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Chromatographic investigation on the binding site characteristics of quail egg-white riboflavin binding protein as a chiral stationary phase

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Abstract

Recently we described the use of riboflavin binding protein extracted from quail egg-white, as a new HPLC chiral stationary phase. In this study we show the further results obtained with the use of high-performance affinity chromatography to provide a better understanding of the chiral recognition mechanism for the observed enantioselectivity and to gain a deeper knowledge into the binding site that has been recently characterised by X-ray crystallography for chicken egg-white. High-performance affinity chromatography provides information on the potential protein structural changes occurring upon its immobilisation and enables competitive binding studies as well as the assessment of binding constants through frontal analysis experiments.

Keywords: Chiral stationary phases, LC; Riboflavin binding protein

1. Introduction

The name riboflavin binding protein (RfBP) is applied to several molecular species that are thought to be important in maintaining the supply of the vitamin riboflavin to the developing embryo during pregnancy [1].

Since adequate amounts of riboflavin are essential for normal fetal development, it is not surprising that under these more demanding conditions a specific carrier system has evolved. Pregnancy specific mammalian riboflavin binding proteins found in rat, bovine and human plasma [2–4], appear to share a common similarity to the well known chicken riboflavin binding protein, since antibodies against chicken RfBP have been found to lead to embryonic death in rats, mice and bonnet monkey [5–7], due to acute vitamin deficiency.

X-Ray studies carried out on chicken egg-white RfBP [8] have demonstrated that the binding of riboflavin occurs in a cleft with the isoalloxazine ring stacked between the parallel planes of a tyrosine residue and a tryptophan residue; in particular the binding takes place with the hydrophobic xylene moiety buried deeply into the protein. The major interactions of the natural ligand with the protein are therefore hydrophobic, as five of the six tryptophans present in the protein cluster in the vicinity of the

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binding site. In addition to hydrophobic amino acids, bound riboflavin was found to protect an essential carboxyl group from inactivation by carbodiimide.

In previous papers [9,10], we reported the development of RfBP chiral stationary phases (CSPs) obtained from chicken egg-yolk and quail egg-white and such columns have been studied in respect to their chiral recognition ability. The enantiomers of a wide variety of chiral compounds were separated and a similar chromatographic behaviour was observed between the two different stationary phases, indicating that there is a structural similarity between RfBPs extracted from the eggs of the two avian species.

In this work the quail egg-white RfBP-CSP has been exploited to elucidate the chiral recognition mechanism for the observed enantioselectivity. The use of an immobilized protein in a chromatographic system for the study of biomolecular interactions is a method known as analytical or quantitative affinity chromatography and it is classically carried out by frontal analysis and zonal elution experiments [11–13].

Frontal analysis affinity chromatography has been used to investigate the binding of riboflavin to RfBP at different pH values, in order to verify whether the immobilization procedure alters the conformation and the natural binding properties of the protein.

In order to elucidate the chiral recognition mechanism and following our previous work [10], six racemic compounds of pharmaceutical interest whose enantiomers were successfully resolved by the column were selected as test compounds. The effects of mobile phase pH on retention and enantioselectivity were evaluated and with the aim of assessing the relative contributions of specific and non-specific interactions, the same study was carried out in saturation conditions by adding riboflavin to the mobile phase.

Different concentrations of the natural ligand were also added to the mobile phase and other competitors for the binding site such as (−)-oxazepam hemisuccinate and chlorpromazine were evaluated for displacement studies. As the function of a succinate-binding cleft that was recently characterised for chicken egg white RfBP [8] still remains unidentified, retention and enantioselectivity of the six compounds were also evaluated in the absence and presence of ammonium succinate in the chromatographic system.

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.

2.2. Reagents and materials

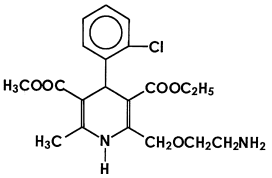
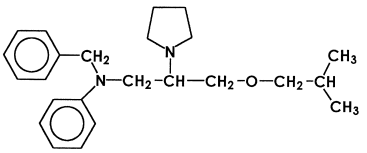
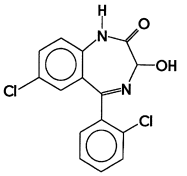
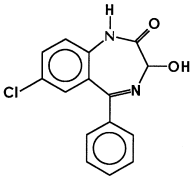
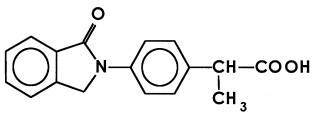
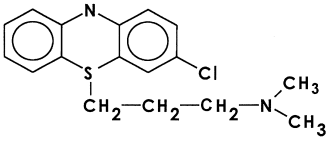
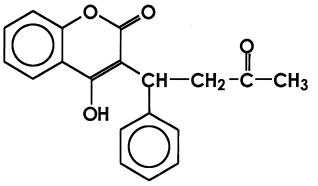
Indoprofen, warfarin, oxazepam, lorazepam, bepridil and chlorpromazine were purchased from Sigma (St. Louis, MO, USA); riboflavin was purchased from Merck (Darmstadt, Germany), amlodipine was kindly supplied by Pfizer (Sandwich, UK); (−)- and (+)-oxazepam hemisuccinate (OXH) were a kind gift from Professor C. Bertucci (CNR, Pisa, Italy). Potassium dihydrogenphosphate and dipotassium hydrogenphosphate and the organic solvents used for the preparation of the mobile phases were of analytical grade and purchased from Merck. Aminopropyl silica packing material (Nucleosil-5NH_2, 5 μm particle size, 100 Å pore diameter) was obtained from Macherey-Nagel (Düren, Germany). The chemical structures of the analytes are reported in Table 1.

