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Enantioselective determination of felodipine and other chiral dihydropyridine calcium entry blockers in human plasma

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SUMMARY

A sensitive method for the enantioselective determination of felodipine in human plasma is described. Following alkaline extraction with dichloromethane–pentane, racemic felodipine and its primary pyridine metabolite are simultaneously assayed using capillary gas chromatography on a DB-1 column, with electron-capture detection. The enantiomers of felodipine are quantitatively separated by high-performance liquid chromatography on a Chiralcel OJ column, containing tris(4-methylbenzoate)-modified cellulose coated on silica, and off-line detection using the same gas chromatographic system is applied. The limits of determination in plasma (and the inter-assay coefficient of variation (C.V.) at levels below 1 ng/ml) were 0.1 ng/ml (C.V. 13%) for felodipine, 0.1 ng/ml (C.V. 15%) for the enantiomers of felodipine and 0.3 ng/ml (C.V. 7%) for its pyridine metabolite. The method has proved to be applicable to several other chiral dihydropyridine calcium entry blockers, including nitrendipine, with comparable sensitivities.

INTRODUCTION

Felodipine, 3-ethyl-5-methyl-1,4-dihydro-2,6-dimethyl-4-(2,3-dichlorophenyl)-3,5-pyridine dicarboxylate (I) (Fig. 1), a potent and vasoselective dihydropyridine calcium entry blocker, has recently been approved in several countries for the treatment of arterial hypertension [1]. Concentrations of racemic felodipine in human plasma are relatively low as a consequence of the low doses (5–20 mg) applied, its rapid presystemic elimination and its high apparent volume of distribution. Maximum plasma concentrations of racemic

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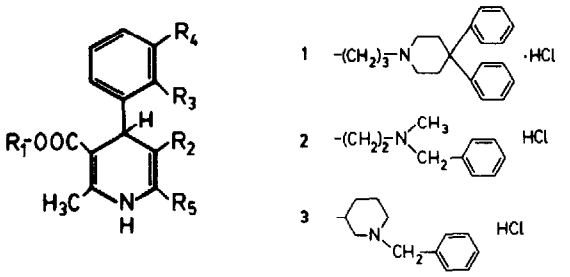

Fig. 1. Structures of dihydropyridine compounds used in these studies.

Compound	R_1	R_2	R_3	R_4	R_5
I Felodipine	C_2H_5	$COOCH_3$	Cl	Cl	CH_3
III Nitrendipine	C_2H_5	$COOCH_3$	H	NO_2	CH_3
V Nimodipine	$CH_2CH_2OCH_3$	$COOCH(CH_3)_2$	H	NO_2	CH_3
VI Nisoldipine	$CH_2CH(CH_3)_2$	$COOCH_3$	NO_2	H	CH_3
VII Bay e 5759	$CH(CH_3)_2$	$COOC_2H_5$	H	NO_2	CH_3
VIII Bay r 9425	$CH_2CHOH(CH_3)_2$	$COOCH_3$	NO_2	H	CH_3
IX Bay k 8644	CH_3	NO_2	CF_3	H	CH_3
X Isradipine	$CH(CH_3)_2$	$COOCH_3$	=N-O-N=		CH_3
XI SDZ 202-791	$CH(CH_3)_2$	NO_2	=N-O-N=		CH_3
XII Niguldipine	1	$COOCH_3$	H	NO_2	CH_3
XIII Nilvadipine	$CH(CH_3)_2$	$COOCH_3$	H	NO_2	CN
XIV FR-7534	C_2H_5	C_2H_5	H	NO_2	CH_2OH
XV Nicardipine	2	$COOCH_3$	H	NO_2	CH_3
XVI KW-3049	3	$COOCH_3$	H	NO_2	CH_3

felodipine are generally ca. 5–10 ng/ml (for a 10-mg dose), whereas plasma concentrations at the end of the usual 24-h dosing interval are below 1 ng/ml [2]. Gas chromatographic (GC) assays with different detection systems for the determination of racemic felodipine in biological material have been reported [3,4]. Reversed-phase high-performance liquid chromatographic (HPLC) methods with UV detection are less suitable for detailed pharmacokinetic studies in humans because of their relatively high limit of determination (ca. 3 ng/ml) (P.A. Soons, unpublished results).

