Table II—Impurities in Trihexyphenidyl Tablets

Lot	Dose, mg	Impurities[a], % II	IV	V
A	2	ND[b]	0.4	ND
B	5	Tr[c]	1.9	ND
C	2	1.0	0.2	ND
D	5	1.6	0.2	ND
E	2	ND	0.2	ND
F	5	0.1	ND	ND
G	2	0.1	ND	ND
H	5	0.1	0.1	ND
I	2	ND	ND	ND
J	5	ND	0.1	ND
K (elixir)	2/5 ml	ND	ND	1.0
Raw material	—	ND	ND	ND
Raw material	—	ND	ND	1.6

[a] Expressed as percentage of the label claim of drug in the hydrochloride form. [b] None detected. [c] Trace.

yield 5.3 g (40%) of crude IV, bp_3 75–79° [lit. (11) $bp_{2.5}$ 74–76°]; IR (film): 1680 (C=O) cm^{-1}.

3-Aminopropiophenone (V)—In a 300-ml, round-bottom flask, 5.3 g (0.1 mole) of ammonium chloride, 4.5 g (0.15 mole) of formaldehyde 37% solution, and 0.2 ml of concentrated hydrochloric acid were dissolved in 40 ml of ethanol (5). To this solution, 12.0 g (0.1 mole) of acetophenone (III) was added. The mixture was refluxed for 1.5 hr, another 9 ml of formaldehyde (37% solution) was added, and the mixture was refluxed for another 1.5 hr.

Compound V was extracted into ether as the free base. The ether phase was dried over sodium sulfate and filtered, and hydrogen chloride gas was passed through the solution. The yield of the hydrochloride salt was 5.0 g (33%) of V, mp (hydrochloride) 125–127° [lit. (11) mp 128°]; IR ($CHCl_3$): 1685 (C=O) and 3440 (NH_2 vibration) cm^{-1}.

RESULTS AND DISCUSSION

The identity of 3-piperidinopropiophenone (II), 1-phenyl-2-propenone (IV), and 3-aminopropiophenone (V) found in raw material and tablet formulations was established by comparison of TLC R_f values, GLC retention times, and mass spectral fragmentation patterns to those of synthetic samples. The authenticity of the synthetic samples was demonstrated by TLC, GLC, and mass spectra.

The structure postulated for the impurities is supported by the mass spectral results, which conform to the fragmentation diagrams presented in Schemes II and III. Detectability limits, R_f values, and GLC retention times for trihexyphenidyl, 3-piperidinopropiophenone, 1-phenyl-2-propenone, and 3-aminopropiophenone are listed in Table I (Figs. 1 and 2). Two lots of trihexyphenidyl hydrochloride, 10 lots of tablets, and one lot of elixir preparation from five manufacturers were screened for impurities (Table II). Five lots contained II at levels from 0.1 to 1.6%, and half of the lots contained IV at levels from 0.1 to 1.9%. Compound V was found in two lots from the same manufacturer at levels of 1.0 to 1.6%.

The presence of V may be the result of ammonium chloride in the piperidine hydrochloride used as a starting material in one synthetic process (5) (Scheme I). Compound II is an intermediate in the synthesis of trihexyphenidyl (5). The presence of IV, which was observed in low levels, may be due, according to previous investigators (12), to the hydrolysis of II or to an elimination reaction in the step involving the Grignard reagent (11).

REFERENCES

(1) G. R. Gordon, D. C. Ghoul, and J. H. Peters, *J. Pharm. Sci.*, **64**, 1205 (1975).

(2) D. C. Chatterji, A. G. Frazier, and J. F. Gallelli, *ibid.*, **67**, 622 (1978).

(3) K. M. McErlane, N. M. Curran, and E. G. Lovering, *ibid.*, **66**, 1015 (1977).

(4) K. M. McErlane, R. J. Wood, F. Matsui, and E. G. Lovering, *ibid.*, **67**, 958 (1978).

