Journal of Chromatography, 644 (1993) 277–283
Elsevier Science Publishers B.V., Amsterdam

CHROM. 25 227

High-performance liquid chromatographic separation of enantiomeric amino acids on bis[carbamoyl(alkyl)methylamino]-6-chloro-*s*-triazine-derived chiral stationary phases

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(First received February 8th, 1993; revised manuscript received April 15th, 1993)

ABSTRACT

The synthesis and chiral recognition ability of a series of four chiral stationary phases (CSPs) containing 2,4-bis[carbamoyl(alkyl)methylamino]-6-chloro-*s*-triazine (designated phase A) are described. The synthesis of these CSPs is achieved through amide formation by bonding 2,4-bis[carboxy(alkyl)methylamino]-6-chloro-*s*-triazine onto 3-aminopropyl silica gel. Such phases are quite effective for high-performance liquid chromatographic separation of a racemic mixture of five typical amino acids. The chromatographic behaviour of these CSPs was studied. Comparison of these CSPs with the *s*-triazine-derived CSP (designated phase B), which bears a tripeptide chiral moiety, is also discussed. The present study clearly indicates that an *s*-triazine-terminated CSP derived from certain L-amino acids (phase A), instead of tripeptide, is effective enough for the separation of enantiomeric amino acids.

INTRODUCTION

In our previous report [1], a highly selective *s*-triazine-modified C_{18} column for HPLC was prepared. The chromatographic efficiency and selectivity of the bonded phase were evaluated from the separability of a mixture of twelve aromatic hydrocarbons. Better performance was achieved on the prepared column than on some commercially available C_{18} columns. Also, the bonded phase so prepared showed better chemical stability. We therefore considered that the presence of an *s*-triazine ring in the bonded phase system not only led to a convenient reproducible synthetic way of introducing a hydrocarbon moiety to the silica surface but also played an important role, possibly due to a $\pi-\pi$ interaction with aromatic analytes, in the separation of aromatic hydrocarbons.

In a study of chiral stationary phases (CSPs) for HPLC, Oi *et al.* [2] reported that the *s*-triazine derivative of tripeptide ester bonded to silica gel (Fig. 1, phase B), when used as a CSP in HPLC, gave good enantioselectivity for dinitrobenzoyl (DNB)-derivatized enantiomers of amino acids. It is possible that, by alternative bonding with the *s*-triazine terminus, an L-amino acid derivative instead of a tripeptide ester derivative (Fig. 1, phase A) may be effective enough in the chiral recognition process owing to the increasing rigidity of this terminal moiety. Furthermore, from the viewpoint of the synthetic advantage, as we described previously [3,4], an amide linkage could be formed effectively

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0021-9673/93/$06.00

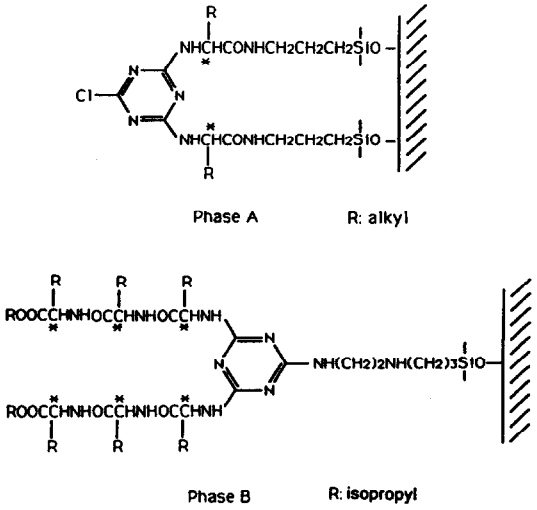

Fig. 1. The structures of chiral stationary phases.

through an active ester transformation. Thus, an amide formation would be more favourable than a nucleophilic substitution for introducing the chiral moiety of the *s*-triazine derivative to aminopropylsilanized silica.

The aim of this work was to evaluate the performance of *s*-triazine linking to different positions of silanized silica in the separation of enantiomeric amino acids by HPLC. Thus, bis(L-amino acid)-substituted *s*-triazine-derived CSPs were prepared and the chiral recognition ability of these sorbents was investigated. It was found that not only is the preparation of the *s*-triazine-terminal CSPs favourable, but these phases also exhibit excellent recognition ability for DNB-derivatized racemic amino acids in reversed-phase chromatography.

