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Chiral chromatography of amino acids on porous graphitic carbon coated with a series of N-substituted L-phenylalanine selectors Effect of the anchor molecule on enantioselectivity

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Abstract

Six chiral stationary phases were prepared by coating the surface of porous graphitic carbon (PGC) with a series of N-substituted L-phenylalanine chiral selectors. The N-substituents served as anchor molecules for immobilization of chiral selectors on the support material. The effect of the alkyl (C_7, C_9, C_{12}) and aryl (methoxybenzyl, naphthylmethyl, anthrylmethyl) anchor molecules on retention and enantioselectivity was studied using 36 amino acid enantiomers as probe compounds. The surface concentrations of the chiral selectors, determined using the breakthrough method, were found to be in the range 0.55–1.26 μmol/m^2. The coated PGC phases all showed appreciable enantioselectivity for both non-polar and acidic amino acids, but basic amino acids were predominantly unretained. The order of retention of a pair of amino acid enantiomers was L>D on the alkyl-L-phenylalanine phases but a reversed retention order (D>L) was observed on the aryl-substituted L-phenylalanine phases. The chromatographic properties of each of the chiral stationary phases were compared in terms of retention order, enantioselectivity and diastereomeric complex stability. The reversal of the elution order on these two types of phases is discussed in relation to competitive intramolecular interactions involved in transient diasteriomeric complexes.

Keywords: Enantiomer separation; Stationary phases, LC; Porous graphitic carbon; Enantioselectivity; Amino acids

1. Introduction

Chiral ligand-exchange chromatography is a well established method for resolving a variety of racemic mixtures, including amino acids, hydroxy acids, amino alcohols and amines [1,2]. The method is based upon exploiting the differences between the stability constants of diastereomeric pairs. The separation of enantiomers may be accomplished by the formation of transient ternary copper complexes with an optically active amino acid derivative either covalently bonded or permanently adsorbed to a solid support [1]. The chromatographic support used for such separations has typically been chromatographic silica, but recent work has indicated that porous graphitic carbon (PGC) is also a suitable support matrix for chiral ligand-exchange chromatography [3]. PGC possesses a crystalline surface that is essentially free of any ionizable groups [4–6] and may therefore result in fewer secondary interac-

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tions with solutes. Chiral properties can be conferred on PGC by the surface adsorption of an appropriate selector molecule, covalently bonded to a suitable non-polar hydrocarbon anchor molecule. Successful chiral separation of amino acids and hydroxy acids, based upon copper complexation, has been achieved using PGC coated with adsorbed N-(2-naphthalene-sulphonyl)phenylalanine [3].

The enantioselectivity of a chiral ligand-exchange system is known to depend, primarily, upon the structure of the amino acid derivative used, the surface concentration of the chiral selector and the length and type of linkage of the spacer/anchor molecule used to attach the chiral ligand to the support surface [7–10]. Previous studies, using silica supports, have demonstrated the profound effect of the alkyl spacer length on enantioselectivity [7–10]. This suggests that hydrophobic interactions with the spacer molecule play a role in enantioselectivity. However, due to the potential secondary interactions of solutes with residual silanols on the silica support, and the lack of information on the surface coverage of the chiral selector, no firm conclusions were drawn by the authors regarding the effect of spacer chain length on the chiral retention mechanism. We believe that the more predictable reversed-phase interactions observed on PGC [6] will lead to a clearer understanding of the structural aspects of spacer length and type on chiral ligand-exchange interactions, especially with regard to the role of hydrophobic interactions in enantioselectivity.

In this investigation, six chiral stationary phases for the separation of amino acid enantiomers were prepared by coating the surface of PGC with N-substituted L-phenylalanine. The effects on retention and enantioselectivity of a series of different aryl and alkyl anchor molecules were studied using amino acid enantiomers as probe compounds.

2. Experimental

2.1. Apparatus

The chromatographic system consisted of a Gilson 305 pump (Villiers le Bel, France), a Gilson 805 manometric module, a Gilson 231 XL sampling injector, a Gilson 401 diluter and an ABI 759A absorbance detector (Gilson, Foster City, CA, USA) connected to a Gilson HPLC 715 system controller via a Gilson 506B interface. Porous graphitic carbon or "Hypercarb" columns (100×4.6 mm I.D., particle diameter 7 μm, specific surface area 119 m^2/g, batch number 123/R3) were supplied by Hypersil (Runcorn, UK). The same batch of the packing material was used throughout.

2.2. Chemicals

Glycine and the enantiomers and racemates of alanine, norvaline, norleucine, valine, leucine, isoleucine, methionine, proline, serine, threonine, asparagine, glutamine, ornithine, lysine, arginine, aspartic acid and glutamic acid were purchased from Sigma (Poole, UK). Sodium cyanoborohydride, copper(II) acetate (analytical grade), heptyl aldehyde, nonyl aldehyde, dodecyl aldehyde, and 4-methoxybenzaldehyde, 2-naphthaldehyde and 9-anthraldehyde were purchased from Aldrich (Gillingham, UK). Methanol (HPLC grade) was obtained from Fisons (Loughborough, UK). All solvents used as reaction media were of HPLC or analytical grade.

2.3. Synthesis of chiral selectors

Six chiral selectors derived from L-phenylalanine were synthesized by a one-step reductive N-alkylation of the amino acid, as described by Ohfune et al. [11] (Fig. 1). The typical experimental procedure is briefly described here. To a suspension of L-phenylalanine (1.65 g, 10 mmol) and $NaBH_3CN$ (0.44 g, 7 mmol) in methanol (15 ml) was added the appropriate aldehyde (11 mmol) over a 15-min period at room temperature. The reaction mixture was stirred for 18 h at room temperature. The product precipitated during the reaction and was collected on a filter and washed thoroughly with 1 *M* HCl and then with methanol. The product was dried in vacuo at 60°C for 16 h.

