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Determination of felodipine and its metabolites in plasma using capillary gas chromatography with electron-capture detection and their identification by gas chromatography–mass spectrometry

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ABSTRACT

A novel method for the determination of felodipine and its metabolites in plasma by capillary gas chromatography (GC) with electron capture detection was developed. Felodipine and its oxidized metabolite were assayed by capillary GC after solid-phase extraction with the aid of a cool on-column injection technique. Acid metabolites, methyl monoacid and ethyl monoacid and diacid, were extracted with diethyl ether and propylated with 1-*n*-propyl-3-*p*-tolyltriazene before being submitted to capillary GC. These methods were very sensitive and useful for the pharmacokinetic study of felodipine. Felodipine and its metabolites were identified by GC–mass spectrometry. The mass spectral patterns of the peaks of extracts from human plasma samples after oral administration of felodipine were in good agreement with those of reference compounds.

INTRODUCTION

Felodipine [4-(2,3-dichlorophenyl)-1,4-dihydro-2,6-dimethyl-3,5-pyridinedicarboxylic acid, 5-ethyl-3-methyl ester] is a new type of a calcium antagonist which is currently being investigated for its clinical efficacy in lowering blood pressure. Sensitive and accurate methods for the determination of felodipine and its metabolites in plasma are therefore required for such pharmacokinetic studies. Ahnoff *et al.* [1,2] reported a useful capillary gas chromatographic (GC) method for monitoring M-I and M-II (see Fig. 1) in human plasma. We have modified and improved the procedure by using a solid-phase extraction method. Although several investigations concerning felodipine's metabolites in body fluids have al-

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ready been published [3–5], in this paper we propose a new method for monitoring low plasma levels of felodipine's acid metabolites, M-III, M-IV and M-V (see Fig. 1) using GC with electron-capture detection (ECD).

