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Enantioselective determination of felodipine in human plasma by chiral normal-phase liquid chromatography and electrospray ionisation mass spectrometry

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Dedicated to Professor Dr Gottfried Blaschke on the occasion of his 65th birthday.

Abstract

An analytical method was developed for the determination of the enantiomers of felodipine, a dihydropyridine-type calcium antagonist, in human blood plasma. Felodipine was extracted from plasma using toluene as extraction solvent. The enantiomers were separated on a cellulose tris(4-methyl benzoate) stationary phase (Chiralcel OJ-R) using 2-propanol–*iso*-hexane (11:89) as mobile phase. Post-column addition of ammonium acetate in ethanol–water (95:5) allowed sensitive detection of the ammonium adduct by electrospray ionisation and selected reaction monitoring. Deuterated felodipine racemate was used as internal standard. Within-run repeatability was determined and a coefficient of variation below 2% was achieved at 22 nmol/l and below 10% at 0.27 nmol/l. Between-day precision was evaluated and a coefficient of variation of 3.6% at 4 nmol/l plasma was obtained. Limit of quantification (LOQ) was set at 0.25 nmol/l (0.10 μg/l). The method proved adequate for pharmacokinetic studies of *R*- and *S*-felodipine after oral administration of therapeutic doses of felodipine.

Keywords: Enantioselective determination of felodipine; Felodipine enantiomers in plasma; Chiral normal-phase LC–MS

1. Introduction

Felodipine is a dihydropyridine calcium antagonist widely used as a potent antihypertensive drug with therapeutic concentrations in blood plasma often below 20 nmol/l. Commonly, analytical methods for dihydropyridine calcium antagonists in biological samples have been based on gas chromatography (GC) with electron capture (ECD) or mass spectrometric (MS) detection [1]. The dihydropyridine ring of felodipine has a chiral carbon atom in the 4-position due to asymmetric substitution at the ring, like other dihydropyridine drugs developed after nifedipine. Enantioselective methods have been developed,

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based on liquid chromatographic (LC) resolution of dihydropyridine enantiomers and subsequent gas chromatographic determination of the separate LC fractions. Chiral stationary phases developed by Okamoto and coworkers [2] have been employed for LC enantioseparation of chiral dihydropyridine derivatives. Chiralcel OJ, cellulose tris(4-methyl benzoate) was used for the resolution of felodipine [3–5], nimodipine [6] and isradipine [7]. Fractions were analyzed by GC with ECD [3] or nitrogen selective detection [7] or by MS detection [4–6]. Amlodipine enantiomers were determined as diastereomeric derivatives by GC–ECD [8] or by coupled-column LC with electrochemical [9] and ultra violet (UV)-detection [10]. UV-detection was also used in two recent applications for nicardipine after enantioseparation on Sumichiral OA-4500 stationary phase [11] and for nimodipine on Ultron ES-OVM after preceding non-chiral separation [12]. A method for nimodipine using chiral LC with on-line MS detection was reported by Mück [13]. It had an estimated limit of quantification (LOQ), of 0.5 ng/ml (1.2 nmol/l). Recently the enantiomers of clevidipine, a new ultrashort-acting calcium antagonist, were quantified by on-line MS detection after separation on Chiralcel OD-H with an LOQ of 0.2 ng/ml (0.5 nmol/l) [14].

It has been shown for several dihydropyridines that plasma levels after oral administration of the racemate are higher for the *S*-enantiomer than for the *R*-enantiomer [15]. Further, it has been demonstrated that intake of grapefruit juice may enhance bioavailability of dihydropyridines (measured as the sum of the two enantiomers) [16]. An enantioselective assay for felodipine was needed for a bioavailability study where the effect of grapefruit juice on plasma levels of the individual enantiomers was investigated [17].

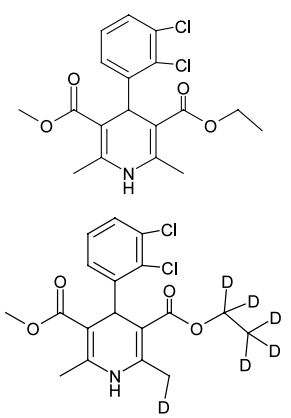

Fig. 1. Structural formulae of felodipine and the internal standard, D_6-felodipine.

