Biochemical Pharmacology, 1966, Vol. 15, pp. 1595–1600. Pergamon Press Ltd., Printed in Great Britain.

METABOLISM OF GRISEOFULVIN-^{14}C; STUDIES *IN VIVO**

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(*Received* 28 *January* 1966; *accepted* 21 *April* 1966)

Abstract—The rate of griseofulvin excretion and its metabolic fate were studied in rat, by means of biosynthetically prepared ^{14}C-labeled drug. It was shown that after i.v. administration about 55 per cent, and after oral administration about 31 per cent, of the radioactivity was excreted in urine over a period of 96 hr. Practically all the radioactivity was present in urine samples collected for the first 24 hr after administration of griseofulvin. Several metabolites of griseofulvin were detected in ether extracts of urine, of which two major ones were identified by paper chromatography as the 6- and the 4-demethyl derivatives. The relative ratio of these metabolites changed with time and both were present in free and conjugated forms. After i.v. injection of griseofulvin-^{14}C, about 37 per cent of the radioactivity was found in the intestines, indicating an appreciable enterohepatic circulation of this drug.

GRISEOFULVIN,† (7-chloro-4,6,2′-trimethoxy-6′-methylgris-2′-en-3,4′-dione), is widely used in treatment of fungal infections in man and animals. The metabolism and excretion patterns *in vivo* of this drug in rat have not as yet been adequately elucidated. Barnes and Boothroyd[1] found that after the oral administration of griseofulvin-^{36}Cl to the rat, only 14·6 per cent of the total radioactivity was excreted in the urine over a 48-hr period. A single metabolite, 6-demethylgriseofulvin, accounted for 65 per cent of the total radioactivity in urine, while unchanged griseofulvin accounted for less than 1 per cent of the excreted radioactivity. In the first 24-hr period following the oral administration of griseofulvin to rats, Bedford *et al.*[2] recovered 16 per cent of administered radioactivity in feces, while Davis *et al.*[3] accounted for 30–66 per cent of the drug in feces.

The present study was undertaken to shed more light on the fate of this drug in rat. It will describe the metabolic and excretion patterns of biosynthetically prepared griseofulvin-^{14}C after oral and intravenous administration.

EXPERIMENTAL

Griseofulvin-^{14}C with a specific activity of 95·8 μc/m-mole[4] was used throughout the investigation. Charles River C.D. strain male rats, weighing 150–200 g, received 50 mg doses/kg of this preparation orally, suspended in water containing 0·5 per cent of Tween-80. For intravenous administration, a solution of griseofulvin in 75 per cent of N,N-dimethyl formamide was employed. In this case 20 mg of the drug/kg

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* A preliminary report was presented at the 49th meeting (1965) of the Federation of American Societies for Experimental Biology.

† This drug is formulated and distributed by Schering Corp. under the trade name of Fulvicin U.F

(0·2 ml) was injected via the femoral vein, under ether anesthesia. The rats were then placed in individual metabolism cages. Water was supplied *ad libitum*, but food was withheld for the first 8 hr of the experimental period. Urine samples were collected at specified time intervals and were kept frozen until analyzed.

Aliquots of urine samples containing (whenever possible) about 10,000 counts/min were acidified to pH 1 with 1 N HCl and were extracted twice with ether. In order to determine the degree of conjugation of the drug and its metabolites, an aliquot of the urine sample was adjusted to pH 5 with acetate buffer and incubated with Glusulase* for 60 min at 37°. After incubation, the samples were acidified to pH 1 with 1 N HCl and extracted with ether. The ether extracts and the remaining aqueous phases were analyzed for radioactivity in a Packard liquid scintillation spectrometer, as described previously.[4] The difference in the amount of radioactivity present in ether extracts of Glusulase-treated and of the untreated urine samples was assumed to represent the amount present in conjugated form.

Ascending paper chromatography was used to study the metabolism of the drug. Samples of urine and the ether extracts were spotted on Whatman no. 1 filter paper, and the paper was subjected to ascending chromatography at 25° for 7 hr. Authentic griseofulvin and its 4- and 6-demethyl derivatives were also spotted on each strip for identification purposes. The solvent system consisted of benzene:cyclohexane: methanol:water (5:5:6:4). Glacial acetic acid (0·5%) was added to the organic phase of the solvent after equilibration. On occasion, n-butanol:ammonia (20:1) was used as an alternative solvent system. The chromatographic paper strips were scanned for radioactivity pattern by means of a Vanguard 4π automatic strip counter. The positions of authentic griseofulvin and its derivatives were located under u.v. light.

