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Metabolism of 4-Ethoxy-2-methyl-5-morpholino-3(2H)-pyridazinone(M73101), a New Anti-inflammatory Agent. I. Identification of the Metabolites in Rabbit and Their Pharmacological Studies[1]

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The metabolism of 4-ethoxy-2-methyl-5-morpholino-3(2H)-pyridazinone (M73101) in rabbits and the pharmacological activities of metabolites were investigated. Rabbits orally received the drug in a dose of 100 mg/kg excreted ten metabolites together with unchanged drug in the urine. These metabolites were characterized by their spectrometric data (infrared, mass, ultraviolet, and nuclear magnetic resonance spectra), thin-layer chromatography and gas chromatography. Some of them were unequivocally identified by comparison with synthesized authentic samples.

The following compounds were found to be present in the urine: 4-ethoxy-2-methyl-5-morpholino-3(2H)-pyridazinone (unchanged drug), 4-hydroxy-2-methyl-5-morpholino-3(2H)-pyridazinone (M-1), 5-bis(2-hydroxyethyl)amino-4-ethoxy-2-methyl-3(2H)-pyridazinone (M-2), 4-ethoxy-5-[2-(β-hydroxyethyloxy)ethylamino]-2-methyl-3(2H)-pyridazinone (M-3), 4-ethoxy-5-(2-hydroxyethylamino)-2-methyl-3(2H)-pyridazinone (M-4), 4-ethoxy-5-(2-hydroxy-tetrahydro-1,4-oxazin-4-yl)-2-methyl-3(2H)-pyridazinone (M-5), 4-ethoxy-5-morpholino-3(2H)-pyridazinone (M-6), 4-ethoxy-2-methyl-5-(3-oxo-tetrahydro-1,4-oxazin-4-yl)-3(2H)-pyridazinone (M-7), 5-(N-carboxymethyl-N-2-hydroxyethylamino)-4-ethoxy-2-methyl-3(2H)-pyridazinone (M-8), 5-[2-(carboxymethyloxy)-ethylamino]-4-ethoxy-2-methyl-3(2H)-pyridazinone (M-9), 5-carboxymethylamino-4-ethoxy-2-methyl-3(2H)-pyridazinone (M-10).

From these results, it was concluded that the major metabolic route in rabbits was oxidative cleavage of C–N and C–O of morpholino group followed by further degradation. Deethylation and demethylation were found to be minor reaction.

In the pharmacologocal studies of metabolites, no activities were observed, which indicated that the pharmacological activities of M73101 were not due to these urinary metabolites.

Keywords——anti-inflammatory drug; drug metabolism; identification and determination; rabbit urine; metabolism of morpholino group; pharmacological study of metabolites

Introduction

4-Ethoxy-2-methyl-5-morpholino-3(2H)-pyridazinone(M73101) is a new analgesic and anti-inflammatory agent[3] which has been recently synthesized and now is being developed as a new drug in the authors laboratories.

The drug consisted of the basic structure of 3(2H)-pyridazinone has been so far scarcely developed, and, therefore, little information about their metabolic fate in lives has been known. Moreover, the metabolic changes of morpholino group have not been exhaustively studied in spite of its structure which is speculated to undergo easily various biodegradative conversions and so it seems very interesting to study the metabolism of 3(2H)-pyridazinone ring substituted with a morpholino group.

1) A part of this work was presented at the 96th Annual Meeting of Pharmaceutical Society of Japan, Nagoya, April 1976.
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3) T. Yuizono, T. Fukuda, H. Tanizawa, M. Sato, and S. Konishi, *Japan. J. Pharmacol.*, suppl. **26**, 122 (1976).

In another point of view, the increasing importance of metabolic studies is now accepted because a bioactivation or bioinactivation of drug administered have great influences on toxicological and pharmacological actions in actual uses. In this paper, the authors deal with the metabolic fate of M73101 in rabbits and toxicological and pharmacological studies of its main metabolites.

Experimental

Metabolic Studies

Materials——M73101 was white needle crystal (mp 89—92°) synthesized according to the method described by Takahashi.[4]

Thin-Layer Chromatography (TLC)——Thin-layer plate of silica gel (Kiesel gel GF_{254}, Merck; activated at 100° for 60 min) was used for the spot test and preparative chromatography. Solvent system for TLC: Solvent A; ether–chloroform–ethanol (15: 10: 2, v/v), Solvent B; ethylacetate–isopropanol–water (20: 3: 1, v/v), Solvent C; chloroform–ethanol–acetic acid (25: 1: 1, v/v). The resulting chromatograms were visualized under ultraviolet (UV) light (2536 Å).

Gas-Liquid Chromatography (GLC)——The analytical conditions were as follows; column: 2% OV-17 on 60—80 mesh chromosorb W (AW-DMCS) packed in glass column (2 m × 3 mm i.d.), temperature of oven: 175—250° (programming rate, 2°/min), temperature of injection part and detector: 280°, nitrogen flow rate: 40 ml/min. Samples of acidic fraction were injected after esterification with diazoethane.

Administration of the Compounds——Male albino rabbits weighing about 3.0 kg were fasted for 16 hr before dosing and M73101 dissolved in water was administered orally in a dose of 100 mg/kg. The urine was collected for 24 hr and stored in a freezer (−20°) until used.

