RESOLUTION OF CERTAIN RACEMIC PHARMACEUTICALS BY LIQUID CHROMATOGRAPHY

Ph.D. THESIS

by

SHIV KUMAR ALWERA

DEPARTMENT OF CHEMISTRY
INDIAN INSTITUTE OF TECHNOLOGY ROORKEE
ROORKEE-247667 (INDIA)
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ROORKEE

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"RESOLUTION OF CERTAIN RACEMIC PHARMACEUTICALS BY LIQUID CHROMATOGRAPHY"** in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the **Department of Chemistry** of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period from July, 2013 to November, 2017 under the supervision of Dr. Ravi Bhushan, Professor, Department of Chemistry, Indian institute of Technology Roorkee, Roorkee.

The matter presented in the thesis has not been submitted by me for the award of any other degree of this or any other Institution.

(SHIV KUMAR ALWERA)

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

Signature of Supervisor

The Ph.D. Viva-Voce Examination of Shiv Kumar Alwera, Research Scholar, has been held on 13th April 2018.

Chairman, SRC **Signature of External Examiner**

This is to certify that the student has made all the corrections in the thesis.

(Ravi Bhushan)
Signature of supervisor **Head of the Department**
Dated:

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ABSTRACT

It is well noticed in both academia and industry that a pair of enantiomers show different activity in the 'chiral environment of our body' and, in many countries, the regulatory agencies ask the industries to present full information on pharmacodynamics and pharmacokinetics of both the enantiomers including the stereoselective analytical methods before permitting a new drug to be registered and also insist on bringing (preferably) only the active enantiomer of a chiral drug to market. With such an awareness of these issues among those involved in the drug development, marketing and law enforcement efficient methods for verification of enantiomeric purity or to monitor stereoselective synthesis are increasingly required. Among the various available methods for establishing enantiomeric purity, liquid chromatographic techniques especially high performance liquid chromatography is commonly used.

In recent years, applications of surfactant based aq. mobile phase for HPLC analysis (micellar liquid chromatography; MLC) of certain compounds has gained attention. But, the present thesis describes the application of the MLC for the first time for enantioseparations.

From among the large number of β-adrenolytics in use, (*RS*)-propranolol, (*RS*)-metoprolol, (*RS*)-atenolol, (*RS*)-betaxolol, (*RS*)-carvedilol, (*RS*)-salbutamol and (*RS*)-bisoprolol, were selected for the development of sensitive methods of enantioseparation. Besides, (*RS*)-selenomethionine, (*RS*)-methionine, **(***RS*)-cysteine and (*RS*)-penicillamine, were selected from among the pool of the amino acids considering the importance of sulphur containing amino acids specially. A chapter wise description is given below.

Description of chapters

The **first chapter** deals with preamble to present studies including introduction to enantiomers and their importance, their separation approaches and separation techniques. The chapter also includes present work, selection of chiral chromophoric moieties and experimental approach.

The **second chapter** presents pharmaceutical importance and literature survey on enantioseparation of chosen β-adrenolytics and amino acids. Besides, physical and chemical properties of the chiral moieties and justification for choosing them for their application in presents studies has been described.

The **third chapter** presents description of materials, equipment, preparation of stock solutions and extraction of active pharmaceutical ingredients from the commercial tablets, liquid chromatographic techniques, synthesis of chiral derivatizing reagents, and synthesis of diastereomeric derivatives (of racemic β-adrenolytics and amino acids) and their RP-HPLC, open column chromatography, detagging of diastereomeric derivative, characterization, calculation of chromatographic data and method validation.

The **fourth chapter** deals with the enantioseparation of chosen racemic drugs, after derivatization with (*S*)-levofloxacin based chiral derivatizing reagents, using RP-HPLC and micellar liquid chromatography. It has been divided into three sections.

***Section A*:** Describes enantioseparation of (*RS*)-propranolol. "Diastereomeric derivatization of (*RS*)-propranolol were synthesized using (*S*)-levofloxacin-based new chiral derivatizing reagents (CDRs). Levofloxacin was chosen as the pure (*S*)-enantiomer for its high molar absorptivity ($\varepsilon_o \sim 24000$) and availability at a low price. Its -COOH group had *N*-hydroxysuccinimide and *N*-hydroxybenzotriazole, which acted as good leaving groups during nucleophilic substitution by the amino group of the racemic (*RS*)-propranolol; the CDRs were characterized by UV, IR, ^{1}H-NMR, high resolution mass spectrometry (HRMS) and carbon, hydrogen, nitrogen, and sulphur elemental components analyser (CHNS). Diastereomeric derivatives were separated quantitatively using open column chromatography; absolute configuration of the diastereomeric derivatives was established and the reagent moiety was detagged under microwave-assisted acidic conditions. (*S*)- and (*R*)-propranolol as pure enantiomers and (*S*)-levofloxacin were separated, isolated and characterized. Optimized lowest-energy structures of the diastereomeric derivatives were developed using Gaussian 09 Rev. A.02 program and hybrid density functional B3LYP with 6-31G* basis set (based on density functional theory) for explanation of elution order and configuration. In addition, RP-HPLC conditions for separation of diastereomeric derivatives were optimized with respect to pH, concentration of buffer, flow rate of mobile phase and nature of organic modifier. HPLC separation method was validated as per International Conference on Harmonization guidelines. With the systematic application of various analytical techniques, absolute configuration of the diastereomeric derivatives (and the native enantiomers) of (*RS*)-propranolol was established".

***Section B*:** Describes "An effective and simple method that successfully leads to liquid chromatographic enantioseparation of racemic β-adrenolytics by derivatization approach

(with an example established with (*RS*)-metoprolol, and verified by using (*RS*)-atenolol). Chiral derivatizing reagents (CDRs) were synthesised using (*S*)-levofloxacin as the chiral moiety. The effectiveness of this method is not limited to enantioseparation but also to determine absolute configuration of diastereomeric derivatives. The method describes (i) synthesis of CDRs by reaction of (*S*)-levofloxacin with *N*-hydroxysuccinimide and *N*-hydroxybenzotriazole in presence of coupling reagent dicyclohexylcarbodiimide, (ii) synthesis of diastereomeric derivatives of racemic β-adrenolytics under microwave irradiation using the CDRs so synthesized, (iii) separation of diastereomeric derivatives by HPLC and open column chromatography, and (iv) determination of absolute configuration of diastereomeric derivatives. The (*S*)-Lfx based CDRs are efficient in chromatographic separation and provide very low limit of detection (LOD) and limit of quantitation (LOQ) they can be successfully used in trace analysis of several other racemic compounds which contain amino group in their structures (e.g., β-adrenolytics, amino acids etc)".

***Section C*:** "The enantioseparation of a few commonly administered racemic β-adrenolytics (namely, betaxolol, carvedilol, salbutamol, and bisoprolol) has been achieved using a water micellar mobile phase containing surfactants (SDS and Brij-35) without organic solvents as a new approach in RP-HPLC. Two CDRs based on enantiomerically pure (*S*)-(−)-levofloxacin were synthesized using *N*-hydroxysuccinimide and *N*-hydroxybenzotriazole as the activation auxiliaries. Diastereomeric derivatives of the chosen β-adrenolytics were synthesized under microwave irradiation in a very short reaction time. The (*S*)-(−)-levofloxacin moiety enhanced molar absorbance of the diastereomeric derivatives resulting into very low LOD (1.618 ng mL^{-1} and 4.902 ng $mL^{-1,}$ respectively, for diastereomeric derivatives of (*RS*)-Bxl and better resolution with lower retention times (for all the analytes), in comparison to literature reports. There was 15-20 times less consumption of mobile phase because of lower retention time".

The **fifth chapter** deals with the enantioseparation of chosen racemic drugs, after derivatization with (*S*)-ketoprofen based chiral derivatizing reagents, using RP-HPLC and micellar liquid chromatography. It has been divided into two sections.

***Section A*:** Describes "the diastereomeric derivatives of racemic β-adrenolytic drugs [namely (*RS*)-propranolol, (*RS*)-metoprolol and (*RS*)-atenolol] were synthesized under microwave irradiation with (*S*)-ketoprofen based chiral derivatization reagents (CDRs) newly synthesized for this purpose. (*S*)-Ketoprofen was chosen for its high molar

absorptivity (ε_o ~ 40,000) and its availability as a pure (*S*)-enantiomer. Its -COOH group was activated with *N*-hydroxysuccinimide and *N*-hydroxybenzotriazole; these were easily introduced and also acted as good leaving groups during nucleophilic substitution by the amino group of the racemic β-adrenolytics. The CDRs were characterized by UV, IR, ^{1}H-NMR, HRMS and CHNS. Separation of diastereomeric derivatives was achieved by RP-HPLC and open column chromatography. Absolute configuration of the diastereomeric derivatives was established with the help of ^{1}H-NMR supported by developing their optimized lowest energy structures using Gaussian 09 Rev. A.02 program and hybrid density functional B3LYP with 6-31G* basis set (based on density functional theory), and elution order was established. RP HPLC conditions for separation were optimized and the separation method was validated. The limit of detection values were 0.308 and 0.302 ng mL^{-1}".

***Section B*:** In this section "Micellar liquid chromatographic method has been developed for enantioseparation of four β-adrenolytics, namely, (*RS*)-salbutamol, (*RS*)-carvedilol, (*RS*)-bisoprolol, and (*RS*)-betaxolol. Both sodium dodecyl sulfate and Brij-35 were used as the surfactants in water as the mobile phase. Advantages for using both the surfactants in combination were investigated. Two (*S*)-ketoprofen-based activated esters were synthesized by activating its carboxylic group with *N*-hydroxybenzotriazole and *N*-hydroxysuccinimide, respectively. The esters were characterized by UV, IR, ^{1}H-NMR, HRMS, and elemental analyses. These reagents were used for synthesis of diastereomeric derivatives of the chosen β-adrenolytics. These diastereomeric derivatives were enantioseparated on C_{18} column by high-performance liquid chromatography. Chromatographic conditions were optimized by varying concentration of surfactant and buffer, and pH. The method was validated according to International Conference of Harmonization guideline and the retention factor (k), selectivity factor (α), resolution factor (R_S), and limit of detection and limit of quantification were calculated".

The **sixth chapter** describes enantioseparation of chosen racemic amino acids, after derivatization with (*S*)-ibuprofen, (*S*)-ketoprofen and (*S*)-levofloxacin based chiral derivatizing reagents, using Micellar liquid chromatography.

"Micellar liquid chromatographic method has been developed for enantioseparation of four racemic amino acids, namely, (*RS*)-selenomethionine, (*RS*)-methionine, (*RS*)-cysteine and (*RS*)-penicillamine. The aqueous solution of sodium dodecyl sulphate and Brij-35 was prepared and used as mobile phase for HPLC analysis. Activated esters of (*S*)-ibuprofen, (*S*)-ketoprofen, and (*S*)-levofloxacin were synthesized by reacting them with *N*-

hydroxybenzotriazole. These esters were characterized by UV, IR, ^{1}H-NMR, HRMS, and elemental analysis. These chiral reagents (activated esters) were used for the synthesis of diastereomeric derivatives of the chosen amino acids. The diastereomeric derivatives were separated on C_{18} column by micellar liquid chromatography. Chromatographic conditions were optimized by varying concentration of surfactant in aqueous solution, and by varying concentration and pH of the buffer. The method was validated according to ICH guidelines and the retention factor (k), selectivity factor (α), resolution factor (R_S), and limit of detection and limit of quantification were calculated".

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Dated: (Shiv Kumar Alwera)

LIST OF PUBLICATIONS

1. **Shiv Alwera** and Ravi Bhushan. (*RS*)-Propranolol: enantioseparation by HPLC using newly synthesized (*S*)-levofloxacin based reagent, absolute configuration of diastereomers and recovery of native enantiomers by detagging. *Biomedical Chromatography*, **30**, 2016, 1223-1233. **(Chapter-4A)**

2. **Shiv Alwera** and Ravi Bhushan. Liquid chromatographic enantioseparation of three beta-adrenolytics using new derivatizing reagents synthesized from (*S*)-ketoprofen and confirmation of configuration of diastereomers. *Biomedical Chromatography*, **30**, 2016, 1772-1781. **(Chapter-5A)**

3. Ravi Bhushan and **Shiv Alwera**. Liquid chromatographic enantioseparation of racemic β-blockers via synthesis of diastereomeric amides and (*S*)-levofloxacin esters as chiral derivatizing agents. *Nature Protocol exchange*, 2018, doi:10.1038/protex.2017.150.

4. **Shiv Alwera** and Ravi Bhushan. RP HPLC enantioseparation of β-adrenolytics using micellar mobile phase without organic solvents. *Biomedical Chromatography*, **31**(11) 2017, e3983. DOI: 10.1002/bmc.3983. **(Chapter-4C)**

5. **Shiv Alwera** and Ravi Bhushan. Micellar liquid chromatography for enantioseparation of β-adrenolytics using (*S*)-ketoprofen-based reagents. *Journal of Liquid Chromatography & Related Technologies*, **40**(14), 2017, 707-714. **(Chapter-5B)**

6. **Shiv Alwera**. Enantiomeric separation of sulphur amino acids and selenomethionine using micellar liquid chromatography and activated esters of some enantiomerically pure pharmaceuticals, *New Journal of Chemistry*, communicated, 2018, Manuscript ID NJ-ART-03-2018-001054. **(Chapter-6)**

7. Mohd Kazmi, **Shiv Alwera,** Poonam Malik and Ravi Bhushan, Application of underivatized L-Leucine for synthesis of diastereomeric derivatives of achiral-esterified (*RS*)-Ibuprofen and RP-HPLC enantioseparation, *Journal of Chromatography A*, communicated, 2017.

LIST OF ABBREVIATIONS USED

1.	Anal. Calcd	Analysis Calculated
2.	Atl	Atenolol
3.	Ar	Aromatic
4.	Bpl	Bisoprolol
5.	Bxl	Betaxolol
6.	Cdl	Carvedilol
7.	CC	Cyanuric Chloride
8.	CDR	Chiral Derivatization Reagent
9.	DANI	(1*S*,2*S*)-1,3-diacetoxy-1-(4-nitrophenyl)-2-propyl-isothioctanate
10.	DCM	Dichloromethane
11.	d	Doublet
12.	dd	Double Doublet
13.	DFDNB	1,5-Difluoro-2,4-Dinitrobenzene
14.	DCU	Dicyclohexyl Urea
15.	DMAP	4-Dimethyl Amino Pyridine
16.	*Hz*	Hertz
17.	ICH	International Conference on harmonization
18.	*k*	Retention Factor
19.	Kpf	Ketoprofen
20.	LEC	Ligand Exchange Chromatography
21.	LC	Liquid Chromatography
22.	Lfx	Levofloxacin
23.	m	Multiplet
24.	Mel	Metoprolol
25.	Min	Minute
26.	mL	Millilitre
27.	mmol	Millimole
28.	MWI	Microwave Irradiation
29.	MLC	Micellar Liquid Chromatography
30.	mM	Milimolar
31.	mp	Melting Point
32.	*N*-Btz	*N*-hydroxybenzotriazole

33.	*N*-Suc	*N*-hydroxysuccinimide
34.	NP	Normal Phase
35.	nmol	Nanomole
36.	PDA	Photodiode array detectors
37.	PenA	Penicillamine
38.	Prl	Propranolol
39.	Q	Quartet
40.	RP	Reversed Phase
41.	*Rs*	Resolution
42.	S	Singlet
43.	SD	Standard deviation
44.	T	Triplet
45.	*T*	Retention time
46.	*v*/*v*	Volume/Volume
47.	W	Watt
48.	µmol	Micromole

Chapter-1: Introduction

It is well noticed in both academia and industry that enantiomers of a pair show different activity in the 'chiral environment of our body' and, in many countries, the regulatory agencies ask [1] the industries to present full information on pharmacodynamics and pharmacokinetics of both the enantiomers including the stereoselective analytical methods before permitting a new drug to be registered and also insist on bringing (preferably) only the active enantiomer of a chiral drug to market. The two enantiomers may act on different receptors and on different pathways. Thus, the two enantiomers should be considered as different drugs and a clear picture of their pharmacodynamics and pharmacokinetic profile cannot emerge until the fate of each enantiomer is established [2, 3]. With such an awareness of these issues among those involved in the drug development, marketing and law enforcement efficient methods for verification of enantiomeric purity or to monitor stereoselective synthesis are increasingly required. Development of efficient methods for control of enantiomeric purity and separation of enantiomers on analytical scale is increasingly required (and becoming more and more important) in the fields of chromatography, pharmacology, medicine, asymmetric synthesis, mechanistic studies, life sciences etc.

A large number of commonly used drugs, such as **β-adrenolytics**, are being administered and marketed as racemates though only one of the enantiomers has desirable pharmaceutical effect. The β-adrenolytics are commonly used worldwide to manage cardiac arrhythmias, to treat hypertension and to control acute panic symptoms in anxiety-provoking situations; pharmaceutical applications of β-adrenolytics are well documented [4] and it is now known that (*S*)-(−)-enantiomer of β-adrenolytics is pharmacologically effective, showing about 50–500 fold higher activity in comparison to (*R*)-isomer. Based on their activity for blocking the receptor sites these are designated as β_1, β_2 and β_3 receptors. β_1-Adrenergic receptors are located mainly in the heart and in the kidneys [5], β_2-adrenergic receptors are located mainly in the lungs, gastrointestinal tract, liver, uterus, vascular smooth muscle, and skeletal muscle [5], and β_3-adrenergic receptors are located in fat cells [6]. Beta receptors are found on cells of the heart muscles, smooth muscles, airways, arteries, kidneys, and other tissues that are part of the sympathetic nervous system and lead to stress responses, especially when they are stimulated by epinephrine (adrenaline). β-Adrenolytics interfere

with the binding to the receptor of epinephrine and other stress hormones, and weaken the effects of stress hormones.

The stereochemistry of amino acids (AAs) plays an important role in their biological and pharmacological properties including in humans. AAs are also used as drugs, dietary supplements, raw materials or precursors or enantiomer pool for many enantioselective syntheses of other molecules. Therefore, development of efficient methods for enantioseparation of DL-amino acids (by both direct and indirect approaches) using different chromatographic methods continues to be a subject of immense importance.

Both direct and indirect approaches of enantioseparation (using LC) have their own advantages and limitations depending upon the situation, particularly, in terms of source and amount of sample available, chemical structure of the analyte and the ease of availability of laboratory facilities. At present chiral stationary phases (CSPs) and bonded phases used for separation of enantiomers of racemic drugs and biologically active compounds (by ***direct approach***) are by no means cheap since the development costs are formidable. Many times, the CSP columns may not be stable enough and have relatively limited applications and restricted performance. A lot of CSPs have been developed for of direct enantioseparation. These include CSPs based on proteins, cyclodextrin, polysaccharides, macrocyclic antibiotics, crown ethers, and ligand-exchange. A "three point interaction" (involving electrostatic attraction, π-π interaction, hydrogen bonding and steric repulsion) is required for separation of analyte enantiomers on a chiral stationary phase [7-9].

A large number of papers reporting on the development and application of enantiomeric separations by high-performance liquid chromatography (HPLC) via ***indirect approach*** reflect the importance of HPLC and covalent chiral derivatization in the solution of practical problems in pharmaceutical and biomedical analysis. The primary aim of derivatization in enantioseparation is the formation of easy to separate diastereomeric derivatives and at the same time to improve the detectability by introducing chromophoric or fluorophoric groups into enantiomeric molecules. Thus, (in the indirect approach), the chiral derivatizing reagents (CDRs) having high molar absorptivity (ε) or high fluorescence quantum yield (φ) make available this property to the enantiomeric pair via coupling and provide ultra violet/ visible absorption, or fluorescence, or chemiluminescence for highly sensitive on-line detection (of diastereomeric derivatives) commonly during HPLC separation. Literature shows a comprehensive discussion on enantioseparation of amino acids and several pharmaceuticals using chiral CDRs [10, 11].

The recovery of native enantiomers from diastereomeric derivatives constitutes a difficult proposition because of the possibility of decomposition of enantiomers or chances of racemization during hydrolysis. Determination of absolute configuration and elution order of the diastereomeric derivatives derived from the CDR is not easily predictable while using conventional achiral C_{18} stationary phases because most of the time diastereomeric derivative corresponding to pure enantiomer of the analyte is not available.

Application of RP-HPLC for enantioseparation of pharmaceutically active compounds is well established in its own way. To make HPLC analysis more useful and sensitive, development of new CSPs, application of new CDRs and mobile phase additives and reduction in particle size/column size have passed through a stage of progress and evolution. Nevertheless, little attention has been paid to modify the mobile phase for reducing analysis time and the solvent consumption by deviating from established practices of using organic solvents (mixed with TFA).

According to "*Transparency Market Research*" [12] the global market of chiral technology was $47.5 million in 2010 and is anticipated to generate $58.3 million in 2017; it includes developments in chiral synthesis, chiral resolution, and chiral analysis. The search for new and effective methods of separating a wide variety of enantiomeric compounds is an ongoing process.

Present Work

With the above mentioned background of pharmaceutical importance of β-adrenolytics (and amino acids) and importance of enantioseparation for drug industry and regulatory agencies and application of HPLC for this purpose the outline of the work undertaken for this thesis is mentioned below.

Selection of analytes: Taking into account the quantum of racemic β-adrenolytics commonly used for clinical purposes and importance of their enantioseparation for drug industry and regulatory agencies certain β-adrenolytics were chosen for enantioseparation studies. These are (*RS*)-propranolol, (*RS*)-metoprolol, (*RS*)-atenolol, (*RS*)-betaxolol, (*RS*)-carvedilol, (*RS*)-salbutamol and (*RS*)-bisoprolol (structures are shown in **Fig. 2.1)**. (*RS*)-propranolol, (*RS*)-metoprolol, (*RS*)-atenolol, (*RS*)-bisoprolol and (*RS*)-betaxolol are β_1-adrenolytics; (*RS*)-salbutamol is a β_2-adrenolytic, and (*RS*)-carvedilol belongs to both β_1 and β_2-adrenolytics class.

Besides, (*RS*)-methionine and **(***RS*)-cysteine as the naturally occurring sulphur-containing proteinogenic amino acids (AAs) and (*RS*)-selenomethionine, which is also a naturally occurring amino acid, were chosen for the ease of their availability and as a single pool of nutritionally-equivalent amino acids (structures are shown in **Fig. 2.2**). The sulphur-containing amino acids, methionine and homocysteine, can be converted into each other but neither can be synthesized *de novo* in humans. Likewise, cysteine can be made from homocysteine but cannot be synthesized on its own.

Selection of chiral chromophoric moieties: There were literature reports on successful enantioseparation of certain β-adrenolytics, in the last few years, using ligand exchange chromatography [13] and CDRs based on difluorodinitrobenzene (DFDNB) [14, 15], cyanuric chloride [16], (*S*)-naproxen [17], isothiocyanates [18-21], isocyanates [22], chloroformates [21, 23], acid chlorides [24], and aromatic anhydrides [25]. For DFDNB based derivatives a photochemical decomposition occurs if the solutions are not protected from light and many of the other reagents lead to formation of unstable derivatives, or lack of quantitative yield or poor detection. These reports neither attempted detagging of the chiral reagent to obtain the native enantiomer nor established configuration of diastereomeric derivatives, so separated.

The literature cited above and the references cited therein suggested a need to develop new CDRs for improved enantioseparation of commercial β-adrenolytics. Thus, certain unexplored chiral chromophoric moieties such as (*S*)-(−)-levofloxacin, (*S*)-(−)-ketoprofen and (*S*)-(+)-ibuprofen were chosen for developing new CDRs. There are no literature reports on the application of these chiral moieties in enantioseparation.

Experimental approach: It included the following steps:

a. Isolation and purification of active pharmaceutical ingredient from commercial tablets.
b. Synthesis of CDRs.
c. Synthesis of diastereomeric derivatives including preparative synthesis.
d. Analytical separation of diastereomeric derivatives by RPHPLC and their preparative separation and isolation by open column chromatography.
e. Application of mobile phases in a linear gradient of MeCN (or MeOH) – TEAP buffer from 80-20%, 70-30%, 60-40%, 50-50%, 40-60%, 30-70% and 20-80%, or

isocratic mode or application of water micellar mobile phase, containing both SDS and Brij 35.

f. Optimization of separation conditions with respect to time of mobile phase gradient, flow rate, and concentration of buffer for achieving enantioseparation.
g. ^{1}H-NMR spectra of the diastereomeric derivatives.
h. Developing the lowest energy structures by using Gaussian 09 Rev. A.02 program and hybrid density functional B3LYP with 6-31G* basis set (based on density functional theory).
i. Establishing configuration of the diastereomeric derivatives.
j. Establishing elution order.
k. Determination of stability of CDRs and recovery of diastereomeric derivatives.
l. Detagging of diastereomeric derivatives by hydrolysis and isolation and characterization of native enantiomers.
m. Method validation for linearity, range, accuracy, precision, reproducibility, specificity, limit of detection (LOD) and limit of quantitation (LOQ).
n. Comparison of results obtained with literature reports.

Pharmaceutical importance and literature survey on enantioseparation of the chosen β-adrenolytics, and (*RS*)-amino acids has been described in **Chapter-2**. Besides, pharmaceutical importance along with physical and chemical characteristics of the chromophoric chiral moieties and the relevant literature on their enantioseparation has also been discussed in **Chapter-2**. To maintain the uniformity of nomenclature with CDRs, (*R*) and (*S*) notations have been used for AAs instead of D and L.

Experimental details regarding materials, equipment and methods of synthesis of CDRs, the diastereomeric derivatives, and chromatographic separation along with characterization data have been described in **Chapter-3**.

Results and discussions are systematically described in subsequent chapters.

Chapter-2: The chosen analytes and the chromophoric chiral moieties

A. Pharmaceutical importance and literature survey on enantioseparation of the chosen (*RS*)-β-adrenolytics

1. (*RS*)-Propranolol (Prl)

(*RS*)-Propranolol, is a non-selective, first generation β-adrenolytic (**Fig. 2.1**). Its systematic chemical name is (*RS*)-1-(1-methylethylamino)-3-(1-naphthyloxy) propan-2-ol. It is used for the treatment of hypertension, anxiety, and panic and to prevent migraine headaches and angina. It was the first successful beta blocker developed and is on the World Health Organization's List of Essential Medicines. It is marketed and administered as racemic mixture, although the desirable action is largely confined to (*S*)-(−)-enantiomer showing about 50–500-fold. (*S*)-Propranolol is important in therapy against oxidative stress because it induces the activity of antioxidant and other beneficial enzymes and increases endothelial nitric oxide production, directly protecting cardiovascular cells and tissues against oxidative injury [26]. Besides, (*R*)-(+)-propranolol is also used for the treatment of hyperthyroidism (by inhibiting the conversion of thyroxine to triiodothyronine) and under such situation racemic propranolol mixture cannot be administrated as it may cause serious side effects to the patients due the prominent adrenergic effect of (*S*)-(−)-propranolol [27].

Literature on enantioseparation of (*RS*)-Prl: Direct and indirect separation of β-blockers by chiral TLC has been reviewed by Agbaba and Ivkovic´ [28]. HPLC enantioseparation (*RS*)-Prl by indirect approach has been achieved using (*R*,*R*)-*O*,*O*'-diacetyltartric acid anhydride (DATAAN) [29], *N*-trifluoroacetyl-L-prolyl chloride [30], (+)-1-(9-fluorenyl)ethyl choroformate (Flec) [23], anhydride of tert. butoxy carbonyl-L-leucine [31], (*S*)-flunoxaprofen isocyanate and (*S*)-naproxen isothiocyanate [22], 2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl isothiocyanate [32] and (1*R*,2*R*)-1,3-diacetoxy-1-(4-nitrophenyl)-2-propyl isothiocyanate [19]; dinitrophenyl-L-Pro-*N*-hydroxysuccinimide ester, *N*-succinimidyl-(*S*)-2-(6-methoxynaphth-2-yl) propionate and difluorodinitrobenzene [14, 15], cyanuric chloride [16] and (*S*)-naproxen anhydride [25] as CDRs.

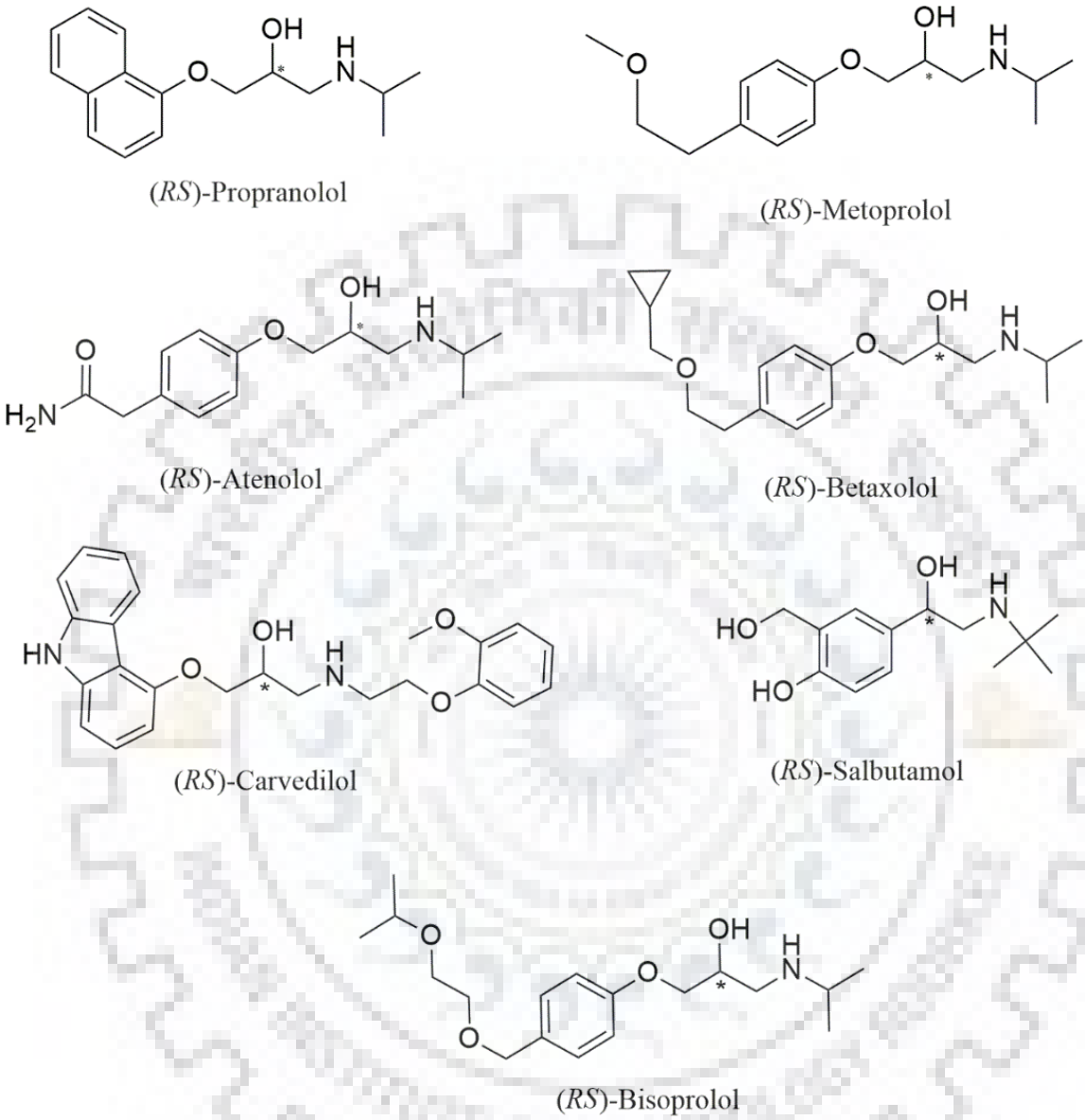


Fig. 2.1. Structures of (*RS*)-propranolol, (*RS*)-metoprolol, (*RS*)-atenolol, (*RS*)-betaxolol, (*RS*)-carvedilol, (*RS*)-salbutamol and (*RS*)-bisoprolol.

