Laboratory tests of antifungal drugs

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SYNOPSIS The procedures evolved in the author's laboratory over the past 20 years for the microbiological assessment of antifungal drugs are described; methods are detailed for the estimation of the sensitivity of pathogenic fungi to therapeutic agents and for the assay of those agents in body fluids. The preparation and maintenance of stock reference solutions of the drugs, the culture media used, and the incubation temperature and time are discussed.

Sensitivity tests by paper disc and by liquid titration for minimal inhibitory and cidal concentrations estimated are described, and the importance of standardized initial inocula is emphasized. Two groups of assay procedures are given, the liquid dilution and the agar diffusion methods, and suitable indicator organisms for both methods are named. The paper concludes with a discussion on the problem of differential assays when two antimycotic agents are in simultaneous clinical use.

The last decade has seen a marked increase in systemic mycoses of iatrogenic origin, an increase that has partly been matched by the recent introduction of new systemic antifungal agents of considerable potency. Earlier almost all effective systemic agents carried prohibitive hazards of toxicity; the only exception was griseofulvin, which had restricted application. Amphotericin B had rightly gained a sinister reputation for almost inevitable renal damage and other unpleasant side effects (Utz *et al*, 1964), although more recently safer regimes were devised with gradually increasing doses on alternate days to a low and clearly defined maximum; these ensured that side effects were minimized (Bindschadler and Bennett, 1969). Symmers (1973) sounded a clear warning that clinicians occasionally withheld amphotericin B therapy from patients on vague suspicion of pre-existent renal impairment and cited five instances where the severe systemic mycosis from which the patients subsequently died could probably have been treated safely with the drug; nevertheless expert laboratory monitoring is essential to ensure that these safe limits are not exceeded.

The present paper outlines the methods evolved in these laboratories during the past 20 years for sensitivity testing and assay of antifungal agents. Among the agents investigated were nystatin (Stewart, 1956), other polyenes, diamidines, and quinoles (Stewart, 1958), and, in the 1960s, natamycin and candicidin. At the end of the decade

<i>Polyenes</i> Nystatin Amphotericin B Trichomycin Candicidin Rimocidin Natamycin	<i>Cycloheximide</i> Actidione <i>Halogenated Quinolines</i> Chloro- and Dichloro- 5-Fluorocytosine	<i>Diamidine</i> Pentamidine <i>Imidazoles</i> Mycopolylicid Clotrimazole Miconazole
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Table Antifungal drugs investigated by this methodology

intensive studies began on clotrimazole (Holt, 1970; Holt and Newman, 1972), miconazole (Holt, 1972, 1973) and 5-fluorocytosine (Holt and Newman, 1973) (table).

The methods fall into two groups, those which establish the sensitivity of pathogenic fungi to the agents likely to be used therapeutically and those to assay drug levels in body fluids when antifungal therapy has been given. A number of technological features are common to both aspects, and these include:

- maintenance of antimicrobial stock solutions
- appropriate culture media and pH
- time and temperature of incubation.

Stock Solutions

Antifungal agents are rarely soluble in water; 5-fluorocytosine is one of the few exceptions in common use. It is soluble at 1% in 0.01 M phosphate

buffer solution at pH 7.0, and aliquots of this have almost indefinite storage life when held at -20°C . Assays at three-monthly intervals up to two years have shown no loss of potency. Dilutions into nutrient culture fluid are entirely clear.

All the polyenes and imidazoles are freely soluble in organic solvents. Dimethylformamide readily dissolves the antifungal polyenes, and stable solutions up to 5% wt/vol can be prepared; these are stable at -20°C . The intravenous presentation of amphotericin B (Squibb) now contains sodium desoxycholate and phosphate buffer and is readily soluble at 50 mg per ml water (1 international unit of amphotericin B equals 1 μg). The imidazoles are soluble to 10% in polyethyleneglycol, and clotrimazole has shown complete stability for two years at room temperature in this vehicle.

On dilution into aqueous nutrient media, the solutions in organic solvents give a definite turbidity which slowly precipitates on standing. Providing the suspensions are freshly mixed before further dilution, there appears to be no loss of antifungal activity.