Extraction of Urinary Metabolites——The urine was adjusted to pH 7 and extracted with chloroform after being saturated with sodium chloride. The chloroform extract, which contained neutral compounds, was dried over anhydrous sodium sulfate and evaporated under reduced pressure below 40° to give a brown

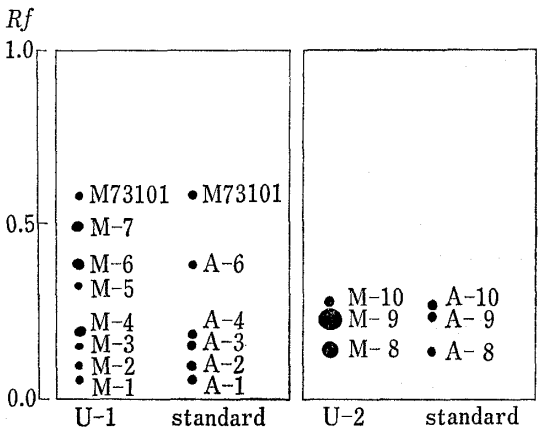


Fig. 1. Thin-Layer Chromatogran of Urinary Extracts and Authentic Samples

U-1: neutral fraction of urinary extract (solvent system A)
U-2: acidic fraction of urinary extract (solvent system C)
A-1: 4-hydroxy-2-methyl-5-morpholino-3(2H)-pyridazinone
A-2: 5-bis(2-hydroxyethyl)amino-4-ethoxy-2-methyl-3(2H)-pyridazinone
A-3: 4-ethoxy-5-[2-β-hydroxyethyloxy)ethylamino]-2-methyl-3(2H)-pyridazinone
A-4: 4-ethoxy-5-(2-hydroxyethylamino)-2-methyl-3(2H)-pyridazinone
A-6: 4-ethoxy-5-morpholino-3(2H)-pyridazinone
A-8: 5-(N-carboxymethyl-N-2-hydroxyethylamino)-4-ethoxy-2-methyl-3(2H)-pyridazinone
A-9: 5-[2-(carboxymethyloxy)ethylamino]-4-ethoxy-2-methyl-3(2H)-pyridazinone
A-10: 5-(carboxymethylamino)-4-ethoxy-2-methyl-3(2H)-pyridazinone

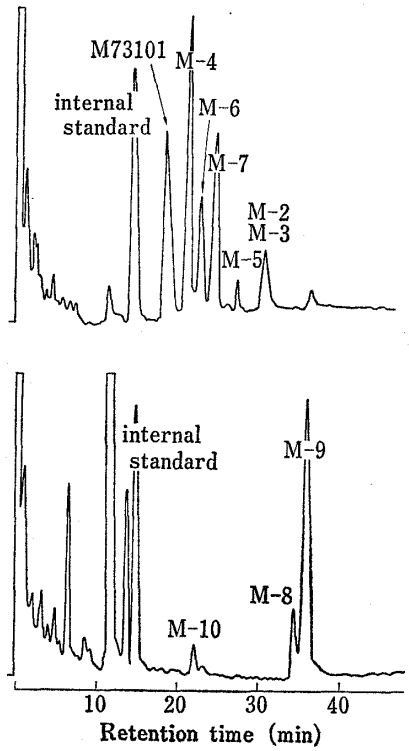


Fig. 2. Gas Chromatogram of Urinary Extracts

Upper chromatogram: neutral fraction, internal standard: aldrin, lower chromatgoram: acidic fraction.

4) T. Takahashi, M. Takaya, Y. Maki, and T. Satoda, Japan Patent 47-24030 (1972) [*C.P.I.* 44179*T* (1972)].

residue. The aqueous solution was then adjusted to pH 2 with 6 N HCl followed by extraction with chloroform. After being dried over anhydrous sodium sulfate, the organic fraction was evaporated under reduced pressure below 40° to give a similar brown residue. Thin-layer chromatograms and gas-liquid chromatograms of neutral and acidic fractions were depicted in Fig. 1 and 2, on which M73101 and ten metabolites were separably visualized.

Isolation of Urinary Metabolites——Neutral fraction dissolved in a small volume of chloroform was applied to alumina column (2×8 cm) using chloroform, chloroform–isopropanol (3: 1, v/v), isopropanol, ethanol and methanol as effluent solvent and collected 10 ml of each fraction. Crude mixture of M73101 and M-7 and M-4 were eluted separately by chloroform, the latter of which was recrystallized from isopropylether to obtain white needles. M-2 was isolated as viscous oil from chloroform–isopropanol (3: 1, v/v) elute. M-5 was isolated as viscous oil from isopropanol fractions and a mixture of M-3 and M-5 was eluted with ethanol. Further separation of M-7 from M73101 was carried out by means of a column chromatography of silica gel using ether–chloroform (4: 1, v/v) as effluent solvent followed by recrystallization from isopropylether to give pure white needles. M-3 and M-5 were further separated and purified by means of preparative TLC using solvent system A. The pure M-3 was obtained as oily matter. M-1 and M-6 were so small amount that they were separated and purified by means of preparative TLC using solvent system A. Acidic fraction dissolved in a small volume of chloroform was applied to silica gel column chromatography (2×13.5 cm) using as effluent solvent chloroform–acetic acid (28: 1, v/v) from fraction No. 1 to 70 and chloroform–acetic acid–ethanol (28: 1: 1, v/v) from fraction No. 71 to 110, collecting 10 ml of each fraction. Crude M-9 was eluted in fraction No. 21 to 85 and obtained as white needles after twice recrystallization from ethylacetate. M-8 and M-10 were eluted in other fractions as crude material, each of which was obtained as colorless needles by means of preparative TLC using solvent system C followed by recrystallization from ethylacetate.

Pharmacological Studies

Eight mice weighing 18—22 g were used for each dose. The LD_{50} and ED_{50} were calculated according to Litchfield and Wilcoxon's method.[5]

Acute Toxicity Test——The test compounds were given intraperitoneally into animals, which were observed for 7 days.

Analgesic Test——Mice were given subcutaneously morphine hydrochloride in a threshold dose of 0.5 mg/kg, and 15 min later the test compounds were given intraperitoneally. The analgesic effect was considered to be positive when the animal did not attempt to remove a clip applied to the base of the tail at 30 min after medication.

