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Evaluation of six chiral stationary phases in LC for their selectivity towards drug enantiomers*

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Abstract: Six chiral stationary phases (CSP) were evaluated for their enantioselectivity towards a series of 45 drugs with different acidic, basic or neutral properties. These CSPs were: a polyacrylamide phase, Chiraspher; two polysaccharide-based phases, cellulose tris-3,5-dimethyl phenylcarbamate (Chiralcel OD) and the *S*-naphthylethylcarbamate derivative of β-cyclodextrin (SN-β-CD; Cyclobond I SN); and three protein-based CSPs — α_1-acid glycoprotein (Chiral-AGP), ovomucoid (OVM) and cellulase. A total of 28 different mobile phases were involved. Chiral-AGP, OVM and Chiralcel OD appeared to be the most promising CSPs for the enantioseparation of the series of structurally different compounds evaluated. Cellulase and Chiralcel OD show particularly high enantioselectivity towards the group of β-blocker drugs. The different protein-based CSPs were used in their usual reversed-phase mode. The other phases were used in combination with apolar mobile phases, except for SN-β-CD, which was evaluated in both modes. Formal optimization strategies were not adopted, although the effect of organic modifier and eluent pH on enantioselectivity was briefly examined for the protein-based phases.

Keywords: *Polymeric chiral stationary phases; β-blocker drugs; calcium-channel blocker drugs; barbiturates; amides; cyclodextrin derivative; α_1-acid glycoprotein; ovomucoid; cellulase; polysaccharide-based phases; liquid chromatography.*

Introduction

The pharmacological, pharmacodynamic and toxicological behaviour of the enantiomers in chiral drugs can differ widely. It is therefore important to develop enantioselective separation methods for studies on chiral pharmacokinetics, stereoselective metabolism and quality control. In the last few years, the number of approaches for the resolution of enantiomers based on liquid chromatography (LC) has increased rapidly, as indicated in recent books and reviews on this subject [1, 2].

In LC, each type of chiral stationary phase (CSP) can be classified according to the principle of its design and/or the molecular size of the chiral selector molecules involved. In general, two main groups can be distinguished: 'brush' type CSPs, where small molecule chiral selectors are immobilized upon silicagel; and polymeric-type CSPs, based for instance on polysaccharides, proteins or on synthetic polymers [3].

In the present work, six different stationary phases (Fig. 1) are evaluated as regards their enantioselectivity towards a range of structurally different chiral drug compounds. The phases have been selected in order to illustrate the principal types available, with emphasis on the polymeric CSPs. The various stationary phases selected are described in Fig. 1 and Table 1. The synthetic polymer phase (Chiraspher) is stable both in aqueous mobile phase systems as well as in normal-phase mode. The enantioselectivity is considered to be attributable to the presence of chiral monomers or chiral suprastructures or cavities in the polymer [4].

In the *S*-naphthylethylcarbamate derivative of β-cyclodextrin (SN-β-CD) [5], the hydroxyl functions on the edge of the toroidal cavity, formed by the cyclic glucose polymer in the well known silica-bonded β-cyclodextrin phase (Cyclobond), are derivatized. The enantioselectivities for the original Cyclobond phase and its derivative are known to be quite different [5]. The SN-β-CD phase can be used either in reversed-phase or in normal-phase mode; the chiral discrimination mechanism is different for each mode [5].

Derivatives of naturally occurring polymers such as cellulose have also been used as chiral stationary phases [1, 2]. The presence of chiral cavities within the polymeric structure is con-

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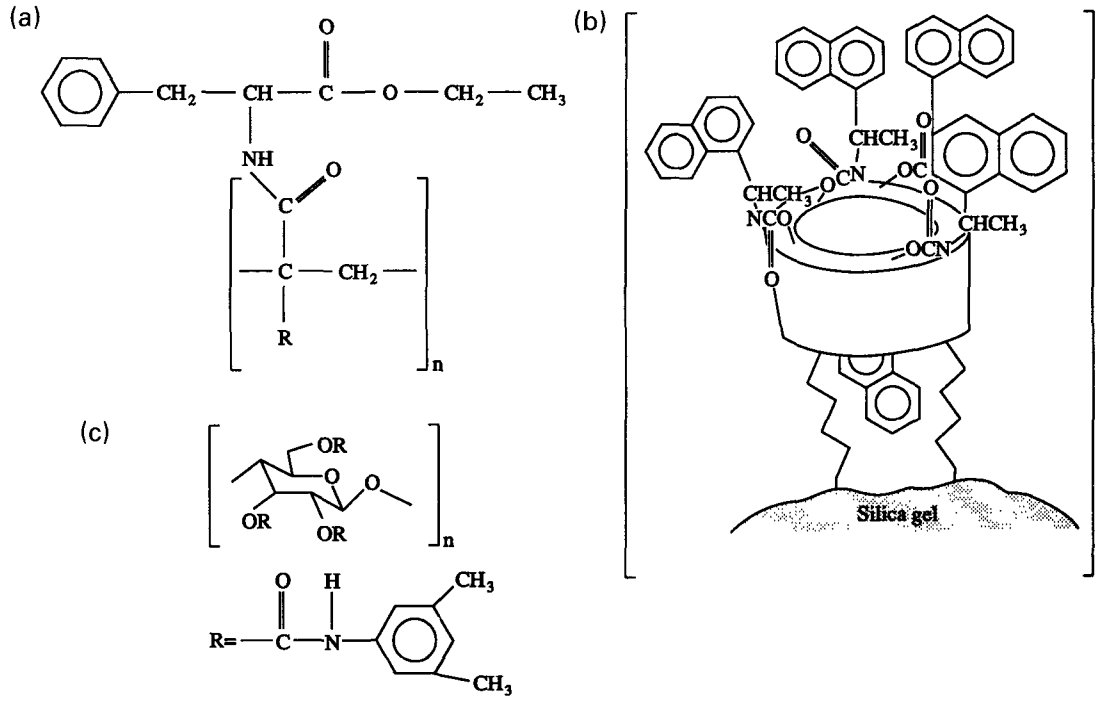

Figure 1
Three of the chiral stationary phases evaluated. (a) *S*-phenylalanine ethylester of polyacrylamide (Chiraspher); (b) *S*-naphthylethylcarbamate derivative of β-cyclodextrin (Cyclobond I SN); and (c) cellulose tris-3,5-dimethylphenylcarbamate (Chiralcel OD). See Table 1 for protein-based phases.

Table 1
Protein-based CSPs

Protein type CSP	AGP*	OVM†	CEL‡
MW	41000	28800	60000–70000
IEP	2.7	3.9–4.5	3.9
Sialic acid residues	14	0.3	—
Disulphide bridges	2	8	12
% carbohydrate	45	30	6

*AGP: α_1-acid glycoprotein (Chiral-AGP).
†OVM: ovomucoid (Ultron ES-OVM).
‡CEL: cellulase.

sidered to contribute to the chiral discrimination mechanism. In this work, a cellulose tris-3,5-dimethylphenylcarbamate derivative, coated upon silicagel (Chiralcel OD) has been used. The separation of a number of β-blocking agents has been reported on this CSP, using non-aqueous mobile phases [6–8].

Finally, a number of protein-based CSPs have been evaluated. Several kinds of protein have been successfully linked to silicagel for use as a chiral stationary phase. Cellulase, ovomucoid (Ultron ES-OVM) and α_1-acid glycoprotein (Chiral-AGP) phases have been selected for this study. An evaluation of the enantioselectivity of these proteins towards β-blocking agents has already been reported previously [9, 10].

