Identification of Felodipine Metabolites in Rat Urine

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After intragastric administration of 100 μmol kg^{-1} [^{14}C]felodipine to rats eight urinary metabolites were isolated. Batch extraction at pH 2.2 and semipreparative reversed-phase liquid chromatography were used for trace enrichment of the metabolites. Trimethylsilylation followed by transesterification with diazomethane blocked the carboxylic acid and alcohol groups selectively before gas chromatography/mass spectrometry (GC/MS) in the electron impact (EI) mode. Deuterated derivatives of the metabolites and chemical ionization measurements added complementary structural information. All metabolites reported in this study were formed from oxidized felodipine by ester hydrolysis. Hydroxylation of the pyridine methyl group represented an important metabolic pathway and metabolites oxidized to the corresponding carboxylic acids were detected as well. Lactone formation from hydroxy acid metabolites in urine as a possible analytical artefact is discussed.

INTRODUCTION

The vasodilator felodipine (3,5-pyridinedicarboxylic acid, 4-(2,3-dichlorophenyl)-1,4-dihydro-2,6-dimethyl-5-ethyl-3-methyl ester) is a potent antihypertensive drug in man[1] and is currently under investigation in patients to establish its clinical efficacy. Orally administered felodipine is completely absorbed from the gastrointestinal tract and undergoes significant presystemic elimination (Ref. 2 and unpublished data). The highly lipophilic drug is mostly cleared from the body by metabolism and unchanged drug is not excreted into urine. Dehydrogenation of the 1,4-dihydropyridine system of felodipine to the corresponding pyridine has been described as the primary metabolic step *in vitro* using rat liver microsomal preparations[3] and this metabolite is present in human plasma.[4] However, oxidized felodipine is still too lipophilic to be eliminated via the kidneys in man, dog, rat and mouse and the metabolic pattern in urine is fairly similar in these species.[5] Three metabolites formed from oxidized felodipine by ester hydrolysis to polar carboxylic acids were identified by gas chromatography/mass spectrometry after acidic extraction and methylation. The reversed-phase gradient elution high performance liquid chromatography (HPLC) system used for species comparison demonstrated the presence of additional radioactive compounds. However, the structure of the unknown metabolites could not be elucidated with the applied work-up procedure. These findings initiated a more thorough study of the metabolism of felodipine in the rat by development of more specific analytical methods for isolation and identification of metabolites.

EXPERIMENTAL

Chemicals

[6-^{14}C]Felodipine of radiochemical purity 96.0% by HPLC and a specific radioactivity of 811 MBq mmol^{-1} (57.1 μCi mg^{-1}), unlabelled felodipine (mol. wt 384) and reference compounds of metabolites were synthesized at the Department of Organic Chemistry at AB Hässle. Diazomethane was prepared from Diazald® (Aldrich, Milwaukee, Wisconsin, USA) and ($^2H_2$)diazomethane by the use of NaO^{2}H and EtO^{2}H (Fluka AG, Buchs, Switzerland). Other derivatizing reagents, BSA (*N,O*-bis(trimethylsilyl)acetamide, Macherey-Nagel, Düren, GFR) and ($^2H_9$)BSA (MSD, Montreal, Canada) were used as received. All solvents were of analytical grade and used without further purification. They were purchased from the following suppliers: diethyl ether (Fluka AG), methylene chloride, methanol (E. Merck, Darmstadt, GFR), pyridine (Macherey-Nagel) and glacial acetic acid, perchloric acid (70% w/v) and hydrogen peroxide (30% w/v) all from Merck. Water was passed through a MilliQ filter (Millipore Ltd, Milford, Massachusetts, USA). Sodium acetate monohydrate (Merck) and sodium hydroxide (EKA, Bohus, Sweden) were of analytical grade.

Animal experiments

Four male Sprague-Dawley rats (Möllegaard, Denmark) (270–280 g) were fasted overnight and dosed intragastrically with 100 μmol kg^{-1} of [^{14}C]felodipine diluted with unlabelled felodipine to give a specific radioactivity of 17.1 MBq mmol^{-1} (1.20 μCi mg^{-1}). The compound was dissolved in absolute ethanol + polyethylene glycol 400 (1 : 1 by vol.). The rats were kept individually in all-glass metabolism cages which allowed separate collection of urine and faeces. The excreta were collected at 24 h intervals and weighed. The cages were rinsed carefully with water after each collecting period and the samples were stored immediately at −18 °C until analysed.

Radioactivity measurements

Urinary samples of 25–35 mg were weighed into plastic vials for liquid scintillation counting of total radioactivity. After addition of 0.50 ml water and 8 ml Lumagel® (Lumac Systems AG, Basel, Switzerland), radioactivity was determined in a Searle Mark III liquid

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scintillation counter with reference to an external standard. Each sample was analysed in duplicate. Aliquots of the rinsing water (0.30 ml) were counted as well. Weighed faeces in plastic tubes were homogenized in two to three parts of water. The homogenate (50–150 mg) was transferred to glass vials for liquid scintillation counting. Perchloric acid (70% w/v) and H_2O_2 (30% w/v), 0.20 ml of each, were added and the mixture was treated at 80 °C for 40 min. After cooling, 8 ml of Lumagel® were added before radioactivity counting. The samples were analysed in triplicate.

Extraction of metabolites

Pooled urine (14 ml) from the first 24 h collection interval following [^{14}C]felodipine dosing was brought from pH 6.1 to 8.9 with 5 M NaOH. The sample was extracted twice with 35 ml of methylene chloride. The separated aqueous phase was acidified with conc. H_3PO_4 to pH 2.2 and extracted with 45 ml of diethyl ether three times. The aqueous layer was acidified further to pH 1.0 and extracted three times with 30 ml of diethyl ether to recover the most acidic fraction of felodipine metabolites. The unextracted material in the aqueous phase was not analysed further. The organic layers from extraction at different pH were concentrated separately by evaporation under reduced pressure followed by evaporation to dryness in a water bath under a stream of nitrogen at 30 °C before derivatization. The recovered amounts were calculated with reference to the molecular weight of felodipine and specific radioactivity of the given dose.

