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Review

Enantiomer separation of drugs by capillary electrophoresis using proteins as chiral selectors

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Abstract

The separation of drug enantiomers using proteins as the chiral selectors in capillary electrophoresis (CE) is considered in this review. The proteins used include albumins such as bovine serum albumin, human serum albumin and serum albumins from other species, glycoproteins such as α_1-acid glycoprotein, crude ovomucoid, ovoglycoprotein, avidin and riboflavin binding protein, enzymes such as fungal cellulase, cellobiohydrolase I, pepsin and lysozyme and other proteins such as casein, human serum transferrin and ovotransferrin. Protein-based CE is carried out in two modes: in one proteins are immobilized or adsorbed within the capillary, or protein-immobilized silica gels are packed into the capillary (affinity capillary electrochromatography mode), and in the other proteins are dissolved in the running buffer (affinity CE mode). Furthermore, the advantages and limitations of the two modes and the factors affecting the chiral separations of various drugs by protein-based CE are discussed.

Keywords: Reviews; Enantiomer separation; Electrochromatography; Capillary electrophoresis; Affinity capillary electrochromatography; Chiral selector; Drug; Protein; Glycoprotein

Contents

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1. Introduction

Enantiomeric forms of a drug can differ in potency, toxicity and behavior in biological systems [1]. Many chiral analytical methods have been developed for the analysis of drug enantiomers. Among those, high-performance liquid chromatography (HPLC) methods based on chiral stationary phases are widely employed for the assays of drug enantiomers in pharmaceutical preparations and biological fluids [2–4]. Recently, capillary electrophoresis (CE) methods using chiral selectors as the running buffer additives or immobilized ligands have been used for the above purposes. Because CE techniques can take advantages of high resolution, relatively fast separation, and small sample and media size. The chiral additives or immobilized ligands so far employed have included chiral ligand exchange, cyclodextrins, crown ethers, chiral micelles, polysaccharides, proteins, macrocyclic antibiotics and molecularly imprinted polymers [5–12].

HPLC chiral stationary phases based on a protein are of special interest because of their unique properties of stereoselectivity and because they are suited for separating a wide range of enantiomeric mixtures. Similarly, CE methods using proteins as the immobilized ligands or running buffer additives are attractive for the separation of enantiomeric mixtures. Although separation efficiencies by CE are generally somewhat higher than those obtained with HPLC, CE methods based on proteins have disadvantage of low efficiencies in addition to low loadability. Low efficiencies are due to slow interaction kinetics, as described below. Thus, some methods have been applied to test the optical purity of pharmaceutical preparations, while none of the methods was applied to biomedical analysis. This review article deals with protein selectors that can be used for the separations of drug enantiomers in CE and the modes of the use of protein selectors, immobilized and in-solution modes, and comparison of general properties of two modes. Further, factors affecting the chiral separations of various drugs by protein-based CE will be discussed.

2. Protein selectors

A protein or glycoprotein consists of amino acids or amino acids and sugars, both of which are chiral. Thus, all proteins have the possibility to discriminate a chiral molecule. However, only a limited number of proteins have been investigated as HPLC chiral stationary phases. Protein-based stationary phases so far developed have included albumins such as bovine serum albumin (BSA) [13] and human serum albumin (HSA) [14], glycoproteins such as α_1-acid glycoprotein (AGP) [15], ovomucoid from chicken egg whites (OMCHI) [16], ovoglycoprotein from chicken egg whites (OGCHI) [17], avidin (AVI) [18], riboflavin binding protein (RfBP) (or flavoprotein) [19], enzymes such as trypsin [20], α-chymotrypsin [21], cellobiohydrolase I (CBH I) [22], pepsin [23], lysozyme [24], and other proteins such as ovotransferrin (or conalbumin) [25] and β-lactoglobulin [26]. Most of proteins described above, except for trypsin and α-chymotrypsin, have been tested for use as chiral selectors in CE as the immobilized ligands or buffer additive. In addition to those proteins, serum albumins from other species, fungal cellulase from fungus *Aspergillus niger*, casein and human serum transferrin (HST) are used as the chiral selectors. The physical properties of proteins used in chiral CE are shown in Table 1. Tables 2 and 3, respectively, show chiral separations by CE using immobilized or adsorbed proteins, and proteins as running buffer additives.

The most extensively investigated protein ligands in chiral CE are BSA [27–37] and HSA [38–44], which are the major serum proteins in bovine and human sera, respectively. In addition, serum albumins from rabbit, chicken, horse, guinea pig, dog and goat have been used [45]. BSA and HSA are closely related proteins and, consequently, the stereoselective binding characteristics of these two proteins are similar. Sometimes, the elution order is reversed between chiral stationary phases based on these proteins; on the HSA-based phases *S*-warfarin elutes before *R*-warfarin whereas on the BSA-based phases the opposite elution order is observed [14]. These observations were consistent with the enantioselectivity of the native proteins [46]. Stereoselective binding characteristics of HSA have been thoroughly examined: stereoselective binding of drugs on HSA occurs principally at two major binding sites; warfarin-azapropazone and indol-benzodiazepine sites (well known as sites I and II) [47]. Site I is seen as a broad binding area that bears overlapping subsites,

Table 1
Physical properties of proteins used for chiral selectors in CE

Protein	Molecular mass	Carbohydrate composition (%)	Isoelectric point	Origin
Albumins				
BSA	66 000	–	4.7	Bovine serum
HSA	65 000	–	4.7	Human serum
Glycoproteins				
α_1-Acid glycoprotein (AGP)	44 000	45	2.7	Human or bovine serum
Ovomucoid (OMCHI)	28 000	30	4.1	Egg white
Ovoglycoprotein (OGCHI)	30 000	25	4.1	Egg white
Avidin (AVI)	66 000	7	10.0	Egg white
Streptavidin (STAV)	66 000	–	7.0	Bacterium
Riboflavin binding protein (flavoprotein) (RfBP)	32 000–36 000	14	4	Egg white
Enzymes				
Fungal cellulase	60 000–70 000	6	3.6	Fungus
Cellobiohydrolase I (CBH I)	64 000	6	3.9	Fungus
Pepsin	34 600	–	<1	Porcine stomach
Lysozyme	14 300		10.5	Egg white
Others				
Casein				Goat milk
α- (70%)	26 200		4.7	
β- (27%)	24 400		4.0–4.5	
γ- (3%)	30 000		5.8–6.0	
Human serum transferrin (HST)	76 500	5.7	5.5	Human serum
Ovotransferrin (conalbumin)	77 000	2.6	6.1	Egg white
β-Lactoglobulin	18 000/36 000		5.2	Bovine milk

while the site II is represented as a narrow hydrophobic pocket with a cationic region [48,49]. In addition, other minor sites were postulated to elucidate the bindings of a drug which could not be explained by the two major binding sites. A variety of acidic and uncharged compounds are resolved in chiral CE based on BSA and HSA.

AGP is one of glycoproteins used in chiral CE [34,50–52]. AGP is the major plasma protein responsible for the protein binding of cationic drugs

Table 2
Chiral separation by capillary electrophoresis using immobilized or adsorbed proteins

Selector	Type	Compound[a]	Reference
BSA	Crosslinked gel	Tryptophan	[27]
BSA	Dextran conjugate	Leucovorin	[28]
BSA	Immobilization on the capillary wall	DNP-alanine, DNP-glutamic acid, DNP-phenylalanine, DNP-proline, lorazepam, oxazepam	[29]
HSA	Adsorption on the capillary wall	Warfarin, tryptophan	[39]
HSA	Packed silica gel	Benzoin, temazepam, oxazepam	[40]
AGP	Packed silica gel	Benzoin, hexobarbital, pentobarbital, ifosfamide, cyclophosphamide, metoprolol, oxprenolol, alprenolol, disopyramide	[50]
CBH I/BSA	Crosslinked gel	Propranolol, metoprolol, pindolol, atenolol	[63]
CBH I/BSA	Crosslinked gel	Acebutolol, atenolol, metoprolol, pindolol, prenalterol, propranolol	[64]
Lysozyme	Adsorption on the capillary wall	Tryptophan, PTH-aspartic acid, PTH-threonine, dansyl-leucine, mephenytoin	[68]

[a] DNP = 2,4-dinitrophenyl; PTH = phenylthiohydantoin.