It is the purpose of the present study to attempt to deduce some general guidelines to characterize the enantioselective abilities of these CSPs. It should, however, be noted that the protein type CSPs can only be used with aqueous mobile phases, whereas the others are used under normal-phase and/or aqueous mobile phase conditions.

A wide range of compounds with different structural characteristics and different acidic, basic or neutral properties were selected for this study. The drugs selected include: a series of strong to weakly basic compounds, viz. 24 β-blocking agents including propafenone, and six calcium-channel blockers of the dihydropyridine type; weakly acidic compounds such as mephenytoin, hexobarbital, methylphenobarbital and thalidomide; and neutral compounds such as chromakaline, its structural analogue CL 89-19-02 and oxazepam, plus some additional amides.

Experimental

Apparatus

A Hewlett–Packard 1090 liquid chromatograph with a diode-array detector was used. The synthetic polymer phase (*S*-phenylalanine

ethyl ester of polyacrylamide) was a Hibar RT 250-4 Chiraspher column (250 × 4 mm i.d.) and was obtained from Merck (Darmstadt, Germany). The mobile phase flow rate used with this phase was 1 ml min^{-1} at ambient temperature. The Cyclobond I SN column (250 × 4.5 mm i.d.) was kindly provided by D.W. Armstrong (University of Missouri–Rolla, USA) and manufactured by Astec (USA). The mobile phase flow rate was 1 ml min^{-1}, both in normal-phase as well as in reversed-phase mode at ambient temperature. The Chiralcel OD column (250 × 4.5 mm i.d.) was obtained from Daicel (Tokyo, Japan); the mobile phase flow rate was 0.6 ml min^{-1} and the experiments were performed at a constant oven temperature of 30°C. The Chiral-AGP column (100 mm × 4.5 mm i.d.) was obtained from ChromTech AB (Stockholm, Sweden). The ovomucoid column (150 mm × 4.5 mm i.d.) (Ultron ES-OVM) was a gift from G. Tamai and manufactured by Shinwa Ltd (Kyoto, Japan). The mobile phase flow rate used with both these phases was 0.8 ml min^{-1}. The cellulase CSP was packed into a small-bore column (100 × 2.1 mm i.d.) and was provided by C. Pettersson (Uppsala, Sweden). The mobile phase flow rate was 0.1 ml min^{-1}. The experiments with all these protein-based phases were performed at a constant oven temperature of 25°C.

Standards and reagents

Heptane, dioxane and methyl *tert*-butyl ether were of Pro-Analysis quality from Merck (Darmstadt, Germany). Acetonitrile, ethanol and isopropanol were of HPLC grade from Merck. Buffer solutions were prepared by dissolving sodium dihydrogenphosphate and disodium hydrogenphosphate in doubly-distilled water. Samples of racemic mixtures of each compound were obtained either from the manufacturers or by synthesis at the University of Graz (Graz, Austria). Solutions of each racemic compound (*ca* 200 μg ml^{-1}) were prepared in the relevant mobile phase. Aliquots of 5 or 10 μl of these solutions were injected, except for the cellulase CSP, where 1 μl was injected.

Results and Discussion

The chromatographic results are described and considered separately for each CSP.

The choice of the initial mobile phase used with each different column was based on literature data and on information given by the manufacturer concerning the enantioseparation of other substances. Changes in the concentration of organic modifier and the pH of the phosphate buffer were systematically studied for some phases in order to evaluate the influence of these parameters on the separation of the enantiomers. Optimization of the mobile phase composition by means of systematic designs, such as the Simplex design [11], was not carried out in view of the large number of different compounds studied. It should be emphasized that the optimum conditions would clearly be expected to differ for each compound on each chiral stationary phase, so to this extent the results obtained for individual racemates may be considered to be of limited value. Nevertheless, it would be entirely reasonable to regard the present initial study as one of the first broad surveys in the field, forming the basis for certain tentative conclusions and indicating further directions for systematic research.

Chiraspher

This CSP can be used with both apolar and polar mobile phases. Most of the enantioseparations described on this CSP were, however, achieved in normal phase mode (cf. Application Guide, Merck, Darmstadt, Germany). In this study, several kinds of apolar eluent composition were used. Aprotic solvents were preferred in order to facilitate formation of possible hydrogen bonds between the chiral solute (chiral selectand) and the chiral stationary phase (chiral selector). Only a few of the compounds studied could be chirally separated on this CSP (Table 2).

The first mobile phase consisted of heptane–dioxane–diethylamine (70:30:0.1, v/v/v; MP 1). DEA was added to the mobile phase to minimize the effects of any residual silanol groups. Retention was decreased when 5% of the protic solvent isopropanol was added (mobile phase 2). As dioxane has strong eluting properties, a further decrease in retention was obtained by increasing its content in the mobile phase (mobile phase 3). The eluent composition methyl *tert*-butyl ether–dioxane–DEA (70:30:0.1, v/v/v; MP 4) resulted in a decrease in k' values and, for the chirally separated compounds, in an increase in the α-values (Table 2). This may be attributable to

Table 2
Enantioselectivity of Chiraspher for four racemates. k'_1 represents the capacity factor of the first eluting enantiomer, α is the separation factor and P is the peak–valley ratio [18]

Compound	Mobile phase*	k'_1	α	P
Mephenytoin	3	0.77	1.09	0.26
	4	0.39	1.13	NM
Oxazepam	1	10.7	1.21	0.98
	2	5.85	1.17	NM
	4	1.74	1.24	NM
Chromakaline	1	6.74	1.07	0.57
	2	2.66	1.04	NM
	4	1.06	1.08	NM
CL 89-19-02	1	6.71	1.09	0.83
Structural	2	2.48	1.07	0.39
Analogue of chromakaline	4	1.08	1.12	NM

*Mobile phases used were: MP 1 = heptane–dioxane–diethylamine (70:30:0.1, v/v/v); MP 2 = heptane–dioxane–isopropanol–diethylamine (70:25:5:0.1, v/v/v/v); MP 3 = heptane–dioxane–isopropanol–diethylamine (60:35:5:0.1, v/v/v/v); MP 4 = methyl *tert*-butyl ether–dioxane–diethylamine (70:30:0.1, v/v/v).
NM: not measured.

the different hydrogen bonding properties of the various solvents.

The presence of amide functions in the vicinity of the stereogenic centre of the select- and seems to be beneficial for enantioseparation on this CSP. To study this more closely, the enantioselectivity of Chiraspher towards some simple chiral amides was evaluated. The structural formulae of these compounds are given in Fig. 2, from which it can be seen that the compounds differ only in their aryl substituents and in the positions of these groups relative to the amide function. Only the dinitro-benzoic acid phenylethylamide enantiomers, which are π-acidic in character, could be resolved on this phase (MP 1: k'_1 = 2.73, α = 1.10, P = 0.56). As all the compounds in Fig. 2 are secondary amides, capable of hydrogen bond formation and/or dipole–dipole interaction, it seems that, under the given eluent conditions, these structural elements are not sufficient to lead to chiral recognition, so that additional strong π–π interactions would seem to be crucial. The separation of the enantiomers of the π-acidic compound D (Fig. 2) may well be an indication that this CSP is serving as a π-base.