Semipreparative HPLC

A semipreparative reversed-phase liquid chromatographic system applying gradient elution was used for the fractionation of felodipine metabolites in pooled rat urine ($n = 4$, 0–24 h). The HPLC system was composed of two Altex model 110 A pumps controlled by an Altex model 421 microprocessor, a Rheodyne M-7010 injection valve fitted with a 20 ml stainless steel sample loop (i.d. 2.2 mm). The stainless steel column (30 cm × 7.6 mm i.d.) was packed with LiChroprep RP-8 5–20 μm particles (Merck) (2-propanol + methylene chloride 1 : 1 by vol. as slurry medium and methanol + water 3 : 2 by vol. as the mobile phase at a pressure drop of 200 bar). The Waters Lambda-Max M 480 UV detector was operated at 280 nm. The mobile phases consisted of 0.35 M acetate buffer, pH 4.0 (ionic strength 0.05) and methanol (phase A: 5% v/v methanol and phase B: 67% v/v methanol) and a flow rate of 4.0 ml min^{-1} was used. The gradient formed by the microprocessor was 0% B during 0–5 min, 0–50% B during 5–10 min and 50–80% B during 12–19 min. After a rapid rise to 100% at 22 min the system was maintained at 100% B to 36 min. Finally, it was reversed to 0% B between 37 and 39 min.

The rat urine was thawed and centrifuged at 3000 rpm for 15 min and 16.0 ml was loaded in the sample loop. The sample was injected onto the column with water as the mobile phase at 4.0 ml min^{-1} for 10 min. The flow through the column was stopped while the pump was vented and filled with mobile phase A via a flush valve. The eluate from the column throughout the analysis was collected in 1 min fractions. Aliquots of 50 μl from each fraction were analysed by liquid scintillation counting. A final methanol wash (100 ml) of the column did not contain significant amounts of radioactivity, indicating complete recovery of all metabolites applied. The collected material was pooled into different fractions (F), F1: 2–9 min, F2: 9–14 min, F3: 14–23 min, F4: 23–27 min, F5: 27–32 min and F6: 32–37 min. The pooled fractions were brought to pH 5–6 with 5 M NaOH to avoid lactonization. The volume was decreased to <5 ml by evaporation at reduced pressure and 40 °C. The fractions were acidified with conc. H_3PO_4 to pH 2.2, diluted to 5.0 ml with water and extracted five times with 5.0 ml aliquots of diethyl ether. The solvent was evaporated under reduced pressure to 1–2 ml and finally under a nitrogen stream at 40 °C to dryness before derivatization.

Derivatization

Reference compounds (dissolved in MeOH), the material extracted at pH 2.2 and the different fractions from the semipreparative HPLC run were derivatized after removal of the solvent under a stream of nitrogen.

Trimethylsilylation was performed with a mixture of pyridine and BSA (1 : 1 by vol.) The samples were heated at 60 °C for 1 h. For methylation of the silyl derivatives, an etheral solution of diazomethane was added directly to the BSA + pyridine solution. After vortex mixing for 30 s, the sample was left at room temperature for 30 min, after which the solution was concentrated under nitrogen. The same method was used to form the deuterated derivatives with ($^2H_9$)BSA and $C^2H_2N_2$ as reagents.

Gas chromatography/mass spectrometry

The final solution after derivatization was analysed directly by GC/MS with a Finnigan MAT 44S quadrupole mass spectrometer which was interfaced to a Varian 3700 gas chromatograph. The fused silica capillary column (20 m × 0.32 mm i.d.) was coated with the bonded stationary phase P-Sil-5 (Chrompack, Middleburg, the Netherlands) and was connected to the mass spectrometer transfer line by an open split interface for venting of the solvent front. Samples were injected by the splitless mode of operation with the column oven held at 180 °C for 1 min (injector temperature 250 °C). The oven temperature was then raised linearly to 260 °C by 5 °C min^{-1}. Mass spectra were recorded in the electron impact (70 eV) or chemical ionization (isobutane 0.4 Torr) mode and were acquired and processed by a Finnigan MAT Spectro System 200 on-line data system.

RESULTS

Excretion of radioactivity

After intragastric administration of 100 μmol kg^{-1} [^{14}C]felodipine to rats, radioactivity was found in both urine and faeces and the results are summarized in Table 1. Over a collection period of 72 h an average of 84%

Table 1. Urinary and faecal excretion of radioactivity following intragastric administration of 100 μmol kg^{-1} [^{14}C]felodipine to four rats. Mean values ± SEM

Collection interval (h)	Urine (% of given dose)	Faeces (% of given dose)
0–24	16.95 ± 1.38	21.74 ± 5.05
24–48	9.15 ± 1.75	28.44 ± 6.05
48–72	1.83 ± 0.28	5.98 ± 1.14
Total	27.93 ± 0.96	56.16 ± 2.31

of the given dose was accounted for. Most of the urinary recovery (17%) occurred during the first collection interval and this fraction was taken for further analysis.

Isolation of metabolites

Both batch extraction and semipreparative reversed-phase liquid chromatography were used to concentrate the metabolites in a solvent suitable for derivatization. Pre-extraction of the sample at pH 8.9 did not cause a significant loss of radioactivity (4.8%, 0.12 mg). The following batch extraction of the urine at pH 2.2 with diethyl ether recovered the bulk of radioactivity (86%, 2.0 mg), indicating the acidic character of the metabolites. Only minor amounts of radioactivity were found in the final extraction at pH 1.0 (2.7%, 0.07 mg). The results from the semipreparative reversed-phase HPLC separation of felodipine metabolites in untreated rat urine are given in Fig. 1. At least six metabolite fractions of different polarity were separated by the gradient system. The recovery of radioactivity applied to the column was 99% and fractions F1–F6 contained 12% (0.33 mg), 11% (0.30 mg), 20% (0.54 mg), 4% (0.11 mg), 52% (1.43 mg) and 1% (0.04 mg), of the applied amount, respectively. The recovery of radioactivity in the final extraction step before derivatization of fraction F1 was approximately 45%, whereas no losses were observed with fractions F2–F6.

