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Enantioselective assay of nisoldipine in human plasma by chiral high-performance liquid chromatography combined with gas chromatographic−mass spectrometry: applications to pharmacokinetics

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Abstract

Nisoldipine, a second-generation dihydropyridine calcium antagonist, is a racemate compound used in the treatment of hypertension and coronary heart disease. This study presents an enantioselective HPLC-GC–MS method for the analysis of nisoldipine in human plasma and establishes confidence limits for its application to pharmacokinetic studies. Plasma samples were basified and extracted with toluene. The enantiomers were resolved on a Chiralcel® OD-H column using hexane–ethanol (97.5:2.5, v/v) and the (+)- and (−)-fractions were collected separately with the diode array detector switched off. For the quantification of the nisoldipine enantiomers a GC–MS with an Ultra 1 Hewlett-Packard column was used with the detector operated in the single-ion monitoring mode with electron-impact ionization (*m/z* 371.35 and 270.20 for nisoldipine and *m/z* 360.00 for the internal standard, nitrendipine). The method proved to be suitable for pharmacokinetic studies based on the low quantification limit (0.05 ng/ml for each enantiomer) and the broad linear range (0.05–50.0 ng/ml for each enantiomer). Low coefficients of variation (<15%) were demonstrated for both within-day and between-day assays. No interference from drugs associated with nisoldipine treatment was observed. The enantioselective pilot study on the kinetic disposition of nisoldipine administered in the racemic form to a hypertensive patient using a multiple dose regimen revealed the accumulation of the (+)-enantiomer with an AUC^{0-24} (+)/(−) ratio of approximately 8. Both enantiomers were quantified in plasma at a time interval of 24 h. This HPLC-GC–MS method is reliable, selective and sensitive enough to be used in clinical pharmacokinetic studies on the enantioselective disposition of nisoldipine in humans.

Keywords: Enantiomer separation; Nisoldipine

1. Introduction

Nisoldipine [(±)-3-isobutyl-5-methyl-1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)pyridine-3,5-dicarboxylate (Fig. 1)], a calcium antagonist of the 1,4-

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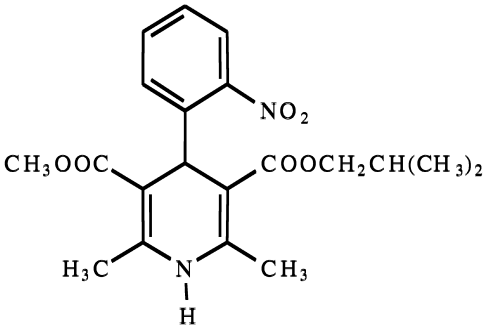

Fig. 1. Structure of nisoldipine.

dihydropyridine type, reduces vascular resistance and blood pressure by inhibiting calcium uptake by the myocardium and smooth muscle cells. Nisoldipine is employed in clinical practice as an antiangina drug and vasodilator [1–5].

Nisoldipine is a chiral drug due to its asymmetric ester function in the 4-position of the 1,4-dihydropyridine system [6]. The antihypertensive activity of the (+)-enantiomer in rats is approximately 20 times higher than that of the (−)-antipode. The (+)/(−) ratios of plasma concentration range from five times in elderly hypertensive patients (multiple dose) to 13 times in healthy volunteers (single dose) orally treated with racemic nisoldipine [7]. In healthy volunteers the bioavailability of the (+)-enantiomer is 6.3 times higher than that of the (−)-enantiomer, probably due to the enantioselective differences in intrinsic clearance [6]. Intravascular administration of racemic nisoldipine does not result in relevant differences in the plasma concentrations of the enantiomers [7].

Nisoldipine (solution) is rapidly (t_{max}=25–46 min) and almost completely (87%) absorbed by the gastrointestinal tract, whereas its oral bioavailability is low (3.9–8.4%) due to high presystemic elimination. Nisoldipine is highly bound to plasma proteins with a free fraction of 0.27. The distribution volume is high, ranging from 1.6 to 7.1 l/kg [8]. The oxidation of 1,4-dihydropyridine to pyridine, catalyzed by CYP3A4, represents the main metabolic pathway [8,9]. A systemic clearance of 0.83–1.17 l/min characterizes nisoldipine as a drug with a high hepatic excretion rate, with its clearance mainly depending on liver blood flow [8]. Nisoldipine shows linear pharmacokinetics in hypertensive patients treated with doses of 30–90 mg [1,9].