Felodipine has two unequal ester moieties and thus has a chiral carbon atom at the 4-position (Fig. 1). The drug is marketed as a racemic mixture. Pharmacodynamic differences between the enantiomers of many dihydropyridine compounds have been reported [5–7], and in some cases the two enantiomers even have opposite effects [8,9]. For only a few racemic dihydropyridines have pharmacokinetic data of their enantiomers been reported, either via enantioselective assay [10,11] or after administration of labelled pseudo-racemates [12].

No pharmacokinetic data on the enantiomers of felodipine in humans have been reported, since no method was available for the selective assay of unla-

belled felodipine enantiomers. Such a method would be very useful to study the pharmacokinetics, metabolism and relationships between kinetics and effects of the enantiomers of felodipine, without the need to synthesize and administer labelled pseudo-racemates or pure enantiomers.

Besides felodipine, at least nine representatives of the group of dihydropyridine calcium entry blockers are marketed and/or are in development as racemic mixtures [13–21]. For most of them a capillary GC assay with electron-capture detection (ECD) has been described for the racemates, but only for nilvadipine has an enantioselective assay been published [10].

This paper describes a sensitive and selective assay, using chiral-column HPLC combined with GC–ECD, for the determination of the enantiomers of felodipine in human plasma, when the drug is administered as a racemate. The method has also proved to be applicable to nitrendipine as well as to several other dihydropyridine compounds, with only minor modifications.

EXPERIMENTAL

Chemicals and glassware

Felodipine (I), its *R*-(+)-enantiomer (H 183/91), its (*S*)-(−)-enantiomer (H 183/96) and its pyridine metabolite [3-ethyl-5-methyl-2,6-dimethyl-4-(2,3-dichlorophenyl)-3,5-pyridinedicarboxylate; H 152/37] (II) were generous gifts of Hässle (Mölndal, Sweden). Nitrendipine (III), its *R*-(+)-enantiomer (Bay p 2695), its (*S*)-(−)-enantiomer (Bay p 2696), the internal standard [3-(2-hydroxy-2-methyl)ethyl-5-methyl-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridine dicarboxylate; Bay r 9590] (IV), nimodipine (V), nisoldipine (VI), 3-ethyl-5-isopropyl-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridine dicarboxylate (Bay e 5759) (VII), 3-(2-hydroxyisobutyl)-5-methyl-1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridine dicarboxylate (Bay r 9425) (VIII) and methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate (Bay k 8644) (IX) were all kindly supplied by Bayer (Wuppertal, F.R.G.). Isradipine (X) and both enantiomers of isopropyl-4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-nitro-3-pyridinecarboxylate (SDZ 202-791) (XI) were gifts of Sandoz (Basel, Switzerland). Niguldipine·HCl (B 844-39) (XII), its (+)-enantiomer (B 859-34) and (−)-enantiomer (B 859-35) were gifts of Byk Gulden (Konstanz, F.R.G.). Nilvadipine (XIII) and diethyl-1,4-dihydro-2-hydroxymethyl-6-methyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate (FR-7534) (XIV) were gifts of Fujisawa Pharmaceutical (Osaka, Japan). Nicardipine·HCl (XV) and 2,6-dimethyl-4-(3-nitrophenyl)-3,5-dicarboxylic acid-1-benzyl-3-piperidinyl ester methyl ester hydrochloride (Benidipine, KW-3049) (XVI) were gifts of Syntex (Palo Alto, CA, U.S.A.) and Kyowa Hakko Kogyo (Tokyo, Japan), respectively. All compounds except II and IV contain a chiral carbon atom at the 4-position. Structures of the dihydropyridine compounds (all except II and IV) are shown in Fig. 1.

Solutions of these substances at suitable concentrations (100–150 ng/ml) were prepared in freshly distilled methanol p.a. and stored at −20°C excluding light. Under these conditions the compounds were stable for at least three months. All the other chemicals used were of analytical grade (Baker, Deventer, The Netherlands). The organic solvents were always distilled twice prior to use. All glassware was rinsed with methanol p.a. and dried at 250°C for at least 12 h.

