GLC Determination of Griseofulvin in Human Plasma

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Abstract □ A specific and quantitative GLC method for the determination of griseofulvin in human plasma is described. The method involves extraction with ether, evaporation, addition of the internal standard dissolved in benzene, and GLC analysis using an electron-capture detector. The sensitivity of the method is 0.05 μg/ml of plasma. The results obtained with this specific GLC method were compared with the results obtained with the more frequently used spectrofluorometric method by analyzing duplicate plasma samples obtained from 12 subjects following a single dose of griseofulvin. It was deduced that the 30% higher plasma levels obtained spectrofluorometrically were due to the coextraction and presence of the metabolite 6-demethylgriseofulvin in the assay solutions.

Keyphrases □ Griseofulvin—GLC analysis in plasma, compared to spectrofluorometric method □ GLC analysis—griseofulvin in plasma, compared to spectrofluorometric method □ Antifungal agents—griseofulvin, GLC analysis in plasma

Griseofulvin is an orally active antifungal agent widely used in the treatment of fungal infections in animals and humans (1).

Several spectrofluorometric methods for the determination of griseofulvin in plasma (2–7) have been described. Most of them are modifications of the original method (2). The latest modification (7), which was to be preferred because of its high sensitivity and low blank values, was also very time consuming.

A GLC method for the determination of griseofulvin in skin was described (7), and a determination in plasma was mentioned (8). These investigators used an electron-capture detection method.

The purpose of the present study was to develop a specific, highly sensitive method which was simple enough to permit the assay of a large number of samples. Work progressed along two lines, simplification of the spectrofluorometric method and adaptation of the previously reported (7) GLC method to plasma samples.

EXPERIMENTAL

Reagents and Chemicals—The following reagents and chemicals were used: sodium chloride[1], anhydrous ether[2], methanol[3], and sulfuric acid[1], all analyzed reagents; benzene[4], glass distilled; OV-17[5]; Chromosorb W[5]; diazepam[6]; and griseofulvin[7]. 6-Demethylgriseofulvin was isolated from human urine (9).

Instrumentation—The gas chromatograph[8] was equipped with a ^{63}Ni electron-capture detector and a 152.4-cm (5-ft) long, 4-mm (0.157-in.) i.d. glass column, packed with 3% OV-17 on 80–100 mesh Chromosorb W. The following conditions were used: column temperature, 270°; injection port temperature, 310°; detector block temperature, 330°; carrier gas (10% methane–90% argon) flow, 150 ml/min; and range, 10 with attenuation factor of 16×. Chromatograms were recorded on a 1-mv recorder[8].

Table I—GLC Analyses of Griseofulvin after *In Vitro* Addition to Human Plasma

Griseofulvin Added, μg/ml	Griseofulvin Recovered[a]	
	μg/ml ± *SD*	%
0.1	0.100 ± 0.013	100.0
0.5	0.483 ± 0.037	96.6
1.0	1.068 ± 0.073	106.8
1.5	1.464 ± 0.120	97.6
2.0	1.942 ± 0.154	97.1
Average recovery		99.6

[a] Average of eight determinations.

Method—A 1-ml aliquot of plasma was pipetted into a glass-stoppered test tube, and 1 ml of saturated sodium chloride solution and 10 ml of anhydrous ether were added. The tube was shaken for 1 min, and a 5-ml aliquot of the ether phase was transferred to a pear-shaped flask and evaporated to dryness *in vacuo*. The residue was dissolved in 1 ml of benzene containing 0.25 μg/ml of diazepam as the internal standard. About 5 μl of this solution was injected.

The heights of the peaks corresponding to griseofulvin and the internal standard were measured, and the ratio of the peak heights, griseofulvin–internal standard, was converted into micrograms of griseofulvin per milliliter of assay solution by means of a standard curve. The plasma concentration was obtained after correction for dilution (concentration); the recovery was assumed to be quantitative.

Standard curves were prepared by injecting standard solutions of griseofulvin in benzene in the 0.1–1.5-μg/ml range, each containing 0.25 μg/ml of internal standard, under the same analysis conditions as the samples. The peak height ratios of griseofulvin–internal standard were plotted against the concentration of griseofulvin in the assay solution.

Fluorometric Determination—The spectrofluorometric method described previously (7) was simplified and modified in the following manner.

To 1.0 ml of plasma in a stoppered test tube was added 1.0 ml of saturated sodium chloride solution followed by 10.0 ml of anhydrous ether. The test tube was shaken for 1 min. A 9-ml aliquot of the ether layer was transferred to a 50-ml pear-shaped flask and evaporated to dryness *in vacuo*. The residue was dissolved in 5.0 ml of 80% methanol (v/v). The fluorescence was read in a spectrophotofluorometer[9] with the following settings: excitation wavelength, 300 nm; detection wavelength, 420 nm; sensitivity, 70; and slits, 4, 5, 4, 2, 2, and 5.

