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Reversed-phase high-performance liquid chromatographic separation of diastereomers of (*R*,*S*)-mexiletine prepared by microwave irradiation with four new chiral derivatizing reagents based on trichloro-*s*-triazine having amino acids as chiral auxiliaries and 10 others having amino acid amides

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ABSTRACT

A new series of chiral derivatizing reagents (CDRs) consisting of four dichloro-*s*-triazine reagents was synthesized by nucleophilic substitution of one chlorine atom in trichloro-*s*-triazine with amino acids, namely L-Leu, D-Phg, L-Val and L-Ala as chiral auxiliaries. Two other sets of CDRs consisting of four dichloro-*s*-triazine (DCT) and six monochloro-*s*-triazine (MCT) reagents were also prepared by nucleophilic substitution of chlorine atom(s) with different amino acid amides as chiral auxiliaries in trichloro-*s*-triazine and its 6-methoxy derivative, respectively. These 14 CDRs were used for the synthesis of diastereomers of (*R*,*S*)-mexiletine under microwave irradiation (*i.e.* 60 s and 90 s at 85% power (of 800 W) using DCT and MCT reagents, respectively), which were resolved by reversed-phase high-performance liquid chromatography using C18 column and gradient eluting mixtures of methanol with aqueous trifluoroacetic acid (TFA) with UV detection at 230 nm. The resolution (R_s), difference between retention times of resolved diastereomers (Δt) and retention factors (k) obtained for the three sets of diastereomers were compared among themselves and among the three groups. Explanations have been offered for longer retention times and better resolution of diastereomers prepared with DCT reagents in comparison of their MCT counterparts and, for the influence of hydrophobicity of the side chain R of the amino acid in the CDRs on retention times and resolution. The newly synthesized CDRs were observed to be superior as compared to their amide counterparts in terms of providing better resolution and cost effectiveness. The method was validated for limit of detection, linearity, accuracy and precision.

1. Introduction

The physiological environment within a living organism is chiral, and therefore the pharmacodynamic, pharmacokinetic and toxicological activities of enantiomers of a drug can differ dramatically. However, many drugs are administered as a racemic mixture which may cause undesired biological processes resulting in catastrophic side effects. Therefore, enantiomeric separation and analysis of chiral drugs are significant areas of research in pharmaceutical chemistry. The resolution of a pair of enantiomers by reacting them with an optically pure chiral derivatizing reagent (CDR), *i.e.* the formation of diastereomers followed by their chromatographic separation in an achiral environment, is considered as an indirect approach. Its advantages include availability of a variety of CDRs either commercially or via simple synthetic sequence, presence of easily derivatizable and compatible functional groups leading to diastereomeric derivatives with excellent separation and detection possibilities and, a relatively wide choice of chromatographic conditions in comparison to direct methods for chiral resolution [1–3].

Mexiletine [1-(2,6-dimethylphenoxy)-2-amino propane, MEX] (Fig. 1) is classified as a class Ib antiarrhythmic drug and clinically administered as antiarrhythmic, antimyotonic, and analgesic agent in its racemic form [4]. Several lines of evidences have shown that mexiletine enantiomers differ in their pharmacodynamic, pharmacokinetic and receptor binding properties [5,6]. The aliphatic and aromatic hydroxylations of mexiletine in human microsomes are reported to be stereoselective; aliphatic hydroxylation is predominant for the (*R*)-enantiomer while the aromatic hydroxylation is favored for the (*S*)-enantiomer [7]. The (*R*)-isomer of mexiletine is also reported to be more potent than the (*S*)-isomer in the treatment of experimental arrhythmias [8].

Indirect enantioseparation of (*R*,*S*)-MEX using high-performance liquid chromatography (HPLC) has been reported with CDRs like (*S*)-1-(l-naphthyl)ethyl isocyanate (NEIC) [9], (1*S*,

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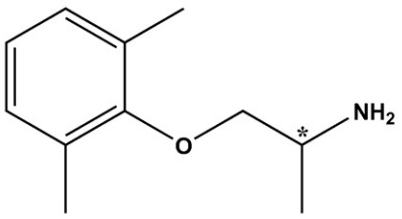
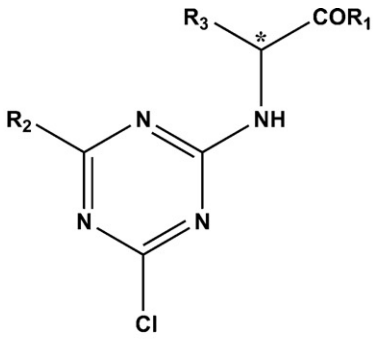


Mexilitine



CDR (1-14)

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-Ala	-OH	-Cl	$-CH_3$
5, *N*-(4,6-Dichloro-[1,3,5]triazine-2-yl)- L-Leu-NH_2	$-NH_2$	-Cl	$-CH_2CH(CH_3)_2$
6, *N*-(4,6-Dichloro-[1,3,5]triazine-2-yl)- D-Phg-NH_2	$-NH_2$	-Cl	$-C_6H_5$
7, *N*-(4,6-Dichloro-[1,3,5]triazine-2-yl)- L-Val-NH_2	$-NH_2$	-Cl	$-CH(CH_3)_2$
8, *N*-(4,6-Dichloro-[1,3,5]triazine-2-yl)- L-Ala-NH_2	$-NH_2$	-Cl	$-CH_3$
9, *N*-(4-Chloro-6-methoxy-[1,3,5]-triazine-2-yl)-L-Leu-NH_2	$-NH_2$	$-OCH_3$	$-CH_2CH(CH_3)_2$
10, *N*-(4-Chloro-6-methoxy-[1,3,5]-triazine-2-yl)-D-Phg-NH_2	$-NH_2$	$-OCH_3$	$-C_6H_5$
11, *N*-(4-Chloro-6-methoxy-[1,3,5]-triazine-2-yl)-L-Val-NH_2	$-NH_2$	$-OCH_3$	$-CH(CH_3)_2$
12, *N*-(4-Chloro-6-methoxy-[1,3,5]-triazine-2-yl)-L-Met-NH_2	$-NH_2$	$-OCH_3$	$-CH_2CH_2SCH_3$
13, *N*-(4-Chloro-6-methoxy-[1,3,5]-triazine-2-yl)-L-Ala-NH_2	$-NH_2$	$-OCH_3$	$-CH_3$
14, *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 MEX and chiral derivatizing reagents (CDR 1–14).

