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Study of retention behaviour and enantioseparation of selected calcium antagonists on cyclodextrin stationary phases in high performance liquid chromatography

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Separation of a series of dihydropyridine calcium antagonists into their enantiomers was attempted with chiral selective columns, β-cyclodextrin (Chiradex) and S-naphthylethyl carbamoyl β-cyclodextrin (Cyclobond). 0.1 to 0.4% triethylamine adjusted to pH 5.0 containing different proportions of an organic modifier (methanol or acetonitrile) was used as mobile phase. The influence of the mobile phase composition on retention and separation is discussed. From a series of five calcium antagonists (nisoldipine, nimodipine, nitredipine, isradipine and amlodipine), only two (nisoldipine and nimodipine) were successfully separated into their enantiomers. It is assumed that in these separations inclusion mechanism plays an important role through the penetration of the benzene ring of the drug into the cyclodextrin cavity. The application of decreased column temperature resulted in a pronounced improvement of nimodipine enantiomer separation on Chiradex. It is proposed that separations at decreased temperatures can be exploited for semipreparative purposes.

La séparation d'une série d'antagonistes de la dihydropyridine calcique en leurs énantiomères a été tentée en utilisant deux colonnes à sélectivité chirale, c'est-à-dire à base de β-cyclodextrine (Chiradex) et S-naphtyléthyl carbamoyl β-cyclodextrine (Cyclobond). Les phases mobiles étaient des solutions de triéthylamine de 0,1 à 0,4% ajustées à pH 5 et renfermant différentes proportions d'un modifiant organique (le méthanol ou l'acétonitrile). L'influence de la composition de la phase mobile sur la rétention et la séparation est discutée. D'une série de cinq antagonistes de la dihydropyridine calcique (nisoldipine, nimodipine, nitrédipine, isradipine et amlodipine), seulement deux (nisoldipine et nimodipine) ont été correctement séparés en leurs énantiomères. Il semble que dans ces séparations le mécanisme d'inclusion joue un rôle important, notamment par la pénétration de l'anneau benzénique de ces produits dans la cavité de la cyclodextrine. L'application d'une température programmée descendante de la colonne a conduit à une meilleure séparation des énantiomères de la nimodipine sur le Chiradex. Il est proposé que la mise en œuvre de la programmation en températures décroissantes puisse être utilisée à des fins semi-préparatives.

Key words : Dihydropyridine calcium antagonists — Enantiomers — Cyclodextrin — Liquid chromatography.

Mots clefs : Antagonistes de la dihydropyridine calcique — Enantiomères — Cyclodextrine — Chromatographie liquide.

Chiral separations of optically active compounds have attained a considerable increase in interest during the past few years. This is reflected quite distinctly in the number of separated drugs and pharmacologically important compounds [1-3].

This is quite understandable as drug enantiomers may be considerably different in their pharmacological action. While one of the enantiomers may be active, the other could be inactive and may cause undesirable side effects or may even be toxic. Reasons for the enantioselective effects of drug action may be found in any phase of the drug interaction with the organism, i.e., in the absorption, distribution, metabolic or excretion phases [4]. Regardless of the phase involved, the main role is played by the enantioselective interaction of a particular drug with the biological receptor.

The new policy of the US FDA requires that newly introduced drugs be subjected to an estimation of the effects and pharmacokinetics of all isomers of the pharmacologically active components [5]. There are already some drugs which are produced as optically pure isomers and it is expected that in the near future a number of other drugs (still marketed as racemates) will be produced in the form of optically pure isomers (« enantiomeric switch »). This concerns e.g. beta-blockers [6] and dihydropyridine calcium antagonists.

The action mechanism of dihydropyridine calcium antagonists involves the inhibition of an L-type calcium channel which results in a decreased amount of calcium penetrating the cells with a consequent relaxation of smooth musculature of the vessel wall.

Dihydropyridine calcium antagonists possess one asymmetric carbon thereby yielding two enantiomers. Different effects of individual enantiomers upon the human organism were proven with most current calcium antagonists. For instance, felodipine [7] is subjected to stereoselective metabolism : (R)-felodipine is metabolized more readily than (S)-felodipine. Soons and Breimer [8] found stereoselectivity in the pharmacokinetics of nitredipine : bioavailability of the more potent (S)-(-)-nitredipine was 75% higher than that of (R)-(+)-nitredipine. The more efficient enantiomer usually exhibits the (S)-absolute configuration [7-10].

