Sensitive Bioassay for Ketoconazole in Serum and Cerebrospinal Fluid

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Ketoconazole is a broad-spectrum antifungal agent which appears promising for treatment of a variety of systemic mycoses. Pharmacokinetic studies are limited due to a lack of readily available methods for quantitation of ketoconazole in serum or cerebrospinal fluid. We developed a rapid, simple bioassay for measurement of ketoconazole alone or in the presence of therapeutic levels of amphotericin B, using an agar diffusion assay incorporating Candida pseudotropicalis. Pairs of 8-mm wells cut in the seeded assay medium were filled with four duplicate ketoconazole standards and duplicate patient specimens. Zones of inhibition were visible after 7 to 8 h of incubation, but were more easily measured after overnight growth. The assay allowed determinations of serum ketoconazole levels as low as 0.3 μg/ml with a 4.4% coefficient of variation. Thirty-five serum samples from patients receiving the drug were assayed by this method, and the results were compared with the Coccidioides immitis endospore assay. The correlation coefficient between the assays was 0.90. This assay allows any microbiology laboratory to easily and safely determine ketoconazole levels in serum or cerebrospinal fluid.

Materials and Methods

Antibiotic standards. Ketoconazole diagnostic powder was kindly supplied by Janssen Pharmaceutica, Inc., New Brunswick, N.J. To facilitate the solubility of ketoconazole for preparation of antibiotic standard solutions, 0.2 N HCl was used as the diluent (20 ml to dissolve 1 g of ketoconazole). Succeeding dilutions were prepared in sterile distilled water; final working standards were prepared with either pooled normal human serum or pooled normal human cerebrospinal fluid (CSF). Working antibiotic standards were either used immediately after preparation or stored at 2 to 8°C until needed.

Test strain. Several candidate microorganisms were initially screened for use in a bioassay system. A strain of Candida pseudotropicalis (kindly supplied by Roy Hopfer, The University of Texas, M. D. Anderson Hospital, Houston, Tex.) was chosen due to its relatively rapid growth characteristics, susceptibility to ketoconazole, and production of sharply defined zones of inhibition in a bioassay system.

Test medium. Preliminary studies indicated that a growth medium composed of (per liter) 6.7 g of yeast nitrogen base (Difco Laboratories, Detroit, Mich.), 10 g of Trypticase peptone (BBL Microbiology Systems, Cockeysville, Md.), 5 g of dextrose (BBL), and 15 g of agar (BBL) was optimal for bioassay of ketoconazole. The medium was prepared by heating all ingredients in distilled water, dispensing the dissolved medium into 30-ml deeps in individual tubes, followed by sterilization at 121°C, 15 to 17 lb/in², for 15 min. After sterilization, the agar deeps were allowed to solidify and were stored at 2 to 8°C until used.

Performance of the ketoconazole bioassay. Seeded agar test plates were prepared by inoculation of molten, cooled (50°C) agar deeps with 300 μl of an overnight growth of the test strain in yeast-nitrogen base broth (yeast-nitrogen base with 0.5% dextrose). The inoculum was mixed by inversion of the tube.
several times; then the seeded medium was dispensed into plastic petri dishes (150 by 15 mm) arranged on a level surface. This provided a seeded agar medium containing approximately $5 \times 10^5$ yeast cells per ml of agar. After the agar solidified and cooled, plates were either used for assay immediately or stored in sealed plastic bags until needed. To perform the assay, wells (8 mm in diameter) were cut in the agar with a number 4 cork borer, followed by vacuum aspiration. Sixty-five-microliter samples of patients' specimens and standards were dispensed into the wells, using two wells for each of four standards and two for each patient's specimen. In this manner, all determinations were performed in duplicate on a given plate, and two assay plates per sample were used to obtain all measurements in quadruplicate. Test plates were incubated at 37°C for 16 to 24 h. After incubation, zones of inhibition around each well were measured with vernier calipers (Analytab Products, Plainview, N.Y.) and recorded to the nearest 0.1 mm. Duplicate zone diameters were averaged, and standard curves relating zone diameter to concentration of ketoconazole were prepared on semilogarithmic paper for each assay plate. Values for samples containing unknown concentrations of antibiotic were then calculated by the standard curve. The mean of sample values obtained from each of the two plates was then derived.

**Standard curve.** The range of linearity in response to ketoconazole was determined by the assay of serum standards containing from 0.10 to 50 µg of ketoconazole per ml.

**Determinations of accuracy and precision.** The ability of the assay to measure ketoconazole accurately and reproducibly was determined by the assay of four replicate serum samples containing a known amount of ketoconazole. These assays were performed on five consecutive days with the same serum samples.

**Measurement of ketoconazole in the presence of amphotericin B.** The ability of the assay to measure accurately the concentration of ketoconazole in the presence of amphotericin B was determined by the assay of sera containing 0.3 µg of ketoconazole per ml plus 0.5 to 5 µg of amphotericin B per ml and by the assay of sera containing only amphotericin B in the same concentrations.

