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Microwave-assisted synthesis and reversed-phase high-performance liquid chromatographic separation of diastereomers of (*R,S*)-baclofen using ten chiral derivatizing reagents designed from trichloro-*s*-triazine

Ravi Bhushan*, Shuchi Dixit

Department of Chemistry, Indian Institute of Technology Roorkee, Roorkee – 247667, India

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ABSTRACT

Four dichloro-*s*-triazine (DCT) and five monochloro-s-triazine (MCT) chiral derivatizing reagents (CDRs) were synthesized by incorporating amino acid amide moieties as chiral auxiliaries in trichloro-*s*-triazine and its 6-methoxy derivative, respectively. Another MCT reagent was synthesized by substitution of two chlorine atoms with two different amino acid amides in trichloro-*s*-triazine. These reagents were used for synthesis of diastereomers of (*R,S*)-baclofen under microwave irradiation (*i.e.* 60 s at 85% power using DCT reagents and 90 s at 85% power using MCT reagents). The diastereomers were separated on a reversed-phase C18 column using mixtures of methanol with aqueous trifluoroacetic acid (TFA) with UV detection at 230 nm. The separation behavior in terms of retention times and resolutions obtained for the two sets of diastereomers prepared with DCT and MCT reagents were compared among themselves and among the two groups. Longer retention times and better resolutions were observed with DCT reagents as compared to MCT reagents. The calibration curves were linear for both (*R*)- and (*S*)-baclofen in the concentration range 50-500 μg/ml. The average regression was 0.999 for both (*R*)- and (*S*)-baclofen. The RSD for (*R*)-baclofen was 0.40-0.86% for intra-day precision and 0.60-1.40% for inter-day precision and these values for (*S*)-baclofen were 0.52-0.75% and 0.64-1.32%, respectively. The recovery was 97.2-98.9% for (*R*)- and 97.0-98.9% for (*S*)-baclofen. The limit of detection was 1.63ng/ml and 1.52ng/ml for (*R*)- and (*S*)-baclofen, respectively.

1. Introduction

The inherent chiral selectivity of biological systems grounds the contrasting pharmacodynamic, pharmacokinetic and toxicological activities of the enantiomers of bioactive substances, particularly the drugs. Hence the development of analytical methods for enantioresolution of chiral compounds and control of enantiomeric purity of pharmaceuticals remains an area of great interest. The indirect method of enantioresolution can be considered to be associated with the following advantages: the commercial availability of a wide variety of chiral derivatizing reagents (CDRs), excellent separation and detection possibilities of the resulting diastereomers, easy optimization of chromatographic conditions, possibility of tailor-made separations, and the prospect of using relatively inexpensive achiral columns [1–3].

Baclofen, [4-amino-3-(*p*-chlorophenyl) butyric acid] (Fig. 1) is a selective $GABA_B$ receptor agonist, which is used as a muscle relaxant in treatments of spasticity associated with cerebral and spinal cord injury, secondary to multiple sclerosis, tardive dystonia, tetanus, cerebral palsy, and complex regional pain syndromes [4]. Although the drug is administered as a racemic mixture both the enantiomers show significant difference in their pharmacological effects and activities. While (*R*)- enantiomer is stereospecifically active for $GABA_B$ receptor; the (*S*)-enantiomer is almost inactive, sometime toxic and even antagonizes the effects of (*R*)- enantiomer [5,6]. Also, the (*R*)-enantiomer decreases heart rate and arterial pressure, whereas the (*S*)-enantiomer increases arterial pressure with no effect on heart rate [7].

Indirect enantioseparation of baclofen by using reversed-phase high-performance liquid chromatography (RP-HPLC) has been reported using CDRs like *o*-phthaldialdehyde combined with *N*-acetyl-L-cysteine (OPA-NAC) [8], (*S*)-naproxen chloride [9] and 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (FDAA) [10]. The direct methods include the use of a chiral mobile phase of aqueous copper (II) acetate and *N,N*-di-n-propyl-L-alanine [11], application of a D-penicillamine based ligand exchange chiral column [12], crown ether based chiral stationary phase (CSP) [13], Crownpak® CR CSP [14] and CSP based on macrocyclic antibiotic teicoplanin [15]. Baclofen enantiomers have been determined in pharmaceutical formulations using enantioselective membrane electrodes based upon maltodextrin [16] and α- and γ -cyclodextrins [17].

