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Analytical Studies on Mepirizole and Its Metabolites. III.[1] Gas Chromatographic Determination of Mepirizole and Its Metabolites in Biological Fluids[2]

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Highly sensitive techniques for the quantitative determination of mepirizole (I) and its metabolites are described. The compounds were first extracted from rat serum with dichloromethane, and they were converted to their respective trifluoroacetyl derivatives in isopropyl ether, which could be detected by a gas chromatograph equipped with an electron capture detector. The approximately linear relationships existed between the quantities of these compounds and the sensitivities to electron capture detector in the range of the serum levels as low as several hundred ng.

The structures of the trifluoroacetylated derivatives of the metabolites were identified by gas chromatography–mass spectrometry.

The distribution of mepirizole (I) (1-(4-methoxy-6-methyl-2-pyrimidinyl)-5-methoxy-3-methylpyrazole) in experimental animals was already studied by Akimoto[4] by using the ^{14}C-labeled compound. Attempts have been made to determine I and its metabolites in serum either by gas chromatograph equipped with a flame ionization detector (FID) or by ultraviolet spectrometer. However, none of these techniques was successful in determining I and the metabolites whose serum levels are very low. In this connection, we have attempted to find a new technique which enables us to determine these compounds by gas chromatography.

We have previously shown[5] that the hydrogen at 4-position of the pyrazole ring of I is easily replaced by deuterium, which suggests that this position might be readily attacked by electrophilic reagents. This leads us to introduce a perhalogeno group to the 4-position of the pyrazole ring in order to have a derivative which could be detected by gas chromatograph with an electron capture detector (ECD). The present study deals with a gas chromatographic method to determine I and its metabolites in rat serum by converting them to their respective trifluoroacetyl derivatives.

Materials and Methods

Materials——Mepirizole (I)[6] and its metabolites[7] were prepared as described previously.

Apparatus and Conditions——A Shimadzu GC-4BM gas chromatograph equipped with an ECD containing a 10 mCi-^{63}Ni ionization source was used. The analytical conditions were as follows; column: 1% OV-17 on 80/100 mesh Chromosorb G packed in silicated glass column (1 m × 3 mm i.d.), temperature of oven: 160—250° (programmed rate, 5°/min), temperature of injection port and detector: 270°, nitrogen flow rate: 60 ml/min, sensitivity: 10 MΩ and range: 160 mV.

1) Part II: Y. Tanaka and M. Sano, *Chem. Pharm. Bull.* (Tokyo), **24**, 804 (1976).
2) This work was presented at the 93rd Annual Meeting of Pharmaceutical Society of Japan (1973), Tokyo.
3) Location: *a) Minamifunabori-cho, Edogawa-ku, Tokyo; b) Muramatsu, Tokai-mura, Naka-gun, Ibaraki.*
4) T. Akimoto, *Tokyo Jikeikai Medical J.*, **86**, 645 (1971).
5) M. Sano and Y. Tanaka, *Chem. Pharm. Bull.* (Tokyo), **23**, 209 (1975).
6) T. Naito, T. Yoshikawa, S. Kitahara, and N. Aoki, *Chem. Pharm. Bull.* (Tokyo), **17**, 1467 (1969).
7) R. Dohmori, R. Yoshimura, S. Kitahara, Y. Tanaka, and T. Naito, *Chem. Pharm. Bull.* (Tokyo), **18**, 1908 (1970).

A Shimadzu GC-5AP gas chromatograph equipped with an FID was also used for the examination of the reaction condition for trifluoroacetylation of I. Analytical conditions were similar to those described above except that the temperature of the column was fixed on 170°.

A Japan Electron & Optics Laboratory JGC-20K gas chromatograph-JMS Ol SG-2 mass spectrometer was used for the structure determination of trifluoroacetylated derivatives of the metabolites.

Trifluoroacetylation of I——A mixture of I (1.0 g) in isopropyl ether (70 ml) and trifluoroacetic anhydride (30 ml) was allowed to stand at room temperature for 17 hr. After evaporation of the solvent, the residue was dissolved in $CHCl_3$ (50 ml) and washed twice with 1N NaOH (50 ml) and then with water (50 ml) three times. The $CHCl_3$-extract was dried with Na_2SO_4 and evaporated to dryness. The oily residue was crystallized from petroleum ether and then recrystallized from isopropyl ether to give trifluoroacetylated I (II), mp 83.8—84.5°. *Anal.* Calcd. for $C_{13}H_{13}O_3N_4F_3$: C, 47.27; H, 3.97; N, 16.97. Found: C, 47.21; H, 3.95; N, 17.35. IR ν_{max}^{KBr} cm^{-1}: 1690 (C=O).

