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Resolution and Determination of Enantiomeric Purity of the Enantiomers of Felodipine Using Chiral-AGP® as Stationary Phase

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ABSTRACT A direct chiral chromatographic reversed phase method for the determination of the enantiomers of felodipine is described. The influence of charged and uncharged modifiers as well as the effect of the mobile phase pH on the enantiomeric resolution is discussed. A high mobile phase pH and the addition of 2-propanol as organic modifier gave the highest separation factor (α = 1.3). The high mobile phase pH (pH = 7.6) is outside the recommended pH limit of silica based columns but was necessary to achieve baseline resolution of (R)- and (S)-felodipine. Improvement of column efficiency by increasing column temperature was utilized for optimization of the enantiomeric resolution (Rs = 1.7). The enantiomers of felodipine and three related compounds were separated within 15 min. The enantiomeric purity of (R)- and (S)-felodipine in injections and (R)-felodipine in bulk substance was higher than 99.5% and no racemization was observed after storage at accelerated conditions. A poor Chiral-AGP® column used for a long period was restored using a simple wash step together with repacking the top of the chromatographic column.

KEY WORDS: direct chromatographic chiral separation of felodipine, mobile phase pH, column temperature, enantiomeric purity, restored chiral column

Felodipine is an effective antihypertensive drug, when given both alone and in combination with other antihypertensive agents.[1–3] It acts as a calcium antagonist with high vascular selectivity,[4] which means that the effect on the vascular smooth muscle is much more pronounced than that on the heart. Thus no negative inotropic effects are seen in therapeutic doses[5] nor does felodipine affect conduction in the heart.[6]

Felodipine is a nonprotolytic 1,4-dihydropyridine derivative with extremely low solubility in water. It is susceptible to oxidation. In this process the dihydropyridine moiety of felodipine is aromatized giving the pyridine analog.

Felodipine is a racemic mixture and the molecule has one chiral center. Both enantiomers of felodipine are hemodynamically active, but in vitro data indicate that the (S) form is slightly more potent than the (R) form.[7]

To elucidate the enantiomeric purity and stability of (R)- and (S)-felodipine injections and the bulk substance of (R)-felodipine, a chromatographic separation method using a protein immobilized on silica as the chiral stationary phase was developed. A minor stability study was initiated to determine the extent of racemization.

Different proteins immobilized on silica particles have been successfully used as chiral discriminators.[8] Albumin,[9] α_1-acid glycoprotein,[10] α-chymotrypsin,[11] and cellulase[12] are some proteins used for resolution of enantiomers. Enantiomers of basic compounds, acids, and aprotic solutes were successfully separated with these protein phases in reversed phase chromatography.[8–12] In view of its superior chromatographic performance in comparison with Resolvosil® and Chiralcel OD R®, Chiral-AGP® was the column mostly used in the present study.

Earlier studies have shown that addition of charged and uncharged modifiers to the mobile phase could effect the enantiomeric separation when using Chiral-AGP® as the chiral solid phase.[13] The mobile phase pH and column temperature were also previously shown to effect chiral recognition.[14] In this study the mobile phase composition and column temperature were varied to optimize the chiral resolution of the enantiomers of felodipine.

MATERIALS AND METHODS

Chemicals

Methanol, acetonitrile, 2-propanol (all solvents of LiChrosolv quality), sodium dihydrogen phosphate (p.a.), and disodium hydrogen phosphate (p.a.) were obtained from Merck (Darmstadt, Germany). Ornithine and cysteine were purchased from Sigma (St. Louis, MO). Cholic acid (as sodium salt) was bought from Fluka (Buchs, Switzerland). (R,S)-, (R)-, and (S)-felodipine (4-(2,3-dichlorophenyl)-1,4-dihydro-

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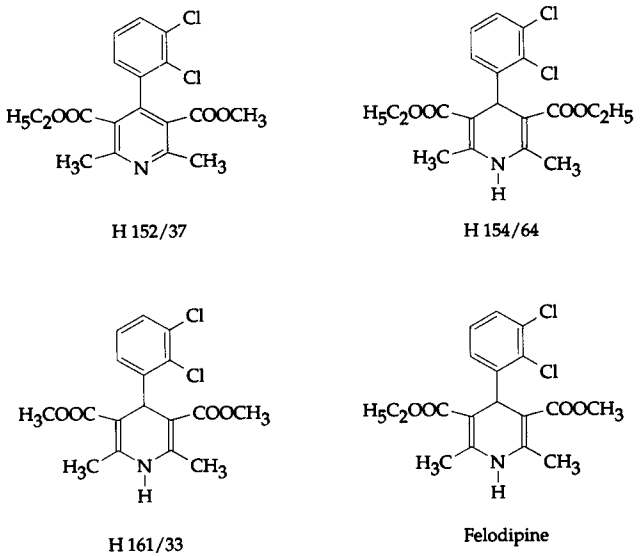


Fig. 1. Structure of solutes.

