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Determination of four carboxylic acid metabolites of felodipine in plasma by high-performance liquid chromatography

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ABSTRACT

A reversed-phase high-performance liquid chromatography method with ultraviolet detection at 220 nm was developed to determine four carboxylic acid metabolites in plasma following therapeutic doses of the calcium antagonist felodipine. After the addition of an internal standard the analytes were isolated by liquid–liquid and solid-phase extraction. The metabolites were applied to a C_2 cartridge in their free acid form, but they were transformed and retained as ion pairs with tetrabutylammonium during a wash with phosphate buffer (pH 7), prior to automated elution and injection by the Varian AASP system onto the analytical C_{18} column. Using a sample volume of 1 ml of plasma, the lower limit of determination for the metabolites was about 20 nmol/l. The influence of the pH of the mobile phase on the retention time of the metabolites and the structural requirements for the internal standard were studied. The method was applied to plasma samples from four dogs collected after an oral dose of felodipine. The plasma concentration–time profiles of the metabolites gave useful information about the mechanisms by which they were formed and eliminated.

INTRODUCTION

Felodipine, 3,5-pyridine dicarboxylic acid, 4-(2,3-dichlorophenyl)-1,4-dihydro-2,6-dimethyl, 5-ethyl 3-methyl ester (Fig. 1), belongs to the group of 1,4-dihydropyridine (DHP)-type calcium antagonists which are widely used for the treatment of various cardiovascular disorders. The pharmacokinetics and biotransformation of ten DHPs, including nifedipine as prototype of these drugs, have been reviewed [1]. The DHPs are all highly lipophilic compounds which can be determined in plasma by both gas and liquid chromatography [2].

Felodipine lowers the blood pressure very effectively by selective peripheral vasodilation [3] and has been approved for marketing in about twenty countries. It is a high-clearance drug which is entirely eliminated by metabolism in animals [4,5] and humans [6]. Depending on the species, between 50 and 75% of a given dose is excreted as metabolites via the kidneys. The bioavailability is about 15% in dogs [7] and humans [8], presumably due to first-pass metabolism in the liver and/or in the gut wall. The primary metabolic step is the cytochrome P-450-mediated [9] oxidation of the dihydropyridine ring to the corresponding pyridine metabolite M2 (dehydrofelodipine, see Fig. 1), which results in the loss of the vasodilating acitivity [10]. As supported by *in vitro* experiments using liver microsomes from rat, dog and humans [11], ester hydrolysis of M2 yields the monoacids M3 and M4 as end-products in terms of phase 1 metabolic reactions. The corresponding hydroxy acids, M6 and M7, are formed by hydrolysis of the lactones M12 and M13, which are intermediates formed from M2. All four carboxylic acid metabolites (M3, M4, M6 and M7) are excreted renally in humans in free and conjugated forms [6].

These four metabolites have been determined in urine by bimodal column-switching high-per-

