[Chem. Pharm. Bull. 19(9)1929—1936(1971)]

UDC 547.787.3.09 : 547.892.09 : 615.31.076.9

Studies on the Metabolism of Oxazolam. II.[1] Isolation and Identification of the Metabolites of Oxazolam in Rats

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(Received March 20, 1971)

^{14}C-Oxazolam (10-chloro-2,3,5,6,7,11b-hexahydro-2-methyl-11b-phenylbenzo[6,7]-1,4-diazepino[5,4-*b*]-oxazol-6-one), randomly labeled with ^{14}C in the 11b-phenyl ring, was administered orally to the rat, and a number of metabolites were detected in the extracts of liver, kidney, brain, blood, urine and feces by thin-layer chromatography. Among those metabolites, N-desmethyl diazepam, oxazepam and 2-(2-hydroxy-*n*-propylamino)-acetylamino-5-chlorobenzophenone (PACB) were isolated from the liver extracts, 7-chloro-1,3-dihydro-5-(4-hydroxyphenyl)-2H-1,4-benzodiazepine-2-on (CDHB) from the bile, and 2-amino-5-chlorobenzophenone (ACB), 2-amino-5-chloro-3-hydroxybenzophenone (ACHB) and ACHB glucuronide from the urine. On the basis of these findings, a possible metabolic pathway of oxazolam was discussed.

In a previous report,[1] it has been shown in mice and rats that oxazolam (10-chloro-2,3,5,6,7,11b-hexahydro-2-methyl-11b-phenylbenzo[6,7]-1,4-diazepino[5,4-*b*]-oxazol-6-one) was rapidly absorbed and widely distributed in various tissues, and that the concentration of drug in various tissues was found to reach to maximum level within one hour after its oral administration and then to fall rapidly to low. Moreover, it has been observed that rats had excreted about 30% of dose in the urine and 61% in the feces after 48 hours, whereas dogs had excreted about 60% of dose in the urine after 48 hours and 40% in the feces after 72 hours.

In the present paper, the biotransformation of oxazolam has been investigated in rats and seventeen metabolites including N-desmethyl diazepam, oxazepam and 2-amino-5-chlorobenzophenone have been isolated.

Experimental

Compounds——Oxazolam, ^{14}C-oxazolam, N-desmethyl diazepam, oxazepam, 2-(2-hydroxy-*n*-propylamino)-acetylamino-5-chlorobenzophenone (PACB), 2-amino-5-chlorobenzophenone (ACB) and 2-amino-4′-hydroxy-5-chlorobenzophenone (4′-OH-ACB) were prepared in our laboratory (Fig. 1).[3] ^{14}C-Oxazolam with a specific activity of 2.40 μCi/mg randomly labelled with ^{14}C in the 11b-phenyl ring was used.

Animal Studies——Male wistar-Imamichi rats weighing about 125 g were used and deprived of food for 15 hours before and 24 hours after the administration of the drug.

^{14}C-Oxazolam dispersed in 0.5% aqueous solution of tragacanth was administered orally in dose of 30 mg/kg (72 μCi/kg). After 30 minutes blood collected from the carotid artery, and then liver, kindeys and brain were quickly excised. Blood and tissues were homogenized in 4 times its weight of 60% aqueous acetone with Potter-Elvejem glass homogenizer respectively, centrifuged and the supenatant fluid was obtained.

The collection of bile was performed with the following procedure. Rats were anaesthetized with ether and the bile duct was cannulated with polyethylene tube (Igarashi No. 10). After the administration of the drug, rats were kept in Bollman cages[4] and bile was collected for 24 hours.

1) H. Shindo, E. Nakajima, A. Yasumura, H. Murata, T. Hiraoka, and K. Sasahara, *Chem. Pharm. Bull.* (Tokyo), **19**, 60 (1971).
2) Location: *1-2-58 Hiro-machi, Shinagawa-Ku, Tokyo.*
3) T. Miyadera, A. Terada, M. Fukunaga, Y. Kawano, T. Kamioka, C. Tamura, H. Takagi, and R. Tachikawa, *J. Med. Chem.*, **14**, 520 (1971).
4) A. Zlatkis, B. Zak, and A.J. Boyle, *J. Lab. Clin. Med.*, **41**, 486 (1953).

