TLC Determination of Griseofulvin in Plasma and 6-Demethylgriseofulvin in Urine

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Abstract □ Fluorometric TLC procedures are described for the determination of griseofulvin in human plasma and 6-demethylgriseofulvin in human urine. Griseofulvin is extracted from plasma with ether, and its metabolite, 6-demethylgriseofulvin, is extracted from urine with benzene. Both compounds are subjected to TLC on silica gel plates. The plates are scanned using the fluorescent mode of a spectrodensitometer. For griseofulvin, the quantitation limit is 20 ng/ml of plasma and the recovery is 100%; for 6-demethylgriseofulvin, the limit is 1 μg/ml of urine and the recovery is 96%. The methods were used to determine the plasma levels of griseofulvin and the amount of 6-demethylgriseofulvin excreted in the urine of human volunteers after a single oral dose of griseofulvin.

Keyphrases □ Griseofulvin—TLC determination in plasma □ 6-Demethylgriseofulvin—TLC determination in urine □ TLC—determination of griseofulvin in plasma and 6-demethylgriseofulvin in urine □ Antifungal agents—TLC determination of griseofulvin in plasma and 6-demethylgriseofulvin in urine

The antifungal agent griseofulvin is metabolized mainly to 6-demethylgriseofulvin and 6-demethylgriseofulvin glucuronide in humans (1–3). Following oral griseofulvin administration to human volunteers, intact griseofulvin and 6-demethylgriseofulvin were measured in plasma (3). In urine, <0.2% of an oral dose was excreted as unchanged griseofulvin (3); the main urinary metabolites were 6-demethylgriseofulvin and its glucuronide, and together they accounted for 65% of an intravenous dose and 34–64% of an oral dose (4).

Plasma levels of unchanged griseofulvin and the urinary excretion of 6-demethylgriseofulvin have been used to estimate griseofulvin bioavailability (4–7). Griseofulvin has been measured in plasma using spectrophotofluorometry (8), GLC (9, 10), and high-pressure liquid chromatography (11, 12). The metabolite 6-demethylgriseofulvin has been measured in urine using UV spectrophotometry (13) and GLC (5, 14). A qualitative TLC procedure also was described and used to identify griseofulvin and its metabolites in urine (15).

This paper describes quantitative fluorometric TLC procedures that have the simplicity of a spectrophotometric method as well as the sensitivity and specificity of a GLC assay.

EXPERIMENTAL

Materials—All reagents and solvents were analytical reagent grade. Commercial silica gel TLC plates[1], 20 × 20 cm (without fluorescent indicator), were used. The plates were divided into 20 channels of 1-cm width with a scoring device[2].

Solutions of griseofulvin and 6-demethylgriseofulvin were prepared in methanol. Acetate buffer (0.1 *M*, pH 4.7) was prepared by dissolving 0.37 g of sodium acetate in 100 ml of distilled water and adjusting to pH 4.7 with acetic acid. Enzyme solutions of β-glucuronidase[3] from beef liver were prepared in this acetate buffer. 6-Demethylgriseofulvin was obtained by extraction from dog urine according to a published procedure (15).

Determination of Plasma Griseofulvin—One milliliter of human plasma was shaken with 5 ml of ether for 15 min to extract the drug. After centrifugation, a 3-ml aliquot of the ether phase was transferred to a clean centrifuge tube and evaporated to dryness under nitrogen[4] at 40–45°. The residue was dissolved in 100 μl of chloroform, and a 40-μl aliquot from this solution was spotted on a TLC plate along with standard griseofulvin solutions.

The plate was developed to a height of 10 cm in a saturated tank containing 100 ml of the ether–acetone (80:20) solvent system. Then the plate was air dried and scanned in a spectrodensitometer[5] containing a 200-w xenon–mercury lamp[6] and coupled to a density computer[7]. The instrument was operated in the fluorescence mode with excitation at 305 nm and emission at 650 nm. Quantitation was achieved by a computing digital integrator[8].

