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Determination of Pyrithioxin and Its Metabolites in Blood and Urine of Dogs by High-Performance Liquid Chromatography[1]

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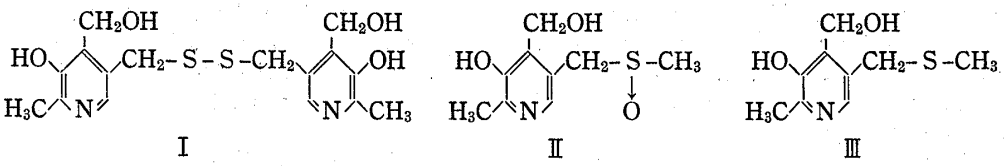
Analysis of pyrithioxin (I) and its metabolites in blood and urine by thin-layer chromatography and high-performance liquid chromatography was described following single oral doses of pyrithioxin dihydrochloride (I·2HCl) to beagle dogs. Pyrithioxin glucuronide (I-G), 5′-desoxy-5′-methylsulfinylpyridoxol (II) and its glucuronide (II-G) were found as metabolites in the urine. 5′-Desoxy-5′-methylthiopyridoxol (III), which was reported in the literature as one of main metabolites of I in the urine, was found to be a reduced product of II during the storage of excreted urine at room temperature. An analytical method of these metabolites in blood and urine was established employing a high-performance liquid chromatography with a cation-exchange resin column.

Keywords——pyrithioxin; metabolism; determination; high-performance liquid chromatography; thin-layer chromatography; dog

Pyrithioxin (I), 3,3′-(dithiodimethylene)bis[5-hydroxy-6-methyl-4-pyridinemethanol], is a derivative of vitamin B_6 having neither vitamin B_6 nor anti-vitamin B_6 activity. A variety of psychotropic actions has been reported for I.[3]



I II III

Chart 1

Nowak and Schorre[4] studied the metabolites of ^{35}S-labelled I in the urine employing thin-layer chromatography(TLC) and radioactivity assay following single oral doses to rats, rabbits, guinea pigs, and dogs. Darge, Liss, and Oeff studied pharmacokinetics and metabolism of ^{35}S-labelled I in rats[5] and man.[6] They reported[4,5] that 5′-desoxy-5′-methylsulfinylpyridoxol (II) and 5′-desoxy-5′-methylthiopyridoxol (III) were excreted in the urine of rats, rabbits, and guinea pigs, but I was not detected. In a preliminary study with dogs,[4] only the metabolite III was detected in the urine. But, in a subsequent experiment with a large number of dogs, they concluded that I is metabolized completely and is excreted in the urine in the form of metabolites II and III. In the urine of man, they found four metabolites

1) Biopharmaceutics of Pyrithioxin. I. A part of this work was presented at the 25th Meeting of Kinki Branch, Pharmaceutical Society of Japan, Kobe, November 1975.
2) Location: *Yamadakami, Suita, Osaka.*
3) R. Hotovy, H.J. Enenkel, J. Gillissen, U. Jahn, H.G. Kraft, H. Müller-Calgan, P. Mürmann, S. Sommer, and R. Struller, *Arzneim.-Forsch.*, **14**, 26 (1964); G. Quadbeck, H.R. Landmann, W. Sachsse, and I. Schmidt, *Med. Exp.*, **7**, 144 (1962); J. Teijeira and M. Martinez-Lage, *Medicina del Estrdio General de Navarra*, **4**, 332 (1960); G. Sierra and F. Reinoso-Suárez, *Med. Exp.*, **9**, 84 (1963); S. Sommer, *Naunyn-Schiedebergs Arch. Exp. Path. pharma.*, **245**, 119 (1963); E.W. Fünfgeld, *Med. Klin.*, **58**, 1197 (1963); D. Adam and H. Hamelmann, *Munch. Med. Wschr.*, **106**, 1045 (1964); E. Sugaya, A. Morifuji, and C. Izumi, *Keio Journal of Medicine*, **16**, 83 (1967).
4) H. Nowak and G. Schorre, *Arzneim.-Forsch.*, **19**, 11 (1969).
5) W. Darge, E. Liss, and K. Oeff, *Arzneim.-Forsch.*, **19**, 5 (1969).
6) W. Darge, E. Liss, and K. Oeff, *Arzneim.-Forsch.*, **19**, 9 (1969).

including two unknown metabolites having a strong hydrophilic property which was proven by an extraction experiment with *n*-butanol.

