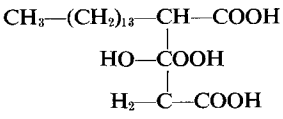
Phytochemical Evaluation of Some Cantharelloid Fungi

E. D. HENRY and G. SULLIVAN

Abstract □ A white crystalline compound was isolated from dried carpophores of *Gomphus kauffmanii* (A. H. Smith) Corner in a yield of 2.0% and identified as norcaperatic acid. The presence of norcaperatic acid in a yield of approximately 4.4% was also reconfirmed in the dried carpophores of *G. floccosus* (Schw.) Singer. Norcaperatic acid was not detected in dried carpophores of *Cantharellus cibarius* Fr., *C. infundibuliformis* Fr., *C. subalbidus* Smith *et* Morse, and *G. clavatus* S. F. Gray. The presence of mannitol was demonstrated in all six cantharelloid fungi.

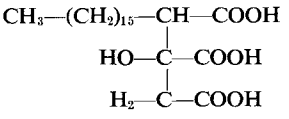
Keyphrases □ Norcaperatic acid isolation, identification—*Gomphus kauffmanii, G. floccosus* □ Mannitol isolation, identification—*Cantharellus* species □ TLC—separation, identification □ IR spectrophotometry—identification

Cantharelloid fungi are generally regarded as edible and nontoxic. *Cantharellus cibarius* Fr. (the "Chanterelle") has been a particular favorite for over four centuries in continental Europe. However, *Gomphus floccosus* (Schw.) Singer (*syn: Cantharellus floccosus* Schw.) has been reported to cause gastrointestinal disturbances in some individuals (1–3). Miyata *et al.* (4) have shown that a constituent of this species, norcaperatic acid (α-tetradecylcitric acid) (I), is probably the toxic principle. Carrano and Malone (5) have indicated that the toxic properties of norcaperatic acid and its homolog, agaricic acid, may be related to the structural chemical relationships of these substances to citric acid. Agaricic acid (α-hexadecylcitric acid) (II) has been isolated from *Polyporus officinalis* Fr. (6) and has been classified as an anhydrotic and parasympatholytic agent (7–9). Carminati and Spina (10) have arbitrarily classed its activity as an atropine-like anticholinergic agent in small animals.



norcaperatic acid

I



agaricic acid

II

A number of phytochemical investigations have been carried out involving cantharelloid fungi and the following compounds have been reported to be present in these fungi: mannitol, choline, dextrose, trehalose, and cellulose (11); a trypsin-type enzyme (12); ergosterol, solid and liquid fatty acids, and a dye (13); thiamine, riboflavin, vitamin D, ascorbic acid, and dehydroascorbic acid (14–16); cobalt (17); carotenes (14, 18–21); lycopene, neurosporene, and phytofluene (20, 21); sodium, potassium, zinc, and aluminum (22); canthaxanthine (18); 18 amino acids (15, 22–25); theleophoric acid (26); and citric acid (27). An appraisal of these constituents isolated from cantharelloid fungi revealed no apparent chemical relationship worthy of chemotaxonomic consideration.

Since the presence of an aliphatic polycarboxylic acid had been reported in but one cantharelloid, it appeared desirable to chemically examine other closely related cantharelloid fungi for the presence of this type of acid and to evaluate its possible role as a chemotaxonomic marker.

EXPERIMENTAL

Materials and Methods—Dried carpophores of *Cantharellus cibarius* Fr., *C. infundibuliformis* Fr., *C. subalbidus* Smith *et* Morse, *Gomphus clavatus* S. F. Gray, *G. floccosus* (Schw.) Singer, and *G. kauffmanii* (A. H. Smith) Corner (*syn: Cantharellus kauffmanii* A. H. Smith) were obtained from Dr. Harry D. Thiers, San Francisco State College, San Francisco, Calif. These carpophores were collected in the San Francisco Bay area in the fall of 1967.