In addition, we have indentified felodipine and its metabolites in human plas-

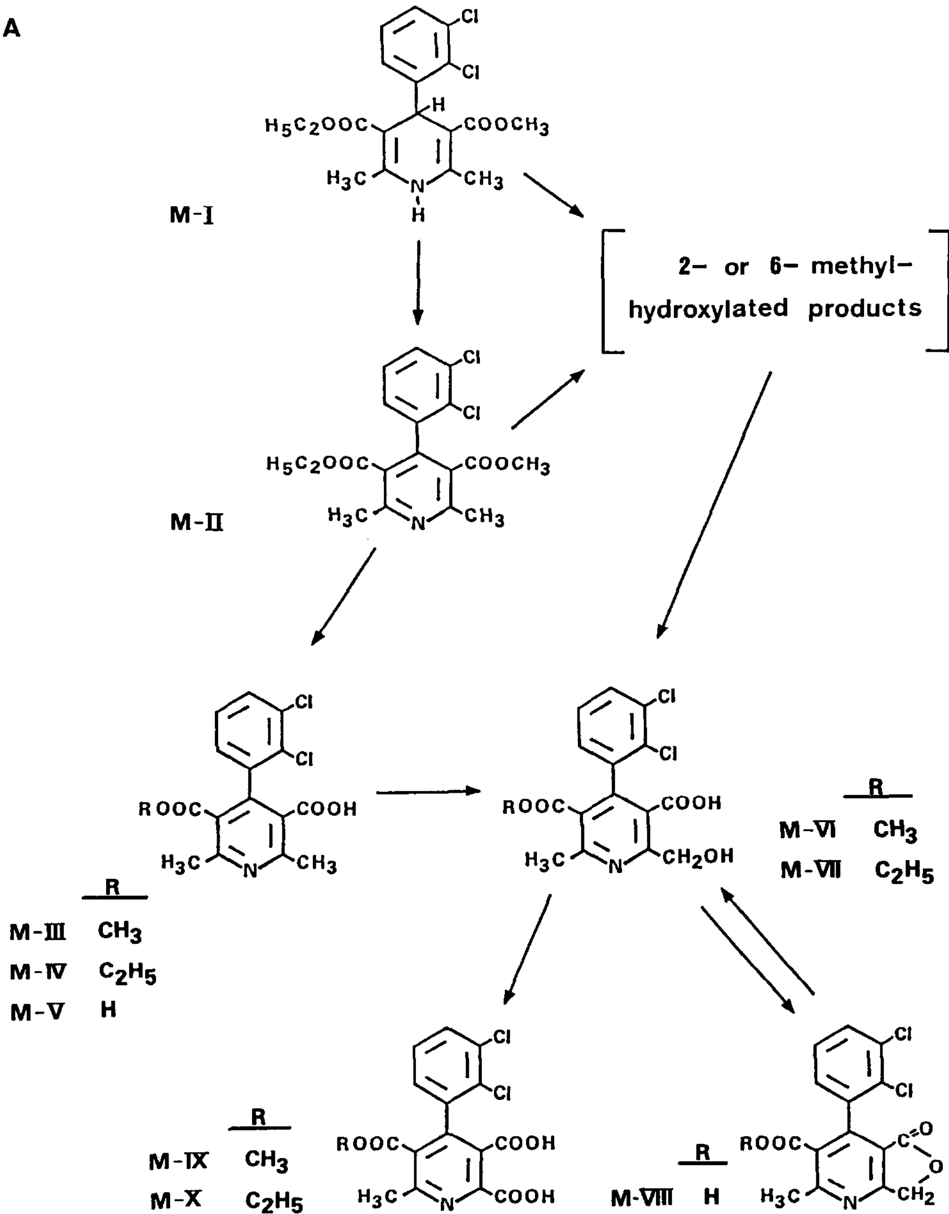

Fig. 1.

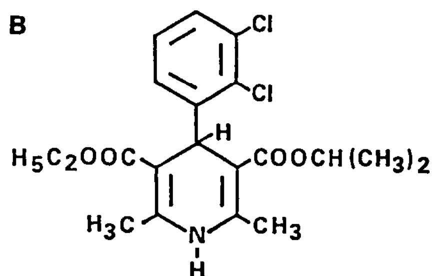

Fig. 1. (A) Metabolic pathway of felodipine in the rat. Reproduced from the paper by Hoffmann and Weidolf [4]. (B) Structure of internal standard (H165/04).

ma by mass spectrometry (MS) by comparison of the mass spectra obtained from plasma extracts with those of reference standards. Lactone-type metabolites, M-VIII (see Fig. 1), M-VI-lactone-body (methyl ester of M-VIII), and M-VII-lactone-body (ethyl ester of M-VIII), were also analysed by GC–MS and their mass patterns were clarified, although they were not determined by GC–ECD.

EXPERIMENTAL

Reagents and chemicals

Standard compounds used in this study, including the internal standard (H165/04), were provided by Hässle (Mölndal, Sweden). Bond-Elut C_{18} disposable extraction columns were purchased from Analytichem International (Harbor City, CA, U.S.A.). 1-*n*-Propyl-3-*p*-tolyltriazene was purchased from Tokyo Kasei (Tokyo, Japan). Hydrochloric acid, methanol, diethyl ether, toluene and *n*-hexane were obtained from Wako (Osaka, Japan) and were all of analytical-reagent grade.

Apparatus and analytical conditions

A Shimadzu Model GC-9APE gas chromatograph equipped with a ^{63}Ni electron-capture detector was used. An ULBON HR-52 fused-silica capillary column (25 m × 0.32 mm I.D.) was purchased from Shinwa Kakou (Kyoto, Japan). The injector and detector temperatures were maintained at 330°C and the column temperature was programmed from 110°C (maintained for 1 min) to 300°C at 10°C/min. The carrier gas was helium and the make-up gas was nitrogen at a flow-rate of 40 ml/min. A Shimadzu OCI-G9 cool on-column injection system was used in the determination of M-I and M-II and a Shimadzu SPL-G9 splitless injection system in the determination of M-III, M-IV and M-V. The column head pressure was 1.0 kg/cm^2 for M-I and M-II and 0.75 kg/cm^2 for M-III, M-IV and M-V.