Culture Media

A working rule was laid down by Fleming in the early days of antibiotic testing that the test organism should be given every chance to grow freely; fungi of medical interest seem able to grow well and sometimes prolifically in many media, and the choice is often, therefore, one of convenience. Much of our early work was done in glucose-peptone broth or agar, sometimes fortified with yeast extract, at a pH of 7.0. More recently, the medium recommended by Shadomy (1969) for tests with 5-fluorocytosine has been adopted as routine for all antifungal agents. It is prepared according to the formula of Wickerham:

6.7 g Bacto-yeast nitrogen base (code 0392)

1.5 g L-asparagine

10 g Dextrose

dissolved in 100 ml glass-distilled water and sterilized by membrane filtration; this is a $\times 10$ concentrate, and for liquid tests 1 volume is diluted with 9 volumes of sterile 0.01 M phosphate phenol-red buffer at pH 7.0; 1% ion agar (Oxoid L.12) in glass-distilled water is melted, cooled to 45°C , and used as diluent for all plate tests. Chromatographic screening of both yeast-nitrogen base and agar revealed no free cytosine nor pyrimidine bases. When bacterial contamination is likely, 100 $\mu\text{g}/\text{ml}$ of gentamicin or chloramphenicol may be incorporated; these agents do not appear to affect the activity of antifungal drugs.

Most of our investigations with antifungal drugs related to their systemic use, and it was decided that

pH 7.0 should be arbitrarily adopted for all media as a reflex of biological conditions. It is well recognized that the activity of antibacterial drugs may be affected by the pH of the medium in which they are tested (Garrod *et al.*, 1973), but antifungal agents seem much less vulnerable to pH variation. This is fortunate, because the addition of glucose to fungal media greatly enhances growth but undoubtedly causes lowering of pH, as acid is produced by carbohydrate metabolism.

The pH of the healthy adult vagina is about 5.0 and that of most skin surfaces around 6.0; since topical preparations of both imidazole derivatives are used extensively for the treatment of candidoses, a series of tests was made to compare the anti-candida activity of these agents in more acid environment with that at pH 7.0. No significant variation in activity was noted between pH 8.0 and 4.0, although microbial growth was far less luxuriant at the latter pH.

Incubation Temperature

In an attempt to simulate body conditions, 37°C was adopted for sensitivity tests on fungi responsible for systemic invasion, and 30°C for dermatophytes. All candida species are tested at 37°C , including many strains from subjects with vaginal candidosis.

Most sensitivity tests, including those of *Aspergillus*, can be reliably read after 18 to 24 hours; it has been found that agar-diffusion assays give clear-cut results after 10 to 12 hours.

Sensitivity Tests

As with bacteria, these tests fall into two distinct categories, and are performed either in liquid or on solid agar surfaces. In the former method a standard inoculum of the fungus under test is challenged by a range of drug concentration in nutrient broth; in agar diffusion methods an even lawn of fungal inoculum encounters a variable zone of drug concentrations diffusing radially from a drug reservoir, usually a filter paper disc impregnated with drug. Intermediate between these is the agar gradient plate, where a suspension of the organism is sown onto an established drug gradient; the emergence of resistant variants can be induced by this method.

DISC SENSITIVITY TESTS

Discs

Dilutions of drug are made in 0.01 M phosphate buffer pH 7.0 at a concentration per millilitre 30 times the required disc content. One drop from a 30 drop/ml pipette is placed on 9 m filter paper discs presterilized in dry air at 160°C for one hour; the

discs are colour coded with dyes previously shown to have no antimicrobial activity. They are immediately vacuum dried at room temperature over silica gel and stored in tightly capped containers at 4°C; under these conditions all disc drug concentrations appear stable when assayed over several months.

Inoculum

For all drugs except 5-fluorocytosine a suspension of the fungus is made directly into 0.01 M phosphate buffer containing 0.1% Triton X. 100, a non-ionic surfactant with considerable dispersant powers. Where the primary cultures appear homogenous, samples of several apparently identical colonies are mixed; when even small differences in colonial morphology are apparent, sensitivity tests are performed individually on each variety. The cell density of suspensions of yeast-like organisms or fungal spores is counted in a Fuchs-Rosenthal chamber, and the suspension is further diluted in surfactant buffer to a final inoculum density of approximately 1×10^5 cells per ml. This may be impossible with filamentous fungi and 1/100 dilutions of well mixed overnight cultures in YNB broth are used in such cases; these dilutions may be homogenized by shaking with small sterile glass shards. Wherever possible the spores are harvested separately and counted.

All 5-fluorocytosine investigations must be made in medium free from cytosine and pyrimidine bases; primary plate culture media are usually rich in these, and it is therefore necessary to subculture first into YNB broth and to incubate for at least 6 to 8 hours to deplete the cultures of all traces of these substances which may have been carried over. Further dilutions are made as described earlier.

An even lawn of 0.2 ml dilute suspension is sown over YNB agar plates; within a few minutes the fluid has been absorbed into the agar, and discs are placed on the surface. Incubation for 24 hours at the specified temperature usually gives zones of inhibition up to 20 to 30 mm in diameter with sensitive organisms. Pre-diffusion plates, where the drug-loaded disc is placed on the agar for one hour and removed before inoculation, show little practical advantage.

