Method B—A solution of IV (0.2 g) in methanol (20 ml) was treated with hydroxylamine (0.1 g) and refluxed gently for 6 hr. Removal of the solvent left a white solid (0.2 g, 90% yield), which was identical (melting point, IR, and NMR analyses) with the material obtained by Method A.

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High-Performance Liquid Chromatographic Analysis of Griseofulvin in Drug Substance and Solid Dosage Forms: Separation of Impurities and Metabolites

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Abstract □ A high-performance liquid chromatographic (HPLC) system, consisting of a methanol–water (3:2 v/v) mobile phase and a Zorbax CN column with *m*-phenylphenol as the internal standard, was utilized to determine the purity of griseofulvin bulk drug substance, to assay griseofulvin in powders, tablets, capsules, and boluses, and to separate griseofulvin from its metabolites. The method was tested on commercial griseofulvin samples, griseofulvin tablets, and a mixture of griseofulvin and its metabolites. The HPLC method is compared to a GLC method.

Keyphrases □ Griseofulvin—high-performance liquid chromatographic analysis, purity and stability of drug substance, powders, tablets, capsules, and boluses, metabolites □ High-performance liquid chromatography—analysis, griseofulvin, purity and stability of griseofulvin dosage forms, metabolites □ Antifungal agents—griseofulvin, high-performance liquid chromatographic analysis of powders, tablets, capsules, and boluses

Griseofulvin has well-established fungistatic activity against various species of *Microsporum, Epidermophyton,* and *Trichophyton.* Usual dosage forms are powders, capsules, tablets, and boluses. Liquid–solid extraction methods for the preparation of griseofulvin for subsequent analysis include extraction of griseofulvin from tablets with boiling alcohol (1) and extraction from tablets with warm chloroform (2, 3).

The drug substance has been determined by several methods, the most common of which is a simple UV analysis (1, 4, 5). Other methods include polarography (6), GLC (2, 3), spectrofluorometry (7, 8), colorimetry (9–11), iodimetry (12), paper chromatography (13), and TLC (14–17). Liquid chromatography has been useful for the analysis of griseofulvin in crude mycelium (18) and in plasma (19).

This report describes a specific, simple, and robust high-performance liquid chromatographic (HPLC) procedure. It is applicable to the drug substance and solid dosage forms and to the separation of griseofulvin from its metabolites. The procedure is offered as an alternative to the GLC method (2), which suffers from the difficulty of drug analysis at high temperatures.

EXPERIMENTAL

Materials—*m*-Phenylphenol[1], reagent grade dichloromethane[2], sodium chloride[3], and anhydrous sodium sulfate[4] were obtained from commercial sources.

Apparatus—The modular high-pressure liquid chromatograph was equipped with a constant-flow pump[5], a valve-type injector[6], a fixed-wavelength (254-nm) UV detector[7], and a strip-chart recorder[8]. Stainless steel columns (4.6 mm × 30 cm) were packed with fully porous 10-μm silica particles to which a monomolecular layer of cyanopropylsilane[9] was chemically bonded. A rotating mixing wheel[10] and centrifuge tubes[11] were used to extract the samples. A data acquisition system[12] was used for both peak height and area measurements.

Chromatographic Conditions—The mobile phase was methanol–water (3:2). This solution was passed through a 0.45-μm filter[13], degassed, and then pumped through the HPLC system at a rate of 1 or 2 ml/min.

Table I—Analysis for Purity and Dechlorogriseofulvin Content in Griseofulvin Batches from Worldwide Sources

Batch	Purity[a], %	Dechlorogriseofulvin Content, %
0672-F2	100[b]	0.6
UGFP-1961	99.4	1.1
UGRB-505	96.6	2.0
GU-4830S	97.0	3.2
GU-4910S	97.0	2.8
26	99.7	0.9
5209	98.9	1.6
15 (805/7)	97.9	1.5

[a] Determined using USP XIX, p. 584. [b] USP reference standard.

