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Evaluation of β-lactoglobulin as a stationary phase in high-performance liquid chromatography and as a buffer additive in capillary electrophoresis: observation of a surprising lack of stereoselectivity

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Abstract

Previous studies have reported that α_1-acid glycoprotein is quite similar in amino acid sequence and disulfide bond arrangements to members of a group of proteins which include β-lactoglobulin (BLG). Since generally homologous proteins retain some similarity in function at the molecular level, we decided to evaluate the enantioselective properties of BLG as an high-performance liquid chromatographic chiral stationary phase (HPLC-CSP), and as an additive in capillary electrophoresis (CE). Two columns with differences in internal diameter and method of immobilisation on epoxide silica were prepared. Chiral acidic, basic and uncharged drugs were chromatographed and mobile phase parameters, namely pH and type of organic modifier, were varied in order to test the column performance. The CE approach has some advantages in that there is no need for immobilisation and only a small amount of protein is required. BLG was therefore tested as a CE buffer additive, using the same analytes as in the HPLC study. Although one would expect that a protein would display some enantioselectivity, BLG did not show any enantioselectivity whatsoever in either system; the protein has fairly weak interaction with the majority of the test solutes, as indicated by both techniques. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

High-performance liquid chromatography (HPLC) is an important tool for the separation of drug enantiomers, and many chiral stationary phases (CSPs) have been developed in the last decade. Protein-based columns have become popular because of their broad applicability in the separation of enantiomers, and several proteins have been investigated as HPLC-CSPs, namely α_1-acid glycoprotein (AGP), ovomucoid, cellobiohydrolase (CBH), bovine and human serum albumins, avidin, conalbumin and riboflavin binding protein [1–3]. These

proteins have also been successfully employed as chiral selectors in solution by adding them to the background electrolyte in capillary electrophoresis (CE), a topic which has recently been reviewed [4].

Both the well studied chiral selector human AGP and a protein known as bovine β-lactoglobulin (BLG) have been reported to belong to an extensive group of proteins called lipocalins who share sequence homologies and structural similarities together with the common ability to bind lipophilic drugs [5]. Comparisons of the sequence of AGP with BLG show positive correlations, giving 18.7% of identical residues and a significant correlation score [5]; the secondary structure profile of AGP, predicted from its amino acid sequence, and the similar constraints imposed by the known disulfide bond arrangements might support the view that its three dimensional structure is closely related to the published structure of BLG [6]. Nevertheless some authors [7], in attempting to predict unknown lipocalin structures by comparison with known lipocalin crystal structures, came to the conclusion that AGP does not fit any of the lipocalin profiles whereas BLG showed marked complementarity with at least two well-resolved lipocalins. Straight model-building studies to fit the AGP sequence into a structure based on that of BLG have not yet been conducted and thus the degree of similarity between AGP and BLG remains controversial. Nevertheless, since AGP does display enantioselectivity in vivo [8,9], and has proved to be a very successful chiral selector both in HPLC and CE [10–12] we decided to evaluate the enantioselective properties of BLG as an HPLC-CSP and as a buffer additive in CE.

Although BLG is very abundant in the whey fraction of milk, its function is still not clear. The identification of a binding site for retinol has suggested a role for BLG in the transport of this vitamin [6,13,14]. Its isoelectric point is 5.2. Above pH 2.8 bovine BLG exists as a dimer [15] and the molecular mass of the subunit is about 18 000, corresponding to a chain of 162 amino acids. It contains two disulphide bridges and is remarkably acid-stable, resisting denaturation at pH 2.0. Two genetic variants commonly called BLG A and BLG B have been described and they differ at positions 64 and 48 where an Asp and a Val in variant A are substituted by Gly and Ala in variant B [16]. The abundance of bovine BLG, its potential similarity with AGP and the existence of a binding site for hydrophobic molecules [17,18] were important factors in its choice as a protein for this study.

