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Studies on Sulfamethoxazole/Trimethoprim. Absorption, Distribution, Excretion and Metabolism of Trimethoprim in Rat

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Absorption, distribution, excretion and metabolism of trimethoprim were studied in rats. Trimethoprim was absorbed rapidly and almost completely from the digestive tract, the half-life for absorption being 18 min. After absorption the drug left the blood rapidly and was taken up by various organs except brain. The highest concentration was found in the kidney. Appreciable levels were found in the bone marrow, thyroid, liver and lung. The lowest drug concentration was noted in the brain.

Approximately 95% of the radioactivity after oral administration of ^{14}C-trimethoprim was recovered from the urine and feces within 72 hr. The urinary excretion is the major excretory route since more than 85% of the total radioactivity recovered appeared in the 72 hr urine. About 30% of the radioactivity excerted in 8 hr urine was present as intact ^{14}C-trimethoprim.

Metabolic pathways of trimethoprim consisted of O-demethylation, ring N-oxidation and α-hydroxylation. 3-Demethyl-trimethoprim was the largest metabolite which accounted for more than 30% of the radioactivity excreted in 8 hr urine. 4-Demethyl-trimethoprim was excerted mainly as glucuronide which accounted for about 19%. Trimethoprim ring N-oxide and α-hydroxy-trimethoprim were excreted in amounts of 7 and 5%, respectively.

Simultaneous administration of sulfamethoxazole did not influence the absorption, distribution, excretion and metabolism of trimethoprim in rat.

Trimethoprim[2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine] is an inhibitor of dihydrofolate reductase which potentiates the activity of sulfonamide against a wide variety of bacterial species.[2,3] This study was designed to elucidate the absorption, distribution, excretion and metabolism of trimethoprim (TMP) in the rat following oral administration. Since this drug is clinically used in combination with sulfonamide 5-methyl-3-sulfanilamido-isoxazole(sulfamethoxazole),[4] effect of simultaneous administration of sulfamethoxazole (SMX) upon the absorption, distribution, excretion and metabolism of TMP was also investigated.

Experimental

^{14}C-TMP and Mode of Administration——TMP labeled with ^{14}C in position 2 of the pyrimidine ring (22 μCi/mg), which is shown in Fig. 1, was obtained from Burroughs Wellcome, England. Male Sprague-

1) Location: *Toda-shi, Saitama.*
2) E. Grunberg and W.F. Delorenzo, "Antimicrobial Agents and Chemotherapy," 1966, pp. 430—433.
3) S.R.M. Bushby and G.H. Hitchings, *Brit. J. Pharmacol. Chemother.*, 33, 72 (1968).
4) D.T.D. Hughes, C.D.M. Drew, T.B.W. Johnson, and J.D. Jarvis, *Chemotherapy*, **14**, 151 (1969).

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Dawley rats weighing about 200 g were fasted for 16 hr before dosing. A 20 mg/kg dose of the labeled drug was given orally to rats in the form of a suspension in 5% gum arabic. A combination of ^{14}C-TMP (20 mg/kg) and SMX (100 mg/kg) was administered in the same manner.

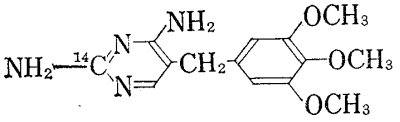

Fig. 1. Structure of ^{14}C-TMP

Absorption——At 10, 15, 20, 30, 60, 90, and 120 min after the oral administration of ^{14}C-TMP or a combination of ^{14}C-TMP and SMX, the gastrointestinal tract was removed and slit open for gastrointestinal contents to be washed out in 20 ml of cold physiological saline containing 5% N HCl. To the washed saline solution were added equal volume of 10% Na_2CO_3 and two volumes of chloroform. After shaking for 5 minutes, an aliquot of the chloroform extract was determined for ^{14}C, and then analyzed for unchanged ^{14}C-TMP by thin-layer chromatography (TLC) in solvent system I of chloroform–*n*-propanol–NH_4OH (80: 20:1).[5]

Distribution——At 0.5, 1, 2, 4, 6 and 24 hr after oral administration of ^{14}C-TMP or the combination of two drugs, blood was collected by cardiac puncture, and then brain, lung, liver, kidney, spleen, adrenal, thyroid, bone marrow, and skin were removed. Plasma and tissues were homogenized with 4 volumes of 0.1N HCl. After centrifugation, an aliquot of the supernatant fluid was determined for ^{14}C and then analyzed for unchanged ^{14}C-TMP as follows. To one volume of plasma or tissue homogenates were added 8 volumes of 5% Na_2CO_3 and 10 volumes of chloroform. After shaking for 5 minutes, the chloroform phase was removed as completely as possible and the extraction repeated twice, using an equal volume of chloroform. The three chloroform extracts were pooled. The contribution of unchanged TMP towards the radioactivity of the chloroform extract was determined by TLC.[5]

Excretion——Rats treated orally with ^{14}C-TMP or the combination of two drugs were kept in metabolic cages to ensure separate collection of urine and feces. Urine and feces were collected at various time intervals up to 72 hr. Urine was diluted with distilled water to a fixed volume and measured for ^{14}C. Feces was homogenized with 10 volumes of 0.1N HCl, and the supernatant was counted for ^{14}C.