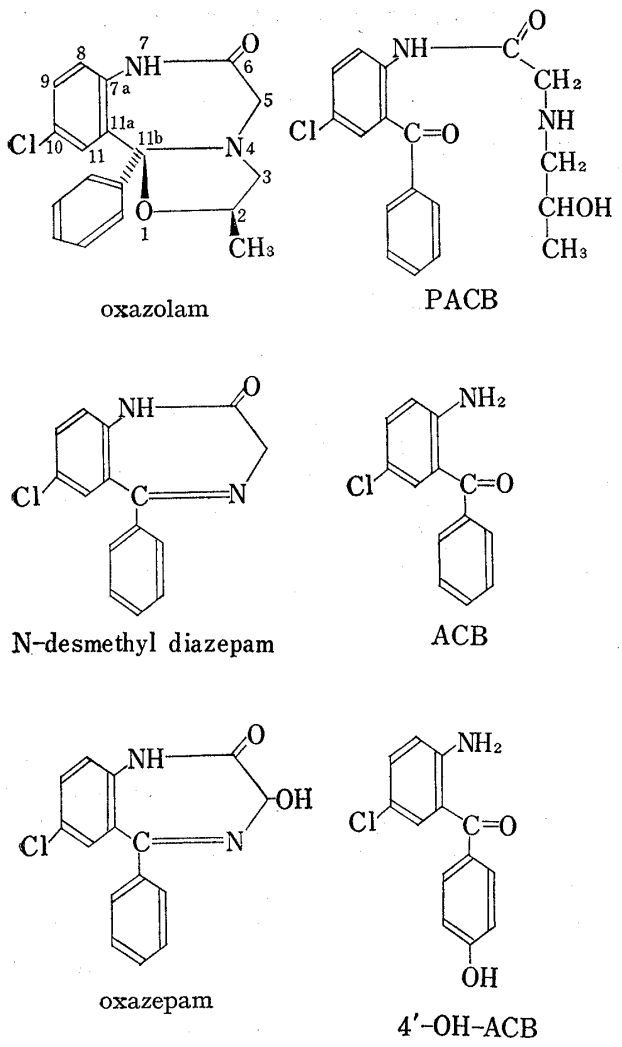

Fig. 1. Chemical Structure of Oxazolam and its Related Compounds

Urine was collected under toluene for 24 hours. Feces was collected for 48 hours, extracted with 4 volumes of 60% aqueous acetone, centrifuged and the supernatant fluid was used.

Thin-Layer Chromatography——Oxazolam and its metabolites were separated by thin-layer chromatography on pre-coated silica gel plates F-254 ("Fertig platte," Merk). For a preparative separation of metabolites, the following plates were used. The slurry was prepared by suspending 30 g silica gel (Kiesel gel H, Merk) in 75 ml of water and set at 0.25 mm thick on a glass plate, which was dried at room temperature and then activated at 110° for 90 minutes.

The following systems were used as developing solvents: System A, benzene–ethyl acetate (3:1); system B, benzene–ethyl acetate–ethanol (18:6:1); system C, benzene–ethyl acetate–ethanol (10:3:1); system D, benzene–ethyl acetate–*n*-butanol–ammonia (6:5:1:2); system E, benzene–*n*-dibutyl ether–ammonia (6:3:1); system F, benzene–*n*-dibutyl ether–*n*-butanol–ammonia (4:14:1:1); system G, benzene–pyridine–*n*-hexane–*n*-propanol (2:2:1:1), system H, *n*-butanol–acetic acid–water (4:1:1); system I, chloroform–ethyl acetete–ethanol (6:4:1); system J, benzene–metanol (9:1); system K, *n*-hexane–*n*-butanol (3:1). The best resolution of metabolites on the plate was achieved by two-dimensional thin-layer chromatography, using system B followed by system H as the developing solvents.

The spot of each metabolite was detected by immersion of the plate in iodine vapor or by spraying the Dragendorff reagent.[5]

A radioautogram was prepared by placing the thin-layer chromatogram in contact with X-ray film for 10—20 days.

