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Journal of Chromatography A, 694 (1995) 285–296

JOURNAL OF CHROMATOGRAPHY A

Protein chiral selectors in free-solution capillary electrophoresis and packed-capillary electrochromatography

David K. Lloyd*, Song Li, Patricia Ryan

Department of Oncology, Pharmacokinetics Division, McGill University, McIntyre Medical Building, Room 717, 3655 Drummond, Montreal, Quebec H3G 1Y6, Canada

Abstract

The use of proteins as chiral selectors in CE is reviewed. The performance of packed-capillary electrochromatography with protein phases is compared with free-solution systems. The use of free-solution CE with protein additives for the determination of protein–ligand binding and ligand–ligand interactions is discussed. Some new results are presented using capillaries packed with immobilized human serum albumin. The measurement by free-solution CE of binding of three cationic phenothiazine derivatives to human serum albumin is also shown, and the potential and pitfalls of this method are discussed. Finally some data on the use of dextran to modify protein mobility are shown, and effects of the dextran co-additive on the binding of some ligands to human serum albumin are shown.

1. Introduction

Proteins may display considerable stereoselectivity in their binding of xenobiotics [1]. This stereoselectivity may usefully be exploited in HPLC to perform chiral separations by immobilizing the protein on a suitable support [2,3]. An attractive feature of protein-based chiral stationary phases (CSPs) is that they often display stereoselectivity for a wider range of solutes than do other CSPs. Furthermore, in many cases the native properties of the free biopolymer are retained, and so HPLC with immobilized proteins can be used to probe pharmacologically important interactions [4].

Over the last few years it has been shown that many of the chiral selectors which have been successfully used in HPLC can also be applied in CE [5,6], although the majority of published work has focused on the use of cyclodextrins as chiral selectors. As with HPLC, CE with protein chiral selectors may be used as an analytical technique for chiral separations, or as a method for determining physico-chemical properties such as binding constants.

In this article the use of proteins as chiral selectors in CE will be reviewed. Some new results will also be presented on the use of immobilized human serum albumin (HSA) in packed-capillary electrochromatography (CEC), and the behaviour of this column is compared with our previous work using capillaries packed with α_1-acid glycoprotein (AGP). The use of HSA as a buffer additive will also be discussed. Addition of viscosifiers such as dextran to alter the mobility of the protein and thus extend its range of application will be considered. It is shown that some caution must be used with this

* Corresponding author.

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SSDI 0021-9673(94)00695-4

approach, since the viscosifier may affect the binding properties of the protein.

2. Capillary electrochromatography

Proteins are not ideal for use as buffer additives in CE because their presence at the detection window elicits a strong detector response. Thus it is necessary for analytical purposes to explore ways of using proteins other than simply adding them to the buffer solution. One option is to use CEC [7] with protein stationary phases. An electrically driven flow of liquid through the chromatographic column can give higher separation efficiencies than a similar flow produced by pressure drive [7], and impressive reversed-phase separations with small-diameter packings and high efficiencies have been reported [8]. We have performed initial studies using both AGP [9] and HSA (work described herein) in CEC with commercially available protein CSPs, as well as a comparison between β-cyclodextrin (β-CD) as an additive and as an immobilized chiral selector in CE [10]. The capillary packing process is described in detail in Ref. [9].

In CEC, electroosmosis replaces the pressure-driven flow of HPLC. Thus, a strong electroosmotic flow is vital to give reasonably short separation times. Because of this, our initial studies with protein-based stationary phases have focused on the electroosmotic flow characteristics of these capillaries. Relatively large packing particles have been used (5 μm for AGP and 7 μm for HSA) because they are readily available and are quite suitable for the investigation of electroosmotic flow properties. However, the ultimate chromatographic performance of CEC systems will only be realized with smaller packings.

Electroosmotic flow is weaker in packed capillaries than in open tubes, and a number of reasons for this including non-alignment of the flow channels with the electric field and the packing porosity are discussed by Knox and Grant [7]. A further effect is the alteration of the ζ potential at the packing particle surface, due to the presence of the immobilized chiral selector (although the coverage of the surface silanol groups by the bonded phase is never complete). The effect of the bonded phase on the electroosmotic flow depends on its charge. In packed capillaries with the neutral bonded phase β-CD, the electroosmotic flow was between 25 and 33% of the measured electroosmosis in an open tubular fused-silica capillary over the pH range 4–7.5 using a sodium phosphate background electrolyte [10]. Using AGP-derivatized silica packings, the electroosmotic flow velocity was 37–38% of the open-capillary flow over the pH range 4.5–7.5 when 1-propanol (2%, v/v) was used as an organic modifier in a 2 m*M* sodium phosphate background electrolyte. However, when the 1-propanol was replaced by 2-propanol in the same system the electroosmosis fell to just 12% of the open tubular value at pH 4.5, increasing to 39% of the open tubular value at pH 7.5 [9]. Clearly the effect of the bonded phase on electroosmotic flow in CEC can be quite significant, and in particular protein phases may display quite major changes in electroosmotic flow after relatively minor alterations in the mobile phase composition. Data on the electroosmotic flow in HSA-packed capillaries are presented in this article.