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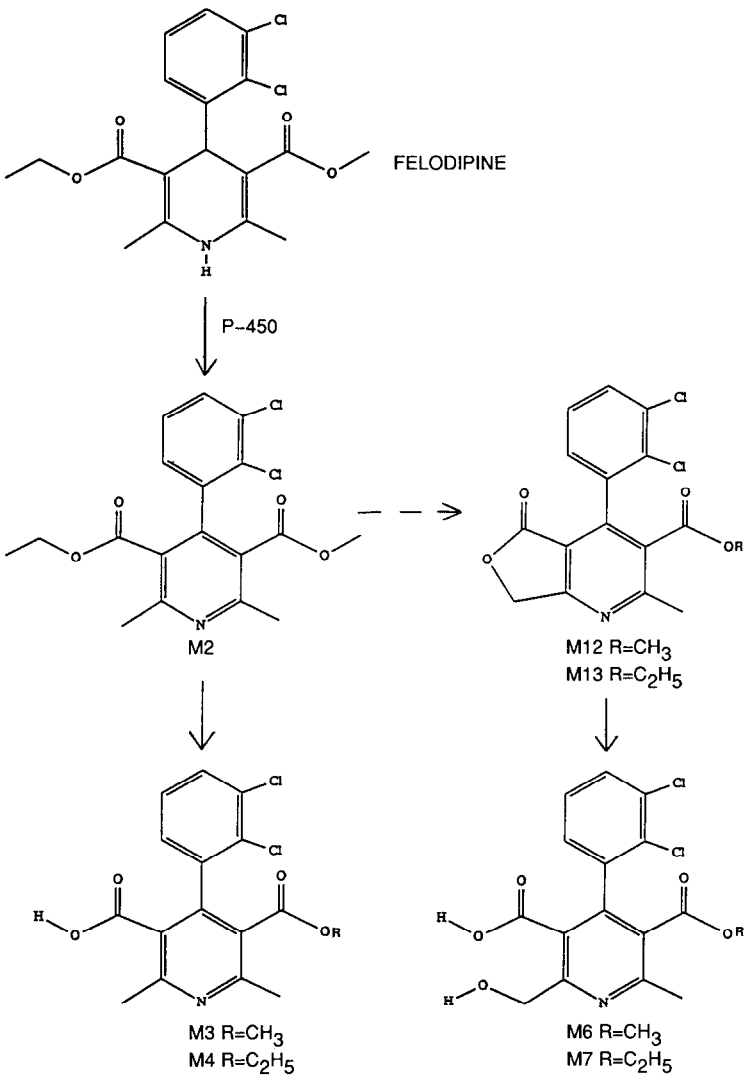


Fig. 1. Structure and proposed metabolic pathways for the formation of the four carboxylic acid metabolites of felodipine determined by HPLC after combined liquid- and solid-phase extractions.

formance liquid chromatography (HPLC) [12]. This method is based on group separation on bare silica of the hydroxy acids and monoacids prior to reversed-phase gradient elution HPLC with radioactivity detection.The aim of this work was to develop a sensitive HPLC method with UV detection for the determination of M3, M4, M6 and M7 in plasma after therapeutic doses of felodipine.

EXPERIMENTAL

Chemicals

Reference samples of the dehydrofelodipine metabolite M3 (methyl monoacid H 152/66, MW 354.2), M4 (ethyl monoacid H 167/87, MW 368.2), the internal standard 3,5-pyridine dicarboxylic acid, 4-(2-chloro-3-methoxyphenyl)-2,6-dimethyl, 3-propyl ester (H 240/95, MW 377.6) and analogues of this compound were synthesized at the Department of Organic Chemistry, Hässle (Mölndal, Sweden), according to a method described previously [13]. The hydroxylated metabolites M6 (methyl hydroxy acid, H 151/72, MW 370.2) and M7 (ethyl hydroxy acid, H 151/73, MW 384.2) were prepared from their corresponding lactones (dissolved in methanol) by the addition of 0.05 *M* sodium hydroxide solution

[14], completing hydrolysis within 60 min. Diethyl ether (May & Baker, Dagenham, UK) and phosphoric acid (E. Merck, Darmstadt, Germany) were of analytical-reagent grade and methanol, methylene chloride and *n*-hexane (Rathburn, Walkerburn, UK) were of HPLC grade. Water was deionized by passing it through a Milli-Q filter (Millipore, Milford, MA, USA). Sodium salts of phosphate, carbonate and hydrogencarbonate (E. Merck) were of analytical-reagent grade. Tetrabutylammonium hydrogensulphate (TBA) was purchased from Synecon Chemicals (Lund, Sweden). The C_2, C_8, C_{18} and phenyl AASP extraction cassettes were manufactured by Analytichem International (Harbor City, CA, USA).

Solutions

Sodium carbonate buffer (pH 10) was prepared by mixing 1 *M* $NaHCO_3$ (25.0 ml), 0.5 *M* Na_2CO_3 (50.0 ml) and 25.0 ml of deionized water. Phosphate buffers (pH 7 and 3.5) were made by diluting 21.0 ml of 1 *M* NaH_2PO_4 and 53.0 ml of 0.5 *M* Na_2HPO_4 or 6.9 g of NaH_2PO_4 and 2 ml of 1 *M* H_3PO_4, respectively, with deionized water to a final volume of 1000.0 ml. TBA (1.7 g) was dissolved in 500.0 ml of phosphate buffer (pH 7), corresponding to a concentration of 10 m*M*.