2. Experimental

2.1. Chemicals and reagents

Felodipine racemate (5-ethyl-3-methyl-4-(2,3-dichlorophenyl) - 1,4 - dihydro - 2,6 - dimethyl - 3,5-pyridinedicarboxylate) (monoisotopic molecular mass M_r 384.3), as well as separate *R*- and *S*-enantiomers and racemic D_6-felodipine (M_r 390.2), (Fig. 1), were obtained within AstraZeneca R&D Mölndal. Toluene, glass distilled grade, methanol, 2-propanol and *iso*-hexane of HPLC grade were from Rathburn (Walkerburn, UK), ammonium acetate of analytical grade from Fluka Chemie AG (Buchs, Switzerland) and 95% ethanol from Kemetyl AB (Haninge, Sweden). Nitrogen of 99.9995% purity used as curtain-and nebulizer gas and argon of 99.9997% purity used as collision-induced dissociation (CID) gas were from AGA Gas AB (Sundbyberg, Sweden).

2.2. Chromatography

The LC system included a Hewlett-Packard 1050 series pump (Waldbronn, Germany), a Jasco Familic-3005 (Tokyo, Japan) make-up pump, a Cluzeau info lab Croco-Cil (Ste. Foy la Grande, France) column heater and a Hewlett–Packard 1050 series autosampler with a 100 μl variable loop. The mass spectrometer was a Sciex API-3^+ triple quadrupole mass spectrometer with electrospray interface (Thornhill, Ont., Canada). The LC column, Chiralcel OJ-R (150 mm × 4.5 mm, 5 μm) from Daicel Chemical Industries (Tokyo, Japan) was kept at 37 °C. The mobile phase consisted of 2-propanol–*iso*-hexane (11:89, v/v). The make-up liquid was 10 mmol/l ammonium acetate in ethanol–water (95 + 5). Flow rates

were 1.0 ml/min through the separation column and 0.25 ml/min for make-up liquid added post-column. Retention times were about 9 min for the *S*-isomers and 13 min for the *R*-isomers of felodipine and deuterated felodipine.

2.3. Mass spectrometry

The effluent from the chromatographic column, mixed with ammonium acetate in ethanol, was splitted in a Valco tee connection so that the liquid flow to the ion source was about 35 μl/min. The orifice voltage was set at 40 V and the collision energy (R0–R2) at 12 V. The collision gas thickness was set at 10^{13} atoms argon per cm^2. Nitrogen was used as nebulizer gas to minimize the risk of accidental ignition in the ion source of solvent vapors which also was vented out with a sample pump. Felodipine enantiomers were monitored as their ammonium adducts at m/z 401, giving a fragment at m/z 338. The enantiomers of D_6-felodipine were monitored at m/z 339, product ion of m/z 407. The dwell time was 1000 ms for each mass transition.

2.4. Sample preparation

Plasma samples and plasma extracts were protected from daylight and excessive exposure of light from fluorescent tubes. Aliquots of 1.00 ml thawed, mixed and centrifuged plasma samples were pipetted into centrifuge tubes, mixed with 1.0 ml of toluene containing the internal standard D_6-felodipine (40 nmol/l) and shaken for 1 h. After centrifugation the aqueous phase was frozen, the supernatant was transferred to clean tubes and the solvent was removed at 35 °C with a gentle stream of dry nitrogen. Extracts were dissolved in 250 μl of 2-propanol–*iso*-hexane (11:89) and 100 μl was injected on the LC column.

Reference plasma samples (six replicates) for daily calibration were prepared freshly by adding 10 μl of standard solution, 4.0 μmol/l of racemic felodipine in methanol, to 1.0 ml of blank plasma, giving a plasma concentration of 20 nmol/l of each enantiomer. A batch of quality control (QC) samples, containing 4 nmol/l of each enantiomer, was prepared separately. Two QC-samples and two plasma blanks were included in each run of authentic plasma samples along with the reference samples.

3. Results and discussion

3.1. Extraction and separation

The extraction procedure was adopted from previous GC methods for felodipine, where toluene was used as extraction solvent with an extraction recovery of 95% [18,19]. The liquid-chromatographic system gave a separation factor α of 1.5 and a resolution R of 1.5 for the enantiomers of felodipine. Chiralcel OJ has been found to be optimal for a number of dihydropyridines, including felodipine, nimodipine, and isradipine, while Chiralcel OD-H was found to give better separation of clevidipine [14]. Baseline separation of the enantiomers of felodipine in the present work facilitated accurate peak integration down to 0.25 nmol/l (0.10 μg/l) for each enantiomer.

