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Design of chiral LC separations for calcium antagonists on α_1-acid glycoprotein and ovomucoid columns*

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Abstract: Three chiral calcium antagonist drugs, gallopamil and two dihydropyridine derivatives, have been successfully separated within short retention times using both the α_1-acid glycoprotein chiral stationary phase (Chiral-AGP) and the ovomucoid column (Ultron ES-OVM). Aqueous buffer at defined pH is modified by the addition of an organic component, in order to modulate the retention properties of each system. Optimization of pH and organic modifier is carried out using the modified simplex method, with Kaiser's peak separation function as a criterion. The influence of pH and percentage of organic modifier on retention, selectivity, resolution and column performance are discussed for the two dihydropyridines analysed on Chiral-AGP and Ultron ES-OVM stationary phases. A new method is proposed as a new chiral system suitability test for these protein-based phases, utilizing a racemic mixture of closely eluting verapamil enantiomers as a probe.

Keywords: *Chiral LC separation; gallopamil; verapamil; dihydropyridines; α_1-acid glycoprotein; ovomucoid; modified simplex method; chiral system suitability test.*

Introduction

Increasing attention has been recently focused on drugs with specific action on ion channels, which are known to be chiral receptors [1]. Good examples of this class of compound widely used in therapy are the calcium channel blockers, which are currently administered as cardiovascular agents. The often dramatic qualitative and quantitative differences in activity seen with the enantiomers of calcium channel drugs have both biomedical and regulatory implications [2]. Chiral drugs are well known to exhibit different clinical properties for each enantiomer [2].

Literature on the enantiomeric separation of this class of compound by LC is not very extensive, and there is very little on the protein-based chiral stationary phases. Previous work has been reported on modified cellulose coated onto silica [3], phenylcarbamates of polysaccharides [4] and the first-generation α_1-acid glycoprotein column [5]. Protein-based chiral stationary phases have become well established for the direct separation of drug enantiomers because of their broad applicability and the use of aqueous buffered mobile phases that are compatible with many biological samples.

The aim of the present work was to examine the factors affecting the direct resolution by chiral LC of gallopamil, an analogue of verapamil, and of two calcium antagonists belonging to the class of 1,4-dihydropyridines, nimodipine and RBH20 (Figs 1 and 2). RBH20 is a Sandoz compound, Isradipine. The substituents are declared in Fig. 2.

The two chiral stationary phases that have been used for this work were the second-generation α_1-acid glycoprotein, Chiral-AGP [6], and the ovomucoid column, Ultron ES-OVM, recently developed by Miwa and co-workers [7].

Experimental

Materials

Isradipine was kindly provided by Sandoz (Milan, Italy); gallopamil was used as received from the Department of Pharmaceutical

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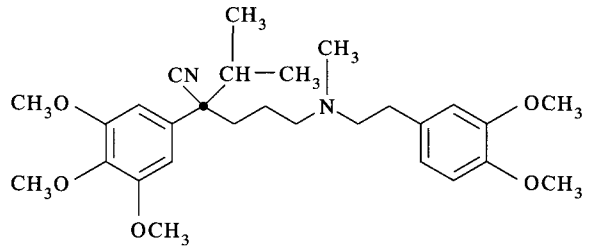

Figure 1
Molecular structure of gallopamil.

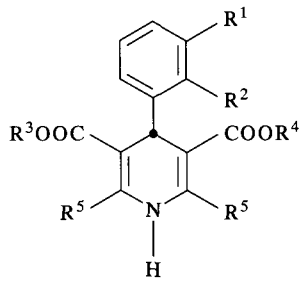

Figure 2
Structure of dihydropyridine compounds. Nimodipine: $R_1 = NO_2$, $R_2 = H$, $R_3 = CH_2CH_2OCH_3$, $R_4 = CH(CH_3)_2$, $R_5 = CH_3$. RBH20: R_1 and R_2 form a five-membered cyclic ring; =N—O—N=; R_3, R_5 are methyl groups; $R_4 = CH(CH_3)_2$.