Standards

The stock solution of metabolite reference samples was made by dissolving the appropriate amount of each compound in 2.0 ml of methanol followed by dilution to 25.0 ml with water. After a 100-fold dilution with water the final concentrations were 1.51 (M3), 1.01 (M4), 1.37 (M6) and 1.19 μmol/l (M7). The solution of internal standard was prepared in the same way to a final concentration of 2.37 μmol/l. The solutions were stored at 4°C under which conditions the compounds were stable for several months.

Chromatograph

The HPLC system consisted of an LKB 2150 pump (Bromma, Sweden), a Spectra Physics (SP 8450) UV detector (San José, CA, USA) operated at 220 nm and an SP 4270 integrator. The advanced automated sample processor (AASP system, Varian) was used for the injection of samples from the solid-phase extraction as described later. Before and after the injection the transfer lines and cartridge were purged with 0.5 ml of water. The valve reset time was 90 s, during which the mobile phase was directed through the cartridge.

A gradient HPLC system was used to separate the metabolites and the internal standard on an LiChrospher 60 RP-select B (5 μm, 250 mm × 4 mm) column purchased from E. Merck. The analytical column was protected by a cyano Brownlee guard column (7 μm, 15 mm × 3.2 mm) from Applied Biosystems (San José, CA, USA).

The mobile phases consisted of 45% (A) and 80% (B) methanol in phosphate buffer (0.05 *M*, pH 3.5). At a flow-rate of 1.15 ml/min the gradient programme, which was formed by an LKB 2152 LC controller via a low-pressure mixing valve, was 100% A for 0–17 min, 0–100% B for 17–22 min and 100% B for 22–27 min. The gradient was reversed for 27–32 min and the system was equilibrated for 8 min before injection of the next sample.

The UV spectra of the metabolites were recorded with a Linear 206 programmable high speed LC detector (Linear Instruments, Reno, NV, USA). The fluorescence data were obtained using a Shimadzu (Kyoto, Japan) RF 5000 spectrofluorimeter (spectra of standard solutions) and RF 535 as HPLC monitor.

Analysis of plasma samples

To 1.0 ml of plasma in a centrifuge tube 100 μl of internal standard and 100 μl of buffer (pH 10) were added (Fig. 2). After mixing, the sample was extracted with 5.0 ml of diethyl ether, which had previously been saturated with water, for 10 min. After centrifugation for 5 min at 1000 *g*, the lower aqueous phase was frozen using dry ice in methanol, and the organic layer was discarded. After the addition of 200 μl of 4 *M* H_3PO_4 the aqueous phase was extracted for 15 min with 5.0 ml of methylene chloride–hexane (1:1, v/v). The tube was centrifuged for 5 min at 1000 *g* and the lower aqueous phase was again frozen. The upper organic layer was poured into another tube and evaporated to dryness under a stream of nitrogen at room temperature. The residue was dissolved in 1.0 ml of 0.1 *M* H_3PO_4 and 1.0 ml of

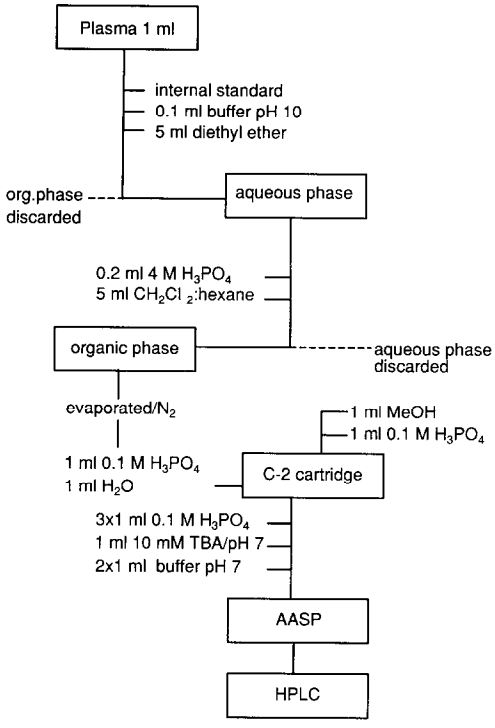


Fig. 2. Flow chart for preparation of plasma samples to determine the acidic felodipine metabolites M3, M4, M6 and M7 by HPLC.