GC apparatus and chromatographic system

A Hewlett-Packard Model 5890A gas chromatograph, equipped with a ^{63}Ni pulse-modified electron-capture detector and a laboratory-made all-glass solid injection system with a moving needle were used. A fused-silica capillary column (30 m×0.32 mm I.D.) with a DB-1 (bonded cross-linked silicon) stationary phase (Durabond, J & W Scientific, Rancho Cordova, CA, U.S.A.) was used.

The operating conditions were: injection port temperature, 260°C; detector temperature, 350°C; column temperature, 250°C for 5.8 min, then rising to 270°C at 40°C/min and remaining at that temperature for 2.0 min. After cooling to 250°C, a stabilization time of at least 2 min was allowed. For the assay of the separated enantiomers the chromatograph was operated isothermally at 250°C. The carrier gas was helium; the flow-rates were 2.0 ml/min through the column and 15 ml/min through the restrictor of the injection system. The flow-rate of the auxiliary gas (argon–methane, 95:5) was 30 ml/min. All gases were of the highest grade available (Hoek-Loos, Schiedam, The Netherlands).

Data were processed using a C-R3A integrator (Shimadzu, Kyoto, Japan) in peak-height mode.

HPLC apparatus and chromatographic system

The HPLC system consisted of an M-45 pump (Millipore Waters, Milford, MA, U.S.A.), a C6W injection valve equipped with a 20-μl fixed-volume sample loop (Valco Instruments, Houston, TX, U.S.A.), and a Spectroflow 757 variable-wavelength UV detector (Applied Biosystems, Ramsey, NJ, U.S.A.) set at 240 nm. The mobile phase was *n*-hexane–2-propanol (87.5:12.5), with a constant flow-rate of 1.0 ml/min.

The analytical chiral column was a 25 cm×4.6 mm I.D. Chiralcel OJ column (Daicel Chemical Industries, Tokyo, Japan), which contains a stationary phase of tris(4-methylbenzoate)-modified cellulose coated on 10-μm particles of silica [22]. A 2 cm×2 mm I.D. precolumn, packed with Perisorb® RP-18 was applied (Upchurch Scientific, Oak Harbour, WA, U.S.A.). The columns were mounted in a laboratory-made water jacket, connected to an F3-CH heating/cooling bath (Haake, Karlsruhe, F.R.G.). The columns and mobile phase were kept at a constant temperature of 37°C.

For calibration purposes, the UV detector was connected to a C-R3A integrator (Shimadzu, Kyoto, Japan) in recorder mode.

Sample preparation

To 1.00 ml of plasma in a centrifuge tube, 5.0 ng of internal standard (IV, 100 ng/ml in methanol) were added. After mixing and equilibration for 10 min, 25 µl of 4 *M* sodium hydroxide were added and mixed. The sample was extracted with 4.0 ml of a mixture of *n*-pentane and dichloromethane (1:1) for 60 min on a CM-9 whirl-mixer (Sarstedt, Numbrecht, F.R.G.). After centrifugation for 5 min at 2000 *g*, the upper organic layer was transferred to another centrifuge tube using Pasteur disposable pipettes, and evaporated to dryness using a vortex vacuum evaporator (Buchler, Fort Lee, NJ, U.S.A.) at 30°C. The residue was dissolved in 50–200 µl of *n*-pentane–dichloromethane (1:1), 2.0 µl were brought on the tip of the glass needle of the GC solid injection system, and the drop was allowed to evaporate for 20 s before the residue was injected into the gas chromatograph.

The remaining organic solution was evaporated to dryness and subsequently dissolved in 40 µl of liquid mobile phase. Of this solution, 20 µl were injected into the HPLC system, and isovolumetric fractions were collected according to predetermined collection times (see calibration). To each collected fraction, 5.0 ng of nitrendipine (100 ng/ml in methanol) were added, and the fractions were evaporated to dryness, using the same vacuum evaporator, at 35°C. The residue was dissolved in 50 µl of *n*-pentane–dichloromethane (1:1), 2.0 µl were brought on the tip of the glass needle of the GC solid injection system, and the drop was allowed to evaporate for 20 s before the residue was injected into the gas chromatograph.

