Chem. Pharm. Bull. 24(7)1433—1438(1976)

UDC 615.282.011.4 : 615.074 : 543.422.062

Metabolism of Piromidic Acid, a New Antibacterial Agent. III.[1] Determination of Piromidic Acid and Its Metabolites in Blood, Urine and Bile of Rats and Humans

Yutaka Sekine, Mie Miyamoto, Masahisa Hashimoto, and Kiyoshi Nakamura

Research and Development Division, Dainippon Pharmaceutical Co., Ltd.[2]

(Received June 11, 1975)

The chemical determinations of piromidic acid (PA, 5,8-dihydro-8-ethyl-5-oxo-2-pyrrolidinopyrido[2,3-*d*]pyrimidine-6-carboxylic acid=pyrrolidino-PPA) and its metabolites in blood, urine and bile of rats receiving a single oral (100 mg/kg) or intravenous (25 mg/kg) dose of PA were performed utilizing thin-layer chromatographic separation, followed by ultraviolet spectroscopic determination. The same determinations were also made for human subjects receiving oral PA of 1 g. The following results were obtained:

(1) PA was rapidly metabolized to M-II (2-hydroxypyrrolidino-PPA) and M-V (3-hydroxypyrrolidino-PPA) in both species after oral and intravenous administrations.

(2) Blood levels of M-II, M-IV (3-hydroxycarbonylpropylamino-PPA), unchanged PA and M-V in order of decreasing amounts in rats all reached their peaks 2 to 3 hr after oral administration. On the contrary, blood levels of M-III (amino-PPA) were too low to be determined. Analogous results were also obtained in humans.

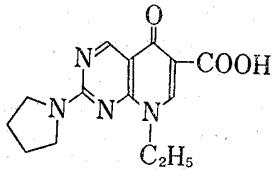
(3) An average 24-hr biliary excretion in rats receiving intravenous PA was about 63% of the dose and a major metabolite was M-IV, accounting for about 23% of the dose.

(4) In man, an average recovery in 24-hr urine was about 42% of the dose after oral administration. M-IV, M-V, M-II, M-III, PA and glucuronides were excreted in order of decreasing amount. In rats, the similar results were obtained after oral and intravenous administrations.

(5) The antibacterial activity in blood, bile and urine was found to be associated almost entirely with M-II, M-V, and unchanged PA in both species after oral or intravenous administration, with significant predominance of M-V in the urine. Urinary or biliary levels of total active materials were found to be high enough to inhibit *Escherichia coli*.

Piromidic acid (PA, 5,8-dihydro-8-ethyl-5-oxo-2-pyrrolidinopyrido[2,3-*d*]pyrimidine-6-carboxylic acid, see Chart 1) is a new antibacterial agent.[3] Absorption, distribution and excretion of PA in rats have been examined utilizing bioassay to show significantly higher levels of antibacterial activity in urine and bile.[4]

The previous study revealed that PA was extensively metabolized in rats and human subjects as evidenced by ten metabolites together with unchanged PA being identified in their urine and bile.[5] The first step in PA metabolism was found to be oxidations in the pyrrolidine ring to afford 2- and 3-hydroxypyrrolidine derivatives, followed by further conversion with the former to give metabolites without retaining pyrrolidine ring.[1] All these metabolites together with unchanged PA were partly converted to their glu-



PA

Chart 1. Chemical Structure of PA

1) Part II: Y. Sekine, M. Miyamoto, M. Hashimoto, and K. Nakamura, *Chem. Pharm. Bull.* (Tokyo), **24**, 437 (1976).
2) Location: *33–94 Enoki-cho, Suita, Osaka, 564, Japan.*
3) M. Shimizu, S. Nakamura, and Y. Takase, *Antimicrob. Ag. Chemother.*, **1970**, 117.
4) *a*) M. Shimizu, Y. Sekine, H. Higuchi, H. Suzuki, S. Nakamura, and K. Nakamura, *Antimicrob. Ag. Chemother.*, **1970**, 123; *b*) M. Shimizu, S. Nakamura, Y. Takase, Y. Sekine, H. Suzuki, and K. Nakamura, *Chemotherapy*, **19**, 387 (1971).
5) Y. Sekine, M. Miyamoto, M. Hashimoto, and K. Nakamura, *Xenobiotica*, **6**, 185 (1976).