water was added. The C_2 disposable extraction column of the AASP cassette was activated in a Varian AASP PrepStation with methanol followed by 0.1 *M* H_3PO_4 (1 ml each) and the sample solution was passed quantitatively through the extraction column. The adsorbed analytes were washed sequentially with 1 ml of 0.1 *M* H_3PO_4 (three times), once with 10 m*M* TBA in buffer (pH 7) and twice with buffer pH 7 followed by automatic AASP injection onto the separation column using mobile phase A as the eluent.

Assay calibration

The assay of four felodipine metabolites was calibrated with standard graphs prepared by spiking blank human plasma samples (1.0 ml) with six concentrations of the standard solution (range 15–750 nmol/l). The samples (n = 5) were processed as described and the ratios of the peak heights of each metabolite to the internal standard were calculated. In the calibration graphs, the ratios were plotted against the known concentrations of the metabolites and the lines were fitted by linear regression analysis.

The extraction yields of the metabolites were determined by processing known concentrations through the assay (range 15–750 nmol/l). The measured peak heights were compared with those obtained after direct injection of standard onto the analytical column.

Experiment with dogs

Plasma samples for the analysis of metabolites were taken from a study in which a deuterated pseudo-racemic mixture of felodipine as a solution was given orally to four female Beagle dogs (3 μmol/kg). The study design and plasma data for the enantiomers of felodipine and the corresponding pyridine metabolite have been reported elsewhere [15]. From the plasma concentration–time graph of the metabolites M3, M4, M6 and M7, the areas under the curves were calculated using the linear trapezoidal rule. The half-lives were determined from the individual regression lines of the log plasma concentration *versus* time plot.

RESULTS

In Fig. 2 the analytical procedure for the isolation of the felodipine metabolites in plasma is outlined schematically. The low acid dissociation constants of 3.1 (M6 and M7) [14], 3.51 (M3) and 3.65 (M4) [16] allowed an initial extraction step at pH 10 to reduce the background interference. Solvent partition at pH 2 excluded non-extractable compounds from further analysis and concentrated the analytes prior to solid-phase extraction. Acidic conditions during the application of samples onto the C_2 cartridge minimized losses of the protonated carboxylic acids. The ion-pairing reagent TBA increased metabolite retention on the C_2 adsorbent, which was crucial during the final wash of the cartridge with phosphate buffer at pH 7.

Representative chromatograms obtained from the assay at 220 nm are given in Fig. 3. The UV spectra of all metabolites showed a maximum at 280 nm and this wavelength was used initially during method development. After extensive purification of the plasma samples before injection

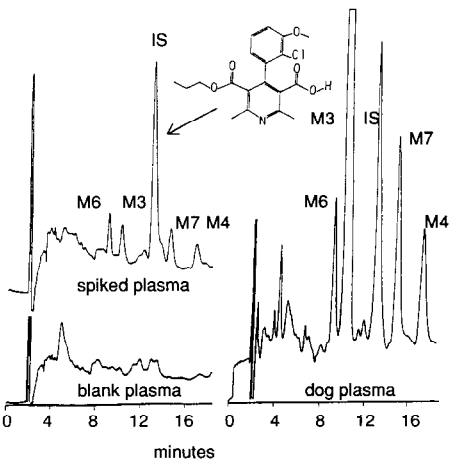


Fig. 3. Separation of four carboxylic acid metabolites of felodipine isolated from plasma by reversed-phase HPLC (220 nm) and the structure of the internal standard H 240/95. The concentration range of the metabolites in spiked plasma was 15–22 nmol/l. The corresponding range in plasma from dogs collected 4 h after an oral dose of felodipine was 147–1786 nmol/l (see Fig. 5).

onto the analytical column (Fig. 2), the number of peaks present in blank plasma decreased and the UV detector response for the metabolites could be enhanced about four times by monitoring at a less selective wavelength of 220 nm. Fluorescence was tested as a means to enhance sensitivity. Following the excitation of standards at 280 nm the dehydrofelodipine structure of the metabolites had poor fluorescence properties with a weak emission maxima at 420 nm for the monoacids and at 390 nm for hydroxy monoacids. If these parameters and the high concentration of standards (> 100 nmol/l) were used for HPLC analysis, the signal-to-noise ratio was below 5.