A Hewlett-Packard Model 5890A–5970B GC–MS system was used in the

identification of felodipine metabolites, and a Hewlett-Packard HP-1 capillary column (12 m × 0.20 mm I.D.) was installed. The operating conditions were the same as described above and an injection port of the splitless type was employed.

Procedure for the determination of M-I and M-II in plasma

A 1-ml volume of plasma sample was applied to a Bond-Elut C_{18} column which was conditioned with methanol (6 ml, twice) and distilled water (6 ml, twice). Subsequently, the column was washed with distilled water (6 ml, twice) and 15% methanol (4 ml, once) and then the sample was eluted with 4 ml of 80% methanol. These procedures of washing and elution from the column were carried out carefully under reduced pressure, which was not controlled exactly but manually controlled so that the rate of solvent passage through the column was about 1 drop per second constantly. The eluate was evaporated to dryness under nitrogen at 40°C and the residue was dissolved in 0.2 ml of toluene containing the internal standard (10 ng/ml). A 2-μl volume of this sample solution was injected into the GC system.

Procedure for the determination of M-III, M-IV and M-V in plasma

A 1-ml volume of 0.1 *M* hydrochloric acid and 3 ml of diethyl ether were added to 0.5 ml of plasma in a 15-ml centrifuge tube. The tube was shaken vigorously for 10 min and centrifuged at 1000 *g* for 5 min. The ether layer was transferred into another tube and the residue was again extracted with 3 ml of diethyl ether. The ether extracts were combined and evaporated to dryness under nitrogen, then 0.5 ml of 1-*n*-propyl-3-*p*-tolyltriazene (0.1 mg/ml in diethyl ether) was added to the residue and the mixture was allowed to stand at room temperature for at least 1 h. The solution was evaporated to dryness under nitrogen and 1 ml of 0.1 *M* hydrochloric acid and 3 ml of *n*-hexane were added. The tube was shaken vigorously for 10 min and centrifuged at 1000 *g* for 5 min. The hexane layer was transferred into another tube and evaporated to dryness and the residue was dissolved in 0.5 ml of toluene containing the internal standard (40 ng/ml). A 2-μl volume of this sample solution was injected into the GC system.

Identification of felodipine metabolites by GC–MS

Mass spectra of the standard compounds were taken; in this case M-III, M-IV, M-V and M-VIII were measured after propylation by 1-*n*-propyl-3-*p*-tolyltriazene. Plasma extracts containing felodipine and its metabolites were concentrated and analysed by GC–MS. M-I and M-II and the other metabolites were specifically determined according to the corresponding extraction methods described above. The patterns of the mass spectra of the plasma extract were compared with those of the standard compounds.

RESULTS AND DISCUSSION

Trace amounts of dihydropyridines in biological samples have been determined previously by GC with negative-ion chemical ionization mass spectrometric detection [6,7], GC–ECD [8,9] and high-performance liquid chromatography with ultraviolet detection [10–12]. Felodipine and its metabolites have a dichlorophenyl ring which has a very high electron-capture capacity and Ahnoff's GC–ECD trace assay technique [1] was of help in our investigations. Ahnoff chose toluene as the extraction solvent [1]; however, we used a simple and rapid solid-phase extraction using Bond-Elut C_{18} and found that this method minimized the interferences of impurities on the ECD gas chromatogram of plasma better than toluene extraction. A detection limit of 0.2 ng/ml was attained for both M-I and M-II. In the GC determination of dihydropyridine, there is a problem with instability against oxidation during the analysis. We employed a cool on-column injection method in order to prevent the oxidation of M-I to M-II on the gas chromatograph, whereas Ahnoff [1] proposed splitless injection with glass material treated by high-temperature silylation.

The determination of the acid metabolites of felodipine in plasma has not been reported previously. Theoretically derivatization by methylation or ethylation of these metabolites is inadequate for the GC assay of M-III, M-IV and M-V when a simultaneous assay is needed. We therefore investigated and established a high-performance simultaneous assay method for M-III, M-IV and M-V by propylating a plasma sample. 1-*n*-Propyl-3-*p*-tolyltriazene was used as the propylating reagent because it has relatively low toxicity and is safer than diazoalkanes for laboratory operation, and the reaction can proceed at room temperature. We found that diethyl ether was the most appropriate solvent for the extraction of M-III, M-IV and M-V from plasma, as ethyl acetate, chloroform and hexane failed to extract M-V even under strongly acidic conditions. It is recommended that diethyl ether extraction is carried out twice in order to improve the recovery.

