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Semi-preparative chromatographic purification of the enantiomers *S*-(−)-amlodipine and *R*-(+)-amlodipine[1]

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Abstract

Pharmacokinetic studies of optically pure compounds after single enantiomer administration are becoming increasingly important. The process of racemization in vivo can diminish all expected advantages of single enantiomer treatment. Amlodipine, one of the calcium channel blockers, currently used in therapy as a racemate, is one of such drugs under study. In order to administer single enantiomers of amlodipine to healthy volunteers both were chromatographically purified and characterised. The two optical isomers of amlodipine, active *S*-(−)- and non-active *R*-(+)-amlodipine, were purified using chromatographic procedure adopted from the analytical separation. Enantiomers were successfully converted to benzenesulphonic salt without any racemization. All semi-preparative purifications were monitored with complementary analytical methods, HPLC and CE, along with the determination of optical activity so that the final product was sufficiently defined for further in vivo studies. The analytical method developed for the determination of plasma concentrations of each enantiomer of amlodipine in these studies is also briefly described.

Keywords: Enantiomer separation; Amlodipine

1. Introduction

Chiral drugs have been used as therapeutic agents for a long time, but in most cases only in the form of racemic mixtures. Until recently, it was neither technically nor economically feasible to separate such mixtures into single enantiomers. Over the last ten years, however, great advances in production technology [1] and quality control techniques have made drug chirality an important issue [2]. Individual properties of different chiral drugs were studied and results are reported in FDA's Policy Statement for the Development of New Stereoisomeric Drugs [3].

The question whether to use racemates or single enantiomers is still under discussion in the pharmaceutical industry [4,5]. In the development process of a new drug entity differentiation due to chirality of the molecule is already an integral part of preclinical and clinical investigations. Regulatory authorities are increasingly requesting detailed investigations regarding potency and toxicity of each optical isomer of the drug. The situation is very well described by Caldwell [6]. The choice of a racemate must be justified and the criteria for this will become more demanding as the time goes by.

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Calcium antagonist amlodipine, 3-ethyl 5-methyl-2-[(2-aminoethoxymethyl]-4-(*o*-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylate, is a racemic drug that has been on the market for a long time. The activity of enantiomers of different calcium antagonists needs to be further investigated, as stressed by Kelly and O'Malley [7]. Only one enantiomer, *S*-(−)-amlodipine, is biologically active [8]. Potential benefits of single enantiomer can only be investigated by studying in vivo effects and correlations between the administration of racemic and single enantiomer drugs to healthy volunteers. A study on the concentrations of each amlodipine enantiomer in human plasma has already been performed, although after administration of the racemic drug [9]. Interaction between optically pure substance and its receptor is an important issue. Nonetheless, attention should also be given to metabolic transformations after single enantiomer administration, since major changes in drug chirality may occur even before the drug reaches the receptor site. Before single enantiomers of amlodipine may be administered to healthy volunteers, both optical isomers of amlodipine, active *S*-(−)- and non-active *R*-(+)-amlodipine, have to be purified and well characterised.

Usually, racemic amlodipine is administered in tablet form as benzenesulphonate. Therefore, it was decided to convert pure enantiomers to the same salt form. At the same time, potential racemization of the optically pure drug must be examined in this preparation step. The analytical chromatographic procedure developed by Flykt et al. [10] proved suitable for semi-preparative separation, since the separation factor on the analytical column was high enough to obtain pure enantiomers with reasonably high yields. All semi-preparative operations were monitored with complementary analytical methods, chiral HPLC and chiral CE, along with the determination of the optical activity in order to make sure that the final product is sufficiently characterised for further in vivo studies.

2. Experimental

2.1. Chemicals

Chemicals and solvents were purchased from Merck (Darmstadt, Germany) except benzenesulphonic acid (98%), which was from Fluka (Buchs, Switzerland), α-cyclodextrin from Sigma (Deisenhofen, Germany) and methylcelullose from Eli Lilly (Giessen, Germany). The raw material used for the preparation of *S*-(−)-amlodipine and *R*-(+)-amlodipine was racemic amlodipine benzenesulphonate from Lek (Ljubljana, Slovenia).

