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Review

Immobilized proteins as chromatographic supports for chiral resolution

SUNANDA R. NARAYANAN

Biochemical Research Laboratory, University of Kansas, Lawrence, KS 66045, USA

Abstract: This review examines the role of protein-bonded chiral stationary phases (CSPs) in enantiomeric separation and investigates the performance characteristics and desired properties of protein CSPs for separation and large-scale operation. The review also discusses the ability of protein-based CSPs to examine the stereochemistry of drug metabolism processes.

Keywords: *Immobilized proteins; chiral stationary phases.*

Introduction

Approximately 25% of the most widely prescribed drugs are sold as racemic mixtures. The individual isomers of such mixtures often differ in their pharmacological properties and exhibit different pharmacokinetic and metabolic fates. Further, the very presence of one enantiomer may affect the fate of another. An often cited example of this phenomenon is the tragedy of thalidomide, where the inactive isomer was responsible for the teratogenic properties of the drug [1]. Yet another example is (+)ketamine which is predominantly responsible for an hypnotic effect whereas the (−)isomer is the main source of side effects [ref 1, table 3]. Perhaps the oldest example is the D and L forms of amphetamine exhibiting significant pharmacological differences [1]. Some of the other examples of drug enantiomers with different pharmacodynamic properties are indacrinone, pentazocine, propranolol, sotalol, tetramisole, pheneturide, ibuprofen and verapamil [1]. Achiral drugs like debrisoquine may also form enantiomeric metabolites [1]. The Food and Drug Administration now considers enantiomers to be impurities even in racemates [2].

Chromatographic analysis of racemates using only achiral systems, which do not give separate results for each of the enantiomers, therefore provides incomplete information. If the pharmacokinetic profiles of active and inactive forms of the drug are different, there may be no obvious relationship between their plasma concentrations and pharmacological effects. These findings have tremendous influence on dosing regimens. Analytical methods to separate and evaluate enantiomers have hence gained widespread attention.

Analysts have developed two different strategies for chromatographic resolution of enantiomers. These involve the use of chiral chromatographic systems based on either chiral stationary phases (CSPs) or chiral eluents, or the use of chiral derivatizing agents to produce diastereoisomers, which are easily separated using standard chromatographic systems [3–7]. However, it is absolutely essential that the chiral derivatizing agent be pure and that no racemization occurs during the reaction. It is also essential that the enantiomers have reactive functional groups suitable for reacting with chiral derivatizing reagents. The reaction process should go to completion without any stereoselectivity or kinetic resolution occuring during the process since it has been shown that enantiomers can react with a chiral reagent at very different rates [8].

Achiral agents have also been used to derivatize but the potential conversion of the analyte during the reaction still remains unsolved. Literature on the separation of enantiomers by LC and GC has been reviewed elsewhere [3, 6, 7]. Enantioselective radioimmunoassay (RIA) using antisera against pure enantiomers and optically pure labelled radioligands has been demonstrated with phenobarbital, hexobarbital and warfarin [9–12]. The first chiral stationary phase for LC

was engineered by Pirkle in 1981 and many more followed soon after [13, 14]. Resolution of enantiomeric drugs on biopolymer-based HPLC-CSPs was recently reviewed by Wainer [15]. These phases are mainly the Pirkle-type HPLC-CSPs based on amino acid derivatives or based on polymers such as methacrylate, cellulose, cellulose triacetate, cyclodextrins, etc. These reports suggest that it is desirable to have a CSP capable of chiral resolution with no prior derivatization of the analyte. Moreover, from the point of view of the pharmaceutical industry, the currently available CSP technology does not appear to be as robust as might be desired.

Proteins

Proteins are complex, high molecular weight polymers composed of chiral subunits (L-amino acids). Some proteins like α_1 acid glycoprotein (AGP), bovine serum albumin (BSA), human serum albumin (HSA) and ovomucoid (OVM) bind small molecules reversibly and these binding interactions can sometimes be stereospecific [16–19]. Stereospecific molecular recognition of such proteins has been exploited to develop protein-based CSPs and these have been found to be useful in chromatographic resolution of pharmaceutically active enantiomeric compounds. These bonded phases appear to have an extremely wide range of applications.

Proteins which tolerate organic solvents as well as high temperatures and which can function over a wide pH range are particularly useful in preparing a CSP. Table 1 enumerates some of the characteristics of these proteins. Of the proteins studied, AGP and OVM respectively contain 45 and 30% carbohydrate content in their structure and this has been reported to play a key role in their stereo-

Table 1
Characteristics of proteins used in chiral stationary phases

Protein	Mol. wt	Isoelectric point	S–S bridges	% Carbohydrate	Sialic acid residues	Mechanism of interaction
AGP	41,000	2.7	2	45	14	Cationic
BSA	66,000	4.7	17	—	—	Anionic–hydrophobic
ACHT	21,600	8.3, 8.7	—	—	—	Hydrogen bonding–hydrophobic
OVM	28,800	3.9–4.5	8	30	0.3	Hydrophobic

