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Determination of felodipine, its enantiomers, and a pyridine metabolite in human plasma by capillary gas chromatography with mass spectrometric detection

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Abstract

Sensitive methods based on capillary gas chromatography (GC) with mass spectrometric (MS) detection in a selected-ion monitoring mode (SIM) for the determination of racemic felodipine, its enantiomers, and a pyridine metabolite in human plasma are described. Following liquid–liquid extraction from plasma, enantiomers of felodipine were separated on a chiral HPLC column (Chiralcel OJ) and fractions containing each isomer were collected on a continuous basis using a fraction collector. These fractions were later analyzed by GC–MS-SIM. A similar method based on GC–MS-SIM detection was developed for the determination of racemic felodipine and its pyridine metabolite with a minor modification of sample preparation. The limits of quantitation in plasma were 0.1 ng/ml for both the *R*(+)- and *S*(−)-enantiomers of felodipine and 0.5 ng/ml for both racemic felodipine and its pyridine metabolite. The stereoselective assay was used to support a clinical study with racemic felodipine, and was capable of analyzing more than 30 plasma samples per day.

1. Introduction

Felodipine, 3-ethyl-5-methyl-1,4-dihydro-2,6-dimethyl-4-(2,3-dichlorophenyl)-3,5-pyridine dicarboxylate (I) (see Fig. 1), is a dihydropyridine calcium antagonist developed for use as a selective vasodilator in cardiovascular disorders, primarily arterial hypertension [1]. Several analytical methods based on gas chromatography (GC) with electron-capture (EC) or mass spectrometric (MS) detection were described for determination of felodipine and its stereoisomers in biological fluids. Enantioselective assays involved chiral HPLC separation, fraction collection of the analytes, and analysis of the isolated isomers using GC–EC [2–4], or, as described in the recent paper [5] published while our studies were in progress, using GC–MS. These laborious and time-consuming procedures allowed determination of enantiomers of I in ca. 50 plasma samples per week [3] with the limit of quantitation (LOQ) greater than 0.1 ng/ml. The LOQ is defined here as the lowest concentration on the standard curve for which intra-day assay precision, expressed as the coefficient of variation (C.V.), was less than 10% and assay accuracy

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was ± 10%. In the case of the GC–MS method [5] both the limit of detection, at signal-to-noise ratio of 3, and the LOQ were claimed to be the same at 0.05 ng/ml, but the C.V. value for the *S*-enantiomer was 11.3% and no precision data for all concentrations below 0.5 ng/ml for both enantiomers were reported. In addition, the chiral separation procedure was not automated, the sample throughput of the method has not been specified, and the long-term performance of the assay was not assessed.

To support large-scale human pharmacokinetic studies involving analyses of more than 500 plasma samples, a partially automated chiral method with high sample throughput and LOQ of 0.1 ng/ml for both enantiomers was required and its development is the subject of this paper. After liquid–liquid extraction from plasma, the enantiomers of I were separated on a chiral HPLC column, fractions containing isomers were collected on a 24 hours-a-day basis using a fraction collector, and, after the fractions were evaporated to dryness, and reconstituted in acetonitrile, the analytes were injected onto the GC–MS system. Standard curves ranging from 0.1 to 5.0 ng/ml for each enantiomer were linear with correlation coefficients of >0.998. Intra-day precision (C.V.) was less than 10% at all concentrations within the standard curve range. This validated method was capable of analyzing more than 30 plasma samples per day and was used to support a clinical study with I.

In addition, GC–MS methodology, instead of GC–EC [2–4] or HPLC-UV [6], was developed for the determination of racemic felodipine and its pyridine metabolite (II) (see Fig. 1) in human plasma. An improvement in the LOQ values from more than 0.75 ng/ml using GC–EC method [3] to 0.5 ng/ml for both analytes, using GC–MS, was achieved largely due to the elimination of plasma impurities coeluting with the peaks of interest by pretreatment of plasma extracts with N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) before analysis. The details of both assay procedures are presented in this paper.