The high pharmacological activity of nisoldipine associated with low therapeutic doses, low bioavailability, high distribution volume and chirality leads to low plasma concentrations, thus requiring analytical methods of high sensitivity working at the limits of the equipment.

The exposure of nisoldipine to light results in photodecomposition. The chemical alterations of the irradiated molecule include oxidation of the dihydropyridine ring and reduction of the nitro to the nitroso group. Depending on the irradiation source, two different photodegradation products can be obtained: nitrophenylpyridine resulting from ultraviolet light and nitrosophenylpyridine resulting from exposure to sunlight. The photodecomposition half-life of nisoldipine in plasma is 11 min [8,10,11]. As a consequence, sample preparation and instrumental analysis require an environment with protection from natural light using yellow light as the only illumination source [7,13].

Studies on the kinetic disposition and metabolism of nisoldipine present limitations with respect to the administration of the individual enantiomers to healthy volunteers or patients. The kinetic disposition may differ in situations of administration of the individual enantiomers and racemates due to possible enantiomer–enantiomer interactions in pharmacokinetics and/or pharmacodynamics. Therefore, the racemates are administered in combination with techniques for the separation of the enantiomers or chiral drugs with one of the enantiomers being labeled with a stable isotope (pseudo-racemate) are used in combination with the GC–MS technique [12].

In 1990, Frost et al. [6] were pioneers in enantioselective studies on the pharmacokinetics of nisoldipine administered orally or intravascularly to healthy volunteers (N=5) in the form of the pseudo-racemate containing four times the (+)-enantiomer labeled with ^{13}C in relation to the unlabeled (−)-enantiomer. The authors determined the enantiomeric ratios of plasma concentration by GC–MS but provided no other details about the analytical procedure. A mass detector permits the differentiation between the masses of the labeled isotope and of the unlabeled drug. This method was also used for the

stereospecific determination of other 1,4-dihydropyridines in plasma and serum such as nitrendipine and felodipine.

Only Heinig et al. [7] described the separation and quantification of nisoldipine enantiomers in human plasma applied to enantioselective studies on the kinetic disposition of nisoldipine administered in the form of the racemate. The (+)- and (−)-enantiomers were separated by HPLC on a chiral Chiralcel® OJ column and 1,4-dihydropyridine was quantified in the HPLC fractions by GC–MS. The authors reported a quantification limit of 0.1 ng for each enantiomer/ml plasma, and were able to quantify the (+)-enantiomer up to 48 h and the antipode up to 24 h after the administration of a single dose (40 mg) of the racemate to healthy volunteers. The analytical difficulties are mainly restricted to the (−)-enantiomer present in human plasma at concentrations approximately ten times lower than the (+)-antipode. Zimmer and Muschalek [13] performed an enantioselective analysis of nisoldipine in the plasma of rats, dogs and mice using HPLC-GC–MS. However, the limit of quantification of the method of 0.5 ng/ml is not compatible with its application to clinical studies.

The objective of the present study was to develop and validate a method for the separation and quantification of nisoldipine enantiomers in human plasma with application to studies on kinetic disposition and metabolism.

2. Material and methods

2.1. Chemicals and reagents

The stock solutions of racemic nisoldipine (BAY K 5552; lot R-146-2, 99.4%; Bayer AG, Wuppertal, Germany) was prepared in toluene (residual-grade; Mallinckrodt, KY, USA) at a concentration of 40 μg/ml. From this solution dilutions at concentrations of 8000, 4000, 1600, 800, 320, 160, 64, 32, 16 and 8 ng racemic nisoldipine/ml toluene were prepared.

Nitrendipine, employed as the internal standard, was prepared in toluene at a concentration of 1 μg/ml.

