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Simplified Bioassay Method for Measurement of Flucytosine or Ketoconazole

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A simple agar-well diffusion bioassay suitable for measurement of flucytosine or ketoconazole was developed by using *Candida pseudotropicalis* ATCC 46764 as the assay organism. A test medium composed of (per liter) 7 g of Trypticase peptone, 7 g of YNB (yeast-nitrogen base), 15 g of glucose, and 15 g of agar was seeded with an inoculum which had been grown to no. 2 McFarland turbidity after 4 to 6 h in YNB-glucose broth. Determinations of flucytosine or ketoconazole were performed without necessity of heating or diluting of serum samples to alleviate amphotericin B interference. A linear relationship between zone diameters and $\log_{10}$ concentration of the drugs was observed over the pharmacologically relevant ranges of 25 to 160 μg/ml for flucytosine and 0.5 to 20 μg/ml for ketoconazole. The mean coefficient of variability for samples measured on 5 separate days was 2.4% for flucytosine and 4.0% for ketoconazole. This assay represents a significant improvement over previous bioassay methods in that a single test system may be used for measurement of either flucytosine or ketoconazole, no serum dilution or pretreatment is required, inoculum preparation is accomplished entirely on the day of the assay, and sharp, clearly defined zones of inhibition are obtained with both drugs.

Monitoring levels of antifungal agents in serum may be helpful in assessing therapeutic efficacy, patient compliance, or risk of toxicity. Ketoconazole is an oral imidazole which has been found to be useful in the treatment of mucocutaneous candidiasis (9), systemic mycoses (8), chronic dermatological infections (18), and in the prevention of candidal infections in neutropenic patients (10, 11). Determinations of drug levels in sera are useful in evaluation of intestinal absorption, as well as patient compliance. Flucytosine, another orally effective antifungal agent (2), is often used in treatment of cryptococcal meningitis (3), systemic candidiasis (9, 21), and chromoblastomycosis (2). However, it has the potential to produce significant bone marrow toxicity, especially when levels in serum exceed 125 μg/ml (15).

Several microbiological (4, 12–14), chemical (17, 20), and chromatographic (1, 7, 19) methods have been used to measure these two antifungal agents in body fluids. Amphotericin B, which is often used in combination with flucytosine, may complicate measurement of flucytosine by bioassay methods (4, 13, 14). This report describes a simple bioassay capable of measuring ketoconazole or flucytosine which improves upon previously described methods and can be performed in the presence of amphotericin B.

MATERIALS AND METHODS

Antibiotic standards. Ketoconazole diagnostic powder (Janssen Pharmaceutica, Inc., New Brunswick, N.J.) was dissolved in 0.2 N HCl (20 ml/1 g of ketoconazole) to facilitate solubilization. Succeeding dilutions were prepared in sterile distilled water. Flucytosine diagnostic powder (Hoffmann-LaRoche, Inc., Nutley, N.J.) and amphotericin B (Fungizone, for infusion, E.R. Squibb & Sons, Princeton, N.J.) were prepared and diluted in sterile distilled water. Antifungal standards were prepared from these stock solutions in commercial pooled human serum (Difco Laboratories, Detroit, Mich.).

Test organism. *Candida pseudotropicalis* ATCC 46764 was maintained by weekly passage on Sabouraud-dextrose agar slants.

Test medium. The test medium, modified after the method of Jorgensen et al. (12), was composed of (per liter) 7 g of YNB (yeast-nitrogen base) (Difco), 7 g of Trypticase peptone (BBL Microbiology System, Cockeysville, Md.), 15 g of glucose, and 15 g of agar, and it had a final pH of 6.0.

Performance of the bioassay. To perform an assay, growth from a 2- to 7-day-old Sabouraud dextrose slant was suspended in YNB broth (Difco) to the turbidity of a 0.5 McFarland opacity standard (88 to 91% transmission at 590 nm) and then was vortexed for 1 min. The inoculum was incubated at 35°C for 4 to 6 h, and the turbidity then was adjusted with sterile distilled water to that of a no. 2 McFarland standard (4×10^6 cells per ml; 65 to 70% transmission at 590 nm).

