ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
15 (1996) 267–277

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

# Coupled-column chromatography on a Chiral-AGP phase for determination of amlodipine enantiomers in human plasma: An HPLC assay with electrochemical detection

Martin Josefsson*, Björn Norlander

*Department of Clinical Pharmacology, Faculty of Health Sciences, Linköping University, S-581 83 Linköping, Sweden*

Received for review 7 February 1996; revised manuscript received 14 March 1996

**Abstract**

A sensitive enantioselective high performance liquid chromatographic assay for determination of the dihydropyridine-type calcium antagonist amlodipine in human plasma samples is described. Chiral chromatography is performed on an $\alpha_1$-acid glycoprotein column (i.e. Chiral-AGP) and the eluted enantiomers are trapped and compressed on two short columns before final achiral chromatography on a narrow bore column (i.e. Zorbax SB-Ph) using electrochemical detection. Both stereoselective quantitative analysis and enantiomeric ratio analysis, for samples with a known total concentration of amlodipine are described. The quantitative assay shows linearity over the range 0.5–10 ng $ml^{-1}$ for the two enantiomers and the limit of detection is about 0.2 ng $ml^{-1}$. The method has been applied to a pharmacokinetic study of the enantiomers of amlodipine in human subjects.

*Keywords:* Amlodipine; Chiral $\alpha_1$-acid glycoprotein; Column switching; Dihydropyridine; Electrochemical detection; Enantiomer; High performance liquid chromatography; Solid-phase extraction

## 1. Introduction

Amlodipine is a potent dihydropyridine calcium channel blocker used in treatment of hypertension and angina pectoris [1]. Like most other calcium channel blockers of this class amlodipine is therapeutically used as a racemate. Since the desirable vasodilating effect only occurs with the *S*-(−)-enantiomer [2,3], information about the pharmacokinetic behaviour of the enantiomers is needed.

In order to measure amlodipine in human plasma, a fast and sensitive assay with a limit of quantitation in the subnanogram per milliliter range is required. The non-enantioselective method most frequently referred to in clinical studies is a sensitive gas chromatography (GC) assay with electron capture detection after derivatization with trimethylacetyl chloride [4] and recently an enantioselective GC assay based on this method has been presented [5]. In this indirect method the enantiomers of amlodipine are con-

* Corresponding author.


*PII* S0731-7085(96)01836-5

verted to their acyl derivatives with the chiral reagent (+)-(*S*)-α-trifluoromethylphenylacetyl chloride (Mosher's reagent) and then separated as diastereoisomers with conventional GC. This assay shows a high sensitivity for the drug in human plasma samples (i.e. 0.02 ng $ml^{-1}$), but the derivatives are not baseline-separated within a reasonable time (i.e. 30 min) and the gas chromatograph is working under rather high temperatures which increase the risk of thermal decomposition of the derivatives or oxidation of the dihydropyridine moiety of amlodipine [6]. Underivatized neutral dihydropyridines require high working temperatures for GC and the derivatized amlodipine is even less volatile [4–6]. Furthermore, since the sample preparation is extensive and time-consuming ( > 3.0 h), a robot has to be used to achieve increased capacity of the assay.

High performance liquid chromatography (HPLC) for direct enantiomeric separation on chiral columns has been reported for several dihydropyridines. Many chiral phases in both normal- (e.g. Pirkle-type and cellulose/amylose) [8–10,12–21,23] and reversed-phase (e.g. proteins, cyclodextrins) [7,10,11,22–26] have been successfully used. The cellulose phases, however, have become the most popular chromatographic tools for stereoselective bioanalysis of these substances since they can be easily combined with GC analysis. The use of mobile phases based on organic solvent allows collection of the enantiomers in fractions that can then be concentrated by evaporation before a final off-line quantitation. Assays suitable for determination of the drug enantiomers in plasma have been developed for felodipine [13–15], manidipine [16], nicardipine [13,17], nilvadipine [13,18], nimodipine [13,16], nisoldipine [13,20,21] and nitrendipine [13]. All these substances have been separated on a Chiralcel OJ column (i.e. tris(4-methyl-benzoate)cellulose) with the exception of nilvadipine, which was separated on a Chiralpak OT(+) column (i.e. polymethacrylate), and all the columns were run in the normal-phase mode. In a majority of these assays off-line detection with GC–electron capture detection (ECD) [5,13,18] or GC–mass spectrometry (MS) [14,19–21] was used. Manidipine, however, was detected off-line with UV in an achiral HPLC system [16].

The application of chiral chromatography to studies of the stereoselective pharmacokinetics of dihydropyridine calcium antagonists in humans has recently been reviewed [23].

Chromatographic materials prepared with the extremely stable human protein $\alpha_1$-acid glycoprotein immobilized on silica (i.e. Chiral-AGP) have been shown to be chiral selectors with a broad range of applications [24]. The enantiomers of dihydropyridines such as felodipine [24,25], isradipine [11], nicardipine [26], nimodipine [11] and nisoldipine [24] have for instance been baseline-separated. However, the AGP material has not yet been used for stereoselective analysis of these substances at human plasma levels due to the lack of sensitive techniques for direct detection in combination with the chiral chromatography. Furthermore, since chromatography is performed in the reversed-phase mode, fractionated collection with off-line GC detection is difficult to apply.