2*S*)-*N*-[(2-isothiocyanato)-cyclohexyl]-pivalinoyl amide (PDITC) and 2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl isothiocyanate (GITC) [10], (−)-4-(6-methoxy-2-naphthyl)-2-butyl chloroformate (NAB-C) [11] and *o*-phthaldialdehyde combined with *N*-acetyl-L-cysteine [12]. Direct HPLC resolution of enantiomers of (*R*,*S*)-MEX and its derivatives has been achieved on various chiral stationary phases (CSPs); these include CSP based on 1,1′-binaphthyl crown ether [13] and (18-crown-6)-2,3,11,12-tetracarboxylic acid [14,15] for (*R*,*S*)-MEX, Pirkle ionic column based on (*R*)-(−)-3,5-dinitrobenzoylphenylglycine for naphthoyl [16] and *N*-anthroyl derivatives [17] and, CSP based on amylose *tris*(3,5-dimethylphenylcarbamate) for resolution of (*R*,*S*)-MEX and its main metabolites in plasma and urine [18]. Chiral separation of mexiletine has also been reported employing cyclodextrins [19] and (18-crown-6)-tetracarboxylic acid [20] as chiral additives using capillary electrophoresis (CE).

Cyanuric chloride (2,4,6-trichloro-1,3,5-triazine; trichloro-*s*-triazine; *s*-triazine chloride; CC) has the prospect of easy and controlled sequential substitution of its chlorine atoms by nucleophiles attributed to its trifunctional high reactivity [21]. It is commercially available and is an inexpensive reagent that makes its applications even more attractive. Enantioresolution of amino acids and amino alcohols has been reported on a series of CSPs prepared by immobilization of chloro-*s*-triazines bearing amines or amino acids as chiral selectors on solid supports such as aminopropylsilica [22–24]. Brückner and co-workers [25,26] synthesized chiral monochloro-*s*-triazine reagents from cyanuric chloride and used them for enantioseparation of only a few selected amino acids by HPLC.

Indirect enantioresolution of *α*-amino acids has been reported from this laboratory using four dichloro-*s*-triazine (DCT) and six monochloro-*s*-triazine (MCT) reagents as CDRs prepared by the nucleophilic substitution of chlorine atom(s) with different amino acid amides moieties as chiral auxiliaries in trichloro-*s*-triazine and its 6-methoxy derivative, respectively [27]. Enantioseparation of 18 proteinogenic and 8 non-proteinogenic amino acids has also been reported with two cyanuric chloride based CDRs having piperidinyl as achiral auxiliary and, L-leucine amide and L-leucine as chiral auxiliaries, respectively [28].

DFDNB (1,5-difluoro-2,4-dinitrobenzene) has a unique feature of allowing the substitution of its fluorine atoms with nucleophiles. For this feature, Marfey prepared the reagent 1-fluoro-2,4-dinitophenyl-5-L-alanine amide (FDNP-L-Ala-NH_2, FDAA, MR) by substituting one of its fluorine atoms with L-Ala-NH_2 [29]. Using DFDNB, several CDRs considered as structural variants of MR (having amino acid amides, amino acids and amines as chiral auxiliaries) have been synthesized and used in this laboratory for indirect enantioseparation of a variety of compounds such as proteinogenic and nonproteinogenic amino acids [30], amino alcohols [31] and (*R*,*S*)-MEX [32,33]. These studies have proved superiority of structural variants of MR having amino acid moieties as chiral auxiliaries over to those having amino acid amide moieties, in terms of providing better resolution of analytes and cost effectiveness. The structures of the diastereomers formed by compounds possessing amino groups with *s*-triazine reagents show similarities with those prepared with Marfey's reagent [1].

Keeping in view the literature mentioned above and the references cited therein, three sets of CDRs were synthesized from trichloro-*s*-triazine, (A) four new DCT reagents having amino acid moieties (viz., L-Leu, D-Phg, L-Val and L-Ala), (B) four DCT reagents having amino acid amide moieties and, (C) six MCT reagents having amino acid amide moieties, as chiral auxiliaries. The CDRs were

used for enantioseparation of (*R*,*S*)-MEX. The separation results obtained with all three sets of diastereomers are compared among themselves and among the three groups. To the best of authors' knowledge this is the first report on microwave (MW)-assisted synthesis of diastereomers of (*R*,*S*)-MEX with the aforementioned 14 CDRs (Fig. 1) followed by their reversed-phase (RP)-HPLC resolution.

2. Experimental

2.1. 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 (800W, 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 methanol). ^{1}H NMR spectra were recorded on a Bruker 500 MHz instrument using dimethyl sulfoxide (DMSO-d_6) as deuterated solvent.

2.2. Chemicals and reagents

(*R*,*S*)-MEX, cyanuric chloride, 2,4,6-collidine, all chirally pure amino acids and 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.

2.3. Preparation of stock solutions

Stock solutions of (*R*,*S*)-MEX (10 mM) were prepared in 0.1 M $NaHCO_3$ and THF (50 mM) for derivatization reactions. Solutions of dichloro-*s*-triazine (DCT) and monochloro-*s*-triazine (MCT) reagents (10 mM) were prepared in methanol and dimethyl sulfoxide (DMSO), respectively. Stock solution of 1 M $NaHCO_3$ was prepared in purified water. All solutions were filtered through a 0.45 μm filter prior to use.