The direct approach has been achieved by using lactobionic acid/D-(+)-xylose-boric acid complexes as chiral selector for RP-HPLC analysis [33]; chirally pure amino acids [34, 35], chiral Cu-II complexes [36-38], macrocylic antibiotics [39] and L-tartaric acid, (*R*)-mandelic acid and (–)-erythromycin [40] as chiral selectors for enantioseparation of (*RS*)-Prl on TLC. Also CSPs based on (+)-(18-crown-6)- 2,3,11,12-tetracarboxylic acid [41], and polyacrylamide and polysaccharide derivatives [42], cellulose tris-(3,5-dimethylphenylcarbamate) [43], cellulose [44] and ovomucoid [45, 46], amylose [47], macrocyclic antibiotic [48] macrocyclic glycopeptide [49], and polysaccharide and Pirkle-type based CSPs [50] have been used for direct separation of enantiomers of (*RS*)-Prl.

2. (*RS*)-Metoprolol (Mel)

(*RS*)-Metoprolol (1-(isopropylamino)-3-[4-(2-methoxyethyl)phenoxy]propan-2-ol) is a β_1-adrenoceptor selective antagonist, clinically used for the treatment of hypertension and ischemic heart disease. (*S*)-(–)-metoprolol has been reported to show significantly greater β_1-adrenergic receptor affinity by 25-fold than (*R*)-(+)-metoprolol [51, 52]. (*S*)-isomers of sustained-release metoprolol are adjuncts to standard angiotensin converting enzyme (ACE) inhibitor and diuretic therapy in congestive heart failure. Metoprolol was shown to protect red blood cells against phenazine methosulfate (PMS) induced toxicity to red blood cells [53].

Literature on enantioseparation of (*RS*)-Mel: HPLC enantioseparation of (*RS*)-Mel by indirect approach has been achieved using (*R,R*)-*O,O*'-diacetyltartric acid anhydride (DATAAN) [26], (*S*)-(+)-1-1(1-naphtyl)ethyl isothiocyanate and 2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl isothiocyanate [32] and (1*R*,2*R*)-1,3-diacetoxy-1-(4-nitrophenyl)-2-propyl isothiocyanate [19], dinitrophenyl-L-Pro-*N*-hydroxysuccinimide ester, *N*-succinimidyl-(*S*)-2-(6-methoxynaphth-2-yl) propionate and difluorodinitrobenzene [14], cyanuric chloride [16] and (*S*)-naproxen anhydride [25] as CDRs.

The direct approach has been achieved by using lactobionic acid/D-(+)-xylose-boric acid complexes as chiral selector for RP-HPLC analysis [33]; chirally pure amino acids [34, 35], chiral Cu-II complexes [36, 38] and macrocylic antibiotics [39] as chiral selectors for enantioseparation of (*RS*)-Mel on TLC. Also CSPs based on (+)-(18-crown-6)- 2,3,11,12-tetracarboxylic acid [41], amylose tris-(3,5-dimethylphenylcarbamate) [54], and polyacrylamide and polysaccharide derivatives [42], and ovomucoid and cellulase [44],

amylose [47], and macrocyclic glycopeptide-based CSPs [49] have been used for direct separation of enantiomers of (*RS*)-Mel.

3. (*RS*)-Atenolol (Atl)

(*RS*)-Atenolol (2-[*p*-[2-hydroxy-3-(isopropylamino)propoxy]phenyl]acetamide) is a selective β_1-adrenoceptor blocker and used in the cardiovascular disorders such as coronary heart disease, angina pectoris, sinus tachycardia, hypertension, arrhythmias and myocardial infarction. Atl acts preferentially upon the β-adrenergic receptors in the heart [55]. Some time it cause side effects like fewer bronchospastic reactions, depression and nightmares. Atl was developed in 1976 for the treatment of hypertension as a replacement of propranolol. It has advantage over propranolol because does not readily pass through blood brain barrier resultant decreases the central nervous side effect.

Literature on enantioseparation of (*RS*)-Atl: Batra and Bhushan presented a review [56] on liquid chromatographic methods for enantioseparation of (*RS*)-Atl by both direct and indirect approaches involving practical applications of several chiral stationary phases (CSPs), chiral derivatization reagents, and ligand exchange and impregnation methods. These include methods using both HPLC and TLC for separation, determination and bioassay of enantiomers of atenolol. Besides, some aspects of enantioseparation under achiral phases of liquid chromatography has been briefly mentioned as applicable to (*RS*)-Atl.

4. (*RS*)-Betaxolol (Bxl)

(*RS*)-Betaxolol, (*RS*)-1-{4-[2-(cyclopropylmethoxy)ethyl]-phenoxy}-3-(isopropylamino)-propan-2-ol], is a β_1-receptor blocking agent and commonly used for hypertension and glaucoma [57, 58]. Bxl shows greater binding affinity for β_1-receptor as compared to metoprolol. Its (*S*)-isomer showing desire effect which (*R*)-isomer reason for side effect like sinus bradycardia, cardiogenic shock, heart block, and overt cardiac failure [59-61]. Dilute solution of betaxolol generally used as eye drop because it reduces intraocular pressure (pressure within eyes) and help to reduce the damage risk of optical nerves.

Literature on enantioseparation of (*RS*)-Bxl: Wang *et al*, presented a review [62] on liquid chromatographic methods for enantioseparation of (*RS*)-Bxl by both direct and indirect approaches involving practical applications of several chiral stationary phases (CSPs), chiral

derivatization reagents, and chiral mobile phase additives. These include methods using both HPLC and capillary electrophoresis for separation, determination and bioassay of enantiomers of Bxl.

5. (*RS*)-Carvedilol (Cdl)

(*RS*)-Carvedilol (1-(9H-carbazol-4-yloxy)-3-{[2-(2-methoxyphenoxy)ethyl]amino}propan-2-ol) is a third generation non selective β-adrenolytic which inhibits β_1, β_2 and α_1-receptor. It is used for congestive heart failure, left ventricular dysfunction and high blood pressure, and also using for dilating vessels by inhibiting α_1-receptor of blood vessels and improves vascular resistance in liver by release of nitric oxide [63]. (*S*)-Carvedilol reduces lipid peroxidation level [64-66]. Cdl also have some side effect for example dizziness, low blood pressure, diarrhoea, slowed heart rate and weight gain etc.

Literature on enantioseparation of (*RS*)-Cdl: HPLC enantioseparation of (*RS*)-Cdl by indirect approach has been achieved using (+)-(*R*)-phenyl ethyl isothiocynate [67], (*S*)-naproxen chloride [24], dinitrophenyl-L-Pro-*N*-hydroxysuccinimide ester, *N*-succinimidyl-(*S*)-2-(6-methoxynaphth-2-yl) propionate and difluorodinitrobenzene [14], cyanuric chloride [16] and (*S*)-naproxen anhydride [25].

The direct approach has been achieved by using chirally pure amino acids [34] as chiral selectors for direct enantioseparation of (*RS*)-Cdl on TLC analysis, and macrocyclic antibiotic [68, 69], macrocyclic glycopeptide [49] and ovomucoid and cellulose [44, 46] based CSPs have been used for direct separation of enantiomers of (*RS*)-Cdl.

6. (*RS*)-Salbutamol (Sbl)

(*RS*)-Salbutamol [4-(2-(tert-butylamino)-1-hydroxyethyl)-2-(hydroxymethyl)-phenol)] is a β_2-agonist and used for treatment of asthma. The both enantiomers of Sbl showing different properties, act as two different drugs [38], (*R*)-enantiomers reason for smooth muscle to relax and (*S*)-enantiomers reason for smooth muscle contraction. Both enantiomers bind with different receptors resulting in the opposing effects and (*R*)-enantiomer is the active bronchodilating enantiomers [70], Sbl also showing some side effect like anxiety, muscle cramps, arrhythmia, headache, dry mouth, and palpitation.

Literature on enantioseparation of (*RS*)-Sbl: Wang *et al*, [62] and Nishi and Kuwahara [71] presented reviews on liquid chromatographic methods for enantioseparation of (*RS*)-

Sbl by both direct and indirect approaches involving practical applications of several chiral stationary phases (CSPs), chiral derivatization reagents, and chiral mobile phase additives. These include methods using both HPLC and capillary electrophoresis for separation, determination and bioassay of enantiomers of Sbl.

7. (*RS*)-Bisoprolol (Bpl)

(*RS*)-Bisoprolol ((+)-1-[*p*-(2-isopropoxyethoxymethyl)phenoxy]-3-(isopropylamino)-2-propanol) is a β_1-selective adrenoceptor antagonist without membrane stabilizing activity or intrinsic sympathomimetic activity [72, 73]. The drug is marketed as a racemic mixture and (*S*)-isomer shows the desired properties. It is used for high blood pressure, myocardial infarction, cardiac ischemia, congestive heart failure but also showing some side effect like hypotension, bronchospasms, low blood sugar and bradycardia [74, 75].

Literature on enantioseparation of (*RS*)-Bpl: HPLC enantioseparation of (*RS*)-Bpl by indirect approach has been achieved using (–)-menthyl chloroformate (MCF) [76], 2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl isothiocyanate [22] and (1*R*,2*R*)-1,3-diacetoxy-1-(4-nitrophenyl)-2-propyl isothiocyanate [19], dinitrophenyl-L-Pro-*N*-hydroxysuccinimide ester, *N*-succinimidyl-(*S*)-2-(6-methoxynaphth-2-yl) propionate and chiral derivatives of difluorodinitrobenzene [14], and cyanuric chloride [16] as CDRs.

The direct approach has been achieved by using lactobionic acid/D-(+)-xylose-boric acid complexes as chiral selector for direct RP-HPLC analysis [33]; chirally pure amino acids [34] as chiral selectors for enantioseparation of (*RS*)-Bpl on TLC. CSPs based on amylose [47], β-cyclodextrine [77], macrocyclic antibiotic [68] and macrocyclic glycopeptide [49] have been used for direct separation of enantiomers of (*RS*)-Bpl.

B. Importance and literature survey on enantioseparation of the chosen (*RS*)-amino acids

A synonym of the natural L-selenomethionine is (*S*)-selenomethionine, L-methionine is (*S*)-methionine, L-cysteine is (*R*)-cysteine and L-penicillamine is (*R*)-penicillamine.

1. (*RS*)-Selenomethionine (SeMet)

(*RS*)-Selenomethionine (2-amino-4-methylselanylbutanoic acid; Fig. 2.2) is a Se-containing naturally occurring chiral amino acid and is well known for its biological and dietary importance. (*S*)-SeMet is used as a source of Se in humans, other mammals and plants has

been covered in books and reviews [78-80]. (*S*)-SeMet is better absorbed and better incorporated into the body than any other known form of selenium [81]. (*R*)-Selenomethionine is degraded to inorganic selenium and returned to the inorganic selenium body pool, and is only one-fifth bioavailable as (*S*)-selenomethionine [82]. The development of pharmaceutical preparations involving Se compounds has become a growing area because of the use of selenium supplementation for cancer chemoprevention [83].

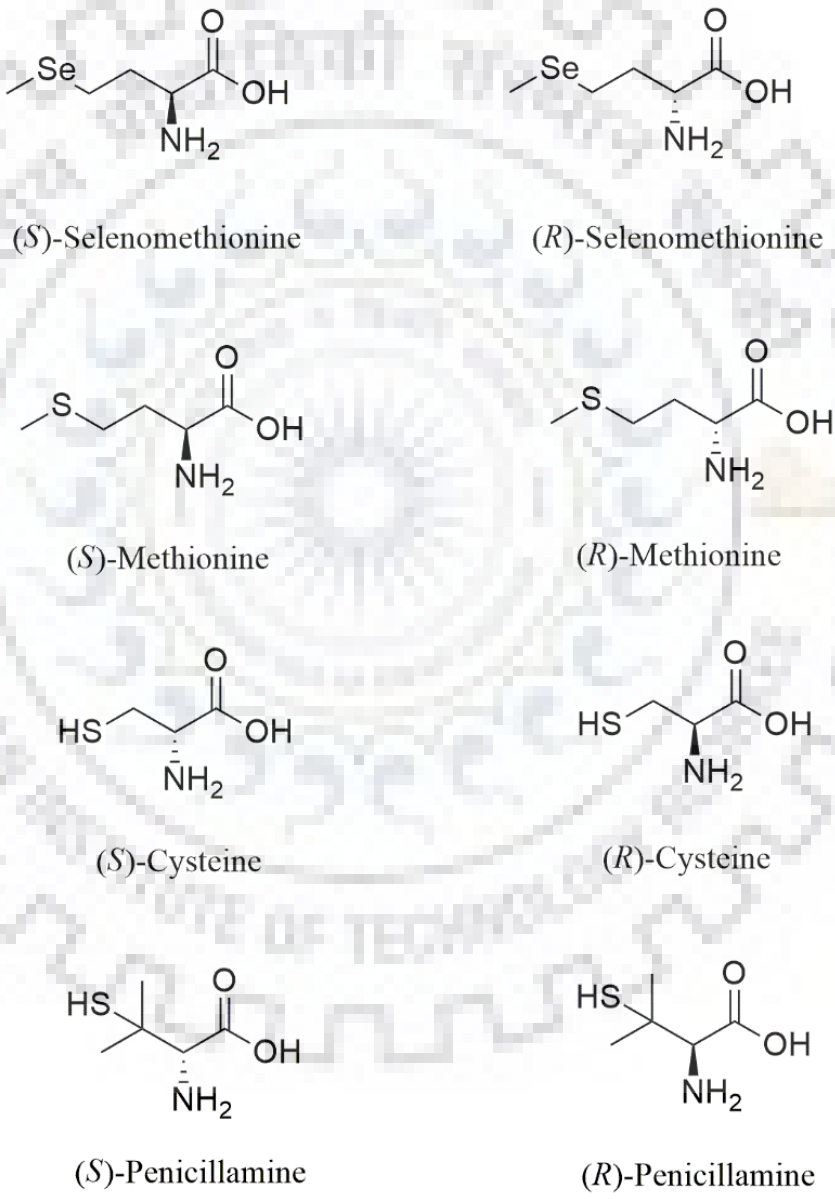


Fig. 2.2. Structures of (*R*)- and (*S*)-amino acids.

Literature on enantioseparation: The enantioseparation of (*RS*)-SeMet has been successful achieved using (*R*)-methyl benzyl isothiocyanate [*(R)*-MBIC] and (*S*)-1-(1-naphthyl) ethyl isothiocyanate [(*S*)-NEIC] [84], difluoro-dinitrobenzene [85], and monochloro- and dichloro-triazine containing amino acid amides [62], *o*-phthalaldehyde and *N*-isobutyryl-L-cysteine [86], (*S*)-naproxen [87], Marfey's reagent based CDRs [88]. Also some commercial available chiral selective columns were used for determination of enantiomeric purity of Se-amino acids [89, 90].

2. (*RS*)-Methionine (Met)

(*RS*)-Methoinine (2-amino-4-(methylthio)butanoic acid), α-amino acid, is an essential AA and its (*S*)-enantiomer helps in growth of blood vessels and prevents liver damage in acetaminophen poisoning, and is used in treatment of depression, copper poisoning, alcoholism, allergies, asthma and Parkinson's disease [91, 92] Methionine also used in biosynthesis of proteins. The oxidation of methionine using pyridinium chlorochromate, pyridininum bromochromate and morpholinium fluorochromate was described by Sharma *et al*, [93-95].

Literature on the enantioseparation: Literature on the enantioseparation of (*RS*)-Met *via* direct and indirect approach using liquid chromatographic techniques has been covered in reviews and book [11, 96, 97].

3. (*RS*)-Cysteine (Cys)

(*RS*)-Cysteine (2-amino-3-mercaptopropanoic acid) is the naturally occurring sulphur-containing proteinogenic acid and used in precursor for biological activities and food additives. Its (*R*)-Cys is indespensible for living beings and (*S*)-Cys is more effective in tumor treatment compared to (*R*)-Cys [98]. The derivatives of (*R*)-Cys have been used as chiral selector in ligand exchange chromatography [99, 100] and conjugates of cysteine with protoporphyrin were separated using HPLC/ESI mass spectrometry [101].

Literature on the enantioseparation: literature on the enantioseparation of (*RS*)-Cys *via* direct and indirect approach using liquid chromatographic techniques has been covered in the reviews and book [11, 96, 97].

4. (*RS*)-Penicillamine (PenA)

(*RS*)-Penicillamine (2-amino-3-mercapto-3-methylbutanoic acid) is naturally occurring chiral amino acid and it is a structural analog of cysteine. It shows chemical properties similar to cysteine (Cys) and is considered as a non-proteinogenic amino acid containing thiol group. (*S*)-Penicillamine is more active pharmacologically enantiomer. (*S*)-PenA is used in treatment of Wilson's disease [102], cystinuria [103], rheumatoid arthritis [104], scleroderma [105], hepatitis and for the prevention of infants' retina disease [106], leukemia and breast cancer [107] and heavy metal poisoning [108]. Because the high toxicity of (*R*)-PenA (distomer), the racemate mixtures restricts its use [109, 110].

Literature on the enantioseparation: Direct enantioseparation of (*RS*)-PenA has been achieved using by L-tartaric acid and (*R*)-mandelic [111], (2*S*, 4*R*, 2′*RS*)-*N*-(2′-hydroxy dodecyl)-4-hydroxy proline [112], copper (II)-L-proline complex [113] as the chiral selector. CSPs like Pirkle-type [114], Teicoplanin [115], β-cyclodextrin columns [115, 116] and *α*-acid glycoprotein (AGP) [117] were used for direct separation of (*RS*)-PenA. Indirect enantioseparation of (*RS*)-PenA has been achieved using Marfey's reagent [118], t-butyloxy-L-leucine-*N*-hydroxy succinimide ester (BOC-L-Leu-SU) [119], 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) [120], 4-(3-isothiocyanato-pyrrolidin-1-yl)-7-(*N*,*N*-dimethyl-aminosulfonyl)-2,1,3 benzoxadiazole (DBD-PyNCS) [121], *N*-succinimidyl-(*S*)-2-(6-methoxynaphth-2-yl) propionate (SINP) [122] as CDRs.

C. Chromophoric chiral moieties

The structures of the chromophoric chiral moieties used for synthesizing CDRs are shown in (Fig. 2.3). The pharmaceutical importance and their physical and chemical characteristics along with the relevant literature on their enantioseparation has also been discussed for each.

1. (*S*)-(-)-Levofloxacin (Lfx)

The systematic chemical name of levofloxacin is (*S*)-(−)-9- fluoro-2,3-dihydro-3-methyl-10-(4-methylpiperazin-1-yl)-7-oxo-7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid (**Fig. 2.3**). It is a broad spectrum antibiotic of the fluoroquinolone drug class. It attracted our attention, to act as chiral moiety, for its high molar absorptivity (Ɛo ≈ 24,000), owing to the substituted aromatic residue and the carboxylic group, and its availability at a low price as a pure (*S*)-enantiomer. Lfx is used in the treatment of respiratory tract infections, cellulitis,

urinary tract infections, prostatitis, anthrax, endocarditis, meningitis, pelvic inflammatory disease, traveler's diarrhoea, tuberculosis and plague [123, 124]. It was considered that its carboxylic group can be activated by reaction with *N*-hydroxysuccinimide and *N*-hydroxybenzotriazole, which are very good nucleophilic acyl substitution reagents. Attachment of such moieties was successful in (*S*)-naproxen (having a similar carboxylic group) to prepare certain CDRs [87, 122, 125, 126].

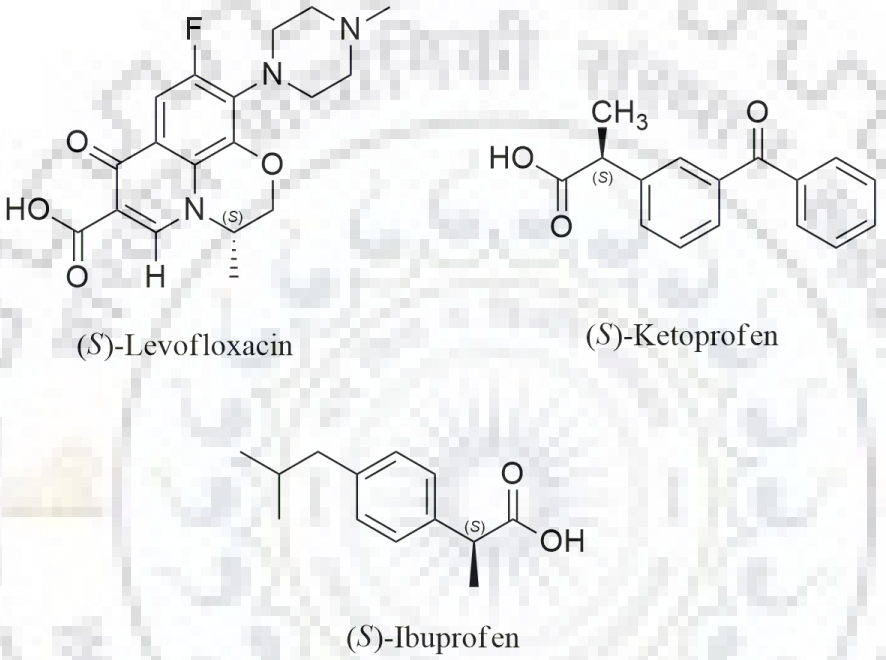


Fig. 2.3. Structures of (*S*)-levofloxacin, (*S*)-ketoprofen and (*S*)-ibuprofen.

2. (*S*)-(+)-Ketoprofen (Kpf)

(*S*)-(+)-Ketoprofen [(*S*)-2-(3-benzoylphenyl)-propionic acid] is one of the propionic acid class of nonsteroidal anti-inflammatory drug. It inhibits the production of prostaglandin in the body. It is generally prescribed for arthritis-related inflammatory pains, toothaches, nerve pain, postherpetic neuralgia etc [127]. It attracted our attention, to act as chiral moiety, for its high molar absorptivity (Ɛo ≈ 40,000), owing to the substituted aromatic rings and the carboxylic group, and its availability at a low price as a pure (*S*)-enantiomer.

Santa et al, [128] described enantioseparation of derivatives of (*RS*)-ketoprofen prepared with 2,2'-dipyridyldisulfide (DPDS) using ESI/MS-SIM.

3. (*S*)-(+)-Ibuprofen (Ibf)

(*S*)-(+)-Ibuprofen [(*S*)-2-(4-isobutylphenyl)propanoic acid] is a nonsteroidal anti-inflammatory drug. It is generally used for the treatment of pain, fever, inflammation, painful menstrual periods, rheumatoid arthritis, and migraines [129]. It attracted our attention, to act as chiral moiety, for its relatively higher molar absorptivity ($\varepsilon_o \approx 4{,}000$) as compared to AAs, since (*S*)-Ibf has the substituted aromatic residue and the carboxylic group, and its availability at a low price as a pure (*S*)-enantiomer.

There are certain reports on enantioseparation of (*RS*)-Ibf and (*RS*)-Kpf by different methods [130-134] but their application as a chromophoric moiety for enhanced detection of β-adrenolytics (or as such, any other pharmaceutical) is being reported for the first time in this thesis and the publications related to the work presented herein.

Chapter-3: Experimental

A. Equipment, chemicals and reagents

1. Equipment

HPLC system: HPLC system (LC-20AD, Shimadzu, Kyoto, Japan) consisted of a DGU-20A5 on-line degasser unit, low-pressure gradient unit, low pressure mixing type gradient, parallel double plunger pump, high pressure mixer, SPD-M20A diode array detector, SPD-20A/20AV (UV-VIS Detector), CTO-20AC column oven and LC solution and DAO (data access objects) 3.5 operating software.

HPLC column: LiChrospher C_{18} column (L x I.D., 25cm × 4.6 mm, 5 μm particle size) was from Merck (Darmstadt, Germany).

Other equipment/instruments used were, Microwave-Multiwave 3000 (800 W, Perkin-Elmer, Shelton, CT, USA), pH meter Cyberscan 510 (Singapore), Milli-Q system of Millipore (Bedford, MA, USA) to obtain purified water (18.2 $M\Omega cm^3$) from double distilled water, Polarimeter (P3001RS Krüss 140, Hamburg, Germany), FT-IR Spectrometer (Nicolet-6700, Thermo Scientific, USA), LC-MS-8030 (Shimadzu Corporation, Kyoto, Japan) Fitted with an ESI-MS (triple quadrupole) Lab solution software, elemental analyzer (Vario EL III, Hanau, Germany), ^{1}H-NMR spectra were recorded on 400 MHz (JEOL Inc., Peabody, USA) and Bruker 500 MHz instrument using $CDCl_3$, MeOD, and DMSO, and UV-2450 Spectrophotometer (Shimadzu; Canada), REMI-PR-24 centrifuge (Maharashtra, India), RUDOLPH Research Analytical Automatic polarometer, commercial TLC (ALUGRAN RP-18W/UV_{254}; Germany) and HRMS [Bruker micrOTOF^{TM}-Q II mass spectrometer (ESI-MS).

2. Chemicals and reagents

N-hydroxysuccinimide, *N*-Hydroxybenzotriazole, Dicyclohexylcarbodiimide (DCC), dimethylaminopyridine (DMAP), sodium dodecyl sulphate (SDS), Brij-35 and NMR solvents ($CDCl_3$-d_1 and DMSO-d_6) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Triethylamine (TEA), phosphoric acid (H_3PO_4), acetic acid (CH_3COOH), concentrated hydrochloric acid (HCl), concentrated sulphuric acid (H_2SO_4), sodium hydrogen carbonate ($NaHCO_3$), sodium carbonate (Na_2CO_3) chloroform ($CHCl_3$), dichloromethane (DCM), tetrahydrofuran (THF), hexane, ethyl acetate (EtOAc), ethanol

(EtOH), acetone of analytical reagent grade, and acetonitrile (MeCN) and methanol (MeOH) of HPLC grade were obtained from E. Merck (Mumbai, India).

Silica gel G with 13% calcium sulphate as binder, having chloride, iron, and lead impurities up to 0.02% and with pH 7.0 in 10% aqueous suspension was from Merck (Mumbai, India).

The analytes and CDR moieties: Following racemates or their enantiomerically pure samples were obtained from Sigma–Aldrich (St. Louis, MO, USA),

(*RS*)-Atenolol: MW: 266.34, assay ≥ 98%;
(*RS*)-Propranolol hydrochloride: MW: 295.80, assay ≥ 99%;

(*RS*)-Selenomethionine: MW: 196.11, assay ≥ 98%,
(*S*)-Selenomethionine [MW: 196.11, assay ≥ 97%, $[\alpha]_D^{25}$ = (+) 18°, c = 2 in 2 M HCl],

(*RS*)-Methionine: MW: 149.21, assay ≥ 99%,
(*R*)-Methionine [MW: 149.21, assay ≥ 98%, $[\alpha]_D^{25}$ = (–) 24.7°, c = 2 in 6 M HCl],

(*RS*)-Cysteine: MW: 121.16, assay ≥ 98%,
D-(*S*)-Cysteine [MW: 121.16, assay ≥ 99%, $[\alpha]_D^{25}$ = (–) 7.6°, c = 5 in 5 M HCl],

(*RS*)-Penicillamine: MW: 149.21, assay ≥ 98%,
D-(*S*)-Penicillamine [MW: 149.21, assay ≥ 99%, $[\alpha]_D^{25}$ = (–) 62.0°, c = 0.5 in 1 M NaOH].

(*S*)-(+)-Ketoprofen: MW:254.28, assay ≥ 99%, $[\alpha]_D^{25}$ = (+) 49°, (c = 1, methanol);
(*S*)-(+)-Ibuprofen: MW: 206.29, assay ≥ 99%, $[\alpha]_D^{25}$ = **(+)** 54°, (c = 0.6 in MeOH);

B. Isolation and purification of active pharmaceutical ingredient from commercial samples

1. (*S*)-Levofloxacin: Levofloxacin as Levoflox-750 tablets (Cipla, Mumbai, India) was purchased from local market. Ten Levoflox tablets, each containing 750 mg of levofloxacin, after removing coating of red oxide iron and titanium dioxide, were grounded to a fine powder and extracted with 50 mL MeOH by sonication at 25 °C. The solution was filtered through Whatman paper (8µm pore). The same procedure was repeated twice with the residue. The combined filtrate was concentrated in vacuum and kept at 4 °C until crystals appeared. The sample was further purified by recrystallization with $CHCl_3$-MeOH. The sample was preserved in tightly closed container, protected from light.

Characterization data: Color, light yellow, solid crystalline; yield, 98% of the quantity reported on the commercial label; m.p., 224 °C; $[\alpha]_D^{25}$ = (−)104° (c= 0.5, $CHCl_3$); FT-IR, characteristic peaks at 3265 (carboxylic group), 2931 (alkene stretching), 1724 (carbonyl stretching), 1294 (amine stretching), and 1085 cm^{-1} (presence of F). The standard stock solution of (*S*)-(−)-Lfx was prepared by dissolving 50 mg of drug in MeOH in 100 mL volumetric flask. Stock solution of (*S*)-(−)-Lfx was further diluted with MeOH to get standard solution with a concentration of 100 µg mL^{-1}. The resulting solution was then scanned between 200-400 nm, UV (λ_{max} in MeOH, 294 nm).

2. (*RS*)-Metoprolol: Capsules of (*RS*)-metoprolol as Metolar XR-100 (Cipla, Solan, Himachal Pradesh, India) were purchased from local market. The granular contents of ten Metolar XR-100 capsule, each containing 100 mg of racemic metoprolol, were grounded to a fine powder and extracted with 50 mL MeOH by sonication at 25 °C. The solution was filtered through Whatman paper (8µm pore). The same procedure was repeated twice with the residue. The combined filtrate was concentrated in vacuum and kept at 4 °C until crystals appeared. The isolated pharmaceutical sample was preserved in tightly closed container, protected from light and was used as reference standard.

Characterization data: Color, white soild crystalline; yield, 96% of the quantity reported on the commercial labels; m.p., 120 °C; FT-IR, characteristic peaks at 3300, 3151, 2960, 2850, 1613, 1534,1240,1180, 1112, 1049, 842 and 714 cm^{-1}. The standard stock solution of (*RS*)-Mel was prepared by dissolving 50 mg of drug in MeOH in 100 mL volumetric flask. Stock solution of (*RS*)-Mel was further diluted in MeOH to get standard solution with a concentration of 100 µg/mL. The resulting solution was then scanned between 200 - 400 nm, UV (λ_{max} 223 nm in MeOH).

3. (*RS*)-Carvedilol, (*RS*)-salbutamol and (*RS*)-betaxolol

(*RS*)-Carvedilol as Carca-25 tablets (Intas Pharmaceuticals, East Sikkim, India), (*RS*)-salbutamol as Asthalin-4 tablets (Cipla, Sikkim, India), (*RS*)-betaxolol as Iobet eye drop (FDC Ltd, Waluj, Aurangabad, India) and (*RS*)-bisoprolol as Concor (Merck Ltd, Waluj, Aurangabad, India) were purchased from the pharmacist's shop in the local market as pharmaceutical medicines.

Ten Carca-25 (uncoated) tablets, each containing 25 mg of carvedilol, were grounded to a fine powder and extracted with 25 mL of methanol by sonication at room temperature. The solution was filtered through Whatman paper (8 µm pore). The same procedure was

repeated twice with the residue. The resulting filtrate was concentrated under vacuum and kept at 4 °C until crystals appeared. The sample was further purified by recrystallization with ethanol. The sample was preserved in tightly closed container, protected from light.

Similar procedure was used for extraction of bisoprolol and salbutamol from Concor and Asthalin-4, respectively, from commercially available tablets. Betaxolol was used directly as sample by diluting Iobet eye drops.