The effect of chiral discrimination on chiral stationary phases is based on the formation of labile diastereomeric complexes between the solute and the stationary phase. The differences in complex stability form the basis for the separation of optically active isomers. The mechanism of chiral stationary phases action, however, has not yet been fully elucidated.

Papers dealing with the chiral separations of some calcium antagonists have almost exclusively exploited modified cellulose as stationary phase in the « normal » mode, i.e., with the mobile phase formed by hexane with an addition of some more polar organic solvent [11,12]. It has also been reported that a stationary phase composed of α_1-acid glycoprotein is able to separate some dihydropyridine calcium antagonists [13].

Nisoldipine and nimodipine have been partially resolved on two 250 x 4.6 mm β-cyclodextrin columns connected in series [14] or on S-hydroxypropyl-β-cyclodextrin stationary phase [15]. Enantioseparation of amlodipine has been reported on S-hydroxypropyl-β-cyclodextrin stationary phase [16]. However, retention and chiral separation of dihydropyridines with β-cyclodextrin as chiral stationary phase has not been systematically studied as yet.

I. EXPERIMENTAL

1. Materials

The following chemicals have been used : triethylamine 99% (Sigma, St. Louis, United States), acetic acid p.a. (Lachema, Brno, Czech Republic), acetonitrile for chromatography (Merck, Darmstadt, Germany), methanol p.a. (Penta, Chrudim, Czech Republic) and de-ionized water. *Table I* specifies the drugs investigated in this study.

2. Methods

2.1. Sample preparation

Stock solutions of the dihydropyridine calcium antagonist were prepared by suspending halved tablets in acetonitrile and removing the insoluble material by filtration. Clear filtrate was used for further study. Concentration of the stock solutions was 200 mg/l of the active substance. For analysis, 0.1 ml of these solutions were taken to dryness and dissolved in 1 ml of the mobile phase ; 0.4 µg of each compound was injected onto the column.

Table I - Specification of drugs used throughout this study.

Active component	Drug name	Content of act. comp.	Provenience
Amlodipine	Norvasc	5 mg/tablet	Pfizer, Brussels, Belgium
Nitredipine	Baypress	20 mg/tablet	Bayer, Leverkusen, Germany
Isradipine	Loimir	2.5 mg/tablet	Sandoz, Basel, Switzerland
Nimodipine	Nimotop	30 mg/tablet	Bayer, Leverkusen, Germany
Nisoldipine	Baymycard	5 mg/tablet	Bayer, Leverkusen, Germany

2.2. High performance liquid chromatography measurement

All measurements were carried out on the Gilson chromatograph (Gilson, Middleton, United States) consisting of Asted autosampler, 115 UV Gilson detector, 805 manometric Gilson module, 305 and 306 Gilson piston pumps and 811C Gilson dynamic mixer of mobile phase. Commercially-available steel columns of either 250 x 4 mm or 250 x 4.6 mm were used, the former packed with Chiradex (native β-cyclodextrin covalently bonded by spacer on 5 µm silica, Merck, Darmstadt, Germany) and the latter with Cyclobond I 2000 SN (S-naphthylethyl carbamoyl β-cyclodextrin covalently bonded by spacer on 5 µm silica, Astec, Whippany, United States).

Mobile phases consisted of 0.1 to 0.4% triethylamine solution, the pH of which was adjusted by acetic acid. Appropriate amounts of either methanol or acetonitrile were added as specified in Section II. Flow rate of the used mobile phases was in all cases 0.8 ml/min.

In separations in which a decreased temperature was used, thermostating was ensured by cooling the mobile phase reservoir, capillaries and columns with a water-ice mixture. Chromatograms were evaluated by using the Apex Extra version 3.1 integration software (Apex, Prague, Czech Republic).

2.3. Measurement of UV spectra

Spectral measurements were done in the interval of 200 to 400 nm using Specord spectrophotometer (Carl Zeiss, Jena, Germany). According to the spectra obtained, the UV detector wavelength for high performance liquid chromatography detection was set to 239 nm.