2.3. Purification, immobilization and packing of quail egg-white RfBP

Quail egg-white RfBP was purified and immobilized in our laboratory according to a previously described method [9] and the resulting stationary phase was packed in a stainless steel column (125 mm×4.0 mm I.D.) from Merck.

The amount of protein immobilized on the 5NH_2 Nucleosil was calculated by elemental analysis and was found to be 54.41 mg/g silica.

Table 1
Chemical structures of analytes

Compound	Formula
Amlodipine	
Bepridil	
Lorazepam	
Oxazepam	
Indoprofen	
Chlorpromazine	
Warfarin	

2.4. Liquid chromatography conditions

All the experiments were carried out at 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. 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.

2.5. Methods

Affinity constants between the protein and its natural ligand, riboflavin, were measured by frontal analysis. In this technique a solution with a known concentration of a given analyte (A) is continuously applied to a column containing a fixed amount of immobilized protein (P). As the protein becomes saturated, the amount of analyte eluting from the column gradually increases forming a characteristic breakthrough curve. The mean position of this curve is related to the concentration of applied analyte, to the amount of protein present and to the association constants for the system. The results obtained in a frontal analysis experiments can be examined by Klotz plots.

$$1/m_{P,app} = 1/K_a m_P[A] + 1/m_P \quad (1)$$

For a system with a single binding site, Eq. (1) predicts that a plot of $1/m_{P,app}$ (apparent moles of solute required to reach the mean position of the breakthrough curve) vs. 1/[A], will give a straight line with slope of $1/K_a m_P$ and intercept of $1/m_P$. The value of K_a (association constant for the binding of A to P) can be determined directly by calculating the ratio of the intercept to the slope from this plot.

Frontal analysis was performed by continuously applying to the column solutions containing riboflavin dissolved in 50 m*M* phosphate buffer–methanol (95:5) at two different pH values (3.5 and 5.0). Riboflavin concentrations ranged from 2.5 μ*M* to 40 μ*M*; the process was followed by monitoring the eluent at 254 nm.

After each set of experiments, retained riboflavin was eluted by applying to the column a 50 m*M* phosphate buffer solution at pH 3.0 [14].

The amount of riboflavin needed to saturate the RfBP column was determined by integration of the resulting breakthrough curves and corrected for the void volume of the system [15]. Similar measurements were carried out on a Nucleosil-NH_2 silica column to study the potential contribution of non specific binding given by the solid support.

Displacement studies were carried out using zonal elution technique. In this technique a known concentration of a competitor (C) is continuously applied to the column containing an immobilized protein (P) while injections of a solute (A) are made.

If C and A compete at a single site on P, and A binds to no other sites on the matrix, Eq. (2) describes the retention of A as [C] is varied.

$$1/k'_A = K_2 V_m[C]/K_3 m_P + V_m/K_3 m_P \quad (2)$$

In this equation k'_A is the capacity factor for a solute A, V_m is the void volume of the column, [C] is the concentration of the competitor applied to the column, K_2 is the affinity constant of C for the protein and K_3 is the affinity constant of A for P.

For a system with single-site competition, this equation predicts that a plot of $1/k'_A$ vs. [C] will yield a linear relationship. By calculating the ratio of the slope to the intercept for such a plot, the value of K_2 can be directly obtained. This equation allows the association constant for C to be measured only at the site at which C and A compete [16,17].

3. Results and discussion

3.1. Frontal analysis

The first part of this work examines the effectiveness of the immobilized RfBP matrix in modelling the binding behaviour of RfBP in solution, as an important factor to consider when using an immobilized protein column is the degree to which this support will model the behaviour of the native protein. This is of potential concern since the immobilization process can affect protein activity through denaturation, improper orientation or steric hindrance of the protein at the binding sites to be studied [11].

In our previous work a comparison between high-

performance liquid chromatography (HPLC) and capillary electrophoresis (CE) data confirmed that immobilized RfBP behaviour reflects the binding behaviour of RfBP in solution, including its enantioselectivity [10].

Frontal analysis was then used to measure the association constants of riboflavin at two different pH values, as described in Section 2.5.

As predicted by theory, the mean position of the curves shifted to shorter breakthrough times as the concentration of applied riboflavin was increased. Frontal analysis curves obtained at pH 3.5 and 5.0, respectively are given in Fig. 1a and b.

