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Review

Selectivity in capillary electrophoresis: the use of proteins

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Abstract

Proteins, by their very diverse nature, provide a wide variety of options for generating selectivity in capillary electrophoresis (CE). Their use in different modes of CE will be considered in this review. Proteins added in solution to the background electrolyte allow separations to be made in a similar fashion to other electrokinetic chromatography methods, e.g., micellar separations. Alternatively, different immobilization schemes can be used to secure proteins within the capillary; these have included capillary electrochromatography with the protein grafted onto a silica support, or immobilization of the protein within a gel structure. Compounds varying in size from small inorganic ions to biopolymers may be bound by proteins. There is the potential for any sort of intermolecular interaction to play a role in the binding process (e.g., hydrophobic interactions, electrostatic interactions, etc.). Very specific high-affinity binding often occurs, but also there is often weaker, non-selective binding. Frequently the interactions of chiral compounds with proteins are stereoselective. Obtaining chiral selectivity has been one of the main applications of protein selectors in CE, and this use will be emphasized here in a discussion structured by type of protein. As well as utilizing the selectivity of proteins to develop separations, the role of CE in investigating ligand–protein interactions will be emphasized.

Keywords: Selectivity; Enantiomer separation; Reviews; Chiral selectors; Capillary columns; Buffer composition; Ligand–protein binding; Capillary electrophoresis; Electrokinetic chromatography; Proteins

Contents

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1. Introduction

Terabe et al. [1] introduced the idea of employing a second phase in solution in capillary electrophoresis (CE) to obtain selectivity other than that based purely on differences in electrophoretic mobility [1]. These first experiments used surfactant additives to the background electrolyte (BGE), and since then a whole variety of other additives have been employed in CE to provide specific selectivity options. Most CE separations using protein selectors take this simple approach, dissolving the protein in the BGE. Of course, the use of specific selectors has a long history in the larger formats of affinity electrophoresis [2]. However, this technique has tended to be used mainly for determination of the strength of intermolecular interactions rather than applications like chiral separations which are popular in CE with protein selectors. A major difference between CE with protein selectors and classical affinity electrophoresis is that in the latter technique, a stabilizing medium such as a gel is used. This is not necessary in CE because of the excellent heat dissipation achieved in capillary tubes [3].

One can borrow other ideas from chromatography in order to introduce extra selectivity into CE separations. It has been demonstrated in larger tubes [4] and in capillaries [5] that electrochromatography could be performed with a retentive phase immobilized on silica. However, despite a recent renaissance of interest in capillary electrochromatography (CEC) [6], its application remains limited compared to the use of electrokinetic chromatography (EKC) with selectors in solution. Alternatively, selectors can be immobilized within a gel inside the capillary; this approach was first applied using cyclodextrins [7], and more recently has shown considerable promise with proteins.

The aim of the present article in this volume devoted to selectivity in CE is to describe the role of proteins in CE as a unique type of selector. CE separations are described in the context of our knowledge of drug–protein binding, and the use of protein selectors in high-performance liquid chromatography (HPLC). The modes of use of protein selectors are discussed, and then particular applications will be reviewed by protein type. We also consider how CE can also be used to probe the binding interactions between proteins and other compounds.

2. Protein selectors

2.1. Binding to proteins

Reversible binding to other macromolecules or to small molecules like drugs is at the core of the physiological role of proteins. The diversity of chemical functions present at the surface of proteins allows them to interact in a variety of ways (the principal interactions being hydrophobic and electrostatic) with a broad range of structurally unrelated substances. The majority of these interactions are relatively weak and non specific but stronger, more specific binding may arise from the combination of multiple interaction points in a well defined, receptor-like area in which only molecules with a particular geometry and chemical functionalities can fit. Most interactions involving small molecules are reversible but there are some documented instances of irreversible binding to proteins [e.g., acetylation of human serum albumin (HSA) by aspirin]. Because of the chiral nature of proteins, they often interact differently with the stereoisomers of chiral molecules [8]. For a chiral compound, non-specific interactions are equivalent for each stereoisomer. However at specific binding sites it is possible that each enantiomer may interact with the protein in a qualitatively (i.e., at different binding sites) and/or quantitatively (i.e., with different affinity or binding capacity) distinct fashion.

These unique qualities of proteins form the basis of their successful utilization as chiral selectors in

HPLC and CE [9]. In recent years, the introduction of new protein phases almost systematically includes the investigation of the number and characteristics of the stereoselective sites [10]. Because binding of drugs is a part of their physiological role, HSA and α_1-acid glycoprotein (AGP) have been more thoroughly examined and the characteristics of their binding sites are better known [8,11,12]. Stereoselective binding of drugs on HSA occurs principally at two major binding areas currently referred to as the benzodiazepine–indole site and the warfarin–azapropazone site. The benzodiazepine site is represented as a narrow hydrophobic pocket with a cationic region while the warfarin site is described as a broad binding area that bears overlapping subsites [11,13]. In addition, the existence of several minor sites has been postulated to account for certain inconsistencies with the two-site model. Several markers have been described for each of the major sites which are primarily used to try and elucidate binding mechanisms and to help identify the primary binding site of new compounds. A unique stereoselective site exists on AGP, at a hydrophobic cleft within the protein part of the molecule [8,12]. For cellulases, enzyme kinetics and X-ray crystallography experiments suggest that the major chiral recognition site is situated in the active site of the enzyme [10]. Some physicochemical properties of proteins used as selectors in CE are summarized in Table 1.

2.2. Use of protein selectors in HPLC

Since the introduction of the bovine serum albumin (BSA) and AGP chiral stationary phases [9,12], there has been a rapid and extensive development of new protein based stationary phases and of their application to the direct separation of enantiomers [10,14–16]. The main advantage of protein phases as a group is their wide applicability [9,10,13–16], and it seems relatively uncommon for a protein to display little or no enantioselectivity although this can occur, and example being β-lactaglobulin (BLG) [17]. Because of the variety of possible interactions, many protein chiral selectors can separate an extremely large number of enantiomeric pairs. The applicability of these columns is further extended by the possibility of changing the protein conformation through simple modifications of mobile phase parameters such as pH, thus producing what are effectively different selectors using the same starting stationary phase [12,18].

Although a few HPLC applications have used

Table 1
Properties of some protein selectors used in CE

Protein[a]	Molecular mass	p*I*	Sialic acid residues	Disulphide bridges	Carbohydrate (%)
AGP	44 000	2.7	14	2	45
AVI	70 000	10–10.5	–	–	20.5
BLG	18 000/36 000	5.2		2	–
CAS a (70%)	26 200	4.7	0.3%	–	0.38
b (27%)	24 400	4.0–4.5	–	–	–
g (3%)	30 000	5.8–6.0	–	–	–
CBH I	64 000	3.9	–	–	6
CON	70 000–78 000	6.1–6.6	–	17	25
Fungal cellulase	60 000–70 000	3.9	–	12	6
HST	76 500	5.5	4	19	5.7
HSA	66 500	4.9	–	17	–
OVM	28 800	3.9–4.5	0.3%	8	30
RfBP	32 000	3.9–4.1	4	9	14

[a] AGP, α_1-acid glycoprotein; AVI, avidin; BLG, β-lactaglobulin; CAS, casein; CBH I, cellobiohydrolase; CON, conalbumin; HST, human serum transferrin; HSA, human serum albumin; OVM, ovomucoid; RfBP, roboflavin binding protein.

proteins as chiral additives in the mobile phase [10], current practice is to use immobilized proteins, most often covalently bound onto a silica-based chromatographic support. Proteins are used exclusively in aqueous buffered media (only water-soluble proteins rather than hydrophobic membrane proteins have been used for HPLC chiral separations) which takes advantage of the natural binding properties of the molecule. The very high affinity for some solutes can lead to excessive retention for analytical purposes, and this is usually overcome by the addition of organic solvents and/or competitors in the mobile phase [9–16]. Protein phases generally suffer from poor efficiency, a problem frequently ascribed to slow mass transfer and low loading capacity relative to most other HPLC stationary phases.

Because immobilization reactions are generally not selective, individual protein molecules may be oriented differently leading to a relatively inhomogeneous stationary phase. Consequently, only a fraction of the immobilized protein is actually useful for enantioselective binding since not all the selective site(s) are accessible to the solute. Another (still unanswered) question is exactly to what extent is the protein structure affected by the immobilization. This is a concern in particular for researchers interested in the application of these phases as biochemical probes of protein binding [11]. Although good correlations between results obtained with immobilized proteins and with proteins in solution (presumably closer to their native state) are often reported, some inconsistencies have also been noted [11,19]. Some immobilizations, e.g., those which cross-link the protein on the support to give extra stability [12], may result in quite different binding properties than those observed in vitro with the protein in solution.

