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Identification of Nifedipine Metabolites and Their Determination by Gas Chromatography[1)]

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The urinary metabolites of nifedipine in a dog were isolated and identified by chromatographic and spectrometric techniques. A new major metabolite, 2,6-dimethyl-4-(2-nitrophenyl)-5-methoxycarbonylpyridine-3-carboxylic acid (M-I), was found in both dog and human urine.

A highly sensitive and specific method is described for the determination of nifedipine, M-I, and a previously reported metabolite. The method for analysis of nifedipine is based on the oxidation of nifedipine to its pyridine analog, benzene extraction, and determination by electron-capture gas chromatography. The method for analysis of the metabolites is based on ethyl acetate extraction of the acidified specimen, derivatization of M-I to its methyl ester, and determination by electron-capture gas chromatography. Quantitative determination was possible down to 5 ng/ml of nifedipine using 1 ml samples of biological specimens.

This method was used to measure plasma levels of nifedipine and its metabolites in dogs, and urinary excretion by humans after oral administration of a nifedipine preparation. It was found that nifedipine was absorbed, metabolized, and excreted rapidly, and that the main metabolite was M-I.

Keywords——nifedipine; metabolism; identification; determination; gas chromatography; plasma level; dog; human

Nifedipine, dimethyl 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylate, is a potent coronary vasodilator widely used for the treatment of angina pectoris.

Medenwald *et al.*[3)] studied the metabolites of ^{14}C-labeled nifedipine in the urine by thin-layer chromatography (TLC) and radioactivity assay following single oral administration to rats and dogs. They reported[3)] that the urinary metabolites in rats and dogs were a hydroxymethyl-pyridinecarboxylic acid (M-II) and the corresponding lactone (M-III). During a study on the bioavailability of nifedipine, however, we found a new major metabolite, dimethyl-pyridinecarboxylic acid (M-I), in the urine of a dog.

1) A part of this work was presented at the 28th Meeting of the Kinki Branch, Pharmaceutical Society of Japan, Nishinomiya, October 1978.
2) Location: *1-3-80, Tomobuchi-cho, Miyakojima-ku, Osaka, 534, Japan.*
3) H. Medenwald, K. Schlossman, and C. Wuensche, *Arzneim.-Forsch.*, **22**, 53 (1972).

Fluorometric[4] and radioisotope[5] techniques are available to determine nifedipine and its metabolites in biological fluids. The former method lacks specificity and sensitivity, while the latter method is sensitive but not applicable to clinical pharmacological studies because of the use of radioactive compounds. Recently, a gas chromatography–mass spectrometric procedure using selected ion monitoring was reported.[6] This procedure involved oxidation of nifedipine to its pyridine derivative before chromatography. In order to study the metabolism and pharmacokinetics, a simpler method which is capable of separating and quantitating nifedipine and its metabolites was required, however. A direct gas chromatographic method for the determination of nifedipine in plasma was reported, using an electron-capture detector.[7] In our study, however, when nanogram amounts of nifedipine were injected into the column of the gas chromatograph, operated under the reported conditions,[7] it was partially oxidized to its pyridine derivative, resulting in two peaks. Therefore, we modified the former method[6] to determine nifedipine and its metabolites by using an electron-capture detector and were able to measure them with good specificity and sensitivity.

The present report describes a new major metabolite, M-I, found in the urine of a dog after administration of nifedipine, together with a procedure for the measurement of nifedipine and its metabolites (M-I and M-III) in biological fluids by electron-capture gas chromatography.

Experimental

Materials——Nifedipine supplied was used without further purification.

Dimethyl 2,6-Dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylate (NPO): NPO was synthesized according to the procedure reported by Kamal *et al.*[8] Pale yellow plates, mp 103—104° (reported[9] 106°). IR ν_{max}^{KBr} cm^{-1}: 2960 (CH of pyridine skeleton), 1730 (C=O of ester), 1525 and 1350 (NO_2).

