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Review

Separation of drug enantiomers by liquid chromatography and capillary electrophoresis, using immobilized proteins as chiral selectors

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Abstract

Proteins display interesting chiral discrimination properties owing to multiple possibilities of intermolecular interactions with chiral compounds. This review deals with proteins which have been used as immobilized chiral selectors for the enantioseparation of drugs in liquid chromatography and capillary electrophoresis. The main procedures allowing the immobilization of proteins onto matrices, such as silica and zirconia particles, membranes and capillaries are first presented. Then the factors affecting the enantioseparation of drugs in liquid chromatography, using various protein-based chiral stationary phases (CSPs), are reviewed and discussed. Last, chiral separations already achieved using immobilized protein selectors in affinity capillary electrochromatography (ACEC) are presented and compared in terms of efficiency, stability and reproducibility.

Keywords: Reviews; Enantiomer separation; Chiral selectors; Proteins, immobilized

Contents

Abbreviations: α, enantioselectivity; ACE, affinity capillary electrophoresis (protein in the running buffer); ACEC, affinity capillary electrochromatography; AEKC, affinity electrokinetic chromatography (protein in the running buffer); AGP, α_1-acid glycoprotein (orosomucoid); AV, avidin; BSA, bovine serum albumin; CBH, cellobiohydrolase; CDI, 1,1′-carbonyldiimidazole; CE, capillary electrophoresis; CSP, chiral stationary phase; DSC, *N*,*N*′-disuccinimidyl carbonate; DSS, *N*,*N*′-disuccinimidyl suberate; FTIR, Fourier-transform infrared spectroscopy; HSA, human serum albumin; *k*, retention factor; Lys, lysine group; OTCEC, open tubular capillary electrochromatography; OTLC, open tubular liquid chromatography; OVM, ovomucoid; PVDF, poly(vinylidene fluoride); RfBP, riboflavin binding protein; TC, tresyl chloride; Trp, tryptophan group; Tyr, tyrosine group; UV, ultraviolet

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1. Introduction

Many therapeutic substances bearing chiral centers are clinically administered as racemic mixtures because of difficulties in stereoselective synthesis and purification. In a symmetric environment, their isomers have nearly identical physical and chemical properties, however, in a stereospecific biological environment, such as the human body, differences in pharmacological properties, pharmacokinetic disposition and metabolic fates have often been observed [1–3]. For instance, the *S*-isomer of propranolol is 100-fold more potent as a β-blocker and is more slowly metabolized than the *R*-form [4]. Therefore, a quantitative and enantioselective study of drug-protein binding is essential for developing racemic drugs. Moreover, the enantiomers of chiral therapeutic substances may exhibit significant differences in toxicity [2]. An example, of this phenomenon is the tragedy of thalidomide in the early 1960s, since the inactive form of this sedative and antinausea preparation was responsible for teratogenic effects. At present, an increasing legislative concern has emerged in the development of chiral compounds of biological interest.

In this context, the pharmaceutical industry is interested in methods capable to rapidly and accurately determine the enantiomeric composition of chiral bioactive molecules and to probe the binding of drugs to proteins. The recent breakthroughs in chiral chromatography and more recently in chiral capillary electrophoresis have given pharmacologists and toxicologists the tools for drug development.

Several strategies have been set up for the differentiation of enantiomers by chromatography [5–7]: (i) indirect methods involving sample derivatization by a chiral reagent prior to injection onto achiral chromatography columns; (ii) direct methods using chiral eluents with standard stationary phases [8]; (iii) direct methods where chiral separations are achieved using a chiral stationary phase (CSP) [9,10].

Chiral stationary phases used in high performance liquid chromatography (HPLC) are classically subdivided into five groups according to the type of chiral selector immobilized on the support [6]. Since proteins are high-molecular mass polymers composed of several chiral subunits, their stereoselective molecular recognition has been exploited to develop protein-based CSPs [9–12]. These phases have the ability to separate a wide range of chiral compounds, especially pharmaceutically active compounds. To date, though, only a limited number of proteins have been evaluated as immobilized chiral selectors. The first part of this review will examine chiral interactions of classical protein-based CSPs and new support materials, with biologically active substances.

More recently, proteins have also received increased attention for use as stereoselective binding agents in capillary electrophoresis (CE) [13]. Electrically driven chiral separations have already been achieved by several methods [14] including (i) addition of a chiral selector to the electrolyte [15,16]; (ii) immobilization of the chiral selector onto the capillary wall [17]; (iii) inclusion of the selector in polymeric or sol–gel matrices [17,18]; (iv) packing capillaries with classical chiral HPLC supports [17]. Several chiral selectors have been employed in chiral CE. Among them, cyclodextrins and their derivatives are the most popular [15]. Proteins have also been used either as additives to the running buffer or as immobilized selectors [13,19]. In the second part of this report, the feasibility of chiral separations by capillary electrophoresis and related techniques using immobilized proteins will be discussed.

2. Immobilized protein selectors

In 1973, for the first time, it was demonstrated that the enantiomers of D,L-tryptophan could be resolved by liquid chromatography using bovine serum albumin onto agarose supports [20]. At present, owing to their high enantioselectivity, a number of proteins have been employed as immobilized chiral selectors in HPLC [10,12] (Table 1). While α_1-acid glycoprotein (AGP) [21] and crude ovomucoid (OVM) [22] probably have the broadest field of applications, numerous reports in the literature have described chromatographic separations and/or binding studies using bovine serum albumin (BSA) [23], human serum albumin (HSA) [24] and cellobiohydrolase I (CBH I) [25] as immobilized chiral selectors. All these columns are commercially available as well as avidin (AV) [26] and pepsin CSPs [27] (Table 1). It is worth noting that proteins, such as riboflavin binding protein (RfBP) [28], ovoglycoprotein (the active fraction of crude ovomucoid preparations) [29] and amyloglucosidase [30] are promising candidates for the chiral separation of basic drugs. Other chiral stationary phases using immobilized trypsin [31], α-chymotrypsin [32], conalbumin (ovotransferrin) [33] and lysozyme [34] have been described, even if their applicability is more limited.

As shown in Table 1, the number of proteins that can be used as immobilized chiral selectors in capillary electrophoresis and related techniques is more limited than in liquid chromatography. Chiral separations by open tubular capillary electrochromatography (OTCEC) with wall-adsorbed proteins (lysozyme [35], avidin [36]) and wall-grafted proteins (BSA [37], AGP [38], HSA [39]) as stationary phases have been described. Parallel to these studies, capillaries filled with BSA–dextran polymer networks [40] and crosslinked gels of BSA and cellobiohydrolase I [41–43] have been evaluated in affinity gel electrophoresis. More recently, chiral separations by capillary electrochromatography using BSA and OVM encapsulated in monolithic sol–gel matrices have been reported by Kato et al. [18]. Finally, HSA and AGP commercially available chiral stationary phases have been used in packed-capillary electrochromatography to resolve racemic mixtures [44,45].

Table 1
Proteins used as immobilized chiral selectors in liquid chromatography and capillary electrophoresis

Proteins	Molecular mass (g/mol)	pI	Liquid chromatography		Capillary electrophoresis (first report)
			First CSP reported in the literature	Commercially available CSPs	
α_1-Acid glycoprotein (orosomucoid) (AGP)	41000	2.7	[21]	CHIRAL-AGP (Chrom Tech)	[44]
	33000 [95]				
Serum albumins					
Human (HSA)	66000	4.7	[24]	CHIRAL-HSA (Chrom Tech)	[45]
Bovine (BSA)	66466	4.7–4.9	[23]	RESOLVOSIL BSA-7 (Macherey-Nagel)-ULTRON ES-BSA (Shinwa Chemical Industries)	[41]
Other type			[156]	–	–
Amyloglucosidase (glucoamylase GA1)	97000	5.0	[30]	–	–
Avidin (AV)	68000	10.0–10.5	[26]	Bioptic AV-1 (GL Sciences Inc)	[36]
Cellobiohydrolases					
CBH I	60000–70000	3.9	[25]	CHIRAL-CBH (Chrom Tech)	[42]
CBH II			[73]	–	
CBH 58			[74]	–	
α-Chymotrypsin	25000	8.1–8.3	[32]	–	–
Conalbumin (ovotransferrin)	70000–78000	6.1–6.6	[33]	–	–
Cytochrome	12400	9.3	–	–	[62]
Lysozyme	14000	11.1	[34]	–	[35]
Ovoglycoprotein	30000	4.1	[29]	–	–
Ovomucoid from chicken (OVM)	28800	3.9–4.5	[22]	Ultron ES-OVM (Shinwa Chemical Industries)	[18]
Ovomucoid from turkey			[75]	–	
Pepsin	34600	<1	[27]	Ultron ES-Pepsin (Shinwa Chemical Industries)	–
Riboflavin binding proteins (flavoproteins) (RfBP)	32000–36000	3.9–4.1	[28,93]	–	–
Trypsin	23800	10.2	[31]	–	–

3. Matrices used for enantiomeric separations

In liquid chromatography, enantiomeric separations have mainly been performed using silica-based chiral stationary phases. Lately, a porous zirconia matrix stable over the pH range from 1 to 14, was successfully evaluated by Park et al. for the preparation of BSA-coated zirconia CSPs [46]. Ideally, in order to obtain efficient separations, the amount of protein immobilized onto the support must be optimal. Therefore, a careful selection of porous materials with adequate physical properties, e.g. pore diameter and specific surface area, must be carried out [21,34,47–50]. In early studies, Hermansson evaluated 10 and 30 nm silica gels with similar surface areas (250 m^2/g) to immobilize AGP [21]. Very small amounts of protein were bound to 10 nm supports (2.5 mg/g), showing that AGP molecules (MW = 41,000 g/mol) were probably excluded from the pores. Nowadays, silica gels with large pores have fewer specific areas. Thus an increase in the pore size generally results in higher surface coverage (nmol of protein/m^2) but lower amounts of protein bound per gram of silica. For instance, ovoglycoprotein (MW = 30,000 g/mol) was immobilized onto Ultron-12 (pore diameter: 12 nm; surface area: 300 m^2/g) and Ultron-30 (pore diameter: 30 nm; surface area: 100 m^2/g) silica gels [47]. The protein surface coverages were equal to 14.4 and 23.1 nmol/m^2, respectively, demonstrating the impact of the pore size, even though the amount of protein immobilized on Ultron-12 was twofold higher than on Ultron-30 (130 mg/g versus 69 mg/g).

Polymeric matrices have also been employed to resolve racemic mixtures by liquid chromatography [51–53]. For instance, enantiomeric separations have been achieved using BSA immobilized onto perfusion chromatographic supports (POROS) [53]. These stationary phases allowed flow rates from 1 to 10 ml/min with no significant decrease in efficiency. As shown in Fig. 1, baseline separation of the enantiomers of ketoprofen was obtained in 2.5 min at a flow rate of 5 ml/min, with a resolution equal to 1.5. Cheap chi-

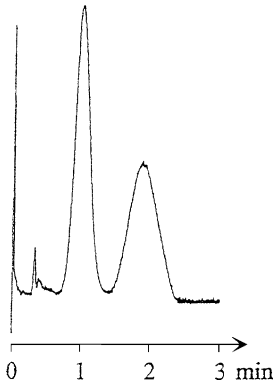


Fig. 1. Enantiomeric separation of ketoprofen on BSA bound to perfusion chromatography supports (POROS) as the chiral stationary phase. Conditions: phosphate buffer, pH = 8.0; flow rate: 5 ml/min; detection at 254 nm (reproduced from [53] with permission, no further details on detection available).

ral stationary phases using CBH I immobilized on continuous polymer beds have also been developed by Hjerten's group [51]. The most efficient polymer beds were obtained by copolymerization of allyl-modified CHB I with piperazine diacrylamide and methacrylamide. After compression of the gels in small columns, the enantiomers of practolol, a β-blocker, were resolved at 5 ml/min on these CSPs within 45 s.

Rapid chiral separations were also achieved by Nakamura et al. [54] using BSA immobilized onto polyethylene porous hollow-fiber membranes (pore size: 0.4 μm). The modules (0.5 cm diameter × 4 cm length) were incorporated into a classical liquid chromatography apparatus. Another membrane process using BSA grafted to activated Nylon membranes, was assessed for large-scale separation of enantiomers [55]. However, a rapid decrease of the enantiomeric excess as a function of time was observed. Recently, a miniaturized membrane chromatography system was developed by Wang et al. [56]. BSA-coated poly(vinylidene fluoride) (PVDF) membranes were inserted between two microfluidic poly(dimethylsiloxane) substrates. The elution of racemic mixtures was achieved at low flow rate, using a syringe pump. With such miniaturized cartridges, the consumption of solutes and solvents was minimized.

Some attempts to miniaturize conventional chiral liquid chromatography systems have also been described [37,57,58]. Beyond the advantages mentioned above, miniaturized systems can be easily interfaced with a mass spectrometer. Chiral separations using either open tubular liquid chromatography (OTLC) with protein-coated capillaries [37] or fused-silica microcolumns packed with protein-supports (BSA [57] and AGP [58]) have been achieved at low flow rates (1–2 μl/min). However, OTLC has given poor efficiency and resolution values.

For a given separation and a given wall-adsorbed protein, efficiency and resolution were generally improved by using open tubular capillary electrochromatography (OTCEC) since the parabolic flow profile observed in OTLC was replaced by a plug profile in capillary electrophoresis [37]. Polymeric matrices have also been used to immobilize proteins and resolve racemic mixtures by capillary electrophoretic techniques [40]. Recently, another approach has been developed by Kato et al. whereby proteins were encapsulated in tetramethoxysilane-based hydrogels by the sol–gel method [18]. In this procedure, a mixture of hydrolyzed silane and protein (BSA and OVM) is sucked into the capillary and allowed to stay for 3–4 days at room temperature. The resulting monolithic capillary columns have been successfully used for chiral separations.

4. Immobilization procedures

Since maintaining the natural conformation of proteins is essential for chiral discrimination, methods allowing the immobilization of proteins must be gentle and quick. To date, three classes of matrices have been employed for enantiomeric separations including silica-based materials (silica particles, fused-silica capillaries), zirconia particles and polymeric supports (chromatography stationary phases, porous hollow-fiber membranes, flat membranes). In any case, the techniques developed are mainly based on physical adsorption and covalent binding to the matrix.

Several different pathways of physical adsorption involving ionic and/or hydrophobic interactions are reported in the literature. This procedure has been employed to immobilize a number of proteins onto naked silica particles [49,57,59–61] and fused-silica capillaries [35,36,62]. In the case of negatively charged proteins (BSA, BSA fragments) [57,59–61], the immobilization was performed near their isoelectric point, at pH = 5.0, while optimal coating of silica matrices with α-chymotrypsin (pI = 8.1–8.3), lysozyme (pI = 11.1), avidin (pI = 10.0–10.5) and cytochrome (pI = 9.3) was obtained at pH values where most silanol groups are ionized (pI = 7.0–7.4) [35,36,49,62]. It was reported that cytochrome-coated capillaries could be used at least for 30 days in open tubular capillary electrochromatography, showing that the wall-adsorbed cytochrome layer was stable under the conditions of utilization [62]. However, a slight increase in the retention of enantiomers has been observed for avidin-coated capillaries, because of a slow loss of protein [36]. In liquid chromatography, chiral stationary phases obtained by physical adsorption of BSA and α-chymotrypsin onto silica particles exhibited a short lifetime [49,61]. To overcome this problem and improve the long-term stability of columns, proteins can be entrapped within the silica pores, using glutaraldehyde [63]. A similar procedure was used to immobilize BSA onto zirconia particles [46]. As for silica-gels, the amount of protein deposited on zirconia supports was maximum at pH = 4.4. However, to obtain a good enantioselectivity with zirconia-based CSPs, the adsorption had to be carried out at pH = 6.9, demonstrating that the

Table 2
Main procedures allowing the covalent binding of proteins to epoxide-, diol- and amino-derivatized silica particles (via their amino-groups)

Derivatized silica	Activation method	Proteins	First report
Epoxide-silica	–	AGP	[21]
		α-Chymotrypsin	[49]
		BSA	[71]
Diol-silica	1,1′-Carbonyldiimidazole (CDI)	BSA	[71]
		HSA	[24]
	Periodic acid oxidation	CBH I, CBH II, CBH 58	[25,73,74]
		α-Chymotrypsin	[49]
		Amyloglucosidase	[30]
	Tresyl chloride	α-Chymotrypsin	[49]
		BSA	[71]
Amino-silica	*N*,*N*′-Disuccinimidyl carbonate (DSC)	Ovomucoid (chicken and turkey)	[22,75]
		Avidin	[26]
		Conalbumin	[33]
		Flavoproteins (chicken and quail egg)	[28,78,79]
		Lysozyme	[34]
		BSA and BSA fragments	[77]
		Ovoglycoprotein	[29]
		Pepsin	[27]
	N,*N*′-Disuccinimidyl suberate (DSS)	Avidin	[82]
	Glutaraldehyde	BSA	[85]
		Other serum albumins	[86]
	Reactive polymer layer	HSA	[87]

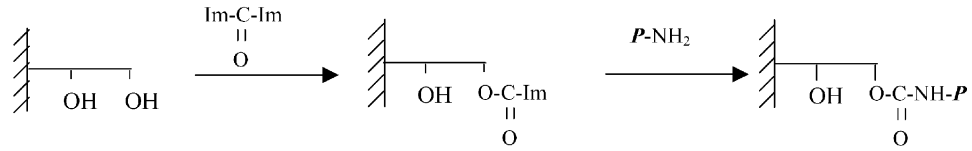
availability of BSA binding sites was different on silica and zirconia matrices.

Some attempts to bind BSA and HSA to anion-exchange silica materials [64–67] and hollow-fibers [68] have been accounted for. In a recent study, HSA was adsorbed to quaternized poly(vinylimidazole)-coated silica particles, resulting in performant chiral stationary phases [67]. It was shown by Fourier-transform infrared spectroscopy (FTIR) that the adsorbed HSA molecules were only unfolded in a small hydrophobic helix corresponding to 3% of the backbone [69]. Thus, the ionic adsorption of proteins to ion-exchange matrices can be considered as a gentle method resulting in minor structural changes. However, in some cases, optimum conditions used for enantiomeric separations may induce stability problems and require crosslinking of the adsorbed protein [68,70].

In another approach, a miniaturized membrane chromatography system was developed by Lee's group, using BSA immobilized by hydrophobic interactions on PVDF membranes [56]. The immobilization procedure resulted in a stable protein layer since no leakage of BSA was detected for at least 1 week. Chiral separations were observed under suitable elution conditions, even if adsorption techniques based on hydrophobic interactions between the protein and the support may entail significant conformational changes for the biomolecule, as it was shown recently by FTIR for HSA molecules adsorbed on reversed-phase silica particles [69].