Table 2
Commercially available protein-based chiral stationary phases

Protein	Trade name	Matrix	Particle size (μm)	Pore size (Å)	Column size (mm)	Manufacturer/distributor
AGP	Enantiopac	Silica	10	250	100 × 4.0	Pharmacia LKB
AGP	Chiral AGP	Silica	5	120	100 × 4.0 100 × 10.0 150 × 10.0	ChromTech AB Sweden/ J.T. Baker
HSA	Chiral protein-2	Silica	7	300	150 × 4.6 150 × 7.8 150 × 10.0 150 × 21.0	SFCC*/ASTEC†
HSA	Chiral HSA	Silica	5	120	10 × 3.0 100 × 4.0 150 × 4.0	ChromTech AB/Sweden
BSA	Resolvosil	Silica	7	100	150 × 4.0	Machery Nagel
BSA	BSA column	Silica	7	300	250 × 4.6 250 × 7.8 250 × 10.0 250 × 21.0	SFCC*/ASTEC†
BSA	BA	Polymer	15–20	500	50 × 8 150 × 8	SHOWA DENKO K.K./ J.M. Science Inc.
OVM	Ultron ES-OVM	Silica	5	120	150 × 4.6 150 × 6.0	Shinwakako, Japan/ MacMod Analytical Inc. SHOWA DENKO
OVM	OV	Polymer	15–20	500	50 × 8.0	SHOWA DENKO K.K./ J.M. Science Inc.

*SFCC = Societe Francaise Chromato Colonne.
†ASTEC = Advanced Separation Technologies Inc.

specificity. CSPs based on BSA, AGP, HSA and OVM are commercially available. Table 2 lists some of the salient characteristics of these bonded phases. Trypsin, chymotrypsin and IgG are some of the other proteins which perform satisfactorily in separating chiral compounds [20–22].

α_1 acid glycoprotein

The α_1 acid glycoprotein stationary phase (AGP-CSP) has been used for the resolution of enantiomers of racemic drugs from many different classes of compounds. For example, primary, secondary and tertiary amines, acids and non-protolytic compounds have been separated on an AGP column [16]. AGP is comprised of a polypeptide of 181 amino acid residues and 14 sialic acid residues incorporated in the sugar parts of the molecule. The sialic acid has been suggested to be involved in the binding of ammonium-type compounds at neutral pH. AGP has a molecular weight of 41,000 and a carbohydrate content of 45%. It has two disulphide bridges and is an acidic protein with an isoelectric point of 2.7. AGP has been suggested to be the main cationic binding protein in the human organism.

Enantiopac and Chiral AGP® are the two commercially available AGP-based columns (Table 2). In Enantiopac, the first generation AGP column, the protein is immobilized by adsorption on a diethylaminoethyl silica which has a particle diameter of 10 μm, a large pore volume (250 Å) and a large surface area. The protein is then crosslinked on the silica support. Chiral AGP® consists of 5 μm spherical silica particles with a much smaller pore volume (120 Å). In this case, AGP is covalently immobilized on silica prior to crosslinking. It has been reported that the differences in the silica and in the immobilization chemistry are responsible for the differences in the performance characteristics and stability of these two columns. Table 3 lists some of the compounds resolved by AGP-CSP.

A detailed study on the stability of the AGP-CSP suggests that it can be used at elevated temperatures and is very stable in the presence of a water–2-propanol mixture or pure 2-propanol [23]. The capacity factors of the test compounds, bupivacaine and mepivacaine, are unaffected after passage of about 40,000 column volumes of mobile phase consisting of 2-propanol–phosphate buffer (0.01 M, pH 7.0) (6:94, v/v). The column has been demonstrated to tolerate very high concentrations of uncharged modifiers like 2-propanol. In fact, pure propanol can be passed through the column for 14 h without affecting the resolving power of the column [24]. However, amine additives which can decrease the life-time of the column are often required in the mobile phase for effective resolution.

With AGP-CSP, retention of isomers is often manipulated by varying the temperature (15–35°C), pH (3–7.5), ionic strength of the buffer electrolyte (such as NaCl), concentration of organic modifier and flow rate. Most drugs are resolved directly on the AGP column with the exception of some β-blockers which

Table 3
Examples of enantiomers separated on α_1 acid glycoprotein chiral stationary phases

Compound	Mobile phase	α*	Reference
alprenolol	2-propanol–phosphate (10 mM, pH 7.0) (7:93, v/v)	1.19	[31]
atenolol	phosphate (10 mM, pH 7.2)	1.31	[31]
bupivacain	2-propanol–phosphate (10 mM, pH 7.0) (6:94, v/v)	1.29	[16]
cyamemazine	acetonitrile–phosphate (10 mM, pH 6.0) (15:85, v/v)	1.55	[16]
dimetidine	2-propanol–phosphate (10 mM, pH 6.0) (10:90, v/v)	1.30	[16]
ephedrine	phosphate (10 mM, pH 7.0)–octanoic acid (5 mM)	1.34	[16]
ketamine	2-propanol–phosphate (10 mM, pH 7.0) (2.5:97.5, v/v)	1.26	[16, 27]
mephenytoin	phosphate (10 mM, pH 7.2)–2-propanol (0.32 M)	1.26	[30]
methylphenobarbital	phosphate (10 mM, pH 7.2)–2-propanol (0.52 M)	1.27	[30]
metoprolol	phosphate (10 mM, pH 7.2)	1.36	[31]
oxaminquine	acetonitrile–phosphate (10 mM, pH 7.0) (3:97, v/v)	1.64	[73]
oxprenolol	phosphate (10 mM, pH 7.2)–ethanol (1.76 M)	1.28	[31]
pheniramine	acetonitrile–phosphate (10 mM, pH 7.0) (7:93, v/v)	1.33	[16]
pindolol	phosphate (10 mM, pH 7.2)–acetonitrile (0.95 M)	1.53	[31]
terodiline	2-propanol–phosphate (10 mM, pH 7.4) (3:97, v/v)	1.27	[31]
tropicamide	acetonitrile–phosphate (10 mM, pH 7.0) (3:97, v/v)	1.38	[16]
verapamil	acetonitrile–phosphate (10 mM, pH 7.0) (10:90, v/v)	1.32	[16, 27]
warfarin	2-propanol–phosphate (10 mM, pH 7.0) (10:90, v/v)	1.39	[16, 27]

