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Determination of Perisoxal and Its Phenolic Metabolites in Rat, Rabbit and Human Urine

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After a single administration of perisoxal citrate to rats (21 mg/kg, *i.v.*), rabbits (15 mg/kg, *i.v.*) and humans (200 mg/man, *p.o.*), urinary excretion of the unchanged drug and its phenolic metabolites and the glucuronide forms of all these compounds was determined by a newly developed specific and simultaneous GC method. Generally, in all the species examined, excretion of the free forms of perisoxal and hydroxyperisoxals (*m*- and *p*-) was low or even undetectable, but that of the glucuronide forms was high. Neither the free nor the glucuronide form of *o*-hydroxyperisoxal was detected. The proportion of total *m*-hydroxy metabolites to total *p*-hydroxy metabolites varied in different species, being about 3 in rats, about 0.4 in rabbits and less than 0.1 in humans.

Keywords——3-(1-hydroxy-2-piperidinoethyl)-5-phenylisoxazole; perisoxal; basic drug; phenolic metabolite; urinary excretion; gas chromatography; species difference

Perisoxal [3-(1-hydroxy-2-piperidinoethyl)-5-phenylisoxazole], an isoxazole analog,[2a,b] possesses analgesic, anti-inflammatory and antitussive activities.[2a,3] In the previous paper,[4] the fate of perisoxal in rats was investigated utilizing isotope-labeled perisoxal, and three kinds of hydroxylated metabolites, 3-(1-hydroxy-2-piperidinoethyl)-5-(4-hydroxyphenyl)-isoxazole (*p*-hydroxyperisoxal), 3-(1-hydroxy-2-piperidinoethyl)-5-(3-hydroxyphenyl)isoxazole (*m*-hydroxyperisoxal) and 3-[1-hydroxy-2-(4-hydroxypiperidino)ethyl]-5-phenylisoxazole (4-hydroxyperisoxal), were isolated after subcutaneous administration of unlabeled perisoxal. These metabolites have also been confirmed to be formed in an *in vitro* study with rabbit liver homogenate.[5] The quantitative determination of metabolites in rat urine[4] and rabbit liver homogenate[5] revealed that aromatic hydroxylation was one of the main metabolic pathways of perisoxal. Therefore, in the present paper, individual determination of these metabolites was done to clarify in detail the metabolism of perisoxal in rats, rabbits and humans.

Experimental

Materials——Perisoxal citrate was synthesized by Kano and Adachi.[2b] Perisoxal, *o*-hydroxyperisoxal, *m*-hydroxyperisoxal and *p*-hydroxyperisoxal were synthesized by Hashimoto *et al.*[5] Dehydroepiandrosterone acetate, an internal standard, was recrystallized from methanol.

Gas Chromatography (GC)——A Shimadzu 4APTF gas chromatograph equipped with a flame-ionization detector was employed. A 1.5 m × 0.4 cm i.d. glass column packed with 1% OV-17 on 80—100 mesh Gas Chrom Q was used. The temperatures of the column, the injection port and the detector were 225°, 240°

1) Location: *a)* *5-12-4 Sagisu, Fukushima-ku, Osaka*; *b)* *3-1 Tanabe-dori, Mizuho-ku, Nagoya.*

2) *a)* H. Kano, I. Adachi, R. Kido, and K. Hirose, *J. Med. Chem.*, **10**, 411 (1967); *b)* H. Kano and I. Adachi, *Shionogi Kenkyusho Nempo*, **18**, 56 (1968).
3) K. Hirose, Y. Kojima, M. Eigyo, and H. Joyama, *Yakuri To Chiryo*, **2**, 1028 (1974); K. Hirose, Y. Kojima, M. Eigyo, and H. Sato, *Oyo Yakuri*, **6**, 1285 (1972).
4) J. Watanabe, H. Okabe, K. Mizojiri, Y. Nakajima, K. Sugeno, and R. Yamamoto, *Chem. Pharm. Bull.*, **27**, 1075 (1979).
5) S. Hashimoto, M. Shizu, and S. Takahashi, *Chem. Pharm. Bull.*, **24**, 1757 (1976).

