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Analysis of pH and Buffer Effects on Flucytosine Activity in Broth Dilution Susceptibility Testing of *Candida albicans* in Two Synthetic Media

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We examined the influences of different pH levels and three different buffers on flucytosine activity against 12 isolates of *Candida albicans* in two synthetic media, yeast nitrogen base (YNB) and synthetic amino acid medium-fungal (SAAMF), using broth dilution techniques and measuring the endpoints of visual MICs and turbidimetric 50% inhibitory concentrations. The two media were originally prepared as follows: YNB, unbuffered, pH 5.6; SAAMF, buffered with morpholinepropanesulfonic acid-Tris, pH 7.4; the resultant geometric mean MIC and 50% inhibitory concentration of 5-FC were 78- and 32-fold higher, respectively, in SAAMF. Raising the pH of YNB or lowering the pH of SAAMF had virtually no effect on these differences in MIC and 50% inhibitory concentration in the two media. In contrast, virtually all of the discrepancy appeared to be due to morpholinepropanesulfonic acid-Tris, which exerted concentration-dependent inhibition of flucytosine activity not evident when *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid or phosphate buffer systems were substituted. In other turbidimetric studies, growth was slowed more than 50% in YNB as the pH was raised to 7.4, regardless of which buffer was used. Based on our studies, we recommend modifying the composition of SAAMF by substituting a nonantagonistic buffer if any buffer is to be used with SAAMF in the testing of flucytosine. With this modification, SAAMF warrants further study as a generally applicable medium for fungal-susceptibility testing.

Broth dilution susceptibility testing of flucytosine (5-FC) against yeasts is influenced by several factors including inoculum size, incubation temperature, and time that endpoints are read (2, 3). Also, the antifungal activity of flucytosine may be competitively antagonized by the action of pyrimidines in various media (11). For this reason, yeast nitrogen base (YNB) (unbuffered, pH usually near 5.6), a synthetic medium low in pyrimidines, has been recommended for use in studies of fungal susceptibility to 5-FC (7, 13, 14).

Recent information suggests that media may have other influences. For example, MICs reported by Galgiani and Stevens with another synthetic medium (synthetic amino acid medium-fungal [SAAMF]) (3) appeared to be considerably higher than those found in studies from the same laboratory in which YNB was substituted in the same method (15). Although this difference was recognized at the time, these separate studies examined different panels of isolates, which precluded direct media comparisons. This apparent difference in results due to media is in agreement with the difference in susceptibility of another isolate of *Candida albicans* tested concurrently in both media (5). That use of these two media might result in different susceptibility test results is plausible since the media differ in composition. Furthermore, SAAMF is compounded with a buffer to pH 7.4.

For these reasons, we believed that comparative studies of 5-FC antifungal activity in these two synthetic media would be of value in achieving the ultimate goal of standardized fungal-susceptibility testing. Various modifications were used to assess the specific effects of the chemical composition, osmolality, pH, and buffering capacity of media on fungal growth and susceptibility tests. In this way, we defined some specific media differences which influence 5-FC activity. The results of these studies are the basis of this report.

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MATERIALS AND METHODS

Media, buffers, and pH adjustment. YNB (BBL Microbiology Systems, Cockeysville, Md.) was prepared by the directions of the manufacturer in a 10× concentration and was refrigerated until use. It contained no buffer and had a pH of 5.6. SAAMF was prepared as previously described (5). Since the amino acid preparation previously used in SAAMF is no longer available, we substituted an aqueous amino acid solution of identical composition to the original (catalogue no. 5831, Difco Laboratories, Detroit, Mich.). SAAMF contained morpholinepropanesulfonic acid (MOPS) at a 0.079 M concentration and Tris at a 0.086 M concentration (total buffer concentration, 0.165 M), and it was adjusted to pH 7.4 before use. Unless otherwise specified below, the media were used in these forms.

Tris, MOPS, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid (HEPES), and sodium phosphate buffers were obtained from Sigma Chemical Co., St. Louis, Mo. Their initial pH in media solution was adjusted to 7.4 with 10 N sodium hydroxide or 12 N hydrochloric acid unless otherwise stated below. All pH measurements were made with an Accumet model 630 pH meter (Fisher Scientific Co., Pittsburg, Pa.).