One limitation of CEC is that ionic analytes will migrate either with or against the electroosmotic flow depending on the sign of their charge, and those migrating against the electroosmosis may have such a low velocity that elution will be impossible within a reasonable time period. With some CSPs it is possible to control the direction of electroosmosis by using the appropriate buffer ions or additives, as we have shown with the β-CD phase [10]; manipulation of electroosmosis is more difficult with protein phases because of the ability of the biopolymers to strongly bind so many different ligands, and the significant effects that additives could have on the binding of analytes. Many cationic and neutral compounds separated by HPLC with the AGP CSP were also separated by CEC, although we had limited success with anions for the reasons mentioned above. The behaviour of the phase in terms of the resolutions achieved and the effect of operating

parameters such as organic modifier type and concentration, mobile phase pH and ionic strength indicate that the electric field in CEC has minimal effect on the immobilized biopolymer. Use of smaller packing materials, and perhaps pressurized systems to avoid bubble formation [8], should allow the full potential of CEC for chiral separations to be realized.

3. Chiral separations using HSA in solution or immobilized in gels

Proteins have been used in CE as buffer additives in solution to separate stereoisomers [11–17], including: bovine serum albumin (BSA) for the separation of leucovorin stereoisomers [11], for tryptophan, benzoin and warfarin [13] and for some amino acids [17]; HSA for benzoin and various phenothiazine derivatives [12], and for kynurinine, tryptophan, 3-indolelactic acid, 2,3-dibenzoyl tartrate and N-2,4-dinitrophenyl glutamate [14]; a cellulase (cellobiohydrolase I) for some β-blockers [15]; AGP for promethazine [13]; a fungal cellulase for pindolol [15]; avidin for leucovorin, warfarin, some profens and other compounds [16]; ovomucoid for chlorphenir-amine and epinastine [16].

To achieve chiral separations, the mobilities of the free and protein-bound ligand must be significantly different (this is discussed at length in the following section on ligand–protein binding). Thus the separation at neutral pH of an anionic ligand such as ibuprofen with a protein such as HSA can be problematic. An approach which offers a solution to this problem has been pursued by Sun et al. [18] using BSA with dextran as a co-additive. By doing this the protein mobility is considerably reduced, while the mobility of small molecules is affected to a much smaller degree. This is advantageous in separations where the protein and analyte would otherwise have too-similar mobilities. In this way a separation of ibuprofen enantiomers was achieved, as well as chiral resolution of dansyl-leucine, dansyl-norvaline and mandelic acid. A refinement of this method was to covalently link BSA to a high-molecular-mass dextran [19], and then pump this BSA–dextran network into the capillary with a syringe. Electroosmotic flow was minimized in the capillaries used by coating them with linear polyacrylamide. This resulted in a system where the protein was practically immobile in the gel which was stable within the capillary because of the elimination of electroosmosis. Thus only analysis of charged species is possible.

As previously mentioned, the potential use of protein additives has been limited as an analytical technique because of the large detector response due to the protein. A solution to this problem has been suggested by Vlatcheva et al. [15]. They filled only part of the capillary with protein, and arranged the experiment so that the protein migrated away from the detector window, while the analyte migrated through the protein, and then went past the detection point. The pH of the background electrolyte was chosen such that the migration of the protein and the analyte were in opposite directions. This was facilitated by the use of a coated capillary to suppress electroosmosis. In principle it might be possible to have both analyte and protein move in the same direction with significantly different mobilities, although this would most likely result in lengthy analysis times. The electrochromatographic approach described above is one alternative possibility for the analytical use of proteins as chiral selectors in CE. Another option with proteins, termed capillary affinity gel electrophoresis (cAGE) was developed by Birnbaum and Nilsson [20]. They used BSA cross-linked with glutaraldehyde in situ in the capillary. The reaction mixture was pumped into the capillary until just before the detection window and then allowed to set, thus detection was not hampered by the presence of the protein. Separation efficiencies of up to 280 000 plates m^{-1} were observed in the analysis of the enantiomers of tryptophan. The use of immobilized proteins in gels allows improved detection, unlike replaceable gels [19], but the potentially limited life of the immobilized gel system may be of concern.