TABLE 1. Effect of uncharged modifiers on enantioselectivity

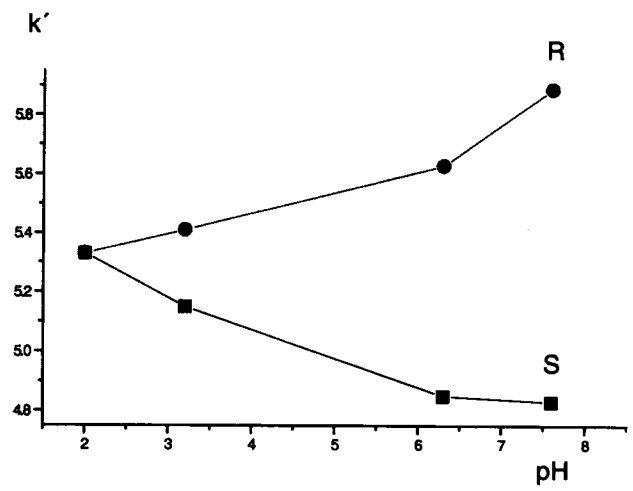
Modifier	t_{R1}	α
2-Propanol	6.71	1.22
Methanol	>50	—
Acetonitrile	2.48	1.0

Stationary phase: Chiral-AGP®.
Mobile phase: phosphate buffer (pH = 7.6): modifier (85:15).

2,6-dimethyl-3,5-pyridinedicarboxylic acid ethyl methyl ester) and related compounds, shown in Figure 1, were synthesized at Astra Hässle AB (Mölndal, Sweden). Felodipine as bulk substance and solutions for injection were also prepared at Astra Hässle AB.

Chromatography

The chromatographic system consisted of an LKB 2248 pump (Pharmacia, Uppsala, Sweden), a PE ISS-200 autosampler (Perkin-Elmer, Norwalk, CT), and a UV detector (Jasco UV-975; Jasco, Tokyo, Japan). The Chiral-AGP® column (150 × 4.6 mm, 5 μm), consisting of α_1-acid glycoprotein as the immobilized protein, was purchased from ChromTech (Stockholm, Sweden). The Resolvosil® column with albumin as the chiral selector was obtained from Machery Nagel (Germany). Chiralcel OD R® with cellulose as chiral selector was purchased from Dionex (Japan). No guard column was used. The temperature of the column and solvent reservoir was maintained at 45°C unless otherwise stated by means of a water bath (Lauda RM 20 T; Lauda, Königshofen, Germany). Flow rate was 1.0 ml/min. Solutes were detected at 225 nm. Injection volumn was 40 μl and the sample concentration 0.0003 to 0.2 m*M*.



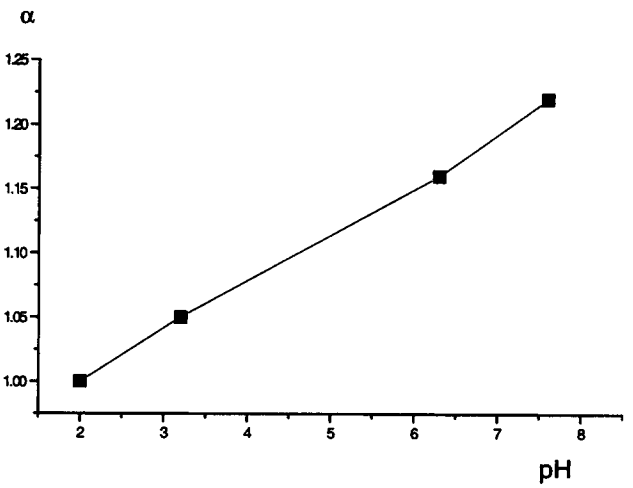


Fig. 2. Enantioselective retention and pH. Stationary phase: Chiral-AGP®. Mobile phase: phosphate buffer (I = 0.01):2-propanol (85:15).

