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# Review

# Proteins and peptides as chiral selectors in liquid chromatography

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ABSTRACT

Considerable progress has been made recently in the application of amino acid-derived chiral selectors to direct enantiomer separations by liquid chromatography. A better understanding of the chiral discrimination exerted by proteins has been achieved through detailed analyses of the adsorption isotherms obtained under various mobile phase conditions and temperatures. Further insight into the complexity of protein–ligand interactions and the effect on retention have come from studies with the use of mobile phase additives causing allosteric interaction, sometimes resulting in dramatic effects on the enantiomeric separation factor. Further, the recent use of small, synthetic peptides as chiral selectors has shown promise for the future. The wide applicability of particularly the protein phases has led to a variety of novel experimental techniques, involving miniaturization (capillary LC and its combination with mass spectrometry), use for electrically driven separations (capillary electrophoresis), immobilization of proteins on so-called continuous gels for rapid enantiomer separations and use for studies of enzyme stereochemistry.

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## 1. INTRODUCTION

One of the most versatile groups of compounds belonging to the so-called chiral pool consists of the natural amino acids. Not surprisingly, amino acid derivatives were among the first selectors to be used in chiral chromatography, forming the starting material of the chiral stationary phases based on charge-transfer interaction, successfully developed over the last decade by Pirkle and his collaborators [1]. However, Nature has made use of the amino acids over the last billion years or so to produce chiral materials of as yet unsurpassed sophistication, *viz.*, the biopolymers known as polypeptides and proteins. This review will treat briefly the most recent advances in the use of the latter as stationary phases for chiral liquid chromatography.

The development, properties and use of chiral stationary phases based on amino acid biopolymers can be found in a number of earlier publications [2–5] and therefore only a brief summary of previous work will be given here. Instead, the main focus will be directed at current developments and research problems within the field. Very little has been done so far on *synthetic* polypeptides, however, so the present state-of-the-art and the possible future potential of this topic will be treated more comprehensively.

While chiral discrimination experiments with the use of small synthetic peptides have usually been performed in non-polar solvents to make use of differential hydrogen bonding, proteins have exclusively been used in aqueous, buffered media which take advantage of the proteins' natural binding resources, notably hydrophobic and electrostatic interactions. This makes the mobile phase selection a fairly complicated issue as a number of parameters can be varied, such as buffer constituents, ionic strength, pH and organic modifier. Optimization of a given separation can therefore be laborious; on the other hand, the probability of achieving the separation of a given enantiomeric pair increases.

## 2. PROTEIN PHASES

### 2.1. Background

A summary of the proteins used for chiral separations and their main properties is given in Table 1.

Owing to their operation in the reversed-phase mode, the protein-based columns have found extensive use for the analytical-scale separation of drug enantiomers. In particular, many racemic protolytes, which are difficult to resolve on other chiral phases, have been successfully resolved into enantiomers.

The very limited capacity of these columns, however, makes them useful mainly for analytical purposes. Owing to the highly complex structure of the stationary phases, it is extremely difficult to obtain any detailed insight into the mechanisms of binding and retention by these phases. During the last few years, however, some studies of fundamental problems associated with capacity and binding phenomena have appeared. Further, interesting work on miniaturization, combination with mass spectrometric detection and electrophoretic applications of proteins as chiral phases have been performed recently. In the following, these and other new developments in the field will be highlighted.

### 2.2. The problem of chiral binding capacity

Extensive work has been carried out on bonded bovine serum albumin (BSA) phases by Guiochon and co-workers [17–21] in order to obtain insight into the mechanism of enantioselective adsorption phenomena. Taking the two enantiomers of N-benzoylalanine as an example, it was shown by frontal analysis that the equilib-

TABLE 1

PROTEIN-DERIVED CHIRAL SORBENTS USED IN LIQUID CHROMATOGRAPHY

| Protein | Molecular mass | Carbohydrate content (%) | Isoelectric point | Column trade name | Main refs. |
|---|---|---|---|---|---|
| Serum albumin | 67 (BSA)<br>68 (HSA) | 0<br>0 | 4.7<br>4.7 | Resolvosil Chiral Protein 2 | 2, 3, 5<br>5–7 |
| Orosomucoid ($\alpha_1$-acid glycoprotein) | 44 | 45 | 2.7 | Chiral-AGP (Enantiopac) | 2, 3, 8, 9 |
| Ovomucoid | 28 | 17–34 | 4.5 | Ultron ES-OVM | 2, 3, 10, 11 |
| Cellobiohydrolase-I | 60–70 | 6 | 3.6 | – | 2, 12, 13 |
| Avidin | 66 | 20.5 | 9.5–10.0 | – | 14 |
| Chymotrypsin | 25 | 0 | 8.1–8.6 | – | 15 |
| Ovotransferrin | 70–78 | – | 6.1–6.6 | – | 16 |

rium isotherms obtained were almost identical with those derived theoretically from a two-site, binary competitive Langmuir adsorption isotherm model (eqn. 1) [17]:

$$q_i = \frac{a_{i,1}C_i}{1 + b_{1,1}C_1 + b_{2,1}C_2} + \frac{a_{i,2}C_i}{1 + b_{1,2}C_1 + b_{2,2}C_2} \quad (1)$$

where the first subscript indicates the enantiomer considered (1 denotes the first eluted) and the second indicates the site (1 denotes the enantioselective site). Table 2 gives the isotherm coefficients ($a$ and $b$) and column saturation capacities ($q_s$) determined. As predicted theoretically, all coefficients of the second (non-selective) site are (almost) equal for the two enantiomers. The excellent agreement between experimental and calculated data is evident from Fig. 1, showing equilibrium data for the individual enantiomers of N-benzoylalanine in the phase system.