Although the use of CEC with protein phases, proteins immobilized in gels or proteins made to migrate away from the detection window are all

interesting possibilities for the development of analytical separation methods, one must consider whether or not protein chiral selectors are the optimum choice for most chiral separations by CE. In HPLC, protein CSPs are attractive because of their broad range of application and, as Wainer [21] noted, AGP has perhaps the broadest utility of any of the current range of CSPs. In CE the situation is somewhat different. Because of the high separation efficiency typical of CE, baseline resolution can be achieved with certain chiral selectors for compounds which our HPLC experience and intuition would say are not amenable for analysis by that type of chiral selector. An example is the enantiomeric resolution of (6*R*)- and (6*S*)-5-methyl-tetrahydrofolate by CE using α, β- or γ-CDs [22]. It is difficult to imagine that the mechanism of inclusion complexation which is almost mandatory for HPLC enantioseparations with CD CSPs can be operating here since the analyte is too large to include into α-CD. Derivatization of cyclodextrins further increases their applicability [5,6], and so it is becoming clear that protein chiral selectors will have a more limited role in CE analysis than they do in HPLC. However, CE with protein additives may become an important tool for the determination of ligand–protein binding, and ligand–ligand interactions, as is described below.

4. Ligand–protein binding and ligand–ligand interactions studied by CE

In any electrokinetic capillary chromatography method the mobility of a solute, μ, is related to its own intrinsic mobility in the background electrolyte, μ_S, and to the strength with which it binds to a buffer additive (for example, a chiral selector) and the mobility of the analyte–additive complex, μ_{CS}, thus [22,23]

$$\mu = ([S]/[S] + [CS])\mu_S + ([CS]/[S] + [CS])\mu_{CS} \quad (1)$$

Where [S] and [CS] the concentrations of the free and bound solute, respectively. For a simple one-to-one liaison the binding constant, K, is

$$K = [CS]/[S][C] \quad (2)$$

where [C] is the free chiral selector concentration, and thus

$$\mu = (\mu_S + \mu_{CS}K[C])/(1 + K[C]) \quad (3)$$

There are several examples of the use of CE for the determination of the binding of chiral compounds to CDs [22–24]. Typically the chiral selector concentration is varied over a wide range, and a non-linear least-squares curve fitting procedure is used to model the data to Eq. 3. Some important points have been emphasized: correction of mobilities for viscosity changes in the solution on addition of chiral selector is vital, and the use of total rather than free concentration of chiral selector in Eq. 3 is only a reasonable approximation at very high selector to analyte concentration ratios. In protein-based separations binding constants are frequently quite high ($>10^4$), and so the optimum protein concentration for maximum resolution is typically <100 μM. Because of the high background, and the limited sensitivity of commonly used absorbance detectors, the solute concentration may have to be similar to the selector concentration. Using this technique, Lloyd et al. [12] investigated the binding of benzoin enantiomers to HSA; in this article, the use and limitations of this approach to determine the binding to HSA of three phenothiazine derivatives, promethazine, propiomazine and thioridazine, is discussed.

To achieve a chiral separation the enantiomers of the analytes must have different binding affinities for the protein, and the bound and free forms of the analyte must have significantly different mobilities (Eq. 1). If the protein is immobilized the second criteria is easy to meet. However, this may not be so simple if the desired separation is of an acidic compound with an acidic protein. As previously indicated, using a simple system with HSA as an additive in a pH 7 phosphate buffer and ibuprofen as the analyte, we found that separation of the enantiomers was

difficult to achieve despite the fact that there is considerable stereoselectivity in this interaction as is known from numerous studies, including HPLC measurements with immobilized HSA [25]. In this case the problem arises because both analyte and selector have similar mobilities. Of course, it should generally be possible to find a pH at which the protein and analyte mobilities are different, but whether this is worth doing depends on what information one wants to obtain from the separation. Moving away from pseudo-physiological conditions removes many of the advantages of using CE to measure binding interactions —although a separation at low pH might be possible, it would be difficult to claim that any measurements made had relevance in vivo.

If co-additives are used along with the protein in the separation buffer then any effect they have on the binding of the ligand to the protein will be directly reflected by an alteration in the mobility of the ligand. The co-additive may take the form of a simple organic modifier such as 2-propanol which generally causes a non-specific reduction in hydrophobic interactions between the ligand and protein. Alternatively, the co-additive may be a ligand which binds to a specific site on the protein, and in this way the CE experiment may be used as a probe of ligand–ligand interactions. For example, the use of co-additives to determine the binding site(s) for benzoin and phenothiazine derivatives has been investigated [12]. Co-additives used included octanoic acid, and enantiomers of oxazepam hemisuccinate and of warfarin.

The use of CE with proteins in solution is particularly attractive for the study of protein–ligand interactions for a number of reasons. Perhaps the foremost advantage is that, unlike chromatographic or conventional affinity electrophoretic methods, in many cases neither the protein nor ligand need be immobilized. Therefore the issue of whether or not the immobilization process has altered the binding never arises. Other useful characteristics are the very small amounts of protein and ligand which are necessary, and for chiral compounds, the fact that enantioselectivity in the binding is revealed as an enantioselective separation, so that pure single enantiomers are not needed. There are limitations to the method, because of the range of mobilities of the analyte/protein combination which may usefully be studied. Although immobilization of the protein or use a co-additive such as dextran which serves the function of entrapment offer useful approaches to achieve separations, some caution should be used if one wishes to apply such systems for binding studies. In such cases, a question which one must ask is how the binding of ligands to the protein might be affected. Results which indicate a change in the binding of some drugs to HSA when using a dextran co-additive will be reported below.

