Table III—Antibacterial Activity

Compound[a]	Bacteriostatic Activity, MIC[b], mcg./ml. *Staphylococcus aureus* ATCC 6538	*Streptococcus hemolyticus* SGA 380[c]	*Streptococcus fecium* SGB[c]	*Escherichia coli* ATCC 9633	*Proteus mirabilis*[c]	*Pseudomonas aeruginosa* ATCC 10145
Ic	78.2	19.6	156.00	625.0	5000.0	5000.0
Id	156.0	78.2	313.0	313.0	2500.0	2500.0
Ie	625.0	156.0	313.0	313.0	5000.0	1250.0

[a] Hydrochlorides of the compounds were used as the test substance. [b] Minimum inhibitory concentration; determined by the method described in P. Klein, "Bakteriologische Grundlagen der Chemotherapeutischen Laboratoriumspraxis," Springer-Verlag, Berlin, Germany, 1957, pp. 53ff; and R. Brunner and G. Macheck, "Die Antibiotica," 2 Bde, Verlag Hans Carl, Nuremberg, Germany, 1962. [c] Culture collection of Microbiology Department of Farbwerke Hoechst AG., Frankfurt, Germany.

mixture of II*b* (16.3 g., 0.48 mole), methanol (100 ml.), and sodium hydroxide solution (5 ml. of 50%) was refluxed for 15 hr. On cooling, the mixture was diluted with water and extracted with methylene chloride. The aqueous solution was acidified with hydrochloric acid and the obtained precipitate was filtered, washed with water, dried, and crystallized from toluene–ligroin, yielding 9 g. (Table II); IR: ν_{max} 1720 (CO), 1634 (C=C), 3350, and 3400 (OH) cm.$^{-1}$; NMR: δ 3.19 (s, 2H, CH_2), 3.99 (s, 3H, OCH_3), 7.4 (m, 8 aromatic protons), and 11.8 (broad s, 1H, COOH); mass spectroscopy (180°/70 ev.): M^+ = 327.

2-Methoxy-3-methyl-4-phenyl-6-chloroquinoline (II*d*)—Compound II*c* (32.7 g., 0.1 mole) was refluxed with quinoline (100 ml.) and copper powder (4.5 g.) for 3 hr. and worked up according to the method of Burness (12); IR: ν_{max} 1630 (C=C), 1400, 1435, 1480, 1500 (aromatic C=C), and 2825 (weak, OCH_3) cm.$^{-1}$; NMR: δ 2.29 (s, 3H, CH_3), 3.98 (s, 3H, OCH_3), and 7.4 (m, 8 aromatic protons); mass spectroscopy (170°/70 ev.): M^+ = 283.

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Determination of 6-Demethylgriseofulvin in Urine

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Abstract □ A simple UV assay is reported that is capable of measuring 1 mcg. 6-demethylgriseofulvin/ml. urine. The method utilizes the difference in the absorbance of the ionized and unionized forms of this molecule at 327 nm.

Keyphrases □ Griseofulvin metabolites—UV spectrophotometric determination of 6-demethylgriseofulvin in urine □ 6-Demethylgriseofulvin—UV spectrophotometric determination in urine □ UV spectrophotometry—determination of 6-demethylgriseofulvin

In man (1) as well as rabbit (2) and dog (3), *O*-demethylation of the antifungal agent, griseofulvin, to 6-demethylgriseofulvin is a significant route of elimination. Griseofulvin, ingested as a solution in polyethylene glycol 300, was almost quantitatively recovered (4) in the urine as 6-demethylgriseofulvin in man. While griseofulvin was readily seen in the plasma following oral administration of standard 250- and 500-mg. doses,

less than 0.1% of the dose appeared unchanged in the urine (1, 5). Urinary excretion measurement of 6-demethylgriseofulvin therefore offers convenient and supplemental information when studying bioavailability profiles of griseofulvin under various conditions. This report describes a simple method for measuring this metabolite in urine, utilizing the difference in the spectrum between the ionized and unionized forms of 6-demethylgriseofulvin at 327 nm.

EXPERIMENTAL[1]

Citrate Buffer—Citric acid, 1 *M*, is adjusted to pH 3.9 with 1 *M* sodium citrate.

