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Evaluation of quail egg white riboflavin binding protein as a chiral selector in high-performance liquid chromatography and capillary electrophoresis

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Abstract

A new chiral stationary phase for high-performance liquid chromatography of quail egg white riboflavin binding protein is presented. Several chiral acidic, basic and uncharged drugs were analysed and the influence of the mobile phase's parameters on the retention times and enantioselectivity was evaluated. On the basis of the results obtained, the same protein was studied as a background electrolyte additive in free solution capillary electrophoresis, in order to evaluate if capillary electrophoresis (CE) could be used as a rapid scouting technique for screening the enantioselectivity of novel proteins without immobilisation on a solid support. To investigate if it is possible to directly compare the results obtained by each technique, the CE experiments were planned on the basis of both the findings and ideas originated in liquid chromatography. © 1997 Elsevier Science B.V.

Keywords: Enantiomer separation; Chiral stationary phases, LC; Proteins

1. Introduction

In the past decade, several proteins have been successfully used as high-performance liquid chromatography (HPLC) chiral stationary phases as they have been shown to have stereospecific binding interactions with numerous chiral molecules of pharmaceutical interest [1–6]. Protein-based columns are thus frequently used because of their versatility and broad applicability in the analysis of aqueous biological samples.

In our laboratories, we have undertaken the evaluation of riboflavin binding proteins (RfBPs) as potential chiral selectors in liquid chromatography. The name riboflavin binding protein is applied to several molecular species that are thought to be important in maintaining the supply of the vitamin to the developing embryo. Typical physico-chemical properties of RfBPs (data taken for chicken egg white) are summarised in Table 1 [7]. The first protein of this family to be isolated and studied was

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Table 1
Physico-chemical properties of chicken egg white RfBP

Molecular mass	32 000/36 000
Isoelectric point	3.9–4.1
Disulphide bridges	9
Amino acids	219
Carbohydrates (%)	14

hen egg white RfBP [8], which is also the first family member whose three-dimensional structure has been determined by X-ray diffraction [9]. Chicken egg white and yolk RfBP share the same amino acid sequence but have undergone different post-translational modifications, i.e. their carbohydrate chains are different and the latter lacks the last eleven–thirteen amino acids, which are proteolytically cleaved. Both proteins have been tested as HPLC stationary phases [10–12] and, in our previous work, we also showed that their chromatographic behaviour is different [12].

Following our previous research, we present here a new chiral column based on quail egg white riboflavin binding protein (qRfBP); although the overall structures of these two proteins of the same family are believed to be very similar, subtle differences have been described between chicken and quail RfBPs by comparing their near UV circular dichroism and fluorescence properties [13]. In this paper, we examine the retention and enantioselectivity characteristics of a qRfBP column for a number of chiral drugs. We also discuss the optimum conditions for one of the compounds tested and attempt to decide whether or not some bind at the vitamin-binding site by performing displacement studies using riboflavin in the mobile phase.

Since the procedure to obtain protein-based HPLC columns, from extraction to immobilisation, is time-consuming, expensive and requires a considerable amount of purified protein, an alternative method for investigating protein enantioselectivity was examined. Capillary electrophoresis (CE) was evaluated as a potentially rapid technique for screening the stereoselective binding properties of ligand to protein, requiring only a few milligrams of the binding protein, which does not need to be immobilised on a solid support.

Similarities between liquid chromatography and CE have been demonstrated for other systems, such as chiral resolution using cyclodextrins [14,15] and various proteins have already been studied as chiral selectors in free solution CE, namely bovine and human serum albumins [16,17], α_1-acid glycoprotein [17,18], ovomucoid [17–19], cellobiohydrolase [20], and avidin [21]. Although differences in selectivity have occasionally been observed between separations with the same protein used in HPLC and CE [20], generally, the selectivity observed in both techniques is rather similar, and it has been shown in the case of analytes binding to human serum albumin that a simple quantitative relationship can be drawn between retention in HPLC and CE [22,23]. Thus, it was expected that RfBP would interact in solution with ligands and discriminate between enantiomeric pairs in a way that was similar to RfBP covalently bound to a silica matrix.

In planning the CE experiments, both results and ideas originated in LC were considered and comparison of experimental data with those from LC shed light on the potential of CE to compete with well-established LC analyses based on chiral-bonded columns. This approach can lead one to make a rational choice regarding the proteins to prepare in large quantities to be used as the immobilised phase in HPLC.

2. Experimental

2.1. Apparatus

2.1.1. Liquid chromatography

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 a HP Vectra Q5/165 workstation was used. A stainless-steel column (100×4.6 mm I.D.) was packed with quail egg white RfBP-conjugated silica gel by Hypersil (Runcorn, UK).

2.1.2. Capillary electrophoresis

A Unicam (Cambridge, UK) model Crystal 310 capillary electrophoresis system was used, with a Spectra 100 (Thermo Separation Products, San Jose, CA, USA) variable-wavelength UV detector, which was set at 247 nm. Data were analysed using a

Unicam 4880 chromatography data handling system. Electrophoresis was performed in a 72 cm long×50 μm I.D., 360 μm O.D. fused-silica capillary (MicroQuartz, Munich, Germany); the length of the capillary to the detector window was 55 cm.

2.2. Reagents and materials

Ibuprofen (IB), ketoprofen (KE), flurbiprofen (FL), indoprofen (IN), suprofen (SU), fenoprofen (FE), carprofen (CA), warfarin (WA), lormetazepam (LM), oxazepam (OX), lorazepam (LO), verapamil (VE), bepridil (BE), propranolol (PP), fenfluramine (FN) bupivacaine (BU) and nicardipine (NC) were purchased from Sigma (St. Louis, MO, USA). Gallopamil (GA) was purchased from Schiapparelli (Turin, Italy), isradipine (IS) was kindly donated by Sandoz (Milan, Italy), amlodipine (AM) was kindly supplied by Pfizer (Sandwich, UK), nimodipine (NM) was a gift from Bayer (Milan, Italy) and manidipine (MA) was kindly donated by Takeda (Osaka, Japan); lercanidipine (LE) was provided by Recordati (Milan, Italy), practolol (PC) was used as received from the Institute of Pharmacology of the University of Pavia, Italy, NaH_2PO_4, Na_2HPO_4 and the organic solvents used for the preparation of the mobile phases and of the background electrolyte were of analytical grade and were purchased from Merck (Darmstadt, Germany). Sodium dodecyl sulphate, acetone and sodium hydroxide anhydrous pellets (98%) were from Carlo Erba (Milan, Italy). DEAE–cellulose was purchased from Whatman (Maidstone, UK) and Sephadex G-100 was from Pharmacia (Uppsala, Sweden). Unless otherwise stated, all of the reagents used were of analytical grade.