2.4. Synthesis of chiral derivatizing reagents

The DCT reagents (CDR 1–8) were synthesized by nucleophilic substitution of one of the chlorine atoms in trichloro-*s*-triazine with L-Leu, D-Phg, L-Val, L-Ala, L-Leu-NH_2, D-Phg-NH_2, L-Val-NH_2 and L-Ala-NH_2, respectively. The MCT reagents (CDR 9–13) were synthesized by nucleophilic substitution of one of the chlorine atoms in 6-methoxy derivative of trichloro-*s*-triazine with L-Leu-NH_2, L-Met-NH_2, D-Phg-NH_2, L-Val-NH_2 and L-Ala-NH_2, respectively. The MCT reagent, CDR 14 was obtained by the substitution of two chlorine atoms with L-Val-NH_2 and L-Phe-NH_2, respectively. Representative synthetic procedure for CDR 1 and characterization data for all four newly synthesized reagents is given below. Data for the remaining reagents (CDR 5–14) was in agreement with earlier reports [27].

2.4.1. N-(4,6-dichloro-[1,3,5]triazine-2-yl)-L-leucine (CDR 1)

L-Leucine hydrochloride (838 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 MeOH): 231 (λ_{max}); IR (KBr): 3416, 2964, 1685, 1610, 1549, 1323, 1240, 1153, 840, 620 cm^{-1}; ^{1}H NMR (500 MHz, DMSO d_6) δ 0.84–0.91 (dd, 6H, –2CH3), 1.50–1.68 (m, 3H, –CH2–CH), 4.28–4.36 (m, 1H, –CH–N), 9.18–9.23 (d, 1H, –NH); Anal. Calcd. for $C_9H_{12}C_{l2}N_4O_2$: C, 38.73%; H, 4.33%; N, 20.07%. Found: C, 38.44%; H, 4.27%; N, 20.01%.

2.4.2. N-(4,6-dichloro-[1,3,5]triazine-2-yl)-D-phenylglycine (CDR 2)

Yield: 93%; color: white; UV (nm, in MeOH): 231 (λ_{max}); IR (KBr): 3413, 2971, 1660, 1618, 1545, 1320, 1242, 847, 612 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6) δ 5.49–5.55 (m, 1H, –CH–N), 7.33–7.59 (m, 5H, aromatic ring of side chain), 9.10–9.15 (d, 1H, –NH); Anal. Calcd. for $C_8H_{10}Cl_2N_4O_2$: C, 36.25%; H, 3.80%; N, 21.13%. Found: C, 36.19%; H, 3.62%; N, 21.02%.

2.4.3. N-(4,6-dichloro-[1,3,5]triazine-2-yl)-L-valine (CDR 3)

Yield: 92%; color: white; UV (nm, in MeOH): 231 (λ_{max}); IR (KBr): 3435, 2960, 1654,1553, 1310, 1239, 847, 612 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.85–0.94 (dd, 6H, –2CH_3), 2.21–2.27 (m, 1H, –CH), 4.18–4.25 (m, 1H, –CH–N), 9.03–9.10 (d, 1H, –NH); Anal. Calcd. for $C_8H_{10}Cl_2N_4O_2$: C, 36.25%; H, 3.80%; N, 21.13%. Found: C, 36.23%; H, 3.77%; N, 21.11%.

2.4.4. N-(4,6-dichloro-[1,3,5]triazine-2-yl)-L-alanine (CDR 4)

Yield: 94%; color: white; UV (nm, in MeOH): 232 (λ_{max}); IR (KBr): 3415, 1687, 1601, 1550, 1334, 1245, 611 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.30–1.34 (d, 3H, –CH_3), 4.31–4.36 (m, 1H, –CH–N), 9.19–9.25 (d, 1H, –NH); Anal. Calcd. for $C_6H_6Cl_2N_4O_2$: C, 30.40%; H, 2.55%; N, 23.64%. Found: C, 30.32%; H, 2.49%; N, 23.58%.

2.5. Microwave-assisted synthesis of diastereomers

The diastereomers of (*R*,*S*)-MEX with all 14 CDRs were synthesized according to the literature reported for the diastereomerization of amino acids [27]. Separate sets of reaction mixture were irradiated in the microwave oven for 50, 55, 60, 70, 80, 90 and 100 s at 75–90% power of 800 W. A 10 μL volume of the resulting solution, containing diastereomers, was diluted 10 times with MeOH, and 20 μL of it was injected onto the column. The reaction conditions for derivatization were optimized by derivatizing (*R*,*S*)-MEX with CDR 1 and 9 (as representatives of diastereomers prepared with DCT and MCT reagents, respectively).

2.6. 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 [MeOH (100 mL) + H_2O (900 mL)] and eluent B [MeOH (800 mL) + H_2O (200 mL)], both containing 0.15% TFA; gradient 100% A to 100% B in 45 min, and (II) eluent C [MeCN (100 mL) + H_2O (900 mL)] and eluent D [MeCN (800 mL) + H_2O (200 mL)], both containing 0.15% 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 examined.

2.7. Validation procedure

Method validation was performed using diastereomers of (*R*,*S*)-MEX prepared with CDR 1 following ICH guidelines [34]. Linearity was established by injecting the samples in triplicate, containing (*R*,*S*)-MEX in the concentration range of 0.1–200 μg mL^{-1}. Intra-day precision was established by making triplicate injections of five concentrations in the above range (72, 96, 120, 144 and 168 μg mL^{-1}). These studies were also repeated on three consecutive days to determine inter-day precision. Limits of detection and quantification were determined by the calculation of signal to noise ratio. Signal to noise ratio of 3:1 and 10:1 were used for estimating the detection and quantification limit, respectively.