For some years a *Saccharomyces* sp (*S. cerevisiae* ATCC 9763) served as a sensitive control organism; more recently, *Candida pseudotropicalis* Carshalton has been used with most tests and always with each new batch of discs.

LIQUID TESTS: ESTIMATION OF MINIMAL INHIBITORY AND CIDAL CONCENTRATIONS
Concentrations of drug are incorporated into 2 ml

tubes of YNB broth, usually in the range 0.05-50 µg/ml, although this range is often shortened as required; a broth tube without drug is included to ascertain that free growth results from the inoculum. *Candida* and other yeast-like organisms are added to give an initial concentration of 1×10^6 cells/ml, and filamentous fungi are added as a very light inoculum; all tubes are well mixed. At this stage it is found helpful to subculture a sample of the inoculum onto nutrient agar containing 10 µg/ml amphotericin B; this 'purity plate' gives immediate warning of bacterial contamination, with its risk of misleading minimal inhibitory concentration (MIC) results. After 24-48 hours' incubation the MIC is noted as the tube with the weakest concentration of drug able to inhibit growth.

The minimal cidal concentration (MCC) is estimated by subculturing standard volumes of each tube showing no visible growth into an excess volume of YNB broth or onto YNB agar (fig 1). In most of the tubes where growth is inhibited the residue of initial inoculum can be seen; this is washed three times in sterile phosphate buffer by centrifugation and resuspension before subculture to remove any drug still bound to the fungal structure. Unpublished observations during work with halogenated oxyquinolines had suggested that some antifungal drugs might otherwise be carried over into subcultures and thus give fallaciously low MCC results. Residual 5-fluorocytosine is inactivated by subculturing into media to which 0.1% cytosine had been added (fig 2).

Fig 1 *Aspergillus fumigatus*. MCC of clotrimazole on wort agar.

Fig 2 *Candida albicans*. MCC of 5FC on cytosine-rich agar.

Detection of Emergent Resistant Mutants

Light inocula of fungal strains are successively passaged across increasing drug gradients on YNB agar plates (Szybalski, 1952). In most cases the series is started on plates with a gradient of 0.5 µg/ml drug; two or three days are allowed for growth on each plate, and subcultures are taken from the growth edge farthest up the drug gradient. This is usually continued for 10 to 15 passages, and liquid MICs and MCCs on the most resistant growth are compared with the values obtained with the original cultures before the sequence was started.

Assays

Assays may be made on two quite distinct principles, by either chemical or microbiological methods:

(1) Chemical procedures usually depend on extraction of the drug in a suitable solvent with subsequent photocolourimetric estimation or measurement of specific absorption bands by spectrophotometry or spectrofluorimetry, often in the ultraviolet range. These methods offer considerable precision and can be completed within an hour or so, but they do not distinguish between the microbiologically active drug and its inactive metabolites; the proportion of the latter may greatly exceed the active fraction, and consequently chemical assay results may be much higher than those from microbial assays. For clinical antimycotic purposes, it seems clear that the antimicrobial level is the realistic value in therapy control, although it must be remembered that meta-

bolites inactive against fungi may still be toxic to the recipient; here measurements of both active and inactive components are necessary. This situation is exemplified in the apparent discrepancies in early studies with the imidazole derivative clotrimazole; German workers used only photocolourimetric methods (Plempel *et al*, 1969; Marget and Adam, 1969) and recorded high drug levels in the serum and urine of their subjects, whereas British work using microbiological methods reported much lower levels on similar dosage (Holt, 1970). Plempel and his colleagues later showed that 2-chlorotriptylcarbenol derivatives appear in considerable concentration in body fluids during continuous administration of clotrimazole, and they regarded these derivatives as microbiologically inactive.

(2) Microbiological methods for assaying drug concentrations depend on comparison between the growth inhibition of a suitably sensitive indicator microorganism by unknown levels of drug in the sample and the predictable inhibition caused by standard drug levels; in essence, two MICs are estimated against a mutual indicator strain.

The comparison can be made either by a liquid dilution method or by agar diffusion techniques; both methods require indicator strains and reference standard solutions of the drug under investigation.

ASSAY INDICATOR STRAINS

Many indicator strains are in use for antifungal assays and each organism should meet certain essential criteria. Ideally, they should be very sensitive to the drug under investigation and should exhibit stable sensitivity on repeated subculture; they should be readily cultivable on relatively simple media, reasonably fast growing during incubation, and not likely during storage to throw up mutants with increased drug resistance. Unless there is no alternative, the strain should not be pathogenic to man. Often the ultimate choice is somewhat of a compromise; the strain of *Candida pseudotropicalis* used in this laboratory for many investigations is very stable and very sensitive to several antimycotic agents but is probably feebly pathogenic.