RESULTS

Urinary excretion patterns of radioactivity

Urine was collected at 4, 8, 24, 48, 72, and 96 hr after the administration of griseofulvin-^{14}C. The amount of radioactivity excreted in each time period was determined by counting aliquots of each sample in the liquid scintillation spectrometer. The cumulative excretion pattern of radioactivity in urine is shown in Fig. 1. Each point represents the mean value obtained from six rats, assayed on three separate occasions. The results show that after an oral dose of 50 mg/kg, about 31 per cent of the radioactivity was excreted in urine, over a period of 96 hr. During a similar period, about 55 per cent of the dose was excreted after i.v. injection of griseofulvin (20 mg/kg). Almost 50 per cent of the total radioactivity found in the urine was excreted during the first 4 hr after i.v. and 12 hr after oral administration of the drug.

The radioactivity found in urine at the early time periods consisted largely of nonconjugated material, as judged by the amount of radiotracer extractable with ether at pH 1 (Fig. 2). The relative amounts of conjugates, which were low initially, increased sharply during the 8–24 hours of collection and became the major urinary constituents during the 24- to 48-hr period. Each urinary fraction contained some radioactivity which could not be extracted with ether even after treatment with Glusulase. This fraction, though negligible at first (about 5 per cent), increased over

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* Obtained from Endo Lab.; each ml contained 100,000 units of β-glucuronidase and 50,000 of sulfatase.

threefold with time. As shown in Fig. 2, the urinary pattern of drug excretion was similar, regardless of the route of griseofulvin administration (oral or i.v.).

Metabolic pattern in urine

Urine samples applied directly to chromatographic paper strips showed essentially two radioactive peaks: one close to, and the other at, the origin. The resolution of these peaks was very poor and, therefore, no further evaluation of whole urine samples

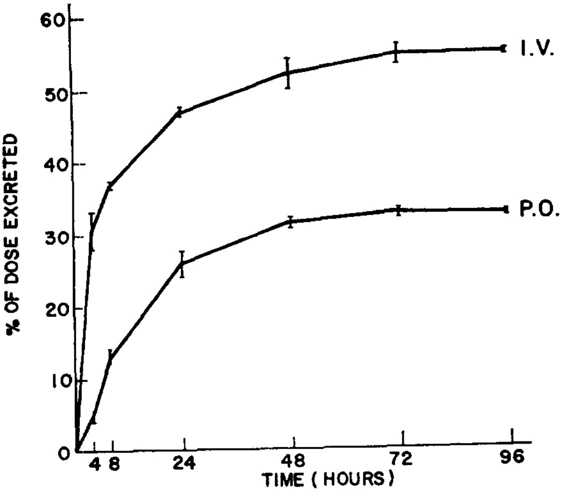

Fig. 1. Excretion of radioactivity in urine after oral and i.v. administration of griseofulvin-^{14}C. Each point represents mean value ± S.E. of six experiments.

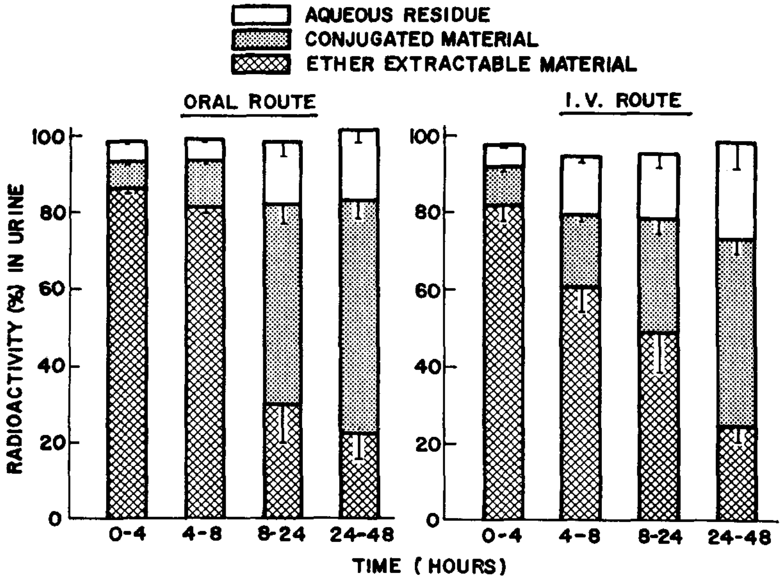

Fig. 2. Metabolic pattern of radioactivity recovered in urine after i.v. and oral administration of griseofulvin-^{14}C. Each value represents the mean ± S.E. of six experiments.

was attempted. As shown in Figs. 3 and 4, however, the radioactivity of ether extracts of urine samples could be separated by paper chromatography into well-defined areas. Spots marked on paper strips 1, 2, and 3 correspond to the positions of authentic griseofulvin (R_f 0·89), 4-demethylgriseofulvin (R_f 0·78), and 6-demethylgriseofulvin (R_f 0·21) respectively.

