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Metabolism of Cloxazolam. I. Distribution, Excretion and Biotransformation in Rats and Mice

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The distribution, excretion and biotransformation of ^{14}C-cloxazolam were studied in rats and mice. The tissue concentration reached the maximum at about 1 and 3 hr after oral administration to rats and mice, respectively. The brain concentration was higher and lasted longer in mice than in rats. In the whole-body autoradiograms of mice, no significant radioactivity was detected in the tissues except the liver after 24 hr and the radioactivity disappeared almost completely after 72 hr. The recovery of radioactivity was considerably higher in the feces than in the urine and was about the same regardless of the route of administration in both rats and mice. In the *in situ* cannulated rats, the most of the oral dose was recovered in the bile in 24 hr period. Eighteen radioactive metabolites were detected in the liver, urine and feces after oral administration of ^{14}C-cloxazolam. Among them, eight metabolites including 7-chloro-2,3-dihydro-5-[*o*-chlorophenyl]-2H-1,4-benzodiazepin-2-one, 7-chloro-5-[*o*-chlorophenyl]-1,3-dihydro-3-hydroxy-2H-1,4-benzodiazepin-2-one (COX) and 2-amino-3-hydroxy-2′,5-dichlorobenzophenone (ADCB-OH) were isolated and identified. The main metabolites in the mouse urine were the glucuronides of COX and ADCB-OH, while in the rat urine two unidentified metabolites in addition to the above two. In the mouse feces, COX and ADCB-OH and their conjugates were the main metabolites, while in the rat feces a much larger number of metabolites were detected.

Cloxazolam, newly synthesized in our laboratories, is 10-chloro-2,3,5,6,7,11b-hexahydro-11b-[*o*-chlorophenyl]benzo[6,7]-1,4-diazepino-[5,4-*b*]-oxazol-6-one.[2] It is an analogue of oxazolam, of which the metabolic fate has been already reported,[3–5] and has been shown to be much more potent than oxazolam as an anticonvulsant, muscle relaxant and taming agent in animals.[6] In addition, it has been reported to be clinically effective against schizophrenia, depression, epileptic psychopathia and neurosis.[7]

The present report deals with the distribution, excretion and biotransformation of cloxazolam in rats and mice.

Material and Method

Materials——^{14}C-Cloxazolam labeled at the carbon 11b was prepared according to the method described previously by Tachikawa, *et al.*[2] using ^{14}C-2-amino-5,2′-dichlorobenzophenone (^{14}C-ADCB) as labeled precursor.[8] The preparation was 97.08% radiochemically pure as assayed by the reverse isotope dilution method and had a specific activity of 10.86 μCi/mg. ^{14}C-7-Chloro-2,3-dihydro-5-[*o*-chlorophenyl]-2H-1,4-benzodiazepin-2-one labeled at the carbon 5 (^{14}C-CND) with specific activity of 9.54 μCi/mg was prepared

1) Location: *Hiromachi 1-chome, Shinagawa-ku, Tokyo.*
2) T. Miyadera, A. Terada, M. Fukunaga, Y. Kawano, T. Kamioka, C. Tamura, H. Takagi, and R. Tachikawa, *J. Med. Chem.*, **14**, 520 (1971).
3) H. Shindo, E. Nakajima, A. Yasumura, H. Murata, T. Hiraoka, and K. Sasahara, *Chem. Pharm. Bull.* (Tokyo), **19**, 60 (1971).
4) A. Yasumura, H. Murata, K. Hattori, and K. Matsuda, *Chem. Pharm. Bull.* (Tokyo), **19**, 1929 (1971).
5) H. Shindo, T. Komai, K. Tanaka, and K. Kawai, *Chem. Pharm. Bull.* (Tokyo), **19**, 2085 (1971).
6) T. Kamioka, H. Takagi, S. Kobayashi, and Y. Suzuki, *Arzneim.-Forsch.*, **22**, 884 (1972).
7) T. Maeda, *Medical Consultation and New Remedies*, **8**, 1498 (1971).
8) Purchased from Daiichi Chemicals Inc., Tokyo.

by the method of Sternbach, *et al.*[9] using ^{14}C-ADCB as labeled precursor. ^{14}C-7-Chloro-5-[*o*-chlorophenyl]-3-hydroxy-1,3-dihydro-2H-1,4-benzodiazepin-2-one (^{14}C-COX) with specific activity of 3.16 μCi/mg was prepared by the method of Bell, *et al.*[10] using ^{14}C-CND as labeled precursor. Both preparation showed a single radioactive spot on two-dimentional thin-layer chromatogram.

Unlabeled cloxazolam, ADCB, CND, COX, 5,2′-dichloro-2-[2-hydroxyethylamino]acetaminobenzophenone (HEAB) and 7-chloro-2,3-dihydro-1-methyl-5-[*o*-chlorophenyl]2H-1,4-benzodiazepin-2-one (CD) were all prepared in this laboratories. Other chemicals were of reagent grade and used without further purification.

Animals——All experiments were performed using male rats of Wistar-Imamichi strain and male mice of ddY strain.