* Corresponding author. Tel.: +91 1332 285795; fax: +91 1332 286202.
E-mail address: rbushfcy@iitr.ernet.in (R. Bhushan).

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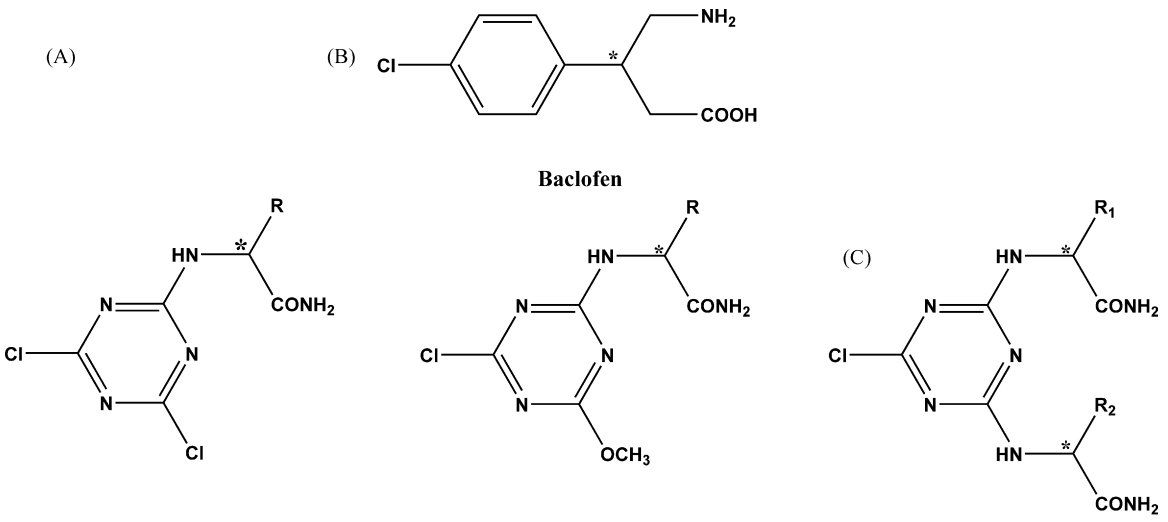

CDR	Structure	R
1, *N*-(4,6-Dichloro-[1,3,5]-triazine-2-yl)-L-alaninamide	A	$-CH_3$
2, *N*-(4,6-Dichloro-[1,3,5]-triazine-2-yl)-L-valinamide	A	$-CH(CH_3)_2$
3, *N*-(4,6-Dichloro-[1,3,5]-triazine-2-yl)-D-phenylglycinamide	A	$-C_6H_5$
4, *N*-(4,6-Dichloro-[1,3,5]-triazine-2-yl)-L-leucinamide	A	$-CH_2CH(CH_3)_2$
5, *N*-(4-Chloro-6-methoxy-[1,3,5]-triazine-2-yl)-L-alaninamide	B	$-CH_3$
6, *N*-(4-Chloro-6-methoxy-[1,3,5]-triazine-2-yl)-L-valinamide	B	$-CH(CH_3)_2$
7, *N*-(4-Chloro-6-methoxy-[1,3,5]-triazine-2-yl)-D-phenylglycinamide	B	$-C_6H_5$
8, *N*-(4-Chloro-6-methoxy-[1,3,5]-triazine-2-yl)-L-methioninamide	B	$-CH_2CH_2SCH_3$
9, *N*-(4-Chloro-6-methoxy-[1,3,5]-triazine-2-yl)-L-leucinamide	B	$-CH_2CH(CH_3)_2$
10,*N*-(4-(*S*)-1-Carbamoyl-2-methyl-propylamino)-6-chloro-[1,3,5] triazine-2-yl] -L-phenylalanine	C	R_1: $-CH(CH_3)_2$ R_2: $-CH_2C_6H_5$

Fig. 1. Structures of baclofen and chiral derivatizing reagents (CDR1-10).

