Amino Acids (2012) 42:1371–1378
DOI 10.1007/s00726-011-0832-3

ORIGINAL ARTICLE

Application of cyanuric chloride-based six new chiral derivatizing reagents having amino acids and amino acid amides as chiral auxiliaries for enantioresolution of proteinogenic amino acids by reversed-phase high-performance liquid chromatography

Ravi Bhushan · Shuchi Dixit

Received: 7 October 2010 / Accepted: 6 January 2011 / Published online: 19 January 2011

Abstract Six dichloro-*s*-triazine (DCT) reagents having L-Leu, D-Phg, L-Val, L-Met, L-Ala and L-Met-NH_2 as chiral auxiliaries in cyanuric chloride were introduced for enantioseparation of 13 proteinogenic amino acids. Four other DCTs and six monochloro-*s*-triazine (MCT) reagents having amino acid amides as chiral auxiliaries were also synthesized. These 16 chiral derivatizing reagents (CDRs) were used for synthesis of diastereomers of all the 13 analytes using microwave irradiation, which were resolved by reversed-phase high-performance liquid chromatography (RP-HPLC) using C18 column and gradient eluting mixture of aqueous TFA and acetonitrile with UV detection at 230 nm. It required only 60–90 s for derivatization using microwave irradiation. Better resolution and lower retention times were observed for the diastereomers prepared with CDRs having amino acids as chiral auxiliaries as compared to counterparts prepared with reagents having amino acid amides as chiral auxiliaries. As the best resolution of all the 13 analytes was observed for their diastereomers prepared using the DCT reagent having L-Leu as chiral auxiliary, this CDR was further employed for derivatization of Lys, Tyr, His and Arg followed by RP-HPLC analysis of resulting diastereomers. The results are discussed in light of acid and amide groups of chiral auxiliaries constituting CDRs, electronegativities of the atoms of achiral moieties constituting CDRs and hydrophobicities of side chains of amino acids constituting CDRs and analytes.

R. Bhushan (✉) · S. Dixit
Department of Chemistry, Indian Institute of Technology Roorkee, Roorkee 247667, India
e-mail: rbushfcy@iitr.ernet.in

Keywords Cyanuric chloride · Amino acids · Chiral derivatizing reagents · Reversed-phase high-performance liquid chromatographic separation · Enantioseparation

Introduction

The sequential and controlled substitution of chlorine atom(s) by nucleophiles in CC (cyanuric chloride; 2,4,6-trichloro-1,3,5-triazine; *s*-triazine chloride; trichloro-*s*-triazine) provides chiral monochloro-*s*-triazine (MCT) and dichloro-*s*-triazine (DCT) reagents. Brückner and coworkers synthesized chiral MCT reagents and used them for enantioseparation of a few selected amino acids by reversed-phase high-performance liquid chromatography (RP-HPLC) (Brückner and Strecker 1992; Brückner and Wachsmann 2003a, b). Enantioresolution of carbonyl compounds using tailor-made chiral derivatizing reagents (CDRs) based on CC has been reported in literature (Kempter et al. 2000). CC has also been applied to bind different amines or amino acids as chiral selectors on solid supports to prepare a series of chiral stationary phases (CSPs) for enantioresolution of amino acids and amino alcohols (Brückner and Wachsmann 1996; Chen and Lin 1995; Iuliano et al. 1997; Lecci and Iuliano 2005; Li et al. 2005; Lin et al. 1996a, b, c, 2001; Lin and Lin 1994; Lin and Yang 1993; Oi et al. 1984, 1996). Chiral separation of racemic mixtures of amino acids by means of micellar electrokinetic chromatography after derivatization with 3-(4,6-dichloro-1,3,5-triazinylamino)-7-dimethylamino-2-methylphenazine (DTDP) has also been reported (Ma et al. 2002).

Certain MCT and DCT reagents have been used as CDRs for indirect enantioresolution of α-amino acids (Bhushan and Agarwal 2010; Bhushan and Kumar 2008),

(*R*,*S*)-baclofen (Bhushan and Dixit 2010a) and (*R*,*S*)-mexiletine (Bhushan and Dixit 2010b) at this laboratory. The diastereomers, of compounds possessing amino groups, prepared with *s*-triazine reagents show structural similarities with those prepared with Marfey's reagent (Bhushan and Martens 2010).