The estimation of radioactivity of each metabolite on the plate was carried out as below. The silica gel containing each metabolite was scraped from the two dimensional thin-layer chromatogram and the metabolite was eluted with 0.5 ml of ethyl acetate, followed by counting radioactivity in a liquid scintillation spectrometer.[1]

Paper Chromatography for the Detection of Glucuronic Acid——Two ml of samples dissolved in 0.2M acetate buffer (pH 5.0) was incubated with 10 mg of β-glucuronidase preparation (N.B.C., 60,000 units per g) at 37° for 40—48 hours. An aliquot of the above incubation mixture was spotted on Toyo No. 51A filter paper and developed with *n*-butanol–acetic acid–water (4:1:1) by the ascending or descending method. The detection of glucuronic acid on the paper chromatogram was carried out using the alkaline-silver nitrate reagent and the benzidine reagent.[6]

Mass Spectrometry and Nuclear Magnetic Resonance (NMR)——The mass spectra were run on Mass spectrometer (JEDL JMA OISG) and the NMR spectra were obtained in deuterio dimethyl sulfoxide solution with tetramethylsilane as an internal standard by a Varian HA-100 spectrometer.

Result and Discussion

Blood, brain, liver and kidneys were removed at 30 minutes after the oral adminisrtation of ^{14}C-oxazolam and the extracts of these tissues were prepared as described in experi-

5) E. Stahl,(ed.), "Thin-Layer Chromatography," 2nd ed., Springer-Verlag, New York, N.Y., 1969, pp. 855—905.
6) I. Smith (ed.), "Chromatographic Techniques," William Heinemann Medical Books LTD., London, 1958, pp. 164—177.