In this paper, the results of experiments carried out in order to investigate the potential chiral recognition properties of BLG are reported. BLG was immobilised on silica with two different HPLC columns being prepared by different immobilization methods, both without crosslinking (although most of the HPLC literature refers to enantioseparations on a crosslinked AGP phase, noncrosslinked AGP has also been used successfully as a chiral selector [19,20]). Chiral acidic, basic and uncharged analytes were chromatographed, and the influence of mobile phase pH and percentage and different types of organic modifier were studied. BLG was also tested as a CE buffer additive, using the same analytes as in the HPLC study. CE has some advantages in that there is no need for immobilisation and only a small amount of protein is required. The CE approach was therefore investigated in order to establish whether this technique could represent a useful tool for preliminary evaluation of the enantioselective properties of a protein.

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 were used. Two stainless-steel columns (100 mm×3 mm I.D. and 100 mm×4.6 mm I.D.) were packed by Hypersil (Runcorn, Cheshire, UK) with BLG conjugated to silica gel.

2.1.2. Capillary electrophoresis

A Unicam (Cambridge, UK) model Crystal 310 capillary electrophoretic system was used, with a Spectra 100 (Thermo Separation Products, San Jose, CA, USA) variable wavelength UV detector at 247 nm. Data were analysed using a Unicam 4880 chromatography data handling system.

Electrophoresis was performed in 72 cm (length to the detector window 55 cm) ×50 μm I.D., 360 μm O.D. fused-silica capillaries (MicroQuartz, Munich, Germany).

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), nicardipine (NC), practolol (PA), disopyramide (DS), promethazine (PM), thioridazine (TH), propranolol (PR) and retinol acetate (all *trans*) 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). Bovine BLG (A and B) was purchased from Sigma (cat. No. L 0130). 3-glycidoxypropyltrimethoxysilane (GOPS) was obtained from Aldrich (Milwaukee, WI, USA). LiChrospher Si 300 (10 μm 300 Å), NaH_2PO_4, Na_2HPO_4 and ethanol (HPLC grade) were purchased from Merck (Darmstadt, Germany). Sodium dodecyl sulphate (SDS) was from Carlo Erba (Milan, Italy). Unless otherwise stated, all chemicals used were of analytical grade.

2.3. Preparation of BLG columns

The immobilisation was carried out in batch by Hypersil. An alternative in situ immobilisation method was also considered, and a second column with a larger I.D. (4.6 mm) was prepared. As both columns showed almost identical chromatographic behaviour, only results obtained with the column prepared using the first immobilisation are reported.

2.3.1. Immobilisation method
LiChrospher Si 300 (5 g) was dried for 16 h at 130°C under vacuum. The dry silica was suspended in 25 ml of dry toluene and 2.5 ml of GOPS were added; this slurry was refluxed for 4.5 h. After cooling and filtering the reaction mixture, the deriva-

tised silica was washed with 250 ml of the following solvents, in order, toluene, hexane, acetone–water (50:50, v/v) and acetone. The epoxide-activated silica was dried overnight at 60°C.

The epoxide-silica (1 g) was suspended in 50 ml of 0.1 *M* borate buffer in a rotary evaporating flask, the BLG solution (100 mg of protein in 5 ml of 0.1 *M* sodium borate buffer at pH 8.5) was added, and the mixture was gently stirred for 48 h. The BLG-silica was packed into a 100 mm×3 mm I.D. column.

2.4. Liquid 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 each chiral drug in *n*-propanol and each solution was diluted with buffer to a concentration of 0.1 m*M*. A 20-μl volume was injected into the HPLC column (normally 2–4 nmol of analyte is recommended).

