Plate Assays for Griseofulvin in Pharmaceutical Preparations and Body Fluids

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A microbiological assay for griseofulvin in pharmaceutical preparations using either cylinders or paper disks is described. A modification to assay griseofulvin concentrations in body fluids is also presented. Both assays offer more precision in assaying griseofulvin than existing microbiological methods.

GRISEOFULVIN, an orally effective antifungal agent, produced by *Penicillium griseofulvin* and other species of *Penicillium*, was first isolated in pure form in 1946 (1). Since that time it has been assayed by spectrophotometric (2), polarimetric (3), isotope dilution (3), and microbiological methods (1). In the latter method, solutions containing griseofulvin are serially diluted in nutrient broth which is later seeded with *Botrytis alli*. The greatest dilution causing distortion or curling of the hyphae of *Bortrytis alli* is recorded and used to estimate the potency of the solution. However, this procedure lacks precision and reproducibility, as do most methods employing observation of morphogenetic responses in microorganisms.

A microbiological assay was developed for griseofulvin in pharmaceutical preparations and in serum. The method is a modification of the cylinder-plate agar diffusion assay for penicillin (4). The response is a well-defined zone of mold inhibition which can be accurately measured by its diameter.

MATERIALS AND METHODS

Medium I.—Dextrose 40.0 Gm., peptone 10.0 Gm., and distilled water sufficient to make 1000 ml. The pH should be 5.65 ±0.05 after sterilization.

Medium II.—Dextrose 40.0 Gm., peptone 10.0 Gm., agar 15.0 Gm., chloramphenicol U.S.P. 0.05 Gm. (activity), and distilled water sufficient to make 1000 ml. The pH should be 5.65 ±0.05 after sterilization.

Medium III.—Dextrose 40.0 Gm., peptone 10.0 Gm., agar 15.0 Gm., chloramphenicol U.S.P. 0.05 Gm. (activity), and distilled water sufficient to make 1000 ml. The pH should be 5.65 ±0.05 after sterilization. To each 100 ml. of this agar at approximately 50° aseptically add 2 ml. of a sterile solution of cycloheximide. The solution contains 10 mg./ml. and is prepared by dissolving cycloheximide in distilled water. The solution is sterilized by filtering through a membrane filter having a porosity of 0.22 μ.

Preparation of Spore Suspension.—Grow *Microsporum gypseum* (ATCC 14683) for 3 weeks at 25° in four 3-L. Erlenmeyer flasks, each containing 200 ml. of *Medium I*. Remove the floating mat from the flask with a sterile wire loop and place in a sterile blending jar. Aseptically add 200 ml. of sterile distilled water and blend for approximately 30 seconds, allow to settle, then decant about 150 ml. of the supernatant into 100-ml. sterile, capped centrifuge tubes. Add the amount of distilled water equal to the amount removed each time and repeat this process four to five times to assure maximum recovery of spores from the mycelial mat. Centrifuge the tubes containing the spores at 4000 r.p.m. for 15 minutes and discard the supernatant. Wash the residual spores from all tubes, using a minimum of sterile distilled water (2–3 ml. per tube) and pool the washings into a sterile flask containing a few sterile glass beads. (This procedure usually yields about 25–35 ml. of spore suspension.) The spore suspension is stable for at least 2 months at 5°. Prepare test plates using a 6-ml. base layer of *Medium II* covered with a 4-ml. seed layer of *Medium III* inoculated with varying volumes of spore suspension, *e.g.*, 1 or 2%. Determine the proper per cent of inoculum to be used by observation of the largest and clearest zones of inhibition given by 5 mcg./ml. of griseofulvin on the test plate.

Add 6 ml. of *Medium II* to a 20 × 100-mm. Petri dish and allow to harden for use as a base layer. Cover the Petri dishes with sterile porcelain lids glazed on the outside. Add the proper amount (as previously determined) of the spore suspension to each 100 ml. of *Medium III* which has been cooled to approximately 50°. Mix the culture and agar thoroughly and add 4 ml. to each plate containing the uninoculated base agar. Tilt each plate back and forth to spread the inoculated agar evenly over the surface. When the agar has hardened place six cylinders on the surface so that they are at approximately 60°-intervals on a 2.8 cm. radius. Use stainless steel cylinders that have an outside diameter of 8 ±0.1 mm., an inside diameter of 6 ±0.1 mm., and a 10 ±0.1-mm. length.

Assay of Pharmaceutical Preparations.—Store the griseofulvin working standard at room temperature under desiccation. Dissolve about 50 mg. in sufficient N,N-dimethylformamide (DMF) to give a concentration of 1000 mcg./ml. This solution may be kept for 3 months under refrigeration. Prepare the standard curve by diluting the 1000 mcg./ml. griseofulvin solution to 64, 80, 100, 125, and 156 mcg./ml. in DMF. Then further dilute one part of each of these solutions with 19 parts of 0.1 *M* phosphate buffer pH 8.0 to give final solutions of 3.2, 4.0, 5.0, 6.25, and 7.8 mcg./ml.