Previously prepared 25-ml aliquots of the test agar were melted, allowed to cool to 48°C, inoculated with 0.5 ml of the adjusted suspension, and then gently mixed by inversion and poured into 150-by-15-mm round plastic disposable petri dishes placed on a level surface.

Paired 10-ml aliquots of drug standards or unknowns were placed in opposing sets of 5-mm-diameter wells cut in the agar bed around the periphery of the plate. Assay plates were prepared in duplicate, resulting in quadruplicate wells for each drug standard and unknown sample. Preliminary experiments established that appropriate flucytosine standards were 160, 80, and 40 μg/ml; ketoconazole standards were 20, 5, 2, and 0.5 μg/ml. Completed assay plates were incubated for 15 to 20 h at 35°C. Zones of inhibition formed by the standards and unknowns were carefully measured with calipers to the nearest 0.1 mm. Zone diameters were plotted versus drug concentration on semilogarithmic paper to prepare standard curves.

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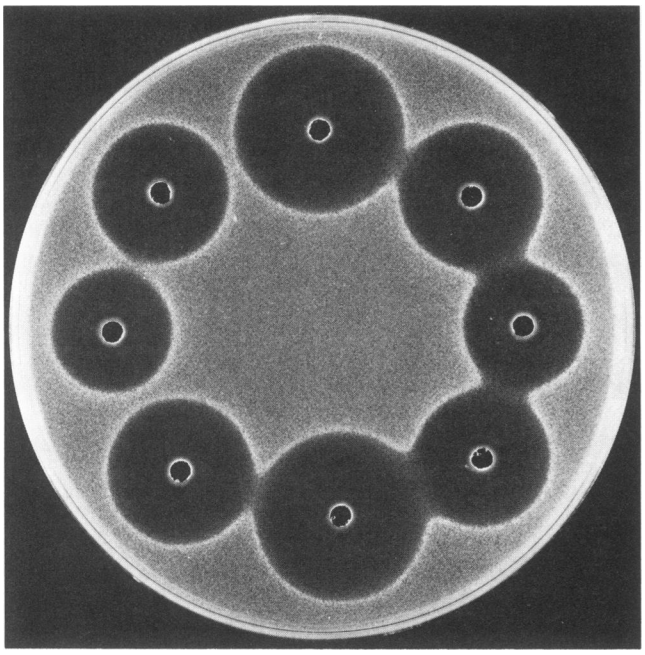

FIG. 1. Bioassay plate for flucytosine. Wells contain (clockwise from 12 o'clock and from 6 o'clock) standards of 160, 80, and 40 μg/ml and an unknown.

Determinations of accuracy and precision. Determinations of zones of inhibition were performed in quadruplicate on 5 consecutive days. Each flucytosine plate included spiked sera containing 25, 40, 60, 80, 120, and 140 μg/ml. Each ketoconazole plate incorporated duplicate samples of spiked sera containing 0.5, 1, 2, 5, 10, and 20 μg/ml. Standard deviations and coefficients of variability were determined for the 5 days.

Determination of the effects of amphotericin B. Zones of inhibition resulting from representative concentrations of either flucytosine or ketoconazole included in the standard curves were evaluated both with and without 5 μg of amphotericin B per ml.

RESULTS

Zones of inhibition were clearly demarcated and free of inner zones of serum precipitation around the assay wells (Fig. 1 and 2). The resulting zone diameters accommodated testing of one flucytosine sample or two ketoconazole samples per assay plate. Although not well depicted in the figures, the zone margins produced by ketoconazole were slightly sharper and thus more easily measured than those produced by flucytosine. As the age of the yeast from the slant culture used for the inoculum increased between 2 and 7 days, zone sizes increased slightly, but concentration-dependent linearity was maintained.