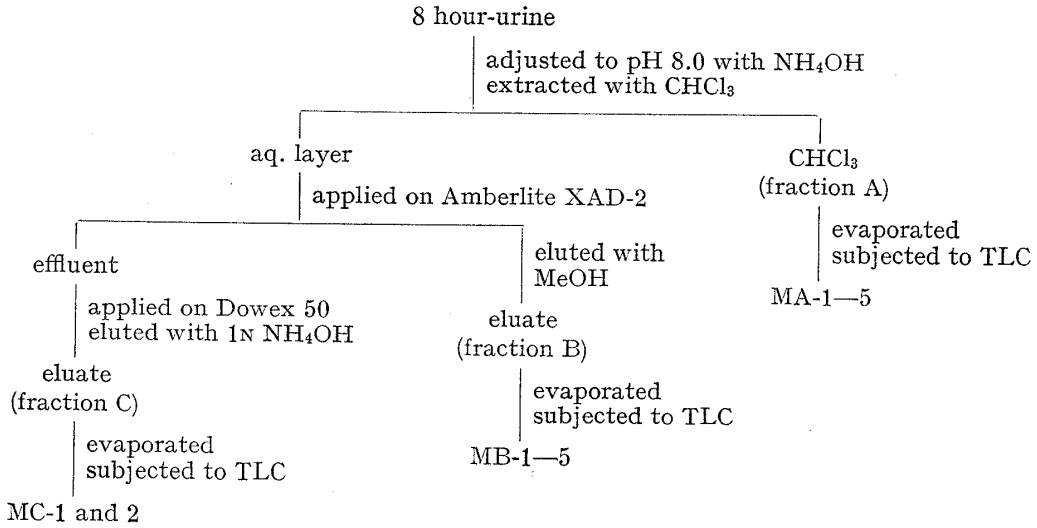

Chart 1. Major Steps used to Separate Urinary Metabolites

Isolation of Urinary Metabolites——^{14}C-TMP which was diluted with nonradioactive TMP was administered orally to 20 rats in a dose of 200 mg/kg (The oral LD_{50} for TMP was more than 2 g/kg in rat.[3]). Eight hour urine samples were used for isolation of metabolites. The procedure for the separation of urinary metabolites is outlined in Chart 1. The urine sample (200 ml) was adjusted to pH 8 with NH_4OH, and then extracted three times with equal volume of chloroform. The residual aqueous solution was retained for analysis of hydrophilic metabolites. Chloroform extract (fraction A) was concentrated to a small volume and spotted on Silica gel GF_{254} plates (250 μm thick, activated at 110° for 60 min). Thin-layer plates were developed by the ascending technique with the solvent systems described in the legend to Table II. Radioactive metabolites were detected by scanning chromatograms with an Aloka thin-layer chromatograms scanner TRM-1B. Color development of metabolites were carried out with Ehrlich reagent[6] and Folin–Ciocalteau reagent.[7] After scanning the chromatograms, the various radioactive zones were separately

5) D.E. Schwartz and W.H. Ziegler, *Postgrad. Med. J. Suppl.*, **45**, 32 (1969).
6) M. Shimizu and S. Ichimura, *J. Pharm. Soc.*, **78**, 1183 (1958).
7) D. Waldi, "Thin-Layer Chromatography," Springer-Verlag, Berlin, 1965, p. 498.

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scraped from the plates, and the metabolites were eluted from the silica gel with methanol. The eluates were concentrated to dryness under vacuum, and the residues were extracted with chloroform or chloroform–methanol (1:1). The solvent was removed under reduced pressure and the white or slightly yellow coloured residues were dried under vacuum. These residues were used for spectroscopic analyses.

The residual aqueous solution above mentioned was passed through the column (2.5×30 cm) of Amberlite XAD-2, the effluent being retained for analysis of conjugated metabolites. After washing the column with distilled water, methanol was used to eluate adsorbed metabolites. Methanol eluate (fraction B) was concentrated to a small volume under reduced pressure and then subjected to TLC with solvent systems I, II, and III.

The effluent from the column of Amberlite XAD-2 was applied on the column (1×30 cm) of Dowex 50W×4 (H^+). After washing the column with distilled water, 1N NH_4OH was used to eluate adsorbed metabolites. The eluate (fraction C) was concentrated to a small volume under reduced pressure and then subjected to TLC with solvent systems I, II, and III. Enzymatic hydrolysis of conjugated metabolites was made with use of β-glucuronidase (obtained from Sigma Chemical Co.).

Identification——Identification of isolated metabolites was accomplished by spectroscopic analyses including ultraviolet (UV), infrared (IR), nuclear magnetic resonance (NMR), and mass spectroscopy. UV spectra were taken of TMP and its metabolites dissolved in methanol, 0.1N HCl or 0.1N NaOH. The mass spectra of TMP and its metabolites were taken on a RMS-4 mass spectrometer (Hitachi Co.) with a direct solids inlet operating at 200°. NMR spectra of TMP and its metabolites were taken on JEOL-C60 NMR spectrometer (Japan Electron Optics Laboratory Co.) in $(CD_3)_2SO$ solution, with tetramethylsilane as an internal standard.

Quantitative Determination of Urinary Metabolites——Eight hour urine samples of rats given ^{14}C-TMP (20 mg/kg) or a combination of ^{14}C-TMP (20 mg/kg) and SMX (100 mg/kg) were used for quantitative determination of metabolites. Separation of metabolites was carried out in a similar manner as described for isolation of urinary metabolites. However, the procedure of application of the conjugated metabolites on Dowex 50W×4 column was omitted, since the ion exchange resin of Dowex 50W×4 was not useful for quantitative adsorption and elution of metabolites. Therefore, the effluent containing conjugated metabolites from Amberlite XAD-2 column was concentrated to a small volume and then subjected to TLC with solvent system III. After scanning thin-layer chromatograms, radioactive spot areas were scraped into counting vials and extracted with 1 or 2 ml methanol. Fifteen ml of solution of 7 g 2,5-diphenyloxazole and 50 mg 1,4-*bis*-2-(5-phenyloxazolyl)-benzene in one liter of 50% ethanol–toluene was added and the vials were counted in an Aloka liquid scintillation spectrometer LSC-502 equipped with an automatic quenching monitor system. This procedure resulted in quantitative detection of TMP and all metabolites originally spotted on the plates.

Result

Absorption

Figure 2 illustrates the rate of disappearance of ^{14}C-TMP from the gastrointestinal tract after oral administration of ^{14}C-TMP or the combination of ^{14}C-TMP and SMX to rat. Nearly one half of the administered dose had disappeared within 20 minutes and after 2 hours the recovery was less than 5%. When the data were plotted semilogarithmically a straight line was obtained; the half-lives of TMP for the absorption were estimated to be 18 min after oral administration of ^{14}C-TMP and 19 min after oral administration of the combination of ^{14}C-TMP and SMX. Total amounts of TMP absorbed during 2 hours were not significantly influenced by the simultaneous administration of SMX.

