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Pharmacokinetic behaviour of *R*-(+)- and *S*-(−)-amlodipine after single enantiomer administration[1]

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Abstract

Amlodipine, 3-ethyl 5-methyl-2-[(2-aminoethoxymethyl]-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylate, is a chiral calcium antagonist, currently on the market and in therapeutic use as a racemate. The pharmacokinetic behaviour of *R*-(+)- and *S*-(−)-amlodipine after single enantiomer administration to healthy male human volunteers together with comparative administration of the racemic mixture of both enantiomers were studied. Plasma levels were studied as a function of time and assayed using an enantioselective chromatographic method (coupled chiral and achiral HPLC) with on-line solid-phase extraction and UV absorbance detection. The method was validated separately for the *R*-(+)- and *S*-(−)-enantiomer, respectively. Results of the study indicate that the pharmacokinetic behaviour of *R*-(+)- and *S*-(−)-amlodipine after single enantiomer administration is comparable to that of each enantiomer after administration of the racemate. No racemization occurs in vivo in human plasma after single enantiomer administration. © 1997 Elsevier Science B.V.

Keywords: Enantiomer separation; Amlodipine

1. Introduction

Amlodipine, 3-ethyl 5-methyl-2-[(2-aminoethoxymethyl] - 4 - (2 - chlorophenyl) - 1,4 - dihydro-6-methyl-3,5-pyridinedicarboxylate, is a potent dihydropyridine calcium channel blocker used in the treatment of hypertension and angina pectoris [1,2]. Like most other calcium channel blockers of this class, amlodipine is therapeutically used as racemate.

The *R*-(+)- and *S*-(−)-enantiomers do not have the same biological activity. Only *S*-(−)-amlodipine posses vasodilating properties [3,4].

Experiments of the racemic drug administered to one human subject, a beagle dog and a rat followed by enantioselective determination of plasma concentrations by gas chromatography with electron capture detection were reported by Scharpf et al. [5].

The separation of amlodipine stereoisomers by HPLC was successfully carried out on an α_1-acid-glycoprotein (AGP) column on the analytical [6,7] as well as on the semipreparative scales [8]. Proper sample preparation and the use of a column-switching technique has led to an additional concentration of single enantiomers on a reversed-phase (RP)

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column, after their separation on a chiral column. This allowed the detection of amlodipine enantiomers at their physiological concentrations in human plasma. Since amlodipine binds strongly to the plasma proteins, biological samples require sample preparation aimed at dissociating these complexes [9]. Amlodipine stereoisomers were also separated by high-performance capillary electrophoresis (CE). In such a separation, α-cyclodextrin and methylcellulose were added to a pH 3.2 buffer [10]. Owing to the relatively low detection sensitivity of CE, this technique cannot be used for the detection of amlodipine stereoisomers in biological samples, at least at present. However, this method is very useful for the control of the purity of amlodipine stereoisomers, which have been separated by some other method (cross-checking).

The study of pharmacokinetics of each optical isomer of amlodipine after single enantiomer administration to healthy human subjects would give answers to the question whether the racemization occurs in vivo or not, and furthermore, whether it is worth developing the enantiomerically pure final dosage form.

Recently, we have succeeded in separating these stereoisomers by semi-preparative HPLC [8], which made possible the in vivo study of single enantiomers administered to healthy human volunteers.

2. Experimental

2.1. Preparative chromatographic separation of R-(+)- and S-(−)-amlodipine

The preparative HPLC separation was described previously [8]. A 150×10 mm I.D. semi-preparative Chiral-AGP column was used to prepare single enantiomers from racemic amlodipine, this separation was followed by HPLC, CE and optical activity determinations. The single dose of *R*-(+)-amlodipine in the form of benzenesulphonate contained 10.4 mg in 20 ml of 20% of ethanol, while that of *S*-(−)-amlodipine in the form of benzenesulphonate contained 7.2 mg in 20 ml of 20% of ethanol. Racemic amlodipine was prepared by dissolving pure standard substance in 20 ml of 20% of ethanol. The single dose contained 16.0 mg of racemic amlodipine in the form of benzenesulphonate. Acute toxicity screening of *R*-(+)-, *S*-(−)- and racemic-amlodipine preparations was done prior to administration.