Among the organisms in common use are:

Saccharomyces cerevisiae NCYC 87 or ATCC 9763

Paecilomyces varioti

Rhodotorula rubra

Torulopsis glabrata Roche (Basle)

Candida albicans H29 or 3153A (Institut Pasteur, Paris)

Candida albicans 2606 (Carshalton)

Candida pseudotropicalis (Carshalton)

The last strain was selected after more than 100 isolates of *Candida*, *Torulopsis*, and *Rhodotorula* had

been screened; it is used routinely for the assay of 5-fluorocytosine and the imidazoles, clotrimazole, miconazole, and econazole. *C. albicans* 2606 is preferred in the assay of amphotericin B and other polyenes.

The indicator strains are subcultured weekly into YNB broth and are plated out on each occasion for purity and for recognition of colonial variants.

DRUG STANDARDS

Dilutions are made from the stock reference solution described earlier; they may be diluted in pooled normal human serum for serum assays or in 0.01 M phosphate buffer at pH 7.0 for the assay of urine, cerebrospinal fluid or other body fluids and exudates.

LIQUID DILUTION

A series of metric dilutions of the test sample and of a reference standard solution of the drug are made in nutrient broth, usually to a final volume of 2 ml; YNB broth is suitable. The final dilutions in the standard series should span the clinically possible levels, starting at zero, and the dilutions of the test sample must extend far enough to ensure that the essential end point of no growth/growth occurs. pH indicators can be incorporated in the broth, or tetrazolium salts can be included to indicate growth, but these agents are rarely necessary.

Probably the single most important factor in liquid dilution assay methods is the inoculum concentration; as described earlier for sensitivity testing, this should be as accurately controlled as practically possible at a final concentration of 1×10^5 cells/ml.

Results by this method are often just visible after 6 to 8 hours' incubation and are clearcut in 14 to 18 hours. Liquid dilution methods can be set up quickly for single test samples but become increasingly tedious when many samples are to be assayed simultaneously.

Agar Diffusion Methods

In these methods the growth of indicator organisms sown evenly over the surface of nutrient agar is proportionately inhibited by drug diffusing radially outwards from reservoirs of standard solution and test sample placed on the agar surface. For most purposes 25 × 25 cm assay plates are suitable; an uninoculated base of 300 ml YNB agar is poured and allowed to set. This is followed by a layer of 100 ml YNB agar to which has been added 0.5 ml of overnight broth culture of the selected indicator organisms. For some purposes 13.5 cm Petri dishes are adequate; these require 50 ml base and 20 ml overlay agar. When the overlay has firmly set the plate is thoroughly dried; with some care this can

be done face downward, but with a plate of the larger size it may be necessary to dry it face upwards. Bacterial contaminants from the air present fewer problems in antifungal assays than in antibacterial assays, but should they become troublesome, gentamicin can be incorporated in the system.

The reservoir may be either a well-hole bored into the agar or a metal or porcelain cylinder placed onto the surface. The well is cut with a 1 cm diameter cork-borer, previously sterilized in a flame; the bottom of the well must be sealed with a single drop of molten agar to prevent the irregular creep of fluid from the well. A measured volume of each sample is carefully pipetted into the well, which at 1 cm diameter holds about 0.2 ml.

Fish-spine beads (Lightbown and Sulitzeanu, 1957) can be used as surface reservoirs and are particularly suitable for assays on limited amounts of fluid from infants or where blood samples have to be collected by peripheral skin punctures. The size commonly used is designated No. 3 and takes up between 0.020 and 0.022 ml per bead.

The wells or beads are usually placed on the large square assay plates in mirror distribution about a diagonal so that each standard and sample is assayed at least twice; 8 × 8 or even 9 × 9 reservoirs can be used, permitting up to 30 individual double estimations in addition to the standards (fig 3). For greater accuracy a 'Latin square' distribution can be used, and each dose is then placed the same number of times in each row and in each column. By this method a 3 × 3 or a 4 × 4 point assay is made, but such extreme accuracy is rarely warranted in clinical assays. On the smaller 13.5 cm diameter plate eight or nine reservoirs are set up at optimal positions equidistant from the centre of the plate (fig 4).

The plates are held for 1 to 2 hours at room temperature to permit prediffusion, and then incubated for 18 hours at 37°C; with fast growing indicator organisms, zones of inhibition may be measurable within 6 to 8 hours' incubation.

