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Note

Assay of itraconazole in leukemic patient plasma by reversed-phase small-bore liquid chromatography

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Itraconazole (R51211; (+)-*cis*-4-[4-[4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-yl-methyl)-1,3-dioxolan-4-yl]methoxy]phenyl]-1-piperazinyl]-phenyl]-2,4-dihydro-2-(1-methylpropyl)-3H-1,2,4-triazol-3-one) is a new broad-spectrum triazole antifungal agent. When compared to ketoconazole, a structurally related, orally active imidazole antifungal compound, itraconazole displays greater antifungal activity against most *Aspergillus* species [1–3]. In addition, itraconazole was more potent than ketoconazole in the treatment of experimental *Candida* infections [4]. Itraconazole is also active against a variety of other fungal species in vitro and in vivo [5–9]. Unlike ketoconazole, itraconazole does not appear to inhibit testosterone biosynthesis or suppress adrenal function in humans [10].

We have found our acute leukemic patients undergoing intensive chemotherapy to be highly colonized with *Candida* organisms in cultures taken from throat, urine and stool, and occasionally there is autopsy evidence of systemic candidiasis and aspergillosis [11]. These findings suggest that itraconazole would be a valuable addition to the prevention and treatment of life-threatening fungal infection in patients with leukemia. Preliminary results from Tricot et al. [12] indicate

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that itraconazole was more efficacious than ketoconazole when given for aspergillosis to patients with severe granulocytopenia. Therefore, we have undertaken evaluation of itraconazole as fungal prophylaxis in patients with acute leukemia undergoing intensive chemotherapy. In previous studies, we found that the measurement of ketoconazole serum concentrations was essential for evaluating the antifungal efficacy and has led us to a better understanding of how subtherapeutic concentrations occurred from pH-dependent dissolution [13].

We have developed a sensitive analytical method for itraconazole in human plasma by high-performance liquid chromatography (HPLC). Other groups have measured serum concentrations of itraconazole in animals by an agar well diffusion microbiological assay [6–8,14]. The lowest reported limit of detection by this method was 0.03 μg/ml [7]. However, one report suggested that only unbound drug may be measured by this method [14]. Recently a reversed-phase HPLC assay for itraconazole with a conventional bore column and UV detection was published by Woestenborghs et al [15]. Their analytical method was quite sensitive, however, a relatively long and complex liquid–liquid extraction scheme was employed. Their extraction efficiency was only 71%. Warnock et al. [16] recently compared a slight modification of this method to the agar diffusion method. They found the HPLC method to be more sensitive (0.01 μg/ml) and much more reproducible than the microbiological method. Our method employs a reversed-phase small-bore column with UV detection at 263 nm with a simplified solid-phase extraction procedure. The assay is currently being used to monitor the plasma concentrations of itraconazole in leukemic patients enrolled in a clinical trial to study the effectiveness of itraconazole for prophylaxis against fungal infections.

EXPERIMENTAL

Reagents and materials

Itraconazole and the internal standard R51012 (I.S.; see Fig. 1) were gifts from Janssen Pharmaceutica (Beerse, Belgium). Stock solutions of itraconazole and I.S. were prepared at a concentration of 0.1 mg/ml in HPLC-grade methanol. These solutions were stable for at least three months at 4°C. HPLC-grade methanol and acetonitrile were from EM Sciences (Gibbstown, NJ, U.S.A.). Drug-free serum (Q-Pak®, Hyland Diagnostics, Deerfield, IL, U.S.A.) or leukemic plasma from patients not on itraconazole were used for the preparation of standard curves. All other chemicals were at least reagent grade or better.

Octadecylsilane solid-phase extraction columns (Baker 10 SPE) were from J.T. Baker (Phillipsburg, NJ, U.S.A.). The analytical column was a 5-μm, 150 mm×2 mm I.D. Spherisorb ODS2 column from Phase Separations (Norwalk, CT, U.S.A.). A microbore guard column (Upchurch Scientific, Oak Harbor, WA, U.S.A.) dry packed with LC-18 pellicular packing (Supelco, Bellefonte, PA, U.S.A.) or a 2-μm in-line filter (Rheodyne, Cotati, CA, U.S.A.) were placed between the injector and the analytical column.

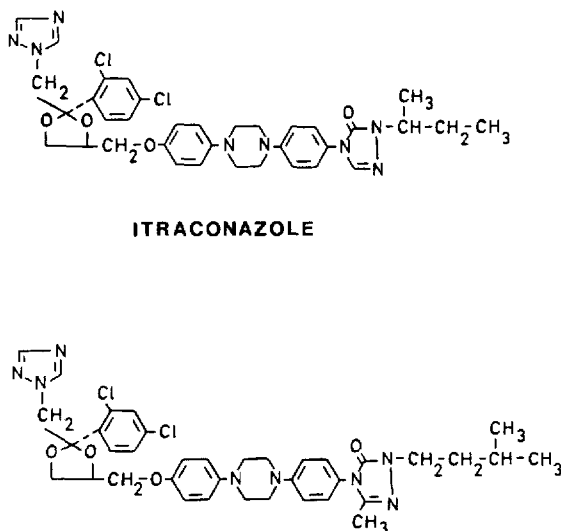

Fig. 1. Structures of itraconazole and the internal standard (R51012).