The chromatographic pattern in urine after oral administration of griseofulvin is presented in Fig. 3, which also shows the amount of radioactivity (per cent of total found on paper strip) associated with each peak. As shown, before hydrolysis with Glusulase, two major radioactive peaks appeared in all samples, one corresponding to the 6-demethylgriseofulvin and the other, an unidentified substance remaining at the origin. During the last period studied, a small additional peak was clearly visible; it corresponded in R_f to 4-demethylgriseofulvin. After hydrolysis with Glusulase (Fig. 3), the radioactive peak corresponding to 4-demethylgriseofulvin was present at all time periods, and the relative amount increased with time from about 14 to 83 per cent. These results indicate that this metabolite appears in urine largely in conjugated form and that it constitutes the major metabolite of the drug excreted in the later time periods. As evident from Fig. 3, 6-demethylgriseofulvin appeared prominently in urine (over 80 per cent) during the first 8-hr period, mostly in the nonconjugated form. It should be pointed out that the chromatographic data presented do not indicate the presence in urine of any measurable amount of free griseofulvin.

The results obtained after i.v. administration of griseofulvin (Fig. 4) were very similar to the pattern described for the oral route. Here again the 6-demethylgriseofulvin appeared in high concentration (over 70 per cent) at the early time intervals, while the 4-demethyl derivative was excreted at the later time intervals largely in conjugated form (97 per cent).

Intestinal content of radiotracer

As shown earlier (Fig. 1), only about 55 per cent of radioactivity was excreted in urine during a 96-hr period after i.v. injection of griseofulvin. In order to determine the fate of the remaining drug, the following experiment was performed. Four rats were placed in individual metabolism cages overnight and supplied with water only. Prior to i.v. injection of griseofulvin (20 mg/kg), the anus of each rat was ligated by suture to eliminate contamination of feces with urine. Urine was collected for a 28-hr period, after which the animals were sacrificed and the intestines, including their contents, were removed. The intestines (from the pyloric end of the stomach to the end of the rectum) were cut into small pieces and homogenized (VirTis homogenizer) with water, and aliquots of the homogenate were analyzed for radioactivity. The results (Table 1) show that 28 hr after i.v. injection of griseofulvin, about 37 per cent

TABLE 1. EXCRETION OF RADIOACTIVITY IN RAT AFTER INTRAVENOUS INJECTION OF GRISEOFULVIN-^{14}C

	% of Dose
Urine	51·3 ± 1·7
Intestines and feces	36·8 ± 1·3
Total	88·1

Results are expressed as mean values ± S.E. of four experiments.

