Metabolism of Griseofulvin in Dogs

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Abstract □ Quantitative and qualitative aspects of griseofulvin metabolism in the dog are presented. The plasma disappearance curves of griseofulvin in dogs after intravenous injection were biexponential. In two dogs the average half-lives were 4 and 47 min. for the fast and slow exponential components, respectively. Plasma clearance values were 487 and 588 ml./min. in these two dogs. After intravenous administration a metabolite of griseofulvin in the urine was isolated and confirmed to be 6-demethylgriseofulvin. Approximately 40 and 57% of the dose was found to be excreted as 6-demethylgriseofulvin in two dogs given doses of griseofulvin ranging from 100 to 280 mg. An additional 25% of the dose was excreted as 6-demethylgriseofulvin glucuronide. Biliary excretion of intact griseofulvin was absent and less than 3% of the dose was excreted in the bile as 6-demethylgriseofulvin. Based on these studies, it appears that the dose of griseofulvin presently used in dogs for dermatomycoses is inadequate. A new dosage calculated to be 90 mg./kg./day is probably required to maintain therapeutically effective body levels of griseofulvin.

Keyphrases □ Griseofulvin—metabolism □ Metabolism, dogs—griseofulvin □ Plasma levels—griseofulvin, i.v. administration □ Metabolites, griseofulvin—isolated, identified □ TLC—separation, identification □ NMR spectroscopy—identity □ IR spectrophotometry—identity

Griseofulvin (7-chloro-4,6,2′ trimethoxy-6′-methylgris-2′-en-3, 4′-dione) was introduced in 1959 for the treatment of dermatophytic infections. It is widely used in both man and animals. Absorption of griseofulvin after oral administration of tablets, capsules, or suspensions is variable and incomplete in all species studied (rats, rabbits, man, and cats) (1–4). The metabolism of griseofulvin has been studied in man, rats, and rabbits (4–8). It is interesting to note that while metabolic or absorption studies on dogs have not been reported in the literature, griseofulvin is commonly used for dermatophytic infections in dogs (9). Findings on the metabolism of griseofulvin in dogs are reported here since this subject has not been previously studied. These results may have clinical implications in the treatment of fungal infections in dogs. The authors' interest in griseofulvin metabolism does not stem from the clinical veterinary usage of griseofulvin. Rather, it comes from an interest in using the dog as an experimental animal for *in vivo* model metabolism studies of drugs which included griseofulvin.

MATERIALS AND METHODS

Materials—Griseofulvin and 6-demethylgriseofulvin (6-DMG) were used.[1] The 4-demethylgriseofulvin (4-DMG) was synthesized by a method reported by Arkley *et al.* (10). Polyethylene glycol 300 NF,[2] bacterial β-glucuronidase, Type II, and the buffer, pH 6.8, 0.075 *M* phosphate, used with the β-glucuronidase,[3] were also used. Intravascular catheters consisted of silicon rubber tubing.[4] The bile duct catheter was polyethylene tubing.[5] McIlvaine's buffer was prepared by adjusting a 0.2 *M* Na_2HPO_4 solution to pH 8 with a 0.1 *M* citric acid solution. Silica Gel H,[6] less than 0.08 mm., to which was added a fluorescent indicator, 4% w/w, was used to coat glass plates for TLC. The indicator was luminescent Green No. 2282.[7]

Dose Formation—Solutions of griseofulvin for intravenous administration were prepared in the following manner. The appropriate quantity of griseofulvin was placed in a multiple-dose vial and wetted with 1 ml. ethyl alcohol. Then a given volume of polyethylene glycol 300 was added. The mixture was heated in a steam cone for about 30 min. with intermittent shaking to effect solution. The concentrations used in these experiments for large doses administered in short time periods ranged from 10 to 50 mg. griseofulvin/ml. in polyethylene glycol 300. The higher concentrations (>30 mg./ml.) must be used within 2 to 3 hr. since the griseofulvin tends to nucleate and precipitate visibly after this time period. For long constant intravenous infusions of griseofulvin the polyethylene glycol 300 solution was diluted to obtain 60% polyethylene glycol 300 in water. Concentrations up to 0.8 mg. of griseofulvin/ml. in this solvent system have been used.

