Studies on the Metabolic Fate of Felodipine (N) : Identification of New Metabolites of Felodipine in Rat

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Summary

Two novel metabolites of felodipine were isolated from rat bile or in an *in vitro* system using rat liver preparations. Their chemical structures were identified by comparative analysis with authentic samples.

Both metabolites were formed by ester hydrolysis of felodipine. One was a 3-carboxyl derivative and the other a 5-carboxyl derivative ; both were identified as having dihydropyridine structures and were shown to be major metabolites in rat plasma.

INTRODUCTION

Felodipine (INN), ethylmethyl 4-(2,3-dichlorophenyl)-1,4-dihydro-2,6-dimethyl-3,5-pyridinedicarboxylate is a dihydropyridine type calcium antagonist. The absorption, distribution, metabolism and excretion of radioactivity in rats after single or repeated administration of ^{14}C -felodipine have been reported previously^{1,2,3)} and two major metabolites (named UM1 and UM2) have been identified in plasma and bile, however, their structures have not been identified, due to an insufficient availability of these metabolites. The two metabolites were therefore isolated from rat bile or in an *in vitro* system using rat liver preparations, and their structures were investigated.

MATERIALS AND METHODS

1. Labelled Compound

^{14}C -felodipine (synthesized by AB Hässle (Sweden)) was supplied by CIBA-GEIGY (Japan) Ltd. The structure with labelled position is shown in Fig. 1. The specific radioactivity was 1.7 MBq/mg. The radiochemical purity, determined by thin-layer chromatography as described previously¹⁾, was more than 99%.

Fig. 1 Chemical structure and labelled position of ^{14}C -Felodipine

2. Reagent and Solvent

Authentic samples of expected metabolites and felodipine were synthesized by AB Hässle (Sweden) and supplied by CIBA-GEIGY (Japan) Ltd. NADPH was purchased from Sigma Co., Ltd. Glusulase (Helix pomatia origin, glucuronidase 116,000 unit/ml, sulfatase 14,500 unit/ml) was purchased from Du Pont NEN Research Products. Other reagents and solvents of analytical grade were obtained commercially.

3. Experimental Animals

Sprague-Dawley strain SPF rats (Charles River Japan, Inc.) were acclimatized and used under non-fasting conditions as described in our preceding paper¹⁾. Female rats of 7~8 weeks of age, weighing about 210g were anesthetized with ether and their bile ducts were cannulated with polyethylene tubes. Male and female rats of 8~11 weeks of age were used in other tests.

4. Administration

^{14}C -felodipine was diluted with unlabelled felodipine [Lot No. 107/85 JD (J. 12-2)] and suspended in 0.5% CMC sodium solution. The suspension was administered orally to rats by gavage at a dose of 1 mg/about 37 kBq/2ml/kg.

5. Collection of Biological Samples

Plasma samples obtained 2hr after oral administration of ^{14}C -felodipine to 2 male rats and 3 female rats were separately combined. Biliary samples obtained in the first 24hr interval after oral administration to 20 bile-duct cannulated female rats were combined.

6. Preparation of Rat Liver Homogenate and $9,000\times g$ Supernatant (S9) Fraction

After decapitation and exsanguination of female or male rats, 10 g of the livers were excised, homogenized in 50ml of cold 50 mM Tris-HCl/0.154M KCl buffer. The homogenate was centrifuged ($9,000\times g$, 15min, 5 °C, SCR20BA, HITACHI) and the S9 fraction obtained.

7. Analytical Procedures

1) High Performance Liquid Chromatography (HPLC)

The HPLC system was equipped with a LC-6A pump (SHIMADZU), fitted with a radioisotope detector (RS-8000, TOSOH), and UV detector (SPD-6A, SHIMADZU) or a fluorometer (820-FP, JAPAN SPECTROSCOPIC, Ex 368nm, Em 434nm). Two sets of conditions were employed.

Condition I :

An ODS column (100mm $\times$ 20mm ID, KUSANO KAGAKU KIKAI) was employed using the following mobile phases, with a flow rate of 4 ml/min : (a) acetonitrile/water, 50 : 50 v/v, (b) acetonitrile/water, 40 : 60 v/v, (c) acetonitrile/water, 30 : 70 v/v, or (d) acetonitrile/0.5% acetic acid, 40 : 60 v/v.