The assay of I has been conducted by ultraviolet spectroscopy,[7] colorimetric assay with indophenol,[8] and gas chromatography.[9] But, little was reported for the analysis of I and its metabolites in biological fluids except for a radioautographic identification with TLC.[5]

The present report describes that three metabolites, I, II-glucuronide (II-G), and I-glucuronide (I–G), are found in the urine of dogs and the metabolite II is reduced to III during the storage of excreted urine at room temperature. I and its metabolites in blood and urine following single doses of I to dogs are assayed employing a high-performance liquid chromatography(HPLC) with a cation-exchange resin column.

Experimental

Materials——I (mp 218—220°) was obtained through the courtesy of the Chugai Pharmaceutical Co., Ltd. I dihydrochloride (I·2HCl) (mp 184°) was obtained following known procedures in which ethanol solution of HCl was added to ethanol suspension of I with precipitation of I·2HCl with ethyl ether. The precipitate was used after recrystallization from hot ethanol. II·HCl (mp 179°) and III·HCl (mp 154—155°) were synthesized following Nowak and Schorre.[4] β-Glucuronidase (Bacterial, Type I) and arylsulfatase (Limpets) were purchased from Sigma Chemical Company. All other chemicals were analytical reagent grade.

TLC——TLC was performed according to the method of Nowak and Schorre[4] employing Kiesel Gel HF (Merck Co.) plates, 20×20 cm, 0.25 mm. The solvent system used was acetone–28% ammonia (17: 3, v/v). One tenth percent methanol solution of 2,6-dibromoquinone-4-chlorimide was used for the development of spots.

HPLC——A Varian HPLC Model 4000 equipped with a 280 nm ultraviolet (UV) detector and a recorder was used. A stainless steel column, 1.8×500 mm, packed with a cation-exchange resin, Zipax SCX, was attached to the apparatus. The column temperature was ambient. The electrometer range was 0.04 mV full scale and a recorder chart speed 2.5 mm/min. Analyses were performed using Mobile Phase A for I and Mobile Phase B for II and III. Mobile Phase A was 0.05 M pH 5.65 citrate–phosphate buffer with 0.3 M sodium perchlorate and 2% isopropanol and Mobile Phase B was 0.05 M pH 4.00 citrate–phosphate buffer with 0.2 M sodium perchlorate.

The mobile phases were degassed by applying vacuum (about 20 mmHg) to the solvent reservoir for approximately 3 min before use. 2-Aminopyridine (AP) and *m*-chloroaniline hydrochloride (MCA) were used as internal standard (IS) for Mobile Phases A and B, respectively.

Preparation of Standard Solutions——Each standard solution of I·2HCl, II·HCl, and III·HCl was prepared by dissolving the compound, accurately weighed equivalent to 50 mg of the respective base, in a 50-ml volumetric flask being brought to volume with methanol. The equivalent amounts of I·2HCl, II·HCl, and III·HCl were 59.9, 58.5, and 59.1 mg, respectively.

Calibration Curves——To produce final solutions containing a desired amount of I·2HCl, aliquots of the I·2HCl standard solution were taken into each 10-ml glass-stoppered test tube. After being evaporated to dryness under reduced pressure, the residue in each tube was dissolved with 1 ml of 0.02% methanol solution of AP. II·HCl and III·HCl solutions with various concentrations were likewise prepared by dissolving with 5 ml of 0.1% methanol solution of MCA. Five microliters of the resulted solutions was injected into the column with a 10-μl syringe.