2.2. Chromatographic semi-preparative separation of enantiomers

Semi-preparative high-performance liquid chromatographic (HPLC) separation was carried out with a liquid chromatograph consisting of an AS 100 autosampler (Bio-Rad, Vienna, Austria), equipped with a 1-ml sample loop, a Constametric 4000 pump and a SpectroMonitor 4000 UV detector (both from Thermo Separation Products, Darmstadt, Germany). The signal was acquired and processed by the Millennium 2010 software (Waters, Milford, MA, USA). For semi-preparative work, the Chiral-AGP column, 150×10.0 mm I.D. (ChromTech, Hægersten, Sweden) was used. Operating conditions for semi-preparative separation were a modification of those described in the analytical procedure according to Flykt et al. [10]. The mobile phase was prepared by mixing 10 m*M* sodium acetate buffer (pH 4.5) with 1% (v/v) of *n*-propanol. The flow-rate was 4.0 ml min^{-1}. A 800-μl volume of racemate solution at a concentration of 1.3 mg ml^{-1} was applied to the column. The chromatographic run was monitored at 240 nm and 1-ml fractions were obtained.

2.3. Determination of optical rotation of pooled fractions

Optical rotation was measured on the Perkin Elmer polarimeter (Norwalk, CT, USA) equipped with a Na lamp, at a wavelength of 589 nm. The volume of the measuring cell was 1 ml and the length of the optical path was 10 cm. The system was thermostated at 20°C. The optical activity of individual fractions obtained from one chromatographic run per day was determined. Other runs from the same day were monitored by UV absorbance detection at 240 nm.

2.4. HPLC determination of R-(+)- and S-(−)-amlodipine

HPLC analysis of *R*-(+)- and *S*-(−)-amlodipine was performed according to Flykt et al. [10] with an HPLC system (Waters) consisting of a photodiode-array (PDA) detector, model 996, a solvent delivery system, model 616, an autosampler, model 717, a degasser and the Millennium 2010 software. The mobile phase was 10 m*M* sodium acetate buffer (pH 4.5) with 1% (v/v) *n*-propanol. A 150×4.0 mm I.D. Chiral AGP analytical column (ChromTech) was used. The flow-rate was 0.9 ml min^{-1}, the injection volume was 20 μl. UV spectra were monitored from 200–400 nm.

Analytical HPLC with photodiode-array detection was used to follow the content of each enantiomer in fractions after semi-preparative chromatography. UV spectra were monitored from time to time to make sure that the peaks collected corresponded to amlodipine.

2.5. Capillary electrophoresis determination of amlodipine enantiomers

Capillary electrophoresis (CE) determination was used as a complementary analytical method to chiral HPLC for the determination of enantiomers in all stages of preparation.

CE analyses were carried out on the BioFocus 3000 CE instrument in uncoated silica capillary, 50 cm×50 μm I.D. (Bio-Rad) at applied voltage 15 kV. The signal was detected at a wavelength of 200 nm. The injection constant was 68.9 kPa×s. The CE method by Small et al. [11] was employed. To the electrophoretic buffer consisting of 20 m*M* Tris–HCl (pH 3.2) and 18 m*M* α-cyclodextrin, 0.05% (m/v) of methylcelullose was added. For normalization of areas with their migration times, the method of Altria [12] was used.

2.6. Concentration of pooled fractions of both enantiomers

Fractions from the semi-preparative column were pooled and evaporated in a rotary evaporator at temperatures not higher than 35°C. The dry substance was dissolved in 25 ml of chloroform and the same volume of water was added for extraction. After phase separation, the organic phase was dried and used for benzenesulphonation.

2.7. Benzenesulphonation of single enantiomers and racemate

Amlodipine in pharmaceutical formulations is usually in the form of benzenesulphonate. Benzenesulphonation was performed on single enantiomers and racemate to examine the possibility of racemization in this procedure.

The dry substance was dissolved in ethanol. Separately, an ethanolic solution of benzenesulphonic acid was prepared and added to each enantiomer and racemate, respectively.

2.8. Bioanalytical method for determination of R-(+)- and S-(−)-amlodipine in human plasma

2.8.1. Plasma sample preparation

To 5 ml of human plasma sample, 250 ng of internal standard chloroamlodipine (Lek) was added, 250 μl of 1 *M* NaOH p.a. (Kemika, Zagreb, Croatia) and 5 ml of chloroform (Merck). The mixture was shaken for 10 min and centrifuged at 3500 *g* in a Centric 322 PLC centrifuge (Tehtnica, Železniki, Slovenia). The lower phase was carefully transferred to another test tube and dried in a vacuum centrifuge Univapo 150H (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 [mixture of acetic buffer (pH 4.5) and methanol, 4:6]. A 50-μl volume was injected in HPLC system.