2.3. Preparation of the quail egg white RfBP column

2.3.1. Purification of quail egg white apo RfBP

Quail egg white RfBP was purified by a suitable modification of a method that is widely used for chicken RfBP [24]. The whites from 450 quail eggs were diluted 1:1 with 0.05 *M* acetate buffer, pH 5.5, and 100 mg of excess riboflavin were added to the sample. After blending and filtering through cheesecloth, the diluted material was left stirring overnight at 4°C. The material was then passed slowly through a DEAE–cellulose column (50×3 cm) that had been equilibrated with 0.05 *M* acetate buffer, pH 5.5. The column was washed extensively with this buffer and the proteins that did not bind to it were discarded. The bound RfBP was eluted by making the acetate buffer 0.5 *M* in NaCl. The fractions that contained the best A_{280}/A_{455} ratios (the closest to a ratio of six, measured for pure RfBP as described by others [24]) were pooled, concentrated and dialysed against 0.050 *M* Tris–HCl, pH 7.5. The next step was gel filtration through a Sephadex G-100 column (120×4 cm). After this step, the sample was considered to be sufficiently pure since its A_{280}/A_{455} ratio was close to six. As in the case of many other species, analytical isoelectric focusing of this material revealed the presence of different isoforms, which are believed to differ in their carbohydrate and/or phosphate content. The different isoforms, which can be separated by preparative isoelectric focusing, present roughly the same A_{280}/A_{455} ratio and, therefore, the pooled isoforms were used for the column. The riboflavin molecule bound to the protein was eliminated by extensive dialysis at pH 3.0 and the column was prepared with the apoprotein.

2.3.2. Immobilization and packing of quail egg white apo RfBP

Immobilization was carried out in our laboratory following a previously described method [12,25]. In brief, $5NH_2$ Nucleosil was slurried in HPLC-grade acetonitrile and *N*,*N*-disuccinylimidyl carbonate was added. After stirring, filtering and washing, this activated silica was added to the protein previously suspended in buffer. The obtained stationary phase was gently mixed using the rotary evaporator and then packed in a stainless steel column (100×4.6 mm I.D.) by Hypersil.

2.4. Liquid chromatography conditions

All of 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 the chiral drug in *n*-propanol and each solution was diluted with buffer to a con-

centration of 0.1 m*M*; 20 μl were injected into the HPLC column. Normally 2–4 nmol of analyte are recommended.

2.5. Capillary electrophoresis conditions

After installation, the capillary was rinsed for about 30 min with 1 *M* NaOH, then with water for 15 min, and background electrolyte (BGE) for 15 min. Between runs, the capillary was rinsed with 50 m*M* sodium dodecyl sulphate (SDS), water and run buffer for 2, 2 and 3 min, respectively. Rinsing with SDS helps to remove adsorbed protein and to prevent capillary blockages [26]. All washes and rinses were performed using a pressure of 2000 mbar applied to the capillary inlet (dynamic compression injection). Analyses were performed at 20 kV, which resulted in a running current of 30–60 μA, depending on the pH conditions. The oven temperature was thermostated at 8°C and the tray temperature at 10°C. Samples were introduced into the capillary by dynamic compression injection for 0.25 min, with a pressure of 25 mbar.

All buffer solutions were prepared fresh daily using bidistilled water. Prior to use, all solutions were filtered through a 0.45-μm membrane filter and degassed by sonication. Phosphate buffers were prepared by mixing 50 m*M* solutions of analytical grade dibasic sodium hydrogen phosphate and sodium dihydrogen phosphate to give the desired pH. An appropriate amount of protein was dissolved in the 50 m*M* sodium phosphate buffer to obtain a 30 μ*M* solution of qRfBP.

3. Results and discussion

3.1. Liquid chromatography

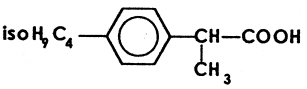
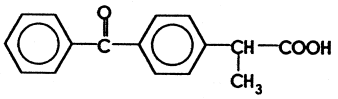
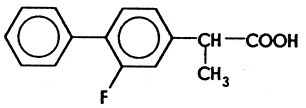
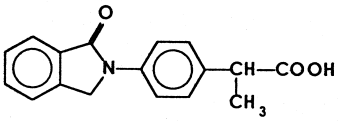
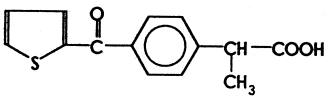
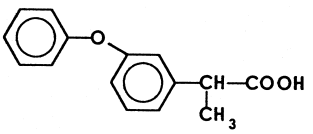
The molecular structures and the pK_a values of the compounds tested are depicted in Table 2. The retention (k') and the enantioselectivity (expressed by α and R_s) of the tested compounds were studied by changing the mobile phase conditions, such as buffer pH, kind and amount of organic modifier. Fig. 1 shows the best resolutions obtained for some of the chiral compounds analysed.

3.1.1. Influence of pH on retention

The effect of the pH on retention and enantioselectivity was investigated using acidic, basic and neutral drugs in the pH range between 3.5 and 6.5 and the results are presented in Table 3. The pH value is a very important factor since it affects both the degree of charge of the solute and the chiral bonding properties of the protein. It is known that pH is a crucial factor that affects the affinity of the protein for its natural ligand, riboflavin, indeed, the standard method used to remove the vitamin from the holoprotein dialyses the macromolecule extensively against a solution buffered at pH 3.0, which disrupts the binding [27]. In general, the affinity for the stationary phase increased as the compounds became less ionized. All of the arylpropionic antiinflammatory drugs and warfarin showed a maximum k' value at pH 4.5, where both protein and analytes are uncharged, and a reduction in the capacity factors was observed on increasing the pH from 4.5 to 6.5. The k' values of the dihydropyridines, LE, MA, NC and AM, decreased on decreasing the pH, while those of IS and NM were almost constant. This behaviour can be explained easily by observing the structures of the compounds; the first compounds are basic (pK_a~9) while NC and IS are neutral. The other calcium channel antagonists (VE, GA and BE), the β-blockers (PP and PC) and the other two basic analytes (BU and FN) follow a trend similar to that of the basic dihydropyridines, although they present much shorter retention times.

These results could be expected considering that the isoelectric point of qRfBP is about four. Therefore, as the pH is increased, there was a corresponding increase in the net negative charge of the protein and a corresponding change in the Coulombic interactions between the qRfBP and the charged solutes. At high pH values, the capacity factors of anionic solutes are lower than their values at pH 3.5, due to increased anion–anion repulsion. The opposite was observed for cationic solutes, which had lower k' values at low pH values, due to cation–cation repulsion. The fact that the retention of uncharged solutes, such as IS, NM and benzodiazepines, was almost independent on the pH of the mobile phase confirms the assumption that Coulombic interactions occur between the charged compounds and the charged protein.