Whole-body Autoradiography——Five mice weighing about 20 g were administered orally with 50 mg/kg (*ca.* 5.8 μCi/body) of ^{14}C-cloxazolam dispersed in 0.5% Tragacanth solution. One, 3,6,24 and 72 hr after administration, animals were slightly anesthetized with ether and sacrificed by immersion in a mixture of hexane and solid carbon dioxide at about $-70°$. After a frozen animal was embedded on a microtome stage with aqueous carboxymethylcellulose gel, sagittal 30 μ sections through the whole animal were cut by means of tape sectioning (Scotch Magic Mending No. 810, Minnesota Mining) with Yamato Type 1111 microtome in a freezing room at $-20°$ and were dried at the same temperature. The dried sections were brought into contact with Sakura Type-N industrial X-ray film and exposed for 20 days.

Experiments on Distribution and Excretion——Rats weighing about 130—150 g and mice weighing about 30—35 g were administered orally with 10 mg/kg of ^{14}C-cloxazolam dispersed in 0.5% Tragacanth solution by stomach tube. At various time after the administration, the animals were sacrificed by bleeding from carotid and various organs and tissues were removed. In the separate experiments, the urine and feces were collected over 2 days period after the administration. For intravenous administration, the compound was solubilized by dissolving in a small amount of 4N HCl and raising the pH to 2.5 with 1N NaOH and injected from the tail vein (10 mg/kg).

The blood and tissues were homogenized in 4 volumes of 70% aqueous ethanol with Polytron (Kinematica GMBH, Luzern-Schweiz) and the aliquots were solubilized by warming in 0.5 ml of 2N NaOH at 80° for 90 min. After the solutions were decolored by addition of 0.2 ml of 30% H_2O_2, they were assayed for radioactivity. A part of the liver homogenates was centrifuged and the supernatant was used for the separation of metabolites by thin-layer chromatography (TLC). The whole gastro-intestinal tract and the carcass were solubilized by warming in 30% KOH at 80° for 2 hr and the solutions were assayed for radioactivity.

The urine was used without any treatment for radioassay and TLC. The feces were homogenized in 70% aqueous ethanol and, after centrifugation, the residue was reextracted twice with the same solvent. The combined extracts were suspended in 1N NaOH and assayed for radioactivity. A part of the extracts was evaporated to dryness *in vacuo*, dissolved in a small amount of 70% ethanol and subjected to TLC.

Experiments on Biliary Excretion——Rats weighing about 150 g were anesthetized with ether and the bile duct was cannulated with polyethylene tube (Igarashi No. 10). About 30 min after the operation, the rats were administered orally with 10 mg/kg of ^{14}C-cloxazolam. The animals were kept in Bollman cages[11] and the bile was collected for 24 hr period. The bile was used without any treatment for radioassay and TLC.

Estimation of Metabolites in Urine and Feces——The urine and the ethanol extracts of feces were subjected to two-dimentional TLC and the chromatogram was placed in contact with X-ray film (Agfa-Gevaert) for 20 days to detect the radioactive spots. Each spot was quantitatively transferred into the counting vials by scratching carefully with a spatula, followed by estimation of radioactivity.

Thin-Layer Chromatography——Precoated fluorescent silica gel plate (E. Merck) of 0.25 and 2.0 mm thickness were used. Chromatograms were developed with the following solvent systems: A) benzene: methanol=9:1, B) *n*-butanol: acetic acid: water=4:1:1, C) benzene:ethylacetate:ethanol=18:6:1, D) chloroform:ethyl acetate=3:1, E) benzene:*n*-butanol=9:1, F) benzene:ethanol:ammonia=45:5:1, G) *n*-butanol: acetic acid=9:1, H) benzene:isopropanol=9:1, I) benzene:acetic acid=3:1, J) chloroform: methanol=9:1, K) benzene:dioxane=4:1, L) isopropanol:acetic acid:water=4:1:1, M) ethanol:acetic acid:water=4:1:1. The two-dimentional thin-layer chromatograms were developed with solvent A, followed by solvent B. The spots on the chromatogram were detected with ultraviolet light (2536 Å), by immersion of the plate in iodine vapor or by spraying the Dragendorff reagent.

Treatment with β-Glucuronidase——Samples in 0.05M acetate buffer (pH 5.0) were incubated with β-glucuronidase (Sigma, 600000 units/g) at 37° for 48 hr. In most experiments, 5 mg of the preparation was added per ml solution. An aliquot of the incubation mixture was subjected to TLC with solvent systems,

9) L.H. Sternbach, R.I. Fryer, W. Metesics, E. Reeder, G. Sach, G. Saucy, and A. Stempel, *J. Org. Chem.*, **27**, 3788 (1962).
10) S.C. Bell and S.J. Childress, *J. Org. Chem.*, **27**, 1691 (1962).
11) J.L. Bollman, J.C. Cain, and J.H. Grindlay, *J. Lab. Clin. Med.*, **33**, 1349 (1948).