Gas chromatography/mass spectrometry

The mass spectrum of unchanged felodipine is given in Fig. 2(a). The compound can be analysed by gas chromatography without derivatization and upon EI ionization only a weak molecular ion at m/z 383 was seen. The base peak at m/z 238 originates from bond cleavage between the two ring systems with charge retention on the dihydropyridine moiety. Oxidation of felodipine changed the mass spectral characteristics appreciably (Fig. 2(b)). In this pyridine analogue the $[M]^{+\cdot}$ ion is absent and the linkage between the ring systems was stable towards EI ionization as the ion at m/z 236 was missing. The base peak was formed by loss of one chlorine atom but still contained the typical chlorine isotope ion cluster at m/z 346/348. This is an important feature enabling for recognition of mass spectra of unknown metabolites in complex biological samples.

Most of the urinary felodipine metabolites are acids which have to be derivatized in order to improve their gas chromatographic properties. In Fig. 3 a total ion current chromatogram is given from the analysis of a rat urine extract at pH 2.2 after reaction with BSA. Inspection of all recorded mass spectra showed that seven compounds were structurally related to oxidized felodipine as indicated by the presence of the chlorine isotope cluster. In this analysis metabolites were not completely separated from interfering endogenous compounds. EI mass spectra of metabolites **3–9** are summarized in Tables 2 and 3, together with those from similar runs after subsequent treatment of the silylated samples with diazomethane. The number of derivatized functional groups in each metabolite was determined by

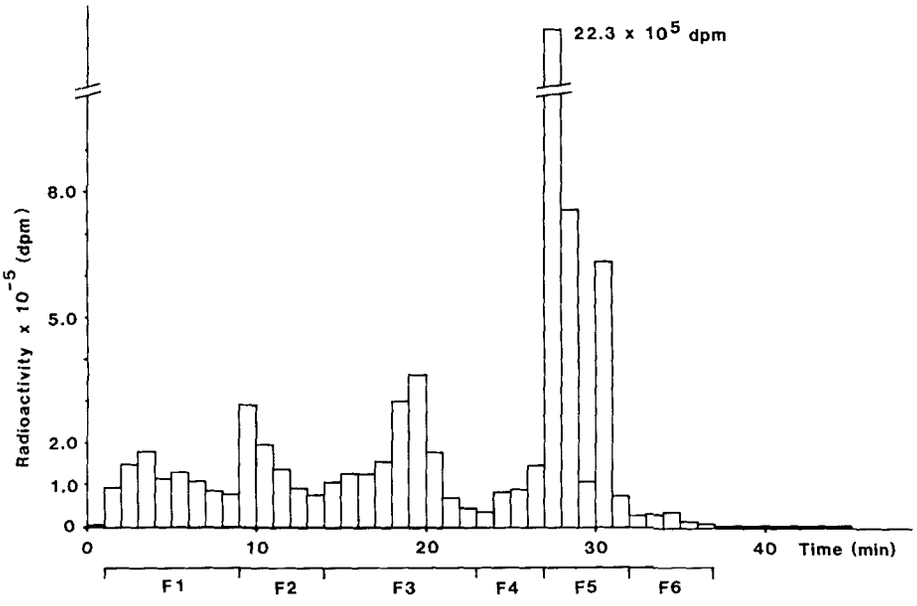


Figure 1. Semipreparative reversed-phase HPLC separation of metabolites in 0–24 h urine after an oral dose (100 μmol kg^{-1}) of [^{14}C]felodipine to rats. F indicates fractions pooled for further analysis.

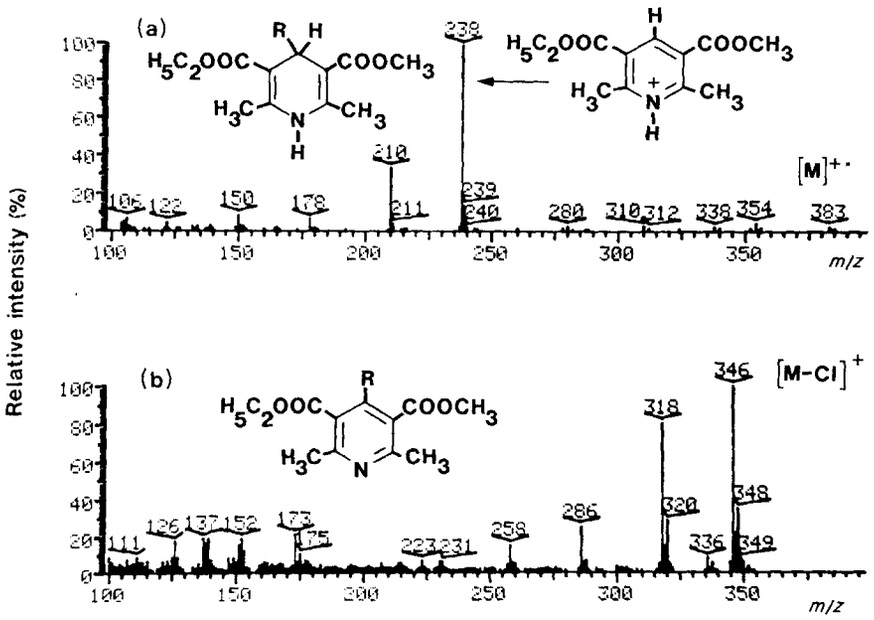


Figure 2. EI mass spectra of (a) felodipine and (b) oxidized felodipine. R=2,3-dichlorophenyl.

using deuterium labelled reagents and the mass spectra are also given in Tables 2 and 3. In Fig. 4 the applicability of this approach is demonstrated for metabolite **5** after silylation with BSA (4(a)) and ($^2H_9$)BSA (4(b)). The observed shift by 18 u in the stable isotope experiment is consistent with two carboxylic acid groups. A second example of a labelled derivative is given in Fig. 5. The $[M-Cl]^+$ ion of metabolite **9** after silylation/methylation was found to be at m/z 376 (Fig. 5(a)), whereas the use of deuterated reagents increased the molecular weight by 6 u, indicating the presence of two carboxylic acid groups. Since isolation of the metabolites by batch extraction also enriched endogenous compounds in the sample, the resolution of the capillary column was too low in some cases to separate metabolites from interfering compounds. An example of this is given in Fig. 5(b), where an interfering compound gave rise to the base peak at m/z 130 in the mass spectrum of **9**.