Studies of the temperature dependence of adsorption isotherms also permitted the determination of the enthalpy of adsorption ($\Delta H_a^0$) and the isosteric heat of adsorption ($\Delta H_{st}^0$) [18]. As the temperature dependence of the $b$ coefficients in eqn. 1, which are equal to the equilibrium constants of the respective adsorption processes, can be expressed by eqn. 2,

$$\frac{\partial(\ln b)}{\partial\left(\frac{1}{T}\right)} = -\frac{\Delta H_a^0}{R} \quad (2)$$

it can be easily shown that a plot of ln $b$ as a function of $1/T$ will give a slope equal to

TABLE 2

ISOTHERM COEFFICIENTS AND SATURATION CAPACITIES DERIVED FOR THE N-BENZOYLALANINE ENANTIOMERS

From ref. 12.

| Site | L-Form | | | D-Form | | |
|---|---|---|---|---|---|---|
| | $a$ | $b$ (l mol$^{-1}$) | $q_s$ (mol l$^{-1}$) | $a$ | $b$ (l mol$^{-1}$) | $q_s$ (mol l$^{-1}$) |
| 1 | 14.16 | 7570 | $1.875 \cdot 10^{-3}$ | 35.09 | 17 236 | $2.036 \cdot 10^{-3}$ |
| 2 | 4.41 | 222 | $19.95 \cdot 10^{-3}$ | 4.25 | 214 | $19.86 \cdot 10^{-3}$ |

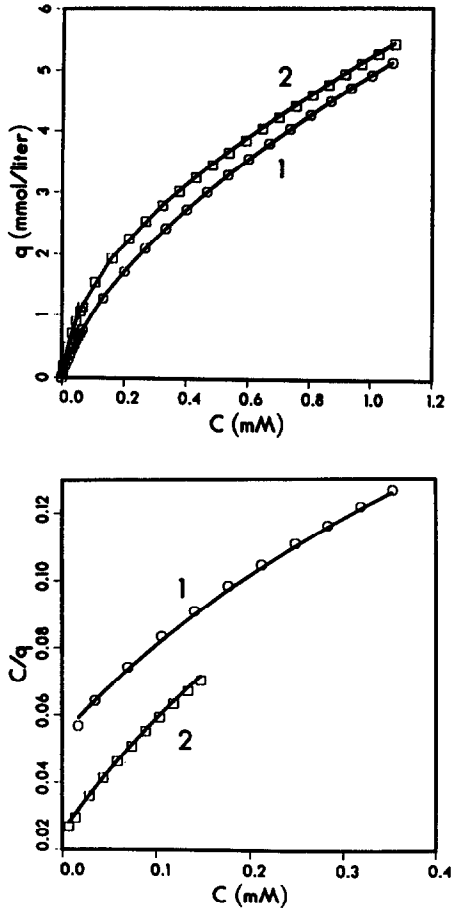



Fig. 1. Experimental (symbols) and calculated (solid lines) data for the equilibrium of the N-benzoylalanine enantiomers in the phase system BSA-silica–3% 1-propanol in 10 m*M* phosphate buffer (pH 6.7). Top, high concentration range, *q vs. C*; bottom, low concentration range, *C*/*q vs. C*. See eqn. 1 for isotherm representation. From ref. 17 (© 1990 American Chemical Society).

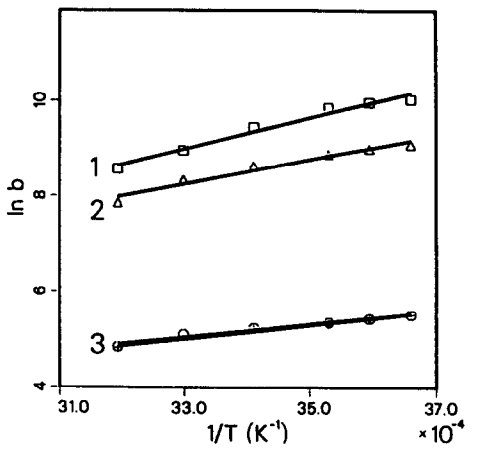



Fig. 2. Van 't Hoff plot giving the enthalpy of adsorption of the N-benzoylalanine enantiomers at the selective and non-selective sites, respectively, of immobilized BSA. From ref. 18.

$-\Delta H_a^0/R$. Fig. 2 shows the result from such a plot, where the different adsorption of the enantiomers at the selective site (1 is the D-form and 2 the L-form) throughout the temperature range is evident. The adsorption at the non-selective site (3, equal for both enantiomers) is also given. It is interesting to compare the enthalpy and entropy data obtained for the adsorption of the enantiomers. For the selective sites the difference in $\Delta H_a^0$ between the more retained D-enantiomer and the L-form ($\Delta\Delta H_a^0$) amounts to $-1.5$ kcal/mol (1 kcal = 4.184 kJ). The corresponding $\Delta\Delta S_a^0$ value is of the order $-3.6$ cal/mol·K (e.u.). The larger negative entropy of adsorption for the D-form at the selective site means that a more ordered adsorption complex with fewer degrees of freedom is formed in this instance, as expected. A comparison of data for the adsorption of the D-enantiomer at the selective and non-selective sites, respectively, reveals a large difference. Here, $\Delta\Delta H_a^0 = -3.8$ kcal/mol and $\Delta\Delta S_a^0 \approx -5$ e.u. This is in complete agreement with the assumption of a multi-point interaction between this enantiomer and the selective site causing a conformationally more rigid adsorption complex.