Mechanisms of retention and selectivity are highly complex and still mostly unknown especially for the most recently developed phases. A proof of the complexity and subtlety of protein interaction mechanisms can be found in the comparison of serum albumins from different species [19,20]. Even though these proteins have a very high degree of similarity in their amino acid sequence they still exhibit remarkable differences in their ability to separate enantiomeric pairs. Some attempts to immobilize fragments of proteins (ovomucoid and BSA) to try to isolate the enantioselective binding site and reduce non selective binding, thus overcoming some of the limitations of protein phases, have met limited success. In almost all cases, the whole protein exhibited better enantioselectivity than fragments [21,22]. Equilibrium binding investigations to determine thermodynamic parameters [23], competitive interactions [24], molecular modeling [13] or nuclear magnetic resonance (NMR) [21] have given some insight into the binding mechanisms of certain protein phases but the optimization of separations on protein phases remains largely empirical.

Protein based liquid chromatographic phases have also been used to study/predict binding interactions between small molecules (drugs in particular) and proteins of biological relevance (mostly human and animal serum albumins) [11,19,23]. This application is now at the centre of much of the research on the serum albumin-based chiral stationary phases.

3. Modes of use of protein selectors in CE

3.1. Proteins in solution

3.1.1. Complete-filling techniques

Perhaps the simplest and most common way of using protein selectors in CE is just to dissolve the protein in the BGE. The solution completely fills the separation capillary and is present in both buffer reservoirs [25–28]. This separation is analogous to a micellar or other sort of EKC experiment, and can be understood in the same way as EKC separations using other sorts of selectors [1,29–34]. Assuming that there is electroosmotic flow (EOF) in the system, the free analyte will migrate at a mobility μ which is the sum of both the EOF mobility (μ_{eo}) and, if it is charged, its own effective mobility (μ_{effA}). Binding to the protein will likely result in a complex which has a different effective mobility (μ_{effC}) from that of the free analyte. Since the analyte will spend part of the time free in solution, and part of the time complexed with the protein selector, the overall analyte effective mobility with the selector present in the BGE (μ_{eff}) will be given by the time-weighted average of its mobility in these two states. This can be related to the concentrations of the free [A] and bound [AP] analyte

$$\mu_{eff} = \frac{[A]}{[A]+[AP]}\mu_{effA} + \frac{[AP]}{[A]+[AP]}\mu_{effC} \quad (1)$$

From Eq. (1), it is clear that there will only be a change in μ if $\mu_{effA} \neq \mu_{effC}$, i.e., when $\mu_{effA} = \mu_{effC}$ there is no relation between the amount of binding and the analyte mobility. Similarly, the largest changes in μ_{eff} for a given degree of analyte–protein binding will come about when μ_{effA} and μ_{effC} are very dissimilar, such as when the analyte is a cation while the overall analyte–protein complex is anionic. Most of the reported separations with protein BGE additives have employed proteins with isoelectric points (p*I*s) in the range ≈2.5–6, with neutral buffers. Under these conditions the protein migrates towards the anode, against the EOF which is transporting all species towards the cathode. This experimental set-up is most favourable for the separation of basic or neutral solutes. Anions may be analysed under these conditions, but the similarity between μ_{effA} and μ_{effC} will weigh against the success of the separation.

The effect of the protein selector can also be considered in terms of the capacity factor, k'. This can be calculated from electrophoretic mobilities, and can be related to the concentration of selector in the system and the equilibrium binding constant K_{EKC} [35–38]

$$k' = \frac{\mu_{effA} - \mu_{eff}}{\mu_{eff} - \mu_{effC}} = K_{EKC}[P] \quad (2)$$

where K_{EKC} is the binding constant for the equilibrium $[A]+[P] \rightleftarrows [AP]$, and [P] is the concentration of the free protein.

There are some problems in accurately determining k' in CE with protein selectors: the EOF and μ_{effC} can be difficult to measure accurately [39]. In micellar EKC, the mobility of the analyte when included in the micelle is generally taken to be the same as that of the micelle (which itself is usually measured by using a totally-included marker compound). With a low-mass neutral analyte binding to a protein selector it seems safe to assume that the mobility of the analyte–protein complex is the same as that of the free protein [28]. However, binding of a charged analyte will likely result in a significant difference between μ_{effC} and the mobility of the free protein [40].

One interesting comparison between proteins and many of the other selectors used in CE is that the strength of the analyte–protein binding interaction is often relatively high. As in HPLC, in EKC it is generally desirable to have moderate values of k' in order to obtain optimum resolution, although in EKC the exact values will depend on the elution range [41]. From Eq. (2), it is apparent that there are two ways of tuning k': either the selector concentration can be varied, or the strength of the binding interaction can be weakened. This is rather different than the situation in HPLC where the amount of selector on the column is fixed, and so only tuning of the binding affinity (e.g., by adding organic modifiers) is possible. In contrast, in EKC it is probably more common to alter k' by varying the selector concentration. Since K_{EKC} is generally quite high with protein selectors, it follows that [P] should be rather low, and typically the protein concentrations used in EKC are <1 m*M* (often <100 μ*M*). In the limit, though, it is not desirable to use selector concentrations which are very low relative to the analyte concentration. In such a case (which would occur for a strongly-bound analyte) it may be preferable to operate with a higher concentration of protein, and use an organic modifier additive to reduce the binding interaction. The effect of organic modifier is shown in Fig. 1, where the separation of the enantiomers of propiomazine is illustrated using 35 μ*M* HSA as a chiral selector with either no organic modifier, or with 6% (v/v) 1-propanol. As well as a reduction in retention upon adding the modifier, there is also an increase in efficiency.

The sort of analysis presented above is frequently used to describe the binding of a single analyte molecule to a single small selector such as a cyclodextrin. It should be emphasised that this can be a gross approximation in the case of proteins, which may have more than one site where an analyte can bind. Still, the most common case is that of a single specific binding site with moderate to high affinity for the analyte, and supplementary, non-specific binding which is considerably weaker.

The major disadvantage of filling the buffer reservoirs and the whole capillary with the protein is that it will always be present in the detection region. Due to the variety of functional groups present in each protein molecule it is highly likely that the

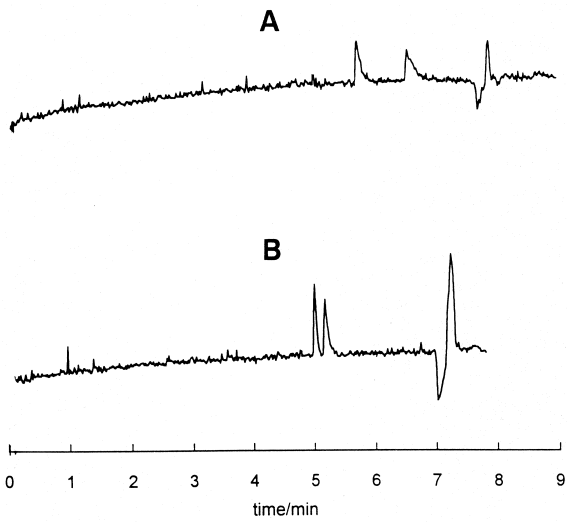

Fig. 1. Effect of organic modifier on the separation of propiomazine enantiomers. BGE: (A) 50 m*M* phosphate, pH 7 with 35 μ*M* HSA; (B) as A, with 6% (v/v) 1-propanol. Capillary 72 cm (50 cm effective length)×50 μm I.D., *V*=30 kV. Binding of propiomazine to HSA is reduced upon adding the modifier, and peak-shape is also improved. The peak at 7–7.5 min is due to the EOF.

protein will elicit some response from almost any detector unless the analyte has rather unusual properties (e.g., a dye which absorbs strongly at visible wavelengths). Detection of the analyte on top of a large background signal from the protein severely compromises complete-filling techniques from the viewpoint of trying to develop useful analyses. The situation is helped somewhat by the relatively low concentration of protein usually used, but even 100 μ*M* of a large molecule such as protein in solution equates to a significant concentration of groups present which will elicit a detector response. When using UV absorbance detection, there is usually some wavelength which provides the best compromise for signal-to-noise ratio, when the analyte response is large or maximized and the protein response is small or minimized. Many proteins have relatively low absorbencies around 250 nm, and this can be a useful wavelength to operate at if the analyte has a reasonable response there.

It is an open question as to whether one should use plain fused-silica capillaries or capillaries coated to reduce protein adsorption when employing proteins as BGE additives. Our own experience (primarily with albumins, but also with BLG and various riboflavin-binding proteins) is that plain fused-silica capillaries are often adequate for such separations. However, many proteins certainly do stick to these surfaces, and this can even be visualized by electron microscopy [42]. It has been shown that when using HSA as a BGE additive the capillary surface quickly becomes coated with a monolayer of protein [43]. Indeed, this coating of protein provides a retentive surface which can even be responsible for chiral resolution, as was illustrated by the separation of the enantiomers of warfarin in a 50 μm I.D. fused-silica capillary which had been pre-treated with a HSA-containing solution [43]. Nevertheless, from an analytical viewpoint this approach is limited since the amount of protein available on the surface is small and thus the total retention and optimal flow velocity ranges for high efficiency operation is very small (this is, of course, the major limitation of any open-tubular electrochromatography performed in capillaries as large as 50 μm [44]). Comparing the retention due to the wall-adsorbed protein, and retention due to dissolved protein, we have estimated that with HSA the retention at the wall is equivalent to that caused by $\simeq 2$ μ*M* of the protein in solution [28]. Clearly this is a relatively small contribution under normal operating conditions.