B, L and M. Glucuronic acid on the plate was detected by alkaline silver nitrate reagent and benzidine reagent.[12]

Radioactivity Measurement——All the extracts, solution, suspension, urine and bile were counted in a Beckman LS-250 liquid scintillation spectrometer using toluene–ethanol scintillator consisted of 8 g PPO, 200 mg dimethylPOPOP, 500 ml of toluene and ethanol and 40 g Cab-O-Sil. The radioactivity of the spots on thin-layer chromatogram was estimated by suspending the silica gel powder from the spot in 0.5 or 1.0 ml of water and 15 ml of dioxane–toluene scintillator consisted of 8 g PPO, 200 mg dimethyl POPOP, 800 ml dioxane, 200 ml toluene and 40 g Cab-O-Sil.

Spectral Measurement——The mass spectra were obtained by JEOL JMA-OISG Mass Spectrometer and the nuclear magnetic resonance (NMR) spectra by Varian HA-100 Spectrometer in deutero-dimethylsulfoxide solution with tetramethylsilane as an internal standard.

Result and Discussion

Whole-body Autoradiography in Mice

Representative autoradiograms from mice 1, 3, 6 and 24 hr after oral administration of ^{14}C-cloxazolam are shown in Fig. 1. At 1 hr after administration (Fig. 1-A), a considerable distribution of radioactivity was observed throughout the body. The highest radioactivity was observed in the stomach and the upper part of small intestine, suggesting an existence of the drug unabsorbed. A high radioactivity was shown in the liver, renal cortex and brown fat, followed by the heart muscle, salivary gland, pancreas and renal medulla. A significant radioactivity was found in the brain, spinal cord and skeletal muscle, while the blood concentration was relatively low. These results indicate that the drug is rapidly taken up by the tissues. A high radioactivity was also seen in the gall bladder, indicating an occurrence of the biliary excretion.

At 3 hr after administration (Fig. 1-B), the tissue concentrations of radioactivity became slightly higher than those at 1 hr. A high radioactivity was observed in the gall and urinary bladders and the intestinal contents, indicating a prominent excretion of the drug through the bile as well as the urine. The lachrymal gland and nasal mucosa also showed a high radioactivity. In the central nervous system, the cerebrum, cerebellum and spinal cord showed an appreciable uptake. It was noted that a slightly higher concentration appears to be persisted in the white matter and that a prominent concentration was seen in the trigeminal nerve.

At 6 hr after administration (Fig. 1-C), the highest radioactivity was shown in the gall bladder and intestinal contents, indicating that the excretion of the drug through the bile

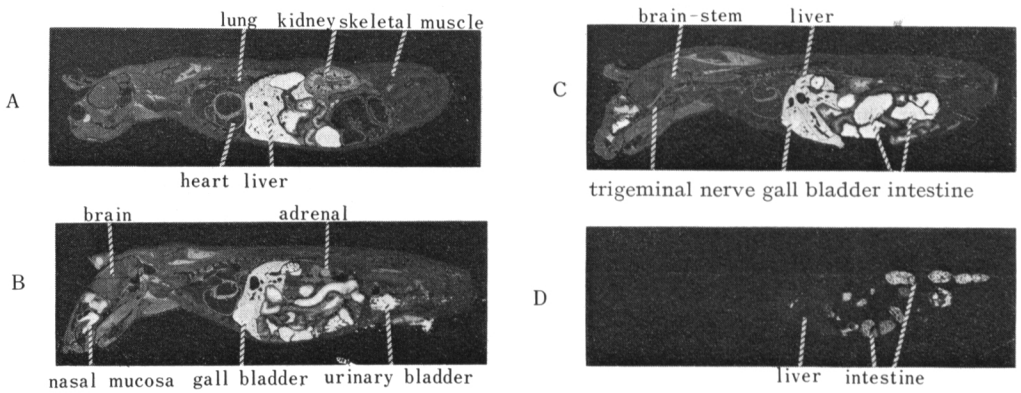

Fig. 1. Autoradiograms of ^{14}C-Cloxazolam in Mice 1 (A), 3 (B), 6 (C), and 24 (D) Hours after Oral Administration

12) I. Smith, "Chromatographic Techniques," 164, William Heinemann Medical Books Ltd., London, 1958.

is highly participating. The concentration in the liver was still high, while that in the muscular tissues and subcutaneous fat decreased to a low level. The concentration in the brain also decreased, but it was noted that an appreciable concentration was still retained in the white matter of the cerebrum, brain stem, trigeminal nerve and pituitary.

At 24 hr after administration (Fig. 1-D), some radioactivity was observed only in the intestinal contents and gall bladder and a very low concentration in the liver. At 72 hr, no appreciable radioactivity was observed throughout the body, except the liver which showed a trace of radioactivity.

Distribution and Excretion in Rats and Mice

The concentration of radioactivity in rat tissues after oral administration of ^{14}C-cloxazolam is presented in Table I. At 30 min after administration, the radioactivity was already distributed in various tissues, such as the liver, kidney, brain and muscle. The concentration in the liver was very high and was about 25 times of the blood concentration, followed by the kidney (5 times). The concentration in the brain was slightly lower than that in the blood. The blood and tissue concentrations appear to reach their maxima at about 1 hr after administration and thereafter decreased gradually. After 24 hr, no significant radioactivity was detected in all the tissues except the liver, kidney and skeletal muscle and after 48 hr a very low level only in the liver and kidney.