The prospect of easy and controlled sequential substitution of the chlorine atoms by nucleophiles in cyanuric chloride (2,4,6-trichloro-1,3,5-triazine; trichloro-*s*-triazine; *s*-triazine chloride; CC) can be attributed to its trifunctionality and high reactivity [18]. It is commercially available and is a very inexpensive reagent, which makes its applications even more attractive. Chiral triazine derivatives have been used to prepare CSPs for direct enantioresolution of amino acids and amino alcohols [19–21]. Triazinyl bonded peptides have been described to serve as chiral solvating agents for the determination of enantiomeric composition of 3,5-dinitrophenyl derivatized compounds such as amines and carboxylic acids by NMR [22]. Brückner and co-workers [23,24] 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 also been reported using ten chiral dichloro-*s*-triazine (DCT) and monochloro-*s*-triazine (MCT) reagents as CDRs prepared by the nucleophilic substitution of chlorine atom(s) in trichloro-*s*-triazine and its 6-methoxy derivative with different amino acid amides [25].

A wide range of pharmaceutically important compounds possessing amino groups have been enantioseparated as their diastereomers prepared with Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide, FDNP-L-Ala-NH_2, FDAA, MR) and its structural variants [10,26,27]; such diastereomers show structural similarities with those prepared with chiral *s*-triazine reagents [1]. Keeping in view the above cited literature and the literature on enantiomeric resolution of (*R*,*S*)-baclofen, its primary amino group and previous work carried out in this laboratory [25], four DCT and six MCT reagents (Fig. 1) were synthesized and used as CDRs for enantioseparation of (*R*,*S*)- baclofen. To the best of the authors' knowledge this is the first report on microwave (MW) assisted synthesis of diastereomers of (*R*,*S*)-baclofen with the aforementioned CDRs synthesized from trichloro-*s*-triazine followed by their 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 equipment 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 [$^2H_6$] dimethyl sulfoxide (DMSO-d_6) as deuterated solvent.

2.2. Chemicals and reagents

(*R, S*)-, (*R*)- and (*S*)-baclofen, cyanuric chloride, 2,4,6-collidine, L-alaninamide hydrochloride (L-Ala-NH_2·HCl), L-phenylalaninamide hydrochloride (L-Phe-NH_2·HCl), L-valinamide hydrochloride (L-Val-NH_2·HCl), L-leucinamide hydrochloride (L-Leu-NH_2·HCl), L-methioninamide hydrochloride (L-Met-NH_2·HCl) and D-phenylglycinamide (D-Phg-NH_2) 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 (18.2MΩ mL) was used throughout.

2.3. Preparation of stock solutions

Stock solutions of (*R, S*)-, (*R*)- and (*S*)-baclofen (100 mM) were prepared in 0.1M $NaHCO_3$ for derivatization reactions. Solu-