[1] Eastman Organic Chemicals, Rochester, N.Y.
[2] Matheson, Coleman and Bell, Norwood, Ohio.
[3] NF grade, J. T. Baker Chemical Co., Phillipsburg, N.J.
[4] NF grade, Fisher Scientific Co., Fair Lawn, N.J.
[5] Model M-6000 A chromatography pump, Waters Associates, Milford, Mass.
[6] Universal injector model U6K, Waters Associates, Milford, Mass.
[7] Model 440 absorbance detector, Waters Associates, Milford, Mass.
[8] Class 19, No. 196711-008-000-506-01, Honeywell, Fort Washington, Pa.
[9] Zorbax CN column, E.I. duPont de Nemours & Co., Wilmington, Del.
[10] Rugged Rotator, Craft Apparatus, New York, N.Y.
[11] Bellco Glass Co., Vineland, N.J.
[12] PDP 11/34 minicomputer, Peak 11 Software Digital Electronics Corp., Maynard, Mass.
[13] Metricel membrane filter DM-450, Gelman Instrument Co., Andover, Mich.

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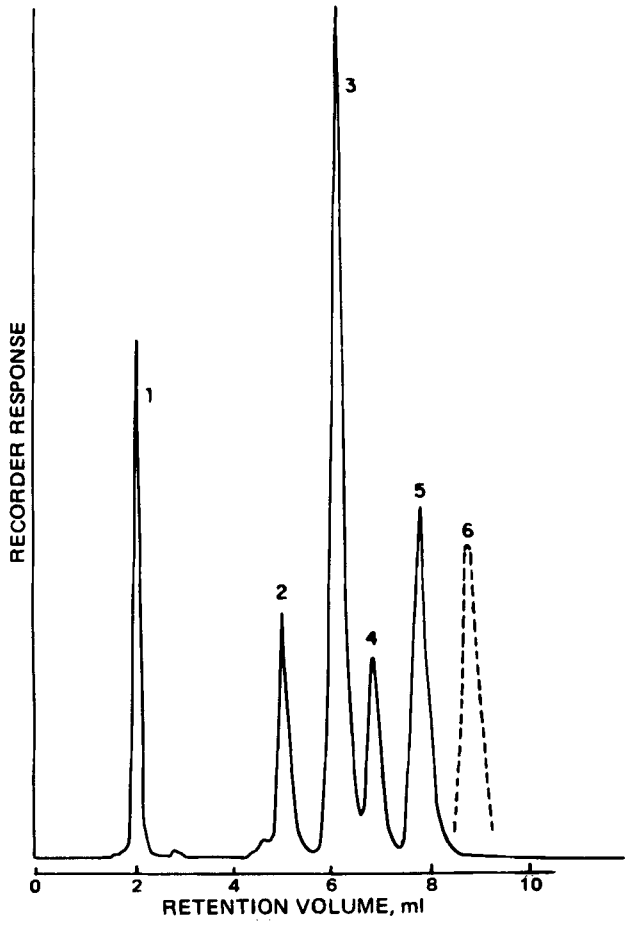

Figure 1—*HPLC separation of biosynthetic impurities (substances identified in the fermentation broth). Key: 1, griseofulvic acid; 2, dechlorogriseofulvin; 3, griseofulvin; 4, isogriseofulvin; 5, tetrahydrogriseofulvin; and 6,* m*-phenylphenol.*

Internal Standard Solution—The internal standard was *m*-phenylphenol prepared as a 1-mg/ml solution in methanol.

Standard Solution—Griseofulvin standard, ~60 mg, was weighed accurately into a 50-ml volumetric flask and was dissolved and diluted to 50 ml with methanol. Five milliliters of this solution was pipetted into a 50-ml volumetric flask. Four milliliters of the internal standard solution also was added by pipet, and the solution was diluted to 50 ml with methanol.

Standard Chromatogram—Twenty microliters of the standard solution was injected into the liquid chromatograph, and the chromatogram was recorded. The peak heights obtained were used in the calculations for griseofulvin.

