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Journal of Chromatography B, 751 (2001) 117–130

JOURNAL OF CHROMATOGRAPHY B

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Properties of a stationary phase based on immobilised chicken liver basic fatty acid-binding protein

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Received 27 April 2000; received in revised form 3 August 2000; accepted 10 August 2000

Abstract

The fatty acid-binding proteins (FABPs) are a class of low-molecular-mass proteins that bind fatty acids and are thought to be involved in their intracellular transport. FABPs have been isolated and studied from several tissues, but their precise function and mechanism of action are still not clear. Chicken liver (basic) fatty acid-binding protein (bFABP) was immobilised on aminopropyl silica and the developed stationary phase was used to examine the enantioselective properties of this protein and to study the binding of drugs to bFABP. The retention and enantioselectivity of the new column for a large number of chiral drugs was investigated. The enantiomers of basic and neutral compounds were poorly retained and not resolved by the bFABP column. On the contrary the resolution of the enantiomers of some acidic compounds was obtained. Therefore the influence of the mobile phase pH and organic modifier on the chromatographic performance of acidic compounds was studied. In order to clarify the retention mechanism, competitive displacement studies were also carried out by adding short-chain fatty acids to the mobile phase as displacing agents and preliminary quantitative structure–retention relationship correlations were developed to describe the nature of the interactions between the chemical structures of the analytes and the observed chromatographic results.

Keywords: Chiral stationary phases, LC; Proteins; Fatty acids

1. Introduction

Basic fatty acid-binding protein (bFABP) is one member of a superfamily of low-molecular-mass molecules collectively called fatty acid-binding proteins (FABPs). The FABPs have been isolated from different mammalian and non-mammalian tissues and it has been shown that they can bind and solubilise fatty acids and may thus participate in their intracellular transport and/or storage, much like albumin does extracellularly [1–3]. Although FABPs are thought to have these biological functions, their precise physiological role is yet to be elucidated. Basic fatty acid-binding protein is present at high

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0378-4347/01/$ – see front matter
PII: S0378-4347(00)00464-3

concentrations in chicken liver and it is known as bFABP because it has an isoelectric point of 9, different from the more widely known FABPs endowed with lower isoelectric points. It consists of 125 amino acid residues and it has a molecular mass of 14 000 [4,5]. The three-dimensional structure of bFABP was solved at intermediate resolution and it was found that its overall architecture is very similar to that of rat intestinal FABP [4,6]. The binding site of bFABP is a hydrophobic pocket and severe steric constraints of the binding site seem to allow only 1 mole of fatty acid to bind to the protein [6,7]. The binding affinity of fatty acids is primarily determined by interactions of the entire aliphatic chain with the binding cavity but also electrostatic interactions take place between the carboxylic group of the fatty acids and an arginine residue present at the binding site [7].

However, systematic binding studies have not been undertaken on bFABP so far and its possible physiological role has not been yet fully investigated.

In this paper, we report the preparation of a stationary phase based on immobilised bFABP in order to use the chromatographic approach to examine the enantioselective properties of this protein and to study the binding of drugs to bFABP. The potential chiral discrimination properties of the bFABP column were evaluated by analysing a large number of chiral drugs with different structures and chemical properties and the effects of the mobile phase pH, type and percentage of organic modifier on retention and enantioselectivity were investigated. Next, the aryl- and aryloxy-propionic acids analysed on this column were also selected to perform preliminary quantitative structure–retention relationship (QSRR) studies using multiparameter regression analysis. QSRR correlations were used to describe the nature of the interactions between the chemical structures of the analytes and the observed chromatographic results.

The developed bFABP column was also used to perform zonal elution studies, this approach was applied to examine the competition between short-chain fatty acids and acidic analytes. These studies should provide information on the general mechanism involved in the retention of molecules by the bFABP column.

2. Experimental

2.1. Apparatus

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 connected to a HPLC ChemStation (Revision A.04.01) was used. Column and mobile phases were temperature-controlled using a HP 1100 thermostat.

Preparative isoelectric focusing (IEF) was performed by means of a Multiphor II System from Pharmacia (Uppsala, Sweden).

2.2. Computational chemistry

Molecular models were created using Insight II (Biosym, San Diego, CA, USA) run on a Silicon Graphics workstation. Energy minimisations were performed using Conformer Analysis and Minimizer (forcefield CFF 95) of Cerius 2 V.3.8 (Molecular Simulations, San Diego, CA, USA) running on a Silicon Graphics workstation.

Molecular descriptors were derived by Cerius 2 V.3.8 and TSAR V.2.4 (Oxford Molecular, UK). Statistical analyses were performed using TSAR V2.4 running on a Silicon Graphics workstation.

2.3. Reagents and materials

Ibuprofen, indoprofen, ketoprofen, carprofen, fenoprofen, suprofen, flurbiprofen, warfarin, amobarbital, phenobarbital, oxazepam, lorazepam, lormetazepam, temazepam, bepridil, verapamil, fenfluoramine, bupivacaine, nicardipine, propranolol, oxprenolol, benfluorex, prilocaine and *n*-octanoic acid were purchased from Sigma (St. Louis, MO, USA). Gallopamil was purchased from Schiapparelli (Turin, Italy), isradipine was kindly donated by Sandoz (Milan, Italy), amlodipine was kindly supplied by Pfizer (Sandwich, UK), nimodipine was a gift from Bayer (Milan, Italy), manidipine was kindly donated by Takeda (Osaka, Japan), lercanidipine was provided by Recordati (Milan, Italy). Practolol was used as received from the Institute of Pharmacology of the University of Pavia. Meflo-

quine was kindly donated by Roche (Basel, Switzerland) and chlorbutinol was from Boehringer Ingelheim (Milan, Italy). Butyric acid was purchased from BDH Italia (Milan, Italy) and *n*-hexanoic acid was purchased from J.T. Baker (Deventer, The Netherlands). The series of structurally correlated aryloxy-propionic acids (Table 2) with their relative methyl esters were synthesized in our department. Potassium dihydrogenphosphate and dipotassium hydrogenphosphate, tris(hydroxymethyl)aminomethane (Tris) and the organic solvents used for the preparation of the mobile phases were of analytical grade and purchased from Merck (Darmstadt, Germany). Aminopropyl silica packing material (Nucleosil-5NH_2, 5 μm particle size, 100 Å pore diameter) was obtained from Macherey–Nagel (Düren, Germany). Diethylaminoethyl-cellulose DE 52 (DEAE-cellulose) and carboxymethyl-cellulose CM 52 (CM-cellulose) were purchased from Whatman (Maidstone, UK) while Sephadex G50, Sephadex G100, Ampholine pH 3.5–10.0 and Ampholine PAGplate 3.5–9.5 were from Pharmacia.