An aqueous sodium hydroxide solution (analytical-grade; Mallinckrodt) was prepared at a concentration of 12.5 *M*. Toluene (residual-grade) used for the sample extraction procedures and for injection into the GC–MS system was obtained from Mallinckrodt; hexane and ethanol (chromatography-grade), used as components of the mobile phase of the HPLC system, were purchased from Merck (Darmstadt, Germany).

All analytical procedures were carried out under yellow light since nisoldipine is photosensitive.

2.2. Sample preparation

Aliquots (2 ml) of plasma were added to a 200-μl solution of 12.5 *M* NaOH and 8 ml of toluene. After shaking for 1 h (horizontal shaker, 220±10 cycles/min) and centrifugation for 10 min (1800 *g*), the organic phases (6.5 ml) were transferred to conic tubes and evaporated to dryness under an air flow at room temperature, and then the residues were reconstituted in 75 μl of the mobile phase and used for HPLC.

2.3. Separation of the enantiomers by high-performance liquid chromatography

The nisoldipine enantiomers were separated by high-performance liquid chromatography (HPLC) on a Shimadzu liquid chromatograph (Kyoto, Japan) consisting of an LC-10AD VP pump, an FRC-10A fraction collector and an SPD-M10AVP diode array detector operating at a wavelength of 230 nm for establishing the retention time and, in the switch-off mode, for sample analysis. The system was controlled by the Class VP program, version 5.03. The Rheodyne model 7125 (Cotati, CA, USA) manual injection system equipped with a 50-μl sampler was used.

The nisoldipine enantiomers were separated on a 250×4.6 mm Chiralcel® OD-H chiral phase column (cellulose Tris–3,5-dimethylphenylcarbamate containing 5-μm silica gel particles) (Diacel Chemical Industries, Los Angeles, CA, USA) equipped with a 4×4 mm CN Lichrospher® 100 precolumn (5-μm particles) (Merck), using a *n*-hexane–ethanol mixture (97.5:2.5 v/v) as the mobile phase at a flow-rate of 1.0 ml/min. The retention times were established by injecting a standard solution of 1.6 μg of each enantiomer/ml toluene (25 μl of the solution were

evaporated until dryness, resuspended in 75 μl of the mobile phase and 50 μl were injected into the HPLC system).

The reconstituted plasma extracts resuspended in 75 μl of the mobile phase were injected into the HPLC system (50-μl sampler) with the detector switched off and the respective fractions of each nisoldipine enantiomer were collected at 17.4–19.3-min (fraction 1) and 19.4–21.4-min intervals (fraction 2). After evaporation of the organic solvent at room temperature, each fraction was resuspended in 50 μl of toluene and 25 μl of the internal standard solution were added. The organic solvent was again evaporated to dryness, reconstituted in 20 μl of toluene and 2-μl volumes were injected into the GC–MS system.

2.4. Quantification by gas chromatography–mass spectrometry of the enantiomers isolated

The isolated enantiomers of nisoldipine were quantified with a gas chromatograph coupled to a model GC–MS QP5000 Shimadzu mass spectrometer with the injector operating at 280°C in the splitless mode. The isolated enantiomers were separated from the internal standard and endogenous plasma components on an Ultra 1 capillary column (0.33-μm particles, 12.5×0.2 mm I.D.) (Hewlett-Packard, Wilmington, DE, USA) with ultrapure helium at a flow-rate of 1.9 ml/min used as the mobile phase with the following temperature program: initial temperature of 100°C for 1 min, increased to 280°C in 35°C steps remaining at the temperature for 3 min for a total of 9 min. The detector operated at 300°C in the single-ion monitoring (SIM) mode with electron-impact ionization. The m/z signals used for the quantification of nisoldipine were 270.20 and 371.35 and 360.00 for the internal standard, nitrendipine.