In conclusion, an advantage of procedures using physical adsorption lies in their simplicity since the protein immobilization is generally carried out by pumping a protein solution through the column or capillary. Such methods can be employed for a quick assessment of the ability of new

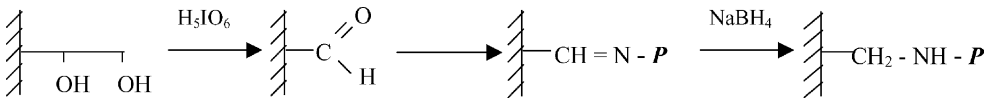
REACTION SCHEMES



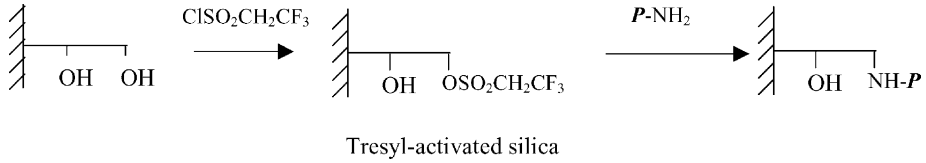
CDI activated diol-silica

Im = imidazole ring ***P*** = protein

Scheme 1. Binding of proteins to CDI-activated diol-silica.



Scheme 2. Binding of proteins to diol-silica after activation with periodic acid.



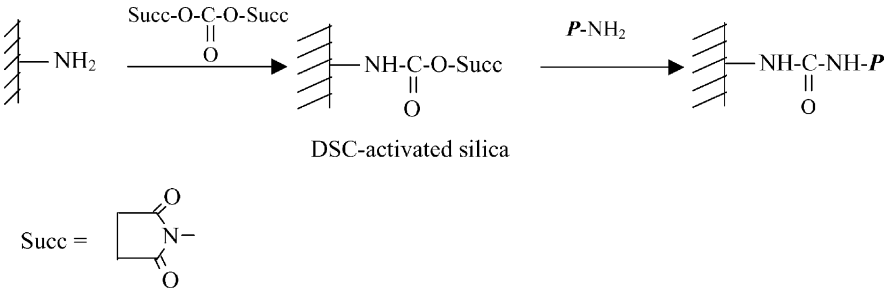
Scheme 3. Binding of proteins to tresyl-activated diol-silica.

protein candidates to perform chiral separations. However, significant stability problems may be encountered since protein molecules are linked to the matrix by weak interactions. Procedures based on the covalent linkage of proteins to the matrix are therefore preferred when long-term stability is required.

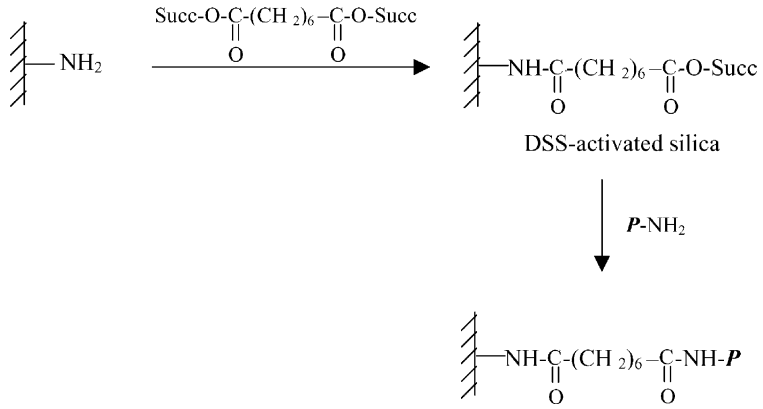
Several different pathways involving amino-, diol- and epoxide-derivatized silica materials have been employed for the covalent linkage of proteins [12]. The use of epoxide-silica gels is advantageous since the binding of proteins to epoxide functions is achieved directly, without activation of the support (Table 2). The reaction time of this method varied from 48 to 24 h whether one chose to perform the coupling in batch [21,49] or to pump the protein solution through the column (in situ method) [71]. A similar procedure has been carried out to bind AGP and HSA to the wall of silica-capillaries [38,39].

A much more rapid method has been developed by Felix, using diol-bonded silica particles activated with 1,1′-carbonyldiimidazole (CDI) (Scheme 1) [71]. The coupling of BSA and HSA to the matrix was carried out in situ in less than 4 h [24,71]. Moreover, the amount of protein immobilized per gram of silica using this method was two-fold higher than with epoxide-derivatized silica [71]. Diol-derivatized silica gels have also been employed, after oxidation using periodic acid, to prepare protein-based CSPs by reductive amination of the resulting aldehyde functions with the protein and sodium cyanoborohydride (Scheme 2) [25,30,49,72–74]. It was reported for α-chymotrypsin, that the above mentioned CSP showed a better stability but lower enantioselectivity than supports prepared by direct coupling to epoxide-silica gels. Another activation process using tresyl chloride (TC) has been tested for in situ immobilization of proteins onto diol-silica particles (Table 2) [49,71] and fused-silica capillaries (Scheme 3) [37]. The coupling reaction was performed quickly (in <30 min.) and the amounts of BSA bound to silica particles by TC and CDI activation were similar [71]. It should be underscored that for α-chymotrypsin-CSPs, tresyl chloride activation resulted in lower enantioselectivity than procedures described earlier [49], showing a possible alteration in the protein structure.

As shown in Table 2, another approach based on the activation of amino-silica gels by *N*,*N*′-disuccinimidyl carbonate (DSC) also permits the coupling of most proteins used as chiral selectors, via urea bonds (Scheme 4) [22,26–29,33,34,75–79]. But Karlsson's group reported that the stability of amyloglucosidase-chiral stationary phases prepared by this method was poor while the coupling to aldehyde-silica resulted in stable supports [30]. According to Haginaka et al., it is possible to improve the long-term stability of ovomucoid columns using an additional cross-linking reaction [80,81], paying attention to the fact that retention properties and enantioselectivity of cross-linked OVM-bonded supports depend on the cross-linking reagent used. Disuccinimidyl suberate (DSS), another more hydrophobic coupling reagent, has been evaluated for the binding of avidin to silica gels (Scheme 5) [82,83]. These avidin-based CSPs permit the direct injection analysis of drug enantiomers in plasma. Plasma proteins indeed, are excluded from the support while drug molecules



Scheme 4. Binding of proteins to DSC-activated amino-silica.



Scheme 5. Binding of proteins to DSS-activated amino-silica.

are retained by interactions with the hydrophobic DSS sublayer [82,83]. According to Oda et al., the retention and enantioselectivity on hydrophobic DSS-avidin are higher compared with those obtained on DSC-avidin columns [83], whereas poor enantioselectivity has been reported by Haginaka et al. for OVM-chiral stationary phases prepared by DSS-activation [84].

A different method has been developed to immobilize serum albumin from different animal species via stable (C–N) functions to amino-derivatized silica gels [60,85,86]. A three-step procedure resulting in stable CSPs is carried out. It involves activation of the matrix using glutaraldehyde, protein coupling via Schiff base formation and reduction by sodium cyanoborohydride.

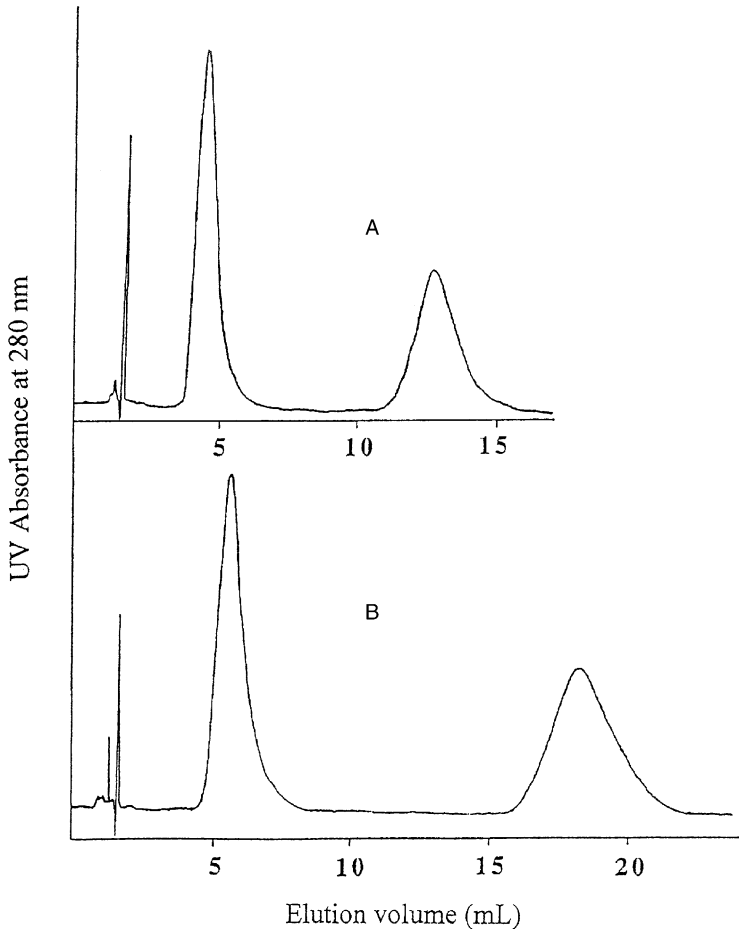


Fig. 2. Enantiomeric separation of *R,S*-oxazepam on HSA bound to polyacryloyl chloride-coated silica via (A) a classical immobilization procedure (B) polymerization inside the pores. Conditions: phosphate buffer, pH = 7.4 with 5% of 1-propanol; flow rate: 1 ml/min; temperature, 20 °C, UV detection at 280 nm (reproduced from [89] with permission, no further details on detection available).

Procedures whereby BSA was immobilized to amino-derivatized silica gels via an intermediate reactive polymer layer have been developed by Millot and coworkers [87–89]. As illustrated in Fig. 2 for *R,S*-oxazepam, polyacryloyl chloride (PAC) coatings formed by polymerization inside the pores give higher selectivity values than polymer films deposited by usual coating procedures. As shown by electron paramagnetic resonance spectroscopy, the mobility of polymer chains obtained by the former method is higher than that of PAC layers deposited by conventional methods, resulting in lower distortion of the protein after immobilization and better enantioselectivity [88].

In all the procedures described earlier, proteins were linked to the matrix via their amino groups. Another immobilization technique involving carboxylic functions of proteins has been reported by Marle et al. [48]. In this method, CBH I was bound at pH = 7.0 to amino-derivatized silica gels using the sodium salt of *N*-hydroxysulfosuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide chloride (EDC). Surprisingly, the resulting CBH I-CSPs showed better enantioselectivity and lower retention for propranolol than CBH I-aldehyde stationary phases although carboxylic groups of the catalytically active site have been found to participate in the chiral discrimination mechanism [90,91].

At present, a number of protein-CSPs are commercially available (Table 1). However, several different immobilization procedures may be carried out to develop new chiral supports. In most cases, proteins are linked to the matrix via amino groups. The method involving coupling of proteins to DSC-activated amino silica-gels has been widely used and leads to performant stationary phases, except for amyloglucosidase [30]. The stability of protein-based chiral materials depends on both the type of protein and the immobilization method. For chiral supports prepared by covalent linkage of the protein to the matrix, it appears that the retention properties and enantioselectivity of resulting stationary phases may be strongly influenced by the hydrophobicity and mobility of the linker layer.

5. Chromatographic applications of protein-based matrices

Protein-based chiral stationary phases have become widely used for the direct separation of drug enantiomers, without sample derivatization. They probably exhibit the broadest range of enantioselectivity among the CSPs, owing to multiple possibilities of intermolecular interactions between chiral compounds and protein surfaces. Some protein-based CSPs have been employed for the resolution of basic, neutral and acidic drugs while other CSPs are best suited to certain types of analytes.

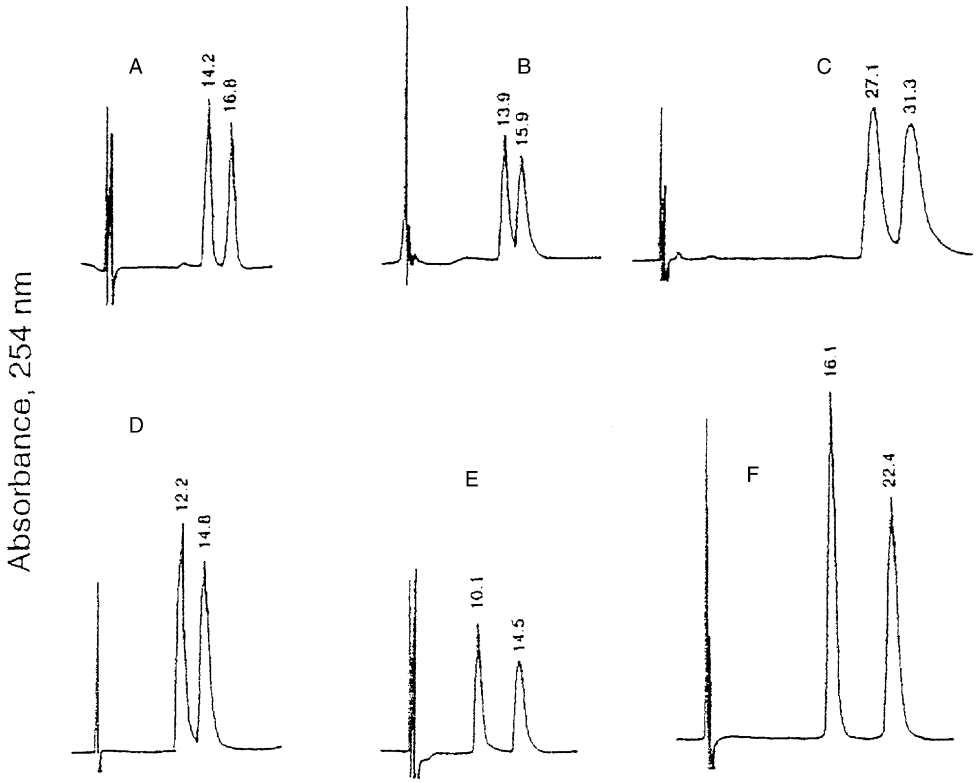


Fig. 3. Representative chromatograms of racemic drugs on avidin-columns with optimized mobile phases at a flow rate of 0.6 ml/min; UV detection at 254 nm: (A) chlortalidone; (B) glutethimide; (C) primaquine; (D) aminoglutethimide; (E) hydroxyzine; and (F) ketoprofen. Numbers at individual peaks refer to retention times (min) (reproduced from [92] with permission, no further details on detection available).

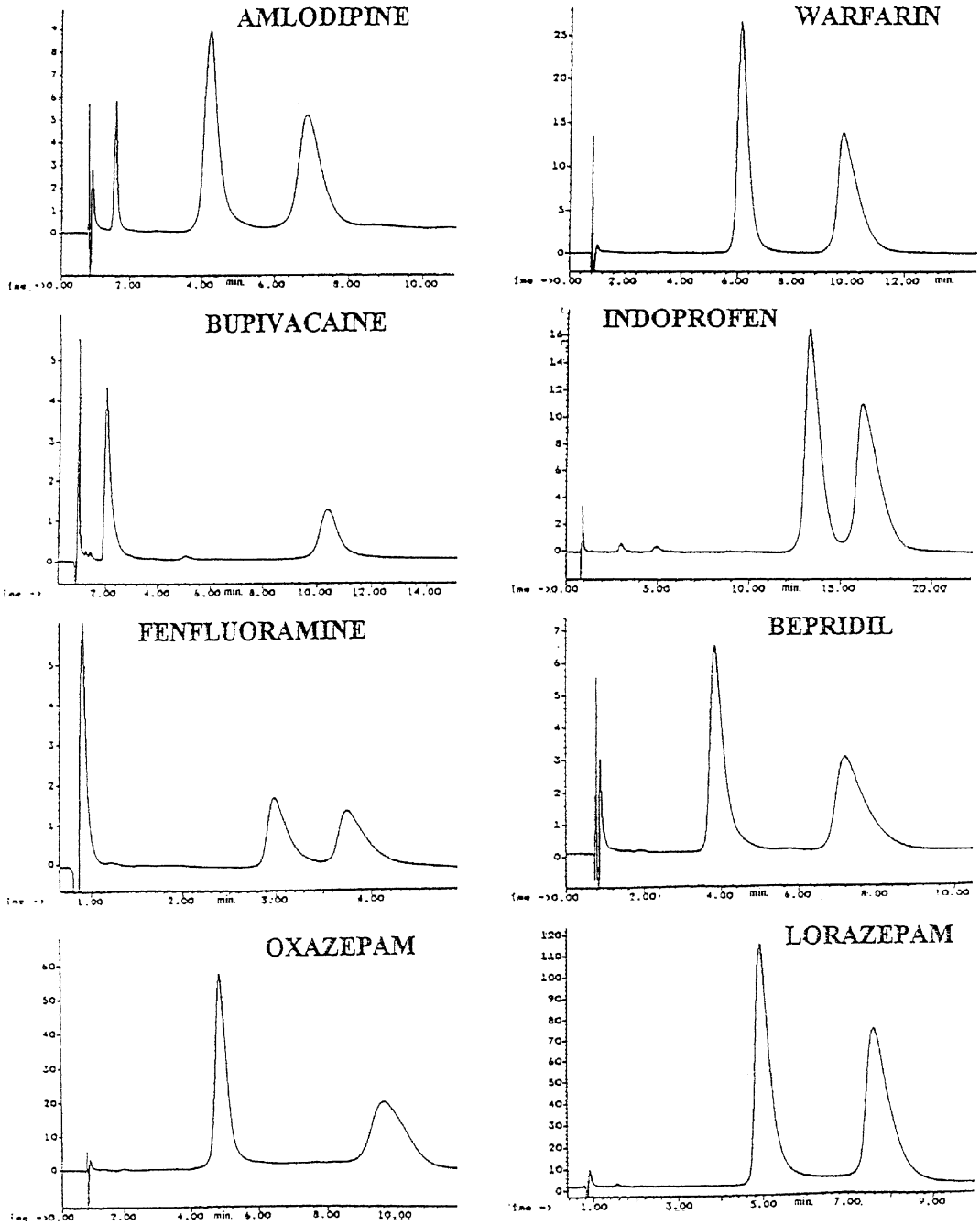


Fig. 4. Representative chromatograms of racemic drugs on a RfBP (quail egg white) column with optimized mobile phase; the operating UV wavelength was fixed at the corresponding maximum for each compound; time in minutes at the *x*-axis (reproduced from [79] with permission).