2. Experimental

2.1. Materials

Racemic felodipine (I) was supplied by Merck Research Laboratories (Rahway, NJ, USA). *S*(−)-Felodipine, *R*(+)-felodipine, felodipine pyridine metabolite [3-ethyl-5-methyl-2,6-dimethyl-4-(2,3-dichlorophenyl)-3,5-pyridinedicarboxylate] (II), felodipine propyl ester [3,5-pyridine dicarboxylic acid, 4-(2,3-dichlorophenyl)-1,4-dihydro-2,6-dimethyl, 5-ethyl-3-isopropyl ester] (III) and deuterated d_6-felodipine (IV) were provided by Astra Hässle (Mölndal, Sweden). HPLC and GC grade solvent were from Baxter (McGaw Park, IL, USA). The N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was from Pierce Chemical (Rockford, IL, USA). The GC column used was a fused-silica capillary column (30 m × 0.2 mm I.D., 0.5 μm film thickness) with methylsilicone stationary phase (HP-1, Hewlett-Packard, Wilmington, DE, USA). A 250 × 4.6 mm I.D. Chiralcel OJ column (Daicel Chemical, Exton, PA, USA), containing 10 μm particles of silica coated with a stationary phase of tris(4-methylbenzoate)-modified cellulose was used as the HPLC chiral column. The chiral column was protected with a Supelco 20 × 2 mm I.D. precolumn packed with Pelliguard LC-18 packing (Supelco, Bellefonte, PA, USA).

A Multi-Tube vortex-mixer (Baxter), and a TurboVap LV Evaporator ZW 700 (Zymark Co., Hopkinton, MA, USA) were used during preparation of plasma samples for analyses.

2.2. Apparatus

GC–MS system

A Hewlett-Packard Model 5890 Series II gas chromatograph equipped with a Model 7673 autosampler and an HP 5971A mass-selective detector (MSD) was used. The operating conditions were: injection port temperature, 290°C; MSD temperature, 300°C; column temperature programming, 220°C for 0.6 min, then

rising to 300°C at 15°/min and remaining at that temperature for 10 min. After cooling to 220°C, an equilibration time of at least 30 s was allowed. The GC conditions were identical for the assay of I, II, and both felodipine enantiomers. Helium was used as the carrier gas, with a flow-rate of 0.365 ml/min. A mass-selective detector in the electron-ionization mode was operated at an ion source pressure of $2.0 \cdot 10^{-3}$ Pa, using helium as a carrier gas. The electron multiplier (EM) voltage was 200–600 V above calibration voltage. Data was processed using HP-Chemstation version B.00. in the selected-ion monitoring mode (SIM).

HPLC chiral separation

The HPLC system consisted of a binary LC 250 pump (Perkin-Elmer, Norwalk, CT, USA), an advanced LC sample processor (ISS 200) equipped with a 100-μl fixed sample loop (Perkin-Elmer), and a Spectroflow 773 variable-wavelength UV detector (Applied Biosystem, Foster City, CA, USA) set at 240 nm. The mobile phase was *n*-hexane–isopropanol (88:12, v/v), with a flow-rate of 1.0 ml/min.

The analytical chiral column and precolumn were thermostatted at 37°C using a Systec CH 1448 thermal jacket from Anspec (Ann Arbor, MI, USA). For calibration purposes, the UV detector was connected to a SP 4240 integrator (Spectra Physics, San Jose, CA, USA).

2.3. Standard solutions

Stock standard solutions of I, II, III and IV at suitable concentrations (100–150 ng/ml) were prepared in acetonitrile, stored at −20°C, and protected from laboratory room light. Under these conditions the compounds were stable for at least three months. These solutions were further diluted to give a series of working standards with concentrations of 100, 50, 20, 10, 5, 2 and 1 ng/ml. The standard curve in the non-stereoselective assay was constructed by analyzing control human plasma samples (1 ml) spiked with standard solutions of racemic felodipine (0.1 ml) and its pyridine metabolite (0.1 ml). The concentrations of the analytes used in the non-stereoselective assay of I and II were 0.5, 1, 2, 5 and 10 ng/ml. The concentrations of the enantiomers used in the stereoselective assay were 0.1, 0.25, 0.5, 1, 2 and 5 ng/ml. Quality control samples were prepared from different standard solutions than used for construction of standard curves. Appropriate aliquots of the diluted solution were added to 60 ml of control human plasma to yield the concentrations indicated in Table 3. Aliquots (1 ml) of the well-mixed plasma were stored at −20°C until taken for assay with a set of test samples.