(5) J. J. Denton, W. B. Neier, and V. A. Lawson, *J. Am. Chem. Soc.*, **71**, 2053 (1949).

(6) "The United States Pharmacopeia," 19th rev., Mack Publishing Co., Easton, Pa., 1975.

(7) "British Pharmacopoeia 1973," Her Majesty's Stationery Office, London, England, 1973.

(8) E. J. Corey, T. H. Topie, and W. A. Wozniak, *J. Am. Chem. Soc.*, **77**, 5415 (1955).

(9) H. Bredereck, F. Effenberger, F. Marquez, and K. Ockewitz, *Chem. Ber.*, **93**, 2083 (1960).

(10) J. Warkentin and L. K. M. Lam, *Can. J. Chem.*, **42**, 1676 (1964).

(11) H. G. O. Becker and E. Fanghaenel, *J. Prakt. Chem.*, **26**, 58 (1964).

(12) A. C. B. Smith and W. Wilson, *J. Chem. Soc.*, **1955**, 1342.

ACKNOWLEDGMENTS

The authors are grateful to Mr. W. Miles and Mr. J. C. Ethier for determination of the mass spectra.

High-Pressure Liquid Chromatographic Assay for Griseofulvin in Plasma

MARVIN C. MEYER ˣ and GURSHARAN RAGHOW

Received January 17, 1979, from the *Division of Biopharmaceutics and Pharmacokinetics, Department of Pharmaceutics, College of Pharmacy, University of Tennessee Center for the Health Sciences, Memphis, TN 38163.* Accepted for publication March 14, 1979.

Abstract □ A high-pressure liquid chromatographic procedure was developed for griseofulvin assay in human plasma. The method utilized warfarin as an internal standard and easily quantitated griseofulvin plasma levels as low as 0.10 μg/ml. The method was compared to two fluorometric assay methods and was more specific for griseofulvin. Assay of 6-demethylgriseofulvin isolated from human urine demonstrated that this material was not responsible for the interferences apparent in the fluorometric procedures.

Keyphrases □ Griseofulvin—analysis, high-pressure liquid chromatography, human plasma, compared to fluorometric assays □ Antifungal agents—griseofulvin, high-pressure liquid chromatographic analysis, human plasma, compared to fluorometric assays □ Fluorometry—analysis, griseofulvin in human plasma, compared to high-pressure liquid chromatography

Griseofulvin is a poorly water-soluble, antifungal agent. This orally administered drug may be subject to reduced bioavailability, and particle-size reduction and preparation of polyethylene glycol dispersions have been employed to improve absorption from the GI tract (1, 2). In view of the potential for griseofulvin dosage forms to exhibit poor bioavailability, a convenient and specific assay method for intact drug measurement in the plasma of patients or volunteer subjects receiving griseofulvin is needed.

The most widely utilized analytical methods have em-

0022-3549/79/0900-1127$01.00/0

ployed plasma extraction followed by fluorescence detection (3, 4). The method of Schwarz *et al.* (3) is a modification of the earlier procedure of Shah *et al.* (4), but the two methods have not been compared directly. Schwarz *et al.* (3) also described a GLC method, using electron-capture detection. They compared their fluorometric and GLC procedures (3) and found that the assayed plasma griseofulvin levels were ~30% higher using the fluorometric method. These authors felt their data indicated an interference in the fluorometric assay by 6-demethylgriseofulvin, a griseofulvin metabolite.

More recent investigators (5) reported a high-pressure liquid chromatographic (HPLC) procedure for plasma griseofulvin levels. The method was very rapid, involving only a protein precipitation step prior to chromatographic analysis, but did not include an internal standard. The drug was quantitated using a fluorescent detector.

The present study investigated the potential interference of 6-demethylgriseofulvin in the fluorometric methods (3, 4). In addition, an HPLC method was developed to provide a procedure utilizing an internal standard that could be applied to the assay of plasma griseofulvin levels in humans.