Assay calibration

The assay of racemic felodipine and its pyridine metabolite was calibrated with calibration curves prepared by spiking control plasma samples (1.00 ml) with racemic felodipine and its pyridine metabolite (0.75, 1.50, 3.00, 6.00, 9.00, 12.0, 15.0 and 22.5 ng/ml). Samples were processed as described, and the ratios of the peak height of the compound to be assayed to the peak height of the internal standard were calculated. Calibration curves were constructed by linear regression analysis.

Subsequently, the assay of the enantiomers was calibrated by calculating the peak-height ratio of the felodipine enantiomer in each fraction to the peak height of nitrendipine. The ratio of the concentrations of the two enantiomers in each sample was calculated as the ratio of peak-height ratios of the two fractions. The ratio in unknown samples was corrected for the mean ratio of the racemic calibration samples.

Method validation

The extraction yields of the internal standard (5.0 ng/ml, $n=6$), racemic felodipine and its pyridine metabolite (3.75 ng/ml, $n=6$) and the separate felodipine enantiomers (3.75 ng/ml, $n=4$) from plasma were determined on two separate occasions with the internal–external standard method using GC only.

The intra-assay variability of racemic felodipine and its pyridine metabolite was determined at two concentration levels (0.75 and 3.75 ng/ml) on two different occasions (for each concentration level and each occasion, $n=6$). The intra-assay variability and precision of the ratio of enantiomers was determined at two concentration levels (1.5 and 7.5 ng/ml) with thirteen different levels of S/R ratio (between 0.1 and 10, for each concentration and S/R ratio, $n=4$).

The inter-assay variability and precision of racemic felodipine and its pyridine metabolite were determined at two concentration levels (0.75 and 7.5 ng/ml, $n=12$). The inter-assay variability of the ratio of enantiomers was determined at the same concentration levels with an S/R ratio of 1.0. Twenty samples (0.2–10 ng/ml felodipine, S/R ratio 2.0–3.5) obtained from patients after administration of racemic felodipine were assayed in duplicate in order to obtain an estimate of the assay performance for real human plasma samples. Results are reported as mean ± S.D.

Preliminary experiment on human subjects

The assay was used in a preliminary study of the pharmacokinetics of felodipine, its pyridine metabolite and its enantiomers, when administered as a racemate, to healthy male volunteers. Racemic felodipine (20 mg) was administered as an oral solution, and blood samples (8 ml) were drawn from a forearm vein using a flexible cannula (Venflon®) or by vena puncture. Samples were drawn in heparinized glass tubes (Vacutainer®) up to 32 h after administration. Plasma was separated within 30 min at room temperature, and samples were stored at −30 °C until analysis. Pharmacokinetic parameters were calculated using standard procedures [23]. Results are reported as mean ± S.D.

RESULTS

Assay of racemic felodipine and its pyridine metabolite in plasma

Fig. 2 shows typical chromatograms obtained from the assay of racemic felodipine and its pyridine metabolite. Although several compounds seem to be co-extracted, none interferes significantly with the determination of felodipine. Blank plasma from some subjects contained a small interfering peak with a retention time similar to that of the pyridine metabolite, thus prohibiting reliable estimation of the metabolite at very low levels. Under the conditions applied no (thermo)degradation of felodipine was observed. The pyridine me-

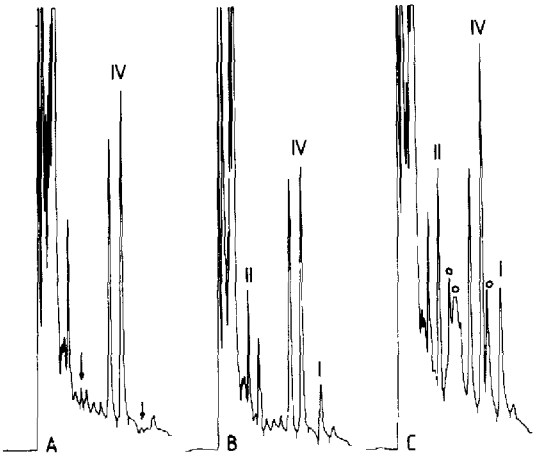

Fig. 2. GC assay of racemic felodipine (I), its pyridine metabolite (II) and internal standard (IV, 5.0 ng/ml). (A) Drug-free plasma; (B) plasma spiked with 1.50 ng/ml I and II; (C) plasma sample containing 2.16 ng/ml I, 2.03 ng/ml II and unidentified felodipine metabolites (o)