Separation was monitored with a radioisotope detector.

Condition II :

An Inertsil ODS-2 column (150mm $\times$ 4.6mm ID, GASUKURO KOGYO) was employed using the following mobile phases, with a flow rate of 1 ml/min : (a) acetonitrile/0.5% acetic acid, 40 : 60 v/v or (b) acetonitrile/0.5% acetic acid, 30 : 70 v/v.

Separation was monitored with UV detector, radioisotope detector or fluorometer.

2) Silica Gel Chromatography

A glass column (250mm $\times$ 0.8mm ID, Ishii Shoten) was filled with silica gel (70~230 mesh, Merck) 10cm in height. The sample dissolved in 2 ml of diethyl ether was applied to the column and eluted with 100ml of diethyl ether.

3) Thin-layer Chromatography (TLC)

TLC was carried out using following two systems. Normal phase : Silica gel 60F₂₅₄ plates (glass plate, 0.25mm thickness, E. Merck) were developed with diethyl ether. Reverse phase : RP-18F₂₅₄S plates (glass plate, 0.25mm thickness, E. Merck) were developed with acetonitrile/water/acetic acid (100 : 100 : 5). The TLC plates were exposed to X-ray films (MARG ^3H type, Konica) or imaging plates (TYPE-BA, Fuji Film) to pre-

pare autoradiograms and to detect the location of radioactive spots or bands.

4) Mass Spectrometry (MS)

Mass spectra were determined with a JMS-1000 mass spectrometer (JEOL), by FAB method with glycerol as matrix.

5) Nuclear Magnetic Resonance (^1H -NMR)

NMR spectra were obtained with a GX-400 spectrometer (JEOL). Each sample was dissolved in 0.5 ml of deuterated methanol (E. Merck).

8. Isolation and Purification of Biliary Metabolites

Biliary samples, (350ml containing radioactivity corresponding to about 1.2mg equivalent to felodipine) collected after administration of ^{14}C -felodipine, were adjusted to pH 5 with 1N HCl and incubated in an equal volume of 0.5M acetate buffer (pH5.0) and 6ml of glucuronidase at 37°C for about 30 min. The mixture was extracted with diethyl ether (3 times with 1300ml). As described in our previous report¹⁾, the metabolites UM1 and UM2 were present in rat bile as conjugates. So, the sample were hydrolyzed by the enzyme before the extraction with diethyl ether. The organic phase was evaporated to dryness and the residue was dissolved in acetonitrile/water (6 ml, 50 : 50 v/v). The solution was filtered through a millipore filter (0.45 μm) and subjected to HPLC (Condition I, Mobile Phase a). The fraction containing UM2 was collected, evaporated to dryness and purified by silica gel chromatography. The eluate was evaporated to dryness and the component was purified further by HPLC (Condition I, Mobile Phase b).

9. Isolation of UM2 from Rat Liver Homogenate

^{14}C -felodipine (5 mg) was added to 250ml of liver homogenate obtained from female rats, and the homogenate incubated at 37°C for 30min. The mixture was extracted with 500ml of diethyl ether and the organic phase was evaporated to dryness. The residue was dissolved in acetonitrile (50ml) and the solution was concentrated to 30ml and washed with n-hexane. After re-concentration of the solution, the UM2 fraction was separated by HPLC (Condition I, Mobile Phase c).

10. Isolation of UM1 from Rat Liver Homogenate 9000 $\times$ g Supernatant (S9) Fraction

^{14}C -felodipine (7.4mg) and NADPH (151mg) were added to 200ml of a liver S9 fraction obtained from male rats and the mixture was incubated at 37°C for 30min, which was then extracted with 400ml of ethyl acetate, the organic phase being evaporated to dryness. The residue was dissolved in acetonitrile/water (2 ml, 50 : 50 v/v) and the UM1 fraction was separated by HPLC (Condition I, Mobile Phase d).