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Use three plates for the determination of each point on the curve, except the 5.0 mcg./ml. solution, a total of 12 plates. On each of three plates fill three cylinders with the 5.0 mcg./ml. solution and the other three cylinders with the concentration of the standard curve under test. There will be thirty-six 5.0 mcg./ml. determinations and nine determinations for each of the other points on the curve. Incubate the plates for at least 48 hours at 30°, and measure the diameters of the circles of inhibition. (Plates may be incubated longer if zones are indistinct.) Average the 36 readings of the 5.0 mcg./ml. concentration and the readings of the other concentrations for each set of three plates. The average of the 36 readings of the 5.0 mcg./ml. concentration is the correction point for the curve. Correct the average value obtained for each point to the figure it would be if the 5.0 mcg./ml. reading for that set of three plates were the same as the correction point. Thus, if in correcting the 4.0 mcg./ml. concentration, the average of the 36 readings of the 5.0 mcg./ml. concentration is 16.6 mm., and the average of the 5.0 mcg./ml. concentration of this set of three plates is 16.4 mm., the correction is plus 0.2 mm. If the average reading of the 4.0 mcg./ml. concentration of these same three plates is 14.8, the corrected value is then 15.0 mm. Plot these corrected values, including the average of the 5.0 mcg./ml. concentration on semilogarithmic paper, placing the griseofulvin concentrations on the logarithmic scale and the diameters of the zones on the arithmetic scale. Draw the line of best fit by inspection or by calculation as described by Deutschberger and Kirshbaum (5).

Dissolve an appropriate aliquot of the griseofulvin powder or suspension in DMF to give a convenient stock solution. Further dilute with DMF to give a concentration of 100 mcg./ml. Then further dilute one part of this with 19 parts of 0.1 *M* phosphate buffer pH 8.0 to give a final estimated concentration of 5.0 mcg./ml. of griseofulvin. For the assay of tablets, pool a representative number of tablets (usually five), blend them in 250 ml. of DMF, and follow the dilution procedure described for powders or suspensions.

Use three plates for each unknown sample. Fill three cylinders on each plate with the standard 5.0 mcg./ml. solution and three cylinders with the sample solution to be tested, alternating standard and sample. Incubate the plates for at least 48 hours at 30° and measure the diameter of each circle of inhibition. To calculate the potency of the sample, average the zone readings of the standard and the zone readings of the sample on each set of plates. If the sample gives larger average zone size than the standard, add the difference between them to the 5.0 mcg./ml. zone on the standard curve. If the average sample value is lower than the standard value, subtract the difference between them from the 5.0 mcg./ml. value on the curve. Read from the standard curve the concentration corresponding to the corrected zone size.

Assay of Potency of Pharmaceutical Preparations Using Paper Disks in Lieu of Cylinders.—In the method described above, paper disks may be substituted for the cylinders if preferred by making the following changes. Use round, blank disks having a diameter of 1/4 in. made of clear-white filter paper. Schleicher and Scheull No. 740-E paper or comparable grade may be used. Place blank disks on aluminum or stainless steel wire mesh which is supported in a manner to allow circulation of air above and below the disks. Prepare the solutions for the standard disks by further diluting the standard stock solution of griseofulvin in DMF to obtain solutions containing the following concentrations: 50, 62.5, 75, 95, and 120 mcg./ml. Pipet 0.02 ml. of each stock solution to three replicate disks, and put in vacuum desiccator for 2–3 hours until perfectly dry. The final concentration of griseofulvin per disk is 1.0, 1.25, 1.5, 1.9, and 2.4 mcg. Disks may be used for assay purposes that have been stored for up to 2 weeks under refrigeration. However, griseofulvin disks stored in stoppered test tubes containing a desiccant showed no loss of potency after 6 months.

Using disks prepared from the five different concentrations of standard described above, proceed as directed for establishing the standard curve by the cylinder-plate method, but in lieu of filling the cylinders, place the disks on the plates with forceps and tap gently to ensure an even seal. Use three plates (prepared as described for cylinder-plate method) for the determination of each point on the curve. On each of three plates, place three 1.5 mcg. disks and three disks containing one of the antibiotic concentrations of the standard curve. There will be thirty-six 1.5 mcg. determinations and nine determinations for each of the other points on the curve.

Prepare the stock solution of the sample as described for the cylinder plate assay. Then further dilute with DMF to a concentration of 75 mcg./ml. Prepare disks from this solution to give a final estimated concentration of 1.5 mcg./disk as described for the standard curves. Incubate the plates for at least 48 hours at 30°. Accurately measure the zones of inhibition with calipers, by projection on a calibrated screen, or by any other suitable method. Plot the standard curve and calculate the potency of the sample as described for the cylinder plate assay.