5.1. Protein-based CSPs for the separation of basic, neutral and acidic drug racemates

A wide range of basic, neutral and acidic drug racemates have been resolved using AGP [21] and OVM [22] as immobilized chiral selectors. Chiral separations using AV have also been reported for some basic drugs, arylpropionic acid derivatives and pharmaceuticals in the unionized state (Fig. 3) [26,92]. The corresponding CSPs are commercially available (e.g. CHIRAL-AGP, Ultron ES-OVM and Bioptic AV-1). It should be underscored that riboflavin binding proteins (RfBPs) have also been described as potential chiral selectors for the enantioseparation of basic, neutral and acidic analytes (Fig. 4) [28,93].

As shown in Table 1, AGP (orosomucoid), OVM and RfBPs are acidic proteins, whereas avidin is a basic protein. AGP has the lowest isoelectric point resulting from a high number of sialic acid residues (14 residues per molecule). It was generally reported that AGP had a molecular mass of 41,000 g/mol and a sugar content of 45% [94]. However,

in a recent study, it was shown using matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometry and size-exclusion chromatography that the molecular mass of AGP could rather be estimated to 33,000 g/mol, with a carbohydrate content of 34% [95].

AGP is thought to be the major plasma binding protein for neutral and basic drugs. The native protein is able to bind a variety of hydrophobic compounds due to interactions with an apolar cavity formed by the folding of the secondary structure of AGP [96].

Ovomucoid is a glycoprotein composed of three tandem homologous domains [97]. Chicken ovomucoid-based CSPs were first described by Miwa et al. [22]. Later, turkey ovomucoid was successfully investigated as a chiral selector for liquid chromatography separations [75,98]. Interestingly, it was reported by Haginaka's group that the good chiral recognition ability of crude chicken ovomucoid stationary phases originated from another protein which was present in crude ovomucoid preparations as an impurity (11% ,w/w) [29,98]. This protein was called ovoglycoprotein [29]. Regarding pure chicken ovomucoid, it was shown that the chiral discrimination ability of the whole protein was negligible [99].

Riboflavin binding proteins extracted from chicken and quail egg (white and yolk) have been evaluated as chiral selectors in HPLC. Their overall structures are very similar although small differences in their tertiary structure have been demonstrated using circular dichroism and fluorescence spectroscopy [100].

AGP, OVM, AV and RfBPs are extremely stable proteins. The corresponding chiral stationary phases have been employed over a wide pH range (3–7.5) and tolerate high concentrations of usual organic solvents (up to 25% for AGP [101], 50% for OVM (manufacturer's recommendations), 40% for AV [102]. It is worth noting that RfBP-CSPs have only been tested at concentrations of organic modifier lower than 10%. AGP-based CSPs have been used at temperatures up to 70 °C while a maximum operating temperature of 40 °C is recommended for OVM columns. In a comparative study on AGP and OVM stationary phases, it was reported by Kirkland et al. that OVM-CSPs exhibited a better long-term stability than AGP columns [103]. It must be noted that OVM-CSPs were employed with a guard column in this study. Even so, Borner et al. also observed an initial loss of retention for AGP columns, with no alteration of resolution [104]. It is worth noting that recently, during a robustness study, problems of column-to-column reproducibility were reported for CHIRAL-AGP columns [105].

Several different classes of hydrophobic chiral drugs have been resolved using AGP and OVM columns and to a less extent avidin and RfBP-CSPs. It has been reported that a limited mobility in the vicinity of the chiral center is generally required to obtain efficient separations with these CSPs [21,78,106]. In another study, Haque and Stewart noticed that pharmaceuticals which were resolved on avidin columns had an aromatic group or a carbonyl function linked to the asymmetric carbon, probably responsible for chiral discrimation [92]. Similarly, it has been reported for β-blocking agents (amino alcohols) that apolar substituents improved enantioselectivity on AGP columns and that hydrogen-bond interactions were also involved in the chiral discrimination mechanism [107]. For example, the presence of an NH function in the aryl part of the molecule of β-blocking agents results in a lack of chiral discrimation because this group probably binds to the active site of AGP instead of the NH group close to the chiral center [107]. Moreover, an increase in enantioselectivity on AGP-CSPs has been reported recently by Gyimesi-Forras et al. for chiral 4-(3*H*)-quinazolone derivatives bearing an amide function near the asymmetric carbon [108]. It is worth noting that, the enantioselective resolution may be strongly impacted by small changes in the molecular structure of racemic drugs [70,107,108]. For example, the enantiomers of oxazepam are well resolved using RfBP columns, whereas the antipodes of lormetazepam (additional methyl and chlorine groups) are not separated on this CSP [78].

Since the recognition mechanisms by these immobilized proteins are not clearly understood, it is difficult to predict the retention and selectivity of a given solute using AGP, OVM, avidin and RfBP columns. For example, it was shown in recent studies that bupropion, a basic antidepressant drug, was more retained on OVM columns than on AGP-CSPs [109,110], while for some other chiral drugs, both a similar and an opposite tendency have been reported [101,103]. Moreover, some enantiomers are resolved using one type of CSP whereas no chiral discrimination is observed using another protein-based CSP [103,111]. For example, the enantioseparation of propafenone is possible using CHIRAL-AGP columns while no separation is obtained on Ultron ES-OVM CSPs [111]. Surprisingly, the opposite phenomenon is observed for the metabolite of propafenone (PPF-5OH), despite a very similar molecular structure (only one additional OH group on the aromatic ring). Moreover, for a given solute the elution order of the enantiomers may be reversed from OVM to AGP-CSP. An interesting application of this phenomenon is the detection of a minor unwanted enantiomer in the presence of large amounts of the corresponding antipode. To avoid interferences coming from peak tailing effects, the impurity must be eluted faster than the major peak. For example, in a recent validation study to determine the enantiomeric purity of an M3 antagonist, Song et al. prefered a CHIRAL-AGP column to an Ultron ES-OVM CSP because the minor *SS*-isomer was eluted before the *R*,*R*-form on the AGP column [105].

The retention of enantiomers and enantioselectivity of protein columns can be regulated by changing chromatographic conditions, e.g. pH value, ionic strength, type and concentration of organic modifier, temperature.

For acidic and basic drugs, a change in the pH of the mobile phase may cause either an increase or a decrease in retention, depending on the charge of the solute. In most cases, the retention factor for basic compounds us-

ing acidic chiral selectors (AGP, OVM and RfBP) displays a decrease with the pH reduction from 7.0 to 3.0 [70,80,93,102,107,109,112–114]. For acidic solutes, such as warfarin ($pK_a = 5.0$) or 2-arylpropionic acid derivatives (pK_a from 4.0 to 4.5), the retention factor (k) generally increases when decreasing the pH [70,102,108,112,113]. A maximum in retention has generally been observed at pH = 4.0–4.5 using OVM and RfBP-CSPs [80,93,113,114]. This retention behavior is consistent with the ionization state of both the drugs and immobilized protein. Over the pH range 7.0–4.0, AGP, OVM and RfBPs bear a net negative charge. However, a pH lowering from 7.0 to 4.0, induces a decrease in the net negative charge of the protein. This results in a reduced electrostatic attraction of cationic compounds by the immobilized protein, whereas a decrease of repulsive effects is observed for anionic drugs. The pH effect on retention indicates that coulombic interactions are involved in the retention mechanism of charged solutes by AGP, OVM and RfBP columns. The maximum retention observed for acidic drugs using OVM and RfBPs is obtained at a pH value where both proteins and solutes are uncharged, suggesting that hydrophobic interactions also play a key-role in the binding between drugs and these CSPs. The bell shape retention curves observed for acidic drugs may also result from conformational changes of the protein. It is known that native OVM can undergo reversible unfolding–refolding processes, depending on the pH [97]. Thus, the decrease in retention observed with OVM columns at low pH values may reflect this conversion.

It should be noted that, using AGP columns, a pH-dependent reversal of the elution order of enantiomers has been reported by Karlsson and Aspegren [115]. At low pH values (pH from 4.2 to 6.0) the *R*-enantiomer of mosapride ($pK_a = 6.2$) was less retained than the *S*-enantiomer whereas a change in the elution order was observed at higher pH, indicating a poor interaction of the unprotonated form of the *S*-enantiomer with the chiral stationary phase. Similar effects have been observed for the main metabolite of mosapride.

Although avidin is a basic protein, the pH effects on the retention of ionic solutes are similar to those observed with acidic proteins (AGP, OVM and RfBPs) [82,83,92,102,113]. However, as reported by Oda et al., the elution of basic compounds is faster on avidin columns than on OVM columns in the high-pH region [113]. These results indicate that coulombic interactions are again involved in the retention mechanism by avidin columns, depending on the positive net charge of the protein and ionization state of the solute.

A change in the pH of the mobile phase may also strongly affect the enantioselectivity. In the case of AGP columns, an increase of α values is generally observed for basic drugs as the pH of the mobile phase is increased [70,101,106,116]. However, despite a normal pH-dependent retention behavior for the enantioseparation of mefloquine (basic drug) on AGP columns, the increase in retention at higher pH values results in a reduced enantioselectivity [117]. This demonstrates that it is important to distinguish between pH effects at the chiral binding site of proteins and general pH effects on the non-selective binding. An increase of α values with the pH was also observed for OVM-CSPs [80,103,113,118], however, the enantioseparation was often less affected by pH variations than when using AGP-CSPs. In the case of RfBP columns, the pH increase had generally no significant effect on the enantioselectivity of basic compounds [93,114], suggesting that coulombic interactions were not the major driving force for enantiodiscrimination by RfBP CSPs.

For acidic chiral compounds, the selectivity generally improves while decreasing the mobile phase pH owing to a diminution of electrostatic repulsion phenomena between the protein and the analyte, allowing better interactions [70,101]. Optimum α values have often been observed at pH = 4.0–5.0 using OVM and RfBP columns [93,113,114] suggesting that hydrophobic interactions are probably involved in the chiral discrimination mechanism, since both the protein and solute are uncharged at this pH value. It should be noted that the enantioselectivity of RfBP-CSPs for arylpropionic anti-inflammatory drugs is generally poor (except for indoprofen) while the warfarin enantiomers have been resolved with a selectivity of 2.59 [119]. Interesting results have been reported by Waters et al. for the enantioseparation of chiral drugs bearing a carboxyl group ($pK_a = 4.5$) and a quinoline moiety ($pK_a = 5.1$) using an AGP-CSP [120]. Surprisingly, high enantioselectivities were observed at a pH beyond 5.0, although AGP and drug enantiomers were both negatively charged. Moreover, an increase in the salt concentration had no impact on the retention and selectivity values. It was concluded by the authors that the chiral discrimination was primarily due to hydrophobic interactions between the quinolinic part of the molecule and the cavity of AGP while coulombic interactions had a minimal effect, as previously suggested by Oda et al. for OVM columns [113].

In some cases, large variations in the enantioselectivity resulting from small variations in the pH may become a problem. For example, it was reported by Abushoffa and Clark that if baseline separation of the enantiomers of oxamniquine was possible using AGP-based CSPs [121], the enantiomeric resolution remained highly dependent on the pH value, making uncertain the regular assays on oxamniquine as the bulk drug and formulations.

Regarding uncharged compounds, the retention and enantioselectivity of enantiomers using OVM- and avidin-CSPs were relatively stable over a wide range of pH [92,118,122]. However, when OVM columns were used for benzoin at pH values lower than 4.5, a significant decrease in selectivity was observed, suggesting again a reversible conformational change in the structure of OVM [118]. When using AGP columns for uncharged compounds, enantioselectivity improvements were reported by Hermansson when the pH changed from 4.5 to 7.5 [70]. This was attributed to a reversible pH-dependent modification of the binding properties of the protein. More complex results were reported

recently by Gyimesi-Forras et al. for the enantioseparation of 4-(3*H*)-quinazolone derivatives at different pH levels, using AGP columns [108]. These compounds are neutral in the pH range investigated (4.0–7.0). Different pH effects have been observed, depending on the structure of the chiral compound, on the concentration of the organic modifier and on its hydrogen donor/acceptor properties. Regarding quail and chicken-egg RfBPs, it is interesting to note that efficient enantioselective separations were obtained for some benzodiazepines [78,79,93,114]. An improvement of the enantioselectivity was observed upon increasing the pH from 3.5 to 6.5 resulting from the drastic increase in the retention of the second enantiomer. It was suggested by the authors that the first eluted enantiomer might be bound to the protein by non-specific interactions while the second antipode might interact with the riboflavin binding site [93,114].

The influence of the eluent pH on the retention of charged chiral drugs by AGP-, OVM- and RfBP-CSPs clearly demonstrates that electrostatic interactions are involved in the retention mechanism. Therefore, ionic strength variations should influence the binding properties of solutes. It appears that the retention of chiral drugs is more or less affected by this parameter, regarding the type of solute and chiral selector [92,102,103,113,116,123]. The effects observed on increasing the buffer concentration depend on the respective contribution of electrostatic and hydrophobic interactions in the retention process. Moreover, Hermansson suggested in the case of arylpropionic acid derivatives, that ion-pair distribution of these compounds, with sodium as counter ion, was the dominant retention mechanism on AGP columns [123]. Regarding the enantioselectivity of AGP, OVM, avidin and RfBP stationary phases, this parameter is generally little affected by the buffer concentration suggesting that coulombic interactions and ion-pair distribution are not the major driving forces in chiral discrimination [92,102,103,113,116,120]. Despite this, non-negligible concentration effects have also been reported by Hermansson's group for AGP columns [123,124].

Different uncharged organic modifiers (alcohols, acetonitrile, THF) have been used to regulate the retention of charged and neutral chiral drugs by protein-based CSPs. In most cases, the retention factors and selectivity decrease in the presence of organic solvents for both AGP [70,105,120,123], OVM [109,113,118], avidin [83,113] and RfBP [93] chiral stationary phases. This can be attributed to a reduction of hydrophobic interactions between the enantiomers and protein-based CSP as the mobile phase becomes less polar. Moreover, the addition of an organic modifier to the mobile phase may cause reversible changes in the secondary structure of immobilized proteins, resulting in a modified enantioselectivity. Interestingly, it was reported that using pure phosphate buffer, the enantiomers of methylphenobarbital were not resolved on AGP columns, while a baseline resolution was observed after addition of 2% of 2-propanol to the mobile phase [70]. Similarly, it was shown by Ceccato et al. that a small amount of organic modifier was necessary to obtain a good enantioseparation of pirlindole on OVM columns [125]. Last, for some solutes, the enantioselectivity of AGP-CSPs improved while increasing the acetonitrile concentration, as reported by Gyimesi-Forras et al. [108]. These unexpected results can be due to the contribution of hydrogen-bonding interactions and/or to reversible changes in the protein conformation, improving the chiral discrimination. Additionally, the influence of uncharged organic modifiers on retention and enantioselectivity clearly depends on the type of solute and modifier. For example, in an evaluation of the enantioselectivity of RfBP columns towards arylpropionic acids and dihydropyridines, Massolini and coworkers reported that modifications of the concentration of organic additives had a greater influence on the more hydrophobic solutes [78,93]. In other studies using OVM- and RfBP-CSPs, chromatographic results obtained with a series of primary alcoholic modifiers demonstrated that the hydrophobicity of the organic modifier also influenced the retention and resolution of the enantiomers [79,113,118,126]. It is moreover suspected that the hydrogen-bonding properties of the organic additive may affect the enantioselectivity to quite a large extent. For example, the enantiomers of pindolol [127] and of verapamil [70] are baseline resolved using AGP columns in the presence of acetonitrile (hydrogen-acceptor) whereas no enantioselectivity is observed with 1-propanol (hydrogen-acceptor and hydrogen-donor) in the mobile phase (Fig. 5). An opposite phenomenon has been observed for propranolol using OVM-CSPs [126]. In some cases, however, the contribution of hydrogen-bonding is negligible, as reported recently by Song and coworkers for the enantioseparation of an M3 antagonist using an AGP column, since similar results can be obtained in the presence of a variety of organic modifiers [105].

Interestingly, the influence of uncharged organic modifiers on the chiral properties may be different on AGP and OVM columns. It was reported by Williams et al. that the enantioselectivity of OVM columns was more affected by the addition of 2-propanol than that of AGP columns [101]. It was also shown that the elution order of *R*- and *S*-warfarin on AGP columns was reversed using acetonitrile and 2-propanol as organic modifiers while no reversal of the retention order was obtained using OVM-CSPs [101]. Opposite effects were occasionally observed when indenoindolic compounds were analyzed on AGP and OVM columns [122]. Thus, it can be assumed that the hydrogen-bonding properties of the organic modifier and the conformational changes of the protein affect to a different degree the enantioselectivity of AGP and OVM columns.