Distribution

Figure 3 illustrates time-course of levels of intact ^{14}C-TMP in the plasma after oral administration of ^{14}C-TMP and the combination of ^{14}C-TMP and SMX. Highest levels of the unaltered drug were noted at 20 minutes after administration, and thereafter the levels of the drug decreased rapidly. After 24 hours the intact drug was scarcely detected in plasma and tissues, although very small amounts of metabolites were present in the plasma, liver and kidney.

Table I shows the distribution of unchanged ^{14}C-TMP in the different tissues after oral administration of ^{14}C-TMP and the combination of ^{14}C-TMP and SMX. The highest concentration of the drug was found in the kidney; appreciable levels were found in the bone marrow, thyroid, liver and lung; somewhat lower level was found in the skin; and very low

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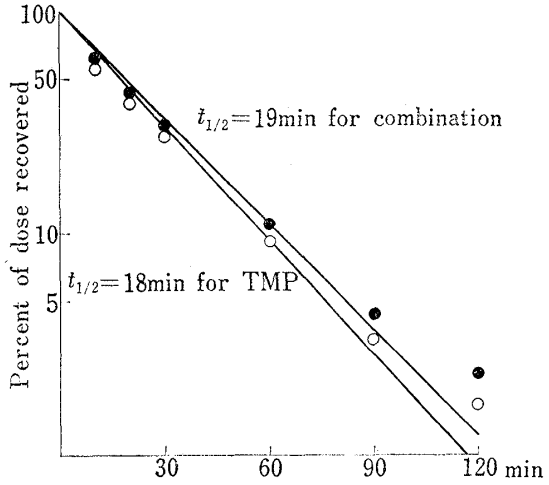

Fig. 2. Rate of Disappearance of ^{14}C–TMP from Gastrointestinal Tract of Rat after Administration of ^{14}C–TMP or a Combination of ^{14}C–TMP and SMX

—○—: TMP —●—: combination
Each point indicates the mean of four animals.

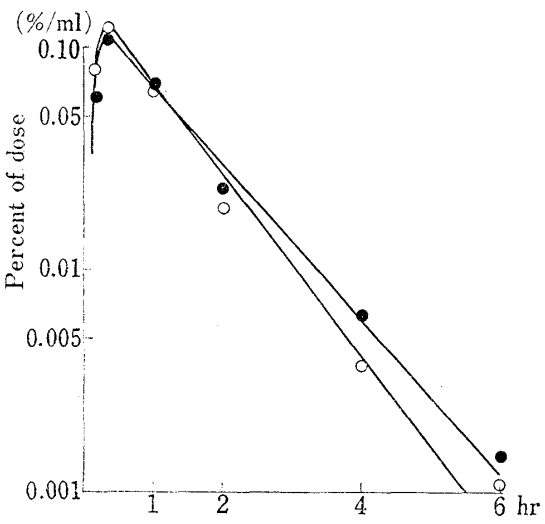

Fig. 3. Plasma Levels of ^{14}C–TMP after Oral Administration of ^{14}C–TMP or a Combination of ^{14}C–TMP and SMX

—○—: TMP —●—: combination
Each point indicates the mean of 3 to 4 animals.

concentration was found in the brain. The concentrations of the drug in various tissues except brain were higher than that in plasma.

The simultaneous administration of SMX did not influence the distribution pattern of TMP.

Excretion

Figure 4 shows the cumulative excretion of ^{14}C in the urine and feces up to 72 hours after oral administration of ^{14}C-TMP and the combination of ^{14}C-TMP and SMX. Eighty

Table I. Distribution of Intact ^{14}C–TMP in Various Tissues of Rat after Oral Administration of ^{14}C–TMP or a Combination of ^{14}C–TMP and SMX

		0.5 hr μg/g	1 hr μg/g	2 hr μg/g	4 hr μg/g	6 hr μg/g
Plasma	(TMP)[a]	4.0±0.7	2.4±0.5	0.7±0.2	0.15±0.03	0.04±0.01
	(combination)[b]	4.0±0.9	2.7±0.4	0.9±0.2	0.25±0.04	0.05±0.01
Liver	(TMP)	16.3±3.8	8.0±1.8	1.9±0.4	0.33±0.07	0.07±0.02
	(combination)	17.6±2.9	11.4±2.0	3.3±0.5	0.74±0.14	0.10±0.02
Kidney	(TMP)	31.0±5.1	20.9±4.3	9.0±2.0	1.80±0.31	0.31±0.06
	(combination)	30.8±4.6	23.6±3.9	11.4±1.8	1.92±0.36	0.34±0.06
Lung	(TMP)	15.5±3.0	8.2±1.7	2.4±0.5	0.39±0.07	0.05±0.01
	(combination)	15.8±3.4	10.4±2.2	4.2±0.7	0.55±0.10	0.09±0.02
Spleen	(TMP)	10.9±2.5	6.0±1.4	1.3±0.2	0.15±0.02	0.04±0.01
	(combination)	12.0±2.0	6.6±1.1	2.1±0.3	0.28±0.04	0.07±0.01
Brain	(TMP)	0.4±0.1	0.3±0.1	0.1±0.03	0.05±0.01	0.02±0.005
	(combination)	0.4±0.1	0.3±0.1	0.1±0.03	0.06±0.01	0.02±0.005
Adrenal	(TMP)	12.9±1.8	7.9±1.2	1.2±0.2	0.12±0.02	0.03±0.01
	(combination)	13.2±1.8	8.5±1.5	1.7±0.3	0.21±0.03	0.05±0.01
Thyroid	(TMP)	18.4±4.0	8.0±1.6	0.9±0.2	0.23±0.04	0.07±0.02
	(combination)	18.0±3.3	8.2±1.6	1.1±0.2	0.30±0.05	0.10±0.02
Bone marrow	(TMP)	19.8±3.4	8.5±2.0	1.6±0.3	0.33±0.06	0.09±0.02
	(combination)	19.4±2.8	8.8±1.6	1.8±0.3	0.48±0.07	0.12±0.02
Skin	(TMP)	3.1±0.6	2.6±0.4	0.5±0.1	0.11±0.02	0.03±0.01
	(combination)	2.9±0.5	2.5±0.5	0.6±0.1	0.17±0.02	0.04±0.01

Data represent the mean of three animals in μg/g of tissues.
a) A single 20 mg/kg dose of ^{14}C-TMP was administered orally.
b) A combination of 20 mg/kg of ^{14}C-TMP and 100 mg/kg of SMX was administered.