*Selectivity factor ($\alpha = k'_2/k'_1$).

require conversion to their cyclic 2-oxazolidines prior to separation [25]. Optical isomers of some cationic drugs, notably cocaine and methadone, are successfully resolved on AGP-CSP [26]. The recommended concentration of the analyte to be separated on an AGP column is in the range 3–5 nmol.

Changes in the mobile phase pH has a strong influence on the retention and enantioselectivity of AGP-CSP for a variety of compouinds [23]. The separation factors for ethotoin (a non-protolytic compound), hexobarbital (a weak acid) and metoprolol (a weak acid) have been reported to increase with increasing pH while the enantioselectivity for the strong acidic compounds like 2-phenoxypropionic acid decreases.

Several different neutral organic modifiers (1–15%) such as methanol, ethanol, 1-propanol, 2-propanol and acetonitrile have been used to regulate retention and enantioselectivity of an AGP column [27]. Verapamil has been successfully resolved using 10% acetonitrile in aqueous buffer while 1-propanol failed to resolve the compound. Diols have also been used to change retention. For example, a separation factor of 3.8 is reported for methylhomatropine with 1,2-butane-diol but is reduced to 2.65 using ethylene glycol [28]. It has been suggested that modifiers compete with the solute enantiomers in binding to groups with different hydrogen bonding properties, such as the carbonyl groups (=CO) and the imido groups (—NH—) in the binding site of the protein.

The presence of cationic modifiers such as *N,N*-dimethyloctylamine (DMOA), tetrapropylammonium bromide (TPrABr) or tetrabutylammonium bromide (TBuABr) in the mobile phase strongly influences the enantioselectivity and retention for several different compounds including uncharged, cationic and anionic drugs [24, 25]. Hydrophobic carboxylic acids such as butyric acid, octanoic acid and decanoic acid also show dramatic effects on the enantioselectivity of the AGP-CSP to several cationic drugs such as ephedrine, atropine, methylphenidate and phenmetrazine [26]. Increasing hydrophobicity of the acid decreases the retention of these solutes on the AGP column. A reversible change in the binding properties of the immobilized AGP is believed to be responsible for the change in the enantioselectivity of the AGP column for these drugs.

The general retention mechanism for protein-based CSPs is a combination of hydrogen bonding and hydrophobic interactions. Studies by Schill *et al.* have shown that under certain conditions charged solutes are retained on the AGP-CSP as ion pairs [26]. Enantioselective separations can be obtained only if the enantiomers of an analyte are bound to the chiral phase with different affinity; a three point interaction is necessary to obtain a stereoselective separation. In the case of AGP, the analyte requires at least one binding group and a ring structure in the vicinity of the chiral center in order to be resolved. Other groups which are important in the binding mechanism are alcohols, amides and carboxylic acid groups [29, 30]. The distance between the binding groups and the chiral center is also found to be important in determining the degree of enantioselectivity. Hydrogen bonding groups are best near the chiral center, not more than two atoms away. Chiral resolution is influenced by substitution of basic nitrogen atoms and by increasing the steric bulk on a basic nitrogen atom [26, 29].

The changes in retention of cationic and anionic drugs on an AGP column can be ascribed to changes in the properties of the AGP molecule and the compounds themselves due to protolysis. In several other cases retention is due to ion pairing [26]. In the cases where 1-octanoic acid is used, the cause of retention is suggested to be because of ion-exchange phenomena. The AGP molecule is believed to change its chiral specificity in the presence of different modifiers, changes in pH, temperature, etc. by changing its conformation. However, evidence recently reported using circular dichroism spectroscopy suggests that while it is possible to induce and increase chiral selectivity of AGP by adding different organic modifiers to the mobile phase, changes in protein conformation cannot be detected even in the presence of 40% 2-propanol [31]. When the effect of the immobilization procedure on the AGP molecule is determined by fluorescence studies, a 20 nm red shift is reported in the fluorescence spectra of immobilized AGP compared to the native form. This suggests the unfolding of the protein molecule exposing the tryptophan residues of the immobilized AGP [31]. However, the effect of *N,N*-dimethyoctylamine on the enantioselectivity for some fentiazine derivatives observed with immobilized AGP is similar to that obtained when AGP is used as a chiral

complexing agent in the mobile phase indicating that there exists some similarities between the chiral binding sites of the two forms of the protein.

The adsorption isotherm data suggest that the cationic compound, (−)terodiline binds to one site of the AGP molecule with high affinity and at least to one other site with low affinity. The enantiomers of amines, acids and non-protolytic compounds compete with (−)terodiline for binding to the same sites. The hydrogen bonding properties of the modifier affects enantioselectivity of AGP-CSP to a large extent [31]. It is possible to induce chiral selectivity for a drug by adding uncharged modifiers to the mobile phase with different hydrophobicities and hydrogen bonding properties. It is reasonable to assume that the modifier competes with the solute enantiomers for binding or, alternatively, the uncharged modifiers cause reversible changes in the protein conformation [31].