Catheterizations—Dogs were anesthetized with pentobarbital (25–30 mg./kg., i.v.) and the left femoral vein and the right femoral artery were catheterized after exposing these vessels through small incisions and blunt dissection. The catheters were advanced into the lower abdominal vena cava and aorta, respectively. After securing the catheters in place, the free ends were exteriorized near the spinal column by running them anteriorly and dorsally around the body to the left side and through the loose subcutaneous connective tissue. The catheters were filled with heparin solution (300 units/ml.), clamped with light plastic screw clamps, and protectively wrapped under stockinette and tape.

The bile duct in one dog was catheterized in the following manner. The dog was anesthetized with pentobarbital and a laparotomy was performed through the linea alba. One end of a polyethylene catheter was placed in the common duct and securely anchored. The free end was exteriorized by a stab incision through the abdominal wall on the lower right side. At the same time the cystic duct was ligated.

Experimental—Unanesthetized, male mongrel dogs weighing between 20 and 25 kg. were used. The experiments were performed several days after the above described catheterizations.

Plasma Disappearance Studies after Intravenous Injection of Griseofulvin—One dog was given a total dose of 50 mg. of griseofulvin and another 100 mg. by injection of the solution into the venous catheter within 1 min. Arterial blood samples were taken over the next 3 hr. Plasma was separated from red cells by centrifugation and analyzed for griseofulvin.

Identification of a Urinary Metabolite—The total urinary output in a dog was collected for 8 hr. following the intravenous administration of 1.5 g. of griseofulvin over a 10-min. period. The main metabolite of griseofulvin in the urine was extracted, partially purified, and identified.

Percent Recovery of 6-DMG from Various Doses—On separate occasions, one dog was given 60, 120, and 240 mg. of griseofulvin

[1] Kindly supplied by the McNeil Laboratories Inc., Fort Washington, Pa., and the Glaxo Laboratories Ltd., Greenford, Middlesex, England, respectively.

[2] Union Carbide Chemicals Co., New York, N. Y.
[3] Sigma Chemical Co., St. Louis, Mo.
[4] Silastic tubing, Dow Chemical Corp., Midland, Mich.
[5] P.E. 330, Intramedic, Clay-Adams Inc., New York, N. Y.
[6] E. Merck, Darmstadt, Germany.
[7] Sylvania Electric Products, Inc., Towanda, Pa.

by constant intravenous infusion over 5 hr. after receiving a 40-mg. intravenous loading dose of griseofulvin by injection. The total doses of griseofulvin given then, were 100, 160, and 280 mg. The total urine production was collected for 18 hr. after each dose and analyzed for 6-DMG.

Attempt at Total Dose Recovery in Urine and Bile—Another dog with a bile-duct catheter was given a total dose of 100 mg. griseofulvin by intravenous injection and the total bile and urine was collected for 18 hr. Urine was analyzed for free and conjugated 6-DMG. Both urine and bile samples were subjected to TLC.

Sample Analysis—Plasma samples were analyzed for griseofulvin spectrophotofluorometrically by the method of Bedford (11) as modified in this laboratory (12). The urine samples were subjected to a spectrophotometric analysis for free 6-DMG according to the method of Rowland and Riegelman (13). The 6-DMG glucuronide in the urine samples was estimated by the same method after hydrolysis with β-glucuronidase. Two-milliliter samples were added to 1 ml. of pH 6.8 phosphate buffer, 0.075 *M*, containing 500 units β-glucuronidase and incubated at 37° for 1, 2, or 5 hr., to effect hydrolysis.