Uncorrected melting points were determined using a Gallenkamp melting point apparatus and the data reported were the average of at least three measurements. Optical rotation measurements were made using a NPL Automatic Polarimeter 143C and the results are given in 10^{-1} deg cm^{-2} g^{-1}. Elemental analyses (CHN) were obtained using a Perkin-Elmer

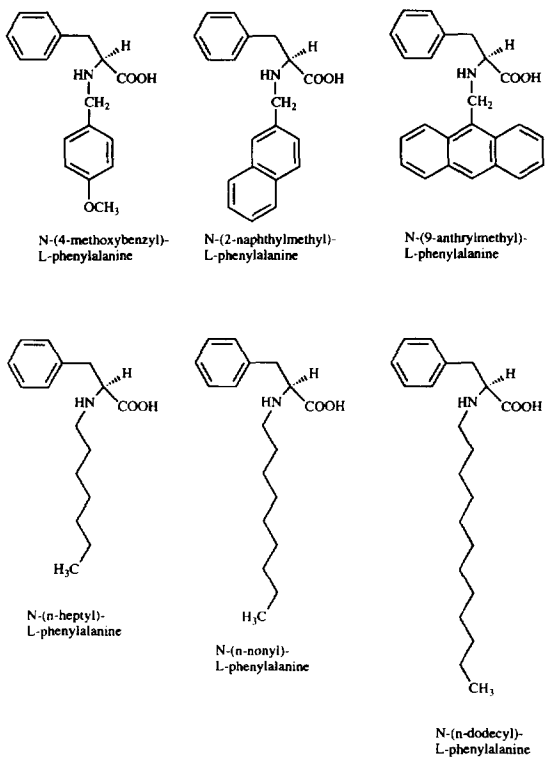

Fig. 1. Chemical structures of the N-substituted phenylalanine chiral selectors.

PE 240B CHN elemental analyzer, at Nottingham University. ^{1}H NMR spectra were recorded where necessary on a Bruker AC 250 at 250 MHz. The sample solution was prepared by dissolving an appropriate amount of the N-substituted amino acid sample in 0.5 ml of [$^2H_6$]dimethyl sulfoxide (DMSO-d_6). Chemical shifts are reported in parts per million (δ).

N-(*n*-Heptyl)-L-phenylalanine (C_7-L-Phe): m.p. 228°C; $[\alpha]_D$+19.8° (c=0.5, 50% methanol–1 *M* NaOH). $C_{16}H_{25}NO_2$ (263.38) requires: C, 72.96%; H, 9.49%; N, 5.32%; found: C, 73.09%; H, 9.82%; N, 5.18%.

N-(*n*-Nonyl)-L-phenylalanine (C_9-L-Phe): m.p. 227°C; $[\alpha]_D$+8.0° (c=0.5, 50% methanol–1 *M* NaOH). $C_{18}H_{29}NO_2$ (291.43) requires: C, 74.18%; H, 10.03%; N, 4.81%; found: C, 73.39%; H, 10.07%; N, 4.35%.

N-(*n*-Dodecyl)-L-phenylalanine (C_{12}-L-Phe): m.p. 207°C; $[\alpha]_D$−190.6° (c=0.5, 50% methanol–1 *M* NaOH). $C_{21}H_{35}NO_2$ (333.51) requires: C, 75.63%; H, 10.58%; N, 4.23%; found: C, 75.43%; H, 10.36%; N, 4.09%.

N-(4-Methoxybenzyl)-L-phenylalanine (MB-L-Phe): m.p. 232°C; $[\alpha]_D$+10.0° (c=0.5, 50% methanol–1 *M* NaOH). $C_{17}H_{18}NO_3$ (285.34) requires C, 71.56%; H, 6.71%; N, 4.91%; found: C, 70.98%; H, 6.72%; N, 4.70%.

N-(2-Naphthylmethyl)-L-phenylalanine (NA-L-Phe): m.p. 227°C; $[\alpha]_D$−16.0° (c=0.5, 50% methanol–1 *M* NaOH). ^{1}H NMR (DMSO-d_6) δ 2.80–3.10 (m, β-CH_2), 3.90 (d, d, J=13.6, 22.6 Hz, ArCH_2), 7.29 (m, PhH_5), 7.51 (m, ArH_3), 7.70 (s, ArH), 7.80–7.92 (m, ArH_3). $C_{20}H_{19}NO_2$ (305.37) requires: C, 78.66%; H, 6.27%; N, 4.61%; found: C, 78.10%; H, 6.21%; N, 4.25%.

N-(9-Anthrylmethyl)-L-phenylalanine (AN-L-Phe): m.p. 226°C; $[\alpha]_D$−14.2° (c=0.5, 50% methanol–1 *M* NaOH). ^{1}H NMR (DMSO-d_6) δ 3.00 (m, β-CH_2), 3.80 (s, α-CH), 4.60 (d, d, ArCH_2), 7.23 (s, PhH_5),7.50 (m, ArH_4), 8.06 (m, ArH_2), 8.28 (m, ArH_2), 8.55 (s, ArH). $C_{24}H_{21}NO_2$ (355.43) requires: C, 81.10%; H, 5.95%; N, 3.94%; found: C, 80.25%; H, 5.69%; N, 3.68%.

2.4. Coating procedures and surface coverage calculations

The coating solution was prepared by dissolving an appropriate amount of chiral selector in a methanol–1.0 *M* aqueous NaOH (1:1, v/v) mixture. To achieve complete dissolution of the chiral selector, the concentration of the chiral selector and the proportion of methanol were varied depending upon the anticipated properties of the chiral selector. Details of the solutions used to coat the PGC columns and the surface coverage obtained are given in Table 1.

The surface concentration (C_s) of the chiral selector coated onto the PGC column from an aqueous solution was determined by the breakthrough method [12]. The PGC column was washed with analytical grade methanol before the coating procedure was performed to ensure that the support surface was clean and the solution contained no UV-absorbing impurities. The solution containing the N-substituted L-phenylalanine at concentration C_m was passed

Table 1
Details of coating solutions and surface coverage

Chiral selector	Concentration used for coating[a] (M)	Surface coverage ($\mu mol/m^2$)
C_7-L-Phe	0.025	0.62
C_9-L-Phe	0.010	0.79
C_{12}-L-Phe	0.005[b]	0.55
MB-L-Phe	0.010	0.94
NA-L-Phe	0.010	1.26
AN-L-Phe	0.010	0.92

[a] In aqueous 1.0 M NaOH–methanol (50:50, v/v).
[b] In aqueous 1.0 M NaOH–methanol (25:75, v/v)

through the PGC column at a flow-rate of 1 ml/min and any change in the absorbance of the eluent was followed by UV detection. Equilibrium in the coating process was indicated by an abrupt rise in the UV baseline. This breakthrough volume (V), which is the volume of the solution (concentration C_m) eluted when the abrupt rise in baseline occurs, was measured from the collected eluent or from the recorded trace of the breakthrough curve. The surface concentration (C_s) of the packing can readily be calculated using the following mass balance equation, where A is the surface area of the packing material:

$$C_s = VC_m/A$$

After the passage of about 10 ml of coating solution through the column, following the observation of the breakthrough curve, distilled water was used to displace the methanolic solution within the column. This was followed by an aqueous solution of copper(II) acetate (0.001 M). The column was then ready for sample injection, once equilibrium between the stationary and mobile phase was attained, which was normally within 30 min.