Chemistry, University of Pavia; nimodipine was kindly supplied by Bayer (Milan, Italy). Buffer salt KH_2PO_4 was purchased from BDH Laboratory Supplies (Poole, UK). Propan-2-ol and acetonitrile were from Rathburn (Walkerburn, UK); ethanol 96% v/v was purchased from Hayman Ltd (Witham, UK). All reagents were of HPLC grade and were used as received.

Equipment

The chromatographic system consisted of a model 302 Gilson HPLC pump equipped with a 802C manometric module (Gilson, Villiers Le Bel, France), a Rheodyne injection valve with a 20-μl loop (Rheodyne, Berkley, CA, USA), a LKB 2151 variable wavelength detector (LKB, Bromma, Sweden) and a chart recorder (Model BD-40, Kipp & Zonen, Delft, The Netherlands).

The second generation α_1-acid glycoprotein analytical column and guard column (Chiral-AGP) (100 × 4.0 mm i.d. and 10 × 3.0 mm i.d., respectively) were kindly provided by ChromTech AB (Norsborg, Sweden).

Ovomucoid-based analytical and guard columns for HPLC (Ultron ES-OVM) (150 × 4.6 mm i.d. and 10 × 4.0 i.d., respectively) were kindly provided by Shinwa Chemical Industries (Kyoto, Japan).

Chromatographic conditions

All separations were performed at ambient temperature (23–25°C). Mobile phase flow rates were 0.9 ml min^{-1} (AGP column) or 1.0 ml min^{-1} (OVM column). The UV detector was set at 277 nm for gallopamil and at 254 nm for nimodipine and RBH20. The phosphate buffer pH value was adjusted with 1 M potassium hydroxide before addition of organic modifier.

Standard solutions were prepared by diluting a 1.00 mg ml^{-1} (gallopamil) or a 0.50 mg ml^{-1} (nimodipine and RBH20) stock solution with mobile phase to obtain concentrations of 0.05 mg ml^{-1} and 0.025 mg ml^{-1}, respectively. The final composition of the mobile phase used is reported in the figure legends.

Results and Discussion

General factors affecting the performance of protein-based chiral columns

It has been reported both for AGP and OVM that parameters such as percentage of organic modifier, buffer pH and molarity, flow rate, column loading and temperature significantly affect retention time, selectivity, resolution and column performance on protein-based stationary phases [6, 8, 10]. However, for optically active drugs with only one chiral centre where there are only two enantiomers, it would be inappropriate to assume that optimization of these factors is necessarily trivial, since the selectivity obtained is often very sensitive to experimental conditions [9]. Furthermore, it is important to note that column temperature can play an important role in the separation of chiral compounds on protein columns, due to the high temperature coefficient often observed for selectivity, α.

Optimization

The optimum separation conditions for

gallopamil on the AGP column were located by a stepwise procedure: the modified sequential simplex method [10]. A simplex is a geometric figure described by a number of points (vertices) equal to one or more than the number of factors, or variables, being optimized. Thus a simplex for two factors is a triangle (Fig. 3). The objective of the sequential simplex procedure is to force the simplexes to move away from regions of poor response towards the region of optimum response.

The simplex movements can be stopped in a relatively simple way, either by limiting the number of experiments carried out, or by specifying a minimum difference in responses between vertices, corresponding to the random noise and error.

Figure 3 illustrates a two-variable modified simplex design used to optimize the pH and organic modifier for gallopamil, on Chiral-AGP, keeping the buffer concentration constant at 30 mM KH_2PO_4. The optimum in the response surface was found after only 11 experiments. It is interesting to observe that a sharp ridge in the pH dimension defines the optimum, illustrating the difficulty of locating the optimum response when employing the traditional 'variation of a single parameter at a time' approach. In other words, separation is rapidly lost as conditions move away the optimum.