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two % of the orally administered radioactivity was excreted in the 72 hour urine, and almost all of the urinary excretion occurred in the first 24 hours. About 13% of the administered radioactivity was excreted in 72 hour feces. Consequently, approximately 95% of the administered radioactivity was excreted in the urine and feces within 72 hours.

The simultaneous administration of SMX did not practically influence the urinary and fecal excretion of the administered radioactivity.

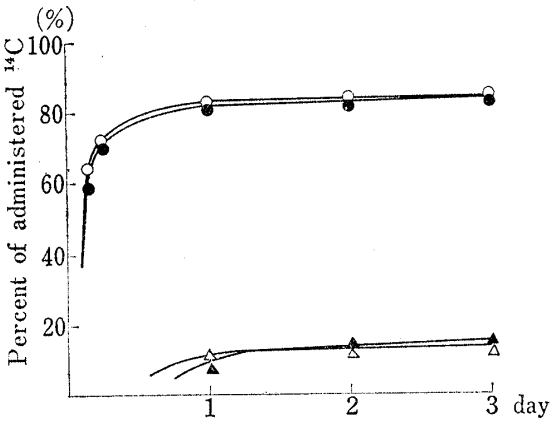

Fig. 4. Cumulative Excretion of ^{14}C in Urine and Feces after Oral Administration of ^{14}C–TMP or a Combination of ^{14}C–TMP and SMX

TMP: —○— urine and —△— feces
combination: —●— urine and —▲— feces
Each point indicates the mean of four animals.

Metabolism

When 8 hour urine samples of rats given ^{14}C-TMP were extracted with chloroform at pH 8, about 35% of the urinary radioactivity was transferred into chloroform phase (fraction A). Next, when the residual aqueous solution was applied on Amberlite XAD-2 column, about 50% of the urinary radioactivity was adsorbed on the column and about 15% appeared in the effluent. The radioactivity adsorbed on the column was eluted quantitatively with methanol (fraction B). When the effluent from Amberlite XAD-2 column was applied on Dowex 50W×4 column, about 90% of the applied radioactivity was adsorbed on the column. Ninty five% of the adsorbed radioactivity was eluted with 1N NH_4OH (fraction C).

When fraction A, B, and C were subjected to TLC in solvent systems I, II, and III, five radioactive peaks were observed in fraction A, five peaks in fraction B, and two peaks in fraction C, as shown in Table II.

Table II. *Rf* Values of TMP and Its Metabolites

	Rf values		
	Solvent I	Solvent II	Solvent III
TMP	0.71	0.64	0.44
Fraction A	0.16 (MA-1)	0.84	0.55
	0.32 (MA-2)	0.68	0.49
	0.49 (MA-3)	0.58	0.47
	0.71 (MA-4)	0.64	0.44
	0.82 (MA-5)	0.68	0.58
Fraction B	0.0	0.02—0.09 (MB-1)	0.03—0.10
	0.0	0.42 (MB-2)	0.40
	0.49	0.58 (MB-3)	0.47
	0.32	0.68 (MB-4)	0.49
	0.16	0.84 (MB-5)	0.55
Fraction C	0.0	0.02—0.09	0.03—0.10 (MC-1)
	0.0	0.14	0.22 (MC-2)

Chromatography was performed on Silica gel GF_{254} with solvent systems: I, chloroform–*n*-propanol–NH_4OH (80:20:1); II, chloroform–ethanol–water–acetic acid (20:20:10:1); III, *n*-butanol–ethanol–water (4:1:1).
Separation of urine sample into fraction A, B and C was described under Experimental.

MA-1——Table III shows prominent peaks of UV spectra and reaction to color reagents for TMP and its metabolites. The UV spectrum taken at acid pH for MA-1 resembled that found for TMP. However, UV absorption for MA-1 shifted to higher wavelength going from

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Table III. Prominent Peaks of UV Spectra and Reaction to Color Reagent for TMP and Its Metabolites

	UV spectra			Color reaction of	
	In 0.1N HCl	In methanol	In 0.1N NaOH	Folin–Ciocalteau reagent	Ehrlich reagent
TMP	272	292	292	−	+
MA-1	274	327	327	+	+
MA-2	263	289	289	−	+
MA-3	270	291	293	+	+
MA-4	272	292	292	−	+
MA-5	267	288	288	−	+
MB-1[a)]	270	291	293	+	+
MB-2	274	282	290	+	+
MB-3	270	291	293	+	+
MB-4	263	289	289	−	+
MB-5	274	327	327	+	+
MC-1[a)]	270	291	293	+	+
MC-2[a)]	274	282	290	+	+

a) MB-1, MC-1 and MC-2 were treated with β-glucuronidase before UV spectra were taken.

acid to base as compared with that of TMP. MA-1 responded positively to Folin–Ciocalteau reagent. These fact suggested that phenolic hydroxyl group was present in MA-1. Table IV shows mass spectral analyses of TMP and its metabolites. The mass spectrum of MA-1 indicated its molecular weight to be 306 which is 16 more than that of TMP. This fact suggested that MA-1 was a oxygenated derivative of TMP. Prominent peaks common to MA-1 and TMP appeared at *m/e* 290 ($C_{14}H_{18}O_3N_4$), 275 ($C_{13}H_{15}O_3N_4$), 259 ($C_{13}H_{15}O_2N_4$), and 123 ($C_5H_7N_4$), the probable structures of which are depicted in Fig. 5. The presence of the fragment ion at *m/e* 139 ($C_5H_7ON_4$), which is formed probably by the loss of trimethoxyphenyl ring, as shown in Fig. 5, suggested that the oxygen was not substituted on the phenyl ring and must be substituted on the pyrimidine moiety. When MA-1 was treated with zinc powder in the presence of acetic acid and then rechromatographed in solvent system I, it was found that MA-1 was converted to original TMP. This fact suggested that the oxygen was not attached to carbon atom of pyrimidine ring but to nitrogen atom. The IR spectrum of MA-1 has a single broad peak at 1650 cm^{-1} as its most prominent feature. This peak is most likely a composite of N-oxide bond. From these data, it may be concluded that MA-1 is TMP ring N-oxide. However, from the present data, the point of attachment of the oxygen to either nitrogen of the pyrimidine ring could not be established. Recently, Rey-Bellet and