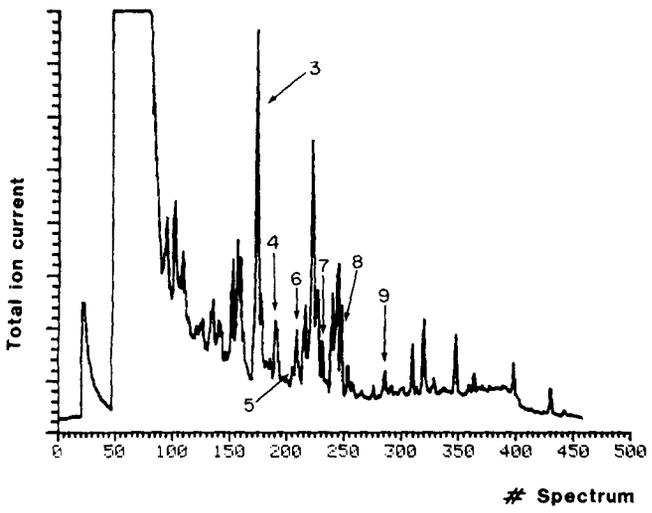


Figure 3. Total ion current chromatogram of trimethylsilylated pH 2.2 urine extract. The hydroxy acid metabolites **6**, **7** and **8** are present as lactones in this sample.

Chemical ionization confirmed the molecular weight of the metabolites and the results are collected in Tables 2 and 3. In Fig. 6 the CI mass spectrum of silylated metabolite **8** in its lactone form is presented together with that of the corresponding deuterated derivative. The $[M+H]^+$ ions were abundant but lack of fragmentation limited further structural information.

The pH 8.9 and 1.0 urine extracts were derivatized and analysed as outlined above. However, no mass spectra of compounds with the characteristic chlorine isotope clusters could be detected.

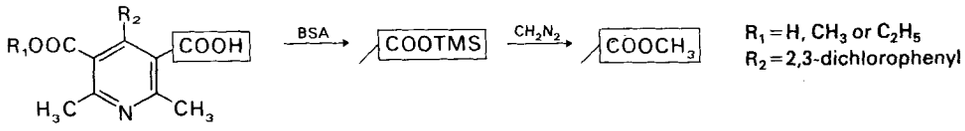
Structural evidence for urinary metabolites

Structures of urinary felodipine metabolites in the rat described in this study are given in Scheme 1. All chemical ionization data for molecular weight information are consistent with the proposed structure of the metabolites both as unlabelled and labelled derivatives. Identification of metabolites was based on identical mass spectra and retention times in gas chromatography and HPLC for both metabolite and reference compound. No reference was available for metabolite **10**.

Metabolite **3** was recovered in fraction F5 (Fig. 1). Its structure as a methyl monoacid was based on a shift by 9 u of the $[M-Cl]^+$ ion from 390 to 399 upon deutero silylation (mass spectrum 1 and 2, Table 2). Consistent data were generated after transesterification of the silyl ester with diazomethane as schematically outlined in Table 2. Also, the $[M-Cl]^+$ ion at m/z 332 was shifted to m/z 335 after reaction with $C^2H_2N_2$ (mass spectra 3 and 4).

Metabolite **4** was recovered in fraction F5 (Fig. 1). after silylation with the $[M-Cl]^+$ ion at m/z 404 as a base peak (spectrum 5) and a shift to m/z 413 in spectrum 6. The loss of 28 u from the $[M-Cl]^+$ ion by McLafferty rearrangement indicated the presence of an ethyl ester in both spectra. Methylation of this compound yielded a molecular structure identical to oxid-

Table 2. Derivatization of urinary carboxylic acid metabolites of felodipine and their corresponding EI mass spectra. Molecular weight was determined in the chemical ionization (CI) mode (isobutane) and the structures of the metabolites are given in Fig. 7



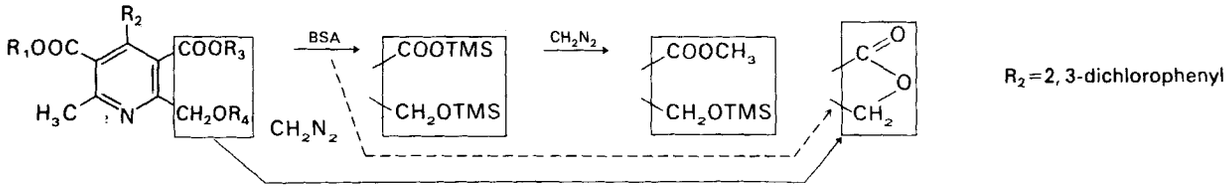
Metabolite	#	Reagent	$[MH]^+$	EI fragment ions (% relative abundance)
3	1	BSA	426	410 (10), 390 (96), 336 (12), 286 (22), 188 (17), 73 (100)
3	2	$(^2H_9)$BSA	435	416 (6), 399 (56), 336 (10), 286 (24), 82 (100)
3	3	BSA/CH_2N_2	368	332 (100), 334 (45)
3	4	$(^2H_9)$BSA/$C^2H_2N_2$	371	335 (100), 334 (40), 337 (35)
4	5	BSA	440	424 (8), 404 (100), 376 (14), 350 (10), 286 (26), 73 (66)
4	6	$(^2H_9)$BSA	449	413 (90), 385 (14), 350 (15), 286 (54), 82 (100)
4	7	BSA/CH_2N_2	382	Mass spectrum given in Fig. 2(b)
4	8	$(^2H_9)$BSA/$C^2H_2N_2$	385	349 (100), 339 (8), 321 (68), 286 (24), 258 (16)
5	9	BSA	484	Mass spectrum given in Fig. 4(a)
5	10	$(^2H_9)$BSA	502	Mass spectrum given in Fig. 4(b)
9	11	BSA	528	512 (14), 492 (40), 73 (100)
9	12	BSA/CH_2N_2	412	Mass spectrum given in Fig. 5(a)
9	13	$(^2H_9)$BSA/$C^2H_2N_2$	418	Mass spectrum given in Fig. 5(b)
10	14	BSA/CH_2N_2	—	$[M-Cl]^+$ ion cluster (390/392), selected ion monitoring.

ized felodipine (**2**). Mass spectral data clearly indicated that the metabolite must be the monoacid and not **2**, since ions in spectrum 7 at m/z 346/348 are increased to m/z 349/351 in spectrum 8.