2.5. Chromatographic measurements

An aqueous solution of copper(II) acetate (0.001 M, pH 5.6) was used as the eluent in all experiments. All separations were performed at ambient temperature with a flow-rate of 1 ml/min and UV detection was at 254 nm. The solute solutions were prepared by dissolving the amino acid enantiomers and racemates in distilled water to give a concentration of 10–100 μg/ml. Injections of 1–10 μl of these analytes produced satisfactory chromatographic peaks. The elution order of a pair of enantiomers was determined by injecting each enantiomer individually. The mobile phase hold-up time, t_M, was taken as the time from injection to the occurrence of the first solvent disturbance peak. The mean retention factors, k_D and k_L for D- and L-isomers, and separation factors, α, defined as the ratio k_D/k_L, were calculated from multiple injections of the racemates. The durability of a PGC column coated with MB-L-Phe under normal chromatographic conditions was investigated. A 5000-ml volume of HPLC mobile phase (equivalent to 3500 column volumes) was pumped through the column and the variation in the retention factors of proline and leucine were monitored.

2.6. Calculation of ligand binding constants

The process responsible for the separation of two enantiomers in ligand-exchange chromatography is the transient formation of transition metal ion complexes, with the chiral selector bonded or adsorbed onto a solid support. Previous investigation of the retention mechanism in chiral ligand-exchange chromatography has demonstrated that retention is inversely proportional to the concentration of copper(II) acetate in the mobile phase, with some deviations in the very low concentration region [3]. This suggests that, at a moderate concentration of copper acetate, almost complete formation of binary copper ion complexes is achieved, both for chiral selectors (S) and enantiomeric analytes (A). Thus, the process may be expressed by the following ligand-exchange equilibrium (where ionic charges are omitted for clarity):

$$CuS + CuA = CuSA + Cu$$
$$K = ([CuSA][Cu])/([CuS][CuA])$$

The equilibrium constant, K, is a measure of the stability of a ternary complex system, as the value of K reflects the coordination tendency of the ligand A for the binary complex CuA on the surface of the support, relative to the copper(II) ion in the mobile phase. The value of K can be calculated from the corresponding retention capacity, k, according to the equation below:

$$K = (k[\mathrm{Cu}])/(\emptyset Q) \quad (1)$$

where $\emptyset$ is the ratio of the surface area of the stationary phase to the void volume within the column and Q is the surface concentration of the chiral selector, Q. The value of $\emptyset$ can be calculated to be $0.85 \cdot 10^{-8}$ m^{-1} using the method of Knox and Wan [13], by dividing the surface area (119 m^2/g for PGC) of the packing material by the void volume of the 100×4.6 mm HPLC column used in these studies.

Enantioselectivity, α, for a pair of enantiomers, D and L, is defined as

$$\alpha = K_D/K_L \quad (2)$$

$$= k_D/k_L \quad (3)$$

It is relevant to note that, unlike the retention factor, both the stability constant and the enantioselectivity are independent of the surface concentration of the chiral selector, provided that retention is solely controlled by the ligand-exchange process described above. However, it should be noted that the equations above assume that the graphite surface of PGC plays no role in the chromatographic enantioselectivity. The equations given above furnish a basis for comparison of retention behaviours of amino acid enantiomers on chiral stationary phases of differing chiral selectors and surface concentrations.

3. Results and discussion

3.1. Stability of adsorbed chiral selectors

The durability of the columns under normal chromatographic conditions was established in a study of the variation of retention capacity with the volume of eluent passed through the column. As shown in Fig. 2, the values of k for proline and leucine enantiomers were essentially unchanged after the passage of up to 5000 ml of the eluent. The excellent stability of the chiral phases is believed to arise from the strong affinity of the chiral selector for the graphite surface as well as the inert nature of the graphitic carbon support.

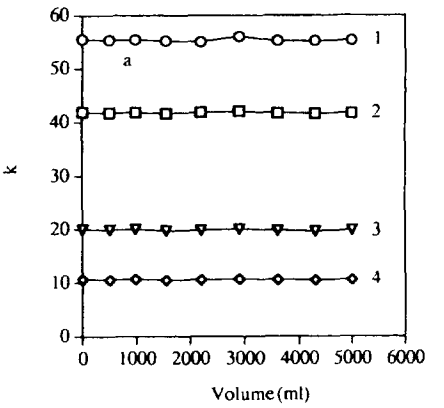

Fig. 2. Stability of a PGC column coated with methoxybenzyl-L-phenylalanine, as indicated by the effect of the volume of the mobile phase on the retention factors of (1) D-leucine, (2) L-leucine, (3) L-proline and (4) D-proline. Chromatographic conditions were as indicated in Section 2.

3.2. Surface concentration of the substituted L-phenylalanine selectors

The surface concentration of the chiral selector coated on PGC was determined by the breakthrough method and found to range between 0.55 and 1.26 μmol/m^2 (Table 1). As expected, the surface concentrations of the aryl-L-phenylalanines were considerably higher than those of alkyl-L-phenylalanines, confirming an earlier observation that aryl compounds serve well as anchor molecules on the surface of graphitic carbon [3]. Previous work with N-(2-naphthalenesulphonyl [3] and 17*H*-tetrabenzo[*a*,*c*,*g*,*i*])fluorene [14] as anchor molecules linked to chiral selectors indicated that the monolayer surface coverages of these compounds on PGC were approximately 1.4 and 0.4 μmol/m^2, respectively. The latter compound has a significantly larger molecular size than naphthalene and hence would be expected to have a lower monolayer surface coverage. The results reported here indicate that the alkyl-L-Phe compounds, with surface coverages of less than 1.0 μmol/m^2, are unlikely to have reached monolayer coverage under the conditions used for coating. An improved surface coverage by the alkyl-L-Phe may be anticipated by increasing the concentration of the compounds in the coating solution or by increasing the alkyl chain length.

