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Metabolism of Afloqualone, a New Centrally Acting Muscle Relaxant, in the Rat[1)]

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The metabolism of afloqualone (6-amino-2-fluoromethyl-3-(*o*-tolyl)-4(3*H*)-quinazolinone, AFQ) was studied in rats. AFQ was extensively metabolized by rats after oral administration of ^{3}H-AFQ. Thirteen unconjugated and three conjugated metabolites were isolated from the 24-h urine. Their chemical structures were identified or characterized by infrared (IR), nuclear magnetic resonance (NMR) and mass spectrometry in comparison with synthetic samples. One of the major metabolic pathways of AFQ was acetylation at the 6-amino group followed by hydroxylation of the methyl carbons of the acetyl and 2′-methyl groups. Another important metabolic pathway was the formation of sulfur-containing metabolites in which the fluorine atom at the 2-position of AFQ is replaced by a methylsulfinyl or methylsulfonyl group. These metabolites may be formed *via* the mercapturic acid conjugate(s) of AFQ.

Five metabolites were detected in plasma 1 h after oral administration. They were the *N*-acetylated and hydroxylated metabolites; the sulfur-containing metabolites found in the urine were not detected in plasma.

Keywords——afloqualone; 6-amino-2-fluoromethyl-3-(*o*-tolyl)-4(3*H*)-quinazolinone; centrally acting muscle relaxant; metabolism; methylsulfinyl metabolite; methylsulfonyl metabolite; *N*-glycolylated metabolite; rat

Afloqualone (6-amino-2-fluoromethyl-3-(*o*-tolyl)-4(3*H*)-quinazolinone, AFQ) is a new centrally acting muscle relaxant.[2,3)] In the previous papers,[1,4)] we reported on its absorption, distribution, excretion and whole body autoradiography in mice, rats and rabbits after oral administration of ^{3}H- or ^{14}C-AFQ. It is essential to identify the metabolites and metabolic pathways of AFQ in various animals in order to evaluate the pharmacological and toxicological characteristics of AFQ in these animal species. The present paper describes the isolation and identification of urinary and plasma metabolites of AFQ in the rat and proposes possible metabolic pathways for the drug in this animal species.

Experimental

Chemicals——^{3}H- or ^{14}C-AFQ was synthesized by the method of Tani *et al.*[2)] from ^{3}H- or ^{14}C-labelled *o*-toluidine hydrochloride (^{3}H at the 4-position or methyl-^{14}C at the 2-position, Fig. 1). The specific radioactivities of ^{3}H-AFQ and ^{14}C-AFQ were 1.3 mCi/mg and 9.0 μCi/mg, respectively. Their radiochemical purities were more than 98% as determined by thin-layer chromatography (TLC). ^{14}C-*N*-Acetyl AFQ was synthesized by the reaction of ^{14}C-AFQ with acetic anhydride in tetrahydrofuran (THF). Its specific radioactivity was 0.4 μCi/mg and the radiochemical purity was more than 99% by TLC. In order to confirm the identities of the metabolites, whose structures were deduced from spectrophotometric analyses, some of them were synthesized.[5)] β-Glucuronidase (bovine liver) was obtained from Tokyo Zoki Chemicals (Tokyo) and arylsulfatase (limpet, type IV) and D-glucaric acid 1,4-lactone from Sigma (U.S.A.). *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) was purchased from Gasukuro Kogyo (Tokyo). Other reagents and solvents were of the best grade commercially available.

Animal Experiments——Male Wistar rats weighing 200 to 250 g were used. The animals were fasted for about 16 h before and 4 h after drug administration, while water was given *ad lib.* The labelled drug was suspended in a 0.5%

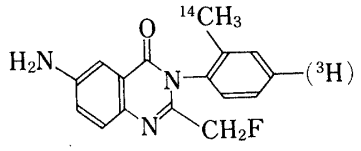


Fig. 1. Chemical Structure and Labelled Positions of ^{3}H- or ^{14}C-AFQ

carboxymethylcellulose solution. ^{3}H-AFQ or ^{14}C-*N*-acetyl AFQ was administered orally to rats at the dose of 20 mg/kg. After administration, the animals were housed in stainless steel metabolism cages. Urine and feces were collected for 72 h after dosing.

In order to examine plasma metabolites, heparinized blood specimens were taken from the abdominal aorta 1 h after oral administration of ^{14}C-AFQ (20 mg/kg). The plasma was separated by centrifugation. The urine and plasma samples were stored at $-20\,^{\circ}C$ until analysis.

Since AFQ is light-sensitive, all procedures were carried out in an area protected from light as much as possible.

Measurement of Radioactivity——Radioactivity was measured in a liquid scintillation spectrometer (Aloka, LSC-652) equipped with an automatic quenching monitor system. The samples of urine and ethyl acetate extracts were dissolved in naphthalene–dioxane scintillator (PPO 5 g, dimethyl POPOP 0.3 g, naphthalene 100 g, dioxane 730 ml, toluene 135 ml and methanol 35 ml) and counted. Aliquots of the plasma and fecal homogenate were combusted in a sample oxidizer (Aloka, ASC-112), and the resulting $^{14}CO_2$ or $^{3}H_2O$ was counted.[4a] Radioactive areas on TLC plates were scraped off into counting vials and extracted with THF or methanol, and then the naphthalene–dioxane scintillator was added to the vials and the radioactivities were measured.

Isolation and Identification of Urinary and Plasma Metabolites——The urine and plasma were fractionated according to the following procedure. The 24-h urine samples of rats orally given ^{3}H-AFQ were passed through a column of Amberite XAD-2. The column was washed with water, and the absorbed radioactivity was eluted with methanol followed by methanol–conc. NH_4OH (95 : 5). The eluates were combined and concentrated to dryness *in vacuo*. The residue was dissolved in water, and then adjusted to pH 9 with 2 N NH_4OH or ammonium carbonate.[6] The alkaline solution was extracted three times with two volumes of ethyl acetate (AcOEt), and this extract was designated as the neutral and basic metabolite fraction.

The residual aqueous layer, after the removal of traces of AcOEt and NH_3 *in vacuo*, was diluted with an equal volume of 0.2 M acetate buffer (pH 5). The solution was incubated with β-glucuronidase and arylsulfatase at 37 °C for 24 to 48 h. In some cases, the enzymatic hydrolysis was conducted in the presence of D-glucaric acid 1,4-lactone, a known inhibitor of β-glucuronidase. After the incubation, the mixture was extracted with AcOEt (hydrolysate fraction) as described above. The residual aqueous solution was adjusted to pH 2 with 2 N HCl and extracted with AcOEt (acidic metabolite fraction). The remaining aqueous layer was designated as the polar metabolite fraction. The 1-h plasma samples of rats orally given ^{14}C-AFQ were diluted with two volumes of water saturated with ammonium carbonate and extracted with AcOEt. The 24-h urine samples of rats orally given ^{14}C-*N*-acetyl-AFQ were diluted with water and extracted with AcOEt at pH 9.