TABLE I

EXTRACTION RATIOS AND INTRA-ASSAY VARIABILITY

Compound	Concentration (ng/ml)	Extraction ratio (mean ± S.D.) (%)	Intra-assay C.V. (%)
(*R/S*)-Felodipine (I)	0.75		6.0
(*R/S*)-Felodipine (I)	3.75	80 ± 5	6.1
(*R*)-Felodipine	3.75	79 ± 4	(8)[a]
(*S*)-Felodipine	3.75	81 ± 2	(8)[a]
H 152/37 (II)	0.75		4.7
H 152/37 (II)	3.75	83 ± 3	3.5
Internal standard (IV)	5.00	92 ± 2	

[a]Approximation, calculated assuming ca. 6% intra-assay C.V. for racemic felodipine and ca. 5% intra-assay C.V for the *S/R* ratio.

TABLE II

INTER-ASSAY VARIABILITY AND PRECISION OF FELODIPINE AND ITS PYRIDINE METABOLITE ($n = 12$)

Compound	Actual concentration (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	Percentage of actual concentration	Inter-assay C.V. (%)
Felodipine (I)	0.77	0.79 ± 0.14	103	13
Felodipine (I)	7.68	7.49 ± 0.71	98	9.5
H 152/37 (II)	1.03	1.16 ± 0.08	112	6.6
H 152/37 (II)	7.68	7.71 ± 0.71	100	9.2

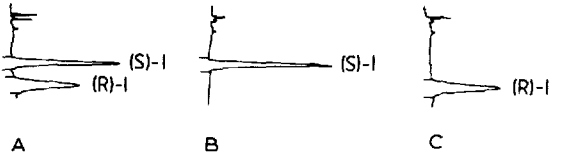

Fig. 3. HPLC after injection of (A) 100 ng of felodipine (I), (B) 50 ng of (*S*)-(−)-felodipine [(*S*)-I] and (C) 50 ng of (*R*)-(+)-felodipine [(*R*)-I].

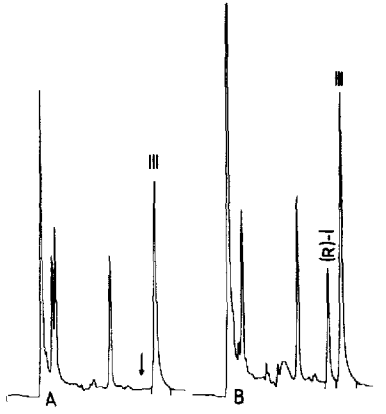

Fig. 4. GC of collected HPLC fractions after addition of 5.0 ng of nitrendipine (III). (A) Second fraction of a drug-free plasma sample; (B) second fraction of a plasma sample containing 4.44 ng/ml (*R*)-(+)-felodipine [(*R*)-I].

tabolite, internal standard and felodipine had retention times of 2.9, 4.5 and 5.2 min, respectively. The total run time was ca. 11 min.

Over 30 calibration curves for felodipine and its pyridine metabolite have been constructed; these were always linear with a correlation coefficient of at least 0.998. On two (6%) occasions the calibration curve for felodipine exhibited a small positive intercept, equivalent to 0.10 and 0.17 ng/ml, respectively. One (3%) calibration curve for the pyridine metabolite had a positive intercept equivalent to 0.32 ng/ml. The results of the method validation procedures are are summarized in Tables I and II. Arbitrarily, it can be concluded that the limit of reliable determination was ca. 0.1 ng/ml of plasma for felodipine and ca. 0.3 ng/ml of plasma for its pyridine metabolite.

