Kornblum (3). The two mixes containing magnesium trisilicate and magnesium stearate result in generation of the freely soluble magnesium salt of aspirin producing a buffer with aspirin (3) of relatively high pH—3.5 and above—which is a poor environment for aspirin. When 1.188 g. Mix *D* was stirred in suspension with 20 ml. water the pH increased from 3.5 to 3.85 in 6 hr. while Mix *E* remained constant at 3.45 for the same time period. Over this interval of time the magnesium titer of *D* goes up to three times its initial value while *E* remains approximately at its original figure. Almost the same values hold for simple aspirin–magnesium stearate and aspirin–magnesium trisilicate suspensions. This helps explain the increased pH as it seems to be a partial result of a large amount of magnesium aspirin in solution. The pH profile of acetylsalicylic acid shows there is a considerable difference in the rate constants for degradation at these two values (3.85 for *D* and 3.45 for *E*) with the ratios being 2:1 at 25° (11). The high water content of magnesium trisilicate USP probably contributes to the degree and rate of Mix *D* relative to the others. It should be pointed out that in systems like these it is extremely unlikely that only one variable is operative but that several factors are working concomitantly to lead to the observed results. The 5% calcium stearate preparation (Mix *B*) exhibits somewhat less aspirin decomposition than 5% magnesium stearate which has previously been shown to be the case with simpler mixtures (3). The 10% aluminum hydroxide mixture (*A*) shows relatively little aspirin breakdown possibly due to the comparative insolubility of the aluminum salt of aspirin thus lowering the pH close to the saturation pH of aspirin (about 2.6) which is a fairly stable range for aspirin (11). Aluminum hydroxide is also well known as an adsorbent which may play some role in preventing aspirin decomposition.

The basic mix (*F*) and the 10% hexamic acid (*C*) show relatively little degradation as might be expected from pH effects (5).

In Table I the milligrams of salicylic acid formed in the tablets and powder mixes are expressed as the amount formed per 1% moisture. The adjustment was made when a variation of water content was found by Karl Fischer analysis (1.0 to 1.8%) rather than the expected 2%. Corrections were based on the assumption that for these systems apparent zero-order rates and therefore amounts of salicylic acid formed were directly proportional to moisture content. The adjustment to equal moisture levels in this manner provided a more successful degree of ranking.

Implicit in work of this nature is the ultimate aim of stability prediction for solid dosage forms from apparent zero-order rates obtained from suspension studies along with other pertinent information concerning the system under investigation. Long-term predictions were not attempted in this study but it is shown that relative results comparing powder mixes, tablets and suspensions exhibit very good correlation in the systems under observation as listed in Tables I and II.

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Microculture Assay for the Rapid Determination of Antifungal Activity

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Abstract ☐ Determination of hyphal growth rates in microslide cultures has been utilized to determine concentrations of griseofulvin. At concentrations between 0.001 mcg. and 0.01 mcg./ml. griseofulvin in Sabouraud liquid medium, the curve showing growth rate as a function of log dose is linear. In this concentration range the rate of hyphal elongation is constant with respect to time, and curling or distortion of hyphae is not seen except at the upper limit of the range. Microculture assays are compared to simultaneous assays performed using the current USP agar cup procedure. The microslide technique requires a combined incubation and reading period of 24 hr. It can be used to determine accurately one thousandth the concentration of griseofulvin required for the official procedure.

Keyphrases ☐ Antifungal activity, determination—microculture slide technique ☐ Griseofulvin activity, assay—agar cup *versus* microculture technique ☐ Hyphal growth rates—griseofulvin concentration

Although chemical assays are available for most antifungal agents, microbiological assays are the accepted standards for potency determinations (1). Microbiological assays use minute quantities of drugs with solvent extraction being the only usual preparative step required for dosage forms or biological specimens. While compounds possessing analogous chemical structure frequently interfere with chemical assay methods, this is not a problem with microbiological methods, unless the analogs also possess biological activity. Microbiological assay techniques have been extended to the determination of compounds possessing antifungal activity. Turbidimetric and agar diffusion methods have been most widely accepted as antifungal assays (1). Although several methods have been developed to observe the growth of fungi microscopically, few have been developed sufficiently to be useful as assay procedures. The objective of this investigation is to develop the microculture slide technique of Elliott *et al.* (2) as a rapid, sensitive, and efficient assay procedure for antifungal agents. Because of its potency

and wide clinical acceptability, griseofulvin was the prototype used for this study.