Table 2
Retention and enantioselectivity of amino acids on N-substituted L-phenylalanine-coated porous graphitic carbon

Analyte	*R*-CH(NH2)COOH	C_7-L-Phe			C_9-L-Phe			C_{12}-L-Phe			MB-L-Phe			NA-L-Phe			AN-L-Phe		
	R-...	k_L	k_D	α	k_L	k_D	α	k_L	k_D	α	k_L	k_D		k_L	k_D	α	k_L	k_D	α
Non-polar																			
Gly	H-...	0.75		–	0.76		–	0.55		–	1.68			1.28		–	9.71		–
Ala	CH_3-...	1.46	0.91	0.62	1.31	0.91	0.69	0.85	0.68	0.80	2.75	3.21	1.17	1.95	2.33	1.19	7.98	9.88	1.24
Nval	$CH_3(CH_2)_2$-	8.46	6.23	0.74	7.63	5.50	0.72	5.88	4.23	0.72	20.53	25.78	1.26	14.39	19.91	1.38	24.46	27.36	1.12
Nleu	$CH_3(CH_2)_3$-	27.78	22.94	0.85	25.01	19.73	0.79	18.53	14.21	0.77	60.48	70.43	1.16	46.07	55.88	1.21	56.14	59.0	1.05
Val	$(CH_3)_2CH$-	5.62	5.18	0.92	4.93	4.56	0.92	3.81	3.47	0.91	16.87	15.29	0.91	8.48	9.93	1.17	13.88	17.30	1.25
Leu	$(CH_3)_2CHCH_2$-	18.16	9.81	0.54	15.51	9.37	0.60	11.36	7.04	0.62	41.74	55.32	1.32	30.68	46.52	1.52	41.25	59.31	1.44
Ile	$CH_3CH_2CH(CH_3)$-	16.34	11.31	0.69	14.13	10.25	0.72	10.53	7.47	0.71	30.59	34.09	1.11	23.13	26.61	1.15	32.64	22.23	0.68
Met	$CH_3S(CH_2)_2$-	24.58	19.61	0.80	20.77	16.88	0.81	15.44	12.71	0.82	53.31	58.08	1.09	42.30	49.08	1.16	65.23	73.98	1.13
Pro	-$(CH_2)_3$-	14.71	4.66	0.32	12.13	4.47	0.37	8.12	3.19	0.39	19.92	10.60	0.53	9.37	9.81	1.17	13.32	21.51	1.62
Polar																			
Ser	$HOCH_2$-	0.87	0.87	1.00	0.86	0.86	1.00	0.65	0.63	0.97	2.54	3.25	1.28	1.63	2.21	1.35	8.54	11.95	1.40
Thr	$CH_3CH(OH)$-	0.91	1.57	1.72	0.96	1.36	1.42	0.71	0.86	1.21	3.31	4.21	1.27	2.52	2.89	1.15	11.12	13.98	1.26
Asn	H_2NCOCH_2-	7.71	1.76	0.23	8.71	1.49	0.17	11.46	1.13	0.10	3.52	4.28	1.21	2.44	2.83	1.16	10.41	12.21	1.17
Gln	$H_2NCO(CH_2)_2$-	4.60	3.51	0.76	3.88	2.86	0.71	3.29	2.41	0.73	10.14	12.96	1.28	7.16	9.19	1.28	19.20	22.71	1.18
Ionizable																			
Orn	$H_2N(CH_2)_3$-	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.06	0.06	1.00	0.07	0.07	1.00	1.54	2.11	1.37
Lys	$H_2N(CH_2)_4$-	0.09	0.09	1.00	0.08	0.08	1.00	0.09	0.09	1.00	0.27	0.34	1.27	0.27	0.33	1.22	1.83	2.32	1.27
Arg	$H_2NC(NH)NH(CH_2)_3$-	2.37	4.00	1.69	1.91	3.18	1.66	0.87	1.38	1.59	6.01	6.69	1.11	2.92	2.92	1.00	1.18	4.25	3.60
Asp	$HOOCCH_2$-	8.35	8.02	0.96	9.00	8.40	0.93	10.76	11.01	1.02	29.55	30.18	1.02	17.60	19.20	1.09	70.43	84.71	1.20
Glu	$HOOC(CH_2)_2$-	23.01	17.53	0.76	21.87	19.26	0.88	25.84	22.32	0.86	66.86	77.57	1.16	51.20	62.27	1.23	141.86	184.71	1.30

Conditions: column, PGC coated with N-substituted L-phenylalanine, 100×4.6 mm I.D.; eluent, 0.001 *M* $Cu(OAc)_2$ aqueous solution; flow-rate, 1 ml/min; detection, UV at 254 nm.

3.3. Retention and enantioselectivity

The PGC columns coated with N-substituted L-phenylalanines were used to separate a series of amino acid enantiomers using a 0.001 *M* aqueous solution of copper(II) acetate as the eluent. The amino acid analytes were classified according to the nature of their side-chains as non-polar, polar and ionizable. Table 2 shows the retention and separation factors obtained on the columns coated with C_7-L-Phe, C_9-L-Phe, C_{12}-L-Phe, MB-L-Phe, NA-L-Phe and AN-L-Phe, respectively. All of the chiral phases showed appreciable enantioselectivity for both the non-polar and acidic amino acids examined here, with selectivity factors ranging between 1 and 10. However, basic amino acids were predominantly unretained. Typical examples of the chiral separations are shown in Fig. 3. These results confirm that the ability of L-phenylalanine to form copper(II) complexes is retained upon transformation of its primary amine group to a secondary amine, caused by the attachment of the anchor molecule. A number of important trends in terms of chiral selectivity and order of retention were obtained from the data and these are discussed below.

3.3.1. Anchor molecule effects

Fundamental differences in retention and selectivity behaviour towards the amino acid solutes exist between the alkyl- and aryl-substituted chiral phases. Firstly, the elution orders on columns coated with alkyl-L-Phe were consistently reversed in comparison to those on columns coated with aryl-L-Phe. For example, the order of retention for amino acids on the alkyl phases C_7-L-Phe, C_9-L-Phe and C_{12}-L-Phe was L>D, with the exceptions of threonine and arginine. However, a reversed retention order, D>L, was observed on the aryl phases, MB-L-Phe, NA-L-Phe and AN-L-Phe, with the notable exceptions of proline on MB-L-Phe and isoleucine on AN-L-Phe. Secondly, as the chain length of the alkyl group was increased, the majority of the analytes underwent a consistent decrease in retention whereas the separation factor remained relatively unchanged. However, on the aryl-substituted phases, there was a slight reduction in retention when the methoxybenzyl group is compared with the naphthylmethyl group, but a large increase in retention was observed with the anthrylmethyl group. The separation factors of the enantiomers on the aryl anchors also remained relatively unchanged, with certain exceptions such as proline, arginine and isoleucine. Thus, the type of anchor molecule has a substantial effect on enantioselectivity.

