Simple Assay for 5-Fluorocytosine in the Presence of Amphotericin B

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A simple method for the measurement of 5-fluorocytosine in the presence of amphotericin B is described. The antifungal activity of amphotericin B is abolished by heating serum at 100 C for 45 min. 5-Fluorocytosine is unaffected by this treatment, and serum levels can be subsequently assayed by either tube dilution or disk diffusion methods.

Combined drug therapy with amphotericin B (AmB) and 5-fluorocytosine (5-FC) has potential usefulness in the treatment of severe fungal infections caused by Cryptococcus neoformans, Torulopsis glabrata, and Candida species (1, 11). Because of its nephrotoxic effects, administration of AmB frequently leads to a diminished creatinine clearance (10). 5-FC is excreted almost entirely by the kidneys (12). In the face of decreasing renal function, excessively high levels of 5-FC may accumulate. To avoid the potentially toxic effects of 5-FC on the bone marrow and other organs (3, 4, 6, 7, 11), it is imperative that 5-FC levels be monitored closely in patients on combination therapy. Block and Bennett have described a method involving separation of 5-FC and AmB by ultrafiltration, allowing quantitation of serum 5-FC levels (3). Kaspar and Drutz have used differential diffusion rates of AmB and 5-FC as a means of assaying for 5-FC in the presence of AmB (5). Reported herein is a simple method of heat inactivation of AmB so that levels of 5-FC can be accurately assayed during combined therapy with both drugs.

MATERIALS AND METHODS

Preparation of standards. A stock solution of 1,000 M of AmB (E. R. Squibb & Sons, Princeton, N.J.) per ml was prepared in sterile distilled water. The 5-FC (Hoffmann-La Roche, Inc., Nutley, N.J.) stock solution, 1,000 µg/ml, was prepared in 0.85% saline. Further dilutions of these standards were prepared with pooled human sera in saline so that a final concentration of 10% serum was achieved.

Effect of heat on antifungal activity of AmB and 5-FC. Standard solutions, prepared as described above in 10% human serum, were heated at different temperatures for varying lengths of time. The temperatures employed were 37, 56, 100 (achieved by using flowing steam at 0 lb/in² in a standard autoclave), and 121 C (21 lb/in²). The antifungal activity of each drug was then tested by the tube dilution method outlined below.

Tube dilution assay for 5-FC and AmB. Saccharomyces cerevisiae (ATCC 9763) was used as the test organism for the assay of both drugs. The inoculum size was 6 x 10³ yeast cells/ml. 5-FC was measured by the method of Shadomy, using yeast nitrogen base broth (Difco Laboratories, Detroit, Mich.) in a standard twofold serial dilution tube assay (9). The S. cerevisiae was inhibited by 0.02 µg of 5-FC per ml. Results were read at 24 h, reading the level as the last tube with no visible growth of the organism. AmB was assayed by a similar tube dilution assay using the same inoculum of S. cerevisiae but employing antibiotic medium no. 20 (Difco Laboratories). S. cerevisiae was inhibited by 0.1 µg of AmB per ml.

Disk diffusion assay for 5-FC. The method of Kaspar and Drutz was employed (5) with the exception that the standards were prepared in 10% rather than 100% serum, the standards and unknown sera were heated as described above, and the results were not read until 24 h. The test organism used was a C. albicans isolated from a patient with disseminated candidiasis. Using this organism, a small zone of inhibition was observed around the amphotericin standards, contrary to the results obtained by Kaspar and Drutz (5).

Preparations of unknowns. Sera to be tested were diluted 1:10 with 0.85% saline and heated at 100 C for 45 min. The 5-FC level was determined by using either the tube dilution assay or the disk diffusion method described above.

RESULTS

AmB in several different concentrations was partially destroyed by heating at 100 C for 15 min, and all antifungal activity was abolished after 15 min at 121 C (Fig. 1). The antifungal activity of several concentrations of 5-FC was unaffected by these temperatures. However, denaturation of proteins was so great at 121 C that the solution was too thick for accurate measurement by using the tube dilution assay.
Therefore, the solutions were heated at 100 C for longer intervals (Fig. 2). After 30 min some residual AmB antifungal activity was present, but after 45 min all antifungal activity was lost. The antifungal activity of 5-FC was not changed by heating at 100 C for 60 min. Although turbid, the solution was pipetted easily.

Varying concentrations of serum were used to determine the effect of serum on the heat inactivation of AmB. When no serum was present, heating to 100 C did not inactivate AmB. When the serum concentration was 20% or greater, heating caused too much protein denaturation, and the solution was very hard to assay. Ten percent serum was the optimum concentration for the inactivation of AmB.

Serum containing both antibiotics (100 μg of 5-FC and 2 μg of AmB) was heated at 100 C for 45 min and assayed for 5-FC and AmB activity by using the tube dilution assay (Table 1). AmB, in the presence of 5-FC, was destroyed by heating to the same extent as AmB alone. 5-FC, whether alone or in the presence of AmB, was not affected by heating.

Sera from patients with disseminated candidiasis and cryptococcal meningitis and treated with both AmB and 5-FC were heated as described above to destroy AmB activity. 5-FC levels were monitored by using either the tube dilution assay or the disk diffusion method. Data from one patient, illustrating the usefulness of monitoring the serum levels of 5-FC and the effect of renal function on 5-FC levels, are shown in Fig. 3.

**DISCUSSION**

A simple accurate assay for 5-FC in the presence of AmB is imperative if one plans to treat patients with serious fungal infections with both drugs. Decreasing renal function, which occurs in almost all patients treated with AmB (10), can cause a rise in the serum levels of 5-FC, a drug almost wholly excreted by the kidneys (12). The suggestion has been made that the toxic effects of 5-FC on the bone marrow are related to high serum levels of drug (3, 4, 7). We also have observed bone marrow depression associated with high serum levels of 5-FC (6; Kauffman et al., manuscript in preparation).

Schiavone et al. first noted that 5-FC antifungal activity was unchanged after heating at 100 C for 15 min (8). We have confirmed that 5-FC is resistant to heating for as long as 60 min and is unaffected by temperatures as high as 121 C. AmB is inactivated by heating for 45 min at 100 C, and thus its antifungal activity can be easily abolished in serum containing both drugs. Since serum is necessary for this heat inactivation to occur, the mechanism of inactivation is probably related to the denaturation of proteins to which the drug is strongly bound (2). Although protein denaturation results in turbidity, this cloudiness does not interfere with the performance of the test or the interpretation of the results.

After inactivation of AmB, 5-FC levels can be assayed easily with either the tube dilution or the disk diffusion methods. The tube dilution method allows accurate measurement of levels

![Graph showing effect of heating on antifungal activity of 5-FC and AmB](http://aac.asm.org/)

### Table 1. Effect of heating at 100 C for 45 min on the antifungal activity of 5-FC alone, AmB alone, and the combination of both drugs

<table>
<thead>
<tr>
<th>Drug</th>
<th>Initial concentration (μg/ml)</th>
<th>Concentration after heating (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-FC</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>AmB</td>
<td>2</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>5-FC + AmB</td>
<td>100 + 2</td>
<td>100 + &lt;0.1</td>
</tr>
</tbody>
</table>
of 5-FC from 1 to 40 μg/ml; however, the disk diffusion method appears to be more accurate in the range from 50 to 150 μg of 5-FC per ml. Since leukopenia has been associated with serum 5-FC levels of 125 μg/ml (Kauffman et al., manuscript in preparation), potentially toxic levels can be monitored best with the use of the disk diffusion assay.

LITERATURE CITED