There are other points to consider regarding coating of the wall by the selector. One is that the ζ-potential of the surface becomes controlled by the properties of the protein. Depending on the p*I* of the protein, the electroosmosis in the protein-coated capillary may be different to that in a clean fused-silica capillary. A more serious problem of protein–wall adhesion is the potential to cause capillary blockages. Our experience has been that some proteins are relatively easy to use in plain fused-silica capillaries (e.g., albumin) while others are more difficult (e.g., AGP) because they quickly lead to capillary blockages. We suspect that these may be related to the build-up of multiple layers of protein adhered to the capillary wall since the frequency of blocking can be greatly reduced by the use of between-run wash procedures designed for protein removal. A suitable procedure would be to rinse with the running buffer solution (without protein), containing 100 m*M* sodium dodecyl sulfate (SDS) [45]. With effective between-run washing it is usually

possible to use one capillary for many days or weeks.

There are also proponents of the use of capillaries which are internally-coated to inhibit wall–protein interactions when using proteins as BGE additives (e.g., [46–48]). A frequently-used coating is the classical non-cross-linked polyacrylamide developed by Hjertén [49], although many alternatives exist such as the hydrophilic poly(acryloylaminoethoxy-ethanol)-coated columns developed by Chiari et al. [50]. It may be worth choosing the exact nature of the capillary coating to minimize adhesion of a particular protein. If a certain protein gives problems when used with plain fused-silica capillaries, then use of such coatings is certainly a worthwhile option. An interesting comparison of the use of a non-coated and coated capillary was described by Barker et al. [25], in the separation of leucovorin diastereoisomers with BSA as a BGE additive. With the bare fused-silica capillary, poor migration-time reproducibility was noted. A poly(ethylene glycol) modified capillary was said to allow up to ten times more separations to be performed using a given capillary before the capillary needed to be replaced (mode of failure not specified). One final point to note with uncharged capillary coatings is that EOF is eliminated. EOF elimination or reversal has been used to reverse the elution order of enantiomers using other selectors [30], and the same effect may be observed with protein selectors [25].

3.1.2. Partial-filling techniques

As a response to the problem of developing useful analytical methods when the protein is present in the detection region, ''partial-filling'' methods have been developed [47,48]. These approaches are quite general, and may also be applied with other selectors which elicit a strong detector response. However, they were first developed for protein-based separations since the protein selectors pose the worst detection problems.

The basis of the partial-filling method is illustrated in Fig. 2. At the beginning of the separation, the protein-containing BGE fills the capillary tube including the detection region. In the example shown, a coated capillary is used to eliminate EOF; the analyte is a cationic racemic mixture, and an acidic protein such as albumin fills the capillary. As the voltage is applied, the protein and the analyte counter-migrate through the capillary. The protein clears the detector window before the analyte enantiomers arrive, and so when they eventually electrophorese past the detector there is no interference from the selector.

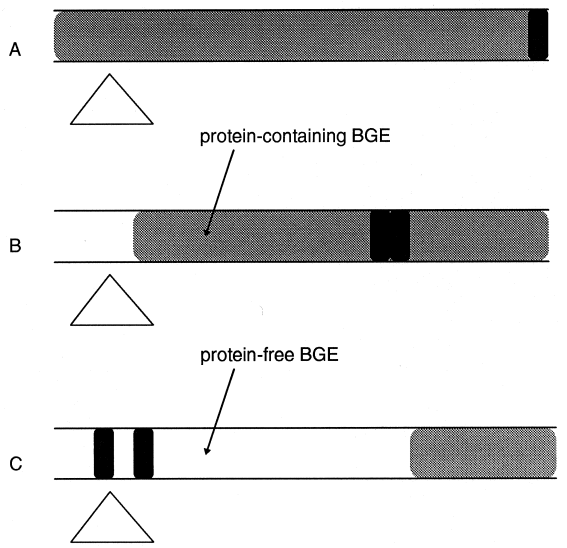

Fig. 2. Schematic of a partial-filling separation system. The capillary is first filled with the protein-containing BGE, and the sample introduced at one end (A). The field is then applied to migrate the sample towards the detector (B). Separation conditions are chosen such that any protein migration is away from the detection zone. Finally, the resolved analytes arrive at the detector window, which is free from interference from the protein (C).

This is an elegant solution to the problem of protein interference with detection. A couple of different formats have been reported. The first was a closed system, using gel plugs at the ends of the capillary and a coated capillary to eliminate EOF [47]. It was later shown that open systems could also be used [48]. The relationship between the mobilities of the analyte and analyte–protein complex and the overall migration time is more complex than in the homogeneous system. In the partial-filling system, the analyte migrates for a certain time with its mobility determined as in Eq. (1) above, but once it leaves the protein zone it migrates at its own free mobility. The amount of time spent in each state clearly depends not only on the analyte and analyte–

protein complex mobilities, but also on the length of the protein zone.

3.2. Immobilized proteins

The simplicity of just dissolving a protein into the BGE is clearly attractive. However, there are a number of limitations to this approach, as the discussion in the previous sections has highlighted. Partial-filling provides a partial solution to the detection problem, but imposes limitations upon the experimental design. Complete-filling approaches offer greater flexibility at the expense of detection problems. In both cases, the analyte and protein mobility must be sufficiently different for solute–protein binding to result in useful analytical selectivity. Nevertheless, the specific selectivity offered by certain proteins makes alternative modes of usage worthy of investigation. Two electrochromatographic approaches which have been taken are to immobilize the protein in a gel within the capillary, or to use capillaries packed with an immobilized-protein stationary phase. Such systems have some significant advantages: the protein can be immobilized such that there is no interference with detection, and the protein mobility is well defined (i.e., zero).

Birnbaum and Nilsson [51] first described CE separations using a protein selector, in the resolution of tryptophan enantiomers. Their protein (BSA) was immobilized within the capillary by cross-linking with glutaraldehyde. The buffered reaction mixture containing BSA and glutaraldehyde was pumped into the capillary from the inlet end until just before the detection window. The mixture was then allowed to gel, and thus a protein gel plug was made, filling the capillary up to the detection point. As detailed in the references to this technique, a reversed-polarity preconditioning is important to ensure stability of the gel-filled capillary. This cross-linking approach avoids the use of other gel-forming polymer additives which may themselves interact with the analytes. The same group have also demonstrated that the formation of gel filled capillaries with binary protein mixtures [52] and antibodies [53] is possible. Binary mixtures can stabilize the gel for proteins which do not readily form stable gels on their own. The selectivity is additive, as it is in HPLC with mixed protein phases [54]. An important point to bear in mind with these capillaries is that EOF is eliminated or negligible. Thus the technique is not applicable to the separation of neutral compounds.

Packed CEC with protein phases has been reported with both AGP [55] and HSA [56]. This work has been carried out with commercially-available silica-based packing materials of 5–7 μm diameter. The packing procedures [55] are similar to those used with other CEC phases, except that the packing is performed in a predominantly aqueous solvent to avoid denaturation of the protein. Addition of a few m*M* Na_2HPO_4, and up to around 10% (v/v) methanol or similar organic solvent minimizes clumping together of the packing particles and consequent capillary blocking during the packing process. Our experience with protein-based phases is that frits can be formed by heating of the packing material; extra glass or silica beads are not necessary.

In CEC, the EOF is determined predominantly by the ζ-potential at the packing surface. Both HSA and AGP are acidic proteins, and thus EOF in these capillaries at neutral pH is in the same direction as that in open fused-silica tubes. In CEC, the magnitude of EOF is important, since it determines how well one can separate neutral compounds. With the AGP capillary, EOF remained fairly constant over the pH range 4.5–7.5 (around 40% of the open-tube EOF) when using 1-propanol (2%, v/v in 2 m*M* phosphate buffer) as an organic modifier. In contrast, with 2-propanol there was far weaker EOF at pH values below 7.5. Acetonitrile generally gave the highest EOF with both AGP and HSA capillaries. The take-home message is that EOF is governed by the protein being used, and relatively subtle alterations in mobile phase (e.g., changing 1- for 2-propanol) can cause significant variations in EOF, probably due to specific effects of the modifier on the protein.