3. Results and discussion

3.1. Synthesis of CDRs

The first set of DCT reagents (*i.e.* set A consisting CDR 1–4) was synthesized by nucleophilic substitution of one of the chlorine atoms in trichloro-*s*-triazine with amino acids, namely L-Leu, D-Phg, L-Val and L-Ala as chiral auxiliaries. The other set of DCT reagents (*i.e.* set B consisting CDR 5–8) was synthesized by nucleophilic substitution of one of the chlorine atoms in trichloro-*s*-triazine with amino acid amides, namely L-Leu-NH_2, D-Phg-NH_2, L-Val-NH_2 and L-Ala-NH_2 as chiral auxiliaries.

The MCT reagents (*i.e.* CDR 9–13 of set C) were synthesized by nucleophilic substitution of one of the chlorine atoms in 6-methoxy derivative of trichloro-*s*-triazine with L-Leu-NH_2, L-Met-NH_2, D-Phg-NH_2, L-Val-NH_2 and L-Ala-NH_2. The MCT reagent, CDR 14 (of set C) was obtained by the substitution of two chlorine atoms with L-Val-NH_2 and L-Phe-NH_2, respectively. Characterization data for newly synthesized amino acid variants of DCT reagents (CDR 1–4) is given under Section 2, while data for remaining reagents (CDR 5–14) was in agreement with earlier report [27]. The chirally pure single enantiomers of the amino acids and amino acid amides (obtained from Sigma–Aldrich) were used for the synthesis of CDRs. The specific rotation value for each of them is characteristic and serves as the test for purity of respective CDRs. Nevertheless, the chiral purity of the CDRs was further verified as per the literature [1,27].

3.2. Synthesis of diastereomers

In all, 14 pairs of diastereomers were synthesized from eight DCT (CDR 1–8) and six MCT (CDR 9–14) reagents under microwave irradiation. These were also synthesized using conventional heating (*i.e.* 3 h at 30 °C using DCT reagents, and 1 h at 80 °C using MCT reagents).

The derivatization of (*R*,*S*)-MEX using aforesaid DCT and MCT reagents under microwave irradiation required much less time (*i.e.* 60 s at 85% power using DCT and 90 s at 85% power using MCT reagents) as compared to the time required with certain other CDRs, e.g., GITC (120 min at RT) [10] and NAB-C (1 h at ambient temperature) [11]. Also, the resultant diastereomers show excellent stability (*i.e.* 1 month at 5 °C) which is much better as compared to the OPA-NAC reagent (where fluorescence is stable for 20–30 min only) [12]. The experimental conditions were optimized for microwave-assisted synthesis as are discussed below.

3.2.1. Role of pH

Use of 1 M $NaHCO_3$ (30 μL, 30 μmol) at a pH around 8.0 provided the best yield for derivatization of (*R*,*S*)-MEX. No derivatization was observed in the absence of $NaHCO_3$ even using MW irradiation for 3–4 min. The increment in pH up to 10 showed no significant change in reaction time and yield of derivatization.

3.2.2. Role of reagent excess

The CDR 1 was used in 1–5-fold molar ratios to find the optimum reagent concentration for derivatization. Derivatization was complete when 1.7-fold (1.7 μmol) molar excess of the reagent was used. Slight kinetic resolution was observed when lower ratios of CDR: MEX (1:1 or 1.5:1) were applied. At higher ratios no significant change in reaction time and yield of derivatization was observed. Therefore, all the CDRs were used in 1.7-fold molar excess for quantitative derivatization and to prevent kinetic resolution.

3.2.3. Microwave heating

MW irradiation for 60 s at 85% power (while using DCT reagents) and 90 s at 85% power (while using MCT reagents) gave complete derivatization.

The course of reaction completion was studied by the observation of areas of diastereomeric peaks which were calculated by system software. The microwave irradiation time and power corresponding to maximum peak areas (representing completion of reaction) were taken as optimized derivatization conditions..

3.3. HPLC analysis

The 14 pairs of diastereomers were separated under reversed-phase conditions by HPLC. Sections of chromatograms showing resolution of the diastereomers of (*R*,*S*)-MEX prepared with all 14 CDRs are shown in Fig. 2. Sharp peaks showing base line resolution were obtained using mobile phase I consisting eluent A [MeOH (100 mL) + H_2O (900 mL)] and eluent B [MeOH (800 mL) + H_2O (200 mL)], both containing 0.15% TFA; gradient 100% A to 100% B in 45 min. MeOH was found to be a better organic modifier in comparison to MeCN as higher resolutions (R_s) were obtained with the former. The retention factors (k), separation factor (α) and resolution (R_s) of the resolved diastereomers are given in Table 1.

Optimization studies using different linear gradients of eluent B (in mobile phase I) revealed a linear gradient from 0 to 100% in 45 min as the successful case. An increment in the amount of organic modifier (using linear gradients of eluent B from 5 to 100%, from 10 to 100%, from 15 to 100% and from 20 to 100% in 45 min) was accompanied by decrease in retention times and

Table 1
Chromatographic parameters of diastereomers of (*R*,*S*)-MEX prepared with different CDRs.