Table 3
Chiral separation by capillary electrophoresis using proteins as running buffer additives

Selector	Capillary[a]	Compound[b]	Reference
BSA	PEG coated	Leucovorin	[30]
BSA	Uncoated	Tryptophan, benzoin, warfarin	[31]
BSA	Uncoated	Leucovorin, ibuprofen, mandelic acid, dansyl-leucine, dansyl-norvaline	[32]
BSA	Uncoated	Ofloxacin, DR-3862, warfarin	[33]
BSA	Linear polyacrylamide coated	Homochlorcyclizine, oxyphencyclizine, propranolol, trimebutine epinastine	[34]
BSA	Uncoated	DNP-Phenylalanie, DNP-glutamic acid, DNP-proline, DNP-alanine, Nε-DNP-lysine, Nδ-DNP-ornithine	[35]
BSA	Uncoated or linear polyacrylamide coated	Folic acid, mandelic acid, N-benzoyl-alanine	[36]
BSA	Uncoated	Pantoprazole, omeprazole, lamsoprazole	[37]
HSA	Uncoated or linear polyacrylamide coated	Kynurenine, tryptophan, 3-indole-acetic acid, DNP-glutamic acid, DBZ-tartric acid	[38]
HSA	Uncoated	Promethazine, propiomazine, thioridazine, benzoin	[39–44]
Other serum albumins	Uncoated	Ofloxacin, tryptophan	[45]
AGP	Uncoated	Promethazine	[31]
AGP	Uncoated	DNP-Phenylalanie, DNP-proline, Nε-DNP-lysine, Nδ-DNP-ornithine	[35]
AGP	Linear polyacrylamide coated	Acebutorol, arotinolol, aatropine, bupivacaine, clorprenaline, denopamine, eperisone, epinastine, etirefline, fenoterol, homatropine, ketamine, metanephrine, metoprolol, mexiletine, nicardipine, oxyphencyclimine, phenylephrine, pindolol, primaquine, promethazine, sulpiride, terbutaline, tolperizone, trihexyphenidyl, trimebutine, trimetoquinol, trimipramine, verapamil	[34,51]
AGP	Methylcellulose coated	Disopyramide	[52]
OMCHI	Uncoated	DNP-Phenylalanie, DNP-proline, Nδ-DNP-ornithine	[35]
OMCHI	Uncoated, linear polyacrylamide coated or PEG coated	Tolperisone, benzoin, eperisone, chlorpheniramine	[55]
OMCHI	Linear polyacrylamide coated	Bunitrolol, pindolol, arotinolol, oxyphencyclimine, tolperisone, verapamil, chlorpheniramine, primaquine, trimebutine	[34,56]
OGCHI	Vinyl coated or linear polyacrylamide coated	Eperisone, tolperisone, chlorpheniramine, verapamil	[57]
AVI	Linear polyacrylamide coated	Abscisic acid, adrenochrome semicarbazone sulfonate sodium salt, 4-bromomandelic acid, 4-fluoromandelic acid, folinic acid, flurbiprofen, ibuprofen, ketoprofen, menadione sodium hydrogensulfite, 2-phenylbutylic acid, 2-phenylacetic acid, 3-phenylacetic acid, 2-phenoxypropionic acid, vanilmandelic acid, warfarin	[56,59,60]
Suc-AVI	Linear polyacrylamide coated	Trimipramine, primaquine, bupivacaine	[60]
STAV	Linear polyacrylamide coated	Chlorpheniramine, trimipramine, primaquine, ibuprofe, flurbiprofen, warfarin, dansyl-valine, dansyl-norvaline, dansyl-aspartic acid	[60]
RfBP	Uncoated	Nicardipine, bepridil, amoldipine, varapamil, oxazepam, lorazepam, practolol	[61]
	PEG coated	Ketoprofen, ibuprofen, flurbiprofen, pranoprofen, proglumide, oxprenolol, aminogluthethimide	[62]
Fungal cellulase	Uncoated	Pindolol	[31]
CBH I	Linear polyacrylamide coated	Propranolol, alprenolol, metoprolol, pindolol, labetolol, warfarin	[65,66]
Pepsin	Linear polyacrylamide coated	Cloperastine, trimipramine, verapamil, promethazine, propranolol oxprenolol	[67]
Casein	Uncoated	DNP-glutamic acid, DNP-proline	[35]
HST	Linear polyacrylamide coated	Tryptophan methyl ester, tryptophan ethyl ester, tryptophan butyl ester	[72]
		bupivacaine, promethazine, propranolol, labetalol, acebutorol	[73]
		buphenine, clofedanol, chlorphenramine	[74]
Ovotransferrin	Linear polyacrylamide coated	Trimetoquinol	[34]
β-Lactoglobulin	Uncoated		[26]

[a] PEG = polyethylene glycol.
[b] DBZ = dibenzoyl; DNP = 2,4-dinitrophenyl; PTH = phenylthiohydantoin.

because AGP has a lower isoelectric point (p*I*) value than BSA and HSA (Table 1). It was thought that drug binding to AGP occurred at a single hydrophobic pocket or cleft within the protein domain of the molecule [53]. However, the role of sugar moieties in enantioselective bindings by AGP has not been investigated precisely. Shiono et al. [54] reported that sialic acid residues influenced the enantioselective binding of basic drugs in different ways. They were not involved in the enantioselective verapamil–AGP binding, but they participated in the binding of *S*-propranolol but not of *R*-propranolol. Further studies are required to clarify the role of sugar moieties in the chiral recognition of AGP. A variety of basic and uncharged compounds can be resolved using AGP as a chiral selector. Other glycoproteins that have been examined for several CE studies include OMCHI. Recently, we found that the OMCHI used in previous studies was crude [17]. In addition, we isolated a glycoprotein from chicken egg whites and termed it OGCHI. It was found that about 10% OGCHI was included in crude OMCHI preparations, and that chiral recognition ability of OMCHI reported previously [16] came from OGCHI and pure OMCHI had no chiral recognition ability [17]. A lot of cationic compounds were resolved using crude OMCHI [34,35,55,56] and OGCHI [57] as chiral selectors. AVI is a basic glycoprotein from egg whites, while streptavidin (STAV) is a neutral nonglycosylated protein from bacterium *Streptomyces avidinii*. Both of them strongly bind biotin with an association constant of $\sim 10^{15}\ M^{-1}$ [58]. The former was useful for enantioseparation of acidic racemates, while the latter was useful for enantioseparation of acidic and basic racemates [56,59,60]. Another glycoprotein used in CE is RfBP. Some acidic and basic racemates were resolved in chiral CE based on RfBP [61,62].

Some enzymes used in CE include fungal cellulase from fungus *Aspergillus niger* [31], CBH I from fungus *Trichoderma reesei* [63–66], pepsin from porcine stomach [67] and lysozyme from egg whites [68]. The fungal cellulases from different organism were used in chiral CE. Most of applications were performed using CBH I, which is used in HPLC stationary phases. CBH I has a structural organization with a terminal, 36 residue-long binding domain connected to the rest of the enzyme (i.e., the core) through a flexible arm [69]. The core is enzymatically active. The dominating enantioselective site for solutes is located on the core, the main part of the enzyme [70]. The three-dimensional structure of the active site of CBH I has been elucidated by X-ray crystallography, and it has been shown that the binding site is a 40 Å long tunnel [71]. Some basic compounds, especially β-blockers, could be resolved in chiral CE based on fungal cellulase [31] and CBH I [63–66]. Pepsin [67] was useful for the enantioseparation of basic compounds, while lysozyme [68] was used for tryptophan and phenylthiohydantoin (PTH)- or dansyl-amino acids.

Some miscellaneous proteins used in chiral CE include casein [35], HST [72–74], ovotarnsferrin [34] and β-lactoglobulin [75]. A mixture of α-, β- and γ-casein from goat milk was used for the enantioseparation of trimetoquinol. HST and ovotarnsferrin belong to the same family of iron-binding proteins. HST was found to be effective for a lot of cationic compounds, while ovotansferrin was used only for the enantioseparation of trimetoquinol. Since β-lactoglobulin was similar in amino acid sequences and disulfide bond arrangements with AGP, the protein was examined for use in chiral CE. However, no solute was enantioseparated.

3. Modes of use of protein selectors in CE

3.1. Affinity electrochromatography

3.1.1. General considerations

For chiral separations in protein-based CE, proteins were immobilized or adsorbed within the capillary, or protein-immobilized silica gels were packed into the capillary. The applied electric fields result in solvent and solute flow through the system. The enantioseparation occurs by differences in interactions with an immobilized or adsorbed protein selector between enantiomers. This system is very similar to HPLC chiral stationary phase system, which is operated by the pressure-driven flow. Thus, this technique is termed affinity capillary electrochromatography (affinity CEC). In addition, the term capillary affinity gel electrophoresis is used when the crosslinked gel is being used. Table 2 shows chiral separations by CE using immobilized or adsorbed

proteins within the capillary, or protein-immobilized silica gels packed to the capillary.

We can assume a simple model for affinity CEC, where the following equilibrium (1:1 solute–protein complex) is quickly established from the viewpoint of the electrophoretic separation process

$$S + P = SP \quad (1)$$

where S, P and SP, respectively, denote a solute, a protein immobilized or adsorbed within the capillary, and a solute–protein complex. Then

$$K_a = [SP]/[S][P] \quad (2)$$

where K_a is the binding constant and [S], [P] and [SP] are concentrations of a solute, a protein, and a solute–protein complex, respectively. The binding constant can be related to the retention factor, *k*, using the expression well known in affinity chromatography [10,11]

$$k = K_a m_p / V_m = K_a [P]_{eff} \quad (3)$$

where m_p is the number of moles of active protein binding sites immobilized or adsorbed within the capillary and V_m is the void volume of a capillary. Thus, $[P]_{eff}$ is the effective protein concentration in affinity CEC. The retention factor, *k*, is expressed by the following equation

$$k = (t_R - t_M)/t_M \quad (4)$$

where t_R and t_M are migration times of retained and unretained solutes: that is, t_R is the migration time of a solute observed in affinity CEC, while t_M is the migration time of the same solute in the absence of any immobilized protein. Eq. (3) indicates that the *k* value (or the migration time) of a solute will increase with an increase in either solute–protein binding interaction or effective protein concentration within the capillary. The usefulness of Eq. (3) was shown by good agreement between the experimental results and those predicted by a model, where *R*- and *S*-warfarin were interacting with the absorbed HSA [39].