Assay of Griseofulvin in Body Fluids.—Prepare the suspension, standard curve, and plates as described for assay of griseofulvin in pharmaceutical preparations by the cylinder-plate method except modify preparation of the plates, and make 1 +19 dilutions for the standard curve using the appropriate body fluid. For example, if the body fluid to be assayed for griseofulvin content is serum, use antibiotic-free normal human serum instead of buffer for the final dilutions of the standard. Add the griseofulvin in DMF directly to the serum and shake immediately to distribute the antibiotic evenly through the serum. Prepare stock griseofulvin solutions in DMF to contain 90, 60, 40, 26, and 18 mcg./ml., and add 0.5 ml. of each solution to 9.5 ml. of serum to obtain final concentration of 4.5, 3.0, 2.0, 1.3, and 0.9 mcg./ml.

To prepare the plates, use heavy flat-bottomed (20 × 100 mm.) glass Petri dishes covered with sterile porcelain lids glazed on the outside. Add 5 ml. of *Medium II* seeded with 1% of the spore suspension previously described and allow to harden. Use one plate for each unknown tested. Test serum samples undiluted unless a concentration of greater than 4.5 mcg./ml. is expected. If so, dilute with serum to an estimated concentration of 2.0

mcg./ml. Add the 2.0 mcg./ml. standard solution to alternate cylinders on each plate and the sample to the remaining cylinders. Incubate the plates for at least 48 hours at 30°, read the zones of inhibition, and calculate the concentration of griseofulvin as described for the cylinder plate assay. The lowest concentration of griseofulvin that this method can detect in body fluids is 0.9 mcg./ml.

EXPERIMENTAL

Griseofulvin bulk material, tablets, and suspensions assayed by this cylinder plate or disk method gave results comparable to those obtained using a spectrophotometric method (2) as shown in Table I. Using the described method, standard curves were run using a commercial bulk sample of griseofulvin as a house standard. Four replicate assays of a bulk sample from a different manufacturer were performed on each of three consecutive days. From the results obtained the 95% confidence limits of a single assay of the method described for pharmaceutical preparations were calculated and shown to be ±10.9%. This range can be reduced of course by performing replicate assays. Different commercial preparations were assayed by this procedure and the spectrophotometric method (2). The results summarized in Table I show good agreement.

TABLE I.—GRISEOFULVIN PHARMACEUTICAL PREPARATIONS ASSAYED BY TWO METHODS

Product	Label Potency	Spectrophotometric	Microbial
Bulk	1,000 mcg./mg.	1000	1000
		978	1008
Tablets	250 mg./tablet	250	255
Oral suspension	250 mg./5 ml.	250	250

Experimental studies on the assay in serum were limited because of lack of clinical facilities. However, one male receiving therapy of griseofulvin (250 mg. every 4 hours, total dose 1 Gm./day) for 1 week volunteered to have his serum assayed for griseofulvin. Blood sample was taken 2 hours after a 250 mg. dose of griseofulvin. His serum assayed 0.9 mcg./ml., whereas the serum from a male volunteer receiving no drug was negative.

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Basic 1,3-Dioxolanes

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The preparation of a series of 2-aminomethyl and 4-aminomethyl-1,3-dioxolanes has been described. A selected number of the products were subjected to preliminary pharmacologic evaluation.

IN RECOGNITION of the various structural aspects assigned to muscarine prior to 1942, Fourneau and associates (1) initiated studies on quaternary ammonium compounds which led to the synthesis of 2-methyl-4-dimethylaminomethyl-1,2-dioxolane methiodide. The compound exhibited strong muscarinic activity which decreased with the substitution of bulkier groups for the C-2 methyl moiety.

Subsequently, extensive investigations on the autonomic pharmacodynamics of basic 1,3-dioxolanes and their quaternary derivatives have been reported (2–8). Recently, Hardie and co-workers (9) have reported on the local anesthetic and spasmolytic properties of a series of 4-(2-piperidyl)-1,3-dioxolanes.

The present work was undertaken to prepare basic dioxolanes with additional structural variations for pharmacologic evaluation. The products represent essentially an extension of the Blicke series (5) of 2-aminomethyl and 4-aminomethyl substituted 1,3-dioxolanes.

The intermediate halodioxolanes (Tables I and II) required for the synthesis of the aminodioxolanes were prepared by two general methods: (*a*) condensation of an α-haloketone with a 1,2-glycol or with glycerol-α-monochlorohydrin in the presence of *p*-toluenesulfonic acid according to the procedure of Salmi (10); (*b*) condensation of a ketone with epibromohydrin in the presence of stannic chloride according to the procedure of Bersin and Willfang (11, 12).

The infrared absorption spectra of a number of the halodioxolanes listed in Table I are shown in Table III. The four bands found in the 990–

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