EXPERIMENTAL

Reagents and Chemicals—The following were used: griseofulvin[1]; glass-distilled methanol[2], ether[2], and hexane[2]; and warfarin sodium[3].

Comparison of Fluorometric Procedures—Pooled plasma samples were prepared containing 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 μg/ml of griseofulvin. These plasma samples were assayed utilizing the procedures of Shah *et al.* (4) and Schwarz *et al.* (3). In both methods, the final fluorescence[4] reading was determined using 5 drops of sulfuric acid for complete sample quenching.

HPLC Method—The procedure utilized warfarin sodium as an internal standard. The extraction method was essentially that of Shah *et al.* (4), except that the plasma was adjusted to pH 5 with hydrochloric acid before extraction to facilitate warfarin removal. One-milliliter aliquots of pooled plasma were spiked with 1-ml aqueous griseofulvin solutions to simulate plasma griseofulvin levels of 0.1, 0.2, 0.4, 0.6, 0.8. 1.0, 1.5, and 2.0 μg/ml. To this mixture were added 1 ml of a 150-μg/ml aqueous warfarin sodium solution, 0.1 ml of 1 *N* HCl, and 10 ml of ether.

The mixture was extracted for 10 min in a 50-ml screw-capped centrifuge tube[5] and centrifuged at 3000 rpm at −10° for 15 min. The tube was then dipped into a dry ice–acetone bath until the aqueous phase was frozen. The ether layer was decanted into a 20-ml screw-capped centrifuge tube and evaporated under nitrogen at 40°. The residue was taken up in 50% methanol and shaken for 5 min with hexane. After centrifuging for 15 min at 3000 rpm and −10°, the hexane layer was removed and discarded. A 50-μl aliquot of the methanolic solution was injected into the chromatograph.

The HPLC system consisted of a sample injector[6]; a mobile phase pump[7] operated at 2.2 ml/min (pressure 2000 psi); a reversed-phase column[8]; a fluorescence detector[9], using 300 and 418 nm as the excitation and emission wavelengths, respectively, and a range setting of 1.0 μamp; and a variable-speed recorder[10] operated at 0.25 cm/min. The mobile phase consisted of 61.5% methanol and 38.5% of 0.5% acetic acid in water. Standard curves were obtained by plotting peak height ratio (griseofulvin/warfarin) *versus* griseofulvin concentration.

Griseofulvin Metabolite Isolation—A 24-hr urine collection was obtained from a volunteer who had ingested two 500-mg griseofulvin tablets[11]. The 2145 ml of urine was adjusted to pH 4 with hydrochloric acid and extracted in ~300-ml portions. Each urine aliquot was extracted twice with 300 ml of ether, and the combined ether extracts were evaporated under nitrogen. The ~100 ml of aqueous residue remaining after ether evaporation was then extracted four times with 100-ml portions of ether. The ~50 ml of aqueous residue remaining after evaporation of the 400 ml of ether extract was then extracted twice with 100 ml of ether. The ~25 ml of aqueous residue remaining after evaporation of the 200 ml of ether extract was further extracted with two 100-ml portions of ether. The 10 ml of aqueous residue remaining after evaporation of the 200 ml of ether extract was finally extracted five times with 50 ml of ether.