Anti-inflammatory Test——Following the method of Srimal *et al.*,[6] mice were given intraperitoneally the test compounds in a dose of 200 mg/kg, and then 30 min later 1% carrageenin solution was injected subcutaneously in a volume of 0.05 ml into the plantar side of the right hind paw. Animals were sacrificed after 4 hr, and the both hind paws were cut at the ankle joint. Edema was estimated by differential weight of both paws.

Results

Characterization and Identification of Urinary Metabolites

M-1——The high resolution mass spectrum showed a molecular ion at *m/e* 211.0986 corresponding to $C_9H_{13}N_3O_2$, which differed from that of M73101 with a decrease in 28 mass units. The nuclear magnetic resonance (NMR) spectrum of this metabolite was very similar to that of M73101 except for a disappearance of peaks assigned to O-ethyl group at 4-position of M73101. From these data, the structure of M-1 was shown to be 4-hydroxy-2-methyl-5-morpholino-3(2H)-pyridazinone. The structure of M-1 was further confirmed from comparison of spectral and chemical properties shown in Table I and II with those of synthesized authentic sample.

M-2——The high resolution mass spectrum showed a molecular ion at *m/e* 257.1349 corresponding to $C_{11}H_{19}N_3O_4$, which differed from that of M73101 with an increase in 18 mass units. The part other than morpholino group of M73101 was left unaltered judging from NMR signals of $-CH_3$ at N-2, $-OCH_2CH_3$ at C-4 and –H at C-6. In NMR spectrum of M-2, there were observed signals of eight protons with the pattern of A_2B_2 which could be assigned to four methylenes stemed from morpholino group of M73101, though their chemical shifts

5) J.T. Litchfield and F. Wilcoxon, *J. Pharmacol. Exp. Ther.*, **96**, 99 (1949).
6) R.C. Srimal and B.N. Dhawan, *Indian J. Pharm.*, **3**, 4 (1971).

TABLE I. NMR[a] Singnals of M73101 and Its Metabolites

Compound	NMR Signal (ppm from TMS)		
M73101[b]	1.38 (3H, t, -$OCH_2\underline{CH_3}$)	3.36 (4H, t, 2×-NCH_2-)	3.76 (3H, s, -NCH_3)
	3.85 (4H, t, 2×-OCH_2-)	4.36 (2H, q, -$O\underline{CH_2}$Me)	7.58 (1H, s, C_6-H)
M-1[b]	3.32 (4H, t, 2×-NCH_2-)	3.80 (3H, s, -NCH_3)	3.84 (3H, t, 2×-OCH_2-)
	6.60 (1H, br, -OH)	7.60 (1H, s, C_6-H)	
M-2[b]	1.32 (3H, t, -$OCH_2\ \underline{CH_3}$)	3.50—3.72 (7H, m, -NCH_3, 2×-NCH_2-)	3.83 (4H, t, -$\underline{CH_2}$OH)
	4.02 (2H, q, -$O\underline{CH_2}$Me)	4.75 (2H, br, 2×-OH)	7.82 (1H, s, C_6-H)
M-3[b]	1.34 (3H, t, -$OCH_2\underline{CH_3}$)	2.56 (1H, br)	3.44 (2H, t, -NCH_2-)
	3.56—3.86 (9H, m, -NCH_3, -$\underline{CH_2}$-O-$\underline{CH_2CH_2}$-OH)		4.38 (2H, q, -$O\underline{CH_2}$Me)
	4.84 (1H, br)	7.55 (1H, s, C_6-H)	
M-4[b]	1.30 (M-4[b] (3H, t, -$OCH_2\underline{CH_3}$)	3.40 (2H, t, -$\underline{CH_2}$OH)	3.72 (3H, s, -NCH_3)
	3.82 (2H, t, -NCH_2-)	4.0 (1H, br)	4.28 (2H, q, -$O\underline{CH_2}$Me)
	5.0 (1H, br)	7.60 (1H, s, C_6-H)	
M-5[b]	1.37 (3H, t, -$OCH_2\underline{CH_3}$)	3.22—3.48 (4H, m, 2×-NCH_2-)	3.64—3.84 (5H, m, -NCH_3, -OCH_2-)
	4.35 (2H, q, -OCH_2-)	5.04 (1H, m, -O$\underline{CH}$(OH))	7.53 (1H, s, C_6-H)
M-6[b]	1.38 (3H, t, -$CH_2\underline{CH_3}$)	3.40 (4H, t, 2×-NCH_2-)	3.85 (4H, t, 2×-OCH_2-)
	4.38 (2H, q, -$O\underline{CH_2}$Me)	7.64 (1H, s, C_6-H)	12.08 (1H, br, -NH-)
M-7[b]	1.40 (3H, t, -$OCH_2\underline{CH_3}$)	3.70—3.88 (5H, m, -NCH_3, -OCH_2-)	4.08 (2H, t, -NCH_2-)
	4.40 (2H, s, -$COCH_2$-)	4.70 (2H, q, -$O\underline{CH_2}$Me)	7.71 (1H, s, C_6-H)
M-8[c]	1.23 (3H, t, -$OCH_2\underline{CH_3}$)	3.20—3.80 (7H, m, -NCH_3, -NCH_2CH_2O-)	4.03 (2H, q, -$O\underline{CH_2}$Me)
	3.2—4.4 (1H, br, -OH)	4.21 (2H, s, -NCH_2CO-)	7.80 (1H, s, C_6-H)
M-9[b]	1.34 (3H, t, -$OCH_2\underline{CH_3}$)	3.50 (2H, t. -NCH_2-)	3.70—3.92 (5H, -NCH_3, -OCH_2-)
	4.20 (2H, s, -OCH_2CO-)	4.31 (2H, q, -$O\underline{CH_2}$Me)	7.66 (1H, s, C_6-H)
	8—11 (1H, br, -COOH)		
M-10[c]	1.29 (3H, t, -$OCH_2\underline{CH_3}$)	3.60 (3H, s, -NCH_3)	4.08 (2H, d, -NCH_2-)
	4.20 (2H, q, -$O\underline{CH_2}$Me)	6.17 (1H, t, -NH-)	7.82 (1H, s, C_6-H)
	12.0—13.5 (1H, br, -COOH)		

a) NMR spectra were measured by JEOL-JMN-PS-100 spectrometer (100 Mc) with tetramethylsilane as internal reference.
b) In $CDCl_3$.
c) In DMSO-d_6.