The standard curve for flucytosine was linear between concentrations of 25 and 160 μg/ml; with a coefficient of variability of 2.4%. Likewise, the standard curve for ketoconazole was linear between concentrations of 0.5 and 20 μg/ml, with a coefficient of variability of 4.0%. Figure 3 plots the mean zone sizes with respective standard deviations for these two standard curves.

Amphotericin B produced detectable zones of inhibition in

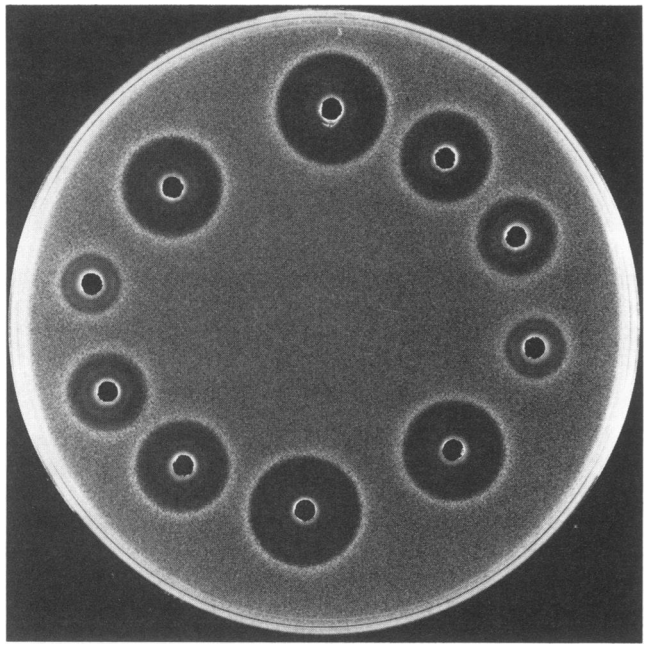

FIG. 2. Bioassay plate for ketoconazole. Wells contain (clockwise from 12 o'clock and 6 o'clock) standards of 20, 5, 2, and 0.5 μg/ml and an unknown.

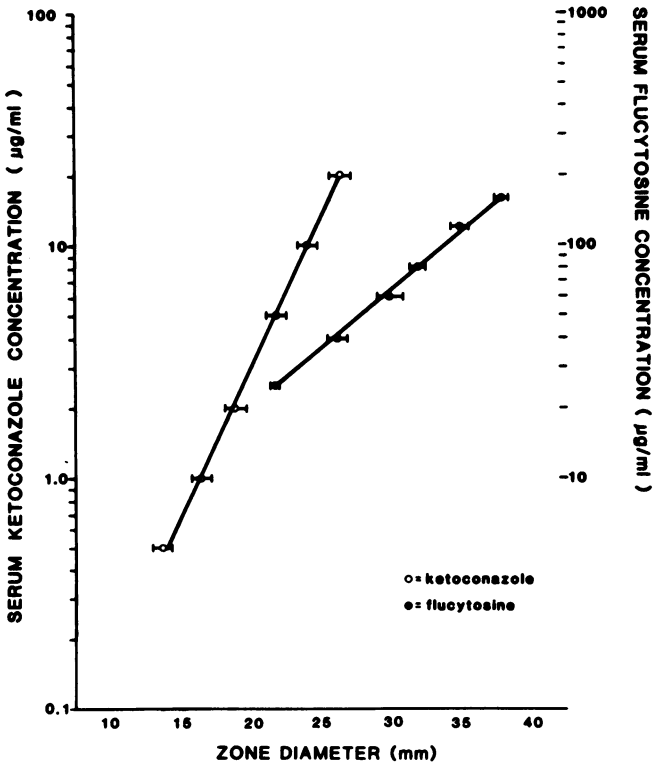

FIG. 3. Graphic representation of zones of inhibition of ketoconazole or flucytosine versus drug concentration in serum. Points represent the mean value of assays done on 5 consecutive days. Bars depict standard deviations. Mean coefficient of variability is 4.0% for ketoconazole and 2.4% for flucytosine.

concentrations greater than 1 μg/ml; however, concentrations as high as 5 μg/ml produced zones of inhibition smaller than those seen with the lowest concentrations of flucytosine or ketoconazole standards. Moreover, addition of 5 μg/ml of amphotericin B to either flucytosine or ketoconazole produced no interference within the limits of the assay method; i.e., zones were equivalent both with and without amphotericin B.

