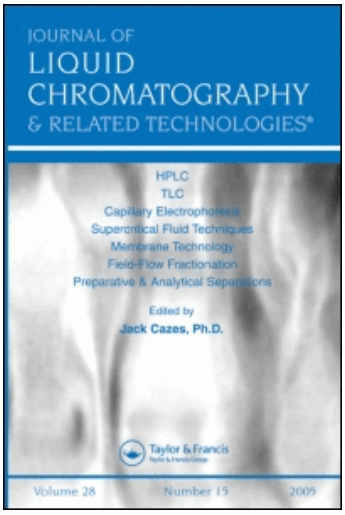

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HPLC Enantioseparation Of Dl-and Tripeptides on Cyclodextrin Bonded Stationary Phases After Derivatization with 6-Aminoquinolyl-N-hydroxysuccinimidyl Carbamate (AQC)

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HPLC ENANTIOSEPARATION OF DI- AND TRIPEPTIDES ON CYCLODEXTRIN BONDED STATIONARY PHASES AFTER DERIVATIZATION WITH 6-AMINOQUINOLYL-N-HYDROXYSUCCINIMIDYL CARBAMATE (AQC)

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ABSTRACT

Enantiomeric separations were performed on a number of di- and tripeptides after their pre-column derivatization with the fluorescence derizatizing agent 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC). It has been demonstrated that the choice of a suitable cyclodextrin bonded phase in conjunction with nonaqueous polar mobile phases offers an effective means to resolve chiral peptides. It was found that the strength of interaction between the cyclodextrin and the solute, and therefore the retention and stereoselectivity, is extremely sensitive to small structural variations of the analyte.

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INTRODUCTION

6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) was recently developed as a new pre-column derivatizing agent for primary and secondary amine containing compounds [1,2]. We reported the first enantiomeric separations of AQC-primary and secondary amino acids on cyclodextrin bonded columns [3]. The derivatization reaction is fast, proceeds without any detectable racemization and provides stable and highly fluorescent derivatives suitable for efficient and sensitive HPLC analysis. The use of nonaqueous systems e.g., different cyclodextrin bonded phases in conjunction with polar organic mobile phases, offered a wide range of possibilities to optimize enantioselectivity. It has been postulated that the enantioseparation has been achieved via external complex formation between the solute and the cyclodextrin molecule. In the case of native cyclodextrin bonded phases the chiral recognition arises from stereoselective hydrogen bonding between donor and acceptor sites of the analyte with the secondary hydroxyl groups at the mouth of the cyclodextrin. The discrimination of enantiomers on derivatized bonded cyclodextrin phases may arise from stereoselective hydrogen bond formation between the solute and the residual secondary hydroxyl groups as well as other polar moieties at the mouth of the cyclodextrin. It has been postulated that the 6-aminoquinolyl moiety provides not only high absorptivity for easy photometric and/or fluorescence detection but also contributes to the overall stereorecognition. It has been found that mobile phase composition controls both retention and enantioselectivity. The addition of methanol can be used to decrease retention while small amounts of triethylamino and glacial acetic acid have been used to optimize selectivity and retention parameters. The addition of water decreases dramatically the retardation and selectivity. In this paper we extend the use

of AQC to the analysis of di- and tripeptides. Also we discuss the effect of solute structure on retention in terms of the overall mechanism. Comparisons with other fluorescent "tagging" agents are beyond the scope of the present paper, but are being made. This information will be reported in a latter work.

EXPERIMENTAL

Chemicals. All peptides and boric acid used in this work were purchased from Sigma (St. Louis, MO). The derivatizing reagent (AQC, Acc Q-Fluor Reagent) was obtained from Waters (Milford , MA). Calcium sodium EDTA was purchased from Aldrich (Milwaukee, WI). All HPLC grade solvents including acetonitrile, methanol, triethylamine and acetic acid were obtained from Fisher Scientific (Pittsburgh, PA).

Methods

Derivatization procedures. The AQC derivatized peptides were obtained according to references 1-3 by dissolving 500 pmol of each compound in 35 µl of sodium borate buffer (0.2 M, pH 8.8) in a vial; vortex several seconds and then add 10 µl of AQC solution to it (3 mg per 1 ml of acetonitrile). The vial was heated in an oven for 10 minutes of 50°C. The resulting solution was injected into a column without further purification. The FMOC-Gly derivatization of Phe and Leu was performed as reported previously in reference 4.

