Liquid–Solid Chromatographic Determination of 6-Demethylgriseofulvin in Urine

E. PAPP [x], K. MAGYAR, and H. J. SCHWARZ

Abstract □ A specific and quantitative liquid–solid chromatographic method for the determination of 6-demethylgriseofulvin in human urine is reported. The method consists of extraction into an organic solvent, addition of internal standard, and analysis by liquid–solid chromatography using a UV detector. Griseofulvin, if present, can be determined simultaneously. The sensitivity of the method is 6 μg/ml of urine. Total 6-demethylgriseofulvin is determined after hydrolysis of the glucuronide conjugate with glucuronidase–sulfatase enzyme solution. The method is well suited for the analysis of a large number of samples.

Keyphrases □ 6-Demethylgriseofulvin—griseofulvin metabolite, liquid–solid chromatographic analysis, urine □ Griseofulvin metabolite—6-demethylgriseofulvin, liquid–solid chromatographic analysis, urine □ Liquid–solid chromatography—analysis, 6-demethylgriseofulvin, urine

6-Demethylgriseofulvin was found to be present in human urine after the administration of the antifungal agent griseofulvin. At the same time, only traces of griseofulvin were found (1). 6-Demethylgriseofulvin and its glucuronide were later shown to be the major urinary metabolites of griseofulvin in humans (2, 3), accounting for 65% of an intravenous dose (4) and 35–64% of an oral dose (2, 4).

The rate of excretion of free or total 6-demethylgriseofulvin was found to be directly proportional to the plasma concentration of griseofulvin (4), and the cumulative amount excreted was a direct measure of the amount absorbed (4, 5). Since less than 0.2% of the dose was excreted as griseofulvin (2), the measurement of 6-demethylgriseofulvin in urine provides a convenient estimation of the absorption and bioavailability of griseofulvin.

Analytical methods for the determination of 6-demethylgriseofulvin in urine by GLC as the trimethylsilyl derivative (5) or by UV spectrophotometry (3) were published previously. The method described in this report is both simple (avoids derivatization) and specific and shows good reproducibility.

EXPERIMENTAL

Reagents and Chemicals—The following reagents and chemicals were used: sodium hydroxide[1], methanol[2], isopropanol[2], acetic acid[2], 1-butanol[2], and griseofulvin[3] (all analyzed reagents); benzene[4], glass distilled; heptane[5], spectrograde; high-speed pellicular column packing[6]; and glucuronidase–sulfatase enzyme solution[7]. 6-Demethylgriseofulvin was isolated from human urine (1), and 4-(*m*-hydroxyphenyl)-1-isopropyl-7-methyl-2(1*H*)-quinazolinone was synthesized.

Instrumentation—A high-pressure liquid chromatograph[8] equipped with a 254-nm UV detector, using a 31.4-cm (3-ft) long and 2-mm i.d. [0.0984-cm (0.25-in.) o.d.] stainless steel column[6], was employed. The following conditions were used: column temperature, ambient; air pressure, 900 psi; gradient rate, 1%/min; function, concave 3; mode, operation and gradient; flow rate, 1.5–2.0 ml/min; initial composition, 0.0% secondary solvent; final composition, 6.0% secondary solvent; attenuation, 16; solvent in primary reservoir, 100% heptane; and solvent in secondary reservoir, methanol–isopropanol (1:1). Chromatograms were recorded on a 1-mv recorder[9].

Method—The pH of 3 ml of urine was adjusted to 5.35 with acetic acid and/or 2 *N* sodium hydroxide, and 0.2 ml of glucuronidase–sulfatase enzyme solution was added. The solution was incubated overnight at 37.5°. The incubated urine was extracted with 10 ml of 20% 1-butanol in benzene (v/v). An 8.0-ml aliquot of the extract was transferred to a pear-shaped flask and evaporated to dryness *in vacuo* on a rotary evaporator. The residue was dissolved in 0.4 ml of methanol containing 0.25 mg/ml of 4-(*m*-hydroxyphenyl)-1-isopropyl-7-methyl-2(1*H*)-quinazolinone as the internal standard. About 5 μl of this solution was injected into the chromatograph.