CDR	Δt	k_1	k_2	α	R_s
1	2.822	11.543	12.551	1.09	5.86
2	2.343	10.789	11.626	1.08	5.43
3	2.221	10.365	11.158	1.08	5.20
4	2.101	9.437	10.187	1.08	4.92
5	2.002	11.866	12.581	1.06	4.85
6	1.923	11.475	12.162	1.06	4.60
7	1.783	10.678	11.315	1.06	4.34
8	1.702	9.912	10.520	1.06	4.18
9	1.151	8.766	9.177	1.05	3.82
10	1.122	8.436	8.837	1.05	3.53
11	1.094	8.321	8.712	1.05	3.13
12	1.005	8.292	8.650	1.04	2.52
13	0.856	8.244	8.549	1.04	2.01
14	1.293	9.703	10.164	1.05	4.02

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, see Section 2, mobile phase (I) consisting eluent A [MeOH (100 mL) + H_2O (900 mL)] and eluent B [MeOH (800 mL) + H_2O (200 mL)], both containing 0.15% TFA; gradient 100% A to 100% B in 45 min; flow rate, 1.0 mL/min; detection, 230 nm; Δt, difference between retention times of resolved diastereomers; k_1 and k_2 retention factors of first and second eluting diastereomers, respectively; α, separation factor; R_s, resolution.

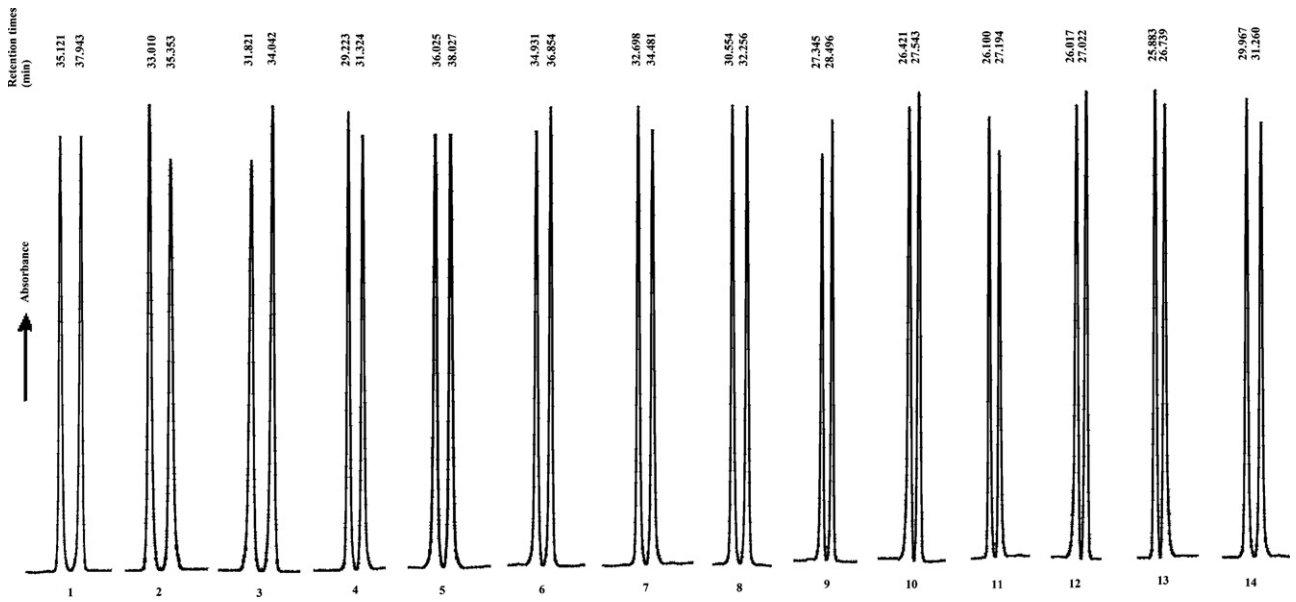


Fig. 2. Sections of chromatograms showing resolution of diastereomers of (*R*,*S*)-MEX prepared with CDR 1–14. Chromatographic conditions: Column, LiChrospher C18 (250 mm × 4.6 mm I.D., 5 μm particle size); eluent, mobile phase (I) consisting eluent A [MeOH (100 mL) + H_2O (900 mL)] and eluent B [MeOH (800 mL) + H_2O (200 mL)], both containing 0.15% TFA; gradient 100% A to 100% B in 45 min; flow rate, 1.0 mL/min; detection, 230 nm.

resolution and, overlapping of peaks as well. The effect of TFA concentration in the range of 0.01–0.3% was studied. While an increment in the concentration of TFA from 0.01 to 0.15% caused an enhancement in separation factor, further increment caused only slight difference. So, a 0.15% TFA concentration was taken as optimized concentration since high concentration could be harmful for column. Experiments with varying flow rate showed that 1 mL/min gave the best results. A decrease in the flow rate from 1 to 0.5 mL/min resulted into increase of retention times with slight broadening of peaks. On the other hand, an increase in flow rate from 1 to 1.5 mL/min resulted in reduced retention times and resolution.

(A) *Separation of the diastereomers prepared with DCT reagents having amino acids as chiral auxiliaries (i.e. set A consisting CDR 1–4)*: The examination of Table 1 clearly indicates that among the four pairs of diastereomers prepared with DCT reagents (having amino acid moieties as chiral auxiliaries) the highest and the lowest Δt was observed with CDR 1 and CDR 4 (having L-Leu and L-Ala moieties as chiral auxiliaries), respectively. In terms of resolution (R_s) the diastereomers prepared with CDR 1 were better resolved than those prepared with other three DCT reagents in this category. The CDRs can be arranged as 1 > 2 > 3 > 4 for the decreasing order of R_s obtained for the corresponding diastereomeric pair.

(B) *Separation of the diastereomers prepared with DCT reagents having amino acid amides as chiral auxiliaries (i.e. set B consisting CDR 5–8)*: Among the other four pairs of diastereomers prepared with DCT reagents (having amino acid amide moieties as chiral auxiliaries) the highest R_s and Δt were observed with CDR 5 (having L-Leu-NH_2 moiety as chiral auxiliary). The CDRs in this category can be arranged as 5 > 6 > 7 > 8 for the decreasing order of R_s obtained for the corresponding diastereomeric pair (Table 1).