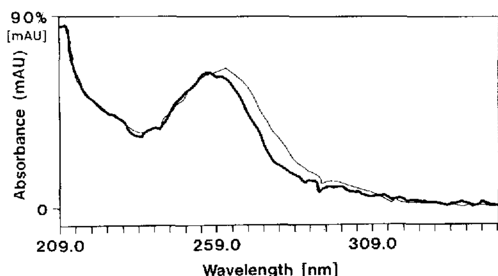

Fig. 2. UV spectra of itraconazole (thick line) and the internal standard (thin line) in mobile phase from a patient plasma sample. The spectra at the upslope, downslope and apex of the itraconazole peak overlayed exactly insuring peak purity.

HPLC apparatus and conditions

The analysis of itraconazole was performed on a Hewlett-Packard 1090A liquid chromatograph (Fullerton, CA, U.S.A.) equipped with a variable-volume automatic injector and a diode array detector. The optimal wavelength for UV detection was 263 nm (see Fig. 2). Peak area or height was determined with either a Shimadzu CR3A integrator or with a DPU integrator (Hewlett-Packard). The column temperature was maintained at 40°C in a heated oven. The mobile phase consisted of acetonitrile–water (65:35). The flow-rate was 0.25 ml/min.

Extraction procedure

Plasma samples (0.5 ml) were spiked with 50 μl of a 0.25 μg/ml solution of I.S. in methanol. For the preparation of standard curves, blank leukemic plasma or drug-free serum was also spiked with known amounts of itraconazole in 20 μl of methanol. The plasma samples were then diluted with 0.5 ml of 0.05 *M* ammo-

nium phosphate buffer, pH 7.8, vortexed for 30 s and loaded onto a pre-conditioned octadecylsilane solid-phase extraction column. The columns were conditioned with 1 ml of HPLC-grade methanol, 1 ml of distilled water and 1 ml of ammonium phosphate buffer, pH 7.8, under vacuum filtration. After the sample was pulled through the column, the columns were washed successively with 1 ml of distilled water and 50 μl of acetonitrile. Itraconazole and the I.S. were eluted into new test tubes with two 1.0-ml aliquots of acetonitrile. The acetonitrile extract was evaporated to dryness under nitrogen at 45°C and the residue was reconstituted in 100 μl of mobile phase and transferred into 2-ml crimp-topped automatic injector vials with 200-μl borosilicate glass inserts (Sun Brokers, Wilmington, NC, U.S.A.). A 25-μl aliquot of the extract was injected by an automatic injector onto the column.

Standard curves were prepared by the addition of 20 μl of diluted stock solutions of itraconazole in methanol to give the following final concentrations: 5, 10, 25, 50, 100, 200 and 500 ng/ml and 1, 2, 5 and 10 μg/ml. I.S. was added and the samples were extracted as described above.

RESULTS AND DISCUSSION

Assay validation

The extraction recovery of 100 ng/ml itraconazole was 93.4 ± 4.2% ($n=6$). The I.S. recovery was 95.0 ± 5.1%. Linear regression analysis of the standard curves showed excellent correlations with r^2 values of ≥0.985. The between-day coefficient of variation based on duplicate standard curves over a five-month period at different plasma concentrations is shown in Table I. Although the standard curve was linear up to 10 μg/ml, the plasma levels in leukemic patients taking up to 400 mg per day were never above 1 μg/ml. The limit of sensitivity of the assay

TABLE I

REPRODUCIBILITY OF THE ITRACONAZOLE ASSAY

The peak-area ratio of itraconazole/internal standard was determined for seven concentrations and the coefficient of variation (C.V.) was calculated for nine duplicate standard curves over a five-month period (between-day) or for one quadruplicate standard curve (within-day).

Concentration (ng/ml)	Between-day			Within-day		
	Peak-area ratio	C.V. (%)	n	Peak-area ratio	C.V. (%)	n
5	0.047 ± 0.012	25.3	17	0.047 ± 0.0096	20.2	4
10	0.074 ± 0.010	13.8	16	0.083 ± 0.0050	6.06	4
25	0.154 ± 0.0096	6.23	17	0.150 ± 0.0115	7.70	4
50	0.297 ± 0.0207	6.97	18	0.258 ± 0.0050	1.94	4
200	1.17 ± 0.123	10.5	16	1.04 ± 0.017	1.67	4
500	2.93 ± 0.238	8.13	18	2.75 ± 0.079	2.87	4
1000	5.87 ± 0.463	7.89	18	5.75 ± 0.057	1.12	4

was 10 ng/ml with a signal-to-noise ratio of greater than 10:1 and an acceptable within-day coefficient of variation of 6.1%.