2.8.2. HPLC system for determination of R-(+)- and S-(−)-amlodipine in human plasma

Determination of *R*-(+)- and *S*-(−)-amlodipine was performed on coupled HPLC system consisting of chiral separation on a Chiral AGP column and trapping each enantiomer on two separate short C_8 columns by column switching, subsequent elution and achiral separation on reversed-phase as used by Flykt et al. [10]. Instrument modules used were autosampler BioRad AS 100 HRLC, two pumps,

LDC Analytical CM 4000 and ConstaMetric 3000, thermostat Mistral Spark Holland (all from Thermo Separation Products), UV–Vis detector and three switching valves (all from 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).

2.8.3. Chromatographic conditions for determination of R-(+)- and S-(−)-amlodipine in human plasma

The mobile phase for chiral separation of amlodipine enantiomers was 10 m*M* acetate buffer (pH 4.5) with 1% (v/v) *n*-propanol. A 150×4.0 mm I.D. Chiral AGP analytical column (ChromTech) was used. The flow-rate was 0.9 ml min^{-1} and the injection volume was 50 μl of plasma extract. Each single enantiomer was trapped on its own short column Supelcosil LC-8 (Supelco, Gland, Switzerland), 20 cm×4.6 mm I.D. The eluting mobile phase consisted of the same acetate buffer (10 m*M* acetate buffer, pH 4.5) and acetonitrile in volume ratio 55:45. The analytical column, used in the second, achiral part of coupled HPLC system, was Symmetry C_8 (Waters), 150 mm long and 4.6 mm I.D. It was thermostated at 30°C. The signal was monitored at 240 nm on a second detector. The first detector (the same wavelength, 240 nm) was used only in the system suitability testing (SST). At the beginning of every new sequence of the analytical runs the chiral separation part of the system was checked and switching times adjusted.

3. Results and discussion

The application of a larger column (10 mm I.D.) allowed a semi-preparative separation of amlodipine isomers. As shown in Fig. 1, good loadability over the range of milligram per run was achieved. Resolution of amlodipine enantiomers on a semi-preparative column was high enough to obtain a pool of fractions containing practically pure single enantiomers. Subsequent analytical separation by HPLC (Fig. 2) showed that fractions with at least 98% enantiomeric purity in the case of *R*-(+)-amlodipine (Fig. 2A) and fractions over 95% enantiomeric purity in the case of *S*-(−)-amlodipine (Fig. 2B) were obtained after semi-preparative chromatography. For comparison, the chromatogram of the fraction containing both enantiomers is shown in Fig. 2C. In

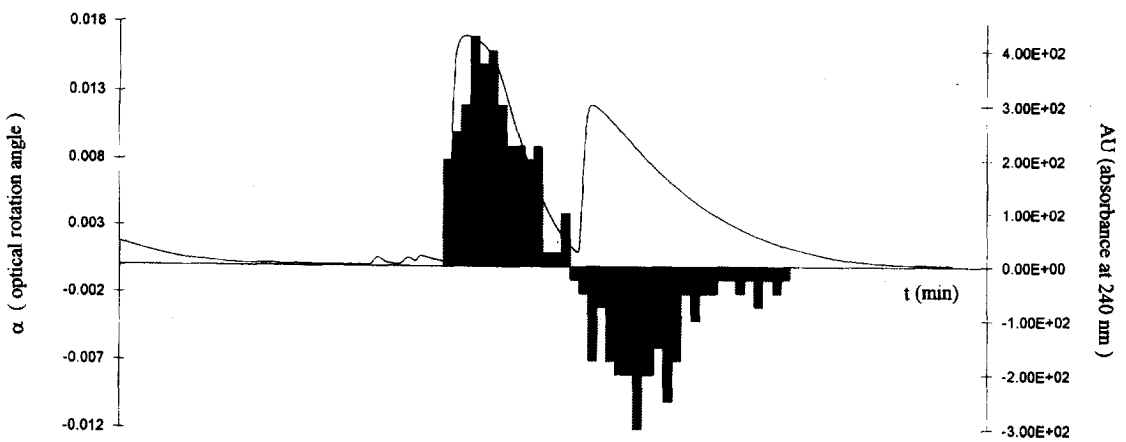

Fig. 1. Chromatographic separation of amlodipine enantiomers with optical rotation of individual fractions. HPLC conditions: Chiral-AGP column, 150×10.0 mm I.D., mobile phase: 10 m*M* acetate buffer (pH 4.5) containing 1% (v/v) of *n*-propanol, flow-rate: 4.0 ml min^{-1}, 800 μl of racemate solution or a concentration of 1.3 mg ml^{-1} was applied onto the column, detection at 240 nm, collection of fractions: per volume (1 ml). Optical rotation measurement: polarimeter, Na lamp, wavelength of 589 nm, 1-ml measuring cell, length of the optical path was 10 cm, the measuring system was thermostated at 20°C.