2.2. Analytical method for the enantioselective determination of R-(+)- and S-(−)-amlodipine in human plasma

2.2.1. Chemicals

All solvents were of HPLC grade, purchased from Merck (Darmstadt, Germany). Sodium hydroxide p.a. was quality from Kemika (Zagreb, Croatia), working standard amlodipine and internal standard chloroamlodipine were prepared in Lek (Ljubljana, Slovenia).

2.2.2. Extraction procedure

To 5.0 ml of human plasma sample 250 ng of internal standard chloroamlodipine, 250 μl of 1 *M* NaOH and 5 ml of chloroform were added. The mixture was shaken for 10 min and centrifuged at 3500 *g* in a Centric 322 PLC centrifuge (Tehtnica, Zelezniki, Slovenia). The lower phase was carefully transferred to another test tube and dried in an Univapo 150H vacuum centrifuge (UniEquip, Martinsried, Germany). The tube was washed twice with 0.5-ml portions of chloroform and dried again. The dry extract was dissolved in 70 μl of solvent (acetic buffer (pH 4.5)–methanol, 4:6, v/v). A 50-μl volume was injected into the HPLC system. The procedure was used for subject, standard and quality control samples.

2.2.3. Standard and quality control preparation

Standards of each enantiomer for the calibration curve were prepared in blank human plasma in the concentration range from 0.2 to 20 ng/ml. Plasma was spiked with adequate quantity of amlodipine racemate (enantiomeric ratio 1:1). Quality control samples were prepared separately for each pure, single enantiomer at two concentrations, 5 and 10 ng/ml, respectively. Five milliliters of blank, heparinised, human plasma was spiked with 25 ng (5 ng/ml) or 50 ng (10 ng/ml) of either *R*-(+)- or *S*-(−)-amlodipine and 250 ng of internal standard chloroamlodipine racemate (Lek) was added and shaken thoroughly.

The extraction procedure was the same as for the plasma samples.

2.2.4. Preparation of validation samples

Quality control samples spiked with single enantiomers for the validation procedure were prepared in blank human plasma at 5 ng/ml and 10 ng/ml of *R*-(+)- or *S*-(−)-amlodipine.

2.2.5. Enantioselective chromatographic determination of R-(+)- and S-(−)-amlodipine

The determination of *R*-(+)- and *S*-(−)-amlodipine was performed by HPLC using coupled columns: chiral separation on the Chiral AGP column and trapping each enantiomer on two separate short C_8 columns by column switching, subsequent elution and achiral separation on a reversed-phase as described by Flykt et al. [6] and Jossefson and Norlander [7]. The instrument modules used were: autosampler BioRad AS 100 HRLC (BioRad, Vienna, Austria), two pumps, LDC Analytical CM 4000 and ConstaMetric 3000, Mistral Spark Holland thermostat (all Thermo Separation Products, Darmstadt, Germany), UV–Vis detector and three switching valves (all Knauer, Berlin, Germany), fast scanning detector UV–Vis Monitor (BioRad). Data acquisition and processing together with switching valves control was done using Millennium 2010 software (Waters, Milford, MA, USA). The coupled system is described schematically in Fig. 1A and the switching scheme is shown in Fig. 1B.

2.2.6. Chiral HPLC separation of R-(+) and S-(−) amlodipine

The mobile phase for the chiral separation of amlodipine enantiomers was a 10 m*M* acetate buffer (pH 4.5) with 1% (v/v) 1-propanol. A 150×4.0 mm I.D. Chiral AGP analytical column (ChromTech, Hægersten, Sweden) was used. The flow-rate was 0.9 ml/min and the injection volume was 50 μl of plasma extract. The separation was carried out at 30°C. The first detector (λ=240 nm) was used only for system suitability testing (SST). At the beginning of each new analytical sequence, the chiral separation part of the system was checked and switching times adjusted. Once the analytical sequence had been running, the detector was switched off.

2.2.7. On-line solid-phase extraction trapping of each enantiomer

Single enantiomers were trapped on a short 20×4.6 mm I.D. Supelcosil LC_8 column of 5-μm particle size (Supelco, Gland, Switzerland). The selective trapping of the optical isomers was done using a column-switching technique.