II. RESULTS AND DISCUSSION

1. The effect of the organic modifier upon retention of dihydropyridine calcium antagonists

The dependence of the capacity factors on the proportion of the organic modifier in the mobile phase was determined using both columns packed with native β-cyclodextrin (Chiradex) and modified β-cyclodextrin (Cyclobond I SN). The mobile phases used were based on 0.1% triethylamine solution, the pH of which was adjusted by acetic acid to 5.0 (*figure 1*).

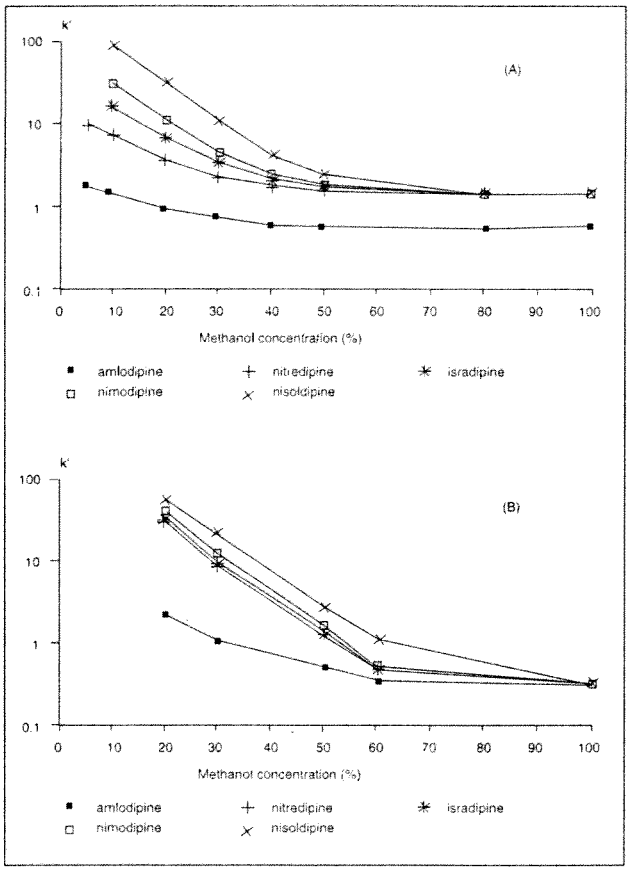

Figure 1 - Capacity factors of calcium antagonists (k') versus methanol concentration for Chiradex (A) and Cyclobond (B) columns.

Dihydropyridine calcium antagonists behave on the cyclodextrin phases in a similar way as on the conventional C_{18} reversed phase, i.e., with an increasing methanol concentration their retention becomes distinctly shorter. This dependence, however, is linear only at a weak elution strength of the mobile phase.

The retention order of different calcium antagonists on the Chiradex and Cyclobond columns depends on the hydrophobicity [17] of the individual derivatives. In *figure 2*, capacity factors obtained in the mobile phase containing 30% methanol are plotted against the constant of hydrophobicity (log Kd) values : the correlation is a linear function, which may be interpreted as if there were a direct interaction with the hydrophobic cavity.

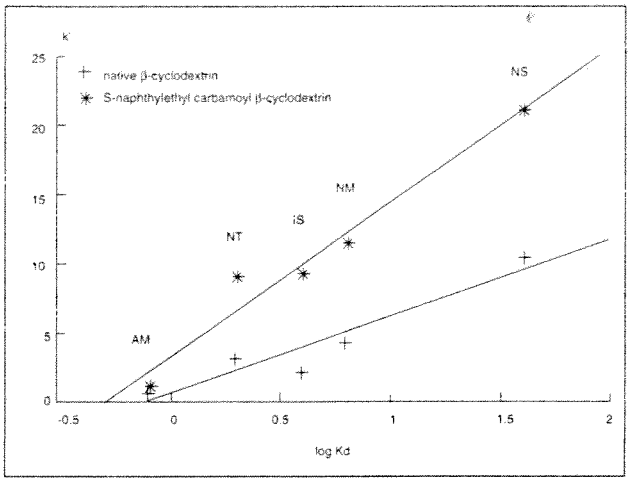

Figure 2 - Correlation between retention of calcium antagonists and log Kd (hydrophobicity).
AM : amlodipine. NT : nitredipine. IS : isradipine. NM : nimodipine. NS : nisoldipine. Hydrophobicity values taken from [17]. Mobile phase : 30% MeOH, 70% 0.1% triethylamine buffer pH 5.0.