curonides with activity abolished. Unconjugated metabolites retaining pyrrolidine rings exhibited potentiated antibacterial activities, whereas other unconjugated metabolites showed markedly decreased activities.[5]

The present study is undertaken to determine blood, urinary and biliary levels of PA and its metabolites, except for the unstable intermediate M-VI[1,5] in urine, in rats treated with oral or intravenous PA, together with humans receiving oral PA, based on a chemical method involving thin-layer chromatographic separation, followed by ultraviolet (UV) spectroscopic determination. Since the antibacterial activities are varied among PA and its metabolites, the results will be discussed to elucidate major material(s) in relation to the antibacterial activity in these biological media examined.

Experimental

Materials——Authentic samples of PA and its derivatives, and β-glucuronidase were the same as those described in the previous paper.[5]

Animal Experiments and Human Volunteer Tests——Studies in Rats: Male Wistar rats weighing 200 to 250 g were used. The procedures used in this study were the same as those described in the previous paper,[5] except following.

Blood was individually withdrawn from rats by cardiac puncture at 1, 2, 3, 4, and 6 hr after oral administration of PA with heparinized syringe. In the case of intravenous administration, PA in 0.01 N NaOH solution (5 mg/ml) was administered at a dose of 25 mg/kg to rats. The urine of rats receiving oral and intravenous PA in metabolism cage was collected from 0 to 3, 3 to 6 and 6 to 24 hr post drug administration. A polyethylene catheter was introduced by the usual surgical procedure into the common bile duct of rats anesthetized with pentobarbital (50 mg/kg, intraperitoneally). After intravenous administration of PA, bile was collected from 0 to 1, 1 to 2, 2 to 3, 3 to 5, 5 to 7 and 7 to 24 hr post administration.

Human Volunteers: Four male healthy volunteers weighing 60 to 75 kg swallowed 1 g[6] of PA. Blood samples were taken at 2, 4, 6 and 8 hr post administration and urine samples were collected for 0—24 hr.

Thin-Layer Chromatography (TLC) and Bioassay——TLC was carried out with silica gel plate (Eastman Chromatogram 6061) and solvent system, chloroform–formic acid–ethyl formate (10: 1: 4). Bioassay was performed by the thin-layer cup-plate method[4b] adopting *Escherichia coli*.

Determination Procedure——Urine: To 1 ml of urine in a 40 ml glass-stoppered centrifuge tube, 2 drops of 1N HCl, 9 ml of 1/5 M Na_2HPO_4-1/10 M citric acid buffer solution (pH 3.0) and 20 ml of $CHCl_3$ were added. The mixture was shaken vigorously for 15 min on a conventional shaker and centrifuged at 3000 rpm for 10 min. Two ml of the chloroform phase transferred to a flask, concentrated *in vacuo* to an appropriate small volume, and subjected to preparative TLC with assending runs for about 2 hr. The plates were dried in air. Spots were located under UV-lamp and scraped off from the plate. Each scraping was suspended in 5 ml of a buffer solution (pH 3.0, 1/5 M Na_2HPO_4–1/10 M citric acid) and extracted with 10 ml of $CHCl_3$. The absorbance in the each chloroform extract at given wave length was measured with UV-spectrometer (PA at 282 nm, metabolite II (M-II, 2-(2-hydroxypyrrolidino)-5,8-dihydro-8-ethyl-5-oxo-pyrido-[2,3-*d*]pyrimidine-6-carboxylic acid=2-(2-hydroxy-pyrrolidino)-PPA) at 276 nm, metabolite III (M-III, 2-amino-PPA) at 254 nm). Determination of unchanged PA, M-II and M-III in the urine was carried out with calibration curve prepared beforehand. *Rf*-values of metabolite IV (M-IV, 2-(3-hydroxycarbonylpropyl-amino)-PPA) and metabolite V (M-V, 2-(3-hydroxypyrrolidino)-PPA) were so closed that combined scraping was subjected to the spectral assay. Separate determinations of M-IV and M-V can be made from absorbances measured at 270 and 280 nm of their chloroform solution, because mixtures of both metabolites behave spectrophotometrically as a simple two-compartment system, where 270 nm and 280 nm are the wave length of maximum absorption for M-IV and M-V, respectively. The system is characterized by a set of two simultaneous equations (1) and (2):

