muscle such as the myocardium, for at least 2 hr following drug administration. This concentration may well have continued beyond 2 hr, as reported by Pluss *et al.* (10), without being detected because of the relatively low sensitivity of whole-body section autoradiography. In any event, these observations are consistent with minoxidil having a direct relaxant effect on peripheral vascular smooth muscle.

REFERENCES

(1) D. W. DuCharme, W. A. Freyburger, B. E. Graham, and R. G. Carlson, *J. Pharmacol. Exp. Ther.*, **184**, 662(1973).

(2) E. Gilmore, J. Weil, and C. Chidsey, *N. Engl. J. Med.*, **282**, 521(1970).

(3) T. B. Gottlieb, F. H. Katz, and C. A. Chidsey, III, *Circulation*, **45**, 571(1972).

(4) L. L. Bennett, Jr., *J. Amer. Chem. Soc.*, **74**, 2432(1952).

(5) J. A. VanAllan, in "Organic Syntheses," coll. vol. 4, N. Rabjohn, Ed., Wiley, New York, N.Y., 1963, pp. 245, 246.

(6) M. Israel, H. K. Protopapa, H. N. Schlein, and E. J. Modest, *J. Med. Chem.*, **7**, 792(1964).

(7) S. Ullberg, *Acta Radiol. Suppl.*, **1954**, 118.

(8) J. I. Peterson, *Anal. Biochem.*, **31**, 204(1969).

(9) T. B. Gottlieb, R. C. Thomas, and C. A. Chidsey, *Clin. Pharmacol. Ther.*, **13**, 436(1972).

(10) R. G. Pluss, J. Orcutt, and C. A. Chidsey, *J. Lab. Clin. Med.*, **79**, 639(1972).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 17, 1974, from the *Research Laboratories, The Upjohn Company, Kalamazoo, MI 49001*

Accepted for publication January 21, 1975.

[x] To whom inquiries should be directed.

Metabolism of Minoxidil, a New Hypotensive Agent II: Biotransformation following Oral Administration to Rats, Dogs, and Monkeys

RICHARD C. THOMAS [x] and HARRY HARPOOTLIAN

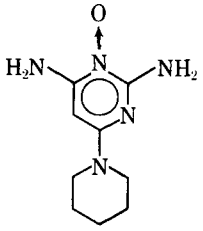
Abstract □ The biotransformation of minoxidil (2,4-diamino-6-piperidinopyrimidine 3-oxide) was studied in the rat, dog, and monkey and compared to reported results in the human. Chromatographic profiles of urinary metabolites show that each species excreted substantially the same metabolites but in quite different relative amounts. The monkey and the human exhibited similar metabolite profiles, whereas the dog and rat were quantitatively different from each other and from the monkey and human. The major excretory product for the monkey and human was a glucuronide conjugate of minoxidil. Substantially smaller amounts of unchanged minoxidil, 2,4-diamino-6-(4′-hydroxypiperidino)pyrimidine 3-oxide, and more polar metabolites also were excreted by these two species. The major excretory product in the rat was unchanged minoxidil. Almost as much (combined) of the two acidic metabolites, 2,4-diamino-6-(4′-carboxy-*n*-butylamino)pyrimidine and its 3-oxide, also were produced. Smaller amounts of the glucuronide of minoxidil, 2,4-diamino-6-(4′-hydroxypiperidino)pyrimidine 3-oxide, its 3′-hydroxy isomer, and 2,4-diamino-6-piperidinopyrimidine also were excreted by the rat. The major metabolite of minoxidil excreted by the dog was the 4′-hydroxy metabolite. Smaller amounts of unchanged minoxidil and polar metabolites and much smaller amounts of the glucuronide of minoxidil, the 3′-hydroxy metabolite, and 2,4-diamino-6-piperidinopyrimidine also were excreted by the dog. Evidence was obtained for a glucuronide conjugate of the 4′-hydroxy metabolite in this species. The major circulatory material in dog plasma was the 4′-hydroxy metabolite, whereas it was the glucuronide of minoxidil in monkey plasma.

Keyphrases □ Minoxidil—biotransformation in rat, dog, and monkey after oral administration, compared to reported human metabolism □ Metabolism, minoxidil—biotransformation in rat, dog, and monkey after oral administration, compared to reported human metabolism

As part of a program to develop minoxidil (2,4-diamino-6-piperidinopyrimidine 3-oxide, I) as an orally active hypotensive agent, metabolism studies with the drug were undertaken in three animal species: the rat, dog, and monkey. Previous reports described studies that showed that minoxidil, following its oral administration, was rapidly and well absorbed, rapidly and widely distributed, and rapidly eliminated, primarily *via* urine, by the rat, dog, and monkey (1) and by the human (2). Tissue distribution studies in the rat using ^{14}C-minoxidil were reported previously (1, 3).

These results, based on measurements of unchanged drug and total drug-related material, were not sufficient for selecting one species as being most like the human in its disposition of minoxidil. Such a selection was of particular importance for this drug because it causes an apparently species-specific, right-atrial lesion in the dog (4). Therefore, studies were undertaken to compare the biotransformation of minoxidil in the three animal species and to see if at least one of them was similar to the human in this aspect of the drug's disposition. These studies were extended to identify the metabolites of minoxidil produced by each animal species and to determine the kinetics of absorption, distribution, and excretion of the unchanged drug and its metabolites by each species.