The samples were read a second time after the addition of three drops of concentrated sulfuric acid, the second reading being used as a blank reading.

Standard solutions of griseofulvin were made up in 80% methanol (v/v) in the 0.05–0.5-μg/ml range. The solutions were read under the identical instrument settings. Linearity was obtained between 0 and 0.5 μg/ml of assay solution (corresponding to 0–2.5 μg/ml of plasma). The lowest measurable plasma concentration was 0.1 μg/ml.

The recovery was determined by adding known amounts of griseofulvin to human plasma in the 0.1–1.5-μg/ml range and ana-

[1] Fisher Scientific Co.
[2] Mallinckrodt, Inc.
[3] Lehigh Valley Chemical Co.
[4] Burdick and Jackson Laboratories.
[5] Supelco Inc.
[6] Roche Laboratories.
[7] Schering Corp.
[8] Hewlett-Packard.
[9] Aminco-Bowman.

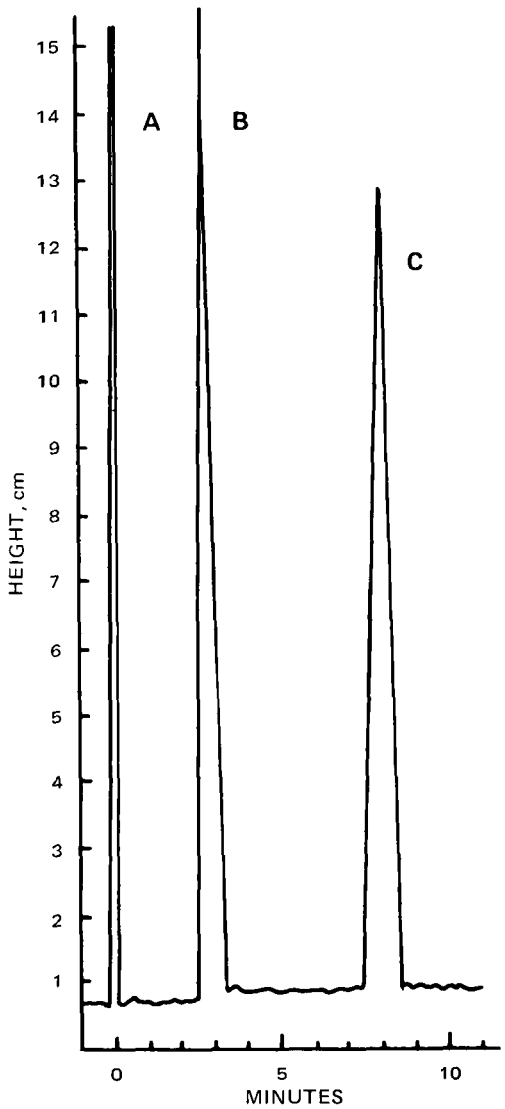

Figure 1—*Gas chromatogram showing a 5-μl injection of a standard solution. Key: A, solvent front; B, internal standard; and C, griseofulvin.*