Peak height ratios were calculated by dividing the peak height of the compound with the peak height of IS.

Recovery of II from Blood——An aliquot (2—64 μl) of the standard solution of II·HCl was taken into a 20-ml screw-capped centrifuge tube. After evaporating methanol, five ml of control blood was taken into the tube. The blood solution was mixed with 9 ml of 0.6 N aqueous solution of perchloric acid and kept in a refrigerator for 1 hr. The tube was then centrifuged for 10 min at 10000 rpm and 0°, and 10 ml of the upper aqueous layer was taken into a 25-ml glass-stoppered centrifuge tube. After adjusting pH at about 6.5 with 2 N KOH. One ml of 1/3 M phosphate buffer (pH 6.5) was added to the tube. The aqueous solution was extracted with three portions of 10 ml of isobutanol shaking for 10 min at 240 strokes per min with a KM Shaker (Iwaki Co., Ltd.) and centrifuging for 10 min at 2500 rpm for each extraction. The organic layer at each extraction was taken into a 20-ml glass-stoppered test tube and evaporated under reduced pressure with

7) T. Kuroda, *Bitamin*, **30**, 431 (1964).
8) J.V. Scudi, *J. Biol. Chem.*, **139**, 707 (1941).
9) M. Shimoda, Y. Sumi, and T. Kuroda, *Bitamin*, **46**, 307 (1972).

a Vapor-Mix (Tokyo Rikakikai Co., Ltd.). Two tenths ml of 0.1% methanol solution of MCA was added into the tube. Five μl of the sample solution (equivalent to 0.089 ml of blood) was injected with a 10-μl syringe.

For the sample of TLC, the evaporated residue of isobutanol extracts was redissolved with 0.1 ml of methanol, and 10 μl (equivalent to 0.356 ml of blood) was spotted on a TLC plate.

Recovery of I from Urine——Five ml of control urine was taken into a 10 ml volumetric flask. After adjusting the pH at about 6.5 with 0.2 N HCl or NaOH, the solution was mixed with 1 ml of 1/3 M phosphate buffer (pH 6.5) and brought to volume with water. An aliquot (0.0125 or 0.1 ml) of the standard solution of I·2HCl was taken into a 25-ml glass-stoppered centrifuge tube and evaporated to dryness under reduced pressure. Two ml of the pH adjusted urine was taken into the tube to dissolve the residue of I·2HCl. The solution was extracted with two portions of 10 ml of ethyl acetate being shaken for 10 min with a KM Shaker and centrifuged for 10 min at 2500 rpm for each extraction. The organic layer was taken into a 20-ml glass-stoppered test tube and was evaporated to dryness with a Vapor-Mix at each extraction. The residue was dissolved with 1 ml of the AP-methanol solution (0.02%). Five μl of the solution (equivalent to 5 μl of urine) was injected into the HPLC column.

Recovery of II and III from Urine——Aliquots of the standard solution of II·HCl and III·HCl was taken into a 25 ml glass-stoppered centrifuge tube and evaporated to dryness under reduced pressure. The residue was dissolved with 1 ml of control urine. The urine solution was extracted with two portions of 10 ml of isobutanol being shaken for 10 min with a KM Shaker and centrifuged for 10 min 2500 rpm for each extraction. The organic layer was taken into a 20-ml glass-stoppered test tube and evaporated with a Vapor-Mix at each extraction and the residue was dissolved with 5 ml of the MCA-methanol solution. Five μl of the solution (equivalent to 1 μl of urine) was injected into the HPLC column.

For the sample of TLC, the evaporated residue of isobutanol extracts was redissolved with 0.5 ml of methanol, and 5 μl of the solution (equivalent to 10 μl urine) was spotted on a TLC plate.