2.4. Purification, immobilisation and packing of chicken liver bFABP stationary phase

Chicken liver bFABP was purified by a modification of a previously described method [4].

Briefly, about 3 kg of chicken liver was homogenised and centrifuged for 1 h at 10 000 rpm in the same volume of a solution of 10 m*M* Tris–HCl, pH 7.5 and 0.1 m*M* phenylmethansulfonyl fluoride was added to inhibit proteolysis. In the first purification step, a batch treatment with CM-cellulose equilibrated with 10 m*M* sodium acetate, pH 5.0 eliminated many unwanted proteins. The extract was chromatographed on a Sephadex G 100 column (50 m*M* Tris–HCl, pH 7.5, 200 m*M* NaCl) and then on a DEAE-cellulose column equilibrated with 10 m*M* Tris–HCl, pH 8.3, developed with a linear gradient from 10 to 50 m*M* Tris–HCl, pH 8.3. The final purification step was a preparative IEF using Ampholine in the pH range 8–10.5. The purity of the protein was monitored by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and IEF using Ampholine PAGplate 3.5–9.5.

The purification was performed at 4°C and the buffers contained 0.02% (p/v) NaN_3.

The purified protein was immobilised on aminopropyl silica in our laboratory following a previously described method [8]. The bFABP stationary phase was packed in a stainless steel column (100×4.6 mm I.D.) from Alltech (Sedriano, Italy).

2.5. Chromatography

All the chromatographic experiments, unless otherwise stated, were carried out at 25°C and the column flow-rate was set at 0.8 ml/min. The operating UV wavelength was fixed at the corresponding maximum for each compound. Stock solutions of each chiral drug were prepared in *n*-propanol at a concentration of 10 m*M* and then diluted with 50 m*M* phosphate buffer to a final concentration of 0.1 m*M*. The loaded amount was 2 nmol.

2.6. Zonal elution

The zonal elution studies were performed on the bFABP column by applying the buffer containing the competing agent. The required volume of displacer was first dissolved in the methanol fraction of the mobile phase (5%, v/v) and then added to the buffer (50 m*M*, pH 4.0). Different concentrations of displacing agent (0.2–8 m*M* for *n*-butyric and *n*-hexanoic acids) were applied to the column. Only one solution of *n*-octanoic acid (0.2 m*M*) was used due to the low solubility at this pH value into the mobile phase. Additional displacement studies adding *n*-octanoic acid to the mobile phase were carried out at pH 3.5, in the concentration range 0.2–2 m*M*, in order to increase the displacer solubility in the methanol fraction. The resulting solutions were passed through the column following the adsorption of the column eluent at 220 nm. Once the column was equilibrated, the detector baseline was re-zeroed and allowed to stabilise. The retention factors for the probes compounds were determined over the range of competing agent concentrations considered and a plot of $1/k'$ versus competitor concentration was made according to the equation reported for a system with a single-site competition [9,10].

