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CHROMATOGRAPHIC ANALYSIS OF GRISEOFULVIN AND METABOLITES IN BIOLOGICAL FLUIDS

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SUMMARY

A simple and accurate assay for the determination of griseofulvin and its metabolites in biological fluids using high-performance liquid chromatography is described. Using a reversed phase column and a mobile phase solvent of 45% acetonitrile in 0.1 *M* acetic acid, baseline separation of griseofulvin and several analogues was obtained. The described method allows one to quantitatively determine griseofulvin, 6-demethylgriseofulvin, and griseofulvic acid, a newly identified metabolite in man, in urine and plasma samples. Treatment of plasma samples prior to the analysis is simply made by deproteinizing the samples with an equal volume of acetonitrile. For urine samples, the procedure involves diethyl ether extraction with subsequent evaporation to dryness and reconstitution with the mobile phase solvent.

INTRODUCTION

Griseofulvin is an orally effective antifungal agent for the treatment of dermatophylic infections. Studies [1, 2] have shown that griseofulvin undergoes metabolic O-demethylation in man and animals to form several metabolites. To date, only two of the metabolites are positively identified as 6-demethylgriseofulvin and 4-demethylgriseofulvin [3]. The unidentified metabolites may include griseofulvic acid, which has been found in incubated fungi solutions of griseofulvin [4], and some dihydroxy-griseofulvin derivatives. These compounds, such as griseofulvic acid which is capable of undergoing methanolysis under mild conditions to form isogriseofulvin [5, 6], may be in part responsible for the toxicity and irregular responses of the

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drug found in clinical studies [7, 8]. Thus, it seems to be desirable to have an accurate method for monitoring concentrations of griseofulvin and its metabolites in biological fluids.

Several methods for the determination of griseofulvin and 6-demethylgriseofulvin in urine and plasma have been reported. These include the spectrofluorometric [9, 10], gas—liquid chromatographic [10—12] and liquid column chromatographic [13, 14] techniques. The spectrofluorometric method is generally considered to be time consuming and non-specific due to its extraction procedures and the inability of separating griseofulvin from its metabolites in fluorescence measurements. The gas—liquid chromatographic method is reported to be sensitive and specific, but the assay procedure involves tedious derivatization of the drug and often gives inconsistent results when using an electron-capture detector. The use of liquid column chromatography in the analysis of griseofulvin in plasma and 6-demethylgriseofulvin in urine has been reported by Nation et al. [13] and by Papp et al. [14], respectively. Improvement of these methods can be made by changing the chromatographic conditions to achieve better separations, so that a simple procedure may be obtained for simultaneous measurement of griseofulvin and its metabolites. Under this guideline, a method for the determination of griseofulvin, 6-demethylgriseofulvin, and griseofulvic acid in urine and in plasma has been developed in this laboratory. Based on this method, a previous study [15] has shown that griseofulvic acid is also a urine metabolite of griseofulvin in man.

EXPERIMENTAL

Materials

Griseofulvin (I) (Imperial Chemical Industries, Macclesfield, Great Britain) and boron tribromide (Alfa Division, Ventron, Danvers, Mass., U.S.A.) were used as obtained. 6-Demethylgriseofulvin (II), 7-chloro-6-hydroxy-4,2′-dimethoxy-6′-methylgris-3,4 -dione, was isolated from dog urine as a griseofulvin metabolite according to the method of Harris and Riegelman [16]. Griseofulvic acid (III), 7-chloro-4,6-dimethoxy-6′-methylgrisan-3,2′,4′-trione, was synthesized by selective boron tribromide ether cleavage of griseofulvin at —60°. 4-Demethylgriseofulvin (IV), 7-chloro-4-hydroxy-6,2′-dimethoxy-6′-methylgris-3,4′-dione, was prepared according to the method of Arkley et al. [5]. 7-Chloro-4-hydroxy-6,4′-dimethoxy-6′-methylgris-3′-ene-3,2′-dione (V) was prepared with selective boron tribromide ether cleavage of griseofulvin at room temperature followed by quenching the reaction mixture with methanol. Compounds II—V were positively identified as named by melting point, nuclear magnetic resonance and mass spectroscopic measurements. These data and the synthetic procedures are published elsewhere [6].

The solvents used in this study were chromatographic grade. All other chemicals used were reagent grade.