Preparation of Blood and Urine Samples——Adult beagle dogs weighing 8—12 kg (mean 10.3 kg) were kept fast for about 12 hr prior to the experiment with water given *ad libitum*. I·2HCl, filled in hard gelatin capsules (Matsuya Co., Ltd. No. 0), was administered orally at a load of 59.9 mg/kg (equivalent to 50 mg of I/kg). Thirty ml of water was given to force dogs to swallow capsules. Control urine was collected just prior to the drug administration. The animals were kept in metabolic cages during the experiments. Urine was collected in a glass reservoir which was kept at 2° with ice during the experiments. Urine samples were stored in a refrigerator until analyzed. Blood samples of 5 ml were drawn from the jugular vein with a 5-ml disposable syringe, proviously flushed with 10% ethylenediaminetetraacetic acid (EDTA) solution, using a 22-gauge needle. Immediately following the withdrawal of blood, the samples were taken into 20-ml screw-capped centrifuge tubes and stored in a refrigerator until analyzed. No samples were stored longer than 30 hr.

The urine and blood samples were handled following the methods described previously and IS-methanol solution were prepared for an HPLC assay.

Hydrolysis of I- and II-Conjugates in Urine——Acid Hydrolysis: Urine was collected during the experimental period of 24 hr after an oral administration of I·2HCl to a dog. The urine was filtered with filter paper (Toyo Roshi Co., Ltd. No. 131). Five ml of the urine and 5 ml of 1 N HCl were taken into a 25 ml volumetric flask. The solution was heated for 1 hr at 90°. After cooling the solution to ambient temperature, the solution was adjusted in pH at about 6.5 with 1 N NaOH and mixed with 1 ml of 1/3 M phosphate buffer. Then, the solution was made to volume with water. The analyses of I and II in the urine were performed following the procedures with HPLC and TLC for urine samples described previously. The amounts of I- and II-conjugates were obtained from differences of concentrations of I and II between before and after the hydrolysis, respectively.

Hydrolysis with Enzymes: Two ml of the urine sample which was collected in 24 hr following an oral administration of I·2HCl to a dog and heated in boiling water for 3 min, was taken into a 25-ml glass-stoppered test tube. The urine was extracted with three portions of 10 ml of isobutanol. The organic layer was evaporated under reduced pressure with a Vapor-Mix at each extraction, and the residue was dissolved with 4 ml of water. The solution was mixed with 5 ml of buffer solution and 1 ml of β-glucuronidase or arylsulfatase solution. For β-glucuronidase hydrolysis, 1/3 M phosphate buffer (pH 6.5) and aqueous enzyme solution of 1000 units/ml were used. For arylsulfatase hydrolysis, 1/5 M acetate buffer (pH 5.0) and aqueous enzyme solution of 25 units/ml were used.

Change of II to III in Urine——II·HCl (58.5 mg; equivalent to 50 mg of II) was taken into a 100-ml volumetric flask and brought to volume with urine. Approximately 50 ml of the solution was taken into a 100-ml volumetric flask. Two flasks were kept at ambient temperature (about 20°) and at 2° in a refrigerator. Fifty ml of II·HCl solution with the same concentration was prepared and taken into a 100-ml volumetric flask employing sterilized urine which was treated at 100° for 1 hr in a sterilizer. This solution was kept at ambient temperature. Aliquots of the three solutions were chronologically taken and assayed as to the concentrations of II and III with HPLC.