The concentration of radioactivity in mouse tissues after oral administration of ^{14}C-cloxazolam is shown in Table II. Like rats, the liver concentration was very high and about 17 times of the blood concentration at 30 min after administration. Unlike rats, on

Table I. Distribution of Radioactivity in Rats Tissues after Oral Administration of ^{14}C-Cloxazolam (10 mg/kg)

Tissue	μg/g wet tissue						
	0.5 hr	1 hr	2 hr	4 hr	7 hr	24 hr	48 hr
Blood[a]	0.73	1.48	1.40	0.98	0.74	—[b]	—
Brain	0.66	1.12	0.84	0.51	0.31	—	—
Lung	1.24	2.16	1.57	1.12	0.52	—	—
Heart	1.01	2.12	1.52	0.80	0.35	—	—
Liver	18.00	21.10	17.10	10.83	8.09	2.84	1.15
Spleen	0.46	1.22	1.02	0.88	0.36	—	—
Kidney	3.83	7.25	6.69	5.28	4.16	2.01	1.22
Fat[c]	0.80	1.86	1.64	0.97	0.78	—	—
Testis	0.40	0.72	0.77	0.44	0.27	—	—
Muscle[d]	0.97	1.02	1.01	0.82	0.33	0.51	—

Each value represents the mean of 2 animals.
Radioactivity was converted to μg equivalents of cloxazolam.
a) μg/ml *b*) undetectable *c*) subcutaneous *d*) thigh

Table II. Distribution of Radioactivity in Mice Tissues after Oral Administration of ^{14}C-Cloxazolam (10 mg/kg)

Tissue	μg/g wet tissue						
	0.5 hr	1 hr	2 hr	4 hr	7 hr	24 hr	48 hr
Blood	0.99	1.91	2.08	1.60	1.18	1.25	0.18
Brain	1.83	3.13	4.36	4.52	2.17	0.78	0.55
Liver	17.08	22.09	26.77	23.21	21.56	14.59	3.76
Kidney	4.31	7.60	8.99	11.02	6.41	3.13	1.15

Each value represents the mean of 2 animals.
Radioactivity was converted to μg equivalents of cloxazolam.

the other hand, the brain concentration was about 2 times higher than that in the blood for the period from 30 min to 7 hr. The concentration in the brain appears to reach the maximum at around 4 hr and the blood and tissue concentrations in mice appear to last longer than those in rats. After 48 hr, however, the tissue concentrations fell off to a very low level. These results are well consistent with those observed by autoradiography.

The excretion of radioactivity in rats after oral and intravenous administration of ^{14}C-cloxazolam is shown in Table III. After oral administration, about 13 and 65% of the dose were recovered in the urine and feces, respectively, in 48 hr period, while after intravenous administration about 20 and 58%, respectively, indicating a significant secretion of radioactivity into the intestinal tract, most probably through the biliary excretion. In a separate experiment with cannulated rats, it was found that during 24 hr after oral administration about 91% of the dose was recovered in the bile (Table IV), indicating that cloxazolam and/or its metabolites are excreted considerably through the bile. It seems probable, therefore, that

Table III. Excretion and Recovery of Radioactivity in Rats following Oral or Intravenous Administration of ^{14}C-Cloxazolam (10 mg/kg)

Sample	% of dose		
	Oral administration		*i.v.* injection
	0—24 hr	0—48 hr	0—24 hr
Urine	10.87	12.56	19.55
Feces	31.17	64.90	57.55
G.I. tract[a)]	48.97	14.30	13.51
Carcass	2.71	2.40	2.37
Recovery	93.72	94.25	92.98

Each value represents the mean of 2 animals.
a) tissue+contents

Table IV. Biliary Excretion of Radioactivity in Rats following Oral Administration of ^{14}C-Cloxazolam (10 mg/kg)

Sample	% of dose 0—24 hr	Sample	% of dose 0—24 hr
Bile	91.30	G.I. tract[a)]	4.83
Urine	4.92	Carcass	2.18
Feces	0.82	Recovery	104.05

Each value represents the mean of 2 animals.
a) tissue+contents

Table V. Excretion and Recovery of Radioactivity in Mice following Oral or Intravenous Administration of ^{14}C-Cloxazolam (10 mg/kg)

Sample	% of dose		
	Oral administration		*i.v.* injection
	0—24 hr	0—48 hr	0—48 hr
Urine	15.46	39.47	35.41
Feces	19.97	45.57	56.80
G.I. tract[a)]	41.18	4.67	1.78
Carcass	15.73	3.61	2.44
Recovery	92.34	93.32	96.43

Each value represnts the mean of 2 animals.
a) tissue+contents

a considerable fecal excretion observed after oral administration was mostly derived from the absorbed radioactivity which was brought back into the intestinal tract through the biliary excretion. In mice, during 48 hr after oral and intravenous administration, about 40 and 35% of the dose were excreted in the urine and about 46 and 56% in the feces, respectively (Table V). Thus, the fact that both in rats and mice the urinary excretion of radioactivity was almost the same regardless of the route of administration suggest that cloxazolam is well absorbed from the gastrointestinal tract. This is in consistent with the fact that no detectable amount of unchanged drug was found in the feces of rats and mice after oral administration of ^{14}C-cloxazolam, but a number of metabolites are excreted (Table VII, VIII).

