Determination of Griseofulvin in Skin, Plasma, and Sweat

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Abstract □ A procedure is described for the quantitative determination of griseofulvin in skin, plasma, and sweat. Griseofulvin is extracted with ether from skin and is measured quantitatively by GLC and an electron-capture detector, using diazepam as an internal standard. Griseofulvin from plasma and sweat is extracted with ether and measured spectrophotofluorometrically.

Keyphrases □ Griseofulvin—GLC determination in skin, spectrophotofluorometric determination in plasma and sweat □ GLC—determination of griseofulvin in skin □ Spectrophotofluorometry—determination of griseofulvin in plasma and sweat

Griseofulvin is an orally effective antibiotic, antifungal agent against a number of important and debilitating superficial fungal infections. The kinetics describing the blood level studies of griseofulvin were reported earlier (1). Roth and Blank (2), using a bioassay procedure, demonstrated the presence of griseofulvin in various depths of the stratum corneum within 72 hr. after oral treatment, and it took anywhere between 7 and 56 days for the antibiotic to appear in higher levels of the stratum corneum. They stated that the concentration of griseofulvin was very low but made no attempts to estimate the relative concentration of the antibiotic in the skin.

This paper describes a quantitative determination of griseofulvin in skin, an improved procedure for fluorometric assay of the drug in plasma, and its determination in sweat.

EXPERIMENTAL

Samples—Subjects received tablets of microcrystalline griseofulvin at 12-hr. intervals. Skin samples were collected by scraping the palm with an open dermal curet preceding, during, and posttreatment. About 1–5 mg. of stratum corneum was collected per sample. Each palm was divided into four regions, and scraping was restricted to one region at a time. At the same time as the scrapings, 5–10 ml. of blood was collected in heparinized tubes. Sweat samples were obtained from the subjects by covering their body with plastic bags and subjecting them to sauna baths for 15–20 min.; 75–250 ml. of sweat was collected in the plastic bags.

Equipment and Reagent—*For Skin Samples*—An aerograph[1] equipped with a ^{63}Ni electron-capture detector was used. The following conditions were found satisfactory for the separation of griseofulvin and diazepam from compounds found in the skin which are detected by the electron-capture detector: chromatographic column, 1.83-m. × 0.31-cm. (6-ft. × 0.125-in.) glass tubes packed with 3% OV-17 on Chromosorb W; column temperature, 270°; injection port temperature, 310°; detector block temperature, 340°; and carrier gas (5% methane, 95% argon) flow, 40 ml./min.

Sensitive settings were range 10^{-10} with an attenuation factor of 16×. The reagents were spectral grade benzene and anhydrous ether (freshly opened can). The retention time under these conditions was 3 min. for diazepam and 8.25 min. for griseofulvin (Fig. 1). The ratio of the height of the griseofulvin peak to the height of the diazepam peak obtained from standard mixtures of 1, 2.5, 5, 7.5, 10, 20, 50, and 100 ng. of griseofulvin, each containing 50 ng. of diazepam, was plotted against the griseofulvin concentration to define the standard curve.

For Plasma and Sweat Samples—A spectrophotofluorometer[2] was used for fluorescence determination. The following settings were found to yield maximum fluorescence for griseofulvin: wavelength: excitation, 300 nm.; emission, 420 nm.; slit openings: excitation, 6, emission, 12. A standard curve was obtained by plotting the fluorescence emission against griseofulvin concentration (0.1, 0.2, 0.3, 0.5, 1.0, 1.3, 1.5, and 2.0 mcg. of griseofulvin/ml. of plasma). The reagents were spectral grade methanol and hexane and a freshly opened can of anhydrous ether.

Electron-Capture–GLC Method for Assay of Griseofulvin in Skin—A GLC assay procedure was developed for the determination of griseofulvin in skin samples. About 1–5 mg. of skin was dried over silica gel in a vacuum desiccator and weighed accurately on a balance[3].