3.1.2. Immobilization or adsorption technique

There are several techniques for affinity CEC. Birnbaum and Nilsson [27] prepared capillaries filled with gels consisting of BSA crosslinked with glutaraldehyde for the resolution of tryptophan enantiomers. In this case, the detection window of the capillary was free from the crosslinked BSA gel in order to avoid the ultraviolet (UV) absorbance due to the crosslinked BSA gel. That was attained by pumping a mixture of BSA and glutaraldehyde just before the detection window and by allowing it to gel. Furthermore, the gel-filled capillary was subsequently preconditioned in the reversed-polarity mode to eliminate bubble formation in the capillaries. As shown in Fig. 1, tryptophan enantiomers were resolved in the migration order of D/L with resolution value of 6.0 and the number of theoretical plates (*N*) of 91 000 for a 32-cm gel capillary. The *N* value was significantly higher than that obtained with HPLC chiral stationary phases based on BSA [75]. The method could have the potential to be applicable for all types of affinity-based separations that include cross-linkable specific binding partners. The same group [63,64] has also demonstrated the applicability of the method by making a mixed protein gel based on CBH I and BSA. Since it was difficult to obtain a stable gel with CBH I alone, it was copolymerized with BSA. Another advantage of the mixed protein gel is that they could enantioseparate a wider range of compounds because of two protein ligands. However, since electroosmotic flow (EOF) is eliminated or negligible with these capillaries, the technique is not applicable to the separation of uncharged compounds.

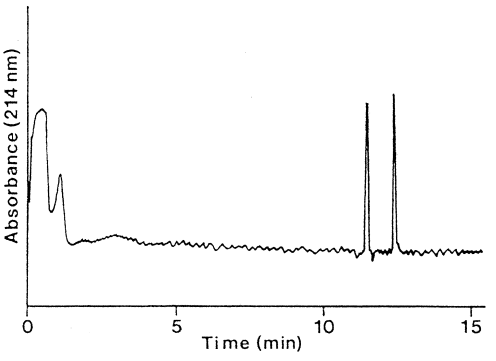


Fig. 1. Electrophoretic separation of tryptophan enantiomers. Reproduced from Ref. [27] with permission. The sample was DL-tryptophan (10 μ*M* in 25 m*M* potassium phosphate, pH 7.5). Conditions: constant applied electric field, 125 V/cm; gel length, 32 cm; total length, 40 cm; buffer, 50 m*M* potassium phosphate, pH 7.5; sample injection, 100 V/cm, 3 s.

A second technique in affinity CEC is to use capillaries packed with protein-immobilized silica particles. Lloyd and coworkers packed HSA- [40] and AGP-immobilized [50] HPLC silica gels into fused-silica capillaries. This work was performed using commercially available silica packing materials of 5–7 μm diameter. In CEC, the magnitude of the EOF is important, since it determines whether uncharged compounds can be separated or not. As shown in Fig. 2, the EOF is generally weaker in these packed capillaries than in open capillaries [50]. Furthermore, it is governed by the protein used and mobile phase composition. Since both AGP and HSA are acidic proteins, the EOF in these capillaries at neutral pH is in the same direction as that in open tubular capillaries. If basic proteins were used, the direction of EOF is reversed. The significant variations in EOF dependent on organic modifier used could be due to specific effects of the modifier on the protein [50]. Fig. 3 shows the enantiomeric separations of disopyramide, pentobarbital, hexobarbital, cyclophosphamide and benzoin by affinity CEC with an AGP-packed capillary. For the separation of benzoin enantiomers as shown in Fig. 3E, N was ≈5800 for a 17-cm length capillary packed with AGP stationary phases. Separation efficiencies are generally somewhat higher than those seen with HPLC separations [50].

A third method employed in chiral CE is to coat a protein dynamically in the capillary. Yang and Hage [39] coated the capillary with HSA, and found that a ~0.7 monolayer of HSA adsorbed on a typical uncoated silica capillary at pH 7.4. Further, the protein coating slowly desorbed in the presence of an electric field. However, the desorbed protein could automatically be replaced by adding a small amount of soluble protein to the running buffer. The advantages of the method are that it does not require the use of any packing material or the immobilization of a protein in the capillary, and that the same capillary can be used for work with additional proteins. Although enantioseparation of warfarin showing strong bindings to HSA ($K_a \geq 10^5\ M^{-1}$) was achieved using the above method, no enantioseparation of tryptophan showing weaker bindings to HSA ($K_a \leq 10^4\ M^{-1}$) was achieved. This is due to that the effective protein concentration is low on the capillary wall. Lloyd et al. [41] estimated that with HSA the retention at the wall was equivalent to that caused by ≈2 μM of the protein in solution. In addition to low effective concentration of adsorbed protein, a more serious problem of the protein adsorption is the potential to cause capillary blockage because of multilayer adsorption of the protein to the capillary wall [10]. Liu et al. [68] adsorbed lysozyme to the capillary wall and attained enantioseparation of four amino acids and mephenytoin with $N = 2.7 \sim 11.4 \cdot 10\,000\ m^{-1}$. Since lysozyme is a basic protein, whose pI is 11.4, it might be bound tightly to the capillary by electrostatic interaction in the neutral running buffer (pH 7.2). The reproducibility of the migration time of PTH-threonine during 27-time runs was good without addition of a small amount of soluble protein to the running buffer.

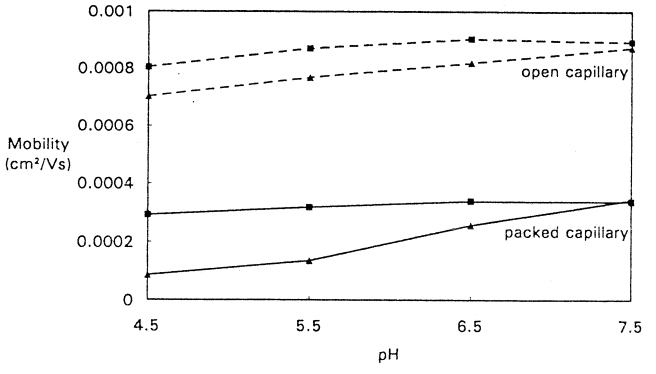


Fig. 2. Dependence of electroosmotic mobility on running buffer pH. Reproduced from Ref. [50] with permission. Conditions: capillary, 42 cm×50 mm I.D., 17 cm packed with AGP stationary phase; running buffer, 2 mM phosphate buffer–2% 2-propanol; applied voltage, 18 kV. ■, 1-Propanol; ▲, 2-propanol.

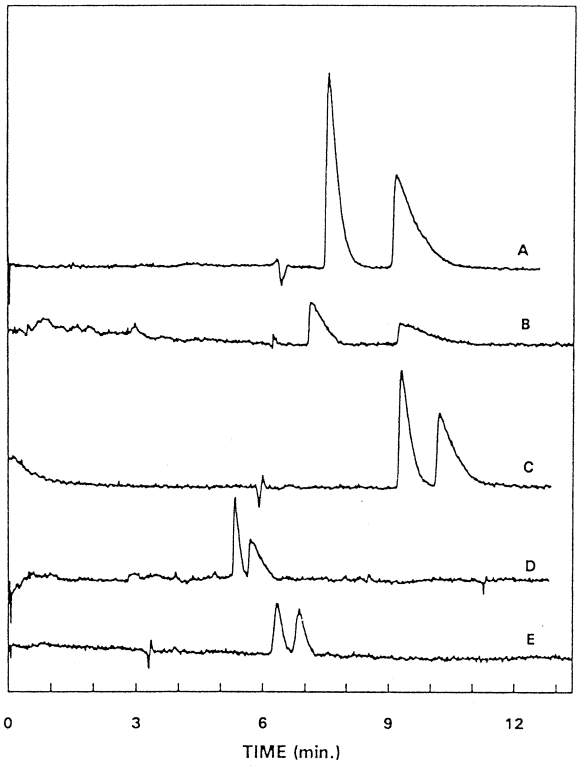


Fig. 3. Electropherograms showing the enantiomeric separations of disopyramide (A), pentobarbital (B), hexobarbital (C), cyclophosphamide (D) and benzoin (E). Reproduced from Ref. [50] with permission. Conditions: (A) disopyramide (15% 2-propanol–4 m*M* phosphate, pH 6.8, applied voltage 12 kV, current 2 μA); (B) pentobarbital (2% 2-propanol–2 m*M* phosphate, pH 5.5, applied voltage 20 kV, current 2 μA); (C) hexobarbital (2% 2-propanol–2 m*M* phosphate, pH 5.5, applied voltage 18 kV, current 2 μA); (D) cyclophosphamide (3% 2-propanol–2 m*M* phosphate, pH 6.5, applied voltage 25 kV, current 2 μA); (E) benzoin (5% 1-propanol–5 m*M* phosphate, pH 6.5, applied voltage 15 kV, current 3 μA).

A fourth technique for affinity CEC is to chemically immobilize a protein to the inner surface of fused-silica capillaries. Hofstetter et al. [29] bound BSA to the capillary wall as shown in Fig. 4. The method includes etching the capillary wall with sodium hydroxide, epoxy-diol coating with 3-glycidoxypropyltrimethoxysilane followed by hydrolysis with hydrochloric acid, activation with tresyl chloride and BSA coupling. The advantage of the method is small consumption of chiral selector with the possibility of UV detection without limitations of protein absorption.

Another approach is the use of protein–dextran polymer networks for chiral CE application. Sun et al. [28] covalently linked BSA to a high-molecular-mass dextran (M_r 2000 000) using cyanogen bromide, and applied to the separation of leucovorin enantiomers. Since the capillary was coated with linear polyacrylamide and a small EOF would exist, opposite in direction to EOF of the protein complex, the polymer would not flow out of the capillary during the course of several hours of the run. It was found that the amount of protein in the polymer network could be varied by dilution with non-derivatized dextran. This can be useful for optimizing the enantioseparation of a solute that adsorbs too strongly to the ligand. The BSA–dextran polymer network can be removed and replaced by means of syringe or by applying a fresh polymer mixture to the capillary.