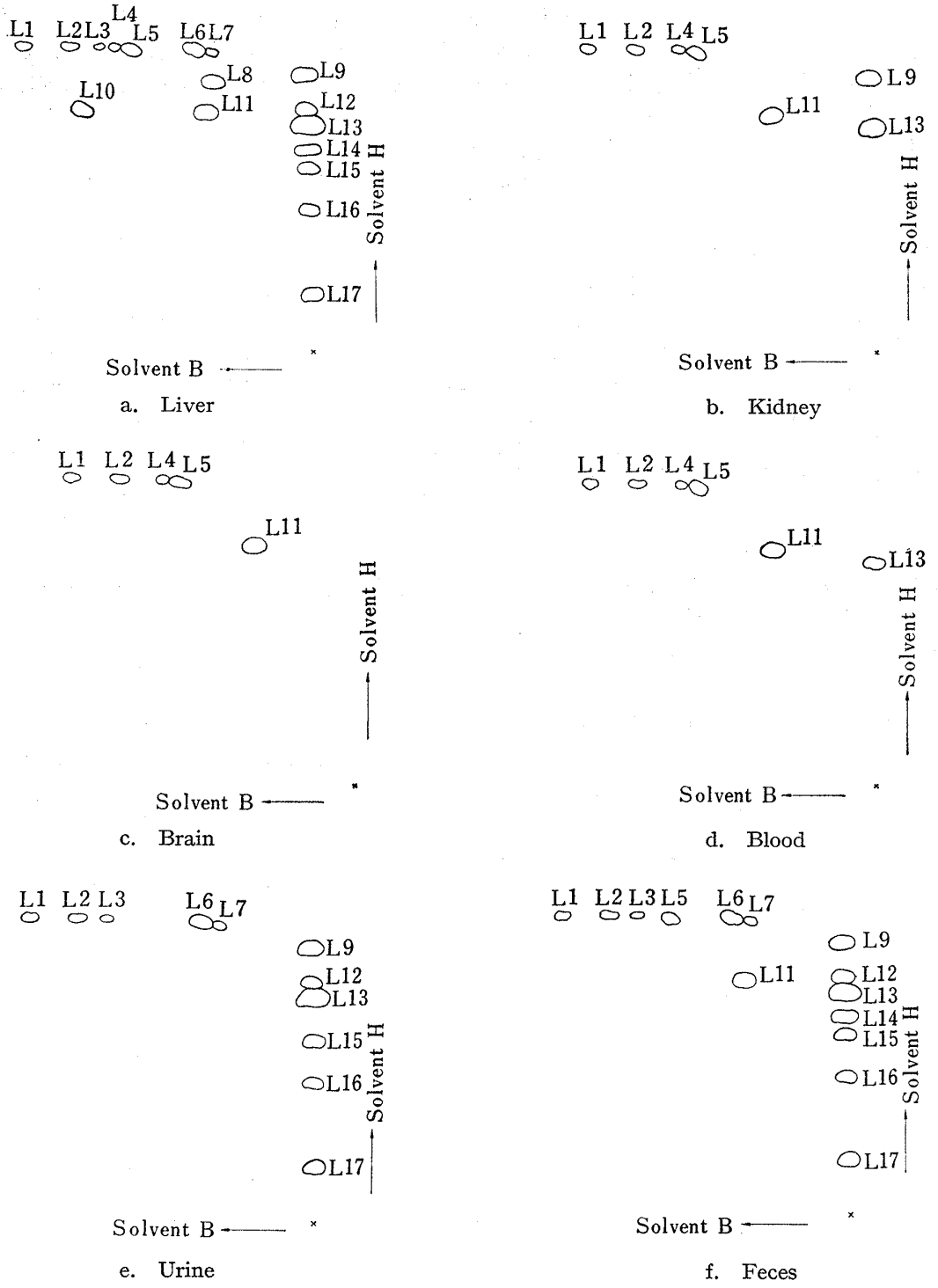

Fig. 2. Radioautograms of Urine, Feces and Various Tissue Extracts Showing the Presence of Metabolites of Oxazolam

mental. The urine was collected during 24 hours after its administration, and the feces during 48 hours. The metabolites of oxazolam contained in these tissues, urine and feces were examined by thin-layer chromatography as indicated in Fig. 2a—f, and seventeen metabolites (L1—L17) including oxazolam (L10) were detected in the liver.

Isolation of L5, L8 and L11 from Rat Liver

Oxazolam was given orally to 50 male Wistar rats weighing about 300 g in dose of 200 mg/kg. At 30 minutes after the administration the liver was removed, homogenized in

4 volumes of 60% aqueous acetone and centrifuged. The supernatant fluid was extracted with chloroform and the extract was concentrated to a small volume *in vacuo* after drying over Na_2SO_4, followed by subjecting to one-dimensional thin-layer chromatography with solvent system A. The areas on the plate corresponding to L5 (*Rf*, 0.30), L8 (*Rf*, 0.18) and L11 (*Rf*, 0.060) were scraped off, respectively, and each metabolite was eluted with chloroform and subsequently with acetone. The combined chloroform and acetone eluates were concentrated *in vacuo*.

L5 (N-Desmethyl Diazepam)

The concentrated eluate of L5 was applied to a column of silica gel (1×10 cm) and successively eluted with 400 ml of benzene and 200 ml of benzene–ethyl acetate (3 : 1). The benzene–ethyl acetate eluate was concentrated to a small volume and the crystal of L5 was obtained by standing it at room temperature. After the recrystallization of L5 from benzene, about 7 mg of L5 was obtained as colorless plate (mp 216—217°). The identity of L5 with the authentic specimen of N-desmethyl diazepam was confirmed by both the mixed melting point determination and the comparison of the infrared spectrum.

L8 (Oxazepam)

The spot L8 in Fig. 2a was found to correspond to that of oxazepam. The concentrated eluate of L8 obtained as above was employed for thin-layer chromatography with solvent system A, H, J and K, and it was observed that the *Rf* values of L8 were completely agreed with those of oxazepam in all solvent systems.

L11 (PACB)

The concentrated eluate of L11 was rechromatographed on thin-layer plate with solvent system B. The purified L11 and the authentic compound, PACB, exhibited the same ultraviolet absorption maxima at 241 and 338 mμ in ethanol. L11 was located as a single spot on the thin-layer plates with solvent system A, C and I, and the *Rf* values of L11 well agreed with those of the authentic PACB (Table I).

Table I. *Rf* Value of L 11 and Reference Compound of PACB

	Solvent system		
	A	C	I
L 11	0.06	0.42	0.53
PACB	0.06	0.42	0.53

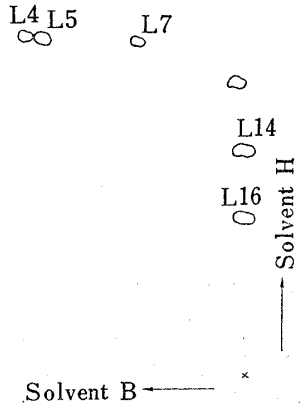

Fig. 3. Two-dimentional Thin-Layer Chromatogram of Bile Showing the Presence of Metabolites of N-Desmethyl Diazepam

Isolation of L7 from Rat Bile

L7 was detected in the liver extracts as a metabolite of oxazolam and also detected in bile after the oral administration of N-desmethyl diazepam (Fig. 3).