Table II. Summary of Analytical Data of M73101 and Its Metabolites

Compound	mp (°)	Formula	Mass[a] M^+ (*m/e*)	UV[b] λ_{max} (mμ)	IR[c] (cm^{-1})	GLC[d] (Relative t_R)	TLC[f] (*Rf*)
M73101	90—91	$C_{11}H_{17}N_3O_3$	239.1251	312	ν_{max}^{KBr} 1610 (CO)	1.00	0.58
M-1	229—231	$C_9H_{13}N_3O_2$	211.0986	303	ν_{max}^{KBr} 3080 (OH) 1605 (CO)	—	0.05
M-2	Oil	$C_{11}H_{19}N_3O_4$	257.1349	304	ν_{max}^{film} 3400—3300 (OH) 1610 (CO)	1.64	0.09
M-3	Oil	$C_{11}H_{19}N_3O_4$	257.1348	302	ν_{max}^{film} 3600—3000 (-NH, -OH) 1600 (-CO-)	1.64	0.15
M-4	101—103	$C_9H_{15}N_3O_3$	213.1133	304	ν_{max}^{KBr} 3260—3200 (OH) 1605 (CO)	1.14	0.18
M-5	Oil	$C_{11}H_{17}N_3O_4$	255.1276	310	—	1.47	0.32
M-6	185—187	$C_{10}H_{15}N_3O_3$	225.1107	318	ν_{max}^{KBr} 1630 (CO)	1.22	0.38
M-7	110—112	$C_{11}H_{15}N_3O_4$	253.1052	282	ν_{max}^{KBr} 1650, 1605 (CO)	1.28	0.49
M-8	127—129	$C_{11}H_{17}N_3O_5$	271.1113	306	ν_{max}^{KBr} 3160 (OH) 3000—2400 (COOH) 1725, 1600 (CO)	1.82[e]	0.13
M-9	121—123	$C_{11}H_{17}N_3O_5$	271.1135	304	ν_{max}^{KBr} 3240 (NH) 3000—2200 (COOH) 1715, 1615 (CO)	1.89[e]	0.23
M-10	180—181	$C_9H_{13}N_3O_4$	227.0906	298	ν_{max}^{KBr} 3320 (NH) 3000—2200 (COOH) 1715, 1615 (CO)	1.17[e]	0.26

a) Mass spectra were measured by JMS-01SG-type double focusing mass spectrometer.
b) In H_2O.
c) IR spectra were measured by model DS-701G, JASCO, diffraction grating spectrometer.
d) GLC spectra were measured by Shimazu gas chromatograph GC-4B, M73101 showed t_R 18 min.
e) Ethylesterified sample.
f) M73101 and M-1—M-7: solvent system A; M-8— M-10: solvent system C.

were slightly different, and two deuterizable protons newly came out at δ 4.75. From these NMR data, it was surely speculated that this metabolite contained the partial structure of bis(β-hydroxyethyl)amino group. These spectral data mentioned above indicated that the structure of metabolite M-2 should be 5-bis(2-hydroxyethyl)amino-4-ethoxy-2-methyl-3(2H)-pyridazinone. Furthermore, the structure of this metabolite was established by comparison with the synthesized authentic sample.[7]

M-3——The high resolution mass spectrum showed a molecular ion at *m/e* 257.1348 corresponding to $C_{11}H_{19}N_3O_4$, which differed from that of M73101 with an addition of 28 mass units. In NMR spectrum of M-3, signals of $-CH_3$ at N-2 (s, at δ 3.73), $-OCH_2CH_3$ at C-4 (t, at δ 1.34 and q, at 4.38) and –H at C-6 (s, at δ 7.55) indicated the presence of the partial structure of M73101 except for morpholino group, whereas eight protons originated from four methylenes of morpholino group of M73101 were also remained in this metabolite with significantly different splitting pattern, and only two of them could attributed to a methylene adjacent to N. Further, two labile protons appeared at δ 2.56 and 4.84 (easily deuterated with D_2O). From such close inspection of NMR spectrum, this metabolite was suggested to contain 2-(β-hydroxyethyloxy)ethylamino group ($HOCH_2CH_2OCH_2CH_2NH-$). On the basis of these data and by the comparison of spectral data (UV, mass, and NMR) of this metabolite with those of synthesized authentic sample, the structure of metabolite M-3 was concluded to be 4-ethoxy-5-[2-(β-hydroxyethyloxy)ethylamino]-2-methyl-3(2H)-pyridazinone.