TABLE I

DAILY VARIABILITY AND ABSOLUTE RECOVERY OF FELODIPINE AND ITS METABOLITES

Mean values, $n = 9$ (three replicate trials at three concentrations).

Compound	Relative standard deviation (%)	Absolute recovery (%)
M-I	6.5	83.7
M-II	10.6	55.4
M-III	4.8	60.4
M-IV	4.1	65.8
M-V	5.9	89.6

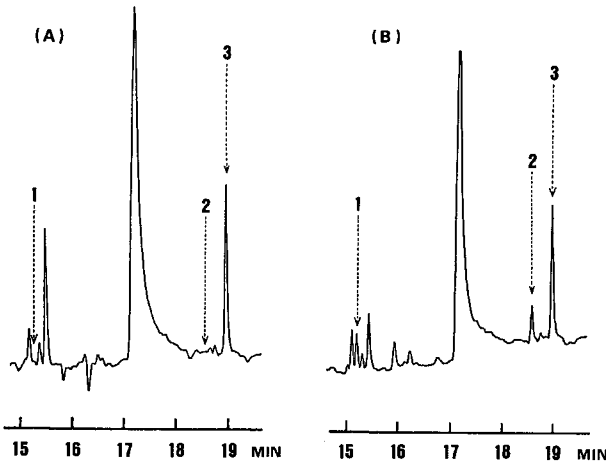

Fig. 2. Typical chromatograms of M-I and M-II in plasma from a volunteer (A) before administration and (B) 0.5 h after administration of 10 mg of felodipine. Peaks: 1 = M-II; 2 = M-I; 3 = internal standard. Peaks 1 and 2 in (B) correspond to 1.8 ng/ml (M-II) and 2.5 ng/ml (M-I), respectively.

The oxidation metabolites having a pyridine ring are stable and were injected into the splitless injection port which was mounted with an autosample injector.

The detection limit was 2 ng/ml for M-III, M-VI and M-V. The reproducibility and recovery of each compound using these methods are given in Table I. The

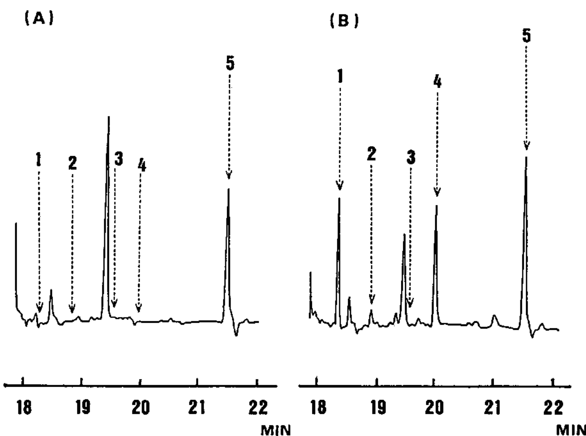

Fig. 3. Typical chromatograms of M-III, M-IV and M-V in plasma from a volunteer (A) before administration and (B) 3 h after administration of 2.5 mg of felodipine. Peaks: 1 = M-III; 2 = M-IV; 3 = M-V; 4 = M-VII-lactone-body; 5 = internal standard. Peaks 1 and 2 in (B) correspond to 39.1 ng/ml (M-III) and 6.1 ng/ml (M-IV), respectively. The peak of M-V is absent at all times and M-VII-lactone-body is not quantified by GC–ECD.

absolute recovery of each compound is not very high, especially for M-II (*ca.* 55%). However, this is not a problem because the calibration graphs were obtained by processing control plasma spiked with corresponding reference standards of various concentrations. The methods are reproducible, with relative standard deviations of 4.1–10.6% for each compound, which are within acceptable precision limits for the determination of drugs in body fluids by GC–ECD. The recovery of the internal standard was less than 40%, so that the accuracy of the method decreases when the internal standard is added before the extraction.