This report is concerned with comparing the biotransformation of minoxidil in the three animal species and humans, including characterization, isolation, identification, and quantification of metabolites in urine and blood plasma. A subsequent report will deal with the kinetics of absorption, distribution, and excretion of minoxidil and its metabolites by each animal species.



I

EXPERIMENTAL

Radioactivity Measurements—Samples were counted with liquid scintillation spectrometers[1] interfaced to keypunches[2]. Routine calculations were made with a computer[3]. Duplicate (usually 0.1- or 0.5-ml) aliquots of solutions were counted in 15 ml of counting solvent [toluene–dioxane–methanol (350:350:210 v/v) containing 73 g of naphthalene, 4.6 g of 2,5-diphenyloxazole, and 0.080 g of 1,4-bis-2-(5-phenyloxazolyl)benzene/liter]. Counting efficiencies were determined by the internal standard technique using toluene-^{14}C.

Radiochromatogram Scanning—Paper and thin-layer chromatograms, when they contained sufficient radioactivity, were scanned for radioactivity using chromatogram scanners[4] having Geiger detectors. The resulting chart-strip tracings were integrated with a mechanical planimeter[5] or with an electronic digitizer system[6] to determine quantitatively the relative distribution of radioactivity along the length of the chromatogram.

When a chromatogram did not contain sufficient radioactivity for quantification by Geiger detection, it was cut into sequential segments (1 cm in the case of paper chromatograms and 0.5 cm in the case of thin-layer chromatograms). Then the segments, each in a vial with 10 ml of counting solvent containing 3% water, were counted by liquid scintillation spectrometry. The resulting raw data were processed by the computer system to give a plot of the distribution of radioactivity along the length of the chromatogram, in a format similar to that of a chart-strip tracing, together with a tabular printout of derived data. Based on the plot, the tabular data were used to quantify the relative distribution of radioactivity along the length of the chromatogram.

Preparation of Samples for Chromatography—Samples of urine and blood plasma containing sufficient radioactivity were applied directly to the paper or thin-layer medium for chromatography, usually as 2.5-cm streaks. Samples containing lower levels of radioactivity, as well as all enzyme incubation samples, were lyophilized and the residues were reconstituted at a greater concentration with water or were leached three times with methanol. The methanol was removed with a nitrogen stream, and the residue was dissolved in a small volume of 1-butanol–methanol (1:2 v/v) for application to the chromatography medium in a manner similar to that for whole urine and blood plasma. The leaching procedure resulted in chromatographic metabolite patterns quantitatively identical to those obtained by direct chromatography of whole urine and blood plasma.

Enzyme Hydrolysis—Samples of whole urine and extracts of urine and blood plasma were incubated with β-glucuronidase[7] at a concentration of approximately 1000 Fishman units/ml in 0.05 *M*, pH 5.5 sodium acetate buffer for 24 hr at 37°. Occasionally, samples were hydrolyzed under similar conditions with a mixture of β-glucuronidase and sulfatase[8].

Paper Chromatography and TLC—Paper chromatography was carried out on 86 × 15-cm sheets[9] by a descending technique in the 1-butanol–piperidine–water (82:2:16 v/v) system (System I). Occasionally, the 1-butanol–acetic acid–water (4:1:1 v/v) system (System II) was used. TLC was carried out on 20 × 5-cm films of silica gel, 0.25 mm thick, in the benzene–methanol–ammonium hydroxide (100:100:1 v/v) system (System III). Occasionally, solvent systems containing 2% piperidine in water and 10% methanol in chloroform (Systems IV and V, respectively) were used.

Appropriate standard compounds were run on each chromatogram. In certain cases, standard compounds were mixed with samples prior to chromatography to act as internal standards. Following solvent development, the chromatograms were viewed under UV light to locate standards and, when possible, drug-related materials by the UV absorption. In addition, certain chromatograms were sprayed with a 10% solution of ferric chloride in water. Minoxidil and its metabolites containing the *N*-oxide group produced a blue color (against a yellow background), whereas minoxidil metabolites not containing an *N*-oxide group produced no color.

Preparation of 2,4-Diamino-6-(4′-hydroxypiperidino)pyrimidine 3-Oxide (III)—A mixture of 0.160 g (1 mmole) of 2,4-diamino-6-chloropyrimidine 3-oxide and 0.505 g of 4-hydroxypiperidine was heated with stirring under nitrogen in an oil bath at 130° for 2.5 hr. After standing at room temperature overnight, the viscous oil was leached with acetone. The acetone leaching was evaporated *in vacuo* and the residue was taken up in methanol and applied to a 4 × 15-cm silica gel bar[10]. The silica gel bar was developed by the ascending technique in a mixture of acetone–methanol (70:30 v/v).

The UV light-absorbing zone near the origin of the bar was removed and eluted with methanol, the methanol was evaporated *in vacuo*, and the residue was recrystallized from acetone–methanol to yield 0.050 g of a crystalline solid. Its IR, UV, and NMR spectra were reasonable for the subject compound. Mass spectrometry revealed a molecular ion of 225 and a fragmentation pattern consistent with that expected for the subject compound.