$$A_{270} = C^{IV}E^{IV}_{270} + C^{V}E^{V}_{270} \quad (1)$$

$$A_{280} = C^{IV}E^{IV}_{280} + C^{V}E^{V}_{280} \quad (2)$$

where A, C, and E represent the absorbance, concentration and extinction coefficient. So A_{270} and A_{280} mean the absorbance at 270 and 280 nm in the mixture, C^{IV} and C^{V} are the concentrations of M-IV and M-V and E^{IV}_{270}, E^{V}_{270}, E^{IV}_{280}, and E^{V}_{280} represent extinction coefficients of M-IV or M-V at 270 or 280 nm, respectively. These extinction coefficients were obtained with the authentic samples to derive Eq. (3) and (4):

6) The clinical dose of PA for adults is 1.5—3.0 g per day (*New Drugs in Japan*, **24**, 97 (1973)). No appreciable toxicities of PA were observed in mice, rats, dogs and monkeys (H. Senda, K. Onishi, T. Oka, and H. Tatsumi, *Chemotherapy*, **19**, 404 (1971)).

$$A_{270} = 0.1592C^{IV} + 0.1421C^{V} \quad (3)$$

$$A_{280} = 0.0251C^{IV} + 0.1796C^{V} \quad (4)$$

The average single recovery of M-IV (50—400 μg/ml) or M-V (20—100 μg/ml) from the urine was found to be 41.0±0.7% (mean±S.E., $n=8$) or 88.4±0.5% ($n=8$), respectively. Thus the concentration of each metabolite in the urine (x, y μg/ml) can be corrected as in Eq. (5) and (6):

$$x = 100C^{IV}/0.410 \quad (5)$$

$$y = 100C^{V}/0.884 \quad (6)$$

Standard urine mixtures were made of M-IV and M-V in their several varied compositions (each 50—200 μg/ml). After the entire assay procedure as described above and calculation from Eq. (5) and (6), the average recoveries of M-IV and M-V from the mixture were 100.1±2.0% ($n=6$) and 98.7±0.5% ($n=6$), respectively.

Determinations were also carried out of urine samples pretreated with β-glucuronidase.

Bile: Bile samples were diluted ten fold with distilled water and assayed similarly to the urine samples. The average recoveries of M-IV and M-V from the bile, after calculation from Eq. (5) and (6), were 100.2±1.7 % (mean± S.E., $n=6$) and 99.6±1.1% ($n=6$), respectively.

Blood: To 5 ml of whole blood in a 40 ml glass-stoppered centrifuge tube, 5 ml of a buffer solution (pH 4.0, 1/5 M Na_2HPO_4-1/10 M citric acid) and 20 ml of $CHCl_3$ were added. The chloroform extracts (15 ml) were similarly subjected to the assay. Calibration curves were made beforehand of PA, M-II and M-III in blood, and M-IV and M-V in blood (x', y' μg/ml) were calculated by Eq. (7) and (8) derived from the same way as in urine:

$$x' = 33.13C^{IV}/5 \quad (7)$$

$$y' = 12.91C^{V}/5 \quad (8)$$

The recovery examinations of M-IV and M-V in blood (each 0.25—5.0 μg/ml) were similarly made as urine. After calculation from Eq. (7) and (8), the average recoveries of M-IV and M-V from the mixture were 98.9±2.0% (mean±S.E., $n=6$) and 99.5±1.0% ($n=6$), respectively.

