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Evaluation of a penicillin G acylase-based chiral stationary phase towards a series of 2-aryloxyalkanoic acids, isosteric analogs and 2-arylpropionic acids

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Abstract

The chiral recognition properties of a new chiral stationary phase based on immobilized penicillin G acylase were investigated using 35 acidic racemates. Twenty-seven compounds were resolved with high separation factors. The influences of mobile phase pH, type of organic modifier and ionic strength on enantioselective retention were studied. The most important tool for affecting the enantioselectivity was the mobile phase pH and interestingly the retention order of the enantiomers of some analytes could be controlled by this parameter. The analysis time for resolving enantiomers could be adjusted with a minor decrease in enantioselectivity using a high ionic strength mobile phase buffer while both retention and enantioselectivity decreased by adding organic modifier to the mobile phase. Displacement studies have demonstrated that the enzymatically active site and the chiral adsorption site overlap.

Keywords: Chiral stationary phases, LC; Enantiomer separation; Substituent effects; Chiral recognition mechanism; Penicillin G acylase; 2-Aryloxyalkanoic acid; 2-Arylpropionic acid

1. Introduction

The importance to develop efficient enantiomeric separation systems is a topic of interest for many scientific and economic reasons [1]. High-performance liquid chromatography (HPLC) is one of the most common techniques for the analytical separation of stereoisomers and in the last two decades many chiral stationary phases (CSPs) have been developed for the resolution of chiral compounds often sharing analogous or overlapping enantioselectivities. A variety of protein-based stationary phases have been used so far as chiral selectors in HPLC as recently reviewed by Haginaka [2]. The proteins used include albumins such as bovine and human serum albumin, glycoproteins (α_1-acidglycoprotein, ovomucoid, avidin and riboflavin binding protein), ovotransferrin, β-lactoglobulin and enzymes such as trypsin, α-chymotrypsin, cellobiohydrolase, lysozyme, pepsine and amyloglucosidase. More recently

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a new enzyme, penicillin G acylase (PGA), was immobilized on silica supports and evaluated as a reactor and chiral selector in HPLC [3].

Penicillin G acylase of *Escherichia coli* ATCC 11105 (EC 3.5.1.11) belongs to the family of N-terminal-nucleophile hydrolases, comprising enzymes that share a common fold around the active site and that contain a catalytic nucleophilic serine at N-terminal position [4].

PGA catalyzes the hydrolysis of penicillin G to phenylacetic acid and 6-aminopenicillanic acid and it is well known for its industrial application in the production of this β-lactamic nucleus, which is a building block in the synthesis of semi-synthetic penicillins. Moreover the enzyme is capable of accepting a wide range of structurally different compounds including phenylacetylated derivatives of anilines, alcohols, α- and β-amino acids and it is able to selectively recognize the side chain according to its chirality [5–8], indicating that the binding site should have a low specificity [9,10].

The three-dimensional structure of the active site of penicillin acylase from *E. coli* was elucidated by X-ray crystallography [4]. The structure of the enzyme complexed with a product, phenylacetic acid, that acts as strong competitive inhibitor, reveals a deep depression in the surface of the enzyme, at the base of which is a small hydrophobic pocket, lined with mainly aromatic residues and hydrophobic side-chains [9]. It was suggested that a positively charged group would be present close to the entrance of the hydrophobic phenylacetyl binding pocket where it could have an interaction with the negatively charged carboxylate groups of β-lactam nuclei and amino acids.

In a previous work, the development of a PGA-chiral stationary phase (PGA-CSP) has been reported and different ways of immobilizing PGA have been studied [3]. It was shown that the use of an epoxide silica gave the best immobilization ratio and higher enantioselectivity, whereas when comparing silica with different pore size diameter (100 and 200 Å) the best resolution was offered by the 100 Å silica. PGA-based CSP could resolve the enantiomers of acidic compounds while no chiral recognition of basic chiral solutes was observed; the developed column was also used as a chromatographic bioreactor able to hydrolyze esters and separate the substrates from the products.

