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Egg yolk riboflavin binding protein as a new chiral stationary phase in high-performance liquid chromatography

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Abstract

A chiral stationary phase for high-performance liquid chromatography based on hen egg yolk riboflavin binding protein is introduced. The purified protein was immobilized on activated $5NH_2$ Nucleosil silica. Chiral acidic, basic and uncharged drugs were chromatographed and the influence of the mobile phase parameters on the retention times and enantioselectivity was studied. Thirteen out of the twenty compounds tested were partially or baseline resolved. These encouraging preliminary results suggest that this chiral stationary phase may be applicable to a wide range of drug enantiomers in the reversed-phase mode.

1. Introduction

Several proteins, such as α_1-acid glycoprotein (AGP) [1], bovine and human serum albumin (BSA [2] and HSA [3]) and ovomucoid (OVM) [4] have been shown to have stereospecific binding interactions with chiral entities of pharmaceutical interest. This recognition process has been successfully exploited to develop protein-based chiral stationary phases (CSPs), useful in the chromatographic resolution of pharmaceutically active enantiomers in the reversed-phase mode. However, there are still many enantiomers that have not been resolved by protein-based CSPs and, moreover, from the point of view of the pharmaceutical industry, the commercially available CSP technology does not appear to be as robust as might be desired. For these reasons, more efforts should be made to establish which type of proteins can be used in the resolution of racemic compounds and to prepare stable and reproducible protein-based chiral columns. In order to find proteins with the property of chiral recognition and broad applicability, we undertook a wide project for the purification and immobilization of proteins from different sources such as the riboflavin binding proteins (RFBP), fatty acid binding proteins (FABP) and β-lactoglobulin (BLG).

The name riboflavin binding protein is applied to several molecular species that are thought to be involved in the transport and storage of the vitamin [5]. The first protein of this family to be isolated and studied was hen egg white RFBP [6]. The molecule has been well characterized; it has a molecular mass of 32 000, which corre-

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sponds to 219 amino acids, it is phosphorylated in a maximum of nine positions and has a carbohydrate content of about 14%. Its isoelectric point is about 4, depending on the exact isoforms studied. Different isoforms are believed to have different phosphate contents and/or deamidated asparagines and glutamines. Subsequently, three other hen proteins were isolated and characterized to various extents: egg yolk, serum and liver RFBP [7]. Egg white, yolk and serum RFBP appear to be the product of the same gene but to have undergone different post-translational modifications. The amino acid sequence is the same but it is between eleven and thirteen amino acids shorter in the case of yolk RFBP [7]. Yolk and serum RFBP share the same type of asparagine-linked oligosaccharide chains, which are different from the carbohydrates linked to egg white RFBP [8]. All the RFBPs are highly cross-linked molecules, presenting nine disulfide bridges, which explains their very high stability. Only one of the members of this protein family has so far been used as a CSP, viz., egg white RFBP [9]. As the differences among the members of this protein family have been shown to be substantial, we anticipated that the behaviour of a yolk RFBP CSP would be likely to be different from that of egg white RFBP.

This paper reports the results of chromatographic experiments carried out using a column of egg yolk RFBP bound to $5NH_2$ Nucleosil silica. Acidic, basic and uncharged analytes were chromatographed on yolk RFBP silica and the influence of mobile phase pH and percentage of organic modifier on the enantioselective retention was studied. Whenever there were data on the behaviour of the analytes on the egg white RFBP CSP, the two sets of results were compared and are discussed.

2. Experimental

2.1. Apparatus

A Hewlett-Packard HP 1050 liquid chromatograph with a Rheodyne sample valve (20-μl loop) equipped with a Hewlett-Packard HP 1050 variable-wavelength detector connected to an HP Vectra Q5/165 workstation was used. A stainless-steel column (100 mm × 4.6 mm I.D.) was packed with yolk RFBP-conjugated silica gel by Shandon HPLC (Runcorn, Cheshire, UK).

2.2. Reagents and materials

Ibuprofen (IB), ketoprofen (KE), flurbiprofen (FL), indoprofen (IN), suprofen (SU), fenoprofen (FE), carprofen (CA), warfarin (WA), tryptophan (TR), lormetazepam (LM), oxazepam (OX), lorazepam (LO), verapamil (VE), bepridil (BE) and nicardipine (NC) were purchased from Sigma (St. Louis, MO, USA) and gallopamil (GA) from Schiapparelli (Turin, Italy). Isradipine (IS) was kindly donated by Sandoz (Milan, Italy), amlodipine (AM) by Pfizer (Sandwich, UK), nimodipine (NM) by Bayer (Milan, Italy) and manidipine (MA) by Takeda (Osaka, Japan). KH_2PO_4 and ethanol (HPLC grade) were purchased from Merck (Darmstadt, Germany). DEAE-cellulose was purchased from Whatman (Maidstone, UK) and Sephadex G-100 from Pharmacia (Uppsala, Sweden). All the reagents used in protein purification were of analytical-reagent grade.