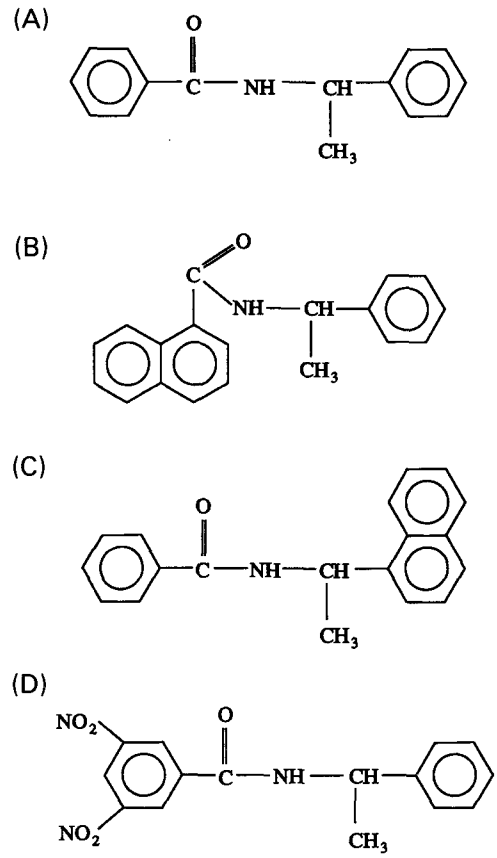

Figure 2
Amide compounds with differing π-acidic and π-basic character: (A) benzoic acid 1-phenylethylamide; (B) naphthalene-1-carboxylic acid 1-phenylethylamide; (C) benzoic acid 1-naphthylethylamide; (D) dinitrobenzoic acid 1-phenylethylamide.

SN-β-cyclodextrin (Cyclobond I SN)

The structure of this stationary phase is illustrated in Fig. 1. In this study, the CSP was used both in normal-phase (NP) and in reversed-phase (RP) mode.

In NP mode, two different mobile phase combinations were used, i.e. heptane–isopropanol–DEA (70:30:0.1, v/v/v) (MP 5) and heptane–ethanol–DEA (70:30:0.1, v/v/v) (MP 6) (MP 11). As ethanol is more polar than isopropanol, retention times are shorter with

mobile phase 6. The enantioselectivity of this CSP towards the selected compounds seemed only minor. Mephenytoin and methylphenobarbital were chirally resolved with α values, respectively, of 1.10 (MP 5) and 2.73 (MP 6).

Hexobarbital did not elute from this CSP under the mobile phase conditions evaluated. Oxazepam enantiomers were resolved with an α value of 1.08 (MP 6). Some of the neutral amide enantiomers (Fig. 2) were separated using MP 5. Their k'_1, α and P values are summarized in Table 3. Hydrogen bonds and dipole–dipole interactions between the amide and the CSP, and also π–π interactions, seem to be important for chiral recognition on this phase. In Table 3, it can be seen that, although enantiomers of compound C are separated, this is not the case for compound B, which may be attributable to the spatial position of the naphthyl ring relative to the amide function. The proximity of this bulky group to the stereogenic centre would also be expected to be significant.

In RP mode, mobile phases comprising 0.1 M ammonium acetate buffer (pH 7.0)–acetonitrile (70:30, v/v; MP 7) or (80:20, v/v; MP 8) were used. Of all the compounds studied, only those listed in Table 4, could be chirally separated. In Table 4, it can be seen that SN-β-cyclodextrin is enantioselective for some weakly acidic compounds. Interestingly, the enantiomers of compound CL 89-19-02 could be separated, while those of its structural analogue chromakaline, could not be resolved under these eluent conditions.

Oxazepam enantiomers can be separated with mobile phase 8, as illustrated in Fig. 3; however, the spontaneous transition of one isomer to the other is observed in aqueous solution (the phenomenon of peak coalescence). This isomerization also takes place in biological matrices such as plasma, so the usefulness of chiral analysis for drugs such as oxazepam is open to question.

Some kind of inclusion complex formation in the 'hydrophobic cavity' of the cyclodextrin toroid is considered to be one of the basic requirements for chiral separations in re-

Table 3
Enantioselectivity of *S*-naphthylethylcarbamate substituted β-cyclodextrin (normal-phase mode)

Amide*	k'_1	α	P
A	0.82	1.10	0.52
B	1.27	1	0
C	1.05	1.13	0.91
D	3.15	2.05	1

*Key (cf. Fig. 2).
A = benzoic acid phenylethylamide; B = naphthalene-1-carbonic acid phenylethylamide; C = benzoic acid naphthylethylamide; D = dinitrobenzoic acid phenylethylamide. The mobile phase used in these experiments was: heptane–isopropanol–diethylamine (70:30:0.1, v/v/v) (MP 5).

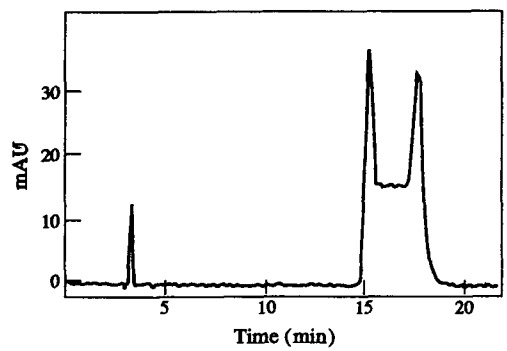

Figure 3
Isomerization of oxazepam enantiomers. Stationary phase: SN-β-cyclodextrin; MP 8: ammonium acetate buffer (pH 7.0; 0.1 M)–acetonitrile (80:20, v/v).

Table 4
Enantioselectivity of *S*-naphthylethylcarbamate substituted β-cyclodextrin (reversed-phase mode)

Compound	MP 7			MP 8		
	k'_1	α	P	k'_1	α	P
CL 89-19-02	0.98	1.04	0.23	1.85	1.06	0.79
Mephenytoin	0.93	1.23	1	1.71	1.36	1
Methylphenobarbital	1.45	1	0	3.01	1.05	0.44
Hexobarbital	1.56	1.11	0.97	3.33	1.15	1
Amide A*	2.01 (*R*)†	1.09	1	—	—	—
Amide B*	3.86	1	0	—	—	—
Amide C*	4.24 (*R*)†	1.04	0.58	—	—	—
Amide D*	0.71	1	0	1.19	1	0

MP 7: ammonium acetate buffer (pH 7.0; 0.1 M)–acetonitrile (70:30, v/v).
MP 8: ammonium acetate buffer (pH 7.0; 0.1 M)–acetonitrile (80:20, v/v).
*For key to amides A, B, C and D, see Table 3 and Fig. 2.
†The *R*-enantiomer elutes first.

versed-phase mode on a native β-cyclodextrin phase [12]. The different stability constant for inclusion formation for each enantiomer in the cavity is considered to be a key factor in leading to chiral separation.

In this study, a decrease in k' and α values is observed when the organic modifier concentration is increased, analogously with the behaviour of a RP packing. This can be explained by the fact that the hydrophobic interactions of the solute within the cyclodextrin cavity are competitively disturbed by the addition of organic modifier to the mobile phase.