Keeping in view the above cited literature, two new DCT reagents (having L-Met and L-Met-NH_2 as chiral auxiliaries) were synthesized and used along with four other DCT reagents (having L-Leu, D-Phg, L-Val, L-Ala as chiral auxiliaries) for the first time for enantioseparation of 13 proteinogenic amino acids. Besides, four DCT and six MCT reagents (Bhushan and Kumar 2008) were also used as CDRs in the present study to compare the separation efficiencies of the newly introduced CDRs. The DCT reagent having L-Leu as chiral auxiliary was further employed for derivatization of Lys, Tyr, His and Arg followed by RP-HPLC analysis of the resulting diastereomers. The separation results are discussed in the light of (a) acid and amide groups of chiral auxiliaries of CDRs, (b) electronegativities of the atoms of achiral moieties constituting CDRs and (c) hydrophobicities of side chains of amino acids constituting CDRs and analytes. To the best of authors' knowledge, this is the first report on microwave (MW)-assisted synthesis of diastereomers of DL-amino acids with the aforementioned 16 CDRs (Fig. 1) followed by their RP-HPLC resolution.

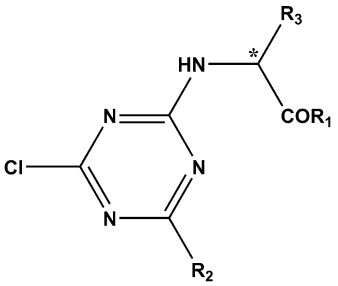
Experimental

Apparatus

The HPLC system consisting of a 10 mL pump head 1000, manager 5000 degasser, photodiode array detection (PDA) system 2600, manual injection valve, and Eurochrom operating software was from Knauer (Berlin, Germany). Other equipments used were Microwave-Multiwave 3000 (800 W, Perkin-Elmer, Shelton, CT, USA), pH meter Cyberscan 510 (Singapore, Singapore), Polarimeter P-3002 (Kruss, Hamburg, Germany), Milli-Q system of Millipore (Bedford, MA, USA), Perkin Elmer 1600 FT-IR spectrometer (Boardman, OH, USA), Vario EL III elementar analyzer, and Shimadzu UV-1601 spectrophotometer (spectra were recorded in acetonitrile). ^{1}H NMR spectra were recorded on a Bruker 500 MHz instrument using dimethyl sulfoxide (DMSO-d_6) as deuterated solvent.

Chemicals and reagents

All racemic and chirally pure amino acids, and chirally pure amino acid amides were obtained from Sigma-Aldrich (St Louis, MO, USA). All other analytical-grade chemicals and HPLC solvents were from E. Merck (Mumbai, India). Double-distilled water purified with a Milli-Q system (18.2 MΩ mL) was used throughout.



CDR	R_1	R_2	R_3
1, *N*-(4,6-Dichloro-[1,3,5]triazine-2-yl)- L-Leu	-OH	-Cl	$-CH_2CH(CH_3)_2$
2, *N*-(4,6-Dichloro-[1,3,5]triazine-2-yl)- D-Phg	-OH	-Cl	$-C_6H_5$
3, *N*-(4,6-Dichloro-[1,3,5]triazine-2-yl)- L-Val	-OH	-Cl	$-CH(CH_3)_2$
4, *N*-(4,6-Dichloro-[1,3,5]triazine-2-yl)- L-Met	-OH	-Cl	$-CH_2CH_2SCH_3$
5, *N*-(4,6-Dichloro-[1,3,5]triazine-2-yl)- L-Ala	-OH	-Cl	$-CH_3$
6, *N*-(4,6-Dichloro-[1,3,5]triazine-2-yl)- L-Leu-NH_2	$-NH_2$	-Cl	$-CH_2CH(CH_3)_2$
7, *N*-(4,6-Dichloro-[1,3,5]triazine-2-yl)- D-Phg-NH_2	$-NH_2$	-Cl	$-C_6H_5$
8, *N*-(4,6-Dichloro-[1,3,5]triazine-2-yl)- L-Val-NH_2	$-NH_2$	-Cl	$-CH(CH_3)_2$
9, *N*-(4,6-Dichloro-[1,3,5]triazine-2-yl)- L-Met-NH_2	$-NH_2$	-Cl	$-CH_2CH_2SCH_3$
10, *N*-(4,6-Dichloro-[1,3,5]triazine-2-yl)- L-Ala-NH_2	$-NH_2$	-Cl	$-CH_3$
11, *N*-(4-Chloro-6-methoxy-[1,3,5]-triazine-2-yl)-L-Leu-NH_2	$-NH_2$	$-OCH_3$	$-CH_2CH(CH_3)_2$
12, *N*-(4-Chloro-6-methoxy-[1,3,5]-triazine-2-yl)-D-Phg-NH_2	$-NH_2$	$-OCH_3$	$-C_6H_5$
13, *N*-(4-Chloro-6-methoxy-[1,3,5]-triazine-2-yl)-L-Val-NH_2	$-NH_2$	$-OCH_3$	$-CH(CH_3)_2$
14, *N*-(4-Chloro-6-methoxy-[1,3,5]-triazine-2-yl)-L-Met-NH_2	$-NH_2$	$-OCH_3$	$-CH_2CH_2SCH_3$
15, *N*-(4-Chloro-6-methoxy-[1,3,5]-triazine-2-yl)-L-Ala-NH_2	$-NH_2$	$-OCH_3$	$-CH_3$
16, *N*-(4-((S)-1-Carbamoyl-2-methyl-propylamino)-6-chloro-[1,3,5]triazine-2-yl]-L-Phe	$-NH_2$	$-NHCH(CH_2C_6H_5)CONH_2$	$-CH(CH_3)_2$