2.3. Purification of yolk RFBP

The hen egg yolk RFBP was purified as follows: after separating the whites from the yolks and adding riboflavin to saturate the protein, the latter were homogenized in a blender with 3 volumes of sodium acetate buffer (0.1 *M*, pH 5.3). The diluted yolks were centrifuged to remove the insoluble material and the supernatant was loaded on a DEAE-cellulose column (50 × 3 cm I.D.) that had been equilibrated with the same buffer. After washing the column extensively until all the bound protein was removed with the same buffer, yolk RFBP was eluted, adding 0.2 M NaCl to the buffer. RFBP is easily detected during the purification by its intense yellow colour and its absorbance at 455 nm. The pooled fractions containing the protein of interest were run through a Sephadex G-100 column using as eluent 0.05 *M* Tris–HCl (pH 7.5). The bound riboflavin was removed by

extensive dialysis at pH 3.0 to prepare the apoprotein that was used in the experiments.

2.4. Preparation of yolk RFBP column

Immobilization was carried out by Shandon HPLC following a previously described method [9]: $5NH_2$ Nucleosil (3 g) was slurried in HPLC-grade acetonitrile and N,N-disuccinylimidyl carbonate (4.5 g) was added. This mixture was stirred gently for 18 h (using a rotary evaporator). The silica was filtered and washed with acetonitrile and 50 m*M* potassium phosphate buffer (pH 7.5). A 300-mg amount of RFBP was suspended in 50 m*M* potassium phosphate buffer and the activated silica was added. After 2 h of gentle mixing using the rotary evaporator the product was collected by filtration and washed with sterile water and 2-propanol–water (1:2). The stationary phase was packed in a stainless-steel column (100 mm × 4.6 mm I.D.).

2.5. Chromatographic conditions

All the experiments were performed at ambient temperature (24–25°C) and the flow-rate was set at 0.8 ml/min. The operating UV wavelength was fixed at the corresponding maximum for each compound. Sample preparation was carried out by dissolving known amounts of chiral drugs in *n*-propanol, each solution was diluted with buffer to a concentration of 0.1 m*M* and 20 μl were injected into the HPLC column. Normally 2–4 nmol of analyte are recommended.

3. Results and discussion

Seven arylpropionic anti-inflammatory drugs, eight calcium channel antagonists with different structures, three benzodiazepines, tryptophan and warfarin were chromatographed on the yolk RFBP column. The structures of all the compounds tested are depicted in Fig. 1. In Table 1 the k', α and *Pi* values corresponding to the best chromatographic results are summarized; the enantioselectivity is expressed by α and *Pi*, where *Pi* is the ratio between the average valley depth and the average peak height (Kaiser's peak separation factor [10]). This parameter was found to give excellent discrimination between good and bad responses and is much more easily measurable than the resolution, R_s. Fig. 2 shows the best resolution obtained for some of the chiral compounds tested. The pH and percentage of organic modifier were changed in order to study the effect on retention and enantioselectivity.

3.1. Influence of pH and mobile phase composition on retention

pH

The influence of mobile phase pH on the enantioselective retention of charged and neutral analytes on the yolk RFBP column was systematically investigated. In general, the affinity for the stationary phase increased as the compounds became less ionized.

For the arylpropionic anti-inflammatory drugs (Fig. 3), increasing the pH above 4.6 decreases k' for most compounds. As the isoelectric point of yolk RFBP is about 4, under these conditions the protein has a net negative charge. Moreover, these compounds have all pK_a values in the range 4.0–5.0 and therefore electrostatic repulsion phenomena between stationary phase and analyte can occur. The fact that the trend is slightly different for the strongly retained IN and CA, where the decrease in k' starts at pH 5.5, may indicate the prevalence of hydrophobic interactions over ionic binding for such bulky structures.

