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Enantiomeric separation of fluorescent, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, tagged amino acids

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ABSTRACT

A new derivatizing reagent, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), has been used successfully for chromatographic enantioseparation of 31 amino acids on cyclodextrin bonded stationary phases. AQC reacts with both primary and secondary amino acids to produce stable and highly fluorescent derivatives suitable for efficient and sensitive chromatographic determinations. The derivatization reaction proceeds without detectable racemization. The detection limit is in the femtomole range and approximately 0.0075% of the D-enantiomer in an excess of the L-enantiomer is detectable. High resolution values are needed when determining trace enantiomeric impurities.

INTRODUCTION

Chemical derivatization prior to chromatography has become a common procedure in amino acid analysis. As widely documented in the review literature, numerous pre-column reactions have been reported that enhance the sensitivity and selectivity of chromatographic amino acid determinations (for reviews, see refs. 1–3 and references therein).

Recently we reported sensitive and effective methods for enantiomeric determinations of both secondary [4] and primary [5,6] amino acids on cyclodextrin bonded columns after pre-column derivatization with 9-fluorenylmethyl chloroformate (FMOC) and 9-fluorenylmethoxycarbonylglycine chloride (FMOC-Gly-Cl) reagents. Labeling with highly fluorogenic reagents enabled the quantitation of enantiomeric trace components in complex biological matrixes [6,7] and the determination of trace enantiomeric impurities in "optically pure" commercial amino acids standards [4].

This paper presents the enantioseparation of a number of amino acids on different cyclodextrin bonded stationary phases using a new "fluorescent-tagging-agent", 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) [8–10]. AQC is a highly electrophilic compound that reacts with nucleophiles such as amines and amino acids. It has been recently used for highly selective and sensitive achiral separation of amino acids in the reversed-phase mode and for quantitation of amino acids from human lysozyme [8–10]. The derivatization reaction is shown in Fig. 1. Both primary and secondary amino acids react rapidly with AQC to produce highly fluorescent and stable urea compounds. The by-products of the derivatization reaction do not appear to interfere

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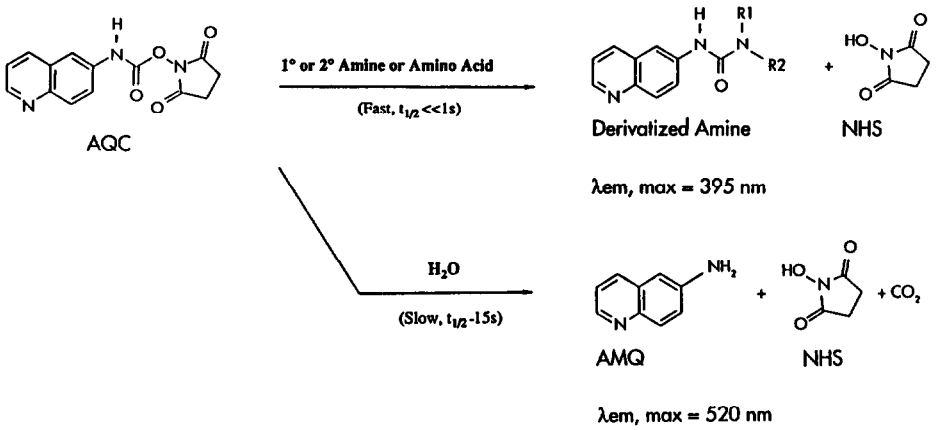

Fig. 1. Derivatization chemistry.

either chromatographically or spectroscopically with the analyte of interest. As can be seen in Fig. 1 the excess reagent is hydrolyzed to yield N-hydroxysuccinimide (NHS) and 6-aminoquinoline (AMQ) which has significantly different fluorescence spectral properties.

EXPERIMENTAL

Chemicals

All native amino acids and boric acid used in this work were purchased from Sigma (St. Louis, MO, USA). The derivatizing reagent AQC was obtained from Waters (Bedford, MA, USA). Calcium sodium EDTA was purchased from Aldrich (Milwaukee, WI, USA). All HPLC-grade solvents including acetonitrile, methanol, triethylamine and acetic acid were obtained from Fisher Scientific (Pittsburgh, PA, USA).