EXPERIMENTAL

Culture—Subcultures from a single stock culture of *Microsporum gypseum* on Sabouraud agar were incubated at 34 ± 1° for 5 days. The cultures were stored at 4° for periods up to 2 months before further subculturing was required.

Media—Basal medium was Sabouraud liquid. The pH after autoclaving was found to be 5.7. Test media were prepared by incorporating griseofulvin solutions after bacterial filtration into the sterile Sabouraud liquid at specified concentrations. A drug solvent consisting of dimethylformamide A.R. (1%) in 0.1 *M* phosphate buffer pH 8.0 was prepared; the control medium was obtained by mixing Sabouraud liquid and 1% drug solvent. A stock solution of authentic griseofulvin[1] (1.00 mg./ml.) in dimethylformamide was prepared. Dilutions of this stock solution were prepared using 0.1 *M* phosphate buffer at pH 8.0. These dilutions were added to the basal medium through a filter (Millipore) to produce test media containing 0.001, 0.0025, 0.005, and 0.01 mcg./ml. griseofulvin. The pH of the test media was found to be 6.2. Test media were stored at 4° for periods up to 2 months. Tests for bacterial contamination were negative.

Inocula—Individual conidial suspensions were prepared for each determination by scraping a number of macroconidia from a 5-day-old culture plate with the open end of a sterile capillary tube. A drop of the test medium was placed in a depression on a sterile spot plate and the conidia were dispersed by stirring with the capillary tube. The sealed end of the tube was then broken and the suspension was drawn into the capillary tube. Mixing was effected by rotating the tube without inverting it.

Equipment—Microculture slides were prepared by cutting two parallel horizontal grooves in a microscope slide with an egg drill. The slides were previously dipped in molten wax to aid in dissipating the heat generated by cutting the glass and thus prevent cracking of the slide. The grooves which extended the full length of the slide were about 1 mm. deep and about 2 mm. apart. On the reverse side, approximately 30 parallel lines were drawn with a diamond pencil perpendicular to the grooves on the opposite face of the slide.

The slides were cleaned to remove all traces of wax by successive soaking in benzene, 0.1 *N* HCl, 0.1 *N* NaOH, and 0.25% detergent.[2] Each soak was followed by rinsing with deionized water. The slides were rinsed several times with distilled water, individually wrapped, and sterilized by autoclaving at 15 p.s.i. for 20 min. Cover slips were rinsed with acetone and autoclaved in individual wrappers. A disposable syringe was filled with melted petrolatum which had been shown to be free of bacteria viable in Sabouraud medium. A sterile No. 22 needle was attached to the syringe and used to place a thin ribbon of petrolatum along two parallel edges of each sterile cover slip and on a line through the middle perpendicular to the edges. Three cover slips were inverted and placed on each slide forming a total of six microculture chambers per slide. Adequate pressure was applied to establish an air-tight seal around three edges of each chamber. The cover slips were situated so as to facilitate the exchange of air between the sealed chamber and the external environment (Fig. 1).

Microcultures—The capillary tube containing the conidial suspension was placed adjacent to the cover slip and a quantity of the inoculum (approximately 0.1 ml.) was drawn into the microculture chamber by capillarity. After inoculation, the remaining exposed edges of the cover slips were sealed with petrolatum, leaving only the grooves open for air exchange.