For the modified simplex procedure it is necessary to define a chromatographic response function (CRF) in a manner suitable for the separation desired. In this case an empirical CRF proposed earlier [8] was used:

$$\mathrm{CRF} = P_i^5/(\log t_r),$$

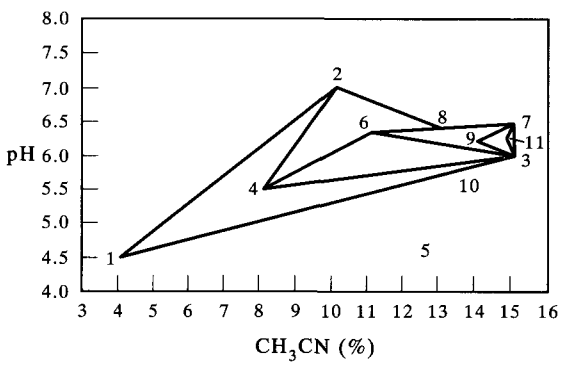

Figure 3
Movements of the modified simplex method in locating the optimum conditions for pH and acetonitrile concentration for the resolution of gallopamil on Chiral-AGP; buffer, 30 mM KH_2PO_4.

where P_i is the ratio between the average valley depth and the average peak height (Kaiser's peak separation function, P_i [12]). This parameter was found to give excellent discrimination between good and bad responses and is much more easily measurable than resolution, R_s.

The result of the simplex optimization for gallopamil on Chiral-AGP is illustrated in Fig. 4(a), where the peak separation function, P_i, is 97%. In Fig. 4(b) the enantiomeric separation of gallopamil on the OVM column is also shown. These conditions are in fact those reported for the separation of the analogue verapamil [10]. It is interesting to note that gallopamil can be separated on OVM under the same conditions as those for verapamil, while this is not possible on AGP.

The relationship of chiral separations with chromatographic operating parameters

Figure 5 shows the chromatograms of nimodipine on AGP and on OVM. As regards the OVM column, for nimodipine the higher the percentage of organic modifier (EtOH), the shorter the retention time; the lower the pH the better the resolution and the shorter

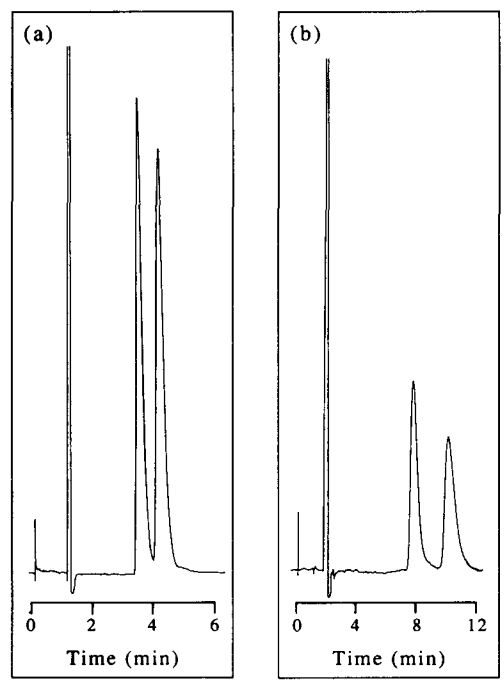

Figure 4
Chromatograms of gallopamil. (a) On chiral-AGP. Mobile phase: KH_2PO_4 (30 mM; pH 6.2)–MeCN (85:15, v/v); AUFS: 0.02 at 277 nm. (b) On OVM column. Mobile phase: KH_2PO_4 (10 mM; pH 6.2)–ethanol (82:18, v/v); AUFS: 0.02 at 277 nm.