Purity of the samples of racemic β-adrenolytics so obtained was confirmed by recording λ_{max} and IR spectrum and determination of melting point. The λ_{max} values and melting points, were in agreement with literature values, and are as follows, carvedilol, 241 nm (115-117 ^{o}C); betaxolol, 254 nm; salbutamol, 223 nm (152-155 ^{o}C); bisoprolol, 222 nm (110-112 ^{o}C).

C. Preparation of stock solutions

Following stock solutions were prepared:

(i) **0.1 M Sodium bicarbonate**: 0.84 g of Sodium bicarbonate was dissolved in 100 mL of purified water.

(ii) β-Adrenolytics and AAs (1 mM): dissolved calculated amount of the respective drug in standard sodium bicarbonate solution (0.1M).

(iii) **CDRs**: 1 mM solution of each of the CDRs (1 to 5) in methanol or MeCN or EtOH.

(iv) **Buffer (10 mM)**: Triethylammonium phosphate (TEAP) buffer (10 mM) by diluting 6.8 mL triethylamine with purified water to 950 mL, the pH was adjusted with 84% phosphoric acid to 3.5 and finally diluted to 1000 mL with purified water.

(v) (*S*)-(−)-levofloxacin (0.2 M), (*S*)-(+)-ketoprofen (0.2 M) and (*S*)-(+)-ibuprofen (0.2 M) in THF were prepared.

(vi) Water micellar mobile phase (WMP) was prepared by dissolving 14.4 g SDS (0.05 M) and 17.8 g Brij-35 (0.015 M) in double distilled water making a total volume of 1000 mL. The resulting solution was sonicated for half an hour to obtain a clear and homogeneous solution. The solution was filtered using 0.45 µm filter. It was then degassed by passing dry nitrogen gas and sonicating again for half an hour. The water-surfactant solution + TEAP buffer (in different ratio) was used as a mobile phase in HPLC.

D. Synthesis of CDRs

1. Synthesis of (*S*)-(−)-Lfx based CDRs

a) CDR-1: *N*-hydroxysuccinimidyl-(*S*)-(−)-Lfx

A solution of DCC (3 mL, 0.36 M) was added, under stirring, to a solution of (*S*)-(–)-Lfx (5 mL, 0.2 M) and *N*-hydroxysuccinimide (5 mL, 0.2 M) in 5 mL THF under nitrogen atmosphere at room temperature. The reaction mixture was stirred for 3 h and a separate set of reaction mixture was sonicated for 30 min; the precipitated dicyclohexylurea was removed by filtration. The filtrate was concentrated under vacuum and the residue was well extracted with 10 mL ethyl acetate. The extract was washed five times with water (5 mL), five times with brine (5 mL) and twice with ice-cold saturated $NaHCO_3$ (5 mL). The washed extract was then dried and recrystallized from hot EtOH to give the reagent as brown solid.

Characterization data: Yield: 428 mg (93.4%); $[\alpha]_D^{25}$ = (–) 92° (c =0.3, MeOH); m.p. 117–118 °C; UV (λ_{max} in MeOH, 298 nm); IR (KBr): 3435, 2927, 2361, 1704, 1628, 1576, 1481, 1305 and 1245 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$-*d*$_1$): δ 1.58 (d, 3H), 2.29 (s, 3H) 2.65 (t, 4H), 2.89 (s, 3H), 3.36 (m, 1H), 3.66 (t, 4H), 4.37-4.52 (dd, 2H, -O-CH_2-), 7.65 (d, 1H, Ar-H), 8.62 (s, 1H, Ar-H); HRMS: Calcd for $C_{22}H_{23}FN_4O_6$: 481.1499 (M^++Na), found 481.2039; anal. Calcd for $C_{22}H_{23}FN_4O_6$: C, 57.64%; H, 5.06%; N, 12.22%.; Found: C, 57.58%; H, 5.18%; N, 12.31%.

b) CDR-2: *N*-hydroxybenzotriazolyl-(*S*)-(−)-Lfx

The procedure was the same as applied to synthesis of CDR-1 using solutions of DCC (3 mL, 0.36 M), (*S*)-(–)-Lfx (5 mL, 0.2 M) and *N*-hydroxybenzotriazole (5 mL, 0.2 M) in 5 mL THF under nitrogen atmosphere at room temperature. The pure recrystallised CDR-2 was obtained as pale yellow solid.

Characterization data: Yield: 450 mg (94.1%); $[\alpha]_D^{25}$ = (–) 93° (c =0.3, MeOH); m.p. 138-139 °C; UV (λ_{max} in MeOH, 294 nm); IR (KBr): 3328, 2927, 2851, 1710, 1626, 1574, 1475, 1398, and 1245 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$-*d*$_1$): δ 1.56 (d, 3H), 2.29 (s, 3H), 2.78 (s, 4H), 3.22 (m, 1H), 3.54 (t, 4H), 4.28-4.40 (dd, 2H), 7.31 (m, 2H, Ar-H), 7.61 (d, 1H, Ar-H), 7.65 (d, 1H, Ar-H), 7.78 (d, 1H, Ar-H), 8.59 (s, 1H, Ar-H); HRMS: Calcd for

$C_{24}H_{23}FN_6O_4$: 501.1662 (M^++Na), found 501.2058; anal. Calcd for $C_{24}H_{23}FN_6O_4$: C, 60.25%; H, 4.85%; N, 17.56%; Found: C, 60.12%; H, 4.74%; N, 17.68%.

The scheme for synthesis of CDR-1 and CDR-2 is shown in **Fig. 3.1.**

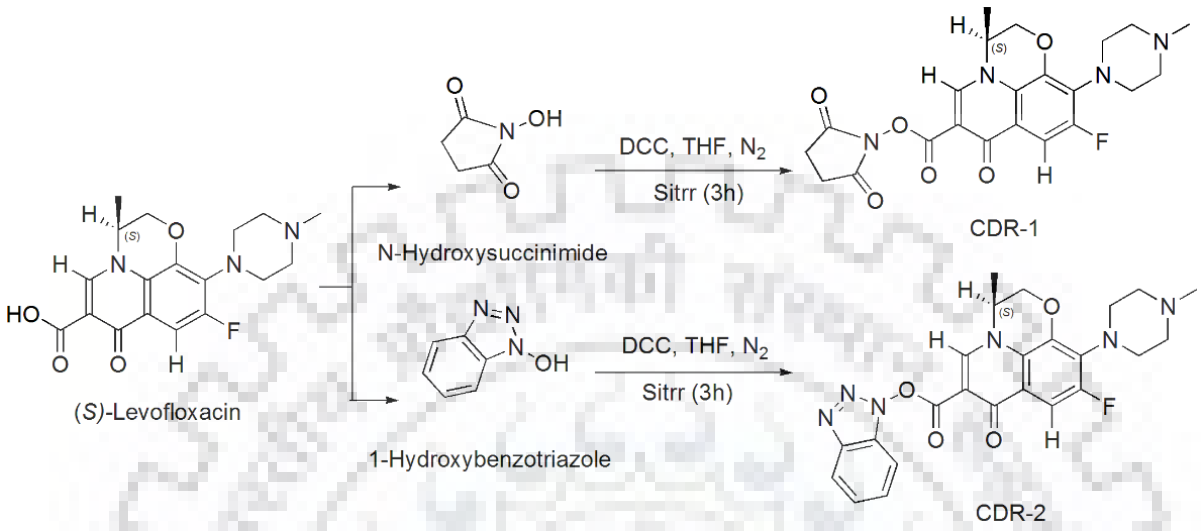


Fig. 3.1. Scheme for synthesis of CDR-1 and CDR-2.

2. Synthesis of (*S*)-(+)-Kpf based CDRs

a) CDR-3: *N*-hydroxysuccinimidyl-(*S*)-(+)-Kpf

A solution of DCC (3 mL, 0.36 M) was added, under stirring, to a solution of (*S*)-(+)-Kpf (5 mL, 0.2 M) and *N*-hydroxysuccinimide (5 mL, 0.2 M) in 5 mL THF under nitrogen atmosphere at room temperature. The reaction mixture was stirred for 3 h and a separate set of reaction mixture was sonicated for 30 min; the precipitated dicyclohexylurea was removed by filtration. The filtrate was concentrated under vacuum and the residue was well extracted with 10 mL ethyl acetate. The extract was washed five times with H_2O (5 mL), five times with brine (5 mL) and twice with ice-cold saturated $NaHCO_3$ (5 mL). The washed extract was then dried and recrystallized from hot EtOH to give the reagent as pale brown solid.

Characterization data: Yield: 331 mg (94.1%); $[\alpha]_D^{25}$ = (+) 41° ± 2 (c =1, MeOH); m.p. 115–117 °C; UV (λ_{max} 254 nm in MeOH); IR (KBr): 3327, 2931, 2850, 1707, 1656, 1597, 1577, 1448, 1312, 1285, 1223, and 1081 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$-d_1): δ 1.56 (d, 3H), 2.79 (s, 4H) 3.82 (q, 1H), 7.41-7.48 (m, 4H), 7.54-7.58 (m, 2H), 7.65 (d, 1H)

and 7.76 (d, 2H); HRMS: Calcd for $C_{20}H_{17}NO_5$: 374.1049 (M^++Na), found 374.1074; anal. Calcd for $C_{20}H_{17}NO_5$: C, 68.37%; H, 4.88%; N, 3.99%.; Found: C, 68.58%; H, 4.78%; N, 3.89%.

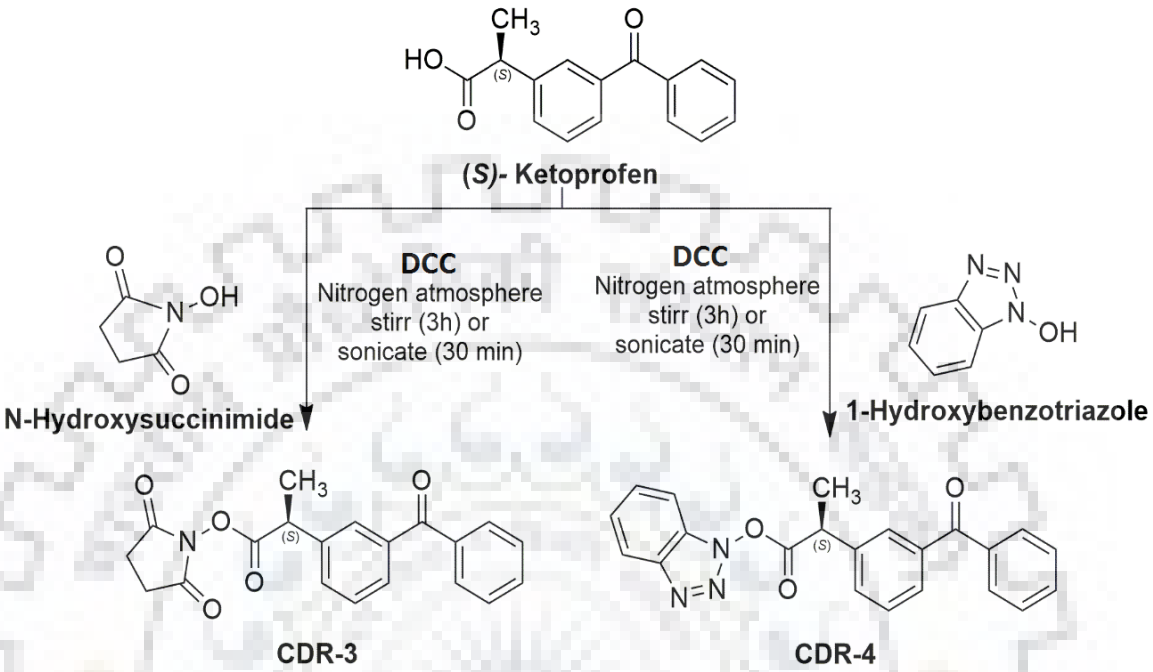


Fig. 3.2. Scheme for synthesis of CDR-3 and CDR-4.

b) CDR-4: *N*-hydroxybenzotriazolyl-(*S*)-(+)-Kpf

The procedure was same as applied to synthesis of CDR-3 using solutions of DCC (3 mL, 0.36 M), (*S*)-Kpf (5 mL, 0.2 M) and *N*-hydroxybenzotriazole (5 mL, 0.2 M) in 5 mL THF under nitrogen atmosphere at room temperature. The pure recrystallised CDR-4 was obtained as yellow-brown solid.

Characterization data: Yield: 350 mg (94.5%); $[\alpha]_D^{25}$ = (+) 39° ± 2 (c =1, MeOH); m.p. 135-138 °C; UV (λ_{max} 254 in MeOH); IR (KBr): 3326, 2930, 2851, 1729, 1656, 1628, 1577, 1449, 1389, 1316, 1284, 1179 and 1080 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$-d_1): δ 1.56 (d, 3H), 3.81 (q, 1H), 7.41-7.47 (m, 4H), 7.53-7.59 (m, 4H), 7.66 (d, 1H), 7.77 (d, 2H), 7.81 (d, 2H); HRMS: Calcd for $C_{22}H_{17}N_3O_3$: 394.1256 (M^++Na), found 394.1267; anal. Calcd for $C_{22}H_{17}N_3O_3$: C, 71.15%; H, 4.61%; N, 11.31%; Found: C, 70.92%; H, 4.70%; N, 11.48%.

Reaction scheme for synthesis of two CDRs based on reaction of (*S*)-(+)-Kpf, with *N*-hydroxysuccinimide (CDR-3), and *N*-hydroxybenzotriazole (CDR-4) is shown in **Fig.3.2**.

3. Synthesis of (*S*)-(+)-Ibf based CDRs

a) CDR-5: *N*-hydroxybenzotriazolyl-(*S*)-(+)-Ibf

CDR-5 is the chiral ester synthesized from (*S*)-(+)-Ibf by its reaction with *N*-hydroxybenzotriazole in presence of DCC. (*S*)-Ibf (0.309 g) and *N*-Btz (0.202 g) were dissolved in THF (5 mL) under nitrogen atmosphere at room temperature. Under ultra-sonication conditions 0.251 g DCC solution in THF was added dropwise into the resulting mixture. After 40 min of sonicating, the reaction mixture was filtered to remove precipitate of dicyclohexylurea. The filtrate was concentrated under vacuum and the residue was well extracted with ethyl acetate (15 mL). The extract was washed five times with water (5 mL), five times with brine (5 mL) and twice with ice-cold saturated $NaHCO_3$ (5 mL). The washed extract was then dried and recrystallized from hot EtOH to give the reagent as whitish solid. Reaction scheme for synthesis of CDR based on reaction of (*S*)-(+)-Ibf, with *N*-hydroxybenzotriazole (CDR-5) is shown in **Fig.3.3**.

Characterization data: Yield: 454 mg (94.1%); $[\alpha]_D^{25}$ = (+) 41° ± 2 (c =1, MeOH); m.p. 115–117 °C; UV (λ_{max} 262 nm in MeOH); IR (KBr): 2952, 2926, 2863, 1717, 1622, 1577, 1510, 1458, 1391, 1225, 1175, 1098 and 1068 cm^{-1}; ^{1}H-NMR (400 MH_Z, $CDCl_3$-d_1): δ 1.32 (d, 6H), 1.56 (d, 3H), 1.78 (m, 1H), 2.41 (d, 2H), 3.82 (m, 1H), 7.08 (d, 2H), 7.26 (d, 2H), 7.56 (d, 2H) and 8.02 (d, 2H); HRMS: Calcd for $C_{19}H_{21}N_3O_2$: 347.1565 (M^++Na), found 347.1571; anal. Calcd for $C_{19}H_{21}N_3O_2$: C, 70.57%; H, 6.55%; N, 12.99%.; Found: C, 70.86%; H, 6.38%; N, 13.15%.

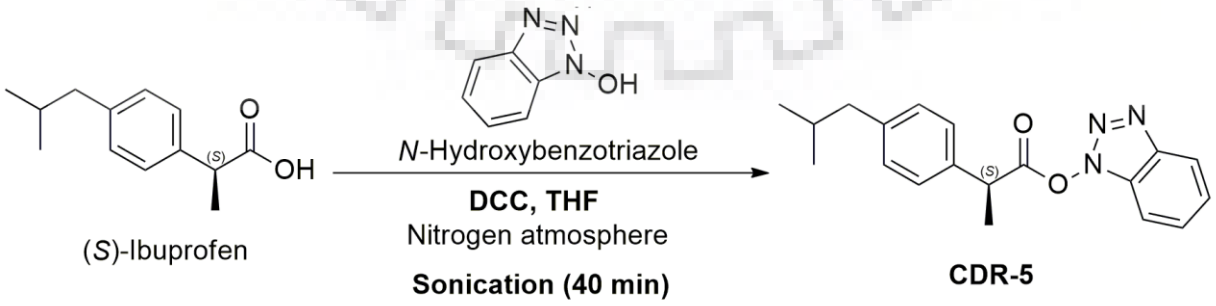


Fig. 3.3. Scheme for synthesis of CDR-5.

E. Synthesis of diastereomeric derivatives of racemic analytes (β-adrenolytics and AAs) using CDRs (CDR 1-5) and their LC separation

1. Synthesis of diastereomeric derivatives of racemic β-adrenolytics with CDRs (1 and 2) and their LC separation

1.1. Synthesis of diastereomeric derivatives of (*RS*)-Prl with CDR-1 and CDR-2

Scheme for synthesis of diastereomeric derivatives is shown in Fig. 3.4. A solution of (*RS*)-Prl (50 µL, 1 mM) was added to the solution of CDR-1 (100 µL, 1 mM) and TEA (10 µL), i.e., in the ratio of 1:2 (Prl: CDR), and pH 3. The resulting mixture was irradiated under microwave for 3 min using 80% power (800 W). 10 µL aliquot of the solution containing mixture of diastereomeric derivatives was diluted 10 times with MeCN and 20 µL of the resulting solution was injected onto the column.

The experimental conditions for synthesis of diastereomeric derivatives were optimized by performing following variations; microwave irradiation (MWI) time (up to 4 min with an interval of 1 min) at 80% power (800 W); pH 9, 10 and 11; and ratio of (*RS*)-Prl:CDR (1:1, 1:1.5, 1:2, 1:2.5). Completion of reaction was monitored by HPLC by recording peak areas of the diastereomeric derivatives. Similar experiments were carried out using CDR-2.

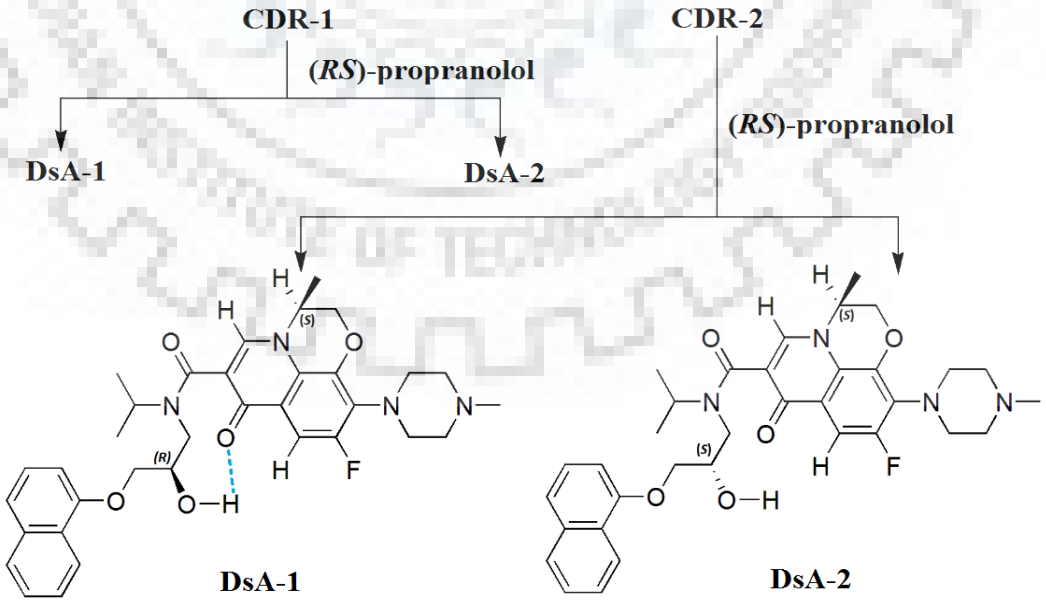


Fig. 3.4. Reactions showing synthesis of diastereomeric derivatives of (*RS*)-propranolol with CDR-1 and CDR-2.

a) Preparative synthesis of diastereomeric derivatives

The optimized conditions of synthesis of diastereomeric derivatives were scaled up to preparative level; the solutions used were (*RS*)-Prl (200 mg, 68 mM) dissolved in 10 mL 0.1 M $NaHCO_3$, and solution of CDR-2 (345 mg, 38 mM) in 20 mL acetonitrile. Similar experiments were carried out using CDR-1; the yields of diastereomeric derivatives were 402 mg (98%) and 397 mg (97%), respectively, using CDR-2 and CDR-1.

b) Open column chromatography for separation of diastereomeric derivatives

A glass column (2.5 x 35 cm) was packed with silica gel in *n*-hexane. The mixture of diastereomeric derivatives (as obtained from preparative synthesis, ~400 mg) was loaded. Solvent system consisting of MeOH-DCM (9:1, *v*/*v*) was found successful for adequate separation of the two diastereomeric derivatives; fractions collected from the column were examined by RP-HPLC for verification of purity of each diastereomeric derivative. The fractions containing single and identical diastereomeric derivative were combined and were concentrated in vacuum. The characterization data of the two separated diastereomeric derivatives synthesized with CDR-2 is presented below. Similar results were obtained for diastereomeric derivatives synthesized by using CDR-1.

First eluting diastereomeric derivative (DsA-1): Yield, 194 mg (96.08%); $[\alpha]_D^{25}$ = (–) 64° (c =0.05, MeOH); color, pale yellow-brown; m.p., 177-179 °C; UV (λ_{max} in MeOH, 294 nm); IR (KBr): 3329, 2925, 2852, 1721, 1628, 1576, 1439 and 1242 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$-d_1): δ 1.45 (m, 6H), 1.57 (d, 3H), 2.05 (s, 1H), 2.38 (s, 3H), 2.58 (s, 4H), 3.22-3.39 (m, 8H), 4.11 (d, 1H), 4.21 (d, 1H), 4.30 (d, 1H), 4.41 (d, 1H), 4.75 (m, 1H), 6.68 (d, 1H, Ar-H),7.27 (m, 1H, Ar-H), 7.37-7.44 (m, 3H, Ar-H), 7.66 (d, 1H, Ar-H), 7.74 (d, 1H, Ar-H), 8.20 (d, 1H, Ar-H), 8.63 (s, 1H, Ar-H); HRMS: Calcd for $C_{34}H_{39}FN_4O_5$: 625.2802 (M^++Na), found 625.3298; anal. Calcd for $C_{34}H_{39}FN_4O_5$: C, 67.76%; H, 6.52%; N, 9.30%; Found: C, 67.22%; H, 6.84%; N, 9.56%.

Second eluting diastereomeric derivative (DsA-2): Yield, 191 mg (95.01%); $[\alpha]_D^{25}$ = (–) 2.8° (c =0.05, MeOH); color, pale yellow-brown; m.p., 175-178 °C; UV (294 nm, λ_{max} in MeOH); IR (KBr): 3329, 2925, 2852, 1721, 1628, 1577, 1439 and 1242 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$-d_1): δ 1.45 (m, 6H), 1.58 (d, 3H), 2.27 (s, 1H), 2.38 (s, 3H), 2.59 (s, 4H), 3.22-

3.40 (m, 8H), 4.12 (d, 1H), 4.21 (d, 1H), 4.32 (d, 1H), 4.41 (d, 1H), 4.86 (m, 1H), 6.67 (d, 1H, Ar-H),7.27 (m, 1H, Ar-H), 7.38-7.45 (m, 3H, Ar-H), 7.65 (d, 1H, Ar-H), 7.74 (d, 1H, Ar-H), 8.22 (d, 1H, Ar-H), 8.65 (s, 1H, Ar-H); HRMS data: Calcd for $C_{34}H_{39}FN_4O_5$: 625.2802 (M^++Na), found 625.3298; anal. Calcd for $C_{34}H_{39}FN_4O_5$: C, 67.76%; H, 6.52%; N, 9.30%; Found: C, 67.21%; H, 6.86%; N, 9.51%.

c) HPLC of diastereomeric derivatives of (*RS*)-Prl

Diastereomeric derivatives of (*RS*)-Prl prepared with CDR-1 and CDR-2 were subjected to HPLC separation. MeCN-water (60:40, *v*/*v*) was used as flushing solvent. The mobile phase was filtered through a 0.45 µm filter and degassed by passing nitrogen and sonication, before use. The following mobile phases were tried:

(A) MeCN-TEAP buffer (10 mM, pH 3.5)

(B) MeOH-TEAP buffer (10 mM, pH 3.5)

These mobile phases were used in a linear gradient of MeCN from 80-20%, 70-30%, 60-40%, 50-50%, 40-60%, 30-70% and 20-80% in 30 min, at a flow rate of 1 mL min^{-1} with injection volume 20 µL with UV detection at 294 nm using PDA detector. Concentration of buffer (in the range of 0.05% to 0.20%) and flow rate (0.5 to 2.0 mL^{-1}) of mobile phase were also varied to optimize separation conditions.

d) Detagging of diastereomeric derivatives of (*RS*)-Prl

Hydrolysis: Fig.3.4 shows that the structures of diastereomeric derivatives synthesized with CDR-1 and CDR-2 are identical, therefore, hydrolysis of the diastereomeric derivatives prepared with CDR-2 only was performed.

DsA-1 and DsA-2 (120 mg each) were first dissolved in a little amount of glacial acetic acid and HCl (10 mL of 20%) was added; the solutions were irradiated under microwave (12 min, 80% power, 800 W).

e) Isolation of enantiomers of Prl and (*S*)-Lfx from hydrolysate

The hydrolysed solutions (of DsA-1 and DsA-2) were evaporated to dryness and the residue was extracted with water; it was expected that only (*R*)- and (*S*)-Prl would go into solution since (*S*)-Lfx is insoluble in water. The solution was centrifuged and (*S*)-Lfx remained as

residue; the residue was further shaken with water, and centrifuged. The combined supernatant was lyophilized; the residue was dissolved in small amount of MeOH and the solution was subjected to a small column (2.5 x 15 cm) packed with silica gel which was eluted with EtOH-DCM (6:4); fractions corresponding to enantiomeric Prl were combined and evaporated to dryness. The residue corresponding to (*S*)-Lfx was air dried and purified by recrystallization with DCM-$CHCl_3$.

The pure enantiomers of Prl so recovered were characterised by recording specific rotation and by spectroscopic techniques like ^{1}H-NMR, HRMS, IR, and UV. The characterization data of isolated Prl and (*S*)-Lfx are presented below:

(*S*)-Propranolol: Yield, 46.5 mg (93%); color, white solid; m.p, 190-192 ^{o}C; $[\alpha]_D^{25}$ = (–) 25.1^o (c =0.01, EtOH); anal. Calcd for $C_{16}H_{21}NO_2$: C, 68.07%; H, 7.50%; N, 4.96%; Found: C, 68.21%; H, 7.81%; N, 4.89%.

(*R*)-Propranolol: Yield, 45.2 mg (90.4%); color: white solid, m.p:192-194 ^{o}C; $[\alpha]_D^{25}$ = (+) 24.5 (c=0.01, EtOH); anal. Calcd for $C_{16}H_{21}NO_2$: C, 68.07%; H, 7.50%; N, 4.96%; Found: C, 68.15%; H, 7.82%; N, 4.91%.

Some of the common characterization data for both the enantiomers of Prl: UV (λ_{max} in MeOH, 289 nm); IR (KBr) 3450, 3200, 3050, 2980, 1630, 1590, 1580, 1500, 1460, 1400, 1340, 1320, 1270 and 1240 cm^{-1}. ^{1}H-NMR (400 MHz, $CDCl_3$-*d*$_1$): 1.47 (t, 6H), 2.03 (s, 1H), 3.26-3.39 (m, 2H), 3.46 (m, 1H), 4.09-4.20 (m, 2H), 4.81 (m, 1H), 6.64 (d, 1H, Ar-H), 7.37 (t, 1H, Ar-H), 7.44 (m, 3H, Ar-H), 7.74 (d, 1H, Ar-H), 8.24 (d, 1H, Ar-H); HRMS: Calcd for $C_{16}H_{21}NO_2$: 282.1470 (M^++Na), found 282.2384.

(*S*)-Lfx: Color, light yellow, crystalline; yield, 66.3 mg (95%) and 65 mg (93%), respectively from DsA-1 and DsA-2; m.p., 224 °C; $[\alpha]_D^{25}$ = (–) 104^o (c= 0.5, $CHCl_3$); UV (λ_{max} in MeOH, 294 nm); IR (KBr) 3265, 2931, 1724, 1294 and 1085 cm^{-1}. HRMS: Calcd for $C_{16}H_{21}NO_2$: 362.1471 (M^++H), found 362.1556. anal. Calcd for $C_{18}H_{20}FN_3O_4$: C, 59.83%; H, 5.58%; N, 11.63%; Found: C, 59.76%; H, 5.67%; N, 11.72%.

1.2. Synthesis of diastereomeric derivatives of (*RS*)-Mel and (*RS*)-Atl with CDR-1 and CDR-2

The synthesis of diastereomeric derivatives of (*RS*)-Mel and (*RS*)-Atl with CDR-1 and CDR-2 were carried out as method described for synthesis of diastereomeric derivatives of (*RS*)-Prl with CDR-1 and CDR-2, and similarly the experimental conditions for synthesis of

diastereomeric derivatives of (*RS*)-Mel and (*RS*)-Atl were optimized. Scheme for synthesis of diastereomeric derivatives of (*RS*)-Mel and (*RS*)-Atl are shown in Fig. 3.5 and Fig. 3.6, respectively.

a) Preparative synthesis of diastereomeric derivatives of (*RS*)-Mel and (*RS*)-Atl

The preparative scale synthesis of diastereomeric derivatives of (*RS*)-Mel and (*RS*)-Atl with CDR-1 and CDR-2 were carried out as described for the preparative synthesis of diastereomeric derivatives of (*RS*)-Prl. The yields of diastereomeric derivatives of (*RS*)-Mel were 262 mg (92%) and 268 mg (95%) respectively, using CDR-1 and CDR-2. The yields of diastereomeric derivatives of (*RS*)-Atl were 270 mg (95%) and 274 mg (97%) respectively, using CDR-1 and CDR-2.

b) Open column chromatography for separation of diastereomeric derivatives of (*RS*)-Mel and (*RS*)-Atl

The open column chromatography for diastereomeric derivatives of (*RS*)-Mel and (*RS*)-Atl were performed as described for open column chromatographic separation of diastereomeric derivatives of (*RS*)-Prl prepared with CDR-1 and CDR-2. The mixture of diastereomeric derivatives of (*RS*)-Mel and (*RS*)-Atl (as obtained from preparative synthesis, ~ 275 mg) was loaded in open column and a solvent system consisting of MeOH–DCM (9:1, *v*/*v*) was used as mobile phase.

The characterization data of first (DsA-3) and second (DsA-4) eluted diastereomeric derivatives of (*RS*)-Mel:

DsA-3: Yield, 110 mg (80%); yellow-brown solid, m.p. 132-136 °C; $[\alpha]_D^{25}$ = (–) 38° (c = 0.5, MeOH at 25 °C); UV (294 nm, λ_{max} in MeOH); IR (KBr): 3300, 2930, 2852, 1709, 1627, 1575, 1440, 1383, 1316, 1244 and 1089 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.22 (d, 6H), 1.59 (d, 3H), 2.06 (s, 1H), 2.36 (s, 3H), 2.54 (s, 4H), 2.75 (t, 2H), 2.92 (m, 1H), 3.11 (d, 1H), 3.20 (m, 1H), 3.30 (s, 3H), 3.35-3.42 (m, 5H), 3.53 (t, 2H), 3.89-3.97 (m, 2H), 4.31-4.43 (m, 2H), 4.48 (d, 1H), 6.81 (d, 2H, Ar-H), 7.09 (d, 2H, Ar-H), 7.68 (d, 1H, Ar-H) and 8.59 (s, 1H, Ar-H); HRMS (m/z) 633.3098 ($[M+Na]^+$, 30%); Analyzed calculated for $C_{33}H_{43}FN_4O_6$ – C, 64.90%; H, 7.10%; N, 9.17%; found C, 64.69%; H, 7.14%; N, 9.22%.