The difference in the chiral recognition mechanism of SN-β-CD in RP and NP mode is illustrated clearly by compound D (Tables 3 and 4). In RP mode, the enantiomers of this amide with π-acidic character cannot be separated, while a baseline separation is obtained in NP mode. This indicates that the naphthyl substituent of the cyclodextrin moiety, which has a π-basic character, might be of importance for the chiral recognition mechanism in NP mode. In this mode, the cyclodextrin cavity is probably filled with the hydrophobic mobile phase, so that inclusion complexes are not favoured, which implies that the naphthyl substituent may become more important in the retention and the chiral discrimination mechanism.

Cellulose tris-3,5-dimethylphenyl carbamate (Chiralcel OD)

In Table 5, the results of these experiments are summarized, based on the use of this CSP in normal-phase mode, using heptane with either isopropanol or ethanol, plus diethylamine (DEA).

It is clear from Table 5, that baseline separation ($P = 1$) can be obtained for most of the β-blocking agents studied (compounds 1–23). For the calcium-channel blockers (compounds 24–29), however, enantioselectivity was found to be limited. As this phase seemed to be rather general in its application, a more detailed investigation on the effect of key factors was carried out, as indicated below.

Effect of the mobile phase composition. Retention can be controlled by changing the concentration and/or the kind of polar modifier added to heptane. An increase in the percentage of polar component in the mobile phase, leads to a decrease in k'. This is illustrated if data in Tables 5 and 6 are compared for a limited number of compounds. As isopropanol is a less polar solvent than ethanol, higher retention times are obtained in mobile phases with isopropanol as the polar component. It is important to note that for most of the compounds studied, enantioselectivity increases with higher retention times.

Effect of solute hydrophobicity. In Fig. 4, a plot is shown of log k'_1 on Chiralcel OD vs log k'_{RP18} for compounds listed in Table 5. The log k'_{RP18} values give an indication of the hydrophobicity of each compound. A significant negative correlation ($P = 0.007$) is obtained between the two sets of data. This means that, with some exceptions, the more polar compounds are better retained by the stationary phase. The value of the slope of the relationship is, however, rather low. One also observes that mepindolol (No. 7 in Fig. 4) is retained more strongly than pindolol (No. 5 in Fig. 4), despite the fact that pindolol is a more polar compound. When apolar groups were substituted on the naphthyl ring of propranolol, an increase in retention on Chiralcel OD was also observed [W. Lindner, unpublished results]. Thus, a clear relationship betwen the enantioselectivity, expressed as α, and the hydrophobicity of the compounds, could not be found in this case.

Effect of structural elements. The position of the asymmetrical stereogenic carbon atom is the same for all the β-blocking agents studied. They differ mainly in the structure of the aryl substituent. Only one chromatographic peak was observed for sotalol (No. 3), prenalterol (No. 2), pindolol (No. 5) and mepindolol (No. 7) in mobile phase 9 (Table 5). The *S*-enantiomers of pindolol and mepindolol, however, did not elute from the column under these conditions. But in a mobile phase with 30% ethanol, pindolol and mepindolol enantiomers were baseline separated (Table 6; MP 13). For sotalol and prenalterol, however, it is not clear whether the CSP is fundamentally not enantioselective towards them, or whether the second enantiomer simply did not elute under the conditions evaluated. It could be that these conditions were unsuitable, due to the lack of optimization discussed above.

Some derivatives of propafenone (No. 41, resolved partially with MP 9; $P = 0.47$) were also studied. For 5-hydroxypropafenone, no separation was observed in MP 9, whereas the

Table 5
Evaluation of the enantioselectivity of cellulose tris-3,5-dimethylphenylcarbamate (Chiralcel OD)

Compound	MP 9				MP 10		
	$\log k'_{C18}$	k'_1	α	P (R_s)	k'_1	α	P (R_s)
1 Atenolol	−0.6	2.85 (*R*)*	1.80	1	1.44	1.41	1
1 Prenalterol	−0.56	4.58	NS		1.43	NS	
3 Sotalol	−0.55	3.18	NS		1.21	NS	
4 Practolol	−0.55	1.93	1.32	1	0.81	1.16	0.92
5 Pindolol	−0.34	2.32	NS		1.16	NS	
6 Nifenalol	−0.29	0.33	1	0	0.22	1	0
7 Mepindolol	−0.24	3.45	NS		1.56	NS	
8 Bunitrolol	−0.09	0.50	1.11	(0.83)	0.27	1	0
9 Acebutolol	−0.08	0.99	1.08	0.28	0.43	1.09	0.18
10 Timolol	0.012	0.20 (*R*)*	1.26	(0.93)	I†		
11 Metoprolol	0.014	0.37	3.52	1	0.24	2.38	1
12 Celiprolol	0.069	1.29 (*S*)*	1.41	1	0.45	1.30	1
13 Carazolol	0.099	4.38	1.22	1	2.21	1.19	1
14 Oxprenolol	0.259	0.57	6.02	1	0.39	3.23	1
15 Metipranolol	0.39	0.24	1.33	(1.12)	0.15	1.17	(0.62)
16 Toliprolol	0.428	0.65	1.09	0.51	0.37	1	0
17 Talinolol	0.468	3.80 (+)*	1.20	1	1.30	1	0
18 Propranolol	0.545	1.12 (*R*)*	1.84	1	0.67	1.40	1
19 Tertatolol	0.598	0.49	6.65	1	0.31	4.11	1
20 Alprenolol	0.603	0.23	4.63	1	0.12	2.42	1
21 Carvedilol	0.654	>15			>15		
22 Bupranolol	0.668	0.10	2.91	1	0.07	1.97	0.90
23 Isopenbutol	1.276	0.09 (*R*)*	3.56	1	I†		
24 Amlodipine	0.149	1.2	1	0	0.76	1.13	(0.60)
25 Nicardipine	0.152	0.67	1.09	0.24	0.43	1	0
26 Nitrendine	0.346	0.46	1	0	0.30	1	0
27 Nimodipine	0.444	0.48	1.09	0.24	0.30	1	0
28 Nisoldipine	0.538	0.43	1.09	0.17	0.27	1	0
29 Felodipine	0.693	0.37	1	0	0.21	1	0
30 Mephenytoin	0.41	0.77	1.05	(0.56)	0.63	1	
31 Chromakaline	0.484	0.66	1.69	1	0.44	1.44	1
32 CL 89-19-02	0.502	0.78	1.42	1	0.50	1.31	1
33 Methylpheno-barbital	0.555	3.84	1.10	(0.89)	2.11	1.16	(1.27)
34 Hexobarbital	0.571	2.24	1.070	0.38	1.69	1.04	0.08
35 Thalidomide	−0.10	>15			—	—	
36 Amide A	0.748	0.75 (*R*)*	1.68	1	0.33	1.40	1
37 Amide B	1.17	1.46 (*R*)*	1.53	1	0.70	1.45	1
38 Amide C	1.3	0.71 (*R*)*	2.06	1	0.34	1.77	1
39 Amide D	−0.09	>15			>15		
40 Oxazepam	0.869	>15			1.86	1.56	1
41 Propafenone	0.966	0.80	1.09	0.47	—	—	

MP 9: heptane–isopropanol–DEA (80:20:0.1, v/v/v).
MP 10: heptane–ethanol–DEA (80:20:0.1, v/v/v).
NS: not separated or one enantiomer not eluted.
R_s values (in brackets) are given instead of P values, when calculation of P was difficult, or when enantiomers were baseline resolved.
*(*R*), (*S*) and (+) indicate the nature of the first-eluting enantiomer, where this is known.
†I: serious interference was observed at the t_0 position in the chromatogram.

enantiomers of 5-methoxypropafenone were separated with α = 1.09. This might indicate that polar groups such as OH in the aryl group do not favour chiral separation. If true, this could be a possible explanation for the low enantioselectivity displayed by this CSP towards the β-blocking agents prenalterol (No. 2), nifenalol (No. 6) and bunitrolol (No. 8).