The k' values of dihydropyridines (Fig. 4) are highly influenced by pH variation except for IS and NM. The extent of retention is directly dependent on the hydrophobicity of the substituents, MA being the most strongly retained compound, followed by NC and AM. Further, the very short retention times at pH 3.8 are ascribable to ionic repulsion as both the amines and RFBP are positively charged. The phenylalkylamines VE, GA and BE (Fig. 5) both follow a trend similar to the other calcium channel antagonists, although the latter shows a much more dramatic increase in the k' value as the pH is raised.

For benzodiazepines and TR the pH does not significantly influence the k' values. The fact that WA shows a decrease in retention time above pH 4.6 might be explained by the delocalized negative charge located at the centre of a largely non-polar molecule which could be responsible for electrostatic repulsion (Fig. 6).

Organic modifier

As expected in a system which works similarly to a reversed-phase mode, the influence of the percentage of organic modifier on the k' parameter is dependent on the hydrophobicity of the molecular structure of the analyte. An increase in the percentage of ethanol results in a large retention time decrease, especially for those compounds which are more retained in the absence of the organic modifier. For example, increasing the ethanol concentration from 5% to 10% (pH 5.5) causes a decrease in k' from 79.44 to 33.25 for IN whereas for the simpler SU the decrease in k' is only from 3.21 to 2.95. The same trend is observed for dihydropyridines.

For arylpropionic acids (Fig. 7), the elution

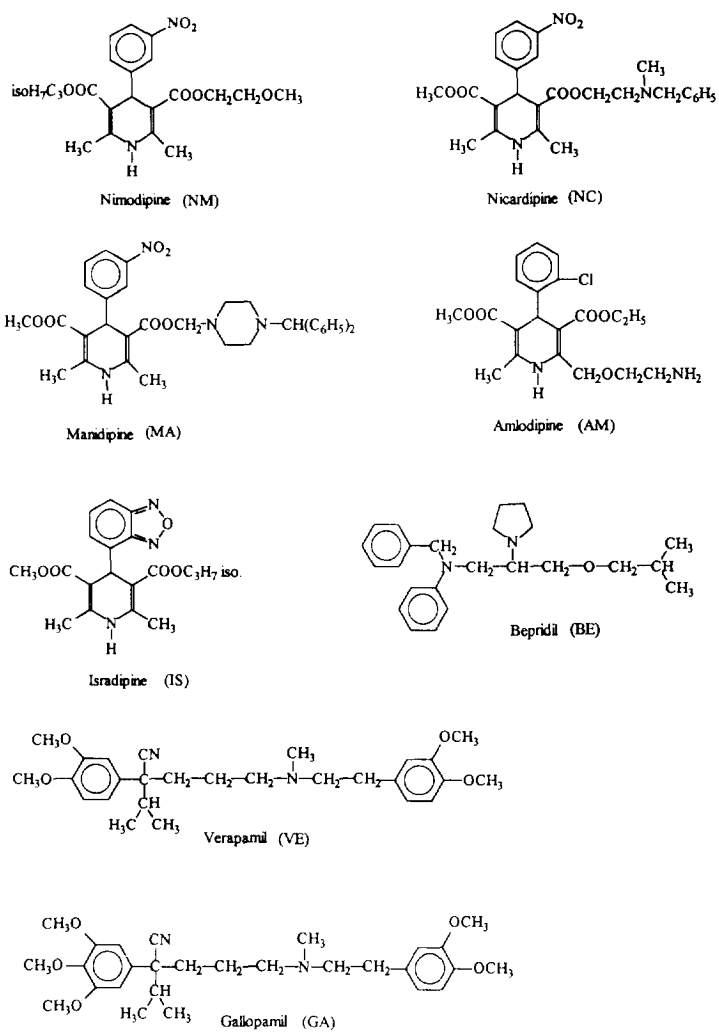
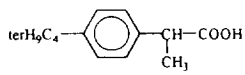
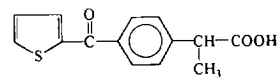


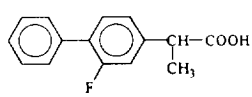
Fig. 1.