2.2.8. Achiral reversed-phase HPLC determination of amlodipine

The mobile phase consisted of 10 m*M* acetate buffer (pH 4.5)–acetonitrile (55:45, v/v). The analytical column used in the second, achiral part of the coupled HPLC system was a 150×4.6 mm I.D. Symmetry C_8 of 4-μm particle size (Waters). The column was thermostated at 30°C and the signal monitored at 240 nm on the second UV absorbance detector.

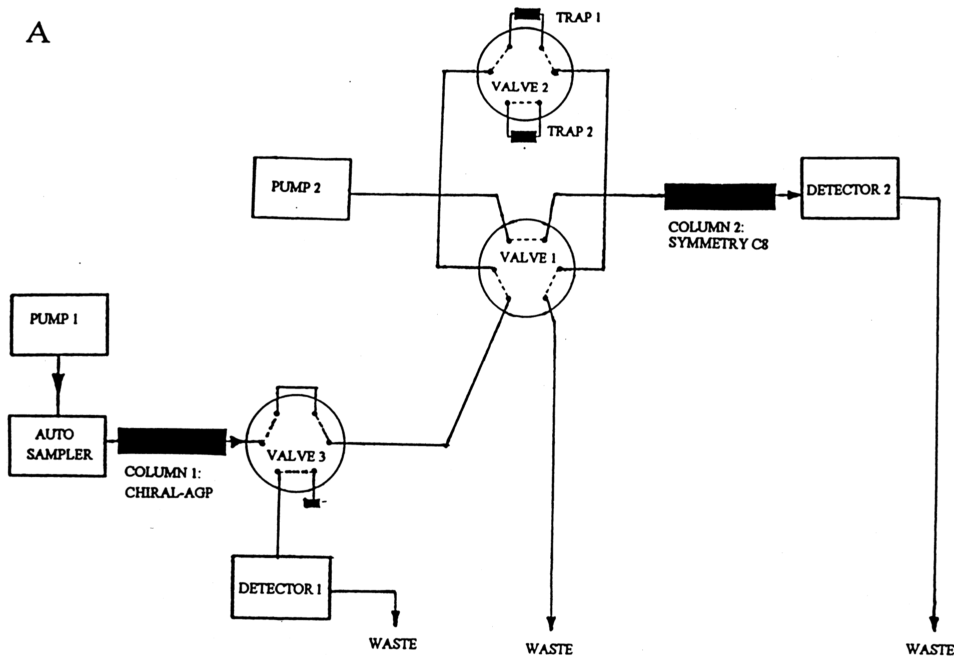
2.3. Single enantiomer administration to healthy human subjects

The clinical study was performed at the Department for Haemodialysis and Nephrology, Internal Clinic, Hospital Sveti duh in Zagreb (Croatia). Ten young healthy male subjects aged between 20 and 45 years were selected according to the protocol. They were orally given amlodipine as a racemate and the *R*-(+)- or *S*-(−)-enantiomer in single oral dose. Drugs were administered with 240 ml of tap water.

Blood sampling was performed according to the following schedule: the samples were taken pre-dose and 1, 2, 3, 4, 6, 8, 10, 12, 24, 48 and 72 h after drug administration. Plasma samples were stored frozen (−20°C) until the analysis.

2.3.1. Pharmacokinetics

Data were calculated by a model-independent method using the TOPFIT 2.0 program (Fischer, 1993, Stuttgart, Germany). Calculation of the terminal eliminations half-life was done by least-squares regression analysis of the terminal part of the concentration–time curves from 8 to 72 h after administration. Areas under concentration curves (AUC) were estimated using the log-linear trapezoidal rule.



