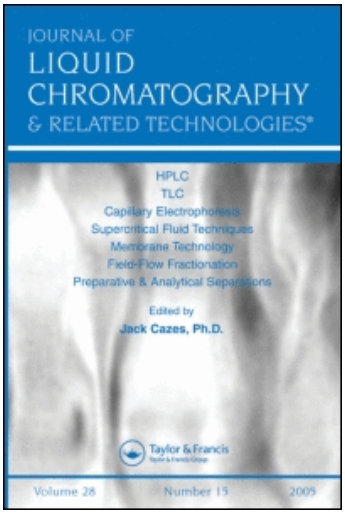

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LC Enantiomeric Separation of Unusual Amino Acids Using Cyclodextrin-based Stationary Phases

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LC Enantiomeric Separation of Unusual Amino Acids Using Cyclodextrin-based Stationary Phases

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AbstractThe use of cyclodextrin based stationary phases was investigated for the enantiomeric separation of 20 unusual amino acids. Mobile phase, pH effects, and flow rate were optimized for each separation. Separations were limited to aqueous mobile phases. Nineteen of the amino acids were separated, with seven having a resolution $\geq$1.5. The highest selectivities came from the alpha, acetylated beta, and 2,6-dinitrophenyl-4-trifluoromethylphenyl derivitized beta-cyclodextrin stationary phases. Amino acids containing a 1,2,3,4 tetrahydroisoquinoline carboxylic acid structure showed great compatibility with the acetylated beta-cyclodextrin. Tyrosine analogues, due to lack of retention, were not well suited to the cyclodextrin stationary phases.

Keywords: HPLC, Cyclodextrin stationary phases, Enantiomeric separation, Amino acids

INTRODUCTION

The need for enantiomerically pure compounds is an increasingly growing concern for active research in pharmacology, biochemistry, etc. Specifically, enantiomerically pure amino acids are needed for peptide synthesis and for the use of pharmocons, among other uses. These synthetic, non-protein amino acids are prepared as racemic mixtures or through the use of asymmetric synthesis, where one enantiomer is produced in excess. In the first case, both analytical and preparative separation

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techniques may be needed, while in the synthetic approach analytical methods alone may suffice.

Previous works in using chromatography for the enantiomeric separation of amino acids includes the use of chiral crown ether stationary phases[1–5] and chiral ligand exchange chromatography.[6–8] More recently, macrocyclic glycopeptide based chiral stationary phases have become the preferred method to separate amino acid enantiomers.[9] For example, most of the amino acids in this study have been separated using the ristocetin A and teicoplanin chiral stationary phases.[10,11] Cyclodextrin based selectors are used in another type of chiral stationary phase and have been successfully used for the enantiomeric separation of a wide array of compounds.[12–19] Cyclodextrins are toroidal shaped molecules consisting of linked gluco-pyranose units. α-, β-, γ-cyclodextrins consist of 6, 7, and 8 gluco-pyranose units, respectively. These molecules provide numerous sites of interaction (hydrogen bonding, steric effects, dipole-dipole interactions, etc.) that may lead to chiral recognition. The most successful native cyclodextrin stationary phase is the β-cyclodextrin, and several derivatives of this stationary phase have been commercialized.[12] Previous attempts at the separation of amino acids using cyclodextrin stationary phases have succeeded only with aromatic amino acids or derivitized amino acids.[20–22]

The purpose of this study was to determine whether or not natural and derivitized cyclodextrin based stationary phases could separate enantiomers of synthetic, non-proteinic amino acids without prior derivatization. Also, a newly available derivative of cyclodextrin (i.e., the 2,6-dinitrophenyl-4-trifluoromethylphenyl derivitized β-cyclodextrin) will be evaluated for the first time for amino acids. For each separation, the following parameters were optimized: flow rate, pH, chiral selector, and mobile phase.

EXPERIMENTAL

Chemicals

Amino acid names, nomenclature, and abbreviations are in agreement with the IUPAC-IUB JCBN recommendations.[23] Apart from **1**, phenylalanine (Phe), **9**, m-tyrosine (m-Tyr), and **11**, α-methyltyrosine (α-MeTyr), which were obtained from Sigma-Aldrich (St. Louis, MO), the amino acids were synthesized using literature methods. These amino acids include **2**, 2′-methylphenylalanine (2′-MePhe),[24] **3**, 4′-methylphenylalanine (4′-MePhe),[25] **4**, 4′-methoxyphenylalanine (4′-MeOPhe),[25] **5**, 2′6′-dimethylphenylalanine (2′6′-diMePhe),[24] **6**, α-methylphenylalanine (α-MePhe),[26] **7**, *erythro*-(2*S*,3*S* and 2*R*,3*R*)-β-methylphenylalanine (*erythro*-β-MePhe),[27] **8**, *threo*-(2*S*,3*R* and 2*R*,3*S*)-β-methylphenylalanine (*threo*-β-MePhe),[27] **10**, 2′6′-dimethyltyrosine (2′6′-diMeTyr),[28] **12**,