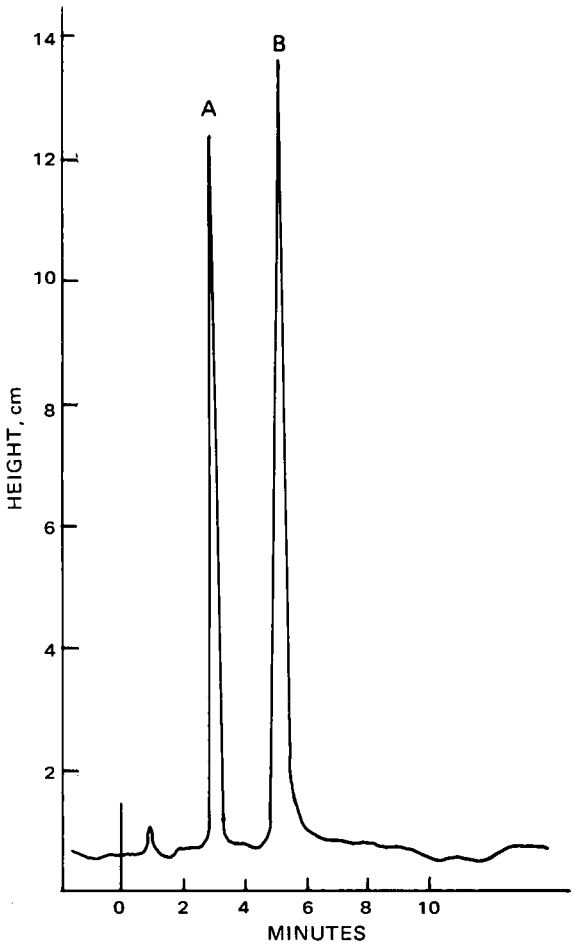

Figure 1—*Liquid chromatogram of a standard solution (5 μl). Key: A, internal standard; and B, 6-demethylgriseofulvin.*

[1] Mallinckrodt, Inc.
[2] Fisher Scientific Co.
[3] Schering Corp.
[4] Burdick and Jackson Laboratories, Inc.
[5] Matheson, Coleman and Bell.
[6] High Speed Pellosil, Reeve Angel.
[7] Glusulase, Endo Laboratories, Inc.
[8] DuPont model 830.
[9] Honeywell.

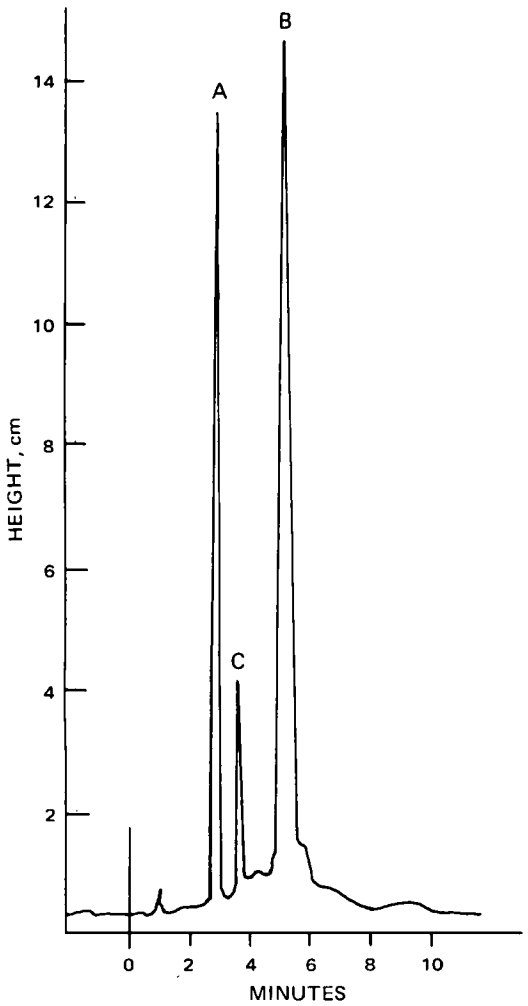

Figure 2—*Liquid chromatogram of a urine extract containing griseofulvin and 6-demethylgriseofulvin. Key: A, internal standard; B, 6-demethylgriseofulvin; and C, griseofulvin.*

