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ORIGINAL ARTICLE

Reversed-phase liquid chromatographic resolution of diastereomers of protein and non-protein amino acids prepared with newly synthesized chiral derivatizing reagents based on cyanuric chloride

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Abstract Two new chiral monochloro-*s*-triazines (MCT) were synthesized [viz *N*-(4-chloro-6-piperidinyl-[1,3,5]-triazine-2-yl)-L-leucine amide and *N*-(4-chloro-6-piperidinyl-[1,3,5]-triazine-2-yl)-L-leucine) (CDR 1 and 2, respectively)] by the nucleophilic displacement of chlorine atoms in *s*-triazine moiety. One of the Cl atoms was replaced with piperidine, and the second Cl atom in the 6-piperidinyl derivative was replaced with amino acid amide (viz L-Leu–NH_2) and amino acid (L-Leu). These reagents were characterized and used as CDRs for chiral separation of protein and non-protein amino acids, and were separated on a reversed-phase C_{18} column. The reaction conditions were optimized for the synthesis of diastereomers using one MCT reagent. The separation method was validated for limit of detection, linearity, accuracy, precision, and recovery.

Keywords Cyanuric chloride · Amino acids · Reversed-phase liquid chromatography · Indirect resolution

Introduction

Enantiomers of amino acids are known to have high stereospecificity, different physiological and biological activities, and differences in metabolic rates as their binding to different receptor types leads to different biological response. Amino acids also play an important role in food industry for their nutritive value and by affecting taste, aroma, and color among other aspects (Hunt 1985). The relevance of amino acid enantiomers in wine gained attention, but gas chromatographic determination of quantities and pattern of D-amino acids, particularly of D-proline, has been found not to be correlated with the storage time of bottled wines (Ali et al. 2010).

Application of different CDRs for indirect enantioresolution is capable of providing better resolution with good sensitivity as the chromatographic conditions can be easily optimized. Several reports (Marfey 1984; B'Hymer et al. 2003; Bhushan and Brückner 2004; Bhushan et al. 2009), and the literature cited therein, on RP-HPLC enantioresolution of certain protein and non-protein amino acids show that indirect resolution has been successful. Recently, D- as well as L-Iva has been detected, and its chiral sequences were evaluated using Marfey's reagent in Integramide A which is a 16-amino acid peptide inhibitor of the enzyme HIV-1 integrase (De Zotti et al. 2010). Triazine derivatives of chiral amines and amino acids have been used to prepare CSP for enantioresolution of chiral amines (Brückner and Wachsmann 1996; Lin and Yang 1993; Wachsmann and Brückner 1998).

Taking advantage of trifuctionality of cyanuric chloride (*s*-triazine, CC), Brückner and Wachsmann (2003) synthesized chiral monochloro-*s*-triazine (MCT) reagents by introducing L-alanine amide as chiral auxiliary along with achiral moieties such as methoxy and phenoxy and used them for chiral separation of a few selected amino acids by HPLC and reported that the CDRs containing $–OCH_3$ moiety gave better separation and resolution in comparison to the CDRs containing other achiral moieties. Bhushan and Kumar (2008) prepared some new dichlorotriazine (DCT) reagents using L-leucine amide and D-phenylglycinamide as chiral auxiliaries and monochlorotriazine (MCT) reagents using the same two chiral auxiliaries along with

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–OCH_3 as an achiral moiety; these reagents were used as CDRs for chiral resolution of protein (except His, Lys, and Arg)and non-protein amino acids. Literature shows that the resolution of diastereomers of amino acids prepared with DCT reagents was better in comparison to those of prepared with MCT reagents (Bhushan and Kumar 2008); the electronegativity difference of O– (in the –OCH_3 of MCT) and Cl atoms (present in DCT reagents) was considered to be responsible for the same.