Chromatographic experiments. Separations were performed at ambient temperature with a Waters dual pump solvent delivery module Model 590. The spectrophotometric detector (Waters, Model 440) with UV wavelength of 254 nm or fluorescence scanning detector (Waters,

Model 470) was used for monitoring the effluent. The excitation and emission wavelengths were 250 nm and 395 nm, respectively. The flow rate in all cases was 1 ml/min. All columns used in this work were obtained from Advanced Separation Technologies (Whippany, NJ). The mobile phases were mixtures of acetonitrile, methanol, triethylamine and acetic acid. The volume ratios of each solvent is reported in the appropriate tables and figures.

RESULTS AND DISCUSSION

Table I shows the chromatographic data for a number of AQC functionalized di- and tripeptides with one chiral center, e.g. containing at least one achiral glycine or β-alanine moiety, obtained under optimal experimental conditions. Table II gives the retention parameters for AQC-di- and tripeptides with two chiral centers. As can be seen from the data presented the chiral recognition of AQC derivatized solutes occurs on native as well as derivatized cyclodextrin bonded phases operated with polar organic solvents. The enantioseparation of the analytes studied can be achieved in some cases also in the reversed phase mode as presented in Figure 1B. However, compared with the nonaqueous system (Figure 1A) the selectivity obtained in a water-rich system is rather poor. The change of the mobile phase composition also changed the elution order of the enantiomers, which is not surprising since the chiral recognition mechanism involved is different for each mode [3,4].

It has been found that cyclodextrin bonded phase columns operated with nonaqueous eluents exhibit enantioselectivity towards AQC functionalized di- and tripeptides which results in baseline resolution for many solutes studied (see Figure 2 and Table I). Obviously enantioseparation depends very strongly on the type of the chiral stationary

TABLE I CHROMATOGRAPHIC DATA FOR SEPARATION OF RACEMIC AQC-DIPEPTIDES AND TRIPEPTIDES WITH ONE CHIRAL CENTER ON CYCLODEXTRIN BONDED PHASES USING NONAQUEOUS POLAR MOBILE PHASES

Compound	k'[a]	Config.	α	R_s	Mobile Phase[b]	Column[c]
AQC-Gly-Leu	4.70	D	1.33	2.97	450/50/4/6	RN
	8.98	D	1.15	1.82	450/50/2/6	SN
AQC-Leu-Gly	3.71		1.29	2.44	450/50/4/6	RN
	5.03		1.24	2.31	450/50/3/6	SN
AQC-Gly-α amino n-butyric acid	3.55	D	1.08	1.01	475/25/4/6	γ
	3.47		1.09	1.01	450/50/4/6	RN
AQC-Gly-Nleu	4.69		1.30	3.00	450/50/4/6	RN
AQC-Gly-Val	3.49	D	1.24	2.29	450/50/4/6	RN
AQC-Gly-Nval	3.66		1.14	1.53	450/50/4/6	RN
AQC-Gly-Met	4.75	L	1.15	1.34	470/30/4/6	AC
	5.05	D	1.27	2.70	450/50/4/6	RN
AQC-Gly-Ser	5.45		1.04	0.55	450/50/3/6	SN
AQC-Gly-Asn	1.83		1.05	0.70	450/50/3/6	SN
AQC-Gly-Asp	7.67	D	1.18	2.81	450/50/4/6	AC
AQC-Gly-Phe	6.85	L	1.12	1.24	450/50/4/6	RN
	10.03	L	1.11	1.37	450/50/3/6	SN
AQC-β-Ala-Leu	8.25		1.12	1.90	460/40/4/6	β
	4.09		1.14	1.71	475/25/3/6	RSP
	5.72		1.36	2.68	460/40/5/3	RN
	5.00		1.21	2.23	450/50/3/6	SN
AQC-β-Ala-Val	3.62		1.10	1.17	475/25/3/6	RSP
AQC-Gly-Gly-Leu	7.48		1.12	1.74	450/50/3/6	γ
	6.71		1.25	2.14	450/50/4/6	RN
AQC-Ala-Gly-Gly	8.65	D	1.12	1.23	450/50/3/6	SN

[a] k' is the capacity factor for the first eluted enantiomer.