Table 2
Molecular structures and pK_a values of the compounds tested

Compound	Formula	pK_a
Ibuprofen		4.4-5.2
Ketoprofen		4.5-5
Flurbiprofen		4-5
Indoprofen		4-5
Suprofen		4-5
Fenoprofen		4.5

(*continued on page 52*)

Table 2. Continued

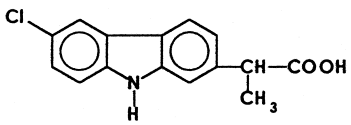
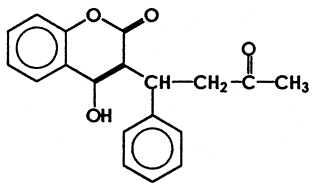
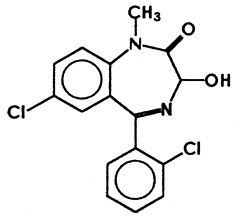
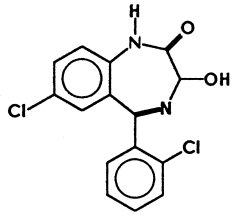
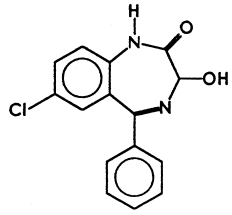
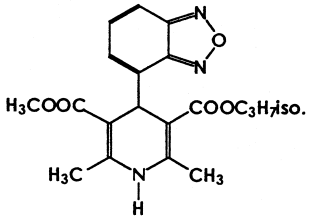
Compound	Formula	pK_a
Carprofen		4-5
Warfarin		5
Lormetazepam		
Lorazepam		
Oxazepam		
Isradipine		

Table 2. Continued

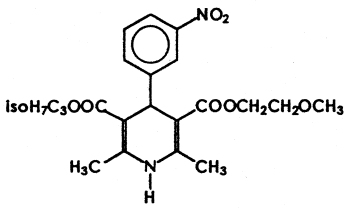
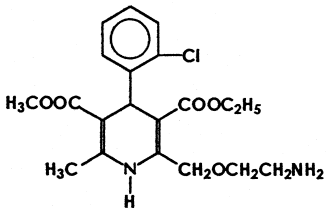
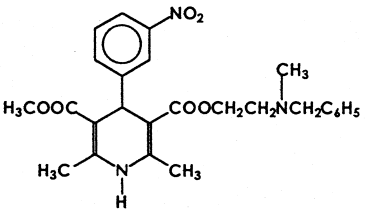
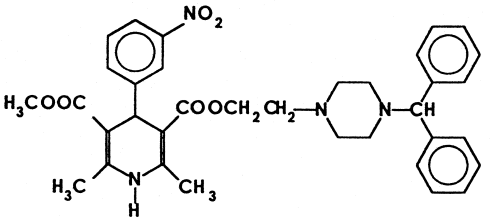
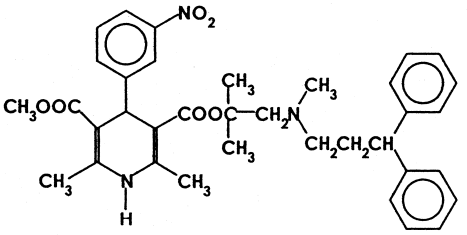
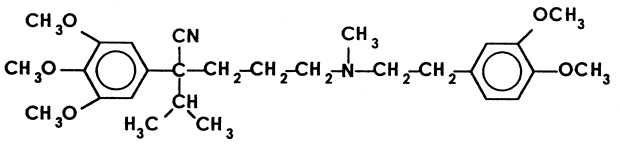
Compound	Formula	pK_a
Nimodipine		7.2
Amlodipine		9
Nicardipine		
Manidipine		
ercanidipine		
Gallopamil		

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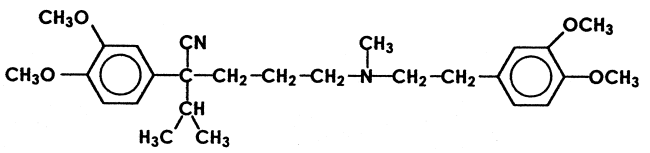
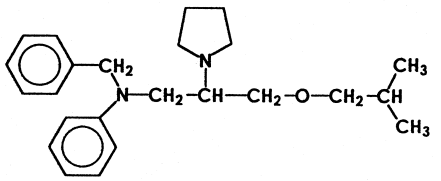
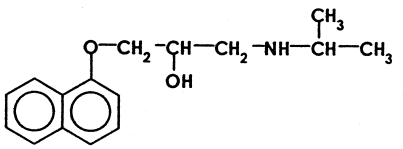
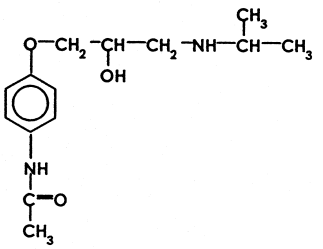
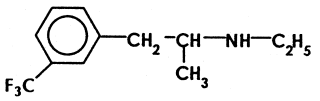
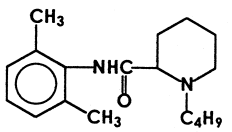
Table 2. Continued

Compound	Formula	pK_a
Verapamil		
Bepridil		
Propranolol		9.4
Practolol		9.5
Fenfluoramine		10
Bupivacaine		8.1