Equipment

A high-performance liquid chromatographic (HPLC) system including a Model 6000A pump and a U6K injector (Waters Assoc., Milford, Mass., U.S.A.) equipped with a Model SF-770 UV and a Model SF-970 fluorescence detector

(both from Schoeffel Instruments, Westwood, N.J., U.S.A.) was used. The outputs of the detectors were displaced on a recorder (Omniscribe, Houston Instruments, Austin, Texas, U.S.A.) having a full scale range of 10 mV. The HPLC analysis was made with a reversed-phase μBondapak C_{18} column (30 cm × 4 mm I.D., particle size 10 μm) (Waters Assoc.).

Fluorometric studies were made with a spectrophotofluorometer (Aminco-Bowman, American Instruments, Silver Spring, Md., U.S.A.) equipped with a 150 W xenon lamp and a 1P21 photomultiplier tube.

Chromatographic conditions

The mobile phase was prepared by degassing a mixture of 450 ml acetonitrile with 550 ml of 0.1 *M* acetic acid. Chromatographic analysis using the above solvent was carried out at a flow-rate of 1.0 ml/min. The column temperature was ambient. The sample injection size was 5 μl for urine samples and 10 μl for plasma samples. The column eluate was monitored by a UV detector at 290 nm and by a fluorescence detector with excitation at 280 nm and emission above 389 nm. The fluorescence wavelength of griseofulvin and derivatives was found to be 430 nm when excited at 310 nm using a spectrophotofluorometer. The UV detector was operated at 0.01 a.u.f.s., the fluorescence detector was operated at 0.2 μA range with an applied potential of 520 mV.

Sample preparations

Stock solutions of 1 mg/ml of compounds I—V were prepared in methanol or acetonitrile. For chromatographic analysis, standard solutions in the concentration range of 0.5—6 μg/ml of these compounds were prepared by transferring desired amounts of the stock solutions, with micro syringes, into test tubes containing 2 ml of the mobile phase solvent.

The analysis of commercial griseofulvin tablets [Schering (microsized), Kenilworth, N.J., U.S.A., Ayerst Laboratories (ultra-micronized), New York, N.Y., U.S.A., McNeil Laboratories (microsized), Fort Washington, Pa., U.S.A.] was carried out as follows: 10 tablets were weighed and reduced to a fine powder, 5—10 mg of the tablet powder were weighed and dissolved in 10 ml methanol. The solutions were filtered and further diluted with the mobile phase solvent for chromatographic analysis. The analyses were performed in triplicate.

Treatment of urine samples prior to the analysis was performed as follows: 4 ml of a urine sample were acidified to pH 3 and extracted with 8 ml of anhydrous diethyl ether; after centrifugation, 6 ml of the diethyl ether layer were transferred into a centrifuge tube containing 6 ml of 0.1 *M* acetic acid, the sample was shaken and centrifuged to remove any acid—water soluble substances from the diethyl ether layer. For chromatographic analysis, 4 ml of the diethyl ether layer were evaporated to dryness and reconstituted with 2 ml of the mobile phase solvent.

For plasma samples, deproteination of the samples using an equal volume of acetonitrile was carried out prior to the analysis. The acetonitrile treated sample was simply vortexed and centrifuged (at 480 *g* for 1 min) to obtain the clear supernate for HPLC injections.