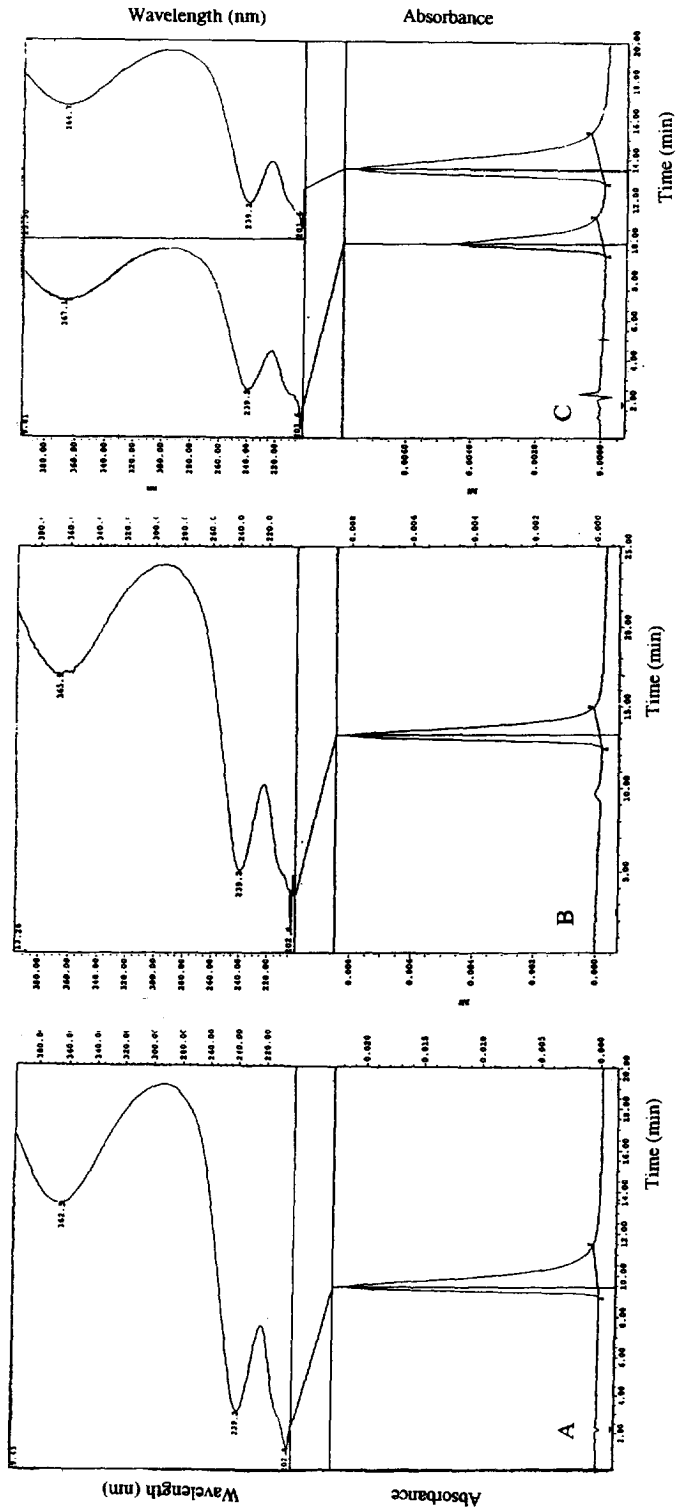

Fig. 2. HPLC of (A) *R*-(+)-amlodipine, (B) *S*-(−)-amlodipine and (C) racemate fractions from semi-preparative (150×10.0 mm I.D.) Chiral-AGP column with UV spectrum at peak maxima. Analytical HPLC conditions: Chiral-AGP column, 150×4.0 mm I.D., mobile phase was 10 m*M* acetate buffer (pH 4.5) with 1% (v/v) *n*-propanol, the flow-rate was 0.9 ml min^{-1}, the injection volume was 20 μl, UV spectra were monitored from 200–400 nm.

