CHROM. 6019

ANALYSIS OF ANTIBIOTICS BY GAS CHROMATOGRAPHY

III. GRISEOFULVIN*

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SUMMARY

Pharmaceutical grade griseofulvin in bulk and dosage form can be assayed by a new gas–liquid chromatographic procedure. The method involves dissolution in chloroform, injection into an OV-17 column, and normalization of the response with tetraphenylcyclopentadienone as an internal standard. Dechlorogriseofulvin, which is also found in all griseofulvin pharmaceutical products, can be determined simultaneously. Both components have been identified by mass spectrometry. The griseofulvin Food and Drug Administration Working Standard was critically analyzed for impurities by gas-liquid chromatography and differential scanning calorimetry.

INTRODUCTION

Griseofulvin (7-chloro-2′,4,6-trimethoxy-6′β-methylspiro-[benzofuran-2(3H), 1′-[2]-cyclohexene]-3,4′-dione) is an antifungal antibiotic obtained as a metabolic product from various species of *Penicillia*. It is used primarily in the systemic treatment of dermatomycoses.

Griseofulvin may be assayed by microbiological[1–3] or by chemical methods. The latter include colorimetry[4]; spectrophotometry either directly[3], after acidic conversion to isogriseofulvin[5], or after partitition chromatographic separation[6]; fluorimetry directly[7] or after thin-layer chromatography[8]; and gas chromatography, as applied to simulated samples[9] and fermentation extracts[10]. The spectrophotometric method is the official method and the method of choice in the United States[3] and in the United Kingdom[11].

This study presents and discusses the analysis of griseofulvin in pharmaceutical bulk and in dosage forms by direct gas–liquid chromatography (GLC) in chloroform and normalization of the detector response with tetraphenylcyclopentadienone. This method is rapid, simple, specific, precise, accurate, and reliable, even when structurally related compounds such as dehydrogriseofulvin, dechlorogriseofulvin, and/or isogriseofulvin, which are biosynthesized concomitantly with griseofulvin, may be present in the final product. The differentiation of griseofulvin from related compounds may be significant if these compounds have pharmacological

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and toxicological properties differing from those of the active principle of interest. The method also provides for the simultaneous determination of dechlorogriseofulvin, which has been found in all griseofulvin products tested by GLC at the National Center for Antibiotic Analysis and whose qualitative characterization has been corroborated by combination gas-liquid chromatography-mass spectrometry (GLC-MS). In addition, the determination of the purity of the FDA Working Standard, as determined by GLC, has been substantiated by differential scanning calorimetry (DSC).

EXPERIMENTAL

Apparatus

Gas-liquid chromatography. A Perkin-Elmer Model 900 gas chromatograph, equipped with an inlet system modified to accommodate a one-piece glass column directly from the site of injection, and a flame ionization detector were used, together with a 1-mV range recorder (Honeywell Electronik 194) at a chart speed of 0.1 in. (2.54 mm)/min, and an electronic integrator (Infotronics CRS-100) for peak area measurements. Column: glass, coiled, 3 ft.(0.914 m) × 4 mm I.D., packed with 1% OV-17 on Gas-Chrom Q, 100–120 mesh (ready-coated from Applied Science Labs., State College, Pa.). Temperatures: column*, 245°; injector, 260°; manifold, 260°. Carrier gas: helium at 60 ml/min and 50 p.s.i.; hydrogen at 22 p.s.i.; and air at 30 p.s.i. (both adjusted for maximal response). Current: 2×10^{-9} A f.s.d., or adjusted to obtain peak height greater than 50% f.s.d. depending upon peak sharpness.

Gas-liquid chromatography-mass spectrometry. An Atlas CH-4 mass spectrometer connected to a GLC column (1.83 m × 4 mm, glass), packed with 3% OV-101 on Gas-Chrom Q via a permeable membrane separator as described by DAMICO AND BARRON[12].