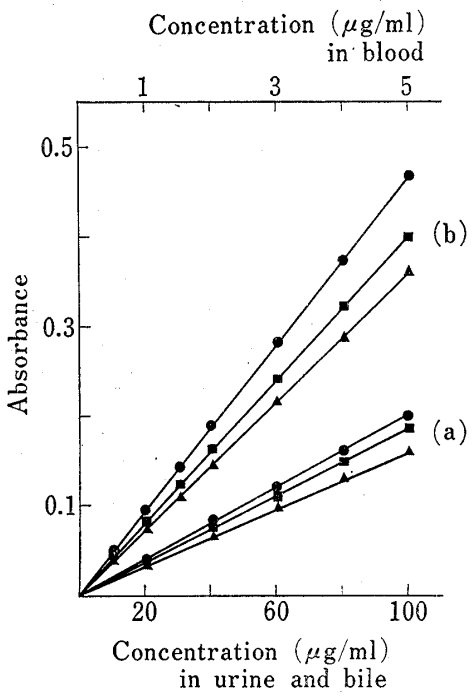


Fig. 1. Calibration Curves of PA, M-II and M-III in Urine, Bile and Blood

(a) urine and bile, (b) blood
—●—: PA
—▲—: M-II (2-(2-hydroxypyrrolidino)-PPA),
—■—: M-III (2-amino-PPA)
PPA: 5,8-dihydro-8-ethyl-5-oxo-pyrido[2,3-*d*]-pyrimidine-6-carboxylic acid

Calibration Curves——Urine : Urine was collected from volunteers and rats prior to drug administration. Standard solutions were prepared of PA, M-II, M-III, M-IV, and M-V (in 0.01 N NaOH). To 1 ml of the urine in a 40 ml glass-stoppered tube, 1 ml of PA (or a metabolite) solution (20—100 μg/ml), 3 drops of 1 N HCl, 8 ml of a buffer solution (pH 3.0, 1/5 M Na_2HPO_4–1/10 M citric acid) and 20 ml of $CHCl_3$ were added. The calibration curves for PA and its metabolites in urine were obtained by the procedure described above as shown in Fig. 1 (a).

Bile : The calibration curves for PA and its metabolites in the bile (20—100 μg/ml) were made similarly those of urine.

Blood: The calibration curves for PA and its metabolites in blood (0.25—5.0 μg/ml) are shown in Fig. 1 (b).

Results and Discussion

I. Blood Levels of PA and Its Metabolites in Rats and Humans

The blood levels of PA and its metabolites were determined after single intravenous (25 mg/kg) and oral (100 mg/kg) dosings of PA to rats. The results are shown in Fig. 2(a) and 2(b). Semi-logarithmic plot of unchanged PA in blood of intravenously treated rats shows two linear phases showing distribution and elimination phases, respectively. Fig. 2(c) illustrates the blood levels of unchanged PA and its metabolites in humans receiving single oral PA of 1 g.