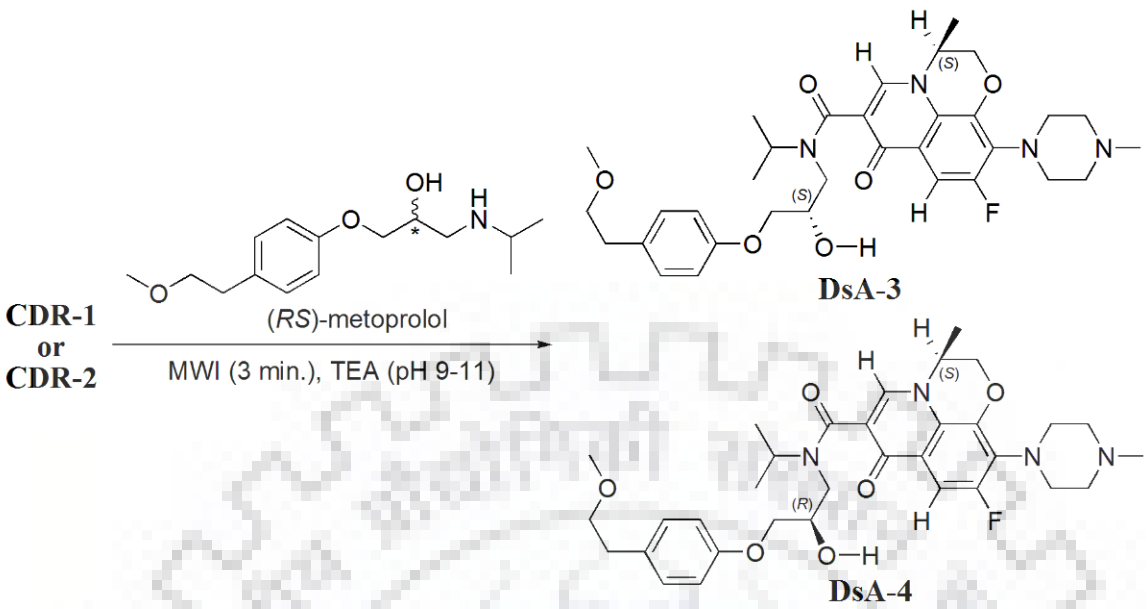


Fig. 3.5. Synthesis of diastereomeric derivatives of (*RS*)-Mel (DsA-3 and DsA-4).

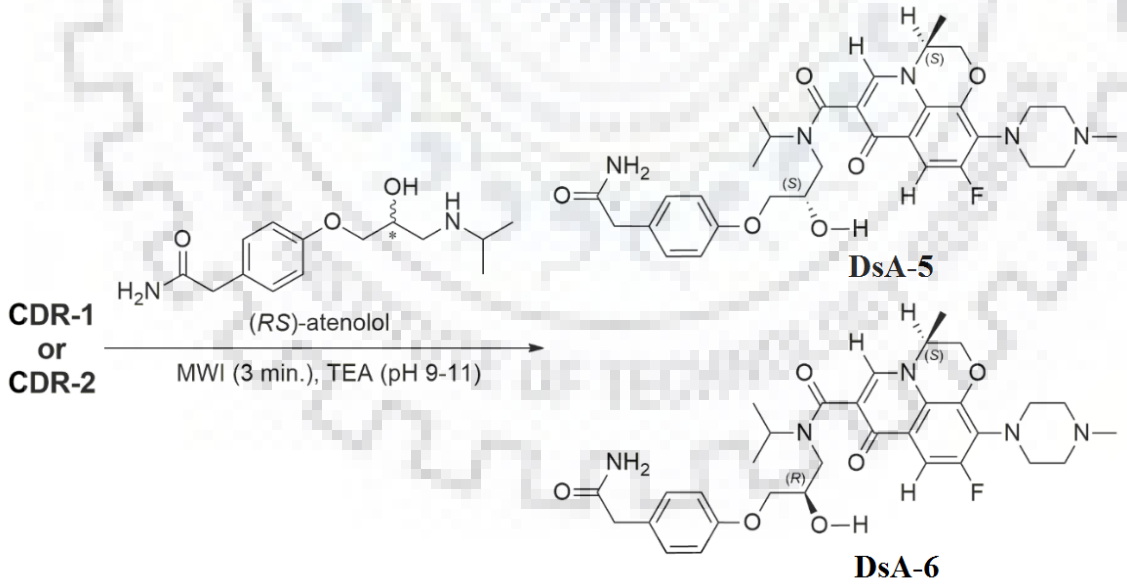


Fig. 3.6. Synthesis of diastereomeric derivatives of (*RS*)-Atl (DsA-5 and DsA-6).

DsA-4: Yield, 115 mg (86%); yellow-brown solid; m.p. 135-138 °C; $[\alpha]_D^{25}$ = (–) 60 º (c = 0.5 in MeOH at 25 °C); UV (294 nm, λ_{max} in MeOH); IR (KBr): 3300, 2928, 2851, 1709, 1627, 1576, 1444, 1383, 1312, 1244 and 1087 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.23 (d, 6H), 1.60 (d, 3H), 2.25 (s, 1H), 2.35 (s, 3H), 2.54 (s, 4H), 2.78 (t, 2H), 2.94 (m, 1H), 3.12 (d, 1H), 3.21 (m, 1H), 3.32 (s, 3H), 3.36-3.41 (m, 5H), 3.53 (t, 2H), 3.89-3.99 (m, 2H), 4.32-4.45 (m, 2H), 4.50 (d, 1H), 6.80 (d, 2H, Ar-H), 7.09 (d, 2H, Ar-H), 7.68 (d, 1H, Ar-H) and 8.60 (s, 1H, Ar-H); HRMS (m/z) 633.3098 ($[M+Na]^+$, 30%); Analyzed calculated for $C_{33}H_{43}FN_4O_6$ – C, 64.90%; H, 7.10%; N, 9.17%; found C, 64.74%; H, 7.02%; N, 9.20%.

The characterization data of first (DsA-5) and second (DsA-6) eluted diastereomeric derivatives of (*RS*)-Atl:

DsA-5: Yield, 119 mg (87%); yellow-brown**;** m.p. 138-140 °C; $[\alpha]_D^{25}$ = (–) 51 º (c = 0.5 in MeOH at 25 °C); UV (294 nm, λ_{max} in MeOH); IR (KBr, cm^{-1}): 3356, 2935, 2847, 1721, 1621, 1582, 1540, 1512, 1489, 1469, 1452, 1358, 1317, 1290, 1241, 1210, 1133, 1110 and 1091 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.10 (d, 6H), 1.59 (d, 3H), 2.09 (s, 1H), 2.35 (s, 3H), 2.53 (s, 4H), 2.68-2.90 (m, 3H), 3.31-3.46 (m, 4H), 3.94-4.07 (m, 4H), 4.32-4.49 (m, 4H), 5.43 (broad, 2H), 6.85 (d, 2H, Ar-H), 7.12 (d, 2H, Ar-H), 7.68 (d, 1H, Ar-H) and 8.61 (s, 1H, Ar-H); HRMS (m/z) 632.2860 ($[M+Na]^+$, 20%); Analyzed calculated for $C_{32}H_{40}FN_5O_6$ – C, 63.04%; H, 6.61%; N, 11.49%; found C, 63.31%; H, 6.28%; N, 11.31%.

DsA-6: Yield, 115 mg (84%); yellow-brown; m.p. 139-142 °C; $[\alpha]_D^{25}$ = (–) 64 º (c = 0.5 in MeOH at 25 °C); UV (294 nm, λ_{max} in MeOH); IR (KBr): 3357, 2935, 2849, 1724, 1620, 1583, 1541, 1517, 1493, 1470, 1452, 1360, 1315, 1292, 1242, 1207, 1136, 1113 and 1090 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.10 (d, 6H), 1.58 (d, 3H), 2.28 (s, 1H), 2.34 (s, 3H), 2.53 (s, 4H), 2.70-2.91 (m, 3H), 3.32-3.46 (m, 4H), 3.93-4.04 (m, 4H), 4.30-4.49 (m, 4H), 5.41 (broad, 2H), 6.87 (d, 2H, Ar-H), 7.15 (d, 2H, Ar-H), 7.69 (d, 1H, Ar-H) and 8.59 (s, 1H, Ar-H); HRMS (m/z) 632.2860 ($[M+Na]^+$, 20%); Analyzed calculated for $C_{32}H_{40}FN_5O_6$ – C, 63.04%; H, 6.61%; N, 11.49%; found C, 63.42%; H, 6.32%; N, 11.20%.

c) HPLC of diastereomeric derivatives (*RS*)-Mel and (*RS*)-Atl

The optimization of RP-HPLC conditions for diastereomeric derivatives of (*RS*)-Mel and (*RS*)-Atl was carried out as described for diastereomeric derivatives of (*RS*)-Prl prepared with CDR-1 and CDR-2.

1.3. Synthesis of diastereomeric derivatives of (*RS*)-Bxl, (*RS*)-Cdl, (*RS*)-Sbl, and (*RS*)-Bpl with CDR-1 and CDR-2

The methodology of synthesis of diastereomeric derivatives of (*RS*)-Bxl, (*RS*)-Cdl, (*RS*)-Sbl, and (*RS*)-Bpl with CDR-1 and CDR-2 are same as used for synthesis of diastereomeric derivatives of (*RS*)-Mel with CDR-1 and CDR-2.

Fig.3.7 shows scheme for synthesis of representative diastereomeric derivatives of racemic Bxl (DsA-7 and DsA-8); the structures of the diastereomeric derivatives of (*RS*)-Cdl, (*RS*)-Sbl, and (*RS*)-Bpl are shown in Fig.3.8 (these are designated as DsA-9, DsA-10 for Cdl; DsA-11, DsA-12 for Sbl, and DsA-13, DsA-14 for Bpl.

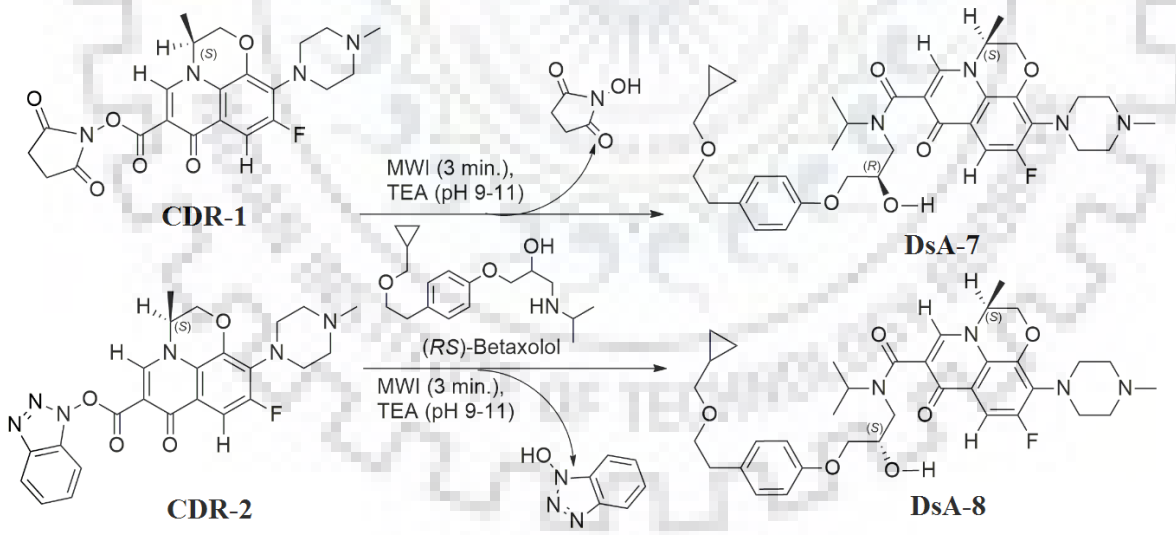


Fig. 3.7. Reaction showing synthesis of diastereomeric derivatives of (*RS*)-Betaxolol (as a representative) with CDR-1 and CDR-2. The structures of the diastereomeric derivatives remain the same (DsA-7 and DsA-8).

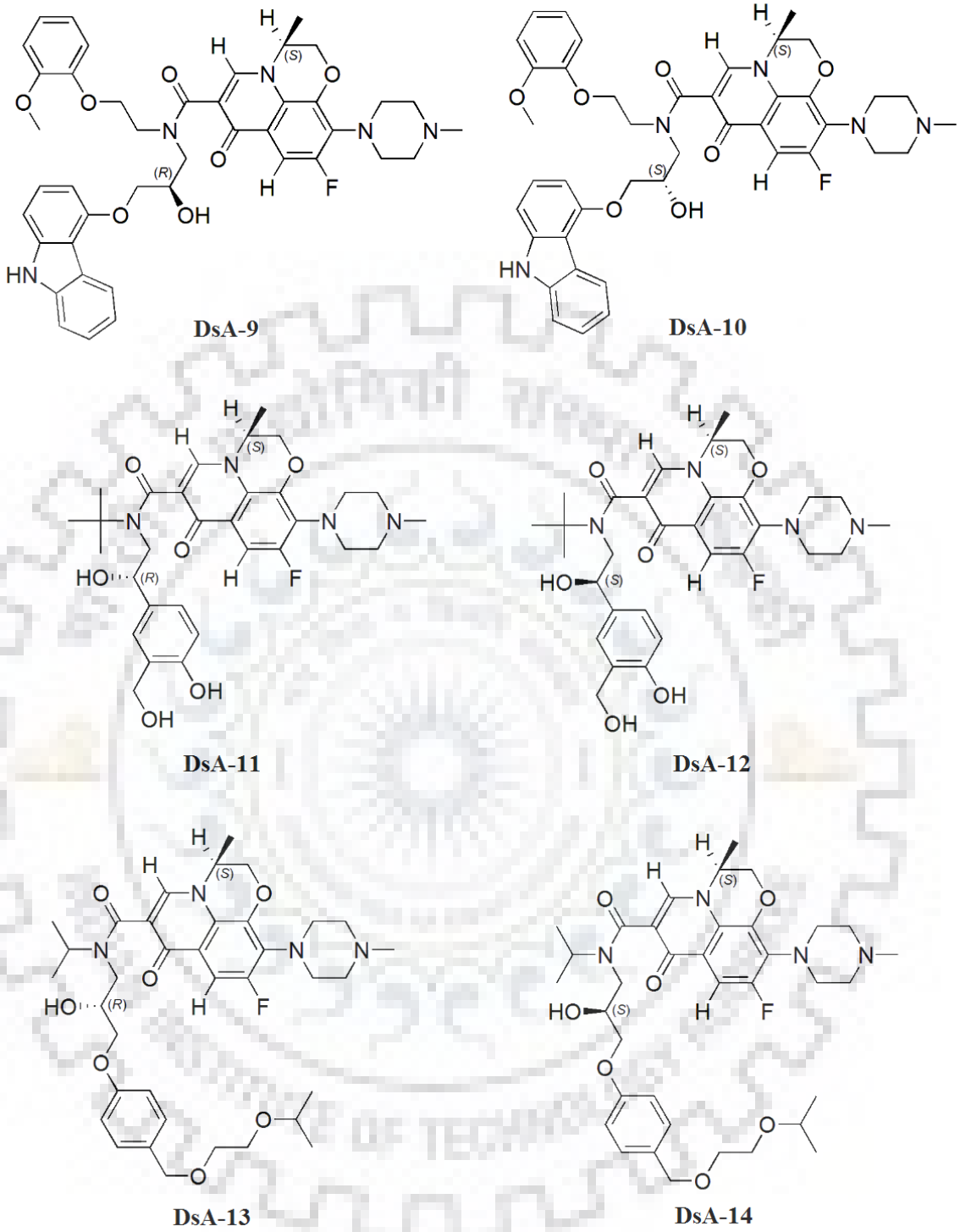


Fig. 3.8. Structures of the diastereomeric derivatives of (*RS*)-Cdl, (*RS*)-Sbl, and (*RS*)-Bpl, (these are designated as DsA-9, DsA-10 for Cdl; DsA-11, DsA-12 for Sbl, and DsA-13, DsA-14 for Bpl).

a) HPLC of diastereomeric derivatives of (*RS*)-Bxl, (*RS*)-Cdl, (*RS*)-Sbl, and (*RS*)-Bpl

10 µL aliquot solution of reaction mixture containing a pair of diastereomeric derivatives of each of the β-adrenolytics was diluted 10 times with ethanol and 20 µL of the resulting sample solution was injected onto the C_{18} column. WMP-TEAP buffer (80:20, *v*/*v*) was used as the flushing solvent.

Separation conditions were optimized by varying mobile phase composition; WMP-TEAP buffer (pH 3.5, 10 mM) was used in an isocratic mode in the ratio of (60:40, 70:30, and 80:20, *v*/*v*) for 60 min, at different flow rates (0.5, 0.7, and 1.0 mL min^{-1}) using PDA detector at 294 nm.

2. Synthesis of diastereomeric derivatives of racemic β-adrenolytics with CDRs (3 and 4) and their LC separation

2.1. Synthesis of diastereomeric derivatives of (*RS*)-Prl, (*RS*)-Mel and (*RS*)-Atl with CDR-3 and CDR-4

The synthesis of diastereomeric derivatives of (*RS*)-Prl, (*RS*)-Mel and (*RS*)-Atl with CDR-3 and CDR-4 and optimization of the synthesis condition were same as described for synthesis of diastereomeric derivatives of (*RS*)-Prl with CDR-1 and CDR-2, only CDR-3 and CDR-4 was used instead of CDR-1 and CDR-2. Scheme for synthesis of diastereomeric derivatives of (*RS*)-Prl (as representative) is given in Fig. 3.9.

a) Preparative synthesis of diastereomeric derivatives of (*RS*)-Prl

The optimized conditions of synthesis of diastereomeric derivatives were scaled up to preparative level by using (*RS*)-Prl (200 mg, 68 mM) dissolved in 10 mL of 0.1M $NaHCO_3$, and CDR-3 (350 mg, 50 mM) dissolved in 20 mL acetonitrile. Similar experiments were carried out using CDR-4. The yields of diastereomeric mixtures of (*RS*)-Prl were 355 mg (91%) and 349 mg (89%), using CDR-3 and CDR-4, respectively. The diastereomeric mixtures so obtained were subjected to open column chromatography.

b) Open column chromatography of diastereomeric derivatives of (*RS*)-Prl

The open column chromatography for diastereomeric derivatives of (*RS*)-Prl prepared with CDR-3 and CDR-4 were performed as described for diastereomeric derivatives of (*RS*)-Prl

prepared with Lfx based CDRs (1 and 2). The mixture of diastereomeric derivatives of (*RS*)-Prl prepared with CDR-3 (as obtained from preparative synthesis, ~ 200 mg) was loaded in open column and a solvent system consisting of MeOH–DCM (9:1, *v*/*v*) was used as mobile phase.

The first (DsB-1) and second (DsB-2) eluting diastereomeric derivatives were characterized. The characterization data is presented under 'Results and Discussion'. Similar results were obtained for diastereomeric derivatives synthesized by using CDR-4.

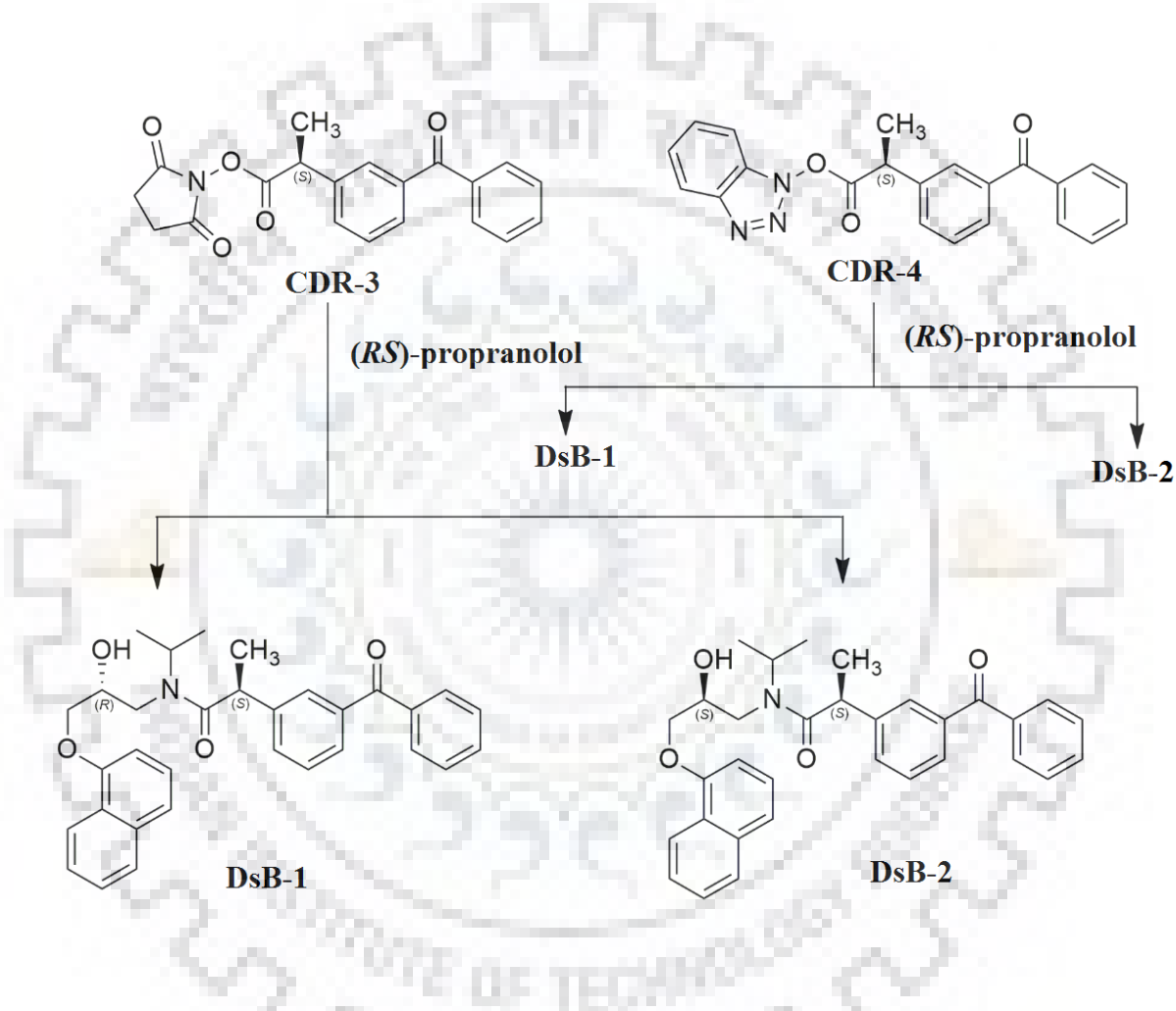


Fig. 3.9. Synthesis of diastereomeric derivatives of (*RS*)-Prl with CDR-3 and CDR-4.

First eluting diastereomeric derivative (DsB-1): Yield, 96 mg (96.08%); $[\alpha]_D^{25}$ = (+) 35.2° (c = 0.5, MeOH); color, pale brown; m.p., 173-175 °C; UV (λ_{max} in MeOH, 254 nm); IR (KBr): 3187, 2927, 2847, 1711, 1652, 1592, 1574, 1443, 1312, 1276, **1221**, and 1085 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$-d_1): δ 1.38 (d, 6H) 1.58 (d, 3H), 2.31 (s, 1H), 3.25-3.40 (m, 3H),

3.94 (q, 1H), 4.09 (d, 1H), 4.20 (d, 1H), 4.76 (m, 1H), 6.68 (d, 1H, Ar-H), 7.25 (m, 1H, Ar-H), 7.35-7.46 (m, 7H, Ar-H), 7.52-7.57 (m, 2H), 7.64 (d, 1H), 7.69 (d, 1H, Ar-H), 7.78 (d, 2H) and 8.20 (d, 1H, Ar-H); HRMS data: Calcd for $C_{32}H_{33}NO_4$: 518.2802 (M^++Na), found 518.3298; anal. Calcd for $C_{32}H_{33}NO_4$: C, 75.12%; H, 6.50%; N, 2.74%; Found: C, 75.19%; H, 6.84%; N, 2.68%.

Second eluting diastereomeric derivative (DsB-2): Yield, 94 mg (94.01%); $[\alpha]_D^{25}$ = (+) 11.5° (c = 0.5, MeOH); color, pale brown; m.p., 174-178 °C; UV (254 nm, λ_{max} in MeOH); IR (KBr): 3329, 2934, 2851, 1718, 1656, 1594, 1578, 1447, 1311, 1279, 1210, and 1082 cm^{-1}; ^{1}H-NMR (400 MH_Z, $CDCl_3$-*d_1*): δ 1.37 (d, 6H) 1.58 (d, 3H), 2.08 (s, 1H), 3.24-3.40 (m, 3H), 3.92 (q, 1H), 4.08 (d, 1H), 4.22 (d, 1H), 4.75 (m, 1H), 6.68 (d, 1H, Ar-H), 7.24 (m, 1H, Ar-H), 7.32-7.45 (m, 7H, Ar-H), 7.50-7.56 (m, 2H), 7.62 (d, 1H), 7.68 (d, 1H, Ar-H), 7.79 (d, 2H) and 8.19 (d, 1H, Ar-H); HRMS data: Calcd for $C_{32}H_{33}NO_4$: 518.2802 (M^++Na), found 518.3298; anal. Calcd for $C_{32}H_{33}NO_4$: C, 75.12%; H, 6.50%; N, 2.74%; Found: C, 75.21%; H, 6.86%; N, 2.54%.

c) RP-HPLC separation of diastereomeric derivatives

The optimization of RP-HPLC conditions for diastereomeric derivatives of (*RS*)-Prl, (*RS*)-Mel and (*RS*)-Atl prepared with CDR-3 and CDR-4 were carried out as described for diastereomeric derivatives of (*RS*)-Mel prepared with Lfx based CDRs (1 and 2).

2.2. Synthesis of diastereomeric derivatives of (*RS*)-Sbl, (*RS*)-Cdl, (*RS*)-Bpl, and (*RS*)-Bxl with CDR-3 and CDR-4

The synthesis of diastereomeric derivatives of (*RS*)-Sbl, (*RS*)-Cdl, (*RS*)-Bpl, and (*RS*)-Bxl with CDR-3 and CDR-4 and their optimization of the synthesis conditions were same as described for synthesis of diastereomeric derivatives of (*RS*)-Prl with CDR-3 and CDR-4. The scheme for synthesis of diastereomeric derivatives of (*RS*)-Sbl, (*RS*)-Cdl, (*RS*)-Bpl and (*RS*)-Bxl with CDR-4 (as a representative) are shown in Fig. 3.10.

a) RP-HPLC of diastereomeric derivatives

Sample was prepared by diluting 10 µL aliquot solution of each of completed reaction mixture with 90 µL EtOH and resulting solution was filtered with 0.45 µm syringe filter, and its 20 µL solution was injected onto the C_{18} column.

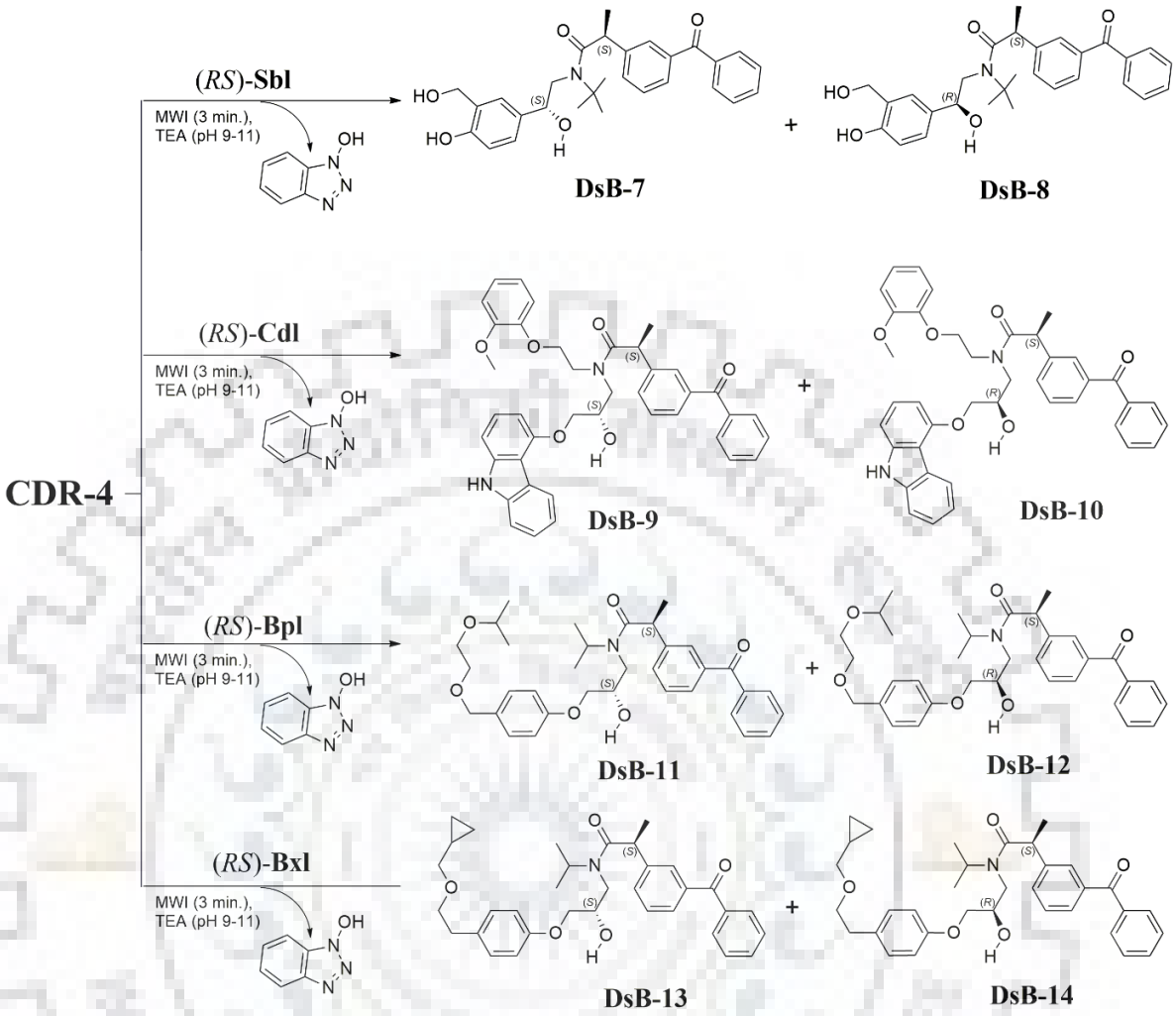


Fig. 3.10. Synthesis of diastereomeric derivatives of (*RS*)-Sbl, (*RS*)-Cdl, (*RS*)-Bpl and (*RS*)-Bxl with CDR-4; the structures of the diastereomeric derivatives are designated as DsB-7**,** DsB-8 for (*RS*)-Sbl; DsB-9**,** DsB-10 for (*RS*)-Cdl; DsB-11**,** DsB-12 for (*RS*)-Bpl, and DsB-13**,** DsB-14 for (*RS*)-Bxl.