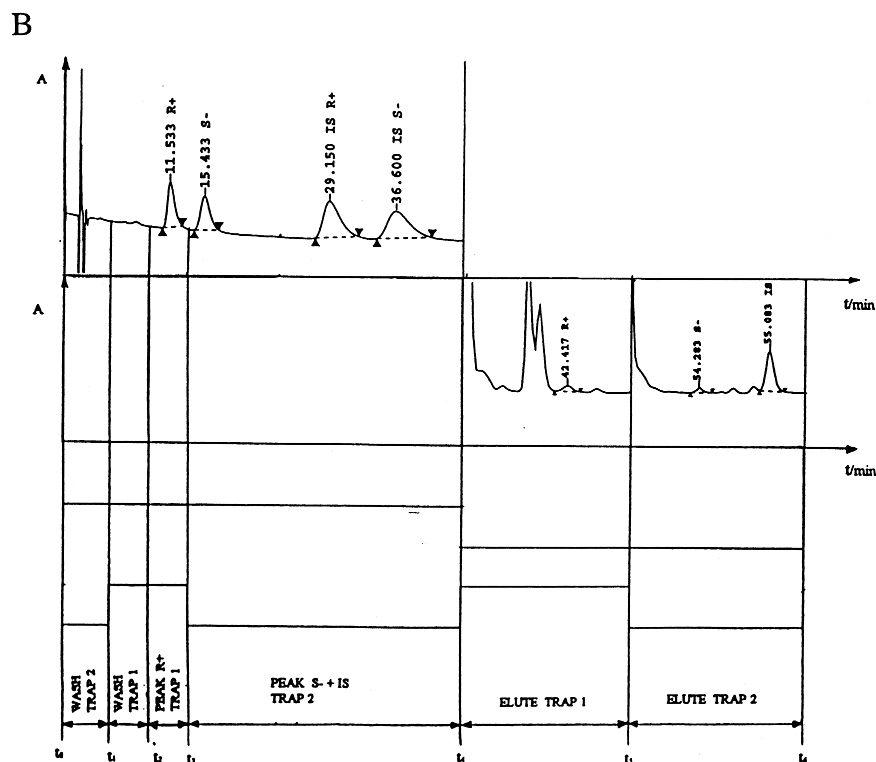


Fig. 1. Schematic of coupling chiral and achiral HPLC system with on line solid-phase extraction using the column switching technique (A) and figure of time events in the coupled system (B).

3. Results and discussion

3.1. Validation of the enantioselective assay method for R-(+)- and S-(−)-amlodipine in human plasma

Validation of the enantioselective assay method for *R*-(+)- and *S*-(−)-amlodipine in human plasma was performed according to the internal standard operating procedures for bioavailability and bioequivalence and the recent publication of Lindner and Wainer [11]. The parameters tested in the validation procedure were: limit of detection and limit of quantitation, linearity and range, precision, accuracy and selectivity of the bioanalytical method. Each parameter was tested for *R*-(+)- and *S*-(−)-amlodipine, respectively.

The limit of detection (LOD) for *R*-(+)- and *S*-(−)-amlodipine was determined at a signal-to-noise ratio of 3:1 and was found to be 0.1 ng/ml. The limit of quantitation (LOQ) for both enantiomers was defined as the concentration which can be determined with the relative standard deviation (R.S.D.) of at least 20% and was found to be 0.2 ng/ml of human plasma (Table 1).

3.1.1. Linearity and range for R-(+)- and S-(−)-amlodipine

The calibration curves established using weighted ($1/x$, x-concentration) linear regression for both *R*-(+)- and *S*-(−)-amlodipine were linear in the range from 0.2 to 20 ng/ml of plasma.

3.1.2. Precision and accuracy for R-(+)- and S-(−)-amlodipine

Precision and accuracy for *R*-(+)- and *S*-(−)-amlodipine were tested at two concentrations of quality control samples (5 and 10 ng/ml) in duplicates on three consecutive days. The precision of the method was expressed as R.S.D. of the measurements. The accuracy was calculated as 100×ratio of measured concentration and known (spiked) concentration. The precision and accuracy results are presented in Table 1.

3.1.3. Selectivity of the bioanalytical method

Six independent human plasma matrices were examined and there was no interference at the retention times of *R*-(+)- or *S*-(−)-amlodipine or the internal standard. Some potentially present exogenous substances, like caffeine, paracetamol and acetylsalicylic acid, were also spiked to human plasma, extracted according to the procedure and chromatographed using the coupled HPLC system. No interferences were observed.

3.2. Pharmacokinetic behaviour of R-(+)- and S-(−)-amlodipine after single enantiomer and racemate administration

All ten subjects regularly terminated the study. No significant clinical and/or laboratory adverse reactions were observed and reported.