Two diameters at right angles of each zone of growth inhibition are measured with callipers to the nearest 0.5 mm; all the diameters for each sample are added together and the average d_1 is taken. The diameter d_2 of the reservoir is subtracted from this; $d_1 - d_2$ is then halved and the result is squared to give $(r_1 - r_2)^2$. The values of $(r_1 - r_2)^2$ for the standards are plotted on the arithmetic axis of semilogarithmic paper and corresponding drug concentrations on the logarithmic axis; the line joining the points constructed from these co-ordinates should be straight or nearly so. The drug concentrations in the test samples are found by plotting the individual $(r_1 - r_2)^2$ values (fig 5).

Fig 3 5 FC assay.
Standards: 4, 20, 100 $\mu\text{g/ml}$ 5 FC in serum
Test sera: A, B, C
Diagonal replication
C. pseudotropicalis

Fig 4 Clotrimazole assay.
Standards: 0.01, 0.05, 0.2, 1.0 $\mu\text{g/ml}$ in serum
Test sera: A, B
C. pseudotropicalis

Fig 5 Assay of 5-fluorocytosine.

DIFFERENTIAL ASSAYS

For a variety of reasons, two systemic antifungal drugs may be in simultaneous clinical use, and this poses the conundrum of differential assay. Microbiological procedures are relatively non-specific because the activity of the agents under investigation overlaps. If, however, one drug can be reliably inactivated, the activity of the other can be estimated

as though it were the sole component; the other obvious procedure depends on the use of an indicator organism sensitive only to one of the drugs.

The activity of 5-fluorocytosine in body fluids is readily inhibited when the assay is performed in medium containing say 0.01% cytosine, and this permits the estimation of amphotericin B and the imidazoles in the presence of 5-fluorocytosine. The combination of amphotericin B and imidazoles presents more difficulty, but limited success has resulted from the use of a coagulase-negative staphylococcus or a β -haemolytic streptococcus Lancefield Group A as an indicator organism. These organisms are moderately sensitive to the imidazoles, especially miconazole (Holt, 1974) whereas amphotericin B and other polyene agents have no activity towards them; it may be necessary to use bore holes as larger drug reservoirs able to hold 0.2 ml of fluid. Alternatively, the imidazoles can be assayed separately by the extraction/chromatographic method first reported by Marget and Adam (1969), although the results record the total imidazole content, not solely the microbiologically active fraction.

Concentrations of 5-fluorocytosine in the presence of other antifungal drugs can be estimated by the fluorimetric method described by Wade and Sudlow (1973); this method is rapid and highly specific for 5-fluorocytosine. Although in my hands it seems far less sensitive than the microbiological method, its accuracy is sufficient to check that the drug is being absorbed from the gut and that cumulative overdosing is not resulting in unnecessarily high drug levels. In these circumstances the speed of the test is valuable.

Lastly, amphotericin B in the presence of 5-fluorocytosine has been differentially assayed in my laboratory by using a strain of *Epidermophyton floccosum* which is very sensitive to amphotericin B (MIC 0.1-0.2 $\mu\text{g/ml}$) but which is virtually resistant to 5-fluorocytosine. This dermatophyte is also very sensitive to the imidazoles, and the assays are best performed in liquid dilution. Amphotericin B can be assayed in the presence of imidazoles by the extraction of the polyene in alkaline n-propanol and the subsequent determination of the absorbance by ultraviolet spectrophotometry at the specific absorption peak 409 μm (Gold *et al*, 1956).

Discussion

The methods detailed in this paper have evolved over the past 20 years from those already in use for the study of the earlier antibacterial antibiotics, penicillin, streptomycin, the tetracyclines, and chloramphenicol, and they are now routine in this laboratory. Although on occasion physicochemical

methods have been used successfully for the assay of antimycotic drugs the results have often been suspect because these methods estimate the active compounds plus their metabolites, and there is no easy way of knowing the proportion or microbiological activity of the derivatives. Preference has always been given therefore to microbiological methods; they may take longer than the physical methods, and in some situations speed is essential, but the clinical reality of the results must be the paramount consideration. These procedures are empirical, and rarely has any attempt been made to achieve unnecessarily high accuracy and sensitivity, largely from a conviction that over-vigorous endeavours to that end are almost certainly counter-productive in terms of time and effort. What is important is that no topical or systemic antimycotic therapy is started without reliable sensitivity tests, nor should systemic therapy continue without laboratory control. The latter will ensure that adequate body fluid drug levels are maintained and that toxic hazards from excessive drug levels are avoided, and indicate when fungal re-isolates during therapy have increased resistance to the drug in use.

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