Organisms. Twelve strains of *C. albicans* were obtained from three sources: five were clinical isolates from the Veterans Administration Medical Center, Tucson, Ariz.;

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TABLE 1. MICs and $IC_{1/2}$ of 5-FC against *C. albicans* in conventional and modified YNB and SAAMF[a]

Strain	Original medium				Modified medium[b]					
	YNB		SAAMF		YNB with MOPS-Tris		Unbuffered SAAMF		SAAMF with HEPES or phosphate[c]	
	MIC	$IC_{1/2}$	MIC	$IC_{1/2}$	MIC	$IC_{1/2}$	MIC	$IC_{1/2}$	MIC	$IC_{1/2}$
1	0.0078	0.0078	1.0	0.25	0.0313	0.0313	0.125	0.0156	0.25	0.0313
2	0.0156	0.0078	2.0	0.125	0.25	0.125	0.125	0.0156	—[d]	—
3	0.0156	0.0156	2.0	0.25	0.5	0.125	0.125	0.0156	—	—
4	0.0156	0.0078	2.0	0.25	0.5	0.25	0.125	0.0156	—	—
5	0.0156	0.0078	2.0	0.25	0.25	0.125	0.25	0.0156	—	—
6	0.0313	0.0078	2.0	0.25	0.5	0.25	0.125	0.0156	—	—
7	0.0313	0.0078	2.0	0.25	1.0	0.25	0.25	0.0156	0.25	0.0313
8	0.0313	0.0313	2.0	1.0	0.5	0.125	1.0	0.0625	1.0	0.0625
9	0.0625	0.0156	8.0	1.0	0.5	0.25	2.0	0.0625	—	—
10	0.25	0.0313	2.0	1.0	32	0.25	4.0	0.25	—	—
11	0.25	0.0313	16	1.0	8.0	0.25	>128	0.25	—	—
12	>32	0.0313	>16	4.0	64	0.25	>128	0.25	>64[e]	0.25
Geometric mean	0.033	0.014	2.6	0.47	1.0	0.17	0.33	0.039		

[a] Concentrations are in micrograms per milliliter.
[b] pH 7.4.
[c] Results were within one tube dilution with either buffer.
[d] —, Study not performed.
[e] Value for HEPES only.

five were clinical isolates from Good Samaritan Hospital, Phoenix, Ariz.; and two were obtained from research laboratories in other states. Each strain was stored at −70°C in YNB containing 10% glycerol. Before studies, thawed cultures were kept refrigerated on Sabouraud-dextrose agar or brain heart infusion agar.

Susceptibility testing. MICs and 50% inhibitory concentrations ($IC_{1/2}$) were determined as previously described (3). Inocula, adjusted to 2×10^3 yeast cells per ml by hemacytometer counting, were mixed with equal volumes of twofold dilutions of 5-FC (Roche Diagnostics, Div. Hoffman-La Roche, Inc., Nutley, N.J.) or drug-free diluent in plastic tubes (final volume, 2.0 ml). Tubes were incubated without agitation at 37°C. All MICs were read by a single observer on the first day that the drug-free control tubes became turbid. The endpoints of the $IC_{1/2}$ were determined by measuring the turbidity of all tubes prepared for MIC studies. Measurements were obtained during log-phase growth of the control by using a Coleman Junior spectrophotometer (Coleman Instruments Corp., Maywood, Ill.). $IC_{1/2}$ were calculated from the turbidimetric data as the lowest drug concentrations that met the following criterion: $\%T \geq (\%T_{control} + 0.5\ [100 - \%T_{control}])$, in which %T equals percent transmission and control equals the turbidity of the drug-free tube. In selected experiments, parallel measurements at 540 and 650 nm resulted in identical $IC_{1/2}$ in both SAAMF and YNB. Replicate studies were performed at 650 nm with selected isolates to confirm reproducibility of the endpoints of the MICs and $IC_{1/2}$. These duplicate values always agreed within one drug dilution and in most instances were identical for all studies with SAAMF and with YNB in its standard formulation. Greater variability occurred in buffered YNB, as described below.