2.5. Calibration curves

For the calibration curves, 2-ml aliquots of blank plasma obtained from volunteers not receiving medication for at least 10 days were enriched with 25 μl of each standard nisoldipine solution (50.0, 25.0, 10.0, 5.0, 2.0, 1.0, 0.4, 0.2, 0.1 and 0.05 ng of each enantiomer/ml plasma). The plasma samples were extracted and the enantiomers were separated by HPLC and quantified by GC–MS. Calibration curves were constructed for each enantiomer by plotting the peak area ratio (standard/internal standard) as a function of plasma concentration and calculating the linear regression equations and coefficients.

2.6. Method validation

Recovery of racemic nisoldipine was initially calculated by comparing the responses of the samples submitted or not to the extraction procedure with toluene. Blank plasma samples were enriched with 0.8, 4.0 and 10.0 ng racemic nisoldipine/ml plasma and analyzed in triplicate. Recovery of the nisoldipine enantiomers was determined by injection of standard solutions of racemic nisoldipine into the HPLC system, collection of the isolated fractions of each enantiomer, addition of the internal standard, nitrendipine, and injection of the isolated fractions into the GC–MS system. These responses were compared with those obtained with the analysis, in triplicate, of blank plasma samples enriched with 0.4, 2.0 and 5.0 ng of each nisoldipine enantiomer/ml. The plasma samples were submitted to the extraction procedure, the enantiomers were separated by HPLC, and the isolated fractions were injected into the GC–MS system after the addition of the internal standard, nitrendipine.

Linearity was determined by analyzing plasma samples spiked with racemic nisoldipine at ten concentrations levels ranging from 0.05 to 50 ng of each nisoldipine enantiomer/ml plasma.

The quantification limit was determined as the lowest concentration for each enantiomer analyzed, reproducible with a precision of 20% and an accuracy of 80–120%. Samples enriched with concentrations as low as 0.05 ng of each enantiomer/ml plasma were analyzed in quintuplicate.

Precision and accuracy were determined in replicate experiments for the individual enantiomers at concentrations of 0.5 and 5.0 ng/ml plasma.

For selectivity evaluation, plasma samples from ten different volunteers were analyzed in order to determine possible interference from endogenous matrix components. Drugs possibly used in combination with nisoldipine were determined in plasma concentrations resulting from therapeutic doses. Standard drug solutions were prepared in methanol and directly injected into the GC–MS system.

2.7. Clinical study

Patient A.D.P. (male, 45 years, 87 kg, 1.80 m tall) with systemic arterial hypertension was included in the study after giving written informed consent according to the research project approved by the Research Ethics Committee of HCFMRP-USP (process 683/99). The patient received one tablet coated with 20 mg of racemic nisoldipine (Syscor® AP, Astra-Zeneca, São Paulo, Brazil) for 15 days. On the 15th day of treatment the patient was admitted to the Nephrology Division Ward of HCFMRP-USP for serial blood collection. The patient received a daily dose of nisoldipine in the morning after a 12-h fast. Blood samples of approximately 5 ml were collected with heparinized syringes at a dose interval of 24 h at times 0, 0.5, 1, 2, 3, 3.5, 4, 4.5, 5, 6, 8, 10, 12, 16, 20, and 24 h. The blood samples were centrifuged at 1800 *g* for 20 min and plasma was stored at −20°C in the dark.

2.8. Pharmacokinetic evaluation

The enantioselective kinetic disposition of nisoldipine was determined in the steady state during the dose interval of 0–24 h [1,2,4,5]. The maximum plasma concentration (C_{max}) and the time to reach C_{max} (t_{max}) were directly obtained from the experimental data. The other parameters were calculated from the log plasma concentration (c) versus time (t) curves. Half-lives ($t_{1/2}$) and rate constants (K) (absorption and elimination) were determined based on the monocompartment model. The rate constants were calculated using the $0.693/t_{1/2}$ equation. The c versus t area under the curve in the steady state was calculated during the dose interval (AUC_{ss}^{0-24}) by the trapezidal method. This parameter was used for the calculation of the apparent clearance ($C_l/f = dose/f/AUC_{ss}^{0-24}$) and apparent distribution volume ($V_d/f = C_l/f/K_{el}$).