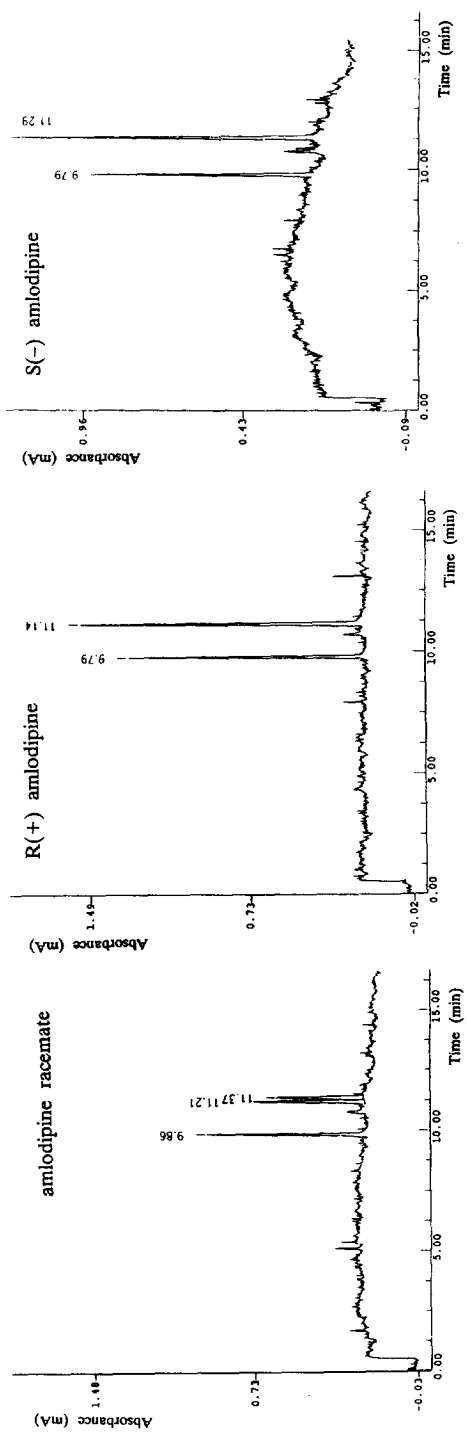

Fig. 3. Electropherograms of amlodipine racemate (0.50 mg ml^{-1}), *R*-(+)-amlodipine (0.52 mg ml^{-1}) and *S*-(−)-amlodipine (0.36 mg ml^{-1}) preparation (all in form of benzenesulphonate, migration time approx. 9.8 min is benzenesulphonic acid). CE operating conditions: BioFocus 3000 CE instrument, uncoated silica capillary, 50 cm×50 μm I.D., applied voltage 15 kV, detection at a wavelength of 200 nm, injection constant was 68.5 kPa×s, electrophoretic buffer: 20 m*M* Tris–HCl (pH 3.2), 18 m*M* α-cyclodextrin and 0.05% (m/v) methylcellulose.

order to control the purity of single substances, a UV spectrum was obtained with PDA at every peak maximum (cf. Fig. 2A to C).

Additional cross-check of single fractions of *R*-(+)- and *S*-(−)-amlodipine from the semi-preparative separation and the racemate was carried out by CE (cf. Fig. 3A to C). Baseline separation was achieved using an uncoated capillary after adding 18 m*M* α-cyclodextrin and 0.05% (m/v) methylcellulose.

The three analytical techniques listed above, measurement of optical activity (cf. Fig. 1), HPLC determination with PDA detection (cf. Fig. 2) and capillary electrophoresis (cf. Fig. 3) were performed in order to thoroughly analyse optical isomers obtained from semi-preparative separation. Usually, only one detection method is used [9,11] sometimes two of them [8]. However, the use of multiple complementary analytical techniques yields more reliable results [13,14]. Complementary principles of monitoring chiral separation of enantiomers comprised interaction with α1-acid glycoprotein (AGP) on a Chiral-AGP HPLC column and chiral recognition of amlodipine enantiomers by α-cyclodextrin