The aim of the present study was to find a reversed-phase HPLC method for enantiomeric separation that could be combined with achiral chromatography in a coupled-column system. The intention was to use a recently presented achiral HPLC assay, based on sensitive electrochemical detection, for the final determination of amlodipine [27]. The enantiomeric separation of *rac.*-amlodipine on an $\alpha_1$-acid glycoprotein column (i.e. Chiral-AGP) was studied. Both enantiomeric ratio analysis of samples with a known total concentration of amlodipine and enantioselective quantitative analysis using internal standardization were evaluated.

## 2. Experimental

### 2.1. Chemicals and materials

Acetonitrile (far-UV) and methanol (HPLC-grade) were purchased from LabScan Analytical Sciences (Dublin, Ireland) and 1-propanol was from Fluka (Buchs, Switzerland). Acetic acid (100%), ammonia (25%), disodium hydrogenphosphate-2-hydrate, sodium acetate and ethylenediaminetetraacetic acid disodium salt dihydrate

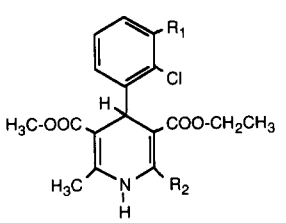
(EDTA), all pro analysi, were purchased from Merck (Darmstadt, Germany). Potassium dihydrogenphosphate and tetrabutylammonium hydrogensulfate (TBAHS) were from Fluka. *R*-(+)-, *S*-(−)- and *rac*.-amlodipine (maleate), and *rac*.-UK52.829 (fumarate) were obtained from Pfizer Central Research (Sandwich, UK). Structures are shown in Fig. 1.

Bond Elut C2 extraction columns, with a sorbent mass of 100 mg in 3 ml cartridges, were purchased from Analytichem (Harbor City, CA) and the same size Isolute C2 and C2 EC (end-capped) columns were from International Sorbent Technology (IST; Hengoed, UK).

### 2.2. Solutions, standards and samples

Stock solutions of *rac*.-, *R*-(+)-, and *S*-(−)-amlodipine and *rac*.-UK52.829 (1 mg $ml^{-1}$) were prepared with 10 mg of the free base in 10 ml methanol. Stock diluted solutions (1:100) to a final concentration of 10 $\mu g\ ml^{-1}$ were prepared in methanol–water 1:1 (v:v) and stored at +4°C. Working solutions (1.0 and 0.1 $\mu g\ ml^{-1}$) were prepared by successive 1:10 dilutions in methanol–water 1:1 (v:v).

Standards and controls were prepared on the day of analysis. Blood samples were collected in EDTA $K_3$ Vacutainers (Becton & Dickinson, Meylan, France) by venipuncture. After centrifugation (2000*g*, 5 min) human plasma samples were stored at −80°C. No loss in recovery of amlodipine in spiked plasma samples was seen after four freeze–thaw cycles.




| | $R_1$ | $R_2$ |
|---|---|---|
| Amlodipine | H | $CH_2$-O-$CH_2$-$CH_2$-$NH_2$ |
| UK52.829 | Cl | $CH_2$-O-$CH_2$-$CH_2$-$NH_2$ |

Fig. 1. Molecular structures.

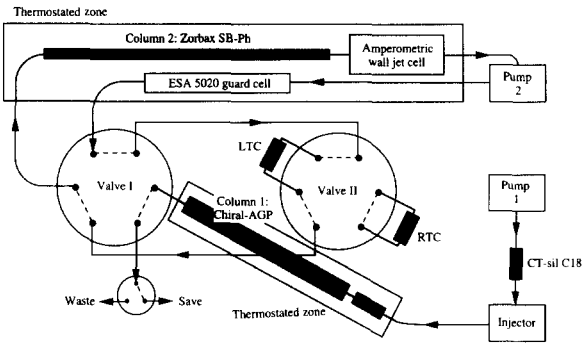



Fig. 2. Schematic diagram of the experimental set-up. Both values (I and II) in off position. LTC = left trapping column, RTC = right trapping column (i.e. CT-sil Phenyl).

### 2.3. Apparatus

The chiral chromatography part of the coupled HPLC system consisted of a Waters 717 autosampler, a Waters 590 programmable solvent delivery module and a Waters WAVS automated valve station with two six-port valves (Milford, MA). Two CT-sil Phenyl trapping columns, 10 mm × 2.1 mm i.d., were mounted on one of the valves and a CT-sil C18 guard column, 10 mm × 3.0 mm i.d. (ChromTech, Hägersten, Sweden) was mounted in front of the autosampler as a mobile phase scavenger (Fig. 2). Chromatography was carried out on a Chiral-AGP column (i.e. $\alpha_1$-acid glycoprotein), 100 mm × 4.0 mm i.d. (ChromTech), with a 10 mm × 3.0 mm i.d. Chiral-APG guard column. The chiral column was housed in a Jones chromatographic column heater/chiller, model 7955 (Hengoed, UK).