TLC analysis of urine and bile samples was performed in the following way. Two-milliliter samples, with and without incubation with β-glucuronidase for 2 hr. were adjusted to pH 1 with 6 *N* HCl and extracted with 10 ml. ethyl ether. Nine milliliters of the ether extract was removed and placed in tapered tubes. After almost completely evaporating the ether at 40° in a water bath, the residue was taken up in 0.1 ml. acetone and applied to glass plates coated with silica gel containing the fluorescent indicator. The thin-layer plates were developed with a solvent system containing chloroform, ether, acetone, and glacial acetic acid in a volume ratio of 65:20:15:0.5. Authentic samples of griseofulvin, 6-DMG, and 4-DMG, were chromatographed simultaneously. The standards were visible as dark spots on the green background under 253-mμ light.

In order to confirm the identity of the metabolite of griseofulvin being eliminated in the urine, the total volume of urine was collected from the dog who received 1.5 g. griseofulvin intravenously. The urine was adjusted to pH 3 with 6 *N* HCl and extracted twice with equal volumes of ether. Since both 4-DMG and 6-DMG (known metabolites in other species) are relatively strong phenolic acids, they can be separated from the ether phase by alkaline extraction. Therefore the ether extracts were combined and extracted twice with one-half volumes of pH 8 McIlvaine's buffer. The buffer solutions were combined and the pH adjusted to 1 with concentrated HCl. The solution was then extracted twice with one-half volumes of an organic solvent consisting of one-half cyclohexane and one-half ethylene dichloride. The organic solvent extracts were combined and dried by the addition of 60 g. anhydrous $MgSO_4$. After removing the $MgSO_4$ by filtration, the organic solvent was removed *in vacuo* at room temperature. The brownish crystalline residue, approximately 500 mg., was dissolved in 10 ml. of boiling ethanol and filtered. The solution was allowed to stand overnight at room temperature during which time an almost colorless material crystallized. These crystals, approximately 100 mg., were separated and dried. A melting point was determined. In addition, a mixed melting point was made with an authentic sample of 6-DMG. The UV and IR spectra of the isolated crystals and of the 6-DMG and 4-DMG standards were recorded. The NMR spectrum of the isolated crystals was also taken.

RESULTS AND DISCUSSION

Plasma Disappearance Studies after Intravenous Injection of Griseofulvin—Figure 1 includes a semilog plot of plasma levels of griseofulvin *versus* time. The curves can be described by the following bi-exponential equation:

$$C_p = Ae^{-\alpha t} + Be^{-\beta t} \qquad \text{(Eq. 1)}$$

where C_p represents the plasma concentration at any time, t, A and B represent the ordinate concentration intercepts of the fast and slow phases at $t = 0$, respectively, and α and β are the rate constants of the fast and slow phases, respectively. The half-life of the slowest component of the curve is 50 min. with the 50-mg. dose in Dog G and 44 min. with the 100-mg. dose in Dog A. The fast component, resolved by the difference in the extrapolated slow component and the actual curve, has a half-life of 4 min. in both animals. It is apparent that griseofulvin disappears from the plasma