An LKB Model 9000 was used as previously cited[13], except that a 1.83 m × 2 mm glass column packed with 1% OV-17 on Supelcoport was operated at an isothermal temperature of 270°.

Cryoscopy. A Perkin-Elmer differential scanning calorimeter Model DSC-1B was used for the purity determination of the FDA Working Standard as described by PLATO AND GLASGOW[14].

Reagents

The solvent chloroform was reagent grade.

The internal standard solution tetraphenylcyclopentadienone is dissolved in chloroform to obtain a solution of about 5 mg/ml.

The reference standard (griseofulvin standard) used in this study was the FDA Working Standard with an assigned potency of 1,000 μg/mg corrected to 903 μg/mg (see text).

Sample preparation

Bulk and standard materials. About 125 mg of sample is accurately weighed into a 25-ml volumetric flask and is dissolved in chloroform with vigorous stirring

* 245° is the preferred temperature for routine testing; 225° was used for some preliminary testing (see Figs. 1 and 3).

(Vortex), and the solution is diluted to volume. A 2.00-ml aliquot is transferred to a conical centrifuge tube and evaporated to dryness on a steam bath under a current of dry air or nitrogen.

Solid dosage forms (capsules, tablets, boluses). An accurately weighed sample from a blended pool of capsule contents, ground tablets, or boluses is stirred with chloroform in a volumetric flask, with gentle heat if necessary, until the active principle is dissolved. The mixture is allowed to cool and settle, and then diluted to volume with chloroform. An aliquot of the supernatant, equivalent to about 10 mg, is transferred to a centrifuge tube and evaporated as above.

Suspensions. A 5-ml portion (equivalent to 250 mg) of the mixed suspension is transferred to a separatory funnel and diluted to about 25 ml with water. The mixture is extracted once with 25 ml of chloroform and then twice more with 10 ml of chloroform. Each extract is sequentially back-washed with 5 ml of water and filtered through a chloroform-moistened wad of glasswool; the filtered extracts are then combined into a 50-ml volumetric flask and diluted to volume with chloroform. A 2.00-ml aliquot is transferred to a centrifuge tube and evaporated as above.

Procedure

To each dried sample, 1.00 ml of the internal standard solution is added and the mixture is stirred vigorously to obtain a uniform solution. One microliter of this solution is injected into the gas chromatograph and the area of each resultant peak is measured by a suitable technique. The potency of griseofulvin is determined by direct comparison of the ratio of the peak areas (griseofulvin/tetraphenylcyclopentadienone) with that of the griseofulvin standard treated in an identical manner.

RESULTS AND DISCUSSION

Preliminary investigation showed that methyl-substituted silicones such as OV-1 used as stationary phases produced chromatograms with excess peak tailing and were of little practical use, especially in the analyses of large numbers of samples. The packing used in this study, OV-17, is a 50 % phenyl-substituted silicone polymer coated on flux-calcined, acid- and base-washed, silane-treated diatomaceous earth. The column was cured at 340° for a minimum of 1 h with no carrier gas flow, and then sample-conditioned at 250° with a helium flow until a stable baseline was obtained. The suitability test for inertness of support showed a single symmetrical peak for injected cholesterol with no evidence of decomposition. This column demonstrated an apparent efficiency of better than 1350 theoretical plates for both griseofulvin and the internal standard, with a symmetry factor at 5 % peak height of about 1.4 for the former and 1.1 for the latter. A resolution factor of better than 3.5 was calculated for the system.