2,6-Dimethyl-4-(2-nitrophenyl)-5-methoxycarbonylpyridine-3-carboxylic acid (authentic sample of M-I): A solution (10 ml) of NPO (2.0 g) in methanol was treated with 25 ml of 1 M methanolic KOH and the mixture was refluxed for 20 min. When cool, the solution was diluted with 25 ml of H_2O and washed with 50 ml of AcOEt. The aqueous layer was acidified with HCl and extracted with 50 ml of AcOEt. The organic layer was dried over $MgSO_4$ and evaporated to dryness. The residue was recrystallized from MeOH–H_2O (1: 1) to afford white plates (1.1 g), mp 233—234°. IR ν_{max}^{KBr} cm^{-1}: 3450 (OH), 1735 (C=O of ester). NMR (DMSO-d_6) δ: 2.56 and 2.60 (6H, two s, ary CH_3), 3.46 (3H, s, CO_2CH_3), 7.2—7.4 (1H, m, arom.), 7.6—7.9 (2H, m, arom.), 8.1—8.3 (1H, m, arom.), 13.2 (1H, broad s, COOH). MS *m/e* (%): 299 (M^+-OCH_3, 3), 284 (M^+-NO_2, 100), 269 ($M^+-NO_2-CH_3$, 3), 253 ($M^+-NO_2-OCH_3$, 6). *Anal.* Calcd for $C_{16}H_{14}N_2O_6$: C, 58.17; H, 4.28; N, 8.48. Found: C, 58.05; H, 4.18; N, 8.44.

5-Methyl-6-methoxycarbonyl-7-(2-nitrophenyl)-4-azaphthalide (authentic sample of M-III): According to the reported procedure[10] for the synthesis of 2-hydroxymethyl-6-methylpyridine, 2 ml of 30% H_2O_2 was added to 10 ml of a solution of NPO (1.0 g) in AcOH and the mixture was heated at 80° for 15 hr. The solution was concentrated, made basic with $NaHCO_3$ solution and extracted with $CHCl_3$. The organic layer was dried over $MgSO_4$ and evaporated to dryness. The residue was chromatographed on 50 g of silica gel with AcOEt–hexane (1: 5). The eluate was evaporated to dryness and the residue was recrystallized from toluene to afford pale yellow plates (0.2 g), mp 173—174°. IR ν_{max}^{KBr} cm^{-1}: 1770 (C=O of lactone), 1735 (C=O of ester). NMR (DMSO-d_6) δ: 2.73 (3H, s, ary CH_3), 3.52 (3H, s, CO_2CH_3), 5.48 and 5.52 (2H, two s, CH_2), 7.3—7.5 (1H, m, arom.), 7.7—8.0 (2H, m, arom.), 8.2—8.4 (1H, m, arom.). MS *m/e* (%): 297 (M^+-OCH_3, 3), 282 (M^+-NO_2, 100), 267 ($M^+-NO_2-CH_3$, 6), 251 ($M^+-NO_2-OCH_3$, 4).[11]

Diethyl 2,6-Dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylate: An internal standard was synthesized by oxidation of the 1,4-dihydro derivative, which was synthesized from *o*-nitrobenzaldehyde, ethyl

4) K. Schlossmann, *Arzneim.-Forsch.*, **22**, 60 (1972).
5) B. Duhm, W. Maul, H. Medenwald, K. Patzschke, and L. Wegner, *Arzneim.-Forsch.*, **22**, 42 (1972); F.A. Horster, B. Duhm, W. Maul, H. Medenwald, K. Patzschke, and L. Wegner, *ibid.*, **22**, 330 (1972).
6) S. Higuchi and Y. Shiobara, *Biomed. Mass Spectrom.*, **5**, 220 (1978).
7) P. Jakobsen, O.L. Pedersen, and E. Mikkelsen, *J. Chromatogr.*, **162**, 81 (1979).
8) A. Kamal, M. Ahmad, N. Mohd, and A.M. Hamid, *Bull. Chem. Soc. Jpn.*, **37**, 610 (1964).
9) Von S. Ebel, H. Schuetz, and A. Hornitschek, *Arzneim.-Forsch.*, **28**, 2188 (1978).
10) H. Kobayashi, S. Furukawa, and Y. Kawada, *Yakugaku Zasshi*, **74** 790 (1954).
11) The mass spectrum was identical with the reported data (ref. 3).