In this paper, the chiral recognition mechanism of PGA-CSP was studied using some 2-arylpropionic acids and a variety of closely related 2-aryloxyalkanoic acids and isosteric analogs. The former is a well-known class of non-steroidal anti-inflammatory drugs (NSAIDs) in which the pharmacological activity resides in the *S*-enantiomers, and the latter are compounds in which the absolute configuration has been shown to exert a strong influence on the herbicidal activity [11], the skeletal muscle membrane chloride conductance [12–16], the prostaglandin-dependent platelet aggregation [12,17], the peroxisome proliferation [18,19] and, more recently, the anti-inflammatory activity [20] and the Peroxisome Proliferator Activated Receptor alpha (PPARα)-mediated hypolipidemic effects [21].

In order to study the influence of molecular structure, hydrophobic, electrostatic and hydrogen binding on chiral recognition, the mobile phase composition (ionic strength, pH and type of organic modifier) was varied. Displacement studies were also carried out adding phenylacetic acid to the mobile phase, which resulted in blocking of the active site of the enzyme.

The derived information shed light on the stereoselective binding characteristics of this enzyme suggesting further applications in the production of substances with high enantiomeric purity.

2. Experimental

2.1. Reagents and materials

Penicillin G acylase crude extract from *Escherichia coli* ATCC 11105 (EC 3.5.1.11) was kindly donated by Recordati (Milan, Italy) and used as received; *rac*-ketoprofen was kindly donated by S.I.M.S s.r.l (Incisa Valdarno, FI, Italy); 2-(4-phenoxyphenoxy) propionic acid (racemate 22) and 2-(4-benzylphenoxy) propionic acid (racemate 23) were a gift from Professor O. Azzolina (University of Pavia). Racemates 1–6, 8–21 and isomers (+)-(*R*)-1, (+)-(*R*)-2, (+)-(*R*)-5, (+)-(*S*)-6, (+)-(*R*)-8, (+)-(*R*)-9, (+)-(*R*)-10, (+)-(*S*)-11, (+)-(*S*)-12, (+)-(*R*)-13, (+)-(*R*)-15 and (+)-(*R*)-16 were prepared according to our previous papers [17,22,23]. Compounds 7 and 24 were obtained by treating a THF solution of 2-(4-chlorophenoxy)acetic and 2-(4-chlo-

rophenoxy)phenylacetic acids with benzyl bromide and methyl iodide, respectively, in the presence of LDA at −78 °C. Compounds 25–28 were prepared by a yield-improving modification of a method reported in literature [24] condensing the suitable phenol or thiophenol with 2-butanone (acids 26–28) or 2-pentanone (acid 25) in the presence of bromoform and KOH.

Rac-ibuprofen, *rac*-indoprofen, *rac*-carprofen, *rac*-fenoprofen, *rac*-suprofen, *rac*-flurbiprofen, were purchased from Sigma (St Louis, MO, USA). Potassium dihydrogenphosphate, dipotassium hydrogenphosphate and the organic solvents were purchased from Merck (Darmstadt, Germany).

Kromasil 5 μm, 120 Å silica gel was from Alltech Italia (Sedriano, MI, Italy). Water was deionized by passing through a Direct-Q™ (Millipore) system (Millipore, Bedford, MA, USA).

2.2. Apparatus

Chromatographic experiments were carried out with a Hewlett-Packard HP 1100 liquid chromatograph (Palo Alto, CA, USA) with a Rheodyne sample valve (20-μl loop) equipped with a Hewlett-Packard HP 1100 variable-wavelength detector and an HP 1100 thermostat. The system was connected to a HPLC ChemStation (Revision A.04.01).

2.3. Methods

2.3.1. Chromatographic conditions for PGA columns

All the chromatographic experiments were carried out at a constant oven temperature of 20 °C and the column flow-rate was set at 0.8 ml/min. The UV trace was followed at 225 nm. When not in use, the columns were stored at 4 °C in a 0.01% (w/v) solution of sodium azide.

Sample preparation was carried out by dissolving known amounts of the chiral drug in 1-propanol and each solution was diluted with the mobile phase buffer to a concentration of 0.1 m*M*.