The achiral chromatography part of the coupled HPLC system connected to the WAVS valve station, consisted of a Gynkotek 480 pump (Munich, Germany), an Antec Decade amperometric detector (Leiden, The Netherlands) and an EZChrom Scientific Software integrator system (San Ramon, CA). The built-in detector oven housed an Antec VT-03 analytical cell with a 50 $\mu$m spacer, an ESA 5020 guard cell (Bedford, MA), an Antec LO-Pulse pulse damper and the analytical column. Chromatography was carried out on a Zorbax SB-Phenyl, 150 mm × 2.1 mm i.d., Rockland Technologies (Chadds Ford, PA) column. The detector cell was attached to the

Table 1
Timetable for the column-switching program

| Row | Time (min.) | Valve I | Valve II | Valve III | Event[a] | |
|---|---|---|---|---|---|---|
| ↓ | 0 | – | – | – | Injection | ← |
| 1 | 0 | On | On | V | AGP phase to waste | |
| 2 | 8 | Off | On | V | Wash of the LTC with AGP phase | |
| 3 | 8.5 | Off | Off | V | *R*-(+)-AML to the RTC | |
| 4 | 13 | Off | On | S | *S*-(−)-AML to the LTC | |
| 5 | 18 | On | Off | S | Elution from RTC to the SB-Ph column | |
| 6 | 23 | On | On | S | Elution from LTC to the SB-Ph column | |
| → | 25 | – | – | – | New injection. Ratio analysis | ↑ |
| 7 | 25 | Off | On | V | UK(I) to the LTC | |
| 8 | 32 | On | On | S | Elution from LTC to the SB-Ph column | |
| → | 32 | – | – | – | New injection. Quantitative analysis | ↑ |
| 9 | 45 | On | Off | S | Resting (AGP mobile phase flow 0.1 ml $min^{-1}$) | |

[a] Left (LTC) and right (RTC) trapping columns, i.e. CT-sil Ph. Amlodipine (AML) and UK52.829 (UK).

analytical column with the shortest possible PEEK capillary.

An IST VacMaster preparation system was used for the extraction and organic solvents were evaporated with a Heto Lab Equipment vacuum concentrator, Hetovac VR-1, CT 110 (Birkerod, Denmark).

### 2.4. Chromatographic conditions

Chiral chromatography was carried out at a flow rate of 0.9 ml $min^{-1}$ and a temperature of 20°C. The mobile phase was prepared in 5l batches by adding 0.3% of 1-propanol to an acetate buffer (pH 3.9; 50 mM) containing 5 mM TBAHS and finally passed through a 100 mm × 4.6 mm Hypercarb-S (Shandon, Runcorn, UK) column ( ≈ 3.0 ml $min^{-1}$) before use. The Hypercarb-S column was restored by washing with pure methanol and water between batches. So far the column has been used for more than 10 batches without deterioration.

Achiral chromatography was a modified version of a previously presented amlodipine assay [27] with a flow rate of 0.4 ml $min^{-1}$ and a detector oven temperature of 30°C. The analytical cell had an applied voltage of + 0.95 V and the nanoampere sensitivity range was used. The applied voltage of the guard cell was + 0.95 V. The mobile phase was prepared by mixing methanol–acetate buffer (pH 4.0; 0.1 M) (55:45, v/v). EDTA was added to a concentration of 10 mg $l^{-1}$ and TBAHS to a final concentration of 5 mM. The mobile phase was passed through a 0.22 μm filter and recycled in the chromatographic system for at least 12 h, with the analytical- and guard cells under working conditions, before analyses.

### 2.5. Switching procedure

The autosampler was working as a master in the chromatographic system initiating the switching program of the valve system when a sample was injected onto the AGP column (Fig. 2). The first-eluted enantiomer of amlodipine (*R*-(+)-) was transferred to the right trapping column by switching valves I and II, after which the second-eluted enantiomer (*S*-(−)-) was transferred to the left trapping column by switching only valve II, according to the timetable described in Table 1. The *R*-(+)-enantiomer was then eluted and applied to the achiral HPLC system by letting the Zorbax SB-Ph mobile phase pass through the right trapping column by switching valves I and II. At the same time the EZChrom integrator system was started. After 5 min, when the first