Anal.—Calc. for $C_9H_{15}N_5O_2$: C, 47.98; H, 6.71; N, 31.09. Found: C, 47.67; H, 6.55; N, 30.65.

Chromatography in Systems I and III revealed a single UV-absorbing zone in each case.

Preparation of 2,4-Diamino-6-(3′-hydroxypiperidino)pyrimidine 3-Oxide (IV)—Compound IV was prepared from 3-hydroxypiperidine in a manner identical to that described for the 4′-hydroxypiperidino compound; 0.030 g of product was obtained. Its IR, UV, and NMR spectra were reasonable for the subject compound. Mass spectrometry revealed a molecular ion of 225 and a fragmentation pattern consistent with that expected for the subject compound.

Anal.—Calc. for $C_9H_{15}N_5O_2$: C, 47.98; H, 6.71; N, 31.09. Found: C, 47.07; H, 6.53; N, 30.14.

Chromatography in Systems I and III revealed a single UV-absorbing zone in each case.

Isolation of Dog Metabolites—The 0–2-hr urine of a dog which had received a tracer dose of ^{14}C-minoxidil was mixed with the urine of four dogs which had received two successive 10-mg/kg oral doses of nonradioactive minoxidil (1). The urine mixture was adjusted to pH 10 with ammonium hydroxide and passed through a 4.8 × 68-cm column of polystyrene polymer resin[11]. The column then was rinsed with three column volumes of water and eluted with three column volumes of acetone.

The acetone eluate was evaporated to dryness *in vacuo*, and the residue was taken up in methanol and applied to a 8 × 90-cm column of silica gel[12] packed with a mixture of benzene–methanol–ammonium hydroxide (75:25:1 v/v). The column was eluted with 3 mixtures of benzene–methanol–ammonium hydroxide while collecting 50-ml fractions as follows: 10 liters of a 75:25:1 (v/v) mixture, 4 liters of a 70:30:1 (v/v) mixture, and 8 liters of a 60:40:1 (v/v) mixture.

Two major zones of radioactivity emerged. Chromatographically, the first zone was a mixture of minoxidil and a more polar material and the second zone was essentially the more polar material alone. The fractions comprising each zone were combined and

[1] Model 3375, Packard Instrument Co., Downers Grove, Ill.
[2] Models 026 and 029, International Business Machines, White Plains, N.Y.
[3] Model 370/145, International Business Machines, White Plains, N.Y.
[4] Model 880 paper and model 885 thin-layer scanners, Vanguard Instrument Co., North Haven, Conn.
[5] Model 700AR Lasico, Los Angeles Scientific Instruments Co., Los Angeles, Calif.
[6] Model 9864 digitizer and model 9810 calculator, Hewlett-Packard Corp., Loveland, Colo.
[7] Ketodase, Warner-Chilcott.
[8] Glusulase, Endo Laboratories.
[9] Whatman No. 2 paper, W. and R. Balston, Ltd., London, England.
[10] Unibar, Analtech Inc., Wilmington, Del.
[11] XAD-2, Rohm and Haas Co., Philadelphia, Pa.
[12] Fifty to 200-μm particles. Available from Brinkmann Instruments, Westbury, N.Y.

Table I—Urinary Excretion of Minoxidil and Metabolites for the 0–24-hr Period following Oral Administration of ^{14}C-Minoxidil to the Rat, Dog, and Monkey

Species	Percent of Total Drug-Related Material in Urine						
	Total Polar	Carboxy Metabolites (VI/VII)	Minoxidil Glucuronide (V)	4′-Hydroxy-minoxidil (III)	3′-Hydroxy-minoxidil (IV)	Minoxidil (I)	Reduced Minoxidil (II)
Rat[a]	38	—[f]	6	12	1	43	<1
Rat (chronic)[b]		—[f]					
Before	43	(34)[g]	4	14	1	29	4
After	38	(28)[g]	2	9	1	35	10
Dog[c]	42	—[f]	5	29	4	13	<1
Dog (chronic)[d]							
Before	27	—[f]	2	47	4	19	<1
After	33	—[f]	4	35	4	18	3
Monkey[e]	21	—[f]	51	8	0	10	<1

[a] A single 5-mg/kg radioactive dose to each of three rats. [b] A 15-mg/kg radioactive dose to each of six rats before and after nine successive, daily, 150-mg/kg nonradioactive doses. [c] A single 0.55-mg/kg radioactive dose to each of three dogs. [d] A 1-mg/kg radioactive dose to each of four dogs before and after 30 successive, daily, 10-mg/kg nonradioactive doses. [e] A single 1-mg/kg radioactive dose to each of three monkeys. [f] Included in Total Polar but not differentiated. [g] Included in Total Polar.

evaporated to dryness *in vacuo*, and the residues were further purified by chromatography on 4 × 12-cm silica gel bars[10] in a benzene–methanol (70:30 v/v) mixture. The material corresponding to the first zone of the silica gel column was recrystallized from ethanol–water, water, and methanol–water (with the aid of charcoal[13]) to yield 0.015 g of white crystals. This material corresponded to minoxidil according to UV, IR, and mass spectrometry.

