Rapid, Simple Bioassay for 5-Fluorocytosine in the Presence of Amphotericin B

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It is important that serum levels of 5-fluorocytosine (5FC) be measured to insure therapeutic levels while avoiding toxicity. This is particularly true in patients with renal insufficiency. The concurrent use of amphotericin B and 5FC complicates the measurement of 5FC in the serum, since fungi used in conventional bioassay systems are uniformly susceptible to amphotericin B. This paper describes the development of a simple, reliable, 6-h bioassay for 5FC in the presence of amphotericin B. The assay is based upon the fact that 5FC diffuses readily through yeast nitrogen base agar, whereas amphotericin B apparently does not. This assay allows rapid adjustments in therapy of patients receiving both 5FC and amphotericin B and has permitted us to maintain 5FC serum levels between limits of 25 and 120 µg/ml in patients with altered renal function.

Clinical and experimental trials have established the efficacy of 5-fluorocytosine (5FC) in fungal infections due to susceptible strains of Candida, Cryptococcus, and Torulopsis spp. (7, 12, 17, 19). The drug is usually well tolerated, producing few side effects. However, it has been suggested that the high serum levels which often accompany impaired renal function may be associated with bone marrow suppression and focal hepatic necrosis (5, 14). Some investigators find it desirable to maintain serum levels of 5FC below 120 µg/ml for this reason (12). Unfortunately, the patients who most often receive 5FC are also those most likely to have significant renal impairment (renal transplant recipients, immunosuppressed patients receiving long courses of aminoglycosides, etc.) (8, 15). Amphotericin B, itself nephrotoxic, may be administered concurrently with 5FC to provide potential synergism and to combat the emergence of 5FC resistant strains (11, 13).

The concurrent use of amphotericin B and 5FC complicates the measurement of 5FC in the serum. Since the fungi used in conventional bioassay systems are uniformly susceptible to amphotericin B, one cannot differentiate the effect of 5FC from that of amphotericin B when they are combined. Attempts to circumvent this problem have included the use of dialysis to separate the two drugs (2). At present, however, no rapid, simple, readily clinically applicable assay for 5FC in the presence of amphotericin B exists. This paper describes the development of a simple 6-h assay based upon the fact that 5FC diffuses readily through yeast nitrogen base (YNB) agar from filter paper disks, whereas amphotericin B apparently does not.

MATERIALS AND METHODS

Preparation of agar. YNB was prepared by adding 6.7 g of YNB (Difco Laboratories, Detroit, Mich.) and 5 g of dextrose to 100 ml of distilled water and filtering through a 0.2-µm Nalgene filter (Nalg Sybron Corp; Rochester, N.Y.). This YNB concentrate (YNB 10 x) was diluted 1:10 in distilled water (YNB 1 x) and used to prepare cultures of the assay organism Candida albicans (ATCC no. 24433).

Ten milliliters of 10 x YNB and 10 ml of 1 x YNB containing 5 x 10^4 microorganisms per ml were added to 80 ml of melted 1.5% agar (agar, granulated; BBL-11849) at 48°C and mixed thoroughly in a 250-ml Erlenmeyer flask. This resulted in a 1 x YNB agar seeded homogeneously with approximately 5 x 10^4 organisms per ml. The seeded agar was dispensed in 25-ml volumes into 150-mm petri dishes (Falcon Plastics no. 1058; Oxnard, Calif.) and allowed to harden.

Preparation of standards. Standard solutions of drugs were prepared in pooled (five donors) normal human AB serum. It was found that concentrations of 5FC of 20 µg/ml or less were most useful in preparing standards, since higher concentrations produced such large zones that they overlapped zones of other antibiotic concentrations on the plate. For the same reason, unknown sera were diluted 1:10 in sterile saline to insure readable zone sizes. This dilution did not interfere with the linearity of the curve. If relatively low (less than 30 µg/ml) concentrations were expected, the serum was not diluted. Samples were run in quadruplicate to insure accuracy.