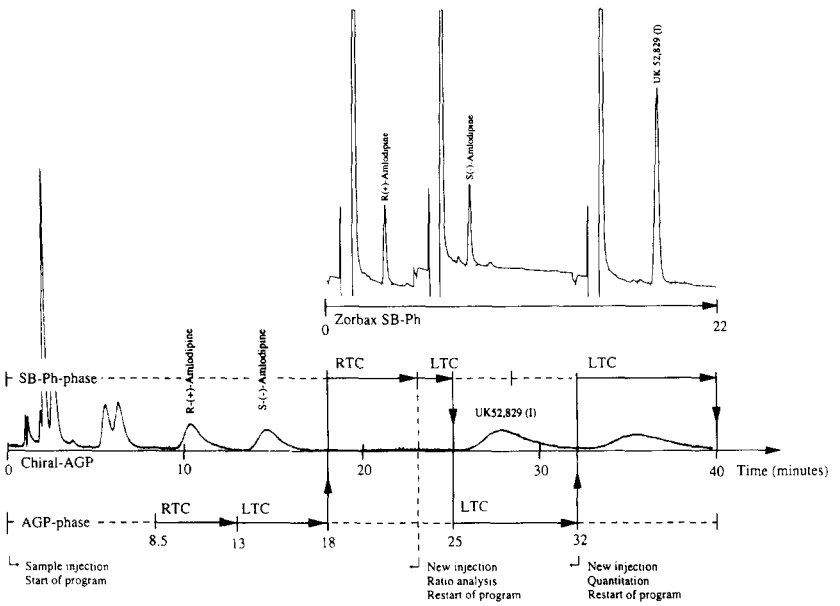



Fig. 3. Chromatograms and timetable of mobile phase flows in the switching system. Bottom: chromatogram from trapping window check analysis with UV detection (240 nm) showing the initial enantiomeric separation on the Chiral-AGP column. Injected amounts are 100 ng *rac.*-amlodipine and 200 ng *rac.*-UK52.829. Top: chromatograms showing the final quantitation of the enantiomers on the achiral Zorbax SB-Ph column. Injected amounts are 5.0 ng *R*-(+)- and *S*-(−)-amlodipine and 50 ng *rac.*-UK52.829 using electrochemical detection (+0.95 V).

chromatogram was completed, *S*-(−)-amlodipine was eluted from the left trapping column by switching valve II. If enantiomeric ratio analysis was performed a new injection onto the AGP column was made now. However, if quantitative analysis with an internal standard was performed, the first-eluted enantiomer of UK52.829 was then transferred to the left trapping column while the second chromatogram was completed according to Fig. 3. The second enantiomer of UK52.829 was directed to waste, the first enantiomer was eluted from the left trapping column and the third, and last, chromatogram was completed. A new injection onto the AGP column was initiated at the same time as the internal standard was eluted from the left trapping column. The three chromatograms of *R*-(+)- and *S*-(−)-amlodipine and the internal standard from the final achiral chromatography are shown at the top of Fig. 3. The complete procedure of chiral chromatography, trapping, elution and final achiral chromatography with detection took less then 35 min.

Retention time windows for sampling from the AGP column were determined beforehand by injecting 100 ng *rac.*-amlodipine and 200 ng *rac.*-UK52.829 with a UV detector (240 nm) coupled directly to the outlet of the AGP column (Fig. 3).

By using one of the low pressure valves on the WAVS station an AGP-mobile-phase-saving arrangement could be established without any risk of cross-contamination of the two mobile phases in the coupled-column system.

### 2.6. Extraction procedure

A solid-phase extraction procedure with high recovery (i.e. ≥ 95%) was used as previously described [27]. The Bond Elut C2 extraction columns were conditioned before use with 2 ml acetonitrile, 1 ml water and 1 ml phosphate buffer (pH 7.0; 0.025 M). To the column cartridge were added 0.5 ml phosphate buffer (pH 7.0; 0.025 M), 50 μl internal standard solution (0.2 μg $ml^{-1}$ UK52.829 in methanol–water, 1:1 (v:v)), 1 ml plasma sample and another portion of 0.5 ml

buffer. The solution was applied to the columns with a gentle underpressure of 2–5 mm Hg. The columns were then washed with 2 ml 20% acetonitrile in water, dried by vacuum suction for 5 min, and finally washed with 1 ml acetonitrile at 2–5 mm Hg. The samples were eluted with 1 ml of a solution of 2.5% ammonia in acetonitrile and the solvent was evaporated with the Hetovac vacuum concentrator.

Samples were dissolved in 50 μl of a solution of 2% 1-propanol in 50 mM acetic acid, and then transferred into microvials for the automated injection system where 40 μl was injected.

## 3. Results and discussion

In chiral HPLC method development it seems natural to focus on the chromatographic considerations and to forget aspects such as the detectability of the analyte in the actual eluting media and the constitution of the injected samples, both of which are crucial to the final result when determination of low concentrations of a drug in complex matrices (e.g. plasma, urine, faeces) is the objective. Many chiral HPLC assays require highly purified biological samples in order to obtain stability for long-term work and the majority of the chiral HPLC columns on the market are run in the normal-phase mode, which can be unfavourable for the detection of the analyte. The current findings, however, show that an achiral HPLC assay with sensitive electrochemical detection can be combined on-line with a chiral separation of amlodipine performed in the reversed-phase mode on a protein column. The solid-phase extraction used allowed a high number of injections on the chiral column without affecting the chromatography.

### 3.1. Detection

Therapeutic plasma concentrations of amlodipine in humans, vary between 1 and 20 ng $ml^{-1}$ and in pharmacokinetic studies, after single-dose administration, plasma levels below 0.5 ng $ml^{-1}$ have to be detected [1]. The UV response or native fluorescense of amlodipine is far from that required for analysis of human plasma sample levels. Furthermore, studies of diastereomeric amlodipine derivatized with the highly fluorescent reagent (+)-1-(9-fluorenyl)ethyl chloroformate ((+)-FLEC) surprisingly showed markedly low response, probably due to quenching between amlodipine and the fluorenyl moiety in the derivatives. Amperometric electrochemical detection, however, has successfully been used by this group in an achiral reversed-phase HPLC assay, on a narrow bore column, for determination of *rac.*-amlodipine in human plasma samples [27]. At an applied voltage of $\approx +1.0$ V the dihydropyridine moiety in amlodipine is readily oxidized and forms the pyridine analogue. A drawback of electrochemical detection in chiral chromatography is that the technique can not be used in the normal-phase mode. Therefore the selection of phases for use in combination with electrochemical detection is restricted to protein, cyclodextrin and antibiotic phases which can be used in the reversed-phase mode.