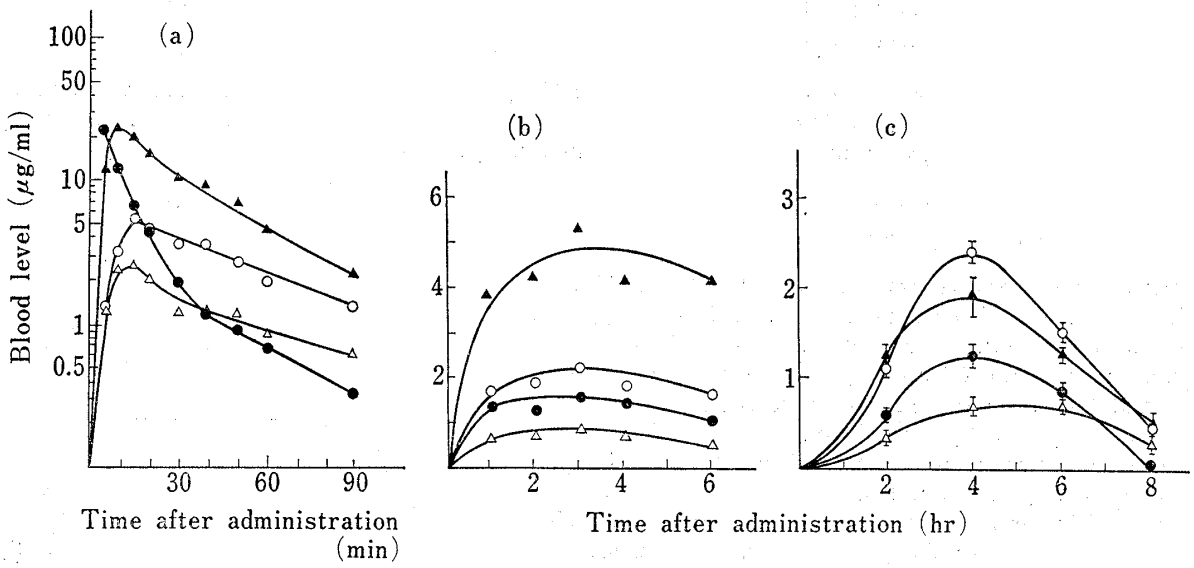


Fig. 2. Blood Levels of PA and Its Metabolites

plotting: (a) mean value (n=3), (b) mean value (n=5), (c) mean value ±S.E. (n=4)
(a) rats, 25 mg/kg (*i.v.*)
(b) rats, 100 mg/kg (*p.o.*)
(c) 1 g for man (*p.o.*)
—●—: PA
—▲—: M-II (2-(2-hydroxypyrrolidino)-PPA),
—○—: M-IV (2-(3-hydroxycarbonylpropylamino)-PPA)
—△—: M-V (2-(3-hydroxypyrrolidino)-PPA)
PPA: 5,8-dihydro-8-ethyl-5-oxo-pyrido[2,3-*d*]pyrimidine-6-carboxylic acid

As is clear from these results, PA was rapidly metabolized to M-II and M-IV in both species. A major metabolite in the blood was M-II in rats treated oral and intravenous PA, while M-IV and M-II in man. Peak levels of unchanged PA and its metabolites in rats and man were attained 2 to 3 hr and about 4 hr, respectively, after oral administration. Blood levels of M-III in both species were too low (0.3 μg/ml) to be determined by the present method.

The previous study revealed that the *in vitro* antibacterial activities were varied among PA and its metabolites.[5] The metabolites retaining the pyrrolidine ring, such as M-V and M-II, exhibit potentiated activities as compared with PA, whereas M-III and M-IV exhibit markedly decreased activities. Thus, combined blood levels of PA, M-II and M-V in either species corresponded to the values obtained by bioassay.

Table I. Biliary Excretion of PA and Its Metabolites in Rats Receiving Intravenous PA at a Dose of 25 mg/kg

Time (hr)	Unconjugates (μg)[a]					Glucuronides (μg)[a]					Total (μg)
	PA	M-II	M-V	M-IV	M-III	PA	M-II	M-V	M-IV	M-III	
0— 1	226.0	1.6	0	326.1	51.7	523.3	49.6	148.1	107.7	56.3	1490.3
1— 2	28.0	19.6	18.0	426.7	49.6	60.3	22.5	97.3	53.7	84.9	860.5
2— 3	6.0	12.0	13.9	241.9	23.4	14.0	5.4	36.3	48.0	32.0	432.9
3— 5	0	14.2	10.1	140.7	21.3	15.0	2.8	17.3	40.8	30.4	292.6
5— 7	0	3.8	9.5	45.6	8.6	6.7	0.7	7.6	20.4	24.3	127.2
7—24	0	4.7	12.0	54.9	20.1	0	0	1.2	69.0	24.3	186.2
Total	260.0	55.9	63.5	1235.9	174.7	619.3	81.0	307.8	340.6	252.2	3389.7
Recovery (%)	4.81	1.04	1.18	22.89	3.24	11.47	1.50	5.70	6.29	4.67	62.79