Figure 3 summarizes the data obtained with acetonitrile as the organic modifier. The dependence shows a shallow minimum at 50% acetonitrile concentration. With the proportion of acetonitrile beyond 50%, the retention starts slowly to increase again. On the contrary to C_{18} phases, with cyclodextrin phases acetonitrile acts as a much stronger eluent ; as a matter of fact, 20% acetonitrile concentration corresponds approximately to a mobile phase containing 40% of methanol.

In spite of various theories attempting to explain the interaction of β-cyclodextrin with different compounds [18,19], it appears evident that at least in part such interactions can be explained on the basis of inclusion. In our particular case, we expect partial inclusion ; this assumption is based on the character and molecular dimensions of investigated solutes (*figure 4*). It appears feasible to expect that for steric reasons it will probably be the substituted benzene ring which will enter the cavity. Nisoldipine exhibits highest retention because the nitro group in the ortho position allows easier inclusion of the benzene ring than with nitredipine or nimodipine, where the nitro group is in the meta position. In the case of nimodipine, a longer and branched side-chain on the pyridine ring (as compared to nitredipine) is likely to cause a longer retention on the column because of the possibility of other interactions with the stationary phase. A behaviour similar to nitredipine and nimodipine can be predicted with isradipine, which contains a similarly voluminous substituent on the benzene ring. Indeed, our experimental data confirmed these expectations.

The investigated dihydropyridine derivatives are structurally closely related. The only member of this family which exhibits more distinct structural differences is amlodipine. Through the nucleophilic Cl substituent on the benzene ring (pKa 8.7), this compound will have considerably different physicochemical

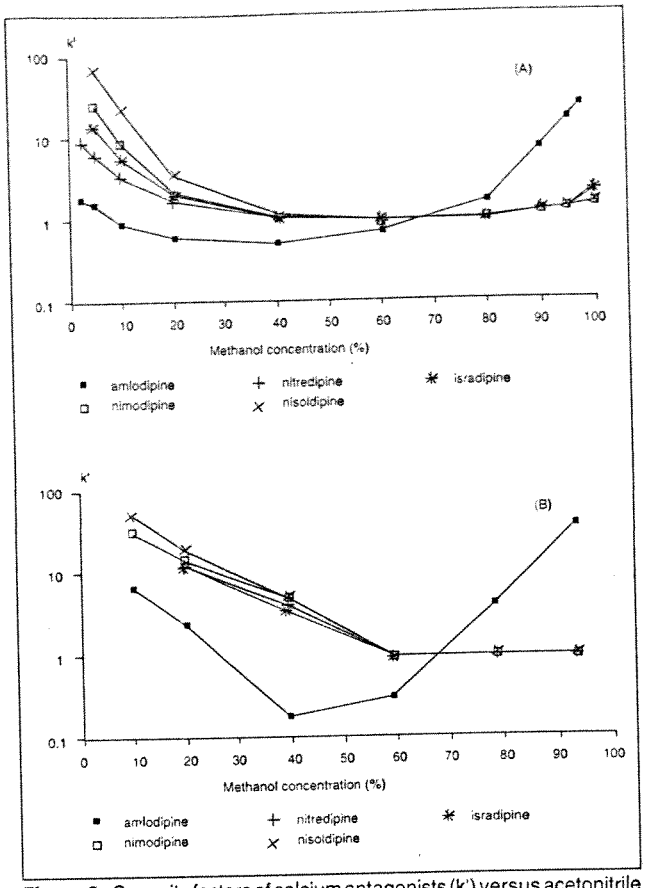

Figure 3 - Capacity factors of calcium antagonists (k') versus acetonitrile concentration for Chiradex (A) and Cyclobond (B) columns.