In the study of the enantiomeric elution order, solutions of (+)- and (−)-isomers with concentration ratio=2, were injected.

2.3.2. Immobilization technique

The epoxide columns (50×4.6 mm I.D.) used for the ''in situ'' immobilization of PGA were prepared following a previously described method [25,3]. Two PGA-CSPs were prepared and the enantioselective performances are compared in Table 1 using ketoprofen as a probe. The amount of immobilized protein, calculated by elemental analysis, was different for the two columns. The retentivity was greater for the stationary phase with higher amount of immobilized enzyme while the variation in enantioselectivity and efficiency was not significant.

2.3.3. Chromatographic responses

The retention factor (k) was calculated using the equation $k = (t_r/t_o) - 1$, where t_r is the retention time of the analyte and t_o is the retention time of an unretained compound; in this study t_o was calculated from the first disturbance of the baseline after injection. The separation factor (α) was calculated using the equation, $\alpha = k_2/k_1$ where k_1 and k_2 are the retention factors for the first and last eluted enantiomers, respectively. The number of theoretical plates was calculated with the half-width method.

2.3.4. Displacement studies

Displacement studies were carried out on the PGA column by applying a buffer mobile phase (50 m*M* pH 7.0) containing phenyl acetic acid as competing agent. Different concentrations of displacing agent (0.01, 0.05 and 0.1 m*M*) were applied to the column, the resulting solutions were passed through the column following the absorbance of the mobile phase at 220 nm. Once the column was equilibrated, the detector baseline was returned to zero and

Table 1
Comparison of the chromatographic performances of the PGA-columns

	Column 1	Column 2
Immobilized amount[a] (mg/g)±RSD	51.23±6.66	133.28±2.33
k_1	1.15	1.59
α	4.3	4.0
Resolution	5.56	5.64
Plates[b]	607	607

Columns 1 and 2: 50×4.6 mm I.D.; analyte, ketoprofen; mobile phase, 50 m*M* KH_2PO_4 (pH 7.0); flow-rate, 0.8 ml/min.

[a] Amount of immobilized enzyme, calculated by elemental analysis ($n=3$).

[b] Calculated on the second eluted enantiomer.

allowed to stabilize and the retention factors for the probes compounds were determined.

3. Results and discussion

3.1. Influence of solute structure on retention and enantioselectivity

Preliminary studies have indicated that immobilized PGA can be used as chiral selector for acid compound [3]. In the context of this study, the applicability of the PGA-CSP for the enantioseparation of an additional 28 closely related 2-aryloxyalkanoic acids, isosteric analogs and seven 2-arylpropionic acids has been evaluated. Moreover, the same analytes were used as model compounds in order to study the influence of the molecular structure on retention and enantioselectivity. Fig. 1 reports the enantiomeric separation obtained for some compounds.

The compounds were chromatographed using a mobile phase of 50 m*M* phosphate buffer pH 7. Tables 2 and 3 summarize the results obtained with the PGA-CSP: 27 out of 35 racemates were chiraly separated and for most of these compounds, the first eluted enantiomer was poorly retained by the PGA-CSP, whereas α-values fell within a fairly broad range. The exceptional enantioselectivity observed for some analytes (racemates 1, 15, 16, 24) is the result of the poor retention observed for the first eluted enantiomer.

An increase in the lipophilicity of the substituent on the benzene ring (compounds 1 and 13 in comparison to 15, 16 and 18–20) leads to an increase in the retention of the first eluted enantiomer while the observed enantioselectivities do not correlate with the hydrophobicity of the analytes. By plotting the separation factors, obtained on PGA-CSP, against the k_1 values no correlation could be found confirming that it is not necessary that the compounds are strongly retained to obtain enantioseparation. If considering the dimensions of the benzene ring substituent of the same racemates, an increase in its size causes a progressive reduction in selectivity suggesting that the binding site at which