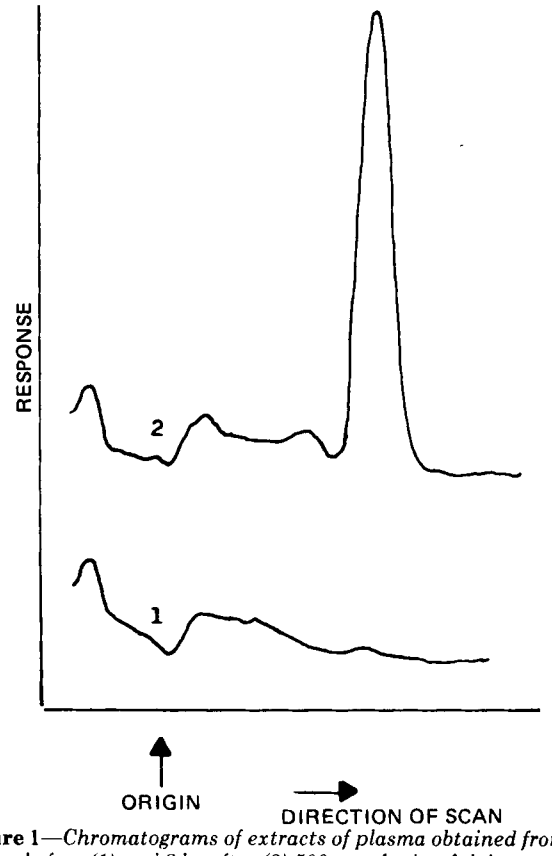

Figure 1—*Chromatograms of extracts of plasma obtained from a volunteer before (1) and 3 hr after (2) 500 mg of griseofulvin po.*

[1] Silplate-22, Brinkmann Instruments.
[2] SDA 320, Schoeffel Instrument Corp.
[3] Worthington Biochemical Corp.
[4] N-Evap, Organomation Associates.
[5] Model SD 3000, Schoeffel Instrument Corp.
[6] Hanovia Lamp Division, Comrand Precision Industries.
[7] Model SDC 300, Schoeffel Instrument Corp.
[8] Autolab System I, Spectra-Physics Corp.

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Table I—Recovery of Griseofulvin from Human Plasma

Amount Spiked, ng/ml	Amount Recovered, ng/ml	Percent Recovery
20	20	100.0
30	30	100.0
40	41	102.5
100	93	93.0
100	100	100.0
100	103	103.0
200	210	105.0
200	217	108.5
300	303	101.0
300	296	98.7
300	297	99.0
300	290	96.7
400	383	95.8
400	379	94.8
400	380	95.0
400	383	95.8
500	500	100.0
1000	1050	105.0
1000	1000	100.0
1000	980	98.0
1000	1020	102.0
1500	1570	104.6
Mean		99.9
SD		3.9

Determination of 6-Demethylgriseofulvin in Urine—For the determination of free 6-demethylgriseofulvin, 1 ml of urine was acidified with 0.1 ml of 1 *N* HCl and shaken with 2 ml of benzene for 15 min. The mixture was centrifuged to obtain a clear benzene phase. Ten-microliter aliquots of the benzene solution were spotted on a TLC plate along with standard solutions of 6-demethylgriseofulvin. The plate was developed to 10 cm in a saturated tank containing 100 ml of the chloroform–ether–acetone–acetic acid (65:20:15:0.5) solvent system. Then the plate was air dried and read in the fluorescence mode of a spectrodensitometer as described.

To assay conjugated 6-demethylgriseofulvin, 1 ml of urine was mixed with 0.5 ml of 0.1 *M* acetate buffer containing 430 units of β-glucuronidase. The solution was incubated at 37° for 90 min. After incubation, 0.1 ml of 1 *N* HCl was added, and the sample was shaken with 10 ml of benzene for 15 min. The mixture was centrifuged to obtain a clear benzene phase, and 20-μl aliquots of this phase were analyzed as described for the determination of free 6-demethylgriseofulvin. This procedure measures total (free and conjugated) 6-demethylgriseofulvin. To determine the amount of conjugated 6-demethylgriseofulvin, the free metabolite is subtracted from the total.