3.2. Affinity capillary electrophoresis

3.2.1. General considerations

The most commonly format using protein selectors in CE is to dissolve the protein in the running buffer. This separation is analogous to electrokinetic chromatography (EKC) using micelles or other kind of selectors. Affinity EKC was previously used for these techniques [7,8]. In this review, the term affinity capillary electrophoresis (ACE) is used. We can assume a simple model for ACE as well as affinity CEC, where the Eq. (1) is valid. The retention factor, *k*, in the ACE system can be written as [10]

$$k = (\mu_s - \mu)/(\mu - \mu_p) = K_a[p] \quad (5)$$

where μ, μ_s and μ_p are the effective mobilities of the solute, free solute and solute–protein complex. The apparent mobility of the solute ($\mu_{app,s}$) is written as [59]

$$\mu_{app,s} = \mu_{eo} + ([S]/([S] + [P]))\mu_s + ([SP]/([S] + [P]))\mu_p \quad (6)$$

where $\mu_{app,s}$ and μ_{eo} are the apparent mobility of the solute and the electroosmotic mobility. Combining Eqs. (2) and (6) gives

$$\mu_{app,s} = \mu_{eo} + (1/(1 + K_a[p]))\mu_s + (K_a[P]/(1 + K_a[p]))\mu_p \quad (7)$$

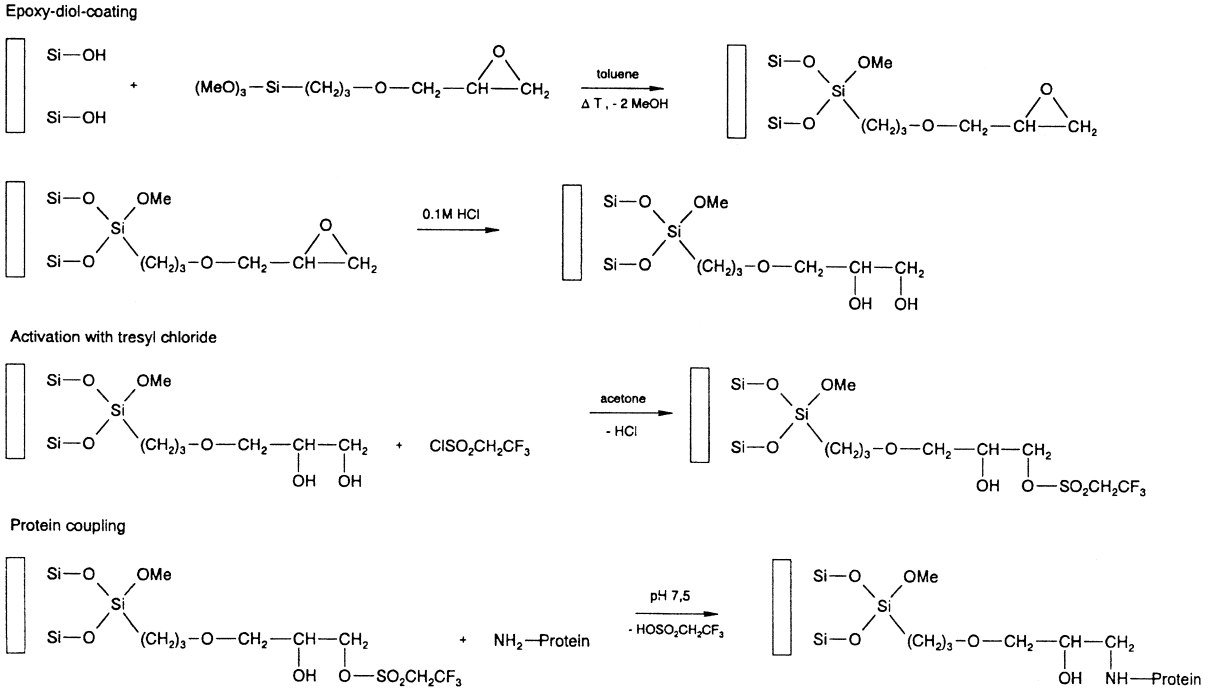


Fig. 4. Procedure for the immobilization of BSA on the capillary wall. Reproduced from Ref. [29] with permission.

For the separation of enantiomers 1 and 2, the difference in apparent mobilities, $\Delta\mu_{app,s}$, between two enantiomers gives [59]

$$\Delta\mu_{app,s} = \frac{(\mu_s - \mu_p)(K_{a2} - K_{a1})[P]}{(1 + K_{a1}[P])(1 + K_{a2}[P])} \quad (8)$$

where K_{a1} and K_{a2} are the binding constants of enantiomers 1 and 2, respectively, and the effective mobilities of the free analyte, μ_{s1} and μ_{s2} are equal to μ_s. Eq. (8) clearly indicates that the difference in apparent mobilities, $\Delta\mu_{app,s}$, becomes larger with an increase in the differences in the binding constants, K_{a1} and K_{a2}, between the enantiomers to be separated, and/or in the electrophoretic mobilities, μ_s and μ_p, between the free and complex solutes. The optimum concentration of protein, $[P]_{opt}$, which gives the maximum resolution, can be calculated as [59]

$$[P]_{opt} = (K_{a1}K_a)^{-1/2} \quad (9)$$

It is difficult to find the optimum concentration by calculation, because the value of the binding constants depends on the running buffer in the case of ACE. In addition, $[P]_{opt}$ represents the best set of the theoretical conditions for chiral separation in ACE. However, it is not easy to experimentally obtain a protein concentration that is equal to $[P]_{opt}$ due to problems with the background signal, solubility or availability of the ligand [11].

One would expect that the separations of various solutes using a protein as a chiral sector in ACE could be similar to those in HPLC chiral stationary phases based on the protein. To a great extent this is the case. However, sometimes one compound separated by ACE cannot always be separated by HPLC, and vice versa. These differences could be explained by taking into account the effective protein concentration between two approaches, ACE and HPLC [42,44]. From Eqs. (3) and (5) [42,44]

$$k_{HPLC}/k_{ACE} = K_{a,HPLC}[P]_{eff}/K_{a,ACE}[P] \quad (10)$$

where the subscripts HPLC and ACE refer to the techniques used. Under the similar conditions, $K_{a,HPLC} = K_{a,ACE}$ is probably a reasonable approxi-

mation, and so the ratio of the retention factors is equal to the ratio of selector concentrations. Generality of the relationship was indicated using racemic benzoin as a test analyte and HSA as a protein selector [42,44]. In HPLC experiments, where the concentration of immobilized HSA is ≈1 m*M*, the modifier is needed to reduce $K_{a,HPLC}$ and thus *k* reaches an acceptable range. In ACE experiments, where the concentration of HSA used is at tens of micromolar concentrations, there is no need to use any modifier. This may explain why selectivity observed in ACE may not always be reproduced in HPLC, since different amounts of modifier will be required to obtain optimum *k* values. Other factors that should be considered are changes of three-dimensional structure and/or binding characteristics of a protein by immobilization, and non-specific interactions with base-silica materials.

The advantages of ACE based on a protein are that no immobilization of a protein to packing materials or capillary walls is required, and that packing procedures, needed for affinity CEC with a packed capillary, are not required. Furthermore, since the binding properties of an immobilized protein are rather different from those of the native protein, it is favorable to use soluble proteins. The disadvantages of the ACE method include (i) use of the larger amount of a protein, (ii) adsorption of the protein to the capillary wall, (iii) absorption of UV light at the detection wavelength and (iv) relatively low purity of the protein.

Uncoated, polyethylene glycol (PEG)-, linear polyacrylamide-, vinyl- and methylcellulose-coated capillaries have been used in protein-based CE as shown in Table 3. With regard to adsorption of a protein to the capillary wall, some proteins (e.g. albumin) are relatively easy to use on uncoated capillaries, while others (e.g. AGP) are more difficult because they quickly result in capillary blockages [10]. The adsorption of proteins on the wall will cause changes in the EOF, which can affect the reproducibility of migration times and peak area [10,11]. When uncoated capillaries were used, it was important to wash the capillary between runs by sodium hydroxide [33,35,42,43] or sodium dodecylsulfate (SDS) [30,61] in order to remove the adsorbed proteins completely. Two approaches to avoid the adsorption of proteins to the capillary wall are the use of coated capillaries and the use of additives to minimize the protein–wall interaction. The most frequently used coating is linear polyacrylamide developed by Hjertén [76]. In addition, PEG- [30] and methylcellulose-coated [77] capillaries are used. The additives to minimize the protein–wall interaction include hydroxypropylcellulose (HPC) [55], dextran [36], *o*-phosphorylethanolamine (PEA) [55], 2-(cyclohexylamino)ethanesulfonic acid (CHES) [55] and 3-[(3-chloramidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO) [56].