Previous studies have also demonstrated the profound effect of the nature of the spacer molecule on enantioselectivity, suggesting a role for hydrophobic interactions between the spacer and the solute in chiral recognition. Davankov et al. [2] and Roumeliotis et al. [8–10] found that the retention of amino acid enantiomers on an N-alkyl-L-hydroxyproline bonded silica chiral stationary phase increased with the chain length of the alkyl spacer molecule. Enantioselectivity was also observed to change as the spacer chain length increased, and a reversal of the retention order was observed for a number of D/L amino acid enantiomers, suggesting a role of hydrophobic interactions with the alkyl spacer in chiral recognition. However, these studies did not determine the surface coverage of the chiral selector and hence it was not possible to draw a firm conclusion regarding the effect of spacer chain length on the chiral retention mechanism.

Our results on PGC support these previous observations with silica-based supports [3,8–10], and provide further evidence for a substantial involvement of the spacer/anchor molecule in the mechanism of enantioselectivity by copper complexation.

3.3.2. Solute effects

For non-polar amino acids, the retention increased with the carbon number in the side chain of the amino acid on all of the chiral columns, pointing to the possibility that strong intermolecular interactions between the chiral selector and the side chain of the analyte are involved in controlling solute retention. The positively charged amino acids (ornithine, lysine) were least retained, suggesting a weak or absent intermolecular interaction between these amino acids and the chiral selector. However, the negatively charged amino acids (aspartic and glutamic acids) were strongly retained, with retention factors that were comparable to those obtained with the corresponding non-polar amino acids, such as

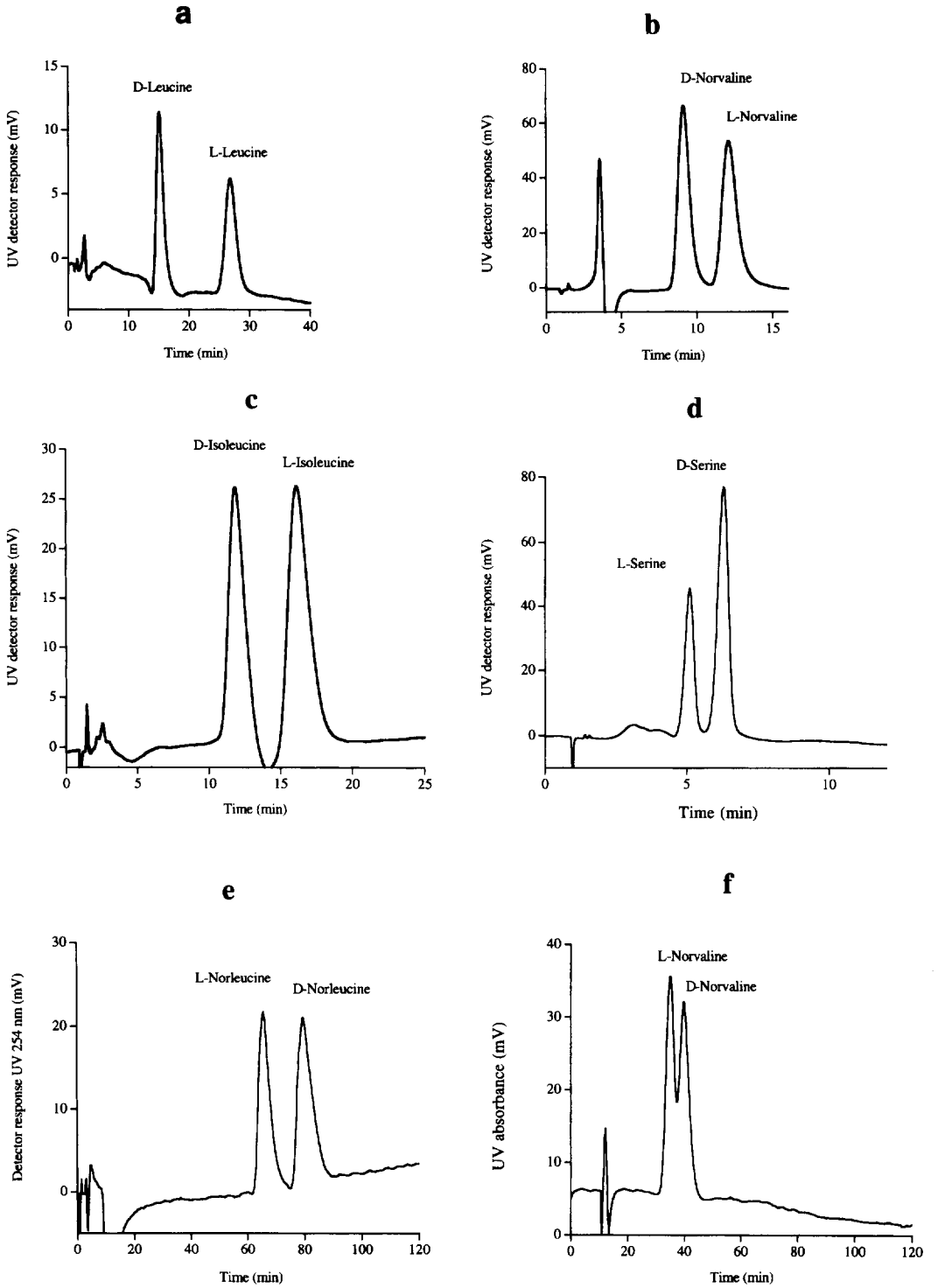

Fig. 3. Chromatograms of amino acid enantiomers separated on porous graphitic carbon coated with N-substituted-L-phenylalanine. (a) C_7-L-Phe, (b) C_9-L-Phe, (c) C_{12}-L-Phe, (d) MB-L-Phe, (e) NA-L-Phe and (f) AN-L-Phe. Chromatographic conditions were as indicated in Section 2.

norvaline. It should be noted that at the mobile phase pH of 5.6, it would be expected that both the carboxylic acid groups of the acidic amino acids and the amino groups of the basic amino acids would be ionized.