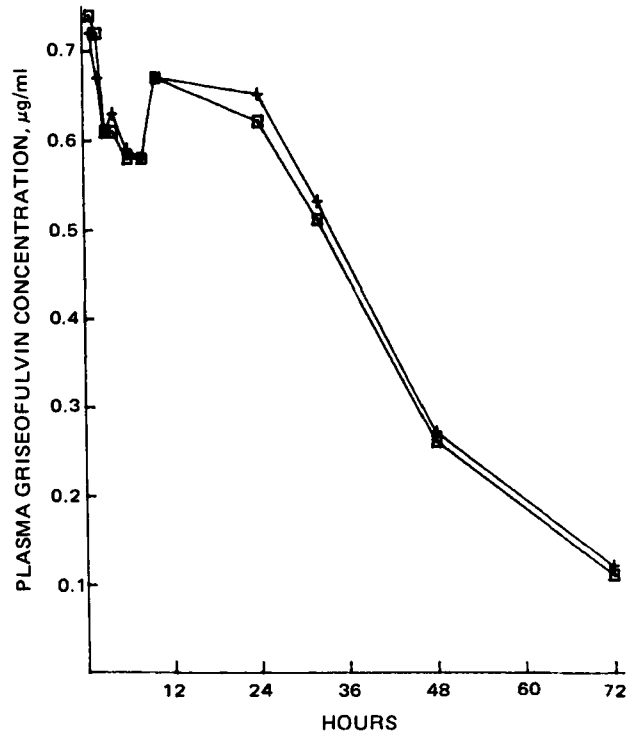


Figure 1—*Plasma griseofulvin levels for a subject receiving a 500-mg tablet. Fluorometric assays were performed using the method of Schwarz* et al. *(3)* (□) *and Shah* et al. *(4)* (+).

The 250 ml of ether was evaporated, and the ~5 ml of deep-red aqueous residue was utilized in the TLC separation. With a 10-μl pipet, the aqueous residue was applied as multiple spots to form a continuous streak on 11 TLC plates[12] (20 × 20 cm, 0.5 mm thick). The plates were developed for 2 hr, using a 1:4 mixture of acetone and chloroform. Even though the solvent front reached the top of the TLC plate before 2 hr, the longer development time was required for optimal separation. At least six well-separated fluorescent bands were observed on each plate when viewed under shortwave UV light. One relatively weak band corresponded to griseofulvin.

Since the fluorescence of 6-demethylgriseofulvin had been reported to be quenched at alkaline pH (6), the strongly fluorescent band that quenched upon exposure to ammonium hydroxide fumes was assumed to be 6-demethylgriseofulvin. This band was carefully scraped from each TLC plate, placed in a 50-ml centrifuge tube, and extracted five times with 30 ml of ether. A total of 87 mg of white powder was obtained after evaporation of the combined ether extracts.

The uncorrected melting point of the material was determined to be 280° with decomposition. The UV spectrum was determined[13] for the compound dissolved in 80% ethanol, to which was added 1% (v/v) of either 2 *N* HCl or 2 *N* NaOH. The fluorescence spectra[4] of the compound dissolved in methanol also were obtained, before and after the addition of 2 drops of ammonium hydroxide. A 5-μl portion of a methanol solution of the material also was spotted on a 0.25-mm TLC plate[14] along with a

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Burdick & Jackson Laboratories, Muskegon, Mich.
[3] Lot R69-522m, provided by Endo Laboratories, Garden City, N.Y.
[4] Aminco-Bowman spectrophotofluorometer, American Instrument Co., Silver Spring, Md.
[5] No. 14-930-10J, Fisher Scientific, Pittsburgh, Pa.
[6] Model U6K, Waters Associates, Milford, Mass.
[7] Model M6000, Waters Associates, Milford, Mass.
[8] μBondapak C_{18}, Water Associates, Milford, Mass.
[9] Model FS 970, Schoeffel Instrument Corp., Westwood, N.J.
[10] Recordall series 5000, Fisher Scientific, St. Louis, Mo.
[11] Fulvicin-U/F, Schering Corp., Kenilworth, N.J.
[12] Silica gel G with calcium sulfate binder, Supelco, Inc., Bellefonte, Pa.
[13] DB-GT spectrophotometer, Beckman Instruments, Irvine, Calif.
[14] NEN-silica gel OF, 5 × 20 cm, 0.25 mm, New England Nuclear, Boston, Mass.