When a protein is added to the running buffer, the background signals due to the protein can interfere with the detection of an analyte, especially if a high concentration of a protein is being used. To overcome this problem, the partial filling technique was developed [34,65]. In the technique, the capillary was partially filled with a solution containing a protein and the protein was not in the detector cell when the analyte reached that cell. Fig. 5 schematically illustrates the operating principle of the technique. At the beginning of the separation, the capillary is partially filled with the solution containing an acidic protein such as BSA or AGP (Fig. 5a). A sample solution of a cationic mixture is introduced at the end of capillary filled with the separation solution (Fig. 5b). A cationic mixture migrates toward the cathode, while an acidic protein migrates in the opposite side. Since in this example, a coated capillary is used to eliminate the EOF, the separation zone or protein does not migrate significantly during the run. In the separation zone, enantiomer separations are attained (Fig. 5c), while the enantiomers migrate at identical velocities outside the separation zone and are detected in the absence of a protein (Fig. 5d). This approach was first introduced by Valtcheva et al. [65] for the separation of β-blockers using CBH I as a protein, where agarose gel plugs should be introduced to prevent hydrodynamic flow. Tanaka and Terabe [34] modified the technique to run automatically using a commercial CE instrument. The partial filling techniques were applied for proteins such as BSA [34], crude OMCHI [34], AGP [34,51,52], AVI [60], CBH I [65,66], pepsin [67], HST [72–74] and ovotransferrin [34]. The technique gave improved detection sensitivity and comparable reproducibilities of mi-

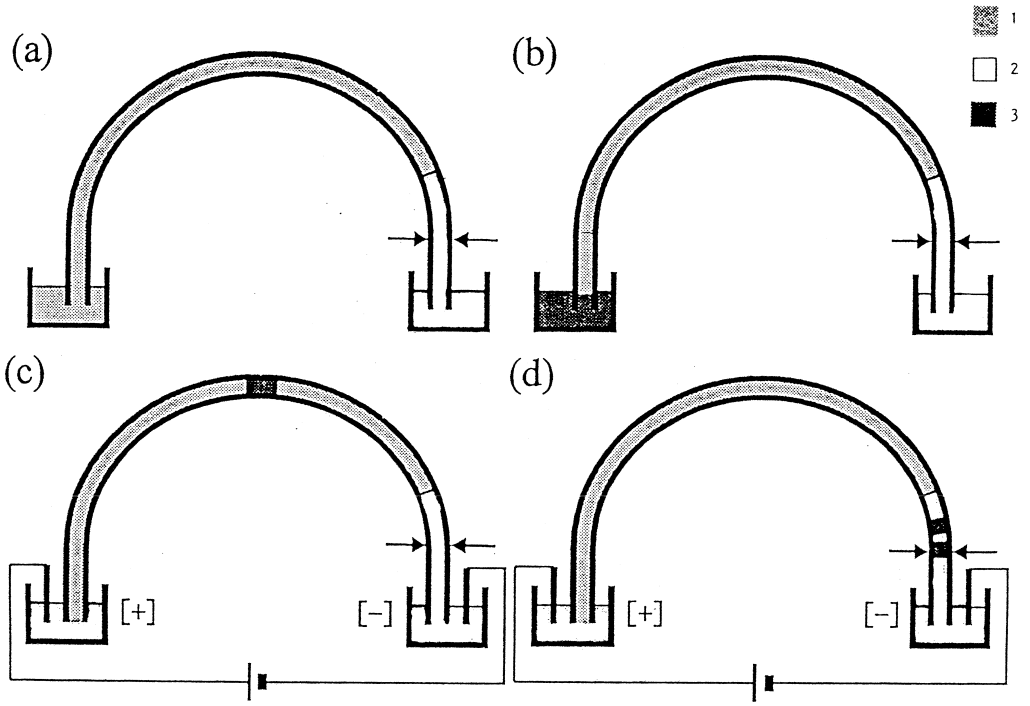


Fig. 5. Schematic illustration of the partial filling technique. Reproduced from Ref. [34] with permission. 1=separation zone; 2=running buffer; 3=sample solution; arrows indicate detection window. (a) The separation zone is introduced from the injection end to a point short of the detector cell, (b) the sample solution is introduced into the capillary; (c) a high voltage is applied between both ends of the capillary after both ends are dipped into the running buffer and the analytes migrate toward the detector; (d) a separated zone reaches the detector cell but the separation zone does not reach this cell.

gration times and peak area, compared with the conventional technique where the protein was completely filled into the separation capillary [34].

3.2.2. Types of protein

Both BSA [30–37] and HSA [38–44] have been used as chiral selectors in ACE using uncoated, PEG- or linear polyacrylamide-coated capillaries. Fig. 6 shows the enantioseparation of leucovorin in BSA-based ACE on uncoated and linear polyacrylamide-coated capillaries [30]. Since by linear polyacrylamide coatings, EOF was eliminated, the elution order was reversed because of the reversal of the polarity. The running buffer pH values used were near neutral, where both BSA and HSA were negatively charged. Thus, under the conditions, cationic and uncharged solutes could be easily resolved because the analyte and protein migrated in opposite directions. However, since anionic solutes migrated in the same direction as the protein, it was sometimes difficult to separate them. Resolution of ibuprofen, an anionic compound, was difficult using only BSA as a running buffer additive. The enantioseparation of ibuprofen was attained by addition of dextran, which modifies the protein mobility [32]. With regard to the N value in HSA-based ACE on uncoated capillaries, for benzoin $N>10\,000$ for a 50-cm capillary, while that value was <400 for a 5-cm HPLC column based on HSA [42]. Generally, protein-based ACE gives higher a N value than typically seen in CEC and HPLC [10,11]. However, protein-based ACE does not provide higher efficiencies because of slow interaction kinetics [8,11].

Other serum albumins from rabbit, chicken, horse, guinea pig, dog and goat were used for the enantioseparations of ofloxacin and tryptophan [45]. The

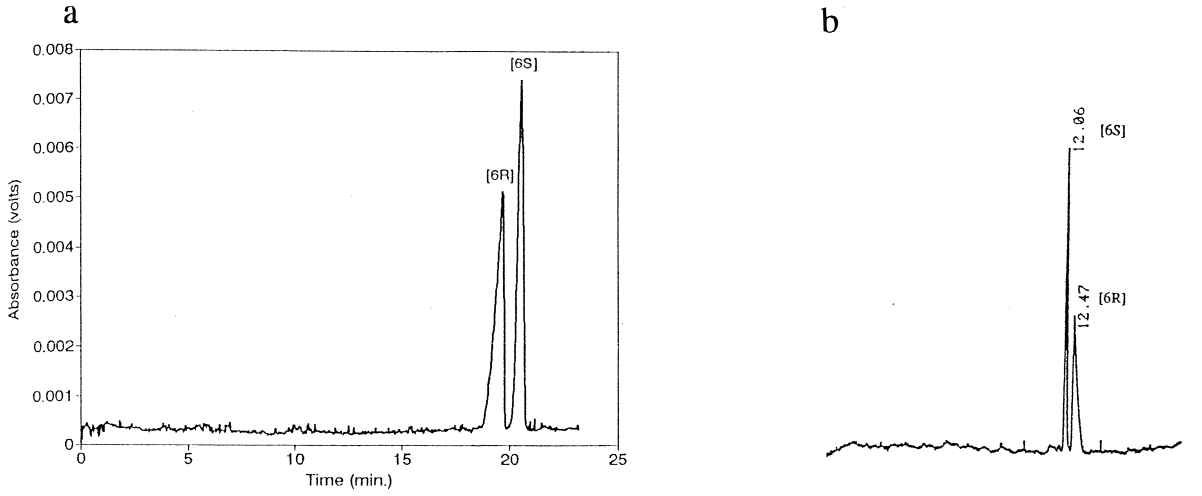


Fig. 6. Electropherograms obtained for leucovorin using affinity capillary electrophoresis on uncoated (A) and PEG-coated capillaries (B). Reproduced from Ref. [30] with permission. Conditions: (A) capillary dimensions, 75 μm I.D.×360 μm O.D.; electric field strength, 325 V/cm; running buffer, 20 m*M* phosphate (pH 7.0) containing 1 mg/ml BSA; detection wavelength, 280 nm. (B) Capillary dimensions, 75 μm I.D.×360 μm O.D., modified by PEG 8M-10; electric field strength, 285 V/cm; running buffer, 20 m*M* phosphate (pH 7.2) containing 1 mg/ml BSA; detection wavelength, 280 nm.

elution orders of the enantiomers were the same among the serum albumins tested, although some serum albumins could not separate them.

AGP had a tendency to stick to the uncoated capillary walls as described above. Since in the early stages, uncoated capillaries were used, only promethazine and DNP amino acids were resolved [31,35]. Recently, a lot of cationic solutes were enantioseparated by partial filling technique on linear polyacrylamide- or methylcellulose-coated capillaries [34,51,52]. Tanaka and Terabe [51] separated twenty-nine cationic racemates by optimizing the separation conditions such as protein concentration, running buffer pH and organic modifier. AGP (50–1000 μ*M*) was used for the separation of those racecmates. Fig. 7 shows electropherograms of atropine, nicardipine, pindolol and sulpiride enantiomers by ACE using bovine serum AGP [51]. Furthermore, they compared bovine serum AGPs from three different suppliers, and found differences in their electrophoretic mobilities. Except for the enantioseparation of acebutolol, all racemates tested were similarly separated with any of the three types of AGP. The reason for the different enantioselectivities of the three types of AGP has not yet been clarified.