It is interesting to note that the retention of polar amino acids that are capable of forming tridentate complexes with copper ions, such as serine and threonine, was not significantly greater than that for the corresponding non-polar amino acids, such as alanine and valine. This suggests that the formation of tridentate complexes alone does not necessarily make a significant contribution to the retention but may have a profound effect on enantioselectivity. This was shown in the case of threonine where the L-enantiomer was eluted before the D-enantiomer on the alkyl-L-Phe-coated phases, in contrast to the reversal of retention order observed for the other amino acid enantiomers.

Roumeliotis et al. [8–10], using L-hydroxyproline-bonded C_1 silica chiral stationary phases, found that basic and hydrophobic amino acids showed the largest retention while acidic amino acids showed the smallest retention, which is the opposite to the retention order that we observed on our PGC chiral phases. However, with a C_8 spacer molecule, the hydrophobic amino acids exhibited the greatest retention while the basic amino acids were eluted with much weaker retention, in agreement with our results. Roumeliotis et al. [8–10] suggested that on the C_1 silica, basic amino acids may be engaged in an ion-exchange process with the ionised surface silanol groups, whereas on C_8 silica the alkyl chains cover the surface with such a dense layer that residual silanols become inaccessible and, therefore, the ion-exchange process becomes virtually absent. Such ion-exchange interactions are essentially absent from the PGC surface, making it easier to interpret the chiral retention data.

3.4. Complex stability

To interpret the retention behaviour in quantitative terms, it is necessary to make a correction for the differences in surface coverage between the six chiral selectors used in this study. This can be achieved by calculating the apparent stability constants (K) of the ternary complexes using Eq. (1). The logarithm of the apparent stability constants (log K) are listed in Table 3 for all of the anchor molecules. For glycine and amino acids with n-alkyl side chains (Ala, Nval, Nleu), the value of log K increases linearly with increasing carbon number of the side chain of the amino acid (Fig. 4). A similar relationship is also observed for two of the aryl anchor molecules (MB-L-Phe and NA-L-Phe), but AN-L-Phe behaves in a markedly different manner, especially with low carbon number amino acid side chains (Fig. 5). For amino acids with a branched alkyl side chain (Val, Leu, Ile), the log K values are generally smaller than those for the corresponding amino acids with n-alkyl chains, and this may be explained in terms of steric effects on the complex formation.

The observation of increased stability and selectivity with carbon number in the amino acid side chain suggests a role for hydrophobic interactions in enantiomer recognition in chiral ligand-exchange chromatography. The stability of copper–phenylalanine complexes with glycine, alanine, aminobutyric acid and norvaline in aqueous solutions has been investigated previously [15,16], and a slight increase ($\Delta\log K<0.04$ per carbon) in the stability constant with the carbon number was reported. The authors attributed this to hydrophobic interactions between the non-polar side chains in mixed ligand amino acid complexes. The increases in log K with amino acid side-chain length observed in this work are more substantial ($\Delta\log K>0.2$ per carbon) than those previously reported, suggesting a significant hydrophobic interaction of the N-substituent in stabilizing the ternary complex. From the above discussion, it becomes evident that the spacer attached to the amine in the chiral selector may have much greater influence on enantioselectivity than was previously believed.

3.5. Role of the anchor molecule in chiral recognition

N-Substituted amino acid enantiomers are widely used as chiral selectors in the chiral chromatography of amino acids, hydroxy acids and aminoalcohols, but the role of the N-substituent in chiral recognition remains unclear. Our experimental data suggest that the nature of the N-substituent of the anchor mole-

Table 3
Stability constants of copper complexes with amino acids determined on L-phenylalanine-based chiral stationary phases

Analyte	*R*-CH(NH2)COOH	C_7-L-Phe		C_9-L-Phe		C_{12}-L-Phe		MB-L-Phe		NA-L-Phe		AN-L-PHE	
	R-	Log K_L	Log K_D	Log K_L	Log K_D	Log K_L	Log K_D	Log K_L	Log K_D	Log K_L	Log K_D	Log K_L	Log K_D
Non-polar													
Gly	H-	−1.85		−1.95		−1.93		−1.67		−1.92		−0.90	
Ala	CH_3-	−1.56	−1.77	−1.71	−1.87	−1.74	−1.84	−1.46	−1.40	−1.74	−1.66	−0.99	−0.90
Nval	$CH_3(CH_2)_2$-	−0.80	−0.93	−0.94	−1.09	−0.90	−1.04	−0.59	−0.49	−0.87	−0.73	−0.50	−0.46
Nleu	$CH_3(CH_2)_3$-	−0.28	−0.36	−0.43	−0.53	−0.40	−0.52	−0.12	−0.05	−0.37	−0.28	−0.14	−0.12
Val	$(CH_3)_2CH$-	−0.97	−1.01	−1.13	−1.17	−1.09	−1.13	−0.67	−0.72	−1.10	−1.03	−0.75	−0.65
Leu	$(CH_3)_2CHCH_2$-	−0.47	−0.73	−0.64	−0.85	−0.61	−0.82	−0.28	−0.16	−0.54	−0.36	−0.28	−0.12
Ile	$CH_3CH_2CH(CH_3)$-	−0.51	−0.67	−0.68	−0.82	−0.65	−0.80	−0.42	−0.37	−0.66	−0.60	−0.38	−0.54
Met	$CH_3S(CH_2)_2$-	−0.33	−0.37	−0.51	−0.60	−0.48	−0.56	−0.18	−0.14	−0.40	−0.34	−0.08	−0.02
Pro	-$(CH_2)_3$-	−0.56	−1.06	−0.74	−1.18	−0.76	−1.16	−0.60	−0.88	−1.06	−1.04	−0.77	−0.56
Polar													
Ser	$HOCH_2$−	−1.78	−1.78	−1.89	−1.89	−1.85	−1.87	−1.90	−1.39	−1.82	−1.68	−0.96	−0.81
Thr	$CH_3CH(OH)$-	−1.77	−1.53	−1.84	−1.69	−1.82	−1.73	−1.38	−1.28	−1.63	−1.57	−0.85	−0.75
Asn	H_2NCOCH_2-	−0.84	−1.48	−0.89	−1.65	−0.61	−1.62	−1.36	−1.27	−1.64	−1.58	−0.87	−0.81
Gln	$H_2NCO(CH_2)_2$-	−1.06	−1.18	−1.24	−1.37	−1.15	−1.29	−0.90	−0.79	−1.17	−1.07	−0.61	−0.54
Ionizable													
Orn	$H_2N(CH_2)_3$-	–	–	–	–	–	–	−3.12	−3.12	−3.18	−3.18	−1.70	−1.57
Lys	$H_2N(CH_2)_4$-	−2.77	−2.77	−2.92	−2.92	−2.71	−2.71	−2.47	−2.37	−2.60	−2.51	−1.63	−1.53
Arg	$H_2NC(NH)NH(CH_2)_3$-	−1.35	−1.12	−1.55	−1.32	−1.73	−1.53	−1.12	−1.08	−1.56	−1.56	−1.82	−1.26
Asp	$HOOCCH_2$-	−0.80	−0.82	−0.87	−0.90	−0.64	−0.63	−0.43	−0.42	−0.78	−0.75	−0.04	+0.03
Glu	$HOOC(CH_2)_2$-	−0.36	−0.33	−0.49	−0.54	−0.26	−0.32	−0.08	−0.01	−0.32	−0.23	+0.26	+0.37