Assay recoveries with the calibration graph data and the reproducibility of the method are summarized in Tables I and II, respectively. In the concentration range of interest the calibration graphs were linear with a correlation coefficient greater than 0.9994. Losses may occur during both liquid- and solid-phase extraction (see Fig. 2). The distribution ratios for the metabolites have been determined by batch extraction using 30% (v/v) *n*-hexane in diethyl ether as the organic phase [14]. The distribution constant for the ethyl monoacid between undiluted diethyl ether and aqueous buffers was greater than 100 [16]. In this study the organic phase was methylene chloride–hexane (1:1, v/v) and the metabolite recovery from the entire sample treatment procedure was greater than 80% (Table I). Except for the lowest concentrations of M3 studied, the relative standard deviation was less than 10% in human plasma in the investigated ranges. No additional peaks were observed in the HPLC patterns of plasma from dogs. The background disturbances were reduced substantially by a gradient wash of the analytical column after isocratic elution of the analytes. It was of interest to note that despite differences in polarity between, for example, the most polar M6 and most lipophilic M4, the metabolites were equally well retained on the C_2 solid-phase extraction column.

The propyl ester analogue of the monoacids, but with a different substituent in the phenyl ring (Fig. 3), was used as the internal standard and was added directly to the plasma sample prior to sample work-up. With the design of the isolation procedure, the physico-chemical properties of the internal standard and analytes had to be similar to avoid losses both during liquid- and solid-phase extraction. Furthermore, it was desirable that the HPLC retention time should be close to those of the four metabolites.

Five compounds were tested as internal standards and their structures and retention times are given in Table III. The pronounced influence of the substituents in the phenyl ring on the retention time was apparent in the series of ethyl esters. The dimethyl analogue (H 240/88) was the most lipophilic and the exchange of a 3-methyl (H 240/84) to a 3-methoxy group (H 240/92) reduced the retention time almost two-fold. The lipophilicity of H 240/92 was increased by substitution to the propyl ester giving the optimum HPLC retention time for H 240/95 as the internal standard. The increase in retention time of H 240/92 by the addition of a CH_2 group was about 4 min, as is seen for M3 compared with M4, or M6 compared with M7.

The influence of the pH of the mobile phase on the capacity ratios of the four metabolites is shown in Fig. 4. The elution order was unchanged with baseline separation in all cases. By lowering the pH from 3.6 to 2.0, longer retention

TABLE I

RECOVERIES OF THE METABOLITES ADDED TO BLANK HUMAN PLASMA SAMPLES AND CALIBRATION GRAPH DATA

Concentration range (nmol/l)	Recovery (mean ± S.D., $n = 5$) (%)			
	M3	M4	M6	M7
15–22	148 ± 41	85 ± 2	96 ± 10	88 ± 9
25–38	108 + 24	90 ± 2	89 ± 12	91 ± 7
51–75	88 ± 5	89 + 3	80 ± 4	87 ± 3
101–151	92 ± 2	96 ± 0.4	87 ± 1	89 ± 0.4
203–301	80 ± 1	95 ± 1	82 ± 2	89 ± 2
507 753	81 ± 1	96 ± 1	80 + 2	89 ± 1
Calibration graph[a]	$y = 3.29 \cdot 10^{-3}x + 3.5 \cdot 10^{-2}$ $r = 0.9994$	$y - 2.58 \cdot 10^{-3}x - 4.61 \cdot 10^{-3}$ $r - 1.000$	$y = 4.02 \cdot 10^{-3}x + 1.91 \cdot 10^{-2}$ $r = 0.9998$	$y = 2.78 \cdot 10^{-3}x - 3.89 \cdot 10^{-4}$ $r = 1.000$

[a] y = Peak-height ration; x = concentration of metabolite.