Organic Solvent—Cyclohexane–ethylene dichloride (50:50) is prepared by successively washing each solvent with 1 vol. 1 *N* NaOH, 1 vol. 1 *N* HCl, and 2 vol. distilled water.

Phosphate Buffer (McIlvaine's Buffer)—Citric acid (0.2 *M*, 27.5 ml.) is diluted to 1000 ml. with 0.2 *M* Na_2HPO_4, final pH 7.9.

Procedure—To 2 ml. of urine (containing 0–10 mcg. 6-demethylgriseofulvin/ml.) are added 1 ml. citrate buffer and 10 ml. organic solvent; the mixture is shaken for 2 min. and centrifuged. Six milliliters of the organic layer is pipeted into another tube containing 6 ml. pH 7.9 phosphate buffer, shaken for 2 min., and centrifuged; then the upper organic layer is aspirated off. Two milliliters of the remaining aqueous layer is pipeted into each of two tubes, one containing 0.2 ml. 5 *N* HCl and the other containing 0.2 ml. McIlvaine's buffer (pH 7.9). The absorbance of the basic solution is read against the acidic solution at 327 nm. The concentration of 6-demethylgriseofulvin in the urine is calculated by reference to a standard curve prepared by taking known concentrations of 6-demethylgriseofulvin (0–10 mcg./ml.) through the same procedure.

The concentration of the glucuronide of 6-demethylgriseofulvin is determined as the difference between the concentration of 6-demethylgriseofulvin before and after incubation of the urine sample overnight at 37° with 100 units of β-glucuronidase in 1 ml. of phosphate buffer (pH 6.8).

RESULTS AND DISCUSSION

Most plasma assays for griseofulvin utilize its native fluorescence in aqueous and hydroalcoholic solutions. It was reasoned and subsequently verified that unionized 6-demethylgriseofulvin also fluoresces with the same activation (315 nm.) and fluorescence (450 nm.) maxima as griseofulvin and, thereby, an avenue should exist for the establishment of a sensitive assay for this metabolite. Surprisingly, a pH fluorescence profile indicated a maximum fluorescent intensity at pH 2.4 equal to 30% of griseofulvin on a molar basis. The decreasing fluorescence, seen with increasing pH beyond 2.4 and virtually absent above pH 7.0, suggests that only the unionized molecule fluoresces. A half-maximal fluorescence at pH 4.5, very similar to the pKa (4.27) of 6-demethylgriseofulvin (6), supports this hypothesis. Below pH 2.4, the fluorescence also declines and it is negligible at pH < 0.5, presumably caused by protonation of 6-demethylgriseofulvin. While subsequent attempts to develop a fluorometric assay for urinary 6-demethygriseofulvin proved unsuccessful because of excessive fluorescent species in urine, fluorescent measurements were used to determine pH partition profiles of this metabolite with various organic solvents. Furthermore, while griseofulvin fluorescence is pH insensitive above pH 2.2, like 6-demethylgriseofulvin it diminishes markedly in a more acidic environment. This last observation provided a useful modification to the usual fluorometric assay for griseofulvin in plasma (7).

By using a 5:1 volume ratio of organic solvent to aqueous buffer, 6-demethylgriseofulvin is extracted quantitatively into ethylene dichloride or ether below pH 3.5. Above pH 3.5, extraction progressively diminishes. Partitioning occurs more favorably into the former solvent, 50% 6-demethylgriseofulvin being extracted into ethylene dichloride at pH 6.9 and 6.0 with ether. Both solvents were investigated in a potential assay in which the urine was adjusted to pH 4.0 (range 3.92–4.30) with strong citrate buffer (pH 3.9) prior to extraction. This pH allows quantitative 6-demethylgriseofulvin extraction while reducing interference from urinary constituents that are extracted in higher proportions from a more acidic urine. 6-Demethylgriseofulvin was determined by differential spectrophotometry, taking advantage of the low absorbance of the unionized phenol at the λ_{max} (327 nm.) for the phenolate ion ($\Delta\epsilon$ = 26,900) (8). One microgram 6-demethylgriseofulvin/ml. aqueous solutions could readily be measured.