Methods

Derivatization procedures. The AQC derivatized amino acids were obtained according to ref. 8 by dissolving 500 pmol of each amino acid 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 min at 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 ref. 7.

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 266 nm or fluorescence scanning detector (Waters, Model 470) were used for monitoring the effluent. The excitation and emission wavelengths were 250 and 395 nm, respectively. For FMOC-Gly derivatives the excitation and emission wavelengths were 266 and 315 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, USA). The mobile phase was a mixture of acetonitrile, methanol, acetic acid and triethylamine.

RESULTS

Selectivity and chiral recognition mechanism

The chromatographic data for enantioseparation of a number of AQC-derivatized amino acids obtained under optimal conditions are collected in Table I. The non-aqueous polar eluents consisting almost entirely of acetonitrile and containing small amounts of glacial acetic acid and triethylamine modifiers (in conjunction with different cyclodextrin stationary phases) have proven to be highly selective for many enantioseparation problems [4–7,11]. The selectivity of the system can be regulated by changing the total amount and relative ratio of the

TABLE I

CHROMATOGRAPHIC DATA FOR THE ENANTIORESOLUTION OF PROTEIN AQC-AMINO ACIDS ON CYCLODEXTRIN BONDED STATIONARY PHASES USING NON-AQUEOUS POLAR MOBILE PHASES

Compound	k' [a]	Configuration	α	R_s	Mobile phase[b]	Column[c]
AQC-Arg	19.11	D	1.11	1.45	400:100:2:10	β
AQC-His	16.03	L	1.06	0.70	450:50:3:6	SN
AQC-Leu	9.51		1.14	1.50	450:50:1:6	α
	5.36	D	1.19	2.44	450:50:2:10	β
	3.74	D	1.11	1.67	475:25:4:6	γ
	2.40	D	1.14	2.04	450:50:4:6	AC
	4.16	D	1.29	3.03	475:25:3:6	RSP
	2.40	D	1.91	6.22	450:50:4:6	RN
	4.26	D	1.69	5.11	450:50:2:6	SN
AQC-Lys	4.73	D	1.10	1.13	430:70:2:10	AC
	3.74	D	1.20	1.27	400:100:2:10	RN
AQC-Met	7.45	D	1.15	2.29	480:20:3:5	β
	3.35	D	1.16	1.94	475:25:4:6	RSP
	2.72	D	1.66	3.69	450:50:4:6	RN
	8.63	D	1.56	4.13	475:25:3:2	SN
AQC-Phe	6.24	D	1.18	2.34	450:50:2:6	β
	3.85	D	1.13	1.74	475:25:4:6	γ
	2.45	D	1.17	2.76	450:50:4:6	AC
	3.21	D	1.20	2.42	450:50:3:2	RSP
	3.22	D	1.55	4.66	450:50:4:6	RN
	4.92	D	1.39	4.05	450:50:2:6	SN
AQC-Thr	4.75	D	1.08	1.00	475:25:2:6	β
AQC-Trp	13.37	D	1.13	2.03	480:20:3:6	β
	5.32	D	1.11	1.52	450:50:3:6	γ
	4.53	D	1.10	1.63	470:30:4:6	AC
	4.88	D	1.21	2.00	450:50:2:4	RN
AQC-Val	5.46	D	1.19	2.76	475:25:4:6	β
	2.35	D	1.12	1.87	470:30:4:6	AC
AQC-Ala	6.23	D	1.10	1.54	480:20:3:5	β
	2.30	D	1.09	1.41	470:30:4:6	AC
AQC-Asp	15.70	D	1.13	1.66	475:25:2:6	β
	4.90	D	1.11	1.22	475:25:3:6	AC
AQC-Glu	7.07	L	1.15	1.77	400:100:2:10	SN
AQC-Tyr	9.84	D	1.14	1.86	450:50:2:6	β
	5.02	D	1.15	2.00	470:30:4:6	AC
	6.67	D	1.14	1.62	475:25:4:6	HP
AQC-Pro	4.02	D	1.26	1.94	475:25:1.5:6	α
	3.05	D	1.23	2.83	475:25:2:6	β
	1.77	D	1.18	2.63	450:50:3:6	γ
	1.24	D	1.28	3.33	450:50:4:6	AC
	2.34	D	1.23	2.17	450:50:3:6	SN
AQC-Ile[d]	6.63	D *allo* 7.59 D	7.90 L *allo*	9.72 L	475:25:2:6	β
	6.06	6.70	7.23	8.21	475:25:3:2	RN