5. Experimental

CE separations were carried out using Applied Biosystems 270-A or 270A-HT (ABI, Foster City, CA, USA) integrated CE systems. Fused-silica capillaries of 375 μm O.D. × 50 μm I.D. were obtained from Polymicro Technologies (Phoenix, AZ, USA) and cut to length. Open-tubular separations were performed in 72-cm capillaries, while packed-capillaries were of 42 cm overall length. The polyamide coating on the capillary was removed 22 cm from one end to provide a detection window. The capillary oven temperature was set at 30°C. Injections were made by applying a vacuum (17 kPa) to the anodic end of the capillary (open-tubular), or electrokinetically (5 kV for 1 s) in packed systems. Detection was by on-capillary UV absorbance. Data were analysed using a Spectra-Physics (San Jose, CA, USA) Datajet integrator, and stored on a personal computer running Spectra-Physics Winner System software. Relative viscosity measurements were made by measuring the elution time for a marker compound to pass through the capillary using the 17 kPa vacuum on the ABI instrument (the accuracy of this method has previously been validated [22]).

Packed capillaries were prepared according to the method described in Ref. [9]. Phosphate buffer was prepared by mixing solutions (usually

4 mM) of analytical grade disodium hydrogenphosphate and sodium dihydrogenphosphate (BDH, Toronto, Canada) to give the desired pH. Deionized water for all solutions was obtained from a Milli-Q[50] system (Millipore, Montreal, Canada). Organic modifiers (Accusolve-grade acetonitrile, 1-propanol, 2-propanol; Anachemia, Montreal, Canada) were added to give the desired volume percentage. A separation potential of 10 kV was generally used for the HSA-packed capillaries. The electroosmotic flow point was measured from the baseline disturbance caused by the sample solvent.

For open-tubular separations, HSA (fatty acid free, fraction V) and dextran (average M_r 267 000) were obtained from Sigma (St. Louis, MO, USA). Phosphate buffer (pH 7) was prepared by mixing solutions of 50 mM sodium dihydrogenphosphate and disodium hydrogenphosphate. HSA was added to this buffer to give the required concentration, typically in the range 10–75 μM. A separation potential of 25 kV resulted in a current of ca. 60 μA without dextran.

Test compounds were obtained from Sigma except for hexobarbital and pentobarbital (U.S.P.C., Rockville, MD, USA) and benzoin (Aldrich, Milwaukee, WI, USA). Solutions were made in water or water–methanol (depending on the solubility of the compound), at a concentration of ca. 1 mg ml^{-1} unless otherwise stated.

6. Results and discussion

6.1. Capillary electrochromatography with a HSA stationary Phase

A HSA CSP which retains many of the ligand-binding properties of the native protein has been developed for HPLC [3,4]. Here, some preliminary CEC results with a capillary packed with this HSA CSP are presented. Packing material (HSA immobilized on 7 μm silica particles) was a gift from Shandon Scientific (Runcorn, UK). A number of chiral solutes could be fully or partially resolved by CEC, including benzoin and some benzodiazepines. Separations of benzoin and temazepam are shown in Fig. 1. In electrokinetic chromatography, the capacity factor, k' can be calculated using the relationship [26]

$$k' = (\mu - \mu_0)/(\mu_p - \mu) \quad (4)$$

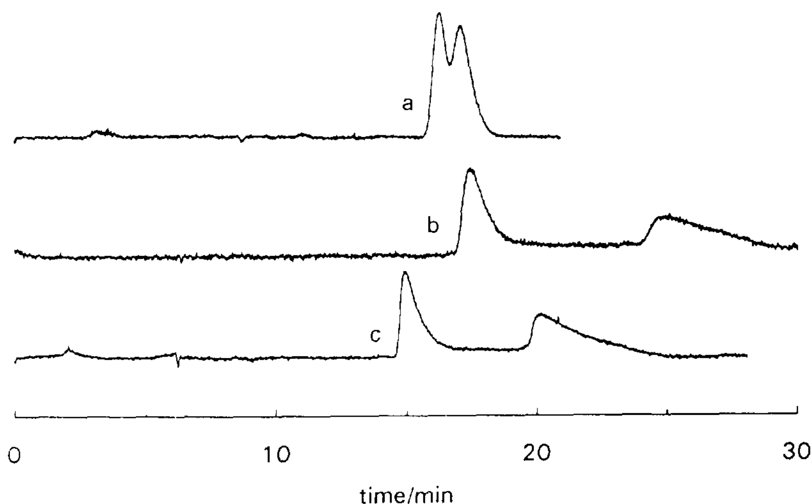

Fig. 1. Chiral separations of benzoin and temazepam by packed-capillary electrochromatography with a HSA CSP. a = Benzoin, modifier 6% 2-propanol; b = temazepam, modifier 5% 2-propanol; c = temazepam, modifier 7.5% 2-propanol. Buffer, 4 mM phosphate, pH 7; separation potential, 10 kV. Capillary, 42 cm overall length (20 cm to the detector) with 17 cm packed with the HSA CSP.