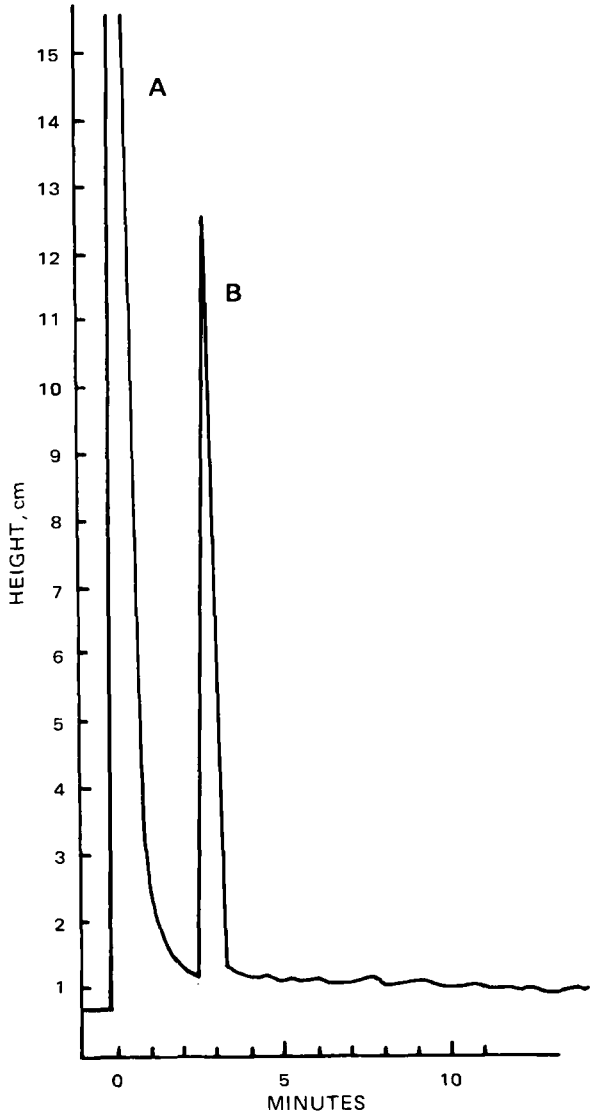

Figure 2—*Gas chromatogram showing a 5-μl injection of blank plasma extract. Key: A, solvent front; and B, internal standard.*

lyzing the samples as already described. The recovery varied from 88 to 102% (average of 93%) and seemed to be independent of the griseofulvin concentration within this range.

Plasma Samples—Twelve normal adult males, 21–50 years of age, received on a fasting stomach one 500-mg tablet of micronized griseofulvin with 120–180 ml (4–6 oz.) of water. Duplicate 10-ml blood samples were obtained by venipuncture at 0, 0.5, 1, 2, 4, 6, 8, 12, and 24 hr after dosing. The plasma was isolated by centrifugation and stored in the frozen state until analyzed.

RESULTS AND DISCUSSION

Standard Curve—A typical recorder tracing of a standard mixture is shown in Fig. 1. A complete separation of the peaks was obtained. The retention times were: internal standard, 3.0 min; and griseofulvin, 8.0 min.

A typical standard curve was smooth but nonlinear in the range of 0–1.5 μg of griseofulvin/ml of assay solution. This nonlinearity was probably due to the nonlinear response characteristics of the electron-capture detector.

Sensitivity—The detection limit (two to three times the noise level) for griseofulvin was 30 pg, corresponding to 6 ng/ml of assay solution, under the conditions of the assay method. Four times this concentration (10 times the noise level) or 0.025 μg/ml of assay solution (peak height ratio = 0.06) was found to be the lowest measurable concentration. This assay sensitivity, corresponding to 0.050 μg/ml of plasma, was sufficient to follow the plasma level after a single dose of 250–500 mg of griseofulvin for 72 hr.

Due to the flattening of the standard curve, the method also has a practical upper limit of usefulness of about 1.5 μg of griseofulvin/ml of plasma. Higher concentrations can be measured by decreasing the volume of plasma or increasing the amount of benzene used in making up the assay solution.

Specificity—The combination of extraction, GLC retention

Table II—Plasma Concentrations of Griseofulvin following a Single Oral Dose of 500 mg of Micronized Griseofulvin in 12 Human Volunteers (Parallel Determinations by the GLC Method and the Spectrofluorometric Method)

Hours	Fluorometric, μg/ml ± *SD*[a]	GLC, μg/ml ± *SD*[a]	Ratio
0.5	0.08 ± 0.10	0.06 ± 0.09	1.33
1	0.29 ± 0.25	0.21 ± 0.21	1.38
2	0.56 ± 0.31	0.48 ± 0.35	1.17
4	0.73 ± 0.21	0.60 ± 0.29	1.22
6	0.73 ± 0.22	0.54 ± 0.26	1.35
8	0.66 ± 0.20	0.48 ± 0.24	1.38
10	0.57 ± 0.16	0.46 ± 0.20	1.24
12	0.56 ± 0.17	0.44 ± 0.18	1.27
24	0.41 ± 0.12	0.28 ± 0.12	1.46

[a] Mean of 12 subjects.