Internal Standard Solution——9.10-Dichloroanthracene (III) in benzene (10.0 μg/ml) and *n*-pentacosane (IV) in benzene (10,0 mg/ml) were used as the internal standards for ECD and FID, respectively.

Derivatization Condition for I——To 10 mg of I was added 1 ml of a mixture of isopropyl ether and trifluoroacetic anhydride at different ratios, and the reaction mixture was allowed to stand for indicated times thereafter. After evaporation of the solvent, the residue was dissolved in 1.0 ml of benzene, and 5 μl of the solution was applied to a GC equipped with an FID.

Determination of the Reaction Rate of the Trifluoroacetylation of I——To 10 mg of I was added 0.3 ml of trifluoroacetic anhydride and 0.7 ml of isopropyl ether, and the mixture was allowed to stand overnight at room temperature. After evaporation of the solvent, 1.0 ml of the internal standard solution was added to the residue, and 5 μl of the solution was applied to a GC equipped with an FID.

The standard solution (5.0 mg of II dissolved in 1.0 ml of the internal standard solution) was applied to a GC in a similar manner as above.

Determination of Gas Chromatographic Sensitivity of II——Three μl of the methanolic solutions of II in concentrations of 1.0 mg/ml and 1.0 μg/ml were applied to GC's equipped with an FID and with an ECD, respectively.

Trifluoroacetylation of the Urinary Metabolites of I——To each 10 mg of authentic UNM-1, UNM-2, UNM-4, UNM-5, methyl esters of UAM-1 and UAM-2[8] was added 0.3 ml of trifluoroacetic anhydride and 0.7 ml of isopropyl ether, and the mixtures were allowed to stand overnight at room temperature with tight capping. After evaporation of the solvent, 1.0 ml of anhydrous benzene was added to the residue, and 1 μl of the solution was immediately applied to a gas chromatograph–mass spectrometer (GC-MS). Care has been taken to prevent from moisture throughout the procedures.

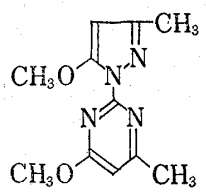
Quantitative Determination of I and the Metabolites in Rat Serum——I was administered orally to rats in a dose of 6.0 mg/kg. A 1.0 ml aliquot of the serum was added to 2 ml of phosphate buffer (pH 6.3), and the mixture was shaken with 5 ml of *n*-hexane for 10 minutes and centrifuged. After the *n*-hexane phase was removed carefully by aspiration, 5 ml of CH_2Cl_2 was added to the aqueous phase and the mixture was shaken for 10 minutes and centrifuged. A 4.0 ml-portion of the CH_2Cl_2 phase was transfered into a 10 ml flask and dried over 2 g of Na_2SO_4. Na_2SO_4 was then removed by filtration and washed twice with 2 ml of CH_2Cl_2. The filtrate and the washings were combined and evaporated to dryness at room temperature. The residue was dissolved in 0.7 ml of isopropyl ether and 0.3 ml of trifluoroacetic anhydride. The reaction mixture was allowed to stand overnight at room temperature with tight capping, and then evaporated to dryness under reduced pressure. The residue was dissolved in 0.5 ml of the internal standard solution, and 0.5 μl of the solution was immediately applied to a GC equipped with an ECD.

Results and Discussion

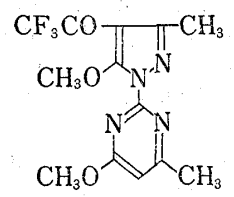
Structure of Trifluoroacetylated Mepirizole

When I was allowed to stand at room temperature for 20 hr in acidic heavy water, the signal (5.5 ppm) for the 4-H of the pyrazole ring disappeared in its nuclear magnetic resonance (NMR) spectrum. This fact suggested that the 4-position is highly reactive to electrophilic reagents. Actually, I could be easily trifluoroacetylated to yield a convenient derivative for the determination by an ECD of gas chromatograph. Its structure was confirmed to be 1-(4-methoxy-6-methyl-2-pyrimidinyl)-5-methoxy-3-methyl-4-trifluoroacetylpyrazole (II)