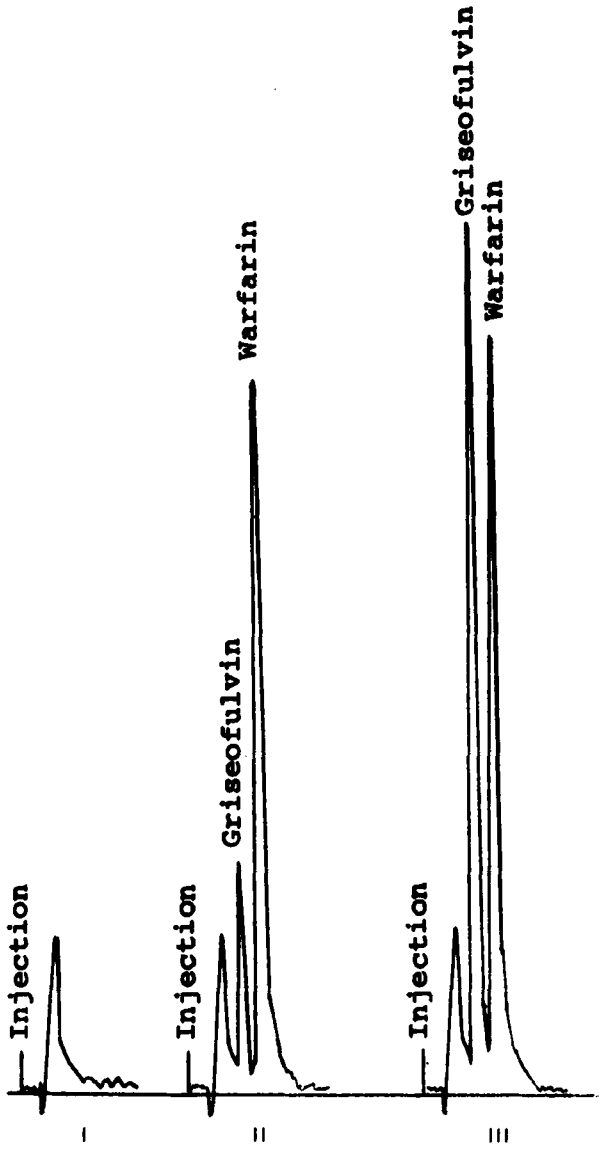


Figure 2—*Typical HPLC chromatograms for the assay of a blank human plasma sample (I) and plasma samples containing 0.1 μg/ml (II) and 0.4 μg/ml (III) of griseofulvin. Warfarin was added to plasma samples II and III as an internal standard.*

griseofulvin sample. The plate was developed for 25 min with acetone–chloroform (1:4).

After development, the plate was viewed under shortwave UV light, followed by exposure to ammonium hydroxide fumes. A diluted methanol solution of the compound also was injected into the chromatograph, using the parameters previously described for the HPLC plasma assay. Finally, the 270-MHz NMR spectra[15] were determined for the material, a sample of griseofulvin, and a sample of 4-demethylgriseofulvin[16]. Deuterated acetone was employed as the solvent for the NMR spectra.

Since interference by 6-demethylgriseofulvin has been suggested as the cause for higher plasma griseofulvin assay values for a fluorometric assay compared to a GLC procedure (3), pooled plasma samples were prepared containing either 1 μg of griseofulvin/ml or 5 μg of 6-demethylgriseofulvin/ml and both 1 μg of griseofulvin/ml and 5 μg of 6-demethylgriseofulvin/ml. These samples were carried through the extraction procedures of Schwarz *et al.* (3) and Shah *et al.* (4). The resulting extracts were assayed fluorometrically, and an aliquot was injected into the chromatograph, using the previously described HPLC system.