Each AcOEt extract was dried over $MgSO_4$, concentrated to a small volume and subjected to preparative TLC. Each radioactive spot was extracted and purified by repeated TLC with various solvent systems as described below until each gave only one spot. Main metabolites were recrystallized from H_2O or THF–benzene.

TLC: TLC was carried out on Silica gel GF_{254} or $60F_{254}$ plates (Merck) employing the following solvent systems; I = benzene–THF (7 : 3), II = benzene–THF (1 : 1), III = THF–$CHCl_3$–acetone–conc. NH_4OH (15 : 10 : 10 : 1) and IV = *n*-BuOH–AcOH–H_2O (4 : 1 : 1) (all ratios by volume). Radioactive areas on the plate were detected by the use of a TLC scanner (Aloka, TRM-101) and autoradiography on X-ray films (Sakura MARG-^{3}H or Kodak NS-5T), and ultraviolet (UV)-absorbing areas under a UV lamp. The Bratton–Marshall diazotation[7] and $K_2Cr_2O_7$–$AgNO_3$[8] reagents were used as TLC spray reagents. Radioactive areas on the plate were scraped off and eluted with THF or methanol.

Derivatization: Trimethylsilyl (TMS) derivatives were prepared by treating the samples with sufficient amounts of MSTFA at 60 °C for 30 min. Methyl derivatives were prepared by treating the samples with a large excess of CH_2N_2 in a mixture of diethyl ether and ethanol.

Spectrometric Analyses: Infrared (IR) spectra were measured in KBr-microtablets with a Hitachi IR-215 spectrophotometer. NMR spectra were recorded on a JEOL JNM-MH-60 II or JNM-PS-100 spectrometer in $CDCl_3$ or $CDCl_3$–d_6-DMSO solutions, with tetramethylsilane as an internal standard. Electron impact mass spectra (direct-inlet system; DIMS) were measured at an ionization voltage of 20 to 70 eV with a Hitachi RMU-6M or RMU-7M mass spectrometer. Chemical ionization mass spectra (CIMS) were obtained at the ionization potential of 70 eV with isobutane or methane as the reagent gas.[9] In gas chromatography-mass spectra (GC-MS) analysis, MS were obtained with a Hitachi RMU-6MG gas chromatograph mass spectrometer equipped with a Hitachi 002B data processing system. A 1 m × 4 mm glass column packed with 3% OV-1 on Gas Chrom Q (100—120 mesh, Applied Science) was heated from 200 to 300 °C at 3 °C/min. The helium flow rate was 30 ml/min.[10] The ionization voltage was 30 eV and the temperature of the ion source was 180 °C.

Quantitative Determination of Urinary Metabolites of ^{3}H-AFQ——Twentyfour-hour urine samples of rats

given ^{3}H-AFQ were fractionated as described for the isolation of urinary metabolites. Metabolites in each fraction were separated by one- or two-dimensional TLC. After detection under a UV lamp and by autoradiography, the amounts of the metabolites on the plate were estimated by measuring the radioactivity after scraping off the appropriate areas of silica gel into counting vials.

Measurement of Liberated ^3H_2O in Urine——Three 1-ml aliquots of the 24-h urine of rats orally given ^{3}H-AFQ were diluted with ten volumes of water and shaken with 100, 300 and 500 mg of activated charcoal, respectively. Each mixture was allowed to stand at room temperature for 30 min, then centrifuged and the supernatant was counted.

Results

Urinary Metabolites of ^{3}H-AFQ

The 24-h urine (accounting for about 50% of the dose[4a)]) was used for the identification and quantification of AFQ metabolites.

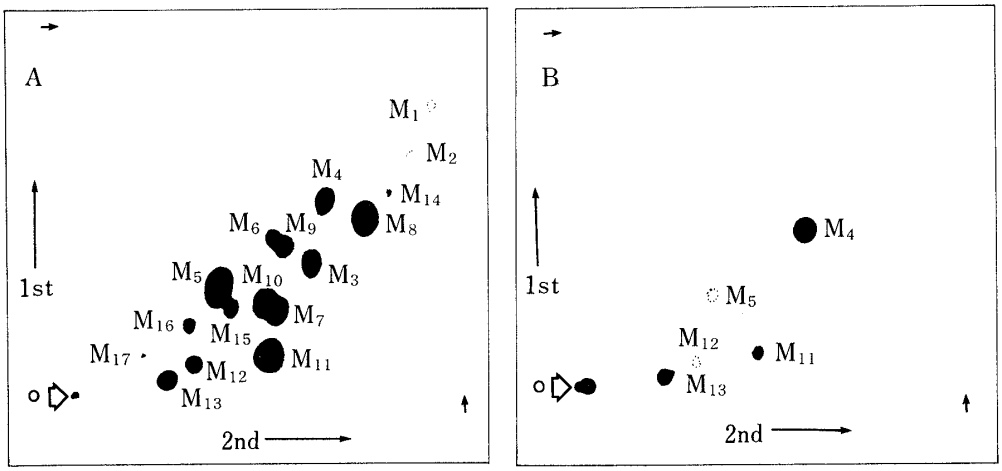


Fig. 2. Two-dimensional TLC Autoradiograms of AcOEt Extracts from the 24-h Urine of Rats after Oral Administration of ^{3}H-AFQ (20 mg/kg)

A) Neutral and basic metabolite fraction.
B) Hydrolysate fraction of glucuronides and sulfates. Solvent systems employed: first dimension (ascending) benzene–THF (1 : 1, v/v) and second dimension (from left to right) THF–$CHCl_3$–acetone–conc. NH_4OH (15 : 10 : 10 : 1, v/v).

TABLE I. MS Data for the Metabolites of AFQ

Metabolites	Selected ion peaks, m/z (Relative intensities, %)	
	M^+	Other ions
AFQ	283 (100)	266 (17), 250 (93), 132 (24), 91 (52)
M_2	325 (55)	292 (62), 283 (45), 250 (100), 132 (16), 91 (49)
M_3	341 (23)	321 (72), 308 (100), 266 (50), 147 (60), 43 (21)
M_4	341 (100)	308 (95), 282 (35), 250 (63), 132 (30), 91 (56)
M_6	339 (100)	308 (71), 250 (35), 248 (14), 132 (20), 91 (77)
M_7	323 (100)	308 (29), 292 (19), 281 (10), 147 (6), 43 (33)
M_9	401 (43)	322 (70), 308 (29), 205 (100), 132 (13), 91 (36)
M_{10}	401 (33)	322 (84), 308 (34), 205 (100), 147 (10), 43 (68)
M_{11}	369 (2)	321 (1), 306 (100), 292 (18), 146 (43), 91 (20)
M_{12}	385 (1)	339 (33), 337 (10), 322 (23), 308 (100), 250 (47)
M_{13}	385 (2)	369 (5), 337 (8), 322 (100), 308 (68), 292 (73)
M_{14}[a)]	467 (62)	452 (91), 364 (17), 73 (100)

a) Di-TMS derivative.