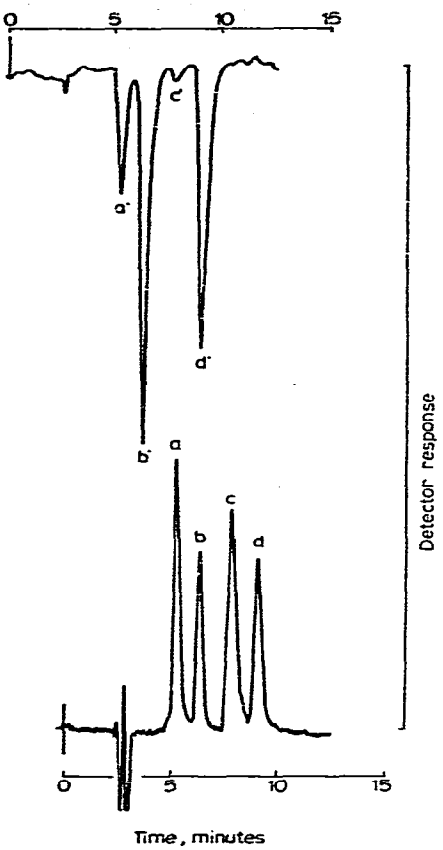
RESULTS AND DISCUSSION

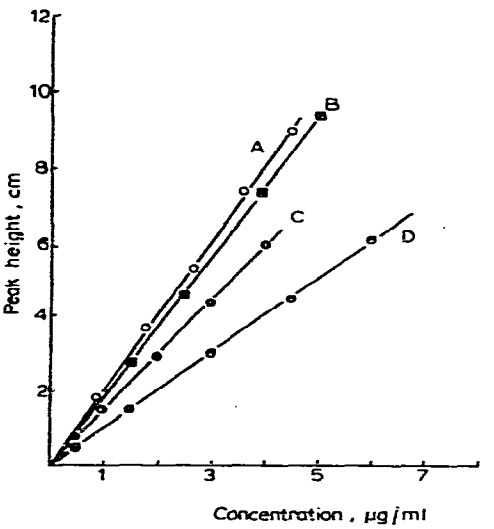
An HPLC assay for the determination of plasma levels of griseofulvin, using a reversed-phase column and a mobile phase of water—acetonitrile (1:1) mixture, has been reported by Nation et al. [13]. This method is found to be adequate for the determination of griseofulvin, but it does not allow one to measure the griseofulvin metabolites. Since the metabolites are the phenolic or hydroxy derivatives of griseofulvin, the pH of the mobile phase plays an important role in the separation of these compounds using reversed-phase chromatography. When a neutral pH solution is used as the mobile phase, the griseofulvin metabolites are essentially not retained by the column due to the fact that these compounds are ionized or partially ionized in the mobile phase solvent. Under acidic conditions, however, the metabolites become protonated, and they can be successfully separated for quantitative analysis. An example of the separation of I (peak d), II (peak a), III (peak b) and V (peak c) is shown in Fig. 1, where the chromatograms from UV (bottom) and fluorescence (top) detections were obtained using a mobile phase of 45% acetonitrile at pH 3.5.

Compound IV, 4-demethylgriseofulvin, which is not shown in Fig. 1, has a retention time of 7.0 min under the same chromatographic conditions. This compound was not included in the chromatogram because it was not found in the biological samples due to its very low abundance [3].

It is interesting to point out that the fluorescence yields of the griseofulvin derivatives depends largely on the substitutions at the 4- and 6-positions of their aromatic ring. Spectrofluorometric studies showed that the replacement of the 6-methoxy group of griseofulvin by a hydroxy group (such as in II), reduces more than half of the griseofulvin fluorescence. When the 4-methoxy group is replaced by a hydroxy group, such as in IV and V, the compound becomes nearly non-fluorescent. Griseofulvin derivatives possessing both 4- and 6-methoxy groups, such as in III, showed approximately equal fluorescence yield with griseofulvin. The same results were also found in the HPLC studies. As it can be seen in Fig. 1, compounds I and III are strongly fluorescent when compared with their UV absorptions, compound II, 6-demethylgriseofulvin, is moderately fluorescent, whereas compound V is nearly non-fluorescent. Compound IV is also non-fluorescent.

The advantage of using both fluorescence and UV detectors is that the identity and purity of a separated component in a chromatogram may be further evaluated with the peak response ratio of the two detections. That is, if the peak response ratio of a component is different from that of the known standard, then either the peak represents a different species, or some impurities may be eluted with the same retention time. In the present study, baseline separations of compounds III and IV were not obtained under the chromatographic conditions described. However, careful studies of the biological samples revealed that these samples do not contain measurable amounts of compound IV based on the fluorescence—UV peak response ratio. Therefore, no attempt was made to separate compounds III and IV at this time.





ig. 1. Chromatograms of griseofulvin (peaks d and d', t_R = 9.0 min, 3 µg/ml); 6-demethyl-riseofulvin (peaks a and a', t_R = 5.2 min, 3.6 µg/ml); griseofulvic acid (peaks b and b', t_R = .3 min, 2.5 µg/ml) and 7-chloro-4-hydroxy-6,4'-dimethoxy-6'-methylgris-3'-ene-3,2'-ione (peaks c and c', t_R = 7.8 min, 6 µg/ml) obtained from fluorescence (top) and UV ıottom) detections.

ig. 2. Calibration curves for compounds I (C), II (A), III (B) and V (D) from UV detection.