Chiral stationary phases with proteins as chiral selector

Since the early work of Allenmark based on bovine serum albumin [2], it has been found that several different proteins can be used for the enantiomeric separation of compounds. It is however difficult to characterize these CSPs as their conformation is known to change under different mobile phase conditions [13, 14]. For ovomucoid, a transition to a more ordered conformation has been described in the low-pH region [14]. The conformation of non-immobilized cellulase is known to change under different conditions [15].

Recently, three protein-based chiral station-

Table 6
Evaluation of cellulose tris-3,5-dimethylphenylcarbamate (Chiralcel OD)

Compound	MP 11			MP 12			MP 13		
	k'_1	α	P (R_s)*	k'_1	α	P	k'_1	α	P
5 Pindolol	—			—			0.54 (*R*)†	5.35	1
7 Mepindolol	—			—			0.75	4.69	1
8 Bunitrolol	0.89	1.14	(1.32)	0.65	1	0	—		
9 Acebutolol	4.36	1.08	(1.02)	1.75	1.1	0.82	—		
15 Metipranolol	0.61	1.26	(1.67)	0.49	1.12	0.69	—		
16 Toliprolol	0.86	1.10	(1.06)	0.71	1	0	—		
24 Amlodipine	—			1.94	1.14	0.28	—		
25 Nicardipine	2.11	1.09	0.77	1.21	1.05	0.15	—		
26 Nitrendine	1.47	1	0	0.88	1	0	—		
27 Nimodipine	1.58	1.09	0.65	0.89	1.05	0.11	—		
28 Nisoldipine	1.37	1.10	0.67	0.77	1.05	0.10	—		
29 Felodipine	1.16	1	0	0.63	1	0	—		
39 Amide D	—			—			1.32	1.49	1

Mobile phase 11: heptane–isopropanol–DEA (90:10:0.1, v/v/v).
Mobile phase 12: heptane–ethanol–DEA (90:10:0.1, v/v/v).
Mobile phase 13: heptane–ethanol–DEA (70:30:0.1, v/v/v).
* R_s values (in brackets) are given instead of P values, when calculation of P was difficult.
†(*R*) indicates the first eluting enantiomer.

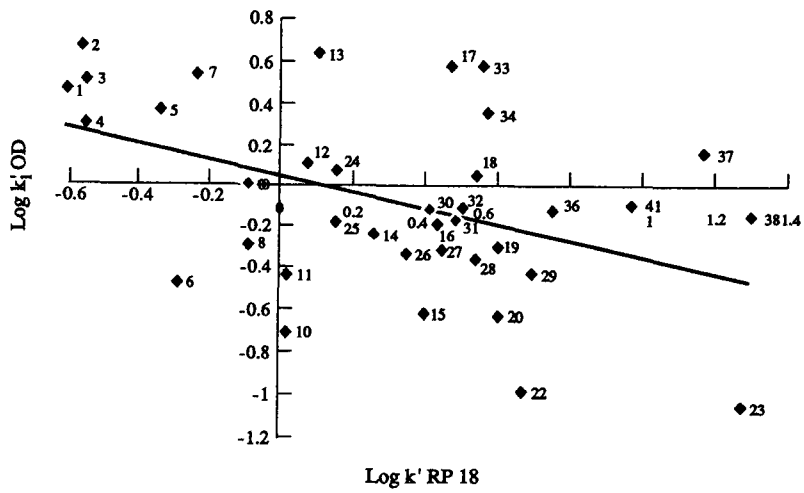

Figure 4
Plot of log k'_1 on Chiralcel OD vs log k'_{RP18}. Slope = −0.3934; intercept = +0.03726; r = 0.44; P = 0.007. Key to compounds as in Table 5.

ary phases were evaluated in the authors' laboratories for their enantioselectivity towards β-blocking agents: α_1-acid glycoprotein (Chiral-AGP) [9], ovomucoid (OVM) and cellulase (CEL) [10]. In the present study, the enantioselectivity of these proteins towards a more heterogeneous group of compounds has been examined.

α_1-acid glycoprotein type CSP (Chiral-AGP)

Table 7 summarizes the results for the evaluation of Chiral-AGP. The neutral racemates with π-basic properties (amides A, B and C in Fig. 2) could be separated, but they showed high affinity to the CSP. The effects of hydrophobicity and mobile phase composition on retention behaviour and enantioselectivity are discussed below.

Effect of hydrophobicity. Figure 5 shows that the retention behaviour of the compounds is correlated with their hydrophobicity, expressed as the retention behaviour on RP C18 stationary phase. This confirms that hydrophobic interactions are important in the retention mechanism on AGP, as observed in an earlier paper concerning β-blocking agents [9].

Effect of organic modifier. As organic modifiers interfere with the hydrophobic inter-

Table 7
Evaluation of Chiral-AGP for basic, acidic and neutral compounds

Compound	Mobile phase	k'_1	α	P
Calcium-channel blockers (weak bases)				
1 Nitrendipine	MP 16	3.26	1.21	0.71
2 Nimodipine	MP 16	2.87	1.27	0.88
3 Nisoldipine	MP 16	7.34	1.38	1
4 Nicardipine	MP 14	4.20	1	0
5 Amlodipine	MP 14	4.04	1.10	0.20
6 Felodipine	MP 16	10.6	1.16	0.59
Others				
7 Oxazepam	MP 14	4.80	1	0
8 Chromakaline	MP 17	2.75	1.19	0.31
10 Omeprazole	MP 14	4.15	1.27	0.94
11 Propafenone	MP 14	3.44	1.15	0.60
Weak acids				
9 Thalidomide	MP 14	0.89	1.12	0.09
12 Mephenytoin	MP 14	0.71	1.25	0.64
13 Prominal	MP 18	1.35	1.18	0.43
14 Hexobarbital	MP 18	0.72	1.31	0.70
Acids				
15 Ibuprofen	MP 17	1.70	1.28	0.83
16 Naproxen	MP 15	6.87	1.13	0.85

Mobile phases used were: MP 14: phosphate buffer (pH 4.6; 0.02 M)–isopropanol (95:5, v/v); MP 15: phosphate buffer (pH 4.6; 0.02 M)–isopropanol (90:10, v/v); MP 16: phosphate buffer (pH 4.6; 0.02 M)–isopropanol (87:13, v/v); MP 17: phosphate buffer (pH 6.5; 0.02 M)–isopropanol (99:1, v/v); MP 18: phosphate buffer (pH 6.5; 0.02 M)–isopropanol (95:5, v/v).