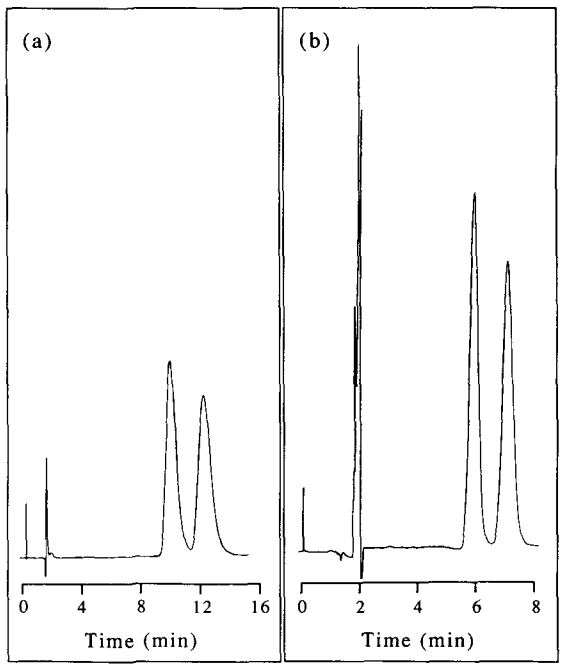

Figure 5
Chromatograms of nimodipine. (a) On chiral-AGP. Mobile phase: KH_2PO_4 (30 mM; pH 4.50)–propan-2-ol (90:10, v/v); AUFS: 0.04 at 254 nm. (b) On OVM column. Mobile phase: KH_2PO_4 (10 mM; pH 4.70)–ethanol (75:25, v/v); AUFS: 0.02 at 254 nm.

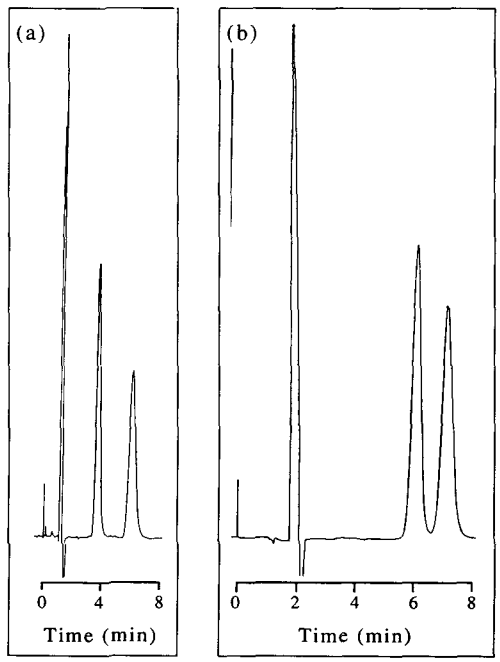

Figure 6
Chromatograms of RBH20. (a) On chiral-AGP column. Mobile phase: KH_2PO_4 (30 mM; pH 6.80)–propan-2-ol (85:15, v/v); AUFS: 0.04 at 254 nm. (b) On OVM column. Mobile phase: KH_2PO_4 (10 mM; pH 4.70)–ethanol (75:25, v/v); AUFS: 0.04 at 254 nm.

the retention time; as pH decreases, selectivity shows a modest increase (from 1.19 to 1.39).

Figure 6 shows the very large resolution obtained for RBH20 on Chiral-AGP. The chromatographic behaviour of nimodipine and RBH20 differ very much on AGP, but are quite similar on OVM. As the very significant difference between the two dihydropyridine molecular structures relates to the steric bulk and the binding properties of the R_1 and R_2 substituents, this would imply that under these conditions AGP can discriminate this structural moiety to a greater extent than can OVM.

The effects of buffer pH and amount of organic modifier on the values of N, α, k' and R_s for the two dihydropyridine derivatives are shown in Figs 7 and 8 (Chiral-AGP) and in Figs 9 and 10 (OVM column). It is generally considered that these two mobile phase variables can induce a reversible alteration in the secondary structure of the protein and subsequently affect the resolution observed [13].