The linear trend of Klotz plots obtained from these frontal analysis data, indicates that the natural ligand binds to a single type of site [18]. From the slopes and intercepts of these lines, the affinity constants of riboflavin for quail egg-white RfBP were found to be $6.6 \cdot 10^4\ M^{-1}$ ($r^2 = 0.998$) at pH 3.5 and $1.2 \cdot 10^5\ M^{-1}$ ($r^2 = 0.99$) at pH 5. A confirmation of these values is not possible as corresponding literature data for quail egg-white RfBP in solution are not available, however the pH dependence of the K_a for the binding of riboflavin to chicken egg-white RfBP has been previously described to follow the same trend [19], that is affinity increases by increasing the pH.

The breakthrough volumes measured on the Nucleosil-NH_2 silica did not reveal specific interactions with the silica support as they are independent on the riboflavin concentration applied.

3.2. Effect of pH on retention and enantioselectivity and influence of column saturation with riboflavin

As the overall charge of the protein and potential conformational changes are pH dependent, the effect of this parameter on retention and enantioselectivity was investigated in the range between 3.5 and 6.5 by using six compounds of pharmaceutical interest namely amlodipine and bepridil (bases), warfarin and indoprofen (acids), oxazepam and lorazepam (uncharged), whose enantiomers were always well resolved.

The isoelectric point of RFBP is about 4 and the pK_a of the acidic compounds warfarin and indoprofen is 5.05 and 5.20, respectively, therefore these compounds are predominantly uncharged when the pH of the buffer is below 5.0 and charged when it is above 5.5. The bell shaped retention curves observed for these compounds are probably due in part to the change in the ionisation status of the drugs and in part to the change of the ionic state of amino and carboxyl groups involved in the interactions between acidic compounds and the protein binding site. However, the maximum in retention and enantioselectivity was obtained in the range between pH 4.5 and pH 5.0, as shown for indoprofen as an example in Fig. 2. As over this range the analytes are mainly uncharged, and the protein bears a slight overall negative charge, this indicates that a key role is played by hydrophobic interactions between these solutes and RfBP. All these findings are in agreement with published results [8,20].

The k' values of basic compounds increased on increasing the pH. These compounds are positively charged throughout the pH range investigated, thus the variation in retention is not due to the ionization status of the analyte but is instead related to the increase in the net negative charge of the protein that leads to an increase of Coulombic interactions with these analytes. A decrease in the pH of the mobile phase towards the isoelectric point of the protein gives a lower degree of negative charge of the protein itself and consequently lower retention for the cationic drugs. The enantioselectivity of basic compounds was not influenced by pH variations.

Both the retention times and the enantioselectivity for the two benzodiazepines increased on increasing the pH, however, the observed relevant improvement in selectivity is due only to the remarkable increase in the retention time of the second eluted enantiomer. These results suggest that the two enantiomers might bind at different loci and it can be hypothesised that the first eluted enantiomer shows a non-specific interaction with the protein, whereas the second enantiomer binds to the specific binding site.

In order to assess the contribution of riboflavin binding site to specific interactions, the whole systematic study was carried out after saturating the chromatographic system with riboflavin.

A 4 μ*M* solution of riboflavin in 50 m*M* phosphate buffer–methanol (95:5, v/v) was applied to the column. As the immobilized protein becomes saturated, the amount of riboflavin eluting from the