The bi-Langmuir adsorption isotherm was also found to be valid throughout a wide range of mobile phase compositions [19]. The saturation capacities for the selective and the non-selective sites were found not to be influenced by the mobile phase when the 1-propanol modifier content was varied between 0 and 10%.

The above results, which were all obtained using columns containing BSA adsorbed on an anion-exchange support, were recently used in a study of optimization of the production rate in preparative enantiomer separations [20]. The low capacity of protein-based columns for such purposes is a limiting factor, however. This has been further elucidated from a study of a covalently immobilized BSA sorbent, which permitted a calculation of the number of enan-

tioselective sites per BSA molecule and which gave an average value of 0.28 [21]. This investigation also led to the conclusion that only *ca.* 12% of the available silica surface was actually covered with protein. Taken together, the results clearly show that there are still some problems to solve with respect to protein immobilization on silica surfaces.

Finally, it was shown recently by Gilpin *et al.* [22] that there is a clear temperature optimum for the $\alpha$-value of DL-tryptophan on immobilized BSA. This effect was shown to be due to a maximum in $k_2'$ (L-tryptophan) at approximately 20–24°C. The decrease in $k_2'$ with decreasing temperature below the optimum is of particular interest. It seems likely that the chiral binding sites are undergoing a slight change due to a temperature-dependent, different conformational mobility and average conformational state of the protein. Whether this phenomenon also exists for the free protein in solution is not known.

### 2.3. *Effects of allosteric interaction on retention*

Liquid chromatography on immobilized protein phases has been used by several groups to study protein-binding interactions of chiral molecules [23–27]. This technique is particularly well suited for studies of allosteric interactions between spatially distinct binding sites and has been applied by Fitos and co-workers [23,24], who used human serum albumin (HSA)-based affinity columns to investigate allosteric protein-binding interactions between the warfarin and benzodiazepine sites. Similar studies have been carried out by Domenici and co-workers [26,27]; however, their experiments were performed with silica-bound HSA as a chiral stationary phase (CSP). A number of benzodiazepinones and their derivatives were chromatographed on immobilized HSA in the presence of (*R*)- or (*S*)-warfarin in the mobile phase. The magnitude of the allosteric effect was found to be strongly dependent on the configurations of both ligands and on the structure of the benzodiazepinone. For oxazepam hemisuccinate (OXH), only (*R*)-warfarin had a detectable effect. Fig. 3a shows the decrease in the retention of (*S*)-OXH on addition of (*R*)-warfarin to the mobile phase (20

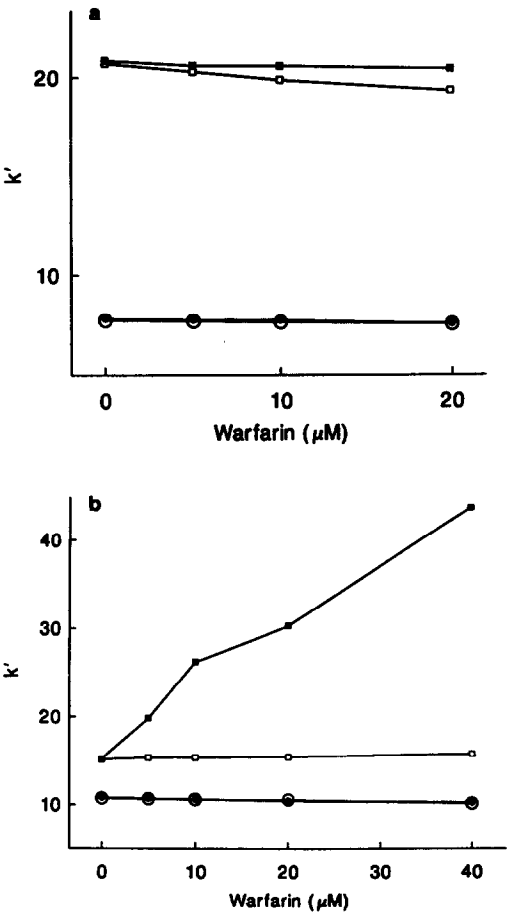



Fig. 3. (a) Effect on the $k'$ values of (*R*)- and (*S*)-oxazepam hemisuccinate (OXH) of the addition of (*R*)- or (*S*)-warfarin (WAR) to the mobile phase [50 m*M* phosphate buffer (pH 7.0) containing 6% 1-propanol; flow-rate: 0.8 ml/min]. ○ = (*R*)-OXH [with (*R*)-WAR in the mobile phase]; □ = (*S*)-OXH [(*R*)-WAR]; ● = (*R*)-OXH [(*S*)-WAR]; ■ = (*S*)-OXH [(*S*)-WAR]. Column: HSA-CSP, 150 × 4.6 mm I.D. (b) Effect on $k'$ values of (*R*)- and (*S*)-lorazepam hemisuccinate (LOH) of the addition of (*R*)- or (*S*)-warfarin (WAR) to the mobile phase [50 m*M* phosphate buffer (pH 7.0) containing 6% 1-propanol; flow-rate: 0.8 ml/min]. ○ = (*R*)-LOH [with (*R*)-WAR in the mobile phase]; □ = (*S*)-LOH [(*R*)-WAR]; ● = (*R*)-LOH [(*S*)-WAR]; ■ = (*S*)-LOH [(*S*)-WAR]. Column: HSA-CSP, 150 × 4.6 mm I.D. From ref. 26 (© 1991 American Pharmaceutical Association).

m*M*). However, the retention of (*R*)-OXH was not significantly affected by addition of (*R*)- or (*S*)-warfarin. This is consistent with results from earlier studies which showed that only (*S*)-OXH is bound to the benzodiazepine site [23]. The most pronounced effect on retention was seen when the *S*-enantiomer of both warfarin and

lorazepam hemisuccinate (LOH) were bound to HSA. The addition of a 40 m*M* concentration of (*S*)-warfarin to the mobile phase resulted in a dramatic increase in the retention of (*S*)-LOH, as shown in Fig. 3b.