acetoacetate, and NH_3 by the Hantzsch synthesis,[12] according to the procedure reported by Kamal *et al.*[8] Recrystallization from AcOEt afforded pale yellow plates, mp 72—73° (reported[8] 73°).

All other chemicals were commercial products of analytical reagent grade.

Identification of Urinary Metabolites——A male beagle dog (10 kg) was fasted overnight and administered orally 1 g of nifedipine dissolved in 40 ml of 50% macrogol 400. After the administration, urine was collected for 24 hr and stored frozen until analysis. The sample was adjusted to pH 1 with HCl and extracted with AcOEt. The organic phase was evaporated to dryness. After addition of hexane to the residue, the solution was subjected to column chromatography on silicic acid with AcOEt–hexane (3: 1) to afford two fractions (fraction-1 and -2). Each fraction was purified by TLC on silica gel 60 F_{254} (Merck 5717). The developing solvent system for fraction-1 was toluene–AcOH–H_2O (5: 5: 1, organic phase), while $CHCl_3$–MeOH (1: 1) was used for fraction-2. The metabolites on the TLC plate were visualized under ultraviolet light at 254 and 361 nm. The spots were scraped off and extracted with EtOH. The substances obtained were then identified by TLC, and nuclear magnetic resonance (NMR) and mass spectrometric analyses. Mass spectrometry was performed on a JEOL JMS-01SG-2 mass spectrometer. The ion source was operated at 180° with an ionizing current of 200 μA and an electron energy of 75 eV.

Gas Chromatographic Determination of Nifedipine in Biological Specimens——The method reported by Higuchi and Shiobara[6] was modified as follows: 1 ml of dog plasma or human urine was taken, then 0.5 ml of an aqueous solution of the internal standard (200 ng/ml), 2 ml of 0.1 N HCl, and 0.3 ml of 1% $NaNO_2$ were added, and the mixture was allowed to stand in a water bath at 45° for 1 hr to oxidize nifedipine to NPO. Up to this point, experiments were carried out in a dark room in view of the high sensitivity of nifedipine to light.[13] When cool, the mixture was extracted with 4 ml of benzene, and the organic layer (3 ml) was evaporated to dryness. The residue was redissolved in 400 μl of AcOEt, and 1—3 μl of this AcOEt solution was injected into the gas chromatograph.

The gas chromatograph (JEOL JGC-20KE) was equipped with an electron-capture detector (10 mCi of ^{63}Ni) operated at 260°. The carrier gas was ultrapure nitrogen at a flow rate of 26 ml/min. Detector setting, attenuation Hi-8. The column was of coiled glass, 2 m × 2 mm i.d., packed with 3% OV-1 on 80—100 mesh Chromosorb W (AW-DMCS). Temperatures during operation were: injection port, 260°; column oven, 230°. Under these conditions, NPO and the internal standard gave retention times of 6.3 and 9.4 min, respectively.