TABLE 2. Effect of charged modifiers on enantioselectivity

Mobile phase additive	t_{R1}	α
—	8.63	1.30
Cysteine (5 mM)	9.18	1.32
Ornithine (5 mM)	9.68	1.33
Cholic acid (5 mM)	7.63	1.29

Stationary phase: Chiral-AGP®.
Mobile phase: phosphate buffer (pH = 7.6, I = 0.01): 2-propanol (9:1).
Column temperature: 37°C.

RESULTS AND DISCUSSION

Chiral Stationary Phase

The enantioselective separation of felodipine was tested using three different chiral stationary phases (CSPs): Chiral-

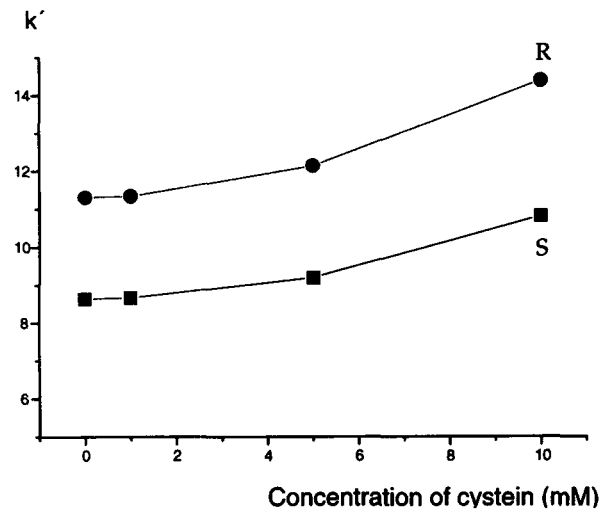


Fig. 3. Influence of the concentration of cysteine on the enantioselective retention of (R)- and (S)-felodipine. Stationary phase: Chiral-AGP®. Mobile phase: phosphate buffer (pH = 7.6, I = 0.01):2-propanol (9:1). Column temperature: 37°C.

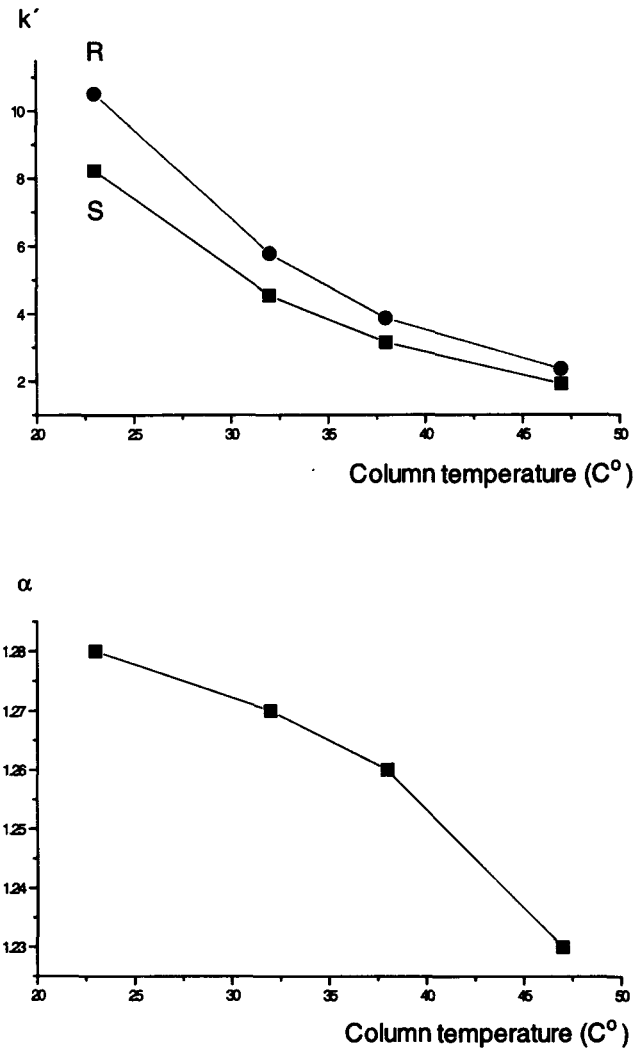


Fig. 4. Column temperature and enantioselective retention. Stationary phase: Chiral-AGP®. Mobile phase: phosphate buffer (pH = 7.6, I = 0.01):2-propanol (85:15).