M-4——The high resolution mass spectrum showed a molecular ion *m/e* 213.1133 corresponding to $C_9H_{15}N_3O_3$, which differed from that of M73101 with decrease in 26 mass units. From the same reasons as mentioned in sections of M-2 and M-3, this metabolite had the

7) M. Takaya, T. Yamada, and H. Shimamura, *Yakugaku Zasshi*, accepted.

partial structure of M73101 other than morpholino group. The NMR spectrum of M-4 was very close to that of M73101, except for a decrease of the number of protons which could be attributed to aliphatic methylene and an appearance of two labile protons easily deuterated; *i.e.* only four protons of two methylenes with a typical A_2X_2 type at δ 3.40 (2H) and 3.82 (2H) and two broad protons at δ 4.0 and 5.0 were shown in this instance. From these data, the structure of metabolite M-4 was concluded tentatively to be 4-ethoxy-5-(2-hydroxyethylamino)-2-methyl-3(2H)-pyridazinone. Moreover, M-4 showed a complete identity to synthetic sample[7] in UV, infrared (IR), mass and NMR spectra as depicted in Table I and II.

M-5——The high resolution mass spectrum showed a molecular ion at *m/e* 255.1276 corresponding to $C_{11}H_{17}N_3O_4$, which differed from that of M73101 with an addition of 16 mass units. From the same reasons as mentioned in case of M-2 and M-3, this metabolite had the partial structure of M73101 other than morpholino group. For protons shown in NMR spectrum of M-5 which could be assigned to aliphatic one originated from morpholino group of M73101, there were observed four methylene protons at δ 3.22—3.48 (m), two methylene protons at δ 3.64—3.84 (m) and one methine proton at δ 5.04, the latter of which shifted to δ 6.00 after acetylation indicated that a hydroxy group was present on the carbon with one proton. From the above spectral data, the presence of hydroxymorpholino moiety was suggested, leaving the position of hydroxy group uncertain. In addition, metabolite M-5 was converted to metabolite M-2 by reduction with sodium borohydride in ethanol. This fact was reasonably interpreted by posturating M-5 to have a intramolecular hemiacetal structure, which was strongly supported by mass and NMR data just mentioned above. After all, the structure of M-5 was determined to be 4-ethoxy-5-(2-hydroxytetrahydro-1, 4-oxazin-4-yl)-2-methyl-3(2H)-pyridazinone.

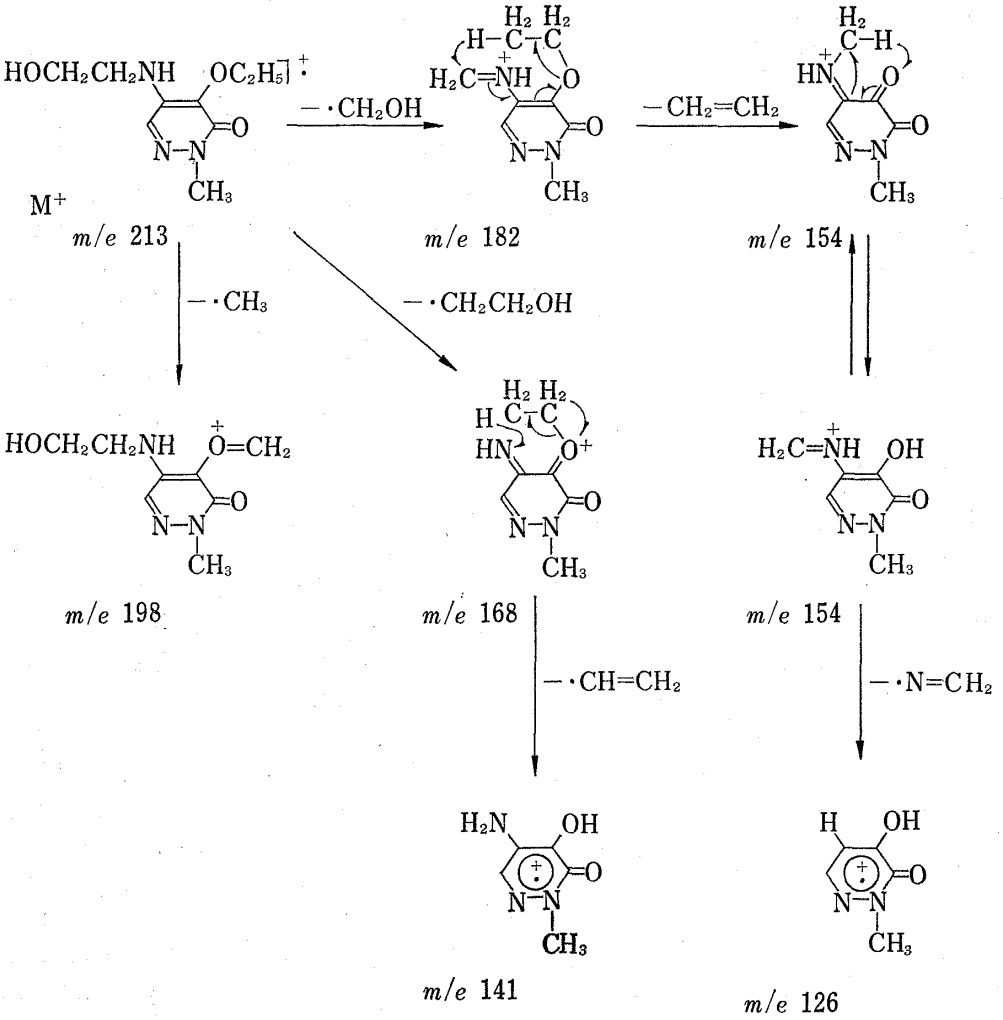
M-6——The high resolution mass spectrum showed a molecular ion at *m/e* 225.1107 corresponding to $C_{11}H_{15}N_3O_3$, which differed from that of M73101 with a decrease in 14 mass units. The NMR spectrum of M-6 was very similar to that of M73101 except for a disappearance of singlet peak stemed from N-methyl group of M73101. From these data, the structure of M-6 should be 4-ethoxy-5-morpholino-3(2H)-pyridazinone. The structure of M-6 was, furthermore, made certain by comparison of UV, IR, mass and NMR spectra depicted in Table I and II with those of synthesized authentic sample.[7]