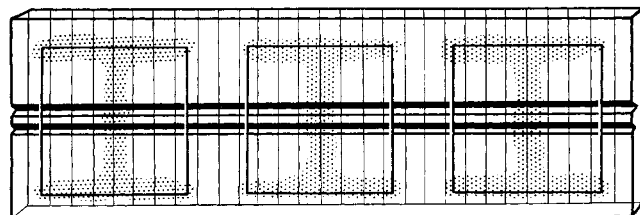

Figure 1—*Microculture slide, see text for explanation.*

[1] Griseofulvin Micronized (B#9589KK) Ayerst Laboratories.
[2] Calgonite, Calgon Corp., Pittsburgh, Pa.

Table I—Relationship between Dose (X) and Mean Growth Rate ($\hat{Y}$) in Microculture Determinations of Griseofulvin

Dose (X), mcg./ml.	Mean Growth[a] Rate ($\hat{Y}$) o.u./hr.	Range of $\hat{Y}$	Estimate of Standard Deviation from Range
0.00	0.87	0.80–0.99	0.067
0.001	0.61	0.50–0.68	0.063
0.0025	0.51	0.43–0.57	0.049
0.005	0.46	0.35–0.51	0.056
0.010	0.31	0.18–0.47	0.102

[a] Based on N = 8 replications of n = 10 hyphae/dose level (X).

Incubation—Inoculated microculture slides were incubated in darkness at 34 ± 1° in a humidity chamber for 16 hr. prior to the determinations.

Measurement—Individual macroconidia were located with respect to the vertical lines on the back of the slide. Only those cultures containing 40–60 macroconidia, at least 75% of which had germinated by the end of the 16-hr. incubation, were used for the determinations. With the aid of an eyepiece micrometer terminal hyphae of 10 macroconidia in each culture were measured every 2 hr. over a 6-hr. period. Rate of hyphal elongation was recorded in ocular units/hr. (o.u./hr.). At the magnification used (objective: 43×; eyepiece: 10×) one ocular unit was equivalent to 0.0175 mm.

Procedure—Using a replicated randomized complete block design, data were collected to construct a standard curve for griseofulvin for the microculture determinations. The standard curve was prepared by plotting log dose *versus* growth rate. Similarly, appropriate dilutions from the original stock solution (10 mcg./ml. griseofulvin in DMF-phosphate buffer) were used to prepare a standard curve for the USP agar cup procedure as modified by Knoll *et al.* (3). Least-squares regression lines for both methods were calculated using a univariate model (Y = random variable) and tested for linearity by analysis of variance. Standard solutions of griseofulvin were prepared and assayed by both techniques simultaneously. In order to test for any effect due to tablet excipients, the DMF-soluble fractions of individual tablets of griseofulvin[3] were assayed by microculture and agar cup methods.

RESULTS AND DISCUSSION

The germination lag period for hyphae in control cultures was found to be 4–6 hr., as determined by the appearance of hyphae in 75% of the conidia in a given culture. The length of this period was not affected by the addition of griseofulvin to the medium at the test concentrations.

Growth rate of the terminal hyphae was found to be constant between 16 and 24 hr. after preparation of the microculture and to show a linear response with respect to the log of gradient concentrations of griseofulvin. Table I shows the quantitation of this response. Homogeneity of variance as determined from an estimate of the standard deviation from the range using the method described by Batson (4) was used to establish the normal distribution of the data. This was done to verify the premise for using parametric statistics in the analysis of the data. Hyphal abnormalities seen at the 0.01 mcg./ml. dose were undoubtedly responsible for a large portion of the increased standard deviation observed at that level.

The pooled means of cultures treated with griseofulvin were used to calculate a regression line for the microculture procedure. The line and its 95% confidence limits are illustrated in Fig. 2. The resultant equation for the least squares line of regression is: $\hat{Y} = 90.75 - 28.5X$, where $\hat{Y}$ is the expected mean value for a series of replications at each level of X. Inspection of this equation

[3] Fulvicin U/F 250-mg. tablets (6AUF4 P96678) Schering Corp., Bloomfield, N. J.