Plasma Level Studies—To obtain plasma samples for evaluation of the fluorometric and HPLC assay methods, a healthy male volunteer received a 500-mg griseofulvin tablet[11] on two different occasions, approximately 4 months apart. Ten-milliliter plasma samples were obtained just before and 1, 2, 3, 4, 6, 8, 10, 24, 32, 48, and 72 hr after the first administration. These samples were assayed using the two previously discussed fluorometric methods. Since the purpose of the second study was to provide plasma samples for a comparison of the HPLC method with the method of Shah *et al.* (4), 10-ml plasma samples were obtained just before and 2, 4, 8, 24, and 48 hr after the second administration. The doses were taken after an overnight fast, and no food or beverage other than water was permitted for 4 hr after dosing.

RESULTS AND DISCUSSION

Comparison of Fluorometric Procedures—In an earlier study (3), plasma griseofulvin levels assayed fluorometrically by the method of Schwarz *et al.* (3) were approximately 30% higher than those from a GLC assay. Since no direct comparison had been made of the two previously reported fluorometric methods (3, 4), it was of interest to determine if the procedures yielded comparable data. Calibration curves obtained for fluorescence intensity, corrected for blank plasma fluorescence, *versus* plasma griseofulvin concentration were essentially superimposable for plasma standards assayed by either method.

The least-squares slope, intercept, and correlation coefficient for one procedure (4) were 24.24, 0.24, and 0.999, respectively; the same values for the other method (3) were 23.88, −0.03, and 0.998, respectively. The recovery of griseofulvin from the plasma samples averaged ~90% for both methods. The blank plasma correction required with the method of Schwarz *et al.* (3) was generally four times that required with the method of Shah *et al.* (4). This difference appeared to be due to the hexane extraction step present in the latter method.

The data shown in Fig. 1 were obtained by assaying plasma samples from a subject who received a 500-mg griseofulvin tablet. The plasma levels at each sampling time differed by 5% or less by the two fluorometric methods.

HPLC Method—Since the two previously described fluorometric methods (3, 4) provided comparable assay data and since one of the methods had been shown earlier (3) apparently to lack suitable griseofulvin specificity, an HPLC assay was developed.

Typical chromatograms for pooled plasma containing 0, 0.1, and 0.4 μg of griseofulvin/ml and 150 μg of warfarin sodium/ml, carried through the extraction and HPLC assay, are shown in Fig. 2. The retention times for griseofulvin and warfarin were 3.1 and 4.3 min, respectively. An additional peak, which also appeared in blank plasma extracts, was well separated from griseofulvin and had a retention time of 2.2 min. No interferences were observed in blank plasma samples obtained from 12 human subjects who participated in a griseofulvin bioavailability study (to be reported later). However, plasma obtained from a subject who was administered three 0.33-g (5-gr) aspirin tablets exhibited a large interfering peak, which would have precluded detection of the griseofulvin peak.

The calibration curve of peak height ratio (griseofulvin/warfarin) *versus* griseofulvin concentration for the assay of pooled plasma samples containing 0–2.0 μg of griseofulvin/ml exhibited a slope of 2.91, an intercept of −0.01, and a correlation coefficient of 0.999. Griseofulvin recovery from plasma averaged 91%, compared to the direct HPLC analysis of methanolic standards. The lowest standard contained 0.1 μg of griseofulvin/ml of plasma. However, a greater sensitivity should be readily attainable by utilizing >1 ml of plasma, diluting the extract with <2 ml of 50% methanol, or injecting >50 μl of sample.

The HPLC assay was compared directly to the fluorometric method of Shah *et al.* (4), using plasma samples obtained from a human volunteer who had been administered a 500-mg griseofulvin tablet. The results of these determinations are shown in Fig. 3. The ratio of griseofulvin concentrations determined by the fluorometric assay divided by the HPLC assay averaged 1.22, which is in agreement with the mean ratio of 1.3 previously reported in a comparison of a fluorometric and a GLC assay method (3).