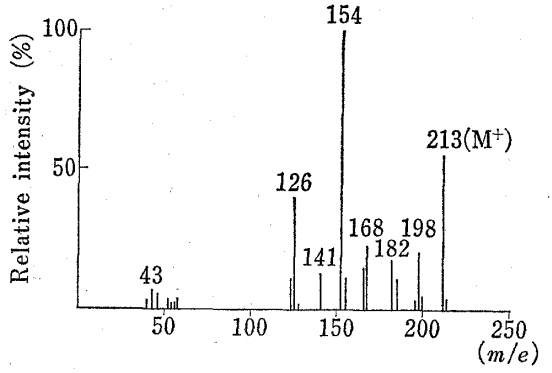
M-7——The high resolution mass spectrum showed a molecular ion at *m/e* 253.1052 corresponding to $C_{11}H_{15}N_3O_4$, which differed from that of M73101 with an addition of 14 mass units. The part other than morpholino group of M73101 was left unaltered judging from NMR signals of $-CH_3$ at N-2, $-OCH_2CH_3$ at C-4 and –H at C-6. Furthermore, the triplet signal observed at δ 3.36 (4H) in case of M73101 which were assigned to two methylenes adjacent to N of morpholino ring shifted to the lower field, at δ 4.08, as triplet one containing two protons in case of this metabolite, and a sharp singlet signal composed of two protons newly arised at δ 4.40. On the other hand, IR spectrum showed a strong absorption at 1650 cm^{-1}, suggesting –CO–NH– group. From these results, the structure of M-7 was identified to be 4-ethoxy-2-methyl-5-(3-oxo-tetrahydro-1,4-oxazin-4-yl)-3(2H)-pyridazinone.

M-8——The high resolution mass spectrum showed a molecular ion *m/e* 271.1113 corresponding to $C_{11}H_{17}N_3O_5$, which differed from that of M73101 with an addition of 32 mass units. Its chromatographic behaviour, its partition properties between the aqueous phase (pH 2) and chloroform, the presence of an IR absorption band at 1725 cm^{-1} and its ability to form an ethylester indicated this metabolite to have a carboxylic acid group. The NMR spectrum of M-8 showed a singlet peak at δ 4.21 which could be assigned to two methylene protons and multiplet peaks of seven protons at δ 3.20—3.80 including three protons of N-methyl group, while the signals seen at δ 3.36 (4H) in spectrum of M73101 which was attributed to two methylene next to N of morpholino ring were lost in case of M-8. The signals of N-methyl and O-ethyl group observed in spectrum of M73101 were also present in the same pattern in case of this metabolite. These inspection of NMR data indicated metabolic change on

the carbon of the morpholino group of M73101, by which the four methylenes of morpholino group in M73101 were diminished to three in this compound, two of which were shifted to the lower field simultaneously with an alteration of splitting pattern. From these facts, the structure of M-8 was concluded to be 5-(N-carboxymethyl-N-2-hydroxyethylamino)-4-ethoxy-2-methyl-3(2H)-pyridazinone and it was finally proved by comparison with authentic sample.[7]

M-9——The high resolution mass spectrum showed a molecular ion at *m/e* 271.1135 corresponding to $C_{11}H_{17}N_3O_5$, which differed from that of M73101 with an increase in 32 mass units. From the same reasons described in case of M-8, it was certain that this metabolite also contained a carboxylic acid group. In NMR spectrum, signals at δ 1.34 (t,





Peak	Obsd.	Calcd.	Molecular formula
M^+ 213	213.1132	213.1113	$C_9H_{15}N_3O_3$
m/e 198	198.0929	198.0879	$C_8H_{12}N_3O_3$
m/e 182	182.0938	182.0930	$C_8H_{12}N_3O_2$
m/e 168	169.0734	169.0773	$C_7H_{10}N_3O_2$
m/e 154	154.0678	154.0617	$C_6H_8N_3O_2$
m/e 141	141.0531	141.0538	$C_5H_7N_3O_2$
m/e 126	126.0435	126.0429	$C_5H_6N_2O_2$

Fig. 3. Fragmentation Pattern of Metabolite M-4

3H), 3.80 (s, 3H), 4.31 (q, 2H), and 7.66 (s, 1H) indicated that the partial structure of M73101 other than morpholino group was left intact. On the other hand, some of peaks which were assigned to methylenes of morpholino ring in case of M73101 shifted to the lower field and had the changed signal pattern in this instance; *i.e.* signals corresponding to one couple of the neighbouring two methylenes on morpholino group of M73101 remained unchanged in M-9 but those assigned to another group of two methylenes of M73101 disappeared in this metabolite, while in return a new singlet signal containing two protons came out at δ 4.20 in M-9. These NMR data and the fact of the presence of carboxylic group in M-9 unequivocally concluded its structure as 5-[2-carboxymethyloxy)ethylamino]-4-ethoxy-2-methyl-3(2H)-pyridazinone, which was strongly supported by mass spectral data just mentioned above. Comparison of the chromatographic and spectral properties of this metabolite M-9, with those of the synthesized authentic sample[7] showed complete identity.

M-10——The high resolution mass spectrum showed a molecular ion at *m/e* 227.0906 corresponding to $C_9H_{13}N_3O_4$, which differed from that of M73101 with a decrease in 12 mass units. From the same reasons described in case of M-8, it was certain that this metabolite, too, contained a carboxylic acid group. The partial structure of M73101 except for a morpholino group was proved to be remained unchanged from NMR signals at δ 1.29 (t, 3H), 3.60 (s, 3H), 4.20 (q, 2H), and 7.82 (s, 1H), but peaks attributed to eight methylene protons of morpholino group in case of M73101 did not observed in this metabolite, while doublet signal containing two protons and triplet peak corresponding to one deuterizable proton newly appeared at δ 4.08 and 6.17, respectively. The only structure compatible with these properties was one which carboxymethylamino group substituted at C-5 instead of morpholino group in M73101, *i.e.* 5-carboxymethylamino-4-ethoxy-2-methyl-3(2H)-pyridazinone.