The necessity to have a mobility difference between the analyte and analyte–protein complex in order to achieve a separation has been emphasized. One of the key parameters in developing a successful HPLC separation with protein phases is pH (see Section 2), affecting not only the ionization of the analyte and protein, but also causing conformational changes of the protein [12,18]. In CE with proteins in solution, changing the pH also alters the mobility of the protein, i.e., not only the fraction of analyte

bound but also μ_{effC} are likely to be a strong function of pH. Clearly in such a situation it is not a simple matter to predict how a pH change will alter the separation. Sun and coworkers [57,58] proposed a simple method to control the protein mobility other than by altering the buffer pH, that is to either covalently link a protein to a replaceable gel support, or to simply use a replaceable gel in the BGE solution to preferentially slow down the protein's mobility. An appropriate use of this approach would be when trying to separate enantiomers of an analyte which has a similar mobility to the protein selector. Fig. 3 shows the effect of an increasing percentage of dextran (M_r 2 000 000) added to a BGE containing 1 mg/ml BSA on the migration time of the stereoisomers of ibuprofen and leucovorin and amino acid derivatives at pH 7.1 [57]. The analytes and protein have similar mobilities in the BGE without dextran, thus little or no resolution is achieved. However, on adding dextran the mobility of the BSA is reduced, while the small molecules hardly interact with the polymer network. The result is an increase in the difference in mobility between the free and complexed species, and an improvement in the separation. Measurements with HSA and dextran additives revealed unexpected effects on the selectivity at high dextran concentrations (10%, w/v, M_r 267 000) indicating that a change in protein conformation or competition at specific binding sites was occurring [56]. Compared to the other immobilization techniques described the use of replaceable gels is relatively simple, although it does not offer any detection advantage compared to the use of free protein additives.

3.3. Performance comparison of proteins in CE and retention relationships between HPLC and CE

One of the big advantages of CE techniques as generally practiced when compared to HPLC in its most common formats is the relatively high number of theoretical plates which can be generated per unit time in CE [3]. HPLC protein phases are notorious for their low efficiency (Section 2.2). Thus, any efficiency advantage which can be gained from CE is likely to be analytically very useful from the viewpoint of achieving good resolution.

With protein selectors in free solution, separations

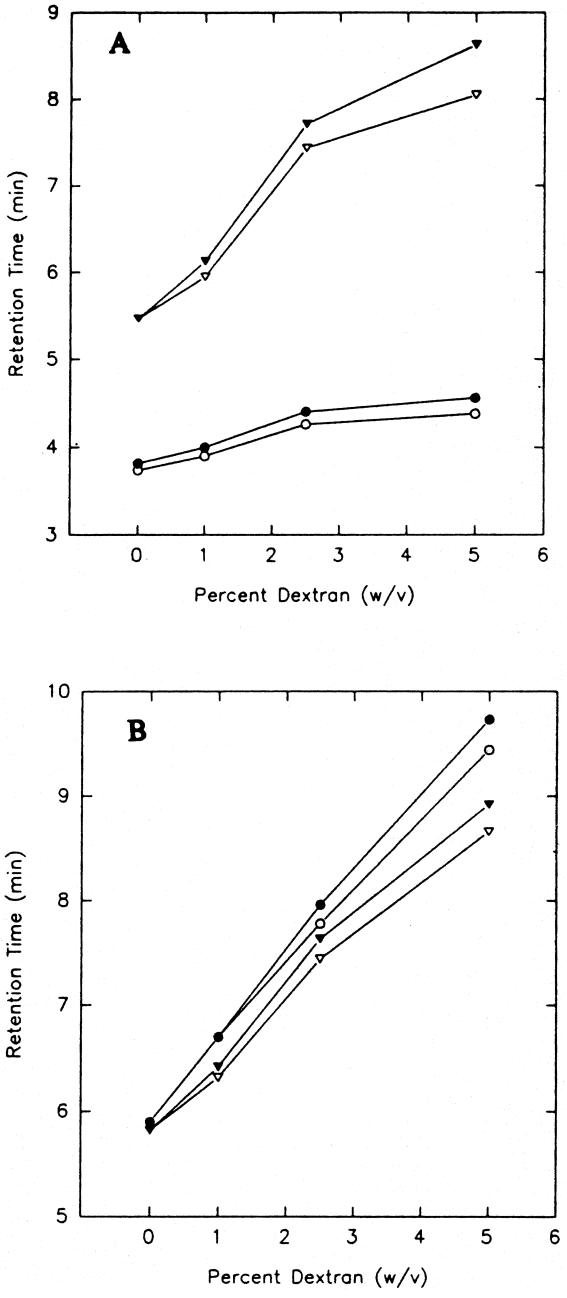

Fig. 3. Effect of percent dextran (w/v) on the retention time of the enantiomers of A, ○ an ●, enantiomers of leucovorin; ▽ and ▼, enantiomers of ibuprofen; B, ○ and ●, enantiomers of dansyl-DL-norvaline; ▽ and ▼, enantiomers of dansyl-DL-leucine. BGE, 10 m*M* phosphate, pH 7.12 with 1 mg ml^{-1} BSA (reprinted from Ref. [57]).

with efficiencies of up to 200 000 plates have been reported (e.g., [25]); similar efficiencies can be observed when using dextran additives as protein mobility modifiers [57]. However, these are probably the best figures. More retained analytes tend to have lower efficiencies. With cross-linked protein gels, efficiencies of around 100 000 plates have been reported [51]. By contrast, with CEC the efficiency is often little better than that seen in HPLC [55], suggesting that extra kinetic problems may be introduced by immobilization, e.g., poor accessibility of a proportion of the binding sites. The limitation does not seem to be with the technique of CEC itself, since much better efficiencies can be achieved with other chiral selectors such as β-cyclodextrins [59]. Another difficulty in CEC is the low ionic-strength buffer used to avoid bubble formation; this favors electromigration dispersion of charged analytes. The use of pressurized systems [6] may help circumvent this problem.

As described in Eq. (2), retention can be related to the amount of protein used in the analytical system. With protein additives, the concentration used is often limited to <100 μM for reliable operation [28], although when using partial-filling techniques the use of higher concentrations is common [48]. With in-situ cross linking around 250 μM is possible [51], and in CEC the amounts may even be higher [55,60]. The rather low capacity of HPLC protein phases is one of the limitations of their use, and clearly with proteins in solution there is a similar potential for problems because of the low concentration of dissolved selector.

Since one would expect that the underlying ligand–protein interactions would be the same no matter what separation technique is employed, it is likely that qualitative and even quantitative similarities exist between separations developed using a given protein in EKC, CEC and HPLC. It is worth emphasizing that for this to be the case, one must really be comparing similar systems. This may not always be so, and an example is the popular Chiral-AGP HPLC stationary phase which uses a cross-linked AGP [12] which has rather different properties to the native protein. More subtle alterations also have the potential to cause variations between separations; for example, the binding properties of albumin vary significantly depending on the method of preparation used [61]. Notwithstanding such differences, it can generally be said that on a qualitative basis separations performed in CE or in HPLC tend to mirror the binding observed with the free protein. Exceptions occur, an example being the analysis of phenothiazine derivatives such as propiomazine. The resolution of its enantiomers can be achieved using HSA as a buffer additive [28], but in HPLC with a HSA stationary phase these analytes have extremely high retention and poor peak shape [62]. The difference comes about in part because of adsorption of basic solutes to the silica support.

The difference in protein concentration in different formats of CE and LC has a direct impact on k'. We have recently shown that in fact quantitative comparisons between EKC and HPLC can be made, relating the capacity factors measured in each technique via the effective selector concentration [60,62],

$$\frac{k'_{LC}}{k'_{EKC}} = \frac{K_{LC}[P]_{eff}}{K_{EKC}[P]} \quad (3)$$

where the subscripts LC and EKC refer to the technique used, and $[P]_{eff}$ is the effective selector concentration in HPLC, determined as the number of moles of available protein in the column divided by the column void volume. Under similar operating conditions $K_{LC}=K_{EKC}$, and so the ratio of the capacity factors is equal to the ratio of selector concentrations. These measurements were made using racemic benzoin as a test analyte and HSA as a protein selector, but similar results have been obtained using cyclodextrin-based systems [63], indicating the generality of the relationship. The relatively high concentration of protein typically found in HPLC phases results in the necessity to use significant quantities of organic modifiers or specific competitors in order to reduce retention to acceptable values. In contrast, CE separations using protein selectors are often performed without modifiers since the low selector concentration will guarantee a reasonably low k'. At high modifier concentrations which are more often found in HPLC there may be significant alterations in the protein conformation [39,64].

4. The "molecular sponges", albumins and AGP

Albumin and AGP are ubiquitous proteins, akin to

molecular sponges with the ability to bind and transport a huge variety of ligands, generally with low to moderate affinity (Section 2.1). Still, even though binding is often not very strong, this does not mean that there are not subtle differences in affinity between structurally-related ligands such as enantiomers. The success of these molecules as chiral stationary phases described above attests to that fact. Because of their similar biological roles, they will be considered together in this section. Almost all of the examples in the literature involve the separation of stereoisomers.