Gas Chromatographic Determination of Metabolites (M-I and M-III) in Biological Specimens——A 1 ml aliquot of dog plasma or human urine was taken, 0.25 ml of 1 N HCl was added, and the mixture was extracted with 5 ml of AcOEt. The organic layer (3 ml) was treated with 1 ml of ethereal CH_2N_2 to derivatize M-I to its methyl ester (NPO), and the mixture was evaporated to dryness. The residue was dissolved in 1 ml of benzene containing 500 ng of the internal standard in the case of the dog plasma sample and 1 μl of this

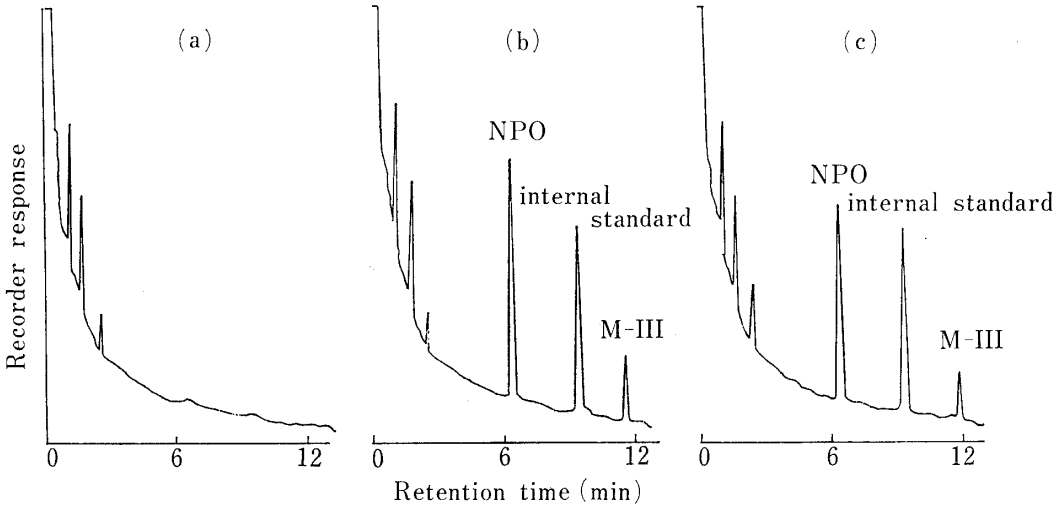

Fig. 1. Gas Chromatograms of Dog Plasma after Acidic Extraction and Derivatization of M-I toNPO

(a) Control plasma, (b) spiked plasma containing 800 ng/ml of NPO and 500 ng/ml of M-III, (c) dog plasma obtained 1 hr after dosing.

12) A. Hantzsch, *Chem. Ber.*, **17**, 1515 (1884); *idem.*, *ibid.*, **18**, 1774, 2579 (1885); J.A. Berson, and E. Brown, *J. Am. Chem. Soc.*, **77**, 444 (1955).

13) A. Kudo, J. Sakai, H. Kono, F. Sueshige, and K. Kamiyama, *Kiso to Rinsho*, **6**, 259 (1972).

benzene solution was injected into the gas chromatograph. The analytical conditions were as described above. The retention time was 6.3 min for NPO and 11.4 min for M-III (Fig. 1).

Plasma Levels of Nifedipine and Its Metabolites (M-I and M-III) in Beagle Dogs——Two beagle dogs (about 10 kg) received 10 mg of nifedipine as a commercial capsule (Adalat, Bayer) with 100 ml of water. The animals were fasted overnight before drug administration. Blood specimens were withdrawn in heparinized syringes. Plasma was separated by centrifugation and aliquots were removed for analysis. Each plasma sample was divided into two portions, one for the determination of nifedipine and the other for the determination of metabolites (M-I and M-III).

Urinary Excretion of Nifedipine and Its Metabolites (M-I and M-III) by Human Subjects——Two volunteers (males, 57 and 59 kg) received 10 mg of nifedipine as a commercial capsule (Adalat, Bayer) with 180 ml of water. A zero time urine specimen was collected immediately before drug administration and subsequent urine specimens were collected 1, 2, 4, 6, and 8 hr after drug administration. The volume of each specimen was recorded, and two aliquots (one for the determination of nifedipine and the other for the determination of metabolites, M-I and M-III) were stored in a frozen state until assayed.