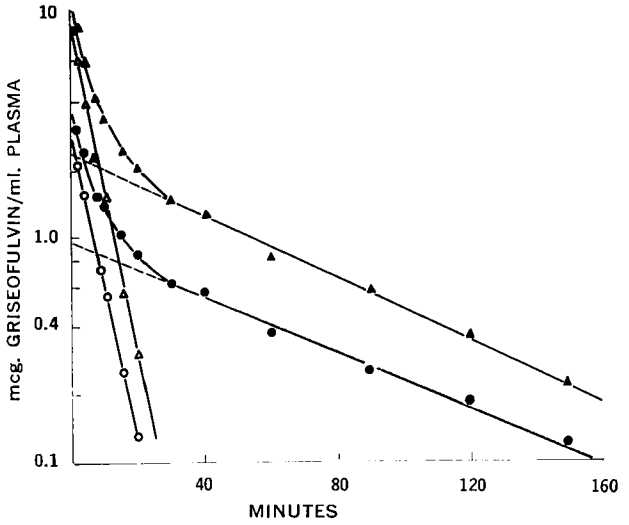

Figure 1—*Plasma concentration-time curves obtained after intravenous injection of 50 and 100 mg. of griseofulvin in two different dogs. Key: ▲, actual points, 100 mg.; △, residuals, 100 mg.; ●, actual points, 50 mg.; ○, residuals, 50 mg.*

of dogs at an extremely rapid rate. If it is assumed that the disposition of griseofulvin in the dog obeys a two-compartment open-system model with elimination occurring from a central compartment, then the plasma clearance[8] from the body can be calculated from these data. It is also assumed that first-order kinetics are followed to and from both compartments.

The method of calculation for clearance, based on the above assumptions, is clearly shown in reports by Mandel *et al.* (14), for *p*-aminohippuric acid and Sapirstein *et al.* (15), for creatinine. The method is based on the following relationship.

$$\dot{V}cl = \frac{\text{dose}}{(A/\alpha) + (B/\beta)} = kel\ V_p \qquad \text{(Eq. 2)}$$

where $\dot{V}cl$ is the plasma clearance and $(A/\alpha) + (B/\beta)$ represents the area under the plasma concentration time curve. The product of the first-order rate constant of elimination, *kel*, and the volume of the central compartment, V_p, also express the plasma clearance. The V_p can be calculated as follows:

$$V_p = \frac{\text{dose}}{C_p^{\circ}} \qquad \text{(Eq. 3)}$$

where C_p° is the sum of A and B and represents the plasma concentration at zero time when no metabolism, excretion, or distribution from the central compartment has yet occurred.

The various parameters obtained after the two injections of griseofulvin are given in Table I. It is to be noted that in these dogs the volume constant of the central compartment, V_p, is several times larger than the total plasma volumes, approximately 1,000 ml. This indicates that griseofulvin penetrates a large volume outside the vasculature within 1 or 2 min. after injection. Furthermore, it is obvious that the elimination rate constant of griseofulvin cannot be represented by the slope of the slow phase of the curve, β. This constant is much smaller than the calculated elimination rate constant, *kel*. That is, of course, to be expected when the disposition of a compound is described by a multicompartmental system in the body. However, the point is emphasized because some authors mistakenly identify the β of some compounds as the elimination rate constant when, in fact, it is a hybrid rate constant whose value is determined by the relative magnitudes of the rates of distribution, excretion, and metabolism. The elimination rate constant and β will be the same only when the compound being studied behaves as if it was distributed in a single-compartmental system.

It has been shown in this laboratory (16) that clearance values calculated as shown above do represent the rate of metabolic conver-

[8] Plasma clearance is defined as the volume of the central compartment cleared of a compound per unit time.

Table I—Kinetic Parameters[a] Calculated from Data Obtained After Intravenous Injection of Griseofulvin

Total Dose, mg.	A, mcg./ml.	B, mcg./ml.	$Cp°$, mcg./ml.	α, min.$^{-1}$	β, min.$^{-1}$	Vp, ml.	kel, min.$^{-1}$	$\dot{V}cl$, ml./min.
50	3.05	0.95	4.0	0.173	0.014	12,500	0.047	588
100	9.5	2.4	11.9	0.173	0.016	8,403	0.058	487

[a] See text for definition of symbols.

sions of griseofulvin in dogs and the metabolism can be described as occurring from a central compartment of which the plasma is a part. Clearance values of griseofulvin in humans can be calculated as shown above from data reported by Rowland and Riegelman (12). These values ranged from 60 to 150 ml./min., which is considerably slower than in dogs.