Assay methods. Several methods were evaluated in an effort to develop the most reliable and useful assay.
(i) Disk diffusion. Filter paper disks (6.35 mm) (Schleicher and Schuell, no. 740-E; Kenne, N.H.) were inoculated with 20 µl of test serum using Eppendorf microliter pipettes (Brinkmann Instruments; Westbury, N.Y.). Disks were then placed on the seeded YNB agar with forceps, using a template such that there were nine evenly spaced disks per plate. Plates were incubated at 37 C and read at 6, 8, and 24 h.

(ii) Cut well. Using a 5-mm diameter cylinder punch, wells were cut into the seeded YNB agar such that there were nine evenly distributed wells per plate. Each well received 40 µl of test serum. Plates were incubated at 37 C and read at 6, 8, and 24 h.

(iii) Kirby-Bauer (1). YNB agar was prepared by adding 10 ml of 10x YNB to 90 ml of 1.5% melted agar at 48 C. Twenty-five milliliters of agar were dispensed into 150-mm petri dishes and allowed to harden. A 3-day growth of C. albicans in YNB broth was swabbed evenly over the plates. Filter paper disks were prepared and placed as described in the disk diffusion assay.

**Standard curved; determination of unknowns.** Standard curves were constructed by plotting zone sizes versus known concentration of 5FC on semilog paper. Five standard concentrations and two pairs of unknowns per plate were used. Unknown concentrations were calculated from the standard curve (Fig. 1).

**Effects of normal serum on assay.** Growth inhibitory factors for candida have been reported in serum (10). To insure that such factors would not interfere with this assay, 30 random samples of fresh sera were obtained from the Clinical Pathology Laboratory. Disks were prepared with serum alone, serum containing 10 µg of 5FC per ml, and serum containing a combination of 10 µg of 5FC per ml and 10 µg of amphotericin B per ml. In all cases serum alone produced no zone of inhibition. In all cases zone sizes of 5FC were identical in the presence or absence of amphotericin B.

**Amphotericin B assay.** Assay of amphotericin B was carried out with a tube dilution method utilizing a clinical isolate of Candida tropicalis resistant to 5FC, but susceptible to amphotericin B (minimal inhibitory concentration, 0.195 µg/ml; minimal fungicidal concentration, 0.39 µg/ml). The technique employed has been previously described (6). In a preliminary study serum was obtained from a patient immediately after an amphotericin B infusion (50 mg intravenously over 45 min). Although the serum contained 3.12 µg of amphotericin B per ml by the tube dilution bioassay, no inhibition zone was obtained when the serum was assayed by the disk diffusion technique described above.

**RESULTS**

Using pour plates of C. albicans and the cut well or disk diffusion methods, zones became interpretable at 6 h, were clearly readable at 8 h, and remained constant after 24 and 48 h. The Kirby-Bauer method, however, required at least 24 h and zones were less well defined.

As shown in Fig. 2, amphotericin B apparently diffuses poorly in YNB agar, and when amphotericin B and 5FC are both present the zone of inhibition reflects only the presence of the latter (Table 1: Fig 2). This is the case even when very high concentrations of amphotericin B are used with relatively low concentrations of 5FC. There is no difference between zones of inhibition for 5FC and 5FC plus amphotericin B (P > 0.10). This is true for all three methods.

In establishing the linearity of the standard curve, concentrations of 20, 15, 10, 5, and 2.5 µg of 5FC per ml were found to be optimal. Unknown sera were diluted 1:10. Results obtained in this manner were highly reproducible. Initially, least square fits were calculated for each line; but lines drawn visually were so similar this was found to be unnecessary. Values determined for unknowns were reasonable and comparable to those obtained in earlier
per ml. The Mg/ml and row) represents diffusion of periphery surrounding discs to left of 5FC.

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kASPAR studies for patients on similar doses of 5FC (2, 3, 7, 19).

In three patients receiving both 5FC and amphotericin B, peak 5FC levels were maintained below 120 µg/ml and valley concentrations were kept above 25 µg/ml for periods of 14 to 29 days. Amphotericin B serum levels were in the usual range of 0.39 to 1.56 µg/ml (6).