Taking into account the role of electronegativity of the atoms of achiral moieties present in the MCT and DCT reagents, it was considered worthwhile to introduce such achiral moieties in CC that have atoms of electronegativity identical to chlorine. Thus, two new monochloro-*s*-triazines having L-leucine amide and L-leucine as chiral auxiliaries and piperidine molecule as an achiral substituent were synthesized since on the Pauling scale the electronegativities are 3.5 for oxygen, 3.04 for nitrogen, and 3.0 for chlorine. These CDRs were characterized and used to synthesize diastereomers of 18 protein and 8 non-protein amino acids. The diastereomers were separated by reversed-phase HPLC. The performance of the two CDRs was compared. The method was also validated for linearity, accuracy, and precision. Limit of detection was determined for all amino acids. To the best of authors' knowledge, this is the first report on use of piperidine as an achiral substituent in CC for preparing CDRs followed by their application in enantioresolution of α-amino acids.

Experimental

Instrumentation and materials

The HPLC system consisting of a 10-mL pump head 1000, manager 5000 degasser, UV–visible detector 2600, Knauer manual injection valve and Eurochrom operating software was from Knauer (Berlin, Germany). Reversed-phase HPLC was performed on a Lichrospher C18 (250 mm × 4.6 mm I.D., 5 μm) column from Merck (Darmstadt, Germany). A pH meter Cyberscan 510 (Singapore) and Incubator CI-65 (Remi, Mumbai, India) were used. The Milli-Q system of Millipore (Bedford, MA, USA) was used to purify double distilled water (18.2 $M\Omega cm^3$). IR spectra were recorded in KBr pellets on a PerkinElmer 1600 FT spectrometer (Boardman, OH, USA). Elemental analysis was carried out using a Vario EL III elementary analyzer. UV–visible spectra were recorded in acetonitrile (MeCN) on a Shimadzu UV-1601 spectrophotometer. 1H NMR spectra were recorded on a Brüker 500 MHz instrument using $CDCl_3$ as deuterated solvent.

All racemic and chirally pure amino acids were obtained from Sigma-Aldrich (Bangalore, India). Cyanuric chloride, piperidine, L-leucine amide hydrochloride (L-Leu–NH_2·HCl), L-phenylalanine amide hydrochloride (L-Phe–NH_2·HCl), D-phenylglycinamide hydrochloride (D-Phg–NH_2·HCl), L-leucine (L-Leu), and L-alanine (L-Ala) were obtained from Sigma-Aldrich (St Louis, MO, USA). All other analytical grade chemicals and HPLC solvents, MeCN and methanol (MeOH) used were from E. Merck (Mumbai, India). Purified water was used throughout all studies.

Synthesis of chiral MCT reagents

Two MCTs were prepared by nucleophilic substitution of chlorine atoms in cyanuric chloride. One of the chlorine atoms was substituted by a piperidinyl group and the second with chiral auxiliaries (L-Leu–NH_2 and L-leucine) (Fig. 1). MCT reagents were synthesized and characterized according to methods given in literature (Wachsmann and Brückner 1998; Bhushan and Kumar 2008). Chiral purity of the CDRs so synthesized was established as described elsewhere (Bhushan and Kumar 2008).

N-(4-Chloro-6-piperidinyl-[1,3,5]-triazine-2-yl)-L-leucine amide (CDR-1)

Yield: 86%; color: white; mp: 145–150°C; UV [λ_{max} (nm), in acetone]: 210; IR (KBr): 3,426, 2,365, 1,676, 1,593, 1,565, 1,407, 1,347, 1,308, 1,017, 621, and 460 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 0.95–0.99 (dd, 6H, –$2CH_3$), 1.41–1.46 (m, 6H, –$3CH_2$–), 1.59–1.68 (m, 3H, –CH_2–CH), 3.74–3.76 (m, 1H, –CH–N), 7.28(s, 1H, –$CONH_2$), 7.72 (s, 1H, –$CONH_2$), 4.12–4.25 (dd, 1H, –NH); analysis: calculated for $C_{14}H_{23}ClN_6O$: C 51.45%; H 7.09%; N 25.71%, found: C 51.51%; H 7.05%; N 25.75%.