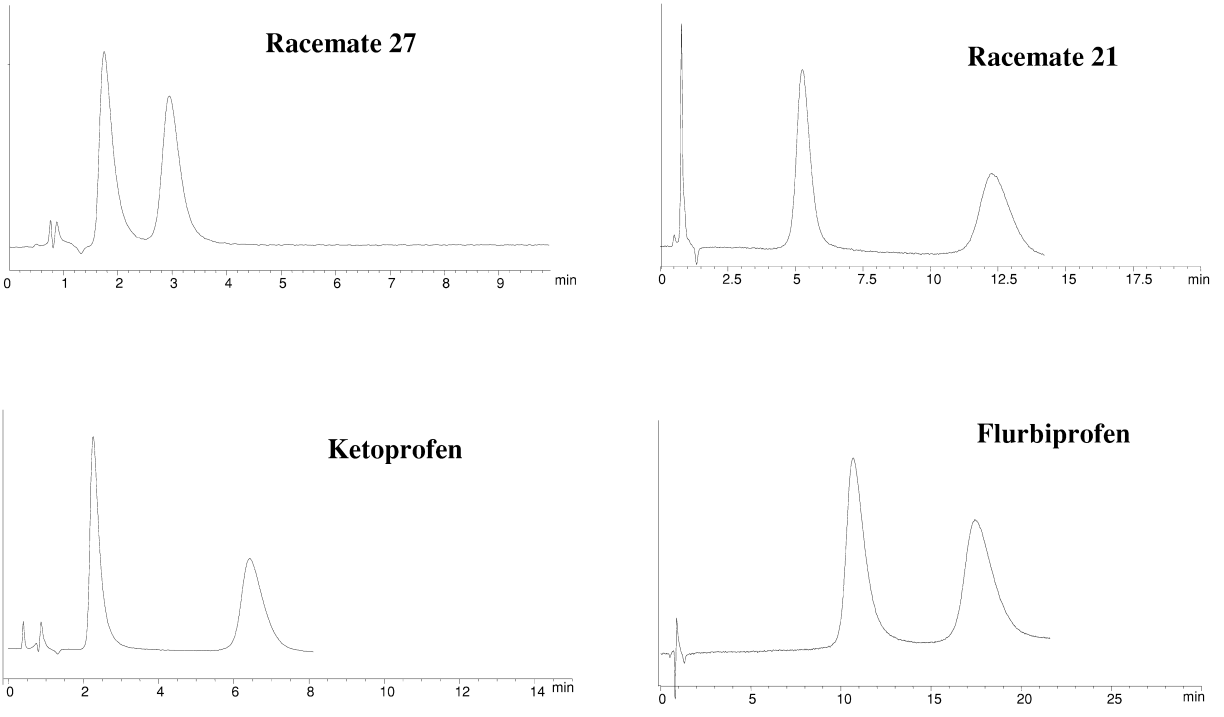
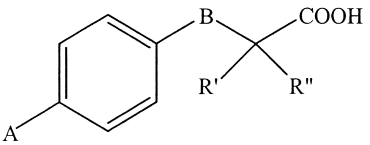


Fig. 1. Chromatograms of racemates 21, 27, ketoprofen and flurbiprofen on PGA-column. Mobile phase: 50 m*M* phosphate buffer (pH 7.0). Flow: 0.8 ml/min. Column 2.

Table 2
Influence of solute structure on retention and enantioselectivity of a series of 28 structurally correlated analytes