Anal.—Calc. for $C_9H_{15}N_5O$: C, 51.66; H, 7.23; N, 33.47. Found: C, 51.55; H, 7.19; N, 34.03.

Chromatography in Systems I and III–V revealed a single UV-absorbing zone corresponding to minoxidil. The material corresponding to the second zone on the silica gel column was recrystallized three times from methanol–acetone (with the aid of charcoal[13]) to yield 0.030 g of white crystals. This material corresponded to 2,4-diamino-6-(4′-hydroxypiperidino)pyrimidine 3-oxide according to UV, IR, and mass spectrometry.

Anal.—Calc. for $C_9H_{15}N_5O_2$: C, 47.98; H, 6.71; N, 31.09. Found: C, 48.02; H, 6.76; N, 30.72.

Chromatography in Systems I and III revealed a single UV-absorbing zone corresponding to the 4′-hydroxypiperidino compound. NMR spectrometry showed that the carbinol hydrogen was not at the C-2′, C-3′, C-5′, or C-6′ position of the piperidino ring, so it must have been at the C-4′ position. The coupling to the C-3′ and C-5′ methylene hydrogens of the piperidino ring indicated that the hydroxy group was equatorial.

Isolation of Rat Metabolites—Each of six female rats[14], weighing approximately 200 g, was given a 3-mg (24.4 μCi) dose of ^{14}C-minoxidil, nine successive 30-mg daily doses of nonradioactive minoxidil, and a final 3-mg (24.4 μCi) dose of ^{14}C-minoxidil by oral intubation of a solution or suspension in water. The rats were placed in individual metabolism cages, and urine was collected at 24-hr intervals and frozen for the duration of the study. Small samples of each urine collection for the 0–24-hr period following administration of the radioactive doses were set aside for analysis. The bulk of the radioactive urine collections was combined with the nonradioactive collections for metabolite isolation.

The composite urine was adjusted to pH 9 with ammonium hydroxide and passed through a 2.8 × 77-cm column of polystyrene resin[11]. The column was rinsed with three column volumes of water, which had been adjusted to pH 9, and then eluted with three column volumes of 50% methanol in water. The methanol eluate was evaporated to dryness *in vacuo* and set aside. The aqueous wash was adjusted to pH 4 with acetic acid and passed through a 2.8 × 77-cm column of polystyrene resin[11]. The column was rinsed with three column volumes of water, which had been adjusted to pH 4, and then eluted with three column volumes of 50% methanol in water. The rinse water was discarded and the methanol eluate was evaporated to dryness *in vacuo*.

A 10% aliquot of the pH 9 polystyrene resin[11] column eluate in 5 ml of benzene–methanol–ammonium hydroxide (50:50:1 v/v) was applied to a 2.5 × 51-cm column of silica gel[15]. The column was eluted with the same solvent mixture while collecting 10-ml fractions. Two major zones of radioactivity emerged. Chromatographically (Systems I–V), the first zone corresponded to 2,4-diamino-6-piperidinopyrimidine and the second zone corresponded to minoxidil. The contents of the tubes corresponding to the second zone were combined and evaporated to dryness, and the residue was recrystallized three times from ethanol–water (with the aid of charcoal[13]) to yield 0.010 g of crystals. This material corresponded to minoxidil according to UV spectrometry and chromatography in Systems I and III–V.

Anal.—Calc. for $C_9H_{15}N_5O$: C, 51.66; H, 7.23; N, 33.47. Found: C, 51.67; H, 7.48; N, 32.94.

This material gave a positive *N*-oxide test with ferric chloride solution, whereas the material that corresponded chromatographically to 2,4-diamino-6-piperidinopyrimidine did not.

The pH 4 polystyrene resin[11] column eluate was subjected to countercurrent distribution in the 1-butanol–acetic acid–water (9:1:10 v/v) system; 100 ml of each phase was used per tube for 20 transfers of the lower phase. The contents of tubes 11–16, which contained the major radioactive zone, were combined and evaporated to dryness in a nitrogen stream. The residue was recrystallized twice from acetone–water (with the aid of charcoal[16]) to obtain 0.026 g of crystals. Mass spectrometry, using an electron-impact technique, revealed an apparent molecular ion of 225 mass units and a fragmentation pattern consistent with 2,4-diamino-6-(4′-carboxy-*n*-butylamino)pyrimidine. UV spectrometry supported this structure.

NMR spectrometry suggested that the material was actually a mixture of the proposed compound and its 3-oxide (in a ratio of approximately 2 to 1) on the basis of comparisons with the spectra of 2,4-diamino-6-piperidinopyrimidine and its 3-oxide (minoxidil). This finding was supported by resolution of the material into two radioactive and UV-absorbing zones, only one of which gave a positive *N*-oxide test with ferric chloride solution, when subjected to chromatography in Systems I (for 6 days) and IV. In this case, the ratio of *N*-oxide to the reduced form was found to be nearly 1:1 based on radioactivity.

Mass spectrometry with a different instrument, using a field-desorption technique[17], revealed two molecular ions, one at 225 mass units, attributable to the initially proposed compound, and the other at 241 mass units, attributable to its 3-oxide. Use of an electron-impact technique and careful regulation of the probe temperature with this same instrument[17] revealed an apparent molecular ion at 241 mass units and a fragmentation pattern consistent with a mixture of the two materials.