As a preliminary step, each species was treated following the procedure of Miyata *et al* (4). The dried carpophores were ground to a 40-mesh powder in a Wiley laboratory mill and a 25-g. sample was placed in a 1-l. beaker and extracted for three 15-min. periods using 200 ml. of boiling 95% ethanol. The ethanolic extracts were filtered while still hot employing suction filtration. The filtered extracts were combined and the marc dried and retained for possible further examination.

The ethanolic extract, in each case, was evaporated to dryness under reduced pressure at a temperature not exceeding 45°, washed with 200–250 ml. of cold distilled water, filtered, and the water-soluble portion evaporated to dryness using a stream of air. The resulting orange-colored solids were washed several times with acetone in order to remove the orange pigments, filtered, washed with five 25-ml portions of cold methanol, filtered, and dried. The water-insoluble solids were solubilized in 500 ml. of boiling water and allowed to cool to room temperature. A white, flocculent precipitate was observed in the cases of *G. floccosus* and *G. kauffmanii*. The precipitates were filtered and dried. Scheme I illustrates the extraction procedure employed.

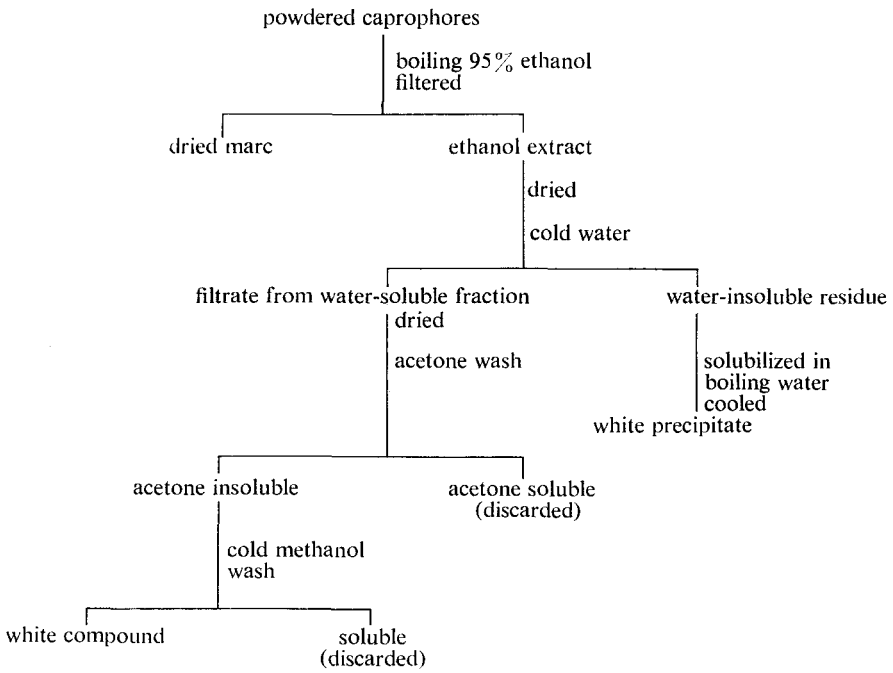
Characterization of Water-Insoluble Compounds—A white, water-insoluble material was obtained from the ethanolic extract of *G. floccosus* (1.1 g.; 4.4% yield) and from *G. kauffmanii* (0.5 g.; 2.0% yield). In each case the material was dried and stored in a desiccator over calcium chloride. A portion of each isolated material was recrystallized three times from boiling water and dried for 48 hr. in a drying pistol at 60° over phosphorus pentoxide. The melting range of both samples was found to be 133–135°.

The material was found to be readily soluble in boiling water and 5% sodium hydroxide solution; slightly soluble in hot ethanol; and insoluble in cold water, 10% sodium bicarbonate solution, ethanol, pyridine, chloroform, and benzene. When solubilized in boiling water a frothing was observed and the solution was acid to litmus. A small amount of each dried sample produced a slight residue when placed on a clean spatula and ignited in the flame of a Bunsen burner which indicated the presence of inorganic elements.

Sodium Fusion—The material was subjected to a sodium fusion test (28) which indicated the absence of sulfur, nitrogen, and halogens.