Table 1
Effect of type of organic modifier and of 2-propanol concentration on k' and α for oxazepam and temazepam using a HSA-packed capillary (42 cm overall length, 20 cm to the detector, 15 cm packed with HSA)

Modifier	Oxazepam: k'_1, k'_2 (α)	Temazepam: k'_1, k'_2 (α)
2% 2-Propanol	2.7, 4.2 (1.6)	2.2, 5.3 (2.4)
5% 2-Propanol	2.1, 2.6 (1.3)	1.7, 2.8 (1.7)
7.5% 2-Propanol	1.6, 2.0 (1.3)	1.4, 2.2 (1.6)
6% 1-Propanol[a]	1.6, 1.7 (1.1)	1.2, 1.5 (1.3)
3% Acetonitrile	2.9, 3.9 (1.4)	2.7, 4.7 (1.8)

Applied potential, 10 kV; buffer, 4 mM pH 7 phosphate, except where indicated otherwise.
[a] 5 mM phosphate.

where μ is the analyte mobility, μ_p is the mobility of the pseudostationary phase and μ_0 is the mobility of the analyte under non-chromatographic conditions. Using this expression, k' may also be calculated for neutral solutes in CEC ($\mu_p = 0$ and μ_0 = electroosmotic flow mobility), but for charged analytes it is impossible to accurately measure μ_0 [9].

With the HSA CSP, 1- and 2-propanol and acetonitrile were used as organic modifiers, with the best enantioselectivity being seen with 2-propanol or acetonitrile (Table 1). Chosson et al. [27] also noted that poorer enantioselectivity was achieved for benzodiazepines using 1-propanol (e.g., $\alpha = 1.3$ for oxazepam) when compared with acetonitrile ($\alpha = 4.5$ for oxazepam) in HPLC with the HSA CSP. This similarity of behaviour between the pressure-driven and electrically driven systems suggests that, as with AGP [9], the electric field (238 V cm^{-1}) has only a small effect if any on the immobilized protein. The applied field was limited such that the current in all CEC separations was <4 μA, so there were no difficulties due to bubble formation in the capillary.

Electroosmotic flow in the HSA-packed capillary is quite weak, similar to that measured with AGP. A comparison of the electroosmotic flow in both capillaries is shown in Table 2. In both cases electroosmosis reduces with increasing organic modifier concentration, and a somewhat lower electroosmotic flow is observed with 2-propanol than with acetonitrile. The variation in electroosmosis as a function of organic modifier concentration is consistent with the alteration of the dielectric constant and viscosity of the background electrolyte [28], and need not be ascribed to any specific interaction of the modifier with the immobilized protein.

Separation efficiencies are, by CE standards, low, with $N \approx 15\,000$ plates m^{-1} for benzoin and $N \approx 7000$ plates m^{-1} for temazepam (Fig. 1), very similar to the efficiency of HPLC columns made with this packing (e.g. see separation of temazepam in Ref. [27], $N \approx 5500$ plates m^{-1} for first eluted enantiomer). Overloading was found not to be a major factor in the performance of the AGP phase under the conditions which we used [9], and so it is assumed that this is also the case with the HSA capillaries. Electrically driven capillaries with AGP immobilized on 5-μm par-

Table 2
Electroosmotic flow in AGP- and HSA-packed capillaries

	EOF mobility in AGP capillary (10^{-4} cm^2 V^{-1} s^{-1})		EOF mobility in HSA capillary (10^{-4} cm^2 V^{-1} s^{-1})	
	Modifier: 2-propanol	Modifier: acetonitrile	Modifier: 2-propanol	Modifier: acetonitrile
2%	3.18	3.42	2.54	2.9 (3%)
7%	2.51	3.06	2.3 (7.5%)	

All mobilities in 10^{-4} cm^2 V^{-1} s^{-1}. Buffer, 4 mM phosphate, pH 6.8 (AGP), pH 7 (HSA). HSA-Packed capillary, 42 cm long, 20 cm to detector, 15 cm packed; applied potential, 10 kV. AGP-Packed capillary, 42 cm long, 20 cm to detector, 17 cm packed with AGP); applied potential, 18 kV (for other details of AGP-packed capillaries, see Ref. [9]). EOF = Electroosmotic flow.

ticles were also found to have efficiencies comparable with the pressure-driven columns [9], and for both HSA and AGP the separation efficiencies are much lower than for CEC with an β-CD phase [10].