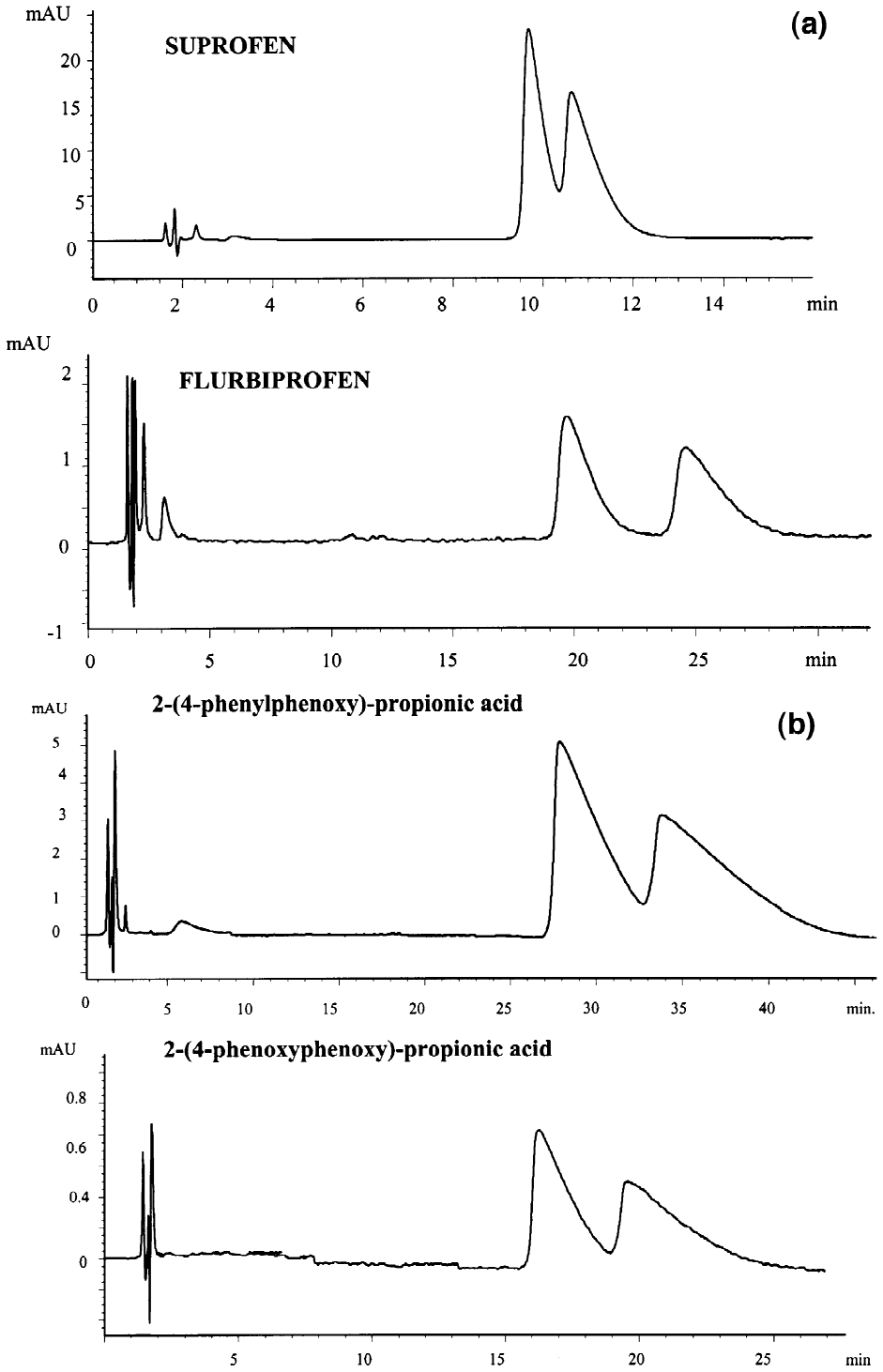


Fig. 1. (a) Resolution of suprofen and flurbiprofen enantiomers on the bFABP CSP. Mobile phase: 50 m*M* phosphate buffer, pH 4.0–methanol (95:5, v/v). (b) Resolution of 2-(4-phenylphenoxy) propionic acid and 2-(4-phenoxyphenoxy) propionic acid enantiomers on the bFABP CSP. Mobile phase: 50 m*M* phosphate buffer, pH 3.5–methanol (95:5, v/v).

3. Results and discussion

3.1. Retention and enantioselectivity on bFABP

The applicability as a chiral stationary phase (CSP) of the bFABP column was tested by analysing several chiral drugs with different chemical characteristics and belonging to different pharmaceutical classes such as calcium antagonists, β-blockers, local anaesthetics, ansiolytics and anti-inflammatory drugs.

The enantiomers of basic and neutral compounds were poorly retained and not resolved by the bFABP CSP. On the contrary the resolution of the enantiomers of some acidic compounds was obtained. Fig. 1a and b) show the chromatograms of the best resolved acidic enantiomers of suprofen, flurbiprofen, 2-(4-phenoxyphenoxy) propionic acid and 2-(4-phenylphenoxy) propionic acid.

This finding was not unexpected considering that the protein used presents a specific binding site for acidic compounds such as natural fatty acids.

3.2. Stability and reproducibility of the bFABP stationary phase

The measurement of chromatographic parameters can be exploited for the study of column reproducibility, providing that the temperature and solvent composition are controlled. The enantioselective performances of two different bFABP stationary phases obtained with two different purification and immobilisation processes are presented in Table 1.

The amount of immobilised protein, calculated by elemental analysis, was different for the two columns. It has been demonstrated that there is a linear correlation between the retention factor [11] and the bound amount of protein, however with bFABP columns the opposite behaviour was observed and enhanced retention of the tested compounds was obtained with the stationary phase with less amount of immobilised bFABP. Since no blocking of aminopropyl groups was carried out, these results can be due to the aspecific interactions of acidic compounds with unreacted aminopropyl groups because of lower protein coverage. These aspecific interactions are also responsible of the poor chromatographic efficiency of this column. On the other hand, the enantioselectivity was greater for the stationary phase with the higher amount of protein immobilised, indicating that an optimum amount of chiral selector is required to achieve enantioselectivity.

The stability of the bFABP stationary phase was checked during the research work and the columns were found to be stable for more than 250 chromatographic runs before observing a significant reduction in enantioselectivity.

Table 1
Chromatographic parameters observed on the two bFABP columns[a]

Compound	Column A, 51.7 bFABP mg/g silica support				Column B, 21.77 mg bFABP/g silica support			
	k'_1	k'_2	α	R_s	k'_1	k'_2	α	R_s
1	3.06	3.06	1.0	–	3.94	3.94	1.0	–
2	4.49	4.66	1.04	0.682	4.90	5.14	1.05	0.615
3	3.97	3.97	1.0	–	4.27	4.27	1.0	–
4	3.27	3.27	1.0	–	3.42	3.42	1.0	–
5	3.43	3.43	1.0	–	3.82	3.82	1.0	–
6	5.88	5.88	1.0	–	7.10	7.10	1.0	–
7	10.41	11.68	1.12	0.99	11.31	12.18	1.08	0.845
8	6.16	7.38	1.19	1.45	7.18	8.43	1.17	1.319
9	7.56	7.56	1.0	–	9.76	9.76	1.0	–
10	5.02	5.02	1.0	–	5.53	5.53	1.0	–
11	4.44	4.44	1.0	–	5.01	5.01	1.0	–
12	3.94	3.94	1.0	–	4.49	4.49	1.0	–
13	4.33	4.66	1.07	1.07	4.78	5.04	1.06	0.801

[a] Mobile phase: 50 m*M* KH_2PO_4, pH 4.0–methanol (95:5, v/v); flow 0.8 ml/min.