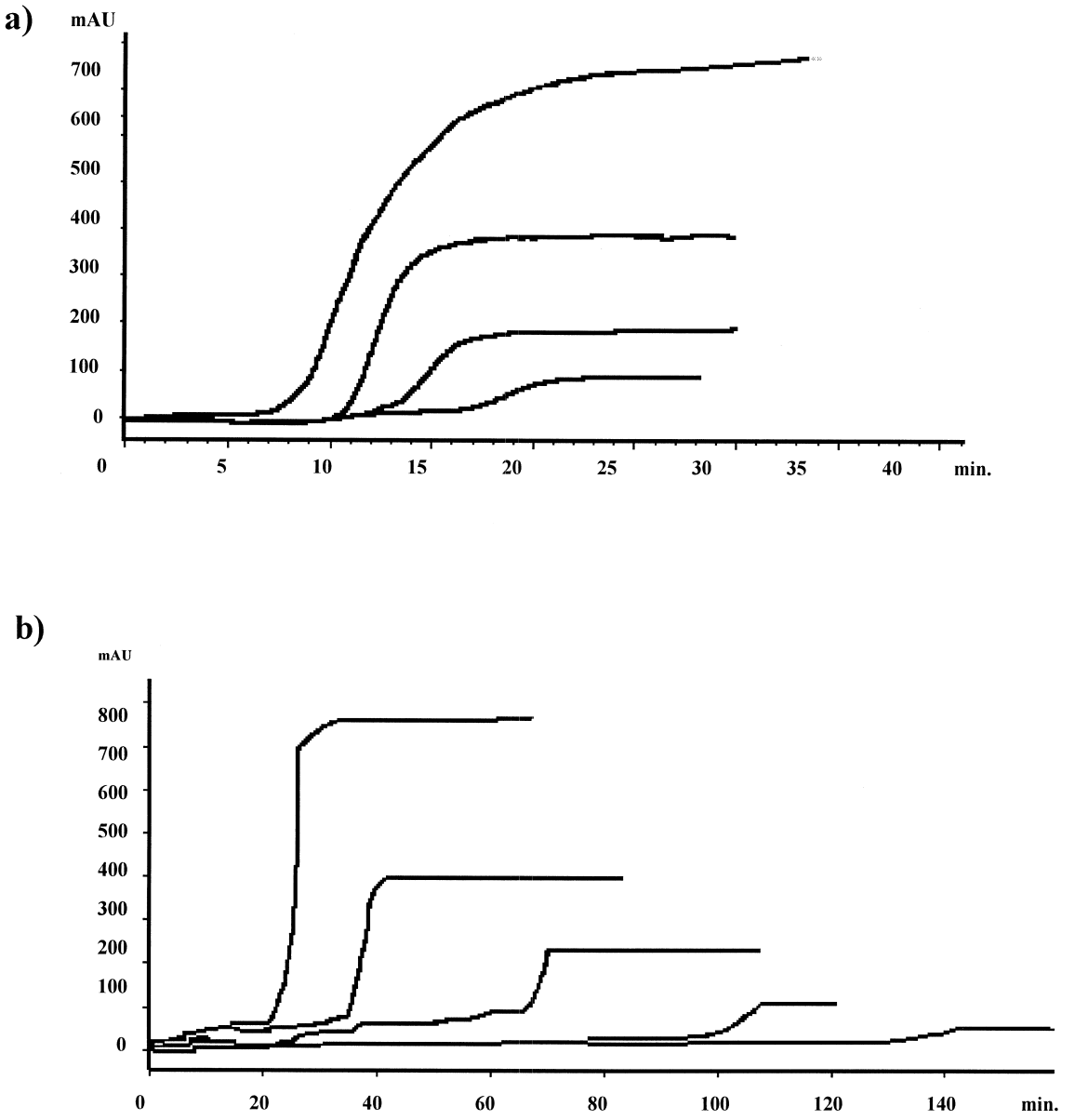


Fig. 1. (a) Frontal analysis curves for riboflavin obtained at pH 3.5 on the quail egg-white RfBP column. The concentrations of riboflavin (from left to right) were 40, 10, 5 and 2.5 μ*M*, respectively. Flow rate: 0.8 ml/min. Temperature: 25°C. (b) Frontal analysis curves for riboflavin obtained at pH 5.0 on the quail egg-white RfBP column. The concentrations of riboflavin (from left to right) were 40, 20, 10, 5 and 2.5 μ*M*, respectively. Flow-rate and temperature as in (a).

column increases; the process was followed by monitoring the eluate at 254 nm, then the detector baseline was re-zeroed and the solutes were analysed in saturation conditions, at each pH value within the range previously considered.

A loss of enantioselectivity for all the compounds tested and a great reduction in retention is induced by the saturation with riboflavin and the characteristic patterns previously observed in absence of riboflavin was lost.

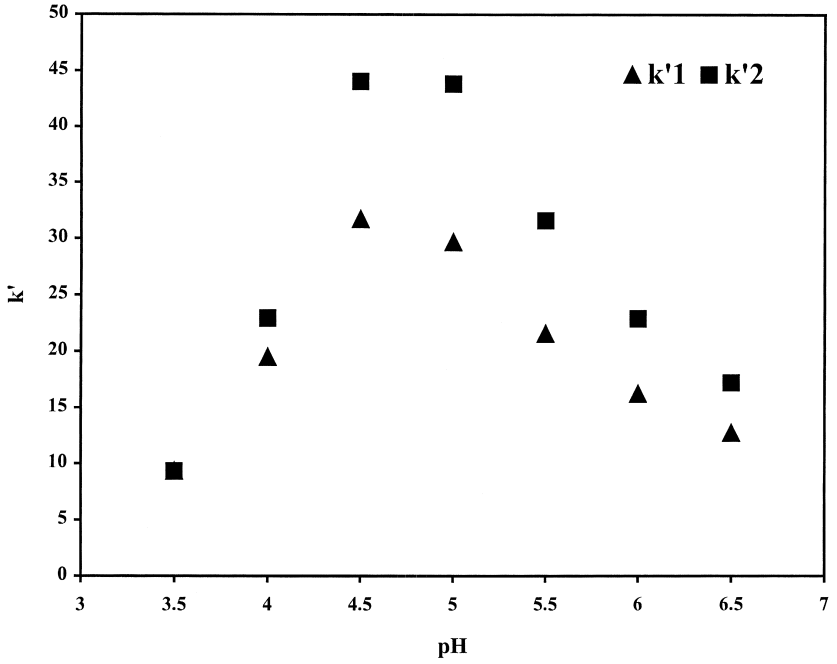


Fig. 2. Effect of pH on the retention of indoprofen enantiomers. Chromatographic conditions: 50 m*M* phosphate buffer–methanol (95:5, v/v). Flow-rate: 0.8 ml/min.