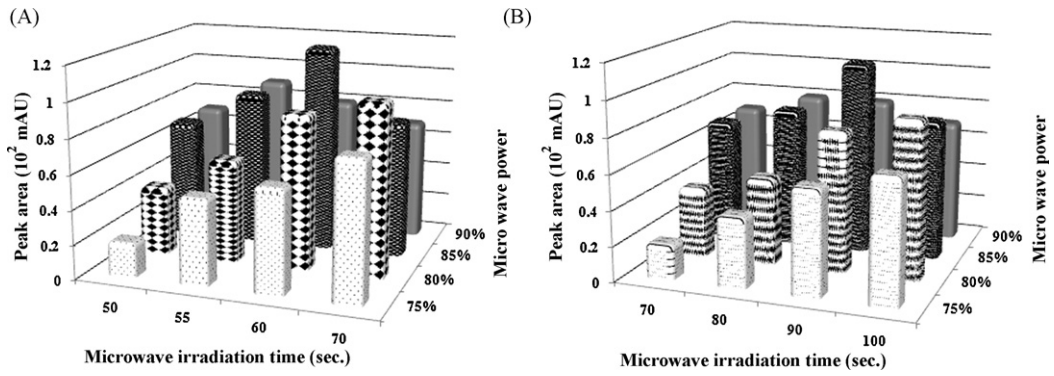

Fig. 2. The effect of microwave power and irradiation time on derivatization process (A in case of DCT reagents and (B) in case of MCT reagents. The peak area represents the area observed for diastereomer corresponding to (*R*)-baclofen prepared with above mentioned CDRs. Chromatographic conditions: Column, LiChrospher C18 (250mm × 4.6mm I.D., 5 μm particle size); eluent, mobile phase (I) consisting eluent A [MeOH(100ml)+ H_2O (900ml)] and eluent B [MeOH(800ml)+ H_2O(200ml)], both containing 0.1% TFA; gradient 100% A to 100% B in 45 min; flow rate, 1.0 ml/min; detection, 230nm. The microwave used was Multiwave 3000 (800W, Perkin-Elmer, Shelton, CT, USA).

tions 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 1M $NaHCO_3$ was prepared in purified water. All solutions were filtered through a 0.45 μm filter prior to use.

2.4. Synthesis of chiral DCT and MCT reagents

The DCT reagents (CDR 1-4) and MCT reagents (CDR 5-10) are the same as described in the previous work [25]. However, the synthesis, characterization and determination of enantiomeric purity of the reagents was carried out for the present studies. The characterization data of all the CDRs has been summarized in Table 2.

2.5. Microwave-assisted synthesis of diastereomers

The diastereomers of (*R,S*)-baclofen with the CDRs 1-10 were synthesized according to literature reported for the diastereomerization of amino acids [25]. In the present case MW irradiation was used for 60s at 85% power (using DCT reagents, CDR 1-4) and 90s at 85% power (using MCT reagents, CDR 5-10). 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*)-baclofen with CDR 4 and CDR 9 (as representatives of DCT and MCT reagents, respectively).

2.6. HPLC analysis

A LiChrospher C18 (250mm × 4.6mm I.D., 5 μm particle size) column from Merck (Darmstadt, Germany) was used for HPLC. The successful mobile phases were (I) eluent A [MeOH(100ml)+ H_2O (900ml)] and eluent B [MeOH(800ml)+ H_2O(200ml)], both containing 0.1% TFA; gradient 100% A to 100% B in 45 min, and (II) eluent C [MeCN(100ml)+ H_2O(900ml)] and eluent D [MeCN(800ml)+ H_2O(200ml)], both containing 0.1% TFA; gradient 100% C to 100% D in 45 min, at a flow rate of 1mL/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, effect of flow rate (1 to 1.5mL/min), and the effect of TFA concentration in the range of 0.01-0.3% was studied.

2.7. Validation procedure

Method validation was performed using diastereomers of (*R,S*)-baclofen prepared with CDR 4 following ICH guidelines [28]. Linearity was established by injecting the samples in triplicate, containing (*R,S*)-baclofen in the concentration range of 50–500 μg/ml. Intra-day precision was established by making triplicate injections of three concentrations in the above range (*i.e.* 300, 375 and 450 μg/ml). These studies were also repeated on three consecutive days to determine inter-day precision. Signal-to-noise ratio of approximately 3:1 and 10:1 were used for estimating the detection and quantification limit, respectively.

3. Results and discussion

3.1. Synthesis of chiral derivatizing reagents

The DCT reagents (CDR 1-4) were synthesized by nucleophilic substitution of one of the chlorine atoms in trichloro-*s*-triazine with L-Ala-NH_2, L-Val-NH_2, D-Phg-NH_2 and L-Leu-NH_2. The MCT reagents (CDR 5-9) were synthesized by nucleophilic substitution of one of the chlorine atoms in 6-methoxy derivative of trichloro-*s*-triazine with L-Ala-NH_2, L-Val-NH_2, D-Phg-NH_2, L-Met-NH_2 and L-Leu-NH_2. The MCT reagent, CDR 10 was obtained by the substitution of two chlorine atoms, respectively, with L-Val-NH_2 and L-Phe-NH_2. All the CDRs were characterized by IR, UV, CHN, and ^1H NMR. The characterization data were in agreement with a previous literature report [25].