Isolation and Identification of Metabolites

The urine, bile and fecal and liver extracts from the rats administered with ^{14}C-cloxazolam were subjected to two-dimentional TLC. As shown in Fig. 2, the largest number of radioactive metabolites (eighteen metabolites, spot 1—18) including cloxazolam (spot 9) were detected in the liver and all of the metabolites found in the urine, feces and bile corresponded to those found in the liver. By the same way, a number of metabolites were detected in the urine and fecal and liver extracts from mice, all of which corresponded to those found in rats, except that spot 6 and 18 were not detected. Among these metabolites, eight compounds were isolated and identified as follows.

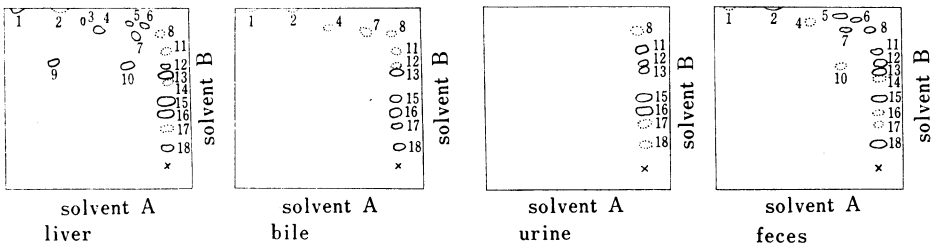

Fig. 2. Schematic Illustration of Autoradiograms of Urine, Bile, Fecal and Liver Extracts of Rats administered with ^{14}C-Cloxazolam

Spot 4 (CND)——The ethylacetate extracts from the rat liver 1 hr after administration of ^{14}C-cloxazolam were concentrated *in vacuo* after drying over Na_2SO_4, followed by subjecting to TLC with solvent A. The spot 4 (*Rf* 0.48) was scraped off and eluted with ethanol. The eluate was concentrated and rechromatographed with the same solvent for further purification. The *Rf* value of the purified sample agreed well with that of authentic CND with solvent A, B, H and I. This was methylated by methyliodide in the presence of sodium methoxide, followed by subjecting to TLC with solvent A, J and K. The product showed the same *Rf* value as that of authentic CD in all the solvents. The identity was further confirmed by the reverse isotope dilution method. An appropriate amount of non-radioactive CD was added to a certain amount of the methylated product and dissolved in ethanol by heating. After cooling, the precipitated crystals were recrystallized from ethanol 4 times. The specific activity determined after each recrystallization gave a constant value (Table VI).

Table VI. The Specific Activity of the Methylated Spot 4 after Recrystallization

Recrystallization	Specific activity dpm/mg	Recrystallization	Specific activity dpm/mg
1	585	3	518
2	502	4	520

Spot 6 (CND-OH) and Spot 7 (COX)——Spot 6 and 7 as well as spot 8, 11, 15, 16, 17 and 18 were also found in the extracts from the rat liver and the bile treated with β-glucuronidase after administration of ^{14}C-CND (Fig. 3). This fact indicates that spot 6 and 7 are the intermediate metabolites formed *via* CND from cloxazolam. Thus, CND was orally administered in a dose of 100 mg/kg to 120 rats weighing about 300 g and about 2500 ml of the bile was collected in 24 hr period. The bile was treated with β-glucuronidase and extracted with chloroform. The extracts were concentrated *in vacuo* after drying over Na_2SO_4 and separated by TLC with benzene: methanol=3 : 1. The area corresponding to spot 6 and 7 (*Rf* 0.46, they are inseparable) was scraped off and eluted with ethanol. The eluate was concentrated *in vacuo* and rechromatographed with the same solvent and then with solvent G by which spot 6 and 7 was separated each other (*Rf* 0.82 and 0.69). The each area was scraped off and eluted with ethanol, followed by their further purification with solvent G.

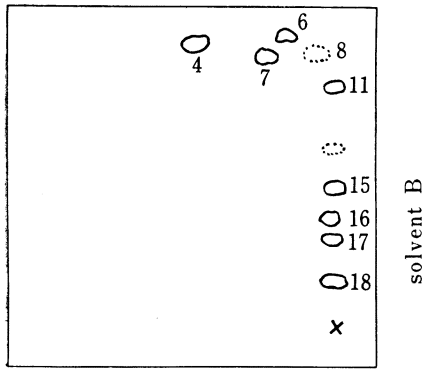

Fig. 3. Schematic Illustration of Autoradiogram of the Rat Liver Extract after Oral Administration of ^{14}C-CND

The *Rf* value of the purified spot 7 agreed well with that of authentic COX on the thin-layer chromatogram with solvent A, B, G and I. The ultraviolet spectrum exhibited maxima at 230 and 320 mμ in ethanol being consistent with those of COX.