Results and Discussion

Medenwald *et al.*[3] stated that high-voltage electrophoresis of the urine from rats given nifedipine showed that the major renal metabolite was a carboxylic acid derivative, while TLC revealed the presence of a minor metabolite. They identified the minor metabolite as a lactone (M-III) from the mass, NMR, and infrared spectral data. In aqueous solution, a pH-dependent equilibrium exists between M-II and M-III. At acidic pH, formation of the lactone (M-III) is favored, whereas in an alkaline medium the acid form (M-II) predominates. Therefore, they considered that the major metabolite was a hydroxymethyl-pyridinecarboxylic acid (M-II) without further study.

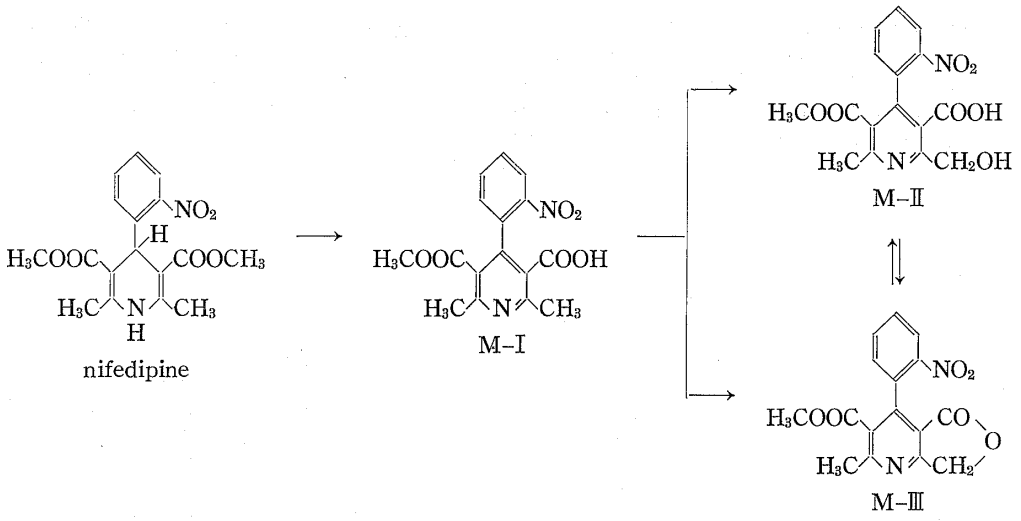

Chart 1

Mass and NMR spectra of the metabolite obtained from fraction-1 in the present study were identical with those of the reported metabolite, M-III[3] (Fig. 2 and Table I). Moreover, it was confirmed by TLC (*Rf* 0.4; plate, Merck 5715; solvent system, toluene–acetic acid–water, 5: 5: 1, organic phase) and by comparison with the mass and NMR spectra of authentic M-III that the metabolite obtained from fraction-1 was M-III. On the other hand, when the NMR spectrum of the metabolite obtained from fraction-2 was taken, the signals of the methyl protons as the C-2 and C-6 positions of the metabolite at 2.56 (3H, singlet) and 2.60 (3H, singlet) ppm suggested that this metabolite might not be M-II. TLC behavior (*Rf* 0.5; plate, Merck 5715; solvent system, $CHCl_3$–MeOH, 1: 1) and mass and NMR spectra of the metabolite obtained from fraction-2 were identical with those of authentic M-I (Fig. 3 and Table I). In the present experiments, M-II was not detected. It was assumed that

M-II was converted into M-III rapidly and completely during the acidic extraction of the urine.

These results confirmed that one of the major metabolites obtained from dog urine is 2,6-dimethyl-4-(2-nitrophenyl)-5-methoxycarbonylpyridine-3-carboxylic acid (M-I). M-I was also found in human urine, as will be described later.