EXPERIMENTAL

Chemicals

The silica gel used was Nucleosil (pore size 100 Å; particle size 10 μm; surface area 350 m^2/g), obtained from Macherey–Nagel.

All reagents used to prepare the bonded CSPs were reagent grade: cyanuric chloride (Fluka), 3-aminopropyltriethoxysilane (APS, Chisso), dicyclohexylcarbodiimide (DCC, Merck), L-amino acids (Sigma) and N-hydroxysuccinimide (Aldrich). The solvents used for HPLC were LC-grade methanol and reversed-osmosis deionized water.

Preparation of s-triazine derivatives of L-amino acids

Three derivatives of *s*-triazine prepared in this study were 2,4-bis(L-alanyl)-6-chloro-*s*-triazine (I), 2,4-bis(L-valyl)-6-chloro-*s*-triazine (II) and 2,4-bis(L-leucyl)-6-chloro-*s*-triazine (III). These compounds were synthesized by a general procedure as follows. Anhydrous sodium carbonate (0.042 mol) and the L-amino acid (0.02 mol) were dissolved in 50 ml of water, then a solution of cyanuric chloride (0.02 mol) in acetone (10 ml) was added with stirring. The mixture was heated at 45–50°C for 30 min. The product was precipitated by acidifying with dilute hydrochloric acid, filtered, and washed with pure water until no more chloride ion could be detected in filtrate by silver nitrate solution, and dried under vacuum in the presence of P_2O_5 to give the expected product quantitatively. The melting points of compounds I, II and III were determined to be 170–172, 166–168, and 70–72°C, respectively. The characteristic IR data (potassium bromide, cm^{-1}) for compounds I–III are summarized: ν(O–H) 2600–3600, ν(C–H) 2970, ν(N–H) 3300, ν(C=O) 1710, ν(*s*-triazine ring) 1500–1600. ^{1}H-NMR($C^2H_3O^2H$) showed peaks at δ1.2(d, 6H) and δ4.4(q, 2H) for compound I; δ0.79(d, 12H), δ2.2(m, 2H) and δ4.4(d, 2H) for compound II; δ0.98(d, 12H), δ1.65(m, 6H) and δ4.6(t, 2H) for compound III. Elemental analysis showed: C:H:N = 3.67:4.21:2.40 (calculated, C:H:N = 3.72:4.15:2.46) for compound I; C:H:N = 45.20:5.81:20.33 (calculated, C:H:N = 45.15:5.79:20.26) for compound II; C:H:N = 48.09:6.33:18.89 (calculated, 48.19:6.43:18.74) for compound III.

Preparation of APS-modified silica gel

Silica gel (3 g) dried at 180°C for 10 h was suspended in dry toluene (100 ml). After APS (3 ml) had been added, the reaction mixture was refluxed under nitrogen for 10 h with stirring. After cooling, the APS-modified silica was collected by filtration and washed exhaustively with toluene, chloroform, methanol and diethyl ether, and then dried under vacuum in the presence of

TABLE I

CHARACTERISTICS OF APS–SILICA AND THE CSPs

	APS–silica	CSP-I	CSP-IIa	CSP-IIb	CSP-III
Elemental analysis					
C (%)	5.69	9.29	9.26	10.96	8.97
N (%)	1.65	2.60	2.87	2.85	2.69
Surface coverage[a]					
(mmol/g)	1.17	0.17	0.17	0.17	0.15
(μmol/m^2)	3.34	0.49	0.49	0.49	0.43
End-capped[b]	–	+	–	+	+

[a] The number of moles of *s*-triazine derivative of L-amino acids grafted per gram or unit surface area of APS–silica (based on N%).

[b] + = End-capped by trimethylchlorosilane; – = not end-capped.

P_2O_5. The result of elemental analysis is given in Table I.