N-Desmethyl diazepam (800 mg) was administered orally to 8 male Wistar rats and about 200 ml of bile was obtained in 0—24 hours. The bile was extracted with chloroform and the extract was concentrated *in vacuo* after drying over Na_2SO_4, followed by subjecting to thin-layer chromatography with solvent system B. The area on the plate corresponding to L7 was scraped off and was eluted with ethanol. The eluate was concentrated *in vacuo* and rechromatographed on thin-layer plate.

In the high resolution mass spectrum of the purified L7 thus obtained, the molecular ion peak, *m/e* 286.048±0.006 corresponded to a molecular formula of $C_{15}H_{11}O_2N_2Cl$ (286.051), suggesting the incorporation of one oxygen atom into N-desmethyl diazepam (L5). L7 developed red-violet colour with ferric chloride. The ultraviolet absorption maximum at 312 mμ of L7 in ethanol shifted to 345 mμ under alkaline conditions and this fact may indicate the existence of a phenolic hydroxyl group in L7.[7]

On heating L7 in 2N HCl–ethanol for 60 minutes at 80°, the formation of 4′-OH-ACB was observed, whose *Rf* values on the thin-layer chromatogram was found to be the same as those of the authentic compound with solvent system A, B and K. The ultraviolet absorption spectrum of the isolated 4′-OH-ACB in ethanol completely agreed with that of the authentic compound under both neutral and alkaline condtions (Fig. 4). From these findings, 7-chlor-1,3-dihydro-5-(4-hydroxyphenyl)-2H-1,4-benzodiazepine-2-one (CDHB) was assigned to the structure of L7.

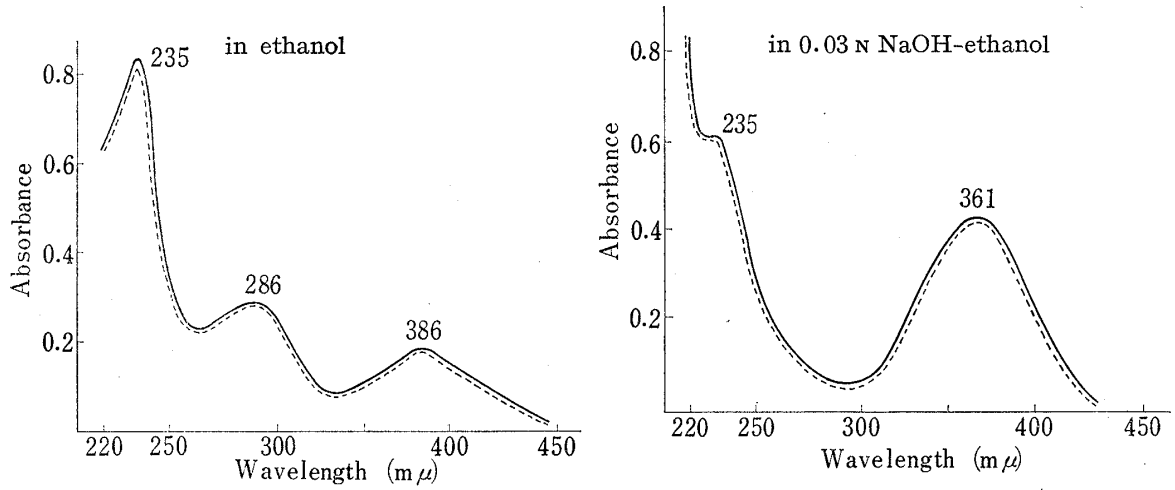

Fig. 4. Ultraviolet Absorption Spectra of 2-Amino-5-chloro-4′-hydroxybenzophenone (4′-OH-ACB)

—— : Isolated 4′-OH-ACB ------ : Authetic compound

Isolation of L1, L2 and L13 from Rat Urine

Total 3 g of oxazolam were administered to 60 male rats and about 1.6 liter of urine were collected during 24 hours. After pH of the urine was adjusted to 7.0—7.4 with ammonia, the metabolites of oxazolam were extracted with chloroform. The extract was concentrated *in vacuo* after drying over Na_2SO_4 and applied to thin-layer chromatography with solvent system D. The area on the thin-layer plate corresponding to L1 and L2 were scraped off, respectively, and they were eluted with chloroform and concentrated *in vacuo*.