Griseofulvin Samples—Griseofulvin samples were obtained from England[14] (Batches UGFP-1961 and UGRB-505), Japan[15] (Batches GU-4830S and GU-4910S), Rumania[16] (Batches 26 and 5209), Austria[17] [Batch 15 (805/7)], and the USP reference file[18] (Batch 0672-F2).

Impurities—Isogriseofulvin[14], dechlorogriseofulvin[14], griseofulvic acid[14], and tetrahydrogriseofulvin[19,20] were obtained. Dihydrogriseofulvin and additional tetrahydrogriseofulvin were synthesized[21] *via* the catalytic reduction of griseofulvin (19). The metabolites, 4-demethylgriseofulvin and 6-demethylgriseofulvin, were obtained as a result of previous research (20, 21). Identities were confirmed by comparison of the IR spectra to

[14] Glaxo Holdings Ltd., London, England.
[15] Nippon-Kayaku Co. Ltd., Chugai Boyeki (America) Corp. Importers and Exporters, New York, N.Y.
[16] Rumanian State Trading Agency; provided by the Jerdan Chemical Corp., Valley Stream, N.Y.
[17] Biochemi Gesmb H-Werk Kundl, Austria; provided by R. W. Greeff and Co., New York, N.Y.
[18] USP XIX, p. 584.
[19] Mr. T. Alexander, HFD-436, Food and Drug Administration, Washington, D.C.
[20] Dr. F. Bailey, I.C.I. Ltd. Pharmaceutical Division, Macclesfield, Great Britain.
[21] Schering-Plough Research, Bloomfield, N.J.

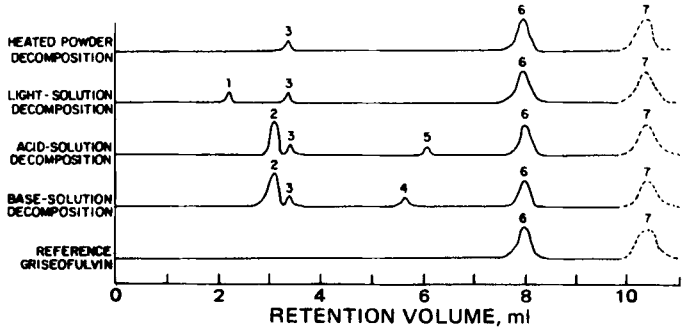

Figure 2—*Retention volume of decomposition impurities (substances resulting from extreme, accelerated decomposition studies). Key: 1, 3, 4, and 5, unknown minor decomposition products; 2, griseofulvic acid; 6, griseofulvin; and 7,* m*-phenylphenol.*

authentic references (isogriseofulvin, dechlorogriseofulvin, and griseofulvic acid) or by mass spectral studies (dihydrogriseofulvin, tetrahydrogriseofulvin, 4-demethylgriseofulvin, and 6-demethylgriseofulvin).

Accelerated Decomposition Studies—Accelerated, extreme degradation of griseofulvin was accomplished by several methods:

1. Griseofulvin drug substance was heated at 75° for 3 weeks.
2. Griseofulvin (0.25 mg/ml) was dissolved in methanol–0.1 *N* NaOH (4:1) and allowed to stand for 7 days.
3. Griseofulvin (0.25 mg/ml) was dissolved in methanol–0.1 *N* HCl (4:1) and allowed to stand for 7 days.
4. Griseofulvin was dissolved in methanol–water (4:1) in a Pyrex volumetric flask and exposed to daylight for 14 days through window glass.