2.5. Capillary electrophoretic conditions

After installation, the capillary was rinsed for about 30 min with 1 *M* NaOH, and then with water for 15 min, and background electrolyte for 15 min. Between runs the capillary was rinsed with 50 m*M* SDS, water and run buffer for 2, 2, and 3 min, respectively. As previously described [21], rinsing with SDS helps to remove adsorbed protein and to prevent capillary blockages. All washes and rinses were performed using a pressure of 2000 mbar applied to the capillary inlet. 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 18°C and the tray temperature at 10°C. Samples were introduced onto the capillary by dynamic compression injection for a time of 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 hydrogenphosphate and sodium dihydrogenphosphate to give the desired pH. Phosphate buffer at pH 3.8 was prepared starting from monobasic sodium dihydrogenphosphate adjusted with 85% phosphoric acid. An appropriate amount of BLG was dissolved in the 50 mM sodium phosphate buffer to obtain a 30 μM solution of BLG.

3. Results and discussion

3.1. Liquid chromatography

Acidic, basic and neutral drugs, namely seven arylpropionic anti-inflammatory drugs and warfarin, eight calcium channel antagonists with different structures, and three benzodiazepines, were chromatographed on the BLG column. In Table 1 the molecular structures and the pK_a values of the compounds tested are shown.

Mobile phase parameters which are known to influence retention, enantioselectivity and column performance were studied. Type and percentage of organic solvent and pH values were varied, in order to induce a different interaction between the chiral solute and the protein by changing the protein conformation and hydrophobic and electrostatic interactions.

Despite the variety of the chromatographic conditions investigated for 19 different racemic drugs, no enantioselective interaction with BLG was observed.

3.1.1. Influence of pH

The influence of mobile phase pH on the retention of charged and neutral analytes on the BLG column was systematically investigated and the results are listed in Table 2.

In the case of arylpropionic anti-inflammatory drugs, the capacity factors decreased on increasing the pH from 4.6 to 6.5 and this is explained by the fact that above the pI value of the protein (5.2) and the pK_a of arylpropionic acids, which is around 4.5–5.0, both BLG and the analytes are negatively charged and electrostatic repulsion phenomena can occur. WA follows the same trend as a delocalised negative charge is located at the centre of its structure and its pK_a value is about 5.0.

The k' values of the neutral dihydropyridines IS and NM as well as of all benzodiazepines, are not greatly influenced by the pH. In contrast the retention time of the dihydropyridines bearing amine functions on the side chains, namely AM, NC and MN, is increased on increasing pH, that is when approaching the pK_b values of these analytes. It may be suggested that hydrophobic interactions also influence retention for these compounds as the increase in k' is much greater for NC and MN which carry one and two additional aromatic rings on the side chains, respectively.

Of the three phenylalkylamines, VE and GA show weak retention with only a slight increase with pH, while BE is more strongly retained and strongly affected by this parameter. Apart from some bulky analytes such as CA and MN, all solutes are weakly retained on the HPLC column as quite low k' values are generally measured.

pH is a very important factor since it affects both the charge of the solute and the binding properties of the protein. In general, the effect of pH on retention is larger for ionizable than for nonionizable solutes. Retention is increased when pH changes maximise the electrostatic attraction between the protein and the analyte. In this way, BLG behaves much as other acidic proteins such as AGP [12].
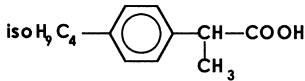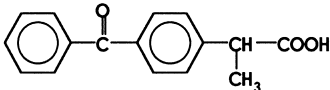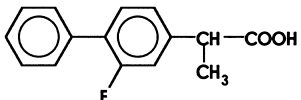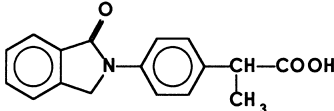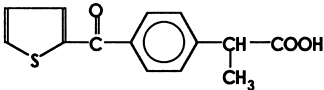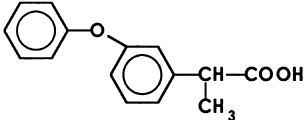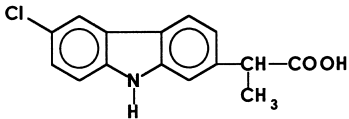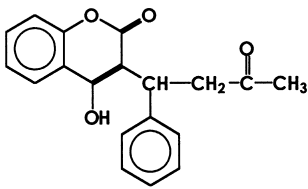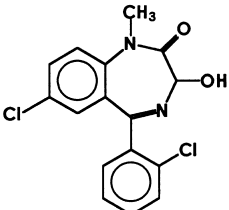
3.1.2. Influence of organic modifier