L1 (ACB)

The concentrated chloroform eluate was again subjected to the same thin-layer chromatography as above. The purified L1 thus obtained exhibited the ultraviolet absorption maxima at 238 and 392 mμ in ethanol, no shift being observed under alkaline conditions (1/10N NaOH–ethanol). L1 developed orange-yellow colour with the Ehrlich reagent, and red-purple by diazo coupling reaction with N-(β-diethylaminoethyl)-α-napththylamine. On the thin-layer chromatogram with various solvent systems, the *Rf* values of L1 agreed with those of the authentic ACB (Table II).

7) A.I. Scott, "Interpretation of the Ultraviolet Spectra of Natural Products," Pergamon Press, New York, N. Y., **1964**, pp. 89—134.

Table II. *Rf* Values of L 1 and ACB

	Solvent system		
	F	E	G
L 1	0.59	0.50	0.89
ACB	0.60	0.50	0.89

L2 (ACHB)

The concentrated chloroform eluate of L2 was applied to a clumn of silica gel (2.5×29 cm) and eluted with 900 ml of benzene and 200 ml of benzene–ethyl acetate (3:1) successively. The benzene–ethyl acetate eluate was concentrated *in vacuo* and the crystal of L2 was obtained. Recrystallization from ethanol gave about 15 mg of pure L2 as yellow needles (mp 167.3—169.5°). *Anal.* Calcd. For $C_{13}H_{10}O_2NCl$: C, 62.23; H, 4.01; N, 5.66; Cl, 14.32. Found: C, 62.81; H, 4.44; N, 5.85; Cl, 14.58. Under alkaline conditions (NaOH), the ultraviolet absorption maxima at 246, 277 and 398 mμ were shifted to 264, 306, and 420mμ, respectively, suggesting the existence of a phenolic hydroxyl group in the structure of L2.[7] The infrared spectrum of L2 showed bands at 1600 and 3300 cm^{-1} indicative of the presence of >C=O and -NH- groups, respectively. In the high-resolution mass spectrum, the molecular ion peak at 247 was sufficiently agreed with the above empirical formula of L2, and the fragment peaks at 170, 142, 105, and 77 were attributable to $C_7H_4O_2Cl^+$, $C_6H_4OCl^+$, $C_7H_5O^+$, $C_6H_5^+$, respectively. These results indicate that the phenolic hydroxyl group was introduced to benzene ring substituted with an amino group. In the NMR spectra, two doublets (J=2.3 cps) were observed at τ 3.19 and 3.27 corresponding to each aromatic one proton. The observed value of the coupling constant agreed closely with the one due to each aromatic proton at the *meta* position. Thus, the hydroxyl and amino groups in L2 were necessarily to be located at *ortho*-position each other. From these findings, 2-amino-5-chloro-3-hydroxybenzophenone (ACHB) was confirmed as the structure of L2.

L13 (ACHB Glucuronide)

To about 1.5 liter of the aqueous layer of urine, from which L1 and L2 were extracted as previously described, 37.5 g of active carbon (Darco G 60) were added and stirred for 30 minutes at 4°. After the active carbon separated was washed with water, L13 was eluted with 80% acetone and concentrated *in vacuo*. Further purification of L13 was carried out by thin-layer chromatography with solvent system H.

After L13 was incubated with β-glucuronidase preparation, the reaction mixture was subjected to thin-layer chromatography with solvent system B and a yellow spot was detected at the position corresponding to ACHB. Both this isolated compound and the authentic ACHB exhibited the same ultraviolet absorption spectra. The *Rf* values of both compounds agreed with each other on the thin-layer plate with solvent system B and H.

The presence of glucuronic acid in the above reaction mixture was examined by paper chromatography, and a spot was detected, whose *Rf* value and the colours developed by both the silver nitrate reagent and the benzidine reagent agreed entirely with those of glucuronic acid.