Typical chromatograms are shown in Figs. 2 and 3. These chromatograms show many concomitant peaks originating from plasma and some of these peaks are superimposed on those of the felodipine metabolites. We succeeded in shifting the unfavourable peaks far enough away so as not to disturb the assay by setting an optimum column head pressure. Linear calibration graphs passing through the origin were obtained in the range 0.2–20 ng/ml for M-I and M-II and 2–150 ng/ml for M-III, M-IV and M-V. These assay methods are simple and reproducible and suitable for routine analyses for pharmacokinetic studies after administration of felodipine.

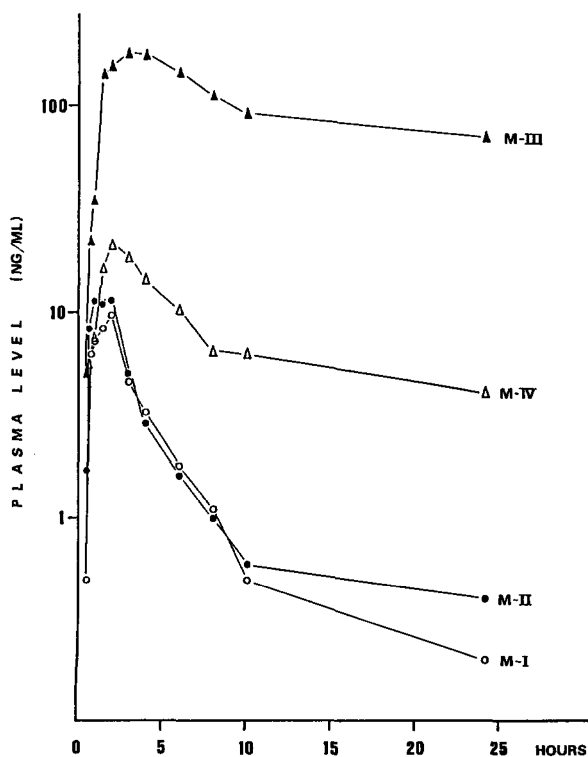

Fig. 4. Plasma levels of M-I, M-II, M-III and M-IV in volunteers after a single oral dose of 10 mg of felodipine.

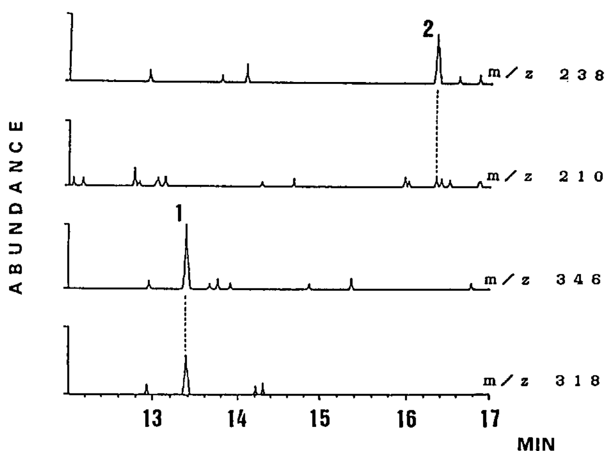

Fig. 5. Mass chromatogram of plasma extract obtained by monitoring typical fragment ions of M-I and M-II. Peaks: 1 = M-I; 2 = M-II. The retention times of these peaks were identical with those for the reference substances.

We also investigated the stability of felodipine and its metabolites in frozen plasma. M-I, M-II, M-III, M-IV and M-V were all stable for at least 50 days at −20°C.

M-I, M-II, M-III and M-IV were the major substances found in human plasma after oral administration of felodipine. Fig. 4 shows their levels in plasma after a single oral dose of felodipine. The concentration of M-V was lower than the detection limit (2 ng/ml) at all times. These substances were identified mass spectrometrically. The mass spectra of the peaks of M-I and M-II in the plasma extract were almost identical with the reference spectra reported by Weidolf *et al.* [3]. The characteristic fragment ions, *m/z* 210 and 239 for M-I and *m/z* 318 and 346 for M-II, were selected for ion monitoring and the mass chromatograms are shown in Fig. 5. M-I and M-II were detected in human plasma with certainty.