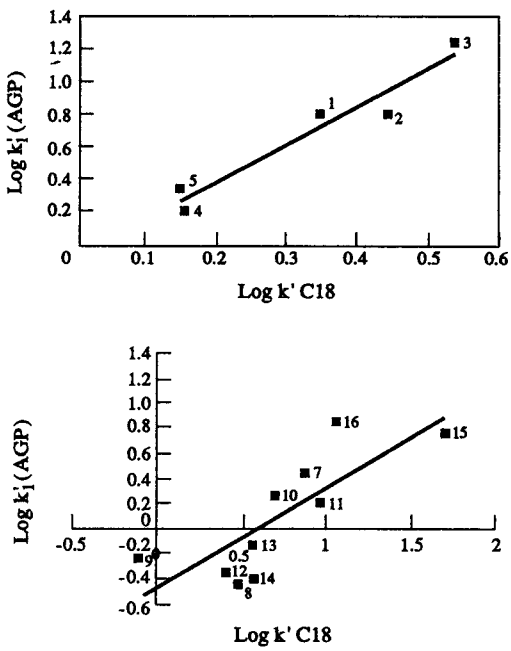

Figure 5
Correlation between retention behaviour on Chiral-AGP and hydrophobicity, expressed as log k'_{C18}. Compounds are numbered as in Table 7.

actions, it is to be expected that an increase in the organic modifier content of the mobile phase would cause a decrease in retention time. This is illustrated in Fig. 6 and Table 7. With some exceptions, the dihydropyridine calcium channel blockers show a relatively high affinity for this stationary phase; in fact the addition of up to 13% (v/v) isopropanol (MP 16) is necessary to obtain reasonable retention times. However, nicardipine and amlodipine are larger molecules and show less affinity for Chiral-AGP, or indeed for RP C18. This difference in behaviour could be attributable to the more basic character of amlodipine, with its primary amine function. Thus amlodipine can only be resolved at low concentrations of organic modifier (MP 14), and even then it is very poorly resolved (Table 7). The structure of nicardipine, which cannot be resolved with this organic modifier (MP 14), differs from the other dihydropyridines in that it has a larger side chain with a benzylaminoalkyl residue esterified in the pyridine nucleus. Since this is close to the stereogenic centre, this might be expected to lead to steric repulsion. In Table 7, it is clear that the enantiomers of the dihydropyridine calcium-channel blockers that contain small alkyl chains substituted in the R1 and R2 positions can be well separated by AGP. This has been confirmed by De Lorenzi *et al.* [16] who proposed a modified Simplex procedure [11] to optimize the organic modifier (acetonitrile) content and the pH of the mobile phase for the enantioseparation of nimodipine and

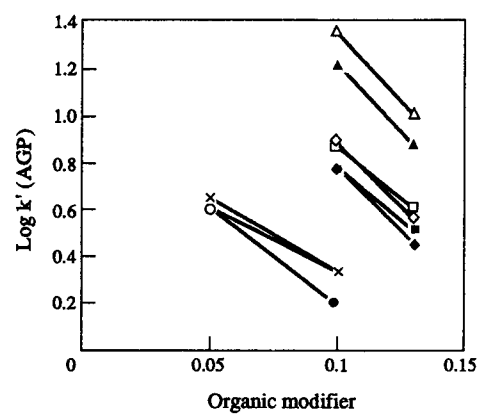

Figure 6
Effect of fraction of organic modifier on the retention of dihydropyridine calcium-channel blockers on Chiral-AGP, using buffer conditions listed in Table 7. ■, nitrendipine 1; □, nitrendipine 2; ◆, nimodipine 1; ◇, nimodipine 2; ▲, nisoldipine 1; △, nisoldipine 2; ●, nicardipine 1; ○, amiodipine 1; and ×, amlodipine 2.

other calcium channel blockers on Chiral-AGP [16].

It has been found for the other compounds in Table 7 that enantioselectivity increases when the organic modifier content of the mobile phase decreases. For example, the enantiomers of the ampholyte omeprazole, a chiral sulphoxide, are separated with 5% (v/v) isopropanol in the mobile phase (MP 14), but enantioselectivity disappears with 10% (v/v) isopropanol. A similar pattern is also observed for thalidomide, hexobarbital and amlodipine and indicates that hydrophobic interactions are involved in the chiral discrimination mechanism. For the other dihydropyridine calcium-channel blockers (except for amlodipine and nicardipine), enantioselectivity remains about the same when organic modifier concentration is changed, as illustrated in Fig. 6. The enantioseparation of some of these calcium antagonists is illustrated in Figs 7 and 8. Although the separation factor α remains about the same when the isopropanol concentration is decreased, the peak–valley ratio, P, increases.

Effect of pH. The influence of pH on retention behaviour and enantioselectivity has

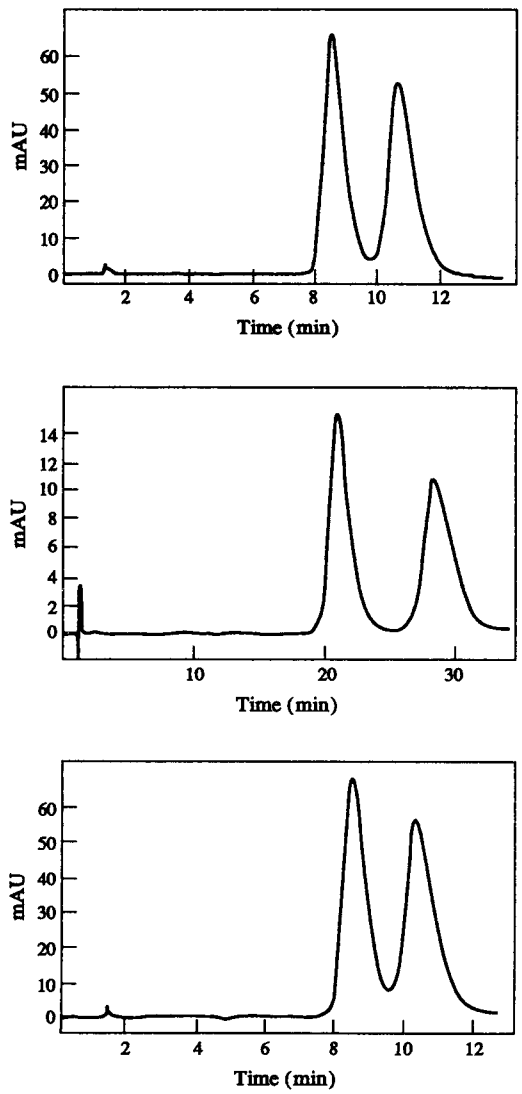

Figure 7
Enantioseparation of calcium-channel blockers on Chiral-AGP with 10% (v/v) isopropanol. (A) nimodipine (α = 1.29, P = 0.94), (B) nisoldipine (α = 1.38, P = 1), (C) nitrendipine (α = 1.26,. P = 0.91). Mobile phase: phosphate buffer (pH 4.6; 0.02 M)–isopropanol (90:10, v/v) MP 15.