As previously described, N-desmethyl diazepam was isolated from the liver extracts as one of the metabolites of oxazolam. On the other hand, it has been observed by thin-layer chromatography that the incubation of oxazolam with the microsomal fraction of rat liver in the presence of NADPH under aerobic conditions resulted in the formation of N-desmethyl diazepam.[8] Oxazepam has already been isolated as the metabolite of diazepam

8) H. Shindo, T. Komai, K. Tanaka, and K. Kawai, *Chem. Pharm. Bull.* (Tokyo), in press.

in dogs and in men,[9] and it has been ascertained that the formation of oxazepam was due to the enzymatic hydroxylation of N-desmethyl diazepam produced by the N-demethylation of diazepam.[10] CDHB could be isolated from bile after the administration of N-desmethyl dizepam, and this indicates that CDHB was formed from N-desmethyl diazepam. The enzymatic formation of ACB from PACB was ascertained by the following experiments. The reaction mixture containing 0.5 μmoles of PACB, 400 μmoles of phosphate buffer (pH 7.4) and the homogenates of 250 mg of rat liver or intestine in a total volume of 5 ml was incubated at 37° for 20 minutes, and extracted with an equal volune of ethyl acetate. The extract was concentrated to a small volume and subjected to thin-layer chromatography with solvent system A. A yellow spot corresponding to ACB was detected on the plate. By incubating with the heated homogenates no other spot than the unchanged PACB was detected by the same procedures. The biotransformation of ACB to ACHB was observed with the oral administration of ACB to the rat as described below. After the administration of total 1.5 g of ACB in dose of 200 mg/kg, about 600 ml of urine were collected during 24 hours and extracted with chloroform. The extract was concentrated *in vacuo* and subjected to two-dimentional thin-layer chromatography. As shown in Fig. 5, a spot (L2) detected on the plate was found to show the same *Rf* value as that of ACHB. The enzymatic formation of ACHB from ACB was observed by the incubation at 37° for 60 minutes under the aerobic conditions of the reaction mixture which contained 3.125 μmoles of ACB, 50 μmoles of phosphate buffer (pH 7.4), 5 μmoles of nicotinamide, 2.5 μmoles of $MgCl_2$, 0.1 μmoles of NADP, 2.5 μmoles of D-glucose 6-phosphate and the homogenates of 125 mg of rat liver in a total volume of 1.0 ml. The reaction mixtur was extracted with chloroform. The extract was concentrated to a small volume and chromatographed on thin-layer plate with solvent system A, the spot of ACHB being detected. From these findings a possible metabolic pathway of oxazolam was shown in Chart 1.

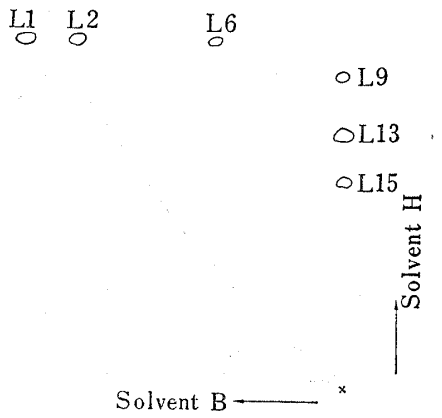

Fig. 5. Two-dimentional Thin-Layer Chromatogram of Urine Showing the Presence of Metabolites of ACB

Table III. Estimation of Metabolites of Oxazolam in the Urine and Feces as % of Administered Dose (30 mg/kg)

Metabolites	Urine	Feces
ACB (L 1)	0.029	1.42
ACHB (L 2)	0.204	2.62
N-Desmethyl diazepam (L 5)	—	2.15
L 6+CDHB (L 7)	0.495	4.24
L 9	1.52	1.72
PACB (L 11)	—	0.49
ACHB-glucuronide (L 13)	13.6	17.7
L 15	2.59	8.29
L 16	0.786	3.56

9) H.W. Ruelius, J.M. Lee, and H.E. Alburn, *Arch. Biochem. Biophys.*, **111**, 376 (1965); M.A. Schwartz, B.A. Koechlin, E. Postma, S. Palmer, and G. Krol, *J. Pharmacol. Exptl. Therap.*, **149**, 423 (1965); J. A.F. de Silva, B.A. Koechlin and G. Bader, *J. Pharm. Sci.*, **55**, 692 (1966).