The organic modifiers methanol, ethanol and acetonitrile were tested to determine their effect on retention. All analytes were chromatographed keeping the concentration of organic modifier constant at 10% and the pH value at 5.5. The results are reported in Table 3.

The elution force of the tested solvents was in the following order: acetonitrile>ethanol>methanol, suggesting a particular role for the aprotic solvent in disrupting H-bonding between the analytes and one element of the binding site.

To determine the influence of the percentage of organic modifier, a study was carried out decreasing the concentration of acetonitrile from 10 to 1% and keeping the mobile phase pH constant at 5.5. CA and MA were not considered as the retention of these bulky structures was shown to be very high even with 10% of organic modifier (see Table 2). The next most retained compounds, IS and NM, showed a

Table 1
Molecular structures and pK_a values of the tested compounds

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
Carprofen		4–5
Warfarin		5
Lormetazepam		

(Cont.)

Table 1. Continued
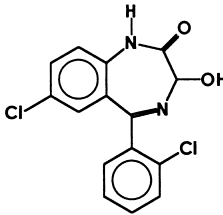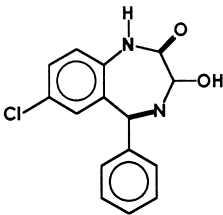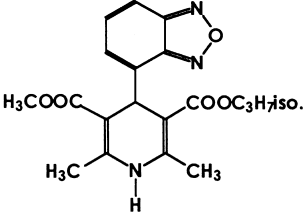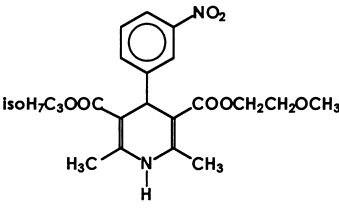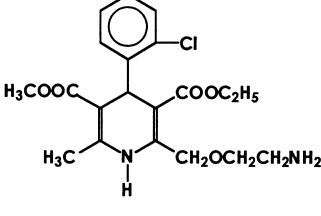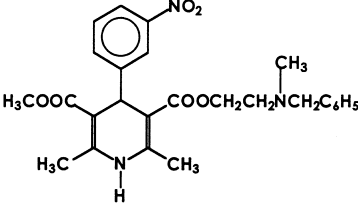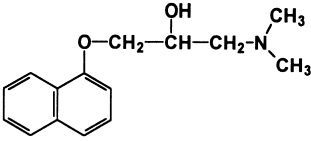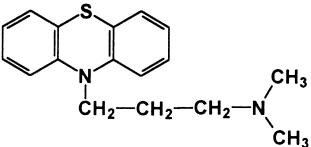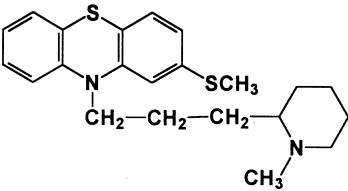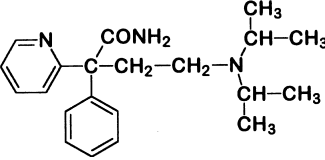
Compound	Formula	pK_a
Lorazepam		
Oxazepam		
Isradipine		
Nimodipine		7.2
Amlodipine		9
Nicardipine		

Table 1. Continued

Compound	Formula	pK_a
Manidipine		
Isradipine		
Gallopamil		
Verapamil		
Bepridil		
Practolol		9.5

(Cont.)