Table 2
Chemical structures of 2-aryl-propionic acids and 2-aryloxy-propionic acids

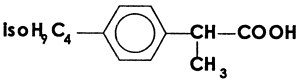
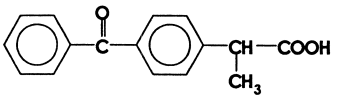
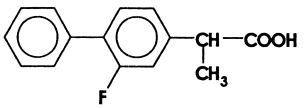
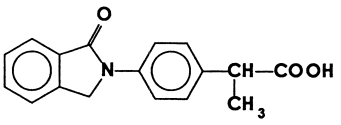
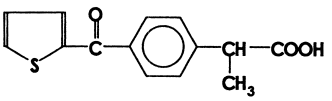
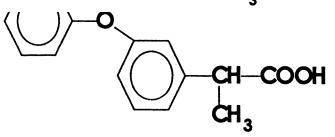
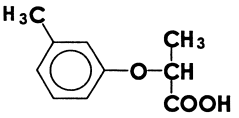
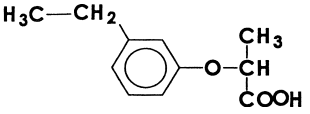
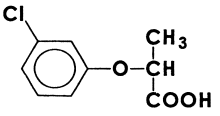
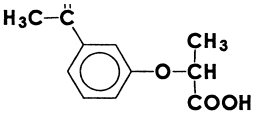
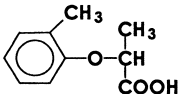
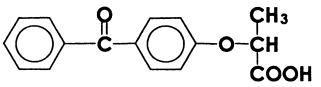
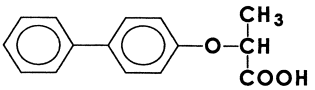
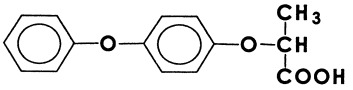
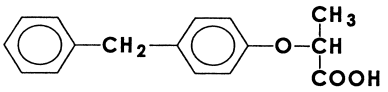
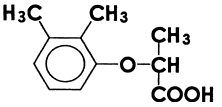
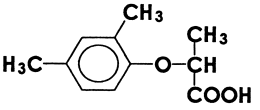
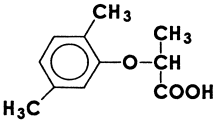
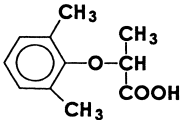
2-Aryl-propionic acids Compound	Formula
Ibuprofen	
Ketoprofen	
Flurbiprofen	
Indoprofen	
Suprofen	
Fenoprofen	
1 2-(3-Methylphenoxy) propionic acid	
2 2-(3-Ethylphenoxy) propionic acid	
3 2-(3-Chlorophenoxy) propionic acid	
4 2-(3-Acetylphenoxy) propionic acid	
5 2-(2-Methylphenoxy) propionic acid	

Table 2. Continued

2-Aryl-propionic acids Compound	Formula
6 2-(4-Benzoylphenoxy) propionic acid	
7 2-(4-Phenylphenoxy) propionic acid	
8 2-(4-Phenoxyphenoxy) propionic acid	
9 2-(4-Benzylphenoxy) propionic acid	
10 2-(2,3-Dimethylphenoxy) propionic acid	
11 2-(2,4-Dimethylphenoxy) propionic acid	
12 2-(2,5-Dimethylphenoxy) propionic acid	
13 2-(2,6-Dimethylphenoxy) propionic acid	

3.3. Influence of chromatographic conditions on retention and enantioselectivity

A series of 2-aryl- and 2-aryloxy-propionic acids were used as model compounds in order to study the effect on retention of the mobile phase pH in the range of 3.5–6.0. The molecular structures of these compounds are reported in Table 2.

The pK_a of 2-aryloxy-propionic acids analysed is around 3 and it is expected that these molecules will be predominantly ionised between pH 3.5 and 6.0, while the isoelectric point of bFABP is 9.0 and the protein will be positively charged under this pH. The decrease of retention observed for these analytes on increasing pH is primarily due to the decreasing positive net charge of the protein (Fig. 2).

The elution pattern of the arylpropionic anti-inflammatory drugs tested was different as they showed a maximum retention factor at pH values close to their pK_a which is between 4 and 5, as shown in Fig. 3. At pH lower than 4 the carboxylic functionality is not ionised and consequently the reduction in retention can be ascribed to a complete loss of electrostatic interactions.

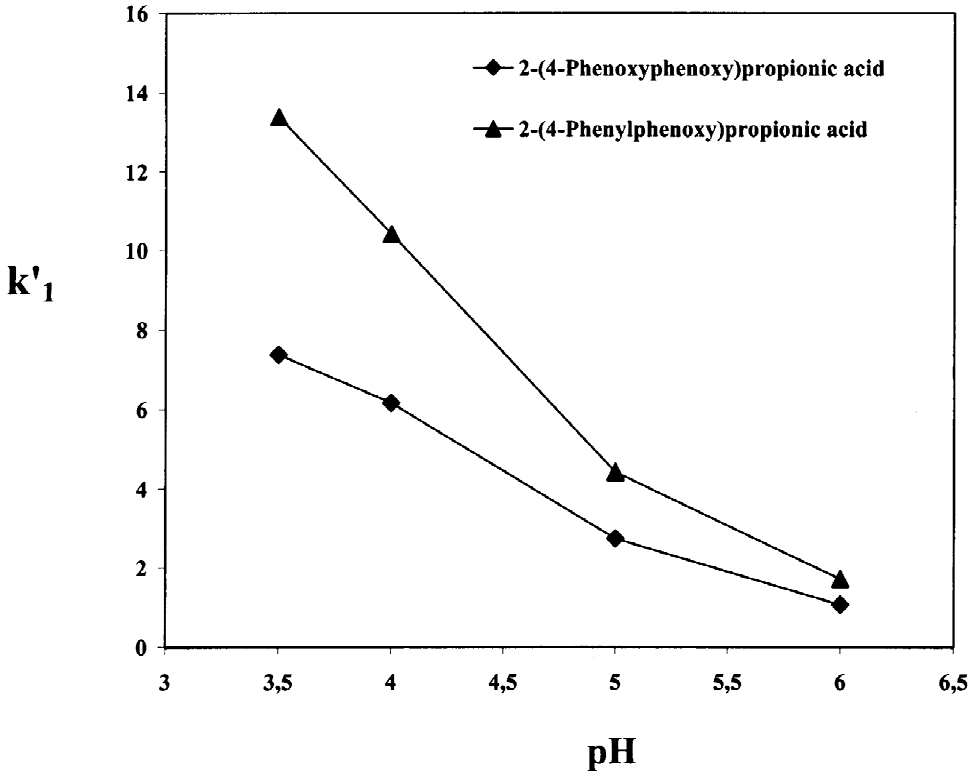


Fig. 2. Effect of pH on the retention of the first eluted enantiomer of 2-(4-phenylphenoxy) propionic acid and of 2-(4-phenoxyphenoxy) propionic acid.