Preparation of CSPs

CSP-I, CSP-IIa, CSP-IIb and CSP-III were prepared by the following general procedure. The *s*-triazine derivative (5 mmol) and N-hydroxysuccinimide (0.01 mol) were dissolved in dimethylformamide (100 ml) and then DCC (0.01 mol) was added. The mixture was stirred first at 0°C for 1 h and thereafter at room temperature for 24 h. After removal of the suspended solid dicyclohexylurea, 3 g of APS–silica gel were added and the mixture stirred gently at 0°C for 1 h and then at room temperature for 48 h. The prepared CSP was collected and washed thoroughly with dimethylformamide, methanol, pure water, methanol and ether, and dried under vacuum in the presence of P_2O_5 to give the uncapped CSP.

The uncapped CSP (3 g) was suspended in toluene (100 ml), and trimethylchlorosilane (3 ml) was added at 0°C and stirred for 1 h, then at 40°C for 4 h under nitrogen. The reaction mixture was filtered and washed with toluene, methanol and ether, and dried under vacuum to give the capped CSP. The results of elemental analyses of all the prepared CSPs are given in Table I.

Chromatographic studies

Stainless-steel columns (250 mm × 4 mm I.D.) were packed by the balanced-density slurry technique with methanol as mobile phase under a head pressure of 720 kg/cm^2. The chromatographic experiments were carried on Waters HPLC system equipped with a Model 6000A pump, a U6K injector and a Model 440 UV ($\lambda = 254$ nm) detector. The analytes were derivatized with 3,5-dinitrobenzoyl chloride by a routine method before injection.

RESULTS

The CSPs were prepared conveniently by a three-step reaction, as shown in Fig. 2. In the first step, acetone–water binary solvents were chosen as reaction solvents and the proper ratio by volume was 1:5. Using this proper ratio of acetone–water binary solvents, L-amino acids without any derivatization were able to react with cyanuric chloride to give the expected disubstituted products in one step with high yield and high purity (supported by elemental analysis and m.p. results). It is noteworthy that the *s*-triazine derivatives must be transferred to an active ester before being bonded to APS–silica gel in the second synthetic procedure. Without active ester formation, the dicyclohexylurea, which was the by-product of amide formation, could not be completely removed from the reaction product and would exist in the prepared silica gel. When this unpurified bonded silica gel was used for column packing, unusually high column pressure and poor chromatographic resolution were obtained.

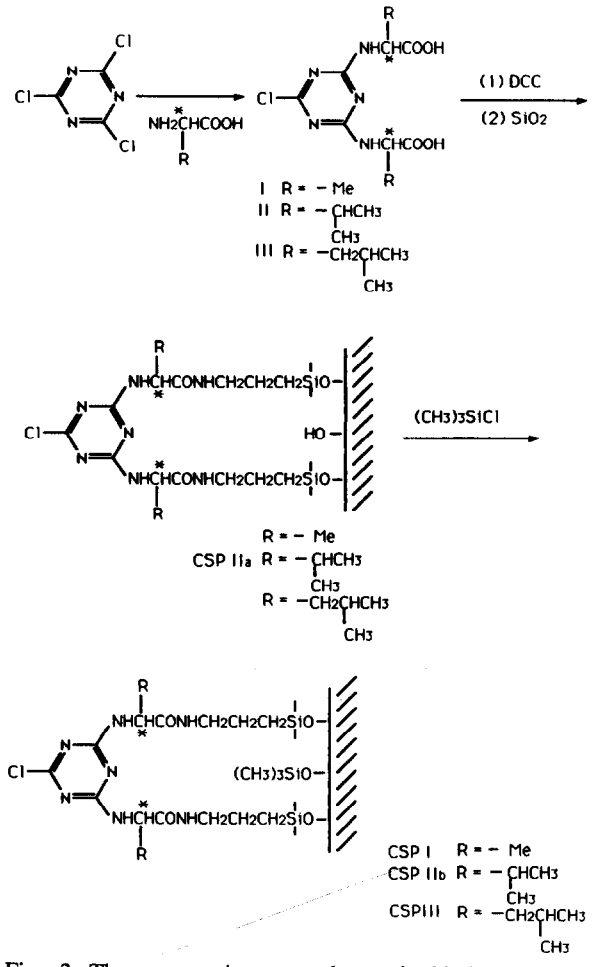

Fig. 2. The preparation procedure of chiral stationary phases.