Standard mass spectral data for the acid and lactone metabolites of felodipine after propylation and GC are given in Table II. In these mass spectra, the fragment ions formed by the loss of one chlorine atom were seen to have a typical isotope pattern. This is a significant criterion in the identification of felodipine metabolites in body fluids by mass spectrometry as suggested by Hoffmann and Weidolf [4].

The mass spectra of M-III, M-IV and M-VII-lactone-body in the plasma extract were almost identical with those of the reference standards. The characteristic fragment ions of each compound were selected, *m/z* 318 and 360 for M-III, *m/z* 332 and 374 for M-IV, *m/z* 346 and 388 for M-V, *m/z* 316 for M-VI-lactone-body, *m/z* 302 and 330 for M-VII-lactone-body and *m/z* 302 and 344 for M-VII, and the ion monitoring of the plasma extract is shown in Fig. 6. The peaks of

TABLE II

ELECTRON-IMPACT MASS SPECTRAL DATA FOR STANDARD COMPOUNDS OF THE ACID AND LACTONE METABOLITES OF FELODIPINE AFTER PROPYLATION AND GAS CHROMATOGRAPHY

Compound	Major fragment ions[a], m/z (relative intensity in parentheses[b])
M-III propyl ester	360[c] (60), 318 (100), 286 (11)
M-IV propyl ester	374[c] (77), 332 (100), 346 (47), 304 (17), 286 (18)
M-V dipropyl ester	388[c] (31), 346 (100), 304 (14), 286 (9),
M-VI-lactone-body	316[c] (100), 301 (9)
M-VII-lactone-body	330[c] (73), 302 (100)
M-VIII propyl ester	344[c] (31), 302 (100)

[a] The molecular ion peak is absent in all compounds.
[b] In %; base peak = 100%.
[c] $[M-Cl]^+$.

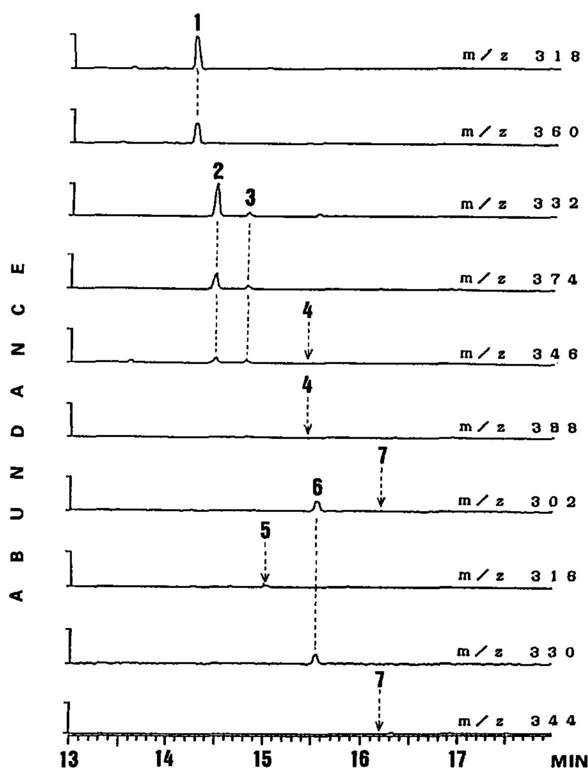

Fig. 6. Mass chromatogram of plasma extract obtained by monitoring typical fragment ions of the acid and lactone metabolites of felodipine. Peaks: 1 = M-III; 2 = oxidized internal standard; 3 = M-IV; 4 = M-V; 5 = M-VI-lactone-body; 6 = M-VII-lactone body; 7 = M-VIII.

M-V and M-VIII did not appear. A small peak was observed at the position of the M-VI-lactone-body but its size was insufficient to give a complete mass spectrum. From this investigation, we conclude that the peaks on the ECD gas chromatogram are identical with those of felodipine and its metabolites, M-II, M-III and M-IV.

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