3. Results and discussion

The treatment of man with low doses of dihydropyridine calcium antagonists associated with high presystemic elimination and a high distribution volume results in extremely low plasma concentrations (pg to ng) and, consequently, analytical studies on kinetic disposition and metabolism are difficult. The carbon atom at the 4-position of the dihydropyridine rings, with the exception of nifedipine, shows chirality due to the asymmetry of the ester groups. Most of these compounds are used in clinical practice as racemic mixtures, although the enantiomers have significantly different pharmacological activities [12].

Studies in man on the phenomenon of enantioselectivity in the pharmacokinetics of these dihydropyridines require methods with limit of quantification of the order of 0.1 ng for each enantiomer per ml of plasma. The stereospecific determination of various dihydropyridine calcium antagonists, such as nivaldipine, nitrendipine, felodipine, nimodipine, manidipine and benidipine among others, in plasma or serum at concentrations as low as 0.01–0.4 ng/ml has been reported using the direct separation of the enantiomers by HPLC followed by quantification using GC–MS or GC–ECD. The HPLC-GC–MS combination has been employed by Heinig et al. [7] and Zimmer and Muschalek [13] for the separation and quantification of nisoldipine enantiomers in plasma from humans and experimental animals, respectively.

Heinig et al. [7] used a Chiralcel® OJ chiral phase column [Tris–(4-methylbenzoate)cellulose in silica] for the separation of the nisoldipine enantiomers with a *n*-heptane–isopropanol (88:12) mixture containing 0.2% trifluoroacetic acid as the mobile phase. In the present study, a Chiralcel® OD-H chiral phase column (cellulose Tris–3,5-dimethylcarbamate) was used since the (+)- and (−)-enantiomers could be eluted as symmetric peaks showing complete resolution using a mobile phase without the addition of acids, thus, reducing the risk of racemization (Fig. 2). The amylose Tris–*S*-α-methylbenzylcarbamate (AS), amylose Tris–3,5-dimethylphenylcarbamate (AD) and cellulose Tris–benzoate (OB-H) chiral phase columns did not show good resolution of the nisoldipine enantiomers regardless of the use of different mobile phases supplemented or not with trifluoroacetic acid.

The enantiomers isolated in the HPLC fractions were determined by GC–MS-SIM with a limit of quantification of 0.05 ng of each enantiomer per ml of plasma using a 12.5×0.2-mm Ultra-1 capillary column (methylsiloxane). Other columns tested such as SPB-1, DB-608, DB-1 (dimethylpolysiloxane),

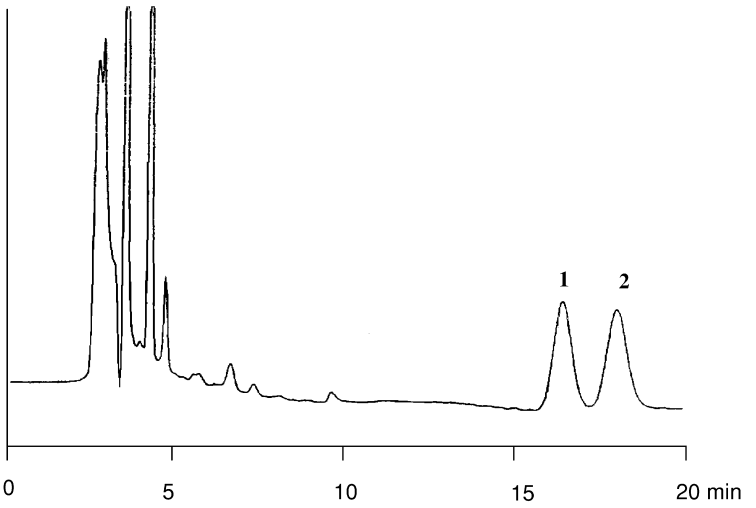

Fig. 2. Separation of the (1) (+)- and (2) (−)-nisoldipine enantiomers (1 μg of rac-nisoldipine/ml solution) by HPLC on a Chiralcel® OD-H column with the mobile phase consisting of an *n*-hexane–ethanol mixture (97.5:2.5 v/v).