Racemate	A	B	R′	R″	k_1 [a]	α
1	Cl	O	CH_3	H	0.37 (*R*)	26.4
2	Cl	O	C_2H_5	H	0.4 (*R*)	3.34
3	Cl	O	n-C_3H_7	H	0.92	1.68
4	Cl	O	i-C_3H_7	H	0.6	1.0
5	Cl	O	n-C_6H_{13}	H	6.43 (*R*)	1.96
6	Cl	O	C_6H5	H	2.19 (*S*)	3.69
7	Cl	O	$C_6H_5CH_2$	H	3.99	1.14
8	Cl	S	CH_3	H	2.76 (*R*)	3.29
9	Cl	S	C_2H_5	H	1.75 (*R*)	4.4
10	Cl	NH	CH_3	H	1.84 (*R*)	2.84
11	Cl	CH_2	CH_3	H	2.92 (*S*)	4.06
12	Cl	CH_2	C_2H_5	H	1.68 (*S*)	3.30
13	Br	O	CH_3	H	1.27 (*R*)	3.78
14	Br	O	C_2H_5	H	0.93	2.09
15	F	O	CH_3	H	0.06 (*R*)	250.79
16	CH_3	O	CH_3	H	0.085 (*R*)	22.05
17	CH_3	O	C_2H_5	H	0.18	1.0
18	CN	O	CH_3	H	0.09	1.0
19	CH_3O	O	CH_3	H	0.07	1.0
20	CH_3CO	O	CH_3	H	0.10	1.0
21	C_6H_5CO	O	CH_3	H	5.56	2.58
22	C_6H_5O	O	CH_3	H	5.89	1.97
23	$C_6H_5CH_2$	O	CH_3	H	4.57	4.33
24	Cl	O	CH_3	C_6H_5	1.08	25.66
25	Cl	O	CH_3	n-C_3H_7	0.8	6.79
26	Cl	O	CH_3	C_2H_5	1.13	1.0
27	Br	O	CH_3	C_2H_5	1.18	2.48
28	Br	S	CH_3	C_2H_5	1.91	1.19

Column 2. Mobile phase: 50 m*M* KH_2PO_4 (pH 7.0); flow-rate, 0.8 ml/min.

[a] The configuration of the isomer corresponding to the first-eluted peak is given in parentheses.

enantioselective binding occurs is a chiral cavity with defined steric restrictions. When the bulkiness of the substituent reaches a critical dimension (racemates 18–20) the solute might fit only partially into the cavity diminishing the ability of the enzyme to discriminate between the two enantiomers of the solute. Compounds with an additional aromatic ring in the A position are the most retained and the above consideration cannot be extended to this group but it is possible to hypothesize that an additional hydrophobic interaction can be involved in the chiral recognition mechanism.

The steric bulk on the stereogenic center was also evaluated. In general, an increase in bulkiness of this substituent causes an increase in retention (racemates 1–7) while enantioselectivity is reduced. When a second substituent is introduced in the α position (compounds 24, 25, 26), the retention is almost unaffected while enantioselectivity increased. In particular the enantioselectivity increased dramatically with an aromatic ring in this position (compound 24). Also in this case hydrophobic interactions may play a role in the chiral recognition mechanism.

As far as the influence of B substituent is concerned, an increase in the lipophilicity causes an increase in retention. The isosteric substitution of the ether oxygen atom with sulfur, amino or methylen groups (compounds 1, 8, 10, 11) had a slight effect on enantioselective retention.

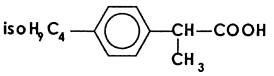
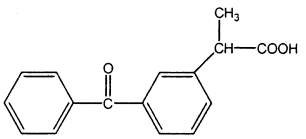
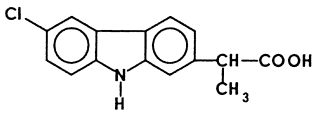
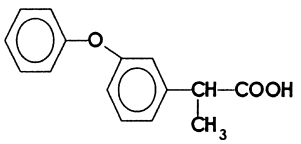
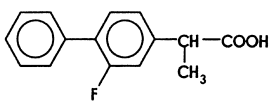
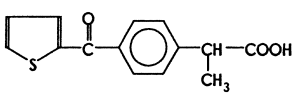
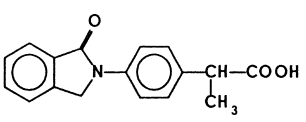
The same considerations can be extended to the arylpropionic acids (Table 3) where a complete loss of enantioselectivity was observed for analytes with bulky (ibuprofen) and rigid aryl moiety (carprofen) that may lead to exclusion of the solute from the chiral binding site.

For those compounds where absolute configuration is known, the elution order *R*/*S* is constant with the exception of compound 6 for which the elution order is reversed. This suggests that the unsubstituted phenyl ring competes with the *p*-chlorophenoxy group for the interaction with the chiral binding site giving rise to an opposite chiral recognition mechanism. Compounds 11 and 12 elute *S*/*R* only as a result of the change in priority of the Cahn, Ingold, Prelog descriptor system for chiral molecules.