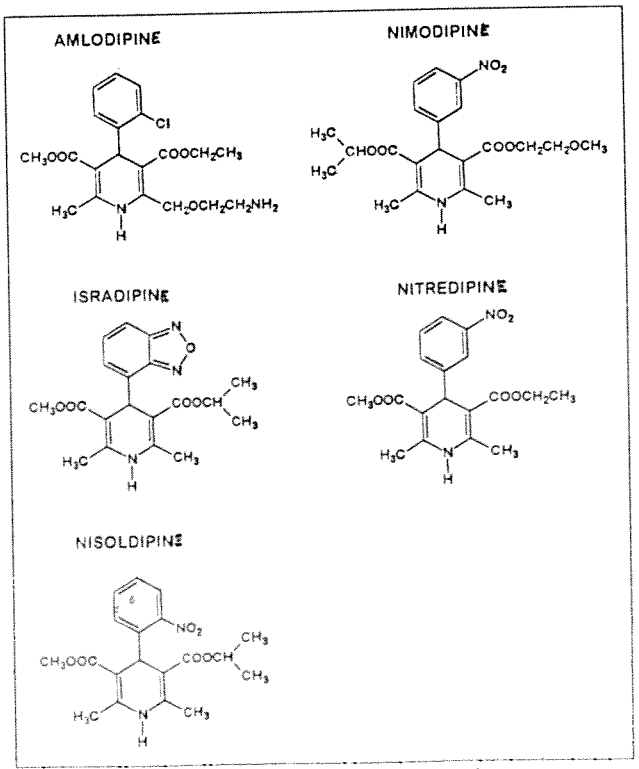

Figure 4 - Chemical structures of drugs investigated.

properties ; in acid media protonization occurs. This explains the low affinity of amlodipine to both the stationary phases used in our experiments at pH 5.0. Compared to other calcium antagonists, amlodipine shows a specific behaviour on β-cyclodextrin. With methanol as the organic modifier, its retention is distinctly lower and a decrease in methanol concentration does not lead to an increase in retention time. The retention behaviour of amlodipine could possibly be influenced at higher pH values ; this, however, is impossible to do because of the limited stability of the stationary phases.

The other difference of amlodipine is the aminoethoxymethyl moiety in the 2 position on the pyridinium ring. This difference manifested itself in a different retention behaviour compared to other dihydropyridine calcium antagonists in a mobile phase containing acetonitrile as organic modifier. The retention of amlodipine increases with the increased content of the organic modifier (from 40 to 90%) in the mobile phase, in spite of the fact that the higher concentration of acetonitrile limits the inclusion effect. This anomalous behaviour of amlodipine was further verified by estimation of the same dependence at pH 4.0.

It can be speculated that, for example, the primary amino group of amlodipine may interact with β-cyclodextrin through an H-bridge formation.

For the modified β-cyclodextrin column, the k' dependence on the methanol concentration in the mobile phase (buffer pH 5.0) is shown in *figure 1B*. When using pure methanol, the respective capacity factors are lower than in the case of Chiradex. With a decreasing methanol concentration, however, the increase in retention is more steeper than that observed with the unmodified (native) β-cyclodextrin column. There are several reasons that may be considered in this context. First, this behaviour may be related to the coverage of the sorbent with the modified cyclodextrin, second, this behaviour may reflect the role of the spacer, or, finally, the substitution of the cyclodextrin ring may affect the size of the cavity and favour the hydrophobic interaction of dihydropyridine calcium antagonists with cyclodextrin in an aqueous environment.

2. Factors influencing the enantioseparation

2.1. The influence of the stationary phase upon the enantioseparation

The selection of a stationary phase naturally influences enantioseparation. However, even with structurally related solutes, the selectivity of a particular chiral phase need not necessarily be better.

In our case, only the two most retained dihydropyridinium derivatives, nimodipine and nisoldipine, were separated on cyclodextrin phases. The α and R_s values obtained with different methanol and acetonitrile concentrations on both types of columns are presented in *table II*.

Table II - Capacity factors (k'_2) of the more retained enantiomer, R_s and α values at corresponding methanol and acetonitrile concentrations in the mobile phase for both types of enantioselective columns investigated.