Table 1. Continued

Compound	Formula	pK_a
Propranolol		9.4
Promethazine		9.1
Thioridazine		9.5
Disopyramide		10.2

dramatic decrease in the k' values from 40.6 to 7.6 and from 35.3 to 8.7 at 1% and 10% of acetonitrile, respectively. In contrast, the capacity factors of GA, VE, LO, LM, OX and NC (Fig. 1a) were affected to a much lower extent compared to IS and NM, and this behaviour may indicate that hydrophobic interactions are not very important in the retention of these compounds.

For IB, KE, FL, SU, FE, WA, BE and AM a surprising increase of the capacity factors was observed on increasing the acetonitrile concentration from 1 to 5% (Fig. 1b). This behaviour may be due to a reversible change of the secondary structure of the protein, induced by a particular concentration of organic modifier, which leads to a change of the binding site characteristics. A similar behaviour was previously described on a CBH column [22] and on an AGP column [23].

3.1.3. Influence of phosphate buffer concentration

The effect of the ionic strength of the sodium phosphate buffer was also studied, at pH 5.5 with 1% acetonitrile and the results are shown in Table 4. On increasing the buffer concentration from 10 mM to 50 mM a modest decrease of k' was observed for acidic compounds such as arylpropionic anti-inflammatory drugs whereas moderate to large increases in retention were obtained with all but one of the basic or neutral compounds. The opposite trend was previously observed for arylpropionic acids binding to AGP [23] where it was suggested that retention was mediated via ion-pairing of the anionic analytes with sodium in the buffer which resulted in increased retention of the overall-neutral species. This is consistent with the model of the AGP binding site with retention driven predominantly by the hydrophobic effect but having a region of negative charge

Table 2
Influence of mobile phase pH on retention

Compound	k'		
	pH 4.6	pH 5.5	pH 6.5
Ibuprofen	4.55	1.91	0.64
Ketoprofen	4.20	1.75	0.87
Flurbiprofen	15.55	9.05	3.85
Indoprofen	2.73	1.38	0.74
Suprofen	3.75	1.69	0.92
Fenoprofen	9.56	4.72	2.25
Carprofen	110.41	52.17	21.56
Warfarin	8.06	5.52	2.39
Gallopamil	0.59	0.83	0.92
Verapamil	0.8	1.09	1.27
Bepridil	3.07	6.69	8.5
Lormetazepam	3.25	4.65	4.67
Lorazepam	3.86	5.31	5.43
Oxazepam	3.81	3.38	3.32
Isradipine	11.86	13.54	14.25
Nimodipine	13.99	17.02	15.79
Amlodipine	2.36	3.08	4.17
Nicardipine	1.98	4.85	12.4
Manidipine	37.83	134.32	220.82

Chromatographic conditions: 50 mM NaH$_2$PO$_4$–ethanol (90:10 v/v).

Table 3
Influence of type of uncharged modifier on retention

Compound	k'		
	Acetonitrile	Ethanol	Methanol
Ibuprofen	1.06	1.91	2.62
Ketoprofen	0.95	1.75	1.83
Flurbiprofen	3.3	9.05	13.23
Indoprofen	4.23	1.38	2.27
Suprofen	0.94	1.69	1.7
Fenoprofen	1.69	4.72	7.27
Carprofen	15.43	52.17	68.15
Warfarin	2.8	5.52	6.82
Lormetazepam	2.33	4.65	6.29
Lorazepam	2.98	5.31	6.78
Oxazepam	2.18	3.38	4.76
Gallopamil	0.5	0.83	0.7
Verapamil	0.65	1.09	1.15
Bepridil	4.61	6.69	7.5
Isradipine	7.6	13.54	24.83
Amlodipine	1.67	3.08	4.35
Nimodipine	8.7	17.02	26.97
Nicardipine	2.19	4.85	5.79
Manidipine	48.6	134.32	143.77