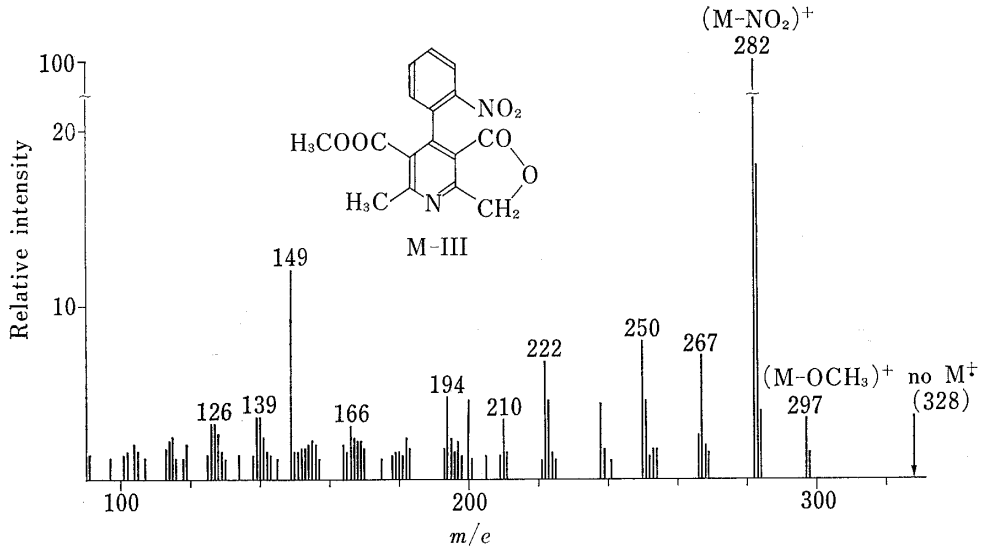

Fig. 2. Mass Spectrum of the Metabolite obtained from Fraction-1

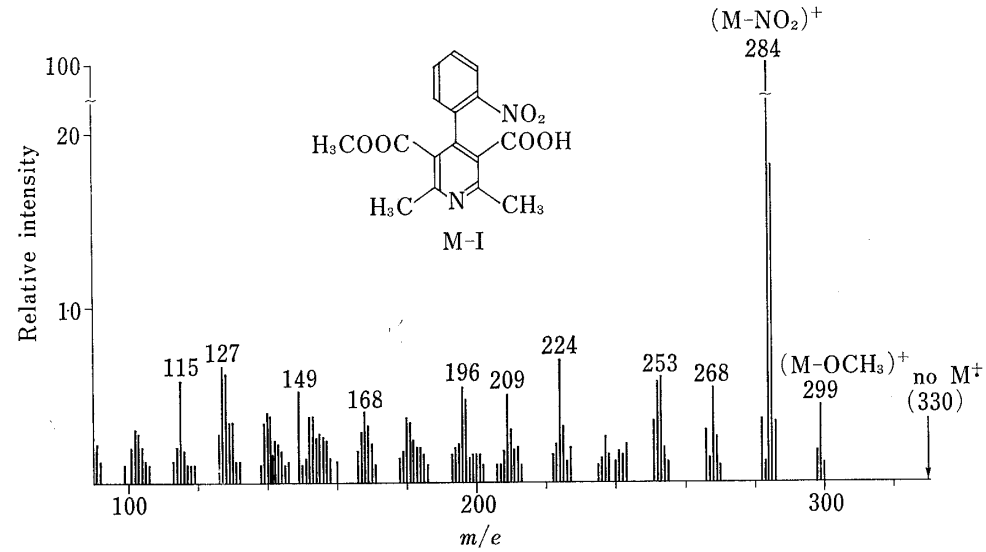

Fig. 3. Mass Spectrum of the Metabolite obtained from Fraction-2

TABLE I. NMR Spectral Data for the Metabolites

Compound[a)]	ppm (in $(CD_3)_2SO$, 100 MHz, TMS)
Fr-1	2.73 (3H, s, ary CH_3), 3.52 (3H, s, CO_2CH_3), 5.48 and 5.52 (2H, two s, CH_2), 7.3—7.5 (1H, m, arom.), 7.7—8.0 (2H, m, arom.), 8.2—8.4 (1H, m, arom.)
Fr-2	2.56 and 2.60 (6H, two s, ary CH_3), 3.46 (3H,s, CO_2CH_3), 7.2—7.4 (1H, m, arom.), 7.6—7.9 (2H, m, arom.), 8.1—8.3 (1H, m, arom.), 13.2 (1H, broad s, COOH)

a) Fr-1=metabolite obtained from fraction-1.
b) Fr-2=metabolite obtained from fraction-2.