2.4. Sample preparation

Non-stereoselective assay of I and II

To 1 ml of plasma in a centrifuge tube, 10 ng of internal standard (10 μl of 1 μg/ml standard solution of III in acetonitrile) was added. After vortex-mixing for 10 s, the sample was extracted with 1 ml of toluene for 20 min on a Multi-Tube vortex-mixer. After centrifugation for 10 min at 3000 *g*, the upper organic layer was transferred to an amber vial, using Pasteur disposable glass pipettes, and evaporated to dryness at 35°C. After addition of 100 μl of acetonitrile and 5 μl of MSTFA the tube was sealed and heated at 98°C for 60 min. After cooling to room temperature the tube was opened, the organic solution was evaporated to dryness and the residue was dissolved in 100 μl of acetonitrile. The extract was transferred to a glass HPLC-insert, evaporated to dryness, and after dissolving the residue in 20 μl of acetonitrile, 2 μl of the solution was injected onto GC–MSD system.

Stereoselective assay

To 1 ml of plasma in a centrifuge tube, 4 ng of internal standard (20 μl of 0.2 μg/ml standard solution of IV in acetonitrile) was added. After vortex-mixing for 10 s, the sample was extracted with 1 ml of toluene for 20 min on a Multi-Tube vortex-mixer and centrifuged for 10 min at 3000 *g*, the upper organic layer was

transferred to an amber vial using Pasteur disposable glass pipettes, and evaporated to dryness using a TurboVap LV Evaporator at 35°C. After reconstitution of the residue in 100 μl of mobile phase (*n*-hexane–2-propanol, 88:12, v/v), the solution was injected onto the HPLC system, and fractions containing *S*(−)- and *R*(+)-enantiomer of I were collected according to predetermined collection times. These times were established daily by injecting I and determining the time interval for the elution of *S*(−)- and *R*(+)-enantiomers of I. A typical collection time for the *S*(−)-enantiomer was between 13.5 and 19.5 min, and for the *R*(+)-enantiomer between 19.5 and 28.5 min. Fraction collection was performed on a 24 hours-a-day basis using a fraction collector allowing unattended separation of the isomers from new batches of plasma samples while previously separated isomers were analyzed using the GC–MS system. The fractions were evaporated to dryness at 35°C, the residue was dissolved in 20 μl of mobile phase and 2 μl of the solution was injected onto the GC–MSD system.

2.5. Precision, accuracy, recovery, and specificity

The precision of the method was determined by replicate analyses ($n = 5$) of human plasma containing I and II at all concentrations utilized for constructing calibration curves. The linearity of each standard line was confirmed by plotting the peak area of drug, its pyridine metabolite, or its enantiomers vs. concentration. Unknown sample concentrations were calculated from the equation $y = mx + b$, as determined by the weighted ($1/y^2$) linear regression of the standard line. The accuracy of the assay was expressed by: [(mean observed concentration)/(spiked concentration)] · 100.

Assay selectivity was assessed by running blank control and patient pre-dose plasma samples. No endogenous interferences were observed. The recovery was determined by comparing the peak area of I and II extracted from human plasma to that of standards injected directly.