Neutral and Basic Metabolite Fraction——This fraction, containing about 70% of the urinary radioactivity, was subjected to two-dimensional TLC. At least seventeen radioactive spots were observed, as shown in Fig. 2. All of the radioactive spots except M_1 gave negative tests with the diazotation reagent, indicating that the 6-amino group was acylated.

M_1 and M_2——These metabolites were present in trace amounts. The *Rf* values on TLC and the MS of M_1 and M_2 were identical with those of unchanged AFQ and *N*-acetyl AFQ, respectively.

M_3 and M_4——In the MS (Table I), M_3 and M_4 had the same molecular ion peaks at *m/z* 341, which were 16 mass units higher than that of M_2, suggesting that M_3 and M_4 are oxygenated derivatives of M_2. The IR spectra of M_3 and M_4 (Table II) exhibited absorption bands assignable to alcoholic hydroxyl groups at 1010 and 1070 cm^{-1}, respectively. In the mass spectrum of M_3, the fragment ion peaks characteristic of the *o*-tolyl moiety at *m/z* 91

Table II. IR Spectral Data for the Metabolites of AFQ

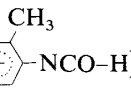
Metabolites	Absorption bands,[a] cm^{-1}						
	$\nu_{C=O}$	$\nu_{S=O}$	ν_{S-O}	ν_{C-F}	ν_{C-O}	Ring	δ_{C-H}
AFQ	1670			1105		835,	755
M_3	1680, 1670			1100	1010	840,	760
M_4	1680, 1670			1098	1070	835,	755
M_5	1685, 1670			1100	1082, 1010	830,	760
M_6	1680, 1670				1075, 1005	840,	760
M_7	1690, 1680				1020	835,	760
M_8	1690, 1680	1310, 1120				840,	760
M_9	1690, 1680	1310, 1120			1080	840,	760
M_{10}	1690, 1675	1310, 1125			1020	840,	760
M_{11}	1695, 1675		1035[b]			840,	760
M_{12}	1690, 1680		1035[b]		1080	850,	770
M_{13}	1690[b]		1030[b]		1030[b]	830,	760

a) KBr microtablet. *b*) Broad.

Table III. NMR Spectral Data for the Metabolites of AFQ

Metabolites	Chemical shifts, ppm[a]
AFQ	2.10 (3H, s, CH_3), 4.95 (2H, d, J=47 Hz, $C\underline{H}_2F$), 5.13 (2H, s, NH_2), 7.0—7.7 (7H, m, arom.)
M_4	2.07 (3H, s, CH_3), 4.15 (2H, d, J=6 Hz, $C\underline{H}_2OH$), 5.00 (2H, d, J=46 Hz, $C\underline{H}_2F$), 5.60 (1H, t, J=6 Hz, OH), 7.40—8.70 (7H, m, arom.), 9.80 (1H, s, NH)
M_7	2.10 (3H, s, CH_3), 2.16 (3H, s, CH_3), 4.35 (2H, d, J=6 Hz, $C\underline{H}_2OH$), 5.05 (1H, t, J=6 Hz, OH), 7.30—8.50 (7H, m, arom.), 10.05 (1H, s, NH)
M_{10}	2.10 (3H, s, CH_3), 3.20 (3H, s, SO_2CH_3), 4.10 (2H, s, CH_2SO_2), 4.35 (2H, d, J=6 Hz, $C\underline{H}_2OH$), 4.95 (1H, t, J=6 Hz, OH), 7.3—8.5 (7H, m, arom.), 10.00 (1H, s, NH)
M_{13}	2.15 (3H, s, CH_3), 2.75 (3H, s, $SOCH_3$), 3.90 (2H, s, CH_2SO), 4.40 (2H, d, J=6 Hz, $C\underline{H}_2OH$), 5.05 (1H, t, J=6 Hz, OH), 7.4—8.5 (7H, m, arom.), 10.15 (1H, s, NH)

a) δ-values in $CDCl_3$–DMSO-d_6 with reference to tetramethylsilane.
Abbreviations: s, singlet; d, doublet; m, multiplet; arom., aromatic protons.

(tropylium) and 132 ( -NCO-H)[11] had disappeared. On the other hand, the nuclear magnetic resonance (NMR) spectrum of M_4 (Table III) showed proton signals assignable to a hydroxymethyl group at δ 4.15 (2H, d; s on addition of D_2O) and 5.60 (1H, t; disappeared on D_2O addition). These chemical shifts are different from those of the 2′-hydroxymethyl group. Thus, M_3 was considered to be *N*-acetyl-2′-hydroxymethyl AFQ and M_4 to be *N*-glycolyl AFQ. Authentic samples were synthesized[5] and their spectra were identical with those of M_3 and M_4.

M_5——The high resolution MS of M_5 (Table IV) gave a molecular ion peak at *m/z* 357.1113, which corresponds to $C_{18}H_{16}FN_3O_4$. This mass number differs from that of M_3 or M_4 by one oxygen atom. This suggested that M_5 was an oxygenated derivative of M_3 or M_4. The IR spectrum showed absorption bands due to the 2′-hydroxymethyl group at 1010 cm^{-1} and the *N*-glycolyl group at 1082 cm^{-1}. In the NMR spectrum (Fig. 3), the signals at δ 4.10

TABLE IV. Compositions of the Fragment Ions of M_5 and M_8 Determined by High Resolution Mass Spectrometry (70 eV)

M_5			M_8		
m/z[a]	Composition	(Δ, mmu)	*m/z*	Composition	(Δ, mmu)
357.1113 (25)	$C_{18}H_{16}FN_3O_4$	(−0.9)	385.1084 (39)	$C_{19}H_{19}N_3O_4S$	(−1.0)
337.1051 (10)	$C_{18}H_{15}N_3O_4$	(−1.0)	306.1240 (100)	$C_{18}H_{16}N_3O_2$	(−0.1)
326.0961 (13)	$C_{17}H_{13}FN_3O_3$	(2.1)	292.1092 (22)	$C_{17}H_{17}N_3O_2$	(0.7)
324.0980 (100)	$C_{17}H_{14}N_3O_4$	(−0.3)	264.1132 (63)	$C_{16}H_{14}N_3O$	(−0.3)
266.0935 (13)	$C_{15}H_{12}N_3O_2$	(0.7)	250.0969 (13)	$C_{15}H_{12}N_3O$	(−0.9)
251.0670 (6)	$C_{11}H_{10}FN_3O_3$	(−3.4)	146.0614 (57)	C_9H_8NO	(0.8)

a) Relative intensities (%) are shown in parentheses.