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and 250°, respectively. The carrier gas (N_2) flow rate was 50 ml/min. Under these conditions, the relative retention times of perisoxal, *o*-hydroxyperisoxal, *m*-hydroxyperisoxal and *p*-hydroxyperisoxal with respect to the internal standard were 0.24, 0.45, 0.52 and 0.70, respectively (Fig. 1).

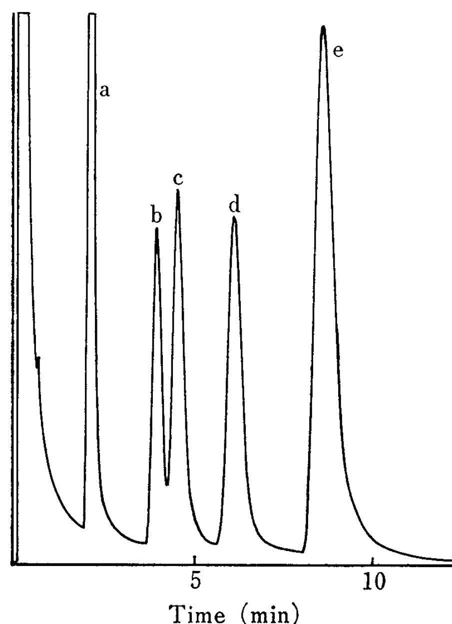

Fig. 1. Gas Chromatogram of Reference Compounds; Perisoxal (a), *o*-Hydroxyperisoxal (b), *m*-Hydroxyperisoxal (c), *p*-Hydroxyperisoxal (d) and Dehydroepiandrosterone Acetate (IS) (e)

Assay Procedure——To determine the free base, 5 ml of urine sample was pipetted into a 50 ml centrifuge tube. Three milliliters of borate buffer (pH 8.9, 2/5 M) was added and the mixture was extracted twice with 15 ml of ethylene dichloride (EDC). The extracts were combined and evaporated to dryness at 45°. The residue was dissolved in 150 μl of ethanol and 100 μl of this solution was applied to a precoated TLC plate (Kiesel gel $F60_{254}$, Merck) which was developed with acetone–28% NH_4OH (20: 0.1). Since the *Rf* values of perisoxal, *o*-, *m*- and *p*-hydroxyperisoxal were very similar (0.61, 0.46, 0.49 and 0.49, respectively), the spots of these compounds were scraped off together from the plate then extracted with 6 ml of ethanol. 4-Hydroxyperisoxal (*Rf*, 0.35), if present, is removed by this step. Five milliliters of the ethanol extract was evaporated to dryness at 45°. The residue was dissolved in 60 μl of EDC, and 20 μl of internal standard solution (5 mg/ml in EDC) and 20 μl of N,O-bis(trimethylsilyl)acetamide (Applied Science) were added. The mixture was maintained at 45° for 75 min, then 2 μl of this solution was injected into a gas chromatograph. To determine the glucuronide, 5 ml of urine sample and 2 ml of β-glucuronidase solution (5000 units/ml, Type I, Sigma) were added to 3 ml of phosphate buffer (pH 6.8, 0.3 M), then the mixture was incubated at 37° for 16 hr. β-Glucuronidase hydrolysis was almost complete in this process (Fig. 2). Thereafter, the hydrolyzed mixture was subjected to the assay procedure described above.