erythro-(2*S*,3*S* and 2*R*,3*R*)-β-methyltyrosine (*erythro*-β-MeTyr),[27] **13**, *threo*-(2*S*,3*R* and 2*R*,3*S*)-β-methyltyrosine (*threo*-β-MeTyr),[27] **14**, 1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid (Tic1),[29] **15**, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic3),[30] **16**, 5′-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (5′-MeTic3),[24] **17**, 6′-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (6′-HO-Tic3),[30] **18**, 7′-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (7′-HO-Tic3),[30] **19**, *erythro*-(2*S*,3*S* and 2*R*,3*R*)-4-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (*erythro*-β-MeTic3),[30] and **20**, *threo*-(2*S*,3*R* and 2*R*,3*S*)-4-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (*threo*-β-MeTic3).[30]

Solvents used for the mobile phase were of HPLC grade and were purchased from VWR (West Chester, PA). A 0.1% buffer was created using HPLC grade triethylamine titrated with ACS grade glacial acetic acid, both of which were acquired from VWR. For aqueous mobile phases, Milli-Q water was employed. Mobile phases were degassed by sonication under vacuum for 1 minute per 100 mL of solvent. Analytes were dissolved in Milli-Q water to give an approximately 1 mg/mL solution.

Equipment

Separations were carried out on a Hewlett Packard 1050 series HPLC using a variable wavelength UV detector. The detection wavelength was set at either 254 or 265 nm. The pump was an LC-6A model from Shimadzu. All separations were carried out at ambient temperature (~22°C).

The cyclodextrin based stationary phases were obtained from Advanced Separations Technologies (Whippany, NJ). The stationary phases evaluated were Cyclobond I (β-cyclodextrin), Cyclobond II (γ-cyclodextrin), Cyclobond III (α-cyclodextrin), Cyclobond I AC (having acetylated β-cyclodextrin), Cyclobond I DM (dimethylated β-cyclodextrin), Cyclobond I DMP (3,5-dimethylphenylcarbamate derivitized β-cyclodextrin), Cyclobond I DNP (2,6-dinitro-3-trifluoromethylphenyl derivitized β-cyclodextrin), Cyclobond I RN (naphtylethyl carbamate derivitized β-cyclodextrin), and Cyclobond I SP (hydroxylpropyl derivitized β-cyclodextrin).

Calculations

The retention factor, (k'), was calculated by $(t_r - t_0)/t_0$, where t_r is the retention time of the eluted peak, and t_0 is the dead time. The selectivity factor, (α), was calculated by k'_2/k'_1, where k'_2 and k'_1 are the retention factors of the second and first eluted enantiomer peaks, respectively. Finally, resolution, (R_s), was calculated by $2(t_2 - t_1)/w_{b1} + w_{b2}$, where t_2

and t_1 are the retention times of the second and first eluted enantiomers, respectively, and w_{b1} and w_{b2} are the base width of the peaks.

RESULTS AND DISCUSSION

Figure 1 shows a retention curve for compound **15** Tic3 on the Cyclobond III stationary phase using water and acetonitrile. The compound shows a U shaped retention curve that is characteristic of amino acids, due to their low solubility in organic solvents. Studying the curve, it seems optimistic to believe that separations could occur in a polar organic mobile phase. However, due to their low solubilities, the amino acids gave very broad and abnormally shaped peaks in non-aqueous mobile phases. Also, for the column used for providing the retention curve (Cyclobond III), all enantioselectivity was lost with the addition of only 10% acetonitrile. Therefore, the separations were limited to the reversed phase mode using a 100% aqueous mobile phase.

Different pH values gave a slight difference in retention time, with the optimal selectivity coming in the pH range of 4.0–5.0. The use of this buffer always gave slightly higher efficiency than a non-buffered mobile phase, possibly due to the protection of the amino acids from adsorption to the free silanol groups or to adsorption sites on the linkage chain.

All stationary phases previously mentioned in the experimental section were tested, but it was found that one of three chiral selectors (Cyclobond III, Cyclobond I DNP, or Cyclobond I AC) gave the best selectivity for each of these amino acids. Data from each of these stationary phases, as well as other stationary phases that showed enantioselectivity, are given in Table 1.