Table 1
Quantitative characteristics of the HPLC-GC–MS method

	(+)-Nisoldipine	(−)-Nisoldipine
Recovery (%)	55.6	51.8
Linearity (ng/ml)	0.05–50.0	0.05–50.0
r^2	0.9912	0.9839
y-Intercept	0.04599	0.04487
Slope	0.1067	0.1107
F	1016	548.4
P-value	<0.0001	<0.0001
Deviation from zero	Significant	Significant
Quantification limit (ng/ml)	0.05	0.05
Precision (C.V.%; $N=5$)	14.7	13.8
Accuracy (% Bias; $N=5$)	−0.6	1.4
Within-day precision (C.V.%)		
0.5 ng/ml ($N=10$)	12.0	13.1
5.0 ng/ml ($N=10$)	12.0	13.8
Between-day precision (C.V.%)		
0.5 ng/ml ($N=5$)	11.6	14.1
5.0 ng/ml ($N=5$)	14.1	14.7
Within-day accuracy (% Bias)		
0.5 ng/ml ($N=10$)	5.0	−1.3
5.0 ng/ml ($N=10$)	0.9	8.4
Between-day accuracy (% Bias)		
0.5 ng/ml ($N=5$)	−10.0	−8.8
5.0 ng/ml ($N=5$)	−3.1	−3.1

DB-5 (5%-phenylmethylpolysiloxane), DB-1301 (6%-cyanopropylphenylmethylpolysiloxane) and LM-1 resulted in the elution of the nisoldipine enantiomers as wide and little symmetric peaks with a consequent low sensitivity. The Ultra-1 column has been previously employed by Heinig et al. [7] for the quantification of nisoldipine using the deuterated compound as the internal standards.

The enantiomers of nisoldipine were extracted from plasma in basic medium using toluene as the extracting solvent. The recovery of nisoldipine in the form of an enantiomer mixture or as isolated/separate enantiomers was close to 60% and was independent of the concentrations used, with precision and accuracy being lower than 10% (Table 1). The blank plasma extracts (ten different matrices of human plasma) did not present interfering endogenous compounds in the elution regions of nisoldipine