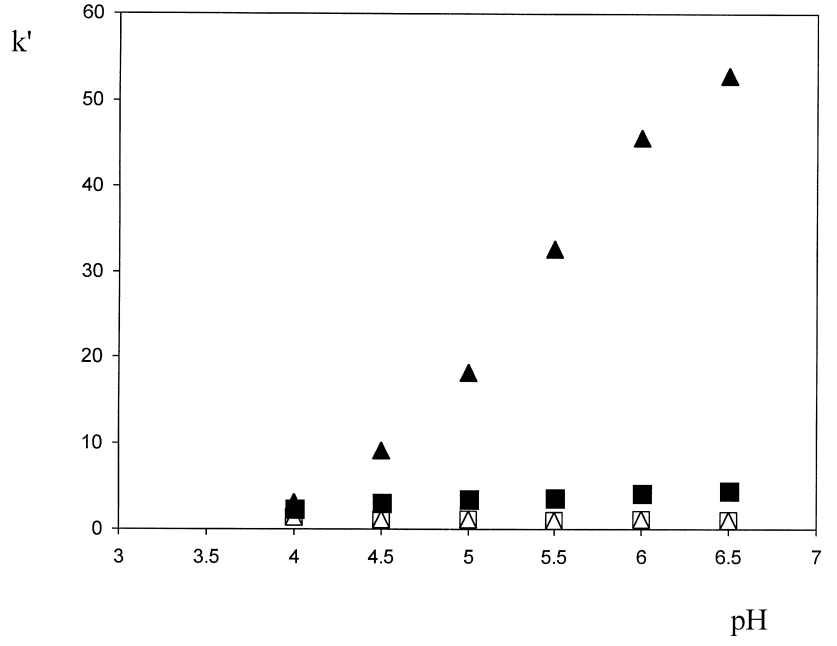


Fig. 3. Effect of pH on the retention of oxazepam enantiomers. ■ and ▲: k_1' and k_2' without addition of riboflavin to the mobile phase; □ and △: k_1' and k_2' after saturation with riboflavin. Other conditions are given in the text.

As an example, Fig. 3 reports the trend obtained for oxazepam by plotting retention factors against pH values in absence of riboflavin in the mobile phase and in saturation conditions. The dramatic reduction of the capacity factors and stereoselectivity compared with non saturation values indicates that the riboflavin binding site might be involved in the interaction mechanism and in the chiral recognition process [21].

The variability of the retention times of oxazepam enantiomers during the saturation process has been monitored by continuously injecting racemic oxazepam. The percentage of saturation was calculated from the ratio between the riboflavin moles at the elution volume of oxazepam enantiomers and the riboflavin moles at the complete saturation of the column. As can be seen from Fig. 4a and b, the saturation of the riboflavin binding site leads to a reduction of the k' values of both enantiomers (68% for the first eluted enantiomer and 95% for the second one). These experiments suggest that the two enantiomers interact at a site affected by riboflavin saturation. The presence of some selectivity after the saturation process indicate that additional binding areas are involved in the chiral separation.

In order to study the mechanism by which RfBP is able to express the enantioselectivity, a series of competitive studies was carried out by monitoring the effects of the mobile phase addition of different concentrations of one enantiomer of oxazepam hemisuccinate on the retention of the other enantiomer [22]. After equilibration of the column with either (−)- or (+)-oxazepam hemisuccinate, the opposite enantiomer was then injected onto the column and its k' was determined. In both cases a slight reduction of the k' values was observed (Table 2) and such a change in retention indicates that some type of competition is taking place between the two enantiomers. Nevertheless by plotting $1/k'$ vs. competing agent concentration, a non-linear curve was observed (Fig. 5a and b) and these results indicate that the stereoselective solute–RfBP interaction is a result of a complex process which involves different binding sites possibly including the riboflavin binding site.

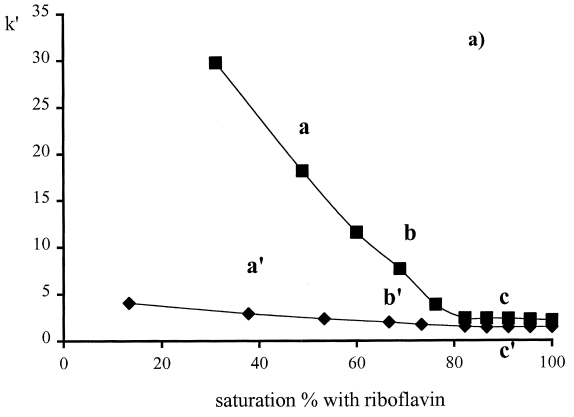


Fig. 4. (a) Changes in the chromatographic retention of oxazepam enantiomers as a result of different saturation conditions with riboflavin. (b) Chromatograms of *rac*-oxazepam recorded at three different steps during the saturation process.

3.3. Displacement studies

Zonal elution studies were initially carried out at two levels of riboflavin concentration (2 μ*M* and 4 μ*M*) at pH 6.5 (Table 3). As expected, by adding the natural ligand to the mobile phase, a large decrease in the elution time and in the enantioselectivity for all tested compounds was observed, while when comparing the k' values obtained at 2 μ*M* and 4 μ*M* of riboflavin concentration it is not possible to find a consistent variation. This lack of concentration-dependent effect on the reduction of k' when riboflavin was added to the mobile phase could be ascribed to the strong affinity of riboflavin for the protein at pH values above 6.0 (K_a $4.9 \cdot 10^8$ M^{-1} at pH 7 [19]) and this implies that riboflavin can not be usefully employed for zonal elution studies [23–25].