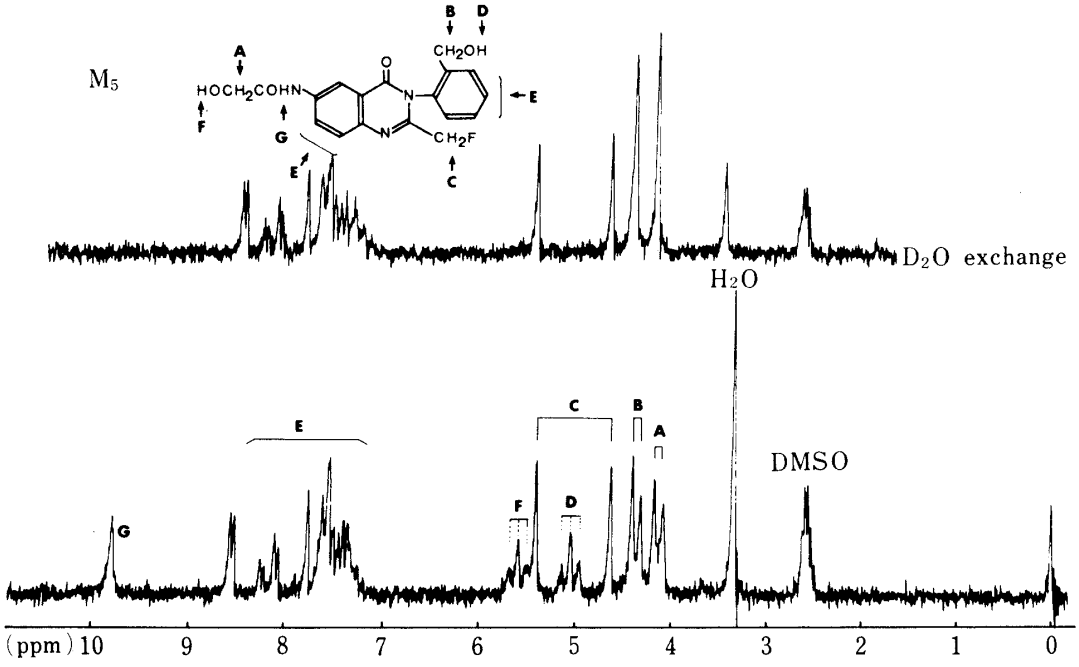


Fig. 3. NMR Spectra of M_5 in a Mixture of $CDCl_3$ and DMSO-d_6 (60 MHz)

(2H, d) and 5.60 (1H, t) were assigned to $NHCOC\underline{H}_2O\underline{H}$ and other signals at δ 4.35 (2H, d) and 5.05 (1H, t) were assignable to $C\underline{H}_2O\underline{H}$ at the 2′-position. Two prominent peaks due to CH_2F also appeared at δ 5.0 with a large coupling constant of 46 Hz. From these results, M_5 was assumed to be *N*-glycolyl-2′-hydroxymethyl AFQ. A synthetic sample was proved to be identical with M_5 by comparison of their IR, NMR and MS.

M_6——The MS exhibited a molecular ion peak at *m/z* 339. Fragment ion peaks appeared at *m/z* 91 and 132, indicating the presence of an intact *o*-tolyl moiety. M_6 was considered to be a defluorinated metabolite, since there were no fragment ions produced by loss of CH_2F which are characteristic of AFQ and its analogues. The IR spectrum also showed no absorption band characteristic of CH_2F at about 1100 cm^{-1}. A broad peak at 3400 cm^{-1} and peaks at 1005 and 1075 cm^{-1} indicated the presence of two alcoholic hydroxyl groups. Thus, M_6 was considered to be *N*-glycolyl-2-hydroxymethyl AFQ as shown in Fig. 9.

M_7——The MS showed the molecular ion peak at *m/z* 323. Fragment ion peaks appeared at *m/z* 308 ($M^+ - CH_3$), 292 ($M^+ - CH_2OH$), 281 ($M^+ - CH_2CO$) and 43 (CH_3CO^+). The MS and IR spectra indicated that M_7 was another defluorinated metabolite. The NMR spectrum revealed the presence of a hydroxymethyl group at the 2′-position (δ 4.35 and 5.05). The peaks at δ 2.10 and 2.16 were attributable to the methyl protons at the 2-position and at the *N*-acetyl moiety, respectively. From these results, M_7 was considered to be *N*-acetyl-2′-hydroxymethyl-2-methyl AFQ.

M_8——The MS of M_8 (Fig. 4) showed the highest ion peak at *m/z* 385, which was subsequently confirmed to be a molecular ion by CIMS (*m/z* 386; MH^+). The high resolution MS (Table IV) also showed a molecular ion at *m/z* 385.1084 which corresponds to $C_{19}H_{19}N_3O_4S$. Elemental analysis indicated the presence of one sulfur atom. This suggested the incorporation of one sulfur atom and loss of the fluorine atom. The IR spectrum showed two strong peaks assignable to a sulfone at 1120 (ν_s) and 1310 cm^{-1} (ν_{as}). The ion peaks at *m/z* 306 and 292 correspond to the fragments $[M - SO_2CH_3]^+$ and $[M - CH_2SO_2CH_3]^+$, respectively. The proton signals at δ 3.25 and 4.06 shown in Fig. 5 (upper), were assigned to the methyl and methylene protons of the methylsulfonylmethyl group. The signals at δ 1.70 and 2.06 were assigned to the methyl protons at the *N*-acetyl moiety and at the 2′-position, respectively. From the above results, M_8 should be *N*-acetyl-2-methylsulfonylmethyl AFQ. The proposed structure was confirmed by comparison of the IR, NMR and MS with those of a synthetic sample.

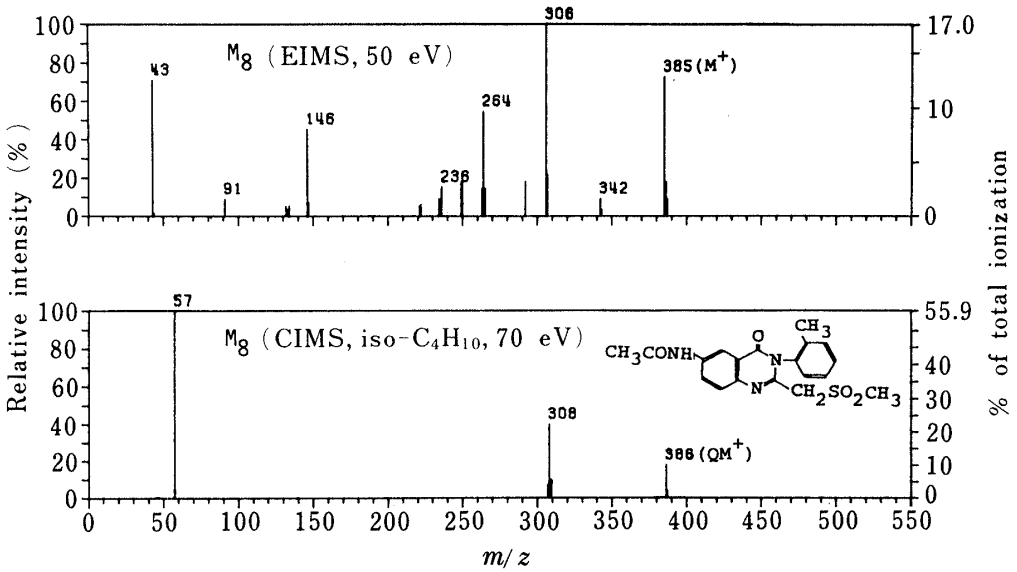


Fig. 4. Mass Spectra of M_8

M_9 and M_{10}——The IR and MS of M_9 and M_{10} (Tables I and II) revealed the presence of the methylsulfonylmethyl group as described for M_8. M_9 and M_{10} had the same molecular ion peaks at *m/z* 401, 16 mass units higher than that of M_8. In the IR spectrum of M_9, an absorption peak due to the *N*-glycolyl group appeared at 1080 cm^{-1}. From these results, M_9 was considered to be *N*-glycolyl-2-methylsulfonylmethyl AFQ.