8) The abbreviations used in this report are quoted from the literature: E. Takabatake and R. Kodama, *Xenobiotica*, 2, 479 (1972). The structures of the metabolites and their trifluoroacetylated derivatives are shown in Chart 1.



mepirizole (I)



TFA−mepirizole (II)

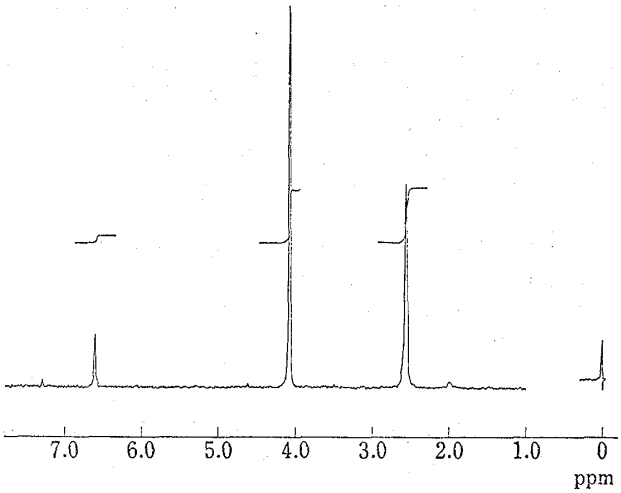


Fig. 1. NMR Spectrum of TFA-Mepirizole (II)

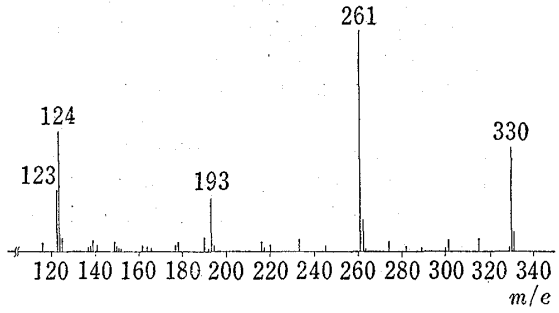


Fig. 2. Mass Spectrum of TFA-Mepirizole (II)

(TFA-mepirizole) which is shown in the following figure. In its NMR spectrum, the proton signal for the 4-position of the pyrazole ring was absent. As shown in Fig. 2, the molecular ion peak at *m/e* 330 and M–CF_3 peak at *m/e* 261 were obtained in the mass spectrum of II. A characteristic ion peak at *m/e* 124 was in accord with the base ion peak of I which was attributed to the fragment of the pyrimidine moiety of I. These facts indicated that trifluoroacetylation occurred at the pyrazole moiety, and not at the pyrimidine ring.

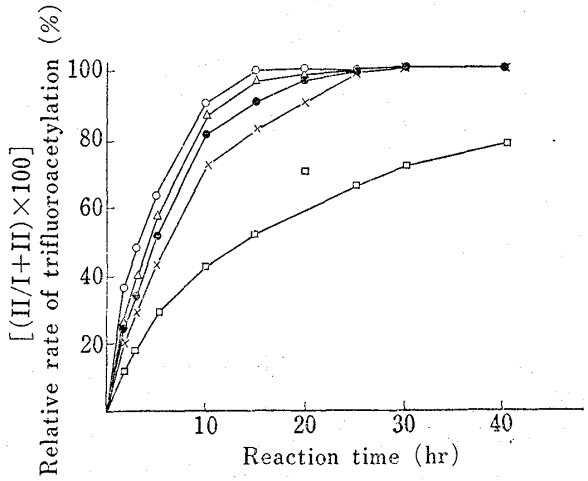


Fig. 3. Relation Reaction Rate of the Trifluoroacetylation of Mepirizole

Derivatization Reaction

The reaction conditions for the derivatization of I were examined. Isopropyl ether proved to be better than other solvents tested. As shown in Fig. 3, the optimum ratio of trifluoroacetic anhydride to isopropyl ether was 3: 7, and the optimum reaction time was 15 hr at room temperature. Though the elevation of the reaction temperature shortened the reaction time, it significantly decreased the yield.

The use of other halogeno compounds as the electrophilic reagent and Lewis acid as a catalyst were examined, but no better results were obtained.

The reaction ratio was estimated to be 49.7±1.3% by comparing the GC peak height of the reaction mixture after trifluoroacetylation of I with that of the standard solution of II (Table I).