Bovine serum albumin

Bovine serum albumin (BSA) is a globular protein of molecular weight 66,210 consisting of 581 amino acids stabilized by 17 disulphide bridges (Table 1). The molecule is cigar shaped and is estimated to be 141 Å long and 41 Å in diameter with an isoelectric point of 4.9 [32]. BSA has been reported to have several hydrophobic binding sites and functions in blood as an 'organic carrier'. Many fatty acids are also known to bind to BSA.

BSA-CSP is commercially available under the trade name Resolvosil® (Table 2). The bonding chemistry of Resolvosil® is not very clear. However, BSA crosslinked by glutaraldehyde has been entrapped on silica and shown to resolve enantiomers [33]. BSA cross-linked onto aminopropylsilica by glutaraldehyde has also been reported to separate chiral molecules [34]. BSA has been immobilized by irreversible adsorption onto silica as well [35]. A very stable BSA-CSP is prepared by covalent immobilization of BSA on polymer-clad silica via a divinylsulphone linkage [36]. Table 4 tabulates the α values of several compounds separated on BSA-CSP.

Recent reports deal with the use of a fragment of BSA as a chiral ligand [34, 37]. BSA is cleaved into fragments by proteolytic degradation and the N-terminal half of BSA is isolated and immobilized on a silica column. This column is capable of resolving enantiomers of oxazepam, benzoin and morpholep while the enantiomers of tryptophan and warfarin are not separated [37]. When a mixture of three peptides obtained by enzymatic cleavage of BSA is isolated and immobilized, it is found that retention is lower than on intact BSA columns and that enantioselectivity is preserved for only a limited number of compounds as is the case with the N-terminal half of BSA [34]. These findings are in agreement with what was previously postulated to be the binding site of tryptophan and warfarin which is situated on the C-terminal part of the BSA molecule and which appears to play a key role in the enantioselectivity of the protein for these molecules. It is also possible that more than one site is involved in these binding interactions or perhaps the enantioselectivity is a function of the three dimensional structure of BSA.

The effect of pH, ionic strength and 1-propanol content of the mobile phase on the ability of the BSA-CSP to resolve enantiomers was studied by Allenmark *et al.* [38, 39]. With decreasing pH, the k' values increase for all

Table 4
Examples of enantiomers separated on bovine serum albumin chiral stationary phases

Compound	Mobile phase	α*	Reference
benzoin	1-propanol–phosphate (50 mM, pH 8.0) (2:98, v/v)	2.70	[33]
benzonal	1-propanol–phosphate (50 mM, pH 7.6) (6:94, v/v)	1.30	[40]
ibuprofen	1-propanol–phosphate (50 mM, pH 7.6) (10:90, v/v) + octanoic acid (1 mM)	2.44	[41]
ketoprofen	1-propanol–phosphate (50 mM, pH 8.0) (10:90, v/v)	1.25	[41]
kynurenine	1-propanol–phosphate (50 mM, pH 8.1) (2:98, v/v)	13.85	[36]
mandelic acid	ammonium dihydrogen phosphate (50 mM, pH 4.94)	1.47	[42]
oxazepam	1-propanol–phosphate (100 mM, pH 6.0) (2:98, v/v)	4.52	[36]
polythiazide	1-propanol–phosphate (50 mM, pH 8.0) (3:97, v/v)	2.10	[41]
temazepam	1-propanol–phosphate (50 mM, pH 7.0) (2:98, v/v)	3.00	[33]
warfarin	1-propanol–phosphate (50 mM, pH 6.79) (3:97, v/v)	2.02	[36]

*Selectivity factor ($\alpha = k'_2/k'_1$).

compounds tested since the negative net charge of BSA also decreases. A decrease in the 1-propanol content increases the k' values, thus indicating the importance of hydrophobic interactions [38, 39].

Several racemic barbiturates have also been chromatographed on the BSA column [40]. The racemic anticonvulsant benzonal and its analogues are strongly retained on the column and the compounds are resolved with α values greater than 1.3. The pH of the mobile phase strongly influences the resolution with significant decrease in k' of both enantiomers with increasing acidity of the mobile phase [40].

Anionic modifiers such as alkanoic acids (C_6–C_{10}) show the maximum influence on retention and capacity ratios. In all cases studied, capacity ratios decrease, thereby affecting the α values. The effects of hexylamine, hexanol and hexanoic acid on the retention and resolution of oxazepam, an uncharged analyte, are not very pronounced; they yield very little influence on the chiral binding site of the BSA molecule [41]. When the effect of increasing concentration of octanoic acid is studied on ibuprofen, α values decrease as modifier concentration increases especially between 2 and 3 mM. A similar effect is observed with octylamine where retention of both enantiomers decreases.