Displacement studies were therefore carried out by adding to the mobile phase a better model compound, such as the most tightly bound enantiomer of oxazepam hemisuccinate. Solutions of (−)-oxazepam hemisuccinate at different concentrations (from 0.005 to 0.050 m*M*) have been applied to the column until equilibrium was reached, following the adsorption of the column eluent at 230 nm. Once the column equilibrated, (−)-oxazepam hemisuccinate could be seen to elute as a sharp front, followed by a plateau, then the detector baseline was re-zeroed and allowed to stabilise. Chlorpromazine, a non chiral drug which presents a molecular structure similar to that of riboflavin and the chiral drugs previously

b)

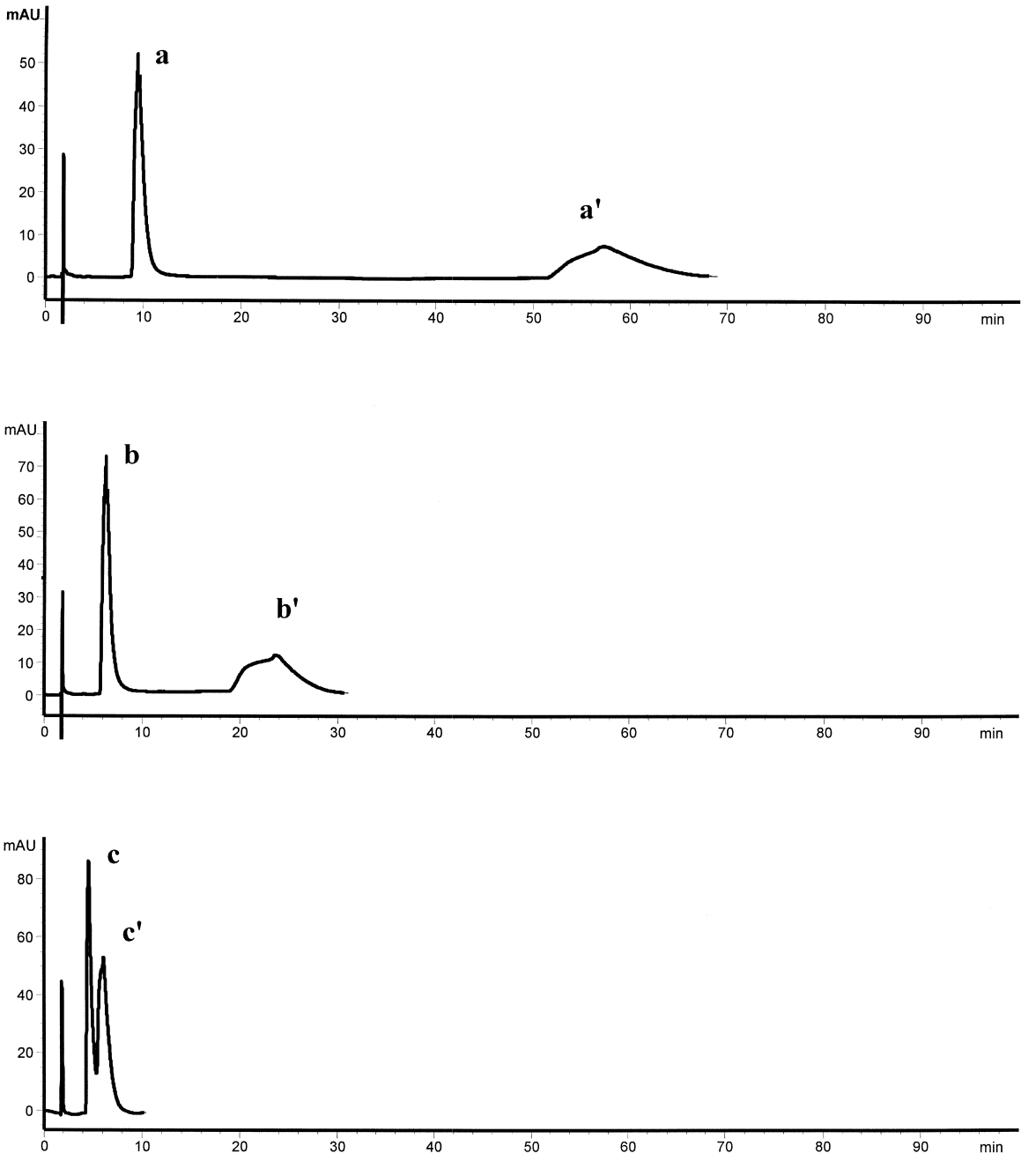


Fig. 4 (*continued*).