6.2. Determination of the binding of the enantiomers of promethazine to HSA, using the protein as a run buffer additive

We have previously shown that CE with a HSA buffer additive could be used to measure the binding of the enantiomers of an uncharged analyte (benzoin), as well as being a useful method to screen for drug–drug interactions [12]. We have now also attempted to use the same method for determination of the binding of the cationic ligands promethazine, propiomazine and thioridazine to HSA (structures of the analytes used in this and the following section are shown in Fig. 2). Fig. 3 illustrates the variation of electrophoretic mobility of the enantiomers of promethazine, propiomazine and thioridazine as a function of the concentration of HSA added to the background electrolyte. Chiral separations are achieved for each compound, indicating stereoselectivity in their binding to HSA. If the protein concentration were further increased the measured mobility would eventually reach that of the protein–analyte complex when the analyte was 100% bound. However, the practical range of protein concentrations is limited because of the background signal and problems with capillary blockages at higher protein concentrations. The mobility difference between the enantiomers can be seen to be function of the protein concentration; for the more strongly bound thioridazine and propiomazine the mobility difference reaches a maximum with ca. 50–100 μM HSA.

For an uncharged analyte such as benzoin it is reasonable to expect that the mobility of the protein–ligand complex is very similar to that of the free protein, and so using the free protein mobility in Eq. 3 will result in a negligible error [12] (the effective mobility of the free HSA was measured to be $-1.82 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}). However, binding of a charged ligand will lead

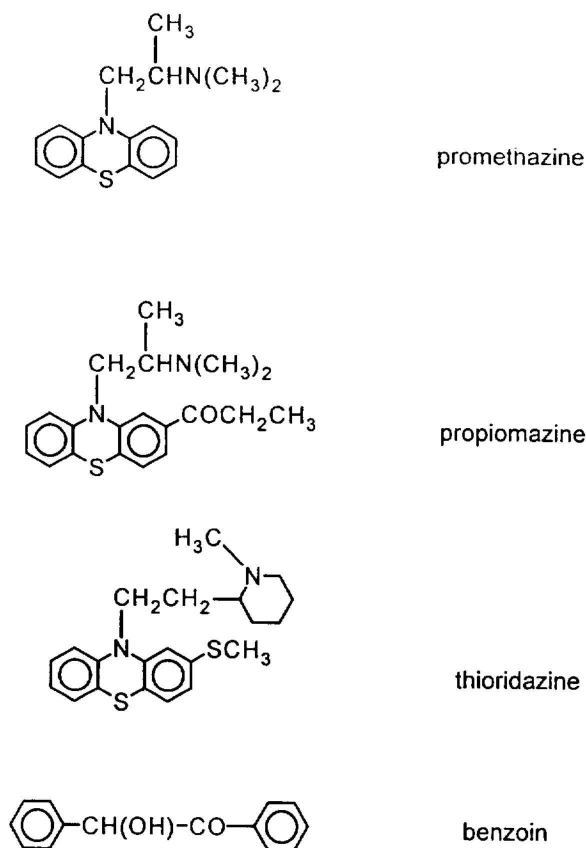

Fig. 2. Structures of the solutes used with HSA as a buffer additive.

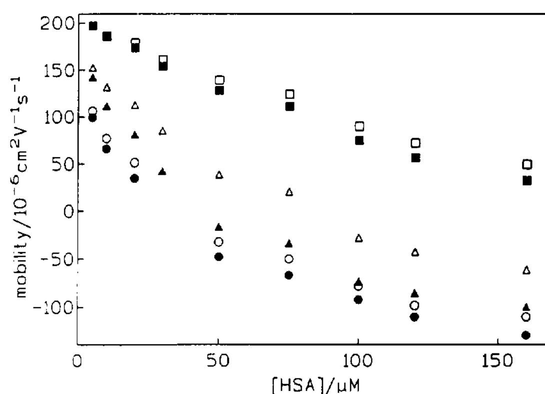

Fig. 3. Variation of analyte effective mobility as a function of the concentration of HSA added to the run buffer. □, ■ = Promethazine; △, ▲ = propiomazine; ○, ● = thioridazine. Open symbols, first detected enantiomer, filled symbols, second detected enantiomer.

to a significant alteration in the protein mobility, and so the effective mobility of the protein–ligand complex must be measured. It is not practical to increase the protein concentration until the analyte enantiomers are so completely bound that their overall mobility approaches that of the analyte–protein complex. However, the mobility can be found by performing a complementary experiment (similar to measurements performed by Avila et al. [29]), using the protein as a sample and adding ligand to the separation buffer. The mobility of a HSA sample (22 μM) was measured in a 50 mM, pH 7 phosphate buffer; the monovalent cationic ligand thioridazine was then added to the buffer at concentrations of 22 and 110 μM, and the protein mobility was measured again. The effective mobility of the HSA–thioridazine complex was found to be 82% of that of the free HSA, with no measurable difference found between the two buffers containing thioridazine. This particular ligand was chosen because it has the strongest binding for HSA, and so it could be used at a relatively low concentration in the buffer (thus there was relatively little interference with detection).