Griseofulvin Metabolite Isolation—6-Demethylgriseofulvin has been reported to be the primary griseofulvin metabolite in humans (6, 7). Thus, it is logical to assume that the major source of interference in the fluorometric assay procedures can be attributed to this metabolite.

The uncorrected melting point of the material isolated from the urine extracts was 280°, in reasonable agreement with the 273–275° melting point reported previously for 6-demethylgriseofulvin (7). The recovery of 87 mg of metabolite after the administration of the 1-g dose is similar to the reported 7% (8) and 10% (2) recoveries 24 hr after administration

[15] Recorded on a custom-built 270-MHz spectrometer, based on an Oxford Instruments solenoid, a Bruker-Nicolet console, and a Nicolet 1180 computer.

[16] Glaxo Laboratories, Greenford, Middlesex, England.

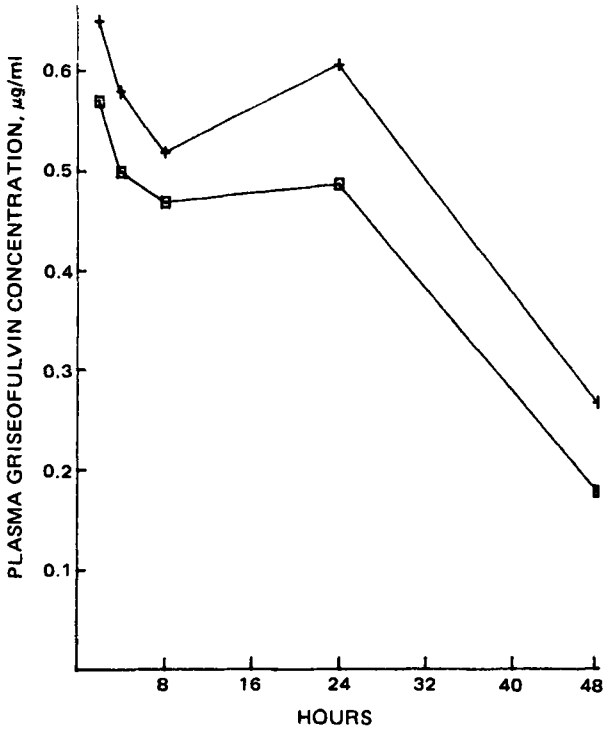


Figure 3—*Plasma griseofulvin levels for a subject receiving a 500-mg tablet. Determinations were carried out using the fluorometric method of Shah* et al. *(4) (+) and an HPLC method (□).*

of a 500-mg dose to a human subject. Similarly, 10.5% of a 1-g dose in a 24-hr urine sample was obtained from a human subject (7).

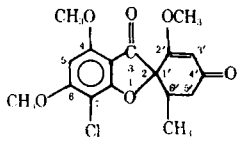
No attempt was made to recover 6-demethylgriseofulvin that might have been present as a glucuronide metabolite in the urine. The UV absorption maxima in alkaline solution were 250 and 300 nm, compared to previously reported values of 249.5 and 330 nm (7). Similarly, the maxima were 236 and 293.5 nm in acidic solution, compared to 235.5 and 293.5 nm. The excitation and emission fluorescence maxima were 320 and 425 nm, respectively, which were nearly identical to the maxima exhibited by griseofulvin. The fluorescence spectrum was totally quenched upon the addition of 2 drops of ammonium hydroxide. When the presumed 6-demethylgriseofulvin was spotted on a 0.25-mm TLC plate along with a sample of griseofulvin and the plates were developed for 25 min, only two distinct spots appeared under shortwave UV light. The R_f values were 0.23 and 0.44 for 6-demethylgriseofulvin and griseofulvin, respectively, and only the former spot quenched after exposure to ammonium hydroxide fumes. When a sample of presumed 6-demethylgriseofulvin was examined using the HPLC system described in the assay procedure, a single sharp peak resulted. The retention time of this peak was 2.2 min, compared to 3.1 min for griseofulvin.