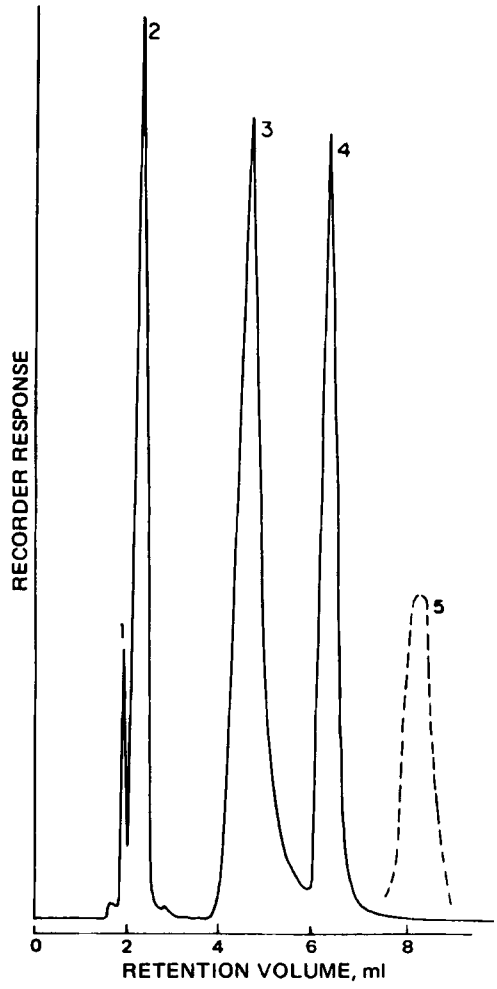

Figure 3—*HPLC separation of 4-demethylgriseofulvin and 6-demethylgriseofulvin metabolites. Key: 1, griseofulvic acid (impurity); 2, 6-demethylgriseofulvin; 3, 4-demethylgriseofulvin; 4, griseofulvin, and 5,* m*-phenylphenol.*

Griseofulvin Drug Substance Purity Determination—Approximately 60 mg of a griseofulvin test sample was weighed accurately into a 50-ml volumetric flask and dissolved and diluted to volume with methanol. Five milliliters of the sample solution was pipetted into a 50-ml volumetric flask. Four milliliters of the internal standard solution was added, and the solution was diluted to volume. Then 20 μl of the sample was injected into the liquid chromatograph operating at the described conditions. The percent purity was calculated from:

$$\text{percent griseofulvin} = \left(\frac{P'_{sple}}{P'_{is}}\right)\left(\frac{P_{is}}{P_{std}}\right)\left(\frac{W_{std}}{W_{sple}}\right) \times 100 \qquad \text{(Eq. 1)}$$