3.2. Synthesis of diastereomers

A total of ten pairs of diastereomers were synthesized from four DCT and six MCT reagents under microwave irradiation. These were also synthesized using conventional heating (*i.e.* 3 hrs at 30°C using DCT reagents, and 1 hr at 80°C using MCT reagents). Nevertheless, experimental conditions were optimized for microwave-assisted synthesis as are discussed below.

3.2.1. Role of pH

Use of 1 M $NaHCO_3$ (30μL, 30μmol) at a pH around 8.0 was found to be optimum to obtain the best yield for derivatization of (*R, S*)-baclofen. No derivatization was observed in the absence of $NaHCO_3$ even by 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. Reagent excess

The CDR 4 and 9 were used in 1-5 fold molar ratios, as representatives of DCT and MCT reagents to find the optimum reagent concentration for derivatization. In both the categories of diastereomers prepared with DCT and MCT reagents derivatization was

complete when 1.7 fold (1.7μmol) molar excess of the reagent was used. Slight kinetic resolution was observed when lower ratios of CDR: baclofen (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

The time of microwave irradiation and microwave power affect the derivatization efficiency, so these parameters were investigated in the study (Fig. 2A and B). 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. 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. Application of MW at a lower power required more time for derivatization.

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

CDR	Δt	k_1	k_2	α	*Rs*
1	1.68	11.74	12.35	1.05	5.43
2	1.72	12.46	13.08	1.05	5.92
3	1.79	13.26	13.90	1.05	6.15
4	2.21	13.39	14.44	1.08	8.20
5	0.87	10.03	10.34	1.03	3.15
6	0.99	10.08	10.43	1.03	3.51
7	1.08	10.11	10.49	1.04	3.61
8	1.05	10.23	10.60	1.04	3.88
9	1.12	10.55	10.95	1.04	4.13
10	1.29	11.49	11.95	1.04	4.84

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 experimental, mobile phase (I); flow rate, 1.0 ml/min; detection, 230 nm; Δt, difference between retention times of separated diastereomers; k_1 and k_2 retention factors of first and second eluting diastereomers, respectively; α, separation factor; *Rs*, resolution.

3.3. HPLC analysis

All ten pairs of diastereomers synthesized from four DCT and six MCT reagents were separated under reversed-phase conditions by HPLC. The retention factor (k), separation factor (α) and resolution (R_S) of the resolved diastereomers are given in Table 1. MeOH was found to be a better organic solvent in comparison to MeCN as higher resolutions (in terms of *Rs*) were obtained with the former. Sharp peaks were obtained using mobile phase (I) consisting eluent A [MeOH(100ml)+ H_2O (900ml)] and eluent B [MeOH(800ml)+ H_2O(200ml)], both containing 0.1% TFA; gradient 100% A to 100% B in 45 min. Sections of chromatograms showing baseline resolution of the diastereomers of (*R, S*)-baclofen prepared with CDR1-10 are shown in Fig. 3. The chromatograms for the separation of diastereomers, which were obtained by aforementioned optimized microwave irradiation conditions and those by conventional heating were in agreement in terms of elution order and retention times.

The successful separation conditions, as optimized, were a linear gradient of eluent B from 0-100% in 45 min (mobile phase I) containing 0.1% TFA at a flow rate of 1 mL/min.

Among the four pairs of diastereomers prepared with DCT reagents the highest and the lowest Δt was observed with CDR 4 and CDR 1 (having L-Leu-NH_2 and L-Ala-NH_2 moieties as chiral auxiliaries), respectively (Table 1). In terms of R_S the diastereomers prepared with CDR 4 were better resolved than those prepared with other three DCT reagents. The CDRs can be arranged as 4 > 2 > 3 > 1 for the decreasing order of R_S obtained for the corresponding diastereomeric pair. On the other hand, among the six pairs of diastereomers prepared with MCT reagents the highest Δt and *Rs* was observed with CDR 10 containing two stereogenic centres (*i.e.* having L-Ala-NH_2 and L-Phe-NH_2 moieties as chiral auxiliaries). The MCT reagents can be arranged as 10 > 9 > 8 > 7 > 6 > 5 for the decreasing order of R_S obtained for the corresponding diastereomeric pair. In conclusion, the highest Δt(2.21), *Rs*(8.20) and α(1.08) were obtained for the diastereomers prepared with CDR 4 having L-Leu-NH_2 moiety as chiral auxiliary.