Figure 1 shows the chromatograms of dog plasma after acidic extraction and derivatization of M-I to NPO. Gas chromatograms of control samples (dog plasma or human urine) did not exhibit any extraneous peaks with retention times similar to those of NPO, the internal standard, and M-III. Linear calibration curves were obtained by analyses of various amounts of nifedipine (5—100 ng), M-I (50—1000 ng), and M-III (50—500 ng) added to control samples. The minimum detectable amount of nifedipine in dog plasma or human urine was 5 ng/ml. The reproducibility of the method was checked by analysis of dog plasma samples to which 100 ng/ml of nifedipine or 1000 ng/ml of M-I had been added. The coefficients of variation for nifedipine and M-I were 2.8 and 1.9%, respectively (n=6). The overall recoveries of nifedipine, the internal standard, and M-I from dog plasma were 96, 92, and 95%, respectively.

If M-II were present in biological specimens, one might expect that it could be assayed by the analytical method for metabolites described above. However, gas chromatograms of dog plasma or human urine after the administration of nifedipine showed only the peaks of NPO, M-III, and the internal standard. It is likely that M-II was converted into M-III during the acidic extraction of the biological specimens.

If NPO were present as one of the metabolites, the values obtained by this assay method would represent the sum of nifedipine, or M-I, and the metabolite (NPO). Higuchi and Shiobara[6] checked this possibility by comparing the assay results for nifedipine in drug-treated human plasma samples after TLC separation with those without TLC separation. The results were the same, and they concluded that NPO itself was not present as a metabolite in human plasma and did not interfere with the assay. However, further study was required to determine NPO in dog plasma or human urine. We therefore measured the concentration of M-I in dog plasma using diazoethane instead of diazomethane to derivatize M-I to its ethyl ester. After the administration of nifedipine to a dog, the plasma sample was divided into two portions. In one, M-I was derivatized to its ethyl ester and in the other, to its methyl ester (NPO). The assay results for the former sample were not appreciably different from those of the latter. This rules out the possibility that any significant amount of NPO was present as one of the metabolites in dog plasma.

The measurement of plasma levels of nifedipine and its metabolites (M-I and M-III) in beagle dogs after single oral administration confirmed the utility of the analytical meth-

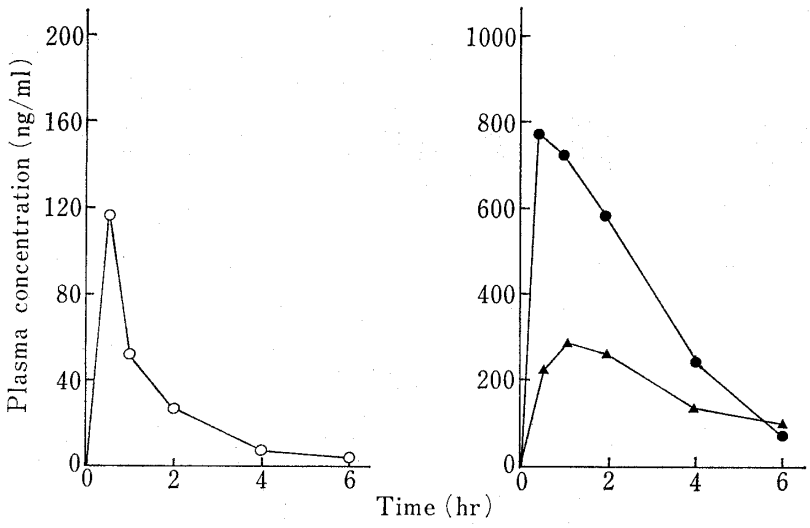

Fig. 4. Plasma Concentration Profiles of Nifedipine, M-I, and M-III after Oral Administration of Nifedipine to Dogs