Using a non-linear regression program (Minsq; MicroMath Scientific Software, Salt Lake City, UT, USA), the data shown in Fig. 3 for promethazine were fitted to Eq. 3. Binding to protein adsorbed on the capillary surface also leads to retention of the analyte; measurement of the solute mobility in a new capillary, and in a capillary washed with a HSA-containing solution reveals a difference in mobility, due to chromatographic interactions with the protein adsorbed on the capillary surface [12]. In the case of promethazine this resulted in a reduction of μ by $4 \cdot 10^{-6}$ cm^2 V^{-1} s^{-1}, and this value was added to the measured mobilities to give a value for μ to be used in the modelling. This is an approximation, since of course two competing equilibria are occurring with analyte binding both to free and surface-adsorbed HSA, but it is a minor effect except at very low dissolved HSA concentrations. The free HSA concentration, [C], was calculated from Eq. 1 (note that in Fig. 3 the total concentration of HSA additive is shown on the x-axis, while the free protein concentration is used in the curve-fitting). The concentration of racemic promethazine was 143 μM. The measured data (after correction for surface interactions) and the fitted data are shown in Table 3. The binding constants were estimated to be $K_1 = 6300 \pm 140$ M^{-1} ($r^2 =$ 0.9994) and $K_2 = 7800 \pm 170$ M^{-1} ($r^2 = 0.9993$). The data for the more strongly bound propiomazine and thioridazine were also modelled, but without success. The difficulty in these cases was accounting for the interactions with protein bound at the capillary surface. As we have previously reported, the average k' for surface interactions (Eq. 4) was only 0.01 for promethazine and propiomazine and 0.17 for thioridazine [12]. However, there was considerable error in some of these measurements, with variations between different capillaries. Uncertainty in the contribution from the surface binding leads to considerable errors in the calculation of the free HSA concentration at low protein additive concentrations and thus makes estimates of the binding constants very unreliable. This seems to be particularly a problem for solutes which are more highly bound. Clearly in these cases there is considerable advantage to be had in using coated capillaries where protein adsorption is minimized.

6.3. Effect of a dextran co-additive on binding to HSA

Use of proteins with a co-additive such as dextran has already been mentioned [18,19]. If one is interested in studying protein–ligand interactions rather than simply making separations then the effect of the co-additive must be determined. We have studied the effect of addition of dextran (average M_r 267 000) in concentrations up to 10% on the separation of benzoin, promethazine, propiomazine and thioridazine, with 50 μM HSA as chiral selector. In Table 4 the relative change in current, electroosmotic flow mobility, viscosity and effective mobility of HSA and the three phenothiazine derivatives is shown as a function of dextran concentration. These data reveal that it is somewhat difficult to

Table 3
Free protein concentration and measured and estimated effective mobilities (and the difference between these two values) for promethazine

[C] (μM)	Measured μ (10^{-4} cm^2 V^{-1} s^{-1})	Calculated μ (10^{-4} cm^2 V^{-1} s^{-1})	Deviation (10^{-4} cm^2 V^{-1} s^{-1})
First eluted enantiomer			
3.09	2.03	2.06	0.03
6.05	1.93	2.00	0.07
14.2	1.84	1.83	−0.01
20.6	1.66	1.72	0.06
36.2	1.44	1.46	0.02
56.9	1.22	1.18	−0.04
76.9	0.97	0.95	−0.02
92.9	0.77	0.80	0.03
128.0	0.54	0.52	−0.02
Second eluted enantiomer			
3.09	2.03	2.04	0.01
5.78	1.92	1.97	0.05
13.2	1.79	1.79	0.00
19.2	1.59	1.66	0.07
34.1	1.33	1.37	0.04
54.5	1.10	1.05	−0.05
73.8	0.81	0.80	−0.01
89.8	0.61	0.63	0.02
125.0	0.37	0.34	−0.03

adequately correct for the effects of the dextran; the proportional changes in HSA mobility, viscosity and current are all different, although the electroosmotic flow and analyte mobilities reduce by similar amounts. To compensate for viscosity effects on addition of the chiral selector measurement of the current [23] or the mobility of a non-bound marker compound [22] have been proposed. When protein additives are used alone the concentrations are so low that viscosity effects are generally negligible [12]. Therefore, in this case it seems most appropriate to measure the analyte mobility in dextran-containing buffers with and without protein, to determine the mobility change due to protein binding.

