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In Vitro Studies of Activities of Some Antifungal Agents against *Candida albicans* ATCC 10231 by the Turbidimetric Method

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Different criteria (the drug concentration which inhibited 50% of growth [$IC_{1/2}$], the lowest drug concentration at which growth was just less than 30% of that in a positive control well [IC_{30}], the visual inhibitory concentration [IC_v], and the minimum fungicidal concentration [MFC]) were applied to study the effects of some antifungal agents against *Candida albicans*. Amphotericin B, flucytosine, and bifonazole produced total growth inhibition. Clotrimazole, itraconazole, ketoconazole, and miconazole produced partial growth inhibition. The values of $IC_{1/2}$ and IC_{30} were similar for all agents and avoided the problems of partial inhibition; the values of IC_v and MFC were higher than those of $IC_{1/2}$ and IC_{30}.

The principal difficulty in interpreting susceptibility test results for antifungal agents is the partial growth inhibition produced by imidazole derivatives (10, 13). There is no unanimous agreement as to the best methodology for interpreting susceptibility test results (5, 7, 8, 19, 21). By the microdilution method, the inhibitory concentration (IC) is the lowest drug concentration that is capable of inhibiting a determined percentage of growth (10, 12, 13, 16, 24–26), and an objective value for assessing the final point of inhibition is achieved. Turbidimetric methods can determine the minimum drug concentration which inhibits 50% of growth ($IC_{1/2}$) (1, 6, 10, 18) or the lowest drug concentration at which growth is just less than 30% of that in the positive control well (IC_{30}) (12, 13).

In this study, the effects of increasing concentrations of seven antifungal agents on the growth of *Candida albicans* ATCC 10231 were studied. The ICs were also evaluated by comparing the criteria mentioned above ($IC_{1/2}$ and IC_{30}), the study of the visual inhibitory concentration (IC_v), and the minimum fungicidal concentration (MFC).

The antifungal agents used in this study were amphotericin B (Squibb), clotrimazole and bifonazole (Bayer), miconazole (Isdin), itraconazole (Janssen) solubilized in dimethyl sulfoxide, flucytosine (Roche) resuspended in water, and ketoconazole (Janssen) solubilized in HCl (0.2 N). The concentrations of antifungal agents ranged from 0.01 to 40 μg/ml and were obtained by dilution in 0.01 M phosphate-buffered saline (PBS) at pH 7.

C. albicans ATCC 10231 was grown in yeast nitrogen base medium (Difco) supplemented with 0.5% glucose in 0.01 M PBS (pH 7) (bYNBG).

Growth kinetics, IC, and MFC studies were carried out in microtiter plates with a final volume of 200 μl. Each row of the microtiter plates contained the different concentrations of the antifungal agents and the strain tested. The final inoculum in each well was approximately 10^5 organisms/ml. Controls of sterility and growth without antifungal agents were carried out. Plates were incubated at 37°C in a humid atmosphere. At 0, 4, 12, 24, 48, and 72 h of incubation, the optical density (OD) at 492 nm was read in a Titertek Multiskan MC photometer. The data obtained after 24, 48, and 72 h were used to establish the $IC_{1/2}$ and the IC_{30}. To determine the MFC, 20 μl was taken from the wells where no visible growth appeared, inoculated into medium without antifungal agent, and examined after 48 h of incubation. The criteria for the MFC corresponded to the lowest concentration that yielded no colonies. All of the experiments were performed five times.

The effects of increasing concentrations of the antifungal agents on the growth of *C. albicans* ATCC 10231 are shown in Fig. 1, 2, and 3. The ODs obtained for the growth controls demonstrated that the culture reached the stationary growth phase after 24 h. The presence of concentrations greater than 1.25 μg of amphotericin B per ml ($IC_{1/2}$ and IC_{30}) brought about sudden growth inhibition. All of the imidazole derivatives except bifonazole showed partial growth inhibition from 24 h of culture for six consecutive concentrations (lower than the $IC_{1/2}$ and the IC_{30}). At 48 and 72 h, inhibition was generally presented as more sudden growth decreases, and the values of both parameters ($IC_{1/2}$ and IC_{30}) remained constant. The *C. albicans* strain studied (ATCC 10231) showed great susceptibility to flucytosine, although it was subsequently shown, by use of subcultures, that in order for flucytosine to act as a fungicide, much greater concentrations are required.

