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Analytical Studies on Mepirizole and Its Metabolites. IV.[1] Quantitative Determination of Mepirizole in Human Serum by Mass Fragmentography, and Isotope Effect on Drug Metabolism

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Mass fragmentographic determination of mepirizole in human serum was carried out with the aid of its deuterium labeled compound as the internal standard. Maximal serum level was observed at 60 minutes after oral administration. Simultaneous observation of isotope effect on metabolic rate revealed that serum level of mepirizole deuterated at the methyl group of the pyrazole ring was slightly higher than that of mepirizole, but the isotope effect was observed on the formation of the metabolites.

Since blood level of a drug is closely related to its therapeutic effect, it is very important to determine its concentration. In the preceding paper,[1] we reported the quantitative determination of mepirizole[3] (1-(4-methoxy-6-methyl-2-pyrimidinyl)-5-methoxy-3-methylpyrazole) in rat serum by gas chromatography. Much attention has been recently focussed on a combination of gas chromatograph (GC) with mass spectrometer (MS) connected with multiple ion detector (MID) as a tool of quantitative analysis. In this paper, we discuss the quantitative determination of mepirizole in human serum by mass fragmentography concurrently with isotope effect on the metabolic rate.

Materials and Methods

Materials——Deuterated mepirizole: mepirizoles deuterated at the methyl group of the pyrazole ring (mepirizole-d_3) and at both of the methoxy group (mepirizole-d_6) were synthesized according to the previous method.[4] These compounds were ascertained to have high isotope purities by measuring nuclear magnetic resonance spectra (NMR).

The internal standard solution was prepared by dissolving mepirizole-d_6 in benzene in a concentration of 1.0 μg/ml.

Apparatus and Conditions——Mass fragmentographic analysis was carried out on Hitachi model RMU-6MG single focussing mass spectrometer combined with Hitachi model 063 gas chromatograph through a glass frit separator. For specific ion detection, the mass spectrometer was programmed with Hitachi model MK-18 multiple ion detector.

Gas chromatographic conditions were as follows; column: 1.5% OV-17 on Chromosorb W (80/100 mesh), temperatures of injection port, column and separator: 250°, 200° and 330°, respectively, inlet pressure of carrier gas (helium): 1.0 kg/cm^2.

Mass spectrometer conditions were as follows; ionizing voltage: 20 eV, total emission current: 100 μA, accelerating voltage: 3.2 kV. Magnetic field was adjusted at m/e 234, 237 and 240 by MID.

Mass spectra of the acidic metabolites were measured on Japan Electron & Optics Laboratory JMS 01 SG 2 mass spectrometer under the following conditions; ionizing energy: 75 eV, emission current: 200 μA, accelerating voltage: 10 kV, ion multiplier voltage: 2.5 kV, main slit: 100 μ, sample temperature: 50→200°.

Preparative thin layer chromatography (preparative TLC) was carried out by using silica-gel plates (Merck Kiesel-gel HF_{254} containing fluorescent indicators, 0.5 mm thick, activated at 120° for 2 hr). The developing solvent used was $CHCl_3$–acetone (9: 1). The acidic metabolites were visualized as fluorescent spots by ultraviolet lamp.

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1) Part III: Y. Tanaka, Y. Esumi, and M. Sano, *Chem. Pharm. Bull.* (Tokyo), **24**, 808 (1976).
2) Location: *Minamifunabori-cho, Edogawa-ku, Tokyo.*
3) This drug was proved to have very low toxicity, the data of which is described in "Studies On Mebron" published from Daiichi Seiyaku Co., Ltd.
4) M. Sano and Y. Tanaka, *Chem. Pharm. Bull.* (Tokyo), **23**, 209 (1975).

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Procedures——An equimolar mixture (200 mg) of mepirizole[5] and mepirizole-d_3 was administered orally to each of two subjects (the authors of this paper). Blood specimens were taken at 45, 60, 90, and 120 minutes after administration. To the serum (1.0 ml) was added phosphate buffer (pH 6.3) (2.0 ml) and *n*-hexane (5.0 ml), and the mixture was then shaken for ten minutes. After removal of the *n*-hexane layer, the internal standard solution (0.5 ml) and benzene (4.5 ml) was added to the aqueous layer. The mixture was shaken for ten minutes. After removal of the aqueous layer, the benzene layer was dried over Na_2SO_4. After filtration of the sodium sulfate and washing it twice with benzene (1.0 ml), the filtrate and washings were combined and evaporated to dryness. The residue was dissolved in MeOH (0.1 ml), 1—2 μl of which was applied to GC–MS system.