These experiments clearly show that chromatography on immobilized HSA reflects the behaviour of the native protein fairly well, and is therefore a versatile tool for studies of ligand–protein and ligand–ligand interactions.

### 2.4. Significant advances in experimental techniques and applications

#### 2.4.1. Miniaturization

Experiments with capillary columns in liquid chromatography are often motivated by the need for special detection techniques, such as laser-induced fluorescence [28], miniaturized electrochemical detection [29] or mass spectrometry [30]. Fused-silica capillaries packed with 5-$\mu$m silica particles covered with BSA [31] and AGP [32] phases have recently been prepared and evaluated. Hermansson *et al.* [32] combined a 150 × 0.18 mm I.D. Chiral-AGP capillary column with a 45 × 0.25 mm I.D. $C_8$ trapping column (Trap-Cap $C_8$) for preconcentration of the dilute analyte solution and was able to achieve a concentration factor of up to *ca.* 330. The column-switching system was also used to generate a mobile phase gradient suitable for elution from the analytical column. Depending on the difference between the two mobile phases used in the system, a variable steepness of the (multiparameter) gradient could be obtained. When properly chosen, a considerable improvement of the chromatographic performance is thereby achieved. Fig. 4 shows a resolution of 0.93 ng of racemic luciferin using the system described. The solution injected and preconcentrated on the trapping column had a concentration of 232 ng/ml (116 ng/ml of each enantiomer).

The same system was also interfaced with a mass spectrometer using electrospray ionization (ESI-MS). A volatile ammonium acetate buffer (3 m*M*) was used for pH regulation with 2-propanol as organic modifier. Fig. 5 shows the operation of the total system as applied to a

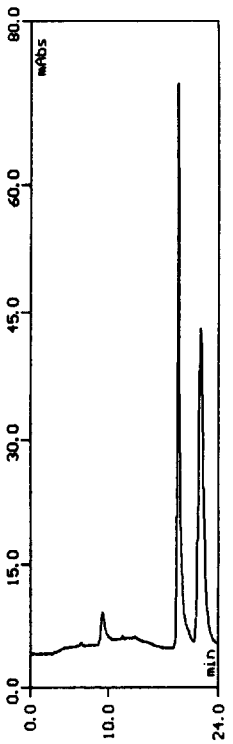



Fig. 4. Resolution of *rac*-luciferin on a Chiral-AGP capillary column (150 × 0.180 mm I.D.). The analytical column used 4% 2-propanol in 3 m*M* ammonium acetate (pH 6.0) at 1.3 $\mu$l/min. The $C_8$ trapping column (45 × 0.25 mm I.D.) used 3 m*M* ammonium acetate (pH 4.5) at 10 $\mu$l/min. UV detection at 327 nm. From ref. 32.

sample of (*E*)-10-hydroxynortriptyline (200 ng/ml) using selected ion monitoring (SIM) at *m/z* 280. As in the previous instances, a total volume of 4 $\mu$l was injected into the system, meaning that each peak in Fig. 5 corresponds to 0.4 ng.

It is evident that capillary LC utilizing protein-based phases is a promising form of chiral chromatography. The low flow-rates (1–2 $\mu$l/min) combined with the high selectivity and sensitivity obtained in SIM are attractive features of an analytical technique directed towards biological samples.

#### 2.4.2. Electrically driven separations

Recently, proteins have also come into use in electrically driven separation techniques. An interesting approach was taken by Birnbaum and Nilsson [33], who performed capillary electrophoresis with the use of fused-silica capillaries

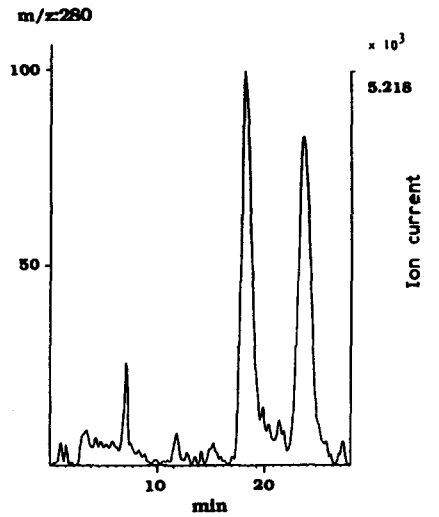



Fig. 5. Resolution of *rac*(*E*)-10-hydroxynortriptyline on a Chiral-AGP capillary column (150 × 0.180 mm I.D.). The analytical column used 16% 2-propanol in 3 m*M* ammonium acetate (pH 6.0) at 1.3 μl/min. The $C_4$ trapping column (45 × 0.25 mm I.D.) used distilled water. Detection by ESI-MS (SIM $m/z = 280$). From ref. 32.

filled with a gel consisting only of BSA cross-linked with glutaraldehyde. Fig. 6 shows the impressive efficiency that can be achieved by the use of this technique. A theoretical plate number of 91 000 was obtained on a 400-mm capillary with a gel length of 320 mm, and the tryptophan enantiomers were separated with a resolution factor of 6. It is assumed that the separation is driven by the electroosmotic flow generated in the gel-filled column at the buffer phase pH of 7.5, under which conditions both the protein (p*I* = 4.9) and the analyte (tryptophan, p*I* = 5.9) possess a positive net charge. The analyte then moves to the cathode where it is detected with a UV detector (214 nm). Because the gel acts like a stationary phase, this kind of technique has often been called electrokinetic capillary chromatography (ECC), since its use with micellar systems (MECC) as first described by Terabe *et al.* [34].