Fig. 1 Structures of chiral derivatizing reagents (CDR 1–16)

Preparation of stock solutions

Stock solutions of all racemic and chirally pure amino acids (100 mM) were prepared in 1 M HCl for derivatization reactions. Solutions of DCT and MCT reagents (10 mM) were prepared in acetonitrile and dimethyl sulfoxide (DMSO), respectively. Stock solutions of $NaHCO_3$ (1 M) and HCl (1 M) were prepared in purified water. All solutions were filtered through a 0.45 μm filter prior to use.

Synthesis of chiral derivatizing reagents

Three sets of CDRs (sets A–C) were synthesized (Fig. 1). Set A consisting of five DCT reagents having amino acids as chiral auxiliaries (CDR 1–5) was synthesized by nucleophilic substitution of one of the chlorine atoms in CC with amino acids, namely, L-Leu, D-Phg, L-Val, L-Met and L-Ala, respectively. Set B consisting of five DCT reagents having amino acid amides as chiral auxiliaries (CDR 6–10) was synthesized by nucleophilic substitution of one of the chlorine atoms in CC with amino acid amides, namely, L-Leu-NH_2, D-Phg-NH_2, L-Val-NH_2, L-Met-NH_2 and L-Ala-NH_2, respectively.

Set C consisted of six MCT reagents (CDR 11–16) having amino acid amides as chiral auxiliaries. Five reagents of this set (CDR 11–15) were synthesized by nucleophilic substitution of one of the chlorine atoms in 6-methoxy derivative of CC with amino acid amides, namely, L-Leu-NH_2, D-Phg-NH_2, L-Val-NH_2, L-Met-NH_2 and L-Ala-NH_2, respectively. CDR 16 was obtained by substitution of two chlorine atoms in CC with L-Val-NH_2 and L-Phe-NH_2, respectively. Synthesis, characterization and determination of enantiomeric purity of the reagents were carried out as per literature (Bhushan and Dixit 2010b; Bhushan and Kumar 2008; Bhushan and Martens 2010). However, representative synthetic procedure for CDR 4 and characterization data for newly synthesized reagents (CDR 4 and 9) are given below.

N-(4,6-Dichloro-[1,3,5]triazine-2-yl)-L-methionine (CDR 4)

L-Methionine (746 mg, 5 mmol) was dissolved in 10 mL of Na_2CO_3 solution (1 M) and maintained at 0–5°C. Acetone (50 mL) was added to the solution and allowed to stand for temperature equilibration (20°C). A solution of CC (922 mg, 5 mmol) in acetone (30 mL) was added with vigorous stirring. The reaction mixture was then stirred at 20°C for 1 h and water (30 mL) was added and acetone was removed under reduced pressure. The product began to crystallize as acetone was removed. The precipitate was filtered and washed with ice cold water. The filtrate was extracted with chloroform and evaporated to dryness in vacuo to give another crop of product.

Yield: 92%; color: white; UV (nm, in MeCN): 231 (λ_{max}); IR (KBr): 3,419, 2,972, 1,698, 1,639, 1,620, 1,573, 1,479, 1,376, 1,218, 1,134, 1,046, 814, 618 cm^{-1}; ^1H NMR (DMSO-d_6): δ 1.96–2.04 (m, 2H, –CH2–), 2.03 (s, 3H, –S–CH3), 2.46–2.51 (m, 2H, –CH2–S), 4.37–4.43 (m, 1H, –CH–N), 8.56–8.68 (dd, 1H, –NH). Anal. calcd for $C_8H_{10}Cl_2N_4O_2S$: C, 32.33%; H, 3.39%; N, 18.85%. Found: C, 32.34%; H, 3.40%; N, 18.88%.

N-(4-Chloro-6-methoxy-[1,3,5]triazine-2-yl)-L-methionine amide (CDR 9)

Yield: 93%; color: white; UV (nm, in MeCN): 232 (λ_{max}); IR (KBr): 3,422, 2,970, 1,694, 1,635, 1,616, 1,578, 1,475, 1,371, 1,219, 1,136, 1,045, 816, 620 cm^{-1}; ^1H NMR (DMSO-d_6): δ 1.99–2.03 (m, 2H, –CH2–), 2.05 (s, 3H, –S–CH3), 2.47–2.52 (m, 2H, –CH2–S), 4.38–4.41 (m, 1H, –CH–N), 7.11 (s, 1H, –CONH2), 7.48 (s, 1H, –CONH2), 8.57–8.69 (dd, 1H, –NH). Anal. calcd for $C_8H_{11}Cl_2N_5OS$: C, 32.44%; H, 3.74%; N, 23.65%. Found: C, 32.46%; H, 3.75%; N, 23.68%.