Urine was collected for 8 hr after administration and combined. An aliquot (50 ml) of the urine was basified with 1N NaOH and shaken with CH_2Cl_2 (50 ml). The separated aqueous solution was acidified with 1N HCl and the acidic metabolites were extracted with CH_2Cl_2 (50 ml) three times. The combined CH_2Cl_2 extracts were dried over $CaCl_2$ and evaporated to dryness. The residue was dissolved in MeOH (2 ml) and to the solution was added large excess of diazomethane in ether. The reaction mixture was allowed to stand for 3 hr at room temperature. After evaporation of the solvent, the residue was applied to preparative TLC. The spot due to 1-(4-methoxy-6-methoxycarbonyl-2-pyrimidinyl)-5-methoxy-3-methylpyrazole (methyl ester of M–COOH) was scratched, and the esterified metabolites in the scratched silica-gel was extracted with $CHCl_2$–acetone (9: 1). The extract was concentrated to dryness and the residue was applied to MS.

Results and Discussion

Mass fragmentography has been widely utilized in quantitative analyses of drugs in biological fluids. In this study, we applied this technique to the determination of mepirizole in human serum. The isotope effect on the rate of metabolism was also investigated using 8 hr urine after oral administration of an equimolar mixture of mepirizole and mepirizole-d_3.[6]

Deuterium Labeled Compounds as an Internal Standard

Use of a stable isotope labeled compound of a drug as its internal standard gives much advantage in quantitative analysis by mass fragmentography, because the labeled compound shows similar behavior to that of the unlabeled drug when it is extracted from biological fluids and treated by other "clean-up" methods.

In our investigation, mepirizole deuterated at both of the two methoxy groups (mepirizole-d_6) was used as the internal standard.

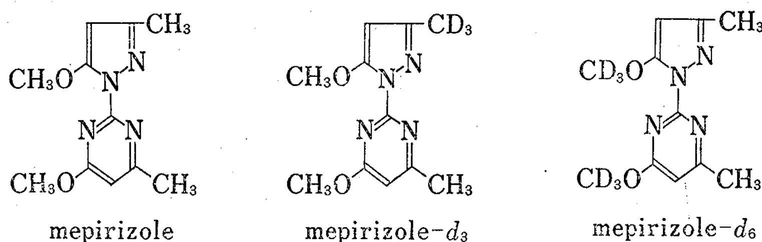

Chart 1

Selection of the Mass Numbers in Mass Fragmentography

Quantitativity in the analysis of a drug in biological fluids depends on the choice of the mass numbers provided for mass fragmentography. Use of the mass number of a base peak is desirable for maximal sensitivity, but it is more important that intensity of a peak arising from the endogeneous components is as weak as possible in the selected mass numbers. From the latter point of view, it is preferable in mass fragmentography to choose a higher mass number, namely, that of a molecular ion which is moderately intense in mepirizole as shown in Fig. 1. In this experiment, MID was alternately set on three mass numbers, *m/e* 234, 237 and 240, which are assignable to mepirizole, mepirizole-d_3 and mepirizole-d_6, respectively.

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5) Dose levels of mepirizole used in clinics are 200—400 mg a day.

6) Toxicity of stable isotopes is described in the literature: D.R. Knapp and T.E. Gaffney, *Clinical Pharmacol. Therap.*, 13, 307 (1972).

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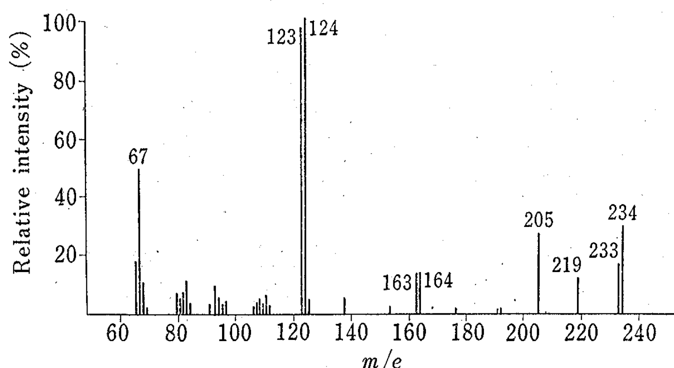

Fig. 1. Mass Spectrum of Mepirizole

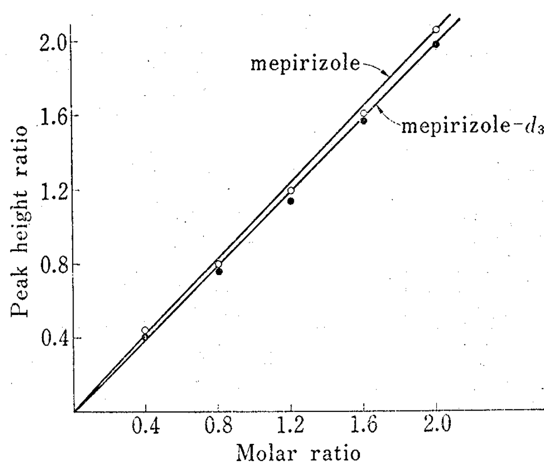

Fig. 2. Standard Curves of Mepirizole (*m/e* 234) and Mepirizole-d_3 (*m/e* 237) to the Internal Standard (Mepirizole-d_6) (*m/e* 240) for Mass Fragmentography