The mechanism of interaction of BSA with chiral molecules is unclear. An interesting retention behaviour is noticed using ±bendroflumethiazide on a BSA column. With increase in the pH of the mobile phase, α increases rapidly with a simultaneous decrease in k'_1 and an increase in k'_2. Similar effects are noticed at 4% 1-propanol. When several α-arylpropionic acids are tested, it is noticed that the mobile phase requires 10% of 1-propanol to obtain useful α values. The presence of octanoic acid further reduces the k' values with an increase in the α values [42]. Increasing the pH and 1-propanol concentration reduces the k' values of both enantiomers in the case of N-acylated amino acids. The pronounced reduction in k' upon *N*-methyl substitution in oxazepam shows that although the amide hydrogen participates in the binding to BSA, this is not a prerequisite for enantioselectivity [42]. These results suggest that BSA-CSP is particularly useful in resolving acidic compounds although the mechanism of interaction is difficult to establish.

In the case of BSA-CSP, both hydrophobic and electrostatic (coulombic) interactions are reportedly involved. Here, the presence of aromatic as well as relatively polar groups has been found to be a prerequisite for optical resolution [17]. Steric effects also appear to be important and account for the largely unpredictable results when analytes are substituted with different groups. With nitro substitution, some degree of charge-transfer interaction between the nitrophenyl and the tryptophan ring systems in the solute and protein respectively is possibly involved [39]. A charge transfer (π–π) mechanism is then suggested to exist between two tryptophanyl residues on the protein and solutes that contain electron-accepting moieties since retention increases with the degree of substitution of nitro groups in *N*-nitrobenzoyl and *N*-nitrophenyl amino acids.

The effect of pH on enantioselectivity is explained by the fact that with decreasing pH, the negative net charge of protein (for example, BSA) also decreases. This decreases the coulombic interaction between amino acids and the stationary phase resulting in lower k' values. Since carboxylic acids have no positive charge carrier in the molecule, a higher k' value is obtained. When the buffer strength is above 100 mM, hydrophobic interactions dominate; below 100 mM coulombic interactions prevail [39].

Human serum albumin

Human serum albumin (HSA) has been widely used to study the binding of drugs to proteins in solution since the mid-seventies [43]. HSA has a molecular weight of 66,500 and has an isoelectric point of 4.8 (Table 1). Since the affinity of binding and stereoselectivities of analytes to HSA and BSA differ when binding studies between these two proteins and drugs are conducted in solution, it is important to compare the chiral resolution capability of immobilized HSA and BSA. Immobilized HSA is commercially available under the trade name Chiral Protein 2 (Table 2). Synthesis and chromatographic properties of Chiral Protein 2 is reported by Domenici *et al.* [18, 44].

HSA is covalently bound to a commercially available diol activated silica-based column [18]. A variety of racemic drugs are resolved on HSA-CSP. Many of the compounds are resolved on both the BSA and the HSA columns. However, the elution order of enan-

tiomers are sometimes reversed on an HSA column compared to a BSA column. Examples of such compounds are *N*-benzoyl-alanine enantiomers, warfarin, etc. The chiral resolution of the benzodiazepine hemisuccinate esters is unique on HSA-CSP since they are not resolved on BSA-CSP. These findings are consistent with the binding studies in solution which show an inversion in the enantioselectivity of the binding of warfarin to HSA and BSA [45].

The effects of mobile phase composition and temperature on the enantioselectivity of the HSA-CSP is studied using racemic oxazepam hemisuccinate ((±)OXH). An increase in the 1-propanol content produces a decrease in the retention of both the enantiomers of (±)OXH, although the effect on the (*S*)-enantiomer is more pronounced. When the concentration of the modifier is increased from 5 to 8%, the α value decreases from 3.58 to 2.27. Similar results are obtained for the BSA column which suggest the existence of non-specific interactions between the modifier and the protein which weakens the solute–protein hydrophobic and hydrogen bonding interactions [17, 18].

An increase in the pH results in a decrease in the k's of both enantiomers of (±)OXH with the effect being greater on the (*R*)-enantiomer. An increase in α from 2.26 to 3.14 is observed as with the BSA column [17]. Increase in buffer molarity decreases the α value as is reported for compounds with a carboxylate moiety on a BSA column [17]. An increase in temperature produces a decrease in the k' of both enantiomers of (±)OXH. Similar results are obtained for warfarin and leucovorin on a BSA-CSP [46, 47].

The capacity factors of both the enantiomers of (±)OXH are compared on a HSA-CSP and the results indicate that the column capacity is relatively high for the first eluted enantiomer, (*R*)-OXH, and low for the second, (*S*)-OXH, suggesting that (*S*)-OXH binds to a stereospecific high affinity site on the HSA molecule while (*R*)-OXH binds to a number of non-specific sites [44]. Further results are awaited to clarify this.

The stability of HSA-CSP has been monitored over 80 days and the results indicate that there is a 30% decrease in the k' of (*R*)-OXH, a 28% decrease in the k' of (*S*)-OXH and a slight increase in α from 2.56 to 2.68. The HSA-CSP is hence quite stable under normal operating conditions. The two chiral stationary phases based on BSA and HSA are comparable with the differences in their stereoselectivities reflecting binding affinities of HSA and BSA in solution.

Ovomucoid

Ovomucoid (OVM), a trypsin inhibitor from chicken-egg white has a molecular weight of about 28,800 (Table 1). It is an acidic mucoprotein with an isoelectric point of 3.9–4.5. The molecule has eight disulphide bridges and 0.3 sialic acid residues incorporated into it. Its carbohydrate moiety constitutes about 30% of the total weight of the protein. Ovomucoid constitutes about 10% of the egg white content, the rest of it being albumin. It is known to have strong hydrophobic interactions with solutes (aromatic) that exhibit hydrophobicity. Since the carbohydrate moiety of the AGP molecule is found to play a key role in its enantioselectivity, it is interesting to explore the stereoselective capability of the ovomucoid molecule. Ovomucoid was bound to aminopropyl silica and used as a chiral stationary phase by Miwa *et al.* since 1987 [19, 48, 49]. Recently, this novel CSP has become commercially available (Table 2).