A similar technique has recently been used by Valtcheva *et al.* [35], although in this case the protein was used in free solution. This puts certain restrictions on the pH of the electrolyte buffer, as it is preferable that the analyte and the protein move in opposite directions. In Fig. 7 the successful use of the technique, as applied to a series of β-blocking drugs, is shown. The protein used was cellobiohydrolase-I (CBH-I, p*I* = 3.6), present in a phosphate buffer of pH 5.1 supplemented with 25% of 2-propanol. As the basic amino alcohol is completely protonated in this medium, it will move towards the cathode, whereas CBH-I migrates to the anode.

### 2.4.3. *New support materials*

A promising new kind of support material, based on polymerization of an acrylamide derivative at high salt concentration followed by

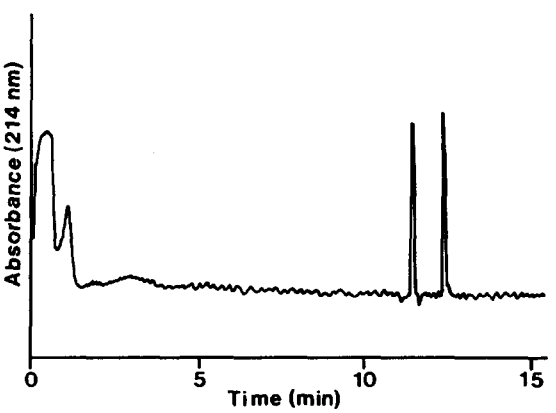



Fig. 6. Electrophoretically driven separation of the enantiomers of tryptophan on a gel consisting of BSA cross-linked with glutaraldehyde and contained in a 400 mm × 75 μm I.D. capillary column. Applied field strength, 125 V/cm; 50 m*M* phosphate buffer (pH 7.5). From ref. 33 (© 1992 American Chemical Society).

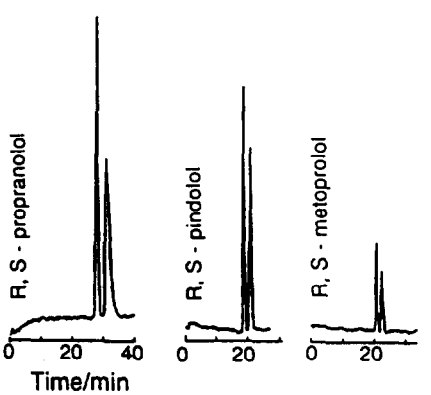



Fig. 7. Separation of the enantiomers of some β-blockers by capillary electrophoresis using free CBH-I as chiral selector. From ref. 35.

compression, was recently introduced by Hjertén *et al.* [13]. Since the material thus obtained is non-disperse and can be regarded as a gel rod permeated by channels, it has been named continuous gel or continuous bed. Columns containing such continuous gels can be operated with high flow-rates even under relatively moderate pressures. As a variety of ligands can be covalently attached to these gels, their application appears to be wide. Of particular interest here is their use as carriers of proteins as chiral stationary phases for rapid direct separation of enantiomers by liquid chromatography. It was reported as an example [13] that *rac*-practolol (a β-blocking compound) was baseline separated within 40 s on a column (40 mm × 6 mm I.D.) containing a continuous gel with immobilized CBH-I at a flow-rate of 7 ml/min (pressure 100 bar).

#### 2.4.4. *Applications to enzyme stereochemistry*

Chromatography in the reversed-phase mode has the inherent advantage of permitting direct injection of aqueous samples on to the column. Therefore, the technique is favourable for investigations of reactions in biological systems or less complex, but water-based media. Thus, biocatalytic conversion of organic substrates are well suited to analysis by protein-based column liquid chromatography and such studies, directed towards the determination of enantioselectivity in enzymatic reactions with racemic substrates, have been initiated recently.

The kinetic resolution of a racemic substrate by an enzyme is expressed by the enantioselectivity ($E$), which, for a Michaelis–Menten type of irreversible reaction, is expressed by

$$E = \frac{(k_{cat}/K_M)_1}{(k_{cat}/K_M)_2} \quad (3)$$

where the subscripts 1 and 2 denote the two substrate enantiomers. It has been shown [36] that $E$ is then only a function of the enantiomeric excess values found for the substrate ($ee_s$) and product ($ee_p$), respectively. These values are, in turn, interrelated by the equation $c = ee_s/(ee_s + ee_p)$, $c$ being the overall degree of conversion. Then, $E$ is most readily expressed by [36]

$$E = \frac{\ln[1 - c(1 + ee_p)]}{\ln[1 - c(1 - ee_p)]} \quad (4)$$

From an analytical point of view, this means that if the chromatographic separation of substrate and product and of either the substrate or product enantiomers can be achieved, $E$ can be easily determined with high precision.

As a wide variety of chiral acids are readily optically resolved on BSA-based columns [37], studies of some lipase-catalysed esterolytic reactions were recently undertaken [38–40] to demonstrate the potential of the analytical method. Fig. 8 presents an illustrative example of the information displayed in a single chromatogram, obtained after just dilution of the sample taken from the reaction mixture. Integration of the three relevant peaks gives $ee_p$ and $c$ from which $E$ is calculated.