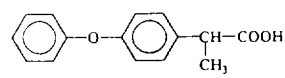
Ibuprofen (IB)



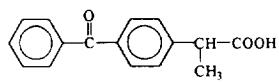
Suprofen (SU)



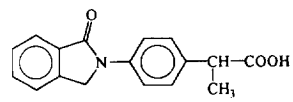
Flurbiprofen (FL)



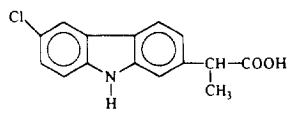
Fenoprofen (FE)



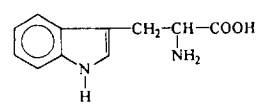
Ketoprofen (KE)



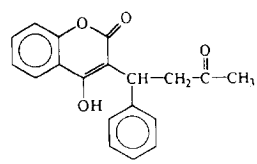
Indoprofen (IN)



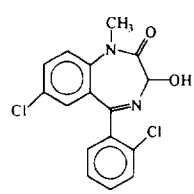
Carprofen (CA)



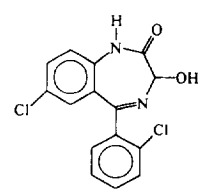
Tryptophan (TR)



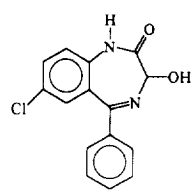
Warfarin (WA)



Lormetazepam (LM)



Lorazepam (LO)



Oxazepam (OX)

Fig. 1. Structures of the compounds tested.

Table 1
Best mobile phase compositions obtained for the compounds tested and corresponding chromatographic parameters (k', α and Pi)

Compound	k'	α	Pi	Mobile phase
Ibuprofen	1.32	1	–	–[a]
Ketoprofen	2.56	1.22	0.61	50 m*M* KH_2PO_4(pH 5.5)
Flurbiprofen	6.10	1.13	0.40	50 m*M* KH_2PO_4 (pH 5.5)
Indoprofen	9.84	1.16	0.77	50 m*M* KH_2PO_4 (pH 6.5)–ethanol (90:10, v/v)
Suprofen	8.27	1.08	0.20	50 m*M* KH_2PO_4 (pH 5.5)
Fenoprofen	1.82	1	–	–[a]
Carprofen	22.09	2.01	1	50 m*M* KH_2PO_4 (pH 5.5)–ethanol (95:5, v/v)
Warfarin	4.67	1.39	0.98	50 m*M* KH_2PO_4 (pH 4.6)–ethanol (90:10, v/v)
Tryptophan	0.31	1	–	–[a]
Lormetazepam	4.35	1	–	–[a]
Lorazepam	4.02	1.63	0.94	50 m*M* KH_2PO_4 (pH 5.5)–ethanol (95:5, v/v)
Oxazepam	3.92	4.55	1	50 m*M* KH_2PO_4 (pH 5.5)–ethanol (95:5, v/v)
Gallopamil	3.20	1	–	–[a]
Verapamil	3.55	1	–	–[a]
Bepridil	11.96	1.21	0.36	50 m*M* KH_2PO_4 (pH 5.5)–ethanol (95:5, v/v)
Isradipine	8.57	1.28	0.76	50 m*M* KH_2PO_4 (pH 5.5)
Amlodipine	5.49	1	–	–[a]
Nimodipine	0.72	1.11	0.08	50 m*M* KH_2PO_4 (pH 5.5)
Nicardipine	10.61	1.32	0.76	50 m*M* KH_2PO_4 (pH 5.5)–ethanol (95:5, v/v)
Manidipine	232.94	1.32	0.50	50 m*M* KH_2PO_4 (pH 5.5)–ethanol (95:5, v/v)

[a] No separation was obtained with any of the mobile phases considered.

order is IN > CA > SU > KE > FL > FE > IB. IN is not shown because in the absence of organic modifier it is not eluted after 3 h. It is likely that the additional carbonyl function present in some of the structures is responsible for a further hydrogen bond with a hydrogen-donating group at the binding site of the protein, and this is clearly demonstrated by the k' values for KE and FE. A further indication of the important role played by the carbonyl function in KE comes from the fact that this compound shows at least a hint of separation in all the chromatographic conditions tested whereas the enantiomers of FE have not been resolved so far. In the dihydropyridine family, with an increase in the percentage of organic modifier the retention times decrease, as expected; the presence of aromatic rings in the MA and NC structures appears to be responsible for their high k' values (followed by NM, IS and AM) (Fig. 8). MA is not shown in Fig. 8 as it was not eluted after 7 h.

Benzodiazepines, TR and WA follow the same rule.

3.2. Influence of pH and mobile phase composition on enantioselectivity

Thirteen out of the twenty compounds tested were partially or baseline resolved, as shown by the chromatographic parameters α and Pi in Table 1. Nevertheless no optimization was carried out and the results are to be considered as preliminary. Data obtained by the systematic study on the influence of the mobile phase parameters on the enantioselectivity showed that on increasing the pH in the range 3.8–6.5 (ethanol concentration 10%) the separation is not generally influenced to a great extent for most of the compounds tested. In contrast, the percentage of organic modifier provided to play an important role with respect to the enantioselective mechanism.