Investigation of Mass Spectra of the Metabolites——The analysis of fragmentation pattern for each metabolite was achieved by high resolution mass spectrum as well as low resolution one of deuterized samples and by the detection of metastable ion by accelerating voltage scan method. Fragmentation pattern of each metabolite contained main peaks at *m/e* 182, 168, 154, 141, and 126 all of which were common to all metabolites (except M-2), and gave strong evidences for elucidation of structure. One example of them was illustrated in Fig. 3 about M-4.

Quantitative Analysis of Urinary Metabolites

Extracts from the urine of rabbits were applied to GLC for quantitative analysis according to the method reported previously.[8] Synthetic samples were used as standard for making caribration curves. Results were summarized in Table III.

Table III. Excretion Percentage of Urinary Metabolites in Rabbits

M73101	M-1	M-3	M-4	M-5	M-6	M-7	M-8	M-9	M-10	Total
1.0± 0.1	Trace		2.3± 0.3	trace	0.5± 0.2	1.0± 0.3	16.4± 2.1	44.8± 1.3	7.8± 0.5	73.8± 2.8

Each value represents mean ± S.E. (n=5).

Toxicological and Pharmacological Studies of Metabolites

As already described, in rabbit, ten metabolites were found, seven of which were identified also in the case of human. Among these metabolites, seven ones which were available synthetically were subjected to such pharmacological tests as described in the preceding section.

8) T. Seki, T. Hayashi, M. Ohki, and T. Kishikawa, *Rinsho Yakuri*, **9**(2), 149 (1978).

Acute toxicity of urinary metabolites were apparently extremely low and only a slight decrease of spontaneous motor activity was observed even at the dose of 1000 mg/kg (*i.p.*), while in the case of M73101, the animals died in a convulsion at the lethal dose and its LD_{50} value was 695 mg/kg (*i.p.*). In analgesic and anti-inflammatory tests, urinary metabolites produced scarcely significant effects, while M73101 did marked significant effects. These results are summarized in Table IV.

Table IV. Pharmacological Activities of Urinary Metabolites of M73101 in Mice[a]

Compound	Acute toxicity LD_{50} (mg/kg, *i.p.*)	Analgesic activity ED_{50} (mg/kg, *i.p.*)	Anti-inflammetory activity inhibitory %[b]
M-1	>1000	>100 (25%)[c]	19.0
M-2	>1000	>100 (25%)	6.2
M-4	>1000	>100 (25%)	−6.7[e]
M-6	>1000	>100 (25%)	3.5
M-8	>1000	>100 (25%)	5.2
M-9	>1000	>100 (12.5%)	4.6
M-10	>1000	>100 (25%)	5.5
M73101	695 (638—758)[d]	42 (26—67)[d]	50.5

a) Eight animals were used for each dose.
b) At a dose of 200 mg/kg (*i.p.*).
c) The percentage shows the portion of mice with positive analgesic response at a dose of 100 mg/kg (*i.p.*).
d) 95% confidence limits.
e) A minus sign expresses the increase of edema.

Discussion

From the results mentioned in the preceding sections, it became evident that the following biotransformation occurred in the metabolism of M73101: N-demethylation, O-deethylation and oxidation of morpholino group, the latter of which was a main route in these changes. The elucidation of structures of ten metabolites provided us with the outline of metabolic pathway of M73101 as illustrated in Fig. 4.

One route of transformation of morpholino group was *via* carbinolamine intermediate (A), which was the product of hydroxylation of carbon atom adjacent to nitrogen atom, and might be in equilibrium with aldehyde with open chain (A′). This posturated intermediate was thought to be very prone to undergo further rapid metabolism. M-7 was probably a product of the direct oxidation of (A) and M-9 might be derived from the oxidation of (A′). On the other hand, the alcohol M-3 was a reductive product of (A′).

Another route of biodegradation of morpholino group was *via* metabolite M-5 (hemiacetal), which was the product of hydroxylation of carbon atom adjacent to oxygen atom, and also might be in equilibrium with aldehydic alcohol (M-5′). Metabolite M-2 and M-8 were probably reduction and oxidation products of (M-5′), respectively. Metabolite M-4 and M-10 were assumed to be derived from further oxidation of M-2, M-3, M-8, or/and M-9, but details of the sequence of biodegradation were not fully clarified. As described above, biotransformation of morpholino group was the most important pathway in the metabolism of M73101.

It has been already reported that morpholino groups in some compounds are converted enzymatically *in vivo* and *in vitro*, *e.g.* in Doxapram,[9] 2-morpholinoethyl group substituted on pyrrolidinone ring is converted to 2-bis(2-hydroxyethyl)aminoethyl, 2-(β-hydroxyethylamino)ethyl, 2-aminoethyl and 2-(3-oxo-tetrahydro-1,4-oxazin-4-yl)ethyl substituents, and in

9) J.E. Pitts, R.B. Bruce, and J.B. Forehand, *Xenobiotica*, 3(2), 73 (1973); T. Fujita, R. Ogawa, A. Ujiie, and T. Takeda, *Masui*, **XXIII(5)**, 424 (1975).