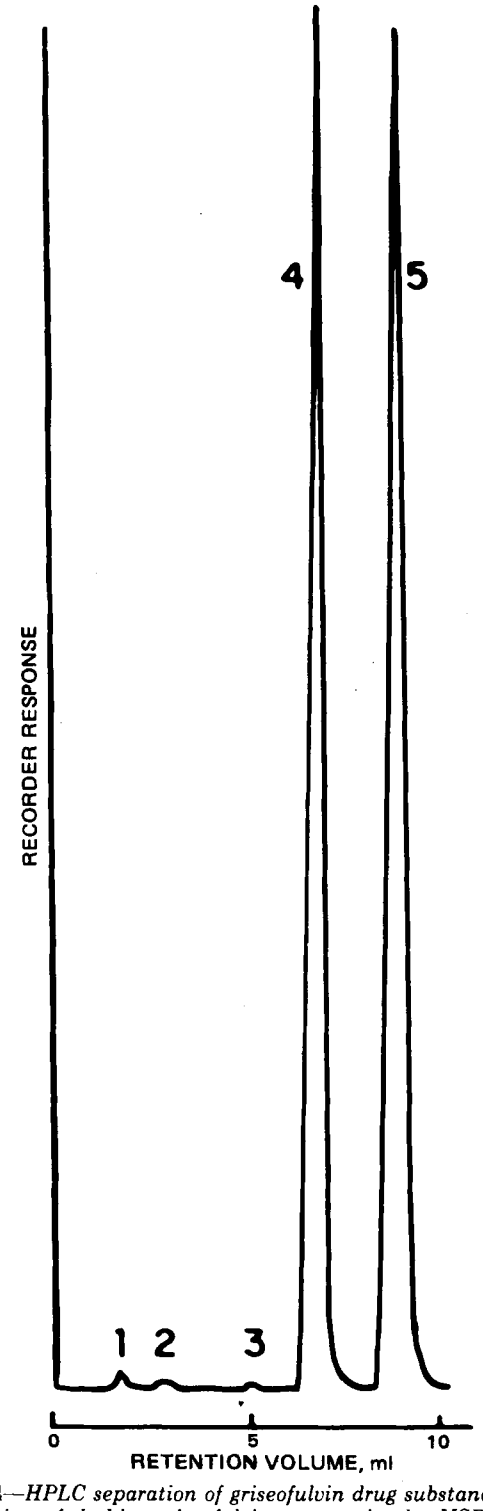

Figure 4—*HPLC separation of griseofulvin drug substance and demonstration of dechlorogriseofulvin content in the USP standard (Batch 0672-F2). Key: 1, solvent; 2, griseofulvic acid; 3, dechlorogriseofulvin; 4, griseofulvin; and 5,* m*-phenylphenol.*

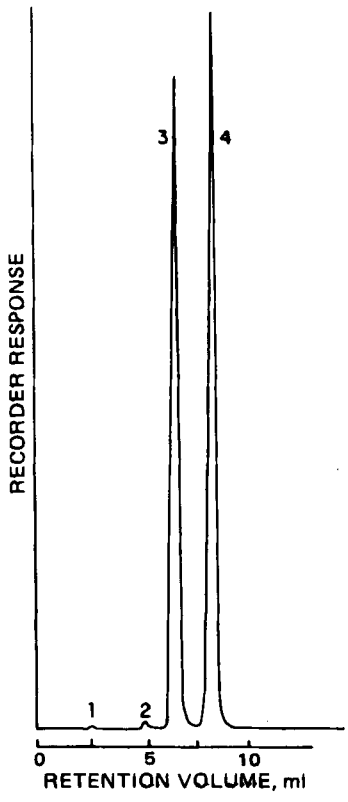

Figure 5—*High-performance liquid chromatogram of griseofulvin extracted from tablets (Batch 7934-45A). Key: 1, griseofulvic acid; 2, dechlorogriseofulvin; 3, griseofulvin; and 4,* m*-phenylphenol.*

where P'_{sple} is the peak height of the sample in the sample chromatogram, P'_{is} is the peak height of the internal standard in the sample chromatogram, P_{is} is the peak height of the internal standard in the standard chromatogram, P_{std} is the peak height of the standard in the standard chromatogram, W_{std} is the weight of the standard, and W_{sple} is the weight of the sample.

Griseofulvin in Solid Dosage Form Determination—A sample of well-mixed ground powder (from powders, tablets, capsules, or boluses) containing 250 mg of griseofulvin was weighed accurately into a 50-ml screw-capped centrifuge tube[11]. Then 20 ml of 10% aqueous sodium chloride solution and 20 ml of dichloromethane were added. Mixing resulted in extraction of the griseofulvin into the dichloromethane after the tube was placed on a rotating wheel[10] for 10 min.

The dichloromethane layer was transferred with a 20-ml syringe and a 16-gauge needle and filtered through ~200 mg of anhydrous sodium sulfate into a 200-ml volumetric flask. The extraction was performed three times and the extracts were combined (total of 60 ml) and diluted to volume. A 5.0-ml portion was transferred to a 50-ml volumetric flask and evaporated to dryness. The *m*-phenylphenol internal standard solution, 4.0 ml, was added, and the griseofulvin residue was dissolved in methanol and diluted to 50 ml. Then 20-μl portions were injected into the liquid chromatograph.

The griseofulvin content was calculated from:

$$\text{milligrams per unit} = \left(\frac{P'_{sple}}{P'_{is}}\right)\left(\frac{P_{is}}{P_{std}}\right)\left(\frac{W_{std}}{W_{sple}}\right)(W_{avg}) \times 4 \qquad \text{(Eq. 2)}$$

where W_{avg} is the average weight of the sample unit (gram, tablet, capsule, or bolus).