Assay of felodipine enantiomers

The enantiomers of felodipine were baseline-separated, as shown in Fig. 3. With UV detection, the detection limit of the separate enantiomers was ca. 1 ng of each enantiomer on-column from methanolic solutions and ca. 5 ng/ml

TABLE III

INTER-ASSAY VARIABILITY AND PRECISION OF S/R RATIO OF FELODIPINE

Concentration (ng/ml)	Actual S/R ratio	S/R Ratio found (mean ± S.D.)	Inter-assay C.V. (%)
1.5–15[a,b]	1.00	0.98 ± 0.05	4.7
0.77	1.00	1.05 ± 0 13	12
7.68	1.00	0.99 ± 0.04	3.6
0.2–10[a,c]		2.02–3.54[a]	12

[a]Range.
[b]Samples of twelve calibration curves, $n = 72$.
[c]Twenty plasma samples assayed in duplicate.

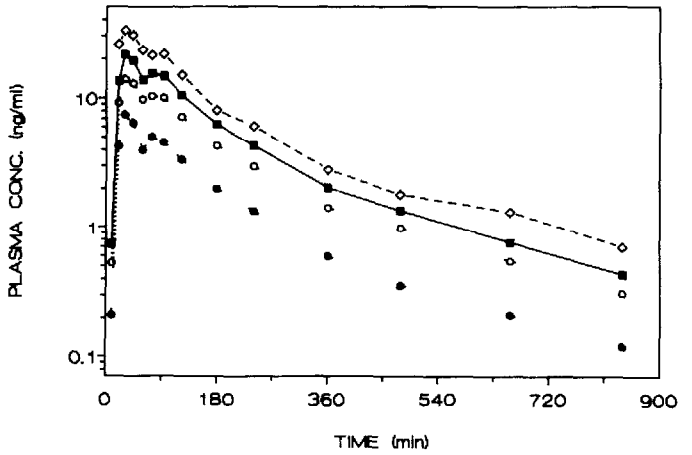

Fig. 5. Representative plasma concentration–time profiles of felodipine (■), (*S*)-felodipine (○), (*R*)-felodipine (●) and the pyridine metabolite (◇), after oral administration of 20 mg of racemic felodipine.

TABLE IV

PHARMACOKINETIC DATA OF FELODIPINE AND ITS ENANTIOMERS AFTER ORAL ADMINISTRATION TO HEALTHY SUBJECTS

Mean ± S.D.; $n = 4$.

Compound	Maximum concentration (ng/ml)	Time of maximum concentration (h)	Elimination half-life (h)	Oral clearance (l/min)
(*R/S*)-Felodipine	32 7 ± 10.5	0.35 ± 0.35	10 3 ± 2.8	3.42 ± 0.88
(*R*)-Felodipine	10.5 ± 3.4	0.35 ± 0.35	9.2 ± 1.0	5.72 ± 1 15
(*S*)-Felodipine	22.3 ± 7.1	0.35 ± 0 35	10.9 ± 2.8	2.42 ± 0.66

from plasma. The sensitivity was substantially improved by using off-line GC–ECD for detection. Fig. 4 shows some typical GC profiles of the GC–ECD of the separate enantiomers. No interfering compounds were present in the blank plasma of any of the subjects studied. The total run-time for HPLC separation of the enantiomers was 24 min.

At both concentration levels tested (1.5 and 7.5 ng/ml) the intra-assay variability of the S/R ratio was below 7% (mean 4.4%) for all S/R-ratios (0.1–10). For S/R ratios between 0.1 and 5 the precision was $100.5 \pm 4.7\%$ of the theoretical value. For S/R ratios between 6 and 10, a tendency was observed to underestimate ($92.3 \pm 2.5\%$ of actual value) the S/R ratio, although this was not significant at the 5% level.

Other results of the validation procedures are summarized in Table III. It can be concluded that the limit of reliable determination was ca. 0.1 ng/ml for each enantiomer of felodipine.

Preliminary experiment on human subjects

Representative plasma concentration–time profiles for felodipine, its enantiomers and its pyridine metabolite after administration of 20 mg of racemic felodipine are shown in Fig. 5. From the data of the four subjects the pharmacokinetic parameters as presented in Table IV were calculated.