MW-assisted synthesis of diastereomers

The diastereomers of DL-amino acids were synthesized under MW irradiation. Reaction conditions were investigated using MW irradiation at 75–90% power in a time range of 50–100 s, one- to fivefold molar ratio of CDRs and pH range of 8–10 maintained by 1 M $NaHCO_3$. For quantitative yield, reactions of representative racemic amino acids, leucine (aliphatic) and phenylalanine (aromatic) were optimized with CDR 1 and CDR 11 as representatives of DCT and MCT reagents, respectively. The optimized conditions for derivatization were pH around 8.0, twofold molar excess of CDRs, and MW irradiation of 60 and 90 s at 85% power (of 800 W) using DCT and MCT reagents, respectively. The diastereomers of all the analytes were also synthesized by conventional heating for 3 h at 30°C using DCT reagents, and 1 h at 80°C using MCT reagents, as per literature report (Bhushan and Kumar 2008) for comparison in the present study. A 10 μL volume of the solution, containing diastereomers, was diluted 10 times with MeCN, and 20 μL of it was injected onto the column.

Literature (Blotny 2006; Ma et al. 2002) reveals that the substitution pattern of three chlorine atoms of CC can be controlled by appropriate reaction conditions (temperature, time, solvent, etc.) and the order of addition of reactants in the reaction mixture. Substitution of a single chlorine atom of DCT reagents under optimized derivatization conditions was confirmed by thin layer chromatography (TLC). The reaction, e.g. of CDR 1 (that contains L-Leu moiety as chiral auxiliary) with DL-Val gives the diastereomers of the

type, [L-L-] and [L-D-]; the first letter refers to the configuration of the chiral auxiliary of the CDR and the second to that of the analyte. An analysis of Table 1 clearly indicates that the CDRs used in the present work gain advantages over several other CDRs in terms of mild derivatization conditions and excellent stability of derivatives.

HPLC analysis

A LiChrospher C18 (250 mm × 4.6 mm I.D., 5 μm particle size) column from Merck (Darmstadt, Germany) was used for HPLC. The successful mobile phases were (I) eluent A [MeCN(100 mL) + H_2O(900 mL)] and eluent B [MeCN(800 mL) + H_2O(200 mL)], both containing 0.1% TFA; gradient 100% A to 100% B in 45 min, and (II) eluent C [MeOH(100 mL) + H_2O(900 mL)] and eluent D [MeOH(800 mL) + H_2O(200 mL)], both containing 0.1% TFA; gradient 100% C to 100% D in 45 min, at a flow rate of 1 mL/min with UV detection at 230 nm. In mobile phase I linear gradients of eluent B of 0–100, 5–100, 10–100, 15–100 and 20–100% in 45 min were applied to separate the diastereomeric pairs. Besides, effects of flow rate (1–1.5 mL/min) and TFA concentration (0.01–0.3%) were also studied.

Validation procedure

Method validation was performed using diastereomers of DL-Leu prepared with CDR 1 following ICH guidelines (ICH 1996). Linearity was established by injecting the samples in triplicate, containing DL-Leu in the concentration range of 0.1–200 μg/mL. Intra-day precision was established by making triplicate injections of five concentrations in the above range (i.e. 78, 104, 130, 156 and 182 μg/mL). These studies were also repeated on three consecutive days to determine inter-day precision. Signal-to-noise ratio of 3:1 and 10:1 were used for estimating the detection and quantification limit, respectively.

Results and discussion

HPLC analysis

All the diastereomers were separated by RP-HPLC. The successful separation conditions, as optimized, were a linear gradient of eluent B from 0 to 100% in 45 min (mobile phase I) containing 0.1% TFA at a flow rate of 1 mL/min. MeCN was found to be a better organic modifier in comparison to MeOH as higher resolutions (R_s) and lower retention times were obtained with the former. The elution order of diastereomers was confirmed by elution of diastereomer of a single enantiomer. The L-L-type diastereomers eluted earlier than L-D-counterparts. The D-D-type of diastereomers eluted earlier than the D-L-type as these were prepared using CDRs having D-Phg or D-Phg-NH_2 moiety as chiral auxiliary (i.e. CDRs 2, 7 and 12). The appearance of two sets of peaks (i.e. the first set consisting of two smaller peaks with baseline separation at 19.419 and 20.487 min and the second set consisting of two larger peaks with baseline separation at 41.888 and 43.064 min) in the chromatogram obtained from resolution of the diastereomers of Lys prepared with CDR 1 can be attributed to monosubstituted derivative (as the result of reaction at α-amino group) and disubstituted derivative (as the result of reaction at α- and γ-amino groups), respectively. The elution of monosubstituted derivatives followed by disubstituted derivatives can be explained on the basis of