cel OD R®, Resolvosil®, and Chiral-AGP®. The first CSP is based on immobilized cellulose and the other two stationary phases utilized two different proteins as the chiral selector. The three CSPs were tested using a mobile phase with a rather high pH (pH = 7.6) and 15% 2-propanol as the organic modifier. The high content of the organic modifier in the mobile phase was necessary to obtain reasonable retention times. The cellulose column gave a long retention with this mobile phase and no enantioselective separation was observed. By using the same mobile phase on the Resolvosil column a tendency to enantioselective retention was obtained. However, due to low column efficiency this column was not an adequate choice for further studies of the separation of the enantiomers of felodipine. An acceptable separation factor (1.3) was observed when using the Chiral-AGP column. Due to the high column efficiency of about 10000–20000 theoretical plates per meter, this CSP was selected for determination of the enantiomeric purity of (R)- and (S)-felodipine.

Effect of Uncharged Modifiers

Previous works have shown that different uncharged organic modifiers can affect the enantioselective separation differently when using immobilized protein phases.[13,15] In this study three common organic modifiers, i.e., methanol, acetonitrile, and 2-propanol, were compared with respect to enantioselective retention (Table 1). The same percentage (15% v/v) of each modifier was added to the mobile phase. Methanol as modifier gave high retention times and the enantiomers of felodipine were not eluted within 1 h. Acetonitrile, however, eluted the enantiomers extremely quickly (both enantiomers eluted within 3 min). However, no enantioselectivity was obtained using this concentration of acetonitrile as the organic modifier. Enantioselective retention was obtained ith 15% (v/v) 2-propanol added to the mobile phase as the organic modifier. The two peaks were eluted within 10 min, with a separation factor of 1.2.

Conformational changes of the immobilized protein induced by the different organic modifiers might explain differences in stereoselectivity.

Effect of pH

The pH in the mobile phase often affects enantioselective resolution when using CSPs with immobilized protein as the chiral selector.[14] In several cases the variation in pH can influence the charge of the solute as well as the charge of the protein. However, in this case the solute, felodipine, is an aprotic uncharged substance which is not influenced by a change of the mobile phase pH. A variation in the enantioselective retention by varying the mobile phase pH must therefore be caused by a change of the protein surface.

The capacity factor for the first eluted enantiomer decreased slightly when increasing the mobile phase pH, whereas the capacity factor for the second eluted enantiomer increased (Fig. 2). This resulted in an increase in the separa-

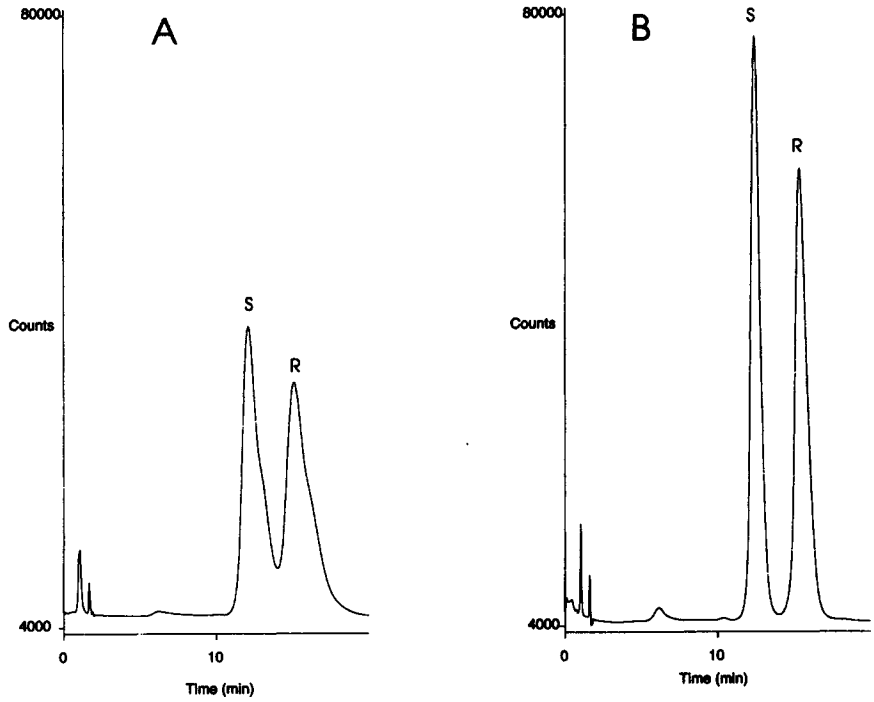


Fig. 5. Enantioselective separation of felodipine before (**A**) and after (**B**) restoring a poor Chiral-AGP® column. Mobile phase: phosphate buffer (pH = 7.6, I = 0.002):2-propanol (9:1). Column temperature: 45°C.