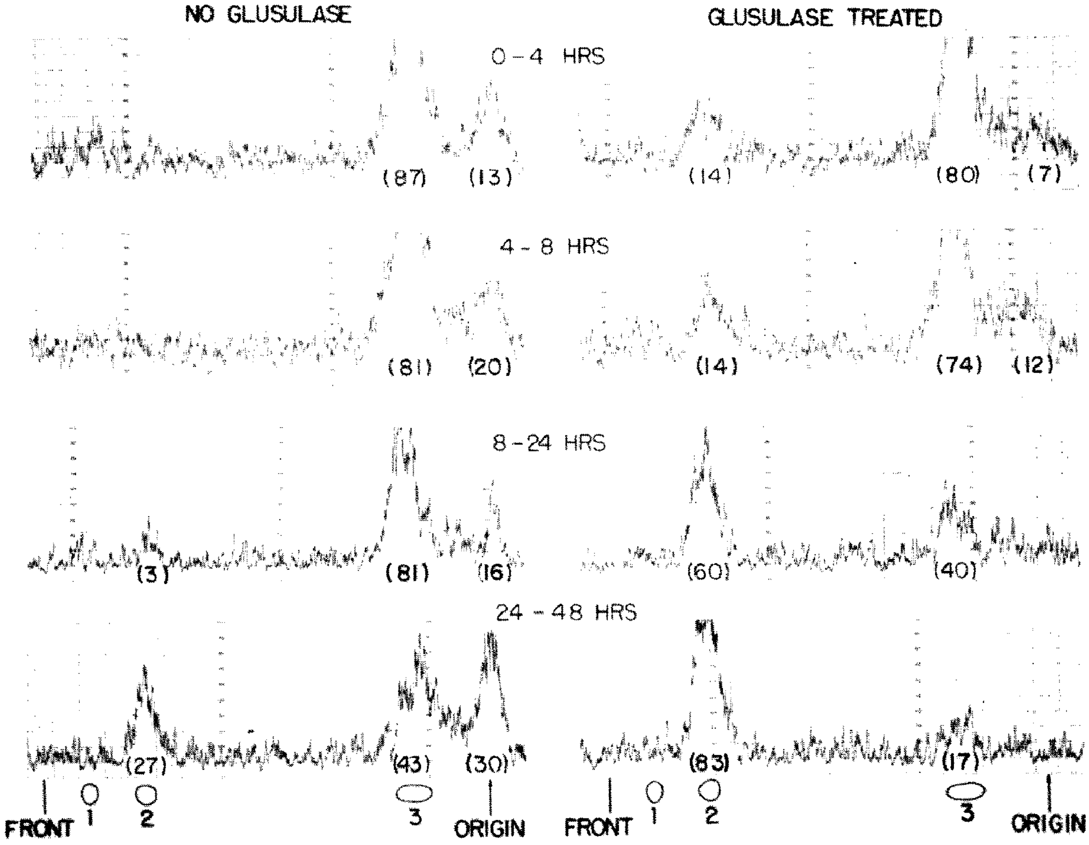

FIG. 3. Metabolic fate of griseofulvin-^{14}C in urine after oral administration of the drug. Zones marked on paper strips 1, 2, and 3 correspond to the positions of authentic griseofulvin, 4-demethylgriseofulvin, and 6-demethylgriseofulvin respectively. The numbers in parentheses represent the percentage of radioactivity associated with each peak.