HPLC chiral stationary phases based on OMCHI showed excellent chiral recognition abilities for various racemates. Busch et al. [31] first carried out some ACE experiments with OMCHI by varying the protein concentration (5–174 μ*M*), the pH over the range 6–8, and the type and concentration of the modifier. No enantioseparation of cationic, anionic or uncharged compounds tested was attained, although a few test solutes showed some interaction with the protein. They supposed that immobilization of OMCHI brought about a change in the tertiary structure of the OMCHI, resulting in an excellent chiral stationary phase for HPLC experiments. Further experiments were subsequently carried out with OMCHI by other investigators [34,35,55,56], and many compounds were successfully separated. Wistuba et al. [35] described OMCHI as the chiral selector in ACE for the separation of 2,4-dinitrophenyl-amino acids with uncoated capillaries. Ishihama et al. [55] achieved optical resolution of some drugs by optimizing the concentrations of both OMCHI and modifier. The addition of a zwitterion, PEA, resulted in an improvement of the peak tailing, but the day-to-day reproducibility was poor because it was unstable at room temperature. Further, better reproducibility of the migration times of tolperisone

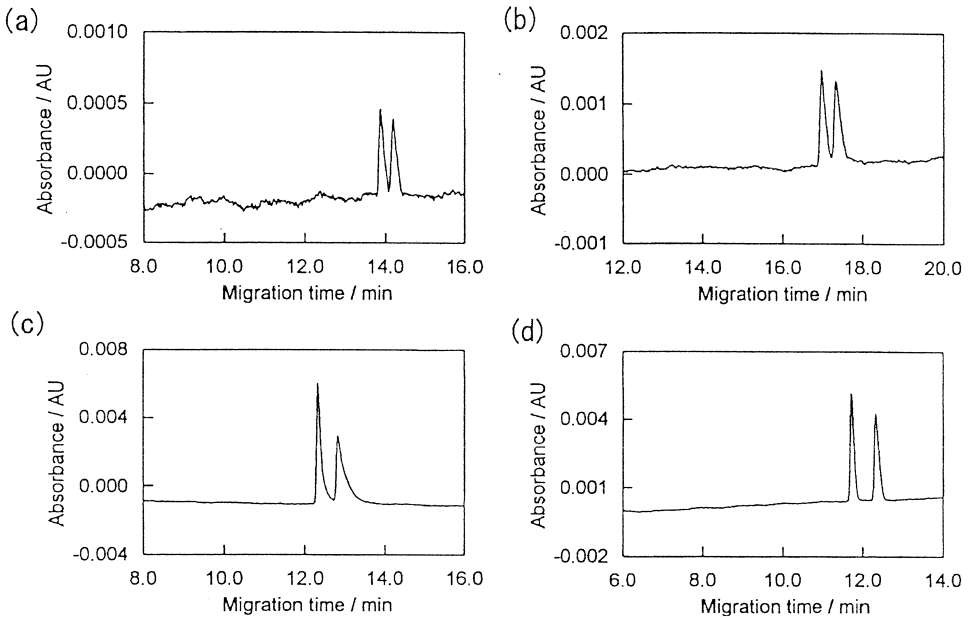


Fig. 7. Representative electropherograms of enantiomer separations by affinity capillary electrophoresis with AGP and employing the partial filling technique. Reproduced from Ref. [51] with permission. Conditions: (a) racemic atropine (AGP concentration, 750 μ*M*; running buffer, 10% 2-propanol/50 m*M* phosphate, pH 6.0); (b) racemic nicardipine (AGP concentration, 200 μ*M*; running buffer, 10% 1-propanol–50 m*M* phosphate, pH 5.0); (c) racemic pindolol (AGP concentration, 100 μ*M*; running buffer, 10% ethanol–50 m*M* phosphate, pH 6.0); (d) racemic sulpiride (AGP concentration, 1000 μ*M*; running buffer, 50 m*M* phosphate, pH 6.0). Sample concentration, 50 μg/ml; injection, 6.9 kPa×2 s; capillary, 50 μm I.D.×36 cm linear polyacrylamide-coated capillary; applied voltage, 12 kV; detection, 210 nm.

enantiomers was observed with the dynamically coated capillaries by addition of 0.25% HPC than PEG-coated capillaries. They concluded that by proper adjustment of concentrations of OMCHI and organic modifier baseline separations of optical isomers could be achieved with high plate numbers. Tanaka and Terabe [34] enantioseparated many cationic compounds using high concentration of OMCHI (500 μ*M*) by partial filling technique. The use of the high concentration of OMCHI with additives such as amphoteric surfactant or organic modifier gave better resolution than the use of the low concentration, although the reason is not clear. Taking account into that OMCHI used in previous studies was crude, that about 10% OGCHI was included in crude OMCHI preparations, and that chiral recognition ability of OMCHI reported previously came from OGCHI and pure OMCHI had no chiral recognition ability, the results described above are easily understood. Haginaka and Kanasugi [57] applied OGCHI as the chiral selector in ACE for the separation of cationic enantiomers. Fig. 8A–C shows the separation of tolperisone enantiomers using crude OMCHI, pure OMCHI and OGCHI, respectively, as chiral selectors in ACE, where a linear polyacrylamide-coated capillaries is employed. The obtained results were consistent with those described above: tolperisone enantiomers were not resolved by using pure OMCHI, slightly resolved by crude OMCHI and completely resolved by OGCHI. Only the use of 50 μ*M* OGCHI as the chiral selector gave good enantioseparation of tolperisone enantiomers. By using OGCHI as the chiral selector, the separation of various drug enantiomers should be achieved by optimizing the concentrations of OGCHI and type and concentration of modifier.

Tanaka et al. [59] described the use of AVI as the chiral selector in ACE for the separation of acidic enantiomers such as 2-arypropionic acid derivatives, vanilmandelic acid and leucovorin. Since AVI is a basic protein, whose p*I* is 10.0, and tends to be adsorbed to the capillary walls because of electro-

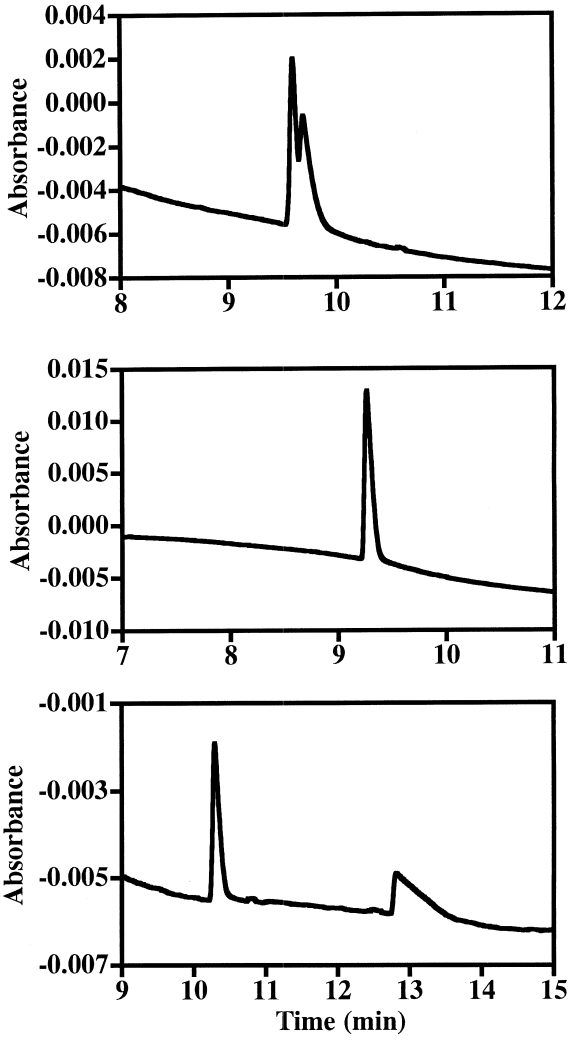


Fig. 8. Separation of tolperisone enantiomers by affinity capillary electrophoresis with ovoglycoprotein on a linear polyacrylamide-coated capillary. Reproduced from Ref. [57] with permission. Conditions: capillary, 75 μm I.D., effective length 30 cm; running buffer, 50 m*M* phosphate buffer (pH 5.0)–2-propanol (95:5, v/v); separation solution, 50 m*M* phosphate buffer (pH 5.0)–2-propanol (95:5, v/v) containing 50 μ*M* crude OMCHI (A), OMCHI (B) or OGCHI (C); sample, 0.1 mg/ml racemic tolperisone hydrochloride; applied voltage, 12 kV; detection, 254 nm.

static interaction, coated capillaries are used. The AVI concentrations higher than 25 μ*M* resulted in detection problems because the complete filling technique was used. Recently, Tanaka and Terabe [60] reported the use of AVI, succinylated AVI (Suc-AVI) and streptavidin (STAV) as the chiral selectors in ACE by partial filing technique. Suc-AVI is a chemically modified AVI with a p*I* of 3.5–6.0, and STAV is a neutral nonglycosylated protein with a p*I* of ~7.0. Basic AVI was useful for the enantioseparation of acidic solutes, while the acidic Suc-AVI was useful for that of basic solutes. STAV was useful for both acidic and basic enantiomer separations. In the experiments, 50–100 μ*M* AVI, Suc-AVI or STAV was used as a chiral selector and the enantioseparations of various solutes were optimized by varying the running buffer pH and additives. It is well known that AVI binds tightly with biotin. Not only enantioselectivity but also the interaction with racemates was significantly lost by the formation of AVI–biotin complex. However, from the results described above it was not concluded that chiral recognition arose from the interaction of biotin binding sites on AVI, because it was reported that the structural change of AVI occurred in complexing with biotin [18].