A fresh aluminum balance pan was used for weighing each skin sample in order to eliminate the potential for cross-contamination. The skin was transferred in an acid-washed tissue grinder. About 1 ml. of water was added, the sample was homogenized, and the plunger was rinsed with 0.5–1 ml. of distilled water. Griseofulvin

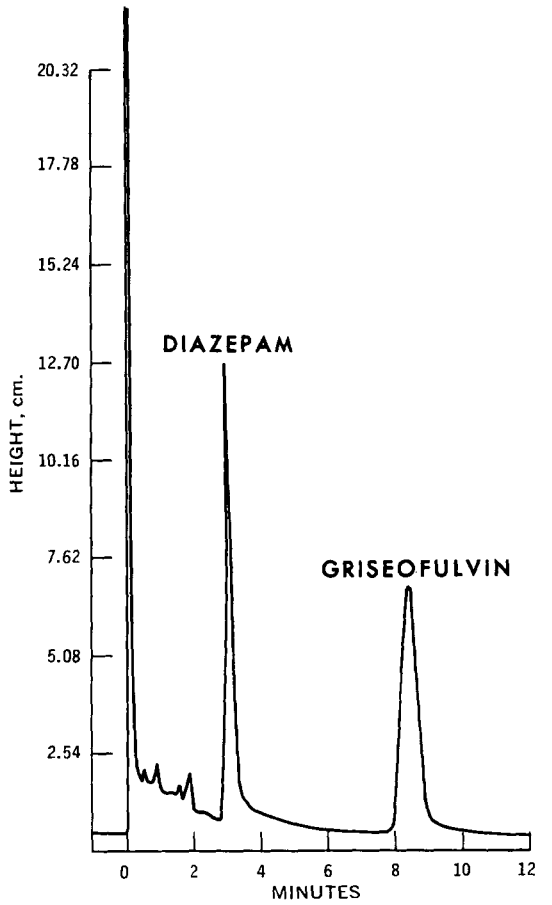

Figure 1—*Gas chromatogram showing pure griseofulvin and diazepam. Curve represents 5-μl. injection of 50 ng. diazepam and 50 ng. griseofulvin in 50 μl. benzene.*

[1] Varian model 1200 series.

[2] Perkin-Elmer model MPF-2A.

[3] Cahn.

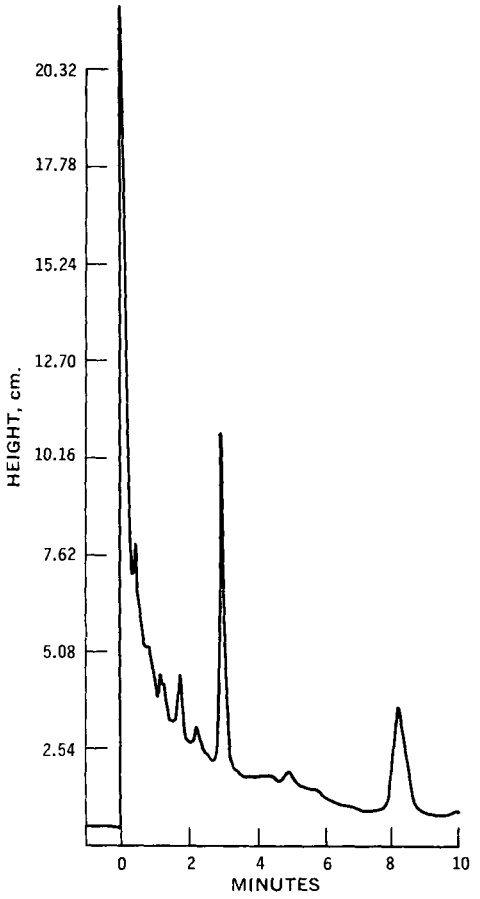

Figure 2—*Gas chromatogram of an extracted skin sample with diazepam. Curve represents 5-μl. injection of 50 ng. diazepam and 3.63 mg. of skin extract in 50 μl. benzene.*

was extracted with 10 ml. of freshly distilled ether. The lower aqueous layer was cooled to −80°, and ether was decanted into another acid-washed tube. One milliliter of ether containing 50 ng. of diazepam was added and evaporated to dryness under N_2. The residue was dissolved in 50 μl. of analytical reagent grade benzene. Duplicate 5-μl. quantities of the resulting solution were injected in GLC, and the average peak height ratio between griseofulvin and diazepam was calculated. The amount of griseofulvin in the sample was calculated from the standard curve. In the initial experiments, the skin was extracted three times with 5 ml. of ether, but later it was proved that one extraction with 10 ml. of ether of homogenized skin was sufficient and improved reproducibility of the measurements.