3.2. Mass spectrometric detection

Initial tests comparing a heated nebulizer (APCI) interface with electrospray gave more promising results with electrospray, although this is less frequently used, judging from literature, than APCI in combination with normal-phase LC [20]. When electrospray was tested with different solvents added post-column (Fig. 2), it was found that much higher ion intensities were achieved in the presence of ammonium acetate (2 mmol/l) than with formic acid (0.2%). By applying a higher declustering potential, the intensity of the protonated molecular ion could be increased at the expense of the ammonium adduct ion, indicating that the formation of the protonated form proceeded via the addition of ammonia rather than by direct protonation. However, the ammonium adduct ion at m/z 401 (m/z 407 for the internal standard) was chosen as precursor ion. Fragmentation resulted in several ions (Fig. 3), the two largest ones being formed by loss of ammonia followed by CH_3OH or C_2H_5OH

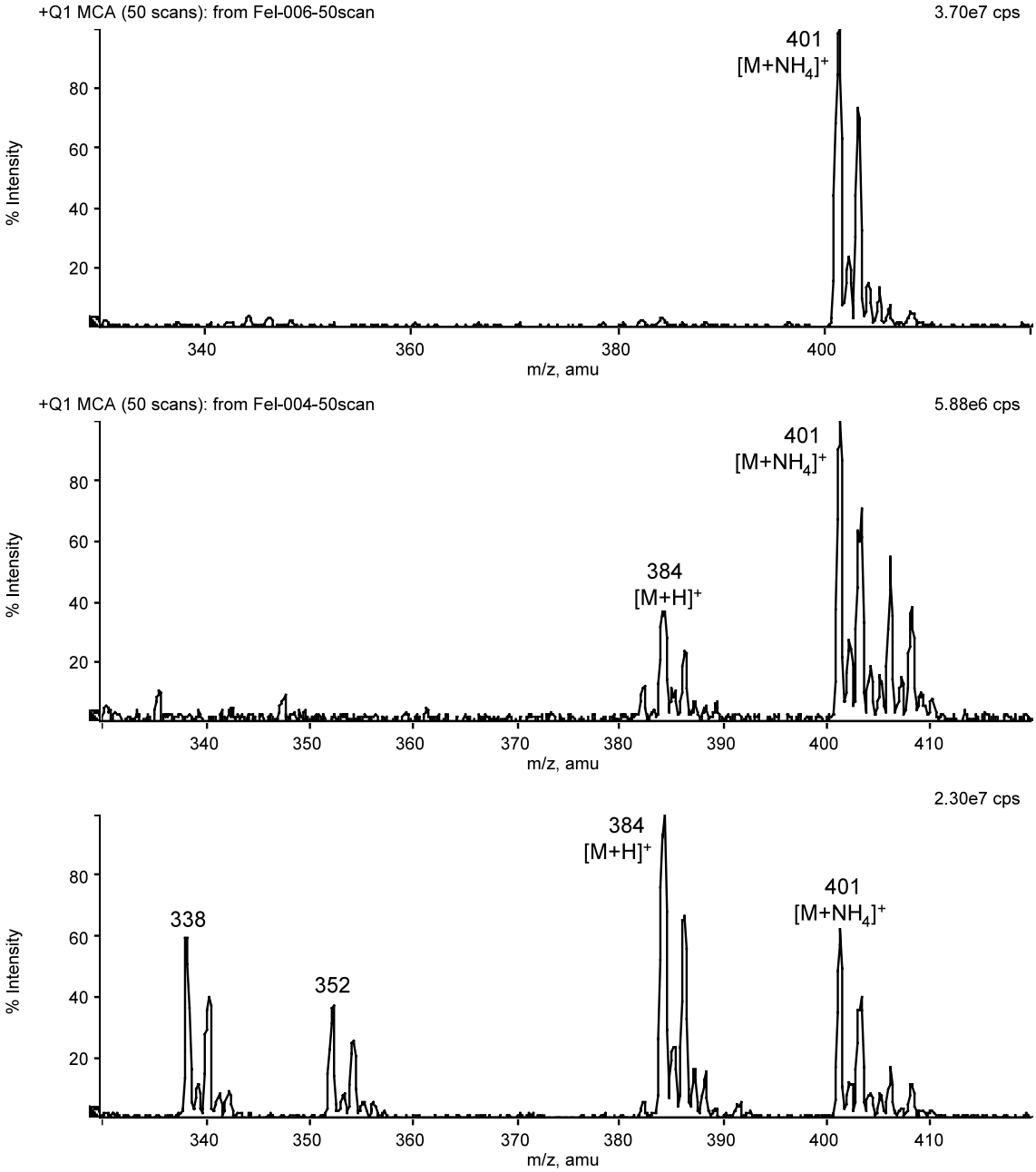

Fig. 2. Mass spectra from an infusion experiment with felodipine in 2-propanol–*iso*-hexane–ethanol–water (9:71:19:1 v/v) plus additive. In A, 2 mM NH_4Ac was added, and a low orifice voltage, 36 V (6 V net potential) was used. In B, NH_4Ac was replaced by formic acid (0.2%), but traces of ammonium still influenced ionisation (orifice potential 36 V). In C, solvent was same as in A, but orifice potential was increased to 60 V. Full scale in A–C was 37, 6 and 23 cps, respectively.