N-(4-Chloro-6-pipredinyl-[1,3,5]-triazine-2-yl)-L-leucine (CDR-2)

Yield: 79%; color: white; mp: 99–110°C; UV [λ_{max} (nm), in acetone]: 210; IR (KBr): 3,439, 2,947, 2,360, 2,134, 1,626, 1,585, 1,547, 1,239, 1,117, 1,104, 1,057, 916, 656, 626, 546, 510, and 449 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 0.94–0.98 (m, 6H, –$2CH_3$), 1.56–1.66 (m, 6H, –$3CH_2$–), 1.71–1.77 (m, 3H, –CH_2–CH), 3.70–3.77 (m, 1H, –CH–N), 4.61–4.65 (dd, 1H, –NH); analysis: calculated for $C_{14}H_{22}ClN_5O_2$: C 51.29%; H 6.76%; N 21.36%. found: C 51.41%; H 6.71%; N 21.40%.

Synthesis of diastereomers of amino acids with MCT reagents

Derivatization of α-amino acids with MCT reagent was carried out according to procedure employed by Bhushan and Kumar (2008). To 30 μL aliquot (3 μmol) of standard

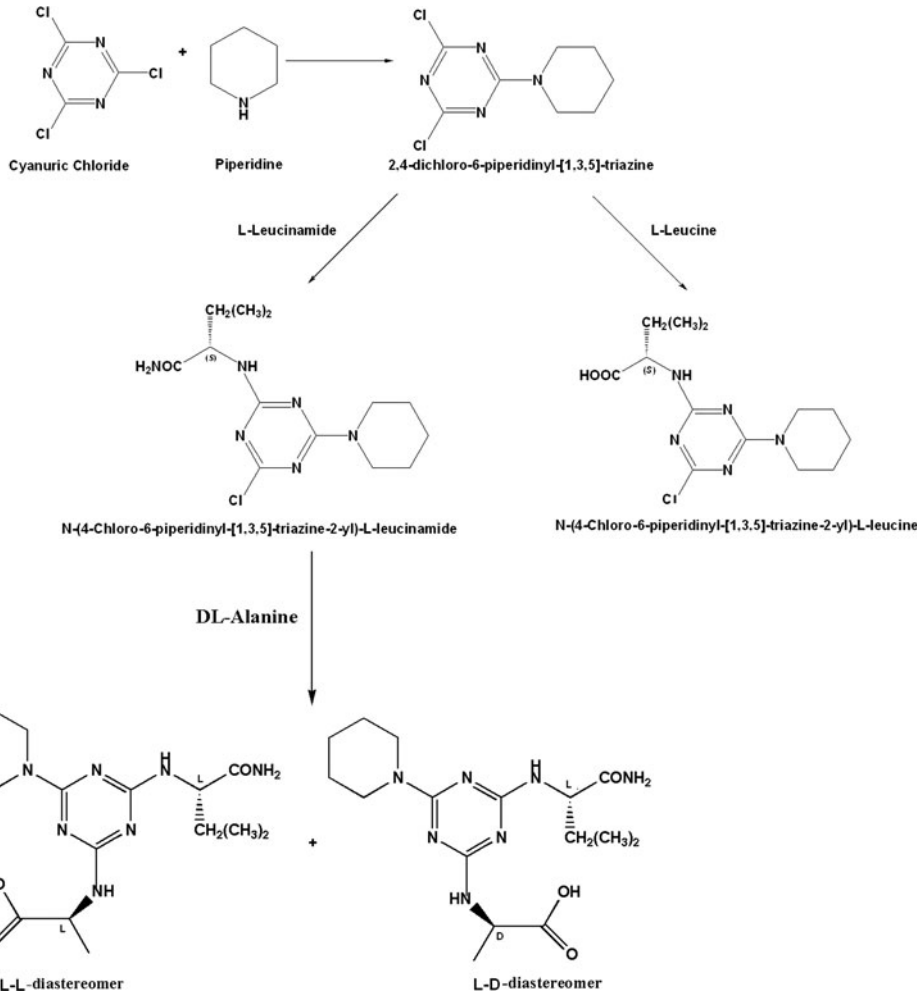

Fig. 1 The general scheme for synthesis of chiral MCT reagents and the diastereomers (*first letter* refers to the configuration of CDR while the *second* refers to that of the analyte)