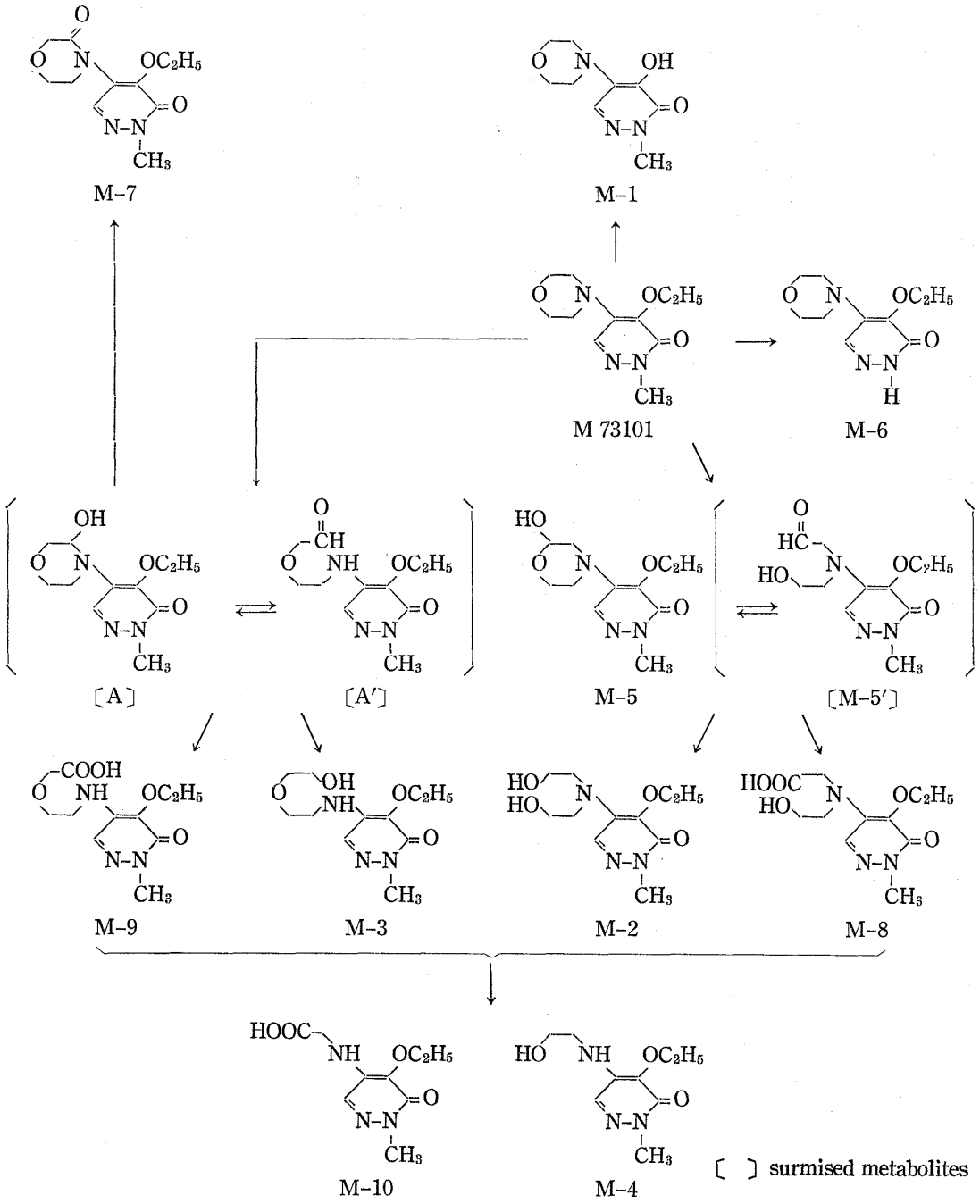


Fig. 4. The Proposed Metabolic Pathways of M 73101

Fomocain[10] morpholino group substituted on alkyl chain is converted to N-oxide and subsequently to carboxylic acid by further oxidative N-dealkylation, while in DS-210,[11] morpholino group substituted on pyrimidine ring undergoes the oxidative cleavage at ether bond. But the entire profile of metabolic sequence of a morpholino substituent is not revealed in each investigation. In the present study, authors identified structures of eight metabolites resulted from biooxidation of morpholino moiety, from which much more details about the metabolic pathway of morpholino group systematically came to light.

10) H. Oelschlaeger, D.J. Temple, and C.F. Temple, *Xenobiotica*, **5(5)**, 309 (1975); H. Oelschlaeger and D.J. Temple, *ibid.*, **7**, 95 (1977); N, Jindrora, *Pharmazie*, **31**, 580 (1976).
11) Y. Nakai, Y. Shirakawa, and T. Fujita, *Japan. J. Pharmacol.*, **22**, 102 (1972).

The quantitative aspects of urinary metabolites which are summarized in Table III indicate that in rabbit, the main metabolite was M-9 which was produced by route *via* carbinolamine intermediate. About 45% of the dose administered was excreted as metabolite M-9, and the percentage of M-9 in total metabolites was about 60%. There was excreted 16.4% of the dose as metabolite M-8 in urine. The metabolites of deethylation and demethylation *i.e.* M-1 and M-6 were in very small amounts. The total excretion percentage of M73101 and its metabolites within 24 hr was 74% of the dose administered. The quantitative results also show that unchanged M73101 was hardly excreted (only 1% or less), which suggests that M73101 was very subject to the metabolic transformation in the rabbit.

As reported previously,[8] main metabolite in human was M-8 which was produced by route *via* M-5 (hemiacetal), and so a great difference between rabbit and human in the metabolism of M73101 was the position of hydroxylation on morpholino ring. We will refer in the subsequent report to details about the species differences including human in metabolism of M73101.

Toxicological and pharmacological activities of seven metabolites found in rabbit or human were studied in mice comparing with the parent compound, M73101. In such pharmacological tests as toxicological, analgesic, and anti-inflammatory experiments, neither of metabolites tested showed a significant action at all though M73101 had pronounced effects in all aspects, which indicated that the pharmacological activity of M73101 was not due to these urinary metabolites.