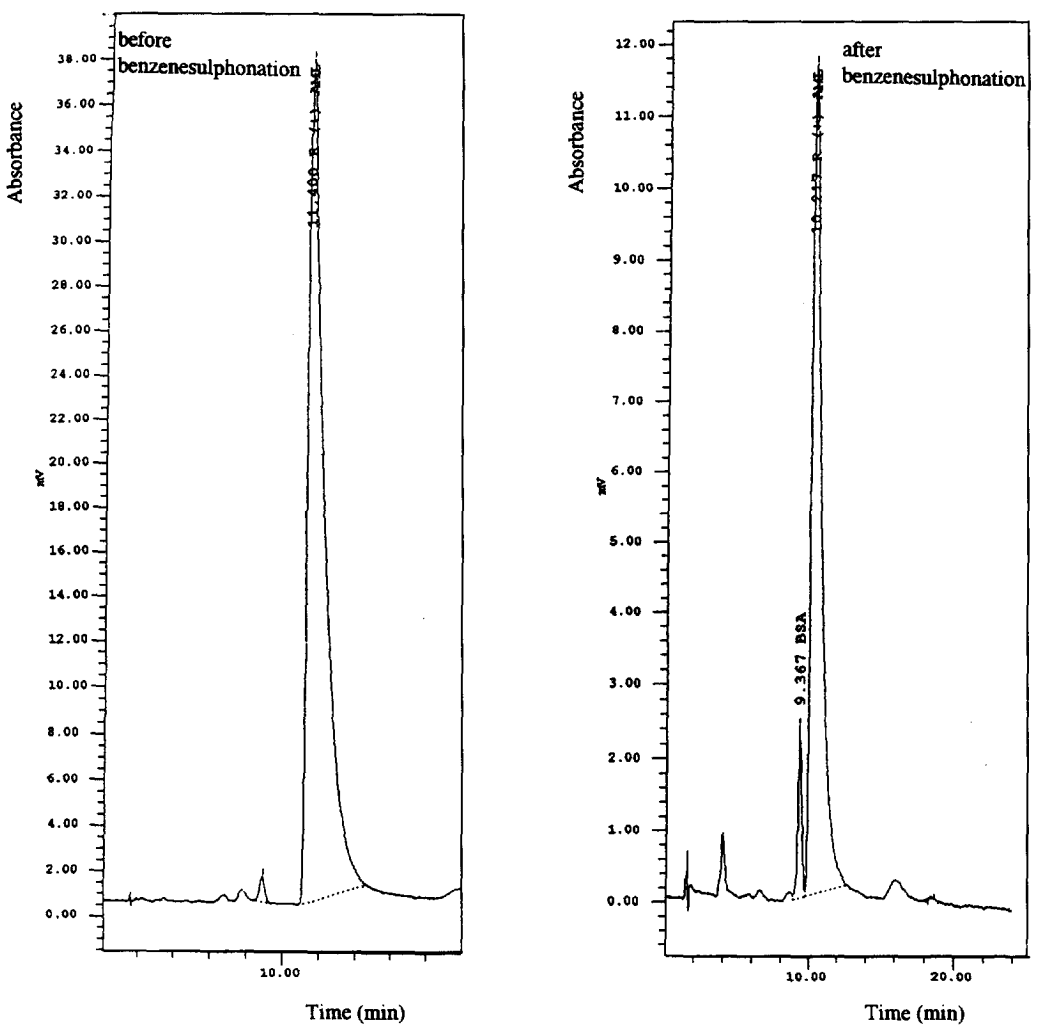

Fig. 4. Chromatogram of pooled fractions of *R*-(+) enantiomer of amlodipine after AGP semi-preparative chromatography before benzenesulphonation and chromatogram of *R*-(+) enantiomer of amlodipine benzenesulphonate after benzenesulphonation (BSA, benzenesulphonic acid). For analytical HPLC conditions see Fig. 2.

combined with electrophoretic mobility of amlodipine. The third complementary technique was the measurement of optical activity. The first, sharper peak exhibited positive optical rotation and was therefore shown to be *R*-(+)-amlodipine. According to Scharpf et al. [9]. this is the biologically less active enantiomer. The biologically more active enantiomer, *S*-(−)-amlodipine, was eluted as the second peak in the chromatogram, as it rotated polarized light in the negative direction (cf. Fig. 1). *R*-(+)-amlodipine benzenesulphonate prepared in ethanol solution had a specific optical rotation of +25.6. *S*-(−)-amlodipine benzenesulphonate had a specific optical rotation of −21.0.