In the mass spectrum of the purified spot 6, the molecular ion peak, *m/e* 320, corresponded to a compound incorporated one oxygen atom to CND. The compound developed redviolet color with ferric chloride and the ultraviolet absorption maxima at 290 mμ in ethanol was shifted to 340 mμ under alkaline condition, suggesting the presence of a phenolic hydroxyl group. Furthermore, an yellowish brown product was obtained on heating in 4N HCl at 100° for 2 hr. The mass spectrum of the product exhibited the molecular ion peak at *m/e* 281 and the fragment peaks at *m/e* 155 and 126, corresponding to a compound incorporated with one hydroxyl group into the chlorophenyl ring of ADCB. Therefore, the structure of spot 6 was assigned as CND-OH (Chart 1). The position of hydroxyl group has not been determined.

Spot 1 (ADCB) and Spot 2 (ADCB-OH)——Cloxazolam was given orally in dose of 200 mg/kg to 60 rats weighing 300—400 g and about 1100 ml of the bile was collected in 24 hr period. After treating with β-glucuronidase, it was extracted with chloroform and the extracts concentrated, followed by subjecting to TLC with solvent A. The areas of spot 1 (*Rf* 0.95) and 2 (*Rf* 0.50) were scraped off and eluted with ethanol. The each eluate was concentrated *in vacuo* and purified by the TLC with the same solvent.

The purified spot 1 developed red-purple color by diazo-coupling reaction with N-(β-diethylaminoethyl)-α-naphthylamine and orange-yellow color with Ehrlich reagent. The ultraviolet absorption showed maxima at 235 and 396 mμ in ethanol, no shift being observed under alkaline conditions. On the thin-layer chromatograms with solvent A, C, D, E, F, I and L, the *Rf* value was agreed well with that of authentic ADCB.

Spot 2 was obtained as yellow needles of mp 155—157° (*ca.* 20 mg). *Anal.* Calcd. for $C_{13}H_{10}O_2N_2Cl_2$: C, 55.32; H, 3.22; N, 4.96; Cl, 25.13. Found: C, 55.41; H, 3.48; N, 5.24; Cl, 24.02. The infrared absorption spectrum (Nujol mull) showed the bands due to –C=O and $-NH_2$ at 1600 and 3500—3400 cm^{-1}, respectively. The ultraviolet absorption maxima at 243, 280 and 400 mμ were shifted to 263, 304 and 435 mμ, respectively, under alkaline conditions, suggesting the presence of a phenolic hydroxyl group. The mass spectrum showed

the molecular ion peak at *m/e* 281 and the fragment peaks at *m/e* 170, 139 and 111, which are attributable to $C_7H_5O_2NCl^+$, $C_7H_4OCl^+$ and $C_6H_4Cl^+$, respectively. These results indicate that the phenolic hydroxyl group was introduced to the benzene ring with an amino group. The NMR spectrum showed two doublets (J=2.4 cps) at 3.17 and 3.58 τ corresponding to two aromatic protons and the coupling constant is consistent with that due to aromatic protons at meta positions. Therefore, the hydroxyl group is necessarily located at the position *ortho* to the amino group. From these findings, the structure of spot 2 was assigned as 2-amino-3-hydroxy-5,2′-dichlorobenzophenone (ADCB-OH).

Spot 10 (HEAB)——The ethanol extracts from the rat liver administered with cloxazolam were extracted again with chloroform and the extracts concentrated *in vacuo* after drying over Na_2SO_4, followed by subjecting to TLC with solvent A. The area corresponding to spot 10 was scraped off and eluted with ethanol. The eluate was concentrated *in vacuo* and rechromatographed with solvent C. The *Rf* value of the purified spot 10 agreed well with that of authentic HEAB on the thin-layer chromatograms with solvent A, B and H. The ultraviolet spectrum showed maxima at 246, 273 and 350 mμ in ethanol, being well consistent with that of authentic HEAB.

Spot 13 (ADCB-OH Glucuronide)——After removing the chloroform extractable metabolites from the bile collected from rats administered with cloxazolam, active carbon (Darco G 60) was added and stirred for 3 hr at 5°. After washing with water, spot 13 could be eluted with 80% acetone and concentrated *in vacuo*, followed by TLC with solvent B for further purification. After treating with β-glucuronidase, the reaction mixture was extracted with chloroform and the extracts were concentrated *in vacuo*, followed by the TLC with solvent A. On the chromatogram a yellow spot was detected at the position corresponding to ADCB-OH. This was isolated, subjected to TLC and the *Rf* value agreed well with that of ADCB-OH obtained before with solvent A, C, D and E. On the other hand, the reaction mixture with β-glucuronidase was separated by TLC with solvent B, L and M and a spot was detected whose *Rf* value and colors developed by the silver nitrate and the benzidine reagents agreed well with those of glucuronic acid.

Spot 15 (COX Glucuronide)——The ethanol extracts from rat liver after administration of ^{14}C-cloxazolam were concentrated *in vacuo*, followed by the TLC with solvent B. The area corresponding to spot 15 (*Rf* 0.58) was scraped off and eluted with ethanol. The eluate was evaporated to dryness and the residue dissolved in a small amount of water, followed by treatment with β-glucuronidase. After extracting with ethylacetate, the extracts were concentrated and subjected to two-dimentional TLC. On the chromatogram a spot corresponding to COX was detected, indicating that spot 15 should be COX glucuronide.