11. Analysis of Plasma Metabolites

Two hours after oral administration of ^{14}C -felodipine (1 mg/kg) to rats, the plasma metabolites were extracted with 3 volumes of diethyl ether. The organic phase was evaporated to dryness, the residue was dissolved in acetonitrile/water (200 μl , 40 : 60 v/v) and the solution was subjected to HPLC (Condition II, Mobile Phase a). The eluates were collected every 30 sec and their radioactivity was counted. While felodipine showed fluo-

Table I HPLC retention times, FAB mass spectral data and NMR spectral data of authentic compounds and metabolites of Felodipine

Authentic Compounds and Metabolites	structure	HPLC tr, min (condition)	FAB mass spectrum m/z major fragment	NMR spectrum ppm (assignment)
Felodipine		34.2 (II)	384 (MH ⁺) 352 ([M-OCH ₃] ⁺) 338 ([M-OC ₂ H ₅] ⁺)	7.32dd(a), 7.27dd(a) 7.14t-like(b) 5.42s(c), 4.03q(e) 3.57s(d), 2.28s(g) 2.26s(g), 1.15t(f)
M- II		31.8 (II)	382 (MH ⁺)	7.60dd(a), 7.12dd(a) 7.33t-like(b) 4.01m(e), 3.54s(d) 2.61s(g), 2.60s(g) 0.91t(f)
S1		15.3 (I)	370 (MH ⁺) 352 ([M-OH] ⁺) 324 ([M-OC ₂ H ₅] ⁺)	7.33dd(a), 7.27dd(a) 7.12t-like(b) 5.43s(c), 4.03q(e) 2.27s(g), 2.26s(g) 1.15t(f)
S2		10.0 (I)	356 (MH ⁺) 338 ([M-OH] ⁺) 324 ([M-OCH ₃] ⁺)	7.32dd(a), 7.26dd(a) 7.12t-like(b) 5.43s(c), 3.56s(d) 2.27s(g)
UM1 from liver S9		14.6 (I)	370 (MH ⁺) 352 ([M-OH] ⁺) 324 ([M-OC ₂ H ₅] ⁺)	7.32(a), 7.24(a) 7.11(b), 5.51(c) 3.99(e) 2.28(g), 1.12(f)
UM2 from rat bile		—	356 (MH ⁺) 338 ([M-OH] ⁺) 324 ([M-OCH ₃] ⁺)	7.32(a), 7.26(a) 7.12(b), 5.44(c) 3.56(d), 2.27(g)
UM2 from liver homogenate		10.0 (I)	356 (MH ⁺) 338 ([M-OH] ⁺) 324 ([M-OCH ₃] ⁺)	7.32dd(a), 7.23dd(a) 7.11t-like(b) 5.50s(c), 3.54s(d) 2.27s(g)

HPLC Column : Inertsil ODS-2 (150mm×4.6mm I.D., GASUKURO KOGYO)

Mobile phase : Acetonitrile/0.5% Acetic Acid (40/60) Flow rate : 1 ml/min

(I) Isocratic elution

(II) Gradient elution to acetonitrile/0.5% acetic acid (70/30) for 15 min
after isocratic elution for 20 minNMR Solvent : CD₃OD

s : singlet, d : doublet, dd : double doublet, t : triplet, q : quartet, m : multiplet

FAB mass Matrix : glycerol

rescence peaking at 434nm with a excitation wave length of 368nm, M-II (oxidized felodipine) did not, suggesting that the fluorescence was originated in the dihydropyridine ring structure ; a fluorometer was therefore used to monitor metabolites.

12. Measurement of Radioactivity

Radioactivity in each test sample was counted for 2min with a liquid scintillation counter (LSC-903, ALOKA). Counting efficiency was corrected by the Channel Ratio method using external standard sources.

RESULTS

The chromatographic (HPLC) and spectrometric (FAB-MS, ^1H -NMR) characteristics of isolated metabolites UM1 and UM2 and authentic samples of felodipine, M-II, S1 and S2 are shown in Table I. HPLC chromatograms of the isolated metabolites UM1 and UM2, plasma metabolites UM1 and UM2, and authentic samples S1 and S2 are shown in Fig. 3 ~ 6 later.

(a) Metabolite UM2

FAB-MS spectra and ^1H -NMR spectra of each UM2 isolated from rat bile and liver homogenates corresponded with each other as shown in Table I. The FAB-MS spectra of UM2, isolated from bile and liver homogenate, showed an MH^+ ion at m/z 356 which was 28mass units (mu) lower than the MH^+ ion of felodipine at m/z 384. Fragment ions were subsequently observed at m/z 338 $[\text{M}-\text{OH}]^+$ and 324 $[\text{M}-\text{OCH}_3]^+$. Diazomethane methylation of UM2 isolated from liver homogenates gave a derivative with an MH^+ ion at m/z 370 which was 14 mu higher than the MH^+ ion, indicating the addition of one methyl group.