Table 1 Comparison of certain derivatization and chromatographic parameters of the diastereomers prepared with different CDRs reported in literature with the diastereomers prepared with CDR 1 used in the present study

CDR	Label	Detection (λ_{max}) (nm)	Derivatization conditions	Stability of diastereomers	LOD	Reference
(*S*)-*N*-(4 Nitrophenoxycarbonyl)phenylalanine methoxyethyl ester (NIFE)	UV	205	20 min at RT	2 weeks (4°C)	1.0 nmol/mL	Péter et al. (2000a, b)
2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC)	UV	250	30 min at RT	24 h (RT)	5.0 ng	Kinoshita et al. (1981)
4-(3-Isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethyl-aminosulfonyl)-2,1,3-benzoxadiazole (DBD-PyNCS)	FL	$\lambda_{exc} = 490$ $\lambda_{em} = 530$	20 min at 55°C	24 h (4°C)	0.16–0.75 pmol	Jin et al. (1998)
1,3-Diacetoxy-1-(4-nitrophenyl)-2-propyl isothiocyanate (DANI)	UV	245	2 h at 60°C	1 week (4°C)	0.16 nmol/mL	Péter et al. (2000b)
N-(4,6-Dichloro-[1,3,5]-triazine-2-yl)-L-leucine (CDR 1)	UV	230	60 s at 85% (800 W) under MW irradiation	1 month (5°C)	0.12–0.14 ng/mL	Present study

RT room temperature

increased hydrophobicity of the latter due to presence of more and bulky hydrophobic groups. Similarly, under the optimized derivatization conditions, Tyr yielded disubstituted, and Arg and His yielded monosubstituted derivatives.

The retention factors (k), separation factor (α) and resolution (R_s) of the diastereomers prepared with set A CDRs are presented in Table 2. Sections of chromatograms showing some representative separations are shown in Fig. 2. The main features for the separation of diastereomers prepared with three sets of CDRs can be summarized as

- *Set A*. The highest R_s was observed for the diastereomeric pair of DL-Leu prepared with CDR 1 (having L-Leu as chiral auxiliary). CDRs can be arranged as $1 > 2 > 3 > 4 > 5$ for the decreasing order of R_s obtained for the diastereomeric pairs.
- *Set B*. The highest R_s was observed for the diastereomeric pair of DL-Leu prepared with the CDR having amide derivative of L-Leu as chiral auxiliary (CDR 6). CDRs can be arranged as $6 > 7 > 8 > 9 > 10$ for the decreasing order of R_s obtained for the diastereomeric pairs.
- *Set C*. Diastereomers prepared with the MCT reagent having L-Leu-NH_2 as chiral auxiliary (CDR 11) were better resolved in comparison to those prepared with the CDRs 12-15. However, the highest R_s under this category was observed for the diastereomers prepared with CDR 16 which has two stereogenic centers (of L-Val-NH_2 and L-Phe-NH_2 present as chiral auxiliaries). CDRs can be arranged as $16 > 11 > 12 > 13 > 14 > 15$ for the decreasing order of R_s obtained for the diastereomeric pairs.

A comparison of resolution values (R_s) and retention factors (k_2) for the diastereomeric pairs of five amino acids (Leu, Phe, Met, Thr and Glu as representatives of aliphatic, aromatic, sulfur containing, basic and acidic amino acids, respectively) prepared with three reagents (CDR 1, 6 and 11 as representatives of set A, B and C, respectively) is shown in Fig. 3a and b, respectively. Evaluation of Fig. 3a indicates that the three sets of CDRs can be arranged as $A > B > C$ for the decreasing order of resolutions for the diastereomeric pairs. On the other hand, examination of Fig. 3b reveals that the three sets can be arranged as

Table 2 Chromatographic data of diastereomers of proteinogenic amino acids prepared with set A CDRs (DCT reagents having amino acids as chiral auxiliaries)