In addition to neutral organic compounds, cationic modifiers (alkylamines, quaternized ammonium salts, terodiline, sparteine) and anionic modifiers (hydrophobic carboxylic acids, alkylsulfonates) have been used to regulate the retention of chiral solutes on AGP and OVM columns. Generally, the retention and enantioselectivity of basic chiral drugs are markedly reduced by the addition of a cationic hydrophobic

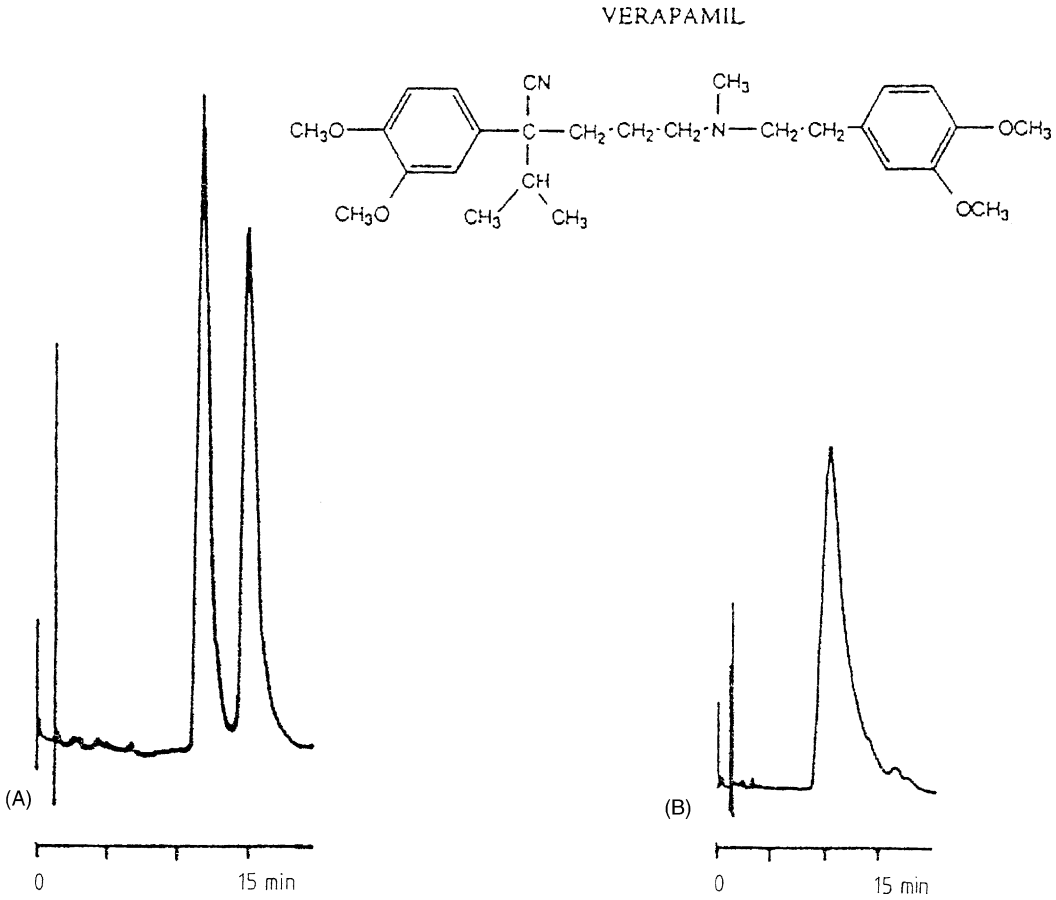


Fig. 5. Influence of the nature of the organic modifier on the enantioselectivity of verapamil. Column: CHIRAL-AGP; mobile phase: (A) 10% acetonitrile in 0.01 M phosphate buffer, pH = 7.0; (B) 4% 1-propanol in 0.01 M phosphate buffer, pH = 7.0 (reproduced from [70] with permission, no details on detection available).

modifier [112,127–129] since this compound may compete with the solutes not only for ionic binding sites, but as well for the hydrophobic part of the binding site and also for non-stereoselective sites of the protein. In addition to that, the modifiers may affect the conformation of the protein. It is interesting to underscore that an improvement of the enantioselectivity was generally observed for anionic drugs after addition of a cationic modifier to the mobile phase [123,130]. According to Hermansson and coworkers, this could be explained by the formation of ion-pairs between the solute and the modifier, fitting better in the chiral center of the protein [123,130]. Attention should be drawn to the fact that addition of an ion of opposite charge to the solute occasionnally results in a decrease in retention. This has been reported by Enquist for the enantioseparation of ibuprofen on AGP columns [127] and by Ceccato et al. for pirlindole, a basic antidepressant drug, on OVM-CSPs [125]. This phenomenon is not consistent with the usual retention behavior observed for an ion-pairing system [130]. Therefore, the decrease in retention might be more readily attributed to the hydrophobic character of the modifier while the increase in enantioselectivity may be due to interactions between the charged organic modifier and the protein, leading to favorable conformational changes in the vicinity of chiral centers.

Temperature is another parameter which has been investigated to adjust retention on AGP, OVM and RfBP columns. Generally, an increase in temperature causes a decrease in retention and enantioselectivity [70,101,105,119,122,126]. Chiral separations are often less affected by changes in temperature when using OVM than AGP CSPs [101,122]. It must be noted that an unexpected improvement of the enantioselectivity with increasing temperature was observed by Munro et al. for the enantiomeric separation of bupropion using AGP columns [110], whereas this unusual effect has not been reported for OVM columns [109]. Additionally, it has been shown by Kirkland and Mc Combs for OVM-CSPs, that variations in enantioselectivity with temperature may depend on the type of solute (acidic, neutral, basic) and on its ionic state [131]. For example, the variations of α with temperature for halofantrine (basic drug) is linear at pH = 7.0 and 6.0 as usual, but not at pH = 5.5 showing that the ability of the immobilized OVM to interact with the fully ionized drug (pH = 5.5) is affected by temperature.

For a given pair of enantiomers, the enantioselectivity α is related to the difference in the free energy of interaction

of the two enantiomers with the immobilized protein by

$$\ln \alpha = -\frac{\Delta\Delta G^\circ}{RT} = -\left(\frac{\Delta\Delta H^\circ}{RT}\right) + \left(\frac{\Delta\Delta S^\circ}{R}\right)$$

From plots of $\ln \alpha$ versus $1/T$, it was shown by Cirilli and La Torre that the separation of benazepril (*S,S* configuration) from its enantiomer (*R,R* form) on AGP columns was enthalpy controlled from 25 to 65 °C but could become an entropy-driven process at temperatures higher than 65 °C, with a reversal of the elution order [132]. In another study, it was reported that Van't Hoff plot of α on AGP columns was non-linear for chiral drugs bearing a carboxyl group and a quinoline moiety [120]. In a temperature range from 35 to 45 °C, the separation was enthalpy-dominated. At temperatures below 30 °C, the enantiomeric separation was entropy-controlled and probably resulted from the inclusion of the chiral drug in the hydrophobic cavity of AGP. The break at 35 °C was attributed to a conformational change of the protein which was confirmed by fluorescence spectroscopy [120]. The change in the retention order observed by Karlsson and Aspegren at high column temperature for mosapride and its main metabolite might be explained in a similar way [115]. It should be underscored that curves with a break around 32.5 °C have also been obtained for the enantiomers of warfarin and oxazepam hemisuccinate on RfBP columns [119]. This phenomenon was also explained by a conformational change of the protein around this temperature.

Interestingly, the resolution of enantiomers on AGP and OVM columns also depends on the mobile phase pH, organic modifiers and column temperature because these parameters may influence both the column efficiency and the peak symmetry. Especially, peak tailing was found to increase at higher pH values for the analysis of basic drugs on AGP and OVM-CSPs [109,110,116], although Kirkland et al. reported in an earlier study that the resolution for basic drugs was improved at higher pH [103]. Such variations in column efficiency with the mobile phase pH are probably due to mass-transfer effects resulting from modifications of the charge density on proteins. Moreover, the addition of organic modifiers to the mobile phase may also influence the peak width [92,122]. For example, it was shown that the efficiency was increased by ca 50% when replacing 2-propanol by acetonitile for the enantioseparation of indenoindolic racemic compounds on AGP and OVM columns [122]. It is also well known that the separation efficiency increases with increasing the column temperature because mass-transfer kinetics are faster at higher temperatures while mobile phase viscosity is reduced [70,101,105,131]. However, due to the harmful influence of high temperatures on enantioselectivity, the evolution of resolution with temperature may be a complex phenomenon.

In conclusion, a wide range of basic, acidic and neutral drug racemates have been resolved using AGP and OVM columns. The applicability of avidin and RfBP columns is probably less promising. Avidin columns are more suitable for the enantiodiscrimination of arylpropionic acid derivatives and unionized chiral compounds. They allow the direct determination of drugs and drug metabolites in plasma, since plasma proteins cannot interact with the hydrophobic inner surface and are rapidly eluted. The retention of pharmaceuticals by AGP, OVM, avidin and RfBP columns results from stereoselective binding and non-selective interactions between the solute and the immobilized protein. Coulombic interactions are involved in the retention mechanism of charged solutes, while hydrophobic and hydrogen-bond interactions are also suspected to play a key-role depending on the type of CSP, on the molecular structure of the solute as well as on the organic modifier in the mobile phase. The chiral discrimination mechanism on AGP, OVM and avidin-CSPs is a complex phenomenon involving hydrophobic interactions and where coulombic and hydrogen-bond interactions also play a key-role depending on the molecular structure of the chiral drug. Regarding RfBP-CSPs, hydrophobic interactions are probably the major driving force for enantioselective discrimination. Trp 156 and Tyr 75 rings as well as Glu 72 might be involved in the chiral discrimination mechanism, as recently suggested by Calleri et al. [119]. It is worth noting that the riboflavin binding site is probably involved in the stereoselective solute-RfBP interaction since a loss of enantioselectivity is observed after saturation of the column using riboflavin. Modification of the eluent pH and column temperature, as well as addition of organic modifiers to the mobile phase are useful tools to regulate the retention of enantiomers, the enantioselectivity and resolution on AGP, OVM, avidin and RfBP columns. The influence of these parameters has been extensively reported, by changing factors one by one. To reduce the time necessary for a complete investigation of these parameters, an experimental design approach can be used, as reported recently by Smet et al. for the enantioseparation of arylpropioic acid derivatives on avidin columns [133]. Small variations of the mobile phase composition and temperature may have wide range effects on the enantioseparation. Thus, these parameters must be carefully controlled for optimum reproducibility and repeatability of intra- and interassays, to avoid a lack of ruggedness which is a negative point in validation procedures [121].

5.2. Protein-based CSPs for the separation of basic and unionized drug racemates

It has been reported that some proteins showed enantioselective properties mainly towards basic drugs and uncharged solutes. Among them, cellulases [25], pepsin [27] and amyloglucosidase (glucoamylase) [30] have proven to be efficient chiral selectors. Besides, cellulase and pepsin-based CSPs are commercially available (Chiral-CBH and Ultron ES-pepsin, respectively). Ten years ago, Haginaka et al. developed a lysozyme-based support that could be used to resolve basic and neutral chiral pharmaceuticals [34]. Despite this, lysozyme-CSPs were not further developed.

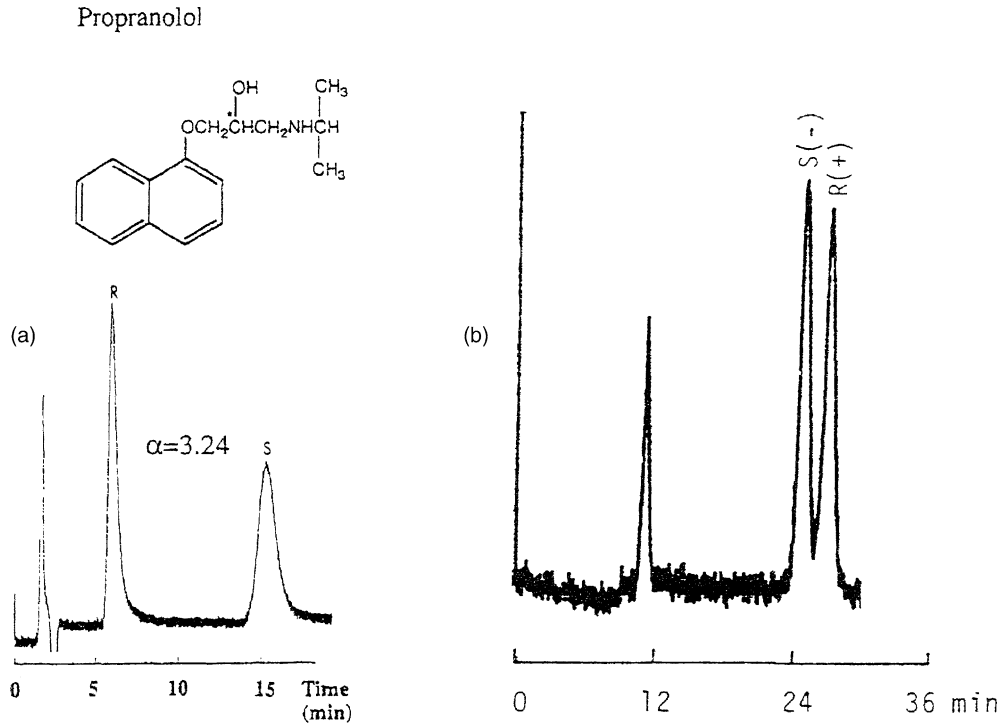


Fig. 6. Chromatography of racemic propranolol on CBH I (a) and OVM (b) columns. Mobile phases: (a) 0.01 acetate buffer (pH = 5.0), (b) 50 mM sodium dihydrogenophosphate (pH = 4.6) containing 12% ethanol; fluorescence detection: λ_{ex} = 297 and 340 nm (reproduced from [134] (a) and [126] (b) with permission, fluorescence arbitrary units, no further details on detection available).

Cellulases are cellulose degradating enzymes. Cellobiohydrolase I (CBH I noted T.r. Cel7 A) produced by the fungus *Trichoderma reesei* was the first cellulase tested for chiral separations [25]. It was found to be an excellent chiral selector towards a set of β-blocking agents. *T. reesei* secretes another stereoselective cellobiohydrolase CBH II (noted T.r. Cel 6A) [73] and two endoglucanases EG I (noted Cel 7B) and EG 2 (noted Cel 6B) [134]. Recently, cellobiohydrolase 58 (P.c. Cel 7D), a close homologue of CBH I produced by *Phanerochaete chrysosporium*, was also found useful to resolve some basic drug racemates [74].

Cellulases are stable acidic enzymes (Table 1) easy to produce in large quantities. They all have a large catalytic domain linked to a small cellulose-binding domain by a highly glycosylated linker region [135]. The three dimensional structure of the catalytically active core of CBH I has been determined by X-ray crystallography [136]. It is composed of an antiparallel β-sandwich and of loops connecting the β-strands, layed out to form a long tunnel (ca. 4 nm long) into which cellulose chains can be threaded and cleaved. Three carboxylic acid residues (Glu 212, Asp 214 and Glu 217) were found to be essential in the catalytic mechanism of CBH I [136,137]. Recently, it was reported that the catalytic site of CBH 58 was similar to that of CBH I [74] whereas the tunnel formed in CBH II is shorter (ca. 2 nm) [73].

It appears that cellobiohydrolase-based supports are probably the best choice for the chiral separation of β-blocking agents because of the higher enantioselectivity obtained compared with other protein-CSPs. For example, as illustrated in Fig. 6, the resolution of a racemic mixture of propranolol is better using a CBH-column than an OVM-CSP [72,106]. It should be noted that the retention order of the amino alcohols is the same on CBH and AGP-CSPs (*S*-enantiomer more retained than the *R*-form) (Fig. 6) [134,138] but not on OVM-supports [139]. Several reports indicate that the chiral recognition center overlaps the enzymatically active site of CBH I. As early as 1993, Marle et al. demonstrated, using both intact and fragmented CBH I-CSPs, that the dominant stereoselective binding site for propranolol was located in the core domain [48]. Later, studies of the inhibition of CBH I by propranolol and alprenolol [134] and enantioseparation data for these compounds on CBH I and CBH 58 CSPs in the presence of cellobiose in the mobile phase (Fig. 7) [51,74,140] confirmed that the enzymatically active site was involved in chiral recognition. Moreover, Henriksson et al. assessed the enantioselective properties of catalytically deficient mutants of CBH I obtained by site-directed mutagenesis (replacement of Glu 212, Asp 214 and Glu 217 by their corresponding amides) [90]. A complete loss of enantioselectivity was observed for E 212 Q and E 217 Q-stationary phases, whereas D 214 N-silica showed residual enantioselectivity, indicating that Glu 212 and Glu 217 were essential for chiral discrimination using CBH I-CSPs. Similarly, as shown in Fig. 8, a great reduction in enantioselectivity was observed by Hedeland et al. [91] after covalent modification of carboxylic groups of CBH I, thus supporting earlier findings. Although *T. ree-*

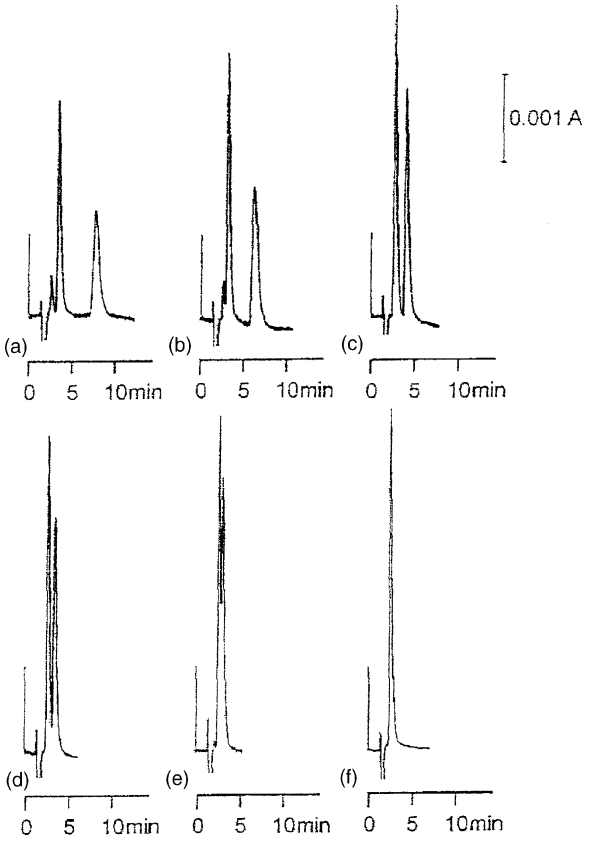


Fig. 7. Chiral separation of racemic atenolol on CBH 58 CSP with cellobiose as a selective competitor in the mobile phase. Mobile phase: sodium acetate buffer (pH = 5.0) with cellobiose in concentrations of: (a) 0 mM, (b) 0.05 mM, (c) 0.25 mM, (d) 0.5 mM, (e) 1 mM, (f) 5 mM UV detection, wavelength not specified (reproduced from [74] with permission).

sei endoglucanase Cel 7B (EG 1) has a very similar active site, it is worth noting that EG-1 CSPs displayed very poor enantioselectivity compared to that of CBH I CSPs [134].

Recently, the structural basis for chiral discrimination of basic drugs by CBH I has been studied by X-ray crystallography [141], using *S*-propranolol as a model compound. Stahlberg et al. concluded that the catalytic residues Glu 212 and Glu 217 interacted tightly with the secondary amino group of propranolol ($pK_a = 9.5$), while the naphtyl moiety of the solute stacked with the indole ring of Trp 376. Moreover, the oxygen atom linked to the asymmetric center was found to form hydrogen bonds to Glu 212 and Gln 175.

Alike other protein-based CSPs, the enantioseparation of drug racemates on CBH-CSPs strongly depends on the molecular structure of the chiral solute [72,106]. A significant correlation has been obtained between the retention behavior of a series of β-blocking agents on CBH-I columns and their hydrophobicity, showing that hydrophobic interactions play a key-role in the retention mechanism on CBH-CSPs [142]. In parallel, a complete loss of enantioselectivity was observed when increasing the distance between the stereogenic center and the amino group [72]. In another study, the presence of hydrogen-bonding groups and primary amino functions were found to result in better chiral discrimination of epinephrine analogues [143]. This indicates that enantiodiscrimination by CBH-phases is a complex mechanism involving hydrophobic, electrostatic and hydrogen bonding interactions.