Table IV. Mass Spectral Analysis of TMP and Its Metabolites

	Molecular ion composition *m/e*	Difference from TMP	Prominent ions common to metabolites *m/e*
TMP	290 ($C_{14}H_{18}O_3N_4$)		275, 259, 243 and 123
MA-1	306 ($C_{14}H_{18}O_4N_4$)	+O	290, 275, 259, 139 and 123
MA-2	306 ($C_{14}H_{18}O_4N_4$)	+O	289, 275, 259, 139 and 123
MA-3	276 ($C_{13}H_{16}O_3N_4$)	$-CH_2$	261, 245 and 123
MA-4	290 ($C_{14}H_{18}O_3N_4$)		275, 259, 243 and 123
MA-5	304 ($C_{14}H_{16}O_4N_4$)	+O, $-H_2$	289, 273, 257 and 137
MB-2	276 ($C_{13}H_{16}O_3N_4$)	$-CH_2$	261, 245 and 123
MB-3	276 ($C_{13}H_{16}O_3N_4$)	$-CH_2$	261, 245 and 123
MB-4	306 ($C_{14}H_{18}O_4N_4$)	+O	289, 275, 259, 139 and 123
MB-5	306 ($C_{14}H_{18}O_4N_4$)	+O	290, 275, 259, 139 and 123

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Reiner[8] reported that *N*-oxide metabolite of TMP was identical in every respect with a synthetic compound of TMP 1-oxide [2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine 1-oxide], of which the crystal structure had been determined by X-ray diffraction method.[9]

MA-2——The UV spectra taken at acid and alkaline pH for MA-2 resembled those found for TMP. MA-2 did not respond to Folin–Ciocalteau reagent. These facts suggested that MA-2 had no phenolic hydroxyl group. The mass spectrum of MA-2 gave a molecular ion

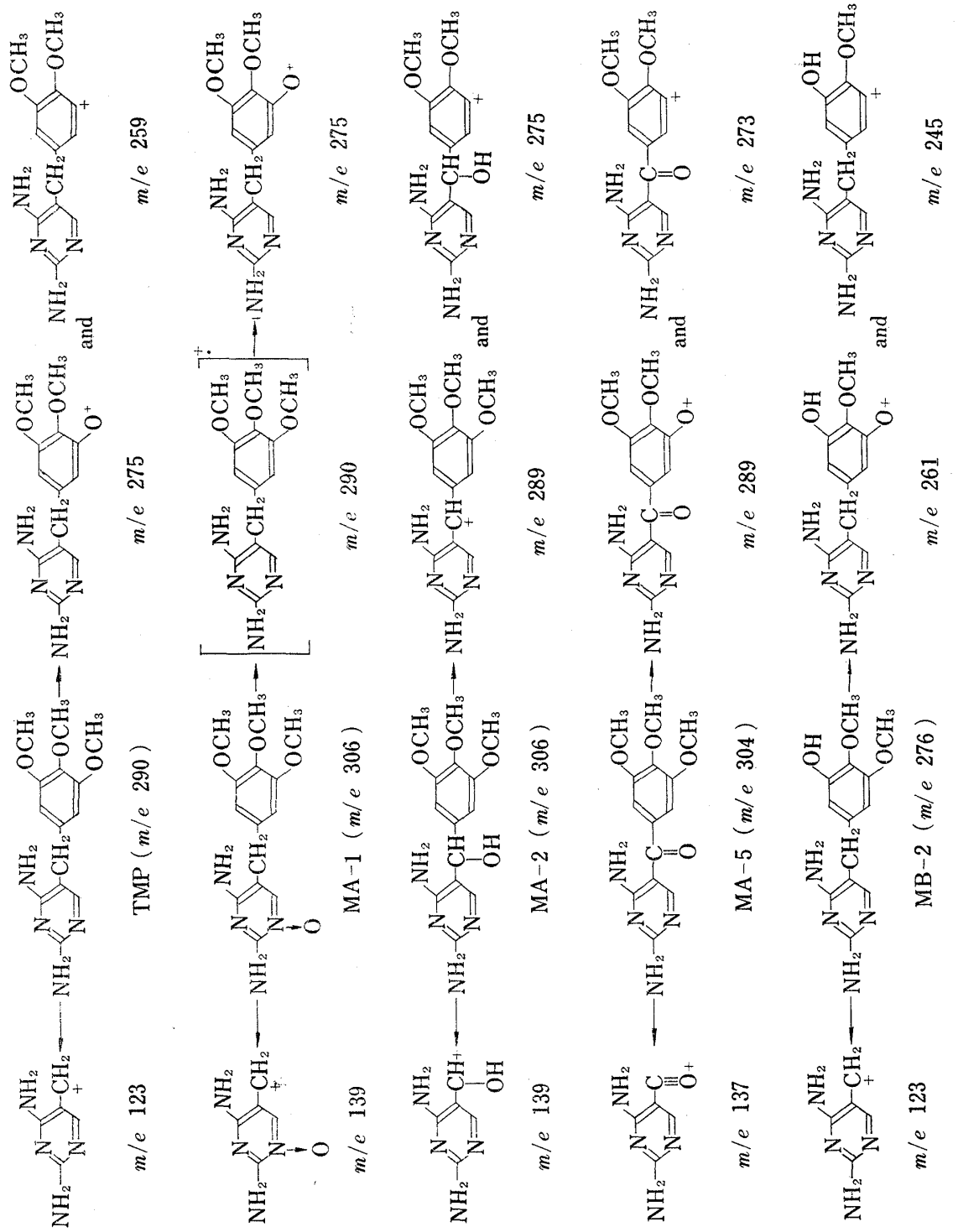

Fig. 5. Structures of Selected Fragments of TMP and Its Metabolites

8) G. Rey-Bellet and R. Reiner, *Helv. Chim. Acta*, **53**, 945 (1970).
9) W.E. Oberhansli, *Helv. Chim. Acta*, **53**, 1787 (1970).