(C_2D_5OH) respectively. The latter ion (m/z 338 for felodipine, m/z 339 for the internal standard) was used for selected reaction monitoring.

3.3. Quantification

A standard curve at six concentrations covering the range 0.50–40 nmol/l (0.20–15 μg/l), ($n = 3$–8 at each concentration level) was run before the start of the study. The following evidence for a simple linear relationship between relative peak heights and concentrations was found, (1) non-weighted linear regression gave correlation coefficients of 0.9993 and 0.9995 for *S*- and *R*-felodipine, respectively, both for regression with and without a constant (line forced through zero). (2) No disturbing peaks were seen in blanks. (3) Accuracy was within ± 15% over the range studied when concentrations were calculated using linear calibration without intercept. Based on these observations, a linear calibration method using plasma standards at one concentration level (20 nmol/l, $n = 6$) was chosen for daily work. A standard curve extended down to 0.25 nmol/l was run at the end of the study, showing linearity within ± 15% over the entire range 0.25–40 nmol/l.

When within-run precision was estimated at a low and a high level, the following coefficients of variation ($n = 6$) were found for *R*- and *S*-felodipine, respectively, 5.5 and 4.1% at 0.27 nmol/l (0.10 μg/l) and 1.3 and 1.5% at 22.1 nmol/l (8.5 μg/l).