The longer retention times and better resolutions (in terms of *Rs*) of the diastereomers prepared with the CDRs having L-Leu-NH_2 moiety as chiral auxiliaries (*i.e.* CDR4 and 9 in case of DCT and MCT reagents, respectively) among the diastereomers prepared with the CDRs containing single stereogenic centre can be explained

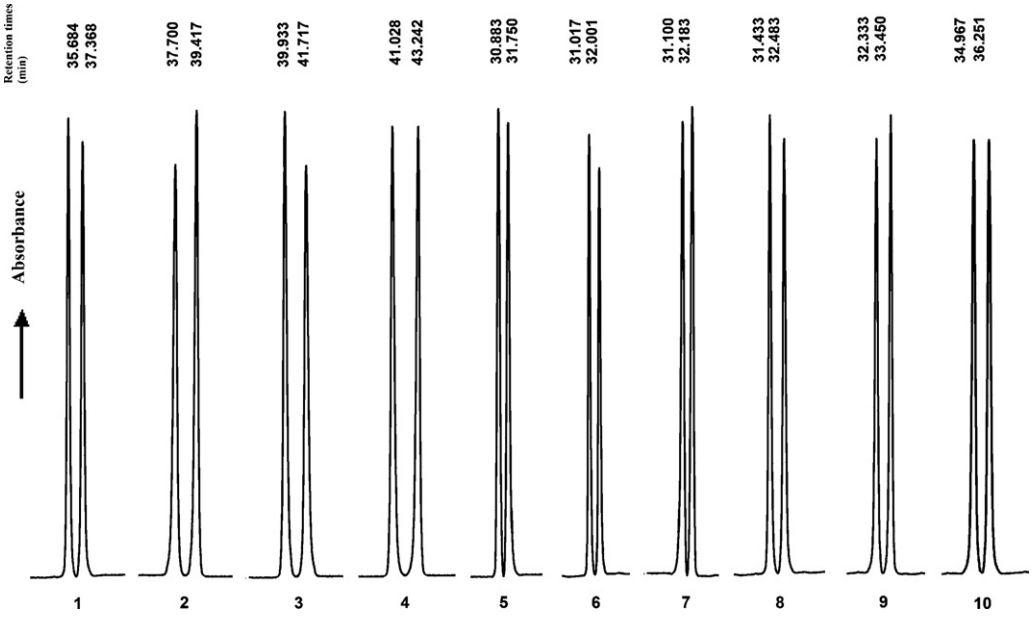

Fig. 3. Sections of chromatograms showing resolution of diastereomers of (*R, S*)-baclofen prepared with CDR1-10. The CDRs are as mentioned in Fig. 1. Chromatographic conditions: Column, LiChrospher C18 (250mm × 4.6mm I.D., 5 μm particle size); eluent, see experimental, mobile phase (I) consisting eluent A [MeOH(100ml)+ H_2O (900ml)] and eluent B [MeOH(800ml)+ H_2O(200ml)], both containing 0.1% TFA; gradient 100% A to 100% B in 45 min;, flow rate, 1.0 ml/min; detection, 230nm.

Table 2
Characterization data of CDR1-10.