Lorenti et al. [61] described the use of quail RfBP as the chiral selector in ACE with uncoated capillaries at running buffer pH 6.5. Uncharged and cationic compounds were enantioseparated, but anionic compounds such as indoprofen and warfarin were not because of their mobilities being too similar to that of the acidic protein. SDS washing between runs was required to remove the adsorbed protein and to prevent capillary blockages. Especially, the protein adsorption was serious at the running buffer pH at 4. On the other hand, Mano et al. [62] reported the use of chicken RfBP as the chiral selector at running buffer pH 4.4–6.8 on PEG-coated capillaries. Under these conditions, anionic and cationic compounds were enantioseparated. At the running buffer pH of 4.4, ketoprofen, an anionic compound, gave the highest enantioselectivity among the running buffer pH values tested. With regard to the chiral recognition sites and mechanism, they assumed that α-helix structure region of RfBP could play an important role in chiral recognition of ketoprofen, and that the critical groups involved could be a tryptophan residue, an amino group, and a carboxyl group of the protein. With an increase in the methanol content, the enantioselectivity was decreased, and lost at >25%. This could be due to the increase of the methanol content in the running buffer resulting in changes in the α-helical structure of the chiral recognition region, decreasing chiral

recognition capacity. Further, at >25% methanol, the α-helical structure of the chiral recognition region being denatured, the chiral recognition ability could be lost.

The use of fungal cellulase from *Aspergillus niger* in ACE was reported by Busch et al. [31]. Only pindolol enantiomers were separated using running buffer (pH 7.4) containing 20 μ*M* fungal cellulase on uncoated capillaries. The use of CBH I from *Trichoderma reesei* in ACE was reported by other investigators [65,66]. Valtcheva et al. [65] separated the enantiomers of β-blockers with CBH I by partial filling technique as shown in Fig. 9, where linear polyacrylamide-coated capillaries were employed. The conditions used were unusual; a high concentration of CBH I (625 μ*M*), a high ionic strength (0.4 *M* sodium phosphate buffer), a high concentration of organic solvent (up to 30%), and a relatively low voltage (1 kV to avoid high temperature in the capillary, resulting in the risks of precipitation of CBH I and bubble formation). Since the running buffer pH was 5.1, the cationic β-blockers and the protein migrated to the opposite direction, and baseline resolution of those enantiomers was attained. Hedeland et al. [66] used CBH I as short plugs of 1.0–10 cm by partial filling technique, where a linear polyacrylamide-coated capillary was employed as an effective length of 20 cm. The enantiomers of oxprenolol and propranolol could be completely resolved with the selector plugs of only 1.0 cm at the running buffer pH of 5.0. Although warfarin, an acidic compound, migrated in the same direction as CBH I at this pH, its enantiomers were separated and detected at the anodic end of the capillary. Furthermore, propranolol was enantioseparated at pH 3.0, where the protein had a positive net charge and migrated in the same direction as propranolol.

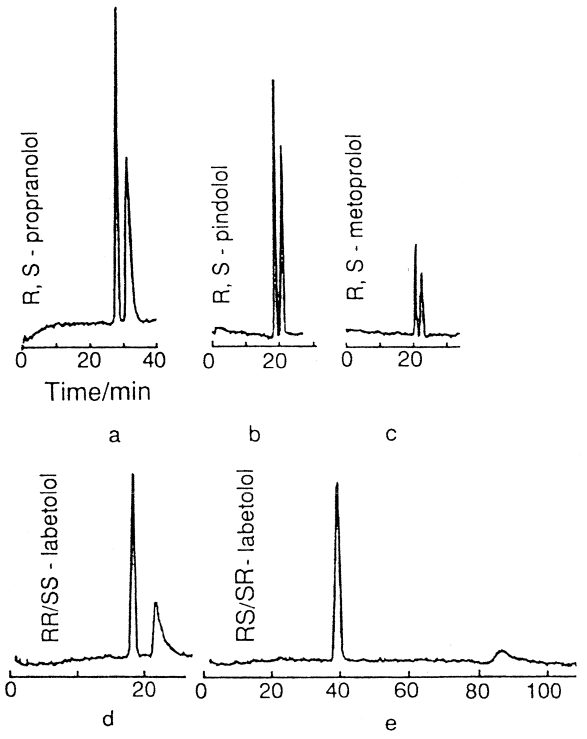


Fig. 9. Separation of (a) *R,S*-propranolol, (b) *R,S*-pindolol, (c) *R,S*-metoprolol, (d) (*RR*/*SS*)-labetolol and (e) (*RS*/*SR*)-labetolol. Reproduced from Ref. [65] with permission. Running buffer, 0.4 *M* sodium phosphate (pH 5.1) supplemented with (a, b, c) 25% and (d, e) 30% 2-propanol. Other conditions as in the text.

Pepsin, whose p*I* is <1, was used as the chiral selector in ACE on a linear polyacrylamide-coated capillary by partial filling technique. The cationic compounds and chiral selector migrated in the opposite direction, keeping the detector cell free of the strong absorbing protein. Enantiomeric separation was achieved for various basic compounds. No resolution for any compounds has been recorded at pH 7, except for oxprenolol, which showed a maximum resolution at such a pH value. This could be due to the denaturing of pepsin at the running buffer pH of 7, as reported in the case of HPLC chiral stationary phases based on pepsin [23].

Casein from goat milk was reported as the chiral selector in ACE, where a concentration close to 1 m*M* allowed the separation of DNP-glutamic acid and DNP-proline [34]. Since the casein used was a mixture of α-, β- and γ-casein, a better chiral resolution may be achieved by using α-, β- or γ-casein alone.

Kilar and coworkers [72–74] introduced the use of HST to ACE. As a chiral selector, 0.64–4.6 m*M* HST in 2-N-(N-morpholino)ethanesulfonic acid (pH 6.0) was used on a linear polyacryamide coated capillary by the partial filling technique. HST, whose p*I* was ~6.0, did not migrate or migrated slowly, but the cationic compounds, tryptophan esters and some drug enantiomers, migrated to the anodic end. Optimal conditions include a ~1–15 cm selector plugs in

a 30–45 cm capillary. As iron-saturated (diferic) HST did not show any enantioseparation, only iron-free HST were used in the experiments. Ovotransferrin, a family of iron-binding proteins, was only applied for the enantioseparation of trimetoquinol [34].

4. Factors affecting chiral separations by protein-based CE

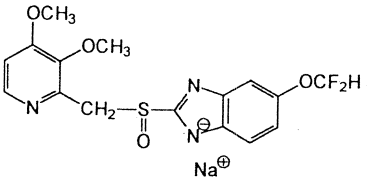
There are various factors affecting the chiral separations of various solutes in CE based on protein selectors; protein concentration, running buffer pH, modifier, ionic strength and displacer.

When using a immobilized protein in affinity CEC, the degree of analyte separation increases with an increase in the k value, which is proportional to the effective concentration of a protein (see Eq. (3)). It is shown that for the binding of *R*/*S*-warfarin and D/L-tryptophan to HSA, *R*/*S*-warfarin that has strong binding to the protein is easily separated with small amounts of the protein, but for the separation of the weakly retained analyte, D/L-tryptophan, large amounts of the protein may be required [11,39]. The situation is different when using proteins in solution. The analytes will migrate between two limits; the mobilities for the free analyte and fully bound analyte–protein complex. From Eq. (9), the optimum concentration of a protein selector is dependent on the binding constants of both enantiomers; the optimum concentration of a weakly binding analyte is higher than that of strongly binding analyte. The analytes with weak-to-moderate binding (e.g. D/L-tryptophan) are more easily resolved, particularly if they have a large difference in binding constants, while the analytes with strong binding (e.g. *R*/*S*-warfarin) tend to coelute [11,39].

The running buffer pH is one of the most important factors to optimize in chiral CE based on protein selectors. It is well known that the binding constants vary with the running buffer pH. This could be due to changes in their net charges and electrostatic interactions, or changes in the conformation of the protein at its binding regions. Furthermore, dependent on the running buffer pH, EOF and electrophoretic mobilities of the analytes and the protein selector could be changed. Thus, altering the running buffer pH can affect the binding and separation of the analytes, but the exact extent of these effects are hard to predict and must usually be determined on a case-by-case basis [11]. Fig. 10 shows effect of the running buffer pH on the enantioseparation of pantoprazole using BSA as the chiral selector in ACE [37]. BSA has a pI of about 4.7 and pantoprazole has a pK value of 8.2 for the deprotonation of the benzimidazole-NH. Thus, in the pH range 7–8 the protein is negatively charged and pantoprazole is uncharged or partly negative charged. Chiral separation of both enantiomers is based on these differences in mobility and on different binding constants of both enantiomers to BSA as described above. Poor separation was found at pH 7.0–7.2. At pH 7.3 the baseline separation of both enantiomers was attained with two narrow peaks (Fig. 10a). Although resolution is markedly improved with higher pH values, peak distortion is started as a consequence of kinetic effects of the stronger interactions of the deprotonated negatively charged enantiomers with the protein (Fig. 10b–d).

Addition of organic modifiers such as 1-propanol, 2-propanol, methanol, ethanol and acetonitrile can improve peak shapes and resolution by decreasing hydrophobic interactions with the analytes and the protein selector [10,11]. However, addition of too much organic modifier can cause denaturation of the protein, resulting in loss in chiral recognition abilities [62]. Fig. 11 shows the effect of 1-propanol content on the separation of pantoprazole using BSA as the chiral selector in ACE [37]. Addition of 1-propanol improved peak shape and resolution at concentrations of 3–10%. For concentrations $>10\%$, peaks became very sharp but enantiomeric resolution decreased. Other modifiers used are zwitterionic compounds such as PEA, CHES and CHAPSO which are used for decreasing adsorption of proteins to the capillary wall and for denaturation of proteins, resulting in the elution of the analyte peaks. However, addition of such modifiers would also alter EOF and electrophoretic mobilities of the analytes and the protein selector.