Chromatography and detection

Itraconazole eluted at 4.1 min and the I.S. eluted at 5.3 min. Representative chromatograms are shown in Fig. 3. No interfering peaks were observed in either drug-free serum or pooled leukemic plasma. Since many of the leukemic patients are on multiple drugs, such as cimetidine, Amphotericin B, other antibiotics and chemotherapeutic agents, interference in drug assays is a potential problem. Other antifungal agents that are given concurrently may interfere with microbiologic assays. Fortunately, itraconazole is a highly lipophilic drug, and most potentially interfering drugs eluted at or near the solvent front in our system. Warnock et al. [16] listed a large number of antimicrobial agents and other drugs that did not interfere with the detection of itraconazole in their system. Two unidentified peaks were occasionally observed in blank or patient plasma (see Fig. 3A), but did not appear in the plasma of every patient. Since they also occurred in some lots of plasma obtained from subjects not taking itraconazole, they do not appear to be metabolites.

Itraconazole was detected at 263 nm with a diode array UV detector. The UV spectrum of itraconazole and its I.S. are shown in Fig. 2. Itraconazole would also be detected with good sensitivity at 254 nm with a fixed-wavelength detector. Both itraconazole and the I.S. are also fluorescent compounds with an excitation maximum of 257 nm and an emission optimum of 342 nm. Fluorescence detection could improve the sensitivity of the assay, however, for the purpose of clinical monitoring the added sensitivity is not necessary and fluorescence HPLC detectors may not be as widely available in clinical laboratories.

The use of a small-bore column (2 mm I.D.) has some important advantages. The flow-rate is considerably lower (0.25 ml/min) which could result in a significant savings in solvent costs. The absolute assay sensitivity is increased [17],

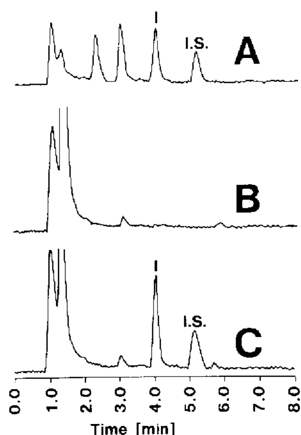

Fig. 3. Chromatograms of extracted plasma samples. (A) Blank plasma spiked with itraconazole (I) and the internal standard (I.S.); (B) blank leukemic plasma; (C) patient plasma sample containing 220 ng/ml itraconazole.

however this gain in sensitivity is dependent upon the ability to concentrate the sample extract so that an equivalent aliquot is injected onto the column. Unfortunately, the small-bore columns also have some drawbacks compared to conventional columns. These columns are more easily contaminated than 4.6 mm I.D. columns. Also, the small surface area causes the front column frit to become plugged easily, resulting in rapidly increasing back pressures. The back pressure can be reduced by periodic reversal of column flow, however, a more practical solution is to use either a microbore guard column or a 2-μm in-line filter. Either system worked well in preventing high back pressures because the frits may be easily exchanged. The microbore guard column afforded better protection for the analytical column, and although it caused some loss in resolution, the loss in resolution was not judged to be a significant problem for this assay. The in-line filter caused only a minimal loss in resolution if microbore tubing was used and if the connections were kept short. When standard guard columns, either the cartridge type or dry packed columns, were used, a significant loss in resolution was observed.

Plasma concentrations in acute leukemics

Plasma itraconazole concentrations in acute leukemics were determined as part of a double blind prophylactic clinical trial. Patients were given either 100 mg per day, 200 mg per day or 200 mg twice daily. The laboratory had no knowledge of what dosages were given to the patients. Of the samples tested, itraconazole peak concentrations (2 h post-dose) varied greatly from a low of 30 ng/ml to a high of 860 ng/ml. The plasma concentration of itraconazole was followed in a single patient within a dosing interval of the drug (see Fig. 4).

In summary, the analytical method described is useful for determining concentrations of itraconazole in acute leukemics. The solid-phase extraction method is relatively rapid, resulting in clean extracts that are free of contamination for this analysis. The microbore column lowers HPLC mobile phase solvent costs and may increase sensitivity, however, a guard column or in-line filter is necessary to prevent back pressure problems. The solid-phase extraction procedure is consid-

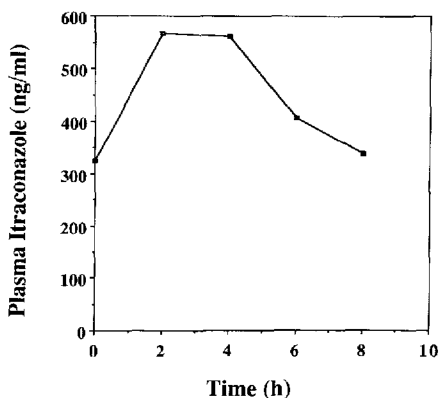

Fig. 4. Concentration of itraconazole in a leukemic patient during a dosing interval.

erably easier and more efficient than the previously reported extraction procedure of Woestenborghs et al. [15]. This assay has good sensitivity and is useful for routine monitoring. It may be sensitive enough for pharmacokinetic evaluations as well, although other investigators may wish to use a fluorescence detector to lower the limit of detection.

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