As shown in Fig. 7, all the values of the key parameters are increased by increasing the pH. The greatest effect is observed on the enantioselectivity and resolution for the RBH20 enantiomers. By increasing the percentage of organic modifier (Fig. 8) all the separation

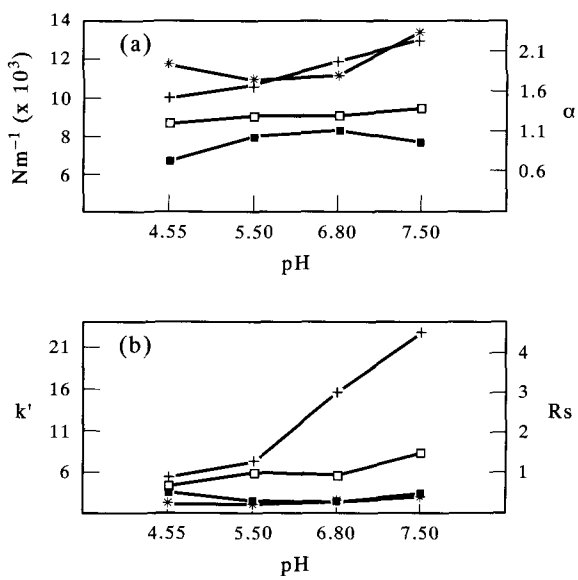

Figure 7
(a) Influence of pH value on N and α for nimodipine and RBH20 on chiral-AGP. ■ RBH20 (N m^{-1}); + RBH20 α; *NIM (N m^{-1}); □ NIM α. Mobile phase: KH_2PO_4 (30 mM)–propan-2-ol (85:15, v/v). (b) Influence of pH value on k' and R_s for nimodipine and RBH20 analysed on Chiral-AGP. ■ RBH20 k'; + RBH20 R_s; *NIM k'; □ NIM R_s. Mobile phase: KH_2PO_4 (30 mM)–propan-2-ol (85:15, v/v).

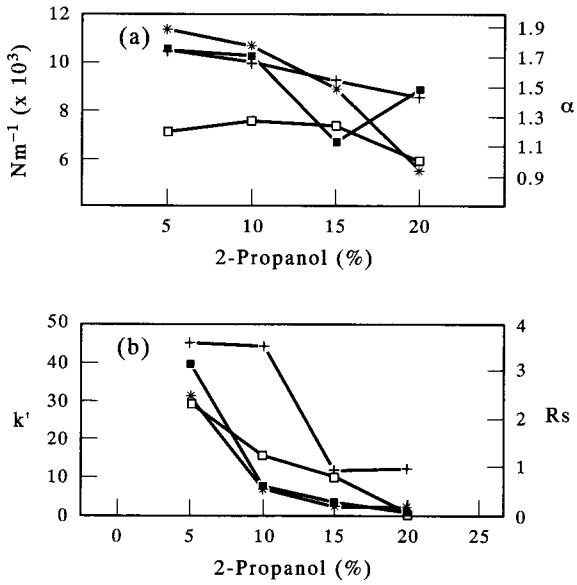

Figure 8
(a) Influence of percentage organic modifier on N and α for nimodipine and RBH20 analysed on Chiral-AGP. ■ RBH20 (N m^{-1}); + RBH20 α; *NIM (N m^{-1}); □ NIM α. Mobile phase: KH_2PO_4 (30 mM; pH 4.55)–propan-2-ol. (b) Influence of percentage organic modifier on k' and R_s for nimodipine and RBH20 analysed on Chiral-AGP. ■ RBH20 k'; + RBH20 R_s; *NIM k'; □ NIM R_s. Mobile phase: KH_2PO_4 (30 mM; pH 4.55)–propan-2-ol.