Recovery Experiments—Samples of human plasma were spiked with solutions of various griseofulvin concentrations, and urine samples were spiked with 6-demethylgriseofulvin. The spiked samples were assayed by the described procedure and compared to absolute standards.

Linearity of Response and Quantitation Limit of Absolute Standards—Solutions with different concentrations of griseofulvin and 6-demethylgriseofulvin were spotted on TLC plates and chromatographed to determine the linear ranges of instrument response. The quantitation limit was set as an "area under the peak" of 25,000 units,

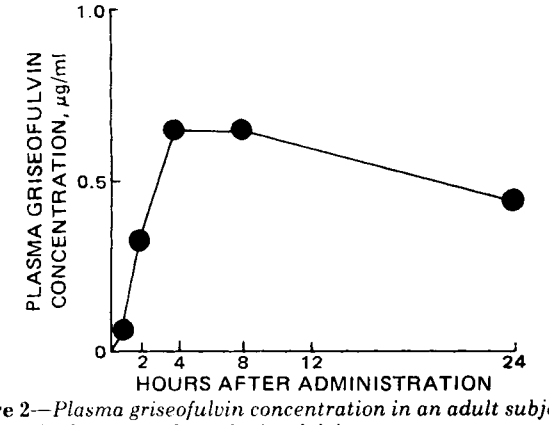

Figure 2—*Plasma griseofulvin concentration in an adult subject following a single 250-mg dose of griseofulvin.*

Table II—Recovery of 6-Demethylgriseofulvin from Human Urine

Amount Spiked, μg/ml	Amount Recovered, μg/ml	Percent Recovery
10	11	110.0
10	10	100.0
20	21	105.0
20	20	100.0
20	20	100.0
40	35	87.5
40	35	87.5
40	44	110.0
60	59	98.3
60	52	86.7
60	62	103.3
80	68	85.0
80	66	82.5
80	69	86.3
Mean		95.9
SD		9.6

which corresponded to 5 ng of griseofulvin or 6-demethylgriseofulvin/spot.

***In Vivo* Experiment**—To demonstrate the applicability of the methods, a plasma level profile of griseofulvin and a urinary excretion profile of 6-demethylgriseofulvin were obtained in human volunteers. One volunteer received 250 mg of griseofulvin[9], and blood samples were drawn before and 1, 2, 4, 8, and 24 hr after medication. Blood was transferred to tubes containing ethylenediaminetetraacetic acid, and the contents were mixed and centrifuged. Plasma was kept frozen until it was analyzed. A second volunteer received griseofulvin as one 500-mg tablet[10], and urine was collected for 72 hr after drug administration in pools at 24,