### 3.2. Chiral separation

The $\alpha_1$-acid glycoprotein column (i.e. Chiral-AGP) has been shown to be a versatile chiral selector. Changes in enantioselectivity and retention on the Chiral-AGP columns are achieved by simple modifications of the mobile phase composition such as changes in the nature and concentration of uncharged modifier (e.g. methanol, ethanol, propanol) [28] or changes in pH [29]. The mechanisms of the stereoselective interaction with the AGP material are still unclear but the protein surface contains many different binding groups (i.e. amino acid and glycoprotein residuals), allowing several possibilities for stereoselective interactions [30].

Baseline separation of the amlodipine and UK52.529 enantiomers was achieved on a 4 mm i.d. Chiral-AGP column with a mobile phase consisting of an acetate buffer, pH 3.9 with 0.3% 1-propanol. The chromatogram is shown at the bottom of Fig. 3. Several organic modifiers were compared (e.g. methanol, ethanol, 1- and 2-propanol, acetonitrile) and 1-propanol was found to have the highest selectivity for the enantiomers

of amlodipine. Furthermore, the pH was found to be the most critical regulator of the capacity and selectivity of the analytes. Even a small increase in pH (0.1 pH units) markedly increased the retention of the analytes, in agreement with the findings of Hermansson and Grahn [29]. They showed that the enantioselectivity of hydrophobic basic drugs increases while the retention markedly decreases, when the pH of the mobile phase decreases from operations at pH 7 to a pH near 4. Furthermore, in the present study it was found that for the AGP column the content of organic modifier (i.e. 1-propanol) needed careful adjustments, typically in the range 0.2–0.6%, to ensure that similar chromatographic conditions were achieved. In order to reduce secondary interactions with the silica matrix and enhance peak shapes a cationic modifier was added to the mobile phase. Since amines are readily oxidized by the electrochemical detector and thereby give an enormous background signal only a quarternary ammonium salt (e.g. TBAHS) could be used.

As in many applications with chiral separation on a protein column the peaks were broad, with some tailing, which is not optimal for quantitative analysis. Even though there was a marked increase in sensitivity when a 2 mm i.d. Chiral-AGP column (narrow bore) was used instead of the 4 mm i.d. column, the signal-to-noise ratio was still too low. Furthermore, there was a marked long-term instability when the narrow bore column was used, with a drifting baseline induced by the injection, even though sample volumes as low as 5 $\mu$l were injected.

### 3.3. Column switching

Column-switching techniques have been used successfully in several assays for optimization of the chromatography of drugs in complex biological matrixes [31]. By combining the Chiral-AGP chromatography of amlodipine with the achiral HPLC mentioned above [27] in a coupled-column system a sensitive on-line assay for determination of the amlodipine enantiomers was obtained. A condition was the access to an effective sample preparation procedure (i.e. single-step solid-phase extraction) that allowed injections of a high number of samples on the AGP column without deterioration of the chromatography. However, since the peaks from the Chiral-AGP chromatography were broad, a direct switching of the enantiomers on to the achiral SB-Ph column would imply large injection volumes (2–5 ml) of mobile phase from the chiral system, disturbing the chromatography and the electrochemical detection in the achiral HPLC system. Walhagen and Edholm [32] have shown that in coupled-column chromatography systems the efficiency and selectivity of the chiral chromatography on protein columns could be improved by peak compression and hydrophobic trapping columns. The low concentration of organic modifier in the AGP mobile phase (i.e. 0.3% 1-propanol) allowed collection of amlodipine on short hydrophobic reversed-phase columns (i.e. CT-sil Ph). With the mobile phase used for the achiral HPLC system (i.e. 55% MeOH) the analytes (i.e. *R*-(+) and *S*-(−)-amlodipine and UK52.829) could be easily eluted from the trapping columns and applied to the SB-Ph column for final chromatography with high sensitivity electrochemical detection. Using this procedure the peak shapes were improved and thereby the sensitivity of the assay increased. Chromatograms are shown at the top of Fig. 3. To configure a suitable automated system, two six-port valves interconnected the two analytical columns (i.e. Chiral-AGP and Zorbex SB-Ph) via the two trapping columns (i.e. CT-sil Ph) according to Fig. 2. As a result of large trapping volumes in combination with a sensitive detection method, interfering peaks were seen in the final chromatogram when pure AGP mobile phase was trapped. By passing the mobile phase, in batches of $\geqslant 2$ l, through the highly hydrophobic Hypercarb-S column, interfering substances could be removed. This procedure caused no further changes to the mobile phase composition, as could be seen by comparing AGP chromatography using the same batch before and after processing.

Decreased separation factors for the analytes were observed after about 50 injections onto the AGP column but chromatography was restored by washing with about 100 ml of a solution of 20% acetonitrile in water.