Both BSA and HSA have been used as selectors in CE. Examples are given in Table 2. It can be seen from the Table that these proteins have been used at near-neutral pH values, under which conditions they are strongly negative (p$I \approx 4.7$, and a net negative charge of around -15 [61]). Despite this negative charge they have a tendency to adsorb to plain fused-silica capillaries under the conditions commonly employed, and suitable washing procedures are recommended in Section 2.1. HSA has a mobility of around $-1.8 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1} at pH 7 [28], and the value for BSA is similar. Thus, under the conditions for the separations reported in Table 2 using these proteins, it should be fairly easy to achieve resolution if the analytes are cationic or neutral, and more difficult for the resolution of anions, particularly those with mobilities similar to that of the protein. In a way this is unfortunate, since albumins are primarily responsible for the transport of anionic species in vivo (see Section 2). The use of replaceable polymer networks (e.g., dextran) to modify the protein mobility has allowed the resolution of chiral compounds such as ibuprofen which are difficult to separate in plain EKC with albumins [57].

It is interesting to note that almost invariably phosphate buffers have been used, as is common in HPLC. pH is usually the most important variable, but also the effects of the buffer type and the ionic strength of the solution should be considered. At least with certain proteins, changes or loss of selectivity may be observed at low (e.g., <10 mM) phosphate concentrations [55]. Relatively high ionic strengths are also favored from a viewpoint of good pH control and to avoid pH changes due to buffer depletion. This can sometimes be a problem with phosphate which has a high conductivity: either the field strength has to be limited to rather low values, or else significant Joule heating will occur. Biological buffers with low conductivity offer an alternative. Although there is a limited literature on the effect of these buffers on the binding properties of proteins in CE [65], in general the effects of changing buffer type are relatively minor compared to changing pH.

From Table 2, it can be seen that while some workers have employed organic modifiers with albumin selectors, others have not. As explained in Section 3.3 above, both the selector concentration and the modifier concentration play a role in determining retention, and the use of a modifier is often necessary when the affinity of the analyte for the protein is high. In general, acetonitrile has the least affect on electromigration [70], and thus is a reasonable choice to use as a modifier. However, specific selectivity effects may be ascribed to other modifiers, e.g., with 1-propanol [39], perhaps due to its strong denaturant effect [64]. With protein selectors another option is to use specific competitors to alter selectivity, as is often done in HPLC (see Section 2). The feasibility of this approach has also been demonstrated in CE [28,66,68].

AGP is more acidic (p$I \approx 2.7$) than albumin, and is primarily responsible in vivo for the transport of cationic ligands (Section 2). Despite the success of the AGP column in HPLC, there has been relatively little interest in this protein as an additive in CE. The separation of neutral or basic compounds is favored, both from the viewpoint of the protein's natural characteristics, and also, in free solution, for reasons of maximizing mobility differences between selector and analyte. Being significantly more acidic than the albumins, it can readily be used at lower pH values while still maintaining a strong negative charge on the protein. This means that there is some flexibility in the use of pH as a variable to control selectivity with using AGP.

In CEC, disopyramide enantiomers were resolved using AGP [55]; this solute is also separated with excellent selectivity by the same phase in HPLC. However, using AGP as a buffer additive, no separation was seen [26]. This is difficult to explain since the free protein is known to stereoselectively bind disopyramide [8]. Modifiers such as 1-propanol and N,N-dimethyloctylamine (which are used with the AGP phase in HPLC) were seen to have quite

Table 2
Uses of albumins and AGP as selectors in CE

Protein	Mode of Analysis	Analyte(s)	Typical BGE composition	Reference
BSA	Gel	Tryptophan	50 m*M* phosphate, pH 7.5	[51]
BSA	EKC	Leucovorin	20 m*M* phosphate, pH 7.2	[25]
BSA	EKC	Tryptophan, benzoin, warfarin	67 m*M* phosphate, pH 7.4 and some organic modifiers	[26]
BSA	EKC	Porphyrin isomers	20 m*M* phosphate, pH 7.6	[46]
BSA	EKC-replaceable polymer network	Leucovorin, ibuprofen, dansyl-amino acids	10 m*M* phosphate, pH 7.12 and dextran	[57]
BSA	EKC-replaceable polymer network	Leucovorin	10 m*M* phosphate, pH 7.12 and dextran	[58]
BSA[a]	EKC	Ofloxacin and tryptophan	20 m*M* phosphate, pH 7.5 and specific competitors	[65]
BSA	EKC	Ofloxacin and a derivative	100 m*M* phosphate, pH 8	[66]
BSA	EKC	Homochlorocyclzaine, oxyphencyclimine, propranolol, trimebutine	50 m*M* phosphate, pH 6	[48]
BSA	EKC	DNP–amino acids	20 m*M* phosphate, pH 7.1 and 1-propanol	[67]
BSA	EKC-replacable polymer network	Folinic acid, mandelic acid, N-benzoylalanine	10 m*M* phosphate, pH 7 and specific competitor additives	[68]
BSA	EKC	Pantoprazole and related sulfoxides	10 m*M* phosphate, pH 7.4 and 1-propanol	[69]
HSA	EKC	Benzoin, promethazine, propiomazine, thioridazine	50 m*M* phosphate, pH 7 and specific competitor additives	[28]
HSA	EKC	Tryptophan	25 m*M* phosphate, pH 7.4	[43]
HSA	CEC	Oxazepam, temazepam	4 m*M* phosphate, pH 7 and various organic modifiers	[56]
HSA	EKC-replacable polymer network	Benzoin, promethazine, propiomazine, thioridazine	50 m*M* phosphate, pH 7 and dextran	[56]
HSA	EKC	Benzoin, propiomazine	50 m*M* phosphate, pH 7 and organic modifiers	[39]
AGP	EKC	Benzoin, warfarin, pindolol, promethazine, disopyramide	67 m*M* phosphate, pH 7.4 and 1-propanol or N,N-dimethyloctylamine	[26]
AGP	CEC	Benzoin, hexobarbital, ifosfamide, alprenolol and others	4 m*M* phosphate, pH 6.8 and organic modifiers	[55]
AGP	EKC	DNP–amino acids	20 m*M* phosphate, pH 7.1	[67]
AGP	EKC	Clorprenaline	50 m*M* phosphate, pH 4	[48]

[a] Albumins from other species were also used, as well as some derivatized albumins including glucosamide-BSA and acetyl-BSA.

specific effects on different solutes in EKC, indicating a specific role at the relevant binding sites, as is observed in HPLC [12].

5. Other protein selectors

Various proteins other than those already men-

tioned have been employed for the separation of enantiomers in CE, mainly as BGE additives in free solution: ovomucoid (OVM) [26,48,67,71], cellobiohydrolase (CBH I) [47,52], fungal cellulase [26], conalbumin (CON) [48], avidin (AVI) [72], casein (CAS) [67], human serum transferrin (HST) [73,74], riboflavin binding protein (RfBP) [75] and BLG [17]. The physico-chemical characteristics of these proteins are summarized in Table 1, and their uses in CE are summarized in Table 3.

As some of these proteins are expensive and furthermore in some cases not even commercially available, the CE approach is of particular advantage because only a small amount of material is required. A wide range of selector concentrations have proved to be effective with these proteins for successful enantioresolution. Since detection problems can occur with high protein concentrations, several researchers [47,48,73,74] used partial filling methods (Fig. 2 Section 3.1.2). The use of coated capillaries is frequently encountered [47,48,71–74], with the aim of reducing electroosmotic flow and preventing

Table 3
CE separations using protein selectors other than albumins and AGP

Protein	Mode of analysis	Separated enantiomers	BGE composition	References
AVI	EKC	Vanilmandelic acid	50 m*M* KH_2PO_4, pH 4.0	[72]
	EKC	Warfarin, flurbiprofen, ibuprofen, ketoprofen, leucovorin	50 m*M* KH_2PO_4, pH 6.0 10% ethanol, methanol or 2-propanol	[72]
BLG	EKC	–	–	[17]
CAS	EKC	DNP–amino acids	20 m*M* phosphate pH 7.1	[67]
CBH I	EKC	Propranolol, pindolol,	400 m*M* NaH_2PO_4, pH 5.1, 20–25% 2-propanol	[47]
	GEL	metoprolol, alprenolol	50 m*M* KH_2PO_4, pH 6.8, 1% 2-propanol	[52]
	EKC	Labetalol	400 m*M* NaH_2PO_4, pH 5.1, 30% 2-propanol	[47]
	GEL	Atenolol	50 m*M* KH_2PO_4, pH 6.8, 1% 2-propanol	[52]
CON	EKC	Trimetoquinol	50 m*M* KH_2PO_4, pH 7.0	[48]
Fungal cellulase	EKC	Pindolol	50 m*M* KH_2PO_4, pH 7.0	[26]
OVM	EKC	Chlorpheniramine	50 m*M* KH_2PO_4, pH 5.0, 8% 1-propanol	[48]
	EKC	Chlorpheniramine	10 m*M* KH_2PO_4, pH 5.0, 9% 2-propanol	[71]
	EKC	Tolperisone	50 m*M* KH_2PO_4, pH 5.0, 10% 2-propanol	[48]
	EKC	Tolperisone	10 m*M* KH2PO4, pH 5.5, 0.1% HPC	[71]
	EKC	Pindolol	50 m*M* KH_2PO_4, pH 5.0, 8% ethanol	[48]
	EKC	Arotinolol, oxyphencyclimine	50 m*M* KH_2PO_4 pH 5.0, 6–8% 2-propanol	[48]
	EKC	Primaquine, trimebutine	50 m*M* KH_2PO_4, pH 5.0, 8% 1-propanol	[48]
	EKC	Benzoin	10 m*M* KH_2PO_4, pH 7.0	[71]
	EKC	Eperisone	10 m*M* KH_2PO_4, pH 5.0, 5% 2-propanol	[71]
	EKC	DNP–amino acids	20 m*M* phosphate, pH 7.1	[67]
RfBP	EKC	Nicardipine, bepridil, oxazepam,	50 m*M* NaH_2PO_4, pH 6.5–7.0	[75]
		lorazepam, practolol, verapamil, amlodipine	1% methanol	
HST	EKC	Bupivacaine, propranolol, promethazine	200 m*M* MES pH 6.0	[74]
	EKC	Tryptophan esters	100 m*M* MES pH 6.0	[73]

protein adhesion to the capillary walls. Those authors using fused-silica capillaries [17,22,67,71,75] generally specified a detailed optimum between-run rinsing procedure in order to obtain reproducible results.