The effective mobilities of benzoin, promethazine, propiomazine and thioridazine were determined in buffers containing 0, 1, 5 and 10%

Table 4
Variation in electroosmotic flow mobility, analyte mobility, HSA mobility, electric current and solution viscosity as a function of % dextran, all values taken relative to 0% dextran

	1% Dextran	5% Dextran	10% Dextran
μ_{EOF} (10^{-4} cm^2 V^{-1} s^{-1})	0.91	0.75	0.51
$\mu_{promethazine}$ (10^{-4} cm^2 V^{-1} s^{-1})	0.94	0.77	0.56
$\mu_{propiomazine}$ (10^{-4} cm^2 V^{-1} s^{-1})	0.94	0.77	0.54
$\mu_{thioridizine}$ (10^{-4} cm^2 V^{-1} s^{-1})	0.93	0.69	0.49
μ_{HSA} (10^{-4} cm^2 V^{-1} s^{-1})	0.93	0.62	0.41
Current (μA)	1	0.86	0.70
Viscosity (cP)	1.7	5.0	12

Table 5
Variation of k' as a function of dextran concentration for the first (k'_1) and second (k'_2) eluted enantiomers of benzoin, promethazine, propiomazine and thioridazine

Dextran (%)	Benzoin		Promethazine		Propiomazine		Thioridazine	
	k'_1	k'_2	k'_1	k'_2	k'_1	k'_2	k'_1	k'_2
0	0.25	0.36	0.26	0.33	0.81	–	1.79	2.27
1	0.25	0.36	0.28	0.32	0.73	–	1.77	2.22
5	0.26	0.39	0.28	0.34	0.75	–	1.73	2.07
10	0.20	0.40	0.07	0.12	0.28	0.82	1.75	2.14

Only one value of k'_2 is shown for propiomazine since this peak eluted close to the electroosmotic flow point at the other dextran concentrations, making accurate determination of its migration time impossible.

dextran, with and without 50 μM HSA. k' as a function of dextran concentration is shown for these solutes in Table 5. k' for the enantiomers of the solutes were then calculated using the Eq. 4, where μ_0 is the mobility of the analyte without chiral selector and μ_p is the mobility of the solute–selector complex; μ_p was taken as being a constant fraction of the protein mobility as determined above for the three phenothiazine derivatives, or equal to the protein mobility for the uncharged benzoin. The k' values are shown in Table 5. For promethazine and propiomazine there is a considerable decrease in k' at 10% dextran. Benzoin and thioridazine are practically unaffected even with 10% dextran. Since all four compounds do not behave similarly, it seems that these effects are not due to a general decrease in non-specific binding, but rather that they are due to a specific alteration or blocking of one binding site on HSA. We have previously shown that the binding of promethazine and propiomazine share many common properties, and that they probably bind to the same region, which is distinct from the binding areas for thioridazine and benzoin [12]. This lends support to the suggestion that the dextran is in this case affecting specific binding, since two ligands which share the same site are similarly affected.

7. Conclusions

CEC can produce high-efficiency chiral separations [10], but with protein phases the efficiency seen so far is rather disappointing ([9], and work reported therein). Optimum performance will only be realized with smaller packings, but it is difficult to foresee CEC with protein phases rivalling the efficiency of other chiral selectors used in CE in solution. Nevertheless, the wide applicability of protein chiral selectors, and the difficulty in their use as additives in CE make their use in CEC an area worthy of further investigation.

A number of reports have dealt with the use of proteins as chiral buffer additives or in gels. In only a couple of these articles has the problem of detection been addressed [15,20], and for a variety of reasons the applicability of these solutions to the detection problem may be somewhat limited. Although analytical applications appear to be somewhat limited, the use of proteins in free-solution CE has a bright future as a method for the determination of protein–ligand binding, and as a method to screen for ligand–ligand interactions. Although not limited to chiral ligands, the utility seems greatest for these compounds since, if the experiment is devised correctly, enantioselectivity in the binding is revealed as a chiral separation. Thus individual enantiomers are not needed. One further attractive feature of this method is that the binding properties of the free protein are measured —when making binding measurements using immobilized proteins the question of whether the binding is affected by the immobilization needs to be answered. Certainly there are instances where the properties of solution or

bound proteins seem different [15]. However, as we have described in this article, co-additives in free solution may also have a significant effect on the protein's binding properties and thus must be used with some degree of caution. A further question to be answered is what would be the ideal medium in which to perform such measurements, after all a pH 7, 50 m*M* phosphate buffer bears only a limited resemblance to the protein's milieu in vivo. Avila et al. [29] have investigated the protein-binding of some non-chiral ligands by free solution CE with organic buffers, and it has been suggested that organic buffers may offer a more realistic environment for the protein than inorganic buffer ions such as the phosphate used here. Whether or not this is really the case requires further study.

Acknowledgements

This work was funded in part by the Natural Sciences and Engineering Research Council of Canada. D.K.L. would also like to thank the Fonds de Recherche en Santé du Québec for a Chercheur–Boursier scholarship.

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