IR Spectra—The IR absorption spectra of the material isolated from both *G. floccosus* and *G. kauffmanii* were obtained on a Beckman IR 8 spectrophotometer in a potassium bromide pellet. The spectrum revealed a hydroxyl peak at 3450 $cm.^{-1}$, an aliphatic CH_2 peak at 2900 $cm.^{-1}$, and a carbonyl signal at 1720 $cm.^{-1}$. These spectra compared favorably with the spectrum reported by Miyata *et al.* (4) for norcaperatic acid and were identical with the spectrum obtained with reference norcaperatic acid.

Color Reactions—A few milligrams of the crystalline compound (m.p. 133–135°), when warmed to 100° for a few minutes with acetic anhydride, gave a cherry-red color. This color reaction indicated



Scheme I—Flow Diagram for the Extraction of Dried Carpophores

the possible presence of an aliphatic polycarboxylic acid salt (29–31). Since at least a limited presence of an acid salt was suggested, the material was converted to the free acid. A portion of the material was dissolved in a minimum volume of 5% sodium hydroxide, acidified with a slight excess of hydrochloric acid, and the resulting white gelatinous precipitate removed by suction filtration. The precipitate was recrystallized three times from boiling water, filtered, air dried, and placed in a drying pistol for 16 hr. The melting point of the compounds obtained from both *G. floccosus* and *G. kauffmanii* was found to be 137–138°. An admixture with reference norcaperatic acid did not depress the melting point.

The free acid, in each case, was subjected to a modified Fürth-Herrmann color reaction (29–31). A small amount of the compound was dissolved in a few drops of pyridine, 3 ml. of acetic anhydride added, and the mixture allowed to stand. After a few minutes a cherry-red solution with a strong green fluorescence was observed, again indicating the presence of an aliphatic polycarboxylic acid.

These data suggested that the water-insoluble compounds obtained from *G. floccosus* and *G. kauffmanii* were identical. Therefore further characterization of only the compound obtained from *G. kauffmanii* was conducted.

Potassium Salt—The potassium salt of the water-insoluble compound obtained from *G. kauffmanii* was prepared. A solution of 100 mg. of the compound in 30 ml. of warm 95% ethanol was treated with 15 ml. of an ethanolic solution of 0.5 g. of potassium acetate and the resulting flocculent precipitate recrystallized three times from boiling water. After an additional recrystallization from acetone, the melting point was observed to be 173–174°. This melting point was identical with the reported melting point of the potassium salt of norcaperatic acid (4) and an admixture with the potassium salt of reference norcaperatic acid was not depressed.

Quantitative Elemental Analysis[1]—The compound isolated from *G. kauffmanii*, which had been converted to the free acid, was subjected to quantitative elemental analysis and was found to have the quantitative composition expected for norcaperatic acid.

Anal.—Calcd. $C_{20}H_{36}O_7$: C, 61.83; H, 9.34. Found; C, 61.83; H, 9.52.

Cantharellus cibarius, *C. infundibuliformis*, *C. subalbidus*, and *G. clavatus* were subjected to the same isolation procedures as previously described but failed to yield a precipitate from boiling water. A frothing action was observed in this procedure and since this behavior was observed with aliphatic polycarboxylic acid-type compounds the extracts merited further investigation for possible minute amounts of norcaperatic acid or its homologs.

Color Reactions—The residues obtained from the ethanolic extracts of the remaining four fungi gave a negative reaction to the Fürth-Herrmann color test, indicating the probable absence of any aliphatic polycarboxylic acids.

TLC—Following the procedure of Sullivan and Albers (32) for the separation of aliphatic polycarboxylic acids, plates of silicon-ized Silica Gel HF were prepared, developed in a solution of glacial acetic acid–dioxane–water–formic acid (4:1:1:6), sprayed with 10% phosphomolybdic acid dissolved in ethanol, and heated. Concentrated ethanolic solutions of the four fungi produced a positive phosphomolybdic spot at R_f 0.33. Reference norcaperatic acid gave an R_f value of 0.53, as did the original compounds obtained from *G. floccosus* and *G. kauffmanii*. Reference agaricic acid produced a positive phosphomolybdic spot at R_f 0.39, and reference citric acid produced a positive 3% potassium permanganate-concentrated sulfuric acid spot at R_f 0.87.

