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Quantitation of Imidazoles by Agar-Disk Diffusion

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A simple, reliable, and inexpensive assay for quantitation of the imidazole drugs miconazole, RV 40,500, and RV 41,400 was tested. The assay, similar to a Kirby-Bauer test, was sensitive to less than or equal to 0.2 μg of drug per ml and linear from less than or equal to 0.2 to 10.0 μg/ml. Concentration of inoculum and agar depth in test plates was not as critical as the type of medium, amount of inoculum, or type of drug used.

The mainstay of antifungal therapy since 1956 has been amphotericin B. However, several serious problems are associated with amphotericin B therapy (2, 6). Recently, several dioxolane imidazole derivatives have been evaluated in animal and human trials (4, 5, 7, 8; J. P. Sung, G. D. Campbell, and J. G. Grendahl, Arch. Neurol., in press). Miconazole, the most extensively tested of these imidazoles, is administered by intravenous infusion. However, the newer imidazoles have been structurally altered to improve absorption by oral administration. Evaluation of blood levels of miconazole has been accomplished primarily by using Sabouraud-dextrose (Sab-dex) plates overlaid with agar containing *Coccidioides immitis* endospores (5). This technique has not been commonly performed by general clinical laboratories, and hence mailing of specimens to a few reference laboratories has been necessary. With the development of oral imidazoles, the measurement of drug concentrations in blood will become more important.

The procedure presented herein would allow any clinical laboratory to evaluate blood levels of miconazole or the newer oral imidazoles by a simple, reliable, and economical test.

Organisms. Four isolates of *Candida albicans* and one of *Cryptococcus neoformans* were tested. All cultures were inoculated on Sab-dex plates for 24 to 48 h before the assay was run. The 24- to 48-h cultures were used to prepare saline suspensions from 2×10^6 to 2×10^7 cells per ml using a Neubauer hemacytometer.

Standards. The three drug standards used (miconazole, RV 40,500, and RV 41,000) were obtained from Janssen Pharmaceutica. Miconazole was in liquid form at 10 mg/ml (7). The other imidazoles were in powder form and were reconstituted in sterile water to 10 mg/ml. The drugs were serially diluted in either pooled human serum or pooled horse serum (Grand Island Biological Co.) to give concentrations ranging from 10.0 to 0.16 μg/ml. Antibiotic assay disks (Schleicher and Schuell no. 740-E; 12.7-mm diameter) were completely filled by absorption with the standards. Disks were dried for 2 h at 37°C before use.

Plates. The following standard Difco bacteriological agar media were tested: antibiotic medium no. 1, brain heart infusion, Eugon, nutrient, and Sab-dex. Quantities from 25 to 45 ml were used in 150-mm petri dishes. All media were prepared on the assay day and allowed to dry for 4 to 6 h at room temperature before use.

Assay procedure. Agar plates were inoculated with a saline suspension of the test organism by means of a saturated cotton swab. Inoculation was in three planes at 120° to each other (1). After the agar surface was seeded, plates were allowed to dry for 1 h at room temperature. Standard disks were positioned on the agar plates, and all standards were in replicates of five with no duplications per plate. Plates were incubated at 4°C for 18 h, then at 25°C for 24 h. Diffusion of antibiotics at 4°C prior to incubation at 25°C increased sensitivity at the low standard levels. After incubation, inhibition zones were measured to the nearest 0.5 mm. Datum points were analyzed using the formula $Y = a + b \log X$, which is a linear equation for Y (inhibition zone) and $\log X$ (concentration). Using a least-squares formula, a best-fit linear line was obtained (3).

Of the five media tested, Sab-dex agar gave the best results at the lowest standard level (Fig. 1). Sab-dex agar was chosen for the remainder of the testing because zones of inhibition were more easily measured due to increased growth of test organisms, clarity of the medium, and lack of contamination. Varying the amount of agar per plate from 25 to 45 ml did not significantly alter the results; however, a consistent trend did appear (Fig. 2). Possibly more significant than the volume of medium used on the