Separation conditions were optimized by varying (i) mobile phase composition as follows, WMP-TEAP buffer (10 mM, pH 3.5) in an isocratic mode in the ratio of (50:50, 60:40, 70:30, and 80:20, *v*/*v*) for 60 min, and (ii) flow rates, 0.5, 0.7, and 1.0 mL min^{-1} for each mobile phase composition. Detection was at 254 nm, using PDA detector.

3. Synthesis of diastereomeric derivatives of AAs [(*RS*)-SeMet, (*RS*)-Met, (*RS*)-Cys, and (*RS*)-PenA] using CDR-2, CDR-4, CDR-5 and their LC separation

Diastereomeric derivatives of each of the racemic AAs (0% *ee*) were prepared using **CDR-2, CDR-4, CDR-5** under microwave irradiation conditions. Solution of the racemic AA (100 µL, 1 mM) was mixed with 200 µL (1 mM) solution of CDR-**5** and TEA (20 µL), and the resulting mixture was irradiated under microwave for 180 seconds (80%, 800 W). Similarly, diastereomeric derivatives of all the four AAs were prepared using all the three CDRs (2, 4 and 5). Thus, there were 12 pairs of diastereomeric derivatives and shown in Fig. 3.11, Fig. 3.12 and Fig. 3.13.

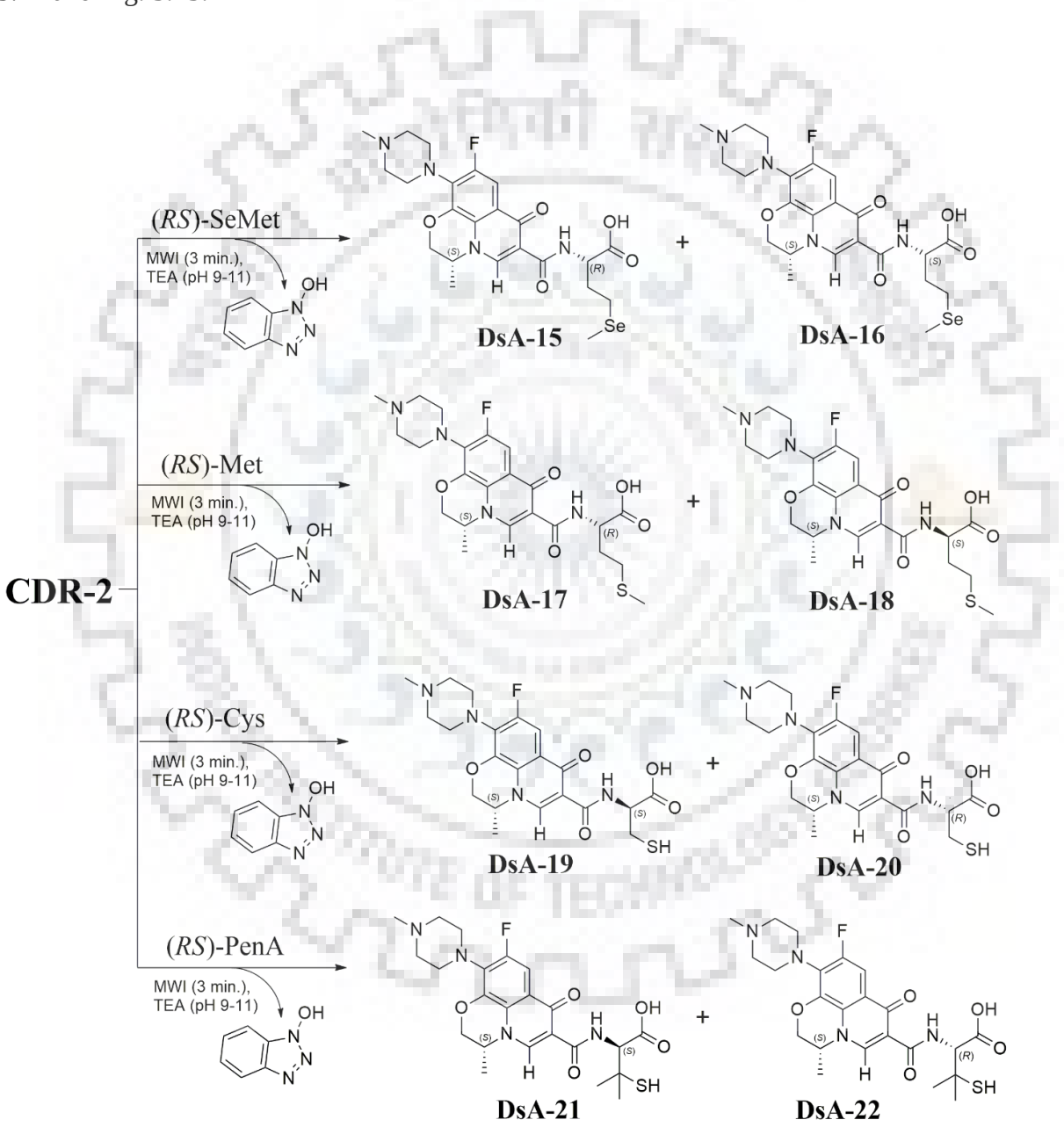


Fig. 3.11. Scheme showing synthesis of diastereomeric derivatives of (*RS*)-amino acids (SeMet, Met, Cys and PenA) with CDR-2; the structures of the diastereomeric derivatives

are designated as DsA-15**,** DsA-16 for (*RS*)-SeMet; DsA-17**,** DsA-18 for (*RS*)-Met; DsA-19**,** DsA-20 for (*RS*)-Cys, and DsA-21**,** DsA-22 for (*RS*)-PenA.

a) RP-HPLC of diastereomeric derivatives of AAs

An aliquot (20 µL) of the diastereomeric derivative mixture (from the reaction vessel) was diluted with 180 µL EtOH and the resulting solution was filtered with 0.45 µm syringe filter, and its 20 µL portion was injected onto the C_{18} column. WMP-TEAP buffer (80:20, *v*/*v*) was used as the flushing solvent. Samples for HPLC analysis were prepared in the similar manner for each of the twelve cases.

Separation conditions were optimized for diastereomeric derivatives of AAs as described for diastereomeric derivatives of (*RS*)-Sbl prepared with (*S*)-Kpf based CDRs (3 and 4).

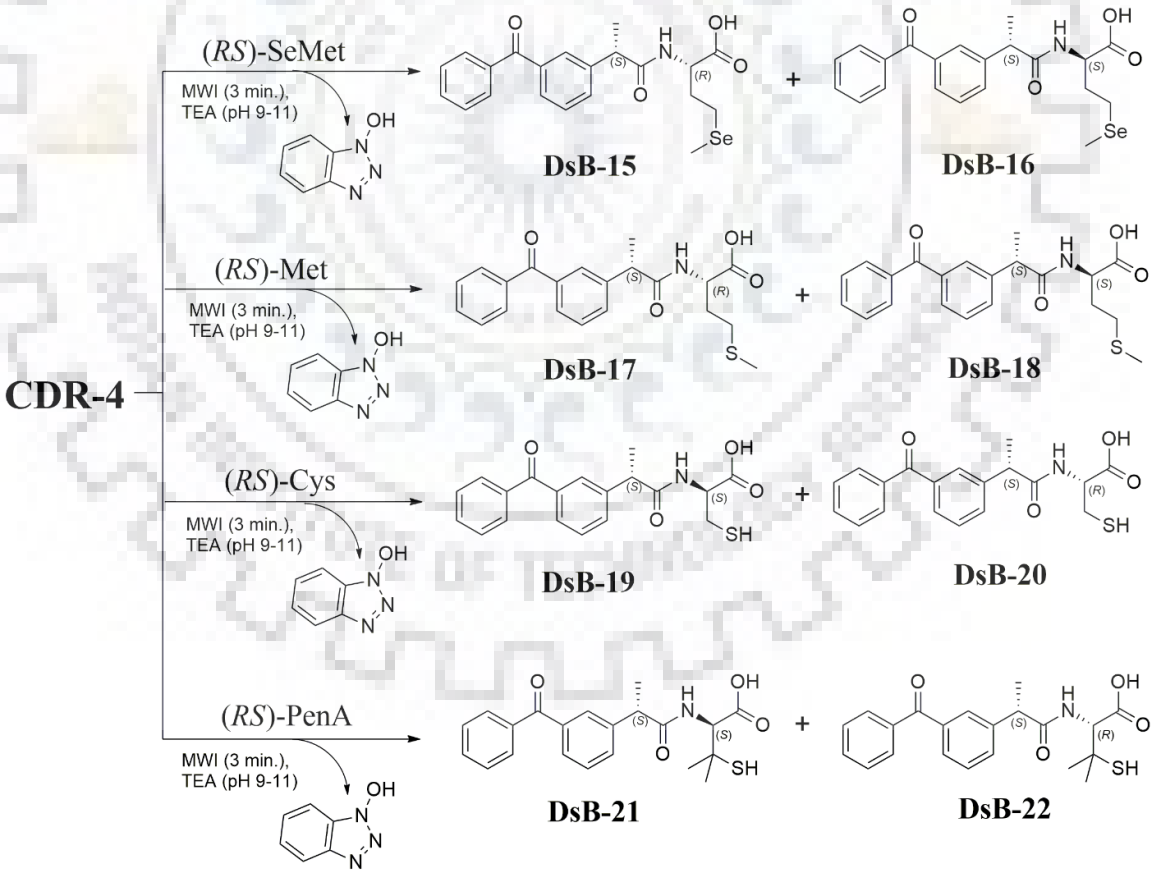


Fig. 3.12. Scheme showing synthesis of diastereomeric derivatives of (*RS*)-amino acids (SeMet, Met, Cys and PenA) with CDR-4; the structures of the diastereomeric derivatives

are designated as DsB-15**,** DsB-16 for (*RS*)-SeMet; DsB-17**,** DsB-18 for (*RS*)-Met; DsB-19**,** DsB-20 for (*RS*)-Cys, and DsB-21**,** DsB-22 for (*RS*)-PenA.

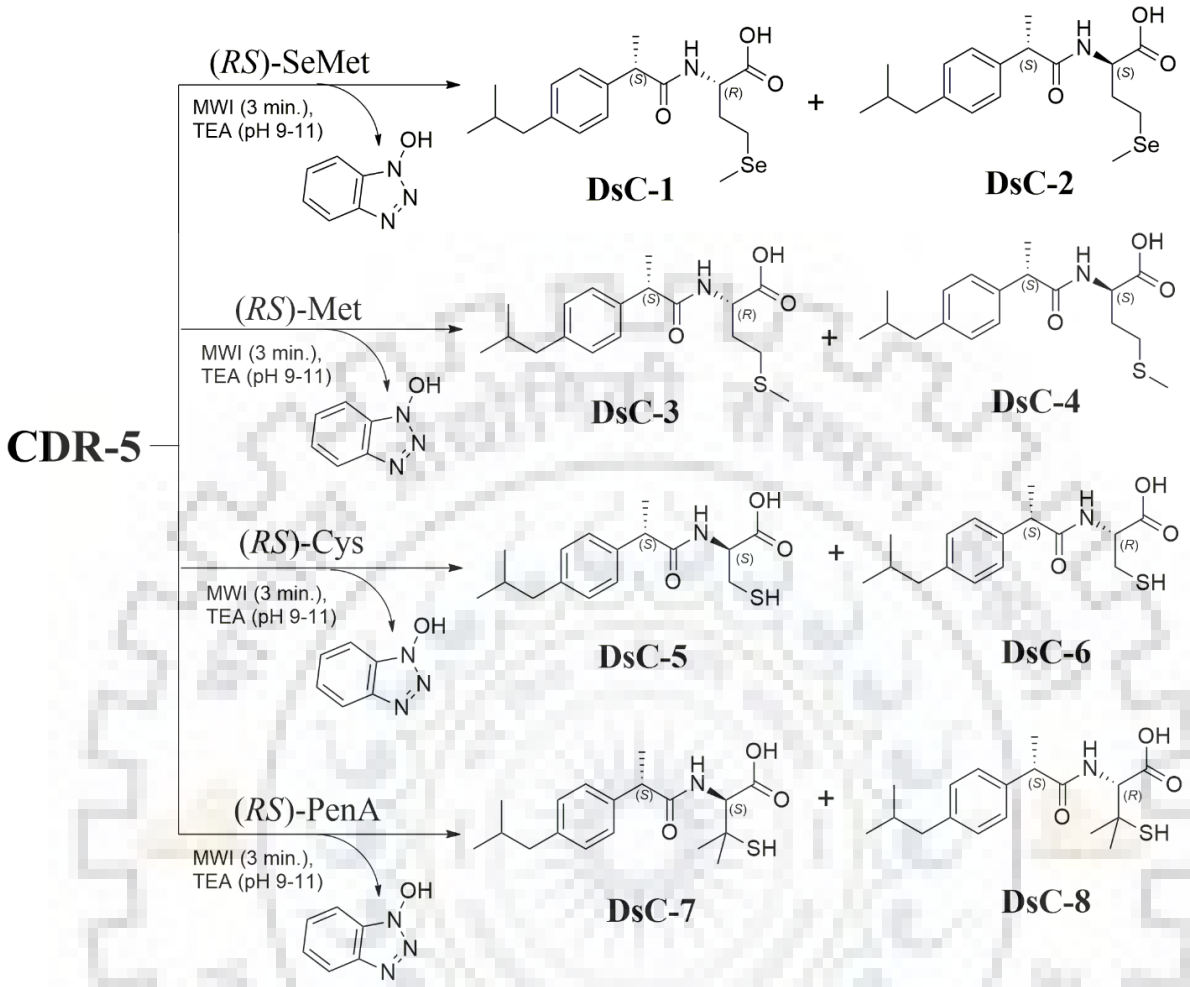


Fig. 3.13. Scheme showing synthesis of diastereomeric derivatives of (*RS*)-amino acids (SeMet, Met, Cys and PenA) with CDR-5; the structures of the diastereomeric derivatives are designated as DsC-1**,** DsC-2 for (*RS*)-SeMet; DsC-3**,** DsC-4 for (*RS*)-Met; DsC-5**,** DsC-6 for (*RS*)-Cys, and DsC-7**,** DsC-8 for (*RS*)-PenA.

F. Liquid chromatography

TLC: Clean, and pre-equilibrated glass chamber with solvent system was used for developing the chromatograms on TLC. Commercially available TLC were used to separate synthesized diastereomeric derivatives. A Hamilton syringe was used for spotting of sample on TLC plate. Experiments were optimized by varying mobile phase (binary, ternary and quaternary mixture of solvents), pH, and temperature. Developed TLC plates were first dried in oven at 40 ^{o}C then spots were located using UV-Visible chamber and iodine vapours chamber.

Open column chromatography: A glass column (2.5 x 35 cm) was packed with silica gel in *n*-hexane was used for preparative separation. After the optimizing separation conditions on TLC, same mobile phase mixtures were used for separation of diastereomeric derivatives on open column. Each fraction of eluted diastereomeric derivatives were collected at a particular time gap, and were examined by RP-HPLC (with already optimized conditions) for verification of purity of each diastereomeric derivatives.

HPLC: Since the present work required a lot of experimental work for optimization of separation conditions of several diastereomeric derivatives using HPLC. The details of such optimization are described in respective chapters.

G. Calculation of retention factor, resolution and selectivity

The chromatographic separation data [retention factor (k), selectivity factor (α) and resolution factor (R_S)] were calculated using the equations $k = (t_x - t_0)/t_0$, $\alpha = k_2 / k_1 = (t_{x2} - t_0)/(t_{x1} - t_0)$ and $R_S = 2\,(t_{x2} - t_{x1})/(W_1 + W_2)$, respectively. Here t_0 is the dead time, t_{x1} and t_{x2} are retention times of first and second eluted peaks, respectively. W_1 and W_2 are the corresponding base peak widths.

H. Method validation

Validation studies were carried out with respect to linearity, accuracy and precision according to ICH guidelines [135] for HPLC separation of diastereomeric derivatives of the analytes under study. Slopes and intercepts were determined by drawing linearity plots for peak area vs concentration and using linear regression equations. To determine precision, inter-day and intra-day assay studies were carried out and the results are represented as relative standard deviation (RSD). Limit of detection (LOD) and limit of quantification (LOQ) were also evaluated. For the studies of stabilities and recoveries, peak areas (as provided by the system software) were used for quantitation.

Results and discussions for separation of diastereomeric derivatives have been given in the subsequent chapters; these are presented on the basis of application of chiral moieties of CDRs, and the analytes. There are subsections in Chapter-4 and Chapter-5 corresponding to application of RP-HPLC and MLC.

Chapter-4

Results and discussion for enantioseparation of diastereomeric derivatives of racemic β-adrenolytics prepared with newly synthesized (*S*)-levofloxacin based CDRs (1 and 2)

Diastereomeric derivatives of (*RS*)-Prl were synthesized with both CDR-1 and CDR-2 and their separation was achieved by RP-HPLC followed by detagging of the derivatives when native enantiomers of Prl were isolated and identified. On the similar lines, synthesis of diastereomeric derivatives of both (*RS*)-Mel and (*RS*)-Atl was carried out using the same two CDRs; these derivatives were also separated by RP-HPLC. The results and discussions for the same are provided below. For all the three analytes there was achieved preparative separation of respective pairs of diastereomeric derivatives using open column chromatography. NMR spectra for all the derivatives, so obtained, were recorded and the absolute configuration for each was established which was supported by developing the lowest energy structures using the program Gaussian 09 Rev. A.02 and hybrid density function B3LYP with 6-31G*.

Diastereomeric derivatives of (*RS*)-Bxl, (*RS*)-Cdl, (*RS*)-Sbl and (*RS*)-Bpl were also synthesized with CDR-1 and CDR-2. The separation of these diastereomeric pairs was achieved using micellar liquid chromatography (MLC) wherein the mobile phase contained simple surfactants in the aq. phase and without any organic solvent.

The experimental details for the synthesis of CDRs and 14 pairs of diastereomeric derivatives are already described in **Chapter-3**.

A. Preparative enantioseparation of (*RS*)-propranolol and recovery of native enantioseparation by detagging

1. CDRs and diastereomeric derivatives

Development of new reagents having high molar absorptivity remains desirable and important at all times since a UV–vis detector is commonly available in most laboratories. (*S*)-(−)-Lfx isolated from commercial tablets was purified and characterized and it was used as an enantiomerically pure chiral auxiliary for synthesis of CDRs. Two new CDRs were synthesized by attachment of succinimidyl and benzotriazole moieties, by nucleophilic acyl

substitution, in (*S*)-(−)-Lfx. CDRs were characterized as described in **Chapter-3**. As no reaction was taking place at the asymmetric carbon, there was no racemization during the synthesis of CDRs. Although the CDRs were considered as enantiomerically pure, their enantiomeric purity was further confirmed as per literature reports [14, 16].

The carboxylic group of (*S*)-(−)-Lfx (Fig. 2.3) was activated by reaction with *N*-hydroxybenzotriazole because esters of 1-hydroxy benzotriazole are extremely potent acylating agents [136], while *N*-hydroxysuccinimide esters are highly reactive and yield the desired amide bond [between amino group of (*RS*)-Prl and the carbonyl of (*S*)-Lfx] with expected ease [137]. The exceptional reactivity of the two esters is due to the effect of neighbouring N-atom (providing anchimeric assistance). Besides being very good nucleophiles, the succinimidyl and benzotriazole moieties served as very good leaving groups when substituted with amino group containing (*RS*)-Prl, leading to easy formation of diastereomeric derivatives. Diastereomeric derivatives (amides) were synthesized under mild derivatization conditions with shorter reaction time. The primary amides synthesis using metal catalyst has been described by Singh *et al*, [138] and there are a few literature reports on synthesis of amides under MWI conditions [139] and their conversion to carbamates under mild and convenient synthesis conditions [140].

HPLC analysis of the diastereomeric derivatives showed only two peaks of similar peak area, which was also a confirmation for the purity of CDRs. Various conditions of mobile phase, flow rate, etc., were used to establish the optimized conditions for separation of diastereomeric derivatives. Microwave-assisted acid-catalyzed hydrolysis of amide reproduced starting materials.

2. Separation of diastereomeric derivatives by RP-HPLC

Mobile phase (A) consisting of MeCN–TEAP (pH 3.5; gradient, 30–70% MeCN) in 30 min, at a flow rate of 1 mL/min was found successful in terms of selectivity and reproducibility; values for retention factor (k), separation factor (α) and resolution (Rs) under the optimized HPLC conditions are given in Table-4.A1 for the diastereomeric derivatives prepared with CDR-1 and -2. Sections of chromatograms showing resolution of two pairs of diastereomeric derivatives of (*RS*)-Prl are shown in Fig. 4.A1. Also, the two diastereomeric derivatives isolated from preparatory open column were independently subjected to RP-HPLC under the same conditions.

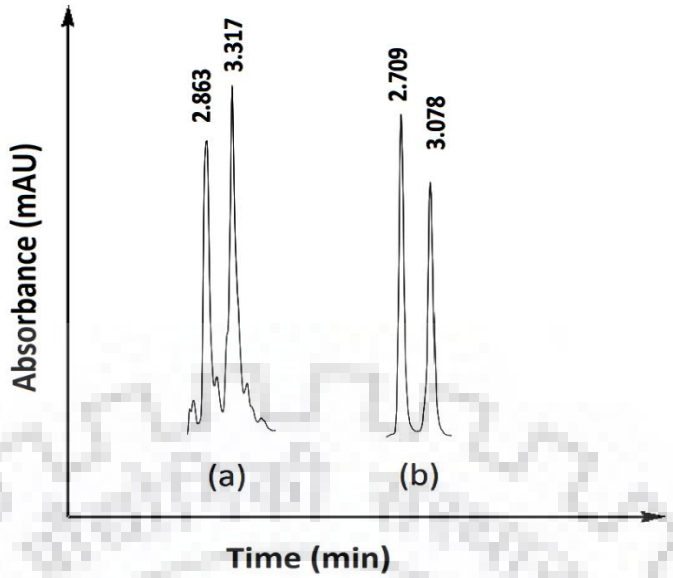


Fig. 4.A1. Sections of chromatograms showing separation of diastereomeric derivatives of (*RS*)-propranolol, (a) separation of diastereomeric derivatives of (*RS*)-Prl prepared with CDR-1; (b) separation of diastereomeric derivatives of (*RS*)-Prl prepared with CDR-2; in both the cases (*S,R*)-diastereomeric derivative (DsA-1) is eluted first. Chromatographic conditions: LiChrospher C_{18} column (L x I.D. 25 cm × 4.6 mm, 5 μm particle size); Mobile phase, MeCN-TEAP (pH 3.5), gradient, 30% MeCN to 70% MeCN in 30 min, at a flow rate of 1 mL min^{-1}.

Separation of diastereomeric derivatives was investigated using MeOH in the mobile phase as organic modifier in place of MeCN; the presence of MeCN provided sharper peaks, enhanced resolution and shorter retention time under the same HPLC conditions. This can be attributed to the characteristics of MeCN such as low UV cutoff, low viscosity (the viscosity of MeOH is 0.59 cP and that of MeCN is 0.38 cP) and high eluting strength. In addition, MeCN possesses unique properties such as dissolving a wide range of solutes, low acidity and minimal chemical reactivity; thus, use of MeCN as the organic modifier is associated with a decrease in retention factor and an increase in peak heights. The UV spectrum corresponding to first- and second-eluting diastereomeric derivative was captured (using PDA detector) at 2.709 and 3.078 min, respectively; these were found to be identical.

The hydrolysate of each diastereomeric derivative was evaporated to dryness and the residue was dissolved in 10 mL MeOH; it was centrifuged and 10 μL of the supernatant was diluted 10 times and 20 μL of the resulting solution was injected on to column. Two peaks were obtained in chromatogram corresponding to Lfx and Prl (**Fig. 4.A2**).

Table-4.A1. Chromatographic separation data of diastereomeric derivatives prepared with CDR-1 and 2.

CDR	β-adrenolytics	Separation data for diastereomeric derivatives prepared with CDR-1 and CDR-2				
		k_1 for [(*S*,*R*)]-, (DsA-1)	k_2 for [(*S*,S)]-, (DsA-2)	*Mean, Rs*	*Confidence interval (95%)*	*Rs*
CDR-1	(*RS*)-Prl	1.392	2.223	2.91	1.334-2.667	1.604
CDR-2	(*RS*)-Prl	1.463	1.799	4.17	1.355-2.334	1.229

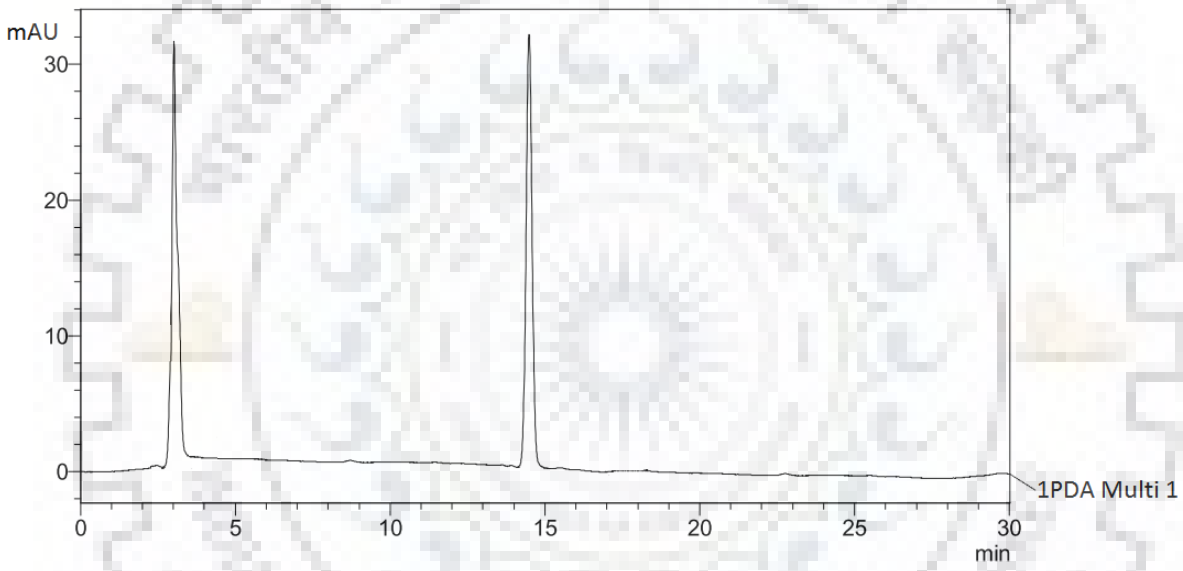


Fig. 4.A2. Chromatogram of acid catalysed hydrolysate of diastereomeric derivative DsA-1; two peaks correspond to Lfx and Prl, respectively (from L to R); Chromatographic conditions: LiChrospher C_{18} column (L x I.D. 25 cm × 4.6 mm, 5 µm particle size); Mobile phase, MeCN-TEAP (pH 3.5), gradient, 30% MeCN to 70% MeCN in 30 min, at a flow rate of 1 mL min^{-1}.

3. Structures of diastereomeric derivatives

The diastereomeric derivatives (Fig. 3.4) were designated as (*S*,*R*)- and (*S*,*S*)-diastereomeric derivatives, respectively, where the first (*S*) corresponds to the configuration of chiral auxiliary and the other (*S*)/(*R*) corresponds to that of (*S*)-Prl or (*R*)-Prl. For determination of structure and absolute configuration, the diastereomeric derivatives were separated and

isolated by open column chromatography; these were designated as DsA-1 and DsA-2 based on elution order. Isolation of enantiomers from the experiments of hydrolysis of diastereomeric derivatives showed that DsA-1 provided (*R*)-Prl and DsA-2 provided (*S*)-Prl.

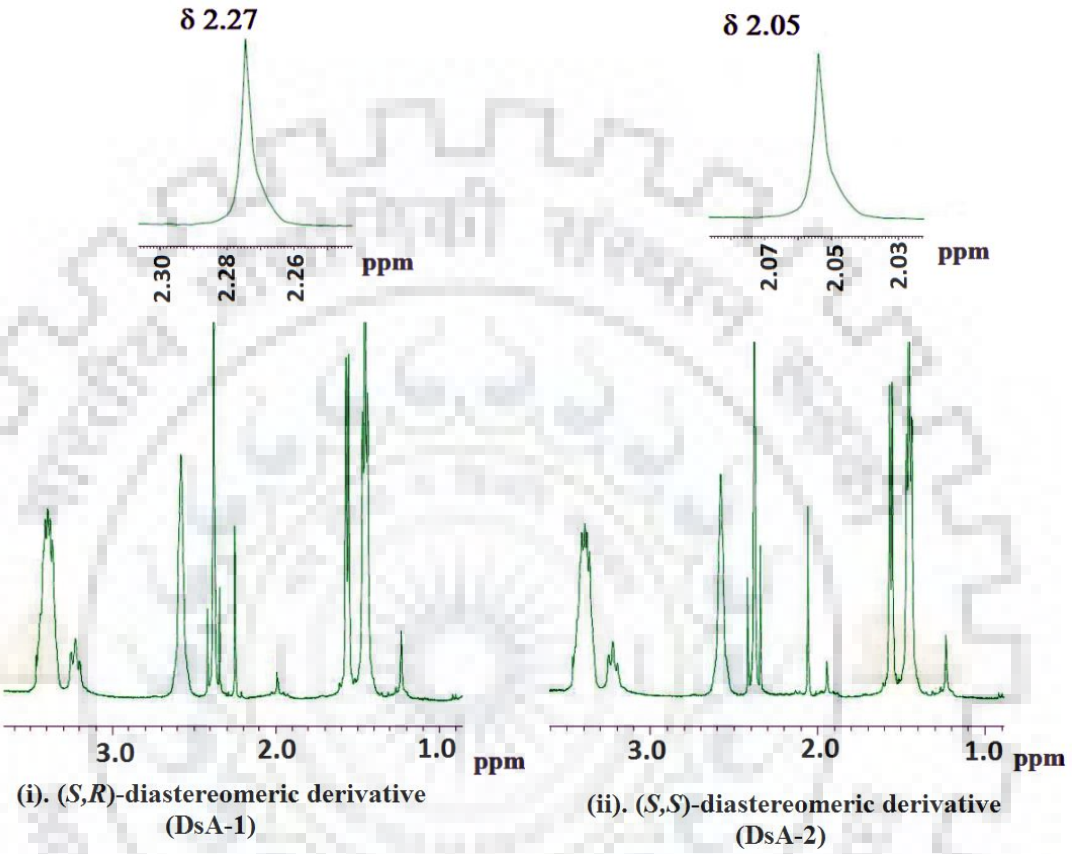


Fig. 4.A3. Sections of ^{1}H-NMR spectra of diastereomeric derivatives illustrating chemical shift values for the peaks of –OH (at the stereogenic center), signal at δ 2.27 in (*S*,*R*)-diastereomeric derivative (i), and at δ 2.05 in ((*S*,*S*)-diastereomeric derivative (ii). This difference in chemical shift of – OH signal is attributed to the formation of H-bonding in (*S*,*R*)-diastereomeric derivative.

NMR spectra of the diastereomeric derivatives and H-bond: There occurs formation of an amide bond (which is a rigid structure) in the diastereomeric derivatives because of the reaction of the amino group of the analyte and the carbonyl of the CDR. Since differences in chemical shifts (Δδ) form the basis of discrimination among the chiral diastereomeric compounds, both diastereomeric derivatives were utilized as representative models for establishing/determining the absolute configuration. ^{1}H-NMR spectra of the purified diastereomeric derivatives (separated through open column) were recorded (Fig. 4.A3). Chemical shift values for the peaks of -OH signal in the two diastereomeric derivatives are

δ 2.27 and δ 2.05. The difference in chemical shift (i.e. $\Delta\delta^{RS}$ value of – 0.22) is large enough for a diagnostic -OH signal which can be represented as [$\Delta\delta^{RS}$(OH) =δ^{R}(OH)–δ^{S}(OH)], where (*R*) and (*S*) descriptors refer to the stereogenic configuration in the Prl moiety. This difference in the chemical shift of the -OH signal may be attributed to the formation of H-bonding in one of the diastereomeric derivatives; whereas in the other diastereomeric derivative H-bonding is not possible (chemical structures are shown in Fig. 3.4). The δ values (in ppm) of the -OH proton corresponding to the asymmetric center in Prl in the (*S*,*R*)- and (*S*,*S*)-diastereomeric derivatives are shown in Fig. 4.A3.