In order to verify the capability of this GLC system to resolve complex mixtures, a qualitative mixture of griseofulvin, dechlorogriseofulvin, dehydrogriseofulvin, isogriseofulvin, and tetraphenylcyclopentadienone in chloroform was injected into the gas chromatograph. The resulting chromatogram (Fig. 1) and those obtained from similar solutions of each component injected individually showed sharp,

symmetrical peaks that were well-resolved, except for dehydrogriseofulvin and isogriseofulvin which merged together. The relative retention time for each of these analogs is listed in Table I. This kind of separation should prove most valuable in quality control from initial crude fermentation materials to finished products. The only detectable impurity peak found in chromatograms of all samples (bulk and dosage forms) tested was tentatively ascribed to dechlorogriseofulvin. However, the chromatogram of a crude bulk material also showed an additional impurity peak which was ascribed to dehydrogriseofulvin, based on retention time. Proof

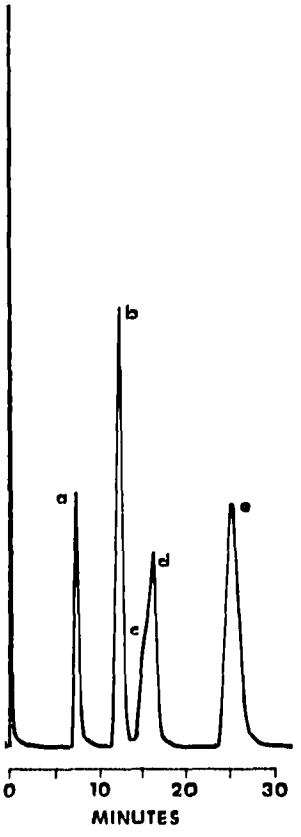

Fig. 1. Gas chromatogram at 225° of a qualitative mixture of griseofulvin and related compounds. (a) Dechlorogriseofulvin; (b) griseofulvin; (c) dehydrogriseofulvin; (d) isogriseofulvin; (e) tetraphenylcyclopentadienone (internal standard).

TABLE I

RELATIVE RETENTION TIMES OF GRISEOFULVIN AND SOME RELATED COMPOUNDS

GLC conditions: 3 ft. (0.914 m) × 4 mm I.D. glass column packed with 1% OV-17 on Gas-Chrom Q (100–120 mesh); column temperature, 225°; helium carrier gas flow-rate, 60 ml/min; recorder chart speed, 0.1 in. (2.54 mm)/min.

Compound	*Relative retention time*
Dechlorogriseofulvin	0.60
Griseofulvin	1.00
Dehydrogriseofulvin	1.20
Isogriseofulvin	1.29
Tetraphenylcyclopentadienone	2.02

of identity may be established further by comparing retention times of unknown and authentic samples on columns of different types and/or polarity. In this case, however, corroboration of identity was obtained by combination GLC–MS as discussed below.

Several solvents were initially tested for applicability in this analysis: benzene, ethyl acetate, pyridine, acetone, and chloroform performed well, whereas various alcohols, cyclohexane, acetonitrile, and dimethylsulfoxide did not. Chloroform was selected because it is immiscible with water and volatile, facilitating sample preparation, and exhibits minimal tailing.

In the preparation of bulk samples or standards, the evaporation step is not really necessary but is recommended so that the procedure is consistent with that of sample preparation for the dosage forms.

Linear detector response was obtained for a hundred-fold range of a total injected sample from 0.40 μg to 40 μg; the normal quantity injected was about 10 μg. Accordingly, a detectable limit of response has been estimated to be better than 1 ng under the operating conditions described. In actual test, a 1-ng injection was well detected with sensitivity to spare.

By using an electronic integrator for peak area measurements, the precision was calculated from five replicates of a standard solution injected at different time intervals between samples during the course of a day. The coefficient of variation was 0.49% for the griseofulvin peak normalized with the tetraphenylcyclopentadienone and about 2% for the dechlorogriseofulvin peak present at the 0.66% concentration level. A total of five injections from two separate weighings of the crude material similarly showed a coefficient of variation of 0.33% for the normalized griseofulvin peak, and 1.6 and 5.4% for the dechloro- and the dehydrocongeners present at the 9 and 0.7% concentration levels, respectively.