Metabolite **5** was recovered in fractions F1 and F2. Convincing evidence for the presence of two carboxylic acid groups after trimethylsilylation is presented in Fig. 4. Subsequent methylation of **5** would generate the same derivative as **3** (spectrum 3). Consequently, it would be difficult to distinguish between these two metabolites in the pH 2.2 extracted fraction after the second derivatization step. However both metabolites were well separated in the liquid chromatographic work-up procedure.

Metabolite **9** was found in fractions F1 and F2. The TMS derivative exhibited $[M-CH_3]^+$ and $[M-Cl]^+$ ions (spectrum 11) and the mass spectral results in Fig.

Table 3. Derivatization of urinary hydroxycarboxylic acid metabolites of felodipine and their corresponding EI mass spectra. Molecular weight was determined in the CI mode and the structures of the metabolites are given in Fig. 7. Spectra 15, 16, 18 and 19 were obtained with synthetic references only, as silylation of biological samples resulted mainly in lactone formation (mass spectra # 17 and 20)



Metabolite	#	Reagent	Derivative R_1	Derivative R_3	Derivative R_4	MH^+	EI fragment ions (% relative abundance)
6	15	BSA	CH_3	TMS[a]	TMS	514	Mass spectrum given in Fig. 7
6	16	BSA/CH_2N_2	CH_3	CH_3	TMS	456	440 (25), 423 (12), 388 (6), 89 (100), 73 (34), 59 (32)
6	17	BSA/CH_2N_2	CH_3	lactone		352	316 (100), 301 (8)
7	18	BSA	C_2H_5	TMS	TMS	528	512 (22), 492 (16), 437 (18), 410 (10), 330 (21), 302 (12), 147 (100), 73 (78)
7	19	BSA/CH_2N_2	C_2H_5	CH_3	TMS	470	454 (90), 437 (41), 402 (22), 330 (20), 302 (24), 89 (100), 73 (46), 59 (40)
7	20	BSA/CH_2N_2	C_2H_5	lactone		366	330 (70), 302 (100)
8	21	BSA	TMS	lactone		410	394 (16), 374 (100), 320 (18), 292 (6), 180 (37), 73 (86)
8	22	$(^2H_9)$BSA	$(^2H_9)$TMS	lactone		419	400 (6), 383 (37), 320 (8), 82 (100)
8	23	BSA/CH_2N_2	CH_3	lactone		352	Same spectrum as #17
8	24	$(^2H_9)$BSA/$C^2H_2N_2$	C^2H_3	lactone		355	319 (100), 318 (40), 301 (22)

[a] TMS = trimethylsilyl.

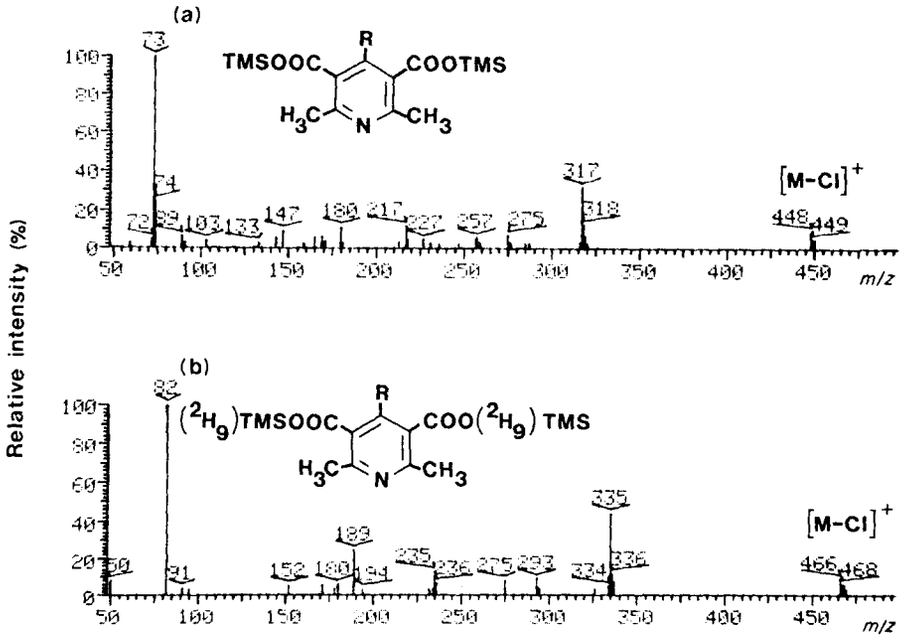


Figure 4. EI mass spectra of metabolite **5** after derivatization with (a) BSA and (b) ($^2H_9$)BSA. R = 2,3-dichlorophenyl.

5 substantiate the proposed structure. It must be emphasized that based on these data, the possible isomeric structure with the carboxy group vicinal to the ester function cannot be excluded, as this isomer was not available as a reference compound.

Metabolite **10** was excreted in trace amounts only and a complete mass spectrum could not be recorded. The structure was assigned tentatively as the ethyl ester analogue of **9** based on similar retention behaviour by liquid chromatography and the highly significant $[M - Cl]^+$ ion cluster of m/z 390/392 after silylation of fraction F2 traced by selected ion monitoring. The gas chromatographic retention time of **10** as compared with **9** was increased in the expected order due to an additional methylene group in **10**.

Metabolite **6** was present in fraction F5 and the mass spectra of the corresponding reference compound after silylation and the following diazomethane treatment are given in Fig. 7(a) and Table 3 (spectrum 16), respectively. As schematically presented in Table 3, reaction with BSA yielded a derivative with both carboxylic acid and alcohol functions. The transesterification with diazomethane is quantitative with the synthetic reference. However, the corresponding metabolite isolated