Characterization of Water-Soluble Compounds—A white, water-soluble compound was isolated from all six fungi in the following amounts: *C. cibarius* (0.35 g.: 1.4% yield), *C. infundibuliformis* (2.0: 8.0%), *C. subalbidus* (0.6:2.4%), *G. clavatus* (1.0:4.0%), *G. floccosus* (2.2:8.8%), and *G. kauffmanii* (2.4:9.6%).

Solubility—The crystalline compound was found to be soluble in water and boiling methanol; very slightly soluble in cold methanol and 95% ethanol; and insoluble in ether, petroleum ether, benzene, chloroform, and ethyl acetate.

Sodium Fusion—The absence of sulfur, nitrogen, and halogens was demonstrated by the sodium fusion test.

TLC—All water-soluble compounds and their methanol washes were spotted on 20 × 20-cm. plates. The method employed was similar to that advocated by Stahl (33) for the separation of sugar alcohols. However, best results were obtained when standard plates of Silica Gel G were used instead of Stahl's activated "Alusil" plates. The plates were spotted and developed in an *n*-propanol–water–ethyl acetate–25% ammonium hydroxide (6:3:1:1) solvent system. The developed plates were sprayed with silver nitrate–acetone solution, treated in an ammonia-saturated tank for 15 min., and then heated in an oven at 110°. All extracts revealed a discreet positive spot at R_f 0.38 but considerable material was observed on the solvent front. Employing this same procedure, the following R_f values for reference compounds were determined: inositol, 0.19; sorbitol, 0.35; glucose, 0.38; and mannitol, 0.38.

Test for Reducing Sugars—Application of Benedict's test for reducing sugars with the isolated compound resulted in a negative response, thereby eliminating glucose from consideration.

[1] Analysis was carried out by the Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.

All isolated compounds were recrystallized five times from boiling methanol, filtered, and dried in a drying pistol for 16 hr. at 60°. A melting point of 165–166° was observed in all instances.

IR Spectra—Preliminary IR spectra revealed minor differences between three of the six isolated compounds and reference mannitol (Eastman). The purity of the reference mannitol was questioned when further purification, employing activated charcoal, failed to alter the spectra of the three compounds.

Column Chromatography—The six isolated compounds and reference mannitol were placed individually on 3.5 × 24.5-cm. columns containing 100–200-mesh silica gel, and the following solvent progression employed: 100 ml. benzene; 500 ml. benzene–methanol (95:5), 400 ml. benzene–methanol (90:10); 400 ml. benzene–methanol (80:20); 500 ml. benzene–methanol (50:50); and 700 ml. methanol. Fourteen 50-ml. fractions of the methanol were collected, and Fractions 10–14, in each case, were combined, evaporated, and the resulting compound recrystallized from hot methanol.

Following treatment on the column, the IR spectra for each of the six isolated compounds and reference mannitol were found to be identical. The spectra revealed: KBr/max. 3250, 3400, 2920, 1730, 1450, 1080, 1010, 920, and 690 $cm.^{-1}$.

Melting Point—The melting point of all six isolated and purified compounds was observed to be 165–166°. Admixtures with reference mannitol were not depressed.

Acetate Derivative—The hexaacetate derivatives of the isolated compounds and of reference mannitol were prepared by mixing 100 mg. of the compound and 100 mg. of fused sodium acetate, adding 10 ml. of acetic anhydride, and heating on a steam bath for 2 hr. with stirring (34). The melting point of the dried derivative was observed to be 121–122° in all instances, which was in agreement with the reported melting point for mannitol hexaacetate. Admixtures of each of the six derivatives with mannitol hexaacetate were not depressed.