[a] k' = Capacity factor for the first eluted enantiomer.
[b] Mobile phases are mixtures of acetonitrile–methanol–acetic acid–triethylamine by volume (v/v).
[c] Columns α, β, γ, RSP, AC, SN, RN stand for α-, β-, γ-, *R,S*-2-hydroxypropyl, acetylated β-, *S*-naphthylethyl-carbamated, *R*-naphthylethyl-carbamated, cyclodextrin bonded stationary phases.
[d] Only k' parameter is shown.

modifiers and by addition of small amounts (2–20%, v/v) of methanol (which reduces the retention time of the analyte to practical levels, if necessary). As can be seen from the results in Table I the AQC derivatives of primary as well as secondary amino acids are easily separated into enantiomers on different bonded chiral stationary phases using the non-aqueous acetonitrile–methanol–acetic acid–triethylamine mixture as eluent. Figs. 2 and 3 show enantioseparations of AQC derivatives of primary and secondary amino acids obtained on different cyclodextrin bonded phases. It should be noted that in previous work, the resolution of the FMOC derivatized nipecotic acid was not possible [4]. However, as shown in Fig. 3, it is easily resolved as the AQC derivative on a γ-cyclodextrin column.

Table I gives the separation data for 15 common protein amino acids. Table II gives analogous data for 16 additional amino acids. In general, one or more columns were found that provided greater than baseline resolution ($R_s > 1.5$). Moreover, as indicated in Tables I and II, the D-enantiomer was eluted prior to the L-enantiomer in all cases except two (His and Glu). Consequently this should be a sensitive and accurate method for the determination of trace level of D-amino acids in the presence of high levels of L-enantiomers [4,12,13].

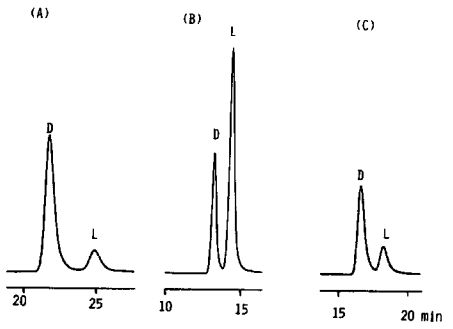

Fig. 2. Enantiomeric resolution of AQC functionalized primary amino acids obtained under optimal experimental conditions in the non-aqueous system. (A) Test compound: AQC-Phe; stationary phase: β-cyclodextrin; eluent acetonitrile–methanol–acetic acid–triethylamine (450:25:3:6, v/v). (B) Test compound: AQC-Met; stationary phase: HP-β-cyclodextrin; eluent acetonitrile–methanol–acetic acid–triethylamine (475:50:2:6, v/v). (C) Test compound: AQC-Trp; stationary phase: β-cyclodextrin; eluent acetonitrile–methanol–acetic acid–triethylamine (470:30:4:6, v/v). All columns were 250 × 4.6 mm, the flow-rate was 1 ml/min and fluorescent detection was used (see Experimental).

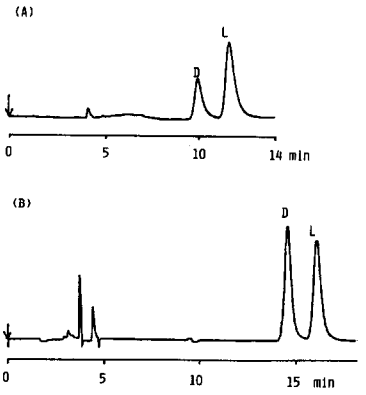

Fig. 3. Enantiomeric resolution of AQC functionalized secondary amino acids obtained under optimal experimental conditions in the nonaqueous system. (A) Test compound: AQC-Pro; stationary phase: SN-β-cyclodextrin; eluent acetonitrile–methanol–acetic acid–triethylamine (470:30:3:6, v/v). (B) Test compound: AQC-nipecotic acid; stationary phase: γ-cyclodextrin; eluent acetonitrile–methanol–acetic acid–triethylamine (450:50:3:12, v/v).