solution of DL-alanine (100 mM in 1 M HCl) were added 45 µL $NaHCO_3$ (1 M) and 500 µL aliquots (5 µmol) of CDR-1 (10 mM in acetone). The mixture was heated for 1 h at 80°C. 10 µL of the resulting solution, containing diastereomers, was diluted ten times with MeCN, and 20 µL of it was injected onto the column.

HPLC conditions

Following eluents were used:

eluent A: MeCN (200 mL) + H_2O (800 mL) + trifluoroacetic acid (1 mL),
eluent B: MeCN (800 mL) + H_2O (200 mL) + trifluoroacetic acid (1 mL),
eluent C: MeOH (900 mL) + H_2O (100 mL) + trifluoroacetic acid (1 mL).

A linear gradient, (I) from 100% A to 100% B and (II) from 100% C to 100% D, in 45 min was employed. The separation was carried out at a flow rate of 0.5 mL/min with UV detection at 230 nm.

Results and discussion

Derivatization of each of the amino acids with each of the two CDRs was successful in presence of 1 M $NaHCO_3$ providing a pH around 8 in 1 h at 80°C and using twofold molar excess of CDR that helped preventing kinetic resolution. Thus, 52 diastereomeric pairs were synthesized. The diastereomers could be stored at 5°C without any decomposition. The first letter denotes the configuration of CDR and the second denotes the configuration of analyte in the diastereomer.

Separation of diastereomers

The chromatographic data for separation of diastereomers are shown in Tables 1 and 2. Sharp peaks of diastereomers were obtained as shown in Figs. 2a, b and 3. The L-D diastereomer eluted with the mobile phase earlier than L-L diastereomer in each case. MeCN was found to be a better organic modifier in comparison to methanol as larger retention times and broader peaks were obtained with the

Table 1 RP-HPLC resolution of diastereomers of amino acids prepared with CDR-1 and CDR-2

No.	Amino acids	CDR-1				CDR-2			
		k_R	k_S	α	R_S	k_R	k_S	α	R_S
1	Alanine	1.85	4.58	2.47	4.197	17.58	18.46	1.05	1.69
2	Valine	10.94	13.17	1.20	1.00	11.06	12.79	1.16	2.31
3	Leucine	14.20	15.84	1.11	1.89	18.79	19.74	1.05	2.20
4	Isoleucine	7.55	8.32	1.10	1.99	14.75	15.64	1.06	2.02
5	Proline	6.56	7.45	1.13	0.78	15.96	16.70	1.05	0.79
6	Threonine	7.41	8.34	1.12	2.06	16.98	18.17	1.07	1.75
7	Serine	6.38	7.01	1.10	0.78	16.40	16.68	1.02	0.58
8	Phenylalanine	15.68	15.84	1.01	0.40	19.39	20.09	1.04	1.53
9	Tyrosine	10.05	11.18	1.11	2.07	12.80	14.56	1.14	2.73
10	Glutamic acid	5.52	5.82	1.05	0.64	15.17	15.51	1.02	0.73
11	Aspartic acid	14.77	17.04	1.15	4.86	16.21	16.38	1.01	0.49
12	Asparagine	6.51	6.79	1.04	1.00	15.36	15.89	1.03	1.08
13	Cystine	10.91	12.93	1.18	2.45	16.04	17.26	1.08	2.29
14	Methionine	6.50	7.51	1.15	1.71	18.03	18.77	1.04	1.49
15	Tryptophan	7.59	8.88	1.17	1.61	13.79	15.09	1.09	1.82
16	Arginine	6.28	6.63	1.06	0.50	3.12	3.27	1.05	0.41
17	Histidine[a]	4.60	5.54	1.20	1.22	NT			
18	Lysine[a]	12.99	17.78	1.37	12.08	NT			