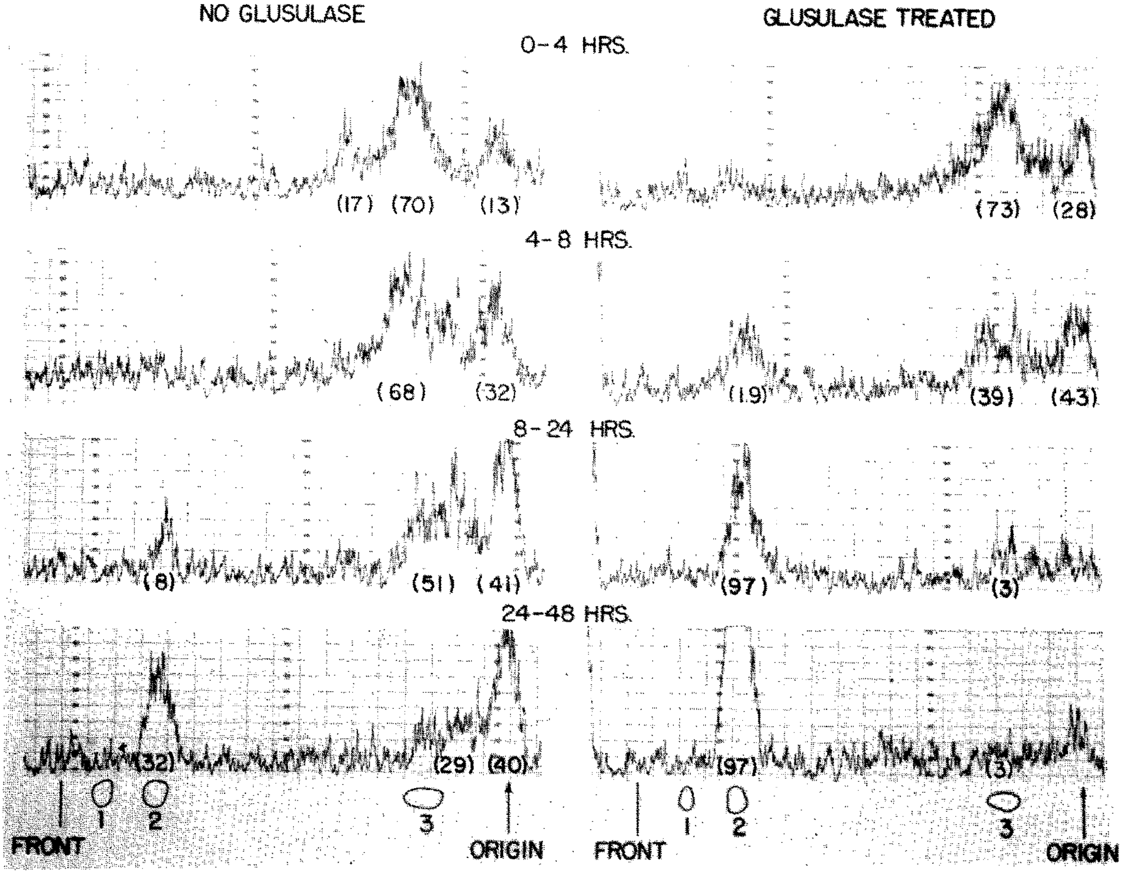

FIG. 4. Metabolic fate of ^{14}C-griseofulvin in urine after i.v. administration of the drug. Zones marked on paper strips 1, 2, and 3 correspond to the positions of authentic griseofulvin, 4-demethylgriseofulvin, and 6-demethylgriseofulvin respectively. The numbers in parentheses represent the percentage of radioactivity associated with each peak.

of the radioactivity was recovered from the intestines and about 51 per cent of the dose was present in the urine. The presence of the radioactive label in the intestines indicates an enterohepatic circulation of this drug.

DISCUSSION

The results presented in this report show that after oral or i.v. administration of griseofulvin, the urinary excretion of radioactivity was 30 per cent and 53 per cent of the dose, respectively, over a period of 48 hr. The amount of the ^{14}C-label found in urine after the oral administration of the drug was about twice that reported for a 48-hr period by Barnes and Boothroyd, employing a higher dose of ^{36}Cl-labeled drug.[1]

At the early time periods, the urinary radioactivity was predominantly present in ether-soluble form. As time progressed, the ether-soluble fraction diminished. Hydrolysis with Glusulase increased the amount of radioactivity extractable with ether, indicating the presence of a conjugated material(such as glucuronides or sulfates), the amount of which was shown to increase with time. In addition, an ether-insoluble fraction was present in urine at all time intervals, and its relative amount also increased with time.

The analysis of urine samples by chromatography revealed several radioactive peaks, two of them corresponding (in two different solvent systems) to 4- and 6-demethylgriseofulvin respectively. Both metabolites were present in the free and conjugated forms. The other metabolites, visible after chromatographic scanning of paper strips, were not identified; one remained at the origin and the other, a minor one, had an R_f value of about 0·25–0·30. At the early intervals, 6-demethylgriseofulvin was the major metabolite but, as time progressed, the relative amount of this metabolite decreased while that of 4-demethylgriseofulvin increased. During the 24- to 48-hr period, 4-demethylgriseofulvin was the predominant metabolite present in urine. It should also be noted that this metabolite was excreted primarily in conjugated form, whereas the 6-demethylgriseofulvin appeared largely in the free form.

The metabolic fate of griseofulvin, as indicated by urine analyses, is qualitatively identical with that found in liver slices.[4] In both cases the same number of metabolites was observed. The 4- and the 6-demethylgriseofulvin were present as major constituents, and both were detected in free and conjugated forms. It is interesting to note that *in vivo*, the 4-demethylgriseofulvin does not appear to any appreciable extent until the later time intervals, while *in vitro*, both the 4- and the 6-demethylgriseofulvin were seen after 2-hr incubation in approximately equal proportions. The reason for the delay in the appearance of the 4-demethylgriseofulvin *in vivo* is not yet clear. As shown in this report, however, after the i.v. administration of griseofulvin, about 37 per cent of the dose was found in the intestines, which suggests an enterohepatic circulation for this drug. It is conceivable, therefore, that the enterohepatic circulation of the drug or its metabolites affects the urinary metabolic pattern. This aspect of the problem is at present being more closely investigated in our laboratory.

Studies reported in the literature[1, 5] showed that only a single metabolite of the drug, 6-demethylgriseofulvin, was excreted in urine. Our data show that in rat urine, 4-demethylgriseofulvin was present in addition to the 6-demethylgriseofulvin (free and conjugated). The minor unidentified metabolites seen on the strips could be postulated as products of further demethylation of the mono-demethylated derivatives of the drug to form, for instance, the 4,6-di-demethylgriseofulvin. Since the presence in rabbit urine of a breakdown product of griseofulvin, namely 3-chloro-4,6-dimethoxysalicylic acid, was shown by Tomomatsu and Kitamura,[6] it is also possible that the unidentified substances may have resulted from splitting of the griseofulvin molecule.

Acknowledgement—The authors wish to thank Dr. I. I. A. Tabachnick for his interest and encouragement in this work and Miss M. Schofield for her excellent technical assistance.

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