It was found previously that chiral recognition of FMOC-derivatized amino acids was dependent on water concentration in the mobile phase [4,5]. Similar behavior was observed in this study for AQC-derivatized amino acids. Fig. 4 shows the change of retention characteristics of AQC-D,L-Leu obtained on the β-cyclodextrin column caused by addition of water to the mobile phase. Both methanol and water diminish the hydrogen bonding interaction between the solute and the cyclodextrin due to the competitive adsorption and solvation effects of these molecules. Hydrogen bonding solvents have considerable influence on both retention and enantioselectivity. The substitution of as little as 5% (v/v) water for methanol causes a significant decrease in the retention and selectivity (Fig. 4B). Further addition of water to the mobile phase negated the enantioselectivity exhibited by native cyclodextrin stationary phases towards almost all amino acids investigated. The only exception found was for AQC-Leu (on the β-cyclodextrin column) which was partially resolved under reversed-phase conditions as shown in Fig. 4C. Unlike the native cyclodextrin bonded phases, the deriva-

TABLE II

CHROMATOGRAPHIC DATA FOR THE ENANTIORESOLUTION OF AQC DERIVATIVES OF OTHER AMINO ACIDS AND THEIR DERIVATIVES ON CYCLODEXTRIN BONDED STATIONARY PHASES USING NON-AQUEOUS POLAR MOBILE PHASES

Compound	k' [a]	Configuration	α	R_s	Mobile phase[b]	Column[c]
Nipecotic acid	4.94		1.10	1.46	450:50:3:2	β
	3.82		1.13	2.26	450:50:3:2	γ
	1.70		1.30	1.35	450:50:1.5:6	AC
AQC-Norval	5.20	D	1.16	2.55	475:25:2:6	β
	2.22	D	1.13	2.02	470:30:4:6	AC
	2.08	D	1.30	2.70	450:50:4:6	RN
AQC-Norleu	3.90	D	1.20	2.31	450:50:2:10	β
	2.64	D	1.16	2.52	470:30:4:6	AC
	3.29	D	1.25	2.75	475:25:4:6	RSP
	2.43	D	1.80	5.95	450:50:4:6	RN
AQC-Homophe	12.14	D	1.28	3.78	475:25:2:6	β
	4.42	D	1.15	1.95	475:25:4:6	γ
	5.00	D	1.29	3.91	470:30:4:6	AC
	5.61	D	1.45	4.57	475:25:4:6	RSP
	7.20	D	1.58	8.54	450:50:3:6	SN
AQC-α-Methyl-Phe	1.92		1.14	1.65	475:25:3:6	AC
AQC-α-Methyl-*m*-methoxy-Phe	3.20		1.09	1.42	450:50:3:2	γ
AQC-α-Amino-*R,S*-Phe-acetic acid	5.36	*R*	1.09	1.47	450:50:3:2	β
AQC-*p*-Nitro-Phe	3.17		1.33	1.77	450:50:2:10	RN
AQC-*o*-Methyl-Tyr	2.22	D	1.13	1.72	450:50:1.5:6	AC
AQC-*m*-Tyr	4.37		1.29	2.40	450:50:2:4	RN
AQC-α-Methyl-*m*-Tyr	2.20		1.12	1.60	475:25:5:6	AC
AQC-*o*-Tyr	3.53		1.16	1.45	460:40:5:3	RN
AQC-6-Fluoro-Trp	8.58		1.10	1.56	450:50:3:2	β
AQC-5-Hydroxy-Trp	6.10		1.18	1.86	450:50:2:4	RN
AQC-7-Methyl-Trp	7.80		1.08	1.43	450:50:3:2	γ
AQC-β-Aminoisobutyric acid	5.85		1.10	1.42	475:25:4:6	β

[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 α, β, γ, RSP, AC, SN, RN stand for α-, β-, γ-, *R,S*-2-hydroxypropyl, acetylated β-, *S*-naphthylethyl-carbamated, *R*-naphthylethyl-carbamated, cyclodextrin bonded stationary phases.

tized cyclodextrin phases exhibited enantioselectivity towards some of the AQC functionalized amino acids in water-rich systems. Table III gives retention data for a number of AQC amino acids obtained on *R,S*-hydroxypropyl derivatized β-cyclodextrin (RSP-β-CD) in the reversed-phase mode. Fig. 5 shows the change of elution order for enantioseparation of DL-Leu on the same RSP-β-cyclodextrin column when operated with nonaqueous and aqueous eluents. As can be seen from the comparison of the results given in Table I and II *vs.* Table III the change in the