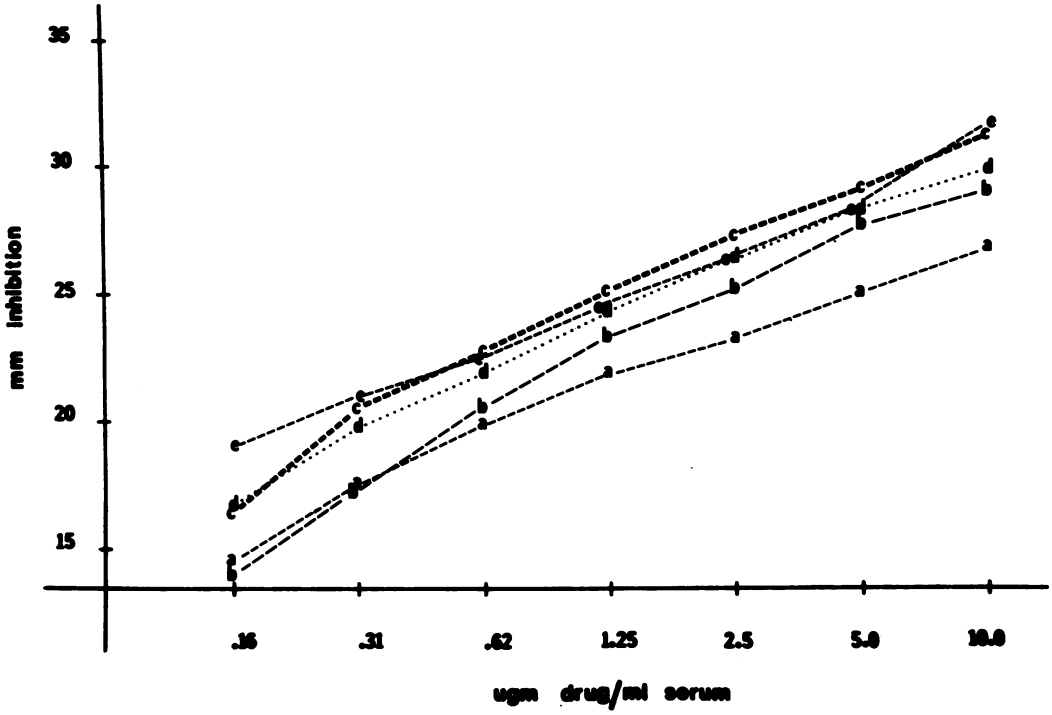

FIG. 1. *Effect of media on inhibition zones using C. albicans 37 and miconazole. Agar media: (a) antibiotic medium no. 1; (b) brain heart infusion; (c) Eugon; (d) nutrient; (e) Sab-dex.*