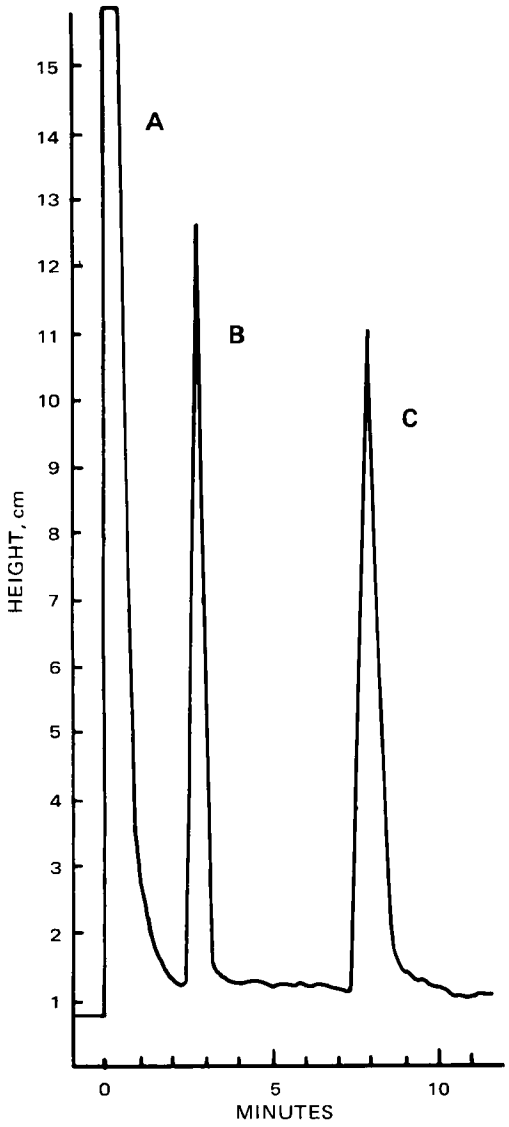

Figure 3—*Gas chromatogram showing a 5-μl injection of plasma extract spiked with griseofulvin. Key: A, solvent front; B, internal standard; and C, griseofulvin.*

time, and sensitivity to electron capture makes this method very specific. The only major plasma metabolite of griseofulvin (8), 6-demethylgriseofulvin, has a much longer retention time and elutes as a very flat, almost indistinguishable band at corresponding concentrations.

Determination in Plasma—The GLC tracing of an extract of a blank plasma sample (Fig. 2) was compared with an extract of a plasma sample containing griseofulvin (Fig. 3). The blank plasma extract showed no interfering peak in the area of the griseofulvin peak. Zero blank values were obtained throughout. Because of variations in the response of the electron-capture detector, standard curves were run daily when plasma samples were analyzed.

Recovery—The validity of the method was verified and the recovery was determined by adding various known amounts of griseofulvin to human plasma. Each plasma standard as well as blank plasma was analyzed eight times according to the described procedure (Table I).

The mean recoveries varied from 97 to 107% of the added amount with an average of 100%, and no correlation with the concentration of griseofulvin was observed.

Comparison of GLC and Spectrofluorometric Results—The duplicate plasma samples obtained from 12 subjects after a single dose of 500 mg of griseofulvin were analyzed by both the GLC and the simplified spectrofluorometric methods (Table II).

The fluorometrically obtained plasma concentrations were consistently higher than the corresponding GLC results. This observation indicated that while the GLC method was specific for griseofulvin, one or several metabolites contributed to the fluorescence. Table II also shows that the ratio of the results obtained by the two methods was constant over the whole time course. From this finding it can be inferred that the ratio of griseofulvin to contributing metabolite(s) is constant at all times, that the disappearance of griseofulvin or fluorescence from plasma would yield the same half-life, and that, therefore, the half-life of disappearance is determined by the metabolism of griseofulvin. In addition, Table II shows that the standard deviation was of the same order for both determinations, indicating that the intersubject variation was the same for both methods.

These results and conclusions are in good agreement with the data obtained (8) with ^{14}C-labeled griseofulvin. Blood contained about equal amounts of griseofulvin and 6-demethylgriseofulvin, the two compounds accounting for 86% of the radioactivity in blood (8). In addition, the disappearance curves of griseofulvin and radioactivity were parallel and yielded the same half-life, indicating that demethylation of griseofulvin was the rate-limiting step in the elimination process.

To tie the two sets of findings together, the fluorescence of 6-demethylgriseofulvin and mixtures of equal amounts of 6-demethylgriseofulvin and griseofulvin was determined at the instrument settings used for the griseofulvin assay. 6-Demethylgriseofulvin did give fluorescence readings, albeit less than griseofulvin, and indeed the 6-demethylgriseofulvin added about 30% to the fluorescence yield of griseofulvin when present at an equal concentration. Thus, the 1.3 times higher plasma levels obtained with the spectrofluorometric method were most likely due to the coextracted 6-demethylgriseofulvin. How far this was the case in other modifications of the spectrophotofluorometric determination method (2–7) cannot be ascertained; however, it is probable that 6-demethylgriseofulvin contributed to the plasma level readings in some or all of them.

SUMMARY AND CONCLUSIONS

The time requirement per sample is about the same for both methods described in this paper if manual injection of the gas chromatograph is employed. However, by using an automatic GLC injector, the output can be doubled or even tripled.

Based on equal sensitivity, better specificity, and the capacity for greater output, it was concluded that the GLC method as described here is the preferred method of assay for griseofulvin in human plasma. Because of its simplicity, it is well suited for the routine analysis of a large number of samples. The method was successfully used in this laboratory in several bioequivalency studies.

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