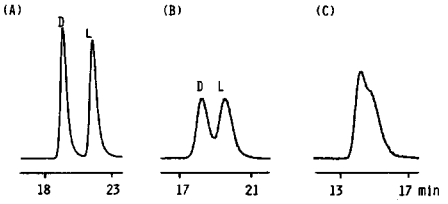

Fig. 4. The influence of the water concentration in the eluent on enantiomeric resolution of AQC-Leu. (A) Eluent: acetonitrile–methanol–acetic acid–triethylamine (475:25:3:6, v/v). (B) Eluent: acetonitrile–triethylammonium acetate (pH 7.1) (95:5, v/v) buffer. (C) Eluent: acetonitrile–triethylammonium acetate (pH 7.1) buffer (5:95, v/v). A β-cyclodextrin column (250 × 4.6 mm) was used. The flow-rate was 1 ml/min. UV detection was used.

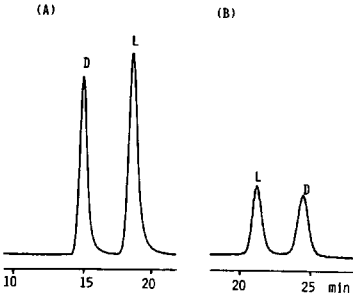

Fig. 5. Enantiomeric resolution of AQC-Leu obtained on a *R,S*-hydroxypropyl derivatized β-cyclodextrin (RSP) column. (A) Non-aqueous mobile phase: acetonitrile–methanol–acetic acid–triethylamine (475:25:3:6, v/v). (B) Reversed-phase mode with a mobile phase consisting of acetonitrile–triethylammonium acetate buffer (5:95, v/v). Notice that elution order has been reversed between two modes. The column was 250 × 4.6 mm. The flow-rate was 1 ml/min and fluorescent detection was used.

operation mode caused changes in elution order for most amino acids resolved with histidine being the only exception.

The retention behavior found in this study for AQC-functionalized amino acids is very similar to that reported previously for FMOC derivatives. In both cases there is little chiral recognition of derivatized amino acids on the native bonded phases under reversed-phase conditions, but the derivatized cyclodextrin phases exhibit enantioselectivity when operated with non-aqueous eluents as well as in the reversed-phase mode.

However, there is a significant difference in the chiral recognition exhibited by native cyclodextrin bonded phases towards FMOC and AQC derivatized amino acids in non-aqueous systems. As shown in Table I AQC derivatives can be easily resolved on native cyclodextrin bonded phases, which was not the case for FMOC-amino acids derivatives. The similarities and differences in retention behavior on the different cyclodextrin phases observed for FMOC- and AQC-functionalized amino acids confirm the chiral recognition mechanism postulated previously [5]. Briefly, this was that inclusion with the hydrophobic fluorescent group

TABLE III

CHROMATOGRAPHIC DATA FOR SEPARATION OF RACEMIC AQC-AMINO ACIDS ON CYCLODEXTRIN BONDED STATIONARY PHASES USING AQUEOUS MOBILE PHASES

Column: *R,S*-2-hydroxypropyl cyclodextrin bonded stationary phase.

Compound	k'	Configuration	α	R_s	Mobile phase[a]
AQC-His	2.23	L	1.08	1.02	90:10
AQC-Leu	2.37	L	1.15	1.76	90:10
AQC-Met	5.07	L	1.15	2.14	95:5
AQC-Val	4.81	L	1.16	1.74	95:5
AQC-Ala	3.38	L	1.10	1.13	95:5
AQC-Ser	2.71	L	1.07	0.88	95:5
AQC-Homoser	2.80	L	1.16	1.86	95:5
AQC-Ile[b]	5.32	D *allo* 5.57 D	5.57	L *allo* 5.82 L	95:5

[a] Mobile phase is a ratio of buffer to acetonitrile (v/v) and buffer is 1% pH 7.1 triethylammonium acetate.
[b] Only the k' for the first eluted enantiomer is shown.