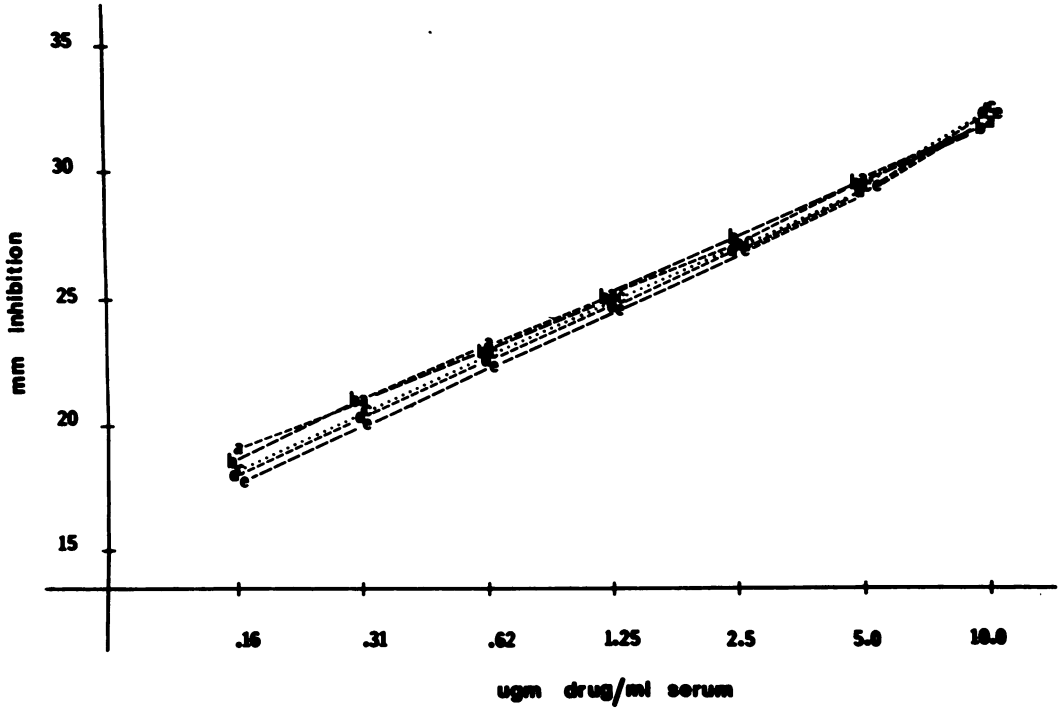

FIG. 2. *Effect of agar depth (milliliters of agar per 150-mm plate) on inhibition zones using Sab-dex agar, C. albicans 37, and miconazole. Symbols: (a) 25 ml; (b) 30 ml; (c) 35 ml; (d) 40 ml; (e) 45 ml.*

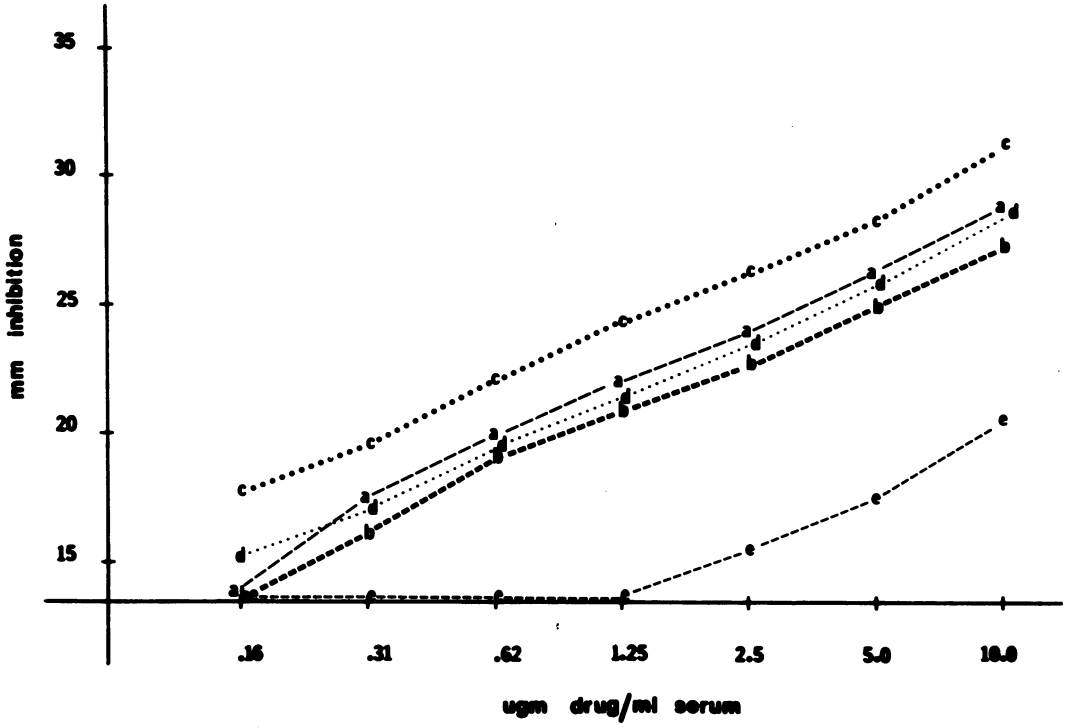

FIG. 3. *Effect of inoculum type on inhibition zones using Sab-dex agar and miconazole. Symbols: C. albicans strains (a) 35; (b) 36; (c) 37; (d) 38; (e) C. neoformans 40.*