Spectrophotofluorometric Assay of Griseofulvin in Plasma and Sweat—The method of Rowland *et al.* (1) for the determination of plasma concentration of griseofulvin was modified to eliminate some of the background problem resulting from decomposition of lipoproteins in the plasma samples during storage, which gives an increase in the plasma blank. These lipoproteins are removed by a differential extraction procedure. A 1-ml. sample of plasma was mixed with 1 ml. of water and 10 ml. of freshly distilled ether in an acid-washed tube, well shaken for 1 min., and centrifuged for 10 min. at 2500 r.p.m. The tube was cooled to −80° in dry ice–acetone for 30 sec. The ether layer was decanted and evaporated to dryness under N_2 at 50°. The residue was dissolved in 5 ml. of 1:1 methanol–water, shaken with 5 ml. of hexane for 1 min., and centrifuged. The emission spectrum of the aqueous alcoholic layer was determined by using an excitation wavelength of 300 nm. (uncorrected) on the spectrophotofluorometer. Three drops of concentrated sulfuric acid were added to the cell, and the emission spectrum was again determined. The absorption difference between the two readings at 420 nm. (fluorescence maximum) was used to calculate the concentration of griseofulvin from the standard curve.

To determine the amount of griseofulvin in sweat, the extraction procedure was slightly modified. Two milliliters of sweat was first treated with 1 ml. of 10% sodium bicarbonate and extracted with 10 ml. of ether. The ether layer was washed with water and evaporated to dryness. Griseofulvin concentration was then determined as already described.

RESULTS AND DISCUSSION

Electron-capture detection in GLC is the most sensitive and specific detector known. Griseofulvin has a halogen in its phenolic ring which increases the capturing properties of the molecule. One can detect griseofulvin at subnanogram levels in the sample. When an ether extract from skin is injected in a GLC equipped with flame-ionization detector, a large number of peaks are seen, making it difficult to estimate the true concentration of griseofulvin in the sample. The electron-capture detector does not detect many of these peaks and, therefore, allows a more accurate estimation of griseofulvin in the sample. Figure 2 shows the chromatogram obtained by injecting an ether extract of skin to which 50 ng. of diazepam was added. By using this procedure, samples of tissue as small as 1 mg. (or less) can be used and drug levels as low as 1–2 ng./sample can be determined accurately. Blank skin, when extracted and injected in GLC, does not give any peak in the region of griseofulvin.

The standard curve was obtained by using the ratio of peak height of griseofulvin to peak height of diazepam (Fig. 3). The electron-capture detector has a limitation on its linear dynamic range. The range of linearity in these studies was found to extend from 100 pg. to 7 ng. If the skin sample contains a larger amount of griseofulvin, its peak height ratio will not fall on the linear range of the electron-capture detector. One would have to add additional internal standard to use GLC. After due consideration, it was decided to assay such skin samples by evaporating to dryness and analyzing for griseofulvin content by the fluorometric procedure described for plasma samples. A correction factor of 10% (loss of 5 μl. from 50 μl. solution) was added to the final fluorometric reading.

After the first extraction, the aqueous layer containing the dispersed skin tissue was reextracted twice with ether, and diazepam was added to it. The residue from these extractions was dissolved in benzene and chromatographed. No chromatographic peak for griseofulvin was detected at the highest sensitivity settings, and this was taken as a proof that the extraction of griseofulvin from skin was complete. When injected into the GLC, pure griseofulvin showed exactly the same retention time as that obtained for the griseofulvin peak arising from skin extract. Several samples of skin were pooled, extracted, and chromatographed. The material obtained in the "griseofulvin peak" was trapped and analyzed with a mass spectrophotometer[4]. The mass spectrum of collected griseofulvin was found to be identical with that obtained from an authentic griseofulvin sample.