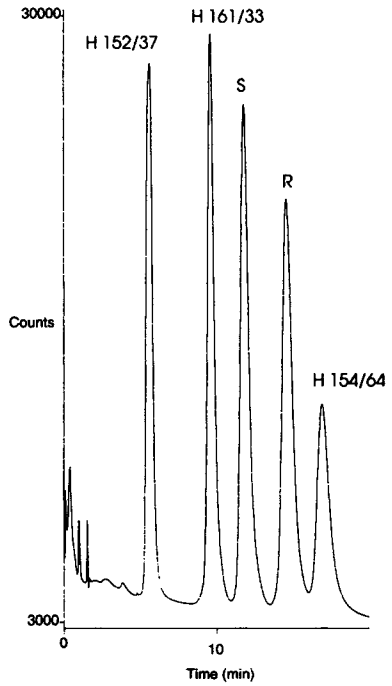


Fig. 6. Separation of the enantiomers of felodipine and related compounds. Chromatographic system as in Figure 5.

tion factor from 1.00 to 1.22 when the pH in the mobile phase was increased from 2.0 to 7.6 (Fig. 2). Because the immobilized protein, α_1- acid glycoprotein, has an isoelectric point between 2 and 3[16] in its native form, the amount of negative charge in the protein increases and the positive charge in the protein decreases when increasing the mobile phase pH from 2.0 to 7.6. This change in charge may cause a change of the three-dimensional structure of the immobilized protein, resulting in improved enantioselectivity for the enantiomers of felodipine. The positive effect on enantioselective retention may also originate from the formation of a new chiral site, arising when the charge changes on the protein surface.

Effect of Charged Modifiers

The effect of some charged modifiers (two polar compounds, ornithine and cysteine, and one hydrophobic compound, cholic acid) on the enantioselective retention were also tested (Table 2). No complex binding between felodipine and the charged additives was expected because of the aprotic and hydrophobic character of felodipine. Thereafter, the likely way for the charged additives to affect the enantioselective retention is to induce a conformational change of the immobilized protein or to compete for the limited number of adsorption sites. If the mobile phase additive mainly competes for the solute in the chiral mechanism, this may decrease enantioselectivity, but if the mobile phase additive mostly competes for achiral adsorption sites, this could increase enantioselectivity.

The two polar additives slightly improved the enantioselectivity and gave higher retention times (Table 2). A further increase of cysteine concentration in the mobile phase increased the retention times but only slightly improved stereoselectivity (Fig. 3). This suggests that the polar additives interact with the chiral stationary phase and create a new adsorption site for the chiral solutes. The hydrophobic addi-