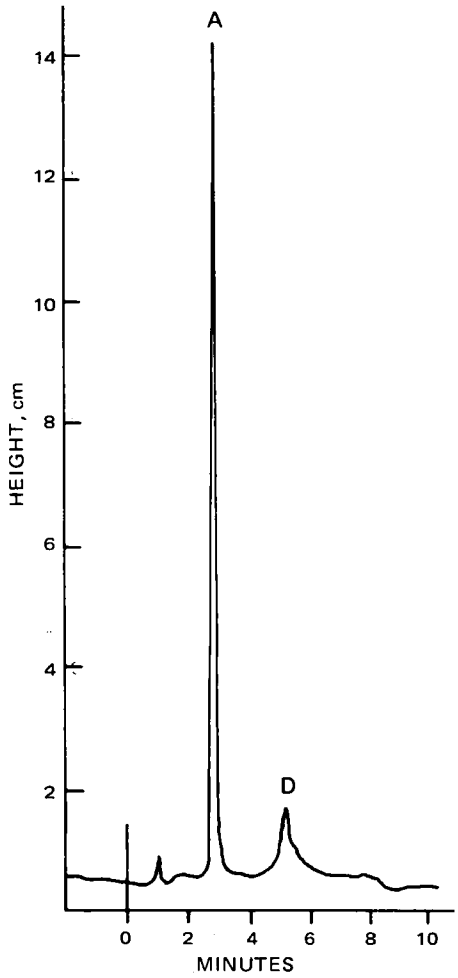

Figure 3—*Liquid chromatogram of a blank urine extract. Key: A, internal standard; and D, interfering blank value.*

The heights of the peaks corresponding to 6-demethylgriseofulvin and internal standard were then measured, and the ratio of the peak heights was converted into milligrams of 6-demethylgriseofulvin per milliliter of assay solution using the slope of the standard curve. The urinary concentration was obtained after correction for blank values, dilution (concentration), and recovery.

Standard curves were prepared by injecting standard solutions of 6-demethylgriseofulvin in methanol in the range of 0.05–1.00 mg/ml, each containing 0.25 mg/ml of internal standard, under the same analysis conditions as the samples. The peak height ratios of 6-demethylgriseofulvin–internal standard were then plotted against the concentration of 6-demethylgriseofulvin in the assay solution.

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; 6-demethylgriseofulvin, 5.2 min; and griseofulvin (Fig. 2), 3.6 min. Linearity between peak height ratio and concentration of 6-demethylgriseofulvin was obtained between 0 and 1.00 mg/ml. The slope of the standard curve changed very little from day to day.

Sensitivity—Under the described conditions, the lowest measurable concentration of 6-demethylgriseofulvin was 0.05 mg/ml of assay solution (peak height ratio = 0.1), which corresponds to a lower limit of 6 μg/ml of urine. This sensitivity was sufficient to follow the excretion of a single dose of 250–500 mg of griseofulvin for 72 hr. The instrument had practically no noise level. Therefore, the detection limit (two to three times signal to noise ratio) could not be determined.

Specificity—The combination of extraction, liquid–solid chromatographic retention time, and low blank values imparts good specificity to this method. However, it is not known whether 4-demethylgriseofulvin, the minor metabolite of griseofulvin in humans (2), has a different retention time from 6-demethylgriseofulvin.

Determination in Urine—The liquid–solid chromagtographic tracing of an extract of a blank urine sample (Fig. 3) was compared with an extract of a urine sample containing griseofulvin and 6-demethylgriseofulvin (Fig. 2). Some blank extracts showed an interfering peak with about the same retention time as 6-demethylgriseofulvin. In such a case, the peak height ratio of the interfering peak to the internal standard was deducted as a blank value in the calculation of the peak height ratios of 6-demethylgriseofulvin–

Table I—Recovery of 6-Demethylgriseofulvin after *In Vitro* Addition to Human Urine

6-Demethylgriseofulvin Added, mg/3 ml	6-Demethylgriseofulvin Recovered[a]	
	mg/3 ml ± *SD*	%
0.10	0.103 ± 0.023	103
0.20	0.224 ± 0.024	112
0.50	0.435 ± 0.044	87
1.00	0.939 ± 0.092	94
1.50	1.47 ± 0.08	98
2.00	1.91 ± 0.13	96
Average recovery		98

[a] Average of eight determinations.

internal standard in the drug-containing samples of the same urine (recovery) or subject.

Recovery—The validity of the method was verified and the recovery was determined by adding various known amounts of 6-demethylgriseofulvin to human urine. Each urine standard, as well as the blank urine, was analyzed eight times according to the method described (Table I).