TABLE II

COEFFICIENT OF VARIATION FOR FELODIPINE METABOLITES M3, M4, M6 AND M7 IN PLASMA AT TWO CONCENTRATIONS

Intra-assay variability is given as median of ten or eleven observations with three or four data points included in each calculation.

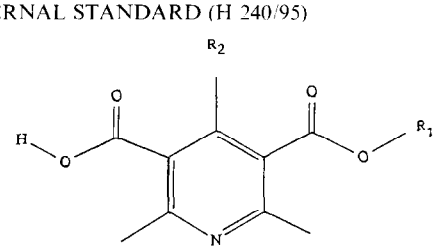
Metabolite	Concentration (nmol/l)	Coefficient of variation (%)	
		Intra-assay	Inter-assay
M3	62	10.0 (n = 4)	16.3 (n = 10)
	186	5.2 (n = 3)	11.2 (n = 11)
M4	62	4.3 (n = 4)	4.7 (n = 10)
	185	2.7 (n = 3)	4.0 (n = 10)
M6	66	7.0 (n = 4)	10.3 (n = 10)
	198	5.0 (n = 3)	8.6 (n = 11)
M7	62	6.8 (n = 4)	12.2 (n = 10)
	185	4.0 (n = 3)	9.1 (n = 11)

times were observed due to the suppression of the ionization of the carboxylic acids at a pH below their pK_a value.

Interestingly, protonation of the pyridine ring at low pH [pK_a 0.85 (M3) and 1.08 (M4)] [16] did not counteract the increasing influence on the retention time due to the protonation of the acid

TABLE III

STRUCTURE AND HPLC RETENTION TIME (t_R) (ISOCRATIC CONDITIONS WITH 45% METHANOL IN PHOSPHATE BUFFER pH 3.5) OF SOME REFERENCE SAMPLES SYNTHESIZED TO FIND THE APPROPRIATE INTERNAL STANDARD (H 240/95)



Compound	R_1	R_2	t_R (min)
H 240/84	C_2H_5	2-Chloro-3-methylphenyl	14.0
H 240/88	C_2H_5	2,4-Dimethylphenyl	19.9
H 240/80	C_2H_5	2-Chlorophenyl	9.4
H 240/92	C_2H_5	2-Chloro-3-methoxyphenyl	8.1
H 240/95	C_3H_7	2-Chloro-3-methoxyphenyl	12.8

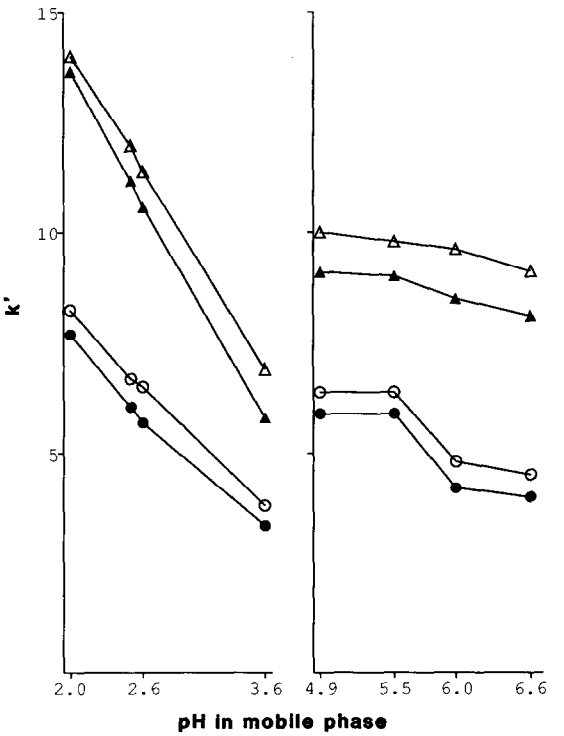


Fig. 4. pH-dependent changes in k' of (○) M3, (△) M4, (●) M6 and (▲) M7. The isocratic analysis was performed on a LiChrosper 60 RP-select B (5 μm) column with 46% methanol (pH 2–3.6) or 40% methanol (pH 4.9–6.6) and phosphate buffer in the mobile phase.

functionality. This might be explained by secondary equilibria in the retention mechanism not accounted for at higher pH, *e.g.* ion-pairing of the pyridinium ions with phosphate anions. By taking both the analysis time and interfering peaks in the blank plasma into account, isocratic conditions at a mobile phase pH of 3.5 were selected for quantitative measurements.