(in the reversed-phase mode) resulted in good retention but poor enantioselectivity (because of insufficient polar interactions between the cyclodextrin hydroxyls and the hydrogen bonding portions of the amino acid) [4,5]. However, when using the polar organic mobile phase (in which most of the solvent is non-hydrogen-bonding acetonitrile) hydrogen bonding interactions are dominant and inclusion is minimized. In the case of AQC-derivatized amino acids there seems to be two benefits. First the smaller AQC moiety does not sterically interfere with hydrogen bonding interactions (as FMOC sometimes does) and the 6-aminoquinolyl group itself contains two amino hydrogen bonding groups as well.

The derivatization of cyclodextrin influences significantly the enantioselectivity exhibited by the cyclodextrin phases in the aqueous as well as non-aqueous systems. Both the cyclodextrin moiety and the substituent can influence the chiral recognition, which results in unique selectivities. Under reversed-phase conditions the mechanism for enantioselectivity with the derivatized cyclodextrin bonded phase is thought to involve not only inclusion complex formation but also additional interactions between the analyte and cyclodextrin substituents. The fact that native cyclodextrins could not resolve amino acids derivatives in the reversed-phase mode indicates that the major contribution to the overall enantioselectivity of the derivatized cyclodextrin stationary phases is caused by the additional interaction of the analyte with the cyclodextrin substituent.

In non-aqueous systems the chiral recognition may arise from stereoselective hydrogen bonding between donor and acceptor sites of the analyte with the residual secondary hydroxyl groups as well as other polar moieties at the mouth of the cyclodextrin cavity. On carbamoylated β-cyclodextrin stationary phases, $\pi-\pi$ interactions between the aromatic substituents on the cyclodextrin moiety and hydrophobic part of the chiral solutes are also possible. The discussion above is supported by the change in peak symmetry observed when changing the operation mode (and chiral recognition mechanism) from non-aqueous to aqueous systems. The measured peak symmetry for both FMOC- and AQC-amino acids in water-free systems was different than that found in the reversed-phase mode. As can be seen from the data presented in Table IV the change of operation mode to reversed-phase conditions improved the symmetry of the eluted peaks for all solutes investigated.

High symmetry factors have been found for FMOC-Pro on RN-β-cyclodextrin column as well as for AQC-Met and AQC-Val on HP-β-cyclodextrin ($a \approx 1$) under reversed-phase conditions; the peak symmetry, a, was determined according to ref. 14 at 1/10 peak height, values <1 indicate tailing. It indicates that the adsorption (binding) sites are essentially of a single type (homogeneous in the adsorption energies) [15,16]. The symmetry observed for AQC-Leu on HP-β-cyclodextrin is significantly lower than that found for AQC-Met and AQC-Val on the same column. The AQC-Leu as shown above in Fig. 4C was the only exception in that it could be slightly resolved into enantiomers on a native β-cyclodextrin column. These findings suggest that in this case both the hydroxypropyl substituents and free hydroxyl groups at the mouth of the cyclodextrin cavity contribute to the chiral recognition.

In water-free systems, where steric discrimination of enantiomers has been achieved due to the external complex formation between the solute and cyclodextrin molecule, several types of adsorption (binding) conformations are possible. They would differ essentially in the accessibility for the analyte and in the type and strength of the interaction with the analyte, which may result in observed symmetry.

Advantages and practical application of the method

Labeling with AQC-reagent converts amino acids into solutes with favorable chromatographic properties. In addition, these derivatives can be resolved into enantiomers on different bonded cyclodextrin phases. AQC reacts rapidly with both primary and secondary amino groups. The by-products of the derivatization reaction are eluted from the cyclodextrin columns close to the dead volume regardless of whether reversed phase or polar organic mobile phases are used.