As far as enantioselectivity is concerned, the best resolutions were obtained at pH 3.5 and 4.0 where the interactions between the acids and the protein appear to be stronger.

The influence of type and percentage of organic modifier on enantioselective retention was also studied using one of the most retained analytes as a model compound, suprofen. The uncharged modifiers methanol and acetonitrile were examined keeping the concentration of organic modifier constant at 5% and the pH value at 4 (50 m*M* phosphate buffer). The addition of acetonitrile to the mobile phase gave lower retention factors (k'_1 4.26 and 3.24 for methanol and acetonitrile, respectively) lower selectivity (α 1.12 and 1.07 for methanol and acetonitrile, respectively) and a higher number of theoretical plates compared to methanol. The influence of organic modifier concentration on the capacity factor and selectivity was also investigated; by increasing the methanol percentage from 1 to 10% a significant reduction of k' and α values was observed, as shown in Table 3. These data confirm the importance of the hydrophobic interactions in the total retention mechanism between the analytes and the stationary phase based on immobilised bFABP.

3.4. Retention mechanism studies

3.4.1. Interactions between the analytes and the protein

The results obtained in the systematic study suggest that the bFABP binding site might be involved in both retention and enantioselective mechanisms. In order to confirm this hypothesis aryloxy-propionic acids were converted into their methyl esters derivatives and chromatographed on the bFABP column, Table 4 lists the retention data for the acids and their corresponding methyl esters. A substantial reduction of the retention factors (ranging from 56.56% to 86.81%) and a loss of

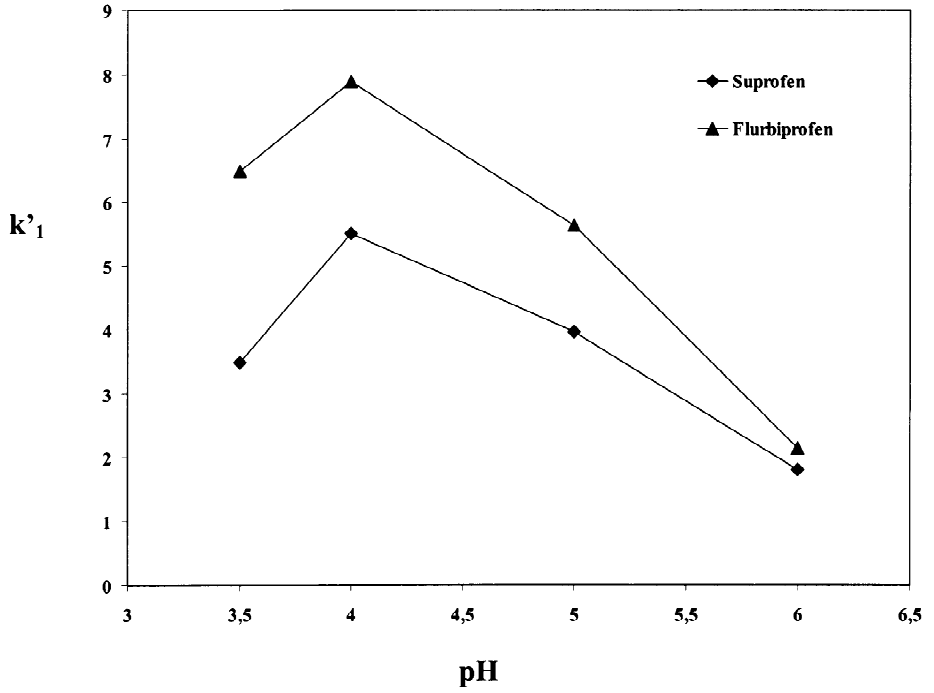


Fig. 3. Effect of pH on the retention of the first eluted enantiomers of suprofen and flurbiprofen.

enantioselectivity was observed for all the esters; these data indicate that electrostatic interactions are involved in the binding of the analytes to the protein and that the area where the interaction take place is a chiral region. However, the esters with larger aromatic systems (esters 6, 7, 8, 9) are still retained with k' values higher than 1.0. These results demonstrate that hydrophobic interactions with the bFABP cavity must play a role in the retention of these solutes.

The effects of solute structure of the aryloxy-propionic acids and aryl-propionic acids on the chromatographic retention were studied by a linear regression analysis [12]. Two molecular descriptors, the total lipole which considers the lipophilicity for the whole molecule and the ellipsoidal volume that can be viewed as a measure of molecular size were considered.

When k'_1 and k'_2 of the enantioselectively resolved solutes and k' values of the unresolved solutes were regressed relative to their total lipole, a statistically significant correlation was observed ($r^2=0.6121$, $P<0.0001$; $r^2=0.6342$, $P<0.0001$ for the first and the second eluted enantiomers, respectively). As can be seen in Fig. 4, the retention increases with increasing

Table 3
Influence of type and percentage of organic modifier on enantioselective retention of suprofen[a]

Organic modifier	Percentage					
	1%		5%		10%	
	k'_1	α	k'_1	α	k'_1	α
Methanol	16.18	1.09	4.26	1.12	1.9	1.03
Acetonitrile			3.24	1.07		

[a] Mobile phase: 50 m*M* phosphate buffer, pH 4.0–methanol (95:5, v/v).