3. Results and discussion

3.1. Mass spectra and SIM chromatograms

The electron-ionization mass spectra of I, II, III (internal standard in a non-stereoselective assay), and IV (internal standard in a stereoselective assay), revealed major fragmentation ions at *m/z* 238, 346, 266 and 243, respectively (Fig. 1). The base peaks in the mass spectra of I, III, and IV at *m/z* 238, 266, and 243 were formed by elimination of the 2,3-dichlorophenyl moiety, whereas the base peak at *m/z* 346 in the mass spectrum of II was formed by elimination of one chlorine atom. In the case of III, in addition to the major fragment at *m/z* 266, an intense fragment at *m/z* 224 was observed. When an attempt was made to monitor III at *m/z* 266, an interference from the plasma extract at the retention time of the analyte was detected. This interference was not present when compound III was monitored at *m/z* 224. Therefore, quantitation of III was performed at *m/z* 224 instead of at *m/z* 266. The major fragmentation ions for I, II, and IV at *m/z* 238, 346, and 243 were used for selected-ion monitoring of these compounds.

Representative SIM chromatograms of racemic felodipine and its pyridine metabolite obtained in a non-stereoselective assay are presented in Fig. 2. Similar chromatograms for *S*(−)-felodipine after extraction from plasma, HPLC separation on a chiral column and GC–MS-EI-SIM analyses are presented in Fig. 3. Chiral HPLC separation of felodipine enantiomers is illustrated in Fig. 4.

3.2. Non-stereoselective assay of I and II

The assay for racemic felodipine and its pyridine metabolite was validated in human plasma in the concentration range 0.5–10 ng/ml for both I and II. Typical equations for the calibration lines for I and II were $y = 4.12x + 0.0000574$ and $y = 0.435x - 0.000453$, with correlation coefficients of 1.000 in both cases. The crux of the non-stereoselective assay was pre-

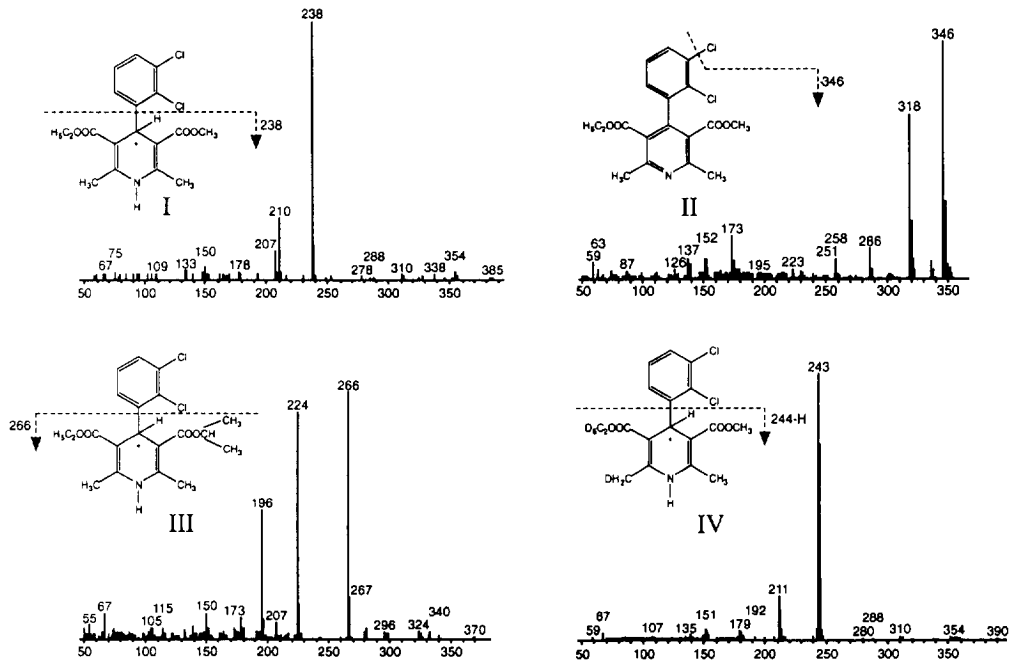

Fig. 1. Chemical structures and electron-ionization mass spectra of felodipine (I), pyridine metabolite (II), isopropyl ester analog of I (III), and d_6-felodipine (IV); (*) denotes the chiral center.

treatment of plasma extract with MSTFA to eliminate an interfering peak coeluting with I. This pretreatment allowed reliable quantitation at 0.5 ng/ml for both I and II. The assay precision expressed as the coefficient of variation (C.V., %) was less than 10% at all concentrations used for constructing the standard curve (Table 1). The accuracy for both I and II in human plasma at all concentrations within the standard curve range was 87–109%. The recovery of I and II from plasma was greater than 90% at all concentrations studied.