TABLE IV

COMPARISON OF PEAK SYMMETRY FOR FMOC AND AQC FUNCTIONALIZED AMINO ACIDS OBTAINED ON DERIVATIZED CYCLODEXTRIN BONDED STATIONARY PHASES IN NON-AQUEOUS AND AQUEOUS SYSTEMS

Solute	Non-aqueous system			Aqueous system		
	Peak symmetry[a]		Chromatographic conditions	Peak symmetry[a]		Chromatographic conditions
	D	L		D	L	
FMOC-Pro[b]	0.66	0.55	RN-β-CD[c]	0.98	0.95	RN-β-CD[d]
AQC-Leu	0.38	0.40	*R,S*-HP-β-CD[e]	0.83	0.63	*R,S*-HP-β-CD[f]
AQC-Met	0.78	0.73	*R,S*-HP-β-CD[e]	1.00	0.94	*R,S*-HP-β-CD[f]
AQC-Val	0.75	0.70	*R,S*-HP-β-CD[g]	0.96	0.93	*R,S*-HP-β-CD[f]

[a] The peak symmetry was determined according to ref. 14 at 1/10 of the peak height, values <1 indicate tailing.
[b] Taken from ref. 4.
[c] Acetonitrile–triethylamine–glacial acetic acid (1000:6:4, v/v). CD = Cyclodextrin.
[d] Water–acetonitrile–triethylamine–glacial acetic acid (850:150:6:4, v/v).
[e] Acetonitrile–methanol–triethylamine–glacial acetic acid (950:50:12:6, v/v).
[f] Acetonitrile–triethylamine acetate buffer (5:95, v/v).
[g] Acetonitrile–methanol–triethylamine–glacial acetic acid (950:50:12:80, v/v).

As shown in Figs. 2 and 3 there is no interference when using fluorescence or UV detection.

Incorporation of the highly fluorescent aminoquinolyl group to amino acids enables high-sensitivity detection. As has been reported recently [10] detection limits for the amino acids in reversed-phase HPLC system range from 40–300 fmol. According to our previous work [4], the applicability of non-aqueous eluents with conjunction with cyclodextrin phases offers several

TABLE V

COMPARISON OF THE DETERMINATION OF ENANTIOMERIC PURITY OF COMMERCIAL "PURE" L-AMINO ACIDS STANDARDS AND THE DETERMINATION OF ENANTIOMERIC RATIO IN THE D,L-MIXTURE USING TWO PRE-COLUMN DERIVATIZING AGENTS: AQC AND FMOC-Gly-Cl

Name	Source	Derivatizing agent			
		AQC		FMOC Gly Cl	
		%D (S.D.)	Chromatographic conditions	%D (S.D.)	Chromatographic conditions
L-Leucine	Sigma	0.049 (0.006)	RN-β-CD[a]	0.045 (0.013)	γ-CD[b]
L-Phenylalanine	Aldrich	<0.0075	RN-β-CD[c]	<0.0075	β-CD[b]
L,D-Phenylalanine	The artificial mixture	4.070 (0.096)		3.8994 (0.065)	

[a] Acetonitrile–methanol–triethylamine–glacial acetic acid (900:100:16:4, v/v).
[b] Acetonitrile–triethylamine–glacial acetic acid (1000:12:3, v/v).
[c] Acetonitrile–methanol–triethylamine–glacial acetic acid (900:100:18:4, v/v).

advantages over water-rich systems including: faster equilibration of the column, more stable base line and more sensitive fluorescence detection due to the lack of quenching effects which occur in aqueous solutions. Consequently we were able to detect as little as 10 fmol of many of the AQC amino acids. In one case as little as 0.0075% of D-Phe in a large excess of L-enantiomer (for $k' = 2.35$) could be determined.

Because of high sensitivity and selectivity this method can be used for trace and ultra-trace determination of enantiomeric impurities. Table V shows the results of an evaluation of enantiomeric purity for L-Phe and L-Leu standards. Approximately 0.049% of D-Leu was found and quantified with a precision of 12.2% (S.D. for 4 measurements). Table V also shows the comparison data for enantiomeric trace analysis obtained after pre-column derivatization with AQC and FMOC-Gly-Cl reagents. As can be seen, excellent agreement was achieved with these two methods. Moreover the results presented in Table V indicated that both of these derivatization reactions proceed without detectable racemization. No change in enantiomeric ratio for AQC-Phe (D,L artificial mixture, see Table V) and AQC-Leu (an L standard, see Table V) was found during a 7-day period (determined both at 21°C and −5°C).

It appears that the new AQC reagent is a very effective "tagging agent" for amino acids. It causes no detectable racemization if used properly. The AQC derivatives have excellent chromatographic properties on cyclodextrin bonded phases. This, along with their spectroscopic properties and stability, make them very useful for determining trace levels of D-enantiomers in an excess of the more common L-amino acids.

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