10) M.S. Schwartz and E. Postma, *Biochem. Pharmacol.*, **17**, 2443 (1968); F. Marcucci, R. Fanelli, E. Mussini, and S. Garattini, *European J. Pharmacol.*, **7**, 307 (1969).

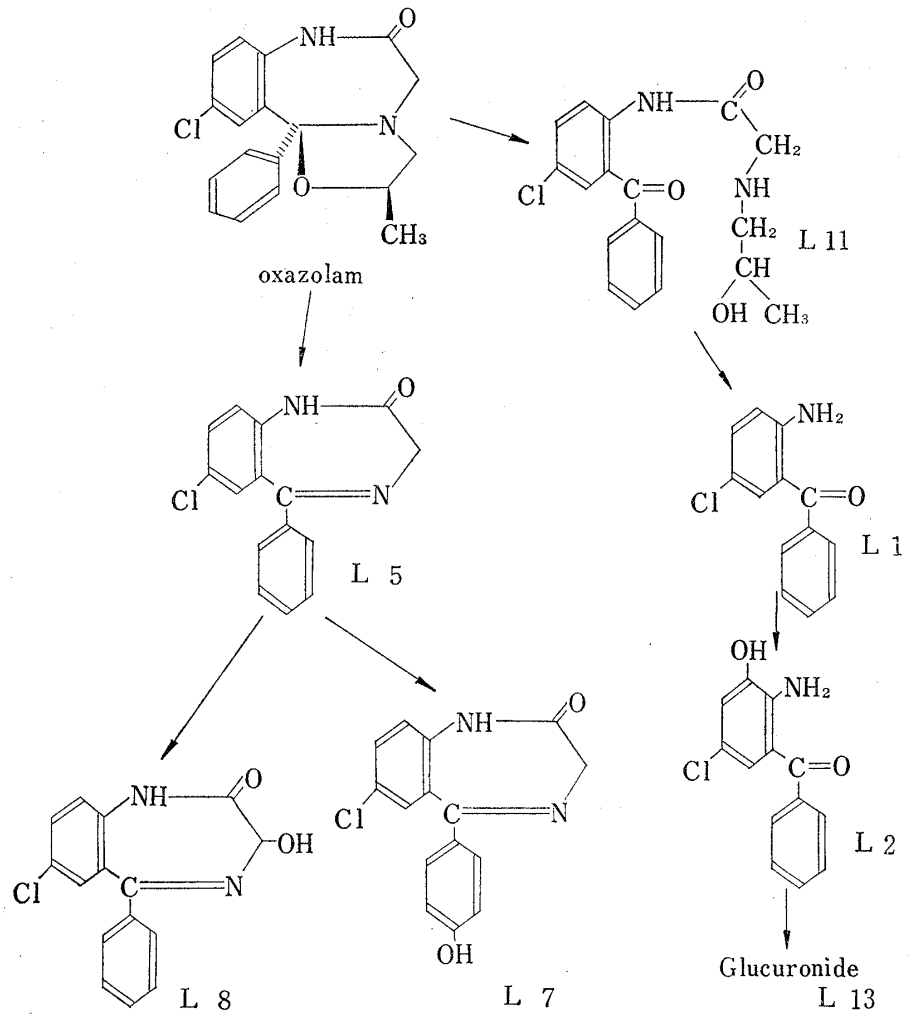

Chart 1. Possible Metabolic Pathway of Oxazolam

As indicated in Fig. 2, not a few metabolites of oxazolam have been detected in the urine and feces, and an attempt has been made to estimate the radioactivity of each metabolite in the urine collected during 24 hours and in the feces during 48 hours after the administration of ^{14}C-oxazolam. The results were indicated in Table III and about 31% of the dose was found to be excreted as ACHB glucuronide in the urine and feces. The intestinal absorption of oxazolam and its metabolism in some organs will be further reported in the subsequent paper.[8]

Acknowledgement The authors wish to express their gratitude to Dr. G. Sunagawa, director of this laboratory, and to Dr. K. Tanabe for their invaluable advice and encouragement. The authors thank Dr. R. Tachikawa, Dr. T. Miyadera, Mr. M. Fukunaga, Mr. Y. Kawano and Mr. T. Tanaka for supplying oxazolam and its related compounds for this study.