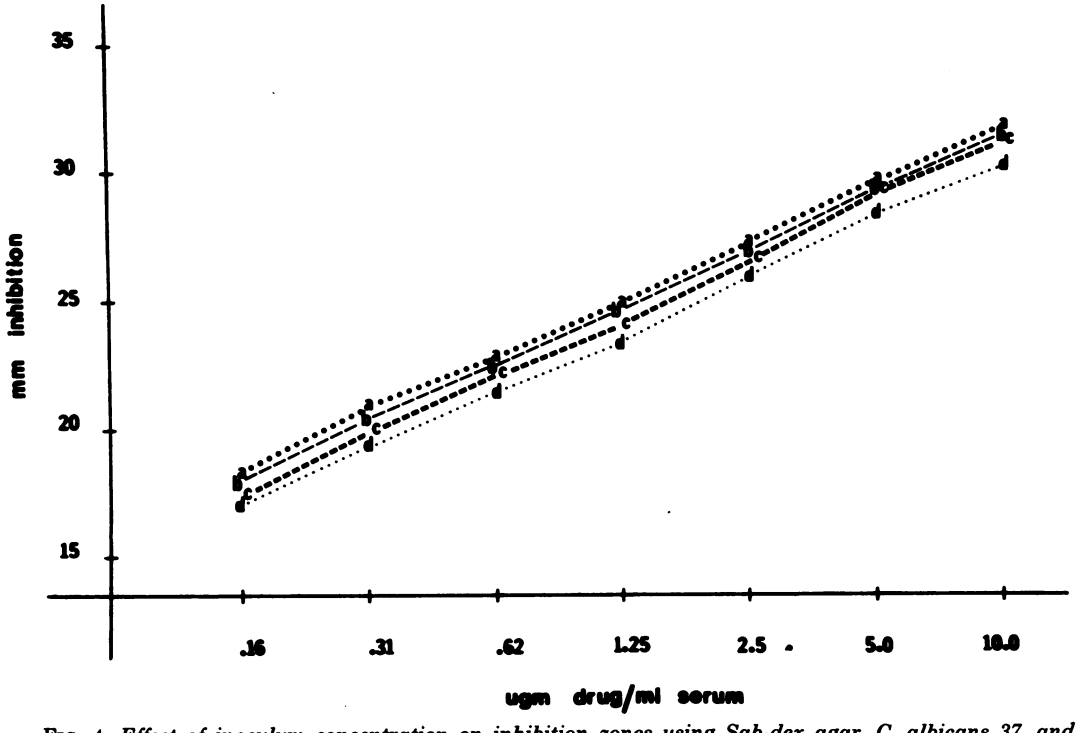

FIG. 4. *Effect of inoculum concentration on inhibition zones using Sab-dex agar, C. albicans 37, and miconazole. Symbols: (a) 2×10^6, (b) 4×10^6, (c) 8×10^6, (d) 1.6×10^7 cells per ml.*