### 3.4. Determination of the enantiomeric ratio

For plasma samples with a known concentration of *rac.*-amlodipine, a simplified and less time-consuming method without internal standard was used for the determination of the *R*-(+)/*S*-(−)-concentration ratio. The detector response was expressed as the peak-height ratio between the enantiomer and the sum of the peak heights of both enantiomers (i.e. $R/[R+S]$ and $S/[R+S]$). Using this procedure losses during sample preparation and injection could be neglected. In these analyses a total cycle of enantiomeric separation, trapping and final achiral chromatography with detection was about 25 min.

Calibration curves were prepared from plasma samples spiked with 2 ng $ml^{-1}$ amlodipine in *R*-(+)/*S*-(−) ratios of 25/75, 50/50 and 75/25. A linear response for the peak-height ratios of *R*- and *S*-amlodipine was seen, with correlation coefficients above 0.999. Controls were prepared by spiking plasma with 2 and 5 ng $ml^{-1}$ amlodipine in *R*(+)/*S*(−) ratios of 40/60 and 60/40. These controls were used regularly throughout the analyses to ensure that the switching windows were correct and that no cross-contamination between the two enantiomers was seen. The controls used in the assay validation are shown in Table 2. The *R*-(+)-/*S*-(−)- ratios of the amlodipine enantiomers in about 250 samples from a pharmacokinetic study, in a concentration range between about 0.5 and 5.0 ng $ml^{-1}$, were determined by this procedure. Plasma concentrations had earlier been determined with the achiral amlodipine assay developed by Josefsson et al. [27]. The pharmacokinetics of *R*-(+)- and *S*-(−)-amlodipine, in four healthy male subjects, after oral administration of 5 mg *rac.*-amlodipine (Norvasc®) are shown in Fig. 4.

Table 2
Assay validation for the enantiomeric ratio analysis of amlodipine. Variation in peak-height ratios of enantiomer versus total in human plasma samples spiked with *R*-(+)- and *S*-(−)-amlodipine added at two fixed ratios

| Spiked *R*/*S* ratio[a] | Total amount (ng $ml^{-1}$) | Number of samples | Mean ratio ± SD (% of total) | RSD (%) |
|---|---|---|---|---|
| 40/60 (a) | 2 | 4 | 40.1/60.9 ± 1.9 | 4.8/3.2 |
| 60/40 (a) | 2 | 4 | 60.6/39.4 ± 2.2 | 3.6/5.6 |
| 40/60 (b) | 2 | 4 | 39.7/60.3 ± 2.4 | 6.1/4.5 |
| 60/40 (b) | 2 | 4 | 60.9/39.1 ± 1.1 | 1.9/2.9 |
| 40/60 (b) | 5 | 4 | 40.0/60.0 ± 1.8 | 4.5/3.0 |
| 60/40 (b) | 5 | 4 | 58.7/41.3 ± 1.5 | 2.5/3.5 |

[a] Intra- (a) and between-day variations (b) for the corresponding *R*/*S* ratios with amlodipine added at different total concentration levels.

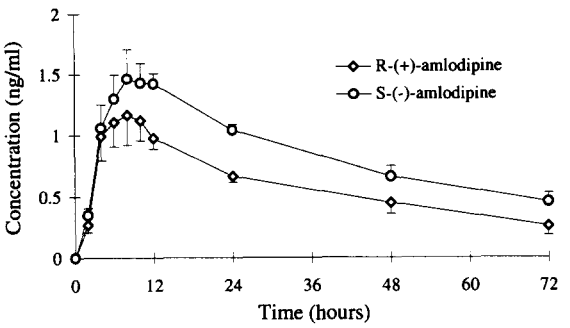



Fig. 4. Plasma concentration–time profiles for *R*-(+)- and *S*-(−)-amlodpine in four healthy male subjects after oral administration of 5 mg *rac.*-amlodipine (Norvasc®). Determination of the enantiomers with ratio analysis.

### 3.5. Internal standardization

In this chiral–achiral coupled-column system it was rather difficult to find a suitable internal standard (IS). The IS either has to be completely separated from amlodipine in the chiral chromatogram and trapped separately or, if co-eluted from the chiral column, the separation must be achieved during the achiral chromatography.

The enantiomers of the IS UK52.829 could be separated from amlodipine with the chiral chromatography and *rac.*-UK52.829 was separated from amlodipine with the achiral Zorbax SB-Ph chromatography. A drawback was the strong retention of UK52-829 on the Chiral-AGP material which increased the total time of the analysis. However, by using a more complex trapping/elution program (Fig. 3, Table 1) the amlodipine enantiomers could be applied consecutively to the final achiral chromatography before the internal standard was collected at one of the trapping