Unfortunately, both ether and ethylene dichloride extracted too many absorbing materials from blank urine. Various combinations of cyclohexane with ether, ethylene dichloride, and chloroform were examined to reduce the urine blank. An equal volume of cyclohexane and ethylene dichloride proved the most suitable solvent. This mixture has a lower specific gravity than water, allowing facile transfer from one tube to another; it also does not form emulsions with urine and quantitatively extracts 6-demethylgriseofulvin at and below pH 4.5. Furthermore, extraction of 6-demethylgriseofulvin into this solvent mixture is negligible at pH 7.5 or greater, so a pH 7.9 buffer was chosen to back-extract this metabolite. The absorbance of the pH 7.9 buffer extract was then measured at 327 nm. against the same solution made acidic with 5 *N* HCl. When using this procedure, blank values were always less than 0.35 mcg. 6-demethylgriseofulvin equivalents/ml. urine. Also, a linear regression was observed between absorbance and concentration in the range of 0–10 mcg./ml. urine. Comparison of the slope of a calibration curve taken through the procedure with an aqueous standard curve indicates an overall recovery of 97.7%.

The presence of 6-demethylgriseofulvin in an ethereal extract of acidic urine was confirmed by TLC on silica gel plates containing a green fluorophore, using chloroform–acetic acid–water (4:1:1) as the developing solvent. A spot at the same R_f value as authentic 6-demethylgriseofulvin (R_f 0.34) was absent in blank urine but present in urine of subjects ingesting griseofulvin. Like 6-demethylgriseofulvin, the native fluorescence of this spot in the acidic environment of the developing solvent was quenched by exposure to ammonia fumes. The presence of another metabolite, 4-demethylgriseofulvin, formed in the rat and rabbit (9), was also sought. This metabolite, synthesized by a literature method (10), gave a different R_f value (0.55) in this thin-layer system. No spot corresponding to 4-demethylgriseofulvin was detected in the urine of subjects receiving griseofulvin before or after incubation with β-glucuronidase. The presence of 6-demethylgriseofulvin glucuronide was assessed by exhaustively extracting 6-demethylgriseofulvin from acidic urine with ether, adjusting to pH 6.8, and incubating with β-glucuronidase in phosphate buffer (pH 6.8) at 37° overnight. The appearance of a spot, absent in control urine, at the same R_f value as 6-demethylgriseofulvin and with identical fluorescent properties was taken as evidence of 6-demethylgriseofulvin glucuronide. Incubation studies under the assay conditions indicated that the glucuronide was hydrolyzed within 4 hr. (11). A GLC assay has been used (11) for the determination of urinary 6-demethylgriseofulvin. The reported (11) failure to find conjugated metabolites conflicts with the present findings. These differences may have arisen from different conditions used in the hydrolysis.

By utilizing the described assay, between 70 and 100% of an intravenous dose of griseofulvin was recovered as the sum of 6-demethylgriseofulvin and 6-demethylgriseofulvin glucuronide in man (12). Also, in man (13), unlike dogs (3, 14), excretion of 6-demethylgriseofulvin is rate limited by the elimination of griseofulvin. Furthermore, the proportionality between the amount of 6-demethylgriseofulvin excreted and the area under the griseofulvin plasma time curve (10) indicates that urinary 6-demethylgriseofulvin may be used to assess the availability of oral griseofulvin.

[1] A Beckman DU spectrophotometer and Beckman pH meter were used. 6-Demethylgriseofulvin was supplied by McNeil Laboratories, Fort Washington, Pa.; β-glucuronidase was purchased from Sigma Chemical, Chicago, Ill. All chemicals and solvents were Baker analyzed grade.

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Quantitative TLC Determination of Primidone, Phenylethylmalonediamide, and Phenobarbital in Biological Fluids

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Abstract □ A rapid and reproducible TLC determination of primidone and its metabolites in plasma and urine is described. The method consists of extracting the drugs into chloroform, evaporating the organic phase to dryness, dissolving the residue in an accurately measured volume of chloroform–acetic acid (9:1), and spotting this solution on a thin layer of silica gel. Quantitation is achieved by comparing the areas under the peaks obtained from scanning the TLC plates in a spectrodensitometer. The limit of detection is 1 mcg./ml. plasma for primidone and phenobarbital and 2 mcg./ml. plasma for phenylethylmalonediamide. Mean recoveries obtained from spiked plasma samples were: primidone, 93%; phenylethylmalonediamide, 97%; and phenobarbital, 102%.