To determine the accuracy of the method, values obtained by GLC were compared with those obtained by the official spectrophotometric method. The results are listed in Tables II and III for bulk and dosage forms, respectively. The GLC analyses are expected to be more reliable because they separate and compare directly only to the griseofulvin peak exclusive of contaminants, whereas the UV method relates to the total absorbance measured at 292 nm, irrespective of the variety and quantity of contaminants that may be present. Thus, highly impure materials, such as the crude bulk sample, would yield grossly aberrant results in the UV method. By comparing both methods in the paired t test performed on results obtained on the certified bulk samples listed in Table II, a statistically significant difference was observed between the official spectrophotometric method and the GLC method. However, when results of the UV method were compared with results obtained with the sum of both GLC peaks, no such difference was noted.

Analysis of impurity

An impurity peak, tentatively identified as dechlorogriseofulvin based on the retention time shown in Fig. 2, was found in the standard reference material and in every batch of bulk or dosage form of the antibiotic analyzed by GLC. The concentration range was found to be about 0.2–1% from one manufacturing source and up to about 2% from another. Corroboration and positive identity of the two peaks was achieved by combination GLC–MS. The electron impact mass spectrograms

TABLE II

GLC DETERMINATION OF GRISEOFULVIN BULK

Sample (No.)	*Dechloro-griseofulvin (%) by GLC*	*Standard (%) by GLC*	*Total (%) by GLC*	*% by official method*	
				Lab. 1	*Lab. 2*
1	0.67	99.3	100.0	100.0	98.0
2	1.09	99.3	98.3	98.1	98.0
3	1.17	98.8	100.0	100.0	98.0
4	1.22	98.7	99.9	97.9	98.3
5	1.04	98.3	99.4	98.1	97.9
6	1.01	97.8	98.8	98.1	98.0
7	0.75	98.0	99.3	99.5	98.3
8	1.77	99.2	98.0	99.3	98.2
9	1.02	98.3	99.3	98.1	99.4
10	0.07	98.0	99.0	98.0	97.8
11	0.09	97.9	98.0	98.2	99.2
12	0.07	99.6	100.0	100.3	98.1
13	<0.15	99.8	100.0	102.0	99.3
14	1.06	98.5	99.5	99.9	100.0
15	0.53	98.4	98.9	98.2	100.0
16	0.33	97.5	97.9	99.2	99.5
17	<0.15	97.6	97.8	100.8	99.4
18	0.60	98.7	99.3	98.2	99.3
19	0.57	99.1	99.7	95.3	98.7
20	0.48	97.9	98.4	99.7	100.0
21	0.51	98.1	98.6	98.7	99.2
22	0.05	98.4	99.0	99.1	99.5
23	0.85	99.7	100.0	98.7	101.0
24[a]	8.85	80.2	98.8[b]	—	99.3

[a] Crude bulk material.
[b] Includes 0.70% dehydrogriseofulvin.

TABLE III

GLC DETERMINATION OF GRISEOFULVIN DOSAGE FORMS

Sample (No.)	*Label claim (mg)*	*Dosage form*	*Found by GLC*	*% of label claim by official method*	
				Lab. 1	*Lab. 2*
1	500	tablets	101.3	100.8	97.9
2	500	tablets	101.6	99.0	98.6
3	250	tablets	102.3	103.6	100.0
4	250	tablets	99.3	99.2	100.0
5	250	tablets	98.2	96.0	101.0
6	250	tablets	97.2	97.6	100.8
7	250	capsules	100.3	104.4	99.6
8	250	capsules	100.1	100.8	98.4
9	125	capsules	93.5	94.8	—
10	50 (per ml)	suspension	98.6	96.2	99.6
11	50 (per ml)	suspension	98.3	97.1	104.6

obtained from two different instruments showed the characteristic mass peaks due to the molecular ions and fragmentation pattern of dechlorogriseofulvin and griseofulvin as described and discussed by BALLANTINE AND FENWICK[15]. The crude