Table I. Reaction Rate of Trifluoroacetylation of I

Peak height ratio (II/I.S.)	Molecular weight correction (II/I.S.) × (234/330)	Peak height ratio of standard II to I.S.	Reaction rate
0.924	0.655	1.311	50.0
0.932	0.661	1.311	50.4
0.888	0.629	1.251	50.3
0.870	0.617	1.251	49.4
0.855	0.606	1.251	48.4
			49.7±1.3

Gas Chromatographic Sensitivity of II

The sensitivity of the detection of the compound II by ECD was shown to be approximately seven hundred fold higher than that by FID as shown in Table II.

Table II. Relative Sensitivity of II to FID and ECD

Detector	Injection volume (ul)	Sample weight injected (ng)	Peak height	Average of peak height	Relative sensitivity
FID	3.0	3000	83.0, 82.9, 84.6	83.5	1.0
ECD	3.0	3.0	62.1, 62.0, 61.9	62.0	743

Structures of the Trifluoroacetylated Derivatives of the Metabolites

Of the known metabolites of I, UNM-1, UNM-2, UNM-4, UNM-5 and the methyl ester of UAM-2 were trifluoroacetylated in a similar manner as I. Since the ester linkages of the trifluoroacetylated derivatives were easily hydrolyzed when exposed to the atmospheric moisture, characterization of these derivatives was carried out by GC-MS.

Table III. Mass Spectral Data of the Trifluoroacetylated Derivatives of the Metabolites

Metabolite	Molecular ion peak, *m/e*	Number of CF_3CO groups introduced	$M-CF_3$ peak, *m/e*	Peak attributable to pyrimidine, *m/e*
UNM-1	442	2	373	236
UNM-2	346	1	n.d.[a]	124
UNM-4	442	2	373	236
UNM-5	346	1	n.d.[a]	124
UAM-2[b]	274	1	305	168

a) not detected
b) methyl ester

As shown in Table III, the mass spectra of both TFA- UNM-1 and TFA-UNM-4 exhibited prominent ion at *m/e* 442 (M^+), *m/e* 373 (M−69, the loss of a trifluoromethyl radical) and *m/e* 236 (attributable to the pyrimidine moiety). These data showed that trifluoroacetylation occurred not only at the 4-position of the pyrazole ring, but at the hydroxyl groups of the pyrimidine ring. In the case of TFA-UNM-5 which exhibited M^+ at *m/e* 346 in the mass spectrum, only the hydroxyl group was esterified by trifluoroacetic anhydride. Though UNM-2 is deduced to be trifluoroacetylated at two sites, that is, at the 4-position of the pyrazole ring and at the hydroxymethyl function, only one trifluroacetyl group proved to be introduced to the molecule as revealed by the molecular ion peak at *m/e* 346. Absence of an intense peak, $M-CF_3$, indicated that only the hydroxymethyl group was trifluoroacetylated. The methyl ester of UAM-2 was trifluoroacetylated at the 4-position of the pyrazole ring to exhibit M^+ at *m/e* 374. These results are shown in Chart 1.