Quantitative Elemental Analysis—Since all six isolated compounds possessed identical solubility behaviors, R_f values, IR spectra melting points, and identical hexaacetate derivatives, it was necessary to characterize further only one of the compounds. The compound isolated from *G. clavatus* was selected and subjected to quantitative elemental analysis. This compound was found to have the quantitative composition anticipated for mannitol.

Anal.—Calcd. for $C_6H_{14}O_6$; C, 39.56; H, 7.74. Found: C, 39.59; H, 7.68.

RESULTS AND DISCUSSION

A white, crystalline, water-insoluble compound was isolated in a 2.0% yield (dry weight basis) from the ethanol extract of *G. kauffmanii* and identified as norcaperatic acid on the basis of its solubility, melting point, qualitative and quantitative elemental analysis, IR spectra, color reactions, TLC, and characterization of the acid salt-derivative. The presence of norcaperatic acid in *G. floccosus*, previously reported by Miyata *et al.* (4), was reconfirmed and a 4.4% yield was obtained. This yield was in excellent agreement with Miyata's findings.

Miyata *et al.* also suggested that norcaperatic acid was probably present in *G. floccosus* as the potassium salt. The authors' results, however, indicated that this compound was probably present as a mixture of both the free acid and the acid salt. This was evidenced by the compounds isolated from both *G. floccosus* and *G. kauffmanii*, which exhibited a melting range, after extensive purification, of 133–135° and also positive color reaction tests for both the salt and free acid.

The absence of norcaperatic acid in carpophores of *C. cibarius*, *C. infundibuliformis*, *C. subalbidus*, and *G. calvatus* was conclusively demonstrated by the negative modified Fürth-Herrmann color reaction and by the employment of a thin-layer chromatographic procedure which possessed a detection limit of 0.5 mcg. for aliphatic polycarboxylic acids.

The presence of the primary metabolite, mannitol, proved to be of no significance as a chemotaxonomic marker. The ubiquitus occurrence of mannitol in varying concentrations provided no basis upon which to draw conclusions.

The results of this investigation are summarized in Table I.

According to Corner's concept of the cantharelloid fungi (35), sufficient morphological differences are present to justify the separation of *G. clavatus* (Sect. Gomphus) from *G. floccosus* and *G. kauffmanii* (Sect. Excavatus). The authors' findings add support to Corner's concept in that norcaperatic acid was found to be absent in *G. clavatus* but present in the latter two species. These data suggest that the accumulation or presence of norcaperatic acid may be indicative of certain infrageneric relationships. It would indeed be interesting to chemically evaluate the two remaining species in Corner's Section Excavatus [*G. bonarii* (Morse) Singer and *G. wilkinsae* (Morese) Corner] for the presence of norcaperatic acid or its homologs.

Table I—Occurrence and Distribution of Norcaperatic Acid and Mannitol in Cantharelloid Fungi

Cantharelloid Fungi	Norcaperatic Acid	Mannitol
C. cibarius	Absent	Present (1.4%)
C. infundibuliformis	Absent	Present (8.0%)
C. subalbidus	Absent	Present (2.4%)
G. clavatus	Absent	Present (4.0%)
G. floccosus	Present (4.4%)	Present (8.8%)
G. kauffmanii	Present (2.0%)	Present (9.6%)

SUMMARY

Norcaperatic acid was found to be present in the dried carpophores of *Gomphus kauffmanii* in a 2% yield and its presence reconfirmed in *G. floccosus*. This acid was found to be absent in *Cantharellus cibarius*, *C. infundibuliformis*, *C. subalbidus*, and *G. clavatus*. The presence of mannitol was demonstrated in all six cantharelloid fungi.

REFERENCES

(1) C. H. Kauffman, "The Agaricaceae of Michigan," vols. I and II, Michigan Geol. and Biol. Survey, Publ. 26, Biol. Ser. 5, Lansing, Mich., 1918.

(2) A. H. Smith, "Mushrooms in Their Natural Habitats," Sawyer's, Portland, Ore., 1949.

(3) A. H. Smith, "The Mushroom Hunter's Field Guide," University of Michigan Press, Ann Arbor, Mich., 1958.