In most cases a systematic study on the optimization of the operating parameters, namely pH of the BGE, selector concentration, temperature, voltage and percentage of organic modifiers was performed. In spite of the high ionic strength and conductivity, phosphate solutions were often chosen for the buffer in which the selector was dissolved. There are obvious advantages to using the same buffer type over a range of pH, and so phosphate is often used at the limit of its buffering range where buffer capacity is limited (Table 3).

As most of the proteins used in CE have already been described as HPLC phases, some authors [17,26,47,71,75] have taken a unified approach and investigated whether it is possible to directly compare the results obtained using these proteins in each technique, in some cases both qualitatively and quantitatively [17,75].

Busch et al. [26] first described the use of fungal cellulase and OVM as chiral selectors in CE. The fungal cellulase used in this study was derived from a different organism (*Aspergillus niger*) than that of CBH I (*Trichoderma reesei*) which is the cellulase employed in the HPLC phase [10], and thus comparison with HPLC separations are probably not useful. By using a fused-silica capillary under neutral conditions (pH 7.4) and with a fungal cellulase concentration of 20 m*M*, the enantiomers of pindolol were resolved. Some explorative experiments were carried out with OVM by varying the protein concentration (up to 174 μ*M*), the pH value (in the range 6–8), and the type and concentration of organic modifier. Despite the fact that OVM is a HPLC chiral stationary phase with wide utility, none of the acidic, basic or neutral compounds tested was enantioseparated, although a few test solutes showed some interaction with the protein.

Further experiments were subsequently carried out with OVM in CE [48,67,71], and successful separations were obtained for many compounds. Ishihama et al. [71] achieved optical resolution of some drugs by optimizing the concentrations of both OVM and organic solvents. The addition of a zwitterion (*o*-phosphorylethanolamine) adjusted with phosphate, led to an improvement of the peak shape although this additive worsened the day-to-day reproducibility as it is unstable at room temperature. A dynamically coated capillary obtained by addition of 0.25% hydroxypropylcellulose (HPC) to the BGE, significantly improved the relative standard deviation (R.S.D.) of the migration times. As an increase in the OVM concentration (up to 250 μ*M*) resulted in a decrease in the apparent mobilities of the isomers with consequent peak tailing but enhanced resolution, a combination of high selector concentration and percentage of organic modifier to optimize resolution and peak shape was suggested. The enantiomeric separation of racemic eperisone, an antispastic agent, is shown in Fig. 4. Wistuba et al. [67] also described OVM as a BGE additive in CE. High concentrations of protein (up to 600 μ*M*) were employed for the enantiomeric resolution of some DNP (2,4-dinitrophenyl) amino acids with a fused-silica capillary. The derivatization of the analytes aided detection. In the same paper the use of another protein, CAS (extracted from goat milk), was also reported for the first time as a chiral selector. A concentration close to 1 m*M* allowed the separation of DNP–glutamic acid and DNP–proline. Despite the baseline disturbances due to the very high protein concentration, a good separation was obtained.

High selector concentrations were generally described when using partial-filling methods [47,48,73,74]. This technique was first introduced by Valtcheva et al. [47] to perform separations with

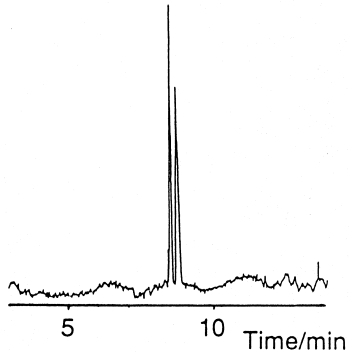

Fig. 4. Resolution of the enantiomers of epirisone by CE using ovomucoid as a chiral selector. BGE, 10 m*M* phosphate, pH 5.0, with 250 μ*M* ovomucoid, 5% (v/v) 2-propanol. Capillary, 57 cm (50 cm effective length)×75 μm I.D.; *V*=18 kV (reproduced from Ref. [71]).

CBH I. By using a coated capillary they resolved the enantiomers of some β-blockers under conditions that are unusual for EKC, namely high ionic strength (up to 400 m*M* phosphate buffer), a high concentration of CBH I (625 μ*M*), a high proportion of organic solvent (up to 30%, v/v) and relatively low voltage (10 kV) to minimize Joule heating. They also introduced a 2–3 mm long agarose plug at one end of the capillary to prevent any hydrodynamic flow. The addition of 2-propanol was important in improving peak symmetry, just as it was in the separation shown in Fig. 1. An HPLC/CE comparison with CBH I was attempted for all the compounds tested, using the same BGE/mobile phase with CBH I used in solution and immobilized on a solid matrix. However, in this case no correspondence was observed between the results.

The use of CBH I was also described by Ljungberg and Nilsson [52] in a protein gel approach based on copolymerization of proteins. The addition of BSA to CBH I was necessary in order to obtain a stable gel. Some β-adrenergic blockers were analyzed. The organic modifier proportion used in this system was limited to 25% (v/v) 2-propanol, as with greater proportions of modifier the gel became unstable. Successful separations were obtained and in Fig. 5 the electropherogram of pindolol is reproduced. The relatively high concentration of protein present in the gel-filled capillary allows a relatively high loading of the analyte (up to 1 m*M* with acceptable resolution of the enantiomers).

The partial filling technique in open tubes was described by Tanaka and Terabe [48] whose account detailed the use of OVM and CON (amongst others) as chiral selectors in CE for the separation of various basic racemates using a capillary coated with linear polyacrylamide. The method was successfully performed automatically with detection at low wavelengths (210 or 220 nm) which resulted in improved sensitivity for the analytes. OVM and CON concentrations were 500 μ*M*. In contrast to CON, with OVM most analytes could not be detected without the addition of organic modifiers to the separation solution. Using CON, only the enantiomers of trimetoquinol were separated.

The highest protein concentration used in EKC so far with the partial filling technique was described by Kilar and Fanali [73] and Kilar [74] who introduced the use of HST in CE. A low ionic strength buffer, 2-(N-morpholino)ethanesulfonic acid (MES) was employed at pH 6.0 which corresponds to the protein p*I*, with a resultant essentially stationary protein zone. Providing that HST does not aggregate, concentrations up to 2.6 m*M* were described for the separation of tryptophan esters [73] and of some drug racemates [74], with a coated capillary. Systematic studies were performed and the concentration of both HST and MES, length of HST and sample zone, applied voltage and temperature were optimized. As the diferric transferrin solution did not give any separation, only iron-free transferrin isoforms were used in these experiments and it is hypothesized that the hydrophilic iron binding site is partially responsible for stereoselective interaction with the solutes. As the length of tryptophan ester alkyl chains influence the enantioseparation, other stereoselective binding sites may be anticipated to have a pronounced hydrophobic nature. A very good separation of bupivacaine enantiomers was obtained with HST [74], showing a surprisingly good baseline, in spite of the very high protein concentration (Fig. 6).

Tanaka et al. [72] described the use of AVI, which has proved to be a powerful selector for the separation of acidic enantiomers. The use of a coated capillary was necessary as AVI is a basic protein and tends to be strongly adsorbed onto the capillary walls by electrostatic attraction. The pH was optimized by considering the pK_a value of the analyte. In order to overcome poor peak shapes and band broadening, the effect of temperature and of type of organic modifier were investigated. Best peak shapes were obtained at 35°C, and at least a 25 μ*M* AVI solution was necessary to observe chiral resolution of the racemic analytes tested, namely antinflammatory profens, vanilmandelic acid and leucovorin. However, protein concentrations higher than 25 μ*M* resulted in detection problems.