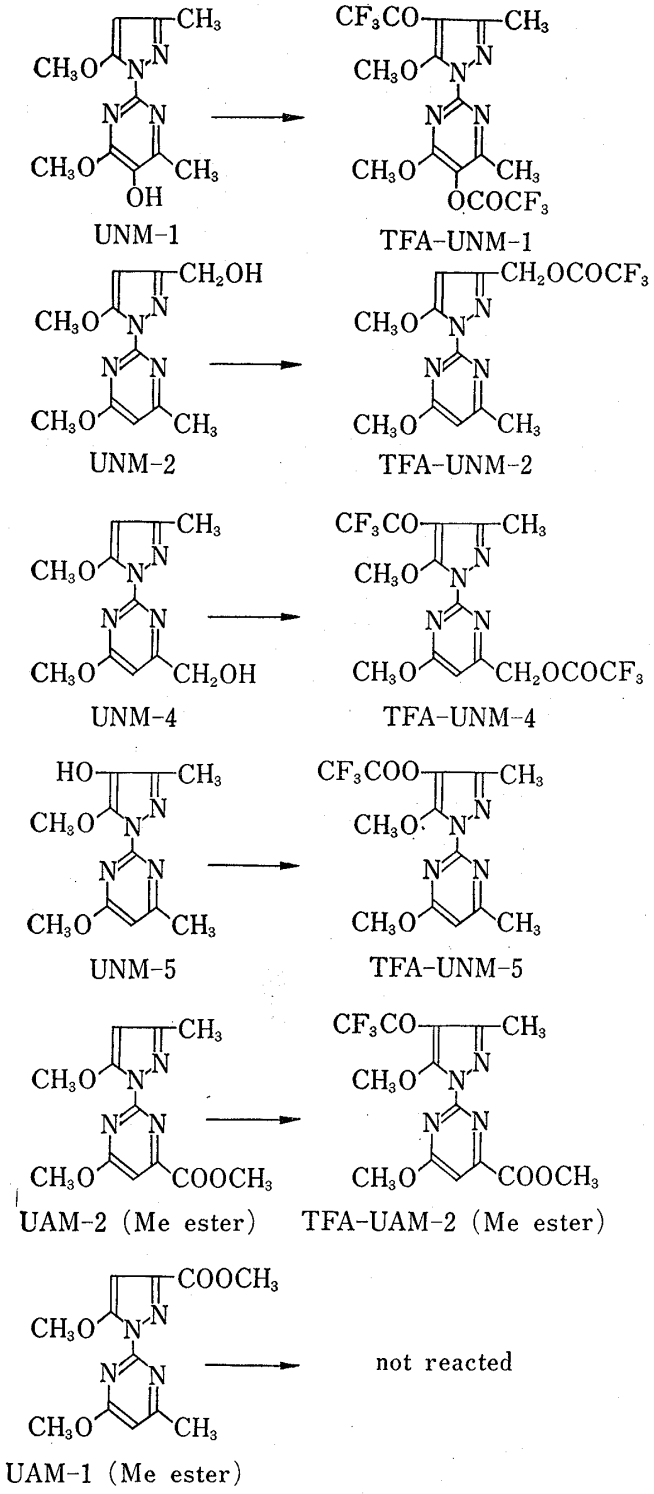


Chart 1. Trifluoroacetylation of the Metabolites

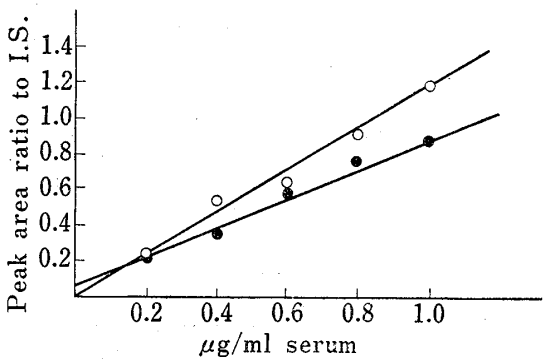


Fig. 4. Calibration Curves for Trifluoroacetylated Derivatives of Mepirizole and UNM-2

○—○ : mepirizole
●—● : UNM-2

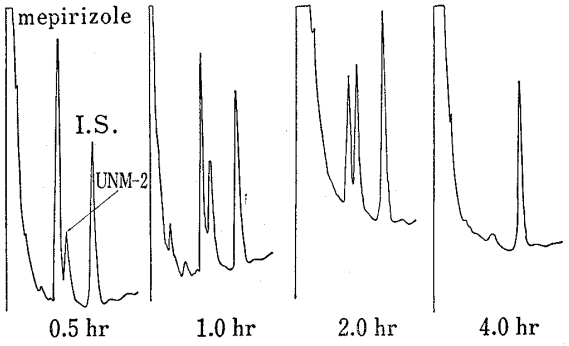


Fig. 5. Gas Chromatograms of the Serum Extracts after Administration of Mepirizole

Table IV. Serum Levels of Mepirizole and UNM-2 in Rat

Metabolite	Serum levels (μg/ml) Hours after administration 0.5	1.0	2.0	4.0
Mepirizole	1.19	0.82	0.44	—
UNM-2	0.49	0.82	0.79	0.11

dose: 6.0 mg/kg

Quantitative Determination of I and Its Metabolites

The blood levels of I and its metabolites were estimated from the calibration curves shown in Fig. 4. The gas chromatograms obtained are shown in Fig. 5, and only I and UNM-2 were detected by the present technique. Though the trifluoroacetyl derivatives of the other metabolites had similar sensitivities to ECD as those of I and UNM-2, these could not be found in the gas chromatograms. The results are shown in Table IV. The blood levels of these compounds estimated by the technique were approximately the same as those obtained by the radioactive tracer method.[4]