[b] Mobile phases are mixtures of acetonitrile/methanol/acetic acid/triethylamine by volume(v/v)

[c] Columns β, γ, RSP, AC, SN, RN stand for , β–, γ–, R,S-2-hydroxypropyl, acetylated β–, S-naphthylethyl-carbamate, R-naphthylethylcarbamate, cyclodextrin bonded stationary phases.

TABLE II CHROMATOGRAPHIC DATA FOR SEPARATION OF RACEMIC AQC-DIPEPTIDES AND TRIPEPTIDES WITH TWO CHIRAL CENTERS ON CYCLODEXTRIN BONDED PHASES USING NONAQUEOUS POLAR MOBILE PHASES

Compound	k'^{b}_{DD}	k'_{LL}	$k'_{DL,LD}$	$k'_{LD,DL}$	Mobile Phase[a]	Column[c]
AQC-Ala-Ala[d]	6.92	7.32	7.72	7.72	450/50/3/5	β
AQC-Ala-Val[d]	4.96	5.38	5.57	5.98	460/40/4/6	β
	2.95	3.73	3.14	3.60	450/50/2.5/5	RN
AQC-Ala-Ser[d]	4.42	4.68	6.08	6.08	490/10/2/6	AC
	4.72	4.72	5.57	6.22	460/40/2/6	SN
AQC-Leu-Ala[d]	3.90	4.05	4.56	4.76	485/15/6/6	AC
	4.22	6.70	5.28	6.43	460/40/2/6	RN
	4.36	5.64	3.52	5.29	450/50/2/6	SN
AQC-Leu-Val[d]	4.02	4.31	5.29	5.63	450/50/3/6	β
	3.54	5.41	4.78	5.14	450/50/3/6	SN
AQC-Leu-Phe[d]	11.13	7.12	8.44	9.56	450/50/3/6	α
AQC-Ala-Phe[d]	11.12	10.09	8.48	9.67	450/50/5/3	β
	15.97	14.65	11.89	12.76	465/35/5/2	AC
	8.27	5.25	4.34	4.68	450/50/4/6	RN
	13.68	10.13	7.28	7.45	450/50/2/6	SN
AQC-Ala-Leu[e]	9.03	10.26	9.29	8.76	460/40/2/6	β
	5.96	8.14	6.89	5.49	460/40/5/3	RN
AQC-Leu-Leu[e]	3.97	7.08	4.97	5.45	450/50/2/6	SN
AQC-Leu-Tyr[e]	10.56	9.17	11.78	12.78	460/40/4/6	β
	5.70	4.84	6.60	7.05	450/50/3/6	γ
AQC-Ala-Met[f]		7.26	5.72	6.02	460/40/2/6	RN
		6.10	4.76	5.04	450/50/2/6	SN
AQC-Leu-Gly-Phe[f]	5.50	6.14	6.54	7.67	450/50/4/6	RN
AQC-Ala-Leu-Gly[f]	7.02	7.34	7.77	8.80	450/50/4/6	β
	4.57	4.95	5.28	5.81	470/30/4/6	AC
	3.58	4.26	4.41	7.02	450/50/4/6	RN
AQC-Gly-Leu-Ala[f]	9.53	9.70	12.53	13.26	490/10/2/6	γ

[a] Mobile phases are mixtures of acetonitrile/methanol/AA/TEA by volume(v/v)

[b] The capacity factor, k', is equal to t_r-t_0 / t_0

[c] Columns α, β, γ, RSP, AC, SN, RN stand for α–, β–, γ–, R,S-2-hydroxypropyl, acetylated β–, S-naphthylethyl-carbamate, R-naphthylethylcarbamate, cyclodextrin bonded stationary phases.

[d] The retention order for the DD and LL enantiomers are known and are correct as shown in this table. However, the retention order for the DL and LD enantiomers is unknown since standards were not available.

[e] The retention order for these peptides is k'_1=DD, k'_2=DL, k'_3=LD, k'_4=LL.