Keyphrases □ Primidone—TLC separation and quantitation with phenylethylmalonediamide and phenobarbital in biological fluids □ Phenylethylmalonediamide—TLC separation and quantitation with primidone and phenobarbital in biological fluids □ Phenobarbital—TLC separation and quantitation with primidone and phenylethylmalonediamide in biological fluids □ TLC—separation and quantitation of primidone, phenylethylmalonediamide, and phenobarbital in biological fluids

Several procedures have been published on the quantitation of primidone and two of its metabolites, phenylethylmalonediamide and phenobarbital, in biological fluids. One chemical method used oxidation of primidone to phenobarbital (1). The other procedures used either TLC (2–4) or GLC (5–11). The TLC procedures were semiquantitative, since they relied on measuring spot size and intensity or colorimetric estimation after elution of compounds from the plates. The GLC methods were generally reproducible, accurate, specific, and sensitive but were relatively time consuming. This disadvantage cannot be neglected when analysis time is important as in a bioavailability study involving several hundred assay samples. This paper describes a fast and quantitative TLC procedure for estimating primidone, phenylethylmalonediamide, and phenobarbital in plasma and urine.

EXPERIMENTAL

Apparatus—A spectrodensitometer[1] equipped with a density computer[2] was used. The light source consisted of a xenon–mercury 200-w. lamp[3]. The instrument was operated in the reflection mode, using the dual-beam system. Samples were scanned at 220 nm. (density computer set at 0.2 o.d.). Scanning and chart speeds were set at 10.2 cm. (4 in.)/min.

Materials and Chemicals—Silica gel TLC plates[4] (20 × 20 cm.) were employed. The plates were divided into 20 equal channels, each 1 cm. wide, with a scoring device[5]. The plates were heated at 100° for 1 hr. before use. Stock solutions of primidone[6], phenylethylmalonediamide[6], and phenobarbital[7] were prepared by dissolving accurately weighed amounts in methanol. Working standards were made by appropriate dilutions of the stock standards. All solvents used were reagent grade. Samples were applied to the plates with 10-, 25-, or 50-μl. syringes[8].

Developing Systems—For primidone and phenylethylmalonediamide analysis, the following developing system was used: ethyl acetate–benzene–acetic acid (90:20:10) (System A). For phenobarbital analysis, the system consisted of benzene–ethyl acetate–acetone–acetic acid (100:25:15:10) (System B). These systems require saturated tanks and an environment where the relative humidity is maintained at less than 25%.

Procedure for Determining Drugs in Plasma—Two-milliliter plasma samples spiked with appropriate amounts of all three drugs were extracted with 30 ml. chloroform in 50-ml. conical tubes. The tubes were shaken on a mechanical device[9] for 15 min. Unspiked plasma samples served as blanks. After extraction, the tubes were centrifuged and 25 ml. of the extracts were transferred to a clean 50-ml. conical tube and evaporated under nitrogen[10]. The sides of the tubes were washed with 1-ml. portions of chloroform, and the solutions were evaporated to dryness. The residues were dissolved in 100 μl. of a solution of chloroform–acetic acid (9:1).

Appropriate aliquots (25–50 μl.) were spotted on scored TLC

[1] Model SD 3000, Schoeffel Instrument Corp.
[2] Model SDC 300, Schoeffel Instrument Corp.
[3] Hanovia Lamp Division, Camrand Precision Ind.
[4] Sil G-25-22, Brinkmann Instruments Inc.
[5] SDA 320, Schoeffel Instrument Corp.
[6] Pharmaceuticals Division, Imperial Chemical Industries Ltd.
[7] USP powder, Mallinckrodt Chemical Works.
[8] Hamilton Co.
[9] Eberbach, Ann Arbor, Mich.
[10] N-Evap, Organomation Association.