Proportion of the org. modif.	Chiradex			Cyclobond I SN		
	k'_2	α	R_s	k'_2	α	R_s
MeOH	nimodipine			nimodipine		
10	29.80	1.14	1.35			
20	10.44	1.13	1.15	38.93	1.00	0.00
30	4.23	1.09	0.71	11.57	1.00	0.00
ACN	nimodipine			nimodipine		
5	25.30	1.12	0.99			
10	8.53	1.09	0.60	19.33	1.00	0.00
20	2.08	1.00	0.00	6.67	1.00	0.00
MeOH	nisoldipine			nisoldipine		
10	82.25	1.04	0.53			
20	28.46	1.03	0.28	51.21	1.06	1.04
30	10.34	1.00	0.00	21.08	1.05	0.42
ACN	nisoldipine			nisoldipine		
5	68.38	1.07	0.80			
10	22.95	1.08	0.60	35.92	1.02	0.30
20	3.68	1.00	0.00	10.25	1.00	0.00

Nimodipine enantiomers are clearly separated on the native β-cyclodextrin column while on the modified cyclodextrin column, under identical conditions, the separation is not even indicated even though the retention is higher in the latter case. On the contrary, better separations of nisoldipine enantiomers were obtained with Cyclobond I SN and methanol as the mobile phase modifier at ambient temperature, while with acetonitrile such behaviour was not observed. Nimodipine separations in several mobile phases with various content of methanol are shown in *figure 5*.

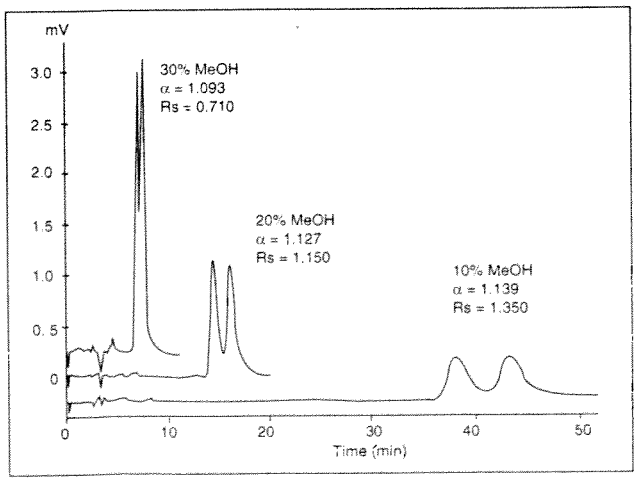

Figure 5 - Separation of nimodipine enantiomers with different proportion of methanol in the mobile phase (buffer 0.1% triethylamine pH 5.0) (Chiradex column).

2.2. The effect of triethylamine content on enantioselectivity

Triethylamine is traditionally used as a polar organic mobile phase modifier in liquid chromatography. Its function is to minimize possible solute interactions with residual silanol groups on the sorbent surface. In selected situations and within a specified concentration range, triethylamine may help enantioseparations [20].

The effect of triethylamine concentration upon the separation factor α was investigated with both columns. With Chiradex as the stationary phase and with mobile phase containing 30% methanol at pH 5.0 and triethylamine concentration varying between 0 and 0.4%, neither the separation of nimodipine nor its retention were changed. With Cyclobond SN and with identical mobile phase as described above, the retention decreases exponentially with increasing concentration of triethylamine (*figure 6*). The separation of nisoldipine into its enantiomers is also influenced by the presence of triethylamine in the mobile phase. The highest resolution was obtained at 0.2% triethylamine concentration in the eluent. Because the separation factor remains unchanged ($\alpha = 1.05$), it appears that the improved separation (increased R_s values) reflects the improved peak symmetry.

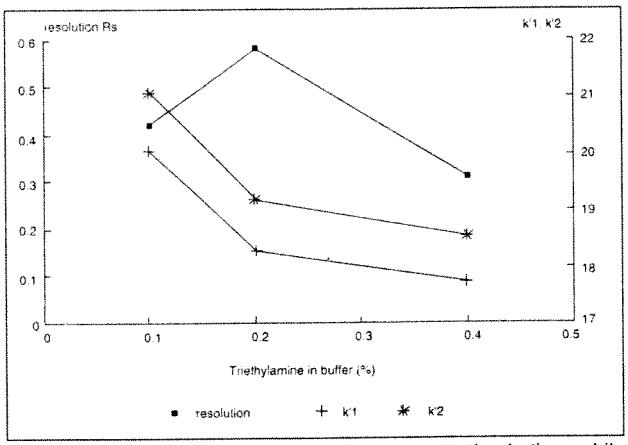

Figure 6 - The influence of triethylamine concentration in the mobile phase buffer (pH 5.0), 30% methanol as organic modifier (Cyclobond column) for nisoldipine.