Fig. 2 shows the calibration curves of compounds I, II, III, and V, where ıe peak heights obtained from UV detection are plotted as a function of their oncentrations. Using the procedures given in the Experimental section, solu-ons containing as low as 0.05 µg/ml of these compounds can be detected. he fluorescence detection gives even better sensitivity for the determination f I and III. Compound V was not observed in biological samples. Therefore, was used in the present study as an internal standard.

Fig. 3 shows the chromatograms of a urine sample from a normal subject ho was given an oral dose of 250 mg griseofulvin (McNeil Laboratories, ıicrosize). The sample was collected 4 h after the oral dose and extracted ac-ɔrding to the procedures given in the Experimental section. Three components ere found in the chromatograms, they are identified as I (peak d), II (peak a), ıd III (peak b), respectively. This sample contains only a trace amount of

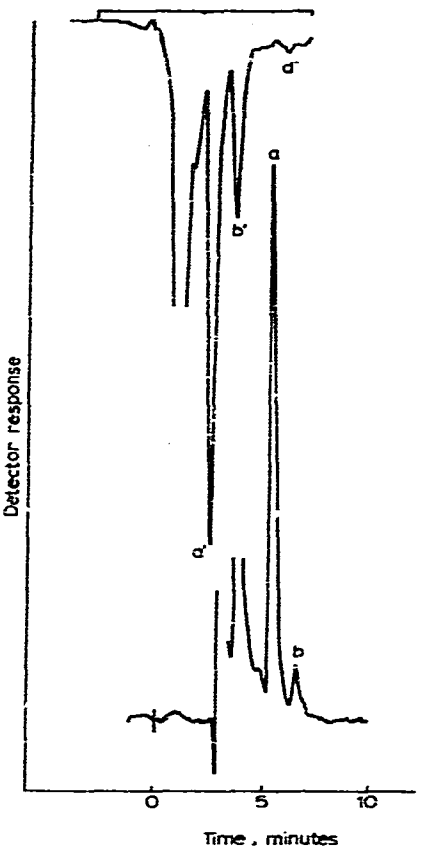


Fig. 3. Chromatogram of a urine sample from a normal subject. Peaks a and a', 6-demethylgriseofulvin; b and b', griseofulvic acid; d', griseofulvin.

griseofulvin which agrees with the literature report that less than 1% of griseofulvin is excreted in the urine [7]. The major metabolite is 6-demethylgriseofulvin with the presence of a significant quantity of griseofulvic acid. 4-Demethylgriseofulvin, which has been reported to be present in the urine in about 1% of the total dose, was not found in this or other samples. The finding of griseofulvic acid (III) in the urine has been reported in a previous communication [15]. In the present study, urine samples from four subjects who were given different brands of griseofulvin tablets were collected and analyzed. All samples showed the presence of griseofulvic acid. The tablets were analyzed prior to the administration and were found to contain griseofulvin in the indicated strengths with the absence of any degradation products or impurities.

The presence of griseofulvic acid (III) in the urine brings an interesting point into the metabolism of griseofulvin. The formation of III is postulated to occur at the 2'-position of griseofulvin via microsomal demethylation and subsequent tautomerization of the 2'-enol to the 2',4'-dione. From the chemical point of view, the formation of III as a metabolite is really not surpising since the vinyl methyl ether at the 2'-position is more readily cleaved than the aromatic ethers at the 4-and 6-positions. Yet both 4- and 6-demethylgriseo-

fulvin, but not griseofulvic acid, have been found to be the metabolites of griseofulvin in man [3]. The fact that griseofulvic acid has been found to be present in incubated agar solutions containing fungi and griseofulvin [4] also supports the finding that griseofulvic acid is a human metabolite of griseofulvin. Griseofulvic acid is chemically reactive, it readily forms isogriseofulvin via methanolysis under mild conditions [5, 6]. Preliminary studies carried out in this laboratory showed that griseofulvic acid is strongly bound to plasma proteins. Thus, this compound may be, at least in part, responsible for the toxicity of the drug. Detailed studies of the protein binding of grisofulvin metabolites and their possible toxicological effects are currently being investigated in this laboratory.