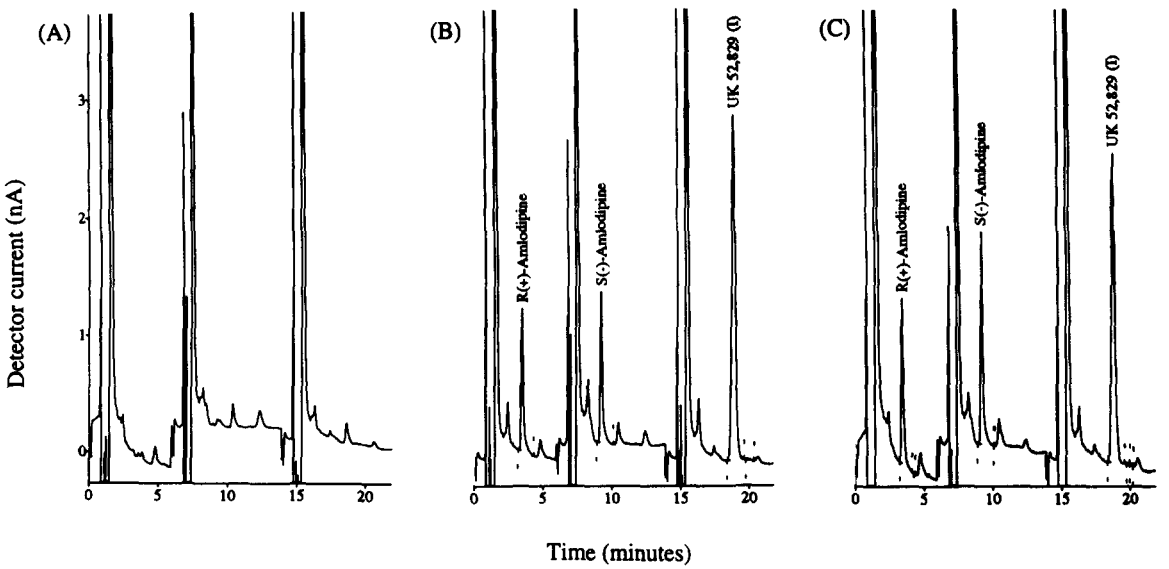



Fig. 5. Chromatograms showing the enantiomeric separation of amlodipine and the internal standard *rac.*-UK52.829: (A) blank plasma; (B) human plasma spiked with 5 ng $ml^{-1}$ *R*-(+)- and *S*-(−)-amlodipine and 40 ng $ml^{-1}$ of the internal standard; (C) 1 ml of a human plasma sample at steady state after once-daily administration of 5 mg *rac.*-amlodipine.

columns and thus the total run time was kept to a reasonable level.

For human plasma samples with unknown amlodipine concentration this procedure was used for the quantitation of *R*-(+)- and *S*-(−)-amlodipine (Fig. 5). A linear detector response for the peak-height ratio of each enantiomer of amlodipine and UK52.829(I) was observed over a range 0.5–10 ng $ml^{-1}$ plasma with a correlation coefficient close to 0.999. The limit of detection was determined to be about 0.2 ng $ml^{-1}$. Calculated relative standard deviations (RSDs) of controls consisting of human plasma samples spiked with amlodipine in *R*-(+)/*S*-(−) ratios of 1.0/4.0 ng $ml^{-1}$ and 4.0/1.0 ng $ml^{-1}$ are listed in Table 3. When *R*-(+)/*S*-(−) ratios of 1.0/8.0 ng $ml^{-1}$ were analysed the RSDs were below 15% for the enantiomers. The determined concentration of the *S*-(−)- enantiomer in the 8.0/1.0 ratio samples, however, was more than 20% too high, probably because of peak tailing of the *R*-(+)- enantiomer in the AGP chromatography. This was not evaluated further since extreme R-(+)/S-(−) concentration ratios, where the *R*-(+)- enantiomer dominates, will probably be of little relevance in studies of human plasma samples after administration of *rac.*-amlodipine.

## 4. Conclusions

The present work shows that chiral chromatography on a protein column (i.e. Chiral-AGP) with broad peak shapes and an accordingly high limit of quantitation can be markedly improved by a coupled-column system. The use of chiral chromatography in the reversed-phase mode, with a low content of organic modifier in the mobile phase, makes a column-switching procedure with trapping of the analytes on hydrophobic columns (i.e. CT-sil Phenyl) possible. In combination with conventional achiral HPLC where the higher content of organic modifier makes on-line elution of the analytes from the trapping columns possible, the peak shape could be enhanced and the detection optimized. Furthermore, thanks to the use of water-based mobile phases throughout the system a sensitive electrochemical detection could be used.

This work also shows that internal standardisation is possible in a more complex coupled system even though there are some difficulties with the optimization of the chromatography. The use of ratio determination of the enantiomers has been shown to be a good alternative when the total amount of the analyte is known.

Table 3
Assay validation of the quantitative analysis of the amlodipine enantiomers. Variation in concentration levels of each enantiomer of spiked human plasma samples with *R*-(+)- and *S*-(−)-amlodipine added at two fixed *R*/*S* ratios.

| Amount *R*/*S* (ng ml$^{-1}$)[a] | Number of samples | Means ± SD (ng ml$^{-1}$) | | RSD (%) | |
|---|---|---|---|---|---|
| | | *R* | *S* | *R* | *S* |
| 1.0/4.0 (a) | 4 | 0.99 ± 0.10 | 4.06 ± 0.24 | 9.7 | 6.0 |
| 4.0/1.0 (a) | 4 | 4.01 ± 0.25 | 1.05 ± 0.10 | 6.2 | 9.1 |
| 1.0/4.0 (b) | 3 × 2 | 1.01 ± 0.07 | 4.01 ± 0.10 | 7.1 | 2.4 |
| 4.0/1.0 (b) | 3 × 2 | 4.14 ± 0.21 | 1.07 ± 0.10 | 5.0 | 9.8 |

[a]Intra- (a) and between-day variations (b) in concentration levels of each enantiomer for the control samples. All with the internal standard *rac*.-UK52.829 added at a total amount of 40 ng ml$^{-1}$.