Table 2
Variation in the chromatographic retention of oxazepam hemisuccinate enantiomers as a result of the addition of the opposite enantiomer to the mobile phase

Solute/competitor	Competitor concentration (m*M*)	*k′*
(+)-OXH/(−)-OXH	0.000	1.46
	0.005	1.40
	0.010	1.33
	0.020	1.25
	0.050	1.17
(−)-OXH/(+)-OXH	0.000	5.06
	0.005	4.89
	0.010	4.81
	0.020	4.74
	0.050	4.33

mentioned were re-injected, and their new *k′* values were calculated.

The addition of (−)-oxazepam hemisuccinate to the mobile phase had a concentration-dependent effect on the *k′* of the test solutes examined and plotting of the chromatographic data did not reveal a linear relationship. This indicates that a simple competitive model does not apply and that allosteric effect or more likely a multisite competition takes place between the analytes and the competitor. The single-site competitive model does fit instead for chlorpromazine, for which a linear plot was obtained ($r^2 = 0.992$). As by means of zonal elution experiments it is also possible to obtain the affinity constant, the K_a value of (−)-oxazepam hemisuccinate for the immobilized RfBP was calculated to be $1.6 \cdot 10^4\ M^{-1}$.

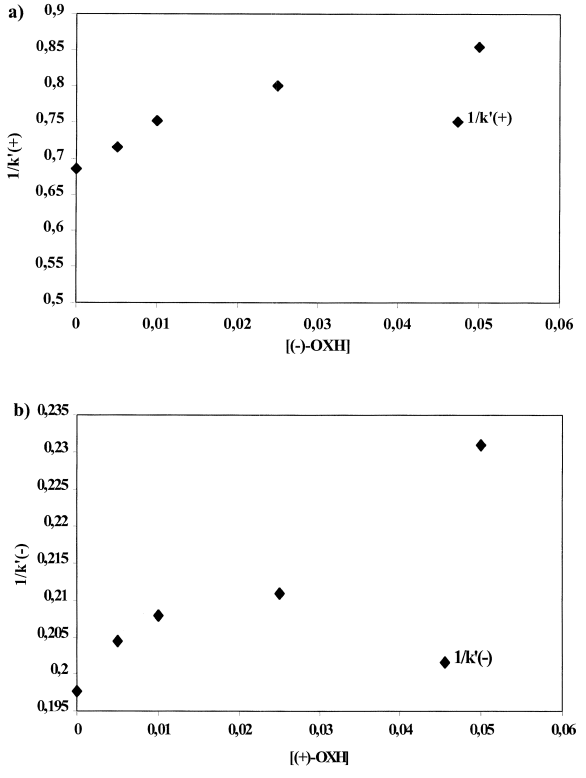


Fig. 5. (a) Result of zonal elution for (−)-OXH in presence of (+)-OXH. (b) Result of zonal elution for (+)-OXH in presence of (−)-OXH.

As chlorpromazine was found to be a good model for further zonal elution experiments, this analyte was added to the mobile phase. Zonal elution studies were performed by applying chlorpromazine solutions at concentrations ranging from 0 to 0.050 m*M* in 50 m*M* phosphate buffer, pH 6.5 (5% methanol) until equilibrium was reached, following the adsorption of the column eluent at 254 nm. All the chiral drugs previously mentioned were re-injected and analysed under equilibrium conditions. The addition of chlorpromazine had a concentration-dependent effect on all the *k′* values of the test solutes examined. Plotting the chromatographic data according to Eq. (2) reveals a linear relationship only for the most retained enantiomer of oxazepam ($r^2 = 0.999$) and gives a K_a for chlorpromazine of 6.6 (± 1.4 SD)$\cdot 10^4\ M^{-1}$.

For all other analytes a direct competition does not occur and non-linear plots were observed as shown in Fig. 6 for amlodipine enantiomers as an example.

As X-ray studies recently revealed the presence of a succinate-binding cleft [8], retention and enantioselectivity of the six compounds were evaluated in the absence and presence of ammonium succinate in the mobile phase. The slight increase in retention observed for both enantiomers of each analyte (Table 4) can be explained with an increase in hydrophobic interactions between the analytes and the protein at higher ammonium succinate concentration (‘‘salting out’’ effect). As regards selectivity, no significant variation was observed (less than 7%) and it is therefore understood that the succinate binding site is not involved in the enantioselective mechanism for the tested analytes.

Table 3
Influence of riboflavin concentration on retention and selectivity

Compound	Riboflavin concentration								
	0 μ*M*			2 μ*M*			4 μ*M*		
	k'_1	k'_2	α	k'_1	k'_2	α	k'_1	k'_2	α
Amlodipine	8.23	12.59	1.53	3.64	4.12	1.13	2.70	2.92	1.08
Bepridil	19.86	44.12	2.22	6.32	6.32	1.0	7.62	8.08	1.06
Warfarin	2.31	5.03	2.18	1.59	1.59	1.0	1.56	1.56	1.0
Indoprofen	12.72	17.21	1.35	1.98	1.98	1.0	1.81	1.81	1.0
Lorazepam	2.43	8.17	3.36	1.35	1.35	1.0	1.43	1.43	1.0
Oxazepam	4.46	52.86	11.86	1.36	1.76	1.27	1.28	1.43	1.11

4. Conclusions

Affinity HPLC studies carried out on RfBP extracted from quail egg-white have provided a wide number of useful information. The riboflavin binding site has been confirmed to be hydrophobic and to be partially involved in the enantioselective discrimination mechanism, at least for the chiral drugs tested. Nevertheless more studies should be carried out to confirm that enantioselectivity arise from the interaction of the analytes on the riboflavin binding site.