Among the arylpropionic anti-inflammatory drugs, the compounds with bulkier structures are those that showed higher retention together with better enantioselectivity. Keeping the ethanol concentration constant (10%), the best sepa-

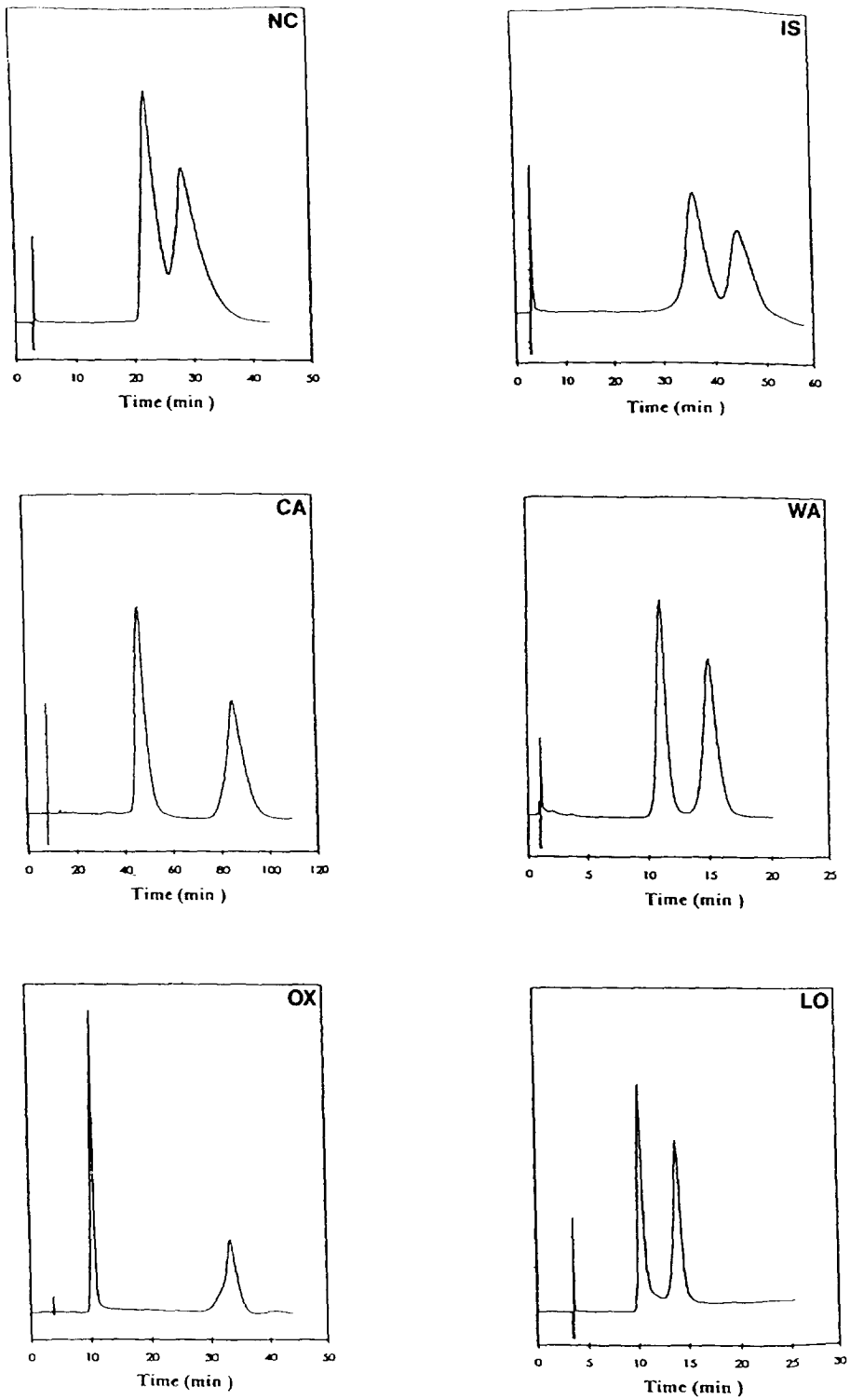


Fig. 2. Chromatograms of some of the compounds tested. The corresponding chromatographic conditions are given in Table 1.