Chromatographic conditions: eluents, see experimental; gradient (I); flow rate 0.5 mL min^{-1}, detection 230 nm

NT not tested; k_1, k_2 retention factors; α separation factor

[a] Mobile phase involving gradient (II) was successful for the diastereomers of protein amino acids 15–17 prepared with CDR-1

Table 2 RP-HPLC resolution of diastereomers of non-protein amino acids prepared with CDR-1 and CDR-2

No.	Amino acids	CDR-1				CDR-2			
		k_R	k_S	α	R_S	k_R	k_S	α	R_S
1	2-Amino octanoic acid	12.45	14.35	1.15	2.27	15.66	18.11	1.16	3.55
2	2-Amino butyric acid	15.89	16.54	1.04	1.07	16.08	17.23	1.07	1.66
3	2-Amino adipic acid	15.43	17.28	1.12	2.00	18.22	18.66	1.02	1.11
4	Norvaline	4.91	6.44	1.31	3.85	12.28	14.66	1.19	3.25
5	Pipecolic acid	15.36	15.89	1.04	1.07	11.90	12.59	1.06	0.72
6	2-Phenyl glycine	12.80	14.56	1.14	2.73	22.79	23.57	1.03	1.62
7	Isovaline[a]	7.76	10.46	1.35	4.26	17.31	17.64	1.02	0.60
8	Cysteic acid	NR				NR			

Chromatographic conditions: eluents, see experimental; gradient (I); flow rate 0.5 mL min^{-1}, detection 230 nm

k_1, k_2 retention factors; α separation factor, *NR* no resolution

[a] Mobile phase involving gradient (II) was successful for diastereomers of isovaline prepared with CDR-1

latter. The best separation was obtained at 45 min for the applied gradient. On varying TFA concentration from 0.005 M to 0.03 M, a slight increase in separation factor was observed.

The basic mechanism for separation of diastereomers of amino acids prepared with MR has been explained by Marfey (1984), Brückner and Keller-Hoehl (1990), and Fujii et al. (1997). It was observed from Table 1 that the resolution values for diastereomers of aliphatic amino acids (serial numbers 1–5) prepared with CDR-2 were higher in comparison to those for the diastereomers prepared with CDR-1, except for alanine; the resolution values for the diastereomers of hydroxyl group containing amino acids (6 and 7) and sulfur containing amino acids (13 and 14) prepared with CDR-2 were lower than those of their diastereomers prepared with CDR-1; for aromatic amino acids (8 and 9) and for acidic amino acids (10 and 11) resolution values for diastereomers prepared with CDR-2 were higher than the diastereomers prepared with CDR-1, except the resolution value of aspartic acid; among the basic amino acids (15–16), the resolution values were higher for the diastereomers of tryptophan prepared with CDR-2 than its diastereomers prepared with CDR-1, while the results were just opposite for diastereomers of arginine.