[f] No standards were available for these compounds. Consequently in those cases the retention order for the stereoisomers is listed from lowest to highest (left to right) and the stereochemical designation at the top of each column may not be correct.

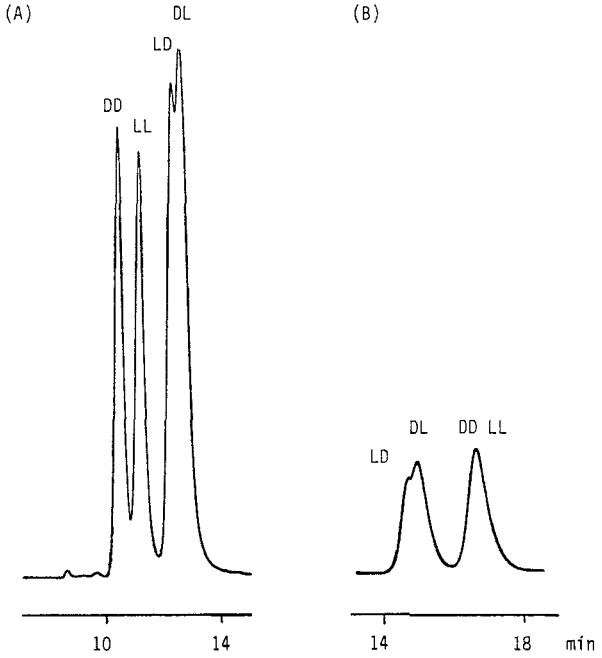

Figure 1. Enantiomeric resolution of AQC-Leu-Leu obtained on (R,S)-hydroxypropyl derivatized β-CD using; a) nonaqueous mobile phase: 475 acetonitrile + 25 methanol + 3 acetic acid + 6 triethylamine (v/v/v/v), b) reversed phase mode with a mobile phase consisting of 5% acetonitrile in 95% triethylammonium acetate buffer (1 % pH = 7.1). Fluorescence detection (λex = 250 nm, λem = 395 nm) was used.

phase used as well as the structure of the analyte. Generally the carbamate derivatives of β-cyclodextrin, where several types of adsorption (binding) sites are available, were found to have the broadest enantioselectivity. The chiral recognition may arise from stereoselective hydrogen bonding between donor and acceptor sites of the analyte with the residual secondary

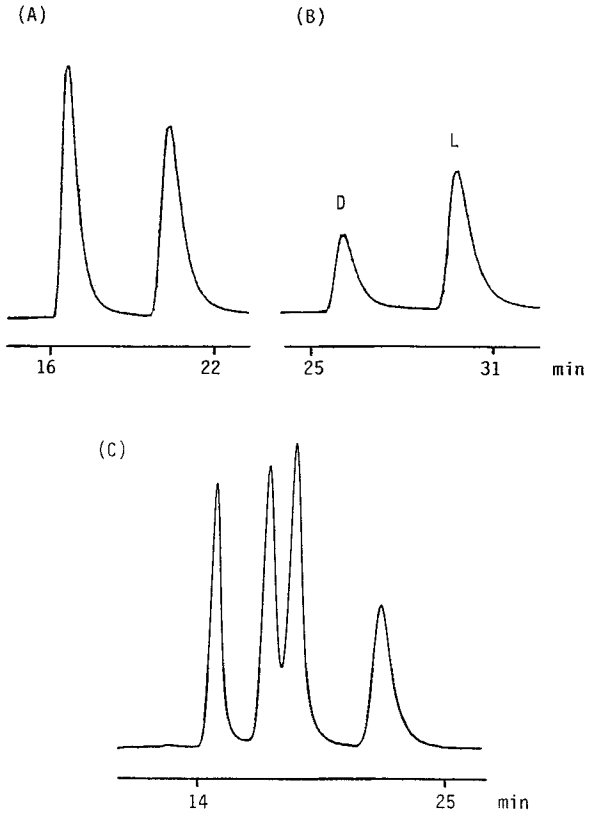

Figure 2. Enantioseparation of selected AQC-dipeptides under optimal chromatographic conditions. a) test compound: AQC-Gly-Nle; column: RN-β-CD; eluent: 450 acetonitrile + 50 methanol + 4 acetic acid + 6 triethylamine (v/v/v/v), b) test compound: AQC-Gly-Asp; column: AC-β-CD; eluent: 450 acetonitrile + 50 methanol + 4 acetic acid + 6 triethylamine (v/v/v/v), c) test compound: AQC-Leu-Leu; column: SN-β-CD; eluent: 450 acetonitrile + 50 methanol + 2 acetic acid + 6 triethylamine (v/v/v/v). Fluorescence detection (λex = 250 nm, λem = 395 nm) was used.