Calculation of Mole Percent Excretion——The excreted amounts of I–G, II, II–G, and their total in the urine were calculated as follows:

$$\text{mole percent of total excretion in urine} = \frac{\text{moles of I-G} + 1/2(\text{moles of II} + \text{moles of II-G})}{\text{moles of I administered}} \times 100$$

$$\text{mole percent excretion of II or II-G in urine} = \frac{1/2(\text{moles of II or II-G})}{\text{moles of I administered}} \times 100$$

Results and Discussion

Figure 1 illustrates a typical example of TLC of I, II, and III as well as blood and urine samples following an oral administration of I·2HCl filled in hard gelatin capsules at a dose level of 59.9 mg/kg to a dog. The blood sample was obtained 1.5 hr after administration and retained in a refrigerator until TLC analysis. The urine sample was collected 24 hr after the drug administration. During the collection, urine was stored in a reservoir which was attached to a urine drainpipe of the metabolic cage and kept at 2° with ice. The urine sample was used for TLC study immediately after the experiment. The blood sample showed only one spot having the same *Rf* value of 0.3 and blue color with those of II·HCl. It was to be noted that only one spot of II·HCl was found in the urine sample, but, after acid hydrolysis, a new spot appeared having the same *Rf* value of 0.38 and violet color with those of I·2HCl. Thus, it may be considered that I was rapidly metabolized during and/or after the absorption and excreted as II and I-conjugate or conjugates in the urine. Acid hydrolysis of the blood sample failed to demonstrate the existence of I-conjugate. Thus, the existence of I and its conjugate in the blood sample was not confirmed in the present study possibly of low concentrations of I and its conjugate.

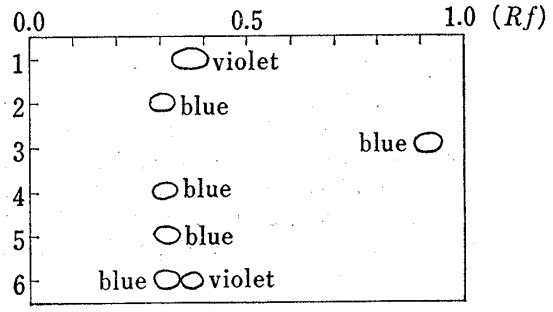


Fig. 1. TLC of I and Its Metabolites in Blood and Urine Samples after Single Oral Administration of I·2HCl

plate: Kiesel Gel HF 20×20 cm, 0.25 mm; solvent system: aceton–28% ammonia (17: 3, v/v); detection: 0.1% 2,6-dibromoquinone-4-chlorimide in MeOH
1: I·2HCl; 2: II·HCl; 3: III·HCl; 4: blood sample; 5: urine sample; 6: urine sample after acid hydrolysis

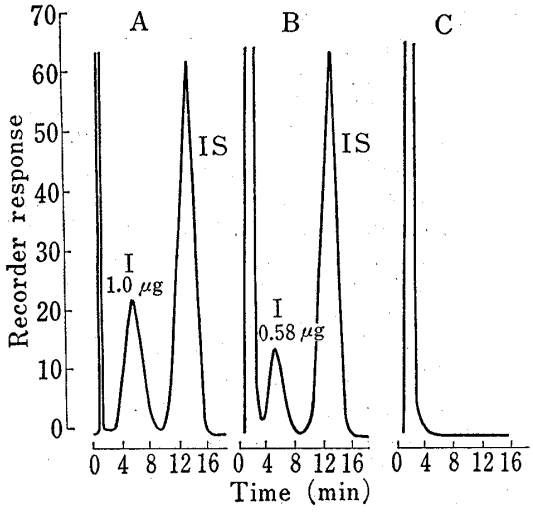


Fig. 2. HPLC of I on a Strong Cation-Exchange Column

Mobile phase was 0.05 M citrate–phosphate buff. (pH 5.65) with 0.3 M sodium perchlorate and 2% isopropanol.
A: standard sample of I with internal standard IS: 2-aminopyridine; B: acid hydrolyzed urine sample after oral administration of I·2HCl; C: acid-hydrolyzed control urine sample

Typical chromatograms of HPLC obtained from the analyses of a standard sample containing 200 μg/ml of each I and AP (A), acid-hydrolyzed urine sample, which was used for the TLC study, with 200 μg/ml of AP (B), and acid-hydrolyzed control urine collected before the drug administration (C) are shown in Fig. 2. The existence of I was not

demonstrated in the urine before acid hydrolysis. This result is consistent with that of TLC.