**DISCUSSION**

Recent studies suggest that amphotericin B may be synergistic with 5FC (11, 13). Further, susceptible fungi may develop rapid resistance to 5FC when the drug is used alone (4, 7, 9, 12, 18). Therefore, it is likely that 5FC will often be used in combination with amphotericin B. Many patients who receive 5FC tend to have preexisting compromise of renal function on the basis of underlying disease (renal transplant recipients) or chemotherapy (aminoglycoside antibiotics). In addition, amphotericin B itself results in impairment of renal function with great regularity (6). 5FC is excreted almost exclusively by the renal route (2, 20); elevated serum levels occur with renal failure (2, 3, 16).

Although bone marrow suppression occurs infrequently with 5FC therapy, its occurrence may be related to the presence of inordinately high serum levels of 5FC (2, 5, 14). Thus, it has been suggested that peak serum levels above 120 µg/ml be avoided (12). On the other hand, it is suggested that minimum serum levels be kept above 25 µg/ml to reduce the emergence of resistant microorganisms (12). Measurement of the serum levels of 5FC becomes critical if therapeutic levels are to be achieved while avoiding toxicity.

**TABLE 1. Reproducibility of zone size data for 5FC, amphotericin B (AMB), and 5FC plus AMB (disk diffusion method)**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Conc (µg/ml)</th>
<th>Zone diameters (mm)</th>
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<th>2nd</th>
<th>3rd</th>
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<td>27</td>
<td>26</td>
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</tr>
<tr>
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<td>23</td>
<td>22</td>
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<tr>
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<td>5</td>
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<td>19</td>
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<tr>
<td>5FC plus AMB</td>
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<tr>
<td></td>
<td>10 + 25</td>
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<td></td>
<td>5 + 12.5</td>
<td>17</td>
<td>18</td>
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*a Each represents an individual plate.*

**FIG. 2.** Paper disks containing 20 µl of test serum on YNB agar seeded with 5 × 10⁶ µg of Candida albicans per ml. The disk at the very top of the plate contains pooled serum alone and serves as a control. Reading from left to right the other disks contain serum with the following drugs: (top row) 5FC, 20, 10, and 5 µg/ml; (middle row) amphotericin B (AMB), 10, 2.5, and 1.25 µg/ml; (bottom row) 5FC, 20 µg/ml and AMB, 10 µg/ml; 5FC, 10 µg/ml and AMB, 2.5 µg/ml; 5FC, 5 µg/ml and AMB, 1.25 µg/ml. The bright white band surrounding each disk represents diffusion of serum into the adjacent agar. An area of enhanced fungal growth is apparent at the periphery of each diffusion zone, lending great clarity to the zone edge. There is no zone of inhibition surrounding discs containing AMB alone (middle row). The presence of AMB does not alter the zone of inhibition of 5FC.

**TABLE 1. Reproducibility of zone size data for 5FC, amphotericin B (AMB), and 5FC plus AMB (disk diffusion method)**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Conc (µg/ml)</th>
<th>Zone diameters (mm)</th>
<th>1st</th>
<th>2nd</th>
<th>3rd</th>
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*a Each represents an individual plate.*
The techniques which we have described for measuring 5FC in the presence of amphotericin B rely on the fact that 5FC readily diffuses from paper disks through YNB agar whereas amphotericin B apparently does not. Thus, even though a serum specimen contains both drugs, the zone of inhibition in the agar reflects only the presence of 5FC. The Kirby-Bauer method is attractive from the standpoint of simplicity and familiarity (1). It has disadvantages in that it requires 24 to 48 h and zone sizes are not absolutely distinct. The cut well and disk diffusion methods are comparable in rapidity and reliability. We prefer the disk diffusion method for its greater simplicity. Since results are available in 6 to 8 h, adjustments in therapy can be made on a day to day basis in the face of rapidly changing renal function. Using this technique, we have maintained serum levels of 5FC between 25 and 120 µg/ml in three patients with candidiasis who were also receiving amphotericin B.

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LITERATURE CITED