Calibration Curve——Known amounts of perisoxal and *o*-, *m*- and *p*-hydroxyperisoxal were added to each control animal and human urine sample and assayed by the method described. The plots of the peak height ratio *versus* the amount ratio of each compound relative to the internal standard were linear in all species. The calibration curve for rabbit urine is shown in Fig. 3 as an example. Linearity was maintained

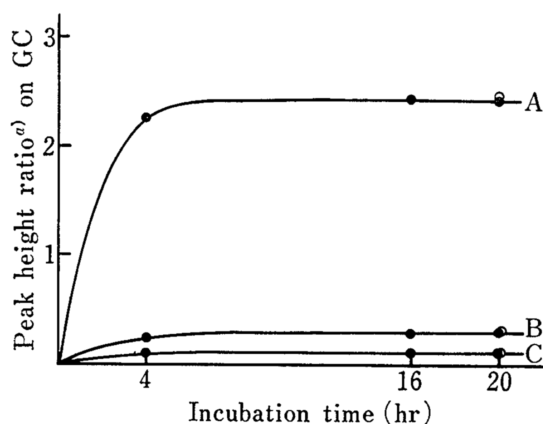

Fig. 2. Increase of Perisoxal (A), *p*-Hydroxyperisoxal (B) and *m*-Hydroxyperisoxal (C) in Rabbit Urine on β-Glucuronidase Treatment

Five milliliters of rabbit urine, taken from an animal given 21 mg/kg perisoxal citrate *i.v.*, was incubated at 37° with 10000 units of β-glucuronidase (●). Supplemental addition of 10000 units of β-glucuronidase was carried out in some experiments at 16 hr (○)
a) Peak height ratio of each compound to that of IS.

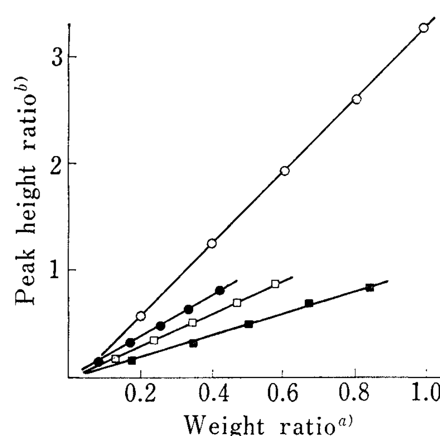

Fig. 3. Typical Calibration Curves for the Determination of Perisoxal (○), *o*-Hydroxyperisoxal (●), *m*-Hydroxyperisoxal (□) and *p*-Hydroxyperisoxal (■) in Rabbit Urine

a) Weight ratio of each compound to that of IS.
b) Peak height ratio of each compound to that of IS.

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Table I. Recovery of Perisoxal, *o*-, *m*- and *p*-Hydroxyperisoxal from Rabbit Urine

Compound	Added (μg/ml)	Found (μg/ml)[a] from non-treated urine	Found (μg/ml)[a] from β-glucuronidase-treated urine[b]
Run 1			
Perisoxal	33.52	34.03±1.95	33.61±1.08
o-Hydroxyperisoxal	13.68	13.69±0.38	13.61±0.09
m-Hydroxyperisoxal	20.43	20.36±0.57	20.55±0.16
p-Hydroxyperisoxal	29.55	28.54±0.84	29.17±0.16
Run 2			
Perisoxal	6.70	7.36±0.38	7.32±0.14
o-Hydroxyperisoxal	2.74	2.85±0.04	2.89±0.12
m-Hydroxyperisoxal	4.09	4.23±0.01	4.16±0.26
p-Hydroxyperisoxal	5.91	6.29±0.11	6.81±0.24

a) Each value represents the mean of 3 determination±S.D.
b) After adding standard compounds, control urine was incubated at 37° for 16 hr with 10000 units of β-glucuronidase (Type I, Sigma).

over concentration ranges of 2.5—30 μg/ml for perisoxal, 0.5—12 μg/ml for *o*-hydroxyperisoxal, 0.75—16 μg/ml for *m*-hydroxyperisoxal and 1.0—24 μg/ml for *p*-hydroxyperisoxal.