TABLE V

SEPARATION OF ENANTIOMERS OF DIHYDROPYRIDINE COMPOUNDS

Compound	Retention times (min)[a]	Separation factor (α)	Resolution factor (R)
Felodipine (I)	15.9/21.9 (S/R)	1.44	2.40
Nitrendipine (III)	19.1/23.1 (S/R)	1.23	1.53
Nimodipine (V)	14.7/19.7	1.43	2.16
Nisoldipine (VI)	10.9/16.6	1.80	4.37
Bay e 5759 (VII)	11.8/16.5	1.58	2.21
Bay r 9425 (VIII)	22.3/47.0	2.36	8.96
Bay k 8644 (IX)	20.9/28.8	1.47	3.26
Isradipine (X)	12.0/15.7	1.48	1.73
SDZ 202-791 (XI)	27.1/30.2 (−/+)	1.12	0.79
Niguldipine (XII)	85.1/88.4 (+/−)	1.04	0.16
Nilvadipine (XIII)	22.0[b]	< 1.1[b]	< 0.3[b]
FR-7534 (XIV)	13.3/14.4	1.13	0.54
Nicardipine (XV)	23.5/25.4	1.10	0.56
KW-3049 (XVI)	21.5[b]	< 1.1[b]	< 0.3[b]

[a]Elution order if known.

[b]Exact values could not be determined because separate enantiomers were not available.

Application to other chiral dihydropyridine calcium entry blockers

The applicability of the present system to separation of other dihydropyridine compounds was evaluated by injection of the racemates (and enantiomers, if available) into the system under the conditions described. The results, expressed as separation factor (α) and resolution factor (R) [24] are summarized in Table V. No attempt was made to optimize the separation of the enantiomers, except for those of felodipine and nitrendipine.

DISCUSSION

This paper describes a sensitive and specific assay of felodipine and its enantiomers, when administered as a racemate, using a combination of GC–ECD and chiral-column HPLC. The sensitivity, accuracy and reproducibility of the assay allow its application in single-dose pharmacokinetic studies of felodipine. In the low concentration range (< 1 ng/ml) the purity of the carrier gas, the make-up gas and the organic solvents appeared to be of critical importance.

Assay of racemic felodipine and its pyridine metabolite

Three methods for the analysis of felodipine have been published previously [3,4], all of them by the manufacturer of felodipine. The capillary GC–ECD method [3] is slightly less sensitive (0.4–0.8 ng/ml) but uses a splitless injector that can easily be automated. The GC–MS method and GC–GC–ECD method with column switching [4] both have approximately the same sensitivity as the present method and their specificity can be expected to be better. However, both methods require rather complicated and expensive apparatus.

HPLC system and assay of enantiomers

A baseline separation of the enantiomers of felodipine was obtained using a column containing a stationary phase of tris(4-methylbenzoate)-modified cellulose coated on silica [22]. Although the column's efficiency was low (typical number of theoretical plates 800–1000), its enantioselectivity was surprisingly high.

As the column temperature was increased, the retention times decreased and the resolution factor increased for all compounds, whereas the separation factor remained essentially unchanged. The operating conditions represent an arbitrary choice and are a compromise to achieve optimal column performance, column stability, run time and ease of handling of the system. Retention times of the enantiomers were very constant and the within-day coefficient of variation (C.V.) did not exceed 0.2% for all the compounds.

Direct quantification of the separated enantiomers using UV detection at 240 nm was possible at concentrations above 5 ng/ml of plasma for each enantiomer. Since this is in no way sufficient for single-dose pharmacokinetic

studies, off-line detection with capillary GC–ECD was necessary. Many dihydropyridine calcium entry blockers are sensitive to UV and visible light. Therefore, in order to avoid potential photodecomposition, the UV detector was switched on only for the daily calibration and check of retention times of the separated enantiomers.

The column's long-term stability was high. A single column has been in continuous use for more than a year, and more than 2000 injections (of which ca. 1200 plasma extracts) have been made, without any signs of worsening column performance. This may also be related to the fact that only very pure, freshly distilled solvents were used throughout all investigations.