We extracted RfBP from quail egg white and tested it either as a BGE additive in EKC [75] or as an HPLC phase [16]. A plain fused-silica capillary was found to be adequate for this study and a between-run rinsing with SDS helped to remove adsorbed protein and to prevent capillary blockages as previously noted [45]. The CE experiments were planned on the basis of findings and ideas originated

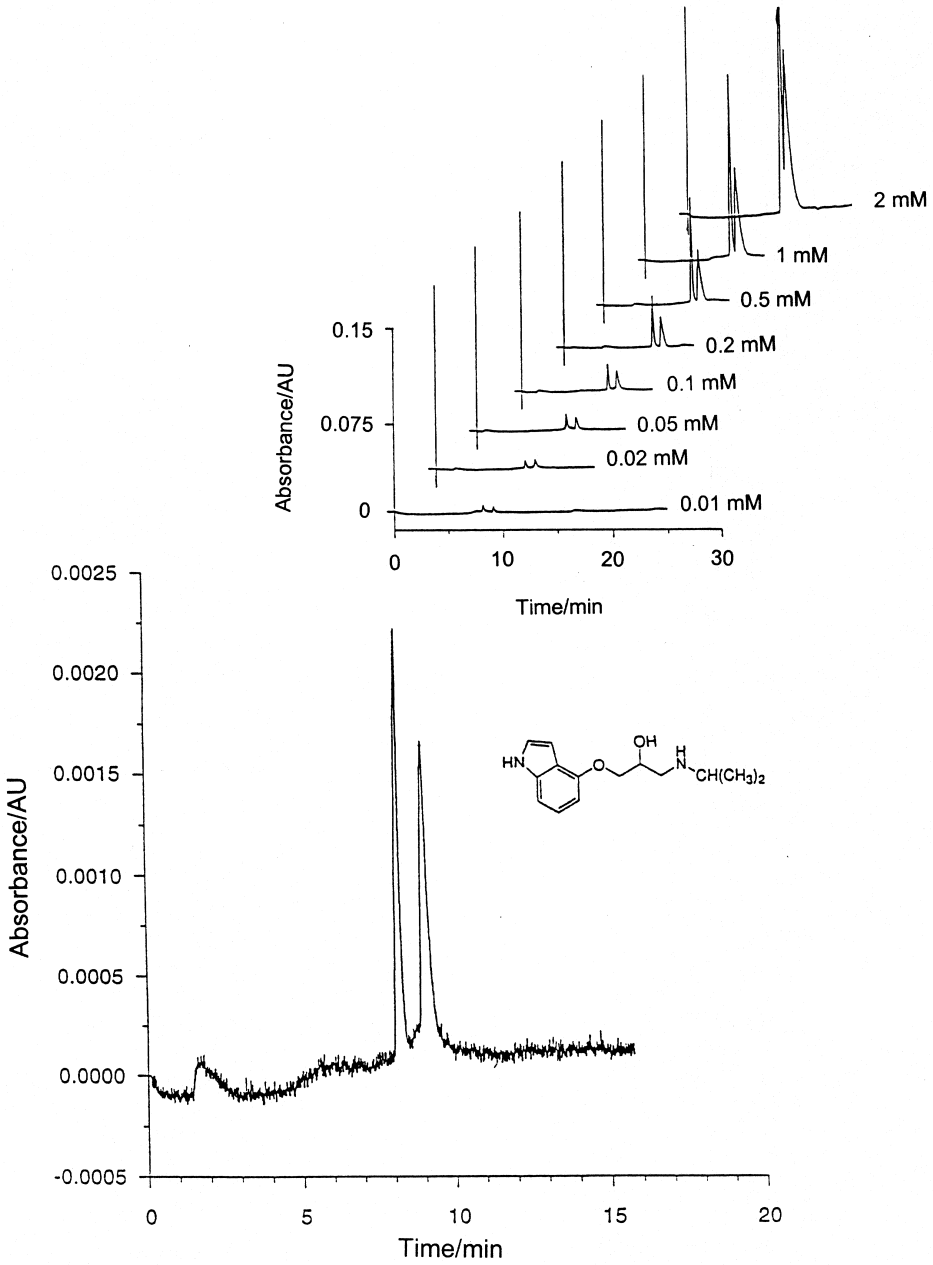

Fig. 5. Capillary gel electrophoresis separation of enantiomers of *rac*-pindolol using a cross-linked cellulase–BSA gel. Gel length, 26.5 cm, total length, 23.5 cm (75 μm I.D.). BGE, 50 m*M* phosphate, pH 6.8 with 1% (v/v) 2-propanol; $V=3.5$ kV. The insert shows the effect of sample loading on the separation (reproduced from Ref. [52]).

in HPLC in order to evaluate whether CE could be used as a rapid scouting technique for screening the enantioselectivity of novel proteins. The same batch of purified protein was used for both the HPLC column and the BGE solution so as to ensure homogeneity of the proteinaceous matrix in each type of experiment. One of the HPLC mobile phases used was chosen as a CE BGE with quail RfBP as an

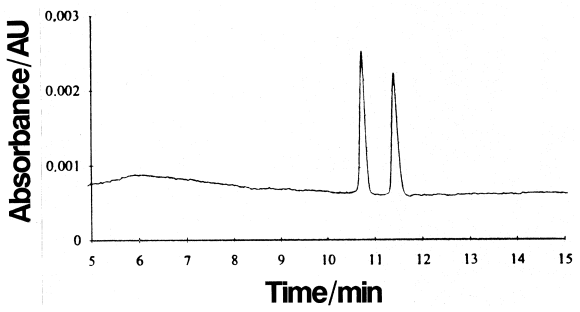

Fig. 6. Separation of bupivicane enantiomers using HST as a BGE additive in partial-filling mode. BGE, 200 m*M* MES, pH 6 with 200 mg ml^{-1} HST. Capillary, 27 cm (23.5 cm effective length)× 50 μm I.D., coated with polyacylamide; *V*=10 kV (reproduced from Ref. [74]).

additive. Because of the relatively low concentration of protein used in the CE experiments (30 μ*M*) and the moderate degree of retention for most of the compounds in HPLC, the proportion of organic modifier (methanol) used in the mobile phase (5%, v/v) was limited to 1% (v/v) in the CE experiments. The electropherogram of bepridil is depicted in Fig. 7. Data obtained with the two techniques are comparable and in particular the correspondence of the retention (k') and enantioselectivity (α) parameters make possible a real prediction of the HPLC results from the explorative experiments carried out in CE (Section 3.3 Eq. (3)). RfBP was also extracted and purified from chicken egg yolk to make a HPLC phase [16] and to test it as a chiral selector in EKC.

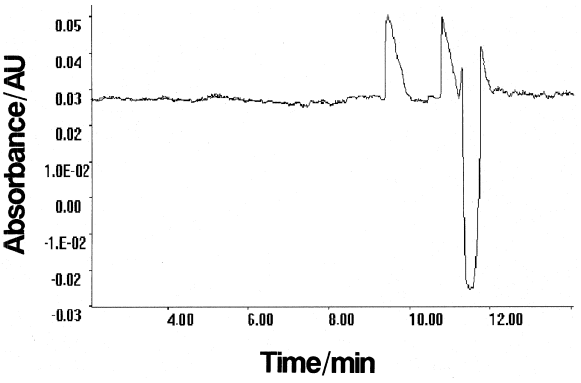

Fig. 7. Separation of bepridil enantiomers by CE using quail RfBP. BGE, 50 m*M* phosphate, pH 7 with 30 μ*M* quail RfBP, 1% (v/v) methanol. Capillary, 72 cm (50 cm effective length)×50 μm I.D.; *V*=30 kV.

Benzoin was separated in both techniques with comparable α values with a mobile phase/BGE composed of pure phosphate buffer (pH 7.0).

We also investigated BLG both as an HPLC phase and as an additive in EKC [17]. This protein was thought to be promising as it is an AGP homologue, having a similar amino acid sequence and disulfide bond arrangements. Contrary to expectations and despite the variety of conditions tested, BLG did not show any enantioselectivity with either technique, and possibly the groups involved in the stereoselective interactions could be sited in a restricted segment of the peptide chain with a different sequence to AGP rather than on the largely homologous part. Both in free solution (EKC) and immobilized (HPLC), the BLG has only weak interactions with the majority of the analytes tested, and the correspondence of the results from each technique further illustrate the strong similarities in the underlying ligand–protein interactions.

6. Investigation of ligand–protein binding

Since this review is primarily devoted to issues of selectivity in CE, the topic of ligand–protein binding studies will only briefly be mentioned here. For detailed reviews see e.g., Refs. [76–78]. However, binding measurements are worthy of some discussion here since an understanding of the binding processes is useful in understanding and developing a separation, and many publications have been devoted to the use of CE to explore ligand–protein interactions.