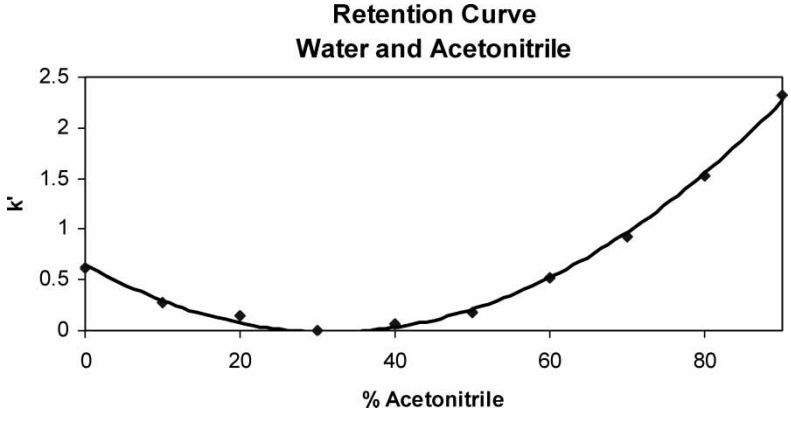
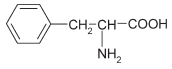
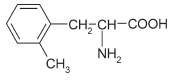
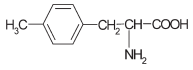
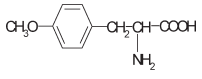
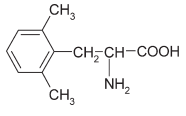
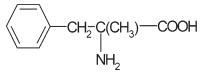


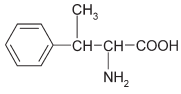
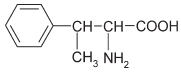
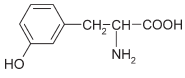
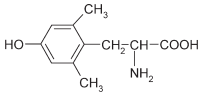
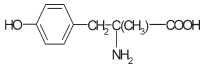
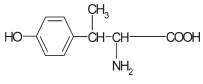
Figure 1. Retention curve for compound **15** Tic 3. At high levels of H_2O the acetonitrile acts an organic modifier and elutes the compound. At low aqueous levels, the compound is less soluble in the mobile phase, and thus retains longer on the stationary phase.

Table 1. Retention factors (k), selectivity factors (α), and resolution (R_s) for unusual amino acids using cyclodextrin based stationary phases

S. No	Compound	Stationary phase	k'_1	k'_2	α	R_s	Flow rate (mL/min)
1	Phe 	Cyclobond I AC	0.58	0.63	1.09	0.80	0.5
		Cyclobond III	0.48				1.0
		Cyclobond I DNP	1.68				1.0
2	2′-MePhe 	Cyclobond I DNP	1.65	1.80	1.09	0.80	0.5
		Cyclobond III	0.56		1.00		1.0
		Cyclobond I AC	0.62		1.00		1.0
3	4′-MePhe 	Cyclobond I AC	1.34	1.46	1.09	0.80	0.5
		Cyclobond III	2.04		1.00		1.0
		Cyclobond I DNP	6.72		1.00		1.0
4	4′-MeOPhe 	Cyclobond III	1.91	2.28	1.19	1.60	1.0
		Cyclobond I DNP	6.50	6.85	1.05	0.50	1.0
		Cyclobond I AC	1.38		1.00		1.0
5	2′,6′-diMePhe 	Cyclobond I DNP	2.42	3.15	1.30	2.10	1.0
		Cyclobond I	0.64	0.71	1.12	1.30	1.0
		Cyclobond III	0.50	0.59	1.18	1.30	1.0
		Cyclobond I AC	0.63		1.00		1.0
6	α-MePhe 	Cyclobond I AC	0.55	0.61	1.11	0.80	0.5
		Cyclobond III	0.50	0.53	1.05	0.50	1.0
		Cyclobond I DNP	1.79		1.00		1.0

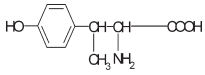
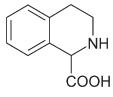
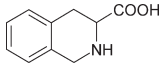
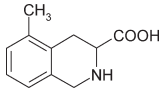
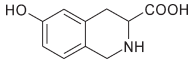
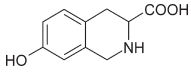
(*continued*)