The values of $IC_{1/2}$ and IC_{30} coincided for all of the antifungal agents studied except bifonazole after 24, 48, and 72 h; bifonazole presented a variation of 1 dilution after 24 h (Table 1). This was to be expected, since theoretical application of the formulae provided endpoints, with variations of ODs never being greater than 0.003 units. The ICs obtained after 48 and 72 h of incubation were similar and were greater than those obtained after 24 h.

After 24 h, the MFCs of all antifungal agents except itraconazole were always greater than the $IC_{1/2}$ and IC_{30}, principally for flucytosine.

The IC_v showed higher values and greater variability; included in Table 1 are the values which were repeated at least three times. Visual reading gave less objective data, and these data were often in disagreement with the spectrophotometric readings. In a study performed by Lefler and Stevens (14), IC_vs showed much variation, whereas the $IC_{1/2}$s were the same for all strains.

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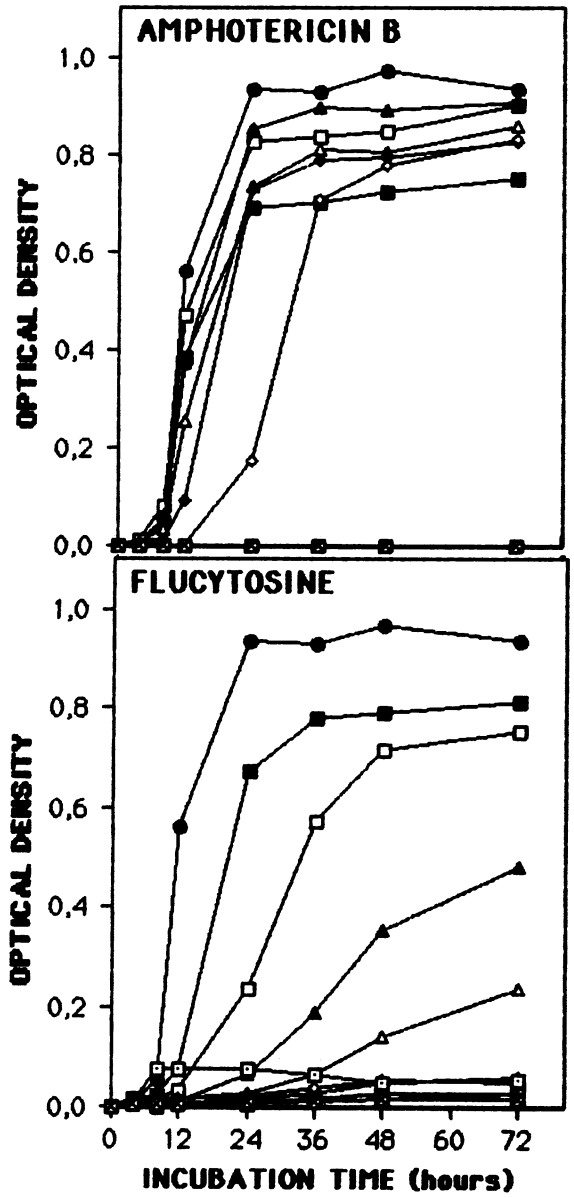

FIG. 1. Effects of increasing concentrations of amphotericin B and flucytosine on the growth of *C. albicans* ATCC 10231. The concentrations (in micrograms per milliliter) were 0 (●), 0.03 (■), 0.07 (□), 0.15 (▲), 0.3 (△), 0.6 (◆), 1.25 (◇), 5 (◘), 10 (⊡), 20 (⊞), and 40 (⊠).

bYNBG medium was chosen for this study because, although unanimity does not exist, it has been used by various investigators in studies of all antifungal agents and because it has been specified that significant variations do not exist with respect to other media (9, 11, 17, 22–24). bYNBG medium was buffered to avoid an acidic pH, which might adversely affect ketoconazole and amphotericin B (4). PBS was used in this study since it is inexpensive and gives ICs similar to those obtained with morpholinepropanesulfonic acid buffer (15). Some investigators have indicated that bYNBG medium produces poorer growth than those produced by other media (9) and that when agar dilution methods are applied, inhibition zones are not well defined (25). In microdilution tests, by using an IC that produces a