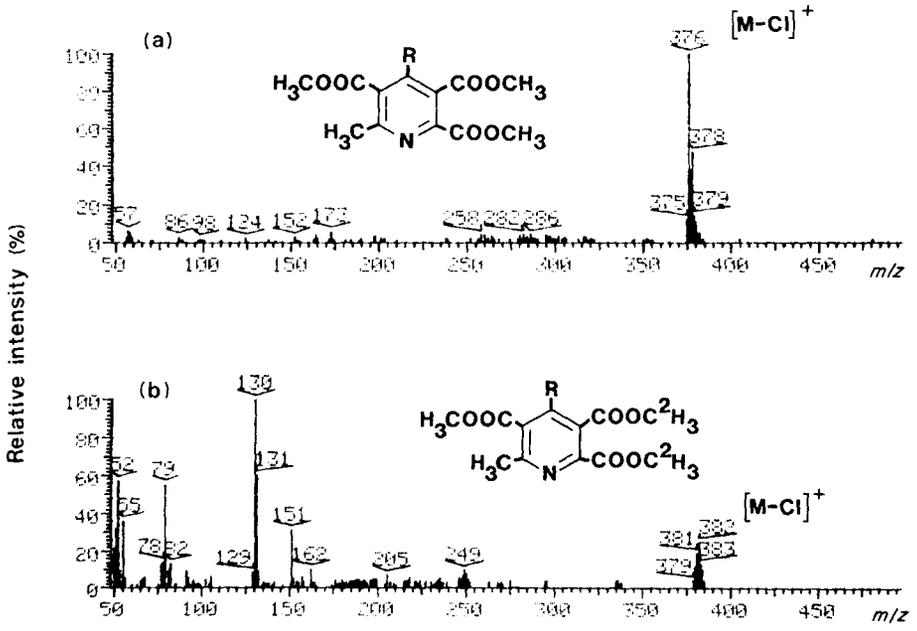


Figure 5. EI mass spectra of metabolite **9** after (a) transesterification of the silyl derivative with diazomethane and (b) reaction with deuterated reagents. R = 2,3-dichlorophenyl.

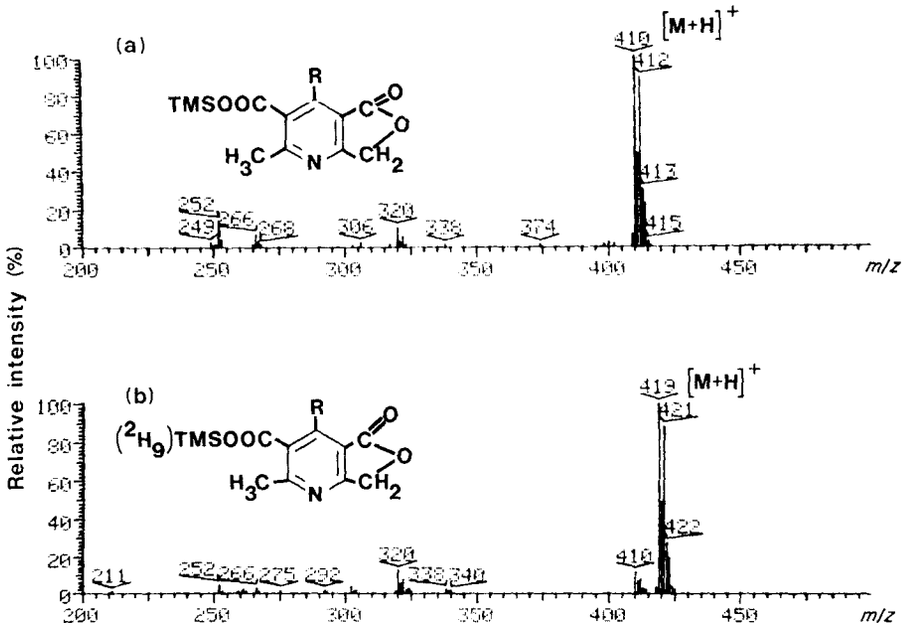
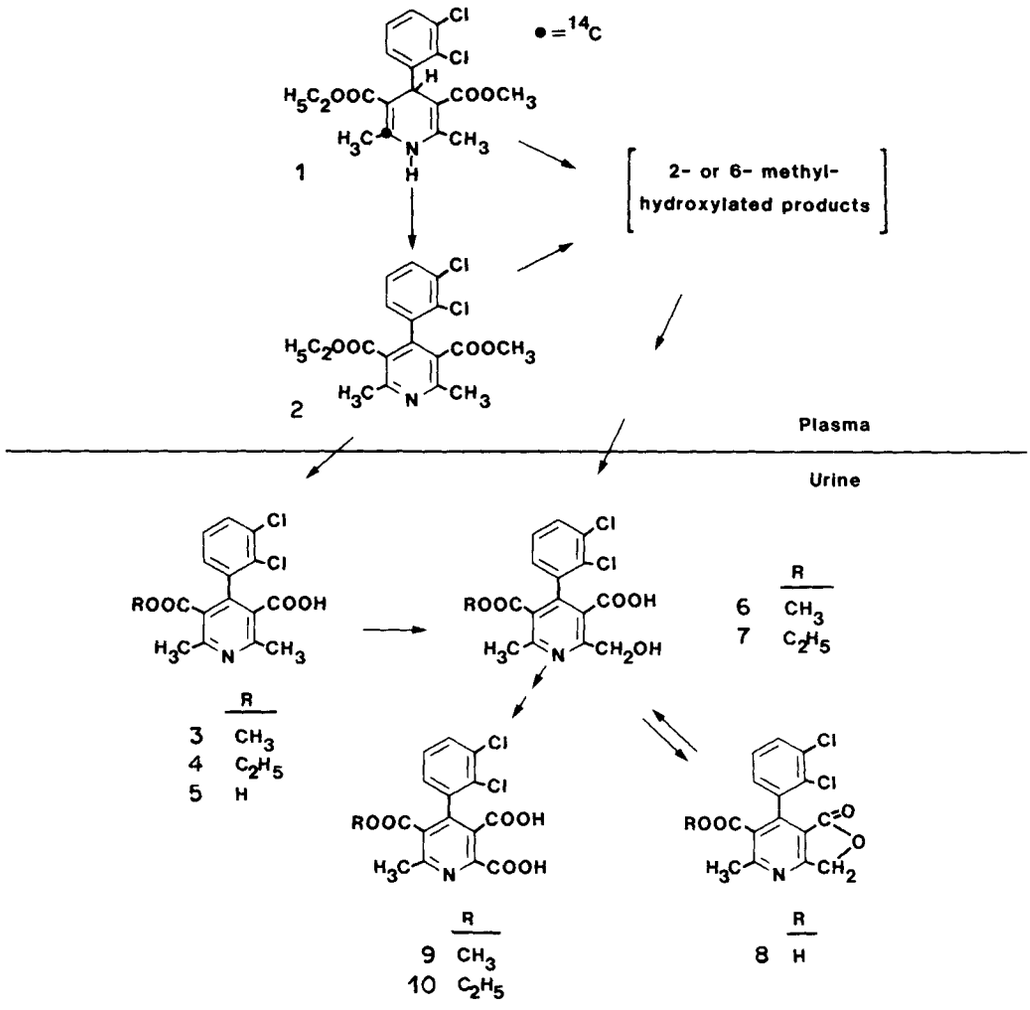


Figure 6. CI (isobutane) mass spectra of metabolite **8** as lactone after silylation with (a) BSA and (b) $(^2H_9)$BSA. R=2,3-dichlorophenyl.