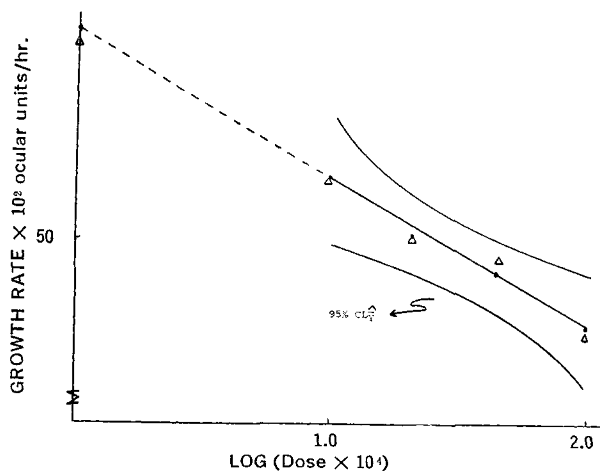

Figure 2—*Standard curve for microculture technique. Key:* $\hat{Y} = 90.75 - 28.5X$ ●, *value expected from regression equation;* △, *experimental value.*

and Fig. 2 indicates that the response of *M. gypseum* to griseofulvin is linear with respect to log dose and that the dosage response curve is continuous when extrapolated to the control. There is good agreement between the mean growth rate observed for control (0 mcg./ml.) cultures with the *Y*-intercept of the regression line. When $X = 0$, the expected value of mean Y ($\hat{Y}$) from the regression equation is 0.91 o.u./hr.; the observed mean value of *Y* for control cultures was 0.87 o.u./hr. This evidence strongly suggests that under the experimental conditions the minimum inhibitory concentration for *M. gypseum* is so small as to be immeasureable.

Previous investigators (5) have reported minimum inhibitory concentrations for griseofulvin with *M. gypseum* in the range of 0.42–0.46 mcg./ml. It should be noted, however, that the criteria used for those determinations were based on complete inhibition of growth for a specified time period in turbidimetric and agar cup determinations. This work demonstrates the physiologic effect of griseofulvin on *M. gypseum* at concentrations much below those required to produce hyphal curling; indicating that the observation of hyphal curling is not necessarily a valid estimate of the onset of antifungal activity. Antifungal activity measured from rates of hyphal growth is manifest before any apparent hyphal distortion occurs.

Analysis of variance was performed to test for linearity of the regression line. *F*, the ratio of the mean square for regression to the mean square for deviations from regression is shown to be highly significant ($p < 0.01$).

Data were obtained for the agar cup determinations of griseofulvin at levels of 1, 5, and 10 mcg./ml. Linearity of the regression line was established by analysis of variance.

The microculture and agar cup techniques were compared with respect to accuracy by replicated simultaneous determinations of standard solutions of griseofulvin. Results of these assays are given in Table II.

Additional assays were performed to observe the effect of tablet additives on each of the assay procedures. Results of these single-tablet assays are shown in Table III. It is apparent by inspection that the two assay methods when applied to Tablet *A* do not differ significantly in results. This is also true for Tablet *B*. Two-tailed "t" tests for paired means were not significant.

Inspection of Tables II and III reveals no apparent difference in the quantitative determination of griseofulvin by the agar cup and microculture methods. The accuracy of both techniques is established by the results of the assays of standard solutions. The precision of the microculture technique compares favorably with that of the agar cup technique as demonstrated by the relative values of the standard deviations. Similarity in the results shown in Table III for each of two tableted products indicates that tablet excipients have no greater effect on the microculture assay than on the agar cup assay.

The microculture technique as an assay procedure is shown to be more rapid than the agar cup technique which is now official. It can be used to measure a concentration of griseofulvin approximately one-thousandth that required for the official technique, and it is more efficient with respect to equipment and effort required to produce equally valid results.