Finally, this concept for sensitive enantioselective HPLC analyses could probably be applied to other dihydropyridines (e.g. nisoldipine, nitrendipine) separated on either protein or cyclodextrin columns used in the reversed-phase mode.

## Acknowledgements

Mrs Eva Hedblom and Mrs Anna-Lena Zackrisson are thanked for their skillful assistance with the column-switching system and sample preparations.

## References

[1] P.A. Meredith and H.L. Elliott, Clin. Pharmacokinet., 22 (1992) 22–31.
[2] J.E. Arrowsmith, F. Camphell, E. Cross, J.K. Stubbs, R.A. Burges, D.G. Gardiner and K.M. Blackburn, J. Med. Chem., 29 (1986) 1696–1702.
[3] S. Goldmann, J. Stoltefuss and L. Born, J. Med. Chem., 35 (1992) 3341–3344.
[4] A.P. Beresford, P.V. Macrae, D.A. Stopher and B.A. Woods, J. Chromatogr., 420 (1987) 178–183.
[5] F. Scharph, K.-D. Riedel, H. Laufen and M. Leitold, J. Chromatogr. B, 655 (1994) 225–233.
[6] M. Ahnoff and B.-A. Persson, J. Chromatogr., 531 (1990) 181–213.
[7] E. Delee, I. Jullien and Le Garrec, J. Chromatogr., 450 (1988) 191–197.
[8] Y. Okamoto, R. Aburatani and K. Hatada, J. Chromatogr., 513 (1990) 375–378.
[9] T. Ohkubo, T. Uno and K. Sugawara, Chromatographia, 33 (1992) 287–288.
[10] C. Vandenbosch, D. Luc Massart and W. Lindner, J. Pharm. Biomed. Anal., 10 (1992) 895–908.
[11] E. De Lorenzi, A.F. Fell, G. Caccialanza, G. Massolini and M. Kitsos, J. Pharm. Biomed. Anal., 10 (1992) 909–915.
[12] T. Ohkubo, T. Uno and K. Sugawara, J. Chromatogr. A, 659 (1994) 467–471.
[13] P.A. Soons, M.C.M. Roosemalen and D.D. Breimer, J. Chromatogr., 528 (1990) 343–356.
[14] T. Sakamoto, Y. Ohtake, M. Itoh, S. Tabata, T. Kuriki and K. Uno, Biomed. Chromatogr., 7 (1993) 99–103.
[15] J.D-Y. Dru, J.Y-K. Hsieh, B.K. Matuszewski and M.R. Dobrinska, J. Chromatogr. B, 666 (1995) 259–267.
[16] M. Yamaguchi, K. Yamashita and I. Aoki, J. Chromatogr., 575 (1992) 123–129.
[17] T. Iwaoka, N. Inotsume, J. Inoue, S. Naomi, Y. Okamoto, S. Higuchi, M. Nakano and T. Umeda, Eur. J. Clin. Pharmacol., 48 (1995) 345–349.
[18] Y. Tokuma, T. Fujiwara and H. Noguchi, J. Pharm. Sci., 75 (1987) 310–313.
[19] C. Fischer, F. Schönberger, W. Mück, K. Heuck and M. Eichelbaum, J. Pharm. Sci., 82 (1993) 244–250.
[20] R. Heinig, V. Muschalek and G. Ahr, J. Chromatogr. B, 655 (1994) 286–292.
[21] D. Zimmer and V. Muschalek, J. Chromatogr. A, 666 (1994) 241–248.
[22] H. Fujitomo, I. Nishino, K. Ueno and T. Umeda, J. Pharm Sci., 82 (1993) 319–322.
[23] Y. Tokuma and H. Noguchi, J. Chromatogr. A, 694 (1995) 181–193.
[24] J. Hermansson, Trends Anal. Chem., 8 (1989) 251–259.
[25] A. Karlsson, K. Pettersson and K. Hernquist, Chirality, 7 (1995) 147–153.
[26] E. De Lorenzi, A.F. Fell, A.L. Holmes, G. Caccialanza, G. Massolini, C. Gandini, M. Kitsos and C. Ponci, Chirality, 5 (1995) 622–626.
[27] M. Josefsson, A.-L. Zackrisson and B. Norlander, J. Chromatogr. B, 672 (1995) 310–313.
[28] M. Enquist and J. Hermansson, J. Chromatogr., 519 (1990) 271–283.

[29] J. Hermansson and A. Grahn, J. Chromatogr. A, 694 (1995) 57–69.
[30] M. Enquist and J. Hermansson, J. Chromatogr., 519 (1990) 285–298.
[31] P. Campíns-Falcó, R. Herráez-Hernández and A. Sevillano-Cabeza, J. Chromatogr., 619 (1993) 177–190.
[32] A. Walhagen and L.-E. Edholm, J. Chromatogr., 473 (1989) 371–379.