CBH I, CBH II and CBH 58 chiral stationary phases may display quite different chiral and chromatographic properties and altogether be complementary for the enantioseparation of basic drugs. If these CSPs are not suitable for the enantiodiscrimination of acidic compounds, it is worth noting that the enantiomers of ibuprofen have been partially resolved on CBH II [73] and those of warfarin, on CBH I columns [72], at pH values where these solutes are mainly uncharged. The best enantioselectivity of β-blockers is generally observed on CBH I columns [73,74]. For example, oxprenolol is baseline resolved on CBH I whereas it is not separated on CBH II. But α values determined for metoprolol and atenolol, are altogether higher on CBH 58 than on CBH I columns [74]. Moreover, mexiletine, a basic compound, can be resolved using CBH II and

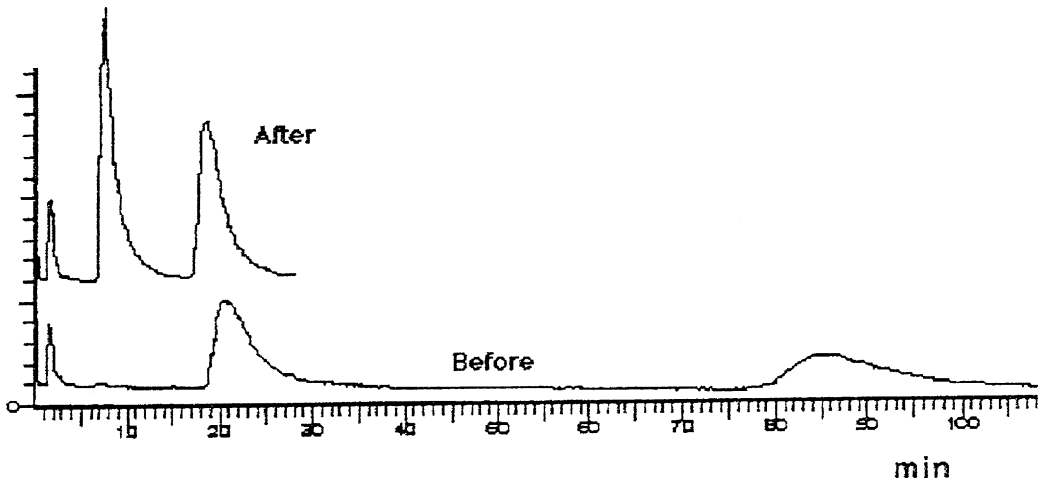


Fig. 8. Enantiomeric separation of *rac*-propranolol on CBH I core-CSP before and after amidation. Conditions: sodium phosphate buffer, pH = 7.0, $I = 0.1$; UV detection (reproduced from [91] with permission, no further details on detection available).

CBH 58 columns whereas, no separation is observed on CBH I CSPSs.

Although less stable than cellulase-based CSPs, pepsin columns [27] are commercially available (Ultron ES-pepsin). The coupling of pepsin to DSC-activated silica is carried out at pH = 4.5 since binding at pH = 6.0 results in a loss of enantioselectivity, while immobilization at lower pH values leads to poor baseline stability [27]. Pepsin columns cannot be employed at pH values higher than 7.0 due to the irreversible denaturation of the enzyme [27]. Haginaka and Miyano reported that the long-term stability of pepsin-stationary phases was improved when crosslinked mixtures of pepsin and purified ovomucoid were used as chiral selectors [144]. Pepsin-CSPs have chiral discrimination properties towards β-blocking agents, antihistaminics, skeletal muscle relaxants and benzodiazepines [27]. Interestingly, basic compounds, such as isoproterenol, salbutamol, atenolol or seproxetine which cannot be resolved on OVM-columns are resolved on pepsin columns [27,145], showing that Ultron ES-pepsin may complement Ultron ES-OVM to achieve separations previously impossible.

Recently, it was reported by Karlsson's group that another enzyme, glucoamylase, could be used for the chiral separation of basic drugs [30]. In native glucoamylase (G1 form) the catalytic domain (434 amino-acids) is connected to the starch-binding domain via an *O*-glycosylated region (residues 513–616). After binding to diol-silica, glucoamylase G1 displays chiral discrimination properties towards amino alcohols [30,146]. However, the enantioselectivity of metoprolol is lower on GA 1 columns compared to that obtained on CBH I CSPs [30,72]. As for AGP and CBH I CSPs, the enantioresolution of amino alcohols is influenced by the number of methylene groups between the chiral center and the nitrogen atom [30,106]. The position of substituents on the aromatic ring is an important parameter too. In contrast to CBH-CSPs, the type of substituent was found to affect only slightly α values [30]. The long-term stability of GA-CSPs is excellent since 12,000 column volumes of mobile phase (pH = 7.0; 15% 2-propanol) were passed through the column without significant decrease in resolution. In a recent study, it was reported that the enantioselective properties of glucoamylase G2 (GA 2) lacking the starch-binding domain and those of glucoamylase G1 were similar [147], suggesting that the catalytic domain is involved in the chiral discrimination mechanism as previously reported for cellobiohydrolase-columns.

As expected, the retention of basic drugs on CBH, amyloglucosidase and pepsin columns increased with the pH value [25,27,72,73,146,147]. From adsorption measurements and modeling of the data using the bi-Langmuir isotherm, Guiochon's group demonstrated that the non-selective adsorption of propranolol to CBH-phases was only weakly dependent on the pH of the mobile phase. By contrast, the enantioselective adsorption was found to depend strongly on this parameter, suggesting that the discrimination mechanism was mainly ionic [148]. In another study, Henriksson et al. reported that retention factors corresponding, respectively, to enantioselective and non-selective binding to immobilized CBH could be determined by addition of a competitor, such as cellobiose to the mobile phase [74]. Thanks to this method, they confirmed Guiochon's findings and suggested that the increase in retention from pH = 5.0 to 7.0 mainly resulted from an increase in the affinity for the enantioselective site, due to deprotonation of one essential carboxylic group (probably Glu 217 for CBH I) in this pH range [74]. The enantioselectivity was also found to increase with the pH for CBH I and CBH 58 supports [25,73,74,106,148] whereas it was only slightly modified on glucoamylase columns [146,147], suggesting that chiral discrimination mechanisms were probably different on both columns.

It is interesting to underscore that an unusual improvement of the selectivity is generally observed for CBH I, CBH 58 and amyloglucosidase chiral stationary phases when increasing the concentration of organic modifier in the mobile phase [72,74,146,147,149]. In most cases, the addition of an organic solvent results in a decreased retention of both enantiomers using CBH columns. However, Hermansson and Grahn have reported for epinephrine analogues, an increase in the retention of the most retained enantiomer on increasing the concentration of 2-propanol in the mobile phase [143], the largest effect being observed for compounds containing a primary amino group in their molecular structure. A similar behavior has been reported by Strandberg et al. for the enantioseparation of amino alcohols on amyloglucosidase-CSPs in the presence of several organic modifiers [146]. This was attributed to a reversible change of the secondary structure of the proteins which could expose other amino acid residues in the binding site. This phenomenon could also be responsible for the reversal in the retention order observed by Karlsson and Aspegren at about 10% of 2-propanol for one amino alcohol, using CBH I columns [106].

Unusual temperature effects have been observed on CBH and glucoamylase CSPs. For example, the retention of the more retained *S*-enantiomer of propranolol increases with rising temperature whereas the retention factor of the antipode is reduced, resulting in an improvement of selectivity at high temperatures (α = 3.5 at 55 °C; α = 1.5 at 10 °C) [138,150]. The thermodynamics of non-specific and chiral selective interactions between *R*- and *S*-propranolol and CBH I has been studied by Guiochon and coworkers [151]. The unusual retention behavior observed for *S*-propranolol was explained by the high entropy of interaction (>11.6 cal/(mol K)) of this antipode with CBH I which compensates for the positive interaction enthalpy (>1.6 kcal/mol). An interesting reversal of the elution order of the enantiomers of *R,S*-sotalol at a temperature around 40 °C has been reported by Fulde and Frahm for CBH I-CSPs [152]. A similar phenomenon has been observed by Karlsson's group at 30 °C for another amino alcohol and has been attributed to conformational changes of the pro-

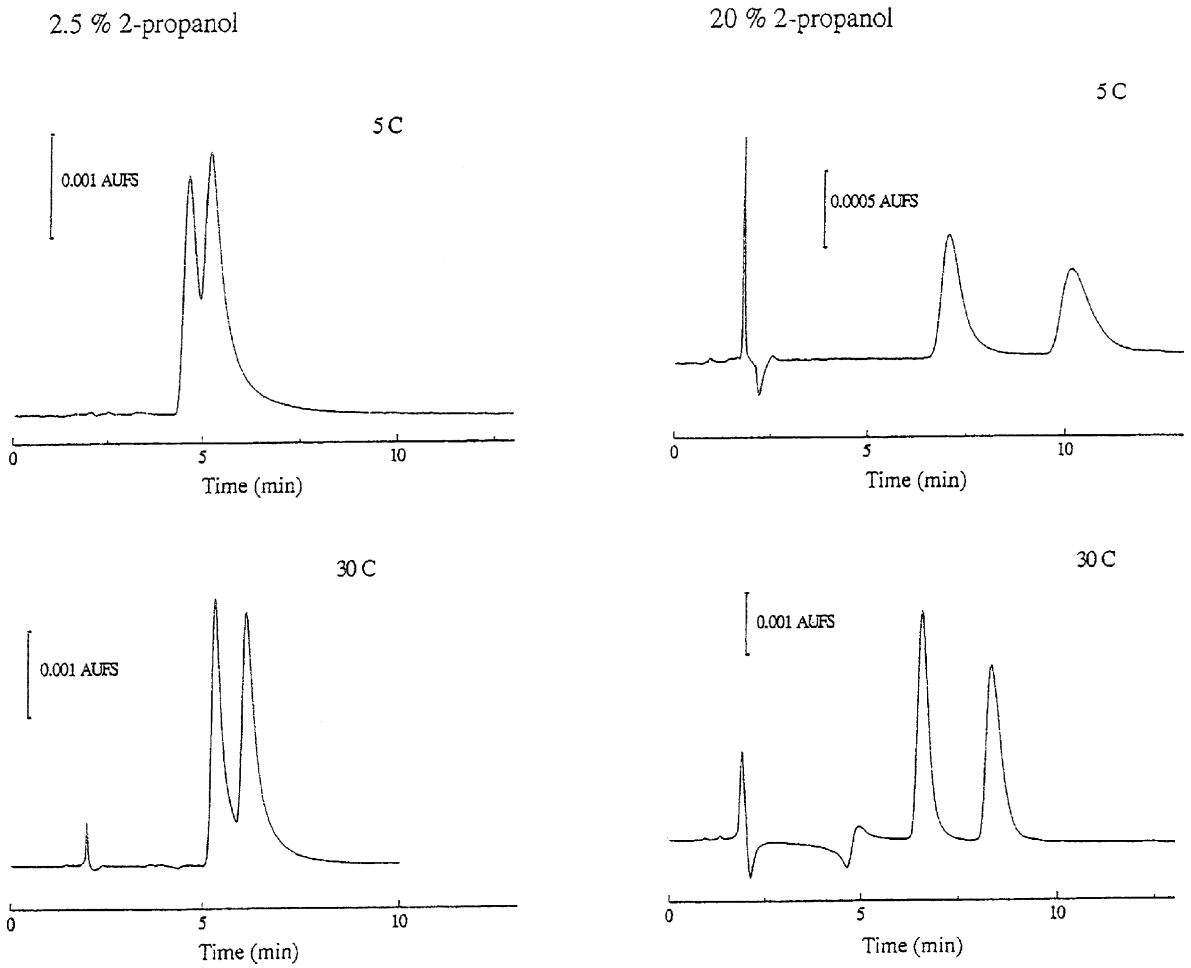


Fig. 9. Effects of 2-propanol and column temperature on the enantiomeric resolution of *R,S*-alprenolol on amyloglucosidase-CSPs. Conditions: phosphate buffer pH = 7.0 with 2.5–20% of 2-propanol; UV detection at 272 nm (reproduced from [146] with permission).

tein and/or adsorption of both enantiomers to different sites [106].

Surprisingly, the temperature impact on the enantioselective retention of amino alcohols using amyloglucosidase CSPs, depends on the concentration of 2-propanol in the mobile phase [146]. As shown in Fig. 9, at low concentrations of 2-propanol (2.5%), the retention of both enantiomers and selectivity increase with the temperature. However, at 20% of organic modifier in the mobile phase, the opposite effect is observed. Although thermodynamic constants determined from van't Hoff plots reflect interactions at both achiral and chiral retention sites, data obtained for amyloglucosidase CSPs suggest that enantioselective retention of amino alcohols is caused primarily by enthalpy effects [146].

In conclusion, cellobiohydrolases, amyloglucosidase and pepsin are principally employed for the enantioseparation of basic drugs. The enantioselectivity of CBH columns for β-blocking agents is remarkable. Although less stable than other CSPs, pepsin-based supports allow the separation of enantiomers which could not be completely resolved by other CSPs. The temperature and pH of the mobile phase must be judiciously adjusted to perform optimal enantioseparations since unusual effects may be observed, depending on the molecular structure of the solutes.

5.3. Protein-based CSPs for the separation of acidic and neutral drug racemates

As demonstrated in several studies, stationary phases based on immobilized serum albumins from human and from a variety of mammalian species, allow the enantiodifferentiation of a number of neutral and acidic drugs [153–156]. Mammalian albumins have nine double loops formed by 17 disulfide bridges [157]. These acidic proteins have very similar structures with 70–80% identities in the amino acid sequences. Despite this, some differences in the retention behavior and enantioselectivity of albumin-based CSPs have already been observed [156]. As reported by Fitos et al., the enantiomers of lorazepam hemisuccinate are not resolved on bovine, pig and rabbit albumin-CSPs, while a high enantioselectivity is observed on HSA columns ($\alpha = 3.25$) [156]. Additionally, a species-dependent reversal of the elution order has been observed for tofisopam and coumarin-type anticoagulants. For instance, human, pig and

rat serum albumins preferentially bind the *S*-enantiomer of warfarin, whereas other albumins interact more strongly with the *R*-form. Regarding benzodiazepine drugs, it should be underscored that the *S*-enantiomer is always more retained than the *R*-antipode [156].

Numerous accounts in the literature describe applications of immobilized human (HSA) and bovine (BSA) serum albumins for the analysis of clinically important drugs and the investigation of drug metabolism. BSA is a globular protein with a molecular mass of 66,466 [158] which displays an overall hydrophobic character. BSA-CSPs have been studied extensively in the 1980s by Allenmark et al. They allow the enantioseparation of a variety of structurally different racemic compounds including aromatic amino acids [159], N-derivatized amino acids [160–162], aromatic sulfoxides and sulfoximine derivatives [163], arylpropionic acids [164], barbiturates [165], benzodiazepine, coumarin and benzoin derivatives [163]. It is worth noting that the enantiomers of prilocain, a basic anaesthetic, have also been resolved on BSA-CSPs, at pH = 8.9 (α = 1.6) [163]. The presence of aromatic as well as polar groups in the molecular structure of the enantiomers appears to be a prerequisiste for enantiodiscrimination [166]. Retention and enantioselectivity can be regulated via pH (4.5–8.0), buffer concentration (0.01–0.2 mol/l) and/or small amounts of 1-propanol (<6%) added to the eluent. The effects observed when changing the mobile phase parameters, clearly demonstrate the contribution of hydrophobic and electrostatic interactions in the overall retention of chiral solutes, although hydrogen bonding and charge-transfer interactions are also probably involved. In a recent study using dansyl derivatives of amino acids as chiral probes, Abe et al. suggested that two binding sites may exist on BSA, which can be distinguished by DNS-L-proline and DNS-D-norvaline [162]. The chiral recognition mechanism of BSA, however, and the structures of its binding sites are still not clear, compared to those of human serum albumin.

HSA-CSPs are characterized by their ability to resolve chiral drugs like warfarin [24], leucovorin [24], benzodiazepines [24,167], 2-arylpropionic acid derivatives [168,169] and indole containing compounds [170]. The relative contribution of ionic binding, hydrophobic interactions and hydrogen bonding depends on the nature of the solute. Since ligand binding properties of HSA are not significantly altered by the immobilization procedure [171], HSA-CSPs have also been widely used to assay the binding of drugs to HSA. This is, indeed, a fast, reproducible and easily automated method which uses minimal quantities of ligand. Two major binding sites responsible for chiral discrimination have been identified on HSA, e.g. the warfarin-azapropazone and indole-benzodiazepine-binding sites (called sites I and II, respectively). New perspectives on their location were gained from the crystal structure of albumin determined in 1992 [172]. According to He and Carter, these two bindind sites reside in hydrophobic cavities in subdomains IIA and IIIA, respectively. The indole-benzodiazepine site of HSA has been described as a hydrophobic cleft, with a cationic region located near the surface at one end of the cleft [173]. In a study of the retention mechanism of some benzodiazepines on HSA-CSPs, Kaliszan et al. reported the existence of two distinct sites for chiral benzodoazepines [167]. The retention of the first and second eluted enantiomer was found to depend to different degrees (i) on the hydrophobicity of the substituent located on the fused benzene ring, (ii) on the excess charge on carbon C(3) of the benzodiazepine ring, (iii) on the excess charge on atoms in the vicinity of C(3) and finally on the width (*w*) of the molecule (Fig. 10). Quantitative

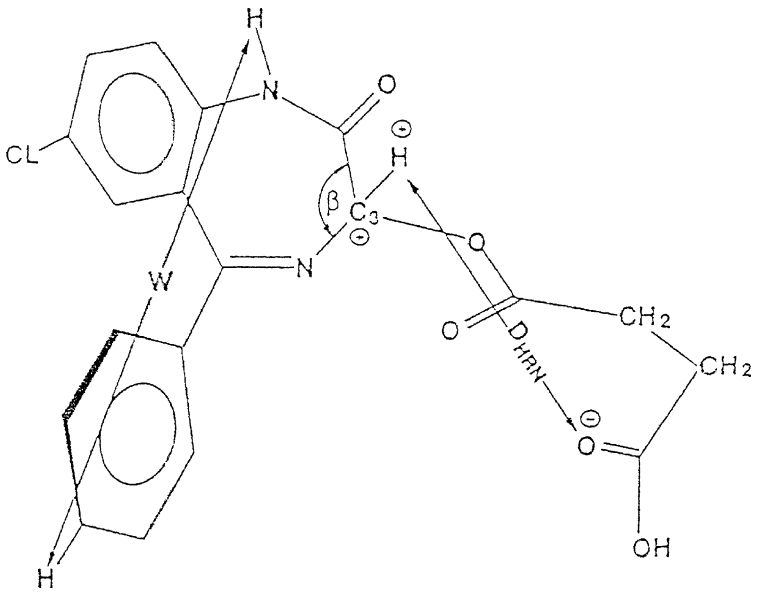


Fig. 10. Representation of oxazepam hemisuccinate (reproduced from [167] with permission).

structure-enantioselective retention relationships have also been reported by Andrisano et al., for the chromatography of a series of arylcarboxylic acid derivatives [174] and 3-hydroxypropionic acids which contain two chiral centers [175,176]. The lipophilicity of the solutes was found to be an important factor influencing mostly the retention of both the first and second eluted enantiomers on HSA-CSPs. In order to explain the enantioselective separation, the authors proposed a two-step chiral discrimination mechanism. First, the chiral acidic compound is assumed to interact with site II through an electrostatic interaction between its carboxylate group and the cationic site on the edge of the cleft. Then, a conformational adjustment of the solute and protein takes place, allowing the insertion of the solute into the hydrophobic cavity at site II and enantiodifferentiation [175,176]. The necessity for an initial conformational adjustment of the solute is supported by the higher α values observed for linear conformers compared to those found for folded conformers [175].