A variety of compounds are resolved on the ovomucoid-CSP (Table 5) [48]. The amount of ovomucoid bound to the bonded phase has been reported to influence the degree of resolution of compounds [19]. The addition of 2-propanol is required to elute compounds such as proglumide from the column. None of the amines tested are resolved in the presence of TBA-HSO_4. Sodium octane sulphonate (SOS), an anionic ion-pairing agent is used instead to resolve amines. All amines studied except chlorphenesin are resolved in the presence of SOS. Fluribiprofen is the only acidic compound that is resolved in the presence of SOS on the OVM-CSP [19].

The OVM-CSP is found to be stable for more than 12 months. Among the profen derivatives evaluated, ibuprofen exhibits the lowest capacity factor and this is attributed to its lower hydrophobicity compared to ketoprofen and flurbiprofen. When the resolution of chlorpheniramine is compared on an AGP (Enantiopac) and an ovomucoid column, the α value differs. This compound is better resolved on the ovomucoid column with an α value of 3.88 compared to 2.34 on the Enantiopac column [25].

Table 5
Examples of enantiomers separated on ovomucoid stationary phases

Compound	Mobile phase	α*	Reference
abscisic acid	2-propanol–phosphate (20 mM, pH 3.5) (2:98, v/v)	1.22	[51]
chlorpheiramine	2-propanol–phosphate (20 mM, pH 6.5) (6:94, v/v)	2.02	[49]
chlorprenaline	ethanol–phosphate (20 mM, pH 6.4) (10:90, v/v)	1.59	[49]
halofantrine	acetonitrile–phosphate (10 mM, pH 5.5) (45:55, v/v)	2.60	[52]
lorglumide	acetonitrile–phosphate (20 mM, pH 6.5) (26:74, v/v)	2.60	[49]
phenylpropanolamine	phosphate (20 mM, pH 6.4)	1.20	[49]
pindolol	ethanol–phosphate (20 mM, pH 5.5) (3:97, v/v)	1.60	[52]
propranolol	2-propanol–phosphate (20 mM, pH 7.75) (20:80, v/v)	1.13	[50]
verapamil	ethanol–phosphate (10 mM, pH 6.2) (18:82, v/v)	1.40	[52]

*Selectivity factor ($\alpha = k'_2/k'_1$).

The influence of pH, ionic strength and organic modifier on retention and enantioselectivity of OVM-CSP is evaluated using propranolol and its ester derivatives [50]. For these cationic analytes, an increase in the organic modifier content and/or a decrease in the pH results in a decreased retention of both enantiomers. The enantiomeric elution order is reversed when ester derivatives are chromatographed in the presence of methanol or acetonitrile. The authors suggest the presence of at least two chiral binding sites which are subjected to conformational changes when the protein is immobilized [50]. The resolution of abscisic acid metabolites has been reported on an OVM-CSP using 2-propanol in the mobile phase [51].

A detailed evaluation and comparison of OVM-CSP and AGP-CSP is reported by Kirkland *et al.* [52]. The authors advocate the use of OVM-CSP without the addition of any cationic or anionic modifiers in the mobile phase for consistent column performance. The OVM-CSP shows higher resolution, greater flexibility in operating parameters and better long-term stability than the AGP column. Up to 45% acetonitrile is used on the OVM column without affecting resolution. The OVM column is also capable of resolving four diastereomers from drugs containing two chiral centers. When the same drug is chromatographed on the AGP column, two of the diastereomers coeluted [52]. On conducting further studies on immobilized OVM, Miwa *et al.* reports that enantioselectivities are lost when carbohydrate moieties are chemically removed from the protein molecule [53]. However, the observed enantioselectivities for chlorpheniramine, a basic analyte and ketoprofen, an acidic analyte are both unchanged when the sialic acid residues of OVM are enzymatically removed [53]. A very elegant study is undertaken by Iredale *et al.* to establish the role played by hydrophobic interactions in the enantioselectivity of the OVM-CSP [54]. Results indicate that the shape and hydrophobicity of the alcohol modifier affect retention and enantioselectivity. A change in pH induces conformational transition even in the immobilized form of OVM and maximum efficiencies are reported at pH 5.0 [54]. The authors suggest the use of *t*-butanol in addition to 1-propanol and ethanol as modifiers in the mobile phase during method development. While Chiral-AGP has been primarily used to resolve basic or cationic compounds and Resolvosil® for acidic compounds, the OVM-CSP is capable of a broader applicability, being able to separate both acidic, basic and neutral enantiomers [52, 54, 55].

Trypsin and chymotrypsin

α-chymotrypsin (ACHT) exists as two isozymes with a molecular weight of 21,600 and isoelectric points of 8.38 and 8.76, respectively. ACHT mediates the hydrolyses of amides and esters of amino acids. ACHT is a chiral polymer composed of 245 L-amino acids. The molecule is ellipsoidal with a number of hydrophobic moieties on its surface. Initial investigations with immobilized ACHT reveal that the column can be used for the resolution of amino acids and amino acid derivatives for which the enzyme displays affinity [56, 57]. ACHT is covalently immobilized on a commercially available silica-based polymer-coated bonded phase which is derivatized with aldehyde groups [56–58].