Anal.—Calc. for a 1:1 mixture of $C_9H_{15}N_5O_2$ and $C_9H_{15}N_5O_3$: C, 46.40; H, 6.49; N, 30.03. Found: C, 46.08; H, 6.52; N, 30.41.

[13] Darco G60, Atlas Powder Co., Wilmington, Del.
[14] Sprague–Dawley (Upjohn strain).
[15] Ten to 40-μm particles containing 13% $CaSO_4$. Available from Brinkmann Instruments, Westbury, N.Y.
[16] Nuchar C-190, West Virginia Pulp and Paper Co., New York, N.Y.
[17] Model CH-5, Varian MAT/USA, Springfield, N.J.

Table II—Areas under Minoxidil and Metabolite Plasma Level Curves for the 0–8-hr Period following Oral Administration of ^{14}C-Minoxidil to the Dog and Monkey

Species[a]	Percent of Total Drug-Related Area				
	Total Polar	Minoxidil Glucuronide (V)	4′-Hydroxy-minoxidil (III)	3′-Hydroxy-minoxidil (IV)	Minoxidil[b] (I)
Dog	14	<1	46	3	23
Dog (chronic)					
Before	12	<1	49	<1	26
After	17	<1	46	<1	27
Monkey	17	64	7	<1	9

[a]See Table I for drug dosages and numbers of animals. [b]Includes possible reduced minoxidil (II).

RESULTS AND DISCUSSION

Initial studies involved comparative paper and thin-layer radiochromatography of urine from rats, dogs, and monkeys which had received ^{14}C-minoxidil orally. In each case, radioactivity zones were detected, indicating extensive metabolism of minoxidil. One of these zones for each species had the same chromatographic mobility as minoxidil. The other zones were attributed to then unidentified metabolites of minoxidil. Judging from the relative amount of radioactivity in each zone, the major metabolite was different for each species. However, each species produced certain amounts of the major metabolites of the others. The urinary metabolite patterns for the three animal species are illustrated in Fig. 1; the metabolite pattern for the human will be discussed later. The peak designated I/II had the same mobility in System I as standard minoxidil.

Attention was next turned to identifying the urinary excretion products of minoxidil in the three animal species. In the case of the dog, the peak designated I/II in Fig. 1 was tentatively identified as unchanged minoxidil on the basis of its paper and thin-layer chromatographic mobilities. Subsequently, mass spectrometry of this material, isolated by preparative paper chromatography and TLC, gave spectra that were identical to that of minoxidil.

Finally, this material was isolated in crystalline form from dog urine and identified as minoxidil by elemental and spectral analyses. Material of the mobility designated I/II in Fig. 1 also was isolated in crystalline form from rat urine and identified as minoxidil. In the case of the monkey, the characterization of peak I/II in Fig. 1 was based entirely on paper chromatography and TLC.

During isolation of the rat urinary minoxidil, a radioactive material was detected with the same mobility as minoxidil in System I but with a greater relative mobility in Systems III–V. In contrast to minoxidil, it did not produce a blue color when treated with ferric chloride solution and, therefore, was considered to lack an *N*-oxide function. Since it had the same mobility in Systems I and III–V as 2,4-diamino-6-piperidinopyrimidine (II), it was characterized as this reduced form (II) of minoxidil. That particular study involved chronic administration of minoxidil to rats [labeled Rat (chronic) in Table I].

In another rat study, involving single 50-mg/kg doses of ^{14}C-minoxidil, II also was found in urine. However, in a third study, using single 5-mg/kg doses of ^{14}C-minoxidil in rats, II was not detected in urine. Thus, at least when large or chronic doses of minoxidil were administered to rats, II was excreted in urine. This also was the case with the dog. This metabolite was not detected in monkey urine.

Mass spectrometry of the material designated III in Fig. 1, isolated by preparative paper chromatography and TLC of urine from dogs that had received ^{14}C-minoxidil, indicated that this material was a hydroxypiperidino form of minoxidil. Therefore, both 2,4-diamino-6-(4′-hydroxypiperidino)pyrimidine 3-oxide (III) and 2,4-diamino-6-(3′-hydroxypiperidino)pyrimidine 3-oxide (IV) were prepared and characterized as standards. Paper chromatography and TLC showed that the 4′-hydroxy standard had the same mobility as the major peak designated III and the 3′-hydroxy standard had the same mobility as the minor peak designated IV.

Subsequently, III was isolated from dog urine in crystalline form and identified as such by elemental and spectral analyses. NMR was particularly valuable in confirming the 4′-position for the hydroxy group and showing that it was equatorial. With the rat and monkey, characterization of peak III in Fig. 1 was based entirely on paper chromatography and TLC. The characterization of IV in Fig. 1 for the dog and rat was based solely on this small peak having the same mobility as standard IV in System I. This metabolite was not detected in monkey urine.

