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Isolation of Perisoxal Glucuronides and Determination of Enantiomeric Ratios of *d*-and *l*-Perisoxal Metabolites excreted in Rabbit Urine

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Perisoxal glucuronides were isolated from rabbit urine after repeated subcutaneous administration of *dl*-perisoxal citrate. Optical rotatory analysis of free bases obtained from the glucuronides indicated that about twice as much *d*-perisoxal glucuronide as *l*-isomer was formed.

Stereoselective metabolism was observed in rabbit urine after *d*- or *l*-perisoxal administration. *d*-Perisoxal glucuronide was excreted predominantly at doses of 1.8 and 9.0 mg/kg. The excretions of *d*- and *l*-isomers of *p*-hydroxyperisoxal glucuronides were similar at the dose of 9.0 mg/kg, but a larger value was obtained for the *l*-isomer at the dose of 1.8 mg/kg.

Keywords——3-(1-hydroxy-2-piperidinoethyl)-5-phenylisoxazole; perisoxal; analgesics; rabbit urine; glucuronide; phenolic metabolites; stereoselective metabolism

Perisoxal [3-(1-hydroxy-2-piperidinoethyl)-5-phenylisoxazole] possesses analgesic, anti-inflammatory and antitussive activities.[1] Previously, phenolic metabolites of perisoxal were detected in rat urine[2] and perisoxal glucuronide was obtained as one of the major metabolites in man and rabbit.[3]

In this study, the isolation of perisoxal glucuronide and the determination of stereoselective glucuronidation and hydroxylation of *d*- and *l*-perisoxal in rabbit were carried out.

Experimental

Materials——*dl*-Perisoxal citrate, *o*-hydroxyperisoxal, *m*-hydroxyperisoxal and *p*-hydroxyperisoxal were synthesized by the same methods as described in the previous paper.[3] *d*-Perisoxal citrate and *l*-perisoxal citrate[4] were supplied by Dr. Adachi. Although these perisoxals were administered as citrate, all doses of the drugs are expressed in terms of the free base.

Animals and Dosing——Commercially obtained male albino rabbits weighing 3.0—4.5 kg were used. For the isolation of perisoxal glucuronide, 22.2 mg/kg of *dl*-perisoxal citrate dissolved in 0.5% NaCl was administered subcutaneously to three rabbits at the dose of 66.5 mg/kg once a day for 6 d. The urine was collected 24 h after each dosing. In order to examine the stereoselectivity of formation of perisoxal glucuronides and hydroxylated metabolites excreted in the urine, *d*- or *l*-perisoxal citrate was administered intravenously at doses of 1.8 and 9.0 mg/kg. The urine was collected until 4 h after dosing *via* a catheter inserted into the blader.

Isolation of Perisoxal Glucuronides and the Free Base——The isolation of perisoxal glucuronides was performed as shown in Fig. 1. The pooled urine was filtered and loaded onto an Amberlite XAD-2 resin (Rohm and Haas) column (5 cm × 45 cm) previously washed with 10 l of water, then eluted with 2.5 l of methanol. The methanolic eluate from the 7th to 12th fractions (200 ml/fraction) was evaporated to dryness at 40°C under reduced pressure. The residue was dissolved in 50 ml of water, and the solution was diluted with 120 ml of methanol, then applied to 22 preparative thin layer chromatography (TLC) plates (20 cm × 100 cm, 0.75 mm thickness, Silica gel F_{254}, Merck) and developed with chloroform–methanol–water–acetic acid (13: 10: 3: 1). The silica gel powder around *Rf* 0.45 (perisoxal glucuronides) was scraped off the plates and extracted with methanol. The methanolic extract was chromatographed with methanol on an Amberlite IRC-50 resin (Rohm and Haas) column (5 cm × 40 cm). Contaminating free base was absorbed by the resins.[5] The effluent was evaporated to dryness at 40°C under reduced pressure. The residue was crystallized from small amounts of methanol to give perisoxal glucuronides as a white powder. *Anal.* Calcd for $C_{22}H_{28}N_2O_8$: C, 58.92; H, 6.29; N, 6.24. Found: C, 58.54; H, 6.33; N, 6.13.