Affinity electrophoresis methods have long been used for determining ligand–protein binding. Typically a ligand is immobilized into a gel matrix, and its affect on the migration of a protein determined; this can then be related to the binding constant [2]. In CE, mobility-shift methods mirror this approach, except that in general the whole process takes place in free solution (no support). The ligand can be dissolved in the BGE, and the selector is the analyte [79]. Alternatively, the selector can be present in the BGE, while the ligand (or ligands) is used as analyte [25,28–34]. Both experimental arrangements should give similar results, although there are some practical points to be borne in mind. The binding must result in an alteration of the mobility of the analyte. With

the protein as analyte, binding of a charged ligand will likely result in a change in its mobility, while binding of a small, neutral molecule will probably not cause an observable change in mobility. Using the protein as a BGE additive will result, upon binding, in a significant change in the mobility of both neutral species and charged species (which have initially a quite different mobility to the protein). In the analysis of a racemic chiral species binding to a protein, one has the advantage when using the protein in the BGE that enantioselectivity in the binding will be revealed by separation of the stereoisomers—thus both the separation and binding measurement are carried out in one step, and individual enantiomers are not needed [25,28].

To determine the affinity constant, one usually varies the concentration of the component present in the BGE, and the effect of this variation on the analyte mobility is measured (in CE it is generally advisable to base calculations on changes in effective mobility rather than migration times, since the migration time is the sum of both electrophoresis of the analyte and EOF). The data on analyte mobility as a function of selector concentration can then be treated in a variety of ways. One can either use a non-linear curve-fitting procedure to Eq. (2) to obtain affinity constants for a simple one-to-one binding interaction [28,30,31], or one can use some type of linear transformation before data fitting [76,78]. Each approach has its merits, and which is the most appropriate treatment for CE data has been a point of discussion [80,81]. It has been suggested that the non-linear fitting method is unreliable because the limiting value of the ligand–protein complex mobility (μ_{effC}) is difficult to determine [80], however the experience of others is that this approach can give binding affinities from CE data which are in accordance with other techniques [28,37], or which are consistent with data obtained after linear transformation [81]. The linear methods are convenient in the sense that they are amenable to graphical analysis (although this is less important with widespread accessibility of powerful microcomputers) and an appropriate linearization may reveal the suitability of the chosen model [81].

A few points are worth stressing when discussing the estimation of binding affinities from CE data. The derivation of Eq. (2) assumes a monovalent equilibrium interaction with kinetics which are rapid relative to the separation time. It should be emphasized that [P] in Eq. (2) is the free protein concentration. When interactions are quite weak one can often approximate this to the total concentration of the selector [30], since the selector is present in large excess over the analyte. This is often not the case with proteins, and either the free concentration should be calculated [28], or at least the degree of error introduced by the approximation of free and total protein concentration can be estimated [62]. Since typically only a short portion of the capillary is filled with the analyte solution (usually $<2\%$ of the capillary length), it should only take a small fraction of the separation time for equilibration of the analyte with the selector in the analyte zone, and so this potential source of error is generally ignored. Where relatively weak analyte–selector affinities require moderate or high concentrations of selector, the addition of the selector can have a significant effect on the solution viscosity and this must be accounted for in any calculation by normalizing data to a standard state, e.g., to the viscosity of the BGE solution without any additive [30,81]. CE instruments with well-controlled pressure or vacuum functions can be used as capillary viscometers to determine changes in relative viscosity [30]. With proteins used at low concentrations the viscosity change upon addition of the selector is quite small [28].

The use of co-additives with specific binding properties is useful not only to modify selectivity, but also for the investigation of ligand–ligand interactions, and to define the specific site on a protein responsible for a given binding interaction [28,66,68]. A direct competition should be revealed by a steady decrease in binding of the analyte as the competitor is added to the BGE. On the other hand, an allosteric interaction is revealed by a change but not necessarily an elimination of binding, as binding of one compound causes a change in the protein's conformation which affects the binding of a second ligand. An example is given in Fig. 8, in which results are presented from both CE and HPLC experiments designed to investigate the binding of tamoxifen to HSA [82]. In the CE experiment, the mobility of tamoxifen is measured as a function of the concentration of a competitor (nitrazepam) in the

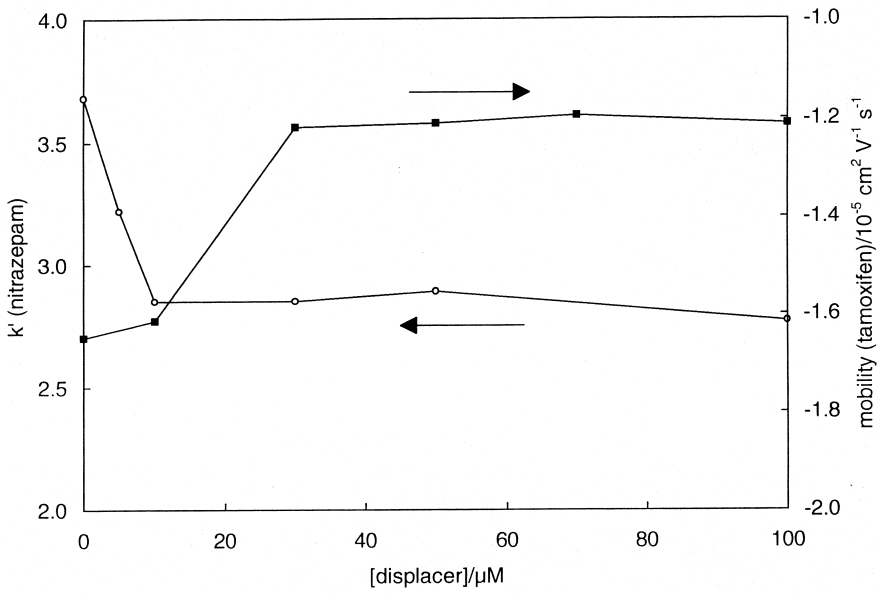

Fig. 8. Use of HPLC with a HSA stationary phase (○) and CE with HSA as a BGE additive (■) to investigate the binding interactions of nitrazepam and tamoxifen with HSA. Arrows indicate which *y*-axis should be read with each line. In HPLC, nitrazepam is used as analyte (tamoxifen is too retained), and tamoxifen is used as an additive to the mobile phase. In CE, the tamoxifen is used as the analyte, and nitrazepam is added into the HSA-containing BGE. Both experiments reveal an allosteric interaction between the two solutes.

BGE. In HPLC, the large quantity of HSA immobilized on the column gives rise to excessively long retentions for tamoxifen (Eq. (3)), and so nitrazepam is used as the analyte and its k' is measured as a function of tamoxifen concentration in the mobile phase. Although the roles of tamoxifen and nitrazepam are reversed in CE and HPLC, the experiments are complimentary. Both reveal an interaction between the two drugs binding to HSA; k' for nitrazepam in HPLC drops initially upon addition of tamoxifen but then stabilises, while in CE the tamoxifen mobility reduces initially upon addition of nitrazepam, but is unaffected upon addition of concentrations of nitrazepam ≈ 20 μM or greater. If the interactions were occurring at the same site, a continued reduction in retention would be expected upon further addition of competitor. As with the determination of affinity constants, an attractive feature of using CE is that the whole process takes place in solution without the need for immobilization of any of the components.

By way of comparison, it should be emphasised that there are other CE options apart from the various mobility-shift methods for determination of drug–protein binding; particularly elegant examples are the use of CE with frontal analysis for the determination of non-enantioselective [83] and enantioselective [84] drug–protein binding. In this method, the protein and ligand are mixed together as sample, and a large aliquot is injected onto the CE system. The separation is arranged such that the protein and ligand migrate in opposite directions. The ligand which migrates out of the injected sample plug is in equilibrium with the protein, i.e., its concentration is determined by the strength of the binding interaction. Thus, the plateau height which can be measured when the zone containing the free ligand arrives at the detector is proportional to the concentration of the free ligand. A series of experiments with different ligand–protein ratios allows a Scatchard analysis to be performed for the determination of the binding constant.

7. Conclusions

A wide variety of protein selectors have been used in CE. The vast majority have demonstrated selec-

tivity towards the stereoisomers of small molecules, mostly drugs and drug candidates. In the simplest experiments, the protein is simply dissolved in the BGE. Detection problems can be circumvented by the use of partial-filling methods, although the range of possible operating conditions is somewhat more limited.

Various immobilization schemes offer alternatives for the use of proteins in CE. From the published separations, protein gels appear to offer excellent analytical characteristics and, with only partial filling of the capillary, avoid detection problems.

There is still considerable interest in developing biomolecules as selectors, whether they be peptides such as teicoplanin [85], proteins as described here, or oligo- and polysaccharides [86]. However, with the success of cyclodextrins as chiral selectors in CE, it is difficult to predict the future role of protein selectors. In HPLC one of the main attractions of protein phases such as BSA and AGP are their wide range of application but in CE a few cyclodextrins can be used to answer many chiral separations problems. As an analytical selector, proteins may be limited to playing a supporting role, providing unique selectivity for certain analytes. Although hardly any non-chiral applications have been reported for protein selectors in CE, their ability to discriminate between very similar molecular structures suggests that they may have some utility in this area. The investigation of ligand–protein binding and ligand–ligand interactions in solution is an interesting application, and it is possible that exploring the selectivity of proteins will be a significant application of CE.

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