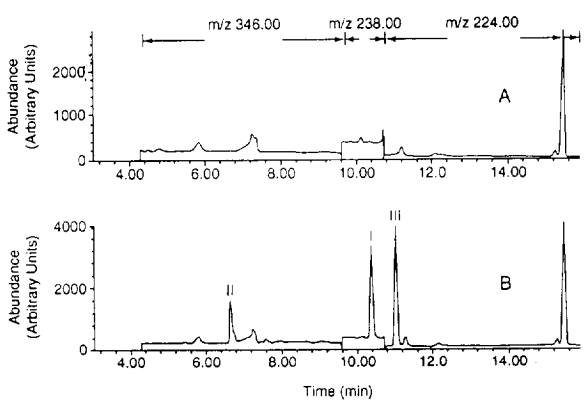

Fig. 2. Representative GC–MS-EI-SIM chromatograms obtained during non-stereoselective assay of felodipine (I), its pyridine metabolite (II) and internal standard (III) in human plasma. (A) Blank control plasma; (B) control plasma spiked with 0.5 ng/ml of I, 2.5 ng/ml of II, and 10 ng/ml of III.

3.3. Stereoselective assay of felodipine enantiomers

The enantiomers of felodipine were baseline-separated on a Chiralcel OJ column (Fig. 4), and the separation was reproducible over a study period. For example, the retention times of both enantiomers measured during a period of one week were 14.6 ± 0.04 min ($n = 27$) and 20.9 ± 0.13 min ($n = 27$), respectively. The assay was validated in human plasma in the concentration range 0.1–5.0 ng/ml for both felodipine enantiomers. Standard curves were prepared daily with study samples. The typical equations for the calibration line were $y = 0.678x - 0.0101$ and $y = 0.672x - 0.00574$, with

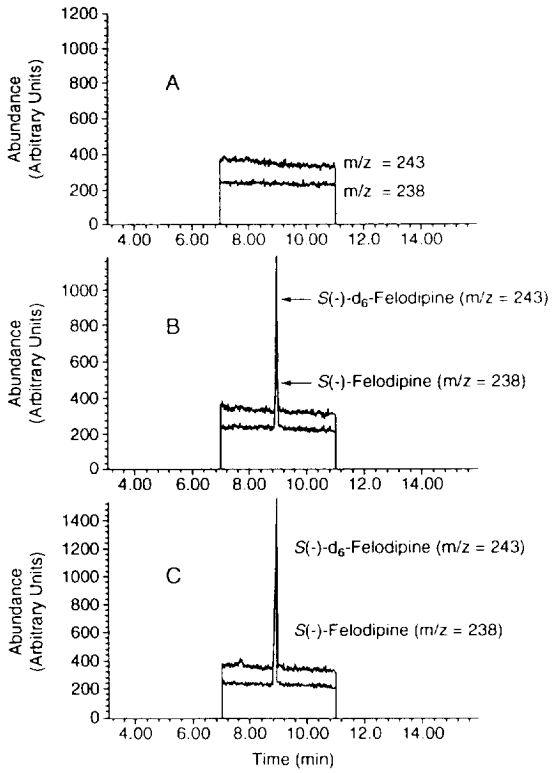

Fig. 3. Representative chromatograms of the *S*(−)-enantiomers of felodipine and d_6-felodipine in human plasma by GC–MS-EI-SIM (similar chromatograms were observed for *R*(+)-enantiomers of I and IV). (A) Blank control plasma; (B) control plasma spiked with 0.25 ng/ml of *S*(−)-enantiomer of I; (C) control plasma spiked with 2 ng/ml of *S*(−)-enantiomer of IV.