Chromatographic conditions: 50 mM NaH$_2$PO$_4$ (pH 5.5)–organic modifier (90:10, v/v).
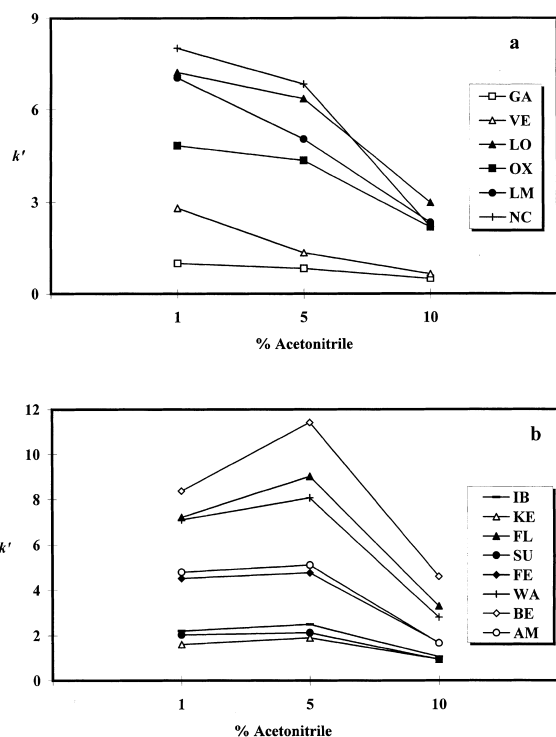

Fig. 1. Influence of the organic modifier concentration (acetonitrile) on retention of (a) GA, VE, LO, LM, OX and NC and (b) IB, KE, FL, SU, FE, WA, BE and AM. The mobile phase pH value was kept constant at 5.5.

Table 4
Influence of buffer concentration on retention

Compound	k'	
	10 mM	50 mM
Ibuprofen	3.73	2.21
Ketoprofen	2.96	1.61
Flurbiprofen	11.11	7.22
Indoprofen	2.79	1.96
Suprofen	3.03	2.03
Fenoprofen	5.89	4.53
Warfarin	7.68	7.11
Gallopamil	0.9	1
Verapamil	1.59	2.8
Bepridil	9.99	8.37
Isradipine	25.3	40.6
Nimodipine	13.01	35.29
Nicardipine	2.4	8.01

Chromatographic conditions: 50 mM NaH$_2$PO$_4$ (pH 5.5)–acetonitrile (99:1 v/v).

at its core which may also influence retention [24]. This difference in the effect of buffer concentration between AGP and BLG further indicates that these analogous proteins are really quite dissimilar, at least in their binding sites. The large effect of the buffer concentration on uncharged analytes with BLG suggests a specific binding process in which a buffer-mediated conformational change at the binding region may be occurring.

3.2. Capillary electrophoresis

Several of the compounds analysed in HPLC and some additional solutes were tested in CE using BLG as an additive in solution. None of the chiral drugs considered was enantioseparated. A concentration of 30 μM BLG was used, since the optimum protein concentration is generally <100 μM [11], with higher concentrations causing detection problems. The racemic solute concentration was fixed at 100 μM, i.e. fairly similar to the selector concentration and high enough to allow easy detection.

Different pH values (7.0, 6.0 and 3.8) were evaluated in the range where phosphate has reasonable buffering capacity. A separation of acidic analytes was not expected at pH values above the pI value of BLG (5.2), when the protein bears a net negative charge. Under such conditions the net mobility of the sample is too similar to that of the protein; to achieve chiral separations the mobility of the free and bound analyte must be significantly different. Nevertheless all chiral drugs were tested at all the pH values mentioned above. Fig. 2a, b and c show the effective mobilities obtained for some selected analytes (at pH 7, 6 and 3.8, respectively) in phosphate buffer and with addition of BLG.