The ^1H -NMR spectrum of UM2 showed three protons at 7.1~7.4ppm due to the aromatics, a six-proton singlet at 2.27 ppm due to the methyl groups at 2 and 6 positions and a proton at 5.50 ppm characteristic of that found at the C-4 position. The signals in spectrum of UM2 corresponded with that of felodipine but not with that of M-II, indicating that UM2 preserves the dihydropyridine ring structure. However, in comparison with the spectrum of felodipine, a three-proton singlet at 3.54 ppm due to the methyl ester was still present while five protons at 1.15 and 4.03 ppm attributed to an ethyl ester were notably absent.

These results suggested that UM2 is formed by hydrolysis of the ethyl ester at the C-5 position of felodipine. The spectra of FAB-MS and ^1H -NMR of UM2 corresponded well with those of the synthetic authentic sample S2. Thus UM2 was identified as 3-methyl 4-(2,3-dichlorophenyl)-1,4-dihydro-2,6-dimethyl-3,5-pyridinedicarboxylate.

(b) Metabolite UM1

Since metabolite UM1 could not be in pure state isolated from rat bile, it was isolated from incubation mixture containing liver S9 fraction and ^{14}C -felodipine.

The FAB-MS spectrum of UM1 showed an MH^+ ion at m/z 370 which was 14 mu lower than the MH^+ ion of felodipine at m/z 384. Fragment ions were observed at m/z 352 $[\text{M}-\text{OH}]^+$ and 324 $[\text{M}-\text{OCH}_2\text{CH}_3]^+$.

The ^1H -NMR spectrum of UM1 showed three protons at 7.1~7.4 ppm due to the aro-

matics, a six-proton singlet at 2.28 ppm due to the methyl groups at the 2 and 6 positions and a proton at 5.51 ppm due to the C-4 position, indicating the preservation of the dihydropyridine ring structure in UM1, similar to that in UM2. In comparison with the spectrum of felodipine, five protons at 1.12 and 3.99 ppm due to the ethyl ester were still present, but the three-proton singlet at 3.57 ppm due to the methyl ester was absent.

In the ^1H -NMR spectrum of the synthetic authentic sample S1 in deuterated methanol, two singlets due to the methyl groups at 2 and 6 positions of the dihydropyridine ring were observed at 2.26 and 2.27 ppm while only one singlet was observed at 2.28 ppm in the spectrum of UM1. However the spectrum of S1 in deuterated methanol containing a small amount of deuterated hydrochloric acid (diluted with heavy water) showed a singlet in the same region as that of UM1 (Fig. 2), suggesting that the single signal due to the methyl groups at 2 and 6 positions of the dihydropyridine ring of UM1 arises in

Fig. 2 ^1H -NMR spectra of S1 and UM1 in CD_3OD or CD_3OD (containing DCl)
A: UM1 in CD_3OD B: S1 in CD_3OD C: S1 in CD_3OD (containing DCl)

a consequence of an effect by admixed acid.

These results suggested that UM1 is formed by the hydrolysis of the methyl ester at the C-3 positions of felodipine. The spectra of FAB-MS and ^1H -NMR of UM1 corresponded well to those of the synthetic authentic sample S1. Thus UM1 was identified as 5-ethyl 4-(2,3-dichlorophenyl)-1,4-dihydro-2,6-dimethyl-3,5-pyridinedicarboxylate.

(c) Chromatographic Characteristics

HPLC chromatograms of UM1 isolated from incubation mixture containing liver S9 fraction and ^{14}C -felodipine, UM2 isolated from incubation mixture containing liver homogenate and ^{14}C -felodipine, plasma metabolites and the synthetic authentic samples S1 and S2 (monitored by fluorescence) are shown in Fig. 3 ~ 6. The isolated metabolites UM1 and UM2 (Fig. 3) showed fluorescence peaks at much the same retention time as S1 and S2 (Fig. 5), respectively. Plasma metabolites also showed fluorescence and radioactivity peaks (Figs. 4, 6) at much the same retention time as S1 and S2. These results indicate that UM1 and UM2 were identical to the plasma metabolites, and both also had a dihydropyridine structure as felodipine. Other metabolites of felodipine (M-II ~ M-X, chemical structures in Fig. 7) had different retention times from those of UM1 (t_R 15 min) and UM2 (t_R 10 min) under the same HPLC conditions.