Further evidence for the formation of H-bonds was provided when geometry optimization structures were developed using the program Gaussian 09 Rev. A.02 and hybrid density functional B3LYP with a 6-31G* basis set (Fig. 4.A4). According to the lowest energy optimized structures of diastereomeric derivatives, there was formation of an intramolecular hydrogen bond between the carbonyl group of Lfx molecule and hydroxyl group of Prl in one of the diastereomeric derivatives; formation of such an intramolecular hydrogen bond was not possible in the other diastereomeric derivative. The chemical shift for the –OH proton in the ^{1}H-NMR spectrum of the diastereomeric derivative is greatly affected by the presence of a hydrogen bond that arises owing to the specific spatial arrangement. The formation of the hydrogen bond causes a downfield shift and we observe a difference in the chemical shift of the -OH protons in the two diastereomeric derivatives.

Based on the downfield shift of the -OH proton in ^{1}H-NMR spectrum of one of the diastereomeric derivatives (Fig. 4.A3) and by developing the minimum energy structural geometry (Fig. 4.A4), a hydrogen bond between the -OH proton (in the Prl moiety) and the carbonyl oxygen (of the Lfx moiety) is envisaged in one of the diastereomeric derivatives only and thus it would be the DsA-1 (as shown in **Fig. 4.A4a**). Owing to the spatial arrangement of groups/atoms at the stereogenic center, such a hydrogen bond formation does not occur in the (*S*,*S*)-diastereomeric derivative (**Fig. 4.A4b**). The $\Delta\delta^{RS}$ value obtained for -OH proton has a negative sign. If the structure of diastereomeric derivative is not as represented in **Fig. 4.A4**, then the $\Delta\delta^{RS}$ value between the diastereomeric pair would not be negative for the -OH proton. The first eluted diastereomeric derivative showed a downfield shift for -OH proton while the second eluted diastereomeric derivative showed an upfield shift in ^{1}H-NMR spectrum; therefore the first eluted diastereomeric derivative has the (*S*,*R*)-configuration.

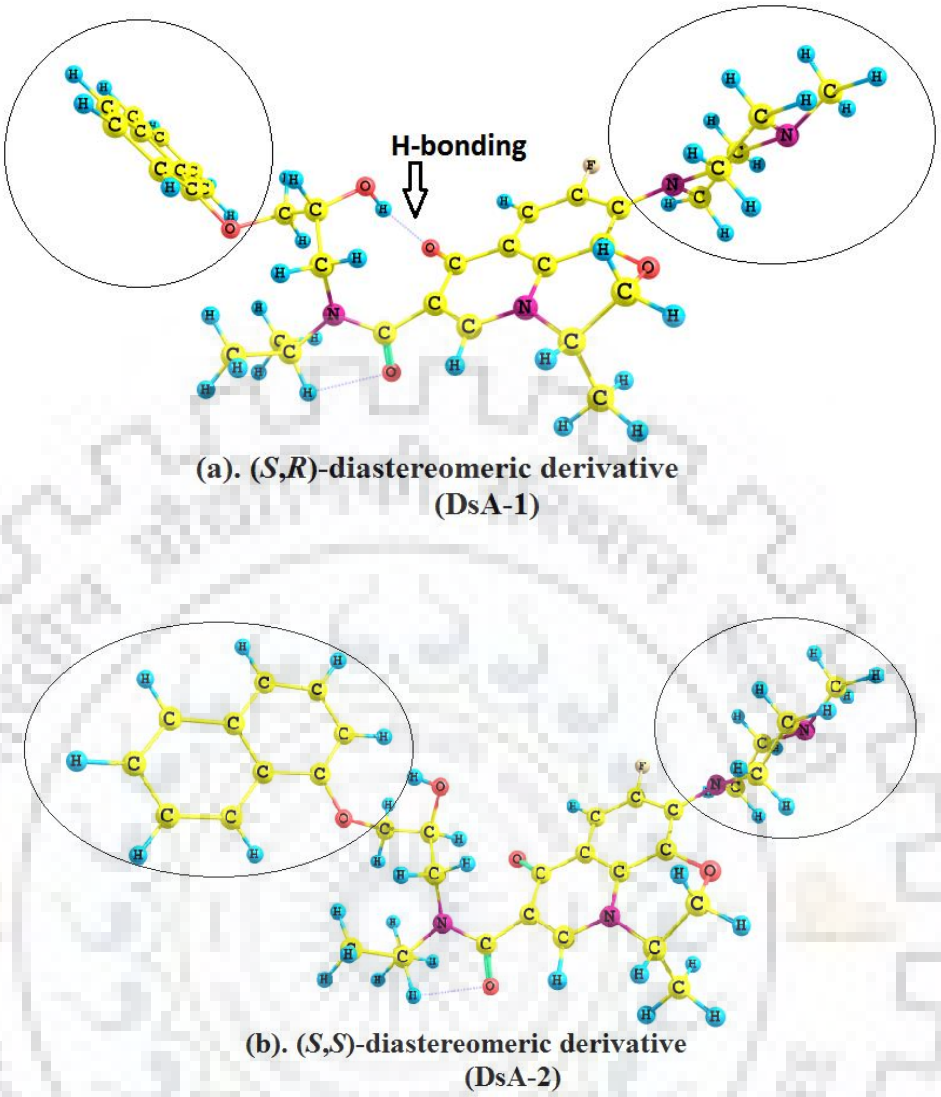


Fig. 4.A4. Structures of diastereomeric derivatives, optimized for lowest energy, developed by using the program Gaussian 09 Rev. A.02 and hybrid density function B3LYP with 6-31G*; (a) the (*S*,*R*)-diastereomeric derivative (DsA-1) shows intramolecular H-bond between the –OH proton (in the Prl moiety) and the carbonyl oxygen (of the Lfx moiety), and, (b) the (*S*,*S*)-diastereomeric derivative (DsA-2) shows no intramolecular H-bonding between the –OH proton and the carbonyl oxygen.

4. Elution order

Hydrogen bonding as described above, and the presence of a piperazinyl group (in Lfx) and a naphthyl group in Prl, contributing to the overall hydrophobicity along with the rheological properties of the mobile phase, are responsible for different partition coefficients and different retention times of the (*S*,*R*)-,and (*S*,*S*)-diastereomeric derivatives. Therefore, the

diastereomeric derivatives elute one after another for these different physical properties. The H-bonding in the (*S*,*R*)-diastereomeric derivative (DsA-1) increases polarity and makes it slightly more polar than the (*S*,*S*)-diastereomeric derivative (DsA-2) and causes a greater affinity, for the molecule as a whole, with the mobile phase (containing polar TEAP) and thus is eluted first.

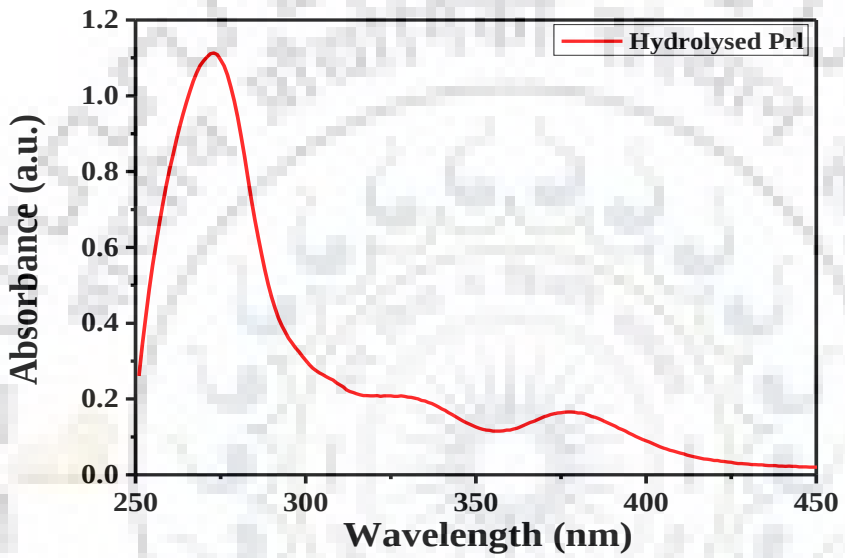


Fig. 4.A5. UV spectra of propranolol from hydrolyse diastereomeric derivatives.

5. Detagging of diastereomeric derivatives and isolation of native enantiomers

The amide bonds in peptides are known to be hydrolysed easily under acidic conditions; therefore, detagging of diastereomeric derivatives (having amide bond) was attempted using acid hydrolysis under MWI in the present studies. HPLC of hydrolysate showed two peaks corresponding to Lfx and Prl (Fig. 4.A2). The presence of only two peaks in the chromatogram confirmed completion of reaction. It was inferred that there was no racemization during the hydrolysis process because no reaction took place at the stereogenic center. Isolation, purification and characterization of both Lfx and Prl from the hydrolysis reaction of diastereomeric derivatives provided the two compounds with the yields of 90–95%, and interestingly (*R*)-, and (*S*)-Prl were obtained as pure enantiomers along with (*S*)-

Lfx. The UV spectrum of isolated Prl given as Fig. 4.B5. The recovery cycle is shown in Fig.4.B6.

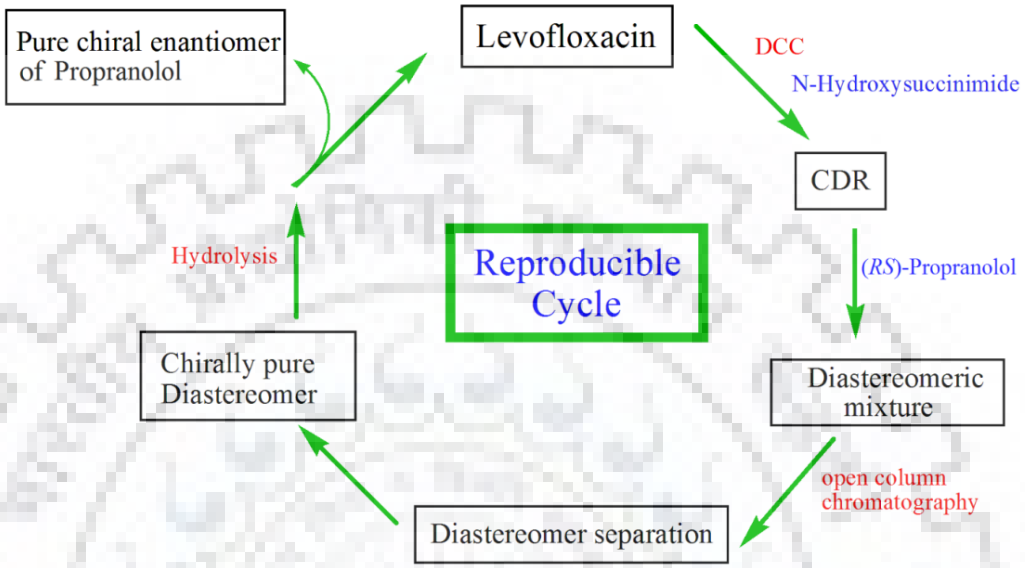


Fig. 4.B6. Reproducible cycle for recovery of pure enantiomers of Prl and (*S*)-Lfx.

6. Stability of CDRs and recovery of diastereomeric derivatives

Stability of the CDRs was investigated after long-term (refrigerated at a temperature of 2–5 °C) and short-term (room temperature) storage. Synthesis of diastereomeric derivatives followed by HPLC separation under optimized conditions was carried out at an interval of 15 days from the day of synthesis of CDRs; CDRs were found to be stable for up to 5 months.

7. Method validation

Linearity, slope and intercept (peak area vs. concentration of diastereomeric derivative, ng/mL) were determined using regression equations y = 4.875x + 644.0 (r^2 = 0.999) and y = 4.792x + 894.2 (r^2 = 0.999) for the first and second eluted diastereomeric derivative, respectively.

The accuracy and precision studies were carried out by replicate HPLC analysis (n = 5) of each of the solutions of mixture of diastereomeric derivatives (expected to contain 100,

200, 300, 400 and 500 ng/mL of individual diastereomeric derivatives) of (*RS*)-Prl prepared with CDR-2 **(Tables-4.A2** and **4.A3)**. The calculated RSD for intra-day assay precision confidence intervals were 1.36–1.59 and 1.54–1.81% for the first and second diastereomeric derivatives, respectively; for inter-day assay, precision was 1.43–1.69 and 1.44–1.64% for the first and second diastereomeric derivative, respectively. The calculated recovery for the first and second eluting diastereomeric derivative, respectively, had confidence intervals of 97.94–100.92 and 97.51–102.47% for intra-day assay and 98.07–101.23 and 97.85–101.05% for inter-day assay (Tables-4.A2, 4.A3).

The LODs were found to be 1.790 and 2.132 ng/mL for diastereomeric derivatives of (*S*)- and (*R*)-Prl, respectively, and LOQs were found to be 5.425 and 6.460 ng/mL for diastereomeric derivatives of (*S*)- and (*R*)-Prl, respectively.

8. Comparison of present work with literature reported.

A comparison of results (in terms of derivatization conditions, resolution, separation factor, and elution time) for diastereomeric derivatives of (*RS*)-Prl prepared with different CDRs (as reported in literature) has been summarized in **Table-4.A4.**

The separation factors (α, 1.23–1.61) for the diastereomeric derivatives prepared with the newly synthesized CDRs were found to be better than the diastereomeric derivatives prepared with CDRs based on (*S*)-naproxen [87, 126], and CDRs having L-amino acids as chiral auxiliary in cyanuric chloride [16], DFDNB [14, 15] and isothiocyanate [18, 20].

The newly synthesized CDRs were found to provide better *Rs* (2.91–4.71) in comparison with the resolution reported in the literature [14, 16, 18-21]; for example, *Rs* was 2.34 and 3.23 using (*R*)-MBIC and (*S*)-NEIC as CDRs, respectively [125]. The retention times were greatly reduced (2–5 min, at a flow rate of 1 mL/min) for the diastereomeric derivatives synthesized with CDR-1 and CDR-2 (in the present report) and there occurred 15–20 times less consumption of organic solvents as compared with those reported in the literature cited above.

The efficiency of Lfx CDRs for diastereomeric separation and enantioselectivity was found to be better, since low retention times, high *Rs* and low LOD were observed for the diastereomeric derivatives in comparison to the resolution reported in the literature. In addition, new CDRs were found to be more stable (>5 months at 2–5 °C) as compared with CDRs based on DFDNB [14, 15] and isothiocyanate [18-20].

Table-4.A2. Method validation for HPLC separation of diastereomeric derivatives of (*RS*)-Prl with CDR-2: Linearity.

Linearity	**First eluting diastereomeric derivative (DsA-1)**	**Second eluting diastereomeric derivative (DsA-2)**
Range (ng mL^{-1})	200-1000	200-1000
Slope	4.875	4.792
Intercept	644	894.2
Determination Coefficient (r^2)	0.999	0.999
SD intercept	2.64	3.09
SE intercept	1.18	1.38
SE slope (m)	0.026	0.019
LOD	1.790	2.132
LOQ	5.425	6.460

Table-4.A3. Method validation for HPLC separation of diastereomeric derivatives of (*RS*)-Prl prepared with CDR-2: accuracy and precision.

Actual conc. of diastereomeric mixture (ng mL^{-1})	**Conc. of each diastereomers (ng mL^{-1})**	**First eluting diastereomeric derivative**				**Second eluting diastereomeric derivative**			
		Found conc. Mean± (t*SD/√n)	**Mean recovery (%)**	**Confidence interval (95%)**	**RSD (%)**	**Found conc. Mean± (t*SD/√n)**	**Mean recovery (%)**	**Confidence interval (95%)**	**RSD (%)**
Intra-day precision									
200	100	99.69 ± 1.17	98.84	98.51-99.69	1.31	100.33 ± 3.45	98.89	96.65-102.75	1.69
400	200	200.92 ± 3.86	99.31	98.25-100.46	1.24	201.54 ± 5.67	100.43	98.18-102.66	1.84
600	300	300.90 ± 5.55	99.51	96.50-100.31	1.71	297.95 ± 5.79	100.14	98.53-102.95	1.36
800	400	395.90 ± 5.76	98.99	98.55-101.37	1.45	398.12 ± 8.41	98.96	97.05-101.92	1.52
1000	500	501.74 ± 8.86	99.87	97.86-102.75	1.68	502.04 ± 9.72	99.52	97.14-101.86	1.98
		Mean	99.31	97.94-100.92	1.48		99.55	97.51-102.42	1.68
Inter-day precision									
200	100	97.64 ± 2.21	97.64	97.69-102.35	1.46	99.86 ± 3.54	99.86	98.61-100.91	1.55
400	200	200.10 ± 5.77	100.05	97.65-102.33	1.35	198.68 ± 3.49	99.34	97.49-100.15	1.46
600	300	298.85 ± 4.42	99.61	98.70-100.11	1.43	293.55 ± 3.74	97.85	98.15-101.73	1.33
800	400	394.85 ± 8.32	98.71	97.78-100.17	1.95	398.72 ± 9.30	99.68	97.83-101.40	1.56
1000	500	495.58 ± 11.4	99.11	98.53-101.19	1.64	485.93 ± 10.67	99.12	97.18-101.06	1.83
		Mean	99.02	98.07-101.23	1.56		99.17	97.85-101.05	1.54

't' represent student 't' value (at 0.05 selected drgree of probability), n (=5) is the number of replicates, SD = standard deviation, RSD = relative standard deviation

Table-4.A4. Comparison of derivatization conditions, resolution, separation factor and elution time for diastereomeric derivatives of (*RS*)-Prl prepared with different CDRs (as reported in the literature) with the diastereomeric derivatives prepared with CDR-2 [having (S)-Lfx as chiral moiety] synthesized for the present studies.

CDR	Detection (λ_{max})	Derivatization conditions	Resolution (*Rs*)	Separation factor (α)	Elution time (min)	reference
1-Fluoro-2,4-dinitrobenzene-D-phenylglyacinamide (FDNP-D-Phg-NH_2)	UV, 357nm	50 s at 200W using microwave irradiation	2.65	1.04	24.61 and 25.61	14
1-Fluoro-2,4-dinitrobenzene-L-valine (FDNP-L-Val)	UV, 357nm	50 s at 200W using microwave irradiation	2.33	1.03	29.17 & 30.05	14
1-Fluoro-2,4-dinitrobenzene-L-Leucine (FDNP-L-Leu)	UV, 357nm	50 s at 200W using microwave irradiation	3.05	1.04	32.87 & 34.12	14
Dinitrophenyl-L-Pro-*N*-hydroxysuccinimide ester	UV, 231nm	3 min at RT using vortex	3.07	1.07	41.03 & 43.87	14
N-(4-Chloro-6-methoxy-[1,3,5]triazine-2-yl]-L-Ala-NH_2	UV, 230 nm	60 s at 680W using microwave irradiation	3.51	1.06	40.01 & 44.25	16
(1*S*,2*S*)-1,3-diacetoxyl-1-(4-nitrophenyl)-2-propyl isothiocyanate [(*S*,*S*)-DANI]	UV, 245 nm	3h at RT	2.32	1.34	11.5 & 14.8	18
(1*S*,2*R*)-1-acetoxy-1-phenyl-2-propyl isothiocyanate [(*S*,*R*)-APPI]	UV, 250 nm	3h at RT	4.22	1.72	10.5 & 15.4	18
N-3,5-dinitrophenzoyl-trans-1,2-diamino-cyclohexane isothiocyanate (DDITC)	UV, 245 nm	2h at 60° C	6.67	1.44	NA	20
2-(6-Methoxy-2-naphthyl)-1-propyl chloroformate (NAP-C)	FL, λ_{max}= 270 nm; λ_{max} =335 nm	0.5h at RT	1.8	1.09	26.8 & 29.6	21
1,3-diacetoxyl-1-(4-nitrophenyl)-2-propyl isothiocyanate [DANI]	UV, 245 nm	3h at RT	2.33	1.22	16.5 & 19.5	19
N-benzotriazolyl-(*S*)-9-fluoro-3-methyl-10-(4-methylpiperazin-1-yl)-7-oxo-3,7-dihydro-2H-[1,4]oxazino[2,3,4-ij]quinoline-6-carboxylate (CDR-2)	UV, 294 nm	3 min at 800W using microwave irradiation	4.17	1.22	2.70 & 3.07	Present study

RT, Room temperature; MWI, microwave irradiation.

B. Preparative enantioseparation of (*RS*)-metoprolol and (*RS*)-atenolol

1. CDRs and diastereomeric derivatives

The explanation for synthesis of CDRs (1 and 2) and diastereomeric derivatives of (*RS*)-Mel and (*RS*)-Atl was same as described in Chapter-4A. Synthesis of Lfx based CDRs (1 and 2) are shown in Fig. 3.1. Fig. 3.5 and Fig. 3.6 respectively shows scheme for synthesis of diastereomeric derivatives of (*RS*)-Mel and (*RS*)-Atl with CDR-1 and CDR-2. Since the succinimidyl and benzotriazole moieties are removed by the amino group of the analyte during synthesis of diasteromeric derivatives, the structures of the diastereomeric derivatives obtained by using either CDR-1 or CDR-2 remain the same (for (*RS*)-Mel, for example, and are designated as **DsA-3** and **DsA-4**). The diastereomeric derivatives of (*RS*)-Atl are designated as **DsA-5** and **DsA-6.**

The CDRs and isolated diastereomeric derivatives were characterized by using spectroscopic technique like ^{1}H-NMR, UV-visible, IR, elemental analysis and HRMS.

2. Separation of diastereomeric derivatives by RP-HPLC

The detail explanation for RP-HPLC separation of diastereomeric derivatives of (*RS*)-Mel and (*RS*)-Atl were same as described for diastereomeric derivatives (*RS*)-Prl in **Chapter-4A**. Mobile phase (A) consisting of MeCN–TEAP (pH 3.5; gradient, 30–70% MeCN) in 30 min, at a flow rate of 1 mL/min was found successful in terms of selectivity and reproducibility; values for retention factor (k), separation factor (α) and resolution (Rs) under the optimized HPLC conditions are given in Table-4.B1 for the diastereomeric derivatives of (*RS*)-Mel and (*RS*)-Atl prepared with CDR-1 and -2. Sections of chromatograms showing separation of diastereomeric derivatives of (*RS*)-Mel and (*RS*)-Atl prepared with CDR-1 and -2 are shown in Fig. 4.B1 and Fig. 4.B2, respectively.

3. Structures of diastereomeric derivatives

The diastereomeric derivatives of (*RS*)-Mel (Fig. 3.5) were designated as DsA-3 or (*S,S*)- and DsA-4 or (*S,R*)-diastereomeric derivatives, respectively, where the first (*S*) corresponds to the configuration of chiral auxiliary and the other (*S*)/(*R*) corresponds to that of (*S*)-Mel

or (*R*)-Mel. Similarly the diastereomeric derivatives of (*RS*)-Atl (Fig. 3.6) were designated as DsA-5 or (*S*,*S*)- and DsA-6 or (*S*,*R*)-diastereomeric derivatives.

DsA-3 and DsA-4 are representing the first eluted diastereomeric derivatives of (*RS*)-Mel and DsA-5 and DsA-6 are representing the first eluted diastereomeric derivatives of (*RS*)-Atl. The characterization data of isolated diastereomeric derivatives of (*RS*)-Mel and (*RS*)-Atl is given in **Chapter-3**.

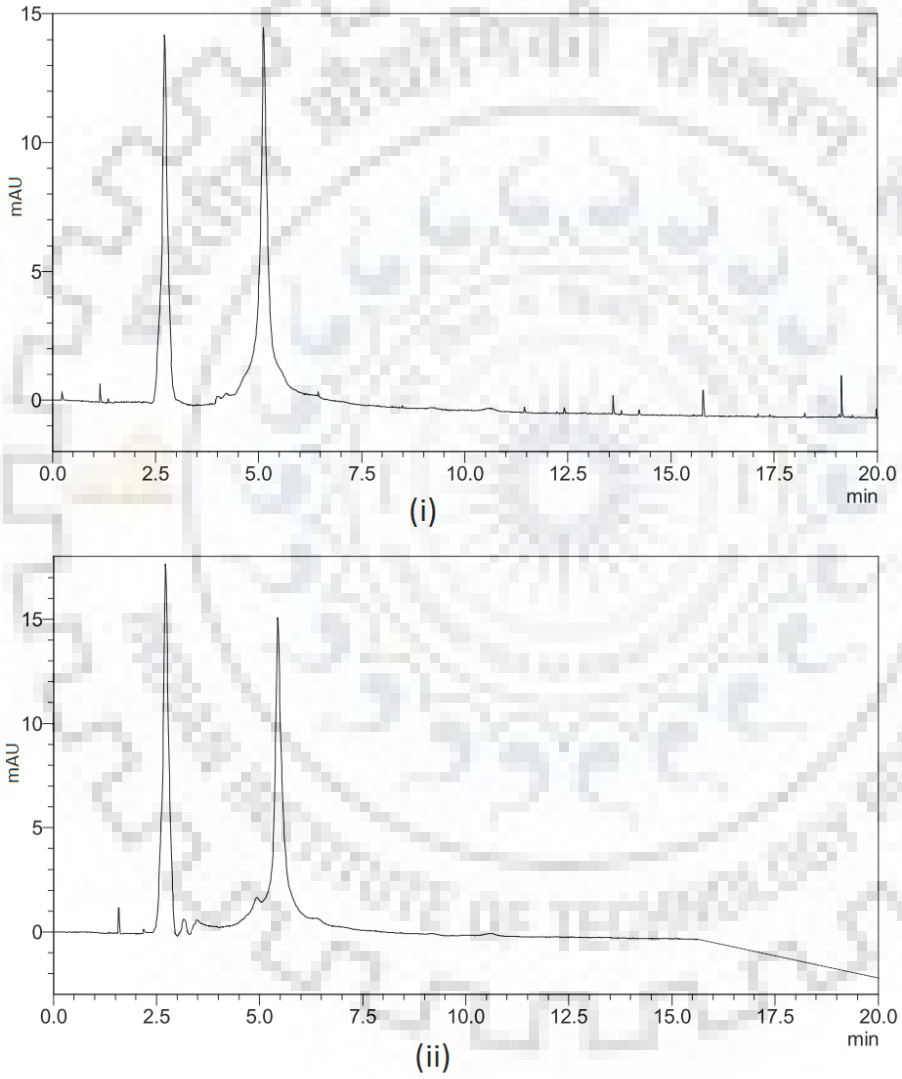


Fig. 4.B1. (i) and (ii) separation of diastereomeric derivatives of (*RS*)-Mel prepared with respectively, CDR-1 and CDR-2. Chromatographic conditions: LiChrospher C_{18} column (L x I.D. 25 cm × 4.6 mm, 5 µm particle size); Mobile phase, MeCN-TEAP (pH 3.5), gradient, 30% MeCN to 70% MeCN in 30 min, at a flow rate of 1 mL min^{-1}.

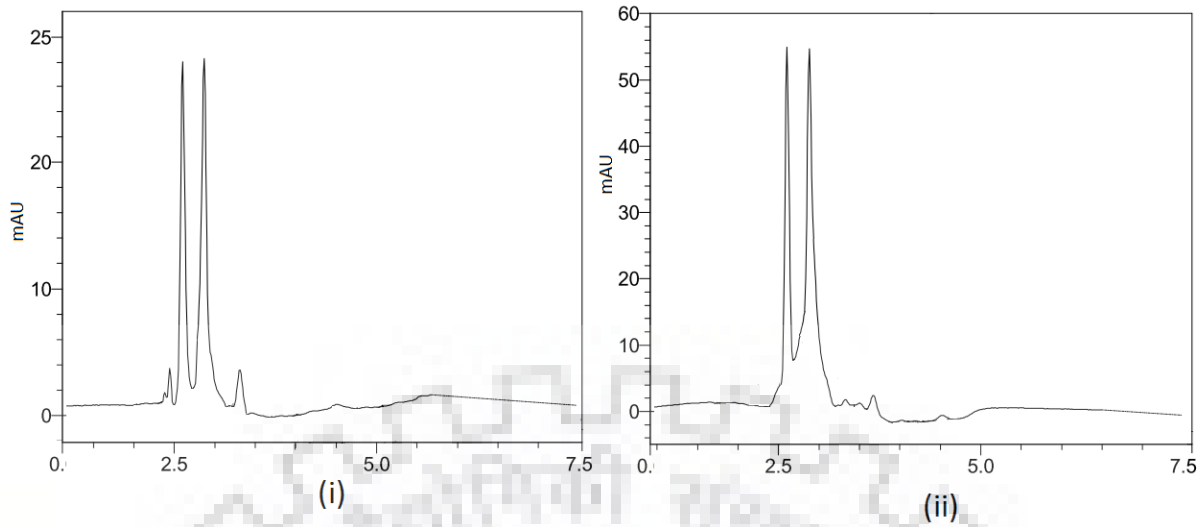


Fig. 4.B2. (i) and (ii) separation of diastereomeric derivatives of (*RS*)-Atl prepared with respectively, CDR-1 and CDR-2. Chromatographic conditions: LiChrospher C_{18} column (L x I.D. 25 cm × 4.6 mm, 5 µm particle size); Mobile phase, MeCN-TEAP (pH 3.5), gradient, 30% MeCN to 70% MeCN in 30 min, at a flow rate of 1 mL min^{-1}.

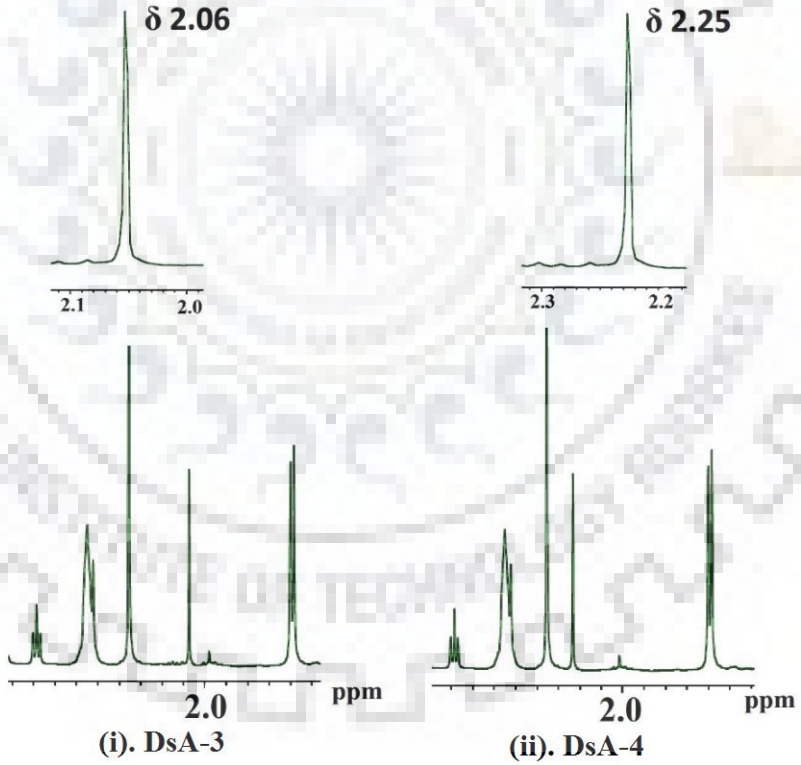


Fig. 4.B3. ^{1}H-NMR spectra represent difference in chemical shift of peaks of –OH of diastereomeric derivatives DsA-3 and DsA-4. [Sections of ^{1}H-NMR spectra of diastereomeric derivatives illustrating chemical shift value for the peaks of –OH (at stereogenic center), signal at δ 2.06 in DsA-3 (i), and at δ 2.25 in DsA-4 (ii). This difference in chemical shift of –OH signal is attributed to the formation of H-bonding in DsA-2.]