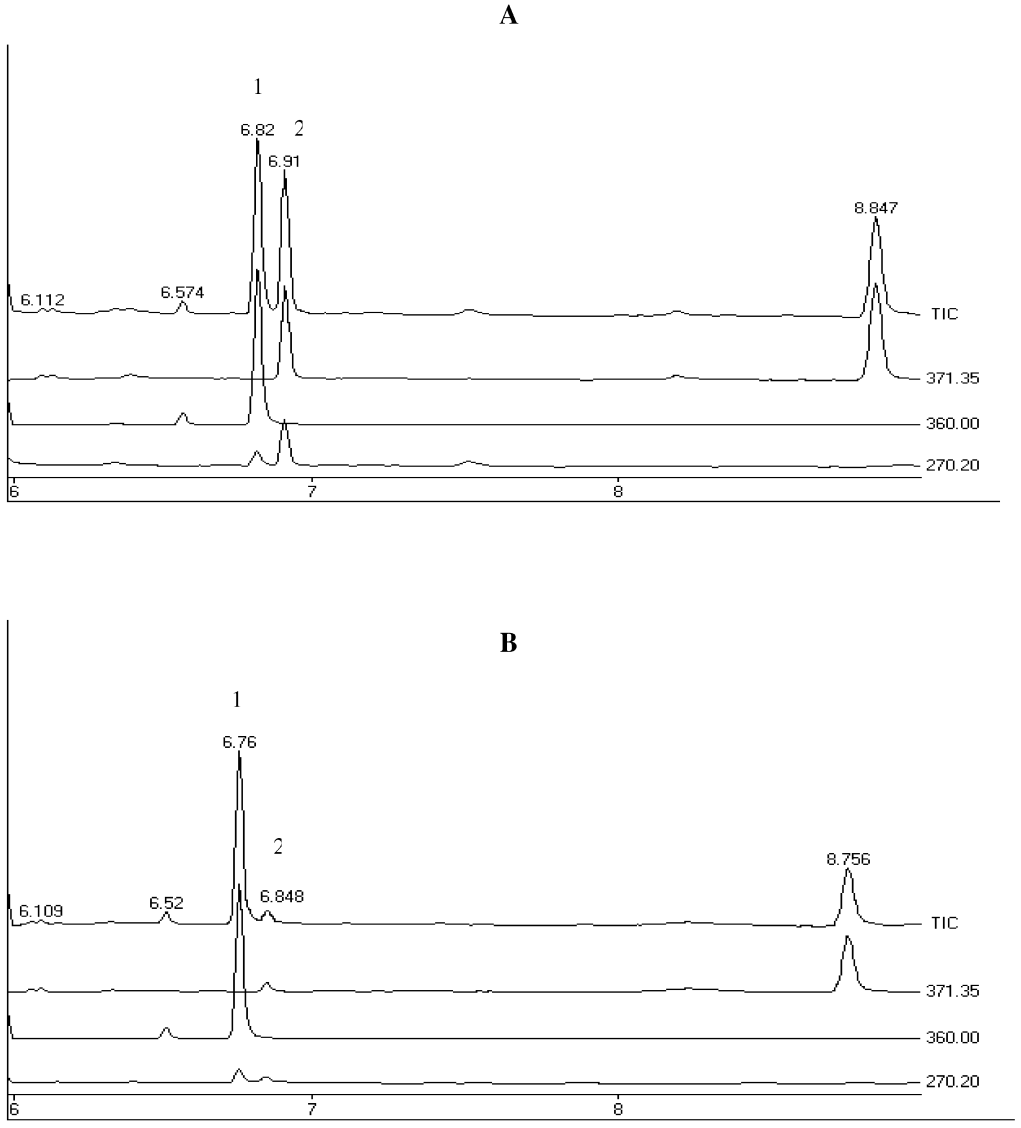

Fig. 3. Chromatograms of plasma from patient A.D.P. (sample collected 12 h after dose administration) using the GC–MS-SIM system: A: (1) IS; (2) (+)-nisoldipine; B: (1) IS; (2) (−)-nisoldipine.

and of the internal standard upon injection of the HPLC fractions into the GC–MS system (Fig. 3). Other extracting solvents tested such as a mixture of pentane and dichloromethane at different proportions, ethyl ether, diisopropyl ether, methyl*tert.*-butyl ether, chloroform and ethyl acetate resulted in a recovery percentage lower than 50% or in chromatograms showing interfering endogenous compounds in the regions of interest.

The enantioselective method was validated in human plasma at concentrations ranging from 0.05 to 50 ng/ml for both nisoldipine enantiomers. All analytical procedures were carried out under yellow light as the only illumination source and using only amber glassware.

The quantification limit of 0.05 ng for each enantiomer/ml plasma permits us to conclude that the method is more sensitive than that reported by Heinig et al. [7]. The high sensitivity is especially important for the quantification of the (−)-enantiomer present in plasma at concentrations about ten times lower than those of the (+)-antipode. The linearity of the method up to 50 ng of each enantiomer/ml plasma included all concentrations tested, reaching values about ten times higher than the maximum plasma concentrations observed in the therapeutic dose range (Table 1).

The coefficients of variation obtained for intra- and inter-assay precision at two different concentrations were below 15%, mainly indicating the reproducibility of the collection of the enantiomer from the HPLC fractions, a phase devoid of an internal standard. The mean concentrations obtained for both nisoldipine enantiomers were within the 15% limit of the real values, ensuring the accuracy of the results (Table 1).

Several drugs that can be used in combination with nisoldipine, such as diuretics, antidepressants, antiepileptics, benzodiazepines and H1 receptor antagonists, did not interfere with the analytical method (Table 2).