Effect of β-Glucuronidase on Recovery——Known amounts of perisoxal and hydroxyperisoxal were added to 5 ml of control rabbit urine samples. Half of these samples were treated with β-glucuronidase according to the described procedure. The recovery of each compound from β-glucuronidase-treated or non-treated control urine samples was determined (Table I). No effect of β-glucuronidase on recovery was observed.

Animal Experiments——Male Wistar rats weighing 220—240 g and male rabbits weighing 2.4—3.2 kg were used. Perisoxal citrate was administered intravenously at a dose of 21 mg/kg to rats and 15 mg/kg to rabbits in 0.9% NaCl solution. The urine was collected for 8 and 24 hr and frozen at −20° until assayed.

Human Experiments——Four healthy male volunteers weighing 58—65 kg were each given one tablet containing 200 mg of perisoxal citrate. Urine was collected for 8 hr after drug administration and frozen at −20° until assayed.

Results and Discussion

Perisoxal citrate was administered intravenously to rats and rabbits and orally to humans. A previous paper reported that the drug was absorbed quickly from the intestinal tract after oral administration to rats, and urinary and fecal excretions of radioactivity were similar to those following an intravenous dose.[4] Moreover, degradation or metabolism of perisoxal in the intestinal tract has not been observed.[6] In the human experiments, the drug was given orally because the level of its safety in intravenous administration was not known. However, the existence of first-pass metabolism in the liver was assumed.

The percentages of the administered doses of perisoxal excreted unchanged and as *m*-hydroxy, *p*-hydroxy and glucuronide forms are given in Table II.

In all the species studied, excretion of the free forms of perisoxal, and *m*- and *p*-hydroxyperisoxal was not detected or was lower than that of their glucuronides. Moreover, neither the free nor the glucuronide form of *o*-hydroxyperisoxal was detected.

Conjugates other than glucuronides, *i. e.*, the HCl-labile unkown conjugate of perisoxal (MX) and the sulfates of perisoxal and hydroxyperisoxals, are excreted by rats.[4] Excretion of MX has also been seen in rabbits[7] and humans.[8] However, only glucuronides were deter-

6) J. Watanabe, H. Okabe, K. Mizojiri, and S. Takahashi, *Shionogi Kenkyusho Nempo*, **22**, 72 (1972).
7) R. Yamamoto, J. Watanabe, and H. Okabe, *Shionogi Kenkyusho Nempo*, **18**, 78 (1968).
8) Unpublished data.

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Table II. Urinary Excretion of Perisoxal and Its Phenolic Metabolites in Rats, Rabbits and Humans after Intravenous or Oral Administration of Perisoxal Citrate[a]

Species	Time (hr)	Urinary excretion (% of dose)[c]					
		Free base			Glucuronide		
		Perisoxal	*m*-Hydroxy-perisoxal	*p*-Hydroxy-perisoxal	Perisoxal	*m*-Hydroxy-perisoxal	*p*-Hydroxy-perisoxal
Rat (M[b])	0—8	0.85±0.03	0.64±0.17	0.57±0.05	0.67±0.32	2.90±0.99	0.66±0.36
	8—24	—	0.57±0.04	—	0.15±0.17	1.46±0.31	0.72±0.83
Rabbit (M)	0—8	0.50±0.28	—	—	7.88±3.63	2.67±0.50	8.04±1.24
	8—24	0.18±0.36	—	—	2.26±2.45	0.87±0.65	0.87±0.25
Man (M)	0—8	—	—	—	16.45±3.72	0.22±0.39	3.43±0.66

a) Doses for rats, rabbits and humans were 21 mg/kg *i.v.*, 15 mg/kg *i.v.* and 200 mg *p.o.*, respectively.
b) Male.
c) Each value represents the mean of 4 determinations±S.D.; —, not detected.

mined in this study since a specific assay method could be established for the glucuronides but not for other conjugates. The β-glucuronidase used in these experiments did not show sulfatase activity for *p*-nitrocatechol sulfate. The appearance of liberated perisoxal and hydroxyperisoxals from rabbit urine reached a maximum with the β-glucuronidase hydrolysis described above and little increase in their quantities was observed on further incubation or supplemental addition of enzyme.