Excretion of Metabolites in Urine and Feces

The amount of metabolites excreted in the urine and feces of rats and mice following oral administration of ^{14}C-cloxazolam are shown in Table VII and VIII.

In the mouse urine, conjugated COX and ADCB-OH were the main metabolites, which accounted for about 35 and 25% of the urinary excretion of radioactivity, respectively. In the rat urine, on the other hand, spot 11 and 16 were excreted in relatively a large amount, which accounted for about 13 and 12% of the excretion of radioactivity, respectively, in addition to the above two metabolites which accounted for about 16 and 13%, respectively.

In the mouse feces, COX and ADCB-OH including their conjugates were the main metabolites, which accounted for about 23 and 21% of the fecal excretion of radioactivity, respectively. On the other hand, in the rat feces, a much larger number of metabolites than that in mice were detected and among them ADCB-OH was the largest in amount, while COX was comparatively small. These results suggest that there might be some difference in the metabolic pattern of cloxazolam between rats and mice. In mice CND formed from cloxazolam appears to be transformed mainly to COX, which is conjugated further. On the other hand, the metabolism of CND in rats appears to be more complicated than that in mice.

Differences in cloxazolam metabolism between rats and mice will be discussed further in the subsequent paper.[13]

TABLE VII. Estimation of Metabolites of Cloxazolam in the Urine and Feces of Rats dosed with ^{14}C-Cloxazolam (10 mg/kg *p.o.*)

Metabolite			% of dose	
			Urine (0—24 hr)	Feces (0—48 hr)
Spot	1	ADCB	——[a]	0.82±0.11 (1.13)
	2	ADCB-OH	——	6.25±0.72 (8.65)
	4	CND	——	0.24±0.12 (0.33)
	5		——	4.81±0.52 (6.66)
	6	CND-OH	——	2.90±0.36 (4.01)
	7	COX	——	1.05±0.15 (1.45)
	8		0.79±0.14 (4.96)[b]	3.34±0.17 (4.62)
	10	HEAB	——	0.85±0.36 (1.18)
	11		2.00±0.26 (12.55)	2.22±0.27 (3.07)
	12		1.15±0.06 (7.08)	4.25±0.17 (5.88)
	13	ADCB-OH-gl.	2.01±0.42 (12.62)	3.25±0.30 (4.50)
	15	COX-conjugate	2.55±0.27 (16.01)	3.56±0.21 (4.93)
	16		1.87±0.25 (11.74)	——
	17		0.57±0.12 (3.58)	——
	18		0.37±0.06 (2.32)	4.01±0.66 (5.55)

Values are the mean±S.E. of 4 animals.
a) not detectable
b) Values in parenthesis are % of the urinary or fecal radioactivity.

TABLE VIII. Estimation of Metabolites of Cloxazolam in the Urine and Feces of Mice dosed with ^{14}C-Cloxazolam (10 mg/kg *p.o.*)

Metabolite			% of dose	
			Urine (0—24 hr)	Feces (0—48 hr)
Spot	1	ADCB	0.19±0.01 (1.07)[a]	0.57±0.06 (0.84)
	2	ADCB-OH	0.05±0.01 (0.28)	10.60±0.12 (15.69)
	4	CND	0.15±0.02 (0.84)	0.72±0.05 (1.07)
	5		0.09±0.03 (0.50)	3.16±0.04 (4.68)
	6	CND-OH	——[a]	——
	7	COX	0.32±0.01 (1.80)	13.00±0.45 (19.24)
	8		——	——
	10	HEAB	——	1.13±0.36 (1.67)
	11		——	——
	12		——	1.87±0.12 (2.76)
	13	ADCB-OH-gl.	4.44±0.74 (24.93)	3.51±0.20 (5.19)
	15	COX-conjugate	6.25±0.67 (35.09)	2.58±0.82 (3.82)
	16		0.61±0.02 (3.42)	——
	17		0.19±0.02 (1.07)	——
	18		——	——

Values are the mean±S.E. of 3 groups.
a) For definition, see footnote to table VII.

Metabolic Pathway of Cloxazolam

In the light of metabolites so far identified, a possible metabolic pathway of cloxazolam is shown in Chart 1. In fact, ADCB (spot 1) and ADCB-OH (spot 2) and its glucuronide

13) H. Murata, K. Kougo, A. Yasumura, and H. Shindo, *Chem. Pharm. Bull.* (Tokyo), to be published.

(spot 13) were detected on the chromatogram of rat liver extracts after administration of HEAB. ADCB-OH and its glucuronide, as well as three unknown metabolites, were detected in the rat liver extracts after administration of ^{14}C-ADCB (Fig. 4).