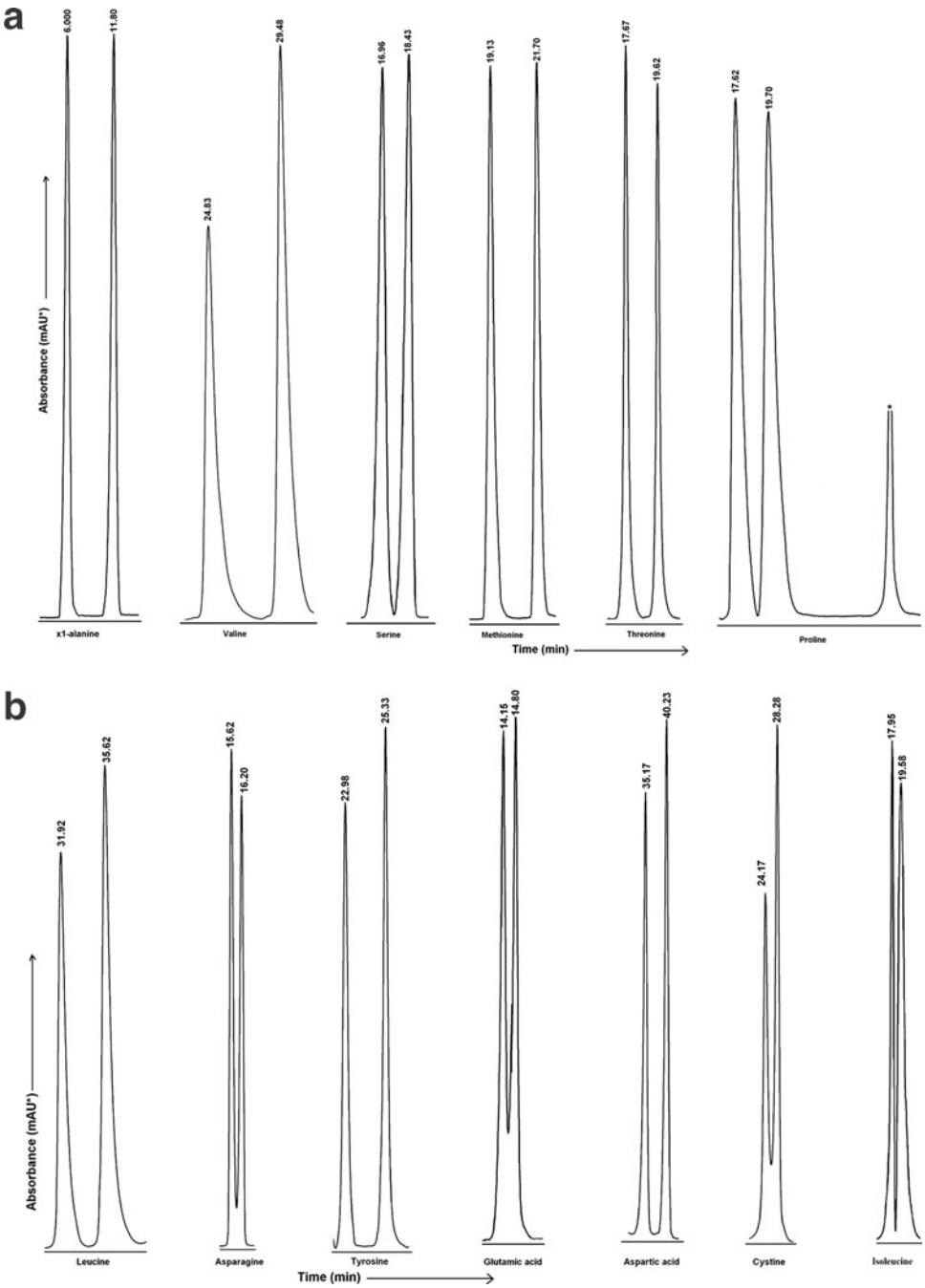

Fig. 2 The sections of chromatograms showing resolution of diastereomers of some protein amino acids prepared with CDR-1. Chromatographic conditions: eluent A: MeCN (200 mL) + H_2O (800 mL) + trifluoroacetic acid (1 mL); eluent B: MeCN (800 mL) + H_2O (200 mL) + trifluoroacetic acid (1 mL); linear gradient (I) from 100% A to 100% B, in 45 min; flow rate 0.5 mL min^{-1}, detection 230 nm. *Reagent-related peaks