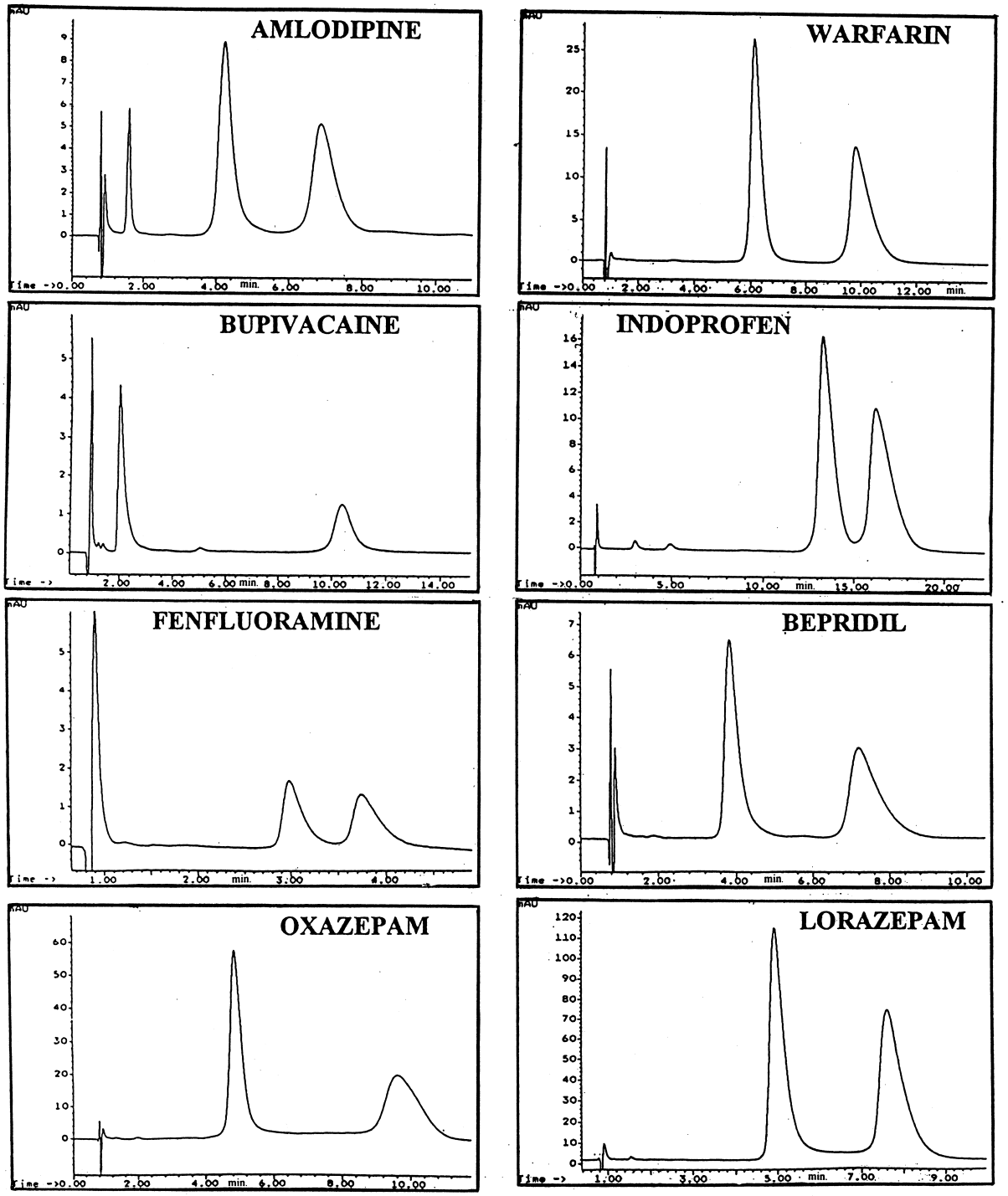


Fig. 1. Chromatograms of some of the compounds tested. Chromatographic conditions: Amlodipine, bepridil and oxazepam, mobile phase, 50 m*M* sodium phosphate buffer (pH 4.5)–methanol (90:10, v/v); lorazepam, mobile phase, 50 m*M* sodium phosphate buffer (pH 4.5)–methanol (95:5, v/v); indoprofen and warfarin, mobile phase, 50 m*M* sodium phosphate buffer (pH 4.5)–acetonitrile (95:5, v/v); bupivacaine and fenfluoramine, mobile phase, 50 m*M* sodium phosphate buffer (pH 5.5)–methanol (95:5, v/v); flow-rate 0.8 ml/min.

Table 3
Effect of the pH on the retention (k'_1) and enantioselectivity (α and R_s)

Compounds	pH 3.5			pH 4.5			pH 5.5			pH 6.5		
	k'_1	α	R_s	k'_1	α	R_s	k'_1	α	R_s	k'_1	α	R_s
Ibuprofen	3.92	1.69	1.58	3.99	1	–	3.88	1.82	–	1.03	1	–
Ketoprofen	5.95	1	–	9.71	1.07	0.41	6.09	1.08	0.39	1.47	1	–
Flurbiprofen	10.29	1	–	11.95	1	–	6.68	1	–	1.43	1	–
Indoprofen	16.51	1	–	53.77	1.31	2.00	40.9	1.42	2.26	17.52	1.31	1.86
Suprofen	5.92	1	–	6.40	1	–	4.37	1	–	1.69	1	–
Fenoprofen	5.41	1	–	7.27	1	–	4.09	1	–	1.07	1	–
Carprofen	33.08	1	–	48.97	1	–	17.70	1	–	8.49	1	–
Warfarin	7.16	1.28	1.60	11.60	2.59	5.48	9.27	3.23	3.57	2.53	2.79	3.27
Lormetazepam	4.01	1.28	1.36	5.37	–	–	5.41	–	–	5.50	–	–
Lorazepam	3.06	1.51	2.30	4.45	1.66	3.04	5.10	2.38	2.32	5.78	2.91	6.55
Oxazepam	3.22	–	–	6.84	2.15	2.42	9.62	5.79	7.3	11.34	8.42	9.22
Isradipine	8.45	1.06	–	14.00	1.15	0.82	14.8	1.07	–	11.42	1.12	0.42
Nimodipine	13.63	1	–	23.62	1.11	0.28	24.45	1.19	0.53	19.53	1.25	0.43
Amlodipine	0.87	2.29	1.37	7.55	1.87	2.61	11.30	1.71	2.5	31.18	1.39	1.72
Nicardipine	0.59	1.46	0.79	8.79	1.18	0.73	20.08	1.53	1.9	67.97	1.72	1.67
Manidipine	9.44	1.25	0.88	53.27	1.94	2.76	[b]	–	–	[b]	–	–
Lercanidipine	2.26	1	–	6.94	1.07	–	31.32	1.25	0.57	101.95	1.2	0.56
Gallopamil	[a]	–	–	0.63	1	–	2.85	1	–	7.08	1.07	–
Verapamil	[a]	–	–	1.08	1.16	0.26	4.42	1.11	0.8	11.64	1.16	0.75
Bepridil	0.78	1.78	1.70	5.49	1.85	3.53	20.20	2.13	2.78	45.45	2.35	2.70
Propranolol	[a]	–	–	2.01	1	–	10.94	1	–	21.67	1	–
Practolol	[a]	–	–	[a]	–	–	0.30	1	–	1.06	1	–
Fenfluramine	[a]	–	–	0.43	1	–	2.31	1.37	1.5	5.93	1.37	1.71
Bupivacaine	[a]	–	–	0.38	1	–	1.21	8.77	9.88	3.72	1	–

Chromatographic conditions: 50 m*M* phosphate buffer–methanol (95:5, v/v); flow-rate, 0.8 ml/min.
[a] Eluted with the solvent front.
[b] Not eluted within 3 h.

Hydrophobic interactions also contribute to the net retention time. It has been shown that riboflavin interacts with chicken egg white RfBP mainly through hydrophobic contacts [9]. It is therefore not surprising that the extent of retention is directly dependent on the hydrophobicity of the substituents for all of the compounds tested, for example, k'CA> k'KE and k'PP>k'PC.

3.1.2. Influence of pH on enantioselectivity

The enantioselectivity of the qRfBP column for arylpropionic anti-inflammatory drugs was poor. In particular, IN was the only compound that was baseline separated, IB enantiomers were resolved only at pH values of 3.5 and 5.5 and KE enantiomers presented a hint of separation at pH values of 4.5 and 5.5. FL, SU, FE and CA could not be separated. The enantiomers of the weak acid, WA, were always well resolved within the pH range considered. The qRfBP column seems to be more suitable for the separation of basic compounds, as only the amino alcohols (PP and PC) were not resolved at all. Despite the short retention time, a very good separation was achieved for BU at pH 5.5 and for FN at pH>4.5, while GA and VE enantiomers gave a hint of separation at pH 6.5 and pH>4.5, respectively. BE could be easily separated within the pH range considered and the best resolution was obtained at pH 4.5 with an R_s value of 3.53. Generally, dihydropyridines and benzodiazepines were the best resolved compounds. The best selectivity factors for benzodiazepines were obtained at pH 6.5, except for LM, which was separated only at pH 3.5. The enantioselectivity of dihydropyridines on a qRfBP column was improved by increasing the pH value from 3.5 to 5.5. The poor resolution obtained for NM at pH 6.5 and for LE at pH 5.5, in spite of the high α values, could be due to the low efficiency of the system (N is about 130 for

both compounds). These results appear to be caused by a pH-dependent change in the nature of the immobilised protein, even though it is still not clear if these conformational changes can occur when the protein is immobilised on a silica support [28].