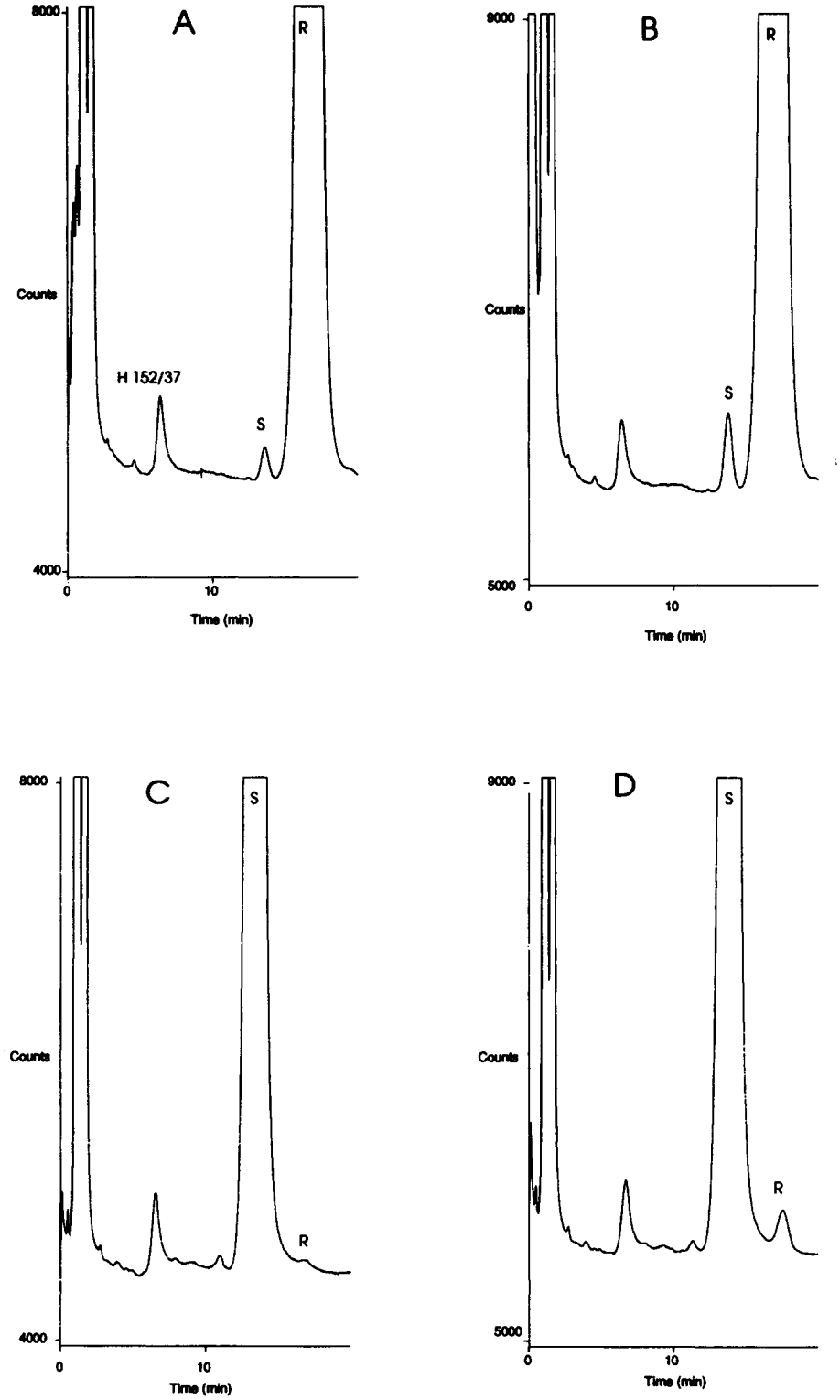


Fig. 7. Determination of the enantiomeric purity of (R)- and (S)-felodipine. Chromatograms of samples containing an added 0.5% of the respective antipode (**B** and **D**) were compared with authentic samples (**A** and **C**). Sample concentration injected 0.1 m*M*. Chromatographic conditions as in Figure 5.

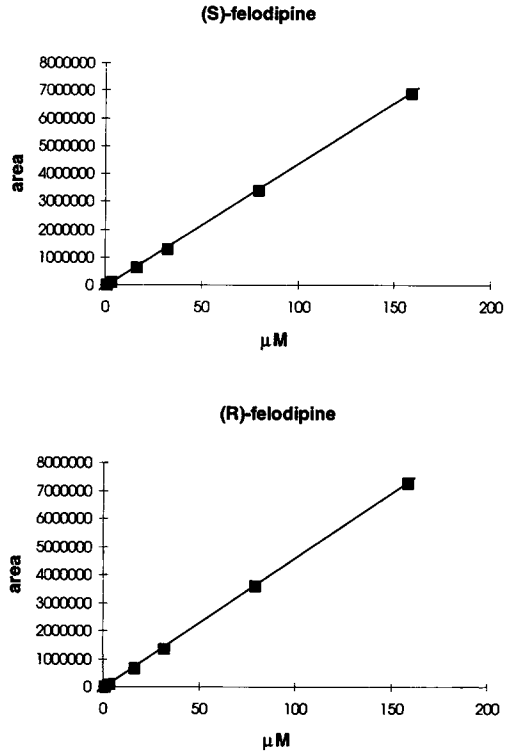


Fig. 8. Linearity. Chromatographic conditions as in Figure 5.

tive, cholic acid, had almost no effect on stereoselectivity but shortened the retention times for the enantiomers of felodipine, which indicated competition for the limited amount of adsorption sites. Conformational changes of the protein induced by the polar and hydrophobic additives might be an explanation for the slight change in enantioselective retention.