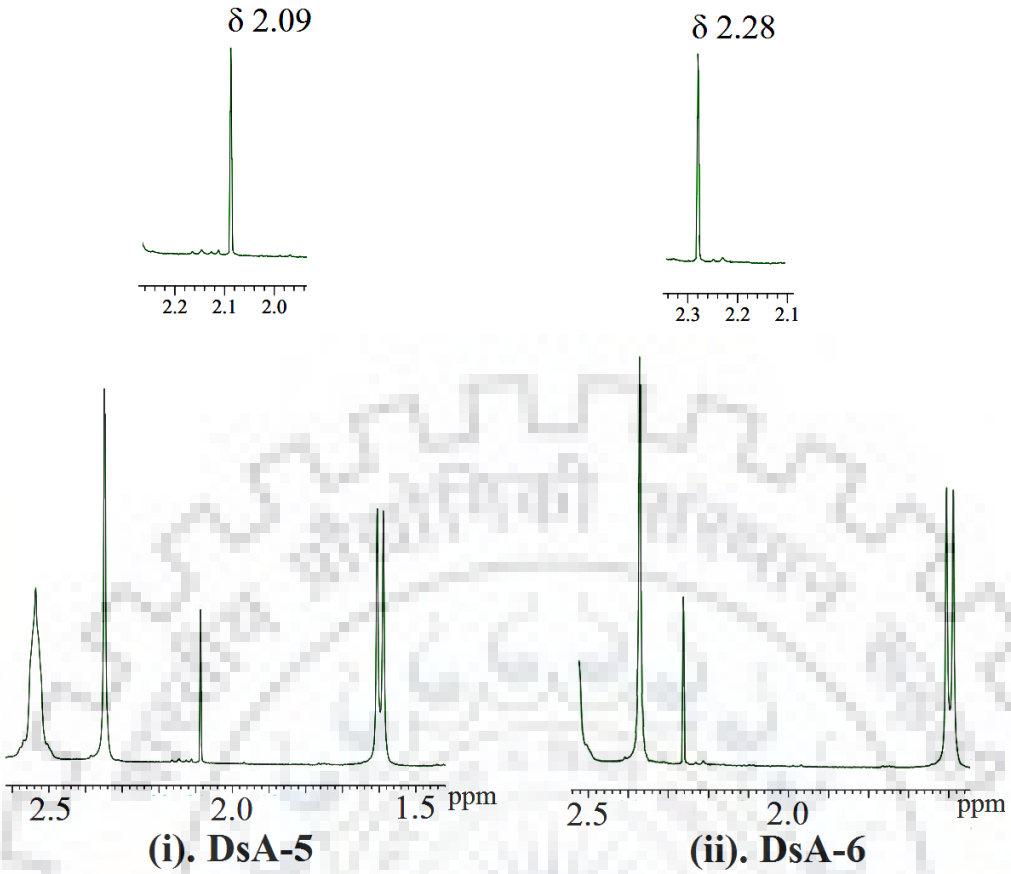


Fig. 4.B4. ^{1}H-NMR spectra represent difference in chemical shift of peaks of –OH of diastereomeric derivatives DsA-5 and DsA-6. [Sections of ^{1}H-NMR spectra of diastereomeric derivatives illustrating chemical shift value for the peaks of –OH (at stereogenic center), signal at δ 2.09 in DsA-5 (i), and at δ 2.28 in DsA-6 (ii). This difference in chemical shift of –OH signal is attributed to the formation of H-bonding in DsB-2.]

NMR spectra and lowest energy optimized structures of the diastereomeric derivatives of (*RS*)-Mel and (*RS*)-Atl: The explanation for NMR spectra and lowest energy optimized structures of diastereomeric derivatives of (*RS*)-Mel and (*RS*)-Atl were same as described in Chpater-4A. ^{1}H-NMR spectra of the diastereomeric derivatives of (*RS*)-Mel and (*RS*)-Atl were given in, respectively, Fig. 4.B3 and Fig. 4.B4.

The lowest energy optimized structures of the diastereomeric derivatives of (*RS*)-Mel and (*RS*)-Atl were developed, using the Gaussian 09 Rev. A.02 program and hybrid density functional B3LYP with 6-31G* basis set (based on density functional theory), and are shown in Fig. 4.B5 and Fig. 4.B6.

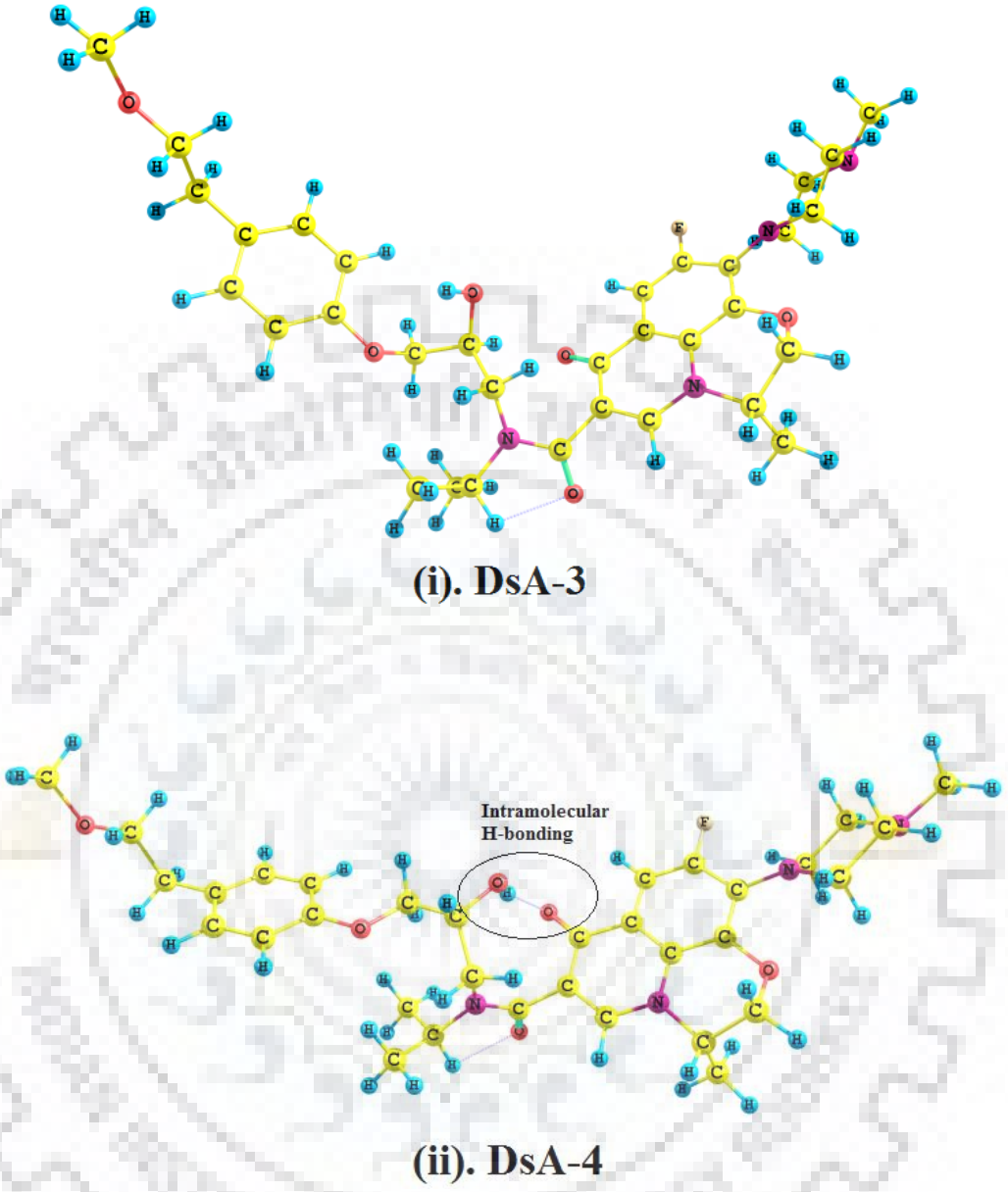


Fig. 4.B5. Lowest energy optimized structures of diastereomeric derivatives of (*RS*)-Mel developed using the program Gaussian 09 Rev. A.02 and hybrid density function B3LYP with 6-31G*; (ii) showing intramolecular H-bond between the –OH proton (of the Mel moiety) and the carbonyl oxygen (of the Lfx moiety), the (*S,R*)-diastereomeric derivative (DsA-2), and, (i) showing no intramolecular H-bonding between the –OH proton and the carbonyl oxygen, the (*S,S*)-diastereomeric derivative (DsA-1).

4. Separation mechanism and elution order

It can be observed (**Fig. 4.B5**) that in (*S*,*R*)-diastereomeric derivative (**DsA-2)** the bulky groups like phenyl (of Mel) and piperazinyl (of Lfx) are present on the same side of the planar amide bond (*cis*-, type orientation) and this planarity further supports the formation of intramolecular H-bond between the –OH proton (in the Mel moiety) and the carbonyl oxygen (of the Lfx moiety). On the other hand, in (*S*,*S*)-diastereomeric derivative (**DsA-1)**, these groups are spatially oriented on the opposite side (*i.e*., *trans* geometry) and there is no intramolecular H-bonding. These differences (in terms of steric arrangement and the H-bond) cause difference in hydrophobicity which would be expected to result into differential interaction of the diastereomeric derivatives with the C_{18} material of the column. The diastereomeric derivative with *cis* orientation is considered to have a stronger interaction with the C_{18} column material; these stronger or weaker interactions lead to different retention times of the two diastereomeric derivatives and their separation. Therefore, the (*S*,*R*)-diastereomeric derivative (**DsA-2**) having *cis*-type orientation would have larger retention time as compared to (*S*,*S*)-diastereomeric derivative (**DsA-1**), having the *trans*-type arrangement. Similarly **DsB-2** (*S*,*R*-diastereomeric derivative of Atl) having *cis*-type orientation would have larger retention time as compared to (*S*,*S*)-diastereomeric derivative (**DsB-1**), having the *trans*-type arrangement.

The *three point rule* [7] proposed for resolution of enantiomers considers H-bond as one of the important factors along with π–π interactions and steric repulsions, between the CSP and one of the enantiomeric forms to distinguish between the two enantiomeric forms. But in the present case, the stationary phase is achiral and the (*S*)-Lfx moiety being chiral is responsible for the formation of diastereomeric derivatives (for example from **CDR-1**) and the differential interaction of the diastereomeric derivatives with the ODS causes separation.

5. Method validation

Validation was performed for diastereomeric derivatives of (*RS*)-Mel prepared with CDR-1 as described in **Chapter-4A**. LOD of the order of 1.85 ng mL^{-1} and 2.22 ng mL^{-1}, and LOQ of the order of 5.62 ng mL^{-1} and 6.75 ng mL^{-1}, are obtained for the diastereomeric derivatives of (*S*)-, and (*R*)-Mel, respectively. Validation data is given in **Table-4.B2**.

Table-4.B1. Chromatographic separation data of diastereomeric derivatives prepared with CDR-1 and CDR-2

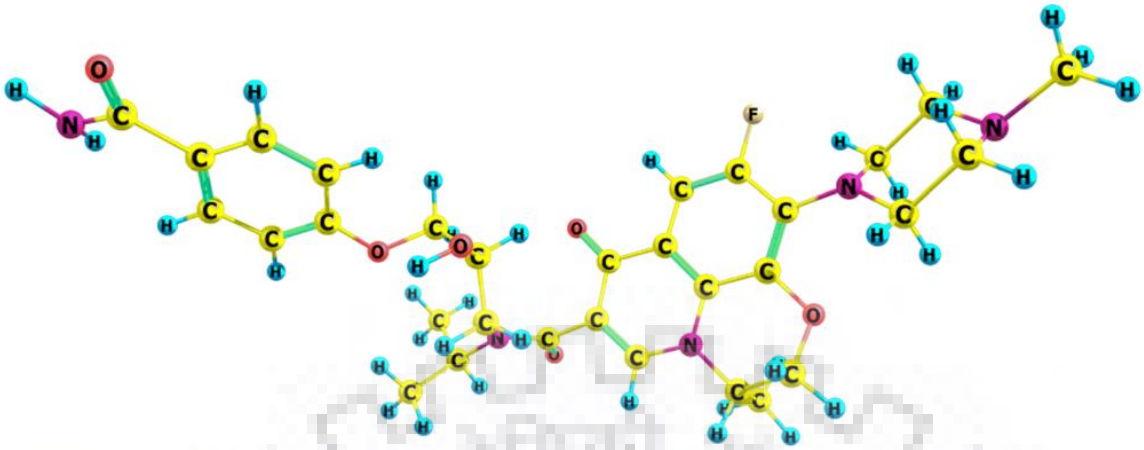
CDR	**β-adrenolytics**	**Separation data for diastereomeric derivatives of (*RS*)-Mel and (*RS*)-Atl prepared with CDR-1 and CDR-2**				
		k_1 for [(*S*,*S*)]-diastereomeric derivative	k_2 for [(*S*,*R*)]-diastereomeric derivative	Mean, *Rs*	Confidence interval (95%)	α
CDR-1	(*RS*)-Mel	1.712	4.438	9.09	1632-4.582	2.592
CDR-2	(*RS*)-Mel	1.719	4.116	7.99	1.651-4.531	2.395
CDR-1	(*RS*)-Atl	1.511	2.021	4.10	1.355-2.291	1.338
CDR-2	(*RS*)-Atl	1.596	1.963	3.41	1.392-2.251	1.230

Chromatographic conditions: column, LiChrospher C_{18} (250 mm × 4.6 mm I.D., 5 µm particle size); mobile phase: MeCN–TEAP (10 mM, pH 3.5) in the gradient of 30% MeCN to 70% MeCN; flow rate, 1.0 mL min^{-1}; k_1 and k_2 = retention factors; α = separation factor; *Rs* = resolution.

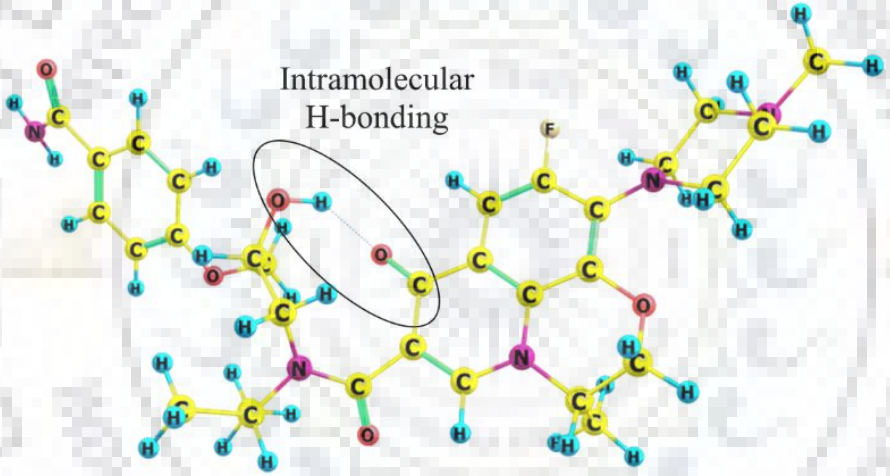
6. Comparison with competing chiral derivatizing agents and applications

The newly synthesized CDRs were found to provide better resolution (3.41-9.09) in comparison to the resolution reported in the literature (**Table-4.B3**). The retention times were greatly reduced (2-6 min, at a flow rate of 1 mL min^{-1}) for the diastereomeric derivatives synthesized with CDR-1 and CDR-2 as compared to those reported in literature. Thus, the developed method reduced significantly (approximately 10-15 times) the consumption of organic mobile phase.

The efficiency of CDR-1 and CDR-2 for diastereomeric separation and enantioselectivity was found to be better in terms of low retention times (2-6 min), high *Rs* (3.14-9.09) and low LOD (1.856 ng mL^{-1} and 2.228 ng mL^{-1}) in comparison to those reported in the literature (Table-4.B3); new CDRs were found to be more stable (5 month at 2-5 ºC) as compared to CDRs based on DFDNB [14], and naproxen [14, 18], and isothiocyanate [19-21]. Also, the separation factor (α, 2.59 and 2.39) for the diastereomeric derivatives prepared with the CDR-1 and CDR-2 were found to be better than the diastereomeric derivatives prepared with reported CDRs (Table-4.B1).



(i). DsA-5



(ii). DsA-6

Fig. 4.B6. Lowest energy optimized structures of diastereomeric derivatives of (*RS*)-Atl developed using the program Gaussian 09 Rev. A.02 and hybrid density function B3LYP with 6-31G*; (ii) showing intramolecular H-bond between the –OH proton (of the Atl moiety) and the carbonyl oxygen (of the Lfx moiety), the (*S*,*R*)-diastereomeric derivative (DsA-5), and, (i) showing no intramolecular H-bonding between the –OH proton and the carbonyl oxygen, the (*S*,*S*)-diastereomeric derivative (DsA-6).

Table-4.B2. Method validation for HPLC separation of diastereomeric derivatives of (*RS*)-Mel prepared with CDR-1

Linearity	**First eluting diastereomeric derivative**	**Second eluting diastereomeric derivative**
Range (ng ml^{-1})	200-1000	200-1000
Slope	4.898	4.943
Intercept	41.2	33.2
Correlation coefficient (R^2)	0.999	0.999
SD intercept	2.74	3.33

Accuracy and precision

Actual conc. Of diastereomeric mixture (ng mL^{-1})	**Conc. Of each diastereomers (ng mL^{-1})**	**First eluting diastereomeric derivative**				**Second eluting diastereomeric derivative**			
		Found conc. Mean± (t*SD/√n)	**Mean recovery (%)**	**Confidence interval (%)**	**RSD (%)**	**Found conc. Mean± (t*SD/√n)**	**Mean recovery (%)**	**Confidence interval (%)**	**RSD (%)**
Intra-day precision									
200	100	99.79 ± 1.11	98.89	97.21-101.40	1.76	100.40 ± 1.04	98.95	97.84-101.10	1.22
400	200	199.83 ± 1.54	99.22	98.25-100.01	0.89	199.42 ± 1.56	99.22	97.95-99.92	0.94
600	300	302.90 ± 2.55	99.15	96.76-100.70	1.71	295.38 ± 2.20	99.40	98.66-100.83	1.01
800	400	395.42 ± 3.69	99.08	97.55-99.80	1.04	397.06 ± 3.37	98.15	98.90-101.30	1.51
1000	500	502.01 ± 4.12	99.17	98.48-99.46	0.91	504.64 ± 4.72	99.36	98.12-102.18	1.98
		Mean	99.10	97.65-100.27	1.26		99.01	98.29-101.06	1.31
Inter-day precision									
200	100	99.59 ± 1.17	98.89	97.11-101.25	1.89	100.50 ± 3.45	99.06	97.51-101.54	1.77
400	200	200.85 ± 1.3	99.34	97.76-102.35	1.92	198.62 ± 4.39	98.56	96.61-100.12	1.54
600	300	301.10 ± 4.12	98.59	96.83-100.16	1.57	296.74 ± 3.45	99.21	97.23-101.82	1.65
800	400	396.84 ± 4.78	98.81	97.94-99.68	1.64	397.49 ± 8.98	98.54	97.03-100.15	1.36
1000	500	499.59 ± 8.09	98.48	97.49-99.36	1.75	501.07 ± 9.76	98.64	97.12-100.02	1.71
		Mean	98.82	97.49-100.56	1.75		98.80	97.15-100.95	1.61

't' represent student 't' value (at 0.05 selected drgree of probability), n (=5) is the number of replicates, SD = standard deviation, RSD = relative standard deviation

Table-4.B3. Comparison of developed RP-HPLC method with competing

S.No	CDR	Detection (λ_{max})	Derivatization conditions #	Resolution (*Rs*) #	Separation factor (α) #	Elution time (min)	Reference
1.	Variants of Marfey's reagent	UV, 357nm	50 s at 200W using microwave irradiation (MWI)	2.00-9.90	1.02-1.13	48.08 and 53.03	14
2.	SINP	UV, 231nm	3 min at RT using vortex	7.78	1.38	35.72 and 38.72	14
3.	Dinitrophenyl-L-Pro-*N*-hydroxysuccinimide ester	UV, 357nm	3 min at RT using vortex	4.32	1.09	35.72 and 38.72	14
4.	Cyanuric chloride based	UV, 230 nm	60 s at 680W using MWI	2.96-8.79	1.06-1.08	36.50 and 39.14	16
5.	DDITC	UV, 245 nm	2h at 60° C	6.67	1.47	7.89 and 12.05	20
6.	GITC	UV, 245 nm	2h at 60° C	2.58	1.21	4.21 and 5.01	20
7.	NAP-C	FL, λ_{max}= 270 nm; λ_{max} =335 nm	0.5h at RT	1.0	1.07	N.M.	21
8.	NAP-IT	λ_{max} =230 nm	0.5h at RT	3.91	1.25	27.01 and 30.15	21
9.	DANI	UV, 245 nm	3h at RT	2.13	1.21	17.05 and 20.12	19
10.	GATC	UV, 276 nm	30 min at RT	5.94	1.25	12.5 and 14.5	32
11.	CDR-1	UV, 294 nm	3 min at 800W using MWI	9.09	2.59	2.71 and 5.44	Present study*

there are different values of *Rs*, α and elution times in the range mentioned since there were different CDRs

* Results described in the thesis

N.M. = Not mentioned
SINP = *N*-succinimidyl-(*S*)-2-(6-methoxynaphth-2-yl)propionate
DDITC = *N*-3,5-dinitrophenzoyl-trans-1,2-diamino-cyclohexane isothiocyanate
GITC = 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate
NAP-C = 2-(6-Methoxy-2-naphthyl)-1-propyl chloroformate
NAP-IT = 1-(6-methoxy-2-naphthyl)ethyl-isothiocyanate
DANI = (1*R*,2*R*)-1,3-diacetoxyl-1-(4-nitrophenyl)-2-propyl isothiocyanate
GATC = 2,3,4,6-tetra-O-acetyl-13-D-galactopyranosylisothiocyanate
CDR-1 = *N*-succinimidyl-(*S*)-9-fluoro-3-methyl-10-(4-methylpiperazin-1-yl)-7-oxo-3,7-dihydro-2H-[1,4]oxazino[2,3,4-ij]quinoline-6-carboxylate

C. Enantioseparation of (*RS*)-betaxolol, (*RS*)-carvedilol, (*RS*)-salbutamol and (*RS*)-bisoprolol using micellar liquid chromatography (MLC) with mobile phase without organic solvents

1. Micellar liquid chromatography (MLC): The RPLC in which the mobile phases are aqueous solutions of a surfactant at a concentration above the critical micellar concentration (CMC), is termed as micellar liquid chromatography (MLC). Surfactant based mobile phase are considered a green non-organic mobile phase because of their lower cost and toxicity and higher biodegradability with respect to hydro-organic mobile phases. Further, the diverse interactions of hydrophobic and electrostatic nature, occurring in the chromatographic column, help elution of compounds of different polarity.

Literature search showed that the mobile phase used for HPLC separation of diastereomeric derivatives (of all the β-adrenolytics prepared with different CDRs) do contain solvents like trifluoroacetic acid (TFA), acetonitrile, methanol, dichloromethane and chloroform. Direct HPLC enantioseparation of carbobenzyloxy derivatives of several amino compounds, including (*RS*)-propranolol and other β-adrenolytics, on polysaccharide and Pirkle-type chiral stationary phases [50] has also been achieved by using several such organic modifiers. A review by Agbaba and Ivković [28] on direct and indirect enantioseparation of β-adrenolytics by chiral TLC reveals the application of such solvents only. Agbaba and co-workers have discussed the effect of nonionic surfactant Brij 35 on solubility and acid-base equilibria of verapamil [141], and protolytic equilibria of sartans in micellar solutions of differently charged surfactants [142].

Ruiz-Angel *et al*. [143], used surfactants based solvents for HPLC analysis of seven tricyclic anti-depressants (TCAs) and two most hydrophobic β-adrenolytics (namely, propranolol and alprenolol); the main goal of the studies was to determine retention behavior, peak profiles, selectivity, and resolution capability of the column and the term ‘resolution’ was used to describe resolution of all peaks in a chromatographic separation while as per concepts of stereochemistry the term ‘resolution’ is used for separation of a racemic mixture into its enantiomers irrespective of the methodology adopted. The report by Ruiz-Angel *et al*. [143] and none of the literature reports cited therein have addressed the issue of enantioseparation by this approach of change in the nature of mobile phase.

The literature reports on enantioseparation of β-adrenolytics and the reports on use of surfactants based solvents for HPLC analysis, as mentioned above, showed a scope for investigating the application of simply aqueous mobile phase containing only surfactant(s) instead of mobile phase containing organic solvents and TFA for enantioseparation of certain β-adrenolytics on analytical scale.

For this purpose a strategy was worked out to prepare water micellar mobile phase by dissolving commonly available surfactants in water to be used for enantioseparation. This resulted into efficient method for enantioseparation and control of enantiomeric purity with a new pathway (to avoid organic solvents and strong acids) for use of water based mobile phase.

2. CDRs and diastereomeric derivatives

The structures of CDR-1 and CDR-2 are shown in Fig.3.1. The scheme for synthesis of representative diastereomeric derivatives of racemic Bxl; the chemical structures of diastereomeric derivatives of (*RS*)-Bxl, shown in Fig. 3.7, are represented to have (*S,R*)- and (*S,S*)-configurations, respectively; the first (*S*) corresponds to the configuration of chiral auxiliary [(*S*)-(−)-Lfx] and the other letter (*S*)/(*R*) corresponds to that of (*S*)-Bxl or (*R*)-Bxl. Since the succinimidyl and benzotriazole moieties are removed by the amino group of the analyte during synthesis of diasteromeric derivatives, the structures of the diastereomeric derivatives obtained by using either CDR-1 or CDR-2 remain the same (for (*RS*)-Bxl, for example, and are designated as Ds**A-7** and Ds**A-8**). The structures of the diastereomeric derivatives of (*RS*)-Cdl, (*RS*)-Sbl, and (*RS*)-Bpl are shown in Fig.3.8 (these are designated as Ds**A-9**, Ds**A-10** for Cdl; Ds**A-11**, Ds**A-12** for Sbl, and Ds**A-13**, Ds**A-14** for Bpl.

Explanation for reactions for synthesis of CDRs and diastereomeric derivatives: The activation of carboxylic group by substitution with strongly electron withdrawing group(s) rendered the carbon atom of the carbonyl group more electrophilic and more prone to nucleophilic attack by the amino group of the amine component (the β-adrenolytics under study) and the *auxiliary nucleophilic moieties* subsequently got substituted with amino group of the analytes (the β-adrenolytics under study). The presence of *auxiliary nucleophile* efficiently shortened the lifetime of the overactivated O-acyl-isourea intermediate and thus diminished the extent of O to N acyl-migration leading to *N*-acylureas. Since *N*-hydroxybenzotriazole is regenerated during acylation its concentration remains almost constant during coupling.

3. Separation of diastereomeric derivatives by RP-HPLC

Application of surfactants based solvents in liquid chromatography (LC) is termed as micellar liquid chromatography (MLC). Addition of simple surfactants in the mobile phase for enantioseparation on simple C_{18} columns is not the prevailing practice. Taking into account the literature reports on separation studies (not involving enantioseparation) on certain β-adrenolytic drugs [143, 144], a reference aqueous mobile phase was prepared containing 0.05 M SDS and 0.015 M Brij-35 in aqueous phase.

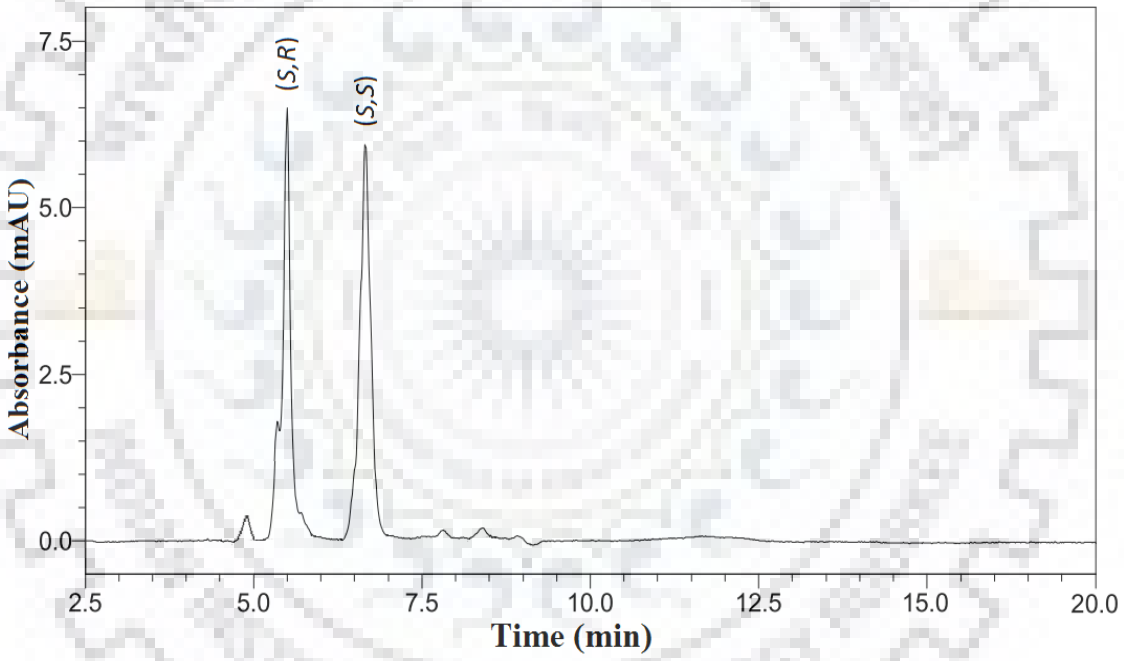


Fig. 4.C1. The chromatogram showing separation of diastereomeric derivatives of (*RS*)-Bxl (DsA-7 and DsA-8).

Mobile phase consisting of WMP-TEAP (pH 3.5, isocratic, 80:20) in 60 min, at a flow rate of 0.70 mL min^{-1} was found successful for separation of diastereomeric derivatives and in terms of selectivity and reproducibility; values for retention factor (k), separation factor (α) and resolution (Rs) under the optimized HPLC conditions are given in Table-4.C1 for the diastereomeric derivatives (of all the four analytes) prepared with CDR-1 and CDR-2. A

representative chromatogram showing separation of diastereomeric derivatives of (*RS*)-Bxl (DsA-7 and DsA-8) is given in Fig. 4.C1; Fig. 4.C2 shows peaks for the separation of the rest of the diastereomeric derivatives. The diastereomeric derivative corresponding to (*R*)-enantiomer eluted before the diastereomeric derivative of (*S*)-enantiomer, in each case.