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at *m/e* 306. The increase in weight of 16 mass units of the molecular ion of MA-2 as compared with TMP indicated that this compound was oxygenated derivative of TMP. Prominent peaks common to MA-2 and TMP appeared at *m/e* 275, 259, and 123, as shown in Table IV. The presence of the fragment ion at *m/e* 139 ($C_5H_7ON_4$), which is also found for MA-1, suggested that the oxygen must be substituted on either amino group of the pyrimidine moiety or the methylene bridge. The NMR spectrum of MA-2 had a prominent peak at 4.30 τ which probably belongs to CH, and the proton count of MA-2 at 4.30 τ corresponded to one proton, as shown in Table V. This fact suggested that the oxygen was not attached to either amino group of the pyrimidine moiety but to the methylene bridge. From these facts, it may be concluded that MA-2 is α-hydroxy-TMP [2,4-diamino-5-(α-hydroxy-3,4,5-trimethoxybenzyl)-pyrimidine]. In fact, the NMR and IR spectra of MA-2 were identical to those of a synthetic sample of α-hydroxy-TMP which was a gift from F. Hoffmann-La Roche, Basel.

Table V. NMR Data of TMP and Its Metabolites

Compound	Signal and number of proton			
	Phenyl τ[a)]	Pyrimidine τ	Methylene τ	O-Methyl τ
TMP	3.25 (2H, s)	2.16 (1H, s)	6.28 (2H, s)	6.10 (3H, s) 6.20 (3H, s) 6.39 (3H, s)
MA-2	3.12 (2H, s)	2.30 (1H, s)	4.30 (1H, s)	6.08 (3H, s) 6.17 (3H, s) 6.26 (3H, s)
MB-2	2.80 (1H, d) 3.07 (1H, d)	2.34 (1H, s)	6.21 (2H, s)	6.05 (3H, s) 6.12 (3H, s)
MB-3	2.70 (2H, s)	2.49 (1H, s)	6.14 (2H, s)	6.03 (6H, s)

a) Chemical shift in τ from tetramethylsilane, solvent $(CD_3)_2SO$.
s: singlet, d: doublet

MA-3 was shown to be identical to MB-3 by UV and mass spectra.

MA-4——MA-4 had the same *Rf* values (in solvent systems I, II, and III) as the authentic sample of TMP. The UV, IR, and mass spectra of MA-4 were identical to those of the authentic sample of TMP. From these data, it could be concluded that MA-4 was unchanged TMP.

MA-5——The UV spectra taken at acid and alkaline pH for MA-5 resembled those found for TMP. The mass spectrum of MA-5 gave a molecular ion at *m/e* 304. The increase in weight of 14 mass units of the molecular ion of MA-5 as compared with TMP suggested that this metabolite might be a monomethylated derivative of TMP. However, when MA-2 was treated with chromic acid for the oxidation of α-hydroxy-TMP, it was found that MA-2 was converted to MA-5. This fact suggested that MA-5 was not methylated derivative of TMP but oxygenated one. The presence of the fragment ion at *m/e* 137, which is formed probably by the loss of trimethoxyphenyl ring, suggested that the oxygen must be substituted on the methylene bridge. The decrease in weight of 2 mass units of molecular ion of MA-5 as compared with MA-2 indicated that MA-5 was a dehydrogenated derivative of MA-2. Furthermore, the IR spectrum of MA-5 showed a carbonyl band at 1660 cm^{-1}. From these data, it seems likely to conclude that MA-5 is carbonyl-TMP [2,4-diamino-5-(3,4,5-trimethoxybenzoyl)pyrimidine]. Indeed, the IR and mass spectrum of MA-5 were identical to those of a synthetic sample of carbonyl-TMP which was a gift from F. Hoffmann-La Roche, Basel.

MB-1——MB-1 responded positively to naphthoresorcinol indicative of glucuronide. When MB-1 was incubated with β-glucuronidase and then rechromatographed in solvent system II, it was found that hydrolyzed product had the same *Rf* value as MB-3. Therefore, it was assumed that MB-1 was a glucuronide of MB-3.

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MB-2——MB-2 which was excreted most largely in urine was assumed to have phenolic hydroxyl group by a bathochromic shift in UV spectra and positive reaction to Folin–Ciocalteau reagent. The mass spectrum of MB-2 gave a molecular ion at *m/e* 276. The decrease in weight of 14 mass units of the molecular ion of MB-2 as compared with TMP suggested that this compound was a monodemethylated TMP. Furthermore, the NMR spectrum of MB-3 had a prominent peak at 6.05 and 6.11 τ which probably belong to -OCH_3, as shown in Table V. The proton count of MB-3 at 6.05 and 6.11 τ corresponded to six protons. This fact strengthened that this compound was O-monodemethylated. The NMR spectrum of the phenyl protons of MB-2 showed resonance for two protons between 2.80 and 3.07 τ while that of TMP showed a singlet at 3.15 τ. The lack of symmetry in the phenyl proton resonance of MB-2 indicates that the hydroxyl group of MB-2 is in the 3 position of the phenyl ring. To confirm the substitution position of the hydroxyl group in MB-2, MB-2 was converted to phenolic acid by treating with $KMnO_4$[10] and then oxidized product was subjected to paper chromatography in solvent system of isopropanol–NH_4OH–H_2O (8:1:1). The phenolic acid derived from MB-2 had *Rf* value of 0.38, which was different from that of syringic acid but was similar to that of 3,4-dimethoxy-5-hydroxybenzoic acid.[11] From these facts, it seems reasonable to conclude that MB-2 is 3-demethyl-TMP [2,4-diamino-5-(3-hydroxy-4,5-dimethoxybenzyl)pyrimidine].