The method for determining the (+)- and (−)-enantiomers of nisoldipine in human plasma was applied to an enantioselectivity pilot study on the kinetic disposition of nisoldipine administered in the racemic form to a patient with systemic arterial hypertension employing a multiple dose regimen (Fig. 4 and Table 3). The data show that the enantiomers were eluted from the OD-H chiral phase column in the (+)- and (−)-sequence, as indicated by the higher plasma concentration of the enantiomer present in fraction 1 collected by HPLC. The AUC^{0-24} (+)/(−) ratio for the patient studied was approximately eight. This result is compatible with the values reported by Frost et al. [6] studying healthy volunteers treated intravascularly or orally with nisoldipine in the form of the pseudo-racemate and by Heinig et al. [7] studying healthy volunteers receiving single doses of racemic nisoldipine and elderly hypertensive patients and patients with liver cirrhosis receiving multiple doses of racemic dihydropyridine. It should be noted that these authors only reported the enantioselective plasma concentrations of nisoldipine without making any inferences about the pharmacokinetic parameters.

Table 2
Selectivity of the HPLC-GC–MS method

Drug	Concentration (μg/ml)	Retention time (min)
Nisoldipine	2.000	6.86
Nitrendipine (I.S.)	1.000	6.77
Carbamazepine	12.000	6.46
Clobazam	0.900	6.31
Clonazepam	0.070	7.33
Diazepam	2.000	6.09
Flurazepam	0.030	7.24
Hydrochlorothiazide	0.430	6.57
Lorazepam	0.240	8.10
N-Dealkylflurazepam	0.060	7.21
N-Demethyldiazepam	0.170	6.20
Nitrazepam	0.070	8.21
Phenytoin	20.000	6.30
Phenobarbital	30.000	6.13
Primidone	12.000	6.52

ND = Not detected in 0–30 min interval: amiodarone, amitriptyline, bromazepam, captopril, cimetidine, diclofenac, digoxin, disopyramide, etidocaine, flunitrazepam, furosemide, imipramine, mexiletine, propanolol, ranitidine and sotalol.

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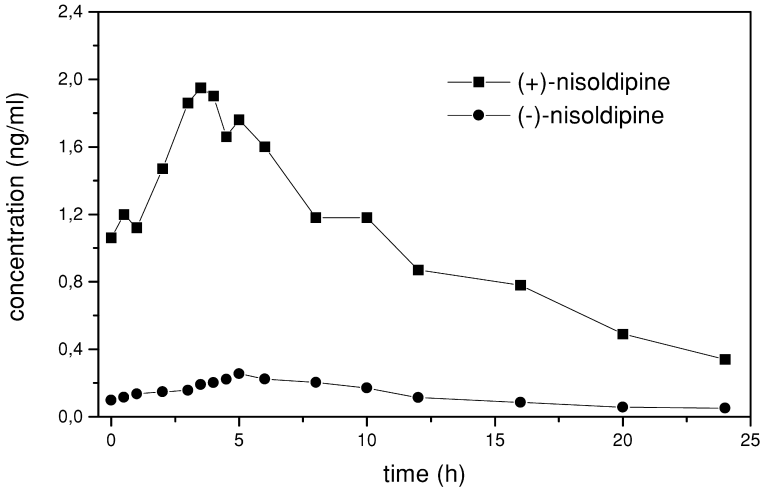

Fig. 4. Plasma concentrations of (+)- and (−)-nisoldipine at a dose interval of 24 h. Patient A.D.P.

Table 3
Enantioselective kinetic disposition of nisoldipine[a]

	(+)-Nisoldipine	(−)-Nisoldipine
C_{max} (ng/ml)	1.95	0.25
t_{max} (h)	3.5	5.0
AUC^{0-24} (ng h/ml)	24.11	3.04
$t_{1/2}a$ (h)	0.9	1.5
k_a (h^{-1})	0.7700	0.4620
$t_{1/2}$ (h)	8.3	8.6
k_{el} (h^{-1})	0.0835	0.0806
C_l/f (l/h/kg)	4.77	37.81
V_d/f (l/kg)	57.1	469.1
$AUC^{0-24}_{(+)/(-)}$	7.93	

[a] Patient A.D.P.

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