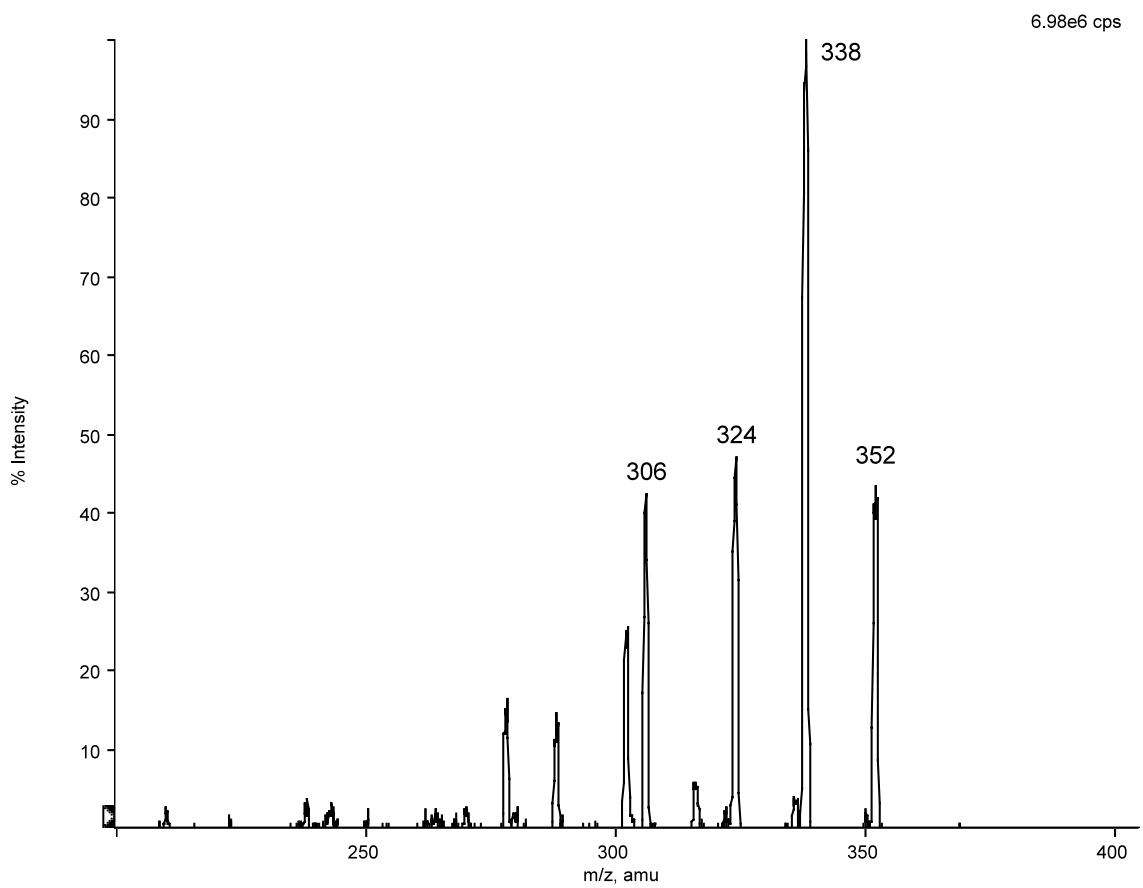

Fig. 3. Product ion spectrum from felodipine obtained by fragmentation of the $(M + NH_4)^+$ ion at m/z 401, using a collision potential of 12 V. Solvent was the same as in Fig. 2.

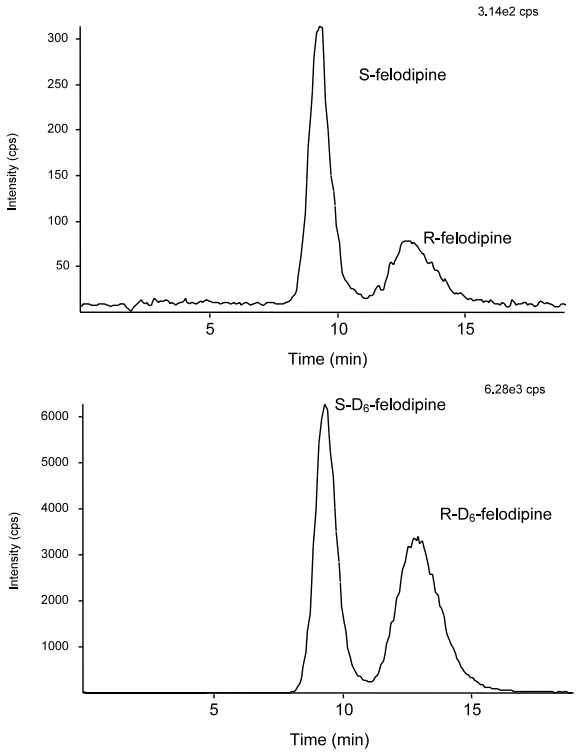

Fig. 4. Chromatogram of an authentic human plasma sample containing 0.70 nmol/l of *S*-felodipine and 0.30 nmol/l of *R*-felodipine.

Day-to-day reproducibility was evaluated with QC samples at 4 nmol/l of each enantiomer giving a CV of 3.6% ($n = 25$) and an accuracy ranging 89.5–100.5%. A chromatogram from an authentic plasma sample is shown in Fig. 4. The lower LOQ was estimated at 0.25 nmol/l. Precision and accuracy below this level were not investigated.

3.4. Pharmacokinetic application

The method was used in a bioavailability study where concentrations of *R*- and *S*-felodipine were measured after oral administration of felodipine racemate [17]. Plasma profiles from a single volunteer are shown in Table 1. The method proved adequate for measuring plasma concentrations of felodipine enantiomers during 24 h after a therapeutic dose (10 mg) at steady-state. The LOQ at 0.25 nmol/l permitted quantification of both enantiomers 24 h after dose in plasma samples from 11 out of 12 subjects. The sensitivity of the method compares favorably with published achiral assays for dihydropyridines.

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Table 1
Plasma concentrations of felodipine enantiomers after oral intake of 10 mg felodipine ER (extended release) at steady-state

Time (h)	Felodipine + water			Felodipine + grape fruit juice		
	S (nmol/l)	*R* (nmol/l)	*S*/*R*	*S* (nmol/l)	*R* (nmol/l)	*S*/*R*
0	0.76	0.31	2.45	0.78	0.29	2.69
0.5	0.75	0.34	2.21	0.71	0.29	2.45
1	0.76	0.36	2.11	0.86	0.30	2.87
2	0.93	0.43	2.16	1.12	0.52	2.15
3	1.87	0.82	2.28	4.19	2.12	1.98
4	4.48	2.02	2.22	9.91	5.24	1.89
5	4.42	2.05	2.16	15.0	7.93	1.89
6	4.41	1.88	2.35	9.43	4.76	1.98
8	2.58	1.10	2.35	5.36	2.49	2.15
10	1.85	0.81	2.28	3.76	1.66	2.27
12	1.64	0.75	2.19	2.87	1.28	2.24
24	0.87	0.40	2.18	0.94	0.38	2.47

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