	CDR 1-(L-Leu)[a]				CDR 2-(D-Phg)[a]				CDR 3-(L-Val)[a]				CDR 4-(L-Met)[a]				CDR 5-(L-Ala)[a]			
	k_L	k_D	R_s	α	k_D	k_L	R_s	α	k_L	k_D	R_s	α	k_L	k_D	R_s	α	k_L	k_D	R_s	α
Leu	13.14	14.65	13.34	1.12	12.93	14.14	10.62	1.09	11.32	12.46	10.10	1.10	10.60	11.91	9.59	1.12	7.84	8.90	7.78	1.13
Ile	12.51	14.02	13.32	1.12	12.43	13.63	10.54	1.09	10.56	12.20	9.88	1.15	10.18	11.44	9.26	1.12	7.48	8.49	7.36	1.13
Val	11.60	12.85	12.09	1.10	11.11	12.21	10.14	1.09	8.85	10.37	9.67	1.17	8.23	9.44	8.89	1.14	5.92	6.87	7.12	1.16
Phe	12.51	13.65	10.04	1.09	12.02	13.10	9.56	1.08	10.83	11.91	9.55	1.09	10.05	11.07	7.49	1.10	7.22	8.06	6.18	1.11
Trp	11.78	12.67	10.01	1.07	11.16	12.19	9.16	1.09	9.32	10.36	8.93	1.11	8.39	9.35	7.04	1.11	6.10	6.75	4.76	1.10
Met	10.24	11.75	9.43	1.14	10.25	11.21	8.38	1.08	8.31	9.47	7.56	1.14	8.29	9.24	7.00	1.11	5.41	6.00	4.28	1.10
Ala	7.52	8.70	8.63	1.15	6.60	7.64	7.60	1.15	5.41	6.51	7.07	1.20	5.29	6.20	6.67	1.17	1.74	2.36	4.15	1.35
Thr	6.39	7.50	8.15	1.17	5.86	6.81	6.96	1.16	3.46	4.46	6.36	1.29	2.87	3.70	6.09	1.28	1.43	1.93	3.61	1.34
Glu	5.85	6.56	6.27	1.12	5.95	6.53	5.92	1.09	3.56	4.23	4.95	1.18	2.97	3.63	4.87	1.22	1.12	1.44	2.38	1.29
Asp	5.96	6.47	3.20	1.08	5.41	5.66	2.20	1.04	3.04	3.51	1.96	1.15	2.58	2.81	1.67	1.08	1.17	1.39	1.50	1.18
Pro	8.83	10.39	10.04	1.17	8.70	10.01	9.22	1.15	6.48	7.86	9.03	1.21	5.58	6.86	7.22	1.22	4.32	5.26	5.76	1.21
Ser	4.90	5.31	3.51	1.08	4.85	5.19	2.56	1.07	2.69	2.99	2.03	1.11	2.28	2.56	1.87	1.12	2.22	2.53	1.70	1.13
Asn	4.77	5.05	1.40	1.05	4.24	4.48	1.35	1.05	3.26	3.47	1.32	1.06	2.37	2.55	1.29	1.07	1.47	1.60	1.20	1.08
His	7.34	8.19	7.00	1.12	NS				NS				NS				NS			
Lys(di)	13.96	14.38	3.47	1.03																
Arg	8.09	8.76	5.50	1.08																
Tyr(di)	14.02	14.80	6.49	1.05																

The CDRs are as mentioned in Fig. 1. Chromatographic conditions: column, LiChrospher C18 (250 mm × 4.6 mm I.D., 5 μm particle size); eluent, mobile phase (I) consisting eluent A [MeCN(100 mL) + H_2O(900 mL)] and eluent B [MeCN(800 mL) + H_2O(200 mL)], both containing 0.1% TFA; gradient 100% A to 100% B in 45 min; flow rate, 1.0 mL/min; detection, 230 nm; k_L and k_D, retention factors of diastereomers of L- and D-amino acid enantiomers, respectively; α, separation factor; R_s, resolution

NS not studied

[a] The chiral auxiliaries constituting the respective CDRs are mentioned in parenthesis