Characterization of the peak designated V in Fig. 1 was based principally on results obtained by incubating urine containing this radioactive metabolite with β-glucuronidase. For each animal species, such incubation resulted in loss of peak V and a concomitant and equivalent increase in peak I/II. Mass spectrometry of eluates of the zone corresponding to I/II from preparative thin-layer chromatograms, following enzyme incubation of monkey urine, confirmed that minoxidil was being released. Metabolite V was thereby characterized as a glucuronic acid conjugate of minoxidil. It is likely that conjugation was with the *N*-hydroxy tautomeric form of minoxidil (Scheme I), giving an *O*-glucuronide[18]. Never-

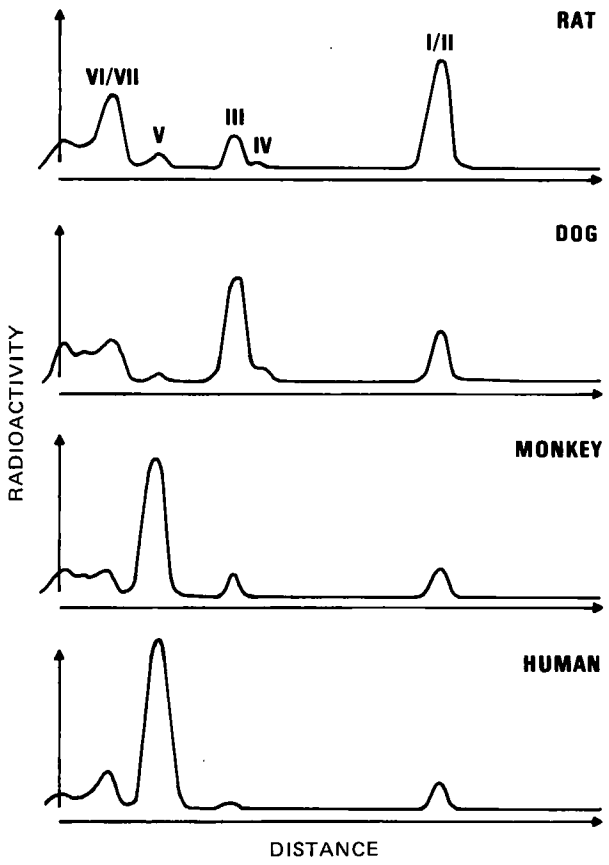
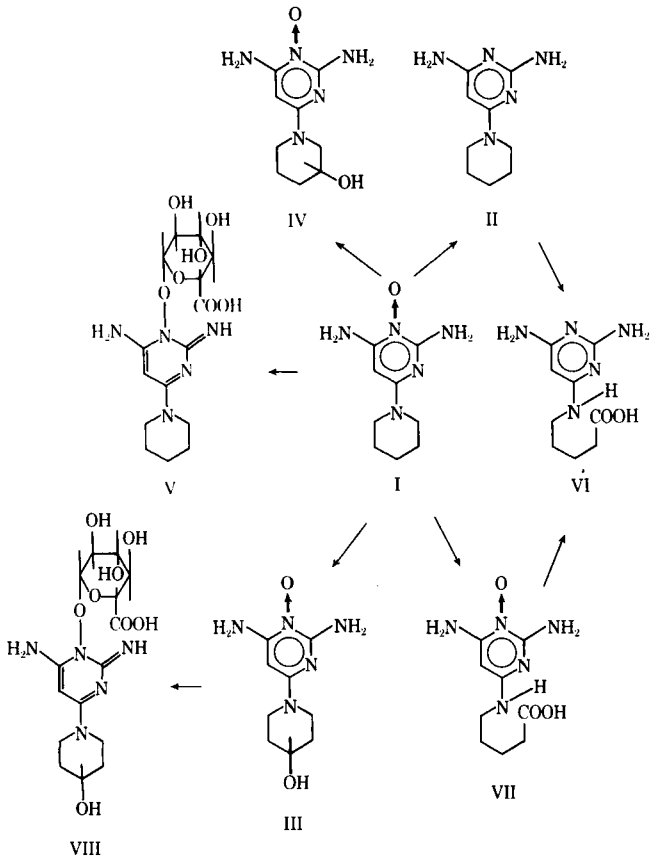


Figure 1—*Composites of radiochromatograms (System I) representing 0–24-hr urine collections for the rat, dog, monkey, and human.*

[18] The glucuronide conjugate is stable to hydrolysis in 0.1 *N* HCl at 35°; *N*-glucuronides generally are hydrolyzed by such treatment.



Scheme I—Biotransformation of minoxidil (the points of attachment of the glucuronic acid moiety to V and VIII have not been definitely established)

theless, conjugation through one amine nitrogen to give an *N*-glucuronide has not been completely ruled out.

Preliminary characterization of the metabolite peak designated VI/VII in Fig. 1 was accomplished by mass spectrometry of appropriate eluates of preparative paper and thin-layer chromatograms of urine from rats that had received ^{14}C-minoxidil. These results indicated that this metabolite was 2,4-diamino-6-(4′-carboxy-*n*-butylamino)pyrimidine (VI), derived from minoxidil *via* oxidative cleavage of the piperidine ring at the C—N bond. Subsequently, the metabolite(s) was isolated in crystalline form from rat urine and shown by elemental analysis and spectrometry to be a mixture of VI and 2,4-diamino-6-(4′-carboxy-*n*-butylamino)pyrimidine 3-oxide (VII) in a ratio of 1:1 or 2:1. The only evidence that these materials were metabolites of minoxidil in dog and monkey urine was that each species produced a urinary metabolite of minoxidil having a mobility corresponding to the carboxy metabolites in System I. Studies are in progress to determine the importance of these metabolites in the dog and monkey.