Table 4
Results from the chromatographic analyses of a series of 2-aryloxy-propionic acids and their methyl esters on the bFABP CSP[a]

Compound	k'_1	k'_2	α
Acid 1	3.94	3.94	1.0
Ester 1	0.52	0.52	1.0
Acid 2	4.90	5.14	1.05
Ester 2	0.72	0.72	1.0
Acid 3	4.27	4.27	1.0
Ester 3	0.73	0.73	1.0
Acid 4	3.42	3.42	1.0
Ester 4	0.46	0.46	1.0
Acid 5	3.82	3.82	1.0
Ester 5	0.59	0.59	1.0
Acid 6	7.10	7.10	1.0
Ester 6	1.38	1.38	1.0
Acid 7	11.31	12.18	1.08
Ester 7	4.03	4.03	1.0
Acid 8	7.18	8.43	1.17
Ester 8	2.98	2.98	1.0
Acid 9	9.76	9.76	1.0
Ester 9	4.24	4.24	1.0
Acid 10	5.53	5.53	1.0
Ester 10	0.90	0.90	1.0
Acid 11	5.01	5.01	1.0
Ester 11	0.91	0.91	1.0
Acid 12	4.49	4.49	1.0
Ester 12	0.84	0.84	1.0
Acid 13	4.78	5.04	1.06
Ester 13	0.68	0.68	1.0

[a] Mobile phase: 50 m*M* phosphate buffer, pH 4.0–methanol (95:5, v/v).

hydrophobicity thus indicating that the retention of acidic compounds appears to correlate with the expected order of hydrophobicity of the molecules.

The relationship between retention factors and ellipsoidal volume for all the solutes used in this study was also considered and in this case no significant relationship was observed (Fig. 5a). Nevertheless, by restricting the correlation to only the most retained compounds, that are those showing k' values higher than 5, a significant correlation could be found. The retention factor values decrease by increasing the ellipsoidal volume (Fig. 5b), and these data suggest that the binding of the analytes takes place in a protein area which presents a steric restriction. The relationship between k' values and ellipsoidal volume has not been found to be significant for the less retained compounds such as methyl and dimethyl aryloxy-propionic acids. It can be hypothesized that the structure of these compounds is small enough to allow a free access of the molecules to the narrow binding site.

3.4.2. Displacement studies

The zonal elution technique has been used to study interactions of the analytes with the bFABP.

These studies were carried out by adding to the mobile phase different concentrations of carboxylic acids with different chain lengths (*n*-butyric acid, *n*-hexanoic acid and *n*-octanoic acid) and monitoring the retention factors and enantioselectivity of some analytes.

A concentration-dependent reduction of the capacity factors and enantioselectivity was observed for all compounds when carboxylic acids were added to the mobile phase and the extent of the reduction of the k' increased as the chain length increased (Table 5). The addition of displacing agent also produced an improvement in peak performance due to the reduction in retention time.

By plotting the chromatographic data in Table 5, according to equation derived for a single-site competition, a linear relationship between the reciprocal of k' and the concentration of competing agent was observed for all the compounds examined only when butyric acid was present in the mobile phase. The linearity of the graphs support a model in which the compounds and butyric acid compete at one site on bFABP. Plots of $1/k'$ versus competing agent concentration generated for hexanoic and octanoic acids did not reveal a linear relationship for any of the tested analytes, indicating that a simple competitive model does not apply. In Fig. 6 the zonal elution profiles for acid 8, as an example, are presented.

For the resolved enantiomers the displacer appears to compete for the binding of each enantiomers at a single site as both retention factors are affected by the presence of the displacer. The retention observed even at high displacer concentration can be due to aspecific interactions as chromatographic retention and selectivity are the result of both non-specific and

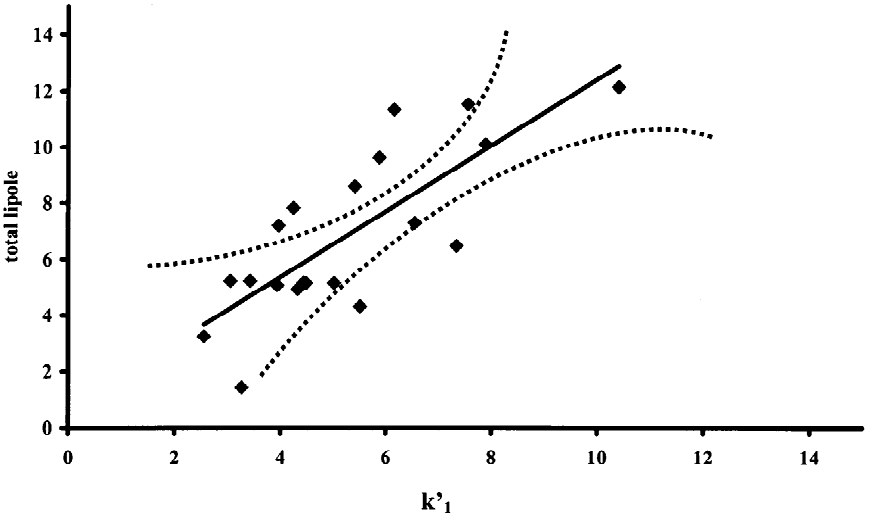


Fig. 4. Correlation between the observed capacity factors and the total lipole.

enantioselective interactions with the stationary phase.

3.4.3. Quantitative structure–retention relationships

Quantitatively comparable retention data of the 13 aryloxy-propionic acids were chosen for QSRR analysis. The data were subjected to multiparameter regression analysis against various molecular descriptors including classical hydrophobic (log *P*, total lipole, etc.), quantum chemical (HOMO, LUMO, etc.) and steric (ellipsoidal volume, accessible surface area, etc.) parameters [13–16]. After consideration of all the available descriptors only the lipophilic and electronic properties of the compounds proved to be correlated with the variation of the

Table 5
Variation in the chromatographic retention and enantioselectivity of some analytes as a result of the addition of displacers to the mobile phase[a]