Several analytes have been studied on ACHT-CSP in order to determine the effect of molecular structure on retention and stereoselectivity. As the hydrophobicity of the

analyte increases, the relative retention increases for the free amino acids. However, tryptophan is the only amino acid that is successfully resolved on the ACHT column. The N-derivatization of the amino acid also increases its hydrophobicity and retention. Derivatization of the carboxylic function of the free amino acids increases both k' and α values.

The enzymatic activity of immobilized ACHT has a strong effect on the resolution and steroselectivity of the CSP [58]. This is studied by inactivating the hydrolytically active site of the enzyme using *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK). When the analyte is not a substrate of the enzyme (for example, N-derivatives), the stereoselectivity is a function of the structure of the analyte. When it is a substrate of the enzyme (the *O*-derivatives), the stereoselectivity is a function of the activity of the enzyme. Since some of the ester derivatives are resolved on the TCPK-inactivated ACHT-CSP, it is suggested that stereospecificity resides in the neighbouring hydrophobic sites as well, at least for certain analytes tested.

The ability of ACHT-CSP to resolve dipeptides is studied on active and inactive columns. The ACHT-CSP is able to stereochemically resolve a number of the enantiomeric D,D- and L,L-dipeptides as well as the diasteromeric D,D-/L,L- and L,D-/D,L-dipeptides [59]. These compounds are also separated on the TPCK inactivated column again suggesting that the stereoselectivity resides away from the active site of the protein. Further, it is observed that the enantiomeric resolutions involving the D,L-/L,D-dipeptides are poor. However, the diastereomeric resolutions of the D,L-/L,D- and D,D-/L,L-dipeptides are significant on both active and inactive forms of the ACHT-CSP. These findings suggest the existence of two types of binding sites on the ACHT molecule each exhibiting different affinities.

The enantioselective and diastereoselective resolutions of the stereoisomers of *N*-α-aspartyl-phenylalanine 1-methyl ester (APME) has been successfully accomplished on ACHT-CSP. The effect of the molarity of the phosphate buffer in the mobile phase on retention and stereoselectivity of the APME stereoisomers differs depending on the presence of L- or D-phenylalanine. The observed enantioselectivities decrease for L,D-/D,L- and for D,D-/L,L- again suggesting the existence of two separate binding sites, the L-Phe and D-Phe sites. However, the effect of pH on retention, affects the binding of all the stereoisomers studied [60].

When the APME stereoisomers are chromatographed on the inactive ACHT-CSP, the retention of each isomer increases relative to the active form of the support. However, the increase in k' is greater for the L,D- and D,D-isomers than that for the D,L- and L,L-isomers, leading to an overall increase in enantioselectivity. ACHT-CSP is able to resolve the L,D-/D,L-APME and D,D-/L,L-APME but the L,D- and D,D-isomers coelutes under the conditions studied. The authors suggest that the observed enantioselectivity is a measure of the difference in the binding affinities at the two sites rather than the consequence of differential binding affinities at a single site. Two binding sites are hence identified on ACHT like for HSA [54]. In the case of ACHT, the structure of the analyte and the activity of the enzyme controls the enantioselectivity of the column. Interactions of hydrophobic nature which reside away from the active site may also be involved in the mechanism of resolution [58].

Similar results are reported for a trypsin-CSP which resolves O- and N,O-derivatized amino acids which are natural substrates of trypsin [21]. Trypsin preferentially catalyses the hydrolysis of ester and peptide bonds involving the carboxyl group of arginine and histidine. The effect of solute structure and other parameters on the enantioselective capability of the trypsin-CSP has been reported elsewhere [21].

Protein stationary phases are extremely complex, involving a plethora of interactions which cause enantiomeric discrimination. The exact nature of interaction is difficult to predict and largely depends on the protein conformation during the chromatographic conditions.

Performance requirements of protein CSPs

The performance requirements of affinity chromatographic supports has been the subject of several reviews [36]. In conventional affinity chromatography, the immobilized ligand and the analyte to be separated are often both large molecules. A typical example of this is the antigen–antibody interaction. In this case, mass transfer is a crucial parameter that dic-

tates the chromatographic efficiency. In chiral stationary phases based on proteins, the molecule to be resolved is always small; this means that mass transfer in the pores of the bonded phase is a faster process.

Mass adsorption often depends on three factors: (1) pore size; (2) particle size; and (3) flow rate. Although the pore size may not be a limiting factor in protein CSPs because of factors mentioned above, the particle size and the flow rate of the elution process have a marked influence on the overall efficiency of the technique. The flow properties of a rigid particle is of little use if the material has no effective capacity at flow rates required for economical process operation. Moreover, slow diffusion or slow adsorption kinetics often result in undesirable characteristics such as band broadening or split peaks. Restricted intraparticle diffusion is also the primary cause of reduced frontal uptake of dynamic systems.