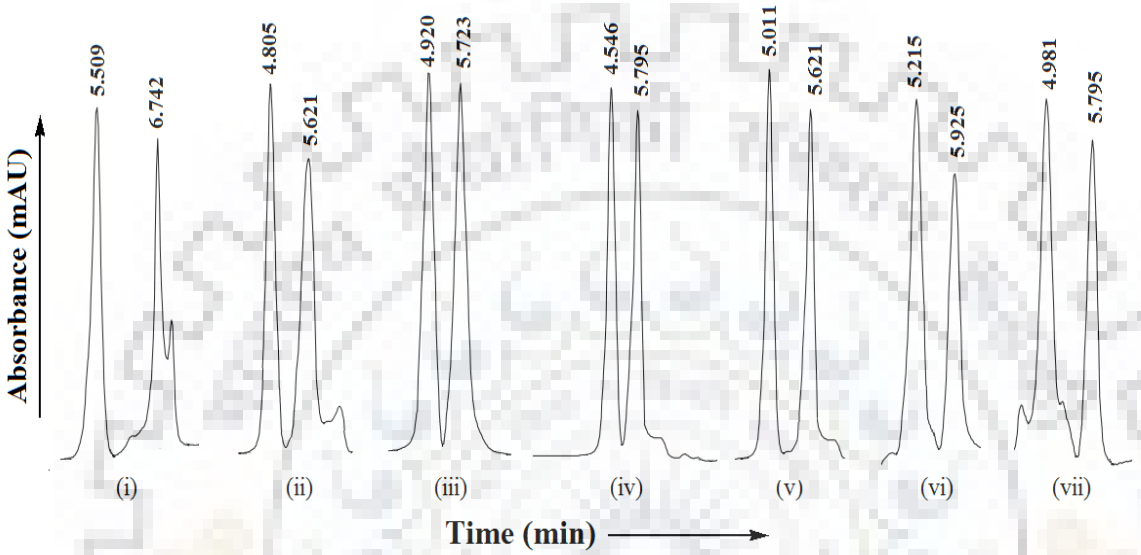


Fig. 4.C2. Sections of chromatograms showing RP-HPLC separation; (ii), (iv), and (vi): chromatograms showing separation of DsA-9 and DsA-10; DsA-11 and DsA-12, and DsA-13 and DsA-14 (the diastereomeric derivatives of (*RS*)-Cdl, (*RS*)-Sbl, and (*RS*)-Bpl, respectively, synthesized with CDR-1); (i), (iii), (v), and (vii) chromatograms showing separation of DsA-7 and DsA-8; DsA-9 and DsA-10; DsA-11 and DsA-12, and DsA-13 and DsA-14 (the diastereomeric derivatives of racemic (*RS*)-Bxl, (*RS*)-Cdl, (*RS*)-Sbl, and (*RS*)-Bpl, respectively, synthesized with CDR-2).

The addition of surfactant(s) causes adsorption of surfactant monomers on the stationary phase and leads to significant reduction in the amount of organic solvent (to be added in the mobile phase). The β-adrenolytic drugs belong to synthetic chiral hydroxyl amine-containing compounds with $pK_a \sim 9.2$; these compounds are basic and are positively charged at the working pH (~3) of the mobile phase. There has been an entirely different consideration and explanation for the observed chromatographic performance for addition of surfactants in the mobile phase.

Separation capacity increases with increasing concentration of SDS in constant-concentration solution of Brij-35 as well as with increasing concentration of Brij-35 in constant-concentration solution of SDS [143].

In the present studies, it was observed that by increasing concentration of SDS (0.005 M to 0.05 M) in the mobile phase containing a fixed concentration of Brij-35 at 0.015 M the retention time of analyte was decreased but peak height was increased. It was also observed that the retention time decreased on increasing the concentration of Brij-35 (0.0015 M to 0.015 M) in the mobile phase having a fixed concentration of SDS at 0.05 M. Thus, the mixed system provided good resolution with lower retention times in comparison to the mobile phase having single surfactant. Successive additions of the non-ionic surfactant gradually reduced the retention time, though to a smaller extent, in comparison to the addition of SDS to a mobile phase containing a fixed amount of Brij-35.

4. Water micellar (mixed SDS-Brij-35) mobile phase system

Literature reveals application of anionic surfactant SDS in MLC particularly in the pharmaceutical field [145-147]. The quantitative structure–activity relationship studies (QSARs) in RPLC suggested Brij-35as an ideal modifier due to its capability to mimic bio-partitioning processes [148, 149].

Micellar system shows a partitioning behaviour. There occur two association equilibria, (i) between solute and stationary phase, and (ii) between solute and micelle [144]. These describe the association of a solute in bulk water with the stationary phase binding sites, and with the surfactant monomers in the micelles dissolved in the mobile phase. This approach is commonly valid for ionic and non-ionic surfactants.

When an anionic (SDS) and non-ionic (Brij-35) surfactants are dissolved in water, they start to form micelles. Their tail part shows hydrophobic interactions and head groups show ion-dipole and hydrophilic interactions. Water micellar mobile phase system increases polarity of the stationary phase of the C_{18} column by modifying the surface of column material. SDS and Brij-35 lead to form micelles. ‘SDS formed micelles’ contain a dodecyl apolar core and ‘Brij-35 formed micelles’ are similar to those of SDS but have relatively polar surface due to oxyethylene chains in micelles.

Table-4.C1. Chromatographic separation data for diastereomeric derivatives prepared with CDR-1 and CDR-2.

The analyte	Separation data for diastereomeric derivatives prepared with CDR-1				Separation data for diastereomeric derivatives prepared with CDR-2			
	k_1	k_2	α	Rs	k_1	k_2	α	Rs
(*RS*)-Bxl	1.945	2.789	1.433	5.40	2.061	2.745	1.332	6.15
(*RS*)-Cdl	1.402	1.811	1.291	4.11	1.463	1.869	1.273	4.25
(*RS*)-Sbl	1.164	1.529	1.314	4.65	1.947	2.306	1.186	3.45
(*RS*)-Bpl	1.732	2.065	1.194	3.12	1.762	2.219	1.259	4.47

Table-4.C2. Literature on elution order of β-adrenolytics.

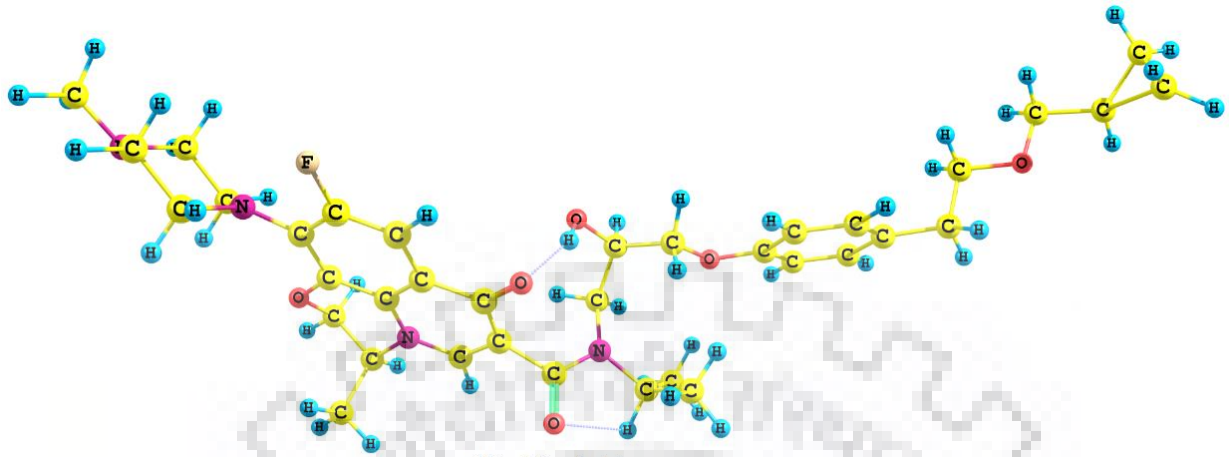
CDR/ impregnation	Approach	β-adrenolytics	First eluting diastereomeric derivatives	Reference
1,5-difluoro-2,4-dinitrobenzene based chiral reagents	Indirect	(*RS*)-Betaxolol (*RS*)-Orciprenaline (*RS*)-Propranolol	Diastereomeric derivatives of (*R*)-β-adrenolytics	[15]
Ligand exchange chromatography	Direct	(*RS*)-Atenolol, (*RS*)-Propranolol (*RS*)-Salbutamol	(*R*)-enantiomer of β-adrenolytics	[37]
Naproxen and dinitro phenyl benzene based chiral reagents	Indirect	(*RS*)-Atenolol	Diastereomeric derivatives of (*R*)- atenolol	[55]
(*S*)-levofloxacin-based reagent (CDR-1 and CDR-2)	Indirect	(*RS*)-Propranolol	Diastereomeric derivatives of (*R*)-Propranolol	[16]
(*S*)-ketoprofen-based reagent (CDR-3 and CDR-4)	Indirect	(*RS*)-Atenolol, (*RS*)-Propranolol (*RS*)-metoprolol	Diastereomeric derivatives of (*R*)- β-adrenolytics	[14]

There occurs 'on surface' interaction between C_{18} material and carbon chain of Brij-35. The polyoxyethylene carbon chain of Brij-35 increases polarity of the stationary phase as it is significantly more polar (in comparison to C_{18} material). Similarly, SDS present on the surface of column material increases polarity of the stationary phase because of the orientation of its negatively charged sulphate groups. Such interactions reduce the retention times of the analyzed compounds and also indicate that there are no specific interactions such as hydrogen bonding between the hydroxyl groups of the adsorbed surfactant and the phenolic group of the analytes [50].

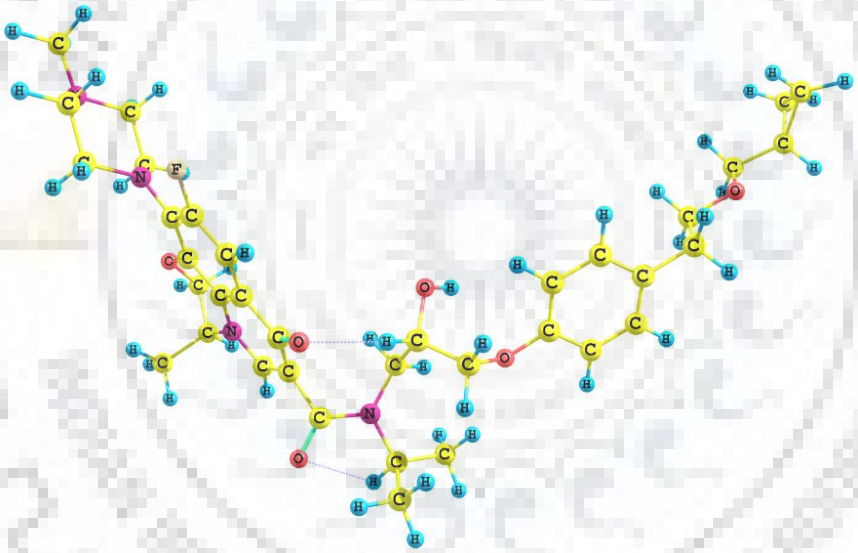
There has been no literature report on the use of mixed surfactants systems for enantioseparation. Surfactant in the mobile phase decreases surface tension and viscosity of the mobile phase. The formation of micelles of diastereomeric derivatives (i.e., during the enantioseparation) is considered responsible for good chromatographic separation along with faster elution. The presence of these surfactants does not interfere with detection as both have low molar absorptivity as compared to (*S*)-(–)-Lfx. The UV spectrum corresponding to first- and second-eluting diastereomeric derivative was captured (using PDA detector) at 5.709 and 6.815 min, respectively; these were found to be identical. The Table-4.C1 clearly shows a very good separation of all the diastereomeric pairs without any organic modifier in the mobile phase. Therefore, the use of organic modifiers in the surfactants systems is not recommended.

5. Optimized lowest energy structures and elution order of the diastereomeric derivatives

Geometry optimized 'lowest energy' structures of diastereomeric derivatives of (*RS*)-Bxl (as a representative) were developed using the program Gaussian 09 Rev. A.02 and hybrid density functional B3LYP with 6-31G* basis set. These are given in **Fig. 4.C3.** The application of the said software is not intended for theoretical calculation of the energies of the different conformations (or the configurations) of each of the isomers which would otherwise require use of two dihedral angles, as variables of the potential energy surface, and other parameters. The default convergence criteria are implemented in Gaussian software itself; the optimization continues simply on submitting the data. There is no consideration of torsional energy (or rotational energy barrier) to decide the relative stability of conformations. The diastereomeric derivatives under question are quite stable species and the configuration is not changing by rotation at any stage (i.e., these are not 'atropisomers').



(i). DsA-7



(ii). DsA-8

Fig. 4.C3. Geometry optimized 'lowest energy' structures of diastereomeric derivatives of (*RS*)-Bxl (as a representative) developed using the software program Gaussian 09 Rev. A.02 and hybrid density functional B3LYP with 6-31G* basis set; (i) the (*S*,*R*)-diastereomeric derivative "DsA-7" and (ii) the (*S*,*S*)-diastereomeric derivative "DsA-8".

Table-4.C3. Method validation for HPLC separation of diastereomeric derivatives of (*RS*)-Bxl prepared with CDR-1.

Linearity		**First eluting diastereomeric derivative (DsA-7)**			**Second eluting diastereomeric derivative (DsA-8)**		
Range (ng mL^{-1})		20-2000			20-2000		
Slope		6.042			5.928		
Intercept		13.65			46.14		
Correlation Coefficient (R^2)		0.999			0.999		
Accuracy and precision							
Actual conc. of Diastereomeric Mixture (ng mL^{-1})	**Conc. of each Diastereomer (ng mL^{-1})**	**First eluting diastereomeric derivative**			**Second eluting diastereomeric derivative**		
		Found conc. Mean ± SD (ng mL^{-1})	**Recovery (%)**	**RSD (%)**	**Found conc. Mean ± SD (ng mL^{-1})**	**Recovery (%)**	**RSD (%)**
Intra-day precision							
20	10	9.87 ± 0.97	98.71	0.22	9.95 ± 1.10	99.52	0.51
100	50	49.78 ± 2.15	99.55	0.89	50.30 ± 3.27	100.61	0.92
200	100	99.77 ± 4.95	99.77	1.08	100.19 ± 5.06	100.19	1.02
600	300	300.77 ± 5.37	100.25	1.26	303.79 ± 5.51	101.26	1.38
2000	1000	999.80 ± 7.72	99.98	3.52	1000.1 ± 5.81	100.01	2.85
		Mean	99.65	1.39		100.31	1.34
Inter-day precision							
20	10	99.81 ± 1.02	99.81	0.31	10.02 ± 1.24	100.19	0.28
100	50	49.16 ± 3.34	98.31	0.98	49.32 ± 2.73	98.62	1.02
200	100	99.05 ± 3.82	99.05	1.17	99.05 ± 3.12	99.05	1.37
600	300	302.15 ± 4.82	100.71	1.81	304.21 ± 4.86	101.40	1.93
2000	1000	999.63 ± 6.24	99.96	2.86	999.02 ± 5.49	99.91	3.12
		Mean	99.56	1.42		99.83	1.54
Sensitivity							
LOD (ng mL^{-1})	1.618			LOD (ng mL^{-1})	1.831		
LOQ (ng mL^{-1})	4.902			LOQ (ng mL^{-1})	5.549		
(n = 5), SD = standard deviation, RSD = relative standard deviation.							

As discussed elsewhere [150, 151] for diastereomeric derivatives of propranolol prepared with (*S*)-Lfx and (*S*)-Kpf based CDRs, these '*lowest energy structures*', show a spatial nearness, due to rigid amide bond between the carbonyl oxygen of the carboxyl group of Lfx and the N of the Bxl (**Fig. 3.7**), and there is a partial double bond character in the amide bond in the diastereomers due to delocalization of lone pair of N atom. This is

evidenced by calculating the C=N bond length from the DFT based software; it comes out as 1.37 A° while the normal C–N bond length (single bond) is known to be 1.47 A°. Such a double bond character gives a geometric configuration in which the benzyl (of Bxl) and piperazinyl (of Lfx) are spatially near to each other in the (*S*,*S*)-diastereomeric derivative (DsA-8), [**Fig. 4.C3(ii**)] in comparison to the (*S*,*R*)-diastereomeric derivative [**Fig. 4.C3(i**)]. These spatial arrangements were verified by making the molecular models of the two geometries using '*orbital molecular building system*' (Cochranes of Oxford Ltd, Leafield, Oxford OX8 5NT, England).

As above given explanation on formation of rigid amide between the benzyl (of Bxl) and piperazinyl (of Lfx) in (*S*,*S*)-diastereomeric derivative (DsA-8); the two hydrophobic groups, thus, cause a relatively more compact situation and the (*S*,*S*)-diastereomeric derivative (Fig. 4.C3), interacts more strongly with hydrophobic ODS material of C_{18} column [87], resulting into higher retention time and its elution after DsA-7 with the mobile phase. Thus, it can be contended that the steric arrangement of the bulky groups at the stereogenic center of diastereomeric derivatives causes a difference in hydrophobicity of the two isomers which results in the differential interaction of the diastereomeric derivatives with the C_{18} material of the column and their separation. Similar studies of other β-adrenolytes show that in most of the cases diastereomeric derivatives of (*R*)-enantiomers elute before the diastereomeric derivative of (*S*)-enantiomers (Table-4.C2).

6. Stability of CDRs

Stability of CDRs was investigated as described in Chapter-4A. The CDRs (1 and 2) were found stable for up to 6 months.

7. Method validation

The method validation for accuracy and precision was carried out for diastereomeric derivatives of (*RS*)-Bxl prepared with CDR-1, as a representative, according to ICH guidelines [135] and the data is given in **Table-4.C3**. The accuracy and precision studies were carried out by replicate HPLC analysis (n = 5) of each of the solutions of a mixture of diastereomeric derivatives (containing 20, 100, 200, 600, and 2000 ng mL^{-1} of individual diastereomeric derivatives) of (*RS*)-Bxl prepared with CDR-1 (**Table-3.C3**). The calculated recovery values for the first and second eluting diastereomeric derivative, respectively, are 99.56 and 99.83% for intra-day assay and 99.65 and 100.31% for inter-day assay**.** The LOD

and LOQ were found, respectively, 1.618 ng mL^{-1} and 4.902 ng mL^{-1} for diastereomeric derivatives of (*RS*)-Bxl.

The resolution (*Rs*) values were found to be 5.40 and 6.15, and separation factor (α) was found to be 1.433 and 1.332, respectively for the first and second diastereomeric derivative (designated as DsA-7 and DsA-8). Linearity, slope and intercept (peak area *vs* concentration of diastereomers in ng mL^{-1}) were determined using regression equations, y = 6.042x + 13.65 (r^2 = 0.999) and y = 5.928x + 46.14 (r^2 = 0.999) for the first and second eluted diastereomeric derivative, respectively. The calculated RSD values for intra-day assay precision are 1.39% and 1.34% and those for inter-day assay precision are 1.42% and 1.54%, respectively for the first and second diastereomeric derivative.

8. Comparison of present work with literature reports

During this work, very good enantioseparation was achieved for racemic Bxl, Cdl, Sbl and Bpl. Literature search revealed few reports for enantioseparation of (*RS*)-Cdl and (*RS*)-Sbl both by direct as well as indirect approaches of enantioseparation. Though certain efforts mentioned in a few reports were not successful for separation of enantiomers of (*RS*)-Cdl and (*RS*)-Sbl [14, 16, 152, 153] and in a few cases enantiomers were resolved, but with a very low resolution [153-157]. The results presented herein clearly show a better enantioresolution of (*RS*)-Bpl and (*RS*)-Bxl as compared with previous literature [16, 157]. Also, CDRs based on (*S*)-(–)-Lfx, reported herein, were found to be more stable (>6 months at 2–5 °C) as compared to CDRs based on DFDNB [14, 15] and isothiocyanate [18-20]. In comparison to literature reports, the method was successful in providing very low LOD and LOQ values, better resolution [14, 16, 18-21, 125, 126]. Low retention times obtained in these studies resulted into lesser consumption of mobile phase as well. The separation factors (α) for the diastereomeric derivatives prepared with the CDR-1 and CDR-2 were found to be better than those for the diastereomeric derivatives prepared with CDRs based on (*S*)-naproxen [87, 126], and CDRs having L-amino acids as chiral auxiliary in cyanuric chloride [16], DFDNB [16, 87] and isothiocyanate [18, 20].

Chapter-5

Results and discussion for enantioseparation of diastereomeric derivatives of racemic β-adrenolytics using newly synthesized (*S*)-ketoprofen based CDRs (CDR-3 and CDR-4)

Diastereomeric derivatives of (*RS*)-Prl, (*RS*)-Mel and (*RS*)-Atl were synthesized with both CDR-3 and CDR-4 and their separation was achieved by RP-HPLC. The results and discussions for the same are provided below. In all of the three analytes only (*RS*)-Prl was used (as representative) for preparative separation of respective pairs of diastereomeric derivatives using open column chromatography. NMR spectra for the diastereomeric derivatives of (*RS*)-Prl, so obtained, were recorded and the absolute configuration for each was established which was supported by developing the lowest energy structures using the program Gaussian 09 Rev. A.02 and hybrid density function B3LYP with 6-31G*.

Diastereomeric derivatives of (*RS*)-Sbl, (*RS*)-Cdl, (*RS*)-Bpl and (*RS*)-Bxl were also synthesized with CDR-3 and CDR-4. The separation of these diastereomeric pairs was achieved using micellar liquid chromatography (MLC) wherein the mobile phase contained simple surfactants in the aq. phase and without any organic solvent.

The experimental details for the synthesis of CDRs and 14 pairs of diastereomeric derivatives are already described in **Chapter-3**.

A. Enantioseparation of (*RS*)-propranolol, (*RS*)-metoprolol and (*RS*)-atenolol and using CDR-3 and CDR-4

1. CDRs and diastereomeric derivatives

The explanation of synthesis of CDRs (3 and 4) and diastereomeric derivatives of (*RS*)-Prl, (*RS*)-Mel and (*RS*)-Atl was same as described in Chapter-4A, only (*S*)-Kpf was used instead of (*S*)-Lfx.

(*S*)-Kpf, being chiral, does not require any other chiral auxiliary for developing a CDR while synthesis of CDRs from DFDNB (providing DNP moiety as a chromophore) and *s*-trichloro triazine requires a chiral auxiliary to be introduced in these moieties.

The synthesis of CDR-3 and CDR-4 are shown in Fig. 3.2 and synthesis of representative diastereomeric derivatives of (*RS*)-Prl with CDR-3 and CDR-4 is shown in Fig. 3.9.

Table-5.A1. Chromatographic separation data of diastereomeric derivatives prepared with CDR-3 and 4.

CDR	**β-adrenolytics**	**Separation data for diastereomeric derivatives prepared with CDR-3 and -4**				
		k_1 **for [(*S*,*R*)]-, (DsB-1)**	k_2 **for [(*S*,S)]-, (DsB-2)**	***Mean, Rs***	***Confidence interval (95%)***	α
CDR-3	(*RS*)-Prl	3.740	4.561	4.54	4.154-4.582	1.219
CDR-3	(*RS*)-Mel	4.062	5.211	6.32	6.042-6.567	1.282
CDR-3	(*RS*)-Atl	2.192	4.214	8.96	8.264-9.124	1.922
CDR-4	(*RS*)-Prl	3.332	4.168	4.55	4.241-4.767	1.250
CDR-4	(*RS*)-Mel	4.106	5.021	5.81	5.334-5.972	1.223
CDR-4	(*RS*)-Atl	2.745	4.742	8.81	8.355-9.034	1.728

2. Separation of diastereomeric derivatives by RP-HPLC

The explanation for RP-HPLC separation of diastereomeric derivatives of (*RS*)-Prl, (*RS*)-Mel and (*RS*)-Atl were same as described in Chapter-4A. TEAP buffer (10 mM, pH 3.5) was used as one of the components of the mobile phase and MeCN or MeOH were used as organic modifiers [the mobile phases, (A) and (B)]. Mobile phase (A) with a gradient, 30% MeCN to 70% MeCN in 30 min, at a flow rate of 1 mL min^{-1} was found successful in terms of selectivity and reproducibility. Table-5.A1 shows values for retention factor (k), separation factor (α) and resolution (Rs) for the diastereomeric derivatives prepared with CDR-3 and CDR-4, under the optimized HPLC conditions. Base line resolution of pairs of diastereomeric derivatives of (*RS*)-Prl, (*RS*)-Mel, and (*RS*)-Atl (prepared with CDR-3 and CDR-4) are shown sections of chromatograms given in Fig. 5.A1. The UV spectrum corresponding to first and second eluting diastereomeric derivative was captured (using PDA detector); these were found to be identical. The open column chromatographic separation of diastereomeric derivatives of (*RS*)-Prl prepared with CDR-3 and CDR-4 and characterization data of first and second eluted diastereomeric derivatives is given in Chapter-3.

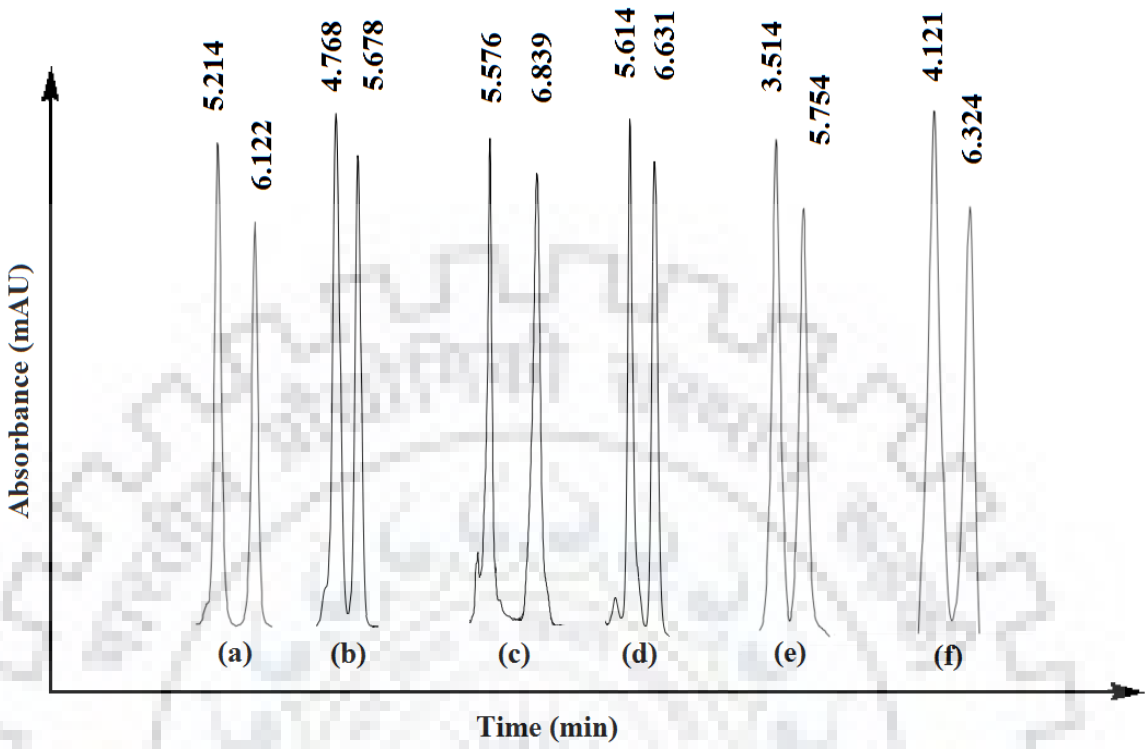


Fig. 5.A1. Sections of chromatograms showing separation of diastereomeric derivatives. Chromatogram (a), (b) and (c) represent, respectively, separation of diastereomeric derivatives of (*RS*)-Prl, (*RS*)-Mel and (*RS*)-Atl, synthesized with CDR-3. Chromatogram (d), (e) and (f) represent, respectively, separation of diastereomeric derivatives of (*RS*)-Prl, (*RS*)-Mel and (*RS*)-Atl, synthesized with CDR-4. Chromatographic conditions: LiChrospher C_{18} column (L x I.D. 25 cm × 4.6 mm, 5 µm particle size); Mobile phase, MeCN-TEAP (pH 3.5), gradient, 30% MeCN to 70% MeCN in 30 min, at a flow rate of 1 mL min^{-1}.

3. Configuration of the diastereomeric derivatives

The representative reaction given in Fig. 3.9 shows that the diastereomeric derivatives contain an amide bond (a rigid structure) formed by the reaction of the amino group of the analyte, (*RS*)-Prl, and the carbonyl oxygen of the carboxyl group of Kpf in CDR-3. Diastereomeric derivatives have been designated as (*S,R*)-, and (*S,S*)-diastereomeric derivatives, respectively, where the first (*S*) corresponds to the configuration of chiral auxiliary and the other (*S*)/(*R*) corresponds to that of (*R*)-Prl or (*S*)-Prl. The diastereomeric

derivatives obtained by 'preparative syntheses' were separated by open column chromatography and were designated as DsB-1 and DsB-1 based on elution order. ^{1}H-NMR spectra of the samples so obtained were recorded (Fig. 5.A2). Besides, the lowest energy structures were developed by using the software Gaussian 09 Rev. A.02 program and hybrid density functional B3LYP with 6-31G* basis set (based on density functional theory).

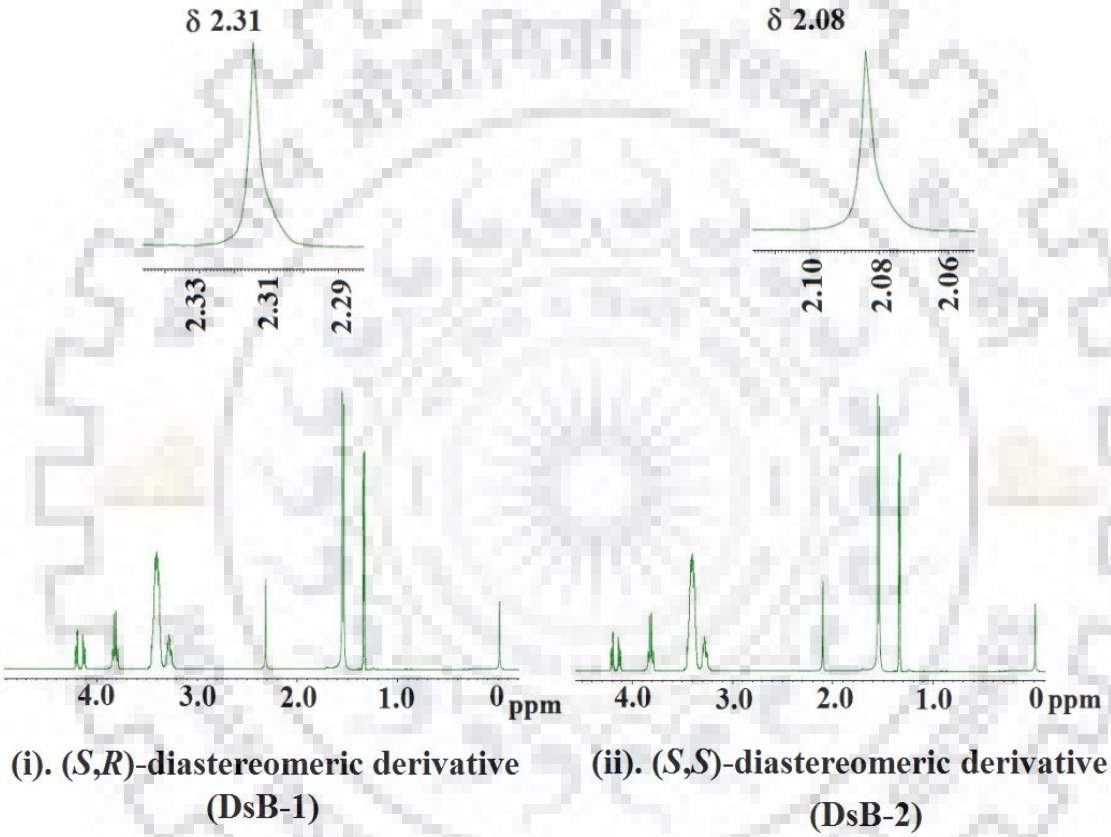


Fig. 5.A2. Sections of ^{1}H-NMR spectra of diastereomeric derivatives illustrating chemical shift values for the peaks of –OH (at the stereogenic center) signal at δ 2.31 and δ 2.08, respectively, in (i) (*S*,*R*)-diastereomeric derivative, and (ii) (*S*,*S*)-diastereomeric derivative. This difference in chemical shift of –OH signal is due to the formation of H-bonding in (*S*,*R*)-diastereomeric derivative