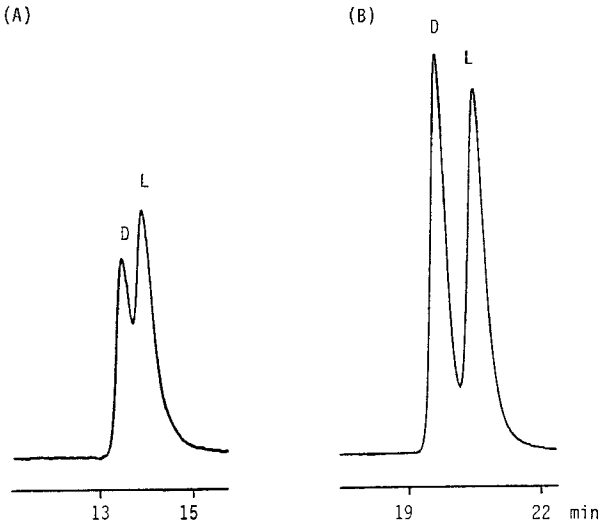

Figure 3. Enantioseparation of AQC-Val (A) and AQC-Gly-Val (B) obtained on a SN-β-CD column with a mobile phase consisting of: 450 acetonitrile + 50 methanol + 3 acetic acid + 6 triethylamine (v/v/v/v). Fluorescence detection (λex = 250 nm, λem = 395 nm) was used.

hydroxyl groups as well as the polar moieties of the chiral naphthylethylcarbamate substituent. Also, π-π interactions between the aromatic substituents at the mouth of the cyclodextrin cavity and the aromatic part of the analytes are possible. All these factors lead to the unique selectivities observed.

Figures 3-5 show the change in the enantioseparation of different AQC derivatives caused by the introduction of a glycine moiety. It was found in all instances that extending the peptide chain length and introducing new hydrogen bonding groups resulted in increased retention.

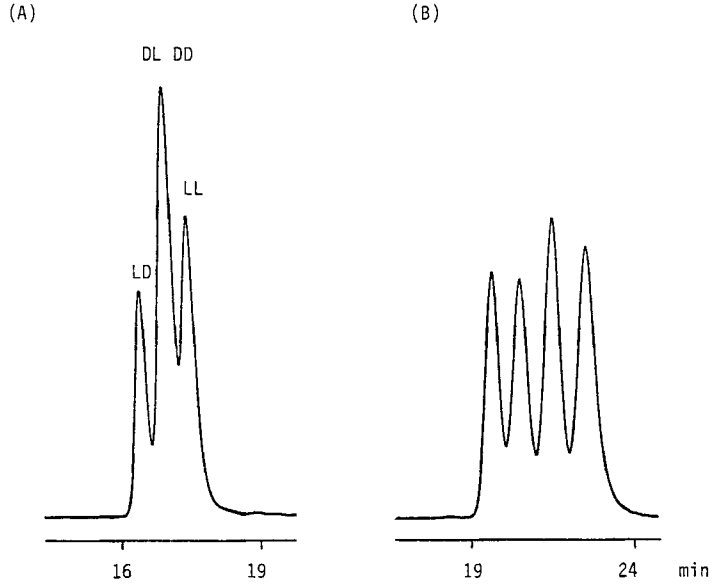

Figure 4. Enantioseparation of AQC-Ala-Leu (A) and AQC-Ala-Leu-Gly (B) on an AC-β-CD column using a mobile phase of 470 acetonitrile + 30 methanol + 2 acetic acid + 6 triethylamine (v/v/v/v). Fluorescence detection (λex = 250 nm, λem = 395 nm) was used.