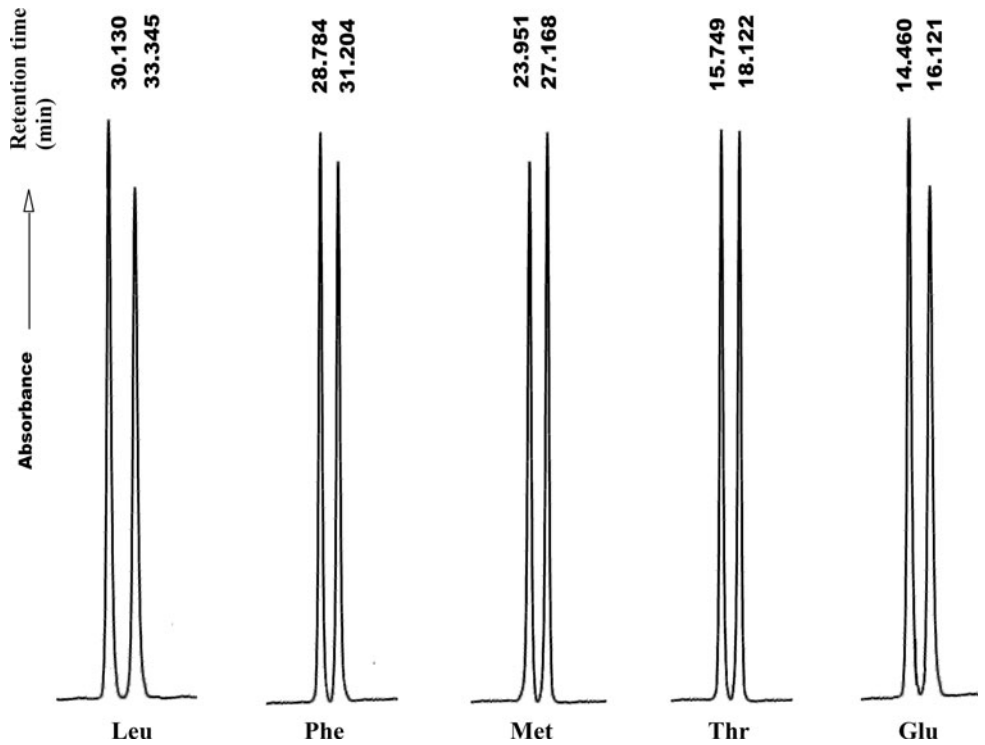


Fig. 2 Sections of chromatograms showing resolution of diastereomers of representative amino acids prepared with CDR 1. The L-L-type diastereomers eluted earlier than L-D-counterparts. Chromatographic conditions: column, LiChrospher C18 (250 mm × 4.6 mm I.D., 5 µm particle size); eluent, mobile phase (I) consisting eluent A [MeCN(100 mL) + H_2O (900 mL)] and eluent B [MeCN(800 mL) + H_2O(200 mL)], both containing 0.1% TFA; gradient 100% A to 100% B in 45 min; flow rate, 1.0 mL/min; absorbance (detection), 230 nm. The *numbers* above the peaks refer to retention times (in min)

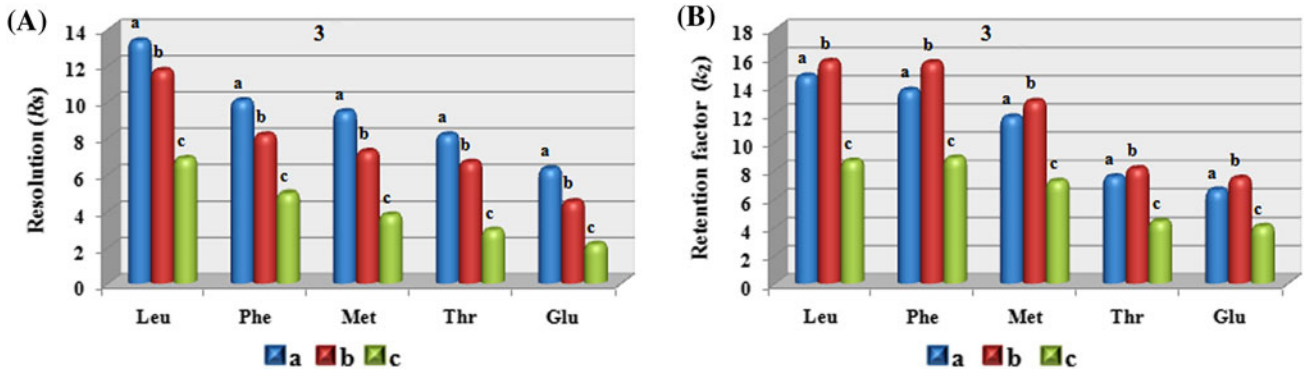


Fig. 3 Comparison of resolution, R_s and retention factor, k_2, for diastereomers of five amino acids (Leu, Phe, Met, Thr and Glu as representatives of aliphatic, aromatic, sulfur containing, basic and acidic amino acids, respectively) prepared with **a** CDR 1 (DCT reagent having L-Leu), **b** CDR 6 (DCT reagent having L-Leu-NH_2) and **c** CDR 11 (MCT reagent having L-Leu-NH_2), as chiral auxiliaries

B > A > C for the decreasing order of retention factors (k_2) obtained for the last eluting diastereomer.

In conclusion, CDR 1 was considered to be the best since the diastereomers (of all the analytes) prepared with it had the highest resolution (R_s) and among all the diastereomers synthesized in the present study, the highest R_s was observed for the diastereomeric pair of DL-Leu prepared with it.

Effect of acid and amide groups of chiral auxiliaries

The superiority of amino acids as chiral auxiliaries over amino acid amides was established as among the two sets of diastereomers prepared with DCT reagents, the diastereomers prepared with CDRs having amino acids as chiral auxiliaries (set A) were observed to have higher resolutions and lower retention times (in terms of k) as compared to

those prepared with CDRs having amino acid amides as chiral auxiliaries (set B). The lower retention times of diastereomers can be attributed to more polarity of acid variants compared to their amides counterparts.