In case of non-protein amino acids (Table 2), it was observed that the resolution values for diastereomers of 2-amino octanoic acid and 2-amino butyric acid prepared with CDR-2 were higher in comparison to those for the diastereomers of rest of the non-protein amino acids prepared with CDR-2. According to Table 2, it is concluded that the R_s values for the diastereomers of isovaline (Iva) prepared with CDR-1 were the highest among the R_s values of the diastereomers of the rest of the non-protein amino acids prepared with CDR-1 and 2. The diastereomers of the same six non-protein amino acids (except Iva) prepared with (*S*)-NIFE required higher run and retention times and showed higher R_s using methanol in the mobile phase (Bhushan and Agarwal 2010) in comparison to the run time, retention times, and R_s of their diastereomers prepared with CDR-1 and 2 using acetonitrile (in the present case) as organic modifier. Use of methanol as organic modifier in the present case showed good R_s for the diastereomers of the six non-protein amino acids, but it was not chosen finally as there were longer retention times.

Hydrophobicity of the alkyl side chain of the amino acids appears to affect the interaction of the diastereomers

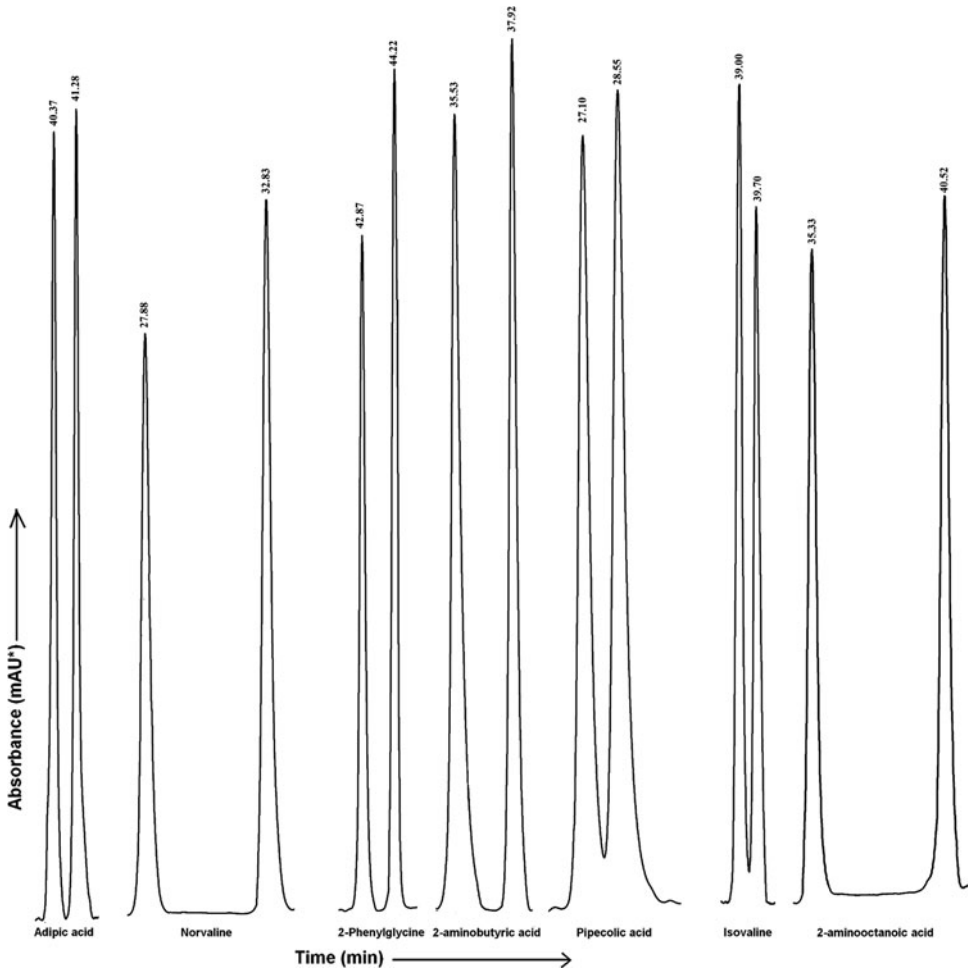

Fig. 3 The sections of chromatograms showing resolution of diastereomers of non-protein amino acids prepared with CDR-2. Chromatographic conditions: eluent A: MeCN (200 mL) + H_2O (800 mL) + trifluoroacetic acid (1 mL); eluent B: MeCN (800 mL) + H_2O (200 mL) + trifluoroacetic acid (1 mL); eluent C: MeOH (900 mL) + H_2O (100 mL) + trifluoroacetic acid (1 mL); linear gradient, (I) from 100% A to 100% B and (II) from 100% A to 100% C, in 45 min; flow rate 0.5 mL min^{-1}, detection 230 nm. *Reagent-related peaks

with the ODS material of the column and the separation. Looking to the alkyl side chain in the structures of analytes, following observations have been made: with increasing alkyl group in the side chain the separation factors of the analytes (diastereomers prepared with CDR-1 and 2) increased particularly for the following sets: Trp > Arg > Asn; Tyr > Thr > Ser; Tyr > Phe; Cys > Met. For diastereomers of Ile, Leu, and Ala prepared with CDR-2, with increasing alkyl group in the side chain the separation factors of the analytes increased particularly for Ile > Leu = Ala.