Identification of a Urinary Metabolite—The purified crystalline material isolated from the urine melted at 281–283°. No significant depression of the melting point occurred when these crystals were mixed with the authentic sample of 6-DMG which melted at 279–281°. The authentic sample of 4-DMG melted at 118–120°. The isolated crystals had the same R_f on TLC plates as the 6-DMG. The NMR spectrum of the isolated crystals in Fig. 2 clearly demonstrates only two methoxy groups indicating that the metabolite is a demethylated product of griseofulvin which contains three methoxy groups. The IR spectra of these crystals, the 6-DMG, and the 4-DMG are shown in Fig. 3. The spectra of the crystals from the urine and 6-DMG are identical while the IR spectrum of 4-DMG differs from them significantly. The UV spectrum of the isolated crystals in acid and base conformed to that of 6-DMG reported by Barnes and Boothroyd (5). Considering all the evidence, there appears to be no doubt that the substance isolated from the dog's urine is 6-DMG.

Percent Recovery of 6-DMG After Various Doses—The percent of the dose excreted as free 6-DMG in the urine after various doses of griseofulvin is shown in Table II. Over nearly a threefold dose range and a fourfold range in infusion rate, the percent of the dose recovered in the urine as 6-DMG was virtually constant. This indicates that saturation of metabolic pathways of griseofulvin did not occur over this dose range in this particular dog. It seems then that the dog has a very large capacity to metabolize griseofulvin as shown by these data as well as a high rate of metabolic clearance. It is unlikely, therefore, that griseofulvin will accumulate in the dog to dangerously high levels after oral administration.

Attempt at Total Dose Recovery in the Urine and Bile—Investigation of the 6-DMG glucuronide was hampered by the difficulty of its hydrolysis without degradation. It was later found that bacterial β-glucuronidase, in relatively high concentrations, was able to hydrolyze the compound. Table III presents data that indicate that hydrolysis is essentially complete in 1 hr. under conditions described previously in this report. In this experiment, approxi-

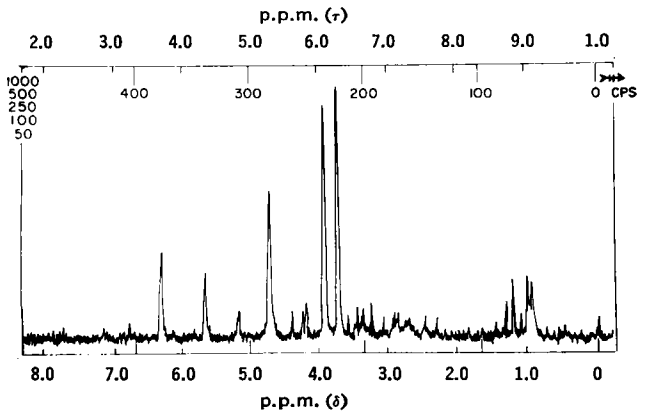

Figure 2—*NMR spectrum of the purified crystals isolated from the urine of a dog given 1.5 g. of griseofulvin. Saturated solution of CD_3OD. Large peak at 4.7 p.p.m. is hydrogen impurity and the phenolic hydrogen of 6-DMG which has exchanged with deuterium in the methanol.*