3.2. Effect of pH on retention and enantioselectivity

It is well known that pH controls the degree of charge present on the protein surface, the tertiary structure of the protein and the ionization status of the analyte; moreover pH is a crucial factor affecting the catalytic activity of this enzyme, for PGA the hydrolytic activity increasing with pH [26]. Therefore, the effect of the mobile phase pH on retention of some selected analytes was studied and the results

Table 3
Retention and enantioselectivity of a series of seven non-steroidal antiinflammatory drugs

Racemate	Structure	k_1	α
Ibuprofen		1.16	1.0
Ketoprofen		1.59	4.0
Carprofen		125.84	1.0
Fenoprofen		4.26	3.89
Flurbiprofen		12.35	1.68
Suprofen		5.77	1.95
Indoprofen		46	1.95

Column 2. Mobile phase: 50 m*M* phosphate buffer, pH 7.0; flow-rate 0.8 ml/min.

are shown in Table 4. Seven mobile phases with pH values ranging from 4.0 to 7.0 were tested.

The considered analytes can be grouped into two distinct categories depending on the chromatographic data. A decrease in retention on increasing the pH was observed for racemates 1, 2, 9, 13, 16, 21, 22, 23 whose pK_a values are between 3 and 3.5. The reduction of the retention factors can be explained by the fact that the negative charge of both the immobilized protein (isoelectric point about 4.0) and the analytes rises as the mobile phase pH increases; therefore anion–anion repulsion increased. In this group an unusual trend (a maximum in retention around pH 4.5) was observed for the second eluted enantiomer of racemates containing a second aryl moiety in their structure (racemates 21, 22, 23). A

Table 4
Influence of mobile phase pH on retentions and enantioselectivities

Racemates	pH 4.0		pH 4.5		pH 5.0		pH 5.5		pH 6.0		pH 6.5		pH 7.0	
	k_1 [a]	α	k_1	α	k_1	α	k_1	α	k_1	α	k_1	α	k_1	α
1	21.2 (*R*)	2.89	13.9 (*R*)	3.45	9.87 (*R*)	4.11	5.51(*R*)	5.5	2.97 (*R*)	7.49	1.28 (*R*)	12.47	0.37 (*R*)	26.4
2	6.53 (*S*)	1.68	4.52 (*S*)	1.58	3.74 (*S*)	1.26	1.97 (*R*)	1.31	1.4 (*R*)	1.66	0.75 (*R*)	2.24	0.40 (*R*)	3.34
9	26.24 (*R*)	16.41	17.3 (*R*)	15.75	12.99 (*R*)	16.50	7.93 (*R*)	18.0	5.37 (*R*)	13.52	3.23 (*R*)	8.62	1.75 (*R*)	4.4
10	5.24 (*S*)	3.35	7.46 (*S*)	2.48	8.71 (*S*)	1.64	8.46	1.0	5.05 (*R*)	1.52	2.80 (*R*)	2.21	1.84 (*R*)	2.84
12	23.2 (*S*)	3.84	29.26 (*S*)	4.27	24.4 (*S*)	4.46	13.7 (*S*)	4.49	7.66 (*S*)	4.22	3.46 (*S*)	3.84	1.68 (*S*)	3.30
13	19.69 (*S*)	1.13	15.76	1.0	10.9 (*R*)	1.21	5.75 (*R*)	1.73	3.61 (*R*)	2.24	2.01 (*R*)	2.97	1.27 (*R*)	3.78
16	3.67 (*R*)	5.37	2.37 (*R*)	6.19	1.44 (*R*)	7.45	0.71 (*R*)	8.87	0.47 (*R*)	9.3	0.52 (*R*)	5.09	0.08 (*R*)	22.05
21	10.02	1.45	8.48	1.99	8.82	2.24	8.34	2.4	7.9	2.46	6.89	2.54	5.56	2.58
22	11.42	1.32	9.19	1.68	9.72	1.80	9.24	1.88	8.52	1.89	7.24	2.04	5.89	1.97
23	14.15	1.47	8.80	2.58	8.96	2.99	7.87	3.5	7.16	3.66	5.69	4.16	4.57	4.33

Column 2. Mobile phase: 50 m*M* KH_2PO_4; flow-rate, 0.8 ml/min.
[a] The configuration of the isomer corresponding to the first-eluted peak is given in parentheses.

possible explanation for this behavior could be a conformational change in the enzyme affecting the availability of an additional lipophilic binding area.