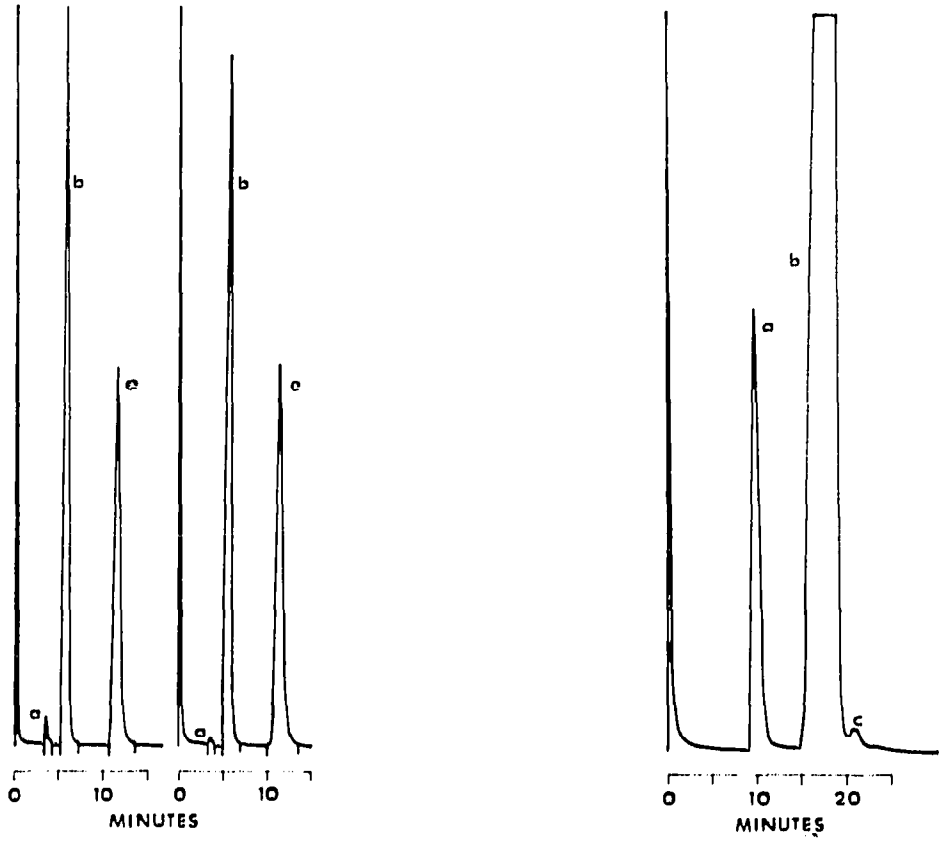

Fig. 2. Gas chromatogram at 245° of griseofulvin from dosage forms. (a) Dechlorogriseofulvin; (b) griseofulvin; (c) tetraphenylcyclopentadienone (internal standard).

Fig. 3. Gas chromatogram at 225° of crude griseofulvin. (a) Dechlorogriseofulvin; (b) griseofulvin; (c) dehydrogriseofulvin.

bulk material exhibited an additional peak which was ascribed to dehydrogriseofulvin based on relative retention time (Fig. 3). These griseofulvin substances have proven to be quite stable as evidenced by the GLC peak shapes and the mass spectrograms.

Standard reference material

Because the FDA Working Standard was found to contain a small amount of dechlorogriseofulvin, it was subjected to closer scrutiny. DSC as demonstrated by PLATO AND GLASGOW[14] was considered an ideal adjunct in this case. The correlation between GLC and DSC was very good. The protocol of analysis, reported in 1963 for this sample, gave a purity of 98.8 %, obtained by solubility analysis. The GLC analysis showed 0.66 area % of dechlorogriseofulvin (uncorrected for a possible differing response factor, but probably well within experimental limits), and no other detectable impurities, giving a maximum purity of 99.34%. These compare to 0.76 mole % of impurity (or a purity of 99.24 mole %) by DSC, which in addition yields a corrected thermodynamic melting point of 216.6°, and a heat of fusion (ΔH) of 12,700 cal/mole.

Because of the high stability of these materials, preparative GLC may be considered as a means of obtaining such reference compounds of very high purity.

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