The NMR spectrum of M_{10} indicated the presence of the 2′-hydroxymethyl group at δ 4.35 and 4.95. Thus, M_{10} was considered to be *N*-acetyl-2′-hydroxymethyl-2-methyl-

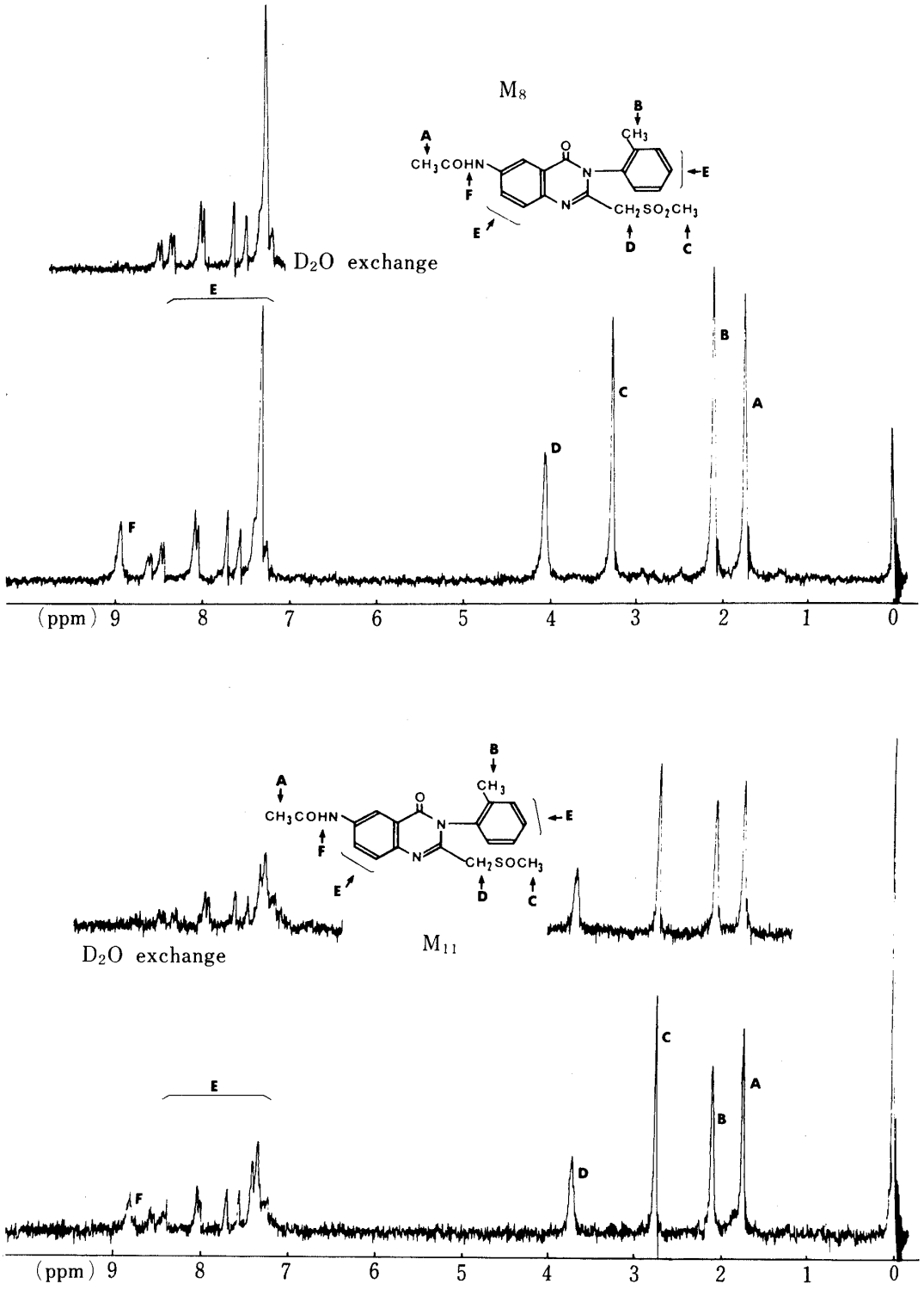


Fig. 5. NMR Spectra of M_8 and M_{11} in $CDCl_3$ (60 MHz)

sulfonylmethyl AFQ.

M_{11}, M_{12} and M_{13}——M_{11}, M_{12} and M_{13} resembled each other in their IR, NMR and MS, suggesting that these were analogous metabolites. In the NMR spectrum of M_{11} (Fig. 5, bottom), the lack of the characteristic peaks of CH_2F indicated that M_{11} was a defluorinated derivative. In its IR spectrum, a broad and intense absorption peak at 1035 cm^{-1} was assigned to a sulfoxide group. The MS (Table I) indicated a molecular ion at *m/z* 369. Fragment ion peaks appeared at *m/z* 321 ($M^+ - SO$), 306 ($M^+ - SOCH_3$) and 292 ($M^+ - CH_2SOCH_3$), suggesting the presence of a methylsulfinylmethyl group. The NMR spectrum also exhibited the presence of a methylsulfinylmethyl group at δ 2.77 and 3.76. The peaks at δ 1.75 and 2.10 were assignable to the methyl protons at the *N*-acetyl moiety and at the 2′-position, respectively. From the above findings, M_{11} was assumed to be *N*-acetyl-2-methylsulfinylmethyl AFQ. The IR, NMR and MS of M_{11} were identical with those of a synthetic sample.

The MS of M_{12} and M_{13} showed similar molecular ion peaks of low intensity at *m/z* 385, 16 mass units higher than that of M_{11}, suggesting that they are oxygenated derivatives of M_{11}. The fragment ion peaks characteristic of the methylsulfinylmethyl group also appeared at *m/z* 322 ($M^+ - SOCH_3$) and 308 ($M^+ - CH_2SOCH_3$) in the MS of both metabolites. The IR spectrum of M_{12} indicated the presence of an *N*-glycolyl moiety at 1080 cm^{-1}. Thus, M_{12} was considered to be *N*-glycolyl-2-methylsulfinylmethyl AFQ.