Scheme 1. Metabolic pathway of felodipine in the rat.

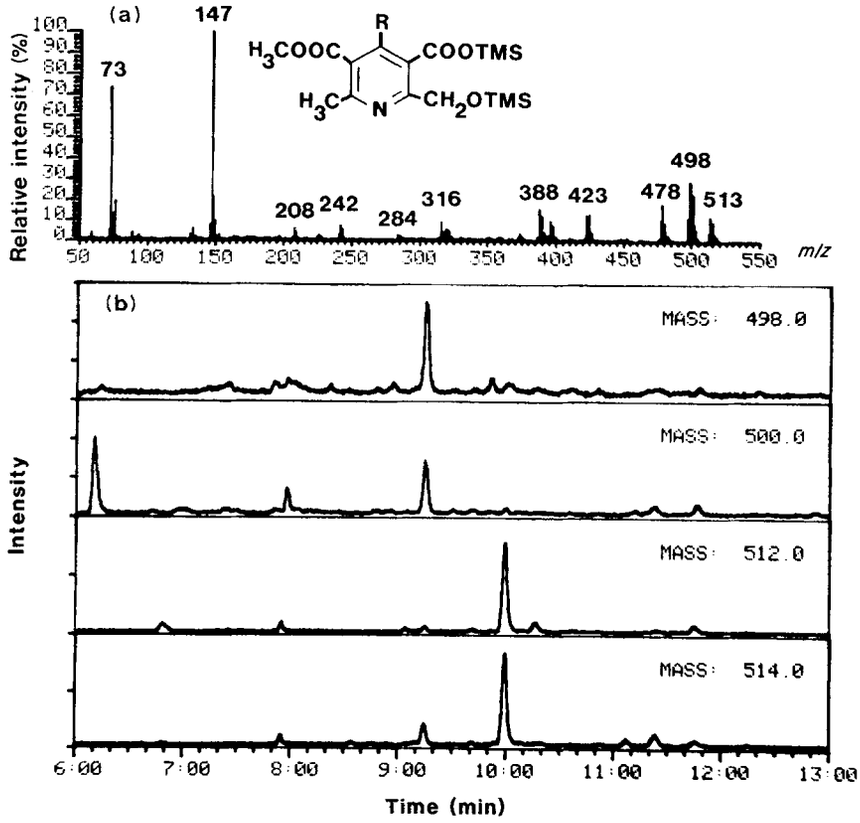


Fig. 7. (a) EI mass spectrum of silylated reference compound **6**. (b) The selected ion monitoring of the silylated liquid chromatographic fraction F5, i.e. the $[M-15]^+$ ion cluster of **6** (m/z 498/500, R_t=9.3 min) and **7** (m/z 512/514, R_t=10.0 min). Gas chromatographic retention times were identical for metabolite and reference. R=2,3-dichlorophenyl.

by batch extraction or liquid chromatography was almost completely converted to a lactone under exactly the same reaction conditions. The mass spectrum of **6** (number 17) exhibited the $[M-Cl]^+$ ions as the base peak at m/z 316 with a weak ion at m/z 301 as the only additional fragment. Despite the different behaviour of metabolite and reference compound upon derivatization, liquid chromatographic data suggested that metabolite **6** was excreted as a hydroxy acid only. Metabolite **6** was isolated from fraction F5 (27–32 min), whereas the retention time of the reference compound of the more lipophilic lactone was greater than 37 min.

Metabolite **7** also eluted from the liquid chromatographic column in the same fraction F5 as **6** and a similar phenomenon of different derivatization characteristics between synthetic reference (spectra 18 and 19) and the isolated metabolite (spectrum 20) was experienced. In the metabolite spectrum the significant ions detected originated from the loss of chlorine (m/z 330) from the molecular ion, followed by elimination of the elements of C_2H_4 (m/z 302).

Metabolite **8** was present in fraction F3 and the mass spectra of the metabolite and deuterated derivatives are presented in Table 3 (spectra 21–24). The $[M-Cl]^+$ ions were prominent in all spectra and the data indicated one free carboxylic acid group. The open hydroxy acid form of **8** with two carboxylic acid groups could be expected to have a polarity comparable to that of **5**. However, the retention time of **8** (F3) was considerably longer than that of **5** (F1 and F2). The synthetic lactone reference of **8** had a retention time identical to that of the isolated metabolite. From this, it was concluded that **8** forms the cyclic ester spontaneously in aqueous media at physiological pH values, as opposed to the hydroxy acid metabolites **6** and **7**.

DISCUSSION

Recently we reported the excretion of radioactivity and the urinary metabolic pattern by HPLC in rats after intragastric administration of 5 μmol kg^{-1} [^{14}C]-felodipine.[5] The same study showed only minor differences in the metabolic disposition of felodipine between different animal species and man. The rat was selected as a suitable animal model to obtain sufficient material for structural identification of minor unknown metabolites. The dose was increased 20-fold to 100 μmol kg^{-1} in this study, resulting in a urinary recovery of total radioactivity within 48 h after dosing of about 27%. This value was somewhat lower than the 41% recovery reported earlier[5] after administration of 5 μmol kg^{-1}. Faecal elimination of the given dose was still the most important one, most probably via biliary excretion. Due to analytical difficulties in isolating metabolites from faeces, urine was used for the isolation procedure.

In agreement with our previous study, most of the radioactive metabolites were extractable from the urine at pH 2.2 with diethyl ether. However, this work-up

procedure yielded a relatively complex sample and even the high separation efficiency of fused-silica capillary gas chromatographic columns was insufficient in some cases to distinguish between endogenous compounds and metabolites of interest (Fig. 3). Pre-separation of the sample on a reversed-phase semipreparative liquid chromatographic column by gradient elution and the combination of different derivatization steps before gas chromatographic/mass spectral analysis were used to circumvent this problem.