The presence of the *s*-triazine derived chiral ligands on the silica surface of the prepared CSPs was characterized by elemental analysis (Table I). The surface coverage of the *s*-triazine chiral moiety on the silica gel was found to range from 0.15 to 0.17 mmol/g (0.43–0.49 μmol/m^2) based on nitrogen percentage. Further surface characterization was done by using the method of trifluoroacetic acid hydrolysis and HPLC identification [5]. The bonded-phase silica gel was hydrolysed in hot trifluoroacetic acid aqueous solution to cleave the amide linkage of the bonded-phases and followed by an HPLC identification of the components of these hydrolysed mixtures with the corresponding authentic samples (I–III).

The chromatographic results for the separation of five racemic amino acids on the prepared CSPs are summarized in Table II. It shows that there is little difference in α values of four CSPs. For convenience, CSP-II was chosen as the representative CSP to be further investigated and the typical chromatograms are shown in Figs. 3 and 4. The enantiomers of five racemic amino acids chosen in this study can be all resolved in these prepared CSPs. The racemic amino acids were reacted with dinitrobenzoyl chloride before being injected into the HPLC system and the chromatographic peaks were identified with the corresponding optically pure L-amino acids.

As shown in Table II, the capacity factor of a

TABLE II

HPLC SEPARATION OF DNB-DERIVATIZED RACEMIC AMINO ACIDS ON CSPs

The separation factor of the enantiomers, α, is the ratio of their capacity factors, and k_1' is the capacity factor for the first-eluted enantiomer (D-form). The analytes were reacted with dinitrobenzoyl chloride before injection.

Analyte	CSP-I[a]		CSP-IIa[b]		CSP-IIb[b]		CSP-III[b]	
	k_1'	α	k_1'	α	k_1'	α	k_1'	α
Alanine	1.53	1.19	5.36	1.15	3.53	1.15	1.29	1.19
Valine	1.69	1.08	8.50	1.08	3.29	1.13	1.76	1.10
Leucine	2.34	1.21	8.86	1.15	4.88	1.15	2.82	1.19
Methionine	2.26	1.18	7.79	1.15	5.00	1.18	2.65	1.18
Phenylalanine	3.84	1.11	9.50	1.10	7.58	1.11	4.76	1.15

[a] Mobile phase: 20% aqueous methanol–0.05 *M* ammonium hydroxide pH 6.8.
[b] Mobile phase: 20% aqueous methanol–0.01 *M* ammonium hydroxide pH 7.0.

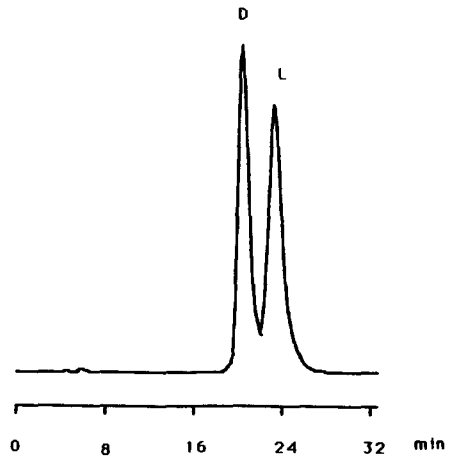

Fig. 3. The chromatogram for the resolution of dinitrobenzoyl-D,L-leucine on CSP-IIb. Mobile phase: methanol–water (20:80), 0.01 *M* ammonium acetate, pH 7.0, flow-rate: 1.5 ml/min.

given analyte on CSP-IIa is larger than that on CSP-IIb under the same chromatographic conditions. Because the structure and the surface coverage of the chiral moiety of CSP-IIa are the same as that of CSP-IIb, the only difference is whether or not the unreacted surface silanol was end-capped by trimethylchlorosilane (Fig. 2).