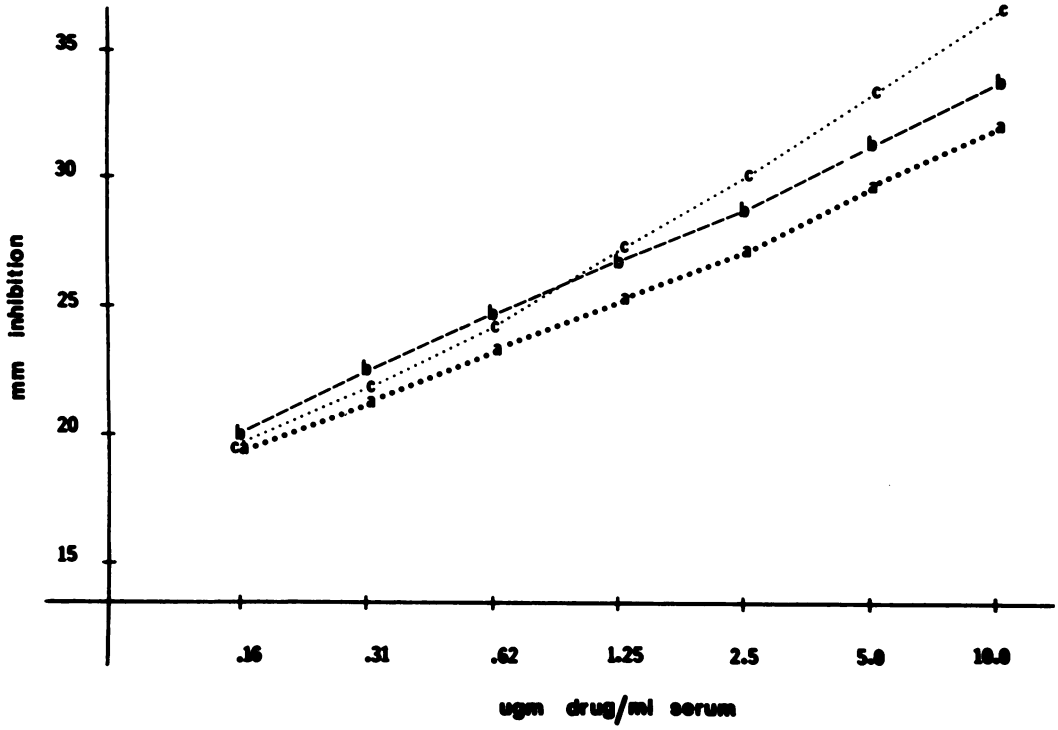

FIG. 5. *Effect of imidazole type on inhibition zones using Sab-dex agar (30 ml) and C. albicans 37. Symbols: (a) miconazole; (b) RV 40,500; (c) RV 41,400.*

effect of agar depth was the shape of the petri dish bottom.

C. albicans 37 (ATCC 28516) was the most susceptible of the organisms tested and gave the best linear response (Fig. 3). When concentrations from 2×10^6 to 16×10^6 cells per ml were tested, the response to standards was not significantly altered (Fig. 4).

All three imidazoles gave linear responses from 0.16 to 10.0 μg/ml. The most water-soluble imidazole, RV 41,400, had the steepest slope (Fig. 5). No significant difference was observed using standards in pooled human serum versus pooled horse serum.

The procedure described is a simple, reproducible, and inexpensive test to monitor serum levels of imidazole drugs. Optimal results were obtained by using 30-ml Sab-dex plates with *C. albicans* 37 inoculum adjusted visually to 4×10^6 to 8×10^6 cells per ml. RV 40,500 was included in this study to test the potential effect of altering drug structure on the test results. This compound was chemically modified to produce RV 41,400 and is no longer in production.

Initially it was hoped that large batches of standard disks could be prepared in advance and stored frozen. After 1 week of storage at −15°C, however, sensitivity at low standard levels was lost. Because of this, standards must be prepared fresh, and patients' sera should be handled identically with the standards.

C. albicans ATCC 28516 can easily be obtained by any clinical laboratory, as well as any other susceptible *Candida* isolate. Suspensions can be adjusted visually with little loss in reproducibility. Standard Sab-dex plates are available for the assay. Approximately 20% of the petri dish bottoms used in this project were concave in shape. This could have been responsible for the apparent increase in zone size for the highest standard (Fig. 2 and 3), as the 10-μg/ml standard was always positioned in the plate center. Elimination of these plates might improve linearity. Drug standards can be prepared in pooled horse sera. With the exception of the 18-h incubation at 4°C, the assay procedure is similar to a Kirby-Bauer test for antibiotic susceptibility testing.

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LITERATURE CITED

1. **Bauer, A. W., W. M. Kirby, J. C. Sherns, and M. Turck.** 1966. Antibiotic susceptibility testing by a stan-

dardized single disc method. Am. J. Clin. Pathol. **45**:493–496.
2. **Bennett, J. E.** 1974. Chemotherapy of systemic mycoses. N. Engl. J. Med. **290**:30–32.
3. **Edwards, A. L.** 1967. Linear regression, p. 91. *In* A. L. Edwards (ed.), Statistical methods, 2nd ed. Holt, Rinehart and Winston, Inc., N.Y.
4. **Levine, H. B.** 1976. R34000, a dioxolane imidazole in the therapy for experimental coccidioidomycosis. Comparison with miconazole and econazole. Chest **70**:755–759.
5. **Levine, H. B., and D. A. Stevens.** 1975. Miconazole in coccidioidomycosis. I. Assays of activity in mice and *in vitro*. J. Infect. Dis. **132**:407–414.
6. **Miller, R. P., and J. H. Bates.** 1969. Amphotericin-B toxicity; a follow-up report of 53 patients. Ann. Intern. Med. **71**:1089–1095.
7. **Sung, J. P., and J. G. Grendahl.** 1977. Clinical experimental therapy with miconazole for human disseminated coccidioidomycosis, p. 293–309. *In* L. Ajello (ed.), Coccidioidomycosis. Current Clinical and Diagnostic Studies. Proceedings 3rd International Coccidioidomycosis Symposium, Tucson, Arizona. Symposia Specialists, Miami, Florida.
8. **Sung, J. P., J. G. Grendahl, and H. B. Levine.** 1977. Intravenous and intrathecal miconazole therapy for systemic mycoses. West. J. Med. **126**:5–13.