The NMR spectrum of M_{13} (Table III) indicated the presence of a 2′-hydroxymethyl group at δ 4.40 and 5.05. Accordingly, it was concluded that M_{13} was *N*-acetyl-2′-hydroxymethyl-2-methylsulfinylmethyl AFQ.

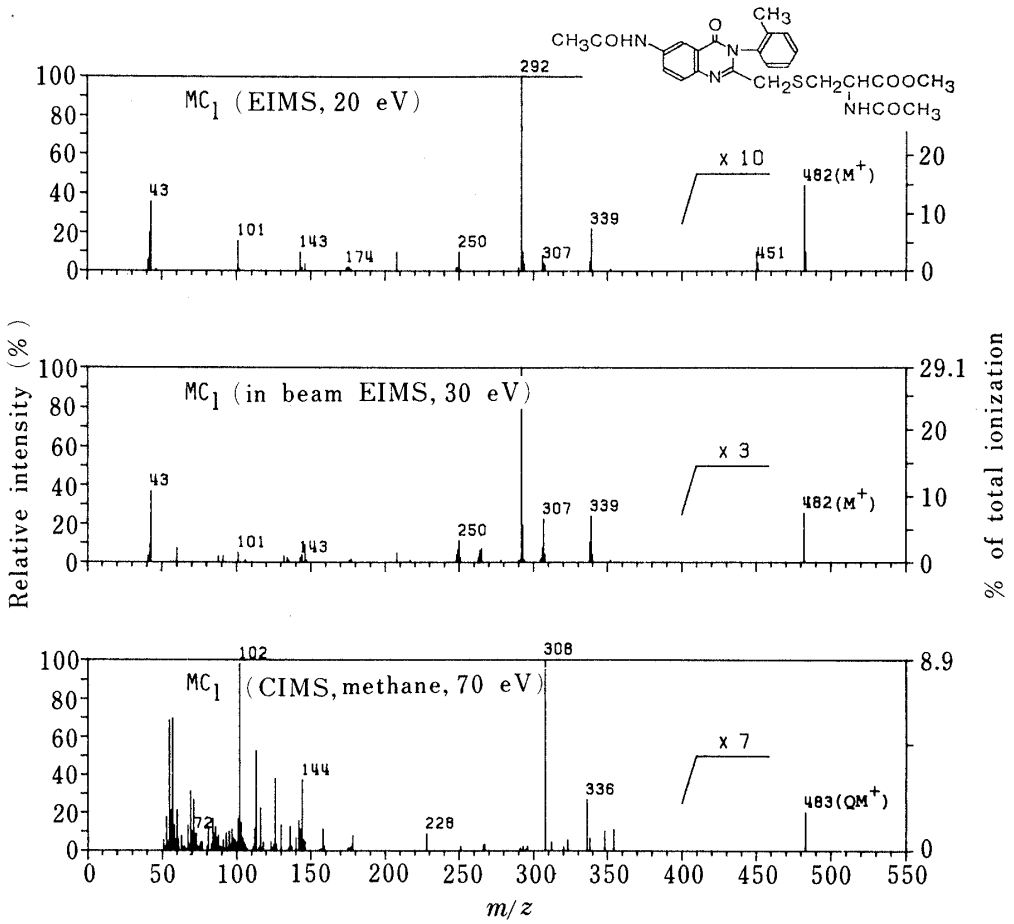


Fig. 6. Mass Spectra of MC_1 Methyl Ester

M_{14}——This metabolite was present in a trace amount. The MS and GC behavior of M_{14} (TMS derivative) were identical to those of the TMS derivative of *N*-acetyl-2-hydroxymethyl AFQ.

Glucuronic and Sulfuric Acid Conjugates——After removal of neutral and basic metabolites, the remaining aqueous solution was incubated with β-glucuronidase and arylsulfatase with or without D-glucaric acid 1,4-lactone, and extracted with AcOEt at pH 9. As a result, glucuronic and sulfuric acid conjugates were estimated to account for 1.7 and 0.3% of the urinary radioactivity, respectively.

When the AcOEt layer obtained after hydrolysis with β-glucuronidase and arylsulfatase was subjected to two-dimensional TLC, five radioactive spots were observed, as shown in Fig. 2. From the TLC properties and the MS of the TMS derivatives, M_4 and M_5 were identical with those in the neutral and basic fraction, respectively. The presence of M_{11}, M_{12} and M_{13} in this fraction was probably a result of incomplete extraction of the neutral and basic metabolite fraction.

Acidic Metabolites——The remaining aqueous layer after the enzyme treatment followed by extraction at pH 9 was extracted at pH 2 with AcOEt. When the extract was spotted on a TLC plate and developed with solvent system IV, two radioactive spots (MC_1, *Rf* 0.6 and MC_2, *Rf* 0.4) were detected. These spots were positive to the $K_2Cr_2O_7$–$AgNO_3$ reagent, suggesting the presence of mercapturic acid conjugates. The MS of the methylated MC_1 obtained by DIMS and CIMS were identical with those of the synthetic compound (Fig. 6). Consequently, MC_1 was identified as *N*-acetyl-2-mercapturoyl AFQ, whose chemical structure is shown in Fig. 9. The chemical structure of MC_2 has not been determined.

Polar Metabolites——Metabolites in this fraction accounted for 13.3% of the urinary radioactivity. Since the mercapturate of AFQ (MC_1) was identified in the acidic metabolite fraction of the urine, glutathione and/or cysteine conjugates of AFQ were considered as possible precursors. Further identification was not attempted.

Amount of Liberated ^3H_2O in Urine

^{3}H-AFQ used in this experiment was labelled at the 4′-position of the *o*-tolyl moiety. If the 4′-position undergoes a metabolic reaction such as hydroxylation, most of the ^{3}H is

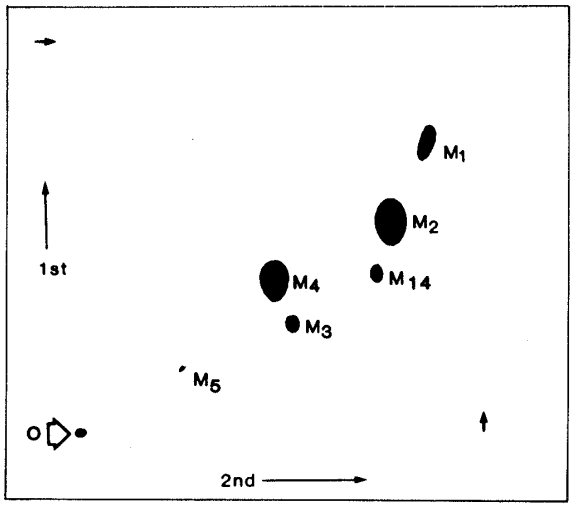


Fig. 7. Two-dimensional TLC Autoradiogram of Neutral and Basic Metabolites in the Plasma of Rats at 1 h after Oral Administration of ^{14}C-AFQ (20 mg/kg)

Solvent systems employed are the same as those described in the legend to Fig. 2.