2.3. The effect of temperature upon enantioseparation

The temperature at which the enantioseparation is carried out may influence the result considerably. There are no general rules stating that either increased or decreased temperature will lead to a better separation. In the case of liquid column chromatography, however, mostly decreased temperatures offer better separation. Indeed, the native β-cyclodextrin column cooled to 0°C showed an improvement in both the separation factor α and resolution R_s of nisoldipine (*figure 7*) and nimodipine (*figure 8*). However, none of the other dihydropyridine derivatives exhibited even an indication of enantiomer separation.

Cooling of the modified cyclodextrin column led to increased retention of nisoldipine, but neither the separation factor nor the resolution were improved. This is probably due to the fact that in enantioseparation H-binding plays an important role ; with decreased temperature, the role of H-binding to native

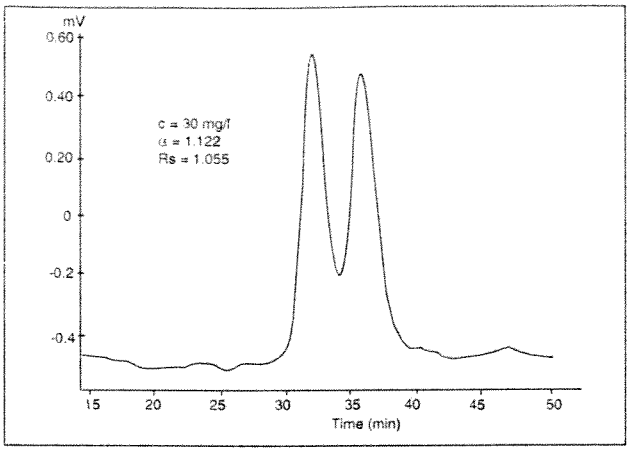

Figure 7 - Separation of nisoldipine enantiomers on the cooled Chiradex column. 15% acetonitrile, 85% buffer 0.1% triethylamine, pH 5.0, 0°C.

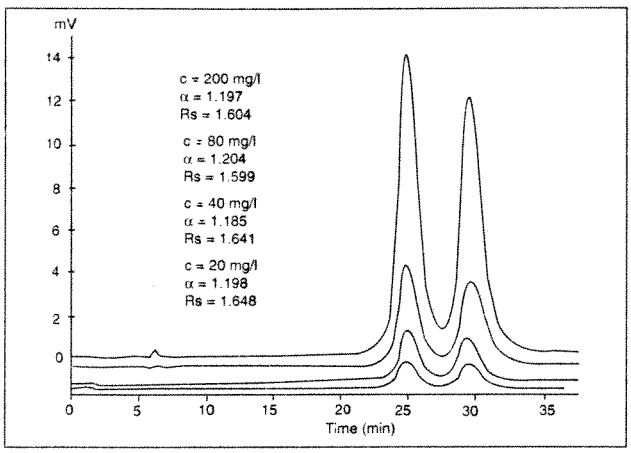

Figure 8 - Separation of nimodipine enantiomers with different amounts injected onto the column. Chiradex column, mobile phase 0.1% triethylamine, pH 5.0, 30% methanol, 0°C.

cyclodextrin apparently becomes more pronounced. On the other hand, H-bridges are not involved in the Cyclobond phase due to derivatization.

2.4. The effect of the injected amount upon separation

These effects were investigated on Chiradex cooled column using a mobile phase containing 30% methanol and 0.1% triethylamine at pH 5.0. *Figure 8* shows typical chromatograms of nimodipine enantiomer separation. Within the investigated range (0.4 to 4.0 µg of the solute) the separation is not worse with increase in the absolute amount injected. Further experiments indicated that the injected amount of nimodipine can be raised up to 40 µg using a 100 µl sample loop without any loss of resolution. It can be concluded that the specified conditions can be used for semipreparative purposes as well.

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