Using the spectrophotofluorometric method of Bedford *et al.* (6), Weinstein (7) has demonstrated peak serum levels of 1–1.6 mcg./ml. griseofulvin. The sensitivity of the microculture assay is at least 10× greater than that of the spectrophotofluorometric procedure. Since the microculture procedure is based on pharmacologic effect and is sensitive in the concentration range at which drugs commonly occur in biological fluids, it would be readily adaptable for application to problems of drug equivalency and as a generalized rapid screening technique for the detection of antifungal activity in a wide range of compounds. Work is in progress to quantitate the effects of other antifungal agents using this procedure. Relative potency among widely different chemical agents can be established by the comparison of linear regression coefficients.

Table II—Comparison of Techniques Using Standard Solutions of Griseofulvin

Standard Solution	Dose Calculated, mcg./ml.	Agar Cup: Dose Found[a] mcg./ml.	Agar Cup: Dose Found, %	Microculture: Dose Found[b] mcg./ml.	Microculture: Dose Found, %
A	10.00	9.80	98.0	9.40	94.0
B	10.00	10.13	101.3	9.80	98.0
C	10.00	10.03	100.3	9.93	99.3
D	10.00	10.83	108.3	10.00	100.0
Means			102.0 ± 4.45[c]		97.8 ± 2.68

[a] Based on 12 measurements at each of three dosage levels. [b] Based on 20 measurements at each of three dosage levels. [c] Standard deviation.

SUMMARY

1. Quantitative determination of the antifungal antibiotic griseofulvin has been performed using a microculture technique.

2. The assay system consists of a suspension of *Microsporum gypseum* macroconidia in Sabouraud liquid medium containing nanogram quantities of griseofulvin.

3. Antifungal activity is determined on specially prepared microculture slides by measuring changes in the rate of hyphal elongation.

4. A linear relationship of log dose to hyphal growth rate (×100) is observed in the range of 0.001–0.01 mcg./ml. griseofulvin. $\hat{Y} = 90.75 - 28.54X$.

5. The useful range of determination using the microculture technique extends to dose levels which are approximately 1000× smaller than those determined by the USP XVII agar cup assay procedure for griseofulvin. In determinations of standard solutions of griseofulvin, the microculture technique exhibited precision at least equivalent to that of the agar cup procedure.

6. Analysis of tableted dosage forms of griseofulvin demonstrated no significant effect of excipients on the microculture procedure.

7. This work indicates that physiologic hyphal abnormalities (curling) may not be accurate indicators of the onset of antifungal activity. Changes in growth rate of hyphae are manifest at concentrations 500× less than the accepted MIC.

Table III—Single Tablet Determination of Griseofulvin[a]

Tablet	Dose Calculated,[b] mcg./ml.	Agar Cup: Dose Found,[c] mcg./ml.	Agar Cup: Dose Found, %	Microculture: Dose Found,[d] mcg./ml.	Microculture: Dose Found, %
A	9.60	10.03	104.48	9.76	101.66
B	9.44	9.30	98.52	9.00	95.34

[a] Fulvicin U/F 250-mg. tablets (6AUF4 P96678) Schering Corp., Bloomfield, N. J. [b] Based on nominal weight. [c] Based on 12 measurements at each of three dosage levels. [d] Based on 20 measurements at each of three dosage levels.

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Urinary Drug Excretion in Dogs During Therapeutic Doses of Different Nitrofurantoin Dosage Forms

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Abstract ☐ Nitrofurantoin, a urinary tract antibacterial, was administered daily in different dosage forms to dogs using a multiple-dose regimen and the urinary drug excretion determined. Results are presented which show that two oral dosage forms, microcrystalline drug in a tablet or macrocrystalline drug in a capsule, are well absorbed. Differences observed in urinary drug recoveries and in urinary drug excretion patterns between these dosage forms suggest a slower rate of absorption for the macrocrystals than for the tablet. Data are also provided which indicate that an intramuscular dosage form is very well absorbed and excreted readily in the urine.