Evidence for urinary excretion of a glucuronide conjugate (VIII) of the 4′-hydroxy metabolite by the dog was obtained. Incubation with β-glucuronidase of urine from dogs that had received ^{14}C-minoxidil resulted in loss of radioactivity from the origin and a concomitant and equivalent appearance of radioactivity in peak III when chromatographed in System I. This could be an *O*-glucuronide of the *N*-hydroxy group or the C-hydroxy group or perhaps an *N*-glucuronide of one amino group of the 4′-hydroxy metabolite. This material was not detected as a metabolite of minoxidil in rat and monkey urine.

The results described thus far are summarized in Fig. 1 and Scheme I. In Fig. 1, an attempt was made to present true, quantitative relationships among the drug and its metabolites for the various species. Scheme I shows the structures of the various metabolites produced by the three animal species as well as their possible relationships to one another. The points of attachment of the glucuronic acid moiety to minoxidil and one of the metabolites are somewhat speculative, but there is substantial evidence in each case for the structure of the aglycone and for its conjugation with glucuronic acid.

Once chromatographic profiles of urinary minoxidil metabolites were obtained and most metabolites were identified, it was possible to quantify the biotransformation of minoxidil for each animal species. Minoxidil and its metabolites in urine were quantified by determining the amount of radioactivity associated with the drug or a particular metabolite following separation by paper chromatography and TLC. The results presented in Table I and Fig. 1 clearly show that the principal drug-related material excreted by the rat was unchanged minoxidil, closely followed by the carboxy metabolites. With the dog, the 4′-hydroxy metabolite was the principal excretion product. The monkey excreted predominantly the glucuronide conjugate of minoxidil.

The various drug-related materials in the circulation of the dog and monkey were characterized and quantified by radiochromatography of blood plasma samples taken sequentially following oral administration of ^{14}C-minoxidil. The results (Table II) are presented as the percent contribution each drug-related material makes to the total area under the plasma radioactivity level *versus* time curve during the 8-hr period following drug administration. This method should take into account the persistence, as well as the concentration, of each drug-related material in plasma.

As can be seen from Table II, the principal circulatory material in the dog was the 4′-hydroxy metabolite; in the monkey, it was the glucuronide of minoxidil. Substantial amounts of minoxidil and the polar group of metabolites were circulated by both species. The major drug-related material in circulation for the human was not identified by Gottlieb *et al.* (2), but it was not minoxidil. Since the major urinary excretion product in the human and the monkey was the glucuronide of minoxidil, it seems reasonable that this was also the major circulatory material in the human.

Gottlieb *et al.* (2) reported that the human excretes four minoxidil-related products in urine following oral administration of ^{14}C-labeled drug. They were characterized as minoxidil, a glucuronide of minoxidil, and two unidentified metabolites, designated M-II and M-I. Their relative amounts in 0–24-hr urine collections were 12, 48, 18, and 21%, respectively, as determined by radiochromatography in System I.

Experience with paper chromatography of monkey urine and urine extracts containing the minoxidil glucuronide has shown this glucuronide to have two apparent migration rates in System I. Although the glucuronide usually migrated to one or the other of two similar R_f values (0.2 and 0.3), at times it would appear as a doublet occupying both locations. Certain samples, particularly those with relatively great solids content, were more susceptible than others to this variation in migration, including doublet formation. Replicate paper chromatograms of such a sample over time could result in the glucuronide displaying either, or both, R_f value unpredictably.

Regardless of the particular chromatographic behavior, however, hydrolysis of each sample resulted in complete loss of the glucuronide and a concomitant and equivalent appearance of minoxidil. The existence of two glucuronides of minoxidil (such as an *O*-glucuronide and an *N*-glucuronide) would not explain these observations. At the same time, these results do not preclude the presence of two such materials. These results can be explained best by a single glucuronide which, depending on its environment, either can be partially modified by application to the paper or can exist in several ionic forms having different migration rates in System I.

Chromatography in System I of human urine samples, collected by Gottlieb *et al.* (2), resulted in separation of three major zones of radioactivity rather than four. These zones corresponded, in order of increasing polarity, to minoxidil, the glucuronide of minoxidil, and a very polar zone, sometimes resolvable into two components. Integration of these chromatograms showed that our glucuronide accounted for the sum of the glucuronide and Metabolite M-II or M-I described by Gottlieb *et al.* (2). Hydrolysis of these urine samples with β-glucuronidase resulted in the complete loss of the material herein designated as the glucuronide and a concomitant and equivalent appearance of minoxidil, as judged by chromatography in System I. Similar hydrolysis studies using System III showed

that minoxidil, not 2,4-diamino-6-piperidinopyrimidine, was released from the glucuronide.