CDR	Yield	Color	Melting point	UV(λ_{max} (nm), in MeCN)
1, *N*-(4,6-Dichloro-[1,3,5]triazine-2-yl)-L-alaninamide	92%	White	177–178 °C	231
2, *N*-(4,6-Dichloro-[1,3,5]triazine-2-yl)-L-valinamide	85%	White	149–150 °C	232
3, *N*-(4,6-Dichloro-[1,3,5]triazine-2-yl)-D-phenylglycinamide	80%	White	80–81 °C	231
4, *N*-(4,6-Dichloro-[1,3,5]triazine-2-yl)-L-leucinamide	85%	White	95–96 °C	231
5, *N*-(4-Chloro-6-methoxy-[1,3,5]-triazine-2-yl)- L-alaninamide	74%	White	177-178 °C	225
6, *N*-(4-Chloro-6-methoxy-[1,3,5]-triazine-2-yl)-L-valinamide	76%	White	198–199 °C	226
7, *N*-(4-Chloro-6-methoxy-[1,3,5]-triazine-2-yl)- D-phenylglycinamide	76%	White	95–96 °C	226
8, *N*-(4-Chloro-6-methoxy-[1,3,5]-triazine-2-yl)- L-methioninamide	83%	White	176–177 °C	225
9, *N*-(4-Chloro-6-methoxy-[1,3,5]-triazine-2-yl)- L-leucinamide	74%	White	187–188 °C	225
10, *N*-(4-((*S*)-1-Carbamoyl-2-methyl-propylamino)-6-chloro-[1,3,5] triazine-2-yl]-L-phenylalanine	93%	White	129–130 °C	223

on the basis of hydrophobicity scale of amino acids prepared by Bull and Breese by calculating their apparent partial specific volume [29]. The amino acids can be arranged in the decreasing order of their hydrophobicity as Leu(0.842) > Val (0.777) > Met(0.709) >Ala(0.691); the values in parenthesis represent the apparent partial specific volume. Among both the categories of diastereomers prepared with DCT and MCT reagents, retention times became longer and resolution became better with increase in hydrophobicities of the chiral auxiliaries of the CDRs.

Examination of Table 1 clearly indicates that the diastereomeric pairs prepared with DCT reagents were better separated and had longer retention times, in comparison to those prepared with MCT reagents. It can be attributed to the higher electronegativity of the oxygen atom in methoxy group of the MCT reagents as compared to the chlorine atom in the DCT reagents (on the Pauling scale the values are 3.5 for oxygen and 3.0 for chlorine). While oxygen may have greater affinity with water present in the mobile phase, the chlorine would have greater affinity with ODS of the column under the reversed-phase conditions, leading to the faster elution of MCT-diastereomers as compared to their DCT-counterparts. The elution order of diastereomers was confirmed by elution of diastereomers of a single enantiomer. The diastereomers of (*R*)-baclofen were found to be eluted earlier than their (*S*)-counterparts for all the reagents except those prepared with the CDRs having D-Phg-NH_2 moiety as the chiral auxiliary (*i.e.* CDRs 3 and 7), where a completely reverse elution order was found.

The derivatization of (*R,S*)-baclofen using aforesaid DCT and MCT reagents under microwave irradiation required much lesser time (*i.e.* 60s at 85% power using DCT and 90s at 85% power using MCT reagents) as compared to the time required for derivatization with FDAA (1 h at 45°C) and GITC (30 min at RT) reagents [25,30]. Also, the stability of the resultant diastereomers (*i.e.* 1 month at 5°C) was found much better as compared to the OPA-NAC (where fluorescence is stable for 20-30 min only) [8], GITC (24h at RT) and FLEC (48h at RT) diastereomers [30]. In addition to the mild derivatization conditions and excellent stability of resultant diastereomers, the resolution (in terms of *R*s) was also significantly enhanced (ranged from 3.15-8.20) as compared to that achieved on a teicoplanin CSP after precolumn derivatization with various alkyl isothiocyanate reagents [*i.e.* CH_3SCN (1.92), C_2H_5SCN (1.13), C_3H_7SCN (1.12), C_4H_9SCN (0.75) and *tert*-C_4H_9SCN (0.67)] [31], and with the CDR (*S*)-naproxen chloride (1.73) [9]; the values in parenthesis represent the resolution (in terms of *R*s) achieved with respective reagent.

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 [25]. The reaction, for example, of the DCT reagent CDR 4 (that contains L-Leu-NH_2 moiety as chiral auxiliary) with (*R*, *S*)-baclofen gives the diastereomers of the type, [L-Leu-(*R*)-baclofen] and [L-Leu-(*S*)-baclofen]; the first letter refers to the configuration of the chiral auxiliary of the CDR and the second to that of the analyte baclofen.