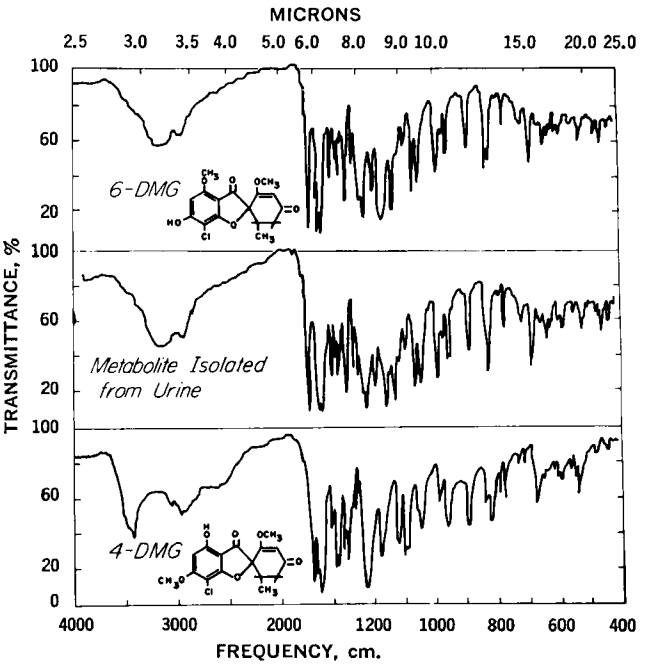

Figure 3—*IR spectra of substances indicated. 1% w/w in KBr.*

mately 40% of the dose was excreted in the urine as free 6-DMG and another 25% as the 6-DMG glucuronide.

The TLC of the same urine samples yielded results consistent with those given in Table III. A spot corresponding with authentic 6-DMG was observed under 253-mμ light. This spot increased in intensity if the sample was previously incubated with β-glucuronidase, indicating again the presence of a hydrolyzable glucuronide of 6-DMG in addition to unconjugated 6-DMG in dog urine after administration of griseofulvin. Within the sensitivity of the experiment there was no detectable griseofulvin, 4-DMG, or conjugated 4-DMG in the dog urine. Quantities as small as 0.25 mcg. of griseofulvin and 10 mcg. of 4-DMG are detectable on the thin-layer plates used here.

A total of 185 ml. of bile was collected in this experiment. Samples of the bile were chromatographed by TLC, but neither griseofulvin nor 4-DMG could be detected nor could any hydrolyzable conjugate be demonstrated. A small quantity of 6-DMG was seen on the TLC plates, however, the total 6-DMG in the bile was estimated to be less than 3% of the dose by visual comparison of the spot intensity with that of a standard.

As noted in Table III, only 66.7% of the dose was recovered from the urine as 6-DMG and its glucuronide. Although no exhaustive study has been carried out to confirm the absence of a sulfate conjugate in the urine, it appears unlikely from the following evidence. Additional quantities of free 6-DMG could not be detected after incubation of urine with sulfatase, an enzyme that hydrolyzes ethereal sulfates. Data reported by Dodgson *et al.* (17) demonstrated that ethereal sulfate conjugates do not form with chlorinated phenols with a pKa of less than 7. The pKa of 6-DMG is 4.5 (18).

On the basis of the above evidence one might propose that either an unknown metabolite is formed or another site of elimination other than the renal and biliary systems exists. There was no detectable unconjugated 4-DMG in the bile or urine; however, the possibility cannot be ruled out that some conjugate of 4-DMG was present which was not cleaved by β-glucuronidase. Symchowicz and Wong have reported (6) that 4-DMG is excreted in the urine of rats mostly as conjugate which was hydrolyzed to free 4-DMG

Table II—Urinary Recovery of Free 6-DMG

Total Dose Griseofulvin, mg.	Infusion Rate, mcg./min.	% Dose Recovered as 6-DMG
100	200	54.9
160	400	51.8
280	800	56.7

Table III—Recovery of Free and Conjugated 6-DMG After Intravenous Injection of 100 mg. Griseofulvin

Incubation Time with β-Glucuronidase, hr.	mcg. 6-DMG/ml. Urine (1,000 ml. Total)	6-DMG Present in Urine as % of Dose
0	38	39.6
1	62	64.6
2	63	65.6
5	64	66.7

by an enzyme preparation containing β-glucuronidase and sulfatase.[9] This report also states that 37% of the dose was found in the rat intestines after intravenous administration suggesting an enterohepatic circulation of griseofulvin or one of its metabolites. In the dog with a bile duct catheter no such evidence was found in this investigation. The authors wish to emphasize that their results in dogs do not contradict the evidence for enterohepatic circulation found in rats. There is, however, the possibility of intestinal elimination of a griseofulvin metabolite after intravenous administration without biliary excretion. In these experiments the feces were not examined for griseofulvin or its metabolites.