A standard sample containing 120 μg/ml of each II and III and 1 mg/ml of MCA was well separated on the described method of HPLC (Fig. 3, A). A blood sample collected at 1.5 hr postadministration of I·2HCl was found to give only one peak corresponding to that of II with the amount of 0.34 μg (equivalent to 2.72 μg/ml of blood). The resolution of II with blood constituents was complete without interference with the calculation of concentration of II (Fig. 3, B). The presence of III was not observed in the blood sample.

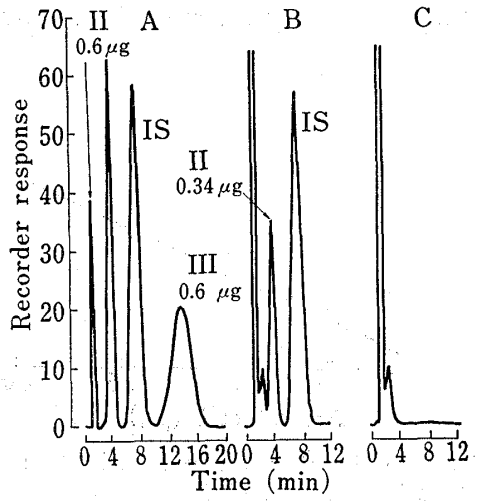


Fig. 3. HPLC of II and III on a Strong Cation-Exchange Column

Mobile phase was 0.05 M citrate–phosphate buff. (pH 4.00) with 0.2 M sodium perchlorate.
A: standard sample of II and III with internal standard IS: *m*-chloroaniline hydrochloride; B: blood sample after oral administration of I·2HCl; C: control blood sample

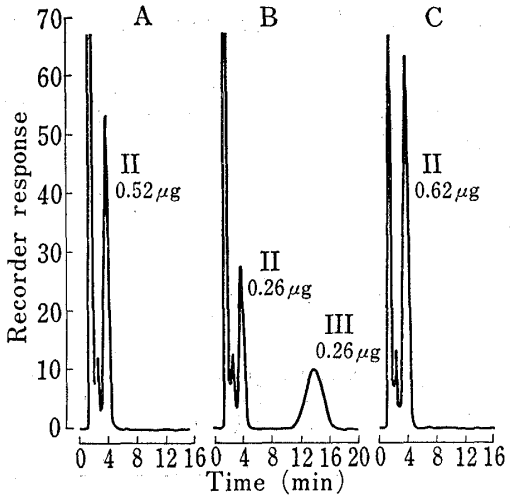


Fig. 4. HPLC of Urine Samples after Oral Administration of I·2HCl on a Strong Cation-Exchange Column

Mobile phase was 0.05 M citrate–phosphate buff. (pH 4.00) with 0.2 M sodium perchlorate.
A: urine sample kept at 2°; B: urine sample kept at ambient temp.; C: after hydrolysis of urine sample kept at 2°

Similar results were obtained with the urine samples kept at 2° during the experiment (Fig. 4, A). But, a urine sample kept at ambient temperature was found to give two peaks, II and III (Fig. 4, B).

It was found that the peak height of II of the urine sample increased significantly after acid hydrolysis, revealing a possible existence of II-conjugate in the urine (Fig. 4, C). The acid hydrolysis of blood samples did not influence the peak height of II owing to a possible low concentration and/or rapid excretion of II-conjugate.

To confirm the type of conjugate in the urine, the urine sample was hydolyzed with β-glucuronidase and arylsulfatase. It was confirmed that a new spot in TLC and a new peak in HPLC corresponding to I appeared in the urine treated with β-glucuronidase, but not with arylsulfatase. The increase in the peak height of I following the treatment of the urine sample with β-glucuronidase was found to be the same as that of acid hydrolysis. The peak height of II also increased with the treatment of β-glucuronidase but not with the treatment of arylsulfatase.