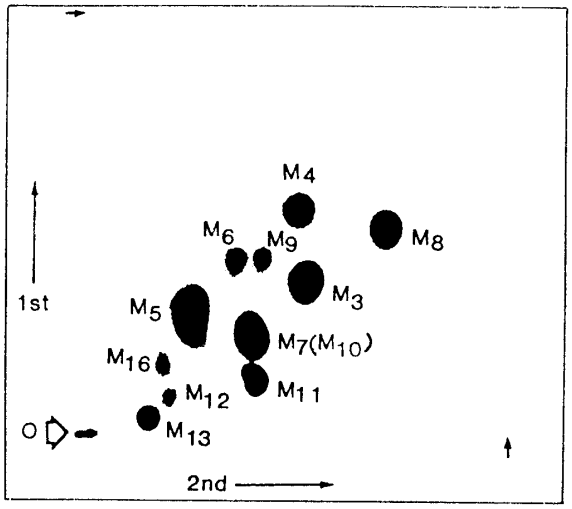


Fig. 8. Two-dimensional TLC Autoradiogram of Neutral and Basic Metabolites in the 24-h Urine of Rats after Oral Administration of ^{14}C-*N*-Acetyl AFQ (20 mg/kg)

Solvent systems employed are the same as those described in the legend to Fig. 2.

eliminated to form ^3H_2O, even if part of the ^3H is transferred to the neighboring 3′- or 5′-position due to the NIH shift. Therefore, the amount of ^3H_2O in the 24-h urine of rats dosed with ^3H-AFQ was measured by the activated charcoal method. The amount of liberated ^3H_2O was less than 0.6% of the urinary radioactivity, indicating that the extent of hydroxylation at the 4′-position was very low.

Metabolites in the Plasma

When the plasma sample obtained at 1 h after oral administration of ^{14}C-AFQ was extracted with AcOEt at pH 9, about 80% of the plasma radioactivity was transferred to the AcOEt layer. The extract, when subjected to TLC, gave six radioactive spots as shown in Fig. 7. These compounds were analyzed as the TMS derivatives by GC-MS and identified as AFQ and its *N*-acetylated and hydroxylated metabolites (M_2—M_5 and M_{14}, Fig. 9), among which M_2 and M_4 were major components. The sulfur-containing metabolites found in the urine were not detected, although the plasma radioactivity level reached the maximum at this time.[4a)]

Urinary Metabolites of ^{14}C-*N*-Acetyl AFQ

Twenty-four hours after oral administration of ^{14}C-*N*-acetyl AFQ to rats, the radioactivity in the urine and feces accounted for 43.2 and 39.9% of the dose, respectively. Upon fractionation of the urine by solvent extraction, about 56% of the urinary radioactivity appeared in the neutral and basic fraction. TLC of this extract showed at least eleven radioactive spots (Fig. 8). By comparison with Fig. 2, each radioactive spot was assigned as shown in Fig. 8. Of these metabolites, M_5 (one of the main metabolites) and M_4 were isolated and their structures were confirmed by MS analysis, demonstrating that these *N*-glycoyl metabolites of AFQ were derived from *N*-acetyl AFQ by hydroxylation at its acetyl–methyl carbon.

Table V. Quantitative Determination of the Urinary Metabolites of AFQ in Rats

Metabolites	Percentage of ^3H in urine[a)]		
	Unconjugated ($68.4 \pm 3.5\%$)[b)]	Hydrolysates of glucuronides/sulfates ($5.0 \pm 0.9\%$)	Acidic metabolites ($11.9 \pm 1.8\%$)
AFQ (M_1)	0.2		
M_2	0.3		
M_3	2.1		
M_4	3.5	1.5	
M_5	12.0	0.2	
M_6	1.2		
M_7	4.0		
M_8	8.6		
M_9	4.6		
M_{10}	6.2		
M_{11}	14.6	0.5	
M_{12}	1.8	0.1	
M_{13}	4.1	0.8	
M_{14}	0.5		
MC_1			5.5
MC_2			2.4
Others	4.7	1.9	4.0

a) Urine samples were collected for 24 h after oral administration of ^3H-AFQ (20 mg/kg).
b) Each value is the mean and S.E. of six rats. Metabolite percentages are for pooled samples.

Quantitative Determination of Urinary Metabolites of ^{3}H-AFQ

Table V shows the quantitative distribution of the urinary metabolites of ^{3}H-AFQ. Urinary excretion of unchanged AFQ was less than 0.2% of the urinary radioactivity. The main metabolites were M_5 and M_{11} which accounted for about 10—15% of the total urinary radioactivity, respectively. The total amount of the six sulfur-containing metabolites (M_8—M_{13}) accounted for about 40% of the urinary radioactivity.

Discussion

When ^{3}H-AFQ was administered orally to rats, about 50% of the administered radioactivity was excreted into the urine within 24 h. Very little unchanged AFQ was excreted in the urine, and this suggests that AFQ is extensively metabolized by rats. All of the metabolites isolated were either acetylated or glycolylated at the 6-amino group of AFQ. *N*-Acetylation is a well-known metabolic pathway for an aromatic amino group. The *N*-glycolylated metabolites were assumed to be formed by *N*-acetylation followed by hydroxylation of the methyl group of the *N*-acetyl moiety. This was confirmed by the detection of *N*-glycolylated metabolites (M_4 and M_5, Fig. 8) in the urine of rats after oral administration of ^{14}C-*N*-acetyl AFQ. Fries *et al.*[12] also reported that the glycolamide and oxamic acid derivatives were identified as major metabolites of sulfanilamide in rabbits, and these metabolites were probably produced by oxidation of the *N*-acetyl derivative of sulfanilamide and its dehydrogenation. Although the present results with AFQ are similar to their results, oxamic acid derivatives of AFQ have not been detected in the urine of rats so far.

In addition to the acetyl–methyl carbon, the side chains such as the 2′-methyl and 2-fluoromethyl groups were hydroxylated in rats. The above substitution of a hydroxyl group for the fluorine atom of the fluoromethyl group is an unfamiliar metabolic reaction. At present, the pathway by which the 2-hydroxymethyl metabolites (M_6 and M_{14}) are formed is uncertain. There are two possible pathways for the formation of these metabolites; one is direct substitution[13] of a hydroxyl group for the fluorine atom of *N*-acetyl AFQ (M_2), and the other is defluorination of M_2 to produce the 2-methyl derivative[14] (such as M_7) followed by hydroxylation of the methyl group.

Nowak *et al.*[15] reported that methaqualone (2-methyl-3-(*o*-tolyl)-4(3*H*)-quinazolinone), a hypnotic of similar structure, was easily metabolized to phenolic metabolites and their glucuronides in animals including the rat. However, in the present study, phenolic metabolites of AFQ were not found in the urine of rats. These results indicate that aromatic ring hydroxylation of AFQ does not occur in rats to a significant extent. Reynolds *et al.*[16] reported that methaqualone was also metabolized to the N_1-oxide derivative in man, accounting for 5—10% of an oral dose after 24 h. In the present work, no evidence for the presence of N_1-oxides of AFQ and its metabolites in the urine was obtained. The metabolism of AFQ seems to be different from that of methaqualone.