The NMR spectra for griseofulvin, 4-demethylgriseofulvin, and the presumed 6-demethylgriseofulvin provided the final confirmation of structure. The data in Table I summarize the assignment of the protons in each spectra. The spectra of all three substances were quite similar, with the correct number of methoxyls being observed in each instance. The replacement of a methoxyl with a hydroxyl slightly shifted the ring proton and remaining methoxyl to a higher field. The methoxyl in 4-demethylgriseofulvin is adjacent to chlorine and is downfield from the methoxyl in 6-demethylgriseofulvin.

To assess the 6-demethylgriseofulvin interference in the fluorometric plasma assay for griseofulvin, samples spiked with griseofulvin and 6-demethylgriseofulvin were assayed by the methods of Shah *et al.* (4) and Schwarz *et al.* (3). With either method, plasma samples containing 5 µg of 6-demethylgriseofulvin/ml exhibited fluorescence values equal to those obtained with blank plasma samples. The fluorescence of plasma spiked with both 1 µg of griseofulvin/ml and 5 µg of 6-demethylgriseofulvin/ml was identical to that resulting from the assay of plasma containing only 1 µg of griseofulvin/ml. Furthermore, when the extracts of the plasma samples containing 6-demethylgriseofulvin were injected into the chromatograph, no peak corresponding to the retention time of 6-demethylgriseofulvin appeared.

Thus, it appears that the higher assay values resulting from the fluorometric assay of plasma samples obtained from subjects ingesting griseofulvin, compared to assay values determined with either a GLC (3) or an HPLC assay, could not be attributed to interference by 6-demethylgriseofulvin as proposed earlier (3). Since the material that interferes in the fluorometric procedures only appears in the plasma of subjects who have received griseofulvin, the material might represent an unidentified griseofulvin metabolite. However, whatever the identity of the interfering material, the use of the fluorometric assays for plasma griseofulvin results in falsely elevated values. The described HPLC procedure provides an alternative assay with good sensitivity and specificity.

Table I—Protons for 270-MHz Signals of Griseofulvin and Metabolites (δ, ppm)



griseofulvin

Proton	Griseofulvin	4-Demethylgriseofulvin	6-Demethylgriseofulvin
2′-OCH_3	3.62	3.62	3.62
3′-H	5.48	5.48	5.48
5′-H	2.77	2.76	2.77
6′-H	2.27	2.28	2.28
6′-CH_3	0.83	0.84	0.84
4-OCH_3	3.93	—	3.80
5-H	6.49	6.49	6.29
6-OCH_3	4.04	3.94	—

REFERENCES

(1) M. Rowland, S. Riegelman, and W. L. Epstein, *J. Pharm. Sci.*, **57**, 984 (1968).

(2) W. L. Chiou and S. Riegelman, *ibid.*, **60**, 1376 (1971).

(3) H. J. Schwarz, B. A. Waldman, and V. Madrid, *ibid.*, **65**, 370 (1976).

(4) V. P. Shah, S. Riegelman, and W. L. Epstein, *ibid.*, **61**, 634 (1972).

(5) R. L. Nation, G. W. Peng, V. Smith, and W. L. Chiou, *ibid.*, **67**, 805 (1978).

(6) M. Rowland and S. Riegelman, *ibid.*, **62**, 2030 (1973).

(7) M. J. Barnes and B. Boothroyd, *Biochem. J.*, **78**, 41 (1961).

(8) P. Kabasakalian, M. Katz, B. Rosenkrantz, and E. Townley, *J. Pharm. Sci.*, **59**, 595 (1970).

ACKNOWLEDGMENTS

Supported in part by a contract from the Tennessee Department of Public Health and FDA Contract 223-77-3097.

The contributions of Dr. James Axelson, Dr. Frank Abbott, and Mr. Roland Burton of the University of British Columbia, in the running and interpretation of the NMR spectra, are gratefully acknowledged.