(C) *Separation of the diastereomers prepared with MCT reagents having amino acid amides as chiral auxiliaries (i.e. set C consisting CDR 9–14)*: Of the total six pairs of diastereomers prepared with MCT reagents the highest R_s and Δt was observed with CDR 14 containing two stereogenic centres (i.e. having L-Ala-NH_2 and L-Phe-NH_2 moieties as chiral auxiliaries). Among the rest five pairs of diastereomers containing single stereogenic centre the diastereomers prepared with CDR 9 (having L-Leu-NH_2 moiety as chiral auxiliary) were better resolved than those prepared with other four MCT reagents in this category. The MCT reagents can be arranged as 14 > 9 > 10 > 11 > 12 > 13 for the decreasing order of R_s obtained for the corresponding diastereomeric pair (Table 1).

The resolution (R_s), difference between retention times of resolved diastereomers (Δt) and retention factor of first eluting diastereomer (k_1) obtained for the three aforementioned sets of diastereomers (A–C) are compared among themselves and among the three groups in Fig. 3a–c, respectively.

Among the three sets, the highest R_s and Δt were obtained for the set of the diastereomers prepared with DCT reagents having amino acids as chiral auxiliaries. The three sets can be arranged as A > B > C for the decreasing order of R_s and Δt obtained for the corresponding diastereomeric pairs (Fig. 3a and b), while for retention time the order was B > A > C (Fig. 3c). In conclusion, CDR 1 was considered to be the best as the diastereomers prepared with it had the highest R_s (5.86), Δt (2.82) and α (1.09).

Based on comparison of R_s and Δt, it is evident that of the two sets of diastereomers prepared with DCT reagents (*i.e.* set A and B) the diastereomeric pairs prepared with the CDRs having amino acids as the chiral auxiliaries were better separated in comparison to those prepared with their amide counterparts (Fig. 3a and b). The slightly lower retention times for the diastereomers prepared with DCT reagents having amino acids as chiral auxiliaries (CDR 1–4) as compared to those prepared with their amide counterparts (CDR 5–8) can be attributed to more polarity of acid variants compared to amides (Fig. 3c).

The analysis of Fig. 3a–c reveals that among the two sets of diastereomers prepared with CDRs having amino acid amides as chiral auxiliaries (*i.e.* set B and C) those prepared with DCT reagents were better separated and had longer retention times, in comparison to those prepared with their MCT counterparts. These results are in agreement with the literature [27] and can be explained on the basis of different interactions of the achiral auxiliaries present in the MCT and DCT reagents with ODS of the column or water present in the mobile phase. The oxygen atom in methoxy group of

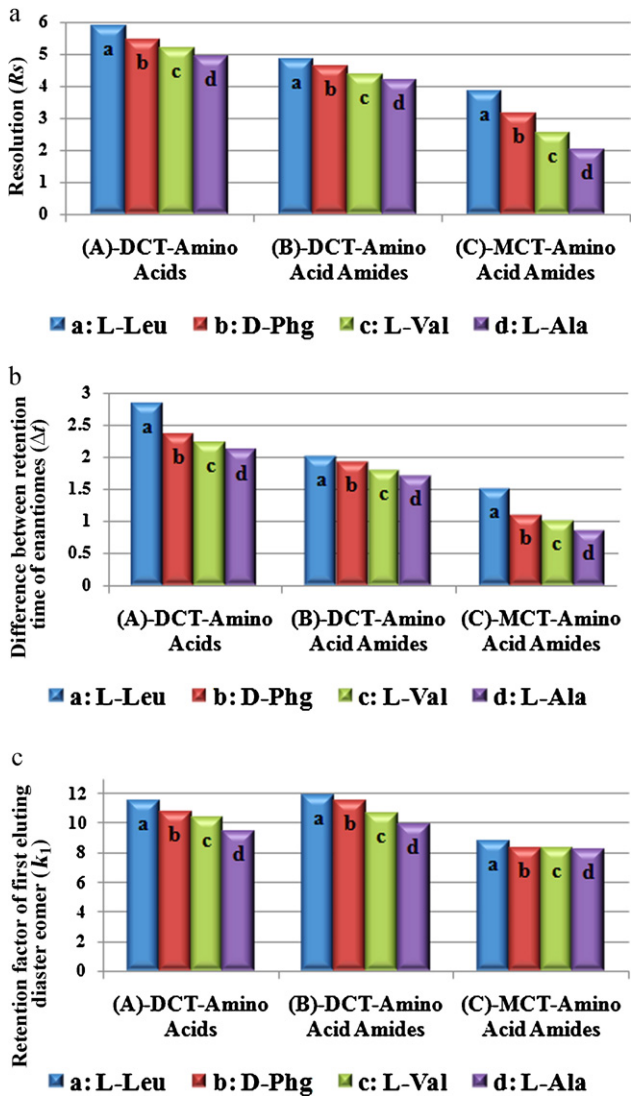


Fig. 3. Comparison of chromatographic data (a) resolution, R_s (b) difference between retention times of resolved diastereomers, Δt (c) retention factor of first eluting diastereomer, k_1 obtained with three sets of diastereomers prepared with (A) DCT reagents having amino acid moieties, (B) DCT reagents having amino acid amide moieties and, (C) MCT reagents having amino acid amide moieties as chiral auxiliaries.

the MCT reagents has higher electronegativity on the Pauling scale as compared to the chlorine atom in the DCT reagents (the values are 3.5 for oxygen and 3.0 for chlorine). Therefore, under reversed-phase conditions chlorine may have greater affinity with ODS of the column and oxygen would have greater affinity with water present in the mobile phase, causing faster elution of MCT-diastereomers as compared to their DCT-counterparts.