Compound		*n*-Butyric acid					*n*-Hexanoic acid					*n*-Octanoic acid				
		0 m*M*	0.2 m*M*	0.8 m*M*	1.6 m*M*	8 m*M*	0 m*M*	0.2 m*M*	0.8 m*M*	1.6 m*M*	8 m*M*	0 m*M*	0.2 m*M*	0.6 m*M*	1 m*M*	2 m*M*
Acid 2	k'_1	5.71	5.92	5.88	5.68	5.13	5.71	5.34	5.06	4.96	4.16	4.79	3.85	3.62	3.42	3.13
	α	1.05	1.07	1.06	1.05	1.03	1.05	1.05	1.03	1.0	1.0	1.06	1.0	1.0	1.0	1.0
Acid 7	k'_1	14.52	14.43	14.10	13.56	12.26	14.52	12.99	11.62	10.67	9.08	13.38	8.65	7.40	6.87	6.06
	α	1.07	1.09	1.08	1.08	1.07	1.07	1.07	1.05	1.04	1.0	1.23	1.16	1.13	1.09	1.07
Acid 8	k'_1	8.39	8.44	8.27	8.04	7.36	8.39	7.65	7.04	6.72	5.79	7.37	5.06	4.41	4.19	3.71
	α	1.21	1.23	1.20	1.20	1.15	1.21	1.16	1.12	1.09	1.0	1.23	1.10	1.04	1.0	1.0
Acid 13	k'_1	5.45	5.34	5.35	5.18	4.61	5.45	3.92	4.50	4.28	3.98	3.56	3.16	3.03	2.91	2.62
	α	1.06	1.06	1.06	1.06	1.04	1.06	1.03	1.0	1.0	1.0	1.08	1.02	1.0	1.0	1.0
Suprofen	k'_1	7.40	7.27	6.81	6.43	4.93	7.40	6.54	5.87	5.41	3.89	3.48	2.93	2.84	2.55	2.23
	α	1.07	1.07	1.06	1.06	1.04	1.07	1.05	1.03	1.0	1.0	1.07	1.0	1.0	1.0	1.0
Flurbiprofen	k'_1	12.92	13.99	12.58	11.64	8.84	12.92	11.64	10.13	8.87	6.08	3.08	2.45	2.32	2.28	1.97
	α	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

[a] Mobile phase: 50 m*M* phosphate buffer, pH 4.0–methanol (95:5, v/v).

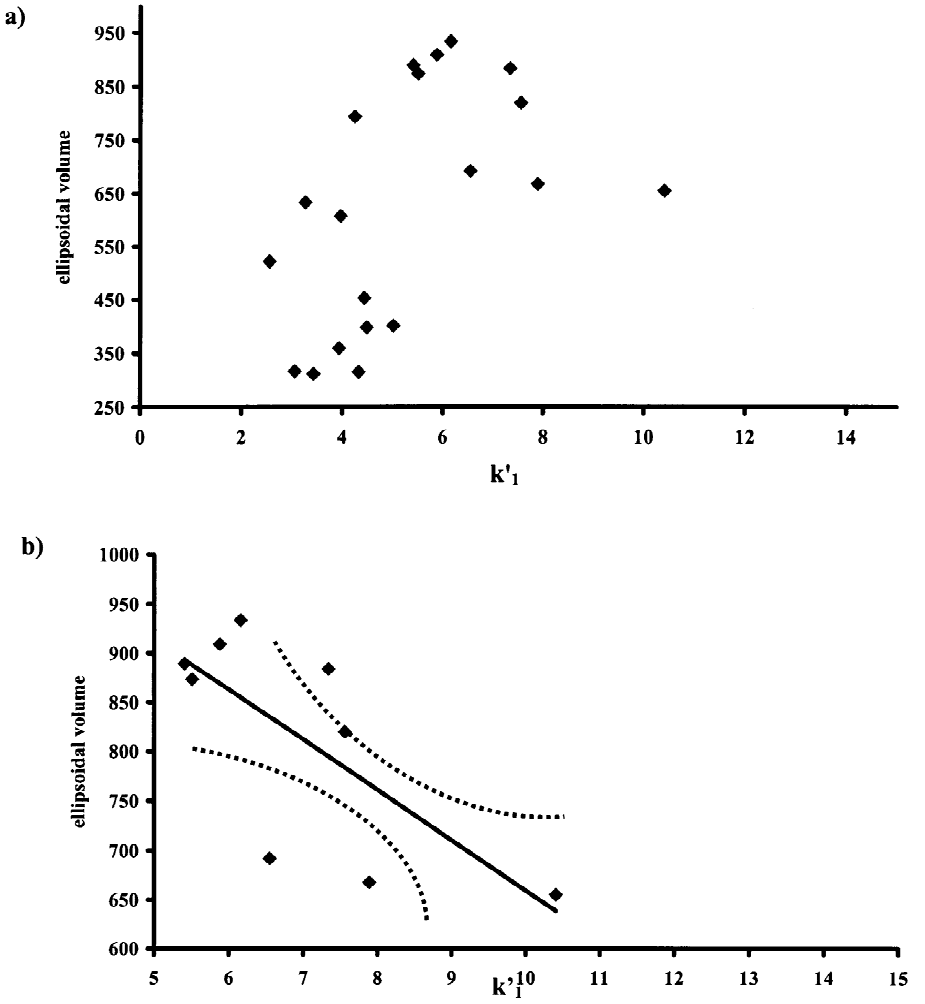


Fig. 5. (a) Correlation between the observed capacity factors and the ellipsoidal volume. (b) Correlation between the observed capacity factors of the most retained compounds ($k' \geq 5$) and the ellipsoidal volume.

retention indices within the series. The relationships between these descriptors were established and are presented in Eqs. (1) and (2):

$$\log k_1' = 0.13\text{HOMO} + 0.025\text{TL} + 2.04;$$
$$n = 13,\ R = 0.91,\ F = 24.46,\ s = 0.068,$$
$$\text{RSD (predictive index)} = 0.7034 \quad (1)$$

$$\log k_2' = 0.14\text{HOMO} + 0.032\text{TL} + 2.2;$$
$$n = 13,\ R = 0.92,\ F = 27.79,\ s = 0.07,$$
$$\text{RSD} = 0.75 \quad (2)$$

where TL is the total lipole and HOMO is the energy of highest occupied molecular orbital related to the ability of the molecule to form charge-transfer complexes.

The total lipole was calculated by TSAR using the centre of gravity of the molecule as an origin:

$$\vec{L} = \sum \vec{r_i} \times l_i$$

where r_i = distance of an atom i from the origin and l_i = lipophilicity of an atom i.

Table 6 reports the retention factors of the aryloxy propionic acids used in QSRR studies along with their total lipole and HOMO parameters.