The impact of the acetylation at Tyr 411 on the retention properties and enantioselectivity of immobilized HSA was reported as soon as 1992 by Noctor and Wainer [177]. This tyrosine residue appears to be located within the indole-benzodiazepine site (site II). In most cases, the retention and enantioselectivity factor of solutes binding at site II, decreased after modification of HSA. For instance, a complete loss of enantioresolution was observed for lorazepam. However, for some other solutes binding at the same site (benaxoprofen, temazepam and oxazepam hemisuccinate), an increase in k and α values was observed after acetylation of Tyr 411. These results suggest that the indole-benzodiazepine binding site should rather be considered as a large, flexible area composed of several subsites, some of them being occluded in the unmodified protein. Conformational changes resulting from the acetylation at Tyr 411 may, therefore, either decrease or enhance the binding of "site II" solutes and may also induce structural changes in remote regions of the flexible HSA molecule. In another study, Bertucci et al. reported that the selective acetylation of Lys 199 located within the warfarin-azapropazone site, affected the binding properties of HSA at both sites I and II [178]. Interestingly, an enhancement of α values was generally observed for drugs binding at site II after acetylation of Lys 199. Lastly, as shown by Hage's group, Trp 214 located in the hydrophobic pocket of subdomain IIA (site I), plays an important role in chiral discrimination of *R,S*-warfarin by HSA, since the racemic mixture was no more resolved on HSA-columns modified at Trp 214 [179]. It should be underscored that the retention of the enantiomers of D,L-tryptophan simultaneously decreased, because of an allosteric-induced change of the indole-benzodiazepine site.

It is worth noting that α-chymotrypsin [32] has also been used as the immobilized chiral selector for the separation of amino-acid derivatives [32,180], dipeptides [181] and acidic solutes with an aromatic group in the vicinity of the asymmetric carbon [49,180].

5.4. Pros and cons of protein-based CSPs

Some protein-based CSPs (HSA, AGP) can be employed to predict drug-protein binding since the retention and enantioselectivity on immobilized proteins are a reflection of the binding behavior of native proteins. However, most biological applications are devoted to the direct enantioseparation of therapeutic substances. A wide range of chiral compounds have been resolved, owing to multiple intermolecular interactions allowed by protein surfaces. It is therefore difficult to predict retentions and selectivities using such chiral phases. However, the respective contribution of selective and non-selective binding to retention can be occasionally determined [74,148]. In some cases, high selectivity values have been reported. For example, the enantioselectivity of CBH-I supports for β-blockers is remarkable ($\alpha = 9.9$ for alprenolol [72]) and the enantiomers of D,L-tryptophan can be resolved on BSA-CSPs with α values up to 14.0 under suitable chromatographic conditions [76]. Retention and enantioselectivity can be easily regulated by mobile phase parameters (pH, ionic strength, organic modifiers) and temperature. However, unexpected and unexplained effects may be observed for some solutes [182]. Although immobilized proteins are probably more stable than native proteins, such chiral stationary phases may suffer from a significant shortening of their lifetime in the presence of organic solvents and at high temperatures. Some reversible pH-dependent conformational changes for usual proteins have also been reported in the pH range 3.0–7.5.

Another drawback of protein-based stationary phases is their low efficiency resulting from slow adsorption/desorption kinetics of the analyte to/from selective and non-selective binding sites on the protein surface. The efficiency may be improved by adjusting the mobile phase composition and column temperature. But, because of the large size of chiral selectors, the major disadvantage of protein supports is their very limited capacity compared to other CSPs. In order to increase the density of chiral recognition center(s) in the columns and to reduce the number of non-selective binding sites which could be responsible for a decrease in efficiency, protein fragments have been evaluated as immobilized chiral selectors [48,59,60,77]. Since different results have been reported by Haginaka's and Allenmark's groups, it seems that the enantioselective properties and loadability of immobilized BSA fragments for a given solute depend on both the purification method and the immobilization procedure [59,60,77]. According to Haginaka and Kanasugi, immobilized BSA fragments showed a better enantioselectivity for benzoin, lorazepam and fenoprofen than BSA-CSPs. But when testing other solutes, better enantioselectivities were observed with intact BSA. As expected, the loadability for benzoin was higher using BSA fragments [77]. On the contrary, BSA fragment-bonded columns prepared by Allenmark's group had less capacity and enantioselectivity than BSA-bonded columns. For instance, no chiral recogni-

tion of warfarin was obtained when using BSA fragments [59,60].

Due to their poor loadability, the main use of protein-based CSPs is limited to analytical separations. In spite of this, semipreparative protein-based columns are commercially available. Recently, the direct semipreparative separation of the enantiomers of pentoxifylline metabolite (M1) has been reported by Nicklasson et al. [183] and some examples of semipreparative separations using commercially available columns are reported on web sites. For example, 4.7 mg of acebutolol can be enantioresolved at pH = 6.25 using CHIRAL-CBH columns (150 mm × 10.0 mm) with a mobile phase containing 5% of 2-propanol [184].

5.5. Recent developments in chiral chromatography

Recentlty, the enantioselective properties of BSA-coated zirconia supports have been studied by Park et al. [46]. This type of matrix can be used over the pH range from 1.0 to 14.0, even if it should be noted that enantioseparations using protein-based chiral stationary phases are generally carried out at pH values between 3.0 and 7.5. Due to the instability of Zr–C and Zr–O–Si bonds in water, BSA was crosslinked inside the pores of zirconia, using glutaraldehyde. Surprinsingly, chiral basic solutes have been resolved on these CSPs with high α values due to the low retention of the first eluted enantiomer. This suggests a low contribution of non-selective interactions between the analyte and this kind of support and a possible denaturation of BSA immobilized on zirconia, resulting in unusual chiral discrimination properties. Other protein-based zirconia materials should be evaluated in the future.

It is interesting to note the recent development of porous membrane chromatography systems. They allow chiral separations with similar α values at any flow rate, since diffusional mass-transfer resistance of solutes is negligible. For example, rapid chiral separations have been achieved by Nakamura et al. using BSA-multilayered porous hollow-fiber membranes with high protein binding capacity (190 mg of BSA/g of membrane) [54]. The enantiomers of D,L-tryptophan were eluted in <1 min (46 s at 0.3 ml/min; 4.6 s at 3 ml/min), with high separation factors (α = 6.6).

More recently, a miniaturized membrane chromatography system has been described by Lee's group [56]. The capillary connections (Fig. 11) allow easy interfacing with a syringe pump and detection instruments. The system has been successfully evaluated for chiral separation of D,L-thiopental and D,L-tryptophan, using BSA-coated hydrophobic membranes. No protein leakage and no decrease in enantioselectivity were detected for at least 1 week. Anyway, in case of alteration, membranes are easy to replace. As for capillary electrophoresis techniques discussed here, the advantage of such miniaturized membrane chromatography systems lies in sample size (50 nl) and mobile phase consumption in comparison with classical HPLC. It should be noted though, that peak tailing might perhaps be reduced by using less hydrophobic membranes.

6. Immobilized proteins as chiral selectors in capillary electrophoresis

Among the chiral selectors that have been used in capillary electrophoresis for the enantioseparation of different classes of compounds, cyclodextrins and their derivatives are the most commonly used. However, in the past decade, proteins have received increased attention to perform chiral separations. Up until now, addition of proteins to the running buffer is the most common method employed in protein-based chiral capillary electrophoresis. This method, called affinity electrokinetic chromatography (AEKC) [16], or affinity capillary electrophoresis (ACE) [13,185], can be used for the separation of chiral therapeutic substances provided that both enantiomers bind the protein to different extents. In this technique, the interaction of the analytes with the protein results in a change in the net mobility of the analyte, on the condition that the solute and solute–protein complex have different mobilities. Thus, when analyzing ionic drugs, the best enantioseparations are obtained with chiral selectors oppositely charged to the solutes [19]. Another approach to enantioseparations by capillary electrophoresis is to use immobilized protein as chiral selectors. The applied electric field is then used to create electrolyte and solute migration through the capillary, while the enantiomers interact with the immobilized protein as it does in liquid chromatography. For these reasons the method is generally called affinity capillary electrochromatography (ACEC) [13,17,185]. Some applications of this technique are reported in this review.

6.1. Chiral separations using immobilized BSA

The feasibility of chiral separations in capillary electrophoresis using immobilized proteins was first demonstrated in 1992, by Birnbaum and Nilsson with capillaries containing cross-linked BSA gels [41]. In this technique called capillary affinity gel electrophoresis, a mixture of BSA and glutaraldehyde was pumped into the buffer-filled capillary and allowed to gel for 10 min. The entrapment of proteins by chemical crosslinking is not only easy to perform but also allows a high concentration of protein to be immobilized (0.25 mM of BSA). To avoid the formation of bubbles within the capillaries, BSA-gel capillaries were preconditioned in the reverse direction. The enantiomers of D,L-tryptophan could be resolved in 12 min (gel length of 32 cm; 150 V/cm) with a resolution value of 6.0 and a theoretical plate number from 85,000 to 91,000 showing the high efficiency of this capillary electrophoretic system compared to HPLC methods using the same immobilized chiral selector (BSA). But, due to the ultraviolet (UV) absorbance of crosslinked protein-gels,

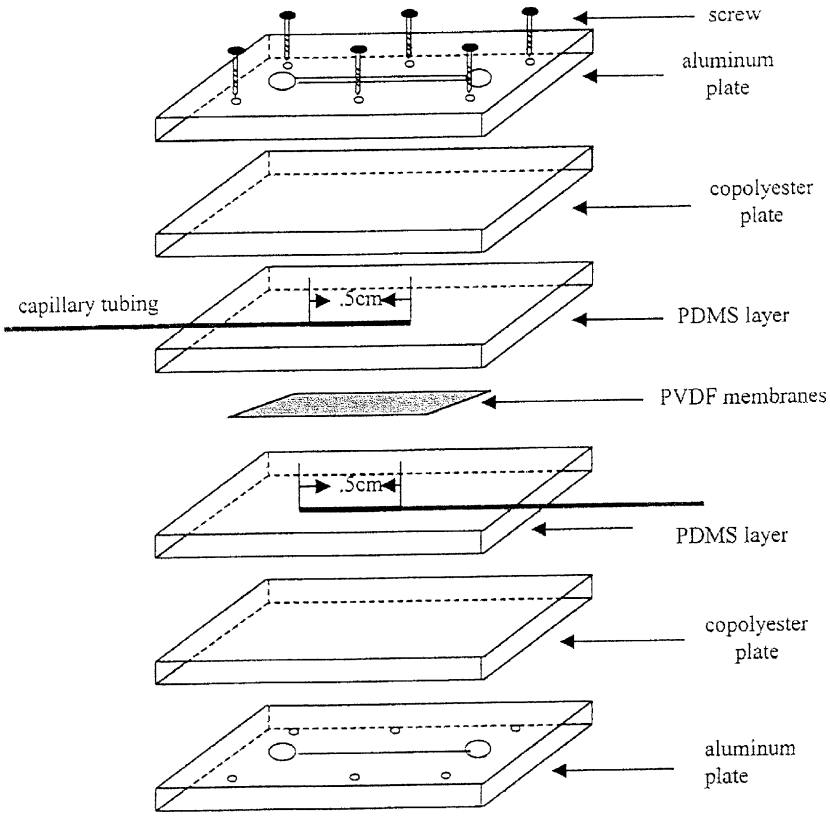


Fig. 11. Miniaturized membrane chromatography system containing preformed PDMS microchannels. Channels are fabricated in the PDMS substrate. The copolyester pieces are used to provide structural support to the soft PDMS substrate (reproduced from [56] with permission).

it is essential that the detection window region be free of gel. To overcome this problem, another approach is the use of capillaries filled with UV transparent BSA–dextran polymer networks, as reported by Sun et al. [40]. The protein is covalently linked to the polymer using cyanogen bromide. Thereafter, polyacrylamide-coated capillaries are filled with the BSA–dextran conjugate, resulting in a stable immobilized gel bed, since the residual electroosmotic flow is opposite in direction to that of the protein–polymer complex. It should be underscored that whenever required, the protein–dextran network can be rapidly replaced by a fresh BSA–polymer gel. Interestingly, for strongly retained solutes, the migration times can be controlled since the phase ratio of the chiral selector can be easily modified by dilution with non-derivatized dextran. Using this technique, the enantiomers of leucovorin were resolved in <9 min (effective gel length of 20 cm) [40]. The analysis time was shorter than in a previous work [186] where BSA was used as the buffer additive, thus demonstrating the superiority of immobilized-protein systems for solutes like leucovorin, which move in the same direction as the protein. Lately, Kato et al. developed a new sol–gel method for the preparation of BSA-encapsulated monolithic columns [18]. The amount of BSA immobilized in the capillary can be easily regulated since it is dependent on the concentration of protein added to the hydrolyzed silane. At BSA concentrations higher than 5% (w/v), the enantiomers of D,L-tryptophan and benzoin have been successfully resolved with a correct run-to-run repeatability. However, the theoretical plate number calculated for D-tryptophan was lower than values reported by Birnbaum and Nilsson for cross-linked BSA gels [41]. Additionally, L-tryptophan showed considerable peak tailing. It is worth noting that BSA bound to the inner surface of fused silica capillaries has also been used in affinity open tubular capillary electrochromatography (OTCEC), to resolve the enantiomers of dinitrophenyl-amino acids and benzodiazepines [37]. The main advantages of this technique are the high efficiency resulting from the plug flow profile at the one hand and the possibility of UV detection over a large range of wavelengths without limitations caused by protein absorption at the other hand. However, the low amount of protein immobilized on the capillary may be considered as a major drawback because of overloading effects.

6.2. Chiral separations using immobilized HSA

Immobilized HSA has also been used to achieve chiral separations in capillary electrophoresis. Hjerten et al. reported the preparation of continuous polyacrylate gels containing immobilized human albumin [187], using a method similar to that described for the preparation of chiral chromatography columnns [51]. In this approach, the gel is synthesized in situ in the capillary. To avoid gel migration during the course of an analysis, the capillary

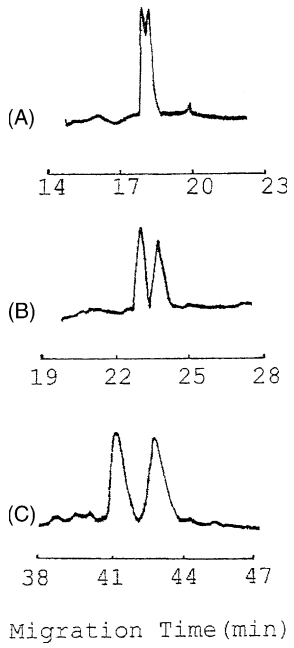


Fig. 12. Chiral separation of AOQ in different capillaries. (A) bare silica capillary; (B) HSA-coated capillary; (C) polyacrylamide-coated capillary, effective length 35.7 cm. Conditions: 67 mM phosphate buffer, pH = 7.4 containing 0.050 mM HSA; applied voltage: >16 kV; UV detection at 244 nm (reproduced from [39] with permission, no further details on detection available).

must be coated by a polyacrylamide layer. Such capillaries (gel length 14 cm) allowed baseline separation of the enantiomers of D,L-kynurenine in <8 min. Capillaries packed with HSA-derivatized silica particles have also been tested for the chiral separation of benzoin and benzodiazepines [45]. In the presence 7.5% of 2-propanol, the enantiomers of temazepam have been resolved in 25 min ($\alpha = 1.6$) with an efficiency similar to that observed in HPLC ($N =$ 7000 plates/m). The amounts of sample and solvents required in capillary electrophoresic techniques are, however, much lower than in HPLC.

Since proteins are known to adsorb to the inner surface of fused silica capillaries, the role played by adsorbed HSA in affinity capillary electrophoresis, using HSA as a buffer additive, has been examined by Hage's group [188]. The amount of HSA immobilized on the capillary wall was estimated to be 0.7 monolayer. There was, however, a slow protein desorption in the presence of an electric field. It is interesting to underscore that the adsorbed protein was the predominant agent involved in the enantioseparation of *R,S*-warfarin, while HSA in the running buffer was responsible for the resolution of the enantiomers of D,L-tryptophan. In another study, Zhang et al. compared the chiral separations of an amino-propionic acid derivative (AOQ) performed in three different kinds of capillaries, using HSA as a buffer additive: (i) a bare capillary, (ii) a HSA-grafted capillary (iii) a polyacrylamide-coated capillary [39]. As shown in Fig. 12, a poor enantioseparation was observed with bare capillaries whereas the enantiomers were baseline resolved using HSA- and polyacrylamide-coated capillaries. This result was attributed to the adsorption of HSA on the capillary wall which could result in a loss of enantioselectivity for AOQ. This phenomenon was not observed with HSA- and polyacrylamide-coated capillaries, since protein adsorption on the inner surface of these capillaries was negligible.

6.3. Chiral separations using immobilized AGP

Although AGP has the broadest field of applications in chiral chromatography, few enantioseparations have been achieved in capillary electrophoresis using immobilized AGP. Capillaries filled with classical CHIRAL-AGP supports were tested in 1993 by Li and Lloyd for the separation of various chiral compounds [44]. Most basic and neutral solutes were resolved with the same elution order as in HPLC, while negatively charged compounds were not eluted from the packed capillary. This problem may be due to the injection mode used in this study (electromigration). The efficiency was generally higher than that found in HPLC using AGP-CSPs. Surprisingly, no significant improvement of the enantioselectivity was observed upon increasing pH, whereas it has been demonstrated that pH played a key-role in chiral chromatography. It is however, difficult to make a direct comparison between separations obtained by both methods, because of the lower buffer concentration range used in the electrophoretic technique [44]. More recently, stable AGP-capillaries have been developed by Hong et al., by covalent grafting of the protein to the inner surface of the capillary wall [38]. The enantiomers of benzoin and promethazine have been successfully resolved in <10 min with these capillaries. Attention should be drawn to the fact that a suitable voltage must be applied to the capillary to obtain high resolution values. The electric field must be low enough to keep the interaction for a certain time before the solutes migrate out of the capillary. However, the decrease in the electric field (values lower than 250 V/cm) simultaneously induces a large increase of the peak width. An intermediate value around 300 V/cm allows good separations.