Quantitative Analysis of Mepirizole and Mepirizole-d_3 in Human Serum

The calibration curves for mepirizole and mepirizole-d_3, obtained by plotting peak height ratios of these compounds to mepirizole-d_6, internal standard, show good linearities as illustrated in Fig. 2.

The serum levels of mepirizole and mepirizole-d_3 were determined from the calibration curves, and the results are shown in Fig. 3. The mass fragmentograms obtained are also shown in Fig. 4. A maximum concentration of mepirizole in human serum was observed at 60 minutes after oral administration.

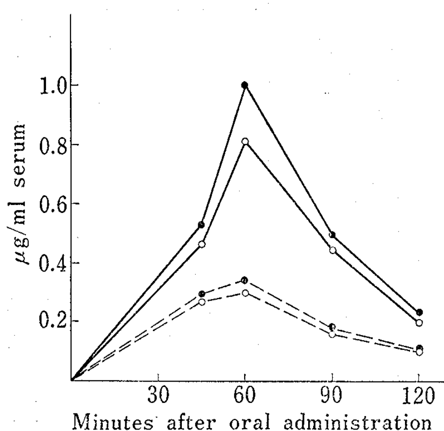

Fig. 3. Human Serum Levels of Mepirizole and Mepirizole-d_3 after Oral Administration

———: volunteer A, -------: volunteer B
○: serum level of mepirizole, ●: serum level of mepirizole-d_3

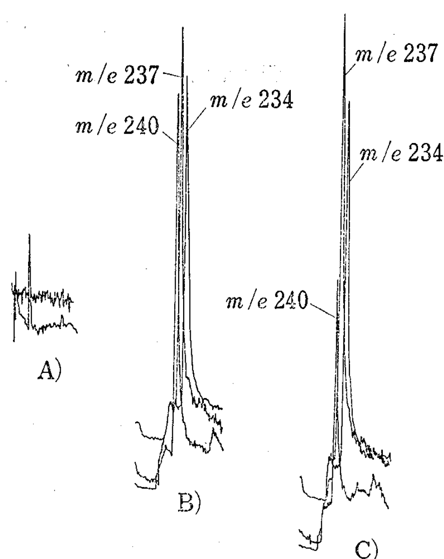

Fig. 4. Mass Fragmentograms of the Extracts of Human Serum

A) the extract of normal human serum
B) and C) the extracts of the human serum after oral administration (B: after 45 min, C: after 60 min) of an equimolar mixture (200 mg) of mepirizole (*m/e* 234) and mepirizole-d_3 (*m/e* 237) The fragmentogram of m/e 240 was derived from the internal standard (mepirizole-d_6).

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Isotope Effects on Drug Metabolism

It has been suggested that deuteration of a drug at the position of substituent which undergoes biotransformation might result in a prolonged pharmacologic action. According to Mitoma, *et al.* who have reported about isotope effects on the metabolic rate, substitution of deuterium for hydrogen in the methyl group of *o*-nitroanisole resulted in approximately 50% reduction in the rate of O-demethylation,[7] and the sleeping time of mice given butethal deuterated at the penultimate carbon atom was approximately twice that of mice given unlabeled butethal,[8] but the isotope effect was slightly observed when methyl group was attached to the aromatic ring (tolbutamide).[9]

The methyl group of the pyrazole ring in mepirizole was shown to be biotransformed to a carboxyl group, especially in man.[10] Delay of the metabolic rate of mepirizole by deuteration