Conditions: column, PGC coated with N-substituted L-phenylalanine, 100×4.6 mm ID; eluent, 0.001 *M* $Cu(OAc)_2$ aqueous solution; flow-rate, 1 ml/min, detection, 254 nm.

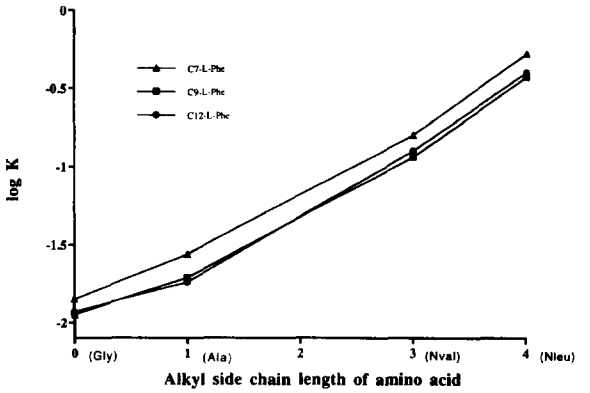

Fig. 4. Log stability constant (K) versus side-chain length of straight chain amino acids on alkyl-L-Phe phases.

cule has a major influence on enantioselectivity when phenylalanine is used as the chiral selector, and that hydrophobic interactions between the anchor molecule and the enantiomer form a critical aspect of the chiral separation mechanism.

It has been established that a mixed ligand copper(II) complex with optically active amino acids can exist in *cis*- and/or *trans*-isomers, depending upon the nature of the side chains [17]. If the two amino acid enantiomers are of the same stereochemical configuration, it follows that both amino acid side chains will be on the same side of the complex in the *trans*-complex, but on opposite sides in the *cis*-complex. In the heterochiral complex, the reverse will be true. It is evident that there are four structural possibilities for mixed ligand complexes containing one fixed amino acid selector (L-enantiomer) and two D and L amino acid analytes (Fig. 6), making the rationale of structural effects difficult. According to Davankov et al. [1,2], a differential distribution of *cis*- and *trans*-isomers is the key to understanding the enantioselectivity for a pair of amino acid enantiomers.

Under normal conditions, the *trans*-isomer is more thermodynamically stable, but the distribution equilibrium of *cis*- and *trans*-isomers can be altered significantly by side chain–side chain interactions or axial binding of the side chain to the central metal ion [15,17]. For example, hydrophobic side chains tend to locate on the same side of the complex or even "stack" one above the other due to intermolecular ligand–ligand hydrophobic interaction. Hydrophobic interactions are known to occur between two aliphatic groups, between two aryl rings (usually referred to as ring stacking), or between aliphatic groups and aryl rings. Axial binding occurs

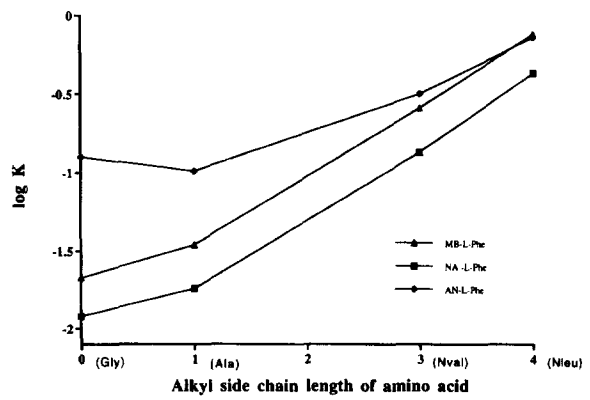

Fig. 5. Log stability constant (K) versus side-chain length of straight chain amino acids on aromatic-L-Phe phases.

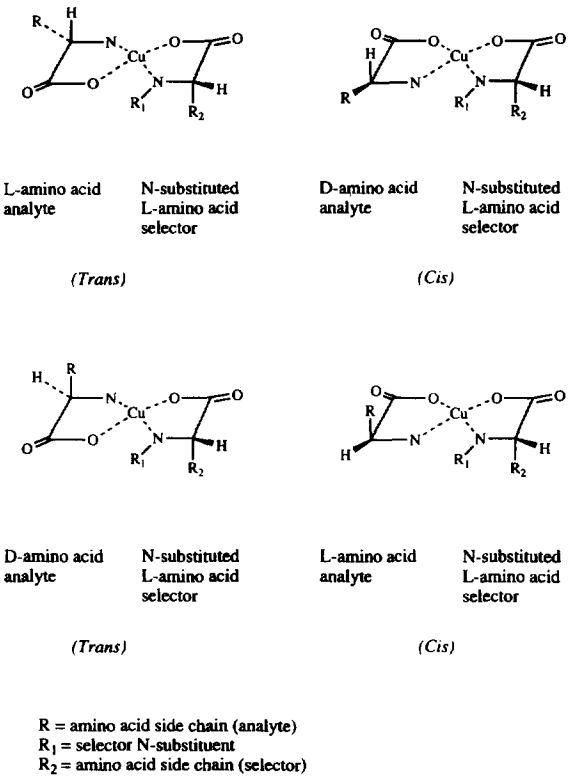

Fig. 6. *trans*- and *cis*-Isomers of ternary copper complexes with D- or L-amino acid analytes.

mainly with groups capable of forming copper(II) complexes in the amino acid side chains, such as hydroxy in serine and threonine, and amide carbonyl in asparagine and glutamine.