3.1.3. Influence of the type of organic modifier

The effects of organic modifiers on retention and enantioselectivity are presented in Table 4. In the case of straight-chain alcohols as organic modifiers (methanol, ethanol and *n*-propanol), all of the analytes investigated showed decreased k' and R_s values when the alkyl carbon number of the primary alcohol was increased. On the other hand, using 2-propanol resulted in higher k' values than those obtained with ethanol and *n*-propanol; this can be explained by the order of the hydrophobic constants of the solvents (−0.31 for ethanol, 0.05 for 2-propanol and 0.25 for *n*-propanol), as previously described by Iredale et al. [29]. Generally, the addition of methanol to the mobile phase resulted in better resolutions than those obtained with other alcohols, and acetonitrile was the best organic modifier for IN and MA, giving the highest number of theoretical plates.

3.1.4. Influence of the percentage of organic modifier

Variations in the capacity factors and resolution of some representative analytes, by alteration of the methanol content, are shown in Table 5. It can be seen that an increase in the amount of organic modifier causes a decrease in k' values; for two of the compounds tested, OX and AM, the capacity factors remained almost constant on increasing the methanol concentration up to 5%. By contrast, the resolution (R_s) increases on increasing the methanol concentration from 2 to 5% for KE, WA, BE and MA, while the retention is reduced. These results indicate that overall, retention on the column is in the reversed-phase mode.

Table 4
Effect of the type of organic modifier on retention (k'_1) and enantioselectivity (α and R_s)

Compounds	Methanol			Ethanol			*n*-Propanol			2-Propanol			Acetonitrile		
	k'_1	α	R_s	k'_1	α	R_s	k'_1	α	R_s	k'_1	α	R_s	k'_1	α	R_s
Ibuprofen	3.99	1	–	4.14	1	–	3.34	1	–	3.76	1.26	1.52	3.80	1	–
Ketoprofen	9.71	1.07	0.41	6.1	1	–	4.26	1	–	5.49	1	–	4.99	1	–
Flurbiprofen	11.95	1	–	9.13	1	–	7.17	1	–	8.10	1	–	6.94	1	–
Indoprofen	53.77	1.31	2.00	20.00	1.16	0.94	12.62	1.15	1.02	17.52	1.18	1.14	13.83	1.24	2.36
Suprofen	6.40	1	–	6.19	1	–	4.64	1	–	5.53	1	–	5.09	1	–
Fenoprofen	7.27	1	–	5.18	1	–	4.36	1	–	4.56	1	–	4.53	1	–
Carprofen	48.93	1	–	33.7	1	–	26.46	1	–	29.62	1	–	22.43	1	–
Warfarin	11.60	2.59	5.48	8.72	1.81	3.40	5.49	1.59	3.23	8.57	1.86	4.50	6.51	1.70	3.21
Lormetazepam	5.37	1	–	3.67	1.12	0.30	2.94	1.15	0.58	3.94	1.16	0.57	3.09	1.14	0.32
Lorazepam	4.45	1.66	3.04	3.63	1.47	1.80	2.65	1.38	1.92	3.30	1.48	2.26	2.64	1.38	0.81
Oxazepam	6.84	2.15	2.42	4.56	1.32	1.65	2.74	1.15	0.8	4.01	1.24	1.40	3.12	1.26	0.55
Isradipine	13.98	1.15	0.82	7.01	1.15	0.52	4.77	1.11	0.51	6.22	1.16	0.79	4.85	1.09	0.36
Nimodipine	23.62	1.11	0.28	12.1	1.10	0.28	7.48	1.05	–	10.44	1.1	0.38	7.98	1.04	0.37
Amlodipine	7.55	1.87	2.61	3.39	2.02	3.12	2.04	1.73	2.74	3.57	1.95	4.08	2.33	1.82	1.54
Nicardipine	8.79	1.18	0.73	3.91	1.17	0.88	2.20	1.30	1.23	3.93	1.24	1.20	1.99	1.12	0.41
Manidipine	53.27	1.94	2.76	24.1	1.40	1.61	17.96	1.35	1.50	24.71	1.45	2.17	17.00	1.38	4.15
Lercanidipine	6.94	1.07	0.22	7.23	1	–	5.4	1	–	8.22	1	–	4.67	1	–
Gallopamil	0.63	1	–	0.5	1	–	0.36	1	–	0.59	1	–	0.41	1	–
Verapamil	1.08	1.16	0.26	0.78	1	–	0.60	1	–	0.74	1	–	0.63	1	–
Bepridil	5.49	1.85	3.53	3.43	2.14	2.73	2.32	2.09	3.53	3.09	2.19	3.63	2.88	1.91	1.18
Propranolol	2.01	1	–	0.66	1	–	0.63	1	–	1.09	1	–	0.62	1	–
Practolol	a	–	–	[a]	–	–	[a]	–	–	[a]	–	–	[a]	–	–
Fenfluramine	0.43	1	–	[a]	–	–	[a]	–	–	[a]	–	–	[a]	–	–
Bupivacaine	0.38	1	–	[a]	–	–	[a]	–	–	[a]	–	–	[a]	–	–

Chromatographic conditions: 50 m*M* phosphate buffer (pH 4.5)–organic modifier (95:5 v/v); flow-rate, 0.8 ml/min.
[a] = Eluted with the solvent front.

Table 5
Effect of methanol content on retention (k_1') and chiral separation (α and R_s)

Compound	2% Methanol			5% Methanol			10% Methanol		
	k_1'	α	R_s	k_1'	α	R_s	k_1'	α	R_s
Ketoprofen	11.80	1	–	9.71	1.1	0.41	5.78	1	–
Indoprofen	45.48	1.9	2.04	53.80	1.3	2.0	23.1	1.3	1.56
Warfarin	18.60	2.2	4.27	11.60	2.6	5.48	9.15	2.2	3.93
Bepridil	7.26	2.2	2.66	5.49	1.9	3.53	3.82	2.1	3.05
Oxazepam	7.47	1.8	2.77	6.84	2.2	2.42	5.08	2.2	3.61
Amlodipine	7.23	1.9	3.01	7.55	1.9	2.61	3.65	1.8	2.82
Manidipine	53.50	1.5	2.11	53.30	1.9	2.76	28.10	1.5	2.51

Chromatographic conditions: 50 m*M* phosphate buffer (pH 4.5)–methanol; flow-rate, 0.8 ml/min