The mean recoveries varied from 87 to 112% of the added amount. Recovery was independent of the concentration of 6-demethylgriseofulvin and averaged 98%.

Because of its simplicity, this method is well suited for the routine analysis of a large number of samples. It measures total, free, and conjugated 6-demethylgriseofulvin. Free metabolite can be determined by omitting the initial hydrolysis step. The method also allows griseofulvin, if present, to be determined simultaneously.

The method was used successfully in several bioavailability studies, and the results will be reported elsewhere.

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CNS Depressant Activity of Pyrimidylthiazolidones and Their Selective Inhibition of NAD-Dependent Pyruvate Oxidation

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Abstract □ Several 1-aryl-3-(2-pyrimidyl)thiocarbamides and their corresponding cyclized 2-arylimino-3-(2-pyrimidyl)thiazolid-4-ones were synthesized and characterized by their sharp melting points and elemental analyses. These thiocarbamides and thiazolidones possessed anticonvulsant activity against pentylenetetrazol-induced convulsions and potentiated pentobarbital-induced hypnosis in mice. Most of these thiocarbamides and thiazolidones selectively inhibited nicotinamide adenine dinucleotide (NAD)-dependent oxidation of pyruvate, where the use of added NAD decreased the degree of inhibition. The NAD-independent oxidation of succinate, on the other hand, remained unaltered. The anticonvulsant activity of thiocarbamides and thiazolidones was unrelated to their ability to inhibit the respiratory activity of rat brain homogenates during oxidation of sodium pyruvate. Cyclization of thiocarbamides to the corresponding thiazolidones in general enhanced their CNS depressant and enzyme inhibitory effectiveness.

Keyphrases □ Thiazolidones—synthesis, evaluation of CNS activity, effect on NAD-dependent oxidation □ Thiocarbamides—synthesis, evaluation of CNS activity, effect on NAD-dependent oxidation □ CNS activity—synthesis and evaluation of several thiocarbamides and thiazolidones □ Enzyme activity—effect of thiocarbamides and thiazolidones on NAD-dependent oxidation □ Anticonvulsants—synthesis and evaluation of several thiocarbamides and thiazolidones

Considerable interest recently has been focused in this laboratory on substituted thiazolidones that have been shown to possess anticonvulsant (1–3), hypnotic (4), and local anesthetic (5) properties. Earlier studies indicated diverse pharmacological profiles of pyrimidine derivatives, including diuretic (5), local anesthetic (6), and anticonvulsant (7, 8) properties. Piperazinothiocarbamides also have been shown to possess effects on the central nervous system (CNS) activity (9, 10). These observations prompted synthesis of substituted thiocarbamides, which were cyclized into the corresponding 2-arylimino-3-(2-pyrimidyl)thiazolid-4-ones.

In the present study, these thiocarbamides and thiazolidones were evaluated for their anticonvulsant activity and their ability to potentiate pentobarbital-induced hypnosis and to inhibit cellular respiratory activity of rat brain homogenates, with a view to studying their biochemical mechanism of action. The various thiazolidones were synthesized by following the methods outlined in Scheme I (3).

EXPERIMENTAL [1]

The various 1,3-disubstituted thiocarbamides (Table I) were prepared by refluxing equimolar quantities of 2-aminopyrimidine and the appropriate arylisothiocyanate in dry benzene. These substituted thiocarbamides were cyclized into the corresponding 2-arylimino-3-(2-pyrimidyl)thiazolid-4-ones by refluxing with chloroacetic acid and anhydrous sodium acetate in acetic acid by the following method.

1-Aryl-3-(2-pyrimidyl)thiocarbamides (I–VIII)—2-Aminopyrimidine (0.01 *M*) was mixed with a suitable arylisothiocyanate (0.01 *M*) in 15 ml of dry benzene, and the mixture was refluxed on a steam bath for 4 hr. The reaction mixture was concentrated by distilling the excess benzene under reduced pressure. The solid mass which separated on cooling was filtered, washed with ether and dilute hydrochloric acid, dried, and recrystallized from ethanol. All thiocarbamides, characterized by their sharp melting points and elemental analyses, are recorded in Table I.

2-Arylimino-3-(2-pyrimidyl)thiazolid-4-ones (IX–XVI)—A

[1] All compounds were analyzed for their carbon, hydrogen, and nitrogen content. Melting points were taken in open capillary tubes with a partial immersion thermometer and are corrected.