RESULTS

Comparison of MICs and $IC_{1/2}$ in YNB and SAAMF. 5-FC in YNB and in SAAMF was tested against 12 strains of *C. albicans*. In YNB, MICs ranged from 0.0078 to >32 μg/ml (Table 1). Against all strains, MICs and $IC_{1/2}$ were higher in SAAMF than in YNB. The geometric mean differences for MICs and $IC_{1/2}$ were 78- and 32-fold, respectively.

A large portion of the difference between susceptibility to 5-FC in YNB and in SAAMF appeared to be related to the MOPS-Tris buffer in SAAMF. When our panel of isolates was tested in YNB buffered with 0.165 M MOPS-Tris (pH adjusted to 7.4), the MICs and $IC_{1/2}$ rose towards those seen previously with SAAMF. The same strains tested in SAAMF prepared without buffer had $IC_{1/2}$ and MICs lower than those found in buffered SAAMF, and $IC_{1/2}$ were frequently within one tube dilution of those obtained in YNB (Table 1).

Although the MOPS-Tris buffer appeared to be an important variable, we could not attribute changes in either MICs or $IC_{1/2}$ to a pH effect. Two strains (1 and 7) with the largest differences in test results in the original two media were tested in YNB and SAAMF, each buffered with MOPS-Tris at a starting pH adjusted to 7.4, 7.2, 7.0, or 5.6. For this buffered SAAMF, the resultant $IC_{1/2}$ varied no more than one drug dilution from those obtained for these same strains in the original SAAMF (Table 2). MICs showed the same trend as $IC_{1/2}$ but varied as much as two drug dilutions in SAAMF. For YNB, the variation of $IC_{1/2}$ or MICs was as much as two drug dilutions; we attribute this variation to difficulties in determining endpoints in buffered YNB, as will be discussed.

Since the alteration of susceptibility to 5-FC appeared to be dependent on the presence of MOPS-Tris buffer rather than on its buffering effect, we examined the effect of varying the concentration of MOPS-Tris buffer on 5-FC activity against a representative isolate (strain 7). Inhibition of 5-FC activity was less at 0.0825 M MOPS-Tris than at the usual 0.165 M. At 1/10 of the usual MOPS-Tris concentration, the $IC_{1/2}$ was 0.0313 μg/ml, which is within one drug dilution of the 0.0156 μg/ml found in unbuffered SAAMF (Fig. 1).

To investigate whether this effect was specific for MOPS-Tris, we selected four strains for susceptibility testing with 0.165 M HEPES or phosphate buffers instead of MOPS-Tris in SAAMF. For all strains, MICs and $IC_{1/2}$ in SAAMF with either of these two buffers were the same as in unbuffered

TABLE 2. $IC_{1/2}$ and MICs in buffered (MOPS-Tris) media at various pH levels

Medium and strain	Results (μg/ml) at pH:							
	5.6		7.0		7.2		7.4	
	$IC_{1/2}$	MIC	$IC_{1/2}$	MIC	$IC_{1/2}$	MIC	$IC_{1/2}$	MIC
YNB								
Strain 1	0.0313	0.0625	—[a]	—	0.0625	0.125	0.0313	0.0313
Strain 7	0.0625	0.25	0.0625	0.25	0.0625	0.5	0.25	1.0
SAAMF								
Strain 1	0.125	0.5	0.25	2.0	0.25	4.0	0.25	1.0
Strain 7	0.125	1.0	0.25	2.0	0.125	2.0	0.25	2.0

[a] —, Study not performed.

SAAMF (Table 1). These results eliminated osmolality as a significant variable since it was equal in these media. Together, these observations indicate a specific concentration-dependent inhibition of 5-FC activity by MOPS-Tris buffer.

Rank order of susceptibility was generally unaltered by media variations. Comparing either the MICs or $IC_{1/2}$ in SAAMF with the MICs in YNB, we found only one isolate that appeared in different rank order. Unbuffered SAAMF showed similar consistency. In contrast, the rank order in buffered YNB was considerably different than that determined in conventional YNB. Possible reasons for this discordance are discussed below.