6.4. Chiral separations using other immobilized proteins

Lysozyme-, avidin-, and cytochrome-coated capillaries have also been tested by Liu et al. to achieve chiral separations in open tubular capillary electrophoresis [35,36,62,189]. Lysozyme-coated capillaries show high chiral selectivity for a number of amino acids including tryptophan, with a reasonable run-to-run reproducibility (17% variation of the migration times after 27 runs) [35]. As shown in Fig. 13, acidic drugs, such as ketoprofen, flurbiprofen and ibuprofen have been successfully resolved with avidin-coated capillaries [36]. Interestingly, the addition of methanol to the running buffer reduced the retention

and increased the efficiency. For instance, the theoretical plate number for the first eluted enantiomer of ketoprofen increased from 7700 plates/m in the absence of methanol to 186,600 plates/m with 15% of methanol in the buffer. These values demonstrate that much higher efficiencies may be attained by open tubular capillary electrophoresis, than with packed capillaries.

Cellobiohydrolase I (CBH I) which showed remarkable enantioselective properties towards β-blocking agents in HPLC, has been tested in affinity gel electrophoresis [42,43]. To obtain stable gels, BSA was used as a matrix component to form a crosslinked gel with glutaraldehyde. The resulting capillaries were found to separate the enantiomers of all the β-blocking agents studied (except for alprenolol), with high resolution values [43]. The efficiencies were between 30,000 and 75,000 plates/m for the first eluted enantiomer, showing that affinity gel electrophoresis is more efficient than capillaries packed with protein-based

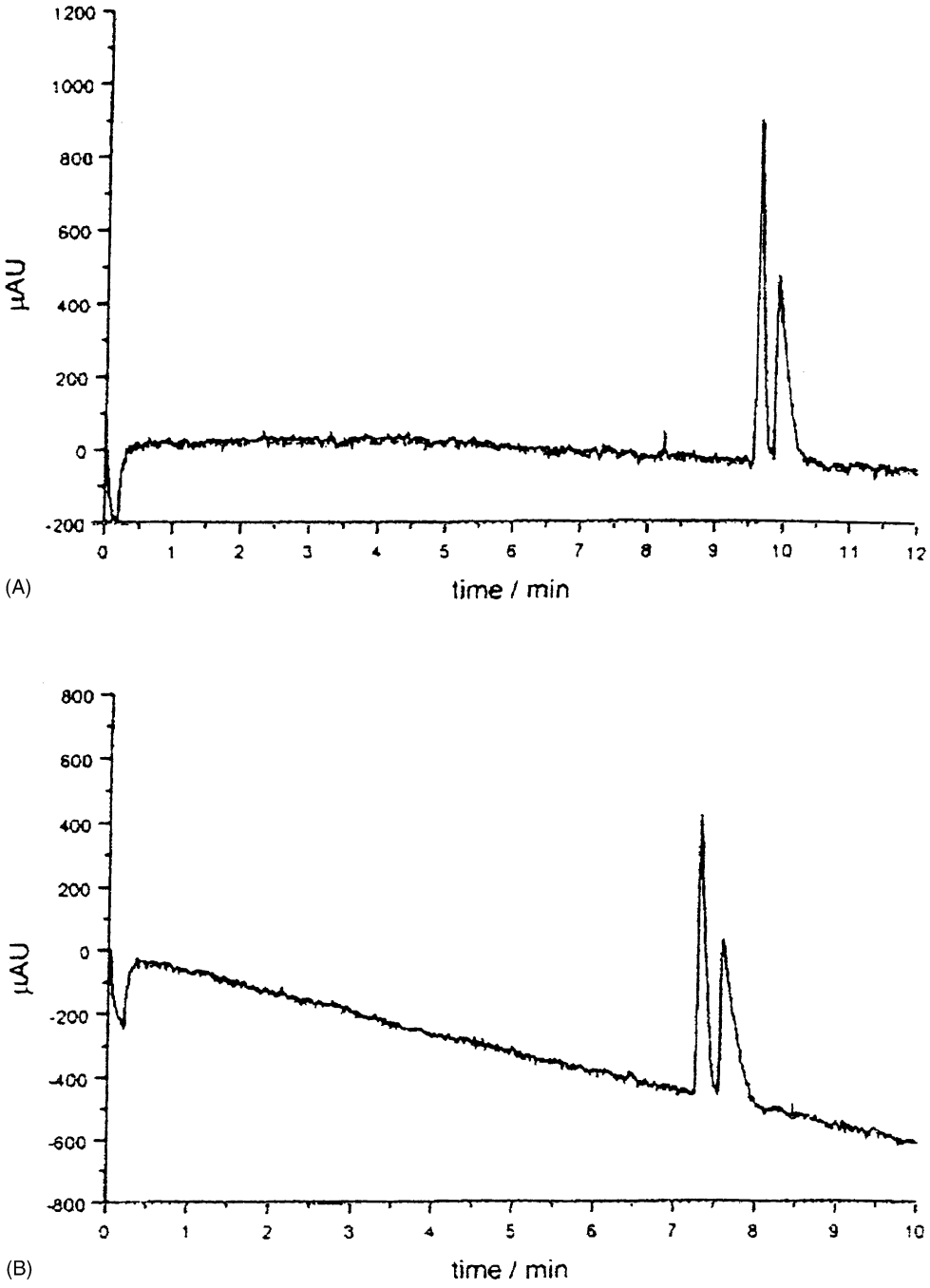


Fig. 13. Enantiomeric separation of: (A) ketoprofen; (B) flurbiprofen; and (C) ibuprofen with avidin-coated capillaries (effective length 50 cm). Conditions: 10 mM phosphate buffer, pH = 5.95 containing different concentrations (v/v) of methanol (A: 15%; B: 5%; C: none); UV detection at 200 nm; applied voltage: −25 kV (reproduced from [36] with permission).

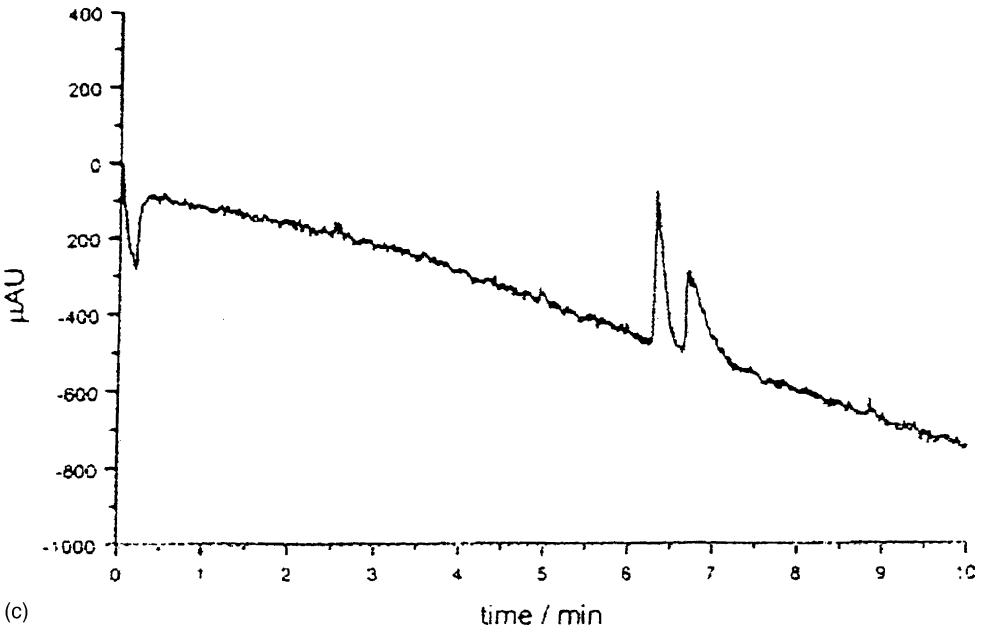


Fig. 13. (*Continued*).

CSPs. In this study, Nilsson et al. compared separations obtained when using CBH I gels and cyclodextrin added to the electrolyte [43]. Even if the efficiency of protein-gels was inferior, the highest selectivities were achieved with immobilized CBH I.

In a recent study, ovomucoid (OVM) has been encapsulated in a tetramethoxysilane-based hydrogel, as described for BSA [18]. The enantiomers of benzoin and some basic drugs have been baseline resolved in less than 30 min with these monolithic OVM-capillaries. It should be underscored that the efficiency for basic drugs was lower than that observed for neutral ones (e.g. 72,000 and 19,000 plates/m for benzoin and eperison, respectively). The observed peak tailing was attributed to electrostatic interactions between positively charged solutes and the matrix.

6.5. Pros and cons of protein-based (immobilized) chiral selectors in capillary electrophoresis

Numerous accounts in the literature describe chiral separations by capillary electrophoresis using proteins as running buffer additives (AEKC), while the number of enantioseparations involving immobilized protein selectors in affinity capillary electrochromatography (ACEC) is much more limited [13,19]. AEKC has, indeed, several advantages over ACEC. Method developments in AEKC are easily performed since this technique does not require protein immobilization. In addition, the use of soluble proteins eliminates the possibility of altering both the structure and the binding properties of the protein. Last, the higher peak efficiency obtained in AEKC is another advantage of this technique over ACEC,

Table 3
Efficiencies measured in affinity capillary electrochromatography (ACEC) techniques

Immobilization procedure	Protein	Chiral solute	*N* (plates/m)[a]
Wall-adsorption (OTCEC)	Lysozyme	Tryptophan	65000 [35]
	Avidin	Ketoprofen	186600[b] [36]
Wall-grafting (OTCEC)	HSA	AOQ	65000 [39]
Packing with protein-based CSPs	AGP	Benzoin	34000 [44]
	HSA	Temazepam	7000 [45]
Crosslinking with glutaraldehyde (capillary gel electrophoresis)	BSA	Tryptophan	280000 [41]
	CBH I + BSA	β-Blockers	30000–75000 [43]
Monolithic sol–gel bed	BSA	Tryptophan	57000 [18]
	OVM	Benzoin	72000 [18]

[a] Calculated for the first eluted enantiomer.
[b] With 15% methanol in the running buffer.

although tailed peaks resulting from the slow kinetics of drug–protein interactions, are even so observed in AEKC, when using protein selectors.

In spite of these advantages, proteins cannot be considered as ideal buffer additives in AKEC and many enantioseparations have been performed using other chiral selectors [17]. A critical problem encountered with proteins in AEKC, is the possible adsorption of proteins onto the capillary wall which may induce (i) changes in both the migration times and the peak area, (ii) instability of the baseline, (iii) peak tailing. Another drawback of AEKC is the low detection sensitivity resulting from the strong UV absorbance of the background electrolyte when concentrated protein solutions are required. For these reasons, the affinity capillary electrochromatography approach using immobilized protein chiral selectors which is described in this review, is one alternative possibility for the enantioresolution of chiral drugs.

For example, a number of proteins including HSA [39], BSA [37], AGP [38], avidin [36], lysozyme [35] and cytochrome [62] have been tested for chiral separations in open tubular capillary electrochromatography (OTCEC). This technique uses immobilized protein selectors either adsorbed or grafted to the inner surface of the capillary. As reported recently by Liu et al., a significant limitation of this technique is the low phase ratio, so that OTCEC is only suitable for the separation of enantiomers that have strong interactions with the protein [36]. Moreover, sample overloading may result in peak broadening and loss of efficiency. Interestingly, OTCEC has several advantages over other ACEC techniques using packed or molded capillaries, including the easy preparation of capillaries, small amounts of protein required for immobilization and short conditioning times.

ACEC methods using capillaries containing either classical protein-CSPs, protein-gels or monolithic sol–gel beds have a number of advantages over OTCEC, including higher loading capacity and higher sensitivity. However, longer conditioning times are generally required and some bubble problems may be encountered. In situ preparation methods whereby no frits are required, are the most attractive procedures. For instance the protein-encapsulation technique using sol–gel is a promising method, although a decrease in non-specific interactions and an improvement of the lifetime and capillary-to-capillary repeatability are still required [18]. Although it would be hazardous to make a comparative study of peak efficiencies obtained in these different formats, it appears in Table 3 that protein-coated capillaries, gel-filled capillaries and monolithic sol–gel columns are more performant than capillaries packed with protein-based silica particles.

7. Conclusion

The enantioselective properties of proteins have been exploited to develop protein-based matrices in liquid chromatography and capillary electrophoresis. Currently, chiral separations using immobilized protein selectors are generally carried out in the HPLC format, since a number of performant protein-based CSPs are commercially available. Despite a shorter lifetime than other chiral supports, protein-based columns have been frequently used in HPLC for the enantioseparation of chiral drugs, owing to their versatility and broad applicability. It should be, however, underscored that affinity capillary electrochromatography involving immobilized protein selectors has a number of potential advantages over HPLC. It is a rapid technique requiring minimum solute, solvent and protein consumption. Moreover, the peak efficiency is generally higher than that found for HPLC separations using analogous proteins as immobilized chiral selectors. However, due to problems of sample overloading and to the slow kinetics of interaction between the solute and the protein, band broadening in capillary electrochromatography is higher than theoretically expected. In conclusion, before capillary electrochromatography could become a powerful technique for routine analysis of chiral solutes and for screening the stereoselective binding properties of drugs to proteins, further investigations are still necessary to develop procedures allowing the immobilization of larger amounts of protein and to improve both the reproducibility and the lifetime of capillaries.

References

[1] G.W. Peng, W.L. Chiou, J. Chromatogr. 531 (1990) 3.
[2] H.Y. Aboul-Enein, L.I. Abou-Basha, The impact of Stereochemistry on Drug Development and Use, Wiley, New York, 1997, p. 1.
[3] M.L. Williams, I.W. Wainer, Ther. Drug Monit. 24 (2002) 290.
[4] B. Silber, N.H.G. Holford, S. Riegelman, J. Pharm. Sci. 71 (1982) 699.
[5] S. Allenmark, Chromatographic Enantioseparation, Methods and Applications, second ed., Ellis Horwood, New York, 1991, Chapter 7, p. 107.
[6] I.M. Mutton, in: G. Subramanian (Ed.), A Practical Approach to Chiral Separations by Liquid Chromatography, VCH, New York, 1994, Chapter 11, p. 329.
[7] O.D. Chandwani, S.R. Dhaneshwar, Indian Drugs 33 (5) (1996) 187.
[8] B.J. Clark, in: G. Subramanian (Ed.), A Practical Approach to Chiral Separations by Liquid Chromatography, VCH, New York, 1994, Chapter 10, p. 311.
[9] D.W. Armstrong, J. Chin. Chem. Soc. 45 (1998) 581.
[10] Y. Okamoto, E. Yashima, Chromatographic Separations Based on Molecular Recognition, Wiley, New York, 1997, Chapter 4, p. 239.
[11] S.G. Allenmark, S. Andersson, J. Chromatogr. A 666 (1994) 167.
[12] J. Haginaka, J. Chromatogr. A 906 (2001) 253.
[13] J. Haginaka, J. Chromatogr. A 875 (2000) 235.
[14] G. Blaschke, B. Chankvetadze, J. Chromatogr. A 875 (2000) 3.
[15] M. Novotny, H. Soini, M. Stefansson, Anal. Chem. 66 (1994) 646A.
[16] H. Nishi, J. Chromatogr. A 735 (1996) 57.
[17] D. Wistuba, V. Schurig, J. Chromatogr. A 875 (2000) 255.
[18] M. Kato, K. Sakai-Kato, N. Matsumoto, T. Toyo'oka, Anal. Chem. 74 (2002) 1915.
[19] Y. Tanaka, S. Terabe, J. Biochem. Biophys. Methods 48 (2001) 103.
[20] K.K. Stewart, R.F. Doherty, Proc. Natl. Acad. Sci. U.S.A. 70 (1973) 2850.