Table II—Comparison of the Accuracy of the HPLC and GLC (2) Methods for Griseofulvin Drug Substance

Batch	Mean Purity, %: HPLC Method (n = 4)	Mean Purity, %: GLC Method (n = 2)
S-03294	99.4 (99.3–99.6)	99.7 (99.6, 99.8)
3-2-58161	98.2 (97.9–98.4)	99.1 (98.9, 99.3)
UGRB-505	96.6 (96.3–96.9)	97.1 (96.8, 97.5)

Table III—Comparison of the Accuracy of the HPLC and GLC (2) Methods for Griseofulvin Tablets

Griseofulvin Tablets, mg	Content, mg/tablet	
	HPLC Method (n = 12)	GLC Method (n = 4)
125	125 (122–127)	126 (125–127)
250	247 (244–253)	250 (249–251)

Dechlorogriseofulvin Content Determination—The area under the dechlorogriseofulvin and griseofulvin peaks was obtained using a suitable area measurement integrator[12]. The percent dechlorogriseofulvin content was calculated according to:

$$\text{percent dechlorogriseofulvin} = \frac{\left[A_{DCG}\left(\frac{a_2}{a_1}\right)\right]}{\left[A_{DCG}\left(\frac{a_2}{a_1}\right)\right] + A_G} \times 100 \quad \text{(Eq. 3)}$$

where A_{DCG} is the area of the dechlorogriseofulvin peak, A_G is the area of the griseofulvin peak, a_2 is the absorptivity of griseofulvin at 254 nm, and a_1 is the absorptivity of dechlorogriseofulvin at 254 nm.

RESULTS AND DISCUSSION

The HPLC separation of biosynthetic impurities that were identified in the fermentation broths (18, 22) is demonstrated in Fig. 1. The HPLC separation of decomposition impurities that result when griseofulvin is subjected to accelerated, extreme decomposition is demonstrated in Fig. 2. The separation of 4-demethylgriseofulvin (23) and 6-demethylgriseofulvin (13) metabolites is shown in Fig. 3. The system is applicable to the determination of nonconjugated metabolites.

The data presented in Figs. 1–3 show that an internal standard eluting with a retention volume of ~10 ml will not interfere with a common biosynthetic impurity, decomposition product, or metabolite. *m*-Phenylphenol, whose retention is demonstrated in Figs. 1–3, meets this criterion.

To ascertain the purity of commercial griseofulvin, eight batches were obtained from worldwide sources and then were assayed by the described method. The purity data are given in Table I. The present study showed, as was reported previously (2, 18), that dechlorogriseofulvin was the major impurity and that it was common to all of the batches tested. The USP standard (Fig. 4) contained 0.6% of this impurity. Dechlorogriseofulvin also was separated by TLC using a silica gel adsorbent and ethyl acetate developing solvent. The absence of other significant impurities was confirmed. Table II compares the accuracy of the HPLC results to those of a GLC method (2) for griseofulvin drug substance.

The analysis of griseofulvin in both fresh and aged samples of powders, tablets, capsules, and boluses was performed by the extraction and HPLC procedures described previously. The analysis of griseofulvin tablets[22], 125 and 250 mg, serves as an example. The sample HPLC curve for griseofulvin extracted from griseofulvin tablets is given in Fig. 5. The extraction efficiency was 98% for a single extraction and 100% for the described method. A linear plot of peak height *versus* griseofulvin concentration response was obtained for griseofulvin in ground tablet powder from 62.5 to 500 mg of griseofulvin/sample. The least-squares coefficient was 0.99995. A system suitability test (reproducibility of six samples analyzed on the liquid chromatograph described) gave a relative standard deviation of ±0.3%.

The accuracy of the method was tested by the addition of griseofulvin to a placebo (procedural amount) and analysis by the described method. Recoveries were 100% with respect to a similarly chromatographed standard. The assay results are compared to those obtained by the GLC method of Margosis (2) in Table III.

[22] Fulvicin P/G griseofulvin ultramicrosize tablets, Schering.

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