DISCUSSION

For drug levels in serum to be used effectively for patient management, the means for determination must be accurate over pharmacologically relevant ranges, there must be absence of interference by drugs likely to be used concomitantly, and the levels must be rapidly available to the clinician. Testing should be simple to perform for use in routine clinical laboratories. The bioassay method described here provides these features with a method suitable for the measurement of either flucytosine or ketoconazole.

Ketoconazole, although well absorbed by the gastrointestinal tract, is subject to wide individual variation in serum levels (5). Consequently, routine monitoring of levels has been suggested (16). Because of the chronicity of fungal infections, ketoconazole determinations in serum are also very useful in monitoring patient compliance with extended dosage regimens.

Recently, the NIH-NIAID Mycoses Study Group reported flucytosine toxicity associated with peak flucytosine levels of ≥100 μg/ml during ≥2 weeks of therapy, and found that 5-fluorouracil levels did not correlate with toxicity (A. Stamm, R. Diasio, W. Dismukes, S. Shadomy, G. Cloud, and the NIH-NIAID Mycoses Study Group. Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 24th, Washington, D.C., abstr. no. 1105, 1984). This was contrary to suggestions based on previous observations that 5-fluorouracil levels might be predictive of toxicity associated with flucytosine (6). Thus, although the metabolite 5-fluorouracil may be detected in patients treated with flucytosine, flucytosine itself appears to have toxic properties, making monitoring of flucytosine levels important in preventing drug toxicity.

Measurement of flucytosine and ketoconazole has been accomplished with a variety of methodologies in the past. Chromatographic techniques are rapid and accurate, but require relatively expensive equipment and specially trained personnel. Likewise, chemical determinations require access to a specific analyzer system which may not be widely available. Bioassays, on the other hand, are more feasible for routine use in clinical laboratories. Interference by amphotericin B with previous bioassay methods for flucytosine has been eliminated only through cumbersome techniques such as dialysis (4) or heat inactivation (14). It has been suggested that differential diffusion of the two drugs in agar serves to eliminate the interference (13).

Although we found that amphotericin B concentrations more than twice the usual clinically achievable serum levels produced small zones of inhibition in our system, there was no interference with measurement of either flucytosine or ketoconazole at the lowest limits of the assay. We also discovered that a white ring of precipitation around the test wells in agar medium described by Kaspar and Drutz (13) could be eliminated by the addition of Trypticase peptone to the test medium. Elimination of this artifact made interpretation of zone sizes easier and, in fact, made it apparent that amphotericin B does diffuse somewhat from the test well, producing a small zone of inhibition. The precipitate appeared to be due at least in part to a pH-dependent phenomenon, since a more acid pH (5.4 to 5.5) enhanced the opaque rings, and alkalinization to a pH of 6.0 with either Trypticase peptone or 1 N NaOH eliminated the ring. Although excessive Trypticase peptone produced less distinct zones of inhibition, 7 g/liter was optimal for this assay.

Bioassays for antifungal agents often require an overnight growth of broth inoculum because of the long doubling time of yeast used in the assays. Our assay uses only a brief period of inoculum preparation, thereby making determinations of drug levels in serum available to the clinician 24 h after the sample is received.

The bioassay method described here eliminates the need for any serum pretreatment, and the abbreviated inoculum preparation makes this bioassay more rapid than those previously described for flucytosine or ketoconazole. Use of a single test medium and organism for either drug determination provides additional convenience for the laboratory. We have also found that this method is suitable for determinations of either drug in cerebrospinal fluid. This bioassay method should prove to be practical for clinical microbiology laboratories, and it should also provide better access to drug levels for use by clinicians.

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