(4) J. T. Miyata, V. E. Tyler, Jr., L. R. Brady, and M. H. Malone, *Lloydia*, **29**, 43(1966).

(5) R. A. Carrano and M. H. Malone, *J. Pharm. Sci.*, **56**, 1611(1967).

(6) H. Thoms and J. Vogelsang, *Ann. Chem.*, **357**, 145(1907).

(7) E. Bergström, *Scand. Arch. Physiol.*, **53**, 278(1928).

(8) M. L. Rodman, *Federation Proc.*, **9**, 311(1950).

(9) T. Sollmann, "A Manual of Pharmacology," 8th ed., W. B. Saunders, Philadelphia, Pa., 1957, p. 394.

(10) G. M. Carminati and G. Spina, *Boll. Chim. Farm.*, **107**, 249(1968).

(11) J. Bares, *Chem. Listy*, **21**, 477(1947); through *Chem. Abstr.*, **22**, 4669(1950).

(12) *Ibid.*, through *Chem. Abstr.*, **22**, 254(1951).

(13) L. Lukacs and J. Zellner, *Monatsh.*, **62**, 214(1933).

(14) M. Grekowicz, S. Berger, and M. Luczakowa, *Przemysl. Spozywczy*, **6**, 379(1952); through *Chem. Abstr.*, **51**, 6906c(1957).

(15) T. A. Kezeli and L. T. Dzhaparide, *Bull. Acad. Sci. Georgian SSSR*, **5**, 933(1944); through *Chem. Abstr.*, **41**, 3172c(1947).

(16) H. Willstaedt, *Svensk Kem. Tidskr.*, **53**, 23(1941).

(17) H. K. Mlodecki, W. Lasoita, and S. Tersa, *Farm. Polska*, **21**, 9(1965); through *Chem. Abstr.*, **63**, 131941g(1965).

(18) F. Haxo, *Botan. Gaz.*, **112**, 228(1950).

(19) J. L. Fiasson and N. Arpin, *Bull. Soc. Chim. Biol.*, **49**, 537(1967).

(20) G. Turian, *Arch. Mikrobiol.*, **36**, 139(1960).

(21) H. Willstaedt, *Svensk Kem. Tidskr.*, **49**, 318(1937).

(22) J. Hadáček, *Časoppi, Českoslov. Lékárnictva*, **18**, 221(1938); through *Chem. Abstr.*, **33**, 18295(1961).

(23) I. Krzeczkowska, S. Burzynski, and Z. Czerniak, *Ann. Univ. Curie-Sklodowska*, **20**, 303(1965); through *Chem. Abstr.*, **64**, 18313g(1966).
(24) M. Wagner, *Zentr. Bakteriol. Parasitenk.*, **115**, 50(1962); through *Chem. Abstr.*, **59**, 4281a(1963).
(25) C. Seelkorf and H. Schuster, *Z. Lebensm. Untersuch Forsch*, **106**, 177(1958); through *Chem. Abstr.* **52**, 609e(1958).
(26) M. Sawadi, *J. Japan Forestry Soc.*, **34**, 110(1952).
(27) T. Thunberg, *Kgl. Fysiograf Sallskap. Lund. Forh.*, **13**, 17(1943); through *Chem. Abstr.*, **41**, 7676e(1947).
(28) R. L. Shriner, R. C. Fuson, and D. Y. Curtin, "The Systematic Identification of Organic Compounds," 5th ed., Wiley, New York, N. Y., 1964, p. 458.
(29) R. Casares-Lopez, *Biochem. Z.*, **284**, 365(1936).
(30) O. Fürth and H. Herrmann, *ibid.*, **280**, 448(1935).
(31) G. Roeder, *J. Am. Pharm. Assoc., Sci. Ed.*, **30**, 74(1941).
(32) G. Sullivan and J. E. Albers, *J. Pharm. Sci.*, **58**, 887(1969).
(33) E. Stahl, "Thin-Layer Chromatography," Academic, New York, N. Y., 1965, p. 553.
(34) V. P. Garg and S. C. L. Verma, *J. Pharm. Sci.*, **56**, 639 (1967).
(35) E. J. H. Corner, "A Monograph of Cantharelloid Fungi," Oxford University Press, London, England 1966, p. 255.