[21] J. Hermansson, J. Chromatogr. 269 (1983) 71.
[22] T. Miwa, M. Ichikawa, M. Tsuno, T. Hattori, T. Miyakawa, M. Kayano, Y. Miyake, Chem. Pharm. Bull. 35 (1987) 682.
[23] S. Allenmark, B. Bomgren, H. Borén, J. Chromatogr. 264 (1983) 63.
[24] E. Domenici, C. Brett, P. Salvadori, G. Félix, I. Cahagne, S. Motellier, I.W. Wainer, Chromatographia 29 (1990) 170.
[25] P. Erlandsson, I. Marle, L. Hansson, R. Isaksson, C. Petterson, G. Petterson, J. Am. Chem. Soc. 112 (1990) 4573.
[26] T. Miwa, T. Miyakawa, Y. Miyake, J. Chromatogr. 457 (1988) 227.
[27] J. Haginaka, Y. Miyano, Y. Saizen, C. Seyama, T. Murashima, J. Chromatogr. A 708 (1995) 161.
[28] N. Mano, Y. Oda, N. Asakawa, Y. Yoshida, T. Sato, T. Miwa, J. Chromatogr. 623 (1992) 221.
[29] J. Haginaka, C. Seyama, N. Kanasugi, Anal. Chem. 67 (1995) 2539.
[30] A. Nyström, A. Strandberg, A. Aspegren, S. Behr, A. Karlsson, Chromatographia 50 (1999) 209.
[31] S. Thelohan, P. Jadaud, I.W. Wainer, Chromatographia 28 (11–12) (1989) 551.
[32] I.W. Wainer, P. Jadaud, G.R. Schombaum, S.V. Kadodkar, M.P. Henry, Chromatographia 25 (10) (1988) 903.
[33] N. Mano, Y. Oda, N. Asakawa, Y. Yoshida, T. Sato, J. Chromatogr. 603 (1992) 105.
[34] J. Haginaka, T. Murashima, C. Seyama, J. Chromatogr. A 666 (1994) 203.
[35] Z. Liu, H. Zou, J.Y. Ni, Y. Zhang, Anal. Chim. Acta 378 (1999) 73.
[36] Z. Liu, K. Otsuka, S. Terabe, J. Sep. Sci. 24 (2001) 17.
[37] H. Hofstetter, O. Hofstetter, V. Schurig, J. Microcol. Sep. 10 (1998) 287.
[38] F. Hong, X.X. Zhang, W.B. Chang, Y.X. Ci, Anal. Chim. Acta 373 (1998) 207.
[39] X.X. Zhang, F. Hong, W.B. Chang, Y.X. Ci, Y.H. Ye, Anal. Chim. Acta 392 (1999) 175.
[40] P. Sun, G.E. Barker, R.A. Hartwick, J. Chromatogr. A 652 (1993) 247.
[41] S. Birnbaum, S. Nilsson, Anal. Chem. 64 (1992) 2872.
[42] H. Ljunberg, S. Nilsson, J. Liq. Chromatogr. 18 (1995) 3685.
[43] S. Nilsson, L. Schweitz, M. Petersson, Electrophoresis 18 (1997) 884.
[44] S. Li, D.K. Lloyd, Anal. Chem. 65 (1993) 3684.
[45] D.K. Lloyd, S. Li, P. Ryan, J. Chromatogr. A 694 (1995) 285.
[46] J.H. Park, J.K. Ryu, J.K. Park, C.V. Mc Neff, P.W. Carr, Chromatographia 53 (2001) 405.
[47] J. Haginaka, H. Takehira, J. Chromatogr. A 773 (1997) 85.
[48] I. Marle, S. Jönsson, R. Isaksson, C. Pettersson, J. Chromatogr. 648 (1993) 333.
[49] I. Marle, A. Karlsson, C. Pettersson, J. Chromatogr. 604 (1992) 185.
[50] V. Tittelbach, M. Jaroniec, R.K. Gilpin, J. Liq. Chrom. Rel. Technol. 19 (1996) 2943.
[51] J. Mohammad, Y.M. Li, M. El-Ahmad, K. Nakazato, G. Pettersson, S. Hjerten, Chirality 5 (1993) 464.
[52] Z. Simek, R. Vespalec, J. Chromatogr. A 685 (1994) 7.
[53] H. Hofstetter, O. Hofstetter, V. Schurig, J. Chromatogr. A 764 (1997) 35.
[54] M. Nakamura, S. Kiyohara, K. Saito, K. Sugita, T. Sugo, Anal. Chem. 71 (1999) 1323.
[55] J. Randon, F. Garnier, J.L. Rocca, B. Maïsterrena, J. Membr. Sci. 175 (2000) 111.
[56] P.C. Wang, J. Gao, C.S. Lee, J. Chromatogr. A 942 (2002) 115.
[57] H. Wännman, A. Walhagen, P. Erlandsson, J. Chromatogr. 603 (1992) 121.
[58] J. Hermansson, I. Hermansson, J. Chromatogr. 631 (1993) 79.
[59] P. Erlandsson, S. Nilsson, J. Chromatogr. 482 (1989) 35.
[60] S. Andersson, S. Allenmark, P. Erlandsson, S. Nilsson, J. Chromatogr. 498 (1990) 81.
[61] K. Harada, Q. Yuan, M. Nakayama, A. Sugii, J. Chromatogr. A 740 (1996) 207.
[62] Z. Liu, H. Zou, M. Ye, J. Ni, Y. Zhang, Chin. J. Chromatogr. 17 (1999) 245.
[63] R.A. Thompson, S. Andersson, S. Allenmark, J. Chromatogr. 465 (1989) 263.
[64] L. Dalgaard, J.J. Hansen, J.L. Pedersen, J. Pharmacol. Biomed. Anal. 7 (1989) 361.
[65] S.C. Jacobson, G. Guiochon, Anal. Chem. 64 (1992) 1496.
[66] S.C. Jacobson, G. Guiochon, J. Chromatogr. 600 (1992) 37.
[67] N.L. Taleb, M.C. Millot, B. Sébille, J. Chromatogr. 776 (1997) 45.
[68] M. Nakamura, S. Kiyohara, K. Saito, K. Sugita, T. Sugo, J. Chromatogr. A 822 (1998) 53.
[69] M.C. Millot, S. Servagent-Noinville, N.L. Taleb, M.H. Baron, M. Revault, B. Sébille, J. Chromatogr. B 753 (2001) 101.
[70] J. Hermansson, Trends Anal. Chem. 8 (1989) 251.
[71] G. Felix, M. Liu, BioSciences 8 (1989) 2.
[72] I. Marle, P. Erlandsson, L. Hansson, R. Isaksson, C. Pettersson, G. Pettersson, J. Chromatogr. 586 (1991) 233.
[73] H. Henriksson, S. Jönsson, R. Isaksson, G. Pettersson, Chirality 7 (1995) 415.
[74] H. Henriksson, I.G. Munoz, R. Isaksson, G. Pettersson, G. Johansson, J. Chromatogr. A 898 (2000) 63.
[75] T.C. Pinkerton, W.J. Howe, E.L. Ulrich, J.P. Comiskey, J. Haginaka, T. Murashima, W.F. Walkenhorst, W.M. Westler, J.L. Markley, Anal. Chem. 67 (1995) 2354.
[76] S. Andersson, R.A. Thompson, S. Allenmark, J. Chromatogr. 591 (1992) 65.
[77] J. Haginaka, N. Kanasugi, J. Chromatogr. A 694 (1995) 71.
[78] G. Massolini, E. De Lorenzi, M.C. Ponci, C. Gandini, G. Caccialanza, H.L. Monaco, J. Chromatogr. A 704 (1995) 55.
[79] E. De Lorenzi, G. Massolini, D.K. Lloyd, H.L. Monaco, C. Galbusera, G. Caccialanza, J. Chromatogr. A 790 (1997) 47.
[80] J. Haginaka, C. Seyama, H. Yasuda, H. Fujima, H. Wada, J. Chromatogr. 592 (1992) 301.
[81] J. Haginaka, T. Murashima, C. Seyama, H. Fujima, H. Wada, J. Chromatogr. 631 (1993) 183.
[82] Y. Oda, N. Asakawa, S. Abe, Y. Yoshida, T. Sato, J. Chromatogr. 572 (1991) 133.
[83] Y. Oda, H. Ohe, N. Asakawa, Y. Yoshida, T. Sato, T. Nakagawa, J. Liq. Chromatogr. 15 (1992) 2997.
[84] J. Haginaka, C. Seyama, T. Murashima, H. Fujima, H. Wada, J. Chromatogr. A 660 (1994) 275.
[85] M. Aubel, L.B. Rogers, J. Chromatogr. 392 (1987) 415.
[86] V. Tittelbach, R.K. Gilpin, Anal. Chem. 67 (1995) 44.
[87] M.C. Millot, B. Sébille, C. Mangin, J. Chromatogr. A 776 (1997) 37.
[88] M.C. Millot, L. Loehman, B. Sébille, H. Hommel, Chromatographia 50 (1999) 641.
[89] M.C. Millot, N.L. Taleb, B. Sébille, J. Chromatogr. B 768 (2002) 157.
[90] H. Henriksson, J. Stahlberg, A. Koivula, G. Pettersson, C. Divne, L. Valtcheva, R. Isaksson, J. Biotechnol. 57 (1997) 115.
[91] M. Hedeland, H. Henriksson, R. Isaksson, G. Pettersson, Chirality 10 (1998) 760.
[92] A. Haque, J.T. Stewart, J. Liq. Chromatogr. Rel. Technol. 21 (1998) 2675.
[93] E. De Lorenzi, G. Massolini, Pharm. Sci. Technol. Today 2 (1999) 352.
[94] J.M.H. Kremer, J. Wilting, L.H.M. Janssen, Pharmaceut. Rev. 40 (1988) 1.
[95] J. Haginaka, H. Matsunaga, Enantiomer 5 (2000) 37.
[96] A. Rojo-Domingues, A. Hernandez-Arana, Protein Seq. Data Anal. 5 (1993) 349.
[97] I. Kato, J. Schrode, W.J. Kohr, M. Laskowski, Biochemistry 26 (1987) 193.
[98] J. Haginaka, C. Seyama, T. Murashima, J. Chromatogr. A 704 (1995) 279.
[99] J. Haginaka, H. Takehira, J. Chromatogr. A 777 (1997) 241.

[100] M. Walker, L. Stevens, D. Duncan, N.C. Price, S.M. Kelly, Comp. Biochem. Physiol. 100B (1991) 77.
[101] R.C. Williams, J.F. Edwards, M.J. Potter, J. Liq. Chromatogr. 16 (1993) 171.
[102] N. Mano, Y. Oda, N. Asakawa, Y. Yoshida, T. Sato, T. Miwa, J. Chromatogr. A 687 (1994) 223.
[103] K.M. Kirkland, K.L. Neilson, D.A. Mc Combs, J. Chromatogr. 545 (1991) 43.
[104] K. Borner, E. Borner, H. Lode, Chromatographia 47 (1998) 171.
[105] S. Song, L. Zhou, R. Thompson, M. Yang, D. Ellison, J.M. Wyvratt, J. Chromatogr. A 959 (2002) 299.
[106] A. Karlsson, A. Aspegren, J. Chromatogr. A 866 (2000) 15.
[107] C. Vandenbosch, T. Hamoir, D.L. Massart, W. Lindner, Chromatographia 33 (1992) 454.
[108] K. Gyimesi-Forras, G. Szasz, A. Gergely, M. Szabo, J. Kökösi, J. Chromatogr. Sci. 38 (2000) 430.
[109] J.S. Munro, T.A. Walker, J. Chromatogr. A 913 (2001) 275.
[110] J.S. Munro, J.P. Gormley, T.A. Walker, J. Liq. Chrom. Rel. Technol. 24 (2001) 327.
[111] P.S. Bonato, L.R.P. de Abreu, C.M. de Gaitani, V.L. Lanchote, C. Bertucci, Biomed. Chromatogr. 14 (2000) 227.
[112] T. Miwa, T. Miyakawa, M. Kayano, Y. Miyake, J. Chromatogr. 408 (1987) 316.
[113] Y. Oda, N. Mano, N. Asakawa, Y. Yoshida, T. Sato, T. Nakagawa, Anal. Sci. 9 (1993) 221.
[114] G. Massolini, E. De Lorenzi, E. Calleri, E. Tabolotti, G. Caccialanza, J. Chromatogr. B 738 (2000) 343.
[115] A. Karlsson, A. Aspegren, Chromatographia 47 (1998) 189.
[116] C. Alvarez, J.A. Sanchez-Brunete, S. Torrado-Santiago, R. Cadorniga, J.J. Torrado, Chromatographia 52 (2000) 455.
[117] A.F. Aubry, F. Gimenez, R. Farinotti, I.W. Wainer, Chirality 4 (1992) 30.
[118] J. Iredale, A.F. Aubry, I. Wainer, Chromatographia 31 (1991) 329.
[119] E. Calleri, E. De Lorenzi, D. Siluk, M. Markuszewski, R. Kaliszan, G. Massolini, Chromatographia 55 (2002) 651.
[120] M.S. Waters, D.R. Sidler, A.J. Simon, C.R. Middaugh, R. Thompson, L.J. August, G. Bicker, H.J. Perpall, N. Grinberg, Chirality 11 (1999) 224.
[121] A.M. Abushoffa, B.J. Clark, J. Chromatogr. A 700 (1995) 51.
[122] B.M. Eriksson, A. Wallin, J. Pharm. Biomed. Anal. 13 (1995) 551.
[123] J. Hermansson, I. Hermansson, J. Chromatogr. A 666 (1994) 181.
[124] J. Hermansson, A. Grahn, J. Chromatogr. A 694 (1995) 57.
[125] A. Ceccato, P. Hubert, P. de Tullio, J.F. Liégeois, A. Felikidis, J. Géczy, J. Crommen, J. Pharm. Biomed. Anal. 18 (1998) 605.
[126] G. Tamai, M. Edani, H. Imai, Biomed. Chromatogr. 4 (1990) 157.
[127] M. Enquist, J. Hermansson, J. Chromatogr. 519 (1990) 285.
[128] H. Makamba, V. Andrisano, R. Gotti, V. Cavrini, G. Felix, J. Chromatogr. A 818 (1998) 43.
[129] J. Hermansson, J. Chromatogr. 298 (1984) 67.
[130] J. Hermansson, M. Eriksson, J. Liq. Chromatogr. 9 (1986) 621.
[131] K.M. Kirkland, D.A. Mc Combs, J. Chromatogr. A 666 (1994) 211.
[132] R. Cirilli, F. La Torre, J. Chromatogr. A 818 (1998) 53.
[133] E. Smet, L. Staelens, Y. Van der Heyden, W.R.G. Baeyens, H.Y. Aboul-Enein, G. Van der Weken, A.M. Garcia-Campana, Chirality 13 (2001) 556.
[134] H. Henriksson, J. Stahlberg, R. Isaksson, G. Pettersson, FEBS Lett. 390 (1996) 339.
[135] P. Tomme, H. van Tilbeurgh, G. Pettersson, J. van Damme, J. Vandekerckhove, J. Knowles, T.T. Teeri, M. Claeyssens, Eur. J. Biochem. 170 (1988) 575.
[136] C. Divne, J. Stahlberg, T. Reinikainen, L. Ruohonen, G. Pettersson, J.K.C. Knolwes, T.T. Teeri, T.A. Jones, Science 265 (1994) 524.
[137] J. Stahlberg, C. Divne, A. Koivula, K. Piens, M. Claeyssens, T.T. Teeri, T.A. Jones, J. Mol. Biol. 264 (1996) 337.
[138] R. Isaksson, C. Pettersson, G. Pettersson, S. Jönsson, J. Stahlberg, J. Hermansson, I. Marle, Trends Anal. Chem. 13 (1994) 431.
[139] C. Vandenbosch, D.L. Massart, G. Egginger, W. Lindner, Trends Anal. Chem. 12 (1993) 168.
[140] H. Henriksson, G. Pettersson, G. Johansson, J. Chromatogr. A 857 (1999) 107.
[141] J. Stahlberg, H. Henriksson, C. Divne, R. Isaksson, G. Pettersson, G. Johansson, T.A. Jones, J. Mol. Biol. 305 (2001) 79.
[142] C. Vandenbosch, D.L. Massart, W. Lindner, Anal. Chim. Acta 270 (1992) 1.
[143] J. Hermansson, A. Grahn, J. Chromatogr. A 687 (1994) 45.
[144] J. Haginaka, Y. Miyano, Anal. Sci. 12 (1996) 727.
[145] D.S. Risley, V.S. Sharp, J.R. Palmer, J. Liq. Chrom. Rel. Technol. 19 (1996) 449.
[146] A. Strandberg, A. Nyström, S. Behr, A. Karlsson, Chromatographia 50 (1999) 215.
[147] A. Karlsson, I. Arfwidsson, Z. Husovic, B. Svensson, Chromatographia 54 (2001) 717.
[148] T. Fornstedt, G. Götmar, M. Andersson, G. Guiochon, J. Am. Chem. Soc. 121 (1999) 1164.
[149] H.Y. Aboul-Enein, V. Serignese, Biomed. Chromatogr. 11 (1997) 47.
[150] S. Jönsson, A. Schön, R. Isaksson, C. Pettersson, G. Pettersson, Chirality 4 (1992) 505.
[151] T. Fornstedt, P. Sajonz, G. Guiochon, J. Am. Chem. Soc. 119 (1997) 1254.
[152] K. Fulde, A.W. Frahm, J. Chromatogr. A 858 (1999) 33.
[153] C. Lagercrantz, T. Larsson, I. Denfors, Comp. Biochem. Physiol. 69C (1981) 375.
[154] G. Massolini, A.F. Aubry, A. Mc Gann, I.W. Wainer, Biochem. Pharmacol. 46 (1993) 1285.
[155] V. Tittelbach, R.K. Gilpin, Anal. Chem. 67 (1995) 44.
[156] I. Fitos, J. Visy, M. Simonyi, J. Biochem. Biophys. Methods 54 (2002) 71.
[157] D.C. Carter, J.X. Ho, Adv. Protein Chem. 45 (1994) 153.
[158] K. Hirayama, S. Akashi, M. Furuya, K.I. Fukuhara, Biochem. Biophys. Res. Commun. 173 (1990) 639.
[159] S. Allenmark, B. Bomgren, H. Boren, J. Chromatogr. 237 (1982) 473.
[160] S. Allenmark, S. Andersson, J. Chromatogr. 351 (1986) 231.
[161] B. Bomgren, S. Allenmark, J. Liq. Chromatogr. 9 (1986) 667.
[162] Y. Abe, S. Fukui, Y. Koshiji, M. Kobayashi, T. Shoji, S. Sugata, H. Nishizawa, H. Suzuki, K. Iwata, Biochim. Biophys. Acta 1433 (1999) 188.
[163] S. Allenmark, J. Liq. Chromatogr. 9 (1986) 425.
[164] S. Allenmark, S. Andersson, Chirality 1 (1989) 154.
[165] S. Allenmark, S. Andersson, J. Bojarski, J. Chromatogr. 436 (1988) 479.
[166] S. Allenmark, B. Bomgren, H. Boren, J. Chromatogr. 316 (1984) 617.
[167] R. Kaliszan, T.A.G. Noctor, I.W. Wainer, Chromatographia 33 (1992) 546.
[168] T.A.G. Noctor, G. Felix, I.W. Wainer, Chromatographia 31 (1991) 55.
[169] W.R.G. Baeyens, G. Van der Weken, J. Haustraete, H.Y. Aboul-Enein, S. Corveleyn, J.P. Remon, A.M. Garcia-Campana, P. Deprez, J. Chromatogr. A 871 (2000) 153.
[170] J. Yang, D.S. Hage, J. Chromatogr. 645 (1993) 241.
[171] T.A.G. Noctor, M.J. Diaz-Perez, I.W. Wainer, J. Pharmaceut. Sci. 82 (1993) 675.
[172] X.M. He, D.C. Carter, Nature 358 (1992) 209.
[173] S. Wanwimolruk, D.J. Birkett, P.M. Brooks, Mol. Pharmacol. 24 (1983) 458.
[174] V. Andrisano, T.D. Booth, V. Cavrini, I.W. Wainer, Chirality 9 (1997) 178.
[175] V. Andrisano, C. Bertucci, V. Cavrini, M. Recanatini, A. Cavalli, L. Varoli, G. Felix, I.W. Wainer, J. Chromatogr. A 876 (2000) 75.
[176] V. Andrisano, R. Gotti, M. Recanatini, A. Cavalli, L. Varoli, C. Bertucci, J. Chromatogr. B 768 (2002) 137.

[177] T.A.G. Noctor, I.W. Wainer, Pharmaceut. Res. 9 (1992) 480.
[178] C. Bertucci, A. Canepa, P. Altemura, Ann. Chim. 87 (1997) 45.
[179] A. Chattopadhyay, T. Tian, L. Kortum, D.S. Hage, J. Chromatogr. B 715 (1998) 183.
[180] G. Felix, V. Descorps, Chromatographia 49 (1999) 595.
[181] P. Jadaud, I.W. Wainer, J. Chromatogr. 476 (1989) 165.
[182] B.A. Persson, S. Andersson, J. Chromatogr. A 906 (2001) 195.
[183] M. Nicklasson, S. Björkman, B. Roth, M. Jönsson, P. Höglund, Chirality 14 (2002) 643.
[184] http://www.chromtech.se/acebutol.htm.
[185] D.S. Hage, Electrophoresis 18 (1997) 2311.
[186] G.E. Barker, P. Russo, R.A. Hartwick, Anal. Chem. 64 (1992) 3024.
[187] S. Hjerten, A. Vegvari, T. Srichaiyo, H.X. Zhang, C. Ericson, D. Eaker, J. Cap. Electrophoresis 5 (1998) 13.
[188] J. Yang, D.S. Hage, Anal. Chem. 66 (1994) 2719.
[189] Z. Liu, H. Zou, M. Ye, J. Ni, Y. Zhang, Electrophoresis 20 (1999) 2891.