As can be seen in Figure 3 and Figure 4 for AQC-Val and AQC-Ala-Leu the additional glycine moiety also causes an enhancement in enantioselectivity. Figure 5 shows the interesting case of AQC-Met. Introduction of glycine resulted in a reversal in the retention order. This shows that the enantioselectivity exhibited by the stationary phase is highly sensitive to changes in the molecular structure of the analyte that are not necessarily near the chiral center. The effects of the structural features of the solute on retention also are shown in Figure 6 and Figure 7. In

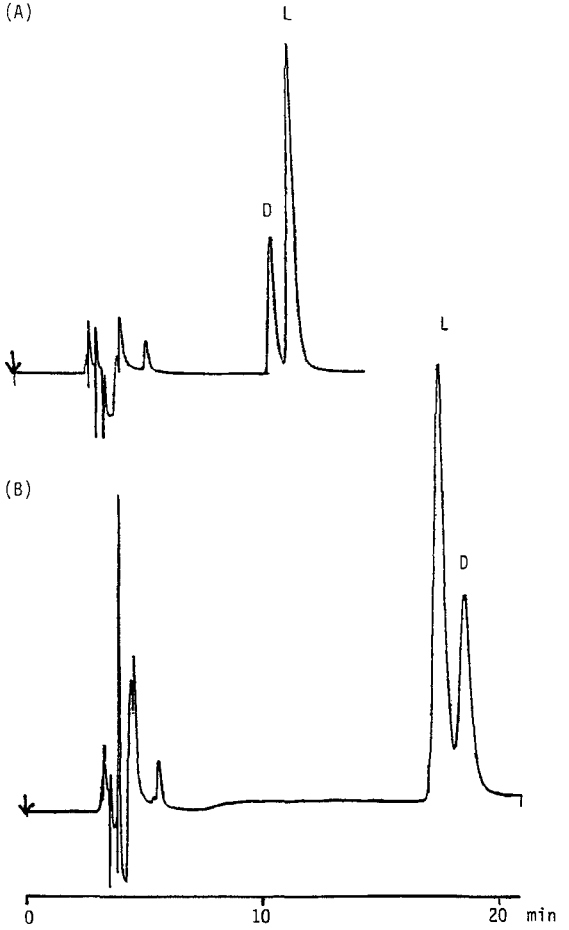

Figure 5. Enantioseparation of AQC-Met (A) and AQC-Gly-Met (B) using a mobile phase of 475 acetonitrile + 25 methanol + 3 acetic acid + 6 triethylamine (v/v/v/v) and an AC-β-CD column. UV detection was used at 254 nm. Both racemic mixtures were spiked with L-enantiomers. Note that elution order has been reversed.

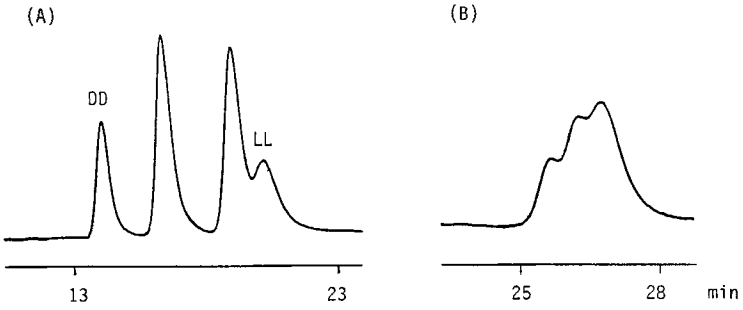

Figure 6. Enantioseparation of AQC-Leu-Ala (A) AQC-Gly-Leu-Ala (B) on a SN-β-CD bonded stationary phase with a mobile phase of 450 acetonitrile + 50 methanol + 1.8 acetic acid + 6 triethylamine (v/v/v/v). Fluorescence detection (λex = 250 nm, λem = 395 nm) was used.

contrast to the results presented above in Figures 3 and 4, the addition of a glycine moiety to the AQC-Leu-Ala resulted in decreased selectivity toward the enantiomers of the resulting tripeptide (Fig. 6). A similar effect is demonstrated in Figure 7. The best enantioseparation is achieved for AQC-Leu. The AQC-based separations are somewhat different from those that use FMOC, in that an added glycine moiety always enhanced the resolution of primary FMOC tagged amino acids [4]. However, in the AQC-case an added glycine can either enhance, decrease or not affect the enantiomeric separation. Currently there is no way to predict which will occur. The retention of the solute always increases with the number of polar groups capable of hydrogen bond formation, however magnitude of this effect depends on the analyte structure. As shown in Fig. 7B and Fig. 7D the retardation of AQC-Gly-Leu and AQC-Leu-Gly (which have the same amino acid residues but in a different order) are significantly different.