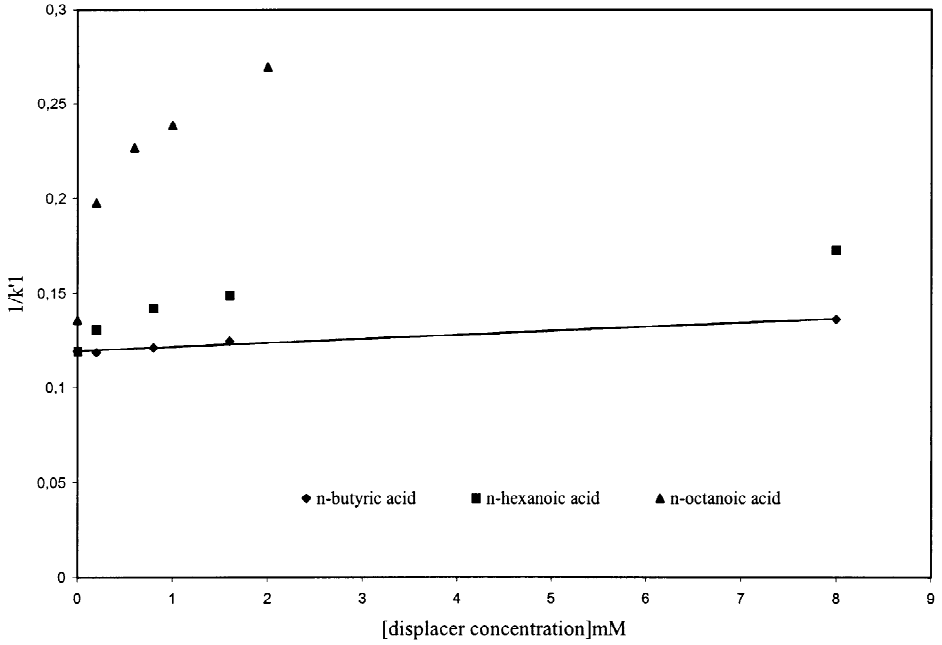


Fig. 6. Zonal elution plots of $1/k'$ of the first-eluting enantiomer of acid 8 versus *n*-butyric acid, *n*-hexanoic acid and *n*-octanoic acid concentration, according to the relationship for competitive displacement from a single site.

The positive sign of TL in both equations demonstrates that the binding of analytes to bFABP increases as their hydrophobicity increases. It is not surprising that QSRR analysis identified hydrophobicity as the key retention parameter. The two equations also show a positive term of HOMO, thus confirming the significance of electrostatic interactions.

Table 6
Results from the HPLC of the series of aryloxy propionic acids on the bFABP CSP, solute molecular descriptors for hydrophobicity (total lipole) and HOMO (energy of the highest occupied molecular orbital)[a]

Compound	k'_1	Total lipole	HOMO
Acid 1	3.06	5.22	−12.137
Acid 2	4.49	5.15	−12.126
Acid 3	3.97	7.19	−12.484
Acid 4	3.27	1.44	−12.374
Acid 5	3.43	5.22	−11.995
Acid 6	5.88	9.63	−12.026
Acid 7	10.41	12.13	−10.9
Acid 8	6.16	11.34	−11.643
Acid 9	7.56	11.52	−11.695
Acid 10	5.02	5.15	−12.022
Acid 11	4.44	5.16	−11.58
Acid 12	3.94	5.06	−11.732
Acid 13	4.33	4.93	−12.148

[a] Mobile phase: 50 m*M* phosphate buffer, pH 4.0–methanol (95:5, v/v).

4. Conclusions

The studies carried out with a chicken liver bFABP stationary phase showed that the protein immobilised on a silica support has limited chiral properties as only acidic compounds, with a chemical character similar to that of the natural ligands and with specific dimensions, were retained by the developed column. The enantiomers of some acidic compounds were resolved, however the low selectivity and efficiency of the bFABP column do not allow the use of this CSP for analytical purposes and for the determination of enantiomeric purity. Nevertheless, the developed bFABP column was used to get more information on the binding mechanism of the protein. These studies reveal that hydrophobic interactions are predominant in the retention mechanism however electrostatic interactions are also

important for the stabilisation of the analyte–protein complex and these conclusions were confirmed by QSRR studies.

The source of the enantioseparation appears to lie in the structure of the binding site on the bFABP molecule, this is illustrated by the fact that the enantioselectivity was lost when acidic displacing agents were added to the mobile phase.

It has been suggested that an arginine is present in the cavity where the interaction between fatty acids and protein takes place [5,7]; a mechanism that could be proposed from our chromatographic results is that the arginine moiety is placed in a cone where the acids solutes enter the outer section of the hydrophobic cavity and there are drown down deeper into the cavity by electrostatic interactions with the positively charged moieties. This cavity should be large enough so that when the directing electrostatic charge is not present (acids converted into esters or higher pH) the acids can still enter the cavity and be retained but orient themselves in a number of random positions eliminating the possibility of enantio-discrimination. The proposed mechanism is consistent with the effects observed in the displacement studies. It can be assumed that the small *n*-butyric acid molecule would only compete with the solutes for the electrostatic interaction with the arginine moieties getting a clean single site competitive interaction profile while *n*-hexanoic acid and *n*-octanoic acid with larger hydrophobic tails can compete for both the electrostatic and hydrophobic binding sites resulting in a more efficient reduction in retention and enantioselectivity, but a more complicated mechanistic relationship.

Nevertheless, in light of these studies, no general rules can yet be given regarding the required solute structural features for HPLC enantioselective separation. Additional structural parameters able to display differences between the conformational structures of bound enantiomers will be considered in further QSRRs and the results will be reported elsewhere.

Acknowledgements

This work was supported by grants from MURST (Programmi di Ricerca Scientifica di Rilevante Interesse Nazionale). The authors are also grateful to Professor O. Azzolina (University of Pavia, Pavia, Italy) for supplying aryloxy-propionic acids and Elena Tabolotti, Ph.D. for supervising the protein purification protocol.

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