The above-said explanation may also hold good for the observation that the diastereomers of Asparagine and Aspartic acid prepared with CDR 10 (DCT reagent having L-Ala-NH_2 as chiral auxiliary) were not resolved, while their diastereomers prepared with DCT reagent having L-Ala as chiral auxiliary (CDR 5) showed a baseline separation.

Effect of electronegativities of the atoms of achiral moieties

Chlorine atom and methoxy group present as the achiral moieties in DCT and MCT reagents, respectively, may be influencing the retention factors; lower retention factors were observed for the diastereomers prepared with MCT reagents (set C) as compared to their DCT counterparts (set B). Under reversed-phase conditions, chlorine (having electronegativity 3.0 on the Pauling scale) may have greater affinity with ODS of the column and oxygen atom in the methoxy group (having electronegativity value of 3.5) would have greater affinity with water present in the mobile phase, causing faster elution of diastereomers prepared with MCT reagents as compared to those prepared with DCT reagents.

Effect of hydrophobicities of amino acid side chains

Bull and Breese (1974) calculated apparent partial specific volumes of amino acids and constituted their hydrophobicity scale; the decreasing order of their hydrophobicity is Leu (0.842) > Ile (0.809) > Val (0.777) > Phe (0.756) > Trp (0.731) > Met (0.709) > Ala (0.691) > Thr (0.655) > Glu (0.632) > Asp (0.558). The values in parenthesis represent the apparent partial specific volume. Examination of chromatographic data reveals that an increment in the hydrophobicities of the side chains of amino acids constituting the analytes and the CDRs as well caused longer retention times and better resolution of resulting diastereomers.

Separation mechanism

The separation mechanism can be considered to be the same as proposed in the literature for diastereomers of certain other analytes (possessing amino group) prepared with CDRs based on CC (Bhushan and Dixit 2010b; Bhushan and Kumar 2008). By the same token, the elution of L–L diastereomer, with the mobile phase, before L–D diastereomer holds good for the diastereomers investigated in this study.

Method validation

Linearity

Method validation was done using diastereomers of DL-Leu prepared with CDR 1. Calibration graphs [peak area (y) vs. concentration of enantiomer (x), μg/mL] were plotted for both diastereomers of DL-Leu prepared with CDR 1 in the range 0.1–200 μg/mL and linear regression equations were used to determine slopes and correlation coefficients. A good linear relationship was obtained over this range. The regression equations were $y = 0.0006x + 0.0013$ ($R^2 = 0.9981$) and $y = 0.0009x - 0.0015$ ($R^2 = 0.9977$) for the first- and second-eluted diastereomer, respectively.

Accuracy and precision

Replicate HPLC analysis ($n = 3$) of diastereomers of DL-Leu (prepared with CDR 1) at five different concentration levels (78, 104, 130, 156 and 182 μg/mL) showed RSD less than 1.5% in all the cases. RSD for first- and second-eluted diastereomer varied from 0.40 to 0.86% and 0.47 to 0.75% for intra-day assay precision and 0.64 to 1.40% and 0.75 to 1.30% for inter-day assay precision. The recovery for first- and second-eluted diastereomer varied from 98.3 to 98.9% and 98.2 to 98.9% for intra-day assay and 97.2 to 97.8% and 97.0 to 97.7% for inter-day assay. LOD was taken as a concentration of the analytes where S/N was 3 and found to be 0.14 and 0.12 ng/mL for first and second eluting diastereomers, respectively.

Accuracy was determined by investigating the standard solutions (100 mM) of L-Leu spiked with D-Leu in the range 0.1–1.0%. The results indicate the ability of this method for the detection of D-Leu in L-Leu up to 0.4% by HPLC.

Conclusion

CDRs based on CC gain advantages over several other CDRs and many CSPs since these CSPs are not as durable. The structural features of CC provide a possibility to vary its hydrophobicity by substituting suitable moieties in it and make the method quite flexible for resolution of DL-amino acids in different situations. It further establishes utility and importance of CC-based CDRs. These made possible the resolution of diastereomeric pairs of DL-amino acids, containing acidic, neutral, basic or aromatic side chains. Besides, CDRs containing opposite enantiomers (e.g. L-Ala and D-Ala) can be easily prepared in laboratory with less expenses, while the commercial CDRs are very costly. Moreover, much less derivatization time using MW irradiation and improved stability of diastereomers (Table 1) as compared to other literature reports (Péter

et al. 2000a, b; Kinoshita et al. 1981; Jin et al. 1998) were observed.

Acknowledgments The authors are grateful to the Ministry of Human Resources Development, Government of India, New Delhi, for the award of a research assistantship (to SD). Thanks are due to Alexander von Humboldt Foundation, Bonn, Germany, for donating Knauer HPLC equipment (to RB). Financial assistance (to RB) from Council of Scientific and Industrial Research (CSIR), New Delhi, India (Grant No. 01(2334)/09 EMR-II) is also gratefully acknowledged.

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