Among each of the three groups of diastereomers prepared with CDRs containing single stereogenic centre the longest retention times and the best resolution (R_S) were obtained with the CDRs either having L-Leu or L-Leu-NH_2 moiety as chiral auxiliaries (*i.e.* CDR 1, 5 and 9 among sets A, B and C, respectively). This trend can be explained on the basis of the hydrophobicity of the side chain R of the amino acid in the CDRs. The amino acids can be arranged in the decreasing order of their hydrophobicity as Leu (0.842) > Val (0.777) > Phe (0.756) > Met (0.709) > Ala (0.691); the values in parenthesis represent the apparent partial specific volume calculated by Bull and Breese [35]. The retention times became longer and resolution became better with increase in hydrophobicity of the side chain R of the amino acid in the CDRs (Table 1) among each of the three groups of diastereomers.

In addition to the mild derivatization conditions and excellent stability of resultant diastereomers, the resolution (R_s) was also significantly enhanced (ranged from 2.01 to 5.86) as compared to that achieved with various CDRs, NEIC (1.5) [9], GITC (1.19) and PDITC (4.34) [10], NAB-C (1.8) [11] and CSPs, Chiral-AGP (1.6), Ultron-Es-OVM (2.6) and Chiralcel OD-H (1.2) [18], CSP based on 1,1′-binaphthyl crown ether (1.43) [13] using HPLC and, with (18-crown-6)-tetracarboxylic acid as chiral additive (2.00) using capillary electrophoresis [20]; the values in parenthesis represent the resolution (R_s) achieved with respective approach (Table 2).

3.4. Separation mechanism

The separation mechanism can be explained by taking into account the mechanism proposed for enantioresolution of amino acids prepared with MCT and DCT reagents by Bhushan and Kumar with the aid of UV and NMR spectral techniques [27]. The reaction, for example, of the DCT reagent CDR 1 (that contains L-Leu moiety as chiral auxiliary) with (*R*,*S*)-MEX gives the diastereomers of the type, [L-Leu-(*R*)-MEX] and [L-Leu-(*S*)-MEX]; the first letter refers to the configuration of the chiral auxiliary of the CDR and the second to that of the analyte (*R*,*S*)-MEX.

Each substituent at the stereogenic centers of CDRs and MEX may remain perpendicular, to the planar molecule of *s*-triazine ring, except for their amino groups. The more hydrophobic substituents namely, isobutyl [$-CH_2CH(CH_3)$] and methyl group [$-CH_3$], respectively, belonging to the chiral auxiliary of the CDR and the analyte MEX, may have *cis* or *trans* type arrangements to each other, resulting in the different hydrophobicities of the diastereomers. The diastereomer having the *cis*-type arrangement of the more hydrophobic substituents would interact more strongly with ODS silica gel of the column and thus would have longer retention time as compared to its counterpart having *trans*-type arrangement. On the grounds of cross steric interactions the aforesaid more hydrophobic substituents belonging to the chiral auxiliary of the CDR and the analyte MEX are likely to stay *cis* in the L–(*R*) diastereomer and *trans* in the L–(*S*) diastereomer (Fig. 4). Therefore, the L–(*S*) diastereomer eluted first with the mobile phase. The explanation holds good for all the 14 diastereomers obtained with CDR 1–14.

3.5. Method validation

Method validation was done using diastereomers of (*R*,*S*)-MEX prepared with CDR 1 and results have been compiled in Table 3.

3.5.1. Linearity

Calibration graphs [peak area *vs.* concentration of enantiomer, $\mu g\,mL^{-1}$] were plotted for both diastereomers of (*R*,*S*)-MEX prepared with CDR 1 in the range of 0.1–200 $\mu g\,mL^{-1}$ 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 $y = 0.0007x + 0.0011$ ($R^2 = 0.9991$) and $y = 0.0011x - 0.0025$ ($R^2 = 0.9987$) for the first and second-eluted diastereomer, respectively.

3.5.2. Accuracy and precision

Replicate HPLC analysis ($n = 3$) of (*R*,*S*)-MEX at five different concentration levels (72, 96, 120, 144 and 168 $\mu g\,mL^{-1}$) showed RSD less than 1.5% in all the cases. RSD for first eluted diastereomer varied from 0.42% to 0.69% for intra-day precision and from 0.60% to 1.40% for inter-day precision; these values were from 0.46% to 0.75% and from 0.63% to 1.37% for second-eluted diastereomer. Intra-day recovery for first and second-eluted diastereomer varied from 98.7

Table 2
Comparison of various approaches for enantioresolution of (*R*, *S*)-MEX (in terms of R_s values).

S. No.	Approach for enantioseparation of (*R*,*S*)-MEX	R_s	Reference
HPLC-Indirect approach (using CDRs)			
1	*N*-(4,6-dichloro-[1,3,5]triazine-2-yl)-L-leucine (CDR1)-(RP)	5.86	Present method
2	(*S*)-1-(l-naphthyl)ethyl isocyanate (NEIC)-(NP)	1.50	Freitag et al. [9]
3	2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl isothiocyanate (GITC)-(RP)	1.19	Kleidernigg et al. [10]
4	(1*S*,2*S*)-*N*-[(2-isothiocyanato)-cyclohexyl]-pivalinoyl amide (PDITC)-(RP)	4.34	
5	(−)-4-(6-methoxy-2-naphthyl)-2-butyl chloroformate (NAB-C)-(RP)	1.80	Büschges et al. [11]
6	(−)-4-(6-methoxy-2-naphthyl)-2-butyl chloroformate (NAB-C)-(NP)	0.80	
HPLC-direct approach (using CSPs)			
7	α_1-acid glycoprotein (α_1-AGP) column-(RP)	1.60	Fieger et al. [18]
8	Ovomucoid (Ultron-ES-OVM) column-(RP)	2.60	
9	Cellulose tris(3,5-dimethylphenylcarbamate) (Chiralcel OD-H) column-(NP)	1.20	
10	CSP based on (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid-(RP)	1.39	Jin et al. [15]
11	CSP based on (−)-(18-crown-6)-2,3,11,12-tetracarboxylic acid-(RP)	1.42	
Capillary electrophoresis (CE)			
12	Using chiral additive (18-crown-6)-2,3,11,12-tetracarboxylic acid	2.00	Nishi et al. [20]

RP, reversed-phase; NP, normal phase.