The R_s values for the diastereomers of glutamic acid prepared with both the CDRs was nearly equal while the R_s value for the diastereomers of aspartic acid prepared with CDR-1 is greater than the R_s value for the diastereomers of the same prepared with CDR-2. It may be due to the polar interactions between COO^- anions present in Asp and NH_2 of the L-Leu–NH_2 present in CDR-1.

Among the specific pairs of diastereomers, it was observed that the diastereomeric pairs of Ser, Pro, Glu, and Arg prepared with both CDR 1 and 2 were poorly resolved; the diastereomeric pair of Phe prepared with CDR-1 was poorly resolved, but its diastereomers prepared with CDR-2 were well resolved ($R_s = 1.53$); diastereomers of Asp, Iva and pipecolic acid prepared with CDR-2 were poorly resolved, but their diastereomers prepared with CDR-1 were well resolved on reversed-phase column.

Linearity, accuracy, and precision

Varying amounts of derivatives in the range 50–500 pmol were injected onto column, and linearity studies were carried out using correlations between the peak areas. The linearity was acceptable in this range for diastereomers of D-Ala ($R^2 = 0.9987$) and for diastereomers of L-Ala ($R^2 = 0.9994$), respectively.

The accuracy and precision studies were carried out by replicate HPLC analysis ($n = 5$) of DL-Ala at five different concentration levels (10, 30, 50, 80, and 100 ng mL^{-1}) and RSD were less than 2%. The relative standard deviation for D- and L-Ala varied from 1.20 to 1.46% and 1.25 to 1.80% for intra-day assay precision and 1.42 to 1.80% and 1.70 to 1.90% for inter-day assay precision. The recovery for D- and L-Ala varied from 96.8 to 97.6% and 96 to 98.5% for

intra-day assay and 95.7 to 97% and 95.8 to 97.8% for inter-day assay. Accuracy of the method was determined by investigating the standard solutions (100 mM) of L-Ala spiked with D-Ala in the range 0.1–1.0%. The results indicate that this method can be applied for detection of D-Ala in L-Ala up to 0.3% by HPLC.

Conclusion

Different reactive and detectable groups can be attached with CC, and new series of CDRs can be prepared that can be used for improved separation of different chiral pharmaceutical drugs and amino acids. It was found that separation and retention times of diastereomers of amino acids depend upon hydrophobicity of amino acids. The results obtained from the piperidine-substituted MCT reagents were comparable to those obtained with other DCT reagents and were better than the other MCT reagents having chiral moieties such as methoxy group.

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