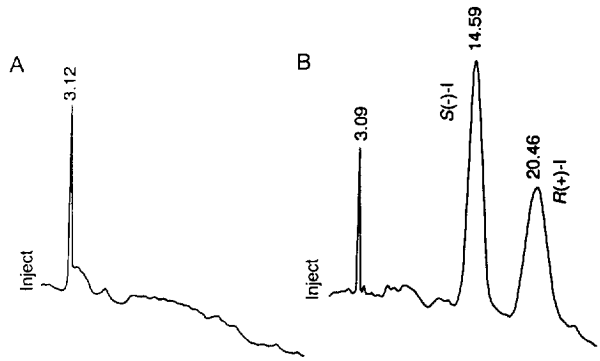

Fig. 4. Chiral separation of the enantiomers of felodipine after extraction from human plasma on a Chiracel OJ column (250 × 4.6 mm I.D., 5 μm). (A) Blank control plasma; (B) control plasma (1 ml) spiked with 100 ng of the racemic mixture of I; all solution (100 μl) after reconstitution of the evaporated extract to dryness was injected onto column.

correlation coefficients of 0.999 for both enantiomers. Intra-day precision data at all concentration within the standard curve range for both enantiomers of I and for the quality control standards are presented in Table 2. The stability was assessed by the analysis of QC standards. This analysis (Table 3) indicated that both enantiomers of I were stable in plasma at −20°C for at least 56 days. Overall recovery of the enantiomers of I including efficiency of extraction from plasma and chiral chromatography was 65 ± 5% and was constant over the whole concentration range studied permitting reliable quantitation of both enantiomers of I.

The five-fold increase in the LOQ from 0.5 ng/ml in the non-stereoselective assay to 0.1 ng/ml in the stereoselective assay was probably due to an efficient additional clean-up of the plasma extract achieved during chiral chromatography and removal of minor plasma interferences leading to the lowering of the GC background noise.

3.4. Analyses of samples from clinical studies

More than 500 human plasma samples from a clinical study with I were analyzed using the stereoselective assay described above. As an example, representative concentrations of both enantiomers of felodipine in human plasma after oral administration of I in selected human subjects obtained using this assay are presented in Table 4.

A significant difference between the concentration of *R*- and *S*-enantiomers of I was observed in post-dose plasma samples. The concentration of the *S*-isomer was about three times higher than that of the *R*-isomer, indicating that the disposition of I was stereoselective. In addition, a significant inter-subject variability in the total concentration of I was ob-

Table 1
Intra-day accuracy and precision data for the non-stereoselective assay of I and II in human plasma

Spiked Concentration (ng/ml)	I			II		
	Mean found concentration (ng/ml)[a]	Accuracy[b]	C.V.[c] (%)	Mean found concentration (ng/ml)[a]	Accuracy[b]	C.V.[c] (%)
0.50	0.50 ± 0.02	100	3.5	0.47 ± 0.04	94	5.4
1.00	0.99 ± 0.03	99	3.2	0.87 ± 0.09	87	9.8
2.50	2.50 ± 0.05	100	1.9	2.46 ± 0.17	98	6.6
5.00	5.02 ± 0.09	100	1.8	5.43 ± 0.60	109	10.0
10.00	9.99 ± 0.47	100	4.7	9.85 ± 0.92	98	9.3

[a] $n = 5$.
[b] Expressed as [(mean calculated concentration)/(spiked concentration)] · 100.
[c] Expressed as coefficient of variation (C.V., %); $n = 5$.

served. Detailed pharmacokinetic data for all subjects in the clinical study, obtained using the stereoselective assay, will be reported in a separate paper.