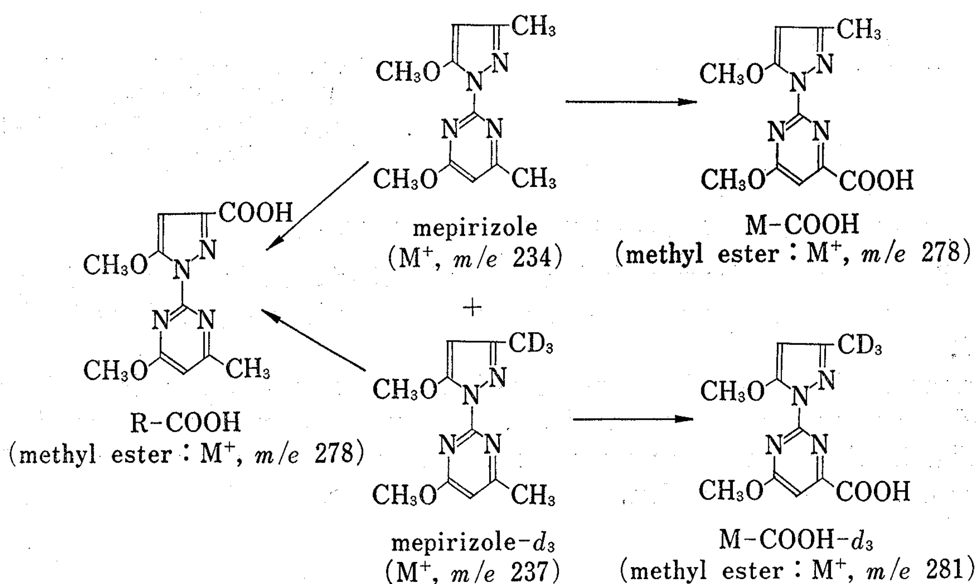

Chart 2. Metabolic Pathways of Mepirizole and Mepirizole-d_3

of the methyl group might result in elevation and duration of its blood levels. In order to evaluate the effect of deuterium labeling on the metabolic rate of mepirizole, an equimolar mixture of mepirizole and mepirizole-d_3 was administered to subjects and their blood levels were determined using mass fragmentography. This method is most suitable for the determination of the slight differences between the blood levels of the two compounds. A comparison of the serum levels of mepirizole and mepirizole-d_3 is shown in Fig. 3, indicating that the rate of oxidation for the deuterated compound is very slightly slower than that of the unlabeled compound. This result agrees with that of tolbutamide described by Mitoma, *et al.*,[9] and

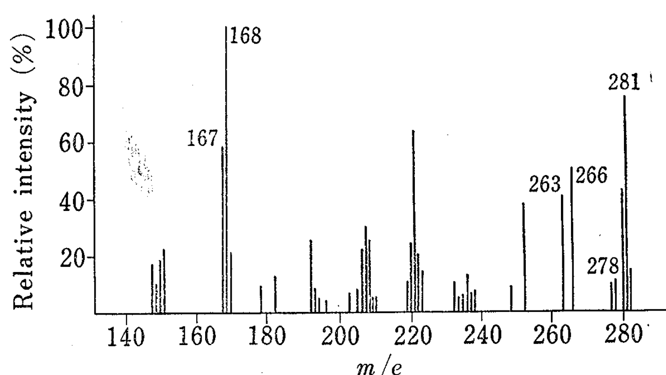

Fig. 5. Mass Spectrum of the Methyl Esters of the Excreted M-COOH and M-COOH-d_3

7) C. Mitoma, D.M. Yasuda, J. Tagg, and M. Tanabe, *Biochem. Biophys. Acta*, **136**, 566 (1967).
8) M. Tanabe, D. Yasuda, S. LeValley, and C. Mitoma, *Life Science*, **8**, 1123 (1969).
9) J. Tagg, D.M. Yasuda, M. Tanabe, and C. Mitoma, *Biochem. Pharmacol.*, **16**, 143 (1967).
10) Y. Tanaka and M. Sano, *Chem. Pharm. Bull.* (Tokyo), **24**, 804 (1976).

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indicates that breaking of the carbon-hydrogen bond is not rate-limiting in the biooxidation of mepirizole.

We next examined the effect of the deuterium lebeling on the formation of the urinary metabolites. As described in the previous paper,[10] the metabolic pathway of mepirizole in man involves the oxidation of the two methyl groups of both rings (Chart 2). The acidic metabolites of mepirizole and mepirizole-d_3, therefore, are R-COOH, M-COOH and M-COOH-d_3. Since R-COOH is a biooxidation product common to both compounds, it is actually indistinguishable whether the metabolite arises from mepirizole or mepirizole-d_3. On the other hand, it is obvious that M-COOH arises from mepirizole and M-COOH-d_3 from mepirizole-d_3. Therefore, the isotope effect on the formation of M-COOH and M-COOH-d_3 was studied by using the 8 hr urine after oral administration of an equimolar mixture of mepirizole and mepirizole-d_3. The acidic metabolites extracted from the urine were converted to their methyl esters using diazomethane. The methyl esters of M-COOH and M-COOH-d_3 were separated from the methyl ester of R-COOH by preparative TLC. The mass spectrum (Fig. 5) of the purified esters of M-COOH and M-COOH-d_3 was measured and the peak intensities were determined by monitoring these molecular ion peaks, m/e 278 and m/e 281, for the methyl esters of M-COOH and M-COOH-d_3 respectively. The peak height of the latter was about five times that of the former. As a result, the isotope effect was observed on the formation of the metabolites.

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