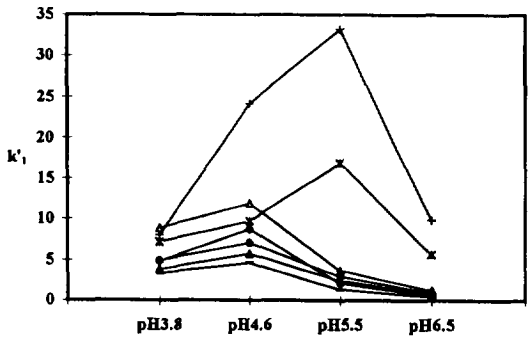


Fig. 3. Influence of pH on k'_1 for arylpropionic acids (ethanol content kept constant at 10%). Sample: – = IB; ▲ = KE; △ = FL; ● = SU; ◆ = FE; ∗ = CA; + = IN.

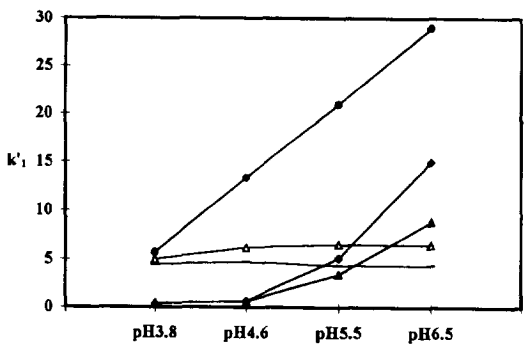


Fig. 4. Influence of pH on k'_1 for dihydropyridines (ethanol content kept constant at 10%). Sample: – = IS; ▲ = AM; △ = NM; ● = MA; ◆ = NC.

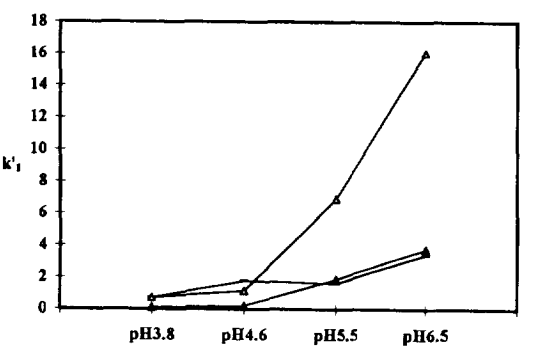


Fig. 5. Influence of pH on k'_1 for phenylalkylamines (ethanol content kept constant at 10%). Sample: – = GA; ▲ = VE; △ = BE.

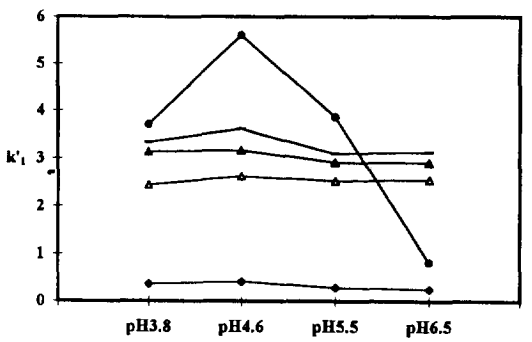


Fig. 6. Influence of pH on k'_1 for benzodiazepines, TR and WA (ethanol content kept constant at 10%). Sample: – = LM; ▲ = LO; △ = OX; ● = WA; ◆ = TR.

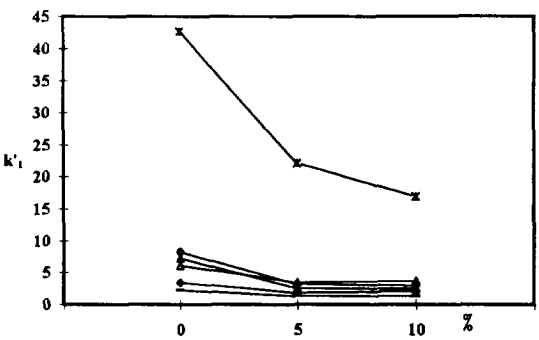


Fig. 7. Influence of percentage of organic modifier on k'_1 for arylpropionic acids (pH kept constant at 5.5). Sample: – = IB; ▲ = KE; △ = FL; ● = SU; ◆ = FE; ∗ = CA.