An ideal support for protein CSP should exhibit minimum non-specific interactions. Residual charge on the surface should be minimum with no hydrophobic binding sites. This is important since the interaction of the immobilized protein and the analyte is itself a combination of hydrophobic and coulombic interactions. The support should be capable of rapid kinetics if the CSP is to be used on a routine scale in the analysis of drugs and their metabolites. The immobilization chemistry should be stable and robust with the protein covalently bound to the support. Good recovery and reproducibility combined with convenient cleaning-in-place procedures would ease the use of these CSPs in routine operations. Loading capacity of the CSP becomes an important consideration in scaling up separation procedures where resolution cannot be sacrificed unlike conventional preparative affinity systems. Protein-based CSPs have a lower binding capacity compared to the other CSPs and as a result the preparative potential for these stationary phases is limited. Efforts to increase the loading capacity of these CSPs are hence important.

Economically, CSPs based on proteins are an expensive alternative. This is largely due to the fact that the commercially available protein-based CSPs are in the 'ready to use' form with the protein already immobilized onto the support. The availability of pre-activated supports for CSPs where the user is able to immobilize the protein or a specific antibody of choice easily and conveniently '*in situ*' sould considerably increase the popularity of these bonded phases and their use in chiral separations.

Stereochemical drug metabolism

The role of protein-based CSPs in elucidating stereochemical drug metabolism processes has been of only limited significance so far. However, drug binding to proteins has been of considerable interest since the degree of protein-binding is a parameter of importance in the evaluation of the pharmacological and pharmacokinetic properties of potential drugs.

As early as 1971, Sjoholm *et al.* studied the specificity of the three binding sites of HSA which was immobilized in microparticles of polyacrylamide [61]. In 1986, Fitos *et al.* demonstrated drug binding to HSA by chromatography on immobilized HSA [62]. Elution volumes indicated the binding strength with the more retained analyte appearing at higher volume. It was then shown that immobilized and dissolved albumins exhibited identical binding properties [62, 63].

Three distinct binding sites are described for HSA: the diazepam site; the digitoxin site; and the warfarin site. Some drugs like tamoxifen and flurbiprofen bind to more than one site [61]. Besides diazepam, site one binds other benzodiazepines, some antibiotics and antidiabetics together with tryptophan, some analgesic agents like flurbiprofen and naproxen (that contain isopropionyl side chains). Site two is found to be quite specific for digitoxin and acetyl digitoxin. Site three is the primary binding site for warfarin and bilirubin and it has a broad specificity with several sulpha and penicillin derivatives and analgesic agents like oxyphenbutazone binding to this site [61].

The differential binding of (±)tofisopam, the active substance of Grandaxin® (a tranquilizer) to the HSA molecule is studied by following the conformational transition by affinity chromatography on immobilized HSA [64]. In order to elucidate the binding stereoselectivity of HSA in detail, the interaction of a number of 2,3-benzodiazepines are investigated by affinity chromatography on immobilized HSA [65]. The HSA molecule shows conformational recognition in the binding of 2,3-benzodiazepines and 1,4-benzodiazepines. The same binding site on HSA is involved in the binding of both these classes of com-

pounds. Based on these findings, it is suggested that the structural configuration and conformation of the species of tofisopam and the strengths of their binding to albumin can be used to identify the multitude of species of the drug present in a solution.

The retention characteristics of a heterogenous group of 23 drugs are compared on a chemically bonded BSA column and a good correlation is found between these data and equilibrium dialysis [66]. The presence of clonazepam and the enantiomers of uxepam affects the binding of warfarin enantiomers of HSA [67]. The binding of (S)warfarin is enhanced in the presence of clonazepam and (S)uxepam. In the clonazepam molecule, it is the 2′-Cl substituent which is responsible for inducing the binding interaction whereas in the (S)uxepam molecule, it is the 4-carbamoyl substituent which is responsible for inducing the binding interaction. Both clonazepam and (S)uxepam have very low binding affinities for HSA. However, they provoke binding interactions which improve resolution of the strongly bound (±)warfarin.

Chromatography on immobilized proteins is able to detect stereochemical binding interactions. This concept is utilized to study the binding interactions of (±)oxazepam hemisuccinate with immobilized HSA in the presence of displacers. Results indicate that retention of HSA-CSP reflects binding to native HSA and the technique can determine enantioselective and competitive binding interactions at specific sites on HSA. The HSA-CSP is able to recognize different binding areas for (*S*)- and (*R*)-oxazepam hemisuccinate. Both allosteric and competitive interactions are identified using warfarin and ibuprofen, respectively [44].

Analytical procedures to reveal and follow drug protein binding is reviewed by Sebille *et al.* [68]. Numerical simulations of the chromatographic processes are applied by Vidal-Madjar *et al.* to determine the equilibrium isotherm of drugs such as phenylbutazone with immobilized HSA using zonal HPLC [69]. The study reveals a specific and non-specific site on the protein molecule. The results from various studies using immobilized protein as CSPs have been compiled into excellent reviews which illustrate the versatility of protein-based chiral stationary phases [70–72]. Possible enantiomeric differences in the biotransformation of oxamniquine to its 6-acid metabolite is recently reported based on studies using the second generation AGP-CSP [73].

Mechanistic studies related to drug–protein chiral recognition are of fundamental interest in molecular pharmacology since optical activity may be directly related to the physiological effect of the drug. Although concentrated physiological samples cannot be used in chromatography, the high speed and accuracy of HPLC and the increased use of automated equipment will certainly make this technique more popular. The concept of isolating proteins with a specific activity towards drugs or metabolites will reveal essential information in immunopharmacology. The possibility of developing an antibody specific to an enantiomeric form of a drug which is difficult to separate by other means opens up a whole new dimension to the role of protein stationary phases in chiral separation.

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