○; nifedipine, ●; M-I, ▲; M-III.
Each point indicates the mean of values from two dogs.

odology (Fig. 4). After oral administration of a preparation containing 10 mg of nifedipine, the drug was absorbed rapidly. The time to maximum plasma concentration of nifedipine was 0.5 hr. Nifedipine was eliminated rapidly with a half-life of about 0.5 hr. The mean peak level of M-I (774 ng/ml) was observed 0.5 hr after drug administration, indicating rapid drug metabolism. The maximum level of M-III (287 ng/ml) was observed at 1 hr after drug administration.

Figure 5 shows the cumulative amounts of nifedipine and its metabolites (M-I and M-III) excreted in the urine after oral administration of a nifedipine preparation (10 mg) to human subjects. The average amounts of nifedipine, M-I, and M-III excreted during 8 hr represented about 0.1, 20, and 3% of the administered dose of nifedipine, respectively.

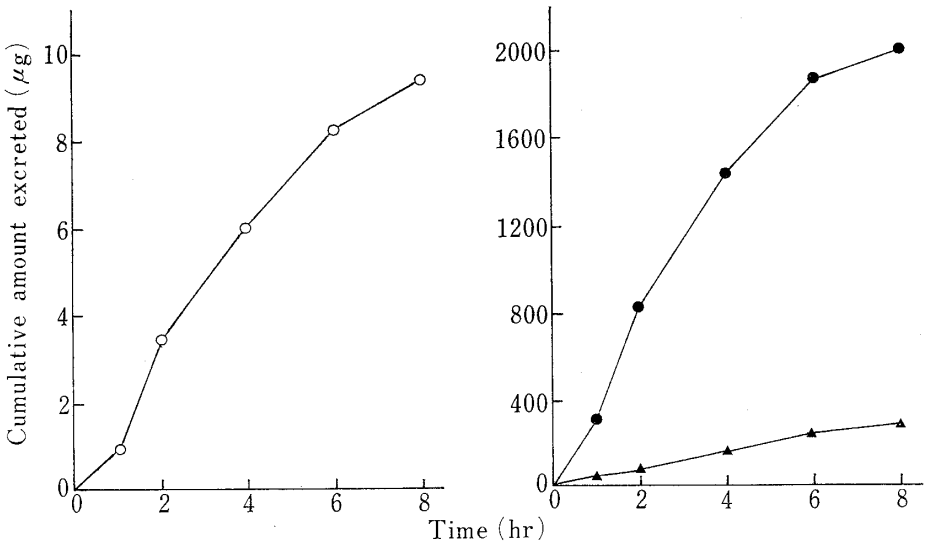

Fig. 5. Cumulative Amounts of Nifedipine, M-I, and M-III in the Urine after Oral Administration of Nifedipine to Man

○; nifedipine, ●; M-I, ▲; M-III.
Each point indicates the mean of values from two human subjects.

The proposed gas chromatographic method can be used for evaluating the metabolism and pharmacokinetics, and for selecting the optimum dosage regimen for nifedipine administration to man.

The results of this investigation indicated that the major metabolite of nifedipine was M-I in a dog and in man, and that M-II or M-III was a minor metabolite. The metabolic pathway of nifedipine is proposed to be as shown in Chart 1; first the dihydropyridine ring is oxidized to a pyridine ring, and at the same time one of the methyl esters is converted into a carboxylic acid by hydrolysis, then the methyl group near the carboxylic acid moiety is oxidized to a hydroxymethyl group.

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