a) calculated as PA
M-II (2-(2-hydroxypyrrolidino)-PPA), M-V (2-(3-hydroxypyrrolidino)-PPA), M-IV (2-(3-hydroxycarbonylpropylamino)-PPA), M-III (2-amino-PPA)
PPA: 5,8-dihydro-8-ethyl-5-oxo-pyrido[2,3-*d*]pyrimidine-6-carboxylic acid
used animal: rats (Wistar, male, body wt. 210—230 g), n=3

II. Urinary and Biliary Excretion of PA and Its Metabolites in Rats and Humans

Biliary excretions of PA and its metabolites in rats receiving an intravenous 25 mg/kg dose of PA are shown in Table I. An average excretion into 24 hr bile of rats was 63% of the dose, in unconjugated (about 53%) and glucuronic-acid conjugated (about 47%) forms. A major unconjugated metabolite in rat bile was found to be M-IV accounting for about 23% of the dose.

Fig. 3 illustrate urinary excretions of PA and its metabolites in rats receiving intravenous or oral PA at a dose of 25 mg/kg or 100 mg/kg, respectively. Urinary excretions of PA and its metabolites in humans receiving oral PA at a single dose of 1 g are shown in Fig. 4. In rats, the average recovery from 24 hr urine after oral and intravenous administrations was about 27.5% and 45% of the dose, respectively, with minor portions in glucuronides. A

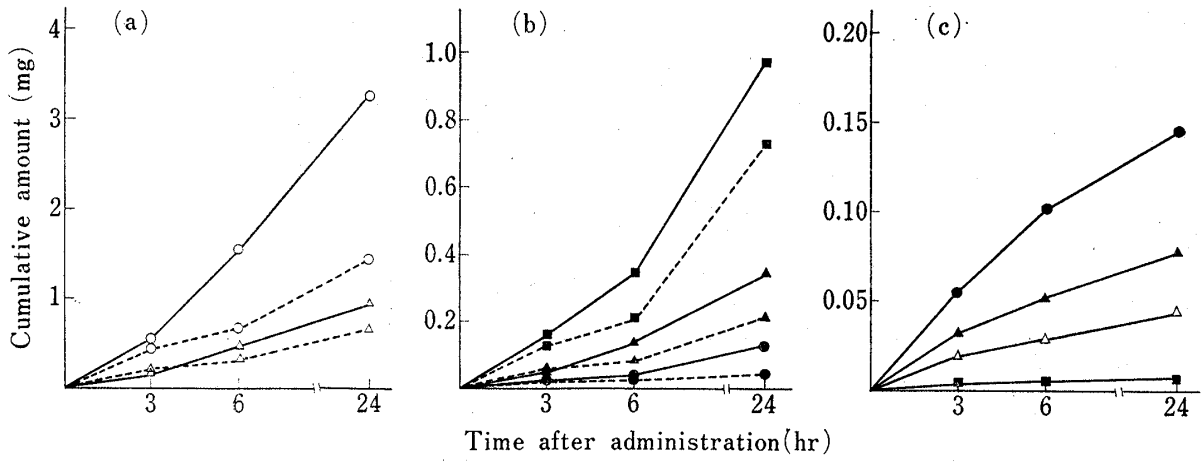


Fig. 3. Urinary Excretion of PA and Its Metabolites in Rats

plotting: ——— 100 mg/kg (*p.o.*), mean value (n=10)
---------- 25 mg/kg (*i.v.*), mean value (n=3)
(a) ○: M-IV, △: M-V
(b) ●: PA, ▲: M-II, ■: M-III
(c) glucuronides of PA, M-II, M-III, and M-V
Amount of each metabolite is calculated as PA.