3.1.5. Optimization

The chiral separation of IS enantiomers on the qRfBP column was optimized by the modified sequential simplex method [30]. It is necessary to define a chromatographic response function (CRF) in a manner suitable for the separation desired and an empirical CRF that had been proposed previously [31] was used: $CRF = P_i^5/(\log t_r)$, where P_i is the ratio between the average valley depth and the average peak height (Kaiser's peak separation function [32]) and t_r is the retention time of the second eluted enantiomer. This parameter was found to give excellent discrimination between good and bad responses and it is much more measurable than resolution, R_s. The simplex movements were stopped when the difference in the CRF between two successive points was found to be less than 2%. The two variables used to optimize the system were the pH of the mobile phase and the percentage of 2-propanol used for IS, with the buffer concentration kept constant at 50 m*M*. The optimum in the response surface (CRF=0.664) corresponded to an eluent composition of 50 m*M* phosphate buffer (pH 4.3)–2-propanol (94:6, v/v). Also, another point in the response surface gave a very similar P_i value, but its CRF was lower (0.594), as the retention time was slightly higher (9.07 min), but was still excellent. This observation indicates that the CRF might not have been the best that could have been chosen, as the retention times were always below 16 min for all of the experiments and, therefore, there was no particular need for an allowance for time to be made.

3.1.6. Displacement studies

In order to elucidate the mechanism of retention on the quail egg white RfBP–chiral stationary phase (CSP), displacement studies were carried out by adding riboflavin, the natural ligand of RfBP, to the mobile phase (2.5 mg/l in 50 m*M* NaH_2PO_4, pH 6.5). WA and IN were chromatographed under these conditions and the capacity factors were compared to those obtained under the same conditions in the absence of riboflavin in the mobile phase. As presented in Fig. 2a,b, showing the chromatograms of IN in the absence and presence of riboflavin in the mobile phase, respectively, the dramatic reduction of the capacity factor obtained in Fig. 2b indicates that the drug tested with riboflavin probably interacts at the same binding site. However, these results do not rule out the possibility that the reduction in retention may be due to an allosteric interaction.

3.2. Capillary electrophoresis

With the aim of exploring a unified approach to chiral separations in HPLC and in CE, we decided to single out one of the HPLC mobile phases that had already been tested and to use it as a BGE in free solution CE with qRfBP as an additive. The HPLC mobile phase chosen as a starting point in the comparison study was 50 m*M* phosphate buffer, pH 6.5, with methanol as the modifier.

In making this choice, we took into account the fact that pH values close to 4 are to be avoided because, at the p*I* value, protein precipitation is likely to occur. Furthermore, we wanted to use the same buffer as in the HPLC system, however, pH values of around 5 gave poor buffering capacity using phosphate. Finally, at pH values below 4, the

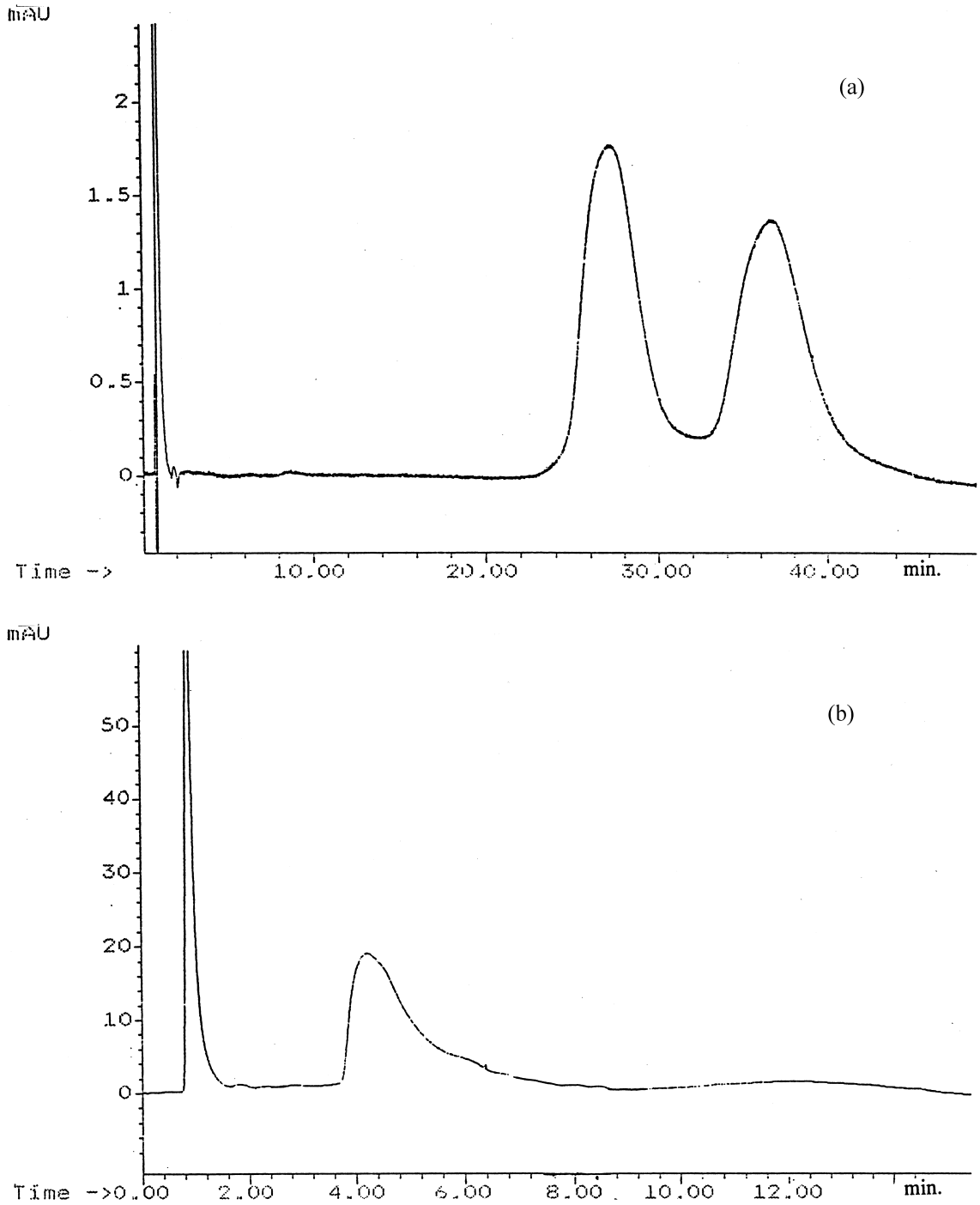


Fig. 2. Chromatograms of indoprofen without (a) and with (b) the addition of riboflavin to the mobile phase (2 mg/l riboflavin in 50 m*M* NaH_2PO_4, pH 6.5).

protein is positively charged and severe adsorption of the protein to the capillary wall can be encountered.