Owing to its sensitivity, precision and ease of operation, the method described is far more attractive than the previously used NMR-based methods.

## 3. SYNTHETIC PEPTIDE PHASES

### 3.1. *Background*

In contrast to the protein-based columns, chiral stationary phases (CSPs) based on synthetic peptides have been used in both reversed- and normal-phase modes. Early work in this field was done by Grushka and Scott [41], who immobilized a polyglycine peptide on chromatographic supports such as glass beads and Porasil C. Grushka and co-workers [42,43] synthesized tripeptides on a silica surface, in a manner analogous to a Merrifield synthesis, for the analysis of isomeric peptides and of phenylthiohydantoin (PTH)-amino acids which result from the Edman degradation of proteins and peptides.

To circumvent the problem of multiple derivative formation, Rogers and co-workers [44,45] synthesized the intact di- and tripeptides before immobilizing them on the silica surface. Similarly, Ôi and co-workers [46,47] reported the separation of racemic compounds on peptide-bonded

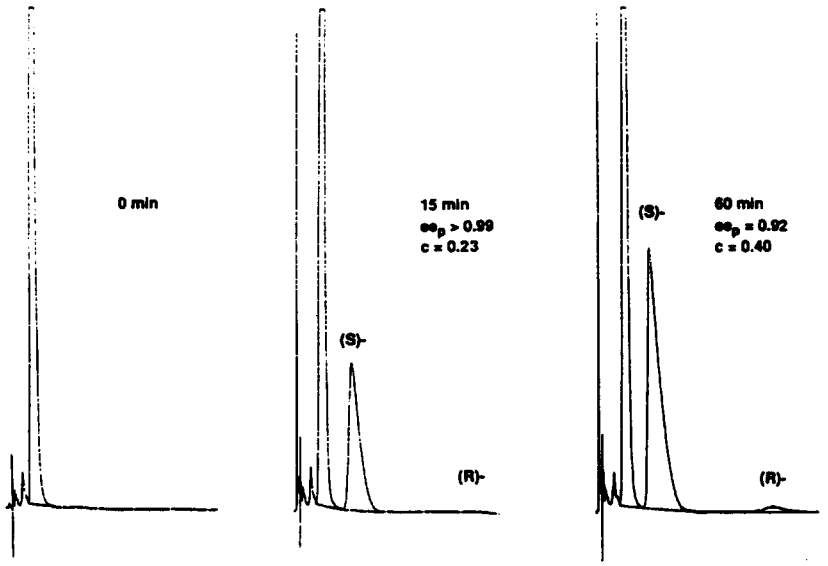



Fig. 8. Analytical chromatograms monitoring the lipase-catalysed hydrolysis of 2-chloroethyl 2-(*p*-isobutylphenyl)propanoate (ibuprofen 2-chloroethyl ester). Column, BSA-silica (Resolvosil), 150 × 4.6 mm I.D.; mobile phase phosphate buffer (20 m*M*, pH 8.0) containing 25% of acetonitrile; flow-rate, 2.0 ml/min; detection at 225 nm. From ref. 37 (© 1992 Harwood Academic Publishers).

CSPs. Recently, Birkinshaw and Taylor [48] reported a new type of chiral sorbent based on cyclic dipeptides synthesized from (*S*)-amino acids containing aromatic or aliphatic side-chains.

### 3.2. Mode of chromatography and retention mechanism

#### 3.2.1. Peptide-based CSPs operated in reversed-phase mode

The analysis of amino acids, peptides and their derivatives is of great importance in the elucidation of the structures of proteins and complex peptides and in the determination of racemization during peptide synthesis. Fong and Grushka [43] and Howard *et al.* [44] investigated in detail the chromatographic properties of an L-Val–L-Ala–L-Pro-bonded stationary phase using water or buffer solutions as mobile phases. A series of isomeric dipeptide solutes were chromatographed in order to characterize their retention behaviour on this tripeptide-bonded stationary phase. The dipeptides eluted in the order of increasing hydrophobicity of the hydrocarbon side-chains of the solutes. Further, the capacity factors of the isomeric dipeptides increased with increasing pH, as shown in Fig. 9.

However, the retention of the dipeptides at high pH, as reported by Fong and Grushka [43], was significantly higher than found by Howard *et al.* [44], even though the carbon contents of both phases were very similar (12.3% [43] and 11.5%

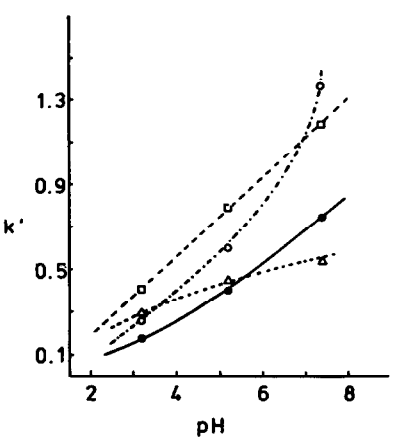



Fig. 9. Effect of pH on the *k'* values of dipeptides in 0.5 *M* McIlvaine buffers: △ = L-Ala–L-Phe; ● = L-Phe–L-Ala; □ = L-Val–L-Phe; ○ = L-Phe–L-Val. Column, L-Val–L-Ala–L-Pro bound to silica, 250 × 3.2 mm I.D.; flow rate, 0.33 ml/min. From ref. 44 (© 1985 American Chemical Society).