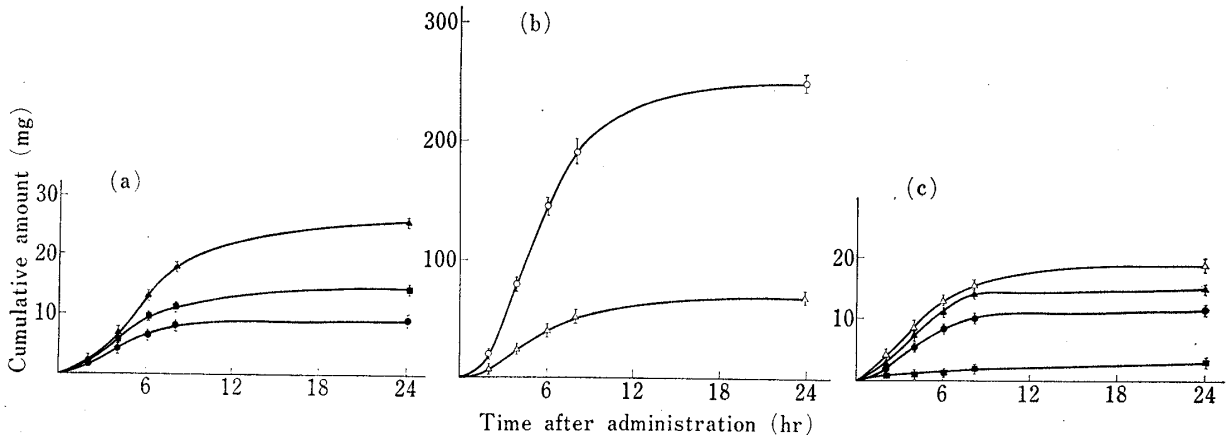


Fig. 4. Cumulative Urinary Excretion Curves of PA and Its Metabolites in Humans receiving Oral PA at a Dose of 1 g

plotting: mean value ±S.E. (n=4)
(a) —●—: PA, —▲—: M-II, —■—: M-III
(b) —○—: M-IV, —△—: M-V
(c) glucuronides of PA, M-II, M-III, and M-V
Amount of each metabolite is calculated as PA.

urinary major metabolite was M-IV in either oral or intravenous treated rats. Following intravenous administration to rats, combined excretion of unchanged PA and its metabolites into 24 hr bile and urine accounted for about 63% and 45% of the dose, respectively, indicating nearly total amounts of the dose excreted into urine and bile within 24 hr. In humans, the average recovery from 24 hr urine was about 42% of the dose after oral administration.

M-III, though trace amounts in blood, was excreted into 24 hr rat urine accounting for about 11% of the dose (correspond to about 24% of total urinary excretion) and about 5% (about 17% of total urinary excretion) after intravenous and oral administrations, respectively. Analogous results were obtained in humans after oral administration. As is clear from the metabolic pathway of PA described in the previous paper,[1] this evidence indicates the elimination rate of M-III might be faster than the biotransformation rate of M-II to M-III *via* M-VI.[1,5] The elimination rate constant of M-III measured in rats receiving intravenous M-III was found to be nearly equal to those of PA, M-II and M-V,[7] suggesting that M-II might be metabolized to M-III with a relatively slow rate.

In both species, the major urinary active-metabolite was found to be M-V and the combined amounts of PA, M-V and M-II in the urine was found to be nearly equal to the amount obtained by bioassay. Total urinary levels of active materials in rats were found to be about hundred fold higher than a minimal inhibitory concentration level against *Escherichia coli* at 3 to 6 hr after oral administration. The similar results were also obtained in humans.

Acknowledgement The authors express their gratitude to Dr. S. Minami for his kind supply of the authentic samples. Thanks are due to Dr. H. Takamatsu, Director of this Division, for his encouragement throughout this work.

7) Y. Sekine, M. Miyamoto, M. Hashimoto, and K. Nakamura, *Chem. Pharm. Bull.* (Tokyo), **24**, 1462 (1976).