The capacity factors of a given analyte on the CSP-IIb were decreased when the pH values of

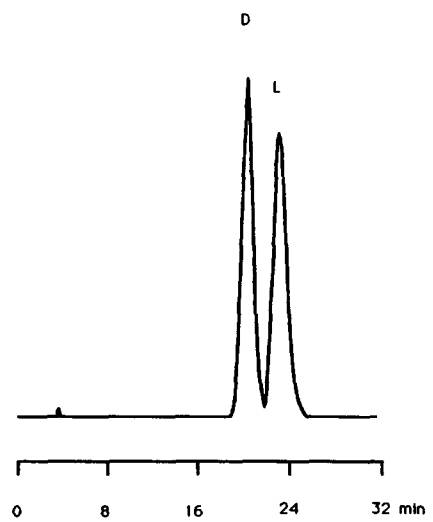

Fig. 4. The chromatogram for the resolution of dinitrobenzoyl-D,L-methionine on CSP-IIb. Mobile phase: methanol–water (20:80), 0.01 *M* ammonium acetate, pH 7.0, flow-rate: 1.3 ml/min.

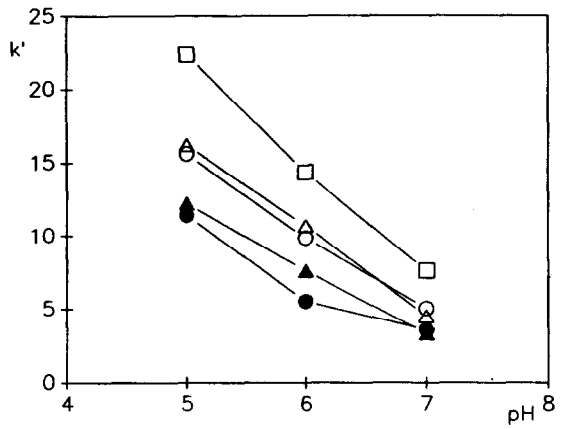

Fig. 5. The effect of the pH of mobile phase on the k_1' of analytes for chromatographic resolution of racemic amino acids on CSP-IIb. Mobile phase: methanol–water (20:80), ammonium acetate 0.01 *M*. Amino acids analytes were reacted with dinitrobenzoyl chloride before injection to HPLC. k_1' is the first-eluted enantiomer of the amino acid (D-form). ○ = Methionine; ● = alanine; △ = leucine; ▲ = valine; □ = phenylalanine.

mobile phases were increased from pH 5.0 to 7.0. Fig. 5 shows the effect of the pH value of the mobile phase on the capacity factor, k_1', of the analytes on CSP-IIb. Five amino acids, after reaction with dinitrobenzoyl chloride, were chosen as analytes. Chromatographic results

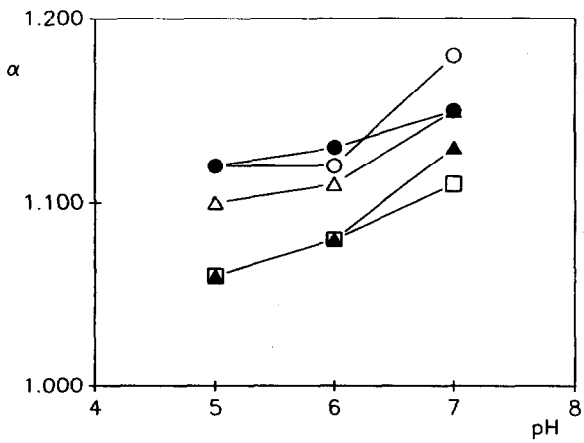

Fig. 6. The effect of the pH of mobile phase on the α-values of analytes for the resolution of enantiomers on CSP-IIb. Mobile phase: methanol–water (20:80), 0.01 *M* ammonium acetate. Amino acids analytes were reacted with dinitrobenzoyl chloride before injection into the HPLC system. Symbols as in Fig. 5.

indicated that the k' of D-valine, for example, was 12.5 when the pH of mobile phase was 5.0, but was only 3.3 when the pH of the mobile phase was 7.0.

However, the separation factor of a given racemic amino acid on the CSP-IIb is slightly increased when the pH of the mobile phase is increased from 5.0 to 7.0. Fig. 6 shows the effect of the pH value of the mobile phase on the separation factors, α, of enantiomers of analytes on CSP-IIb. Chromatographic results indicated that the α-value of the racemic DNB–methionine, for example, was 1.13 when the pH of the mobile phase was 5.0 but increased to 1.18 when the pH of the mobile phase was 7.0.