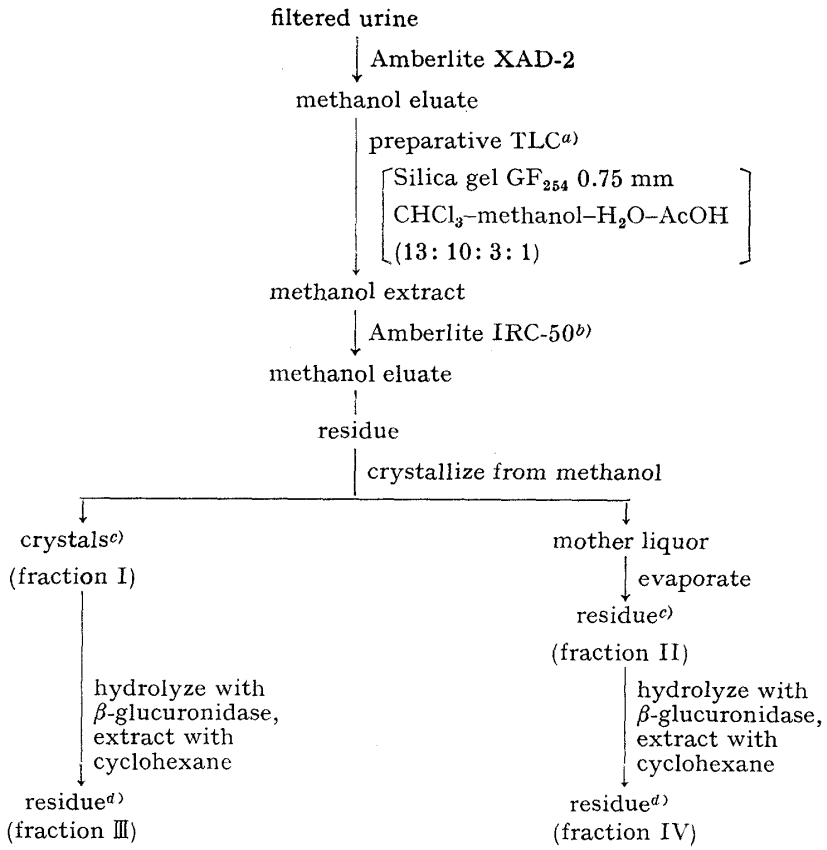

Fig. 1. Isolation of Isomeric Perisoxal Glucuronides from Rabbit Urine after Subuctaneous Administration of *dl*-Perisoxal Citrate

Urine samples from three rabbits were collected (24 h samples) after each dosing. The dose was 66.5 mg/kg once a day for 6 d.

a) A mixture of *d*- and *l*-perisoxal glucuronides was separated from other metabolites and urine components.
b) Contaminating compounds were removed from the glucuronides.
c) A mixture of *d*-perisoxal glucuronide and *l*-perisoxal glucuronide.
d) A mixture of *d*-perisoxal and *l*-perisoxal.

β-Glucuronidase Hydrolysis of Perisoxal Glucuronides——Ten milligrams of crystallized glucuronides (fraction I) and residual glucuronides obtained from the mother liquor by evaporation (fraction II) were dissolved separately in 5 ml each of acetate buffer (0.2 M, pH 4.5). The solutions were each incubated with 1 ml of β-glucuronidase solution (5000 units/ml, Type I, Sigma) at 37°C for 21 h. The incubated media were each extracted twice with 10 ml of cyclohexane after alkalization with 1 N NaOH. The extracts were evaporated to dryness at 40°C under reduced pressure to obtain free bases, fraction III and fraction IV. Recoveries of fraction III and fraction IV from each glucuronide were 86 and 88%, respectively.

Determination of Metabolites in Rabbit Urine——Perisoxal, its phenolic metabolites and their glucuronides were determined according to the GLC method previously reported.[3)]

Results and Discussion

Isolation of Perisoxal Glucuronides and Determination of Enantiomeric Ratio after Subcutaneous Administration of *dl*-Perisoxal Citrate to Rabbits

Excretion of perisoxal glucuronide in rabbits was greater than that of rats, *e.g.*, about 10% of the dose.[3)] Therefore perisoxal glucuronides were isolated from rabbit urine after repeated subcutaneous administration of *dl*-perisoxal citrate.

The glucuronides isolated and purified as described above resisted HCl hydrolysis, but were almost completely hydrolyzed with β-glucuronidase to give a single spot of perisoxal on TLC. Stereoselective hydrolysis of *d*- and *l*-oxazepam glucuronides by β-glucuronidase preparation has been reported,[6)] but in our experiment fraction I, which contained mostly *d*-perisoxal

Table I. Enantiomeric Composition of Perisoxal Glucuronide and Perisoxal in Rabbit Urine after Subcutaneous Administration of *dl*-Perisoxal Citrate

Compound	$[\alpha]_D^{21}$ ($CHCl_3$) or amount	Composition	
d-Perisoxal	+40.2°		
l-Perisoxal	−40.1°		
Fraction III	+36.8°	*d*-Perisoxal	95.8%
		l-Perisoxal	4.2%
Fraction IV	+4.3°	*d*-Perisoxal	55.3%
		l-Perisoxal	44.7%
Fraction I	196 mg	*d*-Perisoxal glucuronide	188 mg
		l-Perisoxal glucuronide	8 mg
Fraction II	387 mg	*d*-Perisoxal glucuronide	214 mg
		l-Perisoxal glucuronide	172 mg

Dosing conditions were as described in Fig. 1.

glucuronide and some *l*-perisoxal glucuronide, and fraction II, which contained about equal amounts of these glucuronides, were hydrolyzed to similar extents of 86 and 88%, respectively. Thus, no enantiomeric difference was apparent in the hydrolysis.