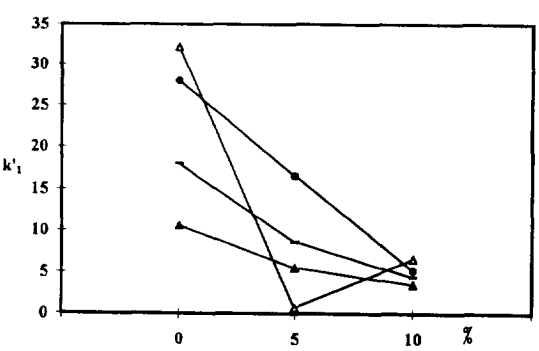


Fig. 8. Influence of percentage of organic modifier on k'_1 for dihydropyridines (pH kept constant at 5.5). Sample: – = IS; ▲ = AM; △ = NM; ● = NC.

ration was obtained for CA, which shows a *Pi* value higher than 0.98 at pH > 4.6; IN was resolved with a *Pi* value of 0.77 at pH > 3.8. A decrease in the percentage of organic modifier results in baseline resolution for CA and in a too strong protein–solute interaction, which leads to unacceptable retention times (no peaks eluted after 3 h in the absence of ethanol in the mobile

phase). The presence of multiple points of attachment to the protein in the IN structure (aromatic ring and carbonyl group) could explain the observed chromatographic behaviour. SU, FL and KE, which were unresolved or partially resolved at all pH values with 10% of ethanol present, showed a slight improvement in enantioselectivity on reducing the concentration of ethanol. FE and IB have not been resolved so far, probably because these compounds do not have the structural prerequisites for obtaining three-point interactions with the binding site of yolk RFBP.

The calcium channel antagonists GA, VE and AM did not show any enantioselectivity, whereas for BE and all the other dihydropyridines the percentage of ethanol was the parameter that influenced the separation most. On decreasing the ethanol concentration from 10% to 5% (at pH 5.5), BE, IS, NC and MA are partially resolved with *Pi* values of 0.36, 0.34, 0.76 and 0.50, respectively. A further improvement in the peak separation function is observed in the absence of the organic modifier (pH 5.5): for example, the *Pi* changes from 0.34 to 0.76 and from 0.76 to 1.0 for IS and NC, respectively, but the long retention times and the low efficiency obtained are not desirable for analytical purposes.

Both benzodiazepines LO and OX gave very good enantioselectivity with 5% of ethanol (pH 5.5) and *Pi* values of 0.90 and 0.94, respectively. Increasing the pH leads to a dramatic increase in the retention time of the second-eluted enantiomer for OX. The fact that LM has not been separated so far under any of the conditions tested clearly demonstrates that small changes in the molecular structure (a methyl group) can influence the separation substantially. The weak acid WA was found to be the best resolved compound so far on the yolk RFBP column. The best separations were achieved in the pH range (4.5–5.5) where this analyte is mainly uncharged, and this leads to the conclusion that the hydrophobic interactions of this bulky and rigid molecule are necessary in the enantioselective interactions with the protein-binding site.

Tryptophan was never separated on the yolk RFBP column and its retention times were very short under any of the chromatographic conditions tested. This is surprising as its indole moiety confers on this molecule a hydrophobicity comparable to that of the anti-inflammatory drugs considered. It can be hypothesized that the amino group which is protonated in the operating pH range is responsible for repulsion phenomena with a positive charge located in the protein hydrophobic binding site.

3.3. Influence of type of uncharged modifier on retention and enantioselectivity

Different organic modifiers, namely methanol, ethanol, *n*-propanol and acetonitrile, were tested with respect to the enantioselective retention of all analytes. The variations in chromatographic parameters observed are shown in Table 2 for a constant concentration of organic modifier of 5% and a pH of 5.5. The alkanols cause a weakening of the hydrophobic interactions between the solute and the protein surface. For a series of 1-alkanols, the effect increases rapidly with increasing length of the alkyl chain. Although acetonitrile gives k' values even lower than *n*-propanol, the enantioselectivity is comparable to that obtained with ethanol.

The small number of compounds tested so far on the egg white RFBP CSP, namely KE, IB, FL, WA, benzoin and α,ε-dibenzoyllysine, makes the comparison between the chromatographic performances of hen egg yolk and egg white RFBP CSPs difficult. The effect of pH and percentage of organic modifier on k' follows a similar trend for both CSPs, that is, with increase in pH, k' decreases for acidic analytes (i.e., KE). For the basic compounds tested on egg yolk RFBP, the pH effect is reversed but comparison with egg white RFBP is not possible as no basic drugs have yet been tested on this phase. The influence of organic modifiers on k' is similar to that of a reversed-phase mode for both columns, but the retention times were generally found to be shorter on egg yolk RFBP under the same conditions.