Blank plasma from both humans and dogs was used during the development of the method and the applicability of the assay was tested on samples from a pharmacokinetic study in dogs. Following an oral dose of felodipine (3 μmol/kg), plasma concentrations of the four acidic metabolites could be monitored over a period of 12–24 h (Fig. 5). The pharmacokinetic parameters derived in this study are shown in Table IV. The monoacids were rapidly formed with t_{max} less than 1 h after a dose and M3 was the most abundant metabolite. The highest plasma concentrations of the hydroxy monoacids were attained 7.5

TABLE IV

PHARMACOKINETIC PARAMETERS IN THE DOG OF FOUR FELODIPINE METABOLITES AFTER ORAL ADMINISTRATION OF 3 μmol/kg OF FELODIPINE IN SOLUTION

Results given as mean ± S.D.

Metabolite	t_{max} (h)	C_{max} (nmol/l)	Area under curve (h μmol/l)	$t_{1/2}$ (h)
M3	0.9 ± 0.4	3279 ± 710	40.4 ± 12.1	9.5 ± 0.6
M4	0.6 ± 0.1	696 ± 157	2.7 ± 0.8	3.6 ± 0.5
M6	7.5 ± 1.0	259 ± 109	7.2 ± 4.2	14.2 ± 4.1
M7	2.6 ± 1	359 ± 153	3.8 ± 2.0	5.7 ± 0.4

h (M6) and 2.6 h (M7), respectively, after the dose and their elimination half-lives were longer compared with the corresponding monoacids.

DISCUSSION

As a result of the common 4-phenyl-substituted 1,4-dihydropyridine dicarboxylate system in the structure of all DHP type calcium antagonists, similar metabolic reactions, *e.g.* ester hydrolysis or aliphatic hydroxylation, are reported [1]. Furthermore, dehydrogenation of the 1,4-DHP ring is consistently described as a primary metabolic step with the relative rate of microsomal oxidation being influenced by the substituents in the aromatic ring [17,18]. The same type of metabolic pathway as described for felodipine in this study is reported for nifedipine [19], nimodipine [20], nisoldipine [21], nitrendipine [22], nilvadipine [23], and isradipine [24]. Consequently, all of these metabolites could be, in principle, amenable for analysis after the adjustment of the conditions outlined in Fig. 2. The same is applicable for amlodipine [24,25], benidipine [26] and nicardipine [27,28], but the amino function in their substituents will exclude the solvent extraction step at pH 2.

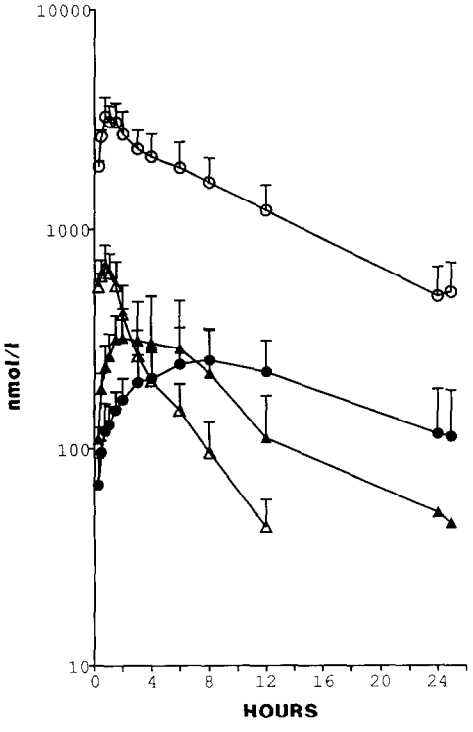


Fig. 5. Concentration of four carboxylic acid metabolites (○) M3, (△) M4, (●) M6 and (▲) M7 in plasma from dogs after oral administration of felodipine (3 μmol/kg, mean ± S.D.; n = 4).