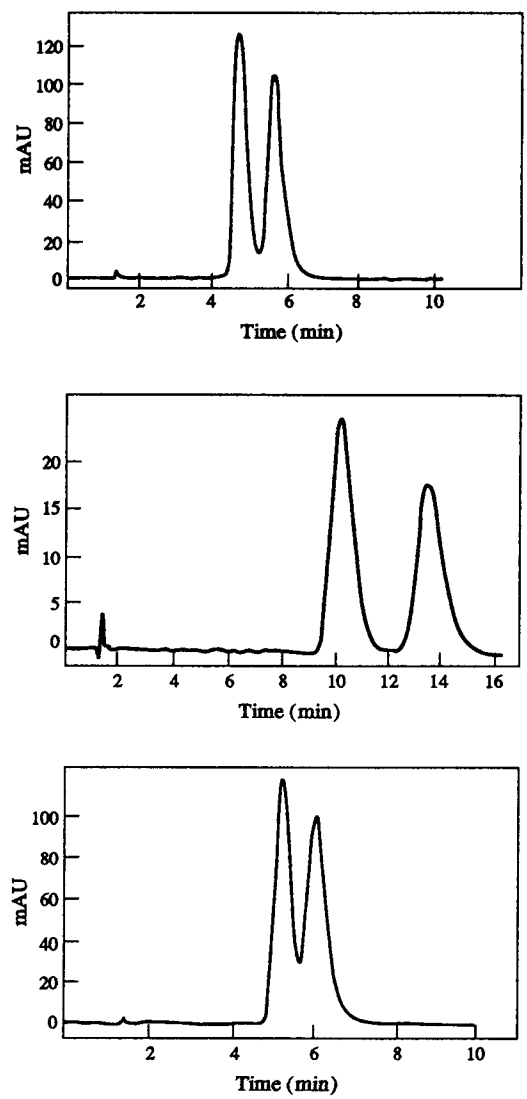

Figure 8
Enantioseparation of calcium-channel blockers on Chiral-AGP with 13% (v/v) isopropanol. (A) nimodipine (α = 1.27, P = 0.88), (B) nisoldipine (α = 1.38, P = 1), (C) nitrendipine (α = 1.21, P = 0.71). Mobile phase: phosphate buffer (pH 4.6; 0.02 M)–isopropanol (87:13, v/v) MP 16.

Table 8
Influence of pH and organic modifier on retention behaviour and enantioselectivity

Compound	MP 14			MP 15			MP 17			MP 18		
	k'_1	α	P	k'_1	α	P	k'_1	α	P	k'_1	α	P
8 Chromakaline	0.71	1	0	0.35	1	0	2.75	1.19	0.31	0.70	1	0
12 Mephenytoin	0.71	1.25	0.64	0.44	1.15	0.06	1.90	1.14	0.22	0.73	1.19	0.29
13 Prominal	1.38	1	0	0.72	1	0	5	1	0	1.35	1.18	0.43
14 Hexobarbital	0.67	1.18	0.24	0.4	1	0	2.34	1.39	0.97	0.72	1.31	0.70
	MP 15			MP 16			MP 17			MP 18		
	k'_1	α	P	k'_1	α	P	k'_1	α	P	k'_1	α	P
15 Ibuprofen	5.67	1.10	0.39	4.37	1.06	0.05	1.70	1.28	0.83	0.68	1	0
16 Naproxen	6.87	1.13	0.85	—			2.44	1.22	0.92	1.40	1	0

For key to mobile phase compositions MP 14–MP 18: see Table 7.

been briefly studied (Table 8), for some representative solutes.

Although pH increase does not have much influence on the retention of the weak acids or chromakaline (cf. MP 14 and 18, Table 8) since these are practically uncharged, the separation factors are, however, affected. For example, the separation of hexobarbital enantiomers improves substantially whereas the chiral discrimination between the mephenytoin enantiomers is significantly diminished when the pH of the mobile phase buffer is increased. Furthermore, the prominal enantiomers are only separated at pH 6.5 (MP 18), although the retention time remains about the same. Surprisingly, however, the prominal enantiomers are no longer separated if the isopropanol content of the mobile phase is lowered (cf. MP 17 and MP 18, Table 8). A similar observation was made by Hermansson, who described the separation of prominal enantiomers when a mobile phase containing 2% (v/v) isopropanol was used compared with pure phosphate buffer [13]. Chromakaline enantiomers are only separated when the retention is increased by decreasing the mobile phase isopropanol content to 1% (v/v) and increasing the pH to 6.5 (MP 17).

The reverse seems to apply for ibuprofen and naproxen, for which a significant decrease of the capacity factors is observed when the mobile phase pH is increased to 6.5 (Table 8). For these compounds, higher enantioselectivity is observed in mobile phases with higher pH and lower content (1% v/v) of isopropanol (MP 17).

The retention times of the basic β-blocking agents increase with increase in the mobile phase pH. As the enantioselectivity of this class of compounds is higher for conditions of high retention, the discrimination between the enantiomers improves at higher mobile phase pH [9].

All these findings concerning the different enantioselectivity of Chiral-AGP towards acids, bases and neutral compounds and the relationship of enantioselectivity with mobile phase changes indicate that the conformation of Chiral-AGP is linked to mobile phase composition. Indeed, there may very well be several stereoselective binding sites, which can be brought into operation under different conditions of mobile phase.

Ovomucoid (OVM)

Table 9 shows the results of the evaluation of the enantioselectivity of OVM towards the same set of substances (excluding propafenone) as for Chiral-AGP.

Neutral amides C and D (Fig. 2) could also be separated but the addition of 50% ethanol to the mobile phase was necessary to obtain reasonable retention times.

Table 9 indicates that the dihydropyridine calcium antagonists also show a strong affinity for OVM; in fact it was necessary to add 30% (v/v) ethanol to the mobile phase (MP 23) in order to obtain reasonable retention times. Felodipine enantiomers were not separated on OVM (by contrast with Chiral-AGP), although their affinity for the stationary phase was high. Ovomucoid shows high enantioselectivity towards the weak acids (mephenytoin, hexobarbital, prominal and thalidomide) and also for chromakaline and omeprazole.

An increase in pH causes an increase in k' and in α for the basic compounds, and a decrease in k' and α for the acidic compounds

Table 9
Evaluation of ovomucoid CSP for basic, acidic and neutral compounds

Compound	Mobile phase	k'_1	α	*P*
Calcium-channel blockers (weak bases)				
1 Nitrendipine	MP 23	0.74	1.18	0.40
2 Nimodipine	MP 23	0.55	1.39	0.88
3 Nisoldipine	MP 23	0.56	1.27	0.65
4 Nicardipine	MP 23	0.32	1	0
5 Amlodipine	MP 20	7.50	1	0
6 Felodipine	MP 23	1.26	1	0
Others				
7 Oxazepam	MP 20	6.48	1	0
8 Chromakaline	MP 20	1.18	1.45	1
10 Omeprazole	MP 20	5.94	1.18	0.76
Weak acids				
9 Thalidomide	MP 19	1.97	1.18	0.72
12 Mephenytoin	MP 20	0.80	2.92	1
13 Prominal	MP 20	2.29	1.25	0.94
14 Hexobarbital	MP 19	2.23	1.50	1
Acids				
15 Ibuprofen	MP 21	7.77	1.21	1
16 Naproxen	MP 22	3.55	1	0

Mobile phases used were: MP 19: phosphate buffer (pH 4.6; 0.02 M)–ethanol (95:5, v/v); MP 20: phosphate buffer (pH 4.6; 0.2 M)–ethanol (88:12, v/v); MP 21: phosphate buffer (pH 5.5; 0.02 M)–ethanol (95:5, v/v); MP 22: phosphate buffer (pH 6.5; 0.02 M)–ethanol (99:1, v/v); MP 23: phosphate buffer (pH 4.6; 0.02 M)–ethanol (70:30, v/v).