The semipreparative liquid chromatographic system gave a metabolic profile comparable to that of the analytical system reported earlier.[5] A larger column was used to increase the injectable sample volume, while the retention times were maintained by operating the system at the same linear mobile phase flow rate as in the analytical method. The performance of the semipreparative column was not affected by the 16 ml urine applied onto the column. Highly purified and concentrated fractions from the HPLC gradient run representing different polarities of metabolites were taken for further analysis. In addition to the increase in sensitivity it was possible to distinguish between two metabolites forming the same gas chromatographic/mass spectrometric derivative (e.g. **6** spectrum 17 and **8** spectrum 25) if they emerged in different liquid chromatographic fractions.

The major fractions of urinary metabolites of felodipine were acidic and consequently the carboxylic acid group had to be blocked to improve the chromatographic properties of the compounds. Trimethylsilylation was found to be an effective procedure for derivatization of all metabolites reported in this study taking hydroxylation of the methyl groups in positions 2 and 6 into account. The reference compounds were converted completely to the expected TMS derivatives and deuterated BSA provided information about the number of reactive functional groups. Reaction of TMS derivatives with diazomethane brought about selective exchange of the TMS esters to form the corresponding methyl esters with formed TMS ethers remaining unchanged (spectra 16 and 19, Table 3). This method could be of general applicability for these γ-hydroxycarboxylic acid metabolites, as the direct reaction with diazomethane resulted in a quantitative conversion to the corresponding lactone as given schematically in Table 3. Surprisingly the two-step derivatization procedure described here was successful only with reference compounds of **6** and **7** (spectrum 15, 16, 18 and 19 in Table 3), and in the biological samples lactone formation was always the major product after initial silylation. The hydroxy acid metabolites shown in the gas chromatogram in Fig. 3 were detected as their cyclic esters. Interfering constituents from the urine extracts might have catalysed lactonization since the remaining reaction conditions were identical. In Fig. 7 strong evidence for the lactone formation as an analytical artefact is presented. The complete mass spectrum of silylated **6** reference is given in Fig. 7(a). The silylated sample analysed originated from fraction F5 containing the metabolites **6** and **7** as hydroxy acids only. The $[M-15]^+$ ion cluster for the derivatized metabolites **6** and **7** were measured by selected ion monitoring and the resulting spectra are shown in 7(b). Both compounds were detectable with retention times of 9.3 and 10.0 min, respectively. However, silylation converted the greatest portion of the metabolites to the corresponding lactones which were easily detected in the scanning mode (spectra 17 and 20, Table 3). Cyclization of the hydroxy acid metabolites during the acidic extraction of fraction F5 preceding silylation can be ruled out. According to our kinetic studies at low pH[6] lactone formation of hydroxy acids should be negligible under the experimental conditions used in the work-up procedure.

Liquid chromatographic data provided convincing evidence that the hydroxy acids **6** and **7** do exist in their open form in urine. It is interesting to note that **8** as a hydroxy diacid metabolite was found to be present in rat urine as a cyclic ester with the same retention time as the synthetic lactone. The finding that **8** forms a lactone spontaneously in urine, in contrast to metabolites **6** and **7**, might be explained by different pK_a values of the acidic groups. In a previous study on the physicochemical properties of **3**, **4** and **5**[7] the pK_a value for one of the two acidic functions in **5** was found to be as low as 1.5. This pK_a value is considerably lower than the corresponding pK_a of 3.1 reported for **6** and **7**.[6] It is reasonable to assume that one of the two free carboxylic acids in **8** exhibits strong acidic properties which might facilitate the chemical formation of the lactone. Even if it is most likely to exclude the possibility that **8** as a lactone is formed as an analytical artefact in the work-up procedure, kinetic experiments remain to be performed in order to fully understand the underlying mechanism.

The isotope cluster technique in drug metabolism studies is widely used.[8,9] Artificially created doublet peaks in the mass spectra of unknown metabolites, formed after administration of drugs enriched to about 50% with a suitable heavy isotope, enhance the confidence with which small amounts of metabolites can be detected. In felodipine, however, the presence of two chlorine substituents in the phenyl ring was valuable from the analytical viewpoint. According to data on oxidized felodipine in Fig. 2(b) the base peak exhibits the chlorine isotope cluster at m/z 346/348. These $[M-Cl]^+$ ions were present in all mass spectra of derivatized metabolites reported in this study. They represented very often the main fragment in the EI mode, thus increasing the selectivity of the method. It must be pointed out that the mass spectra of potential metabolites with an intact dihydropyridine ring cannot be traced with this technique. The chlorine isotope clusters are lost in the EI mode when the bond between the ring systems is cleaved, probably due to the stabilization of the formed pyridinium ion at m/z 238 (Fig. 2). This observation is valid for a series of potential metabolites with a dihydropyridine structure available as reference compounds, e.g. the 2- and 6-hydroxyfelodipine analogues. According to *in vitro* results oxidation of felodipine to the corresponding pyridine analogue is the primary step in the metabolism of the drug.[3] Therefore, it seems unlikely that metabolites with the dihydropyridine ring will be formed or excreted into urine to a great extent.

Felodipine belongs to the group of 1,4-dihydropyridine derivatives with asymmetric ester functions. The metabolic fate of some compounds with a nitro substituent in the 3-position of the phenyl ring and different ester groups has been reported recently, e.g. nicardipine,[10,11] nimodipine[12] and nitrendipine.[13] In animals, the urinary excretion of metabolites as 1,4-

dihydropyridine derivatives has been described for nicardipine only, probably due to a relatively polar ester group carrying a tertiary amine group. All metabolites identified in this study were derived from oxidized felodipine (**2**) by ester hydrolysis, hydroxylation of a pyridine methyl group which then was further oxidized to the corresponding carboxylic acid. According to the total ion current signal in Fig. 3, metabolite **3** appears to be the major one. Appreciable amounts were detected of **4**, **6**, **7** and **8**, while **5**, **9**, and **10** were present in trace amounts only. Similar metabolic pathways have been reported for nimodipine and nitrendipine where the metabolites consisted of pyridine analogues hydrolysed, hydroxylated and oxidized to various extents. Metabolic attack in the phenyl ring of these drugs has not been described which is in good agreement with the results reported in this study with felodipine.

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