[44]). The higher retention was attributed to their being more valine present on the surface of Fong and Grushka's phase, since the stepwise manner in which the tripeptide phase is prepared should lead to the incorporation of a substantial amount of mono- and dipeptide groups, *i.e.*, L-valine and L-Val–L-Ala. The results indicate that the retention mechanism is a combination of electrostatic and hydrophobic interactions. No attempts were made to try to separate enantiomeric peptides of D,L-amino acids.

### *3.2.2. Peptide-based phases operated in normal-phase mode*

It is generally considered that the chiral recognition mechanism is essentially similar in both gas and liquid chromatography. By extending the principle of multiple hydrogen bonding used in the gas chromatographic resolution of enantiomers on *s*-triazine dipeptide and tripeptide ester CSPs [47], Ôi *et al.* [46] showed that some of the *s*-triazine peptide derivatives could be applied to chiral separations in liquid chromatography.

The *s*-triazine derivative of L-Val–L-Val–L-Val isopropyl ester was immobilized on modified silica; the sorbent contained 0.26 mmol of the chiral moiety per gram of support (based on C and N) (Fig. 10). The CSP showed good enantioselectivity for derivatized amino acid enantiomers, giving baseline separations for several N-

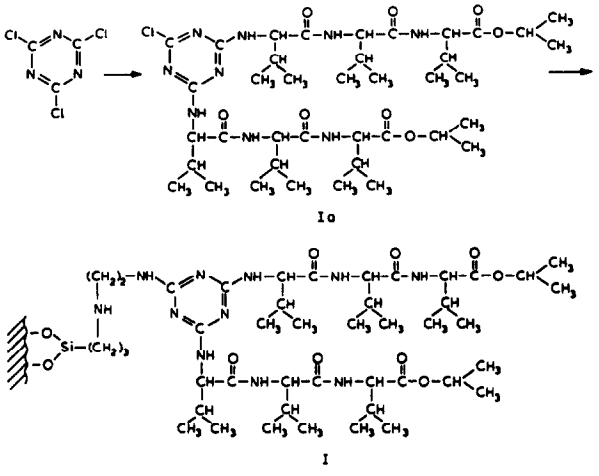


Fig. 10. Synthetic route to the chiral sorbent based on the *s*-triazine derivative of L-Val–L-Val–L-Val isopropyl ester. From ref. 46.

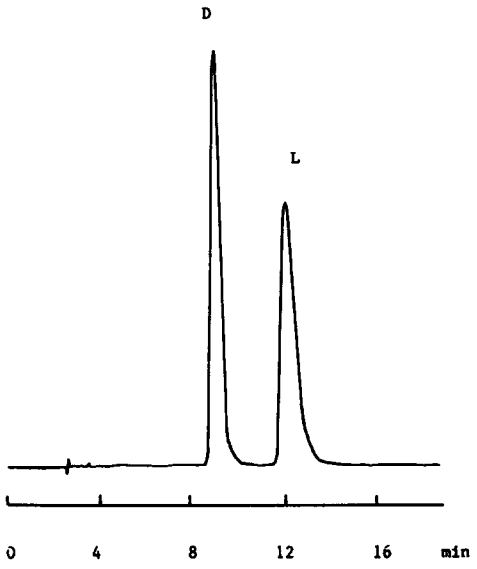


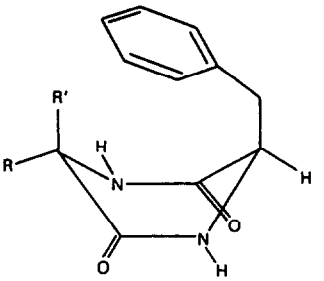

Fig. 11. Chromatographic separation of the enantiomers of the N-3,5-dinitrobenzoylated methyl ester derivative of racemic valine. Mobile phase, hexane–dichloromethane–ethanol (100:20:1); column, silica-bound *s*-triazine derivative of L-Val–L-Val–L-Val isopropyl ester, 250 × 4.0 mm I.D. From ref. 46.

3,5-dinitrobenzoyl methyl ester derivatives in non-aqueous mobile phase systems (hexane–dichloromethane–ethanol mixtures) (Fig. 11).

Hsu *et al.* [45] followed up the work of Fong and Grushka [43] and Howard *et al.* [44]; however, their studies were designed to evaluate the relative contributions of individual amino acids in the tripeptide-bonded stationary phase to separations of optically active compounds. Thus, they constructed chiral stationary phases based on N-*tert.*-butyloxycarbonyl (BOC) derivatives of a single amino acid, a dipeptide or a tripeptide bonded to 4-aminobutyl-derivatized silica and evaluated the phases by comparing the capacity factor, $k'$, and separation factor, $\alpha$, for the enantiomers of (*R*,*S*)-2,2,2-trifluoro-1-(9-anthryl)ethanol (TFAE). It was found that the *R*-enantiomer was the first to elute from all the columns in which the *L*-configuration of the amino acid or dipeptide was bound to silica and the reverse order was found when the opposite amino acid configuration was on the silica surface. Chromatography was performed using hexane containing either 2-propanol or methylene

chloride as the modifier. Generally, an increase in the amount of the polar solvent in the mobile phase decreased the capacity factor; however, the separation factor, $\alpha$, remained essentially constant. The effect of an additional chiral centre was also investigated. The $k'$ values increased with increasing number of amino acids bound to the silica surface. However, addition of the second amino acid had very little influence on $\alpha$, except in the case of BOC–Val–Val columns and with hexane–methylene chloride (80:20) as the mobile phase, as seen from Table 3. Addition of a third chiral centre, even when the amino acid incorporated was valine, had no significant effect on $\alpha$.