Bell-shaped retention curves were observed for both enantiomers of compounds 10 and 12 with a maximum in retention at pH 5.0 and 4.5, respectively, close to their pK_a values (pK_a around 4.5). This can be attributed to the change in the ionization status of the analytes that occurs at pH values higher than 4.

An interesting effect of mobile phase pH was observed for the enantiomers of compounds 2, 10 and 13; a progressive reduction of the mobile phase pH lowered the enantioselectivity to a minimum resulting in a co-elution of the enantiomers at a certain pH value; below this ''crossover'' pH, the enantiomers were again resolved by PGA-CSP.

The availability of isomers (+)-(*R*)-1, (+)-(*R*)-2, (+)-(*R*)-9, (+)-(*R*)-10, (+)-(*S*)-12, (+)-(*R*)-13, (+)-(*R*)-16 allowed the demonstration that for racemates 2, 10 and 13, with limited enantioselectivity, inversion of the elution order took place; the same behavior has previously been observed on AGP-CSP and OVM-CSP [27,28]. One possible explanation for the observed reversal in retention order is that the two enantiomers are retained by different retention mechanisms, i.e. adsorption to different sites.

The enantioselectivity of the racemates 2 and 9 is higher at lower pH values while the opposite trend was observed for compounds 1 and 16, structurally characterized by an oxygen atom in the α position to the chiral center. At low pH, the oxygen can be responsible of an additional hydrogen bond with a protonated basic site, hindering the chiral discrimination.

3.3. Effect of ionic strength on retention and enantioselectivity

The effect of the ionic strength mobile phase on the enantioselective retention was examined. The results are summarized in Table 5. A large decrease in retention was observed on increasing the ionic strength from 0.01 to 0.1 *M*, therefore electrostatic interactions are decreasing faster than hydrophobic interactions are increasing. The enantioselectivity was almost unaffected suggesting that the ion's strength influences mainly the non-enantioselective interactions. This enables adjustment of the retention and shortening the analysis time without sacrificing enantioselectivity for racemates with moderately high separation factors.

Only the compounds with two aromatic systems (ketoprofen, fenoprofen, racemates 21–23) show a significant improvement in enantioselectivity with the decrease in ionic strength.

3.4. Effect of organic modifier on retention and enantioselectivity

The type and the percentage of organic modifier added to the mobile phase may influence enan-

Table 5
Influence of the mobile phase ionic strength on retention factors and enantioselectivities

Racemates	0.1 *M*		0.05 *M*		0.01 *M*	
	k_1	α	k_1	α	k_1	α
Ketoprofen	4.51	1.18	5.84	1.19	12.30	1.72
Fenoprofen	8.64	1.22	11.80	1.19	23.39	1.65
1	9.63	2.85	13.43	2.81	47.68	2.81
2	3.47	1.36	4.68	1.41	14.45	1.52
9	9.90	18.83	14.43	18.01	38.83	[a]
10	2.26	3.24	3.76	3.77	12.45	3.12
12	8.85	4.00	12.72	3.63	43.12	4.13
13	9.08	1.00	11.94	1.13	35.30	1.00
16	1.29	6.62	2.05	6.93	4.84	6.83
21	3.40	1.46	4.91	1.49	15.83	2.22
22	4.89	1.25	7.22	1.28	23.24	1.60
23	6.02	1.34	9.24	1.33	24.22	2.13

Column 1. Mobile phase: phosphate buffer, pH 4.0.
[a] Second enantiomer not observed.

tioselective retention to a great extent when proteins are used as chiral selectors. The retention times were very short for most of the solutes tested, therefore small amounts of organic modifier were used in this study. The effects of organic modifiers on retention and enantioselectivity of PGA-CSP are presented in Table 6; the addition of only 1% organic solvent resulted in a pronounced decrease in k' and α on the PGA column. Two different organic modifiers were tested as mobile phase additives: small differences in enantioselectivity have been observed.