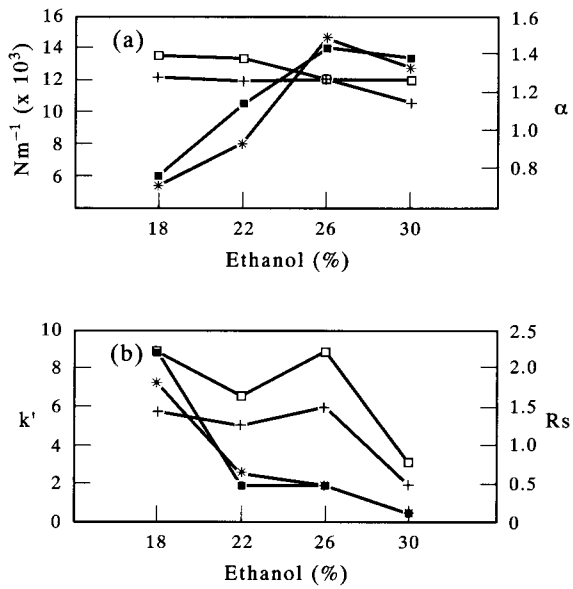

Figure 10
(a) Influence of percentage organic modifier on N and α for nimodipine and RBH20 analysed on OVM column. ■ RBH20 (N m^{-1}); + RBH20 α; *NIM (N m^{-1}); □ NIM α. Mobile phase: KH_2PO_4 (10 mM; pH 4.70)–ethanol. (b) Influence of percentage organic modifier on k' and R_s for nimodipine and RBH20 analysed on OVM column. ■ RBH20 k'; + RBH20 R_s; *NIM k'; □ NIM R_s. Mobile phase: KH_2PO_4 (10 mM; pH 4.70)–ethanol.

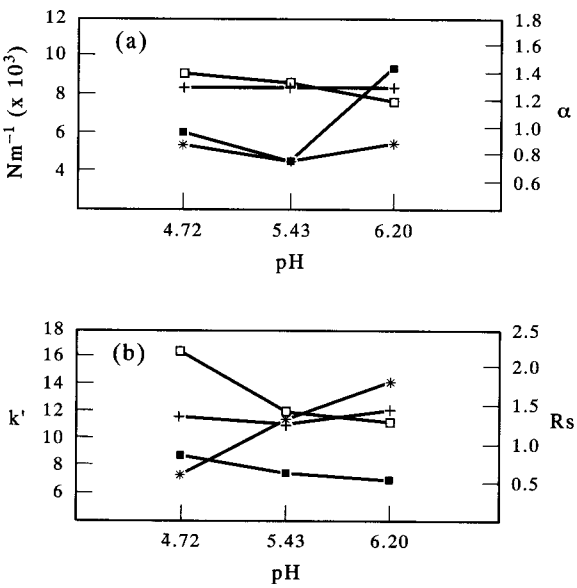

Figure 9
(a) Influence of pH on N and α for nimodipine and RBH20 analysed on OVM column. ■ RBH20 (N m^{-1}); + RBH20 α; *NIM (N m^{-1}); □ NIM α. Mobile phase: 10 mM KH_2PO_4–ethanol (82:18, v/v). (b) Influence of pH on k' and R_s for nimodipine and RBH20 analysed on OVM column. ■ RBH20 k'; + RBH20 R_s; *NIM k'; □ NIM R_s. Mobile phase: 10 mM KH_2PO_4–ethanol (82:18, v/v).

parameters are decreased. At 20% 2-propanol the separation for nimodipine is completely lost. However, despite a dramatic decrease in the R_s value for nimodipine, baseline resolution is still observed for RBH20.

Figure 9 shows that for nimodipine on the OVM column, on increasing the pH, the retention time increases and resolution decreases. For RBH20 the effect of pH on resolution and k' is relatively small over this range. There was a surprising increase in performance (plates m^{-1}) for RBH20 at pH 6.2, where α and R_s remained more or less constant. For nimodipine, α and performance were both approximately constant over this pH range. By increasing the percentage of organic modifier (Fig. 10) the key parameters change in a similar manner for both the two dihydropyridine derivatives. The best column efficiency and resolution are observed at 25% ethanol, where lower k' values are also obtained. Selectivity is the parameter least affected by varying the percentage of organic modifier.