At pH 7 BLG has an effective mobility of $-1.5 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}, at pH 6, $-1.32 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1} and at pH 3.8, $+1.5 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}. The basic analytes all have effective mobilities in the range ~ 1.0–$2.0 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1} at each pH tested, LM and LO are neutral at each pH tested, while the remaining compounds are all acids with effective mobilities in the range -1.5 to $-2.5 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1} at pH values of 6 and 7, and with effective mobilities $\sim -1 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1} at pH 3.8 where their ionisation is incomplete.

At pH 7 (Fig. 2a) the cationic analytes all show slight decreases in mobility on addition of BLG, and the neutral compounds take on a slight negative mobility, consistent with their binding to the negatively-charged protein. The acidic analytes show a reduction in the magnitude of their mobility, which again is consistent with binding to the protein, if one remembers that the protein itself has a fairly weak negative mobility. Retinol acetate, known to bind to BLG [6,13,14] was analysed at this pH and its mobility values in buffer and in protein solution were zero and $-1.9 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}, respectively, indicating that the vitamin is indeed strongly bound to BLG in the CE experiments. At pH 6 (Fig. 2b) the protein has an even smaller negative mobility and this is reflected by a generally greater change in the mobilities of the acidic analytes. This is also consistent with the increase in binding of acids on reducing pH observed in the HPLC measurements (Table 2). Within error, there is little or no effect seen for the basic or neutral analytes.

At pH 3.8, (Fig. 2c), a stronger interaction with BLG was observed for some basic compounds, namely DS, PM and PR. This behaviour may be ascribed to the presence of condensed aromatic rings that may have an optimum steric fit and strong hydrophobic interactions with the binding site of the protein. TH could not be detected at this pH. There was no change in the mobility of neutral analytes whereas the mobilities of each of the anions appear to become surprisingly more negative, which is not consistent with their binding to a protein with a weak positive mobility. It is possible that this apparent change in mobility is in fact due to a reduction in electroosmosis due to increasing adsorption of protein onto the capillary surface during the CE run. The electroosmotic flow (EOF) mobility, as calculated from the time for the EOF marker peak, represents the average EOF during the period of separation up to the appearance of the marker. The anionic analytes migrate after the marker, and thus if EOF is continually decreasing, they actually experience an overall lower EOF than that calculated from the marker. Under these conditions, calculating their effective mobilities in the normal way (i.e. $\mu_{eff} = \mu - \mu_{eo}$) gives an overestimate of their negative mobility.