3.5. Displacement studies

In these studies, racemates 8, 10, 12, and 13 were used as probe compounds. A concentration-dependent reduction of the retention factors of both enantiomers was observed (Table 7) and at high competitor concentration (0.1 m*M*) the stationary phase was unable to retain and to stereochemically resolve all test compounds. Therefore phenyl acetic acid appears to compete for binding with both enantiomers. The results suggest that binding interac-

Table 6
Influence of different organic modifiers on retention factors and enantioselectivities

Racemates			1% methanol		1% 2-propanol	
	k_1	α	k_1	α	k_1	α
Ketoprofen	5.84	1.19	2.95	1.17	3.33	1.00
Fenoprofen	11.80	1.19	5.51	1.22	7.09	1.00
1	13.43	2.81	3.96	2.51	2.37	2.11
2	4.68	1.41	2.33	1.00	1.92	1.00
9	14.43	18.01	4.97	15.09	3.66	8.83
10	3.76	3.77	1.54	2.30	1.27	1.47
12	12.72	3.63	4.85	3.74	3.03	2.44
13	11.94	1.13	4.06	1.00	2.62	1.00
16	2.05	6.93	0.79	4.41	0.79	2.49
21	4.91	1.49	2.29	1.47	2.47	1.19
22	7.22	1.28	3.07	1.31	3.65	1.21
23	9.24	1.33	3.81	1.36	4.74	1.18

Column 1. Mobile phase: 50 m*M* phosphate buffer, pH 4.0.

Table 7
Influence of phenyl acetic acid concentration on retention and selectivity

Compound	Phenyl acetic acid concentration											
	0 m*M*			0.01 m*M*			0.05 m*M*			0.1 m*M*		
	k_1	k_2	α	k_1	k_2	α	k_1	k_2	α	k_1	k_2	α
8	2.76	9.08	3.29	2.23	7.67	3.44	1.62	5.75	3.55	[a]	[a]	–
10	1.84	5.22	2.84	1.37	4.23	3.09	0.47	2.19	4.65	[a]	[a]	–
12	1.68	5.5	3.30	1.21	3.78	3.13	0.77	2.77	3.60	[a]	[a]	–
13	1.27	4.8	3.78	0.95	3.97	4.18	0.62	2.52	4.08	[a]	[a]	–

Column 2. Mobile phase: 50 m*M* phosphate buffer, pH 4.0.
[a] Not retained.

tions between acid compounds and PGA column mainly occur at the active site of the enzyme.

4. Conclusion

Complete chiral resolution of many 2-aryloxyalkanoic acids, isosteric analogs and 2-arylpropionic acids was obtained with the PGA column. The enantioselective retention can be controlled by several mobile-phase parameters, e.g. mobile-phase pH, ionic strength and type of organic modifier. The most important tool for affecting the enantioselectivity and retention is the mobile phase pH and interestingly, the retention order of the enantiomers of some analytes (racemates 2, 10 and 13) could be controlled by this parameter. The increase in mobile phase ionic strength causes a decrease in retention while enantioselectivity is almost unaffected. The addition of an organic modifier to the mobile phase (i.e. methanol and 2-propanol) reduces retention and enantioselectivity.

The results from the displacement studies indicate that interaction with the specific active site is necessary for enantioselective discrimination and for enhanced retention.

Electrostatic interactions between the compounds and the positively charged group present close to the entrance of the binding pocket are the main binding force while hydrophobic interactions contribute to the stabilization of the analyte–enzyme complex and to enantioselectivity. In particular the hydrophobic interaction involves the steric fit of the solute in the chiral cleft which leads to enantiodiscrimination.

From the large set of analytes considered in this work, it was possible to obtain qualitative information on the structural requirement to obtain enantioselective retention. Quantitative structure–enantioselective retention relationship studies will be undertaken to describe the relationships between the chemical structures of analytes and the chromatographic results and to provide information on the chiral recognition mechanism.

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