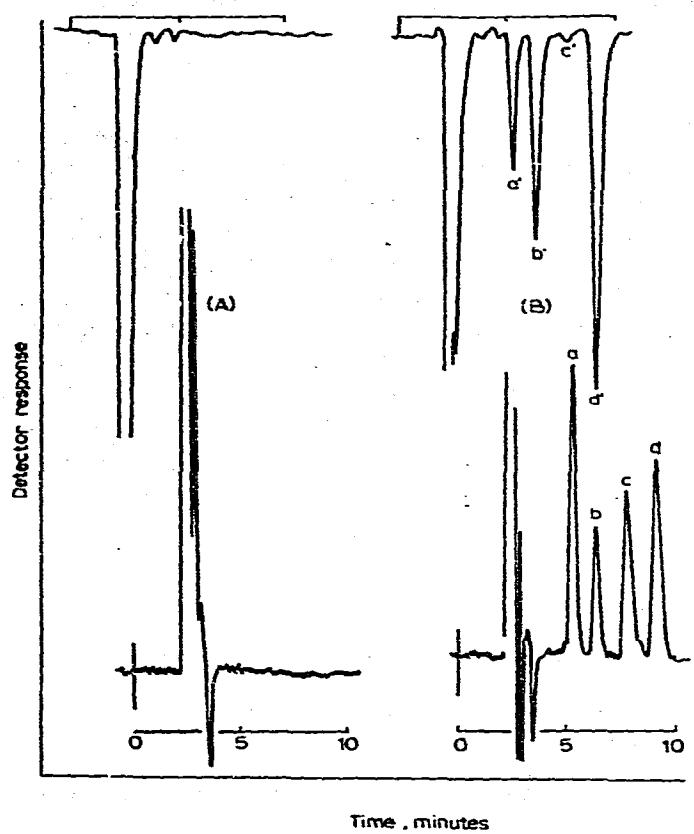


Fig. 4. Chromatograms of a 10-µl injection: (A), a blank plasma sample and (B), a plasma sample containing 2.9 µl/ml of 6-demethylgriseofulvin (peaks a and a'); 2.0 µg/ml of griseofulvic acid (peaks b and b'); 4.4 µg/ml of compound V (peaks c and c', used as an internal standard) and 3.4 µg/ml of grisofulvin (peaks d and d').

Fig. 4 shows the chromatograms of (A) a blank plasma sample, and (B) a plasma sample spiked with griseofulvin and its metabolites. The samples were treated according to the procedure given in the Experimental section. As it can be seen, there is no interference from the plasma ingredients.

In order to evaluate the sample preparation procedures for quantitative

TABLE I

ANALYSIS OF GRISEOFULVIN (I), 6-DEMETHYLGRISEOFULVIN (II), AND GRISEOFULVIC ACID (III) IN IN VITRO URINE SAMPLES

Concentration added (μg/ml)	Recovered*					
	I		II		III	
	μg/ml	(%)	μg/ml	(%)	μg/ml	(%)
0.5	0.50 ± 0.05	100.0	0.52 ± 0.03	104.0	0.48 ± 0.02	96.0
1.0	0.95 ± 0.04	95.0	0.96 ± 0.03	96.0	1.00 ± 0.04	100.0
2.0	1.90 ± 0.08	95.0	2.05 ± 0.07	102.5	1.95 ± 0.05	97.5
3.0	3.10 ± 0.10	103.3	2.86 ± 0.12	95.3	2.85 ± 0.10	95.0
4.0	3.90 ± 0.12	97.5	4.10 ± 0.15	102.5	3.88 ± 0.10	97.0

*Average of 3 determinations.

determination of compounds I, II and III, plasma and urine samples with known concentrations of these compounds in the range of 0.5—4 μg/ml were prepared for HPLC analysis. The results, obtained in triplicate, showed complete recovery of the added compounds in urine samples with an average standard error of ± 5% (see Table I). For the plasma samples, the standard errors were found to be less than ± 5%. Since these samples were simply treated with an equal volume of acetonitrile, no loss of the added compounds was anticipated. The error, however, could come from the non-additive volume changes when the plasma is mixed with acetonitrile. Based on these results, it is seen that this method provides an accurate and simple procedure for the determination of urine and plasma levels of griseofulvin, 6-demethylgriseofulvin and griseofulvic acid.

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