Thus, it may be concluded that the conjugates of I and II in the urine after oral administration of I·2HCl are O-glucuronides of I and II. These two glucuronides are considered to be the two highly hydrophilic metabolites in the human urine found by Darge, *et al.*[5]

Nowak and Schorre[4] reported that II and III are the main metabolites found in the dog urine. But, in the present study, it may be considered that a possible transformation of II to III occurred in the urine after the excretion and during the storage at room temperature. To confirm this consideration, urine solutions of II were kept at various conditions and the

concentrations of II and III were measured (Fig. 5). It was found that II·HCl dissolved in urine decreased in its concentration at room temperature and remained only 16.7% of the initial concentration after a 24-hr experiment, while the concentration of III increased with a decrease of the concentration of II. This reaction was inhibited at 2°. A urine solution of II·HCl prepared with heat-sterilized urine also failed to produce III at room temperature during the 24-hr period. Thus, it may be concluded that the presence of III in the urine is responsible for the contamination of the excreted urine by microorganisms.

Calibration curves of I, II, and III were obtained by plotting the peak-height ratio of compound-IS *versus* the amount of the compound injected into the HPLC column (Fig. 6 and 7). These plots were straight lines having small values of y-intercept in the amount

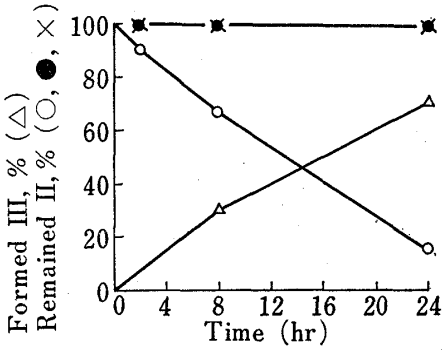


Fig. 5. Transformation of II to III in Urine

key: ○, △, urine solution of II stored at room temp.; ●, urine solution of II stored at 2°; and ×, heat-treated urine solution of II stored at room temp

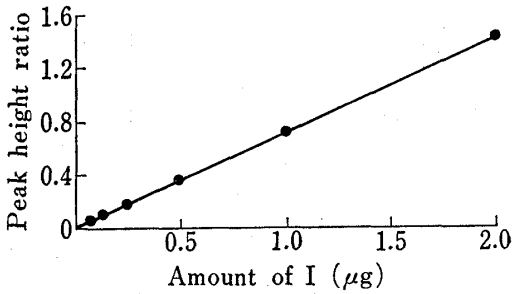


Fig. 6. Calibration Curve for Standard Solution of I by HPLC

slope=0.725, SD=0.035, and y-intercept=−0.011

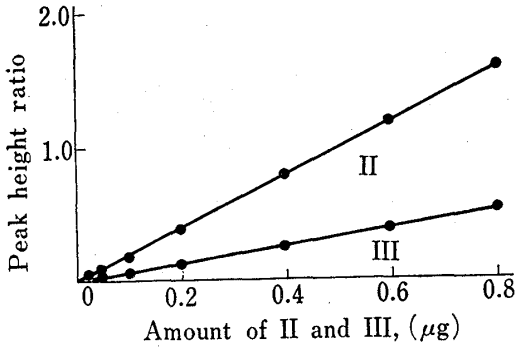


Fig. 7. Calibration Curves for Standard Solution of II and III by HPLC

II: slope=1.961, SD=0.051, and y-intercept=−0.003
III: slope=0.588, SD=0.028, and y-intercept=0.000