A single-site interaction for the natural ligand has been elucidated and affinity constants have been measured under different pH conditions, so to confirm their dependence on this parameter, as described for the non-immobilised RfBP. However, the preci-

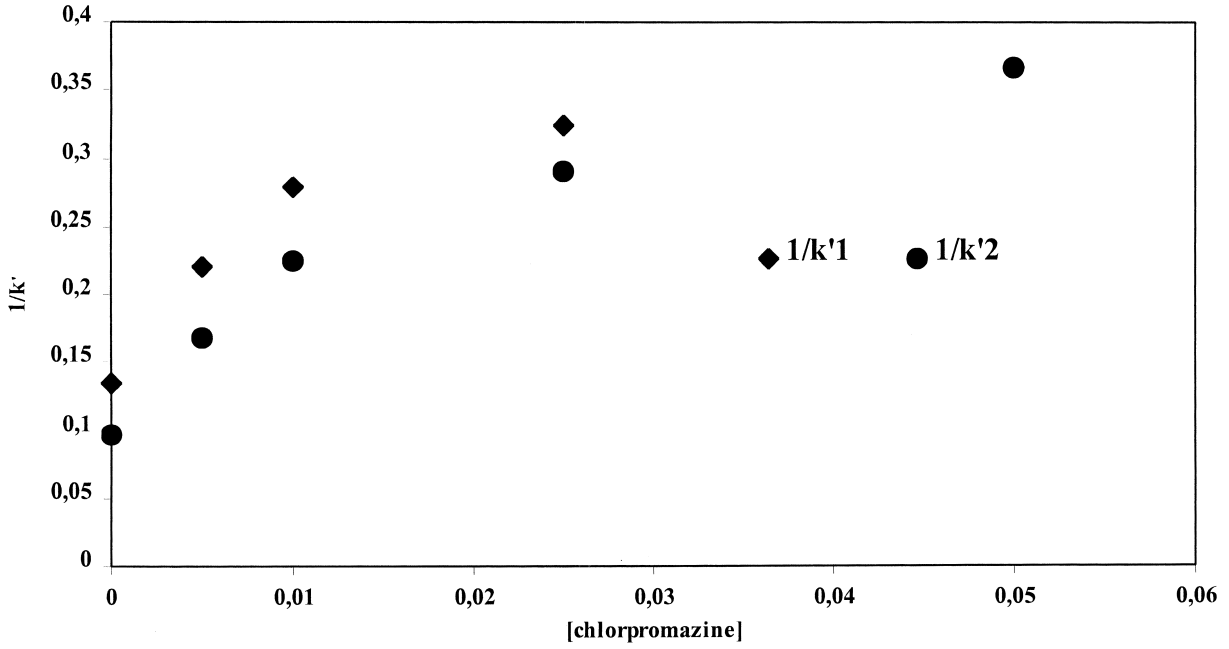


Fig. 6. Result of zonal elution for amlodipine enantiomers in the presence of chlorpromazine.

Table 4
Influence of ammonium succinate concentration on retention and selectivity

Compound		Ammonium succinate concentration				
		0 m*M*	0.020 m*M*	0.040 m*M*	0.060 m*M*	0.1 m*M*
Warfarin	k'_1	1.64	1.68	1.74	1.75	1.98
	k'_2	3.20	3.09	3.28	3.45	3.39
	α	1.95	1.84	1.89	1.97	1.98
Indoprofen	k'_1	5.82	6.1	7.14	7.18	7.04
	k'_2	7.19	7.6	8.94	8.98	8.78
	α	1.24	1.24	1.25	1.25	1.25
Lorazepam	k'_1	2.17	2.59	2.60	2.57	2.44
	k'_2	4.99	5.97	5.93	5.76	5.36
	α	2.30	2.30	2.28	2.24	2.2
Oxazepam	k'_1	3.96	4.21	4.53	4.61	4.58
	k'_2	28.11	28.51	30.92	31.49	31.45
	α	7.09	6.77	6.82	6.83	6.86
Amlodipine	k'_1	8.41	10.31	10.90	11.18	11.60
	k'_2	11.92	14.34	15.46	15.88	16.56
	α	1.42	1.39	1.42	1.42	1.43
Bepridil	k'_1	19.32	21.42	24.96	25.65	24.50
	k'_2	36.66	41.24	45.42	47.31	46.69
	α	1.90	1.92	1.82	1.84	1.90

sion of the association constants measured in this work by zonal elution and frontal analysis still needs to be estimated.

The RfBP-CSP could be successfully used as a quantitative probe of drug binding as well as to investigate drug–drug interactions.

In view of the mentioned physiological role of this protein, HPLC affinity studies with RfBP extracted from bovine plasma will enable a more realistic approach to human plasma RfBP and will shed light on the role of drug intake on vitamin transport during pregnancy.

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