4. Conclusions

A sensitive (0.1 ng/ml), selective and efficient method for the determination of *S*(−)-

Table 2
Intra-day accuracy and precision data for the stereoselective assay of the *S*(−)- and *R*(+)-enantiomers of felodipine in human plasma

Spiked concentration (ng/ml)		Measured concentration (ng/ml)					Accuracy[a]	C.V. (%)[b]
		1	2	3	4	5		
0.10	*S*(−)	0.099	0.099	0.099	0.098	0.104	100	2.3
	R(+)	0.101	0.097	0.102	0.100	0.102	100	2.1
0.25	*S*(−)	0.26	0.26	0.26	0.26	0.22	100	7.2
	R(+)	0.23	0.27	0.24	0.25	0.24	100	6.1
0.50	*S*(−)	0.49	0.49	0.51	0.50	0.50	100	1.7
	R(+)	0.52	0.48	0.47	0.52	0.49	100	4.6
1.00	*S*(−)	1.02	0.97	0.97	1.00	1.02	100	2.5
	R(+)	1.07	0.97	0.96	1.01	0.99	101	4.0
2.50	*S*(−)	2.59	2.51	2.45	2.50	2.54	101	2.1
	R(+)	2.47	2.51	2.56	2.45	2.51	100	1.7
5.00	*S*(−)	4.70	5.06	5.10	4.86	5.15	99	3.8
	R(+)	4.74	5.04	5.15	5.07	5.10	101	3.2

[a] Expressed as [(mean calculated concentration)/(spiked concentration)] · 100 ($n = 5$).
[b] $n = 5$.

Table 3
Inter-day variability for the stereoselective assay of quality control plasma samples spiked with $S(-)$ and $R(+)$-enantiomers of felodipine

Nominal concentration (ng/ml)	Isomer	Initial mean concentration[a] (ng/ml)	Initial intra-day C.V.[a] (%)	Inter-day mean concentration[b] (ng/ml)	Inter-day C.V.[b] (%)
0.4	$S(-)$	0.44	2.3	0.44	5.2
	$R(+)$	0.42	3.2	0.43	2.5
4.0	$S(-)$	4.16	4.7	4.25	4.4
	$R(+)$	4.10	5.0	4.35	2.8

[a] $n = 5$.
[b] Mean of 12 analyses perfomed over a period of 56 days.

and $R(+)$-enantiomers of I in human plasma, based on GC–MSD-SIM, has been developed. This method has been fully validated in the concentration range 0.1–5.0 ng/ml, and allowed analyses of more than 30 plasma samples per day. The method was rugged, reliable and was used routinely for analysis of plasma samples from human subjects dosed with I. A similar but simplified method, i.e. with a minor modification in the sample preparation procedure, was also developed for the non-stereoselective analysis of I and II in human plasma. The non-stereoselective assay was validated in the concentration range 0.5–10.0 ng/ml, with a limit of quantitation of 0.5 ng/ml for both analytes.

Table 4
Concentration of $S(-)$- and $R(+)$-enantiomers of I in plasma of selected human subjects following the administration of 5-mg felodipine tablets once-a-day for seven days

Time (day, h)	Concentration of the $S(-)$-enantiomer of I (ng/ml)		Concentration of the $R(+)$-enantiomer of I (ng/ml)	
	Subject 1	Subject 2	Subject 1	Subject 2
1, 0	0.00	0.00	0.00	0.00
6, 0	3.13	0.45	1.17	0.23
7, 0	3.31	0.32	1.23	0.14
7, 0.5	2.45	0.41	0.91	0.20
7, 1	3.84	0.50	1.56	0.21
7, 1.5	4.43	0.53	1.93	0.21
7, 2	5.55	0.62	2.49	0.23
7, 3	6.47	0.65	2.73	0.26
7, 4	5.78	0.77	2.42	0.28
7, 5	5.71	0.57	2.27	0.21
7, 6	4.01	0.46	1.48	0.21
7, 7	3.68	0.45	1.35	0.18
7, 8	3.51	0.46	1.24	0.20
7, 10	3.03	0.50	1.03	0.24
7, 12	3.14	0.59	1.06	0.30
7, 15	2.68	0.40	0.94	0.19
7, 24	1.94	0.29	0.56	0.15

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