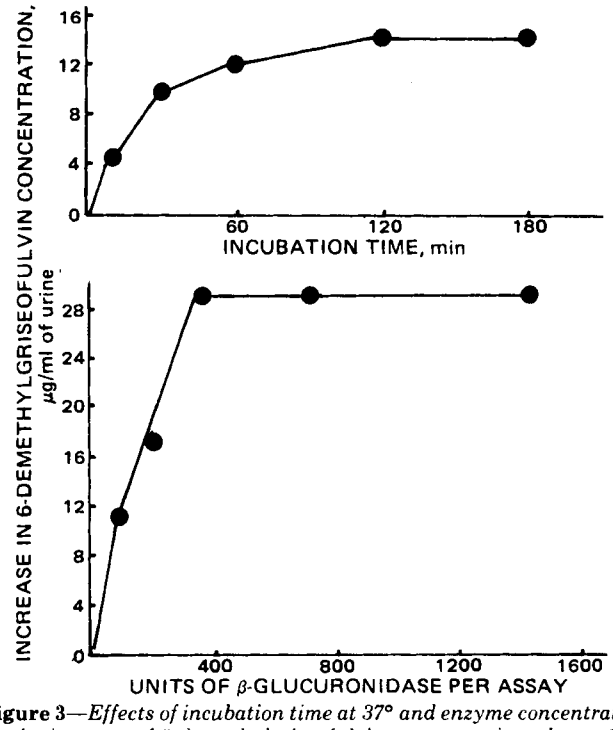

Figure 3—*Effects of incubation time at 37° and enzyme concentration on the increase of 6-demethylgriseofulvin concentration when a 1-ml urine sample was subjected to hydrolysis by β-glucuronidase. Top: time of incubation of urine sample in the presence of 430 units of β-glucuronidase. The urine specimen (0–24 hr) was collected from a volunteer receiving a daily dose of 125 mg of griseofulvin. Bottom: incubation of urine sample at 37° for 90 min in the presence of various concentrations of β-glucuronidase. Enzyme activity is expressed in Fishman units. The urine specimen (0–24 hr) was obtained from a volunteer who had received one oral dose of 450 mg of griseofulvin.*

[9] Grisactin 250, Ayerst Laboratories.
[10] Grisactin 500, Ayerst Laboratories.

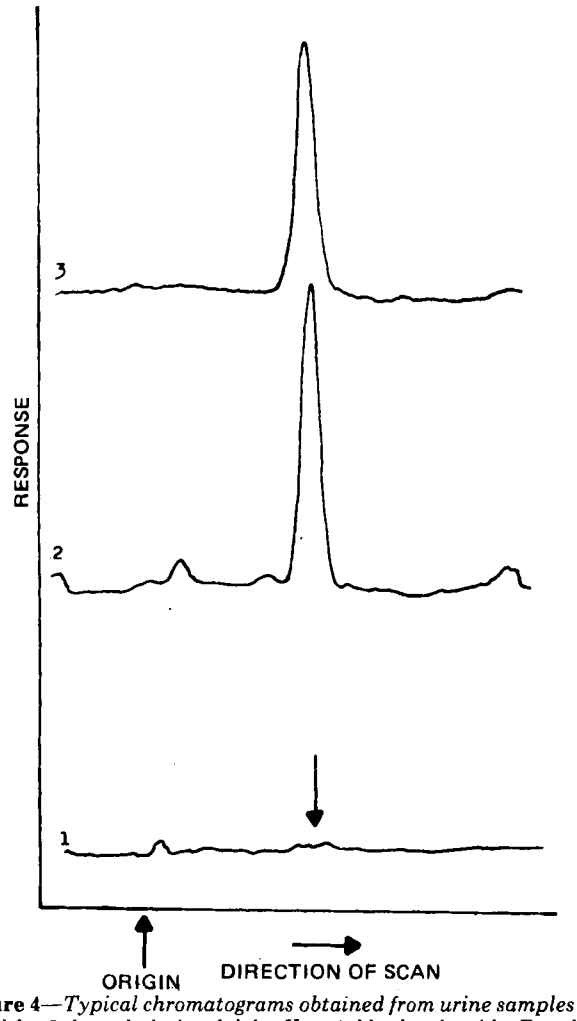

Figure 4—*Typical chromatograms obtained from urine samples analyzed for 6-demethylgriseofulvin. Key: 1, blank urine (the R_f value of 6-demethylgriseofulvin is indicated by the arrow); 2, pooled urine (0–24 hr) from a volunteer who received one single oral 500-mg dose of griseofulvin; and 3, standard of 6-demethylgriseofulvin with 100 ng/spot.*

48, and 72 hr. A preadministration urine sample also was obtained. The samples were kept frozen until they were analyzed.