Each substituent at the stereogenic centers of CDRs and baclofen may remain perpendicular, to the planar molecule of *s*-triazine ring, except for their amino groups. The more hydrophobic substituents namely, isobutyl group [-$CH_2CH(CH_3)_2$] and substituted

Table 3
Summary of HPLC Method Validation Study.

	(*R*)-baclofen			(*S*)-baclofen		
Linearity:						
Range	50-500 μgmL^{-1}			50-500 μgmL^{-1}		
Slope	0.995			0.986		
Intercept	+0.039			−0.092		
Correlation coefficient (r^2)	0.999			0.999		
Accuracy and precision:						
Concentration (actual) (μg/ml)	Mean ± SD (measured) (μg/ml)	Recovery (%)	RSD (%)	Mean ± SD (measured) (μg/ml)	Recovery (%)	RSD (%)
Intra-day precision (n = 3)						
300	294.90 ± 2.53	98.3	0.86	296.70 ± 1.66	98.9	0.56
375	370.87 ± 2.11	98.9	0.57	369.38 ± 2.77	98.5	0.75
450	444.15 ± 1.77	98.7	0.40	441.90 ± 2.29	98.2	0.52
Inter-day precision (n = 3)						
300	291.60 ± 4.08	97.2	1.40	291.90 ± 3.85	97.3	1.32
375	366.00 ± 2.63	97.6	0.72	363.75 ± 3.78	97.0	1.04
450	440.10 ± 2.64	97.8	0.60	439.65 ± 2.81	97.7	0.64
Sensitivity:						
Limit of detection (ng/ml)	1.63			1.52		
Limit of quantitation (ng/ml)	4.89			4.56		

phenyl group [-C_6H_4Cl], respectively, belonging to the chiral auxiliary of the CDR and the analyte baclofen, may have *cis* or *trans* type arrangements, 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. Since the diastereomer corresponding to the (*R*)-baclofen eluted before its corresponding (*S*)-diastereomers it can be concluded that the diastereomer of the (*R*)-baclofen has the *trans*-type arrangement.

3.5. Method validation

3.5.1. Linearity

Method validation was done using diastereomers of (*R, S*)-baclofen prepared with CDR 4. Calibration graphs [peak area (y) vs. concentration of enantiomer (x), μgmL^{-1}] were plotted for both diastereomers of (*R, S*)-baclofen prepared with CDR 4 in the range 50-500 μgmL^{-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 $y = 0.995x + 0.039$ ($R^2 = 0.999$) and $y = 0.986x - 0.092$ ($R^2 = 0.999$) for the diastereomers of (*R*)- and (*S*)-baclofen, respectively.

3.5.2. Accuracy and precision

Replicate HPLC analysis (n = 3) of (*R, S*)-baclofen at three different concentration levels (300, 375, 450 μgmL^{-1}) showed RSD less than 1.5% in all cases (Table 3). The RSD for (*R*)-baclofen was 0.40-0.86% for intra-day precision and 0.60-1.40% for inter-day precision and those values for (*S*)-baclofen were 0.52-0.75% and 0.64-1.32%, respectively. The recovery was 97.2-99.2% for (*R*)- and 96.3-99.0% for (*S*)-baclofen. The limit of detection was 1.63ng/ml and 1.52ng/ml for (*R*)- and (*S*)-baclofen, respectively. The limit of quantitation was 4.89ng/ml and 4.56ng/ml for (*R*)- and (*S*)-baclofen, respectively.

4. Conclusion

The novelty of the present work lies in the microwave-assisted synthesis of diastereomers of (*R,S*)-baclofen with CDRs designed from trichloro-*s*-triazine, which reduced derivatization time to 60-90s as compared to 1-3h under conventional heating. In addition to mild derivatization conditions, these reagents also gain advantages in terms of stability of derivatives and resolution over several other derivatizing reagents as literature reports cited in the paper [8,9,30,31]. The longer retention times and better resolutions of diastereomers prepared with DCT reagents were explained by interaction of achiral substituent with ODS column or with the mobile phase. The separation behavior in terms of retention times and resolutions of diastereomers prepared with various CDRs having different amino acid amide moieties as chiral auxiliaries were explained in the light of their different hydrophobicities. 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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