General—Griseofulvin disappears from dog plasma at least as fast if not faster than in rabbits (2) or in rats (4). Since a large fraction of the dose is metabolized to 6-DMG, the dog, it appears, can demethylate griseofulvin as fast or faster than the rabbit or rat.

It is interesting to compare these *O*-dealkylation results with those obtained by others. Axelrod (19) has studied the enzymatic cleavage of aromatic ethers and has shown that *O*-dealkylation of several substituted phenyl alkyl ethers is accomplished by oxidation with concomitant formation of a phenol and an aldehyde. An oxidative mechanism for griseofulvin demethylation has not been directly demonstrated by measuring the production of formaldehyde and demethylgriseofulvin in the same system. Kaplan *et al.* (8), have shown relationships between griseofulvin and *p*-ethoxyacetanilide, which is oxidatively dealkylated (19), that suggest griseofulvin is demethylated to 6-DMG in the rabbit by the same oxidative system that dealkylates *p*-ethoxyacetanilide.

The demethylation of griseofulvin is probably confined to the liver as substantiated by the lack of griseofulvin metabolism *in vitro* in tissues other than the liver (4, 7). The lack of oxidative dealkylating activity in tissues other than the liver also has been confirmed using the model compound, *p*-ethoxyacetanilide (19). This information coupled with the high metabolic clearance of griseofulvin in the dog indicates that an appreciable fraction of those griseofulvin molecules passing through the liver are metabolized. After absorption from the gastrointestinal tract, the griseofulvin molecules must pass through the liver before reaching the peripheral tissues. A considerable fraction of the absorbed dose will not reach the peripheral tissues before conversion to the inactive metabolites. A reasonable therapeutic dose for dogs can be calculated by considering the rapid rate of metabolism, and high liver clearance of griseofulvin in the dog, approximately 500 to 600 ml./min., relative to an average estimate of 100 ml./min. in man (12). The human dose is approximately 15 mg./kg./day. Since the clearance in dogs is approximately six times larger than in humans, a dose of 90 ml./kg./day is probably required for equivalent response. The clearance in a greater number of dogs should first be established before any firm recommendation of the dose in dogs can be made. Data from other experiments carried out in this laboratory with dogs given griseofulvin indicate that the values reported in this study are representative. Also, consideration must be given to adjustment of clearance values based on the volume from which griseofulvin is being cleared when comparing one animal to another.

CONCLUSIONS

The disappearance half-life of griseofulvin from the plasma of two dogs was 44 and 50 min., based on the slowest phase of the bi-exponential decay curve. Unchanged griseofulvin was not found in the urine or bile, therefore elimination of griseofulvin appears to take place primarily by a very rapid rate of metabolism.

It appears that the primary metabolite of griseofulvin in the dog is 6-DMG. The 6-DMG is excreted in the urine both in the free form and as a conjugate with glucuronic acid. Only a small amount of free 6-DMG could be demonstrated in the bile. However, all of the dose could not be accounted for by urinary 6-DMG and its glucuronide in these experiments. Three possibilities are likely to explain this: (*a*) there is an excretory medium other than urine or bile, (*b*) an unidentified metabolite may exist, (*c*) the authors' methods of detection in bile may be insensitive or obscured.

Demethylation of griseofulvin occurs more rapidly in the dog than in any animal which has been studied to date. This metabolism occurs primarily, if not completely, in the liver. After oral administration of griseofulvin the large hepatic clearance can significantly reduce the amount of absorbed griseofulvin that reaches peripheral tissues. The doses of griseofulvin used in dogs for treatment of dermatomycoses should probably be much higher than are currently used.

[9] Glusulase.

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