Comparison of growth characteristics in YNB and SAAMF. During our susceptibility studies, we noticed marked differences in growth rates associated with media alterations, particularly with YNB. Doubling times (estimated from turbidimetric measurements) for our 12 strains averaged 2.5 h in unbuffered YNB as compared with 6 h in buffered YNB. For more detailed studies, strain 1 was selected because of its large differences in growth rate under these different conditions. When this strain was grown in MOPS-Tris-buffered YNB with the starting pH adjusted to 7.34, 7.11, or 5.6, growth was fastest at the lowest pH (Fig. 2). In unbuffered YNB starting at pH 3.5, growth was only slightly faster than in the same medium or buffered medium starting at pH 5.6. Thus, growth occurred at a nearly maximal level in YNB prepared conventionally. After replacing MOPS-Tris with phosphate, Tris alone, or HEPES buffer (each at concentrations of 0.001, 0.01, and 0.05 M), and initially adjusting the pH to 7.2, we observed slowing of growth to the same degree with all buffers. Also, slowing was increased slightly with a higher buffer concentration. These results suggest that changes in growth rate are related to pH rather than to buffer toxicity.

SAAMF adjusted to a starting pH of 7.4 supported similar growth rates, whether or not any of the buffers were used. Estimated turbidimetric doubling times for six strains in SAAMF were 2.5 h with buffer and 3 h without buffer.

In addition to the above differences in growth rates, marked differences were found in the maximal amount of growth supported by SAAMF or YNB. Growth was maximal in YNB at a %T of 60 to 65 for all strains. Thus, the range of %T over which $IC_{1/2}$ could be measured was only 95 to 70. The narrowness of this range coupled with the lengthened doubling time in YNB at physiological pH interfered with accurate identification of log-phase growth. Consequently, the proper timing of $IC_{1/2}$ measurements was difficult. Furthermore, MICs could not be determined until day 2 of incubation. These problems may account for our discrepant $IC_{1/2}$ in this particular medium. In contrast, maximum growth in SAAMF was reached at a %T of 20 to 25 irrespective of buffer modifications, and $IC_{1/2}$ could be measured at a %T of 95 to 40. Because of these findings, SAAMF was used for the remainder of our studies.

pH control by buffers. The buffering capacities of the three buffers used with SAAMF were evaluated by measuring the pH of the media at maximal growth and by direct titration with 0.1 M HCl. The lowest pH levels attained at maximal growth were approximately the same (6.6 and 6.7) for two strains grown in SAAMF buffered with the same concentration (0.165 M) of HEPES, phosphate, or MOPS-Tris. Titration curves from pH 7.4 to 6.6 of SAAMF with each of the buffers also showed little difference in the amounts of HCl required to reach a specific pH. HEPES was a slightly

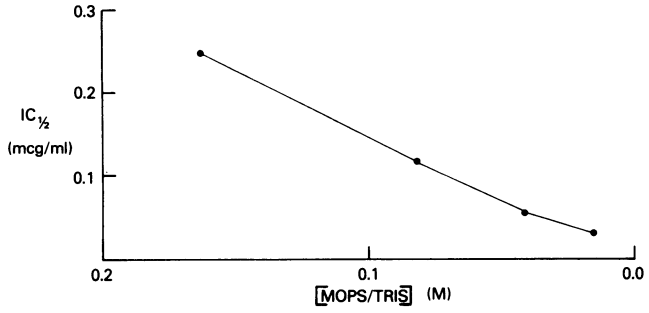

FIG. 1. Inhibition of 5-FC activity against *C. albicans* strain 7 in SAAMF modified by MOPS-Tris buffer at various concentrations.