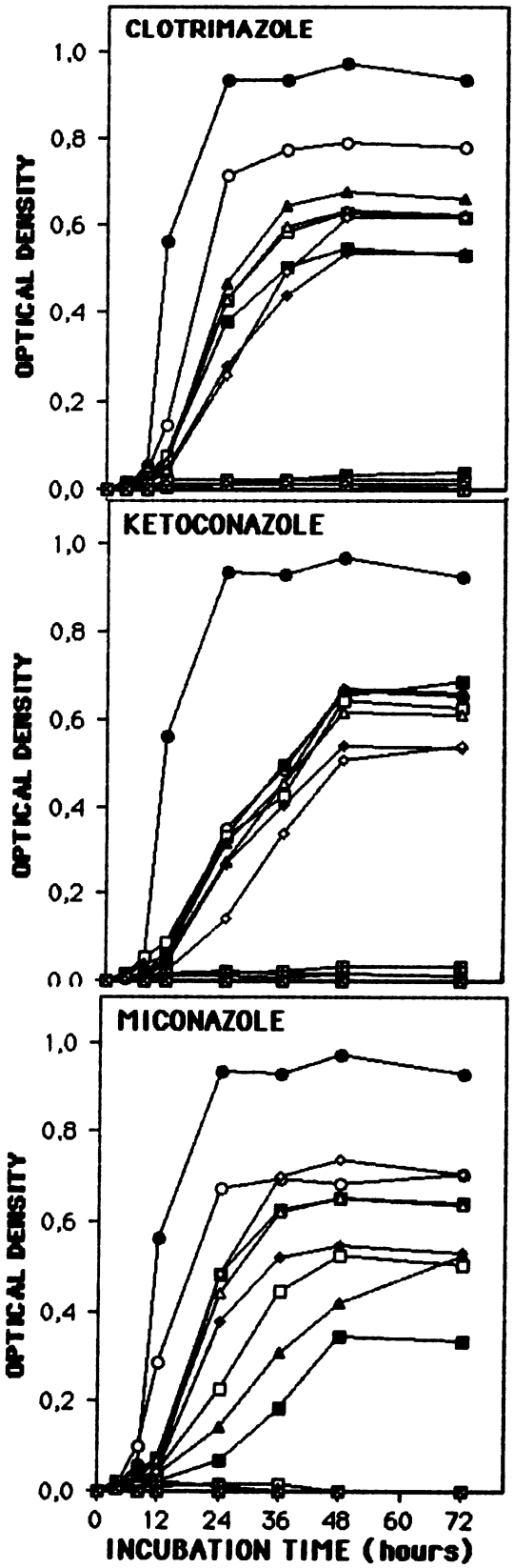

FIG. 2. Effects of increasing concentrations of clotrimazole, ketoconazole, and miconazole on the growth of *C. albicans* ATCC 10231. The concentrations (in micrograms per milliliter) were 0 (●), 0.01 (○), 0.03 (■), 0.07 (□), 0.15 (▲), 0.3 (△), 0.6 (◆), 1.25 (◇), 5 (◘), 10 (⊡), 20 (⊞), and 40 (⊠).