The plasma levels of *R*-(+)- and *S*-(−)-amlodipine after single enantiomer and racemate administration were followed until 72 h after application of the drug. The analytical results of these measurements are presented in Figs. 2–4 and in Table 2. Fig. 2 shows the plasma levels in subjects who received pure *R*-(+)-enantiomer. Fig. 3 represents the plasma levels for each enantiomer for the subjects who were administered pure *S*-(−)-am-

Table 1
Precision and accuracy for *R*-(+)- and *S*-(−) amlodipine

Single isomer	*R*-(+)		*S*-(−)	
	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)
0.2 ng/ml (LOQ)	13.7	95.6	15.3	87.4
5 ng/ml	9.3	89.6	10.3	92.8
10 ng/ml	7.9	96.7	4.2	94.5

Table 2
Pharmacokinetic parameters of amlodipine enantiomers after administration of amlodipine racemate, single S-(−) and single R-(+)-enantiomer to healthy subjects[a]

	Racemate		S-(−)		R-(+)	
	S-(−)	R-(+)	S-(−)	R-(+)	R-(+)	S-(−)
C_{max} (ng/mg)	3.41	2.21	3.70		1.43	
			2.95			
t_{max} (h)	6.0	8.0	3.0		6.0	
			8.0			
$t_{1/2el}$ (h)	31.0	24.0	35.0		24.0	
			16.0			
AUC (ng*h/ml)	76.7	67.5	84.3		68.2	
			76.7			
n	4		2		4	

[a] Average values for racemic amlodipine and R-(+)-enantiomer, and individual values of two subjects receiving S-(−)-enantiomer.

lodipine and Fig. 4 shows graphs for R-(+)- and S-(−)-amlodipine for subjects after administration of the racemate.

The maximal plasma concentrations (C_{max}) and the time to reach the maximal concentrations of the drugs (t_{max}) were experimental values. Because of the limited number of subjects, the calculated numerical data are only approximate, but indicative

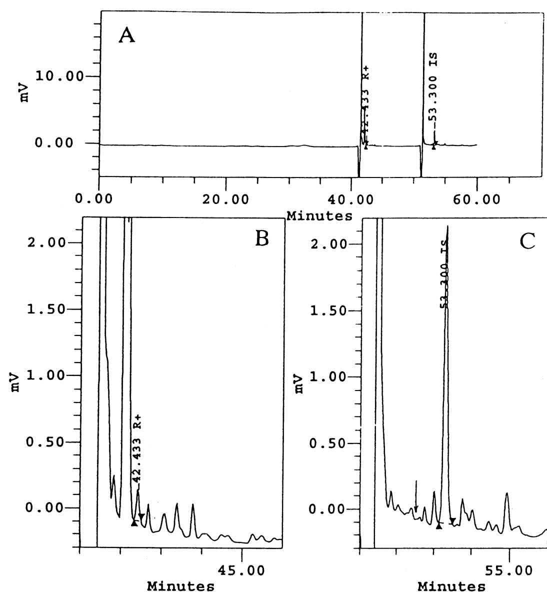


Fig. 2. R-(+)- and S-(−)-amlodipine from the subject who received pure R-(+)-enantiomer (8 h after administration, concentration of R-(+): 1.73 ng/ml); (A) the whole chromatographic run in the coupled system; (B) zoomed insert of a chromatogram when R-(+) is eluted; (C) zoomed insert of a chromatogram when S-(−) would be eluted, if present).

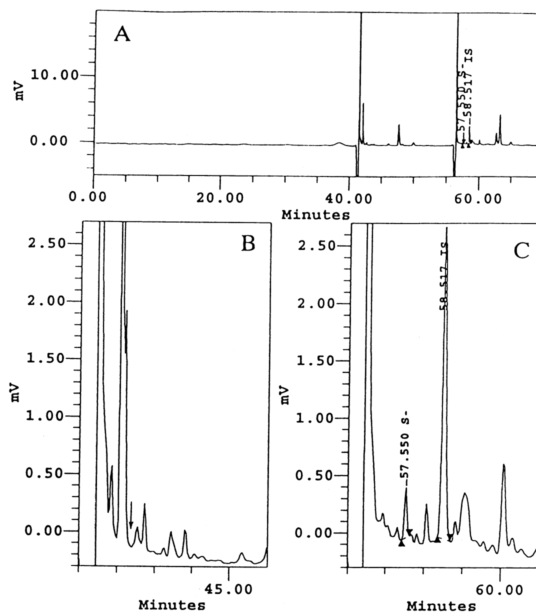


Fig. 3. R-(+)- and S-(−)-amlodipine from the subject who received pure S-(−)-enantiomer (6 h after administration, conc. of S-(−): 2.36 ng/ml); (A) the whole chromatographic run in the coupled system; (B) zoomed insert of a chromatogram when R-(+)- would be eluted, if present; (C) zoomed insert of a chromatogram when S-(−) is eluted).

