of itraconazole (0.1, 0.5, and 1.0 μg/g or μg/ml) that encompass the range of clinically achieved levels in plasma and esophageal tissue and yielded absolute recoveries of 80 to 85 and 85 to 90%, respectively. Six sets of 10 quality control plasma samples at itraconazole concentrations of 0.1, 0.5, and 1.0 μg/ml were analyzed to determine the intra- and interday variabilities of the assay. The coefficients of variation were 4.9, 6.6, and 4.4%, respectively, for intraday measurements and 4.2, 3.3, and 3.7%, respectively, for interday measurements.

A one-tailed, paired *t* test was used to compare itraconazole concentrations in plasma and esophageal tissue specimens from the four evaluable patients. The levels of itraconazole were about three times higher in esophageal tissue (0.69 ± 0.50 μg/g, with a range of 0.12 to 1.34 μg/g) than in plasma (mean ± standard error of 0.24 ± 0.16 μg/ml, with a range of 0.06 to 0.44 μg/ml; $P = 0.04$).

The English literature refers to only one HPLC method for determination of itraconazole levels, originally described by Woestenborghs et al. in 1987 (22) and since adopted by others (2, 8–11, 17, 20–22). Our method is less tedious than that reported method, which involves a total of five extraction steps and requires at least 2 h for completion, and can be completed in only 1 h. Because it entails a single extraction, our method also appears to yield a better recovery of itraconazole from plasma (80 to 85 versus 72%) and body tissues (85 to 90 versus 64 to 73%). The two HPLC methods are equally sensitive, with a detectability limit (0.01 μg/ml) that is 10-fold lower than that of bioassay (0.1 μg/ml) (6, 14, 21).

The levels of itraconazole that we detected in plasma compare well with the relatively wide range of drug levels reported for patients with AIDS (5, 11, 17, 21). The HPLC assay generally underestimates by about threefold the concentrations of bioactive drugs in the plasma of patients receiving itraconazole. This is explained by the fact that hydroxyitraconazole, a microbiologically active metabolite of itraconazole, is twice as concentrated in plasma as itraconazole is (2, 11); because of the limited amount of hydroxyitraconazole powder that is stocked by the manufacturing company, we could not obtain the powder for our experiments. Although the parent compound and its metabolite are equipotent against most fungal pathogens, hydroxyitraconazole is about twofold more active in vitro than itraconazole against isolates of *Candida pseudotropicalis* that are sometimes, but not always, used for the bioassay (11). That is why for patients receiving itraconazole, the levels of bioactive drugs determined by bioassay can be more than three times higher than those measured by HPLC (10, 11, 21).

Decreased bioavailability of itraconazole may result in clinical failure. For instance, patients with hematologic malignancies receiving itraconazole prophylaxis during episodes of prolonged neutropenia have a higher incidence of fungal infection if itraconazole levels in plasma are inadequate (4, 18). Therefore, it may be reasonable to monitor itraconazole levels in patients who (i) have chemotherapy-induced mucosal inflammation of the gastrointestinal tract; (ii) have human immunodeficiency virus-related or drug-induced gastric hypochlorhydria due to antacids, H2-receptor antagonists, buffer-containing

didanosine (zalcitabine), etc.; or (iii) receive medications that increase the hepatic metabolism of itraconazole, such as rifampin and phenytoin. Inadequate drug levels are not, however, solely responsible for poor clinical outcome, which is also likely to be caused by depressed host immunity and drug resistance.

It may be beneficial to use this simplified HPLC method to study the potential relationship between itraconazole levels in esophageal tissue and clearance of *Candida* organisms from infected tissue. Since it is more practical to obtain specimens of plasma than of esophageal tissue, it may also be important to evaluate whether itraconazole levels in serum would accurately reflect drug levels in esophageal tissue for patients receiving antifungal therapy. A strong correlation between itraconazole concentrations in serum and clearance of *Aspergillus fumigatus* from lung tissue of rabbits has, in fact, been demonstrated (3). Provided that a similar correlation can be demonstrated for patients with esophageal candidiasis, it would probably be useful to monitor the concentration of itraconazole in plasma as a guide to adjusting the dosage and optimizing the efficacy of itraconazole in the treatment of this acute infection. Since itraconazole levels may remain detectable in body tissues, such as the epidermis, for up to 4 weeks (8), it may also be reasonable to use this simplified HPLC method to examine the potential relationship between itraconazole levels in esophageal tissue after discontinuation of therapy and recurrence of esophageal candidiasis.

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