Table 1. Continued

S. No	Compound	Stationary phase	k'_1	k'_2	α	R_s	Flow rate (mL/min)
7	*erythro*-β-MePhe 	Cyclobond I DNP	1.05	1.07	1.02	0.40	0.5
		Cyclobond III	0.50		1.00		1.0
		Cyclobond I AC	0.39		1.00		1.0
8	*threo*-β-MePhe 	Cyclobond I AC	0.62	0.73	1.18	1.40	0.5
		Cyclobond III	0.51	0.62	1.21	1.25	1.0
		Cyclobond I DNP	1.35		1.00		1.0
9	meta-Tyr 	Cyclobond I AC	0.20	0.21	1.05	0.40	0.5
		Cyclobond I DNP	0.50		1.00		1.0
		Cyclobond III	0.11		1.00		1.0
10	2′,6′-diMeTyr 	Cyclobond I DNP	0.86	0.99	1.19	1.10	0.5
		Cyclobond I AC	0.14		1.00		1.0
		Cyclobond III	0.13		1.00		1.0
11	α-MeTyr 	Cyclobond I AC	0.17		1.00		1.0
		Cyclobond I DNP	0.60		1.00		1.0
		Cyclobond I III	0.13		1.00		1.0
12	*erythro*-β-MeTyr 	Cyclobond III	0.16	0.19	1.19	0.50	0.5
		Cyclobond I AC	0.18		1.00		1.0
		Cyclobond I DNP	0.50		1.00		1.0

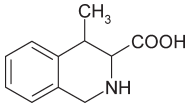
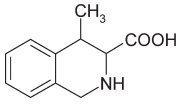
(*continued*)

Table 1. Continued

S. No	Compound	Stationary phase	k'_1	k'_2	α	R_s	Flow rate (mL/min)
13	*threo*-β-MeTyr 	Cyclobond I AC	0.23	0.28	1.22	0.60	0.5
		Cyclobond I DNP	0.57		1.00		1.0
		Cyclobond III	0.21		1.00		1.0
14	Tic-1 	Cyclobond I DNP	1.52	1.83	1.20	1.60	0.5
		Cyclobond I	0.60	0.65	1.08	0.60	1.0
		Cyclobond III	0.75	0.80	1.06	0.50	1.0
		Cyclobond I AC	0.50		1.00		1.0
15	Tic-3 	Cyclobond I AC	0.80	1.05	1.31	3.20	1.0
		Cyclobond I DNP	1.40		1.00		1.0
		Cyclobond III	0.85		1.00		1.0
16	5′-MeTic-3 	Cyclobond I AC	0.95	1.05	1.11	1.21	0.5
		Cyclobond I DNP	3.76	4.28	1.14	0.50	1.0
		Cyclobond III	0.99		1.00		1.0
17	6′-OHTic-3 	Cyclobond I AC	0.34	0.43	1.27	1.50	0.5
		Cyclobond I DNP	0.82		1.00		1.0
		Cyclobond III	0.24		1.00		1.0
18	7′-OHTic-3 	Cyclobond I AC	0.34	0.53	1.56	1.70	0.5
		Cyclobond I DNP	0.82		1.00		1.0
		Cyclobond III	0.22		1.00		1.0

(*continued*)

Table 1. Continued

S. No	Compound	Stationary phase	k'_1	k'_2	α	R_s	Flow rate (mL/min)
19	*erythro*-β-MeTic-3 	Cyclobond I AC	0.98	1.06	1.08	1.10	0.5
		Cyclobond I RSP	0.88	0.95	1.06	0.70	1.0
		Cyclobond I DNP	2.80	2.95	1.05	0.40	1.0
		Cyclobond III	0.87		1.00		1.0
20	*threo*-β-MeTic-3 	Cyclobond I AC	1.06	1.66	1.56	3.50	1.0
		Cyclobond I	1.34	1.53	1.14	1.50	1.0
		Cyclobond I DNP	3.10	3.44	1.11	1.30	1.0
		Cyclobond I DMP	1.93	2.15	1.12	1.30	1.0
		Cyclobond III	0.86		1.00		1.0

The retention data indicates that Cyclobond I DNP gives significantly longer retention than any of the other stationary phases. This is likely due to the stationary phase being derivatized with a π-acidic moiety, leading to increased π-π interactions between the stationary phase and the analytes. None of the other stationary phases are derivatized with such a π-acidic group. This unique capability of increased retention gives this stationary phase the ability to achieve multiple enantiomeric separations on the same chromatographic run, something that the other cyclodextrin stationary phases cannot do as effectively with these analytes. Figure 2 shows an enantiomeric separation of compounds **5**, **10**, and **14**. Note that compound **5** is a phenylalanine analogue, **10** is a tyrosine analogue, and **14** is a "Tic" compound.