Effect of Column Temperature

Several authors have shown that changing column temperature might improve enantioselective resolution.[17–19] In general, increasing the column temperature decreased both enantioselectivity and retention but often increased column efficiency. Depending on the relative changes of these chromatographic parameters, the enantioselective resolution may increase or decrease. By increasing the column temperature from 23°C to 47°C the enantioselectivity and retention slightly decreased (Fig. 4). However, due to an increased column efficiency, a column temperature above ambient was preferred for maximal enantioselective resolution.

The enantiomeric separation of felodipine (Rs = 1.7), using phosphate buffer (pH = 7.6, I = 0.002):acetonitrile (9:1) as the mobile phase and a column temperature of 45°C, is given in Figure 5. This chromatographic system also allowed selective retention of three felodipine-related compounds (Fig. 1) besides the enantioseparation of felodipine (Fig. 6).

The high column temperature and the high pH in the mobile phase decrease the long-time stability of the separation column. However, the column could be restored by washing with 50–100 column volumes of 25% acetonitrile in water and, if necessary, repacking the top of the column with LiChrospher DIOL (Fig. 5). Approximately 500 routine injections were made on the column used throughout this study. During this period the column was repacked twice.

Chromatograms obtained from injections of solutions of (R)- and (S)-felodipine spiked with 0.5% of its antipode and authentic samples are shown in Figure 7. These chromatograms show a high enantiomeric purity of both (R)- and (S)-felodipine, each with less than 0.5% of its antipode, respectively.

Linearity

The chromatographic system showed good linearity when injecting samples in the concentration range from 0.3 μ*M* to 158 μ*M* (Fig. 8). Correlation coefficients for the standard curves were 0.9999 for both (R)- and (S)-felodipine. Linear standard curves for concentration vs. area were obtained despite a slight decrease in peak performance when injecting the highest sample concentrations. These high sample concentrations were necessary for the accurate determination of low amounts of enantiomeric impurities, less than 0.5%, in (R)- and (S)-felodipine.

The minimum detectable quantities, expressed as chromatographic peaks twice the baseline noise, were 2.5 (S) and 3.0 (R) picomoles for the two enantiomers of felodipine.

TABLE 3. Enantiomeric purity after storage at accelerated conditions

	Storage time (months)	Storage conditions[a] (°C/% r.h.)	Enantiomeric purity substance	Enantiomeric purity injection
R-felodipine	0		99.4	99.5
	3	25/65	99.4	99.5
	3	40	99.5	99.5
S-felodipine	0			>99.5
	3	40		>99.5

[a] Temperature in °C/relative humidity.
Stationary phase: Chiral-AGP®.
Mobile phase: phosphate buffer (pH = 7.6): modifier (9:1).

Stability of Felodipine Stored at Accelerated Conditions

The optimized chromatographic system was used for determination of (R)- and (S)-felodipine in bulk substance and injections stored under normal and accelerated conditions. No racemization was obtained in either (R)- or (S)-felodipine injections or in (R)-felodipine bulk substance after accelerated storage (Table 3).

No change in other stability parameters, such as appearance, pH (only injection), and assay performed by achiral liquid chromatography, was observed. The increase of the oxidation product (pyridine) estimated by achiral liquid chromatography was moderate (less than 0.5% w/w of felodipine).

CONCLUSION

The enantiomers of felodipine, a 1,4-dihydropyridine derivative used as an antihypertensive drug, were directly resolved using Chiral AGP as the chiral chromatographic column. Enantiomeric resolution was influenced by the mobile phase pH, type of uncharged modifier, and column temperature. Besides the separation of the enantiomers of felodipine, the optimized chiral chromatographic system also separated three related compounds.

Linear standard curves for (R)- and (S)-felodipine were obtained in a concentration range of 0.3 μM–158 μM using a 40 μl loop. No racemization was observed for (R)- and (S)-felodipine in injections or for (R)-felodipine in bulk substance after storage at accelerated conditions.

A poor Chiral AGP column could be restored using a simple wash together with a repacking of the top of the chromatographic column.