After semi-preparative separation and identification, the fractions containing pure optical isomers were concentrated and the dry substances were benzene sulphonated, as in the case of the pharmaceutical product. In order to examine the possible rate of racemization during the preparation procedure, benzenesulphonation was carried out on *R*-(+)-amlodipine, *S*-(−)-amlodipine and on the racemic amlodipine. Racemic amlodipine crystallized after a few hours of gentle mixing in an icy water bath, but neither the *R*-(+)- nor the *S*-(−)-enantiomer crystallized even after mixing at −20°C for one week. Moreover, HPLC analysis showed that no racemization occurred in the benzenesulphonation step of the enantiomer preparation (Fig. 4).

Fig. 5 shows the detection by HPLC of 5 ng each of *R*-(+)- and *S*-(−)-amlodipine benzenesulphonate in human plasma. In order to detect and identify single stereoisomers, a column-switching technique was applied (see Section 2). The amount of amlodipine, which can be expected at this concentration after administration of 10 mg of substance, is easily quantified by HPLC and the software used. (cf. Fig. 5, bottom).

The optically pure enantiomers, *R*-(+)-amlodipine benzenesulphonate and *S*-(−)-amlodipine benzenesulphonate can be used in further in vivo studies of enantioselectivity of metabolism after individual enantiomer administration to healthy volunteers. The isolated single enantiomers of amlodipine and the optimized analytical methods for the determination of single stereoisomers in human plasma are further prerequisites for a successful conclusion of in vivo investigations.

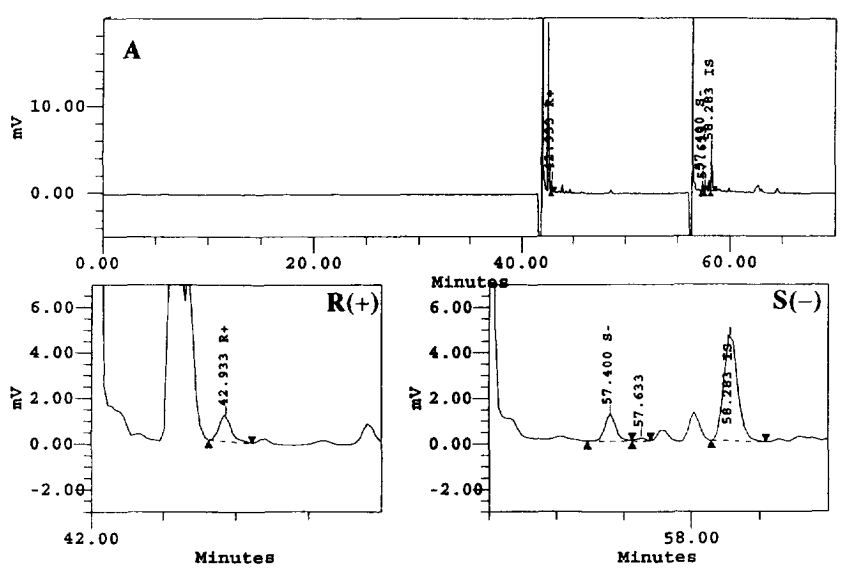

Fig. 5. HPLC (A) of plasma sample spiked with 5 ng of *R*-(+)- and *S*-(−)-amlodipine in the form of benzenesulphonate, respectively, per ml of plasma (approximate concentration expected in plasma samples of healthy volunteers to whom 10 mg of amlodipine in the form of benzenesulphonate is administered). From time zero to 40 min in chromatogram A, enantiomers *R*-(+) and *S*-(−) were trapped on separate short C_8 columns. *R*-(+) assigned chromatogram is the zoomed time region of chromatogram A from 42–44 min when *R*-(+) was eluted and *S*-(−) assigned chromatogram is the zoomed time region of chromatogram A from 56.5–59 min when *S*-(−) was eluted.

4. Conclusions

We have isolated the two enantiomers of amlodipine on a Chiral AGP preparative column and prepared benzenesulphonate salts without any racemization. The pure enantiomers have been thoroughly examined for their purity and optical activity by HPLC and CE analytical methods. After toxicological tests, they will be suitable for single enantiomer administration to healthy human subjects.

The HPLC analytical method and the plasma sample preparation for determination of amlodipine enantiomers developed here will enable us to follow plasma concentrations of each enantiomer after administration of the optically pure substance.

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