Table 10
Evaluation of cellulase CSP for basic, acidic and neutral compounds

Compound	Mobile phase	k'_1	α	*P*
Calcium-channel blockers (weak bases)				
1 Nitrendipine	MP 26	1.26	1	0
2 Nimodipine	MP 26	0.83	1	0
3 Nisoldipine	MP 26	1.50	1	0
4 Nicardipine	MP 24	3.52	1	0
5 Amlodipine	MP 24	3.86	1	0
6 Felodipine	MP 26	3.44	1	0
Others				
7 Oxazepam	MP 24	2.83	1	0
8 Chromakaline	MP 24	0.34	1	0
10 Omeprazole	MP 24	3.34	1.13	0
11 Propafenon	MP 24	2.04	1.51	0.85
Weak acids				
9 Thalidomide	MP 24	0.55	1.36	0.74
12 Mephenytoin	MP 24	0.40	1	0
13 Prominal	MP 24	0.49	1	0
14 Hexobarbital	MP 24	0.25	1	0
Acids				
15 Ibuprofen	MP 25	3.55	1	0
16 Naproxen	MP 26	3.63	1	0

Mobile phases used were: MP 24: acetate buffer (pH 5.0; 0.01 M)–isopropanol (99.5:0.5, v/v); MP 25: acetate buffer (pH 5.0; 0.1 M)–isopropanol (99:1, v/v); MP 26: acetate buffer (pH 5.0; 0.01 M)–isopropanol (85:15, v/v); MP 27: acetate buffer (pH 6.3; 0.01 M)–isopropanol (99:1, v/v).

[10, 14, 17]. For hexobarbital, a slight increase in the capacity factor and an increase in α have been reported when increasing the mobile phase pH [J. Haginaka, unpublished data].

It seems that OVM is well suited for the enantioseparation of acidic, neutral and basic compounds. The chiral separation of benoxaprofen, flurbiprofen, ketoprofen, chlorprenaline and chlorpheniramine has been recently described in the literature [14].

Cellulase

This stationary phase is enantioselective for a large number of the β-blocking agents studied [10, 15]. However, only three of the other selected compounds could be chirally separated on this type of column under the mobile phase conditions evaluated in this study (MP 24): thalidomide, omeprazole and propafenone, which has a β-blocker structure (Table 10). Although the calcium-channel blockers show high affinity for this stationary phase (addition of 15% isopropanol to the mobile phase is necessary, MP 26) they are not chirally separated.

Increasing the pH (MP 27) did not seem to have much influence on the retention time or the enantioselectivity of the weak acidic compounds. For omeprazole, the enantioselectivity was lost at pH 6.8, where the molecule is uncharged [15].

Conclusions

From this limited study in respect of the relatively small number of racemic compounds and from two previous publications from the authors' laboratories [9, 10], it can be concluded that the Chiralcel OD phase, the OVM phase and the AGP phase appear to be the most widely applicable for the set of substances evaluated, including acidic, basic and neutral compounds. Cellulase as a chiral selector shows very good enantioselectivity towards the β-blocking agents, which correspond to basic compounds sharing an aminopropanol structural element. This is illustrated in Table 11, where all the stationary phases have been compared for three selected compounds. The high enantioselectivity of the cellulase CSP is

Table 11
Comparison of the six chiral stationary phases evaluated for three representative chiral compounds

Compound	Chiral-AGP				OVM				CEL			
	k'_1	N^*	α	P†	k'_1	N^*	α	P	k'_1	N^*	α	P
Oxprenolol	0.77	3472	1.11	0.10^1	6.41	1182	1.15	0.33^4	0.53	945	1.89	1^6
Nisoldipine	7.34	1526	1.38	1^2	0.56	1882	1.27	0.65^5	1.50	560	1	0^7
Hexobarbital	0.72	1186	1.31	0.70^3	2.23	912	1.50	1^4	0.25	839	1	0^6

	Chiraspher				SN-β-cyclodextrin (RP mode)				SN-β-cyclodextrin (NP mode)			
	k'_1	N	α	P	k'_1	N	α	P	k'_1	N	α	P
Oxprenolol	0.74	1566	1	0^8	1.32	9585	1	0^{10}	1.30	651	1	0^8
Nisoldipine	1.24	2635	1	0^8	1.97	15602	1	0^{11}	0.89	4234	1	0^8
Hexobarbital	0.97	2526	1	0^9	1.56	11444	1.11	0.97^{12}				

	Chiralcel OD			
	k'_1	N	α	P
Oxprenolol	0.39	8328	3.23	1^{13}
Nisoldipine	0.43	9822	1.09	0.17^{14}
Hexobarbital	2.24	7592	1.07	0.38^{14}

Key to mobile phase used:
[1] Phosphate buffer (pH 4.6; 0.02 M)–isopropanol (95:5, v/v) MP 14.
[2] Phosphate buffer (pH 4.6; 0.02 M)–isopropanol (87:13, v/v) MP 16.
[3] Phosphate buffer (pH 6.5; 0.02 M)–isopropanol (95:5, v/v) MP 18.
[4] Phosphate buffer (pH 4.6; 0.02 M)–ethanol (95:5, v/v) MP 19.
[5] Phosphate buffer (pH 4.6; 0.02 M)–ethanol (70:30, v/v) MP 23.
[6] Acetate buffer (pH 5.0; 0.01 M)–isopropanol (99.5:0.5, v/v) MP 24.
[7] Acetate buffer (pH 5.0; 0.01 M)–isopropanol (85:15, v/v) MP 26.
[8] Heptane–isopropanol–DEA (70:30:0.1, v/v/v) MP 5.
[9] Heptane–dioxane–DEA (70:30:0.1, v/v/v) MP 1.
[10] Ammonium acetate buffer (pH 7.0; 0.1 M)–acetonitrile (80:20, v/v) MP 8.
[11] Ammonium acetate buffer (pH 7.0; 0.1 M)–acetonitrile (60:40, v/v) MP 28.
[12] Ammonium acetate buffer (pH 7.0; 0.1 M)–acetonitrile (70:30, v/v) MP 7.
[13] Heptane–ethanol–DEA (80:20:0.1, v/v/v) MP 10.
[14] Heptane–isopropanol–DEA (80:20:0.1, v/v/v) MP 9.
*N is calculated from the data for peak 1, where there is chiral discrimination.
†Figures in supersciprts refer to the mobile phase used for each experiment.

illustrated for oxprenolol, where a baseline separation is obtained with very low retention. Chiral-AGP and OVM, however, seem to be enantioselective towards compounds with different properties; all three selected compounds could be chirally separated by these two phases.

Chiralcel OD also shows high enantioselectivity towards β-blockers. Table 11 shows that the efficiency, as expressed by the plate number, is high. The column seems to be less enantioselective towards the other compounds examined (Table 11). Chiraspher and the derivative of β-cyclodextrin do not seem to be very enantioselective towards the compounds studied under the mobile phase conditions evaluated (Table 11). Only hexobarbital enantiomers could be practically baseline resolved on the highly efficient SN-β-cyclodextrin CSP in a reversed-phase system.

It should be noted that the protein-based phases are only used in reversed-phase mode, while the other CSPs are used in normal-phase mode, with the exception of SN-β-cyclodextrin, which was used in both modes. Systematic optimization [11, 18] of the 28 mobile phases used in this work was not carried out in these studies on approximately 45 racemic mixtures. Thus the present results for individual racemates may be considered as preliminary in nature, giving a broad overview on some of the points of contrast between the six chiral phases examined. In the field of enantioseparation by LC, new phases and new derivatives of CSPs are appearing regularly. For example, with the new generation of OD phases, aqueous mobile phases can be used. It will be of interest to evaluate the differences in terms of enantioselectivity, compared with the phases studied in this work.

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