**Patient specimens.** Serum and CSF specimens were obtained after receiving written informed consent from patients enrolled in a clinical trial of therapy for a variety of fungal diseases. Patient specimens were either assayed immediately or frozen at −20°C until assayed. Results of ketoconazole determinations by our assay were compared with those obtained by H. B. Levine of the Naval Biomedical Research Laboratory, School of Public Health, University of California, Berkeley, with a *C. immitis* endospore bioassay (3).

## RESULTS

Growth of the assay strain (and zones of inhibition) was evident after 7 to 8 h of incubation, although zones were more clearly demarcated, and therefore more easily measured, after 16 to 20 h of growth (Fig. 1). The ketoconazole standards which were found most useful for determinations involving serum were 15, 5, 1.25, and 0.3 µg/ml (Fig. 2) although standard curves remained essentially linear up to 50 µg/ml. Zones of inhibition with CSF standards were larger than with serum, providing greater sensitivity and allowing use of lower standards, e.g., 5, 1.25, 0.5, and 0.1 µg/ml. The reproducibility of the assay was found to be quite constant when assays of four serum samples were performed on separate days (Table 1). Serum samples containing from 0.6 to 8 µg of ketoconazole per ml yielded an overall coefficient of variability of 4.4% based upon five separate determinations.

Amphotericin B did not produce zones of inhibition when tested at a concentration of 0.5 µg/ml, although a modest zone (approximately 13 mm) was produced by 5 µg/ml. However, combinations of these same amounts of amphotericin B with ketoconazole did not interfere with the measurement of ketoconazole; i.e., the zones of inhibition produced by 0.3 µg of ketoconazole per ml alone or with either concentration of amphotericin B were equivalent.

Assay of 35 serum samples from four patients yielded values which corresponded relatively closely (correlation coefficient = 0.90) with those obtained on the same samples by Levine with the *C. immitis* assay (Fig. 3). However, values...
obtained with our assay were most often slightly higher than those of Levine, especially with sera containing ≥2 μg/ml by our method. After single morning doses of 200 to 400 mg of ketoconazole, serum levels by our method were 2 to 4 μg/ml at 1 to 2 h and at the lower limit of the assay at 24 h. CSF levels at 4 h after 400-mg doses were 0.21 and 0.14 μg/ml in two coccidioidomycosis meningitis patients and 0.56 μg/ml in a third patient at 4 h after receiving an 800-mg dose (concomitant serum level = 13.75 μg/ml).

**DISCUSSION**

The results of this study demonstrate that it is possible to measure rapidly and easily the concentration of ketoconazole in patients’ serum and CSF with a bioassay procedure that can be safely performed in any microbiology laboratory. The use of a ketoconazole-susceptible strain of *C. pseudotropicalis* allows overnight determinations and sharp, easy-to-measure zones of inhibition. The assay was found to be extremely reproducible from day to day with either freshly seeded plates prepared from deeps or previously seeded refrigerated plates 1 to 5 days old. In addition, ketoconazole serum or CSF standards were found to be stable during storage at refrigeration temperature for 1 to 5 days or frozen at −20°C for 1 month or more.

As noted previously (2), amphotericin B appears to diffuse poorly in an agar diffusion system, preventing significant interference with measurement of ketoconazole in the presence of amphotericin B. However, the same is not true for 5-fluorocytosine, which does significantly interfere with the measurement of ketoconazole in this assay system, due to its good diffusion and the susceptibility of *C. pseudotropicalis* to it.

It is noteworthy that ketoconazole was detected in measurable amounts in the CSF of three of our patients receiving the drug. The CSF penetration of ketoconazole has been an important question which has been difficult to assess with previous assay methods. Use of an assay with improved sensitivity should assist in defining the ability of ketoconazole to gain access to CSF and central nervous system tissues.

The rather consistently higher values obtained by our assay compared with those obtained by Levine is not readily explained. Clearly, there are significant methodological differences between the two procedures, including composition of the test medium and use of a different test strain. In addition, prolonged storage and the requirement of sample shipment to H. B. Levine’s laboratory may have resulted in some loss of antimicrobial activity, as measured by the coccidioides assay.

**TABLE 1. Individual measurements of ketoconazole in serum samples performed on five separate days**

<table>
<thead>
<tr>
<th>Actual ketoconazole conc (μg/ml)</th>
<th>Mean of five separate determinations</th>
<th>Standard deviation</th>
<th>Coefficient of variability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>7.58</td>
<td>0.33</td>
<td>4.3</td>
</tr>
<tr>
<td>4</td>
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<td>2</td>
<td>1.92</td>
<td>0.06</td>
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<tr>
<td>0.6</td>
<td>0.62</td>
<td>0.03</td>
<td>4.3</td>
</tr>
</tbody>
</table>

**FIG. 2. Ketoconazole serum bioassay standard curve.** This represents a composite curve with means and ranges indicated for 10 separate standard curves.

**FIG. 3. Regression analysis of concentrations of ketoconazole in serum determined by bioassay with *C. pseudotropicalis* and *C. immitis*.**

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**BIOASSAY FOR KETOCONAZOLE**

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