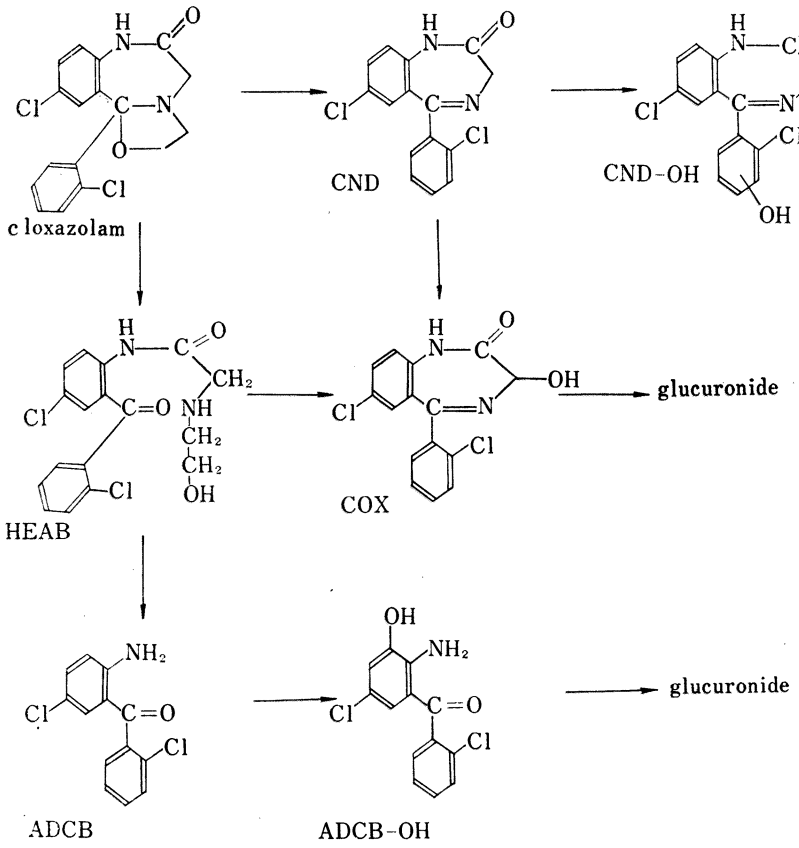

Chart 1. Possible Metabolic Pathway of Cloxazolam

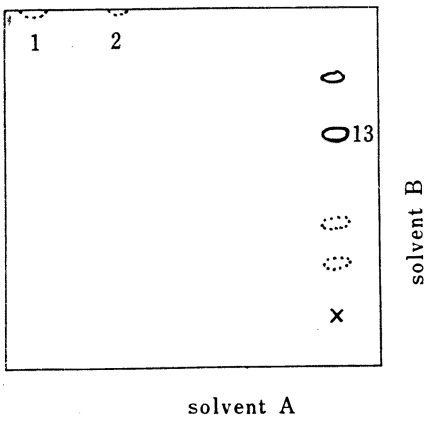

Fig. 4. Schematic Illustration of Autoradiogram of Rat Liver Extract after Oral Administration of ^{14}C-ADCB

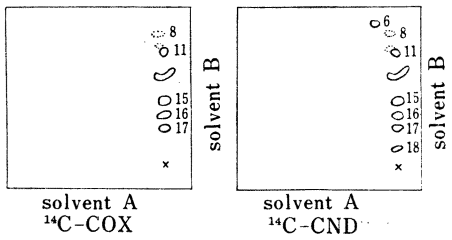

Fig. 5. Schematic Illustration of Autoradiograms of the Rat Urine after Oral Administration of ^{14}C-COX or ^{14}C-CND

As already described, CND-OH (spot 6) and COX (spot 7) and its glucuronide (spot 15), as well as spot 8, 11, 16, 17 and 18 were detected in the liver extracts of rats administered with ^{14}C-CND (Fig. 3). It was found, furthermore, that spot 8, 11, 16, and 17 are also detected in the rat urine after oral administration of ^{14}C-COX and that the relative intensity of these spots appears to be very similar to that observed after administration of ^{14}C-CND (Fig. 5). These facts suggest that spot 8, 11, 16, and 17 are all the metabolites derived from COX which was formed from CND. Recently, Schilling, *et al.*[14] reported that lorazepam, same as COX, was transformed to four metabolites[15] other than the glucuronide in rats. Although these metabolites could not be identified in the present investigation, there is a possibility that there might be the metabolites corresponding to them among the spots 8, 11, 16, and 17.

It was already reported that[4] oxazolam was converted to compounds of aminobenzophenone type *via* a compound of HEAB type and simultaneously to hydroxylated compounds including oxazepam *via* a compound of CND type. Thus, the biotransformation of cloxazolam seems to be very similar to that of oxazolam. However, there appear to be some differences between cloxazolam and oxazolam in the relative amount of excretion of their metabolites in rats. Namely, in the urine and feces after administration of cloxazolam the metabolites of benzodiazepine type including COX and CND were excreted in a larger amount than that of benzophenone type including ADCB and ADCB-OH in contrast to oxazolam. The cause of this difference is not clear, but the fact that cloxazolam is chemically more stable than oxazolam in an acidic solution[16] is of interest in relation to the difference in the metabolism of the two drugs.

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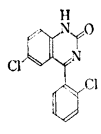
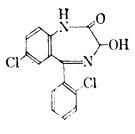
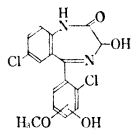
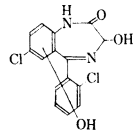
14) R.T. Schillings, S.R. Schrader, and H.W. Ruelius, *Arzneim. Forsch.*, **19**, 1929 (1971).

15)

16) A. Terada and R. Tachikawa, unpublished data in this laboratories.