When comparing separations made in CE and LC, it is useful to know the concentration of selector used in each technique. It has been shown that retention in LC and CE is directly proportional to the concentration of selector used, and that quantitative or semi-quantitative predictions of retention from one technique to the other can be made if concentrations of the selector are known [22,23,33]. For the qRfBP column used in these studies, the amount of immobilized protein was determined by measuring spectrophotometrically the initial and final concentrations of protein in the immobilisation buffer when the stationary phase was being made. In this way, it was found that about 33 mg of qRfBP/g of silica were immobilized. Knowing the column's void volume (0.64 ml), the effective concentration of protein in the column was calculated to be approximately 4.9 m*M*, i.e. up to 150 times higher than the concentration used in the CE experiments (although, in practice, not all of the protein will be available, due to differences in its orientation on immobilization). Because of the relatively low concentration of protein used in the CE experiments (and the moderate degree of retention for most of the compounds in HPLC), the proportion of methanol used in the mobile phase in the CE experiments was limited to l% (v/v), rather than 5% (v/v) as used in HPLC.

The values of k' and α that were determined by HPLC and CE are presented in Table 6 for a selection of cationic (NC, BE, AM, VE), uncharged (NM, OX, LO) and anionic (IN, WA) analytes. In Fig. 3, an electropherogram illustrating the separation of BE enantiomers is shown. Similar mobile phases/BGEs were used, differing only in the proportion of methanol, as described above. The retention factor is calculated using the expression [34] $k'=(\mu-\mu_0)/(\mu_{comp}-\mu)$, where μ is the effective mobility of the analyte in the BGE with the protein additive, μ_0 is the effective mobility of the analyte in the BGE with no protein additive, and μ_{comp} is the effective mobility of the analyte–selector complex. In the case of uncharged analytes, it is reasonable to equate the mobility of the analyte–selector complex with that of the qRfBP. For charged analytes, however, making this assumption will only allow the calculation of a limiting value of k' (in this case, an underestimation for anions and an overestimation for cations). From Table 6, it can be seen that the ratio of k' LC/k' CE varies from 32 to 107, the average value being 73. As noted above, the protein concentration may be up to 150 times higher in the LC experiments. The possibility of a proportion of binding sites being unavailable, and the higher concentration of methanol used in the HPLC measurements would be explanations for the differences in k' values from the values predicted on the basis of differences in the concentration of qRfBP.

Table 6
Comparison of k' and α values obtained in HPLC and CE for the same compounds

HPLC[a]		pH 6.5	CE[b]	
k'_1	α	Compounds	k'_1	α
67.97	1.71	Nicardipine	2.10	1.22
45.45	2.35	Bepridil	0.44	2.05
31.18	1.38	Amlodipine	0.29	
11.64	1.16	Verapamil	0.17	
19.53	1.25	Nimodipine	0.19	
11.34	8.41	Oxazepam	0.20	1.15
5.78	2.91	Lorazepam	0.14	2.61
17.52	1.31	Indoprofen		
2.53	2.78	Warfarin		

[a]Mobile phase: 50 m*M* sodium phosphate buffer (pH 6.5)–methanol (95:5, v/v).
[b]BGE: 50 m*M* sodium phosphate buffer (pH 6.5)–methanol (99:1, v/v).

The acidic compounds IN and WA were not separated and this result can be interpreted based on

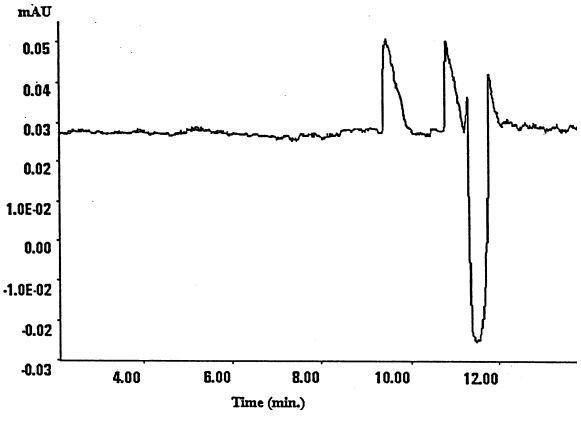


Fig. 3. Electropherogram of 200 μ*M* bepridil (BGE: 50 m*M* sodium phosphate buffer (pH 6.5)–methanol (99:1, v/v).

their mobilities being too similar to that of the acidic protein under these conditions. A drawback of the CE method occurs when the net mobility of the sample is similar to that of the run buffer additive, since, in order to achieve chiral separations, the mobilities of the free and protein-bound analyte must be significantly different.

The cations NC and BE and the neutral compounds OX and LO were separated in CE and showed the highest α values in the HPLC system. Furthermore, the cations NC and BE are also the pair with the highest retention values, whereas, among the neutral compounds, it is more difficult to explain the lack of separation in CE for NM, which is the most retained compound of its class.

Perhaps the explanation is based on a mixture of retention, selectivity and efficiency. With 100-fold less retention, much more efficiency is needed to achieve resolution. In CE with protein additives, the number of theoretical plates (N) is reasonably high, but not high enough to overcome the low k' values. OX and LO can also be separated in CE because of good α values; NM has reasonable retention (also in CE) but it has a poor α value compared to those of the other analytes and, therefore, a very high N is needed to achieve separation.

Considering the expression for the required number of theoretical plates to achieve baseline resolution [35] (i.e. $R_s=1.5$): $N_{req}=[6(\alpha/\alpha-1)(k'+1/k')]^2$ and taking the k' value of NM from the HPLC results, it transpires that the LC separation needs about 1000 plates for baseline resolution, whereas for CE ($k'=0.19$) at least 35 000 plates are required. With other selectors in CE, relatively high concentrations can be used without detection problems, so that k' values can reach the more favoured regime.

On the basis of the results obtained with this BGE and taking into account that the addition of an organic modifier generally reduces the interaction between the protein and the analyte, all compounds were tested in plain buffer and at pH 7.0, where the cations are less ionised. As shown in Table 7, with this BGE, AM, VE and PC enantiomers could also be separated. The selector concentration was kept at 30 μM, whereas the analyte concentration was varied in the range from 400 to 50 μM. By decreasing the analyte concentration and thus approaching a 1:1

Table 7
Capacity factors and enantioselectivities obtained for the compounds separated in CE

Sample name	Concentration of sample (μM)	pH 6.5[a]		pH 7.0[b]	
		k'_1	α	k'_1	α
Nicardipine	200	2.10	1.22	0.40	1.23
	100			0.48	1.25
Bepridil	400	0.44	2.05	0.85	1.63
	200			0.92	1.69
Amlodipine	200	0.29		0.11	1.20
	100			0.14	1.45
	50			0.15	1.52
Verapamil	200	0.17		0.38	1.12
	100			0.20	1.15
	50			0.18	1.20
Oxazepam	200	0.20	1.15	0.54	1.15
	100			0.63	1.17
	50			0.76	1.17
Lorazepam	200	0.14	2.61	0.10	2.17
	100			0.09	2.48
Practolol	100			0.15	1.23
	50			0.20	1.24

The analyte concentration was varied in the range 400 to 50 μM.
[a]BGE: 50 mM sodium phosphate buffer–methanol (99:1 v/v).
[b]BGE: 50 mM sodium phosphate buffer.