DISCUSSION

The two major new metabolites in rat plasma and bile reported in our previous study

Fig. 3 HPLC chromatograms of UM1 and UM2

Column : Inertsil ODS-2 (150mm×4.6mm i.d., GASUKURO KOGYO)

Mobile phase : acetonitrile/0.5% acetic acid = 40/60

Flow rate : 1 ml/min

Detection : Fluorometer Ex 368nm, Em 434nm

UM1 : isolated from rat liver supernatant

UM2 : isolated from rat liver homogenate

Fig. 4 HPLC chromatograms of diethyl ether extract from plasma (2hr) after oral administration of ^{14}C -Felodipine to female rats (dose: 1mg/kg)

Column: Inertsil ODS-2(150mm $\times$ 4.6mm i.d., GASUKURO KOGYO)

Mobile phase: acetonitrile/0.5% acetic acid=40/60

Flow rate: 1 ml/min

Detection: A Fluorometer Ex 368nm, Em 434nm

B liquid scintillation counter (elute was collected in 30 sec fraction.)

¹⁾ have now been identified. It has been reported⁴⁻⁶⁾ that felodipine is metabolized to M-III and M-IV via M-II, thus occurring with oxidation of the dihydropyridine ring. The results in our present study and in our previous study¹⁾, however, suggest that the metabolic pathway (Fig. 7) of felodipine in rats is through metabolites containing a dihydropyridine ring structure. The presumed metabolic pathway of nilvadipine⁷⁾, amlodipine⁸⁾ and nicardipine^{9,10)} through metabolites containing a dihydropyridine structure have also been reported.

In our preceding paper¹⁾, UM1 and UM2 were found as major metabolites in the plasma from 2hr to 24 hr after administration. The total amounts of these two metabolites accounted for more than 48 and 74% of radioactivity in the plasma of male and female rats, respectively. It has been shown^{11,12)} that P-450 takes part in the metabolism from felodipine to M-II, that is, the oxidation of dihydropyridine form to pyridine form. P-450 may also be involved in the oxidation of UM1 and UM2 to the pyridine form. However the elimination of UM1 and UM2 in the plasma was slow¹⁾, which probably indicates that the metabolism of UM1 and UM2 to their pyridine forms is slower than the

Fig. 5 HPLC chromatograms of S1 and S2

Column : Inertsil ODS-2 (150mm×4.6mm i.d., GASU-KURO KOGYO)

Mobile phase : acetonitrile/0.5% acetic acid=40/60

Flow rate : 1 ml/min

Detection : fluorometer Ex 368nm, Em 434nm

metabolism of felodipine to M-II. The structure of the side chain group of the dihydropyridine may influence its oxidation rate to a corresponding pyridine form, as one of factors.

In our previous study¹⁾, there proved to be a sex difference in the elimination of blood radioactivity, due to the difference in the amount of UM1 formed, after oral administration of ¹⁴C-felodipine. It has been reported¹³⁾ that a sex difference in the metabolism of dihydropyridine type drugs is due to the differences in the rate of oxidation from dihydropyridine to pyridine forms, i.e. P-450 activity in female is lower than that of male rats. The plasma concentrations of nifedipine¹⁴⁾ and nilvadipine¹⁵⁾ in female rats were also higher than those in male rats. Therefore a sex difference in pharmacokinetics of felodipine in rats may be due to the difference in the metabolic rate of UM1 to its pyridine form.

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Fig. 6 HPLC chromatograms of diethyl ether extract from plasma (2hr) after oral administration of ^{14}C -Felodipine to male rats (dose : 1 mg/kg)

A : diethyl ether extract only

B : diethyl ether extract was spiked with S1

C : diethyl ether extract was spiked with S2

Column : Inertsil ODS-2 (150mm $\times$ 4.6mm i.d., GASU-KURO KOGYO)

Mobile phase : acetonitrile/0.5% acetic acid = 40/60

Flow rate : 1 ml/min

Detection : fluorometer Ex 368 nm, Em 434nm

supplying labelled felodipine and its metabolites.

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