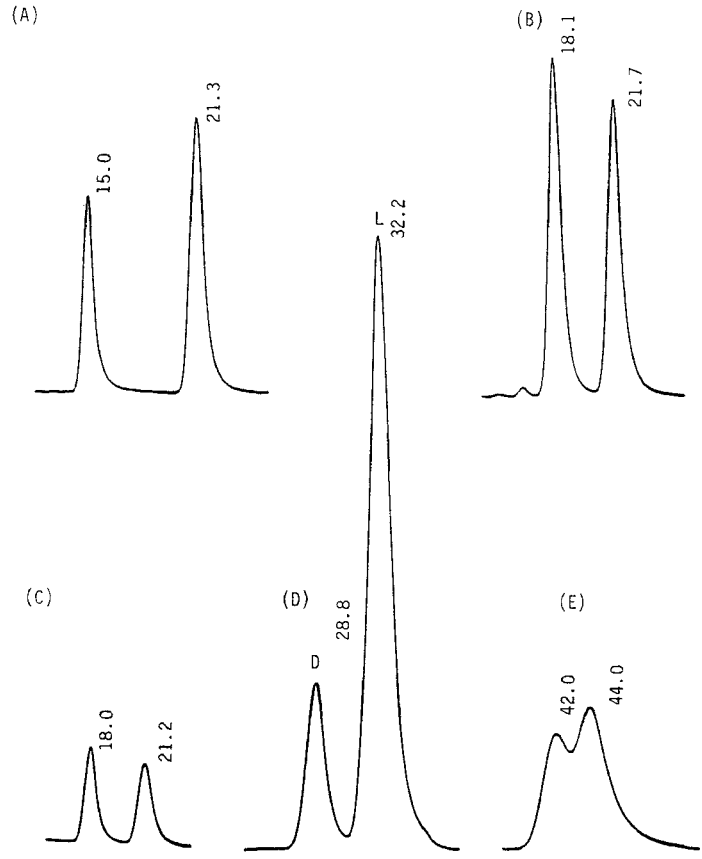

Figure 7. Chromatograms for AQC-Leu (A), AQC-Leu-Gly (B), AQC-β-Ala-Leu, (C) AQC-Gly-Leu (D) and AQC-Gly-Gly-Leu (E) obtained on a SN-β-CD column with a mobile phase of 450 acetonitrile + 5 methanol + 3 acetic acid + 6 triethylamine (v/v/v/v). In the (A) and (D) chromatograms, the L enantiomers have been spiked. Fluorescence detection (λex = 250 nm, λem = 395 nm) was used.

It has been postulated previously from the comparison of retention data obtained for AQC [3] and FMOC derivatives [4] that the 6-aminoquinolyl substituent does not sterically interfere with the chiral recognition process. In fact, it may enhance chiral recognition in some cases. This supposition is confirmed in this study. The elution of AQC derivatized amino acids [1] as well as di- and tripeptides from the cyclodextrin columns requires the addition of methanol to the mobile phase (in some cases as much as 20% which usually was not necessary for FMOC derivatives [4]). It is likely that both the 6-aminoquinolyl moiety containing two nitrogen atoms capable of hydrogen bond formation and the polar amino acids (or their peptide bonds) are closely associated with the cyclodextrin, which results in strong retardations and affects the stereorecognition as well. Addition of too much methanol to the mobile phase usually destroys enantioselectivity.

CONCLUSION

The data presented in this study provide support for the noninclusion model of chiral discrimination on cyclodextrin bonded stationary phases in nonaqueous systems as proposed previously [3,4]. It is demonstrated that the enantiomer separation is extremely sensitive to relatively small structural variations. The strength of the interactions between the cyclodextrin stationary phase and the solutes and therefore the retention and the stereoselectivity are determined both by the structure of the analyte and competitive interaction of the mobile phase components.

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