As far as the enantioselectivity is concerned, KE, IB, FL and WA showed different behaviour

Table 2
Influence of type of uncharged modifier on retention and enantioselectivity

Compound	Methanol		Ethanol		*n*-Propanol		Acetonitrile	
	k'_1	α	k'_1	α	k'_1	α	k'_1	α
Ibuprofen	1.38	1.13	1.32	1	1.12	1	1.07	1
Ketoprofen	3.01	1.22	2.56	1.14	1.52	1	1.36	1.06
Flurbiprofen	3.08	1.14	3.46	1	2.32	1	2.34	1
Indoprofen	115.29	1	79.44	1	12.72	1	7.25	1.06
Suprofen	3.36	1.07	3.21	1	1.58	1	1.44	1
Fenoprofen	1.85	1	1.82	1	1.38	1	1.36	1
Carprofen	30.42	2.20	22.09	2.01	14.45	1	6.67	1.14
Warfarin	6.77	1.99	4.67	1.26	3.11	1	1.97	1.33
Tryptophan	0.38	1	0.31	1	0.27	1	0.26	1
Lormetazepam	5.81	1	4.35	1	3.58	1	0.22	1
Lorazepam	4.91	1.87	4.02	1.63	3.28	1.12	3.07	1.25
Oxazepam	4.87	6.68	3.92	4.55	2.61	1.69	2.5	3.08
Gallopamil	6.42	1	3.2	1	2.91	1	1.13	1
Verapamil	8.28	1	2.55	1	4.32	1	1.32	1
Bepridil	–[a]	–[a]	11.96	1.21	10.02	1.08	16.95	1
Isradipine	10.58	1.18	8.57	1.17	5.21	1	5.07	1
Amlodipine	11.41	1	5.49	1	6.14	1	6.05	1
Nimodipine	21.8	1	14.68	1	8.37	1	6.8	1
Nicardipine	23.71	1.34	16.61	1.32	9.73	1	7.72	1.11
Manidipine	–[a]	–[a]	232.94	1.32	–[a]	–[a]	–[a]	–[a]

Mobile phase: 50 m*M* KH_2PO_4 (pH 5.5)–organic modifier (95:5, v/v)
[a] Not eluted within 5 h.

on egg white and egg yolk RFBP. On egg white RFBP, baseline resolution was obtained for KE and WA and only partial resolution for IB and FL. On egg yolk RFBP, baseline resolution was achieved for WA, with shorter retention times than those on egg white RFBP. On the egg yolk column KE showed only a modest separation and IB and FL were not enantioseparated. On the other hand, IN, CA, LO, OX, IS and NC, not yet tested on the egg white stationary phase, gave very good enantioseparations within reasonable retention times on the egg yolk column. It should be noted that the positive results obtained with IN and CA, in contrast to those obtained with IB and KE, lead us to think that a bulky and rigid structure rather than a simpler molecule is required in order to establish more easily the hydrophobic interactions that seem to be crucial for enantioselectivity. Moreover, the small amount of egg yolk RFBP used for the preparation of this column compared with that used for the egg white RFBP [9], AGP [11] and OVM [12] CSPs could be responsible for the poor resolution of some of the compounds tested. It has already been pointed out for the AGP CSP [13] that a low protein content gives, for hydrophilic drugs, bad resolution owing to a low separation factor, tailing peaks and low separation efficiency.

In order to obtain reproducible results, a very important aspect is the column stability and robustness, which were evaluated on the egg yolk RFBP CSP using WA as the probe. After 250 injections and with the wide changes in mobile phase composition and pH that were required in method development, the stereoselective performance and the efficiency of the column were unaffected. This is consistent with the chromatographic parameters reported in Table 3.

Table 3
Chromatographic parameters of warfarin (A) for initial injection and (B) after 250 injections.

Parameter	A	B
k'_1	4.9	4.64
k'_2	8.5	7.92
α	1.73	1.70
R_s	2.81	2.49
Plates/m	15 020	11 980

Mobile phase: 50 m*M* KH_2PO_4 (pH 5.5)–ethanol (95:5, v/v).

4. Conclusions

The encouraging preliminary results obtained on the egg yolk RFBP CSP suggest its applicability to a reasonably wide range of drug enantiomers in the reversed-phase mode. Further optimization of the chromatographic conditions and a higher protein loading of the column could further improve its applicability, efficiency and selectivity. Although it is well known that egg yolk RFBP has a specific binding site for riboflavin, the data reported here can neither confirm nor disprove whether the drugs tested interact with this protein binding site. Displacement studies are necessary in order to confirm this possibility and to attempt to elucidate the enantioselective mechanisms.

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