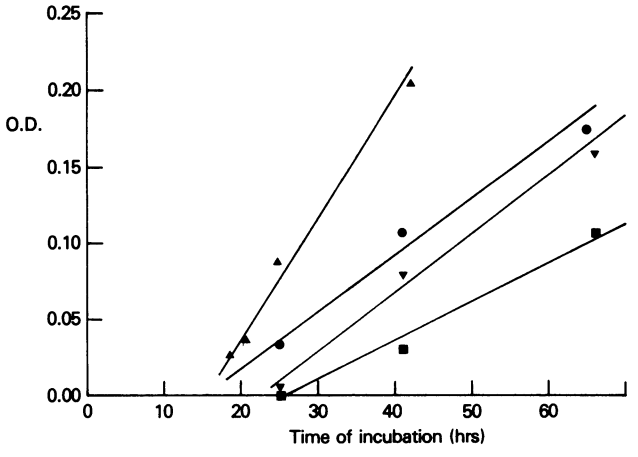

FIG. 2. Growth rates of *C. albicans* strain 1 in YNB modified with MOPS-Tris at pH 5.6 (▲), 7.11 (▼), and 7.34 (■) and with HEPES at pH 7.20 (●). O.D., Optical density.

better buffer than phosphate, which was slightly better than MOPS-Tris. We found unbuffered SAAMF to have essentially no buffering capacity, requiring ca. 0.09 mmol of HCl to lower its pH from 7.4 to 5.6.

DISCUSSION

YNB has been widely used for testing susceptibility to 5-FC (14). The principal advantage of YNB is its lack of pyrimidines, known inhibitors of 5-FC. SAAMF has been used for 5-FC testing for the same reason (12). The present study demonstrates that these two media yield different susceptiblity results and identifies the MOPS-Tris buffer system in SAAMF as the inhibitor responsible for this difference. When this buffer is removed or when other nontoxic buffers are substituted in SAAMF, both MICs and $IC_{1/2}$ are in close agreement with those found in YNB.

The mechanism by which MOPS-Tris buffer inhibits 5-FC activity is not clear. We are unaware of other published studies that compare buffers in microbiological susceptibility testing. In other cellular and acellular systems, however, Tris has been found to inhibit several types of biochemical reactions. Tris inhibited phosphorylation-coupled oxidation of succinate by bean mitochondria, whereas HEPES and phosphate did not (4). In studies of isolated rat vascular smooth muscle, Tris adversely affected contractility, regardless of whether the muscle was stimulated by prostaglandins, epinephrine, potassium chloride, or angiotensin (1, 17). These findings were consistent with Tris binding of calcium as the common requirement for contractility. Support for this possibility is found in an analysis of the stability of the magnesium-ATP complex (10). In this study, Tris buffering at pH 8.0 gave lower stability constants for the complex than did buffering with triethanolamine or *N*-ethylmorpholine. This difference was thought to be due to the binding of Tris to magnesium, although an interaction with the phosphate moiety was not excluded. At physiological pH, unionized Tris constitutes ca. 30% of its total concentration (8). Tris is able to pass through biological membranes, whereas zwitterionic buffers are not (4). Thus, many extracellular and intracellular events could be influenced by this buffer. The specific role of MOPS in the inhibition of 5-FC activity is unknown.

Our results may be useful in future efforts to standardize fungal-susceptibility testing. Since 5-FC activity against *C. albicans* in SAAMF without the MOPS-Tris buffer is comparable to those activities obtained in YNB, these media are, in this respect, equivalent. Further studies are needed to verify that this equivalence is true against other fungi as well. YNB is a traditional medium, commercially available at a cost comparable to other media commonly used for susceptibility testing of bacteria. However, doubling times of yeasts were more than twice as long in this medium when the pH was physiological. Although media pH has received little attention in fungal-susceptibility testing, acidity appears to abrogate the in vitro activity of ketoconazole (9). If physiological pH is deemed necessary, YNB will require longer incubation times to determine MIC endpoints and will be less satisfactory for determining turbidimetric endpoints. SAAMF, on the other hand, is not available from commercial sources, requires more time for preparation, and is more expensive than YNB. These logistical problems could be overcome if usage of SAAMF increased. More importantly, the growth characteristics of SAAMF are quite stable over a wide range of pH. Substituting HEPES or phosphate buffers for MOPS-Tris obviates the problem of buffer interference with 5-FC activity, although buffer effects as specific influences on testing should be assessed with other drugs. Developing a useful reference method for susceptibility testing of antifungal agents will require a full understanding of all variables that influence in vitro measurement of drug activity as well as alignment of test results with in vivo efficacy (16). In that context, our findings encourage further study of SAAMF, either unbuffered or with a nonantagonistic buffer, as a generally applicable medium for such standardization.

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