Table I. Minimum Detectable Concentration of I, II, and III in Biological Fluids

I	II		III
μg/ml Urine	μg/ml Urine	μg/ml Blood	μg/ml Urine
12.5	20.0	0.8	20.0

ranges of 0.05—2.0 μg for I, 0.025—0.8 μg for II, and 0.05—0.8 μg for III. Mean values of the slope were 0.725, 1.961, and 0.588 for I, II, and III, respectively. The minimum detectable quantities were 50, 25, and 50 ng per injection of I, II, and III, respectively. The minimum detectable concentrations of the compounds in blood and urine samples are presented in Table I. The recovery values of I, and II and III from the urine samples are presented in Table II and III, respectively. The recovery values of II from the blood samples are presented in Table IV.

Mean blood levels of II were measured for 24 hr following single oral administrations of 59.9 mg/kg of I·2HCl in hard gelatin capsules to six dogs (Fig. 8). Mean cumulative

Table II. Recovery of I from Urine

Amount of I (mg) added to 2 ml urine	Recovery, %
0.0125	92.8±6.4
0.1	92.7±3.8

Values reported are mean±SD for 5 experiments.

Table III. Recoveries of II and III form Urine

Amount of II or III (mg) add to 1ml urine	Recovery, %	
	II	III
0.025	93.2±12.9	90.8±20.0
0.05	92.2± 1.5	80.6± 6.9
0.8	93.9± 1.0	78.5± 2.5

Values reported are mean±SD for 5 experiments.

Table IV. Recovery of II from Blood

Amount of II (μg) added to 5 ml blood	Recovery, %
2	50.8±9.5
4	46.5±4.0
32	44.9±2.2
64	44.7±1.3

Values reported are mean±SD for 5 experiments.

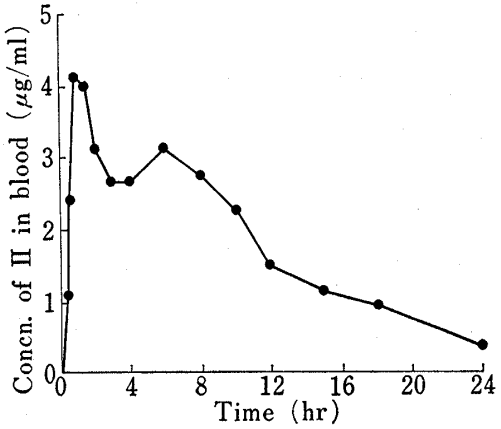


Fig. 8. Mean Blood Concentration of II after Single Oral Administration of I·2HCl to Six Dogs (Dose: 59.9 mg/kg)

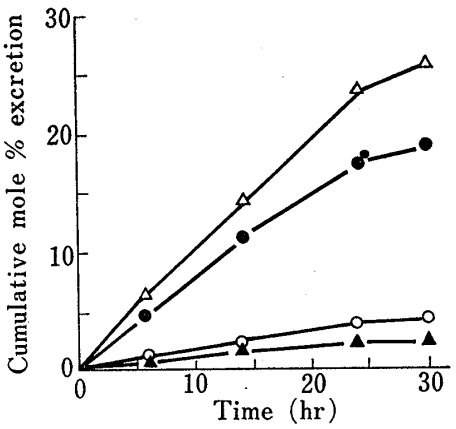


Fig. 9. Mean Cumulative Mole Percent Excretion of I–G, II, II–G, and Total in Urine after Oral Administration of I·2HCl to Four dogs (Dose: 59.9 mg/kg)

key: ▲, I–G; ●, II; ○, II–G; and △, total

values of I–G, II, II–G, and a total excretion of metabolites in the urine collected for 24 hr are presented in terms of mole percent of dose (Fig. 9).

In conclusion, the HPLC procedure described presently provides a favorable method for the determination of pyrithioxin and its metabolites in blood and urine. Thus, the present method can be employed for the study of pharmacokinetics of pyrithioxin on the basis of modification of dosage forms and development of pro-drugs as well as the analysis of pharmaceutical preparations.

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