In the case of alkyl- and aryl N-substituted phenylalanine used in our studies, the number of possible isomers increases considerably due to the fact that co-ordination of an N-substituted amino acid introduces an extra asymmetric centre. Since the amino acid analyte side chain and selector N-substituent both have considerable hydrophobic character, it is likely that intermolecular hydrophobic interactions favour the isomers with both the amino acid analyte side chain and the N-substituent on the same side of the complex. The reversal of the retention order that is observed when the anchor molecule changes from an alkyl to an aryl group indicates that the intermolecular interactions responsible for chiral recognition are not the same on these two types of stationary phases. A detailed analysis of the structure of the ternary copper(II) complexes formed between chiral selectors and analytes is required to understand this phenomenon. Because of the lack of direct structural evidence, it is necessary to make a number of assumptions to facilitate a discussion on the probable structures of the complex:

(1) The preferred conformation of an N-substituted L-phenylalanine is that the substituent is on the side of the complex plane opposite to that of the phenyl group of the chiral selector. This is due to the restriction of the bulky groups on rotation.

(2) The *trans*-(N, O) isomer of the copper complex is more stable than the *cis*-(N, O) isomer, as reported previously in the case of copper(II)–glycine complexes [18].

(3) Hydrophobic interactions between aliphatic and aryl moieties offer greater complex stability than those between aliphatic groups alone [19].

Taking these assumptions into consideration, we propose that the dominant structures for the copper(II) complexes between alkyl- or aryl-substituted L-phenylalanine selectors and D- or L-amino acid enantiomers are as indicated in Fig. 7. It is evident that the side chain of the D- or L-amino acid analyte can engage in intermolecular hydrophobic interactions with either the anchor molecule attached to the amino group or with the phenyl group of the L-phenylalanine selector. These competitive interactions will determine the retention order. Since the

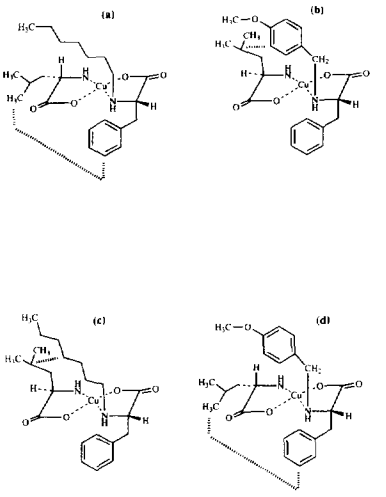

Fig. 7. Proposed copper complexes formed between: (a) L-leucine (analyte) and N-alkyl L-phenylalanine (selector), (b) D-leucine (analyte) and N-aryl L-phenylalanine (selector), (c) D-leucine (analyte) and N-alkyl L-phenylalanine (selector) and (d) L-leucine (analyte) and N-aryl L-phenylalanine (selector). Dotted lines indicate potential hydrophobic interactions which stabilize the complexes and thereby influence enantioselectivity.

anchor molecule and the phenyl group are on opposite sides, the intermolecular interactions will result in two distinct retention orders corresponding with the two distinct spatial orientations. If the interaction of an analyte with the phenyl group of L-phenylalanine is more important than the interaction with the anchor molecule, then the retention order will be L>D; however, if the interaction of the analyte with the anchor molecule is dominant, then the retention order will be reversed i.e. D>L.

The orientation of the chiral selector on the surface of the non-chiral graphite support surface and the possible interactions of the analyte with the underlying surface are important considerations in the mechanism of chiral recognition [20,21]. In keeping with our assumption that the benzyl side group and the N-substituent are on opposite sides of the coordination plane, there are two possible orientations for the N-substituted L-phenylalanine adsorbed on the surface of graphite; one is through the benzyl side group and the other through the N-substituent. The hydrophobic surface of graphite can then be viewed as an extension of the benzyl side group or the N-substituent, depending on competitive interactions of the surface with these two hydrophobic groups in the selector. It appears that

the magnitude of chiral interactions and subsequently the retention of analyte may be modified by interactions involving the underlying surface, but the elution order of a pair of enantiomers should remain unaltered, as shown by the structural analysis mentioned above. This is because the dominant role of hydrophobic interactions of the side group of the analyte with the active site of the chiral selector will not change drastically, due to the involvement of the hydrophobic surface. This explanation is limited to the case of a hydrophobic surface and may be invalid if a hydrophilic surface is involved. Given the important role of the non-chiral surface in regulating the retention and selectivity of chiral chromatography, we consider this as an important area for further exploration.

The differences in the retention order of amino acid enantiomers observed with alkyl- and aryl-L-phenylalanine chiral selectors can be readily explained in line with the structural analysis presented above. On alkyl-L-Phe, the side chain of an amino acid analyte can interact with the phenyl group in L-phenylalanine and with the alkyl chain attached to the amino group, but the interaction with the phenyl group is most likely to be dominant because of the aryl nature of the phenyl group. Thus, the retention order on this type of phase is L>D. However, on aryl-L-Phe, the side chain of the amino acid analyte will interact more strongly with the aryl group (eg. MB-L-Phe) than with the phenyl group, thus producing the retention order D>L. Thus, the N-substituent may contribute positively to chiral recognition by participating in multiple interactions with the analyte. It appears that the aryl groups, which are capable of stronger hydrophobic interactions with the side chain of the amino acid analyte, modify the enantioselectivity more than the alkyl groups do. Thus, the use of the aryl substituents as anchor molecules for the preparation of chiral phases may lead to useful improvements in enantioselectivity. The role of different amino acid selectors and the nature of the amino acid N-substituent chemistry will be examined in subsequent papers.

4. Conclusions

This study confirms the excellent chiral selectivity and column stability of coated porous graphitic carbon for chiral ligand-exchange chromatography. The use of PGC as a support matrix for chiral ligand-exchange chromatography has enabled a more effective investigation of the mechanism of enantioselectivity, without undesirable interference from secondary interactions observed with silica-based supports. The importance of the nature of the anchor molecule in the enantioselectivity mechanism in chiral ligand-exchange chromatography has been confirmed and a mechanism has been proposed to explain this effect.

Acknowledgments

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