MB-3——A bathochromic shift in the UV spectra and positive reaction to spray reagent for MB-3 suggested that phenolic hydroxyl group was present in MB-3. The mass spectrum of MB-3 gave a molecular ion at *m/e* 276. The decrease in weight of 14 mass units of the molecular ion of MB-3 as compared with TMP suggested that this metabolite was a monodemethylated TMP. Comparison of MB-2 and MB-3 spectra showed a striking similarity in the fragmentation and in the variation of intensity with mass number. This suggested that MB-3 was an isomer of MB-2. In the NMR spectrum of MB-3 the two phenolic protons and the six protons of OCH_3 groups appeared as singlets at 2.70 τ and 6.03 τ respectively, indicating a symmetrical substitution of the benzene nucleus. Furthermore, phenolic acid obtained by treating MB-3 with $KMnO_4$ had the same *Rf* value (0.16) as the authentic sample of syringic acid. From these data, it seems reasonable to conclude that MB-3 is 4-demethyl-TMP [2,4-diamino-5-(4-hydroxy-3,5-dimethoxybenzyl)pyrimidine].

MB-4——Since the UV and mass spectra of MB-4 were identical to those of MA-2, it could be concluded that MB-4 was α-hydroxy-TMP.

MB-5——Since the UV and mass spectra of MB-5 were identical to those of MA-1, it could be concluded that MB-5 was TMP ring N-oxide.

MC-1 and 2——MC-1 and 2 responded positively to naphthoresorcinol indicative of glucuronides. When MC-1 and 2 were incubated with β-glucuronidase and then passed through the column of Amberlite XAD-2, more than 90% of both the applied radioactivity was adsorbed on the column. Methanol eluates from the column were subjected to TLC in solvent system II. Hydrolyzed product of MC-1 had *Rf* value of 0.58, which was corresponded to that of MB-3, and that of MC-2 had *Rf* value of 0.42, which was corresponded to that of MB-2. The UV and mass spectra of hydrolyzed products of MC-1, and 2 were identical to those of MB-3 and MB-2, respectively. Therefore, it could be concluded that MC-1 was a glucuronide of MB-3 and MC-2 was a glucuronide of MB-2.

Quantitative Determination of Urinary Metabolites

Table VI shows quantitative determination of urinary metabolites excreted for 8 hours after oral administration of ^{14}C-TMP or the combination of ^{14}C-TMP and SMX. Approximately 75% of the administered radioactivity was excreted in 8 hour urine. About 30% of the urinary radioactivity was present as intact ^{14}C-TMP. Of the radioactivity excreted in

10) D.E. Schwartz, B.A. Koechlin, and R.E. Weinfeld, *Chemotherapy. Suppl.*, **14**, 22 (1969).
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8 hour urine of rat given ^{14}C-TMP, the largest part (more than 30%) was 3-demethyl-TMP (MB-2). TMP ring N-oxide (MA-1 and MB-5) was about 7%, α-hydroxy-TMP (MA-2 and MB-4) was about 5%, and 4-demethyl-TMP (MA-3 and MB-3) was about 1%. Carbonyl-TMP (MA-5) accounted for only 0.1% of the urinary radioactivity. Approximately 19% of the urinary radioactivity was excreted as glucuronide of 4-demethyl-TMP and 6% excreted as glucuronide of 3-demethyl-TMP.

The simultaneous administration of SMX did not significantly influence the urinary excretion of intact TMP and its metabolites.

Table VI. Quantitative Determination of Urinary Metabolites

Metabolites	Corresponding compounds	TMP[a] (75.4%)	Combination[b] (76.1%)
MA-1 and MB-5	TMP ring N-oxide	7.4±0.7	6.6±0.7
MA-2 and MB-4	α-hydroxy-TMP	4.6±0.5	3.4±0.4
MA-3 and MB-3	4-demethyl-TMP	1.1±0.2	1.1±0.2
MA-4	TMP	30.6±4.3	33.9±3.8
MA-5	carbonyl-TMP	0.1±0.02	0.1±0.02
MB-2	3-demethyl-TMP	31.3±3.5	31.8±3.1
MB-1 and MC-1	glucuronide of MA-3 (MB-3)	18.9±2.5	17.1±2.0
MC-2	glucuronine of MB-2	6.0±1.1	6.0±1.2

Data were expressed as the mean percentage of the urinary radioactivity of ^{14}C-TMP and its metabolites found in each of urines of four animals (± standard deviation).
Values in parentheses indicate per cent recovery of the administered radioactivity.
a) The urine samples were collected for 8 hours after oral administration of a single 20 mg/kg dose of ^{14}C-TMP.
b) The urine samples were collected for 8 hours after oral administration of a combination of 20 mg/kg of ^{14}C-TMP and 100 mg/kg of SMX.

Discussion

Kaplan, *et al.*[12] reported that TMP was absorbed completely from the digestive tract of dogs and distributed highly to tissues. In the present study using rats rapid and complete absorption of TMP from the digestive tract was also demonstrated by rapid and almost complete disappearance of the drug from the gastrointestinal tract and the attainment of maximal plasma levels within 30 min. Rapid uptake of TMP into extensive organs except brain was indicated by attainment of highest levels within 30 min after oral administration. Blood levels were low as a result of rapid uptake of the drug by tissues. Release of TMP from tissues was fairly rapid as shown by the very low organ levels 6 hours after administration and by the negligible organ levels at 24 hours.

Studies of the radioactivity of the excreta of rats after an oral dose of ^{14}C-TMP have shown that about 95% of administered ^{14}C can be recovered from the urine and feces within 72 hours. Excretion in the urine seemed to be the main route of elimination of TMP and its metabolites, since more than 85% of ^{14}C excreted within 72 hours was recovered from the urine.

Studies of urinary metabolites showed that about 30% of the radioactivity excreted for 8 hours was present as intact TMP and the remainder was metabolites. The largest metabolite in the urine was 3-demethyl-TMP (MB-2), which accounted for more than 30% of the urinary radioactivity, whereas 4-demethyl-TMP accounted for only about 1%. Contrary to the free form of demethylated metabolites, glucuronide (MC-2) of 3-demethyl-TMP accounted for 6% of the urinary radioactivity whereas that (MB-1 and MC-1) of 4-demethyl-TMP accounted for about 19%. These data indicate that 4-demethyl-TMP is more readily

12) S.A. Kaplan, R.E. Weingeld, S. Cotler, C.W. Abruzzo, and K. Alexander, *J. Pharm. Sci.*, **59**, 358 (1970).