RESULTS AND DISCUSSION

Plasma Griseofulvin—Griseofulvin had an R_f value of 0.25 in the TLC system and could be quantitated to 5 ng/spot. In terms of assay sensitivity in plasma, the limit of reliable quantitation was set at 20 ng/ml. Instrument response, as determined by the area under the peak, was linear from 0 to at least 300 ng/spot. In terms of concentrations in plasma, linearity was observed from 10 to at least 1000 ng/ml. The assay was free of interference from extractable plasma impurities and from the metabolite 6-demethylgriseofulvin (R_f 0.05).

Chromatograms of extracts from human plasma obtained before and after griseofulvin administration are shown in Fig. 1. Recoveries obtained from plasma samples spiked with griseofulvin over the 20–1500-ng/ml range varied between 93 and 109%, with a mean of 99.9% (Table I). The reproducibility was ±3.9% (*SD*), n = 22. The absolute accuracy of the mean was calculated using the expression $\overline{X} - \hat{X}$, where $\overline{X}$ is the mean of the set and $\hat{X}$ is the true value, defined here as 100% recovery. Therefore, the absolute accuracy of the mean was 99.9% − 100.0% = −0.1%. A plasma griseofulvin concentration–time curve obtained in a volunteer following oral administration of a single 250-mg dose is shown in Fig. 2.

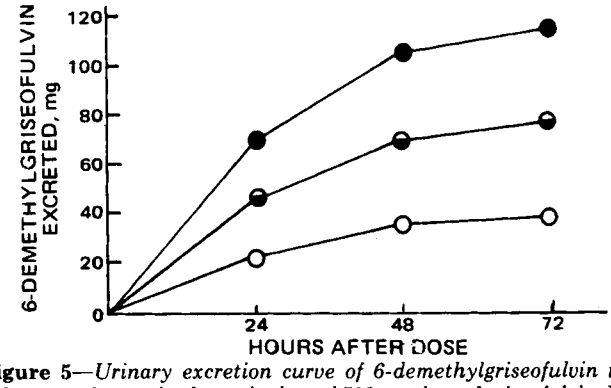

Figure 5—*Urinary excretion curve of 6-demethylgriseofulvin in a volunteer who received one single oral 500-mg dose of griseofulvin. Key: ●, total 6-demethylgriseofulvin; ⊖, free 6-demethylgriseofulvin; and ○, conjugated 6-demethylgriseofulvin.*

6-Demethylgriseofulvin in Urine—In the system used, 6-demethylgriseofulvin (R_f 0.26) was well separated from urine impurities and from the parent drug, griseofulvin (R_f 0.47). Instrument response, as measured by the areas under the peaks, was linear over the range tested, 0–600 ng/spot or 0–120 μg/ml of urine when expressed in concentration units. The detection limit was 5 ng/spot or 1 μg/ml. Recoveries obtained from urine samples spiked with 6-demethylgriseofulvin over the 10–80-μg/ml range varied between 88 and 108%, with a mean of 95.9% (Table II). The reproducibility was ±9.6% (*SD*), n = 14. The absolute accuracy of the mean was −4.1%.

In the determination of total (free and conjugated) 6-demethylgriseofulvin, it was essential to establish that the incubation period and the enzyme concentration used were adequate to hydrolyze all glucuronide present. Since no standard material of 6-demethylgriseofulvin glucuronide was available, urine samples from volunteers dosed with griseofulvin were subjected to hydrolysis using various β-glucuronidase concentrations and incubation times. As seen in Fig. 3, a glucuronidase concentration of 370 units in the 1.5-ml incubation mixture and an incubation time of 1.5 hr were sufficient to liberate the maximum amount of free 6-demethylgriseofulvin from the sample.

Chromatograms of a blank human urine sample and of a postdosing urine sample are shown in Fig. 4.

Figure 5 shows the 72-hr urinary excretion profile of 6-demethylgriseofulvin of a volunteer following oral administration of a single 500-mg dose of griseofulvin.

The described methods are sensitive, specific, and reproducible. In addition, they are simple, relatively rapid, and especially useful when a large number of samples must be analyzed.

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