An increase in ionic strength tends to decrease coulombic interactions through a shielding effect [78], but at the same time may cause an increase in hydrophobic solute adsorption. Raising the ionic



pantoprazole

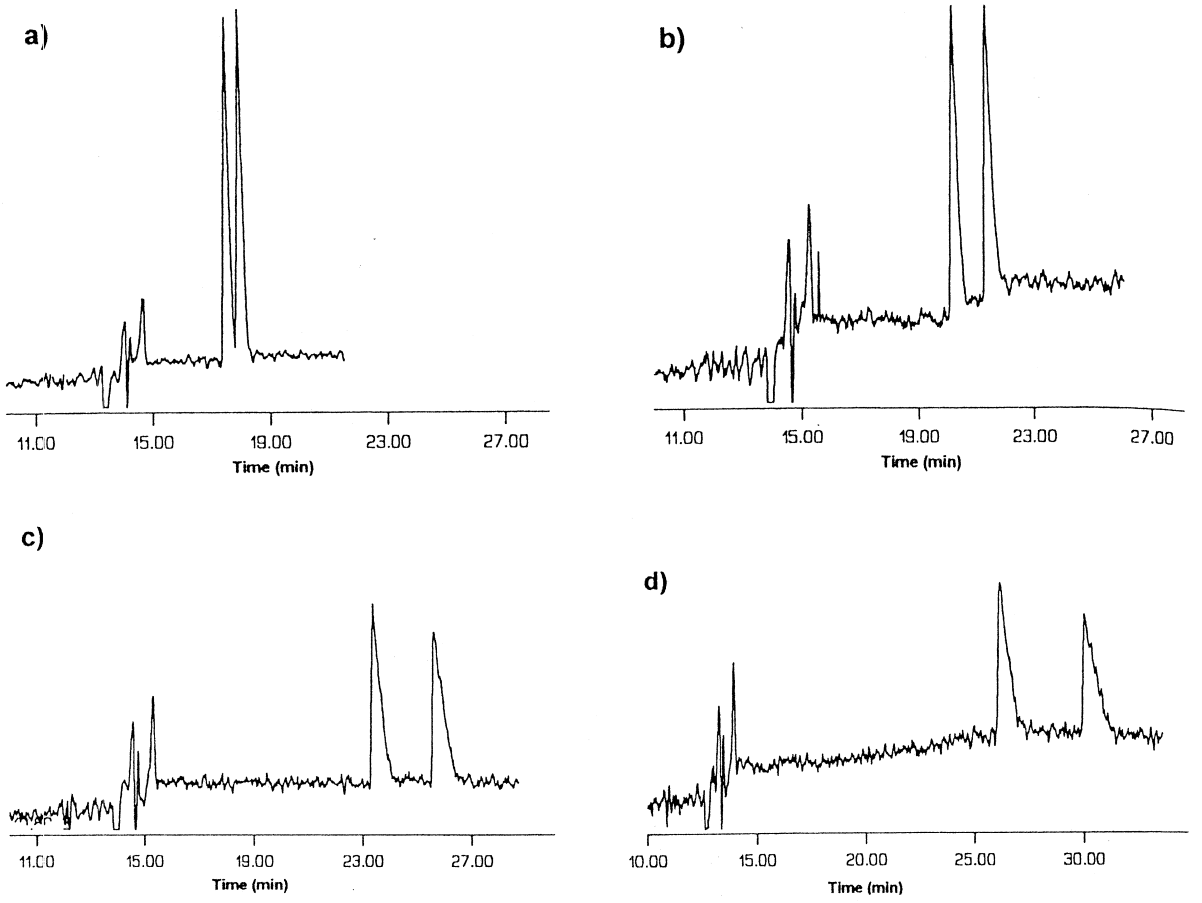


Fig. 10. Effect of pH on the separation of pantoprazole in BSA-based affinity capillary electrophoresis. Reproduced from Ref. [37] with permission. Conditions: running buffer pH, (a) pH 7.3, (b) pH 7.4, (c) pH 7.6 and (d) pH 7.9; capillary, 50 μm I.D.×66 cm uncoated capillary; current, constant current of 35 μA at field strength of ~300 V/cm; running buffer, 10 m*M* potassium phosphate with 55 μ*M* BSA and 5% l-propanol.

strength of the running buffer can be a way of minimizing the adsorption of proteins to the capillary [11]. However, changes in ionic strength would also alter EOF and electrophoretic mobilities of the analytes and the protein selector.

Displacing agents could be used as the coadditive

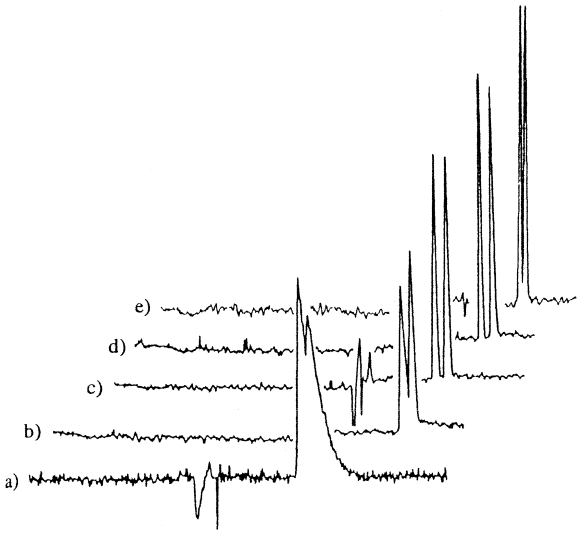


Fig. 11. Influence of the concentration of 1-propanol on the chiral separation of pantoprazole in BSA-based affinity capillary electrophoresis. Reproduced from Ref. [37] with permission. Conditions: capillary, 50 μm I.D.×66 cm uncoated capillary; current, constant current of 35 μA at field strength of ca. 300 V/cm; (a) 0%, (b) 3%, (c) 5%, (d) 8% and (e) 12% l-propanol in 30 m*M* potassium phosphate and 40 μ*M* BSA, pH 7.4.

to modify selectivity in CE [36,41,45]. Direct competition should be revealed by steady decrease in binding of the analyte as the competitor is added to the running buffer. On the other hand, an allosteric interaction is revealed by a change but not necessarily an elimination of binding, as binding of one compound causes a change in the protein's conformation which affects the binding of a second ligand [10]. Zhang et al. [36] found that the salicylate, α-resorcyclic acid, increased retention and stereoselectivity of folinic acid and mandelic acid in BSA-based ACE, while other displacers exhibited a negative effect on chiral resolution. The bindings of folinic acid and mandelic acid to BSA were affected by allosteric interactions caused by α-resorcyclic acid–BSA binding. These results indicate that chiral resolution in protein-based CE may be modified in the presence of displacers.

5. Conclusions

A variety of proteins were used as the chiral selectors in CE for the separation of drug enantiomers. The proteins used include albumins such as BSA, HSA and serum albumins from other species, glycoproteins such as AGP, crude OMCHI, OGCHI, AVI, RfBP, enzymes such as fungal cellulase, CBH I, pepsin and lysozyme and other proteins such as casein, HST and ovotransferrin. Protein-based CE is carried out in two modes: in one proteins are immobilized or adsorbed within the capillary, or protein-immobilized silica gels are packed into the capillary (affinity CEC mode), and in the other proteins are dissolved in the running buffer (ACE mode). The latter method is simpler and easier to operate than the former. However, the ACE method has disadvantages of adsorption of proteins to the capillary wall, and absorption of UV light at the detection wavelength. Adsorption of a protein was overcome by using wall-coated capillaries or additives to minimize the protein–wall interactions, and absorption of UV light at the detection wavelength was by partial filling techniques.

Though HPLC chiral stationary phases based on protein-fragment or protein-domain were prepared, those phases could not always provide excellent chiral recognition abilities [79–81]. Thus, neither protein-fragment nor protein-domain was used for the separations of drug enantiomers as the chiral selectors in CE. Recently, two groups [82,83] reported ACE resolution of DNP-amino acid enantiomers achieved through the cyclopeptide library produced by combinatorial chemistry. In the future, it may be possible to make the most suitable chiral selector, based on the peptide library by combinatorial chemistry, for the target molecule.

6. List of abbreviations

ACE	affinity capillary electrophoresis
AGP	α_1-acid glycoprotein
AVI	avidin
BSA	bovine serum albumin
CBH I	cellobiohydrolase I
CE	capillary electrophoresis
CEC	capillary electrochromatography
CHAPSO	3-[(3-chloramidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate
CHES	2-(cyclohexylamino)ethanesulfonic acid
DBZ	dibenzoyl

DNP	2,4-dinitrophenyl
EKC	electrokinetic chromatography
EOF	electroosmotic flow
HPC	hydroxypropylcellulose
HPLC	high-performance liquid chromatography
HSA	human serum albumin
HST	human serum transferrin
N	number of theoretical plates
OMCHI	ovomucoid from chicken egg whites
OGCHI	ovoglycoprotein from chicken egg whites
PEA	*o*-phosphorylethanolamine
PEG	polyethylene glycol
p*I*	isoelectric point
PTH	phenylthiohydantoin
RfBP	riboflavin binding protein
SDS	sodium dodecylsulfate
STAV	streptavidin
Suc-AVI	succinylated avidin
UV	ultraviolet

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