Chiral system suitability test (SST) for protein-based columns

Based on the authors' experience, it is

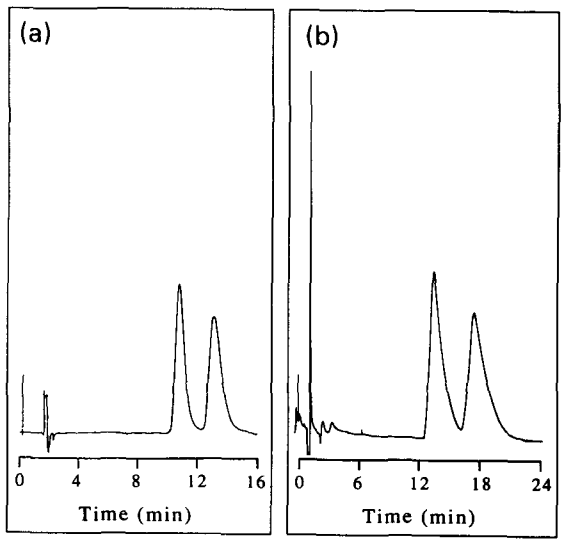

Figure 11
Chromatograms of racemic verapamil in mobile phase (0.050 mg ml^{-1}) with UV-detection at 277 nm, illustrating the chiral SST for: (a) Ultron ES-OVM: KH_2PO_4 (10 mM; pH 6.2)–ethanol (82:18, v/v); R_s = 1.57, P_t = 0.98, α = 1.25, k'_1 = 4.88; and (b) Chiral-AGP: KH_2PO_4 (10 mM; pH 7.0)–CH_3CN (90:10, v/v); R_s = 1.24, P_t = 0.94, α = 1.33, k'_1 = 11.6.

essential to establish a chiral system suitability test (SST) for protein-based columns, because of their susceptibility to the effect of low levels of impurities. In the authors' laboratories the separation of a racemic mixture of verapamil at 0.050 mg ml^{-1} in phosphate buffer is used as a chiral SST with the following mobile phases; this separation involves closely eluting enantiomers and presents an appropriate challenge for these particular chiral columns (Fig. 11). The mobile phases recommended, with UV detection at 277 nm, are: (a) for Ultron ES-OVM, KH_2PO_4 (10 mM; pH 6.2)–ethanol (82:18, v/v); and (b) for Chiral-AGP, KH_2PO_4 (10 mM; pH 7.0)–CH_3CN (90:10, v/v).

Conclusions

Both these protein-based phases were found to be suitable media for the separation of the calcium channel blocker drugs considered. In almost all cases baseline separation was obtained within short retention times. The chiral stationary phases are protein-based but differ widely, both as regards their physicochemical characteristics and their chromatographic behaviour. Furthermore there are many features to be learned by experience in using these columns effectively.

In order to obtain reproducible results, a very important question is the column stability and robustness, which have been found in this laboratory to be broadly comparable for both types of column, provided that a guard-column is used in line. However, in method development studies, wide changes in mobile phase composition and pH are often required, as reported recently by Kirkland *et al.*, [10] and this would certainly be expected to decrease the lifetime of any protein-based column.

It is therefore essential to set up a chiral SST for protein-based columns using, for example, verapamil enantiomers, in order to ensure that adequate stereoselective performance is validated at regular intervals, as noted above. It is of course good practice to establish a chiral SST based on the enantiomeric pair or pairs that are routinely being analysed on a given protein-based column.

It is clear that both of these new protein-based phases are becoming well established as excellent starting points for the development of direct separations of a wide range of chiral drugs and related compounds. It is also apparent that the process of method development can benefit from fundamental studies on the key parameters that modulate separation, coupled with systematic optimization strategies such as those described above, or the more powerful computer-aided methods based on central composite design recently proposed [14].

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