The conjugated metabolites were mainly found as glucuronic acid conjugates. Enzymatic hydrolysis produced only *N*-glycolylated metabolites (M_4 and M_5) but no 2- or 2′-hydroxymethyl metabolites (M_3 or M_{14}). These results indicate that glucuronidation in rats occurs selectively at the hydroxyl group of the *N*-glycolyl moiety.

We also found in this study that the rats excreted large amounts of sulfur-containing metabolites, in which the fluorine atom of the fluoromethyl side chain of AFQ had been replaced by a methylsulfinyl or methylsulfonyl group. This metabolic conversion is a recently discovered, novel metabolic pathway.[17] The formation of metabolites in which an aromatic ring has undergone methylthio-, methylsulfinyl- or methylsulfonyl-substitution was reported with phenacetin,[18] *N,N*-dimethyl-4-aminoazobenzene,[19] bromazepam[20] and naphthalene.[21] However, only a few papers have appeared on these metabolic modifications to the side chain

of an aromatic ring.[22-24] Although methylthio-containing metabolites of AFQ were not found in the present study, the methylsulfinyl and methylsulfonyl metabolites (M_{11}, M_8) are considered to be formed from methylthio intermediates by the monooxygenase of hepatic microsomes, as shown by Watanabe *et al.*[25] Miller *et al.*[19] proposed that the methylthio group was derived from methionine or *N*-acetylmethionine. Tateishi *et al.*[20b,c] reported that the sulfur-containing metabolites of bromazepam might be formed from a cysteine conjugate. Chatfield and Hunter[23] demonstrated that the mercapturic acid conjugate was a precursor of sulfur-containing metabolites of 2-acetamido-4-chloromethylthiazole. Since the mercapturic acid conjugate of AFQ (MC_1) was identified in the urine, the mercapturate is likely to be one of the precursors of the sulfur-containing metabolites. It is well known that mercapturic acid conjugates are formed from glutathione conjugates. The formation route of AFQ metabolites of this new type will be dealt with elsewhere.[26]

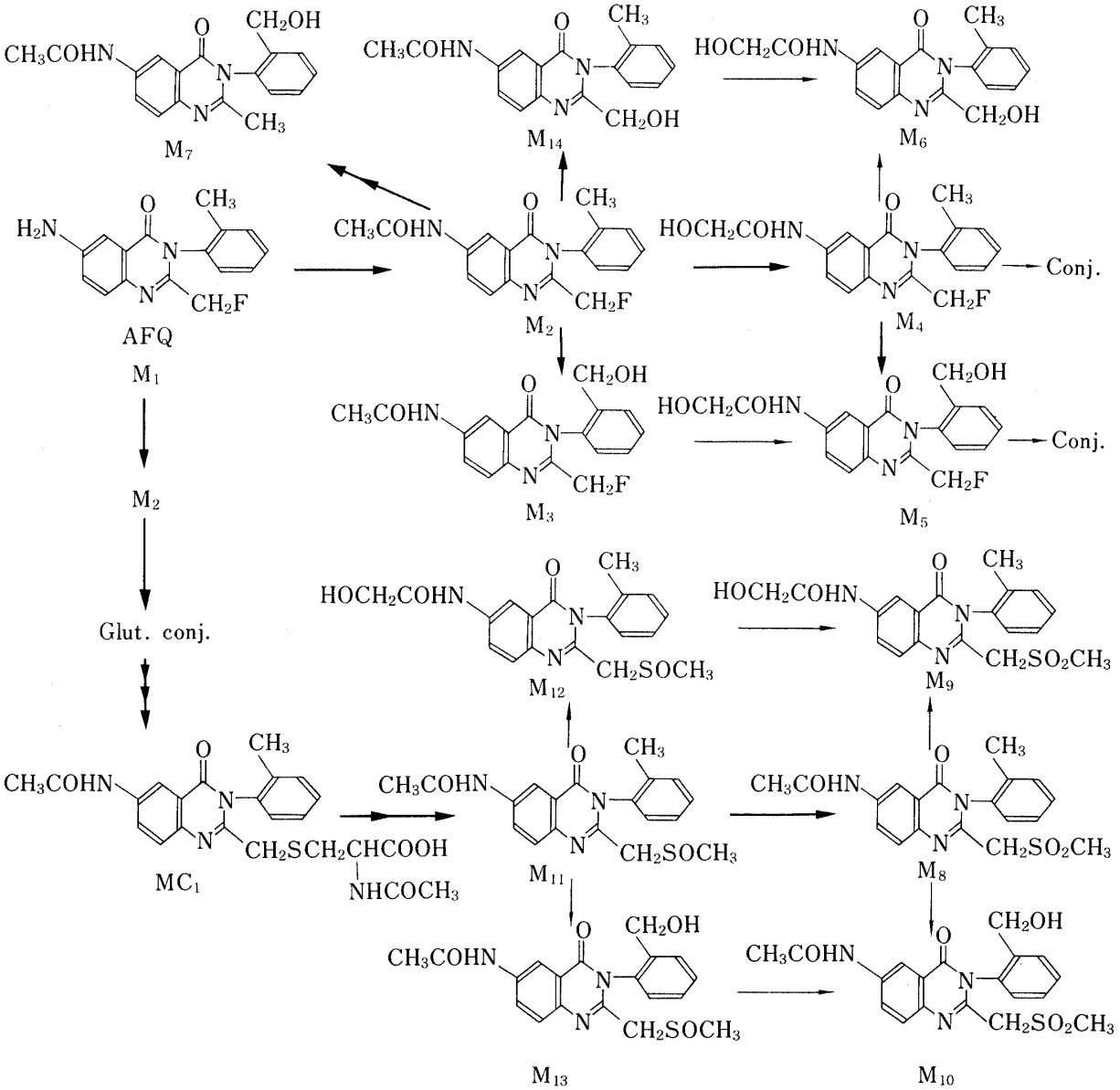


Fig. 9. Possible Metabolic Pathways of AFQ in the Rat

Five metabolites besides unchanged AFQ were detected in the plasma 1 h after oral administration of ^{14}C-AFQ. Among them, the *N*-acetyl derivative (M_2) was a major metabolite (Fig. 7). This suggests that acetylation of the aromatic amino group occurs first. Subsequent hydroxylation presumably occurs rapidly at the methyl group of the *N*-acetyl moiety since the amount of the *N*-glycolyl derivative (M_4) exceeded that of the *N*-acetyl-2′-hydroxymethyl (M_3) or *N*-acetyl-2-hydroxymethyl derivative (M_{14}). The sulfur-containing metabolites found in the urine, however, were not detected in the 1-h plasma, suggesting that these metabolites are formed at a later stage of metabolism.

Taking into consideration all the findings described above, a map of the possible metabolic pathways of AFQ in the rat is presented in Fig. 9.

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