From a purely qualitative viewpoint, it can be seen that in general the mobility changes observed on
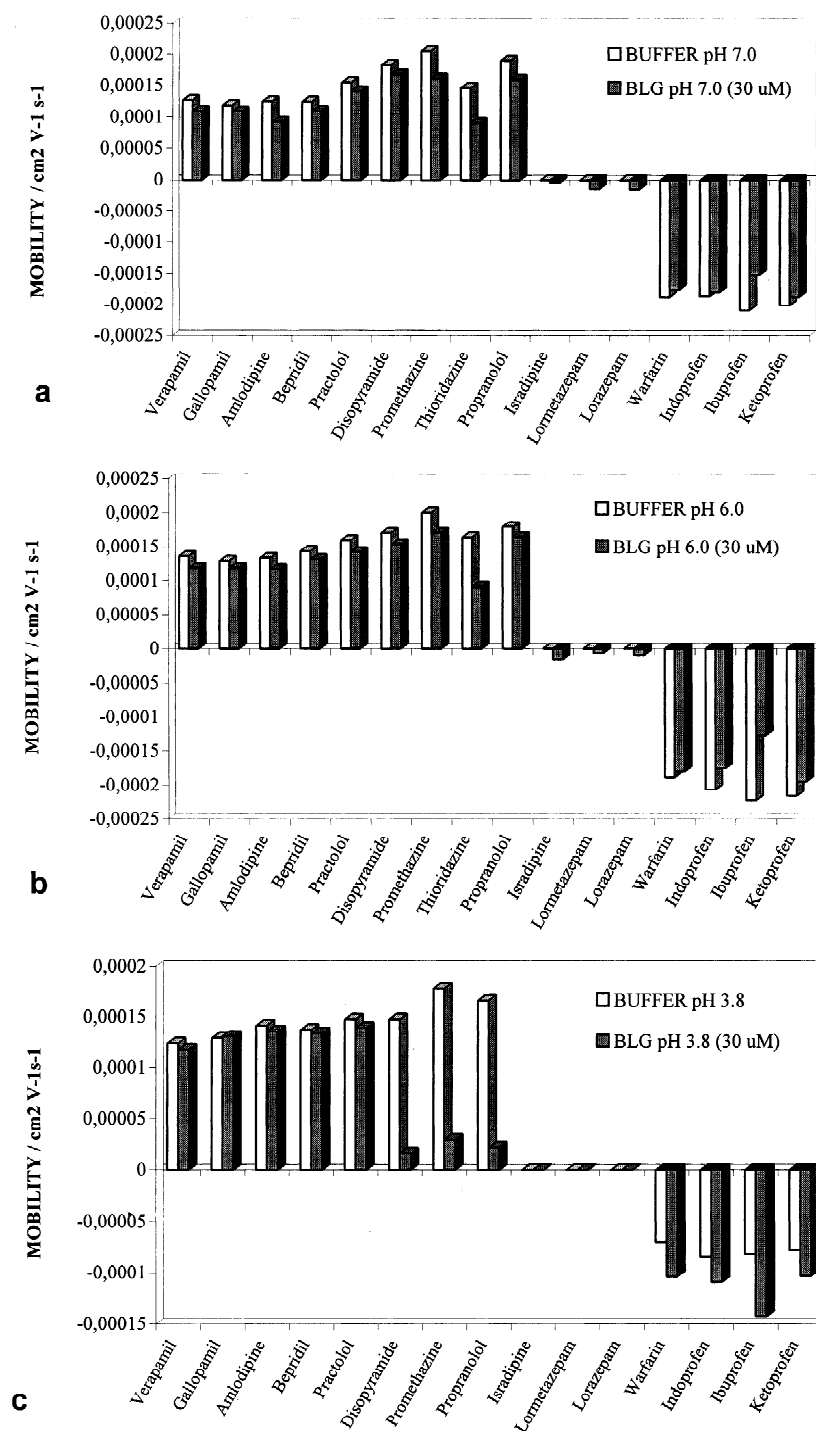
Fig. 2. Effective mobilities of VE, GA, AM, BE, PA, DS, PM, TH, PR, IS, LM, LO, WA, IN, IB, KE in (a) 50 mM phosphate buffer (pH 7.0), and 30 μM BLG in 50 mM phosphate buffer (pH 7.0), respectively; (b) 50 mM phosphate buffer (pH 6.0) and 30 μM BLG in 50 mM phosphate buffer (pH 6.0), respectively and (c) in 50 mM phosphate buffer (pH 3.8) and 30 μM BLG in 50 mM phosphate buffer (pH 3.8), respectively. TH could not be detected at this pH.

addition of BLG are small, indicating that any analyte–protein interaction is quite weak. A quantitative comparison between the two techniques could be made by determining k' values in CE as well as in HPLC [25], but this is not a trivial task in CE since the mobility of the analyte–selector complex needs to be known in each case [26].

4. Conclusions

Contrary to our expectations, this AGP-homologue protein did not show any enantioselectivity, either in HPLC or in CE. Both in free solution (CE) and immobilised (HPLC), BLG has only weak interaction for the majority of analytes tested. The effects of organic modifier and pH on the different classes of compounds were in general consistent with non-specific retention due to the hydrophobic effect and electrostatic attraction between the analytes and the acidic BLG. However, the effect of buffer concentration was quite different from that reported with AGP, and suggests that either a specific binding region which exists on AGP is not present in BLG, or is very significantly modified.

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