Study of human subjects

In a preliminary experiment with four healthy volunteers we observed a 2.4-fold difference in the oral clearance of the enantiomers and a 2.1-fold difference in the maximum plasma concentration. The times of maximum concentration were not different. The small but not significant difference in the terminal elimination half-lives of the enantiomers can be completely accounted for by the higher intrinsic clearance of (*R*)-felodipine.

Only very limited pharmacokinetic data for the enantiomers of felodipine have been presented previously. The (*S*)-(−)-enantiomer seems to be three to five times more potent than the (*R*)-(+)-enantiomer as a peripheral vasidilator [25]. It has been claimed that the pharmacokinetics of (*R*)- and (*S*)-felodipine in the dog are similar [25]. However, the use of a tris-deuterated methyl ester derivative of felodipine may complicate data interpretation, because of the recently discovered in vivo oxidative ester cleavage, a process that is strongly inhibited by deuterium substitution in the ester moiety [26].

The data presented in this paper are the first on stereoselectivity in felodipine pharmacokinetics in humans. There clearly are pronounced differences between the pharmacokinetics of the two enantiomers.

Application to other dihydropyridine compounds

Several other dihydropyridine calcium entry blockers, including nitrendipine, could also be separated into their enantiomers by this method. Interestingly, the enantiomers of nilvadipine could not be separated at all, whereas a clear separation has been reported on Chiralpak OT(+) and OA columns, but not on Chiralpak OP(+), OB, OC and OK columns [10]. Chiral dihydropyridine compounds with basic side-chains [niguldipine (XII), nicardipine (XV) and benidipine (KW-3049; XVI)] or with a modified 2-methyl group [nilvadipine (XIII) and FR-7534 (XIV)] were poorly resolved into their enantiomers with the present HPLC system, but chiral calcium channel activators, such as Bay k 8644 (IX) and SDZ 202-791 (XI), were well resolved into enantiomers by our system. Separation of the enantiomers can be improved by ad-

justment of the mobile phase composition and the HPLC operating conditions. For nitrendipine, a complete assay of the enantiomers, including detection by capillary GC–ECD [12], is fully operational with a limit of determination of ca. 0.1 ng/ml for each enantiomer, and an assay variability comparable with that of the felodipine enantiomers.

There are some scattered reports of resolution of dihydropyridine compounds on chiral stationary phase HPLC columns [10,27–29]. Resolution of dihydropyridine calcium entry blockers on β-cyclodextrin columns as reported by Armstrong et al. [27] could not be reproduced by us. Also the application of several other kinds of cyclodextrin-based HPLC system did not result in separation of these compounds. For off-line GC–ECD, aqueous mobile phases [27,28] must be regarded as disadvantageous compared with the present water-free mobile phase. In addition to stability problems, especially for the α1-AGP columns [28], none of these systems can be applied to a wide range of dihydropyridines, whereas the present system can.

Overall performance of the assay of felodipine enantiomers

The assay sensitivity allowed determination of the S/R ratio for samples with a concentration of racemic felodipine down to ca. 0.2 ng/ml. Taking into account the mean S/R ratio of ca. 2.4 in our healthy volunteers, and the inter-assay variability of the separate analytical steps ($\pm$12% C.V. for the assay of racemate; $\pm$12% C.V. for the assay of S/R ratio), it can be concluded that the limit of determination of the separate enantiomers is ca. 0.1 ng/ml, with an inter-assay C.V. of ca. 15% at this very low level. The assay of the ratio of enantiomers was rather laborious and time-consuming, but required no additional sample clean-up. In one week, a laboratory technician is able to determine the concentrations of racemic felodipine, its enantiomers and its pyridine metabolite in ca. 50 plasma samples.

The present universal methodology makes it possible to assess the kinetic characteristics of several dihydropyridine enantiomers using unlabelled, commercially available racemic drugs. This has advantages over a previous method that involves the use of stable-isotope labelled pseudo-racemates [12] which requires extensive additional testing of purity and evidence of metabolic and pharmacokinetic identity with the commercially available racemate. The possible occurrence of unexpected isotope effects is often neglected [26,30].

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