LITERATURE CITED

1. Saltiel, E., Ellrodt, A.G., Monk, J.P., Langley, M.S. Felodipine, a review of its pharmacodynamic and pharmacokinetic properties, and therapeutic use in hypertension. Drugs 36:387–428, 1988.
2. Campell, L.M., Cowen, K.J., Cranfield, F.R., Goves, J.R., Jones, D.F., Lees, C.T.W., Richardson, P.D.I., Teal, J.A., Timerick, S.J.B. Felodipine-ER once daily as monotherapy in hypertension. J. Cardiovasc. Pharmacol. 15:569–573, 1990.
3. Brun, J., Fröberg, L., Kronmann, P., Olsson, L.B., Skoog, P., Tygesen, G., Bengtsson, C., Sherstén, B., Tibblin, G. Optimal felodipine dose when combined with metoprolol in arterial hypertension: a Swedish multicenter study within primary health care. J. Cardiovasc. Pharmacol. 15(suppl. 4):60–64, 1994.
4. Ljung, B. Vascular selectivity of felodipine. Drugs 29(suppl. 2):46–58, 1985.
5. Culling, W., Ruttley, M.S.M., Sheridan, D.J. Acute haemodynamic effect of felodipine during beta blockade in patients with coronary artery disease. Br. Heart. J. 52:431–434, 1984.
6. Amlie, J.P., Endresen, K., Sire, S. The effect of felodipine on the sinus and atrioventricular node in patients with ischaemic heart disease. J. Cardiovasc. Pharmacol. 15(suppl. 4):25, 1990.
7. Eriksson, U.G., Hoffmann, K.J., Simonsson, R., Regårdh, C.G. Pharmacokinetics of the enantiomers of felodipine in the dog after oral and intravenous administration of a pseudoracemic mixture. Xenobiotica 21:75–84, 1991.
8. Allenmark, S. Protein-bonded phases. In: Chiral Separations by HPLC: Applications to Pharmaceutical Compounds. Series in Medicinal Chemistry. Krstulovic, A.M., ed. Chichester: Ellis Horwood, 1989:285.
9. Allenmark, S., Bomgren, B., Borén, H. Direct resolution of enantiomers by liquid affinity chromatography on albumin-agarose under isocratic conditions. J. Chromatogr. 237:473–477, 1982.
10. Hermansson, J. Direct liquid chromatographic resolution of racemic drugs using α_1-acid glycoprotein as the chiral stationary phase. J. Chromatogr. 269:71–80, 1983.
11. Marle, I., Karlsson, A., Pettersson, C. Separation of enantiomers using α-chymotrypsin-silica as a chiral stationary phase. J. Chromatogr. 604: 185–196, 1992.
12. Erlandsson, P., Marle, I., Hansson, L., Isaksson, R,. Pettersson, C., Pettersson, G. Immobilized cellulase (CBH I) as a chiral stationary phase for direct resolution of enantiomers. J. Am. Chem. Soc. 112:4573–4574, 1990.
13. Hermansson, J., Eriksson, M. Direct liquid chromatographic resolution of acidic drugs using a chiral α_1-acid-glycoprotein column (EnantioPac). J. Liq. Chromatogr. 9:621–639, 1986.
14. Allenmark, S. Chapter 7. In: Chromatographic Enantioseparation, Methods and Applications, 2nd ed. Chichester: Ellis Horwood, 1992:130–132.
15. Balmér, K., Persson, B.A., Lagerström, P.O. Stereoselective effects in the separation of omeprazole and other substituted benzimidazoles on different chiral stationary phases. J. Chromatogr. 660:269–273, 1994.
16. Schmid, K. In: The Plasma Proteins. Putman, F. ed. New York: Academic Press, 1975:184–222.
17. Gilpin, R.K., Ethesham, S.E., Gregory, R.B. Liquid chromatographic studies of the effect of temperature on the chiral recognition of tryptophan by silica-immobilized bovine albumin. Anal. Chem. 63:2825–2828, 1991.
18. Arvidsson, E., Jansson, S.O., Schill, G. Chiral separation of atropine and homatropine on an α_1-acid glycoprotein-bonded stationary phase. J. Chromatogr. 506:579–591, 1990.
19. Jönsson, S., Schön, A., Isaksson, R., Pettersson, C., Pettersson, G. An unexpected temperature effect obtained on enantiomer separation using CBH I-silica as a chiral stationary phase: increase in retention and enantioselectivity at elevated column temperature: a chromatographic and microcalorimetric study. Chirality 4:505–508, 1992.