DISCUSSION

Preparation and characterization of CSPs

In general, non-esterified amino acids are poorly soluble in the organic solvents, while cyanuric chloride is little soluble in water but is soluble in organic solvent. In this study, it was found that using the proper ratio of the acetone–water solvent (1:5), a complete disubstituted reaction of L-amino acid with cyanuric chloride would occur at 40–50°C. In the preparation of CSPs, the presence of N-hydroxysuccinimide not only favours amide formation, but also leads to easy exclusion of dicyclohexylurea, a by-product precipitated from amide formation, before the subsequent addition of APS-modified silica. As a result, it prevents the extreme increase in column pressure caused by the fine particles of the by-product.

The double-bonded *s*-triazine structure on the CSP (Fig. 2) was assumed as a result of the synthetic procedure. But it was also supported indirectly by the chromatographic peak shape in the separation of DNB-derivatized amino acids in the reversed phase. The fact that no peak tailing occurred suggested the absence of carboxylic groups, which would be the other end-moiety of a single-bonded *s*-triazine derivative, on the surface of CSP.

Chromatographic properties of CSPs

The hydrophobic character of the prepared CSPs is illustrated by their chromatographic behaviours. As shown in Table II, the capacity factors of analytes increase with an increase in the carbon number of the analytes. Moreover, the relationship between the capacity factors of analytes and the pH of the mobile phase, as shown in Fig. 5, also indicates that longer retention is obtained for the relatively hydrophobic carboxylic acids in their acid form rather than in carboxylate form of a given analyte on CSP-IIb.

The chromatographic results suggested that the uncapped silanol groups on the silica surface of CSP do not contribute to the discrimination of the enantiomers of analytes chosen in this study. As shown in Table II, the capacity factors on the CSP-IIa, in which silanol groups were not capped, are larger than those on the end-capped CSP-IIb, but the separation factors are almost unchanged on both CSPs. To this is attributed the fact that the free silanol sites on the CSP-IIa contributed to longer retention for the analytes but not to the chiral recognition.

In Fig. 6, the phenomenon of larger α-values of the DNB-amino acids on CSP-IIb in a higher pH mobile phase can be explained by the fact that there is an effective stereoselective interaction between the analyte, in carboxylate form in a high-pH solution, and CSP. Thus, the carboxylate form, which provides a stronger basic site, might form hydrogen bonds with the acidic site (N–H) of the amide linkage in CSP.

Table II shows that the prepared *s*-triazine-terminal CSPs afford effective enantioselectivity for DNB-derivatized racemic amino acids and that the D-enantiomer always elutes first in reversed-phase conditions. Obviously, a $\pi-\pi$ interaction between the analyte (DNB derivative) and the *s*-triazine moiety of the CSP contributes to the chiral recognition.

Comparison of phase A and phase B

Although both of the CSPs (Fig. 1) contain an *s*-triazine moiety, phase A is derived from L-amino acids and phase B is derived from a tripeptide. Moreover, the bonding model of chiral moiety to the silica surface is also different. Phase B contains six chiral centres and four amide functional groups with the *s*-triazine ring on the inner part of the connection arm, while phase A contains only two chiral centres and two

amide groups with the *s*-triazine ring on the terminal of the brush of CSP. It is suggested that the terminal bis(L-amino acid)-substituted *s*-triazine moiety on the CSP seems to be conformationally "stiffer", and therefore more favourable for chiral recognition.

CONCLUSIONS

The present results clearly indicate that the CSP with an *s*-triazine ring on the terminal of the brush (phase A), derived from L-amino acids instead of tripeptide, is effective enough to resolve the enantiomers of amino acids. The preparation of the CSPs is convenient by bonding 2,4-bis[carboxy(alkyl)methylamino)]-6-chloro-*s*-triazine onto APS–silica gel through amide formation. The chromatographic results reveal that the CSPs show hydrophobic character and can recognize DNB-derivatized racemic amino acids in reversed-phase HPLC.

ACKNOWLEDGEMENT

The authors thank the National Science Council, Taiwan, for financial support.

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