There are three ways by which griseofulvin could be present in skin: (*a*) held by physical chemical forces such as tissue adsorption or lipid solubility, (*b*) as a metabolite, and (*c*) through the formation

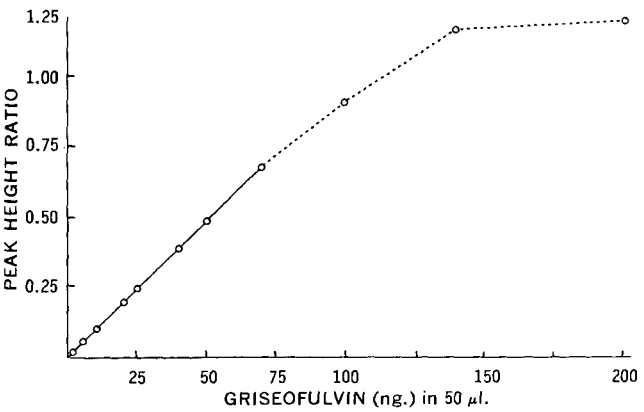

Figure 3—*Standard curve using* x *ng. of griseofulvin and 50 ng. of diazepam in 50 μl. benzene (5 μl. injected in GLC).*

[4] MS902, Associated Electrical Industry.

Table I—Build-Up of Griseofulvin in Skin (ng./mg.) and in Plasma (mcg./ml.)

Subject	0 hr.[a]		4 hr.[a]		8 hr.[a]		30 hr.[a]	
	P	S	P	S	P	S	P	S
Ma	0	0	1.2	1.1	0.8	1.6	2.0	4.1
Bl	0	0	1.6	1.9	1.3	4.8	0.9	6.5
St	0	0	0.5	0.0	0.8	5.0	0.6	5.4
An	0	0	1.5	3.5	1.1	4.7	0.9	7.6
Sm	0	0	1.4	0.0	1.0	1.3	1.2	4.8
Ci	0	0	0.9	0.0	2.1	1.0	2.5	1.7

[a] After first tablet. P = plasma sample. S = skin sample. Subjects were given three tablets at intervals of 12 hr.

of a chemical covalent bond. The known metabolite, 6-desmethyl griseofulvin, is inactive as a fungistatic agent. A sample of authentic 6-desmethyl griseofulvin was injected into the GLC using the same conditions as that used for griseofulvin assay. Its retention time was 6 min., while the retention time for the griseofulvin was 8.25 min. When using the described procedure for extraction, no 6-desmethyl griseofulvin was detected in any skin sample. The chemical structure of griseofulvin is such that if it were chemically bonded to some component of the skin, it would not be possible to convert it back to griseofulvin. Therefore, chemically bonded griseofulvin could not function as an active antifungal agent. It appears, therefore, that the extractable, physically held griseofulvin measured by the assay is the active form of griseofulvin.

Results of the initial skin studies are shown in Table I. Extensive studies on griseofulvin in skin and plasma will be published elsewhere (3). Interestingly, the griseofulvin is easily detected in samples of the palmar horny layer taken from the surface within 4 hr. after oral administration of the drug. This is contrary to the findings of Roth and Blank (2), who reported that it takes 7–56 days for griseofulvin to appear at the various layers of the stratum corneum. Approximately 3 mcg. of griseofulvin/g. of horny layer was detected in 8 hr. The levels built up to 8 ± 2 mcg./g. in the 30-hr. sample and reached 25 mcg./g. of horny layer on prolonged oral administration of griseofulvin.

As noted earlier, a modification of the fluorometric assay was developed. Determination of griseofulvin was carried out in three different solvents: water, 95% distilled ethanol, and 1:1 methanol–water. The latter solvent system was found to be most sensitive. Treatment with hexane allowed extraction of interfering lipids without loss of griseofulvin and reduced the blank value almost to zero. Levels as low as 0.1 mcg./ml. of plasma could be easily detected and determined accurately. The recovery from plasma was checked by comparing the two standard curves: one obtained by adding known amounts in plasma, and the second obtained by taking known amounts in ether. Both were identical. The plasma levels built up to a level of 1–1.5 mcg./ml. of plasma within 4–8 hr. after oral ingestion of the drug when compared to the horny layer samples. This indicates a favorable partition of griseofulvin into skin.

Blank sweat samples showed zero fluorescence at 420 nm. when treated by the described method. The fluorescence spectrum of griseofulvin is not altered when treated by this procedure. The standard curve obtained for griseofulvin determination in plasma was applicable for its determination in sweat also. It was found that 0.2–0.3 mcg./ml. of griseofulvin was lost in sweat. To the ether extract of sweat (after washing with bicarbonate), 50 ng. of diazepam was added; this was evaporated to dryness, and the residue was dissolved in 50 μl. benzene. A 5-μl. quantity was injected in GLC. Once again, no 6-desmethyl griseofulvin was detected in sweat by this procedure.

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