It appears that, as with monkey urine, a single glucuronide best explains the results with human urine. In addition to minoxidil, the glucuronide of minoxidil, and the very polar minoxidil-related material in human urine, small (less than 3%) amounts of 2,4-diamino-6-(4′-hydroxypiperidino)pyrimidine 3-oxide and, in a few samples, traces of 2,4-diamino-6-piperidinopyrimidine were found. The more mobile component of the very polar zone of radioactivity corresponded to the migration of the carboxy metabolites of minoxidil. The urinary metabolite profile for the human is based on the results reported by Gottlieb *et al.* (2) modified by our chromatographic results with human and monkey urine containing the glucuronide of minoxidil as discussed.

The urinary metabolite profile for minoxidil in the human is compared to those in the rat, dog, and monkey in Fig. 1. This comparison shows that each species, including the human, excreted substantially the same metabolites but in quite different relative amounts. The monkey and human exhibited similar metabolite profiles, whereas the dog and rat were quantitatively different from each other and from the monkey and human. Thus, of the three animal species studied, the monkey is most like the human in its biotransformation and disposition (1) of minoxidil.

REFERENCES

(1) R. C. Thomas, R. S. P. Hsi, H. Harpootlian, and R. W. Judy, *J. Pharm. Sci.*, **64**, 1360(1975).

(2) T. B. Gottlieb, R. C. Thomas, and C. A. Chidsey, *Clin. Pharmacol. Ther.*, **13**, 436(1972).

(3) R. G. Pluss, J. Orcutt, and C. A. Chidsey, *J. Lab. Clin. Med.*, **79**, 639(1972).

(4) D. W. DuCharme, W. A. Freyburger, B. E. Graham, and R. G. Carlson, *J. Pharmacol. Exp. Ther.*, **184**, 662(1973).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 17, 1974, from the *Research Laboratories, The Upjohn Company, Kalamazoo, MI 49001*

Accepted for publication January 21, 1975.

The authors are grateful for the contribution of R. W. Judy and W. M. Vanderberg in certain of the studies and to members of Physical and Analytical Chemistry for analytical results. They also thank L. Baczynskyj and R. J. Wnuk for mass spectral results and interpretations and G. Slomp and S. Mizsak for NMR spectral results and interpretations.

[x] To whom inquiries should be directed.

Antiradiation Compounds XV: Condensations of Carbon Disulfide with Amino, Chloro, Cyanomethyl, and Sulfonamido Heterocycles

WILLIAM O. FOYE [x], JOEL M. KAUFFMAN, JOSEPH J. LANZILLO, and EDWARD F. LaSALA

Abstract □ Condensations of carbon disulfide were carried out with amino, chloro, and diamino heterocycles to give condensed ring thiazoline-2-thiones and imidazoline-2-thiones, with cyanomethyl heterocycles to give dithio acid derivatives, and with heterocyclic sulfonamides to give sulfonyldithiocarbamates. Of several examples tested, pyrido[3,2-*d*]thiazoline-2-thione, disodium 2-(5-chloro-2-thienyl)-3,3-dimercaptoacrylonitrile, triethylammonium 4-sulfamoylphenyldithiocarbamate, ammonium β-phenethyldithiocarbamate, and methyl *N*-(thiophene-2-sulfonyl)dithiocarbamate, only the last-named compound showed any radiation protection for mice. Several compounds gave negative tests for antimalarial activity.

Keyphrases □ Antiradiation compounds—condensations of carbon disulfide with amino, chloro, cyanomethyl, and sulfonamido heterocycles □ Carbon disulfide—condensation products with amino, chloro, cyanomethyl, and sulfonamido heterocycles, potential antiradiation agents □ Heterocycles (amino, chloro, cyanomethyl, and sulfonamido)—condensation products with carbon disulfide, antiradiation activity

Condensation products of carbon disulfide with amines (1, 2) and mercaptans (3), as well as with active methylenes adjacent to cyano groups (4), have all shown some degree of protection for animals against ionizing radiation. With the dithiocarbamates, the presence of heterocyclic rings, particularly when strongly basic (5), provided greater protective properties. In the case of the dimercaptoacrylonitriles derived from active methylene compounds, the presence of heterocyclic rings on the carbon alpha to the dimercapto acid function generally resulted in hygroscopic products and sometimes compounds of low stability.

The attempt accordingly has been made to obtain condensation products of carbon disulfide with heterocyclic rings that have greater stability and also a mercapto group two or three atoms distant from a basic nitrogen or heteroatom, to provide an analogy with the more protective aliphatic mercapto amines.

DISCUSSION

Condensations with Amino and Chloro Heterocycles—The finding (6) that 4-chloro-5,6-diaminopyrimidine gave a thiazoline-2-thione (I) with replacement of amino and chloro groups and not the imidazoline-2-thione prompted a trial of this reaction on several suitably substituted heterocycles (Scheme I).

The pyridine analog (II) was prepared previously by Yamamoto and Takahashi (7), who reported a decomposition point of 294° and stated no percentage yield. Their product was obtained from 2-chloro-3-nitropyridine, sodium sulfide, hydrogen sulfide, and carbon disulfide; sulfide reduction of the nitro group was involved. In the present research, II was obtained in 73% yield, mp 310–314° dec., from 3-amino-2-chloropyridine and carbon disulfide, with dimethylformamide as the solvent.