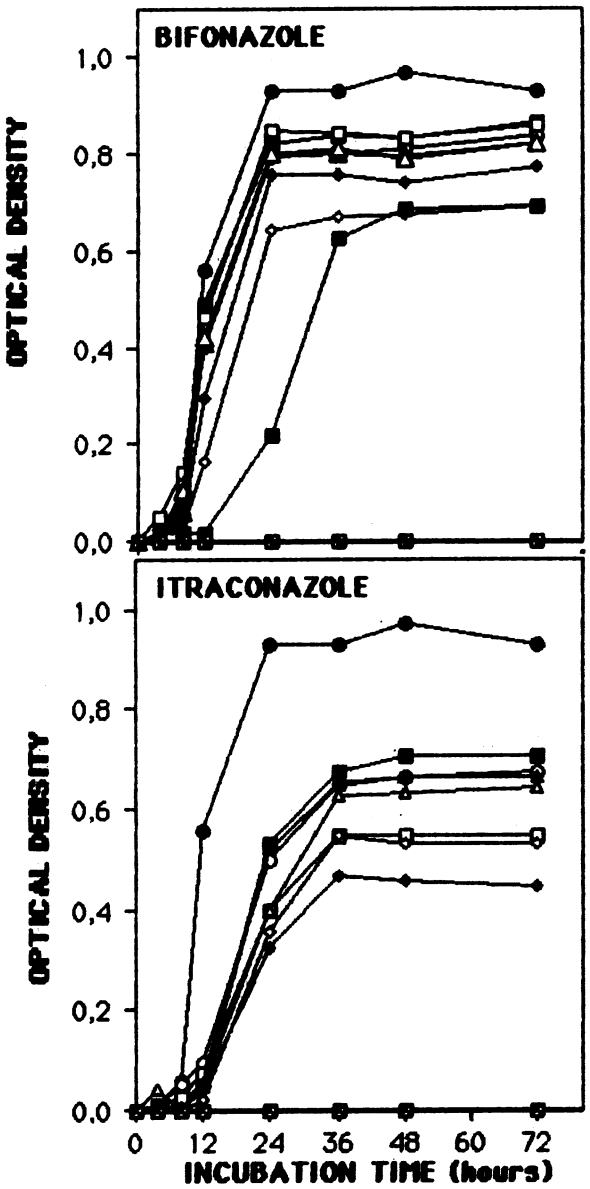

FIG. 3. Effects of increasing concentrations of bifonazole and itraconazole on the growth of *C. albicans* ATCC 10231. The concentrations (in micrograms per milliliter) were 0 (●), 0.01 (○), 0.03 (■), 0.07 (□), 0.15 (▲), 0.3 (△), 0.6 (◆), 1.25 (◇), 5 (◘), 10 (⊡), 20 (⊞), and 40 (⊠).

determined percentage of inhibition of growth with respect to the control, the problem of inhibition zones that are not well defined no longer exists.

The length of incubation has a pronounced effect on antifungal agents; MICs tend to increase with prolonged incubation times (3, 20). Pfaller et al. (23) have pointed out that after 24 h, optimum growth is produced and that the existing inhibition reflects the degree of susceptibility of the yeasts to the antifungal agent. On the other hand, Doern et al. (2) have stated that the reproducibility of the results is greater after 48 h, although they also found that MICs determined after incubation for 48 h were higher.

The partial inhibition produced by the majority of the imidazole derivatives (10, 12, 13) confirms the importance of

TABLE 1. In vitro activities of different antifungal agents against *C. albicans* ATCC 10231 by incubation time and reading criteria

Antifungal agent	Incubation time (h)	IC (μg/ml)			
		$IC_{1/2}$	IC_{30}	IC_V	MFC
Amphotericin B	24	1.25	1.25	5	5
	48	5	5	10	10
	72	5	5	10	10
Flucytosine	24	0.07	0.07	0.15	40
	48	0.3	0.3	0.6	40
	72	0.3	0.3	5	40
Bifonazole	24	10	5	10	20
	48	10	10	10	20
	72	10	10	10	20
Clotrimazole	24	0.6	0.6	5	10
	48	5	5	5	20
	72	5	5	5	20
Itraconazole	24	5	5	5	5
	48	5	5	5	20
	72	5	5	10	40
Ketoconazole	24	0.6	0.6	5	20
	48	5	5	5	20
	72	5	5	5	20
Miconazole	24	0.07	0.07	5	20
	48	5	5	5	20
	72	5	5	5	20

using a growth control and the necessity of carrying out the reading of the test of susceptibility to antifungal agents on the basis of a percentage of growth inhibition. From the results of this study, we found that to evaluate the activity of an antifungal agent, it is advisable to use turbidimetric methods. Thus, it is possible to avoid the problems brought about by the partial inhibition produced by some antifungal agents. Turbidimetric methods also permit the application of objective criteria for the determination of ICs. By this methodology, the IC_{30} and the $IC_{1/2}$ criteria are equally valid.

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