Keyphrases ☐ Dosage forms—nitrofurantoin ☐ Nitrofurantoin—urinary excretion ☐ Absorption, nitrofurantoin—parenteral and oral administration

Usually most drugs are administered clinically according to a predetermined daily multiple-dose regimen. Although much effort has been directed recently toward investigating the relationship between the particle size of various drugs and their *in vivo* absorption (1), few of these studies have been conducted using multiple doses in therapeutic amounts. It seems reasonable to assume that a single dose of a drug may not always yield *in vivo* results which reflect accurately those encountered during a multiple-dose regimen. Nitrofurantoin,[1] 1-(5-nitrofurfurylideneamino)hydantoin, is an antibacterial drug effective in the treatment of urinary tract infections (2). Results are presented in this report concerning urinary drug excretion in dogs during daily multiple therapeutic doses of different nitrofurantoin dosage forms.

EXPERIMENTAL

Oral Drug Administration—Four healthy, adult male beagle dogs were selected on the basis of weight (range 13–16 kg.). A crossover design was used in which two of the dogs received one drug dosage form while the other two dogs received the other dosage form. An interval of 15 days during which drug was not administered was maintained between crossovers. Nitrofurantoin either as microcrystalline drug in a tablet (10- and 50-mg. veterinary tablet[2]) or as macrocrystalline drug (80–200 mesh) in a gelatin capsule (10- and 50-mg. veterinary capsule[3]) was administered orally at 4–5 mg./kg. t.i.d. for 10 days (a therapeutic dose and regimen). The drug was administered at 8 a.m., 12 noon, and 4 p.m., either as two 10-mg. tablets plus one 50-mg. tablet or two 10-mg. capsules plus one 50-mg. capsule, respectively, per dose. Heparinized blood samples were collected by venipuncture on Days 1 and 10 at 2 hr. after the second dose of drug. On Days 1 and 10, urine samples were collected (voided and/or by catheterization) from 0–4, 4–8, 8–12, and 12–24 hr. Urine samples were also obtained just before initial drug dosage on Day 1 to serve as controls and on Day 10 to serve as drug residue samples.

Parenteral Drug Administration—Nitrofurantoin sodium[4] as a solution was administered intramuscularly at about 3 mg./kg. b.i.d. for 10 days (a therapeutic dose and regimen) to the same dogs used in the oral drug study. Drug solutions were prepared by dissolving nitrofurantoin sodium in 5 ml. of sterile water to obtain a drug concentration of 36 mg./ml. (pH 9.1). The drug was injected into the left vastus lateralis muscle at 8 a.m. and into the contralateral muscle at 4 p.m. Heparinized blood samples were collected by venipuncture on Days 1 and 10 at 1 hr. after the second dose of drug. Urine samples were collected (voided and/or by catheterization) from 0–4, 4–8, 8–12, and 12–24 hr. on Days 1 and 10. Urine samples were also obtained just before initial drug dosage on Day 1 to serve as controls and on Day 10 to serve as drug residue samples.

In the intravenous drug study, nitrofurantoin sodium as a solution was administered as a single injection at either 3 or 6 mg./kg. to the same dogs used previously. A crossover design similar to the one described in the oral study was utilized. Drug solutions were prepared by dissolving nitrofurantoin sodium in 15 ml. of sterile 5% dextrose solution to obtain a drug concentration of 12 mg./ml. (pH 8.6). The drug was injected into the cephalic vein of one leg and heparinized blood samples then were collected by venipuncture from the contralateral vein at selected intervals. Samples of urine were collected (voided and/or by catheterization) from 0–4, 4–8,

[1] Furadantin, Eaton Laboratories.

[2] Veterinary 50-mg. nitrofurantoin tablet, Furadantin Ora-Bol, Eaton Laboratories.
[3] Veterinary macrocrystalline nitrofurantoin, Dantamacrin, Eaton Laboratories.
[4] Nitrofurantoin sodium, Furadantin sodium, Eaton Laboratories.