Another retention related observation involves the tyrosine based compounds as compared to the phenylalanine based analogues. Several phenylalanine-tyrosine pairs (**5** and **10**, **6**, and **11**, **7**, and **12**, **8**, and **13**) only differ by the para-substituted hydroxyl group on the aromatic ring. In these pairs, it can be seen that the k'_1 of the phenylalanine analogues are at least twice that of the tyrosine based compounds. This points towards the fact that the hydroxyl group is responsible for the decrease in retention. Generally, in reverse phase mode, the hydrophobic part of the analyte will occupy the cavity of the cyclodextin, forming an inclusion complex.[31]

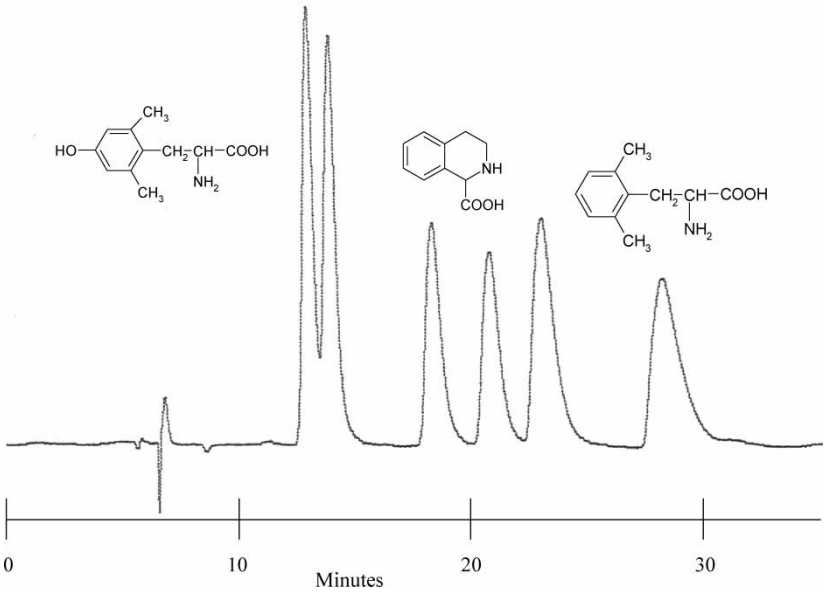


Figure 2. Chromatographic run on the Cyclobond I DNP stationary phase. Mobile phase is 100% water with 0.1% TEAA buffer. The longer retention times for this chiral selector allowed for the enatiomeric separation of multiple compounds, as show here for 2′6′diMeTyr,Tic-1, and 2′6′diMePhe.

Obviously, the addition of a hydroxyl group will make the phenyl ring more hydrophilic, thus leading to a weaker inclusion complex, which in turn leads to decreased retention. Adding this to the fact that amino acids are polar to begin with, it can easily be understood why the tyrosine based group of compounds were more poorly separated than all the other analytes in the study, with only partial separations occurring and with no observed separation for compound **11**, α-MeTyr.

Perhaps the most striking observation is the high enantioselectivity of the "Tic" compounds using the acetylated cyclodextrin (Cyclobond I AC) as compared to the other amino acids. Six of the seven "Tic" compounds were separated using Cyclobond I AC, with five of these being baseline separations ($R_s \geq 1.5$). This is more than likely the combination of several key factors. First, the acetyl groups extend the mouth of the cyclodextrin, creating a larger cavity into which the larger "Tic" compounds can more effectively fit.[12] Also, the "Tic" compounds themselves are more rigid than the phenylalanine or tyrosine analytes, which will lead to better chiral recognition (as compounds that have groups that can rotate freely about the stereogenic carbon generally have lower enantioselectivities). Lastly, the acetyl groups also can act as hydrogen bond acceptors, giving another point of interaction for chiral recognition. This H-bond acceptor site may be especially suitable

for the amine, as the amine is more than likely to be situated around the rim of the cyclodextrin cavity, near the location of the acetyl group(s).[31,32]

CONCLUSIONS

Cyclodextrin based stationary phases can provide quick separations of 19 of the 20 non-protein amino acids, with seven having baseline separations. No derivatization step was necessary. A 100% aqueous mobile phase was needed to provide adequate retention for the separations, as the addition of an organic modifier led to decreased enantioselectivity. Limited results came from the tyrosine analogues, while the greatest success came from the separation of 1,2,3,4 tetrahydroisoquinoline carboxylic acids on the acetylated β-cyclodextrin stationary phase. This work confirmed the conclusions of previous studies, in that mainly aromatic amino acids can be separated on cyclodextrin based chiral stationary phases, and that more rigid compounds separate better than compounds that have higher degrees of rotational freedom.

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