Considerable species differences were observed in glucuronide excretion. In rats, the excretion of glucuronide expressed as a percentage of the dose was 5.74% for hydroxyperisoxals (*m*- and *p*-) and only 0.82% for perisoxal. In rabbits, values of 12.45% for the former and 10.14% for the latter were obtained, while in humans the values were 16.45% for the former against 3.65% for the latter.

Another species difference was noted in the position of ring hydroxylation of perisoxal. The proportion of total *m*-hydroxyperisoxal to total *p*-hydroxyperisoxal excreted by the different species varied significantly. It was about 3 in rats but about 0.4 in rabbits and less than 0.1 in humans. Hashimoto *et al.* have shown that the ratio of the *meta* to the *para* isomer was about 2 in the hydroxylation of ^{3}H-labeled perisoxal by incubation with rabbit liver homogenate for 1 hr.[5] Similar species differences have been recognized in the hydroxylation of aniline,[9] N-2-fluorenylacetamide,[10] and biphenyl,[11] in which the ring hydroxylation occurred mainly in the *para* and *ortho* positions, so the existence of two different enzymes, *para*-hydroxylase and *ortho*-hydroxylase, was suspected. The anilide local anaesthetics, lidocaine,[12] mepivacaine,[12,13] and bupivacaine,[14] are metabolized at the 3'- and 4'-positions of the xylidine ring in different ratios in several species and an epoxide intermediate for the 3'-hydroxy metabolite and an N-hydroxy intermediate for the 4'-hydroxy metabolite were proposed as a possible explanation for the formation of two different phenolic metabolites. From the viewpoint of enzyme systems which are involved in the hydroxylation of the aromatic ring, it is interesting that the excretion of appreciable amounts of *m*-hydroxyperisoxal as well as *p*-hydroxyperisoxal was observed in several species in this study.

In rats, the recovery of radioactivity in the urine after an intravenous dose of 21 mg/kg of ^{14}C-labeled perisoxal was about 40%.[4] Therefore, the excretion of perisoxal and hydroxy-

9) D.V. Parke, *Biochem. J.*, **77**, 493 (1960).
10) J.H. Weisberger, E.K. Weisberger, and H.P. Morries, *Science*, **125**, 503 (1957).
11) P.J. Creaven, D.V. Parke, and R.T. Williams, *Biochem. J.*, **96**, 879 (1960).
12) J. Thomas and P. Meffin, *J. Med. Chem.*, **15**, 1046 (1972).
13) P.J. Meffin and J. Thomas, *Xenobiotica*, 3, 625 (1973).
14) T.J. Coehl, J.B. Davenport, and M.J. Stanley, *Xenobiotica*, 3, 761 (1973).

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perisoxals and their glucuronides determined in this study corresponds to 24% of the urinary excretion. The rest consisted of 5% of the unidentified conjugate MX, 10% of sulfates of perisoxal and hydroxyperisoxals, 16% of other basic methabolites and 45% of unextractable metabolites.[4] In rabbits, 80% of the radioactivity is excreted in the urine after an intravenous dose of 15 mg/kg of ^{14}C-labeled perisoxal,[8] so the sum of the excretions determined here is about 30% of the total urinary excretion. The rest consisted of 15% of the unidentified conjugate MX and other metabolites.[8] In humans, the sum of the excretions was 20% of the dose. A small amount of MX was found in the urine but the total urinary excretion ratio and the qualitative and quantitative characteristics of other metabloites are not known at present.

In this study, a single dose level was administered for each animal species and man, but an investigation on the dose-dependence of metabolism is now in progress.

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