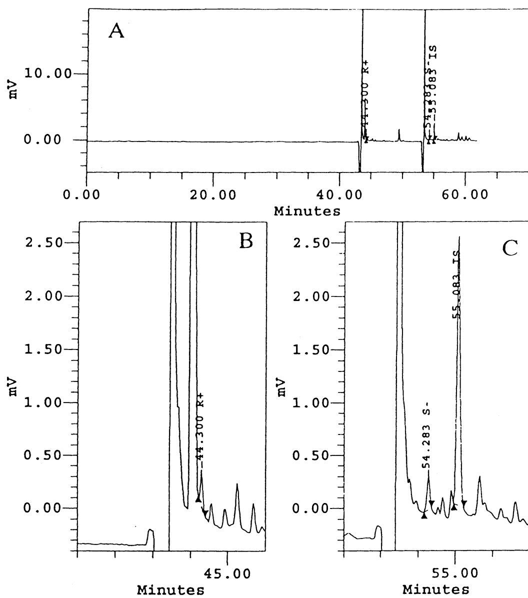


Fig. 4. R-(+)- and S-(−)-amlodipine from the subject who was administered the racemate (7 h after administration, concentration of R-(+): 2.44 ng/ml and S-(−): 2.79 ng/ml); (A) the whole chromatographic run in the coupled system; (B) zoomed insert of a chromatogram when R-(+) is eluted; (C) zoomed insert of a chromatogram when S-(−) is eluted).

enough of the pharmacokinetic behaviour of enantiomers. The mean plasma concentration–time curves after single oral dose of amlodipine racemate and the two enantiomers are shown in Figs. 5–7. The data from ten healthy young subjects indicate that the pharmacokinetic behaviour of enantiomers is comparable and independent of administration of single isomer or racemate, respectively.

The time corresponding to the maximal concentrations of enantiomers (t_{max}) is practically identical for all three groups and is approximately 6 h.

The elimination half-life in subjects who received racemic or one of the enantiomers is practically the same. The elimination half-life after administration of racemic amlodipine is about 31 h for S-(−)-enantiomer and 24 h for R-(+)-enantiomer. After administration of amlodipine R-(+)-enantiomer $t_{1/2}$ is about 34 h. Only two subjects received S-(−)-enantiomer and the corresponding $t_{1/2}$ is between 16 and 35 h. The similarity of the pharmacokinetic behaviour indicates also a non-significant difference in area under the concentration–time curves after administration of the three different forms of the drug.

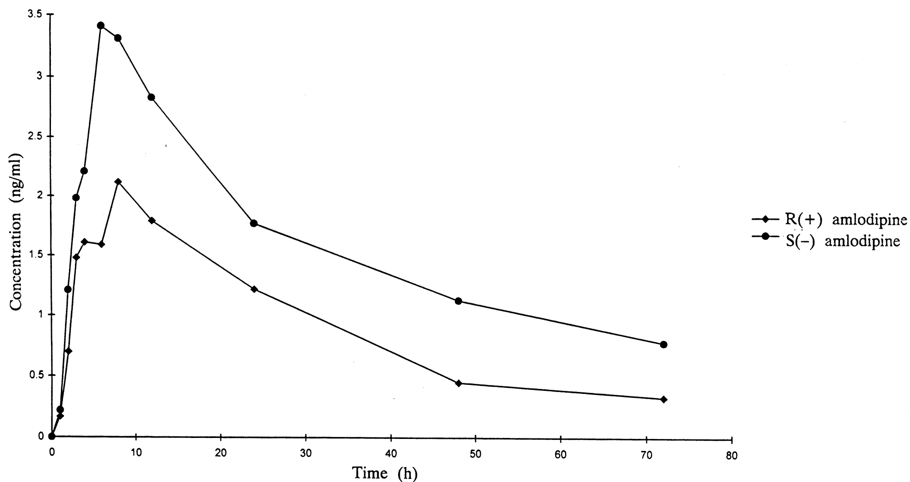


Fig. 5. Mean plasma R-(+) and S-(−)-enantiomer concentration after oral administration of 16.0 mg amlodipine racemate (in the form of benzenesulphonate) from four subjects.