The optical rotations of fraction III (free perisoxals from fraction I) and fraction IV (free perisoxals from fraction II) were measured, then the amount of each enantiomeric compound was calculated (Table I). The amounts of *d*- and *l*-perisoxal glucuronides were 188 and 8 mg in fraction I and 214 and 173 mg in fraction II, respectively. Therefore, the total ratio of *d*- to *l*-perisoxal glucuronide was *d*-(188 mg+214 mg): *l*-(8 mg+173 mg)≒2.2: 1. Thus, stereoselective glucuronidation of perisoxal in rabbits was confirmed.

Urinary Excretion of Metabolites after Intravenous Administration of *d*- or *l*-Perisoxal Citrate to Rabbits

For further investigation of the stereoselective metabolism of perisoxal in rabbits, perisoxal glucuronides and hydroxylated metabolites following intravenous administration of *d*- or *l*-perisoxal citrate were determined. As shown in Table II, there was some variation in the excretion of unchanged perisoxal, ranging from 1.7% to 7.97% for doses of 1.8 and 9.0 mg/kg. The excretions of glucuronide of *d*-perisoxal were larger than those of glucuronide of the *l*-isomer, clearly demonstrating an enantiomeric difference in the glucuronidation of the parent drugs.

Phenolic metabolites were excreted as glucuronides and not as free bases. The position of conjugation of phenolic metabolites with glucuronic acid was considered to be the phenolic

Table II. Urinary Excretions of Perisoxal and Its Phenolic Metabolites in Rabbits after Intravenous Administration of *d*- or *l*-Perisoxal Citrate

Drug and dose[a)]	Perisoxal (%)	Perisoxal glucuronide (%)	Hydroxyperisoxal glucuronide (%)		
			o-	*m*-	*p*-
d- 9.0 mg/kg	6.38	16.14	0.13	3.11	6.44
	7.13	20.17	0.09	1.99	5.95
l- 9.0 mg/kg	7.97	9.01	0.13	0.69	4.71
	3.85	4.15	0.07	1.20	7.58
d- 1.8 mg/kg	7.91	18.34	n.d.	3.22	7.90
	1.70	20.23	n.d.	2.94	6.82
l- 1.8 mg/kg	5.52	5.39	n.d.	2.68	12.13
	4.16	5.23	n.d.	2.52	14.69

The urine was collected for 4 h after dosing *via* a catheter inserted into the bladder.
a) Free base equivalent. n.d.: not detected.

OH, since they were easily hydrolyzed with HCl, liberating almost the same amounts of free base as with β-glucuronidase, while the glucuronide of the intact drug, in which the molecular site of conjugation seemed to be the alcoholic OH, was difficult to hydrolyze with HCl as described above. In the glucuronides of hydroxyperisoxals, the excretion of *p*-hydroxyperisoxal glucuronide was largest, followed by that of *m*-hydroxyperisoxal. Very little *o*-hydroxyperisoxal glucuronide was excreted. The excretion of *p*-hydroxyperisoxal glucuronide of the *l*-isomer was larger than that of the *d*-isomer at the dose of 1.8 mg/kg, but almost the same values were obtained at the dose of 9.0 mg/kg. Excretions of *m*-hydroxyperisoxal glucuronide of the *d*-isomer were larger than those of the *l*-isomer, but these differences may not be significant, since the values were small (about 1—3% of the dose).

In this experiment, the number of rabbits used was only two in any dosage regimen; nevertheless, the percentages of excreted metabolites in the urines of the two rabbits were in good accord. Therefore it is considered that the glucuronidation of *d*- and *l*-perisoxal was stereoselective at both 1.8 and 9.0 mg/kg doses, but the *p*-hydroxylation, followed by glucuronidation, was apparently stereoselective only at the 1.8 mg/kg dose. Therefore, the stereoselectivity of the *p*-hydroxylation of *d*- and *l*-perisoxal in rabbits may be dose-dependent. To confirm this, an experiment with a smaller dose than 1.8 mg/kg is desirable, but could not be performed here because of the inadequate sensitivity of the analytical method used by us.

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