A different approach to the construction of peptides as chiral selectors was developed by Birkinshaw and Taylor [48], who prepared chiral sorbents based on cyclic dipeptides or diketopiperazines synthesized from protected (*S*)-amino acids containing aromatic or aliphatic side-chains. Cyclic dipeptides can adopt a number of conformations depending on the nature of the side-chain substituents, the most interesting conformation being that adopted by diketopiperazines containing an aromatic side-chain functionality as shown below:




The required linear peptide precursors were synthesized from O-prop-1-enyl-(*S*)-BOC-tyrosine and (*S*)-leucine methyl ester and from diprop-1-enyl-(*S*)-glutamate and (*S*)-BOC-phenylalanine. Both the protected peptides were then cyclized and the cyclic peptides bonded to silica, either via a free radical reaction (Fig. 12a) or by catalytic addition (Fig. 12b). The CSPs obtained from radically anchored cyclic dipeptides showed a more than 30% higher surface coverage.

The chromatographic evaluation of the CSPs showed that the 3,5-dinitrobenzoyl esters and

TABLE 3

CAPACITY FACTORS, $k'$, AND SEPARATION FACTORS, $\alpha$, OF TFAE ENANTIOMERS ON CHIRAL STATIONARY PHASES

From ref. 45.

| Stationary phase | Mobile phase | | | |
|---|---|---|---|---|
| | Hexane–dichloromethane (80:20) | | Hexane–2-propanol (99:1) | |
| | $k_1'$ [a] | $\alpha$ | $k_1'$ [a] | $\alpha$ |
| BOC-L-Val[a] | 4.03 ± 0.11 | 1.099 ± 0.011 | 2.84 ± 0.03[c] | 1.076 ± 0.010[c] |
| BOC-L-Val-L-Val | 4.61 ± 0.03 | 1.184 ± 0.014 | 7.63 ± 0.14 | 1.097 ± 0.009 |
| BOC-L-Val-L-Ala | 5.44 ± 0.07 | 1.069 ± 0.011 | 4.91 ± 0.08 | 1.065 ± 0.013 |
| BOC-L-Val-D-Ala | 4.62 ± 0.09 | 1.040 ± 0.009 | 3.67 ± 0.07 | 1.038 ± 0.015 |
| BOC-L-Val-L-Phe | 7.38 ± 0.12 | 1.055 ± 0.015 | 2.18 ± 0.03[d] | 1.038 ± 0.015 |
| BOC-D-Val[b] | 5.87 ± 0.10 | 1.082 ± 0.008 | 4.41 ± 0.07 | 1.094 ± 0.012 |
| BOC-D-Val-D-Val | 6.61 ± 0.18 | 1.158 ± 0.035 | 4.13 ± 0.15 | 1.090 ± 0.006 |
| BOC-D-Val-L-Ala | 4.04 ± 0.28 | 1.062 ± 0.015 | 3.27 ± 0.06 | 1.034 ± 0.013 |
| BOC-D-Val-L-Ile | 8.06 ± 0.09 | 1.010 ± 0.006 | 3.75 ± 0.04[c] | 1.011 ± 0.007[c] |

[a] Capacity factor for the first-eluted enantiomer.
[b] Microbore column.
[c] Hexane–2-propanol (98.5:1.5).
[d] Hexane–2-propanol (98:2).

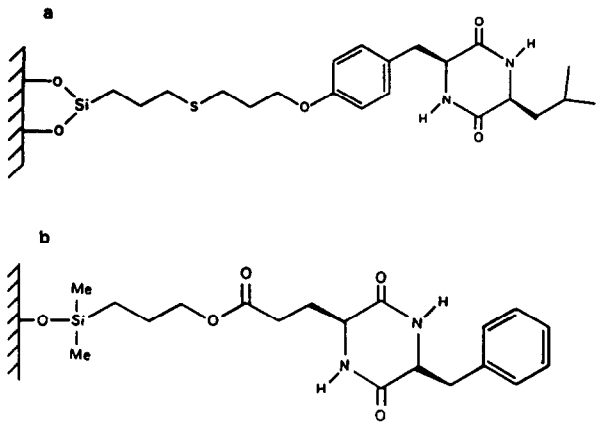



Fig. 12. Structures of chiral sorbents based on dipeptides immobilized on silica (a) via a free radical reaction and (b) by catalytic addition. From ref. 48.

propylamides of various amino acids were separated only on sorbents containing long anchor chains. Further, the incorporation of phenylalanine instead of tyrosine in the immobilized peptide resulted in better peak shapes and resolution, possibly owing to less restriction of the aromatic side-chain in the case of phenylalanine. The racemic 3,5-dinitrobenzoyl propylamides of both aromatic and aliphatic amino acids gave better resolutions than the corresponding esters, suggesting that the extra amide bond may be important in the chiral recognition process.

Hence both the restriction exerted by the anchor chain and the choice of the amino acid residue in the synthesis of the cyclic peptides are factors of importance for the enantioselective properties of these sorbents.

## 4. CONCLUSIONS AND FUTURE PERSPECTIVES

The role of proteins and peptides as components of chiral phase systems continues to grow. Their use is not restricted to liquid chromatographic techniques, but also extends to various types of electrophoretically driven separations. Our knowledge of the mechanisms of retention and enantiodifferentiation is still comparatively rudimentary, however, at least for the longer, folded peptide chains. This means that predictions with regard to selectivity *versus* analyte structure and mobile phase compositions are not yet possible on a rational basis. One could envisage, however, that the enormous developments in the field of polypeptide design and synthesis will soon lead to the point where new polypeptides containing chiral binding sites, constructed to suit a particular racemate to be resolved, can be designed by computer-aided molecular modelling.

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