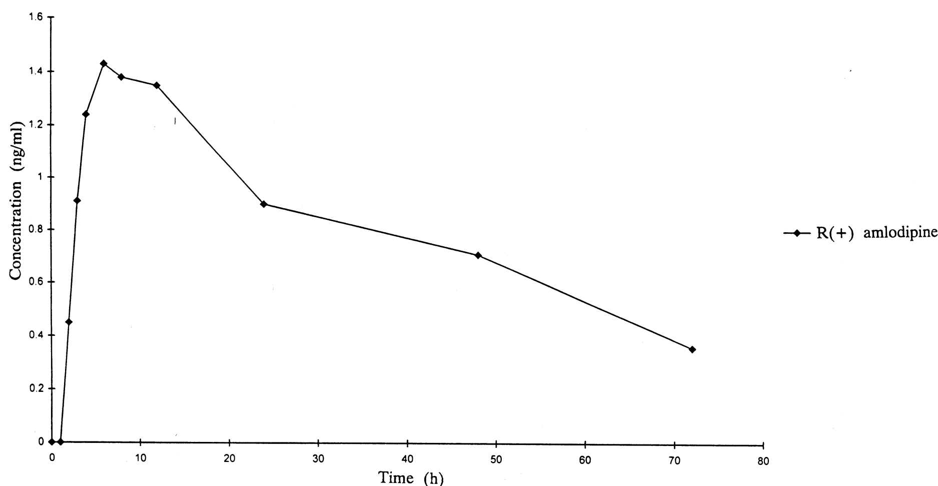


Fig. 6. Mean plasma *R*-(+) enantiomer concentration after oral administration of 10.4 mg amlodipine *R*-(+) enantiomer (in the form of benzenesulphonate) from four subjects.

4. Conclusions

Two healthy subjects received pure biologically active *S*-(−)-amlodipine. In a parallel experiment, four healthy subjects were treated with the pure inactive *R*-(+)-enantiomer. In control experiments, four volunteers received racemic amlodipine. Analytical results were obtained using a validated enantioselective method with improved sample clean-up, which extensively prolongs the life time of the AGP column.

The comparison of the pharmacokinetic behaviour of *R*-(+), *S*-(−) and racemic amlodipine shows no significant differences. Furthermore, our results support the conclusion that there is no in vivo racemization and that it is therefore worth developing the single enantiomer formulation.

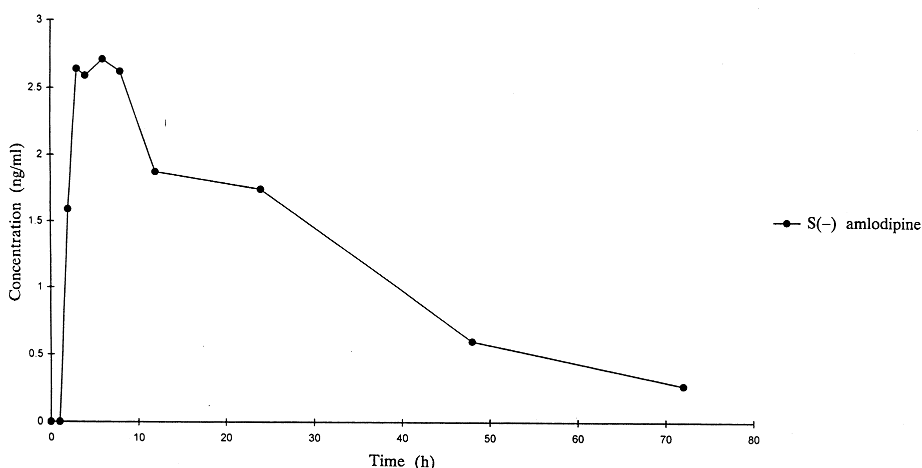


Fig. 7. Mean plasma *S*-(−)-enantiomer concentration after oral administration of 7.2 mg amlodipine *S*-(−)-enantiomer (in the form of benzenesulphonate) from two subjects.

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