Following reaction with diazomethane, the monoacids of nifedipine [29] and isradipine [30] have been determined in plasma and urine by gas chromatography with electrochemical and chemical ionization negative-ion mass spectrometric detection, respectively. These derivatives cannot be distinguished from the primary pyridine metabolite. which therefore has to be removed from the sample before derivatization. This problem has been solved by ethylation and propylation, respectively, of the carboxylic acid of nifedipine [31] and felodipine [32]. No derivatization is required to determine the nifedipine monoacid by reversed-phase HPLC after a single extraction

step at pH 9 with ethyl acetate [33]. More recently, Böcker *et al.* [34] used solid-phase extraction to isolate nitrendipine metabolites formed in rat liver microsomal preparations before separation by HPLC and detection at 234 nm. In this study extensive purification of felodipine metabolites in plasma was required for measurements in the lower nanomolar range (Fig. 2). The most crucial step was to balance the co-extraction of all four acidic metabolites and endogenous compounds by various bonded silica stationary phases. The analytes were effectively retained on C_8, C_{18} and phenyl-bonded columns, but blank plasma components interfered with the analysis. The use of an ion-pairing technique with TBA on the C_2 cartridge increased the retention of the metabolites sufficiently during the final clean-up step at neutral pH. Despite the comprehensive isolation procedure required, this assay using an internal standard was reproducible and efficient due to advanced automated sample processing (analysis time about 40 min).

The HPLC method allowed the simultaneous determination of the monoacids and their hydroxylated analogues. Mechanistically the hydroxy monoacids of felodipine excreted in urine [6] are formed by hydrolysis of the corresponding lactones in plasma (see Fig. 1) and the equilibrium between the two forms is pH-dependent [14]. During the initial extraction step (Fig. 2), plasma was kept at pH 10 for at least 10 min and these conditions should contribute to the alkaline hydrolysis of the lactone metabolite. However, the lactones, formed after an oral dose of felodipine to rats, were stable in alkalized (pH 12) plasma [35]. This indicates that the apparent lactone kinetics and their physico-chemical properties might be different in aqueous solutions [14] compared with plasma, when, for example, the effect of plasma protein binding is taken into account. Further studies will be needed to characterize and measure the lactones in plasma. Interestingly, Schmid *et al.* [31] used acidic conditions to convert the ethylated hydroxy monoacid of nifedipine to the lactone as a means of derivatization prior to analysis by gas chromatography. Attempts to derivatize the acid functionality of the γ-hydroxycarboxylic acids of felodipine through alkylation is, however, not successful due to immediate cyclization to the corresponding lactone [5]. More recently, Roosemalen *et al.* [36] used the pH-dependent equilibrium of the hydroxy monoacid and lactone [14] to convert the corresponding hydroxy metabolite of nifedipine to its lactone, which then was determined in blood, plasma and urine samples by HPLC. Using this approach, the sum of the two metabolites was analysed with a limit of determination in blood of about 160 nmol/l.

The hydroxy and monoacids analysed by this method are major urinary felodipine metabolites in all species studied, including humans [6]. In plasma from dogs, M3 had the highest concentration (Fig. 5) and comparable results have been reported in volunteers by Nishioko *et al.* [32]. Higher plasma concentrations of the methyl monoacid of isradipine than of the corresponding isopropyl monoacid were found in healthy subjects [30]. These plasma data are in good agreement with quantitative results obtained in urine for most DHPs [1], indicating a higher hydrolytic stability of the methyl ester group compared with groups with a larger substituent, *e.g.* an ethyl or isopropyl group. Sequential metabolism of M4 in dogs might be the reason for its shorter half-life compared with M3 if similar renal clearances are assumed for both metabolites. Mechanistically, the late t_{max} of M6 and M7 probably supports their formation via a lactone, as indicated in Fig. 1. The corresponding nifedipine lactone is not detectable in serum from hypertensive patients [33], but the isopropyllactone of isradipine is the major plasma metabolite in humans [30].

In conclusion, this HPLC method allows the determination of four carboxylic acid metabolites of felodipine in the 20 nmol/l range with quantification limits depending on interferences present in the plasma sample. The assay has been successfully applied to about 400 human plasma samples from various pharmacokinetic studies using therapeutic doses of felodipine.

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