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Disposition Kinetics of Griseofulvin in Dogs

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Abstract □ Griseofulvin and its main metabolite, 6-demethylgriseofulvin (6-DMG), have been administered i.v. to dogs. The plasma data of griseofulvin were found to fit biexponential equations while the urinary excretion rate data of 6-DMG after its i.v. dose (and after griseofulvin administration) were found to fit triexponential equations. When increasing doses of griseofulvin were administered intravenously, some dogs followed dose-independent disposition kinetics, while others showed definite evidence of dose-dependent disposition kinetics. The distribution rate constants and the volume of distribution were found changed with dose in dogs showing dose-dependent disposition kinetics. However, the urinary recovery of the main metabolite, 6-DMG, remained almost constant. It is, therefore, postulated that the dose-dependent disposition kinetics of griseofulvin might be attributed to changes in tissue distribution rather than changes in the intrinsic metabolic activity.

Keyphrases □ Griseofulvin—disposition kinetics, dogs □ 6-Demethylgriseofulvin—disposition kinetics, dogs □ Kinetics—dose-dependence, -independence, griseofulvin □ Metabolite excretion—griseofulvin

Griseofulvin (gris), a water-insoluble antibiotic, is widely used in both man and animals for treatment of superficial fungal diseases. Although it has been used in dogs for almost a decade, its metabolic fate in dogs has been only recently reported from this laboratory (1). The purpose of this investigation is to engage in more extensive pharmacokinetic study of this drug in dogs. It is interesting to note that some dogs follow dose-dependent disposition kinetics and some do not. The implication and the possible cause of dose-dependent disposition will be discussed. The absorption characteristics after oral administration of different dosage forms to dogs will be discussed in a future communication.

EXPERIMENTAL

Materials—Gris USP grade,[1] polyethylene glycol (PEG) 400 and 6000,[2] and bacterial beta-glucuronidase, Type II,[3] were used. 6-Demethylgriseofulvin (6-DMG) was isolated from the urine of dogs after administration of large doses of gris.

Formulation of Intravenous Dosage Forms—Pure PEG 400 or 35% PEG 6000 aqueous solution was used as a vehicle for the parenteral administrations of gris. The concentration of the drug ranged from 2–10 mg./ml. The parenteral 6-DMG dosage form was prepared in the aqueous alkaline solution with a strength of about 8 mg./ml. Fifty milligrams of 6-DMG was administered to dogs.

Animal Procedures—Male mongrel, unanesthetized, conditioned dogs weighing between 19–22 kg. were used throughout the study. They were fasted for 16–18 hr. prior to experiments. Food was withdrawn during study, while water was available *ad libitum*. Solutions of gris or 6-DMG were administered i.v. in 1 or 2 min. For intravenous studies of gris, 3–5 ml. of blood samples were usually taken at 2, 4, 6, 8, 10, 15, 25, 35, 50, 70, 90, and 120 min. after administration, while urine samples were usually collected at 20, 40, 60, 80, 100, 120, 150, 180, 210, 240, 300, 360, 420, and 480 min. from the indwelling urethral catheter. The dogs were then returned to metabolic cages. Total urine samples at 24 and 48 hr. were collected. At least 20 ml. of saline was used each time to flush the bladder after initial withdrawing of urine samples. Both the washing and the urine sample were mixed and the total volume recorded. Only a portion was retained in a plastic container for the assay. The plasma was collected after centrifugation of the heparinized blood specimen. Both plasma and urine samples were stored at 5° until assayed.

Assay of Plasma and Urine Samples—The plasma concentration

[1] McNeil Laboratories Inc., Fort Washington, Pa.
[2] Union Carbide Chemicals Co., New York, N. Y.
[3] Sigma Chemical Co., St. Louis, Mo.