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conjugated with glucuronic acid than 3-demethyl-TMP. As shown in Table II, 4-demethyl-TMP had higher *Rf* values in three solvent systems than 3-demethyl-TMP and the former (MA-3) was slightly extracted with $CHCl_3$ but the latter was scarcely done. These facts suggested that the former had a more higher lipid-solubility than the latter. It is of interest to note that more lipid-soluble the metabolite is, more readily is it conjugated with glucuronic acid.

Comparison of O-demethylation of TMP at 3 and 4 positions showed that O-demethylation occurred more greatly at 3 position than at 4 position. Ratcliffe and Smith[13] reported that mescaline, which has trimethoxy group in its molecule like TMP, was demethylated at 4 and 5 positions. Musacchio and Goldstein[14] reported that rat demethylated N-acetyl-mescaline (N-acetyl-3,4,5-trimethoxyphenylethylamine) more largely to N-acetyl-3,5-dimethoxy-4-hydroxyphenylethylamine than to N-acetyl-3,4-dimethoxy-5-hydroxyphenylethylamine whereas Griffiths[12] reported that a major metabolite of 3,4,5-trimethoxybenzoic acid in rat was 3,4-dimethoxy-5-hydroxybenzoic acid.

Harley-Mason, *et al.*[15] isolated 3,4-dihydroxy-5-methoxyphenylacetic acid from urine of human given mescaline. However, the result of the present study showed that further demethylated metabolites of 3-demethyl- or 4-demethyl-TMP could not be detected in the urine of rat given TMP.

TMP ring N-oxide (MA-1 and MB-5) accounted for about 7% of the urinary radioactivity. The biological N-oxidation of aliphatic tertiary amines has already been shown to occur in several drugs,[16–20] but a few reports of the oxidation of aromatic ring nitrogen atom have

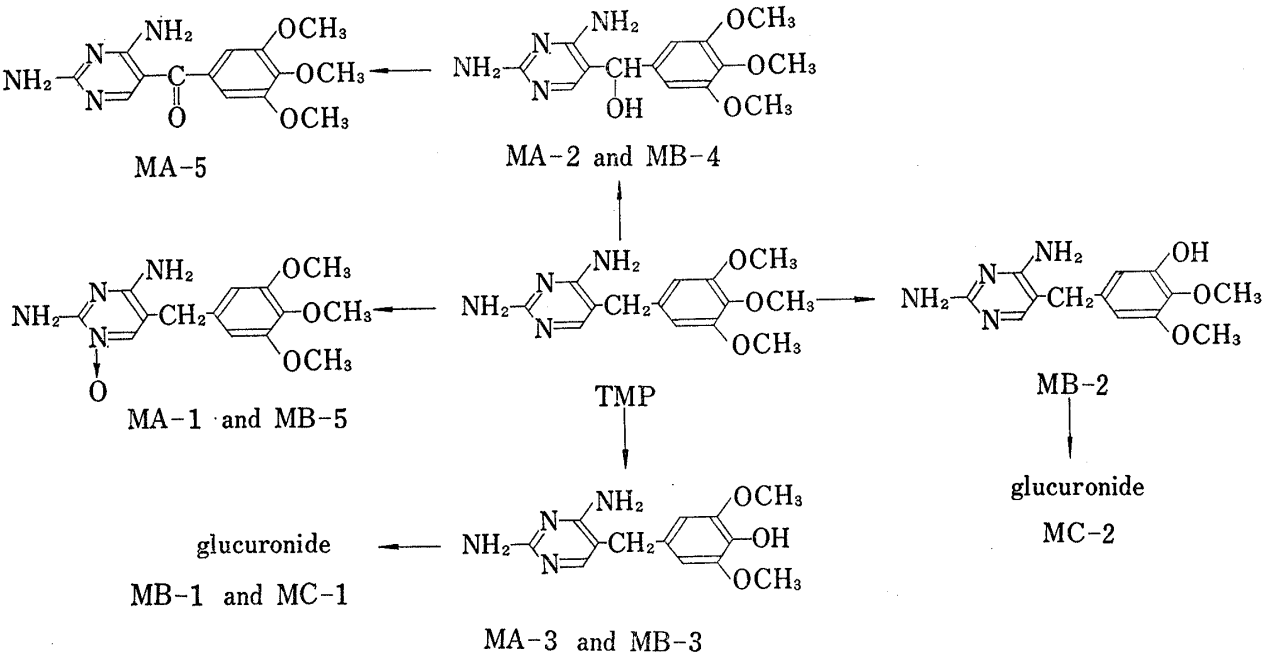

Fig. 6. Possible Metabolic Pathways of TMP

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been found. Bonavita, *et al.*[21] and Chaykin, *et al.*[22] identified one metabolite of nicotinamide as nicotinamide N-oxide. Zins[23] and Zins, *et al.*[24] reported that diallylmelamine[2,4-diamino-6-diallylamino-S-triazine] was metabolized to a ring *N*-oxide of the parent molecule.

α-Hydroxy-TMP (MA-2 and MB-4) accounted for 5% of the urinary radioactivity. Oxidation of alkyl groups is common metabolic pathway during drug metabolism. Therefore, it is clear that α-hydroxy-TMP is formed by the oxidation of the methylene bridge of TMP.

The presence of carbonyl-TMP (MA-5) as the smallest metabolite was demonstrated in the urine of rat given TMP. This metabolite is assumed to be formed by the oxidation of α-hydroxy-TMP, since it is known that secondary alcohols are oxidized to carbonyl compounds.[25]

From a consideration of the facts described above, it seems most reasonable to conclude that the metabolic fate of TMP includes 3 different pathways of O-demethylation, ring N-oxidation, and α-hydroxylation. Therefore, reactions shown in Fig. 6 may be proposed for the metabolism of TMP.

Recently, Schwartz, *et al.*[26] reported that the simultaneous administration of SMX did not influence the metabolic fate and renal excretion of TMP in man. In the present study using rats SMX was found not to significantly influence the absorption of TMP from the gastrointestinal tract, the distribution of intact TMP and the excretion of unchanged TMP and its metabolites.

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