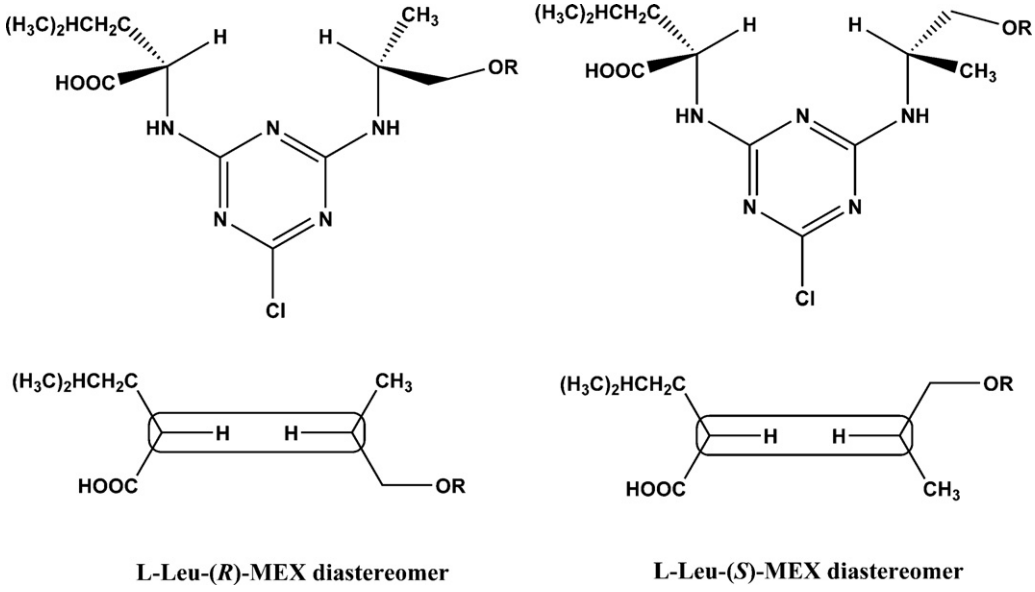


Fig. 4. The plausible conformations of the (*R*)- and (*S*)-MEX diastereomers prepared with CDR 1; proposed *cis* and *trans*-type arrangements of the more hydrophobic groups in L–(*R*) and L–(*S*) diastereomers, respectively. R = the dimethylbenzyl portion of MEX.

Table 3
Summary of HPLC method validation data obtained for diastereomers of (*R*,*S*)-MEX prepared with CDR 1.

	First eluting diastereomer			Second eluting diastereomer		
Linearity						
Range	0.1–200 μg mL^{-1}			0.1–200 μg mL^{-1}		
Slope	0.0007			0.0011		
Intercept	+0.0011			−0.0025		
Correlation coefficient (r^2)	0.9991			0.9987		
Concentration (actual) (μg mL^{-1})	Mean ± SD (measured) (μg mL^{-1})	Recovery (%)	RSD (%)	Mean ± SD (measured) (μg mL^{-1})	Recovery (%)	RSD (%)
Accuracy and precision						
Intra-day precision (n = 3)						
72	71.42 ± 0.29	99.2	0.42	71.49 ± 0.33	99.3	0.46
96	95.04 ± 0.55	99.0	0.58	95.14 ± 0.68	99.1	0.71
120	118.44 ± 0.56	98.7	0.47	118.2 ± 0.74	98.5	0.63
144	142.42 ± 0.84	98.9	0.59	142.27 ± 1.06	98.8	0.75
168	165.98 ± 1.14	98.8	0.69	165.81 ± 1.09	98.7	0.66
Inter-day precision (n = 3)						
72	71.20 ± 0.66	98.9	0.94	71.28 ± 0.44	99.0	0.63
96	94.65 ± 0.68	98.6	0.72	94.84 ± 0.70	98.8	0.74
120	117.72 ± 0.70	98.1	0.60	117.96 ± 1.09	98.3	0.93
144	140.97 ± 1.71	97.9	1.22	140.97 ± 1.93	97.9	1.37
168	164.13 ± 2.29	97.7	1.40	163.96 ± 1.78	97.6	1.09
Sensitivity						
Limit of detection (ng/mL)	0.13			0.13		
Limit of quantitation (ng/mL)	0.39			0.39		

to 99.2% and from 98.5 to 99.3%; the respective values for inter-day recoveries were from 97.7 to 98.9% and from 97.6 to 99.0%. LOD was taken as a concentration of the analyte where S/N was 3 and found to be 0.13×10^{-9} g/mL for both (*R*)- and (*S*)-enantiomer

4. Conclusion

The scientific novelty of the present work is the synthesis of four new DCT reagents by introducing amino acids as chiral auxiliaries in trichloro-*s*-triazine; these reagents provided better resolution and are also cost effective in comparison to DCT and MCT reagents having amino acid amides as chiral auxiliaries. Besides, the microwave-assisted synthesis of diastereomers of (*R*,*S*)-MEX with the 14 CDRs reduced the time of derivatization to 60–90 s as compared to 1–3 h under conventional heating. These reagents gain advantage as CDRs in terms of mild derivatization conditions, stability of derivatives and resolution (R_s) over several other reagents as literature reports cited in the paper. Moreover, structural features of trichloro-*s*-triazine offer possibility of straightforward and cost effective synthesis of a wide spectrum of CDRs with different reactive and detectable groups, thus making it a potential candidate in the field of chiral chromatography.

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