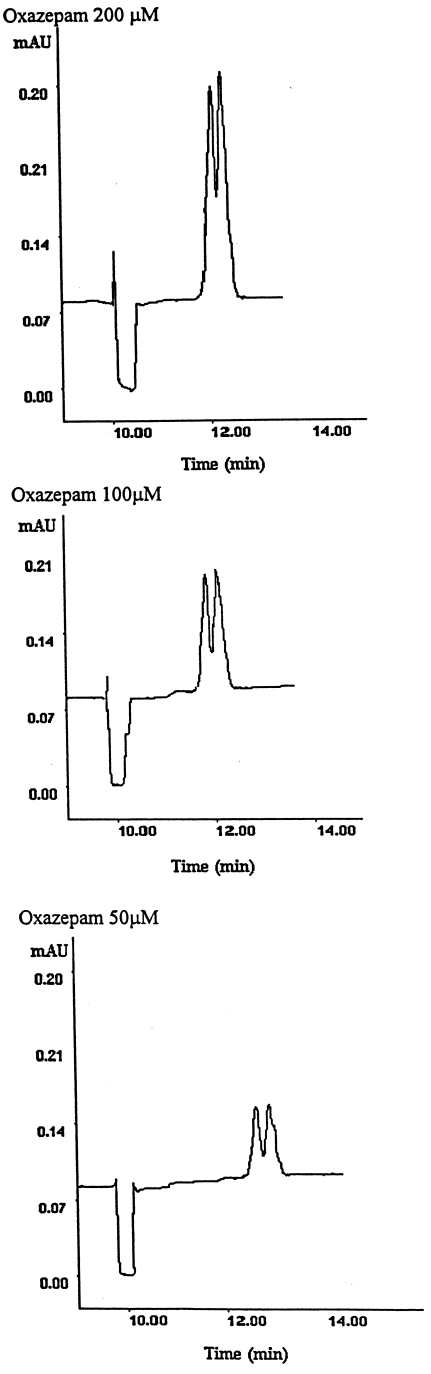


Fig. 4. Electropherograms of oxazepam at three different concentrations (200, 100 and 50 μ*M*, respectively). BGE: 50 m*M* sodium phosphate buffer (pH 7.0).

ratio between the individual enantiomer and the selector concentrations, the α values increased. In Fig. 4, electropherograms showing the separation of OX analysed at different concentrations are presented. Clearly, at higher concentrations of sample, overloading limits the resolution that can be obtained. By comparing the retention behaviour of the analytes in the two BGEs, it is difficult to explain why, for NC, AM and LO, the k' values in plain buffer are lower than in the presence of organic modifier. It seems that the enantiomeric separation of these compounds in plain buffer does not depend on retention but rather on conformational changes induced in the protein by the absence of methanol.

3.2.1. Stability of proteinaceous BGE in free solution

Since the aim of this part of the work was to use CE as a scouting technique to evaluate the potential of qRfBP as a chiral selector by using minimal quantities of protein, a study was carried out to establish how often it was necessary to replenish the protein solution vial (4 ml) to obtain reproducible electropherograms.

In Fig. 5, three electropherograms of OX analysed with fresh BGE, and BGE after 15 and 25 analyses are depicted. The variation in the retention time and resolution can be ascribed to electrolysis phenomena, which cause pH changes in the two electrode reservoirs, with a consequent change in protein conformation. Additionally, the carousel temperature (8°C) might not be optimum for proper storage of the protein for the time necessary to perform 25 analyses. For these reasons, it was necessary to change the solutions in the inlet and in the outlet vial every ten analyses in order to obtain reproducible results.

4. Conclusions

The quail egg white RfBP HPLC chiral stationary phase gave an excellent performance and reproducible chromatograms for about 500 analyses without the use of a pre-column.

In spite of the described inherent limitations of the CE system when trying to transfer separation conditions from HPLC, namely protein concentration and choice of pH values, it seems that CE is a fast

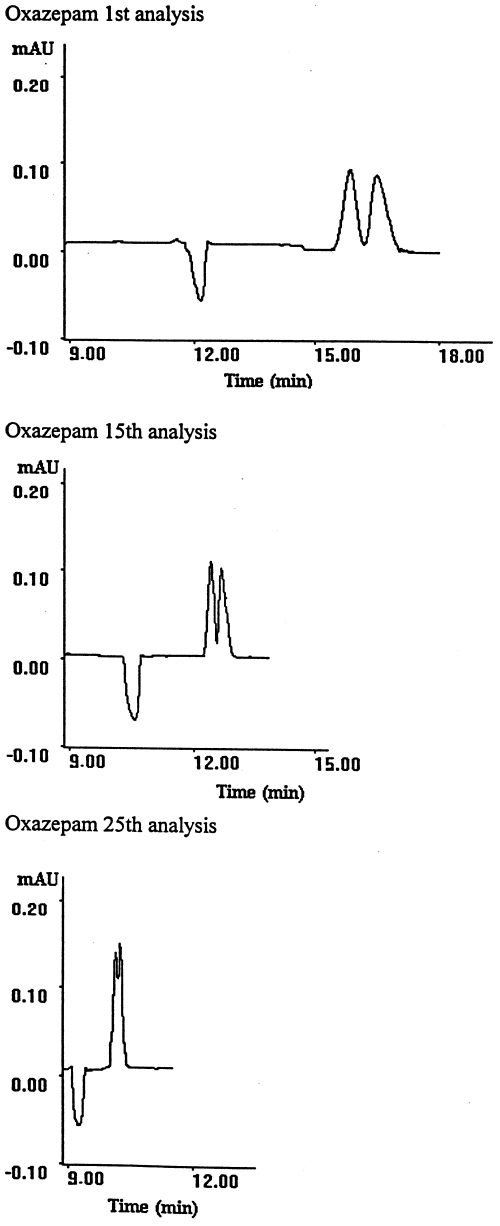


Fig. 5. Electropherograms of oxazepam using a fresh protein batch as the BGE, after 15 analyses and after 25 analyses. BGE: 50 m*M* sodium phosphate buffer (pH 7.0).

pilot tool for scanning enantioselectivity for the planning of further experiments in HPLC with protein selectors. The data obtained with the two techniques are comparable and, in particular, the correspondence of the retention (k') and enantioselectivity (α) parameters make it possible to predict the HPLC results starting from the explorative experiments carried out in CE. It was confirmed that only milligram quantities of protein are needed to obtain enough CE data to get a preliminary evaluation of the enantioselective properties of a new protein as a chiral selector.

In order to make a better comparison between HPLC and CE, the use of the partial filling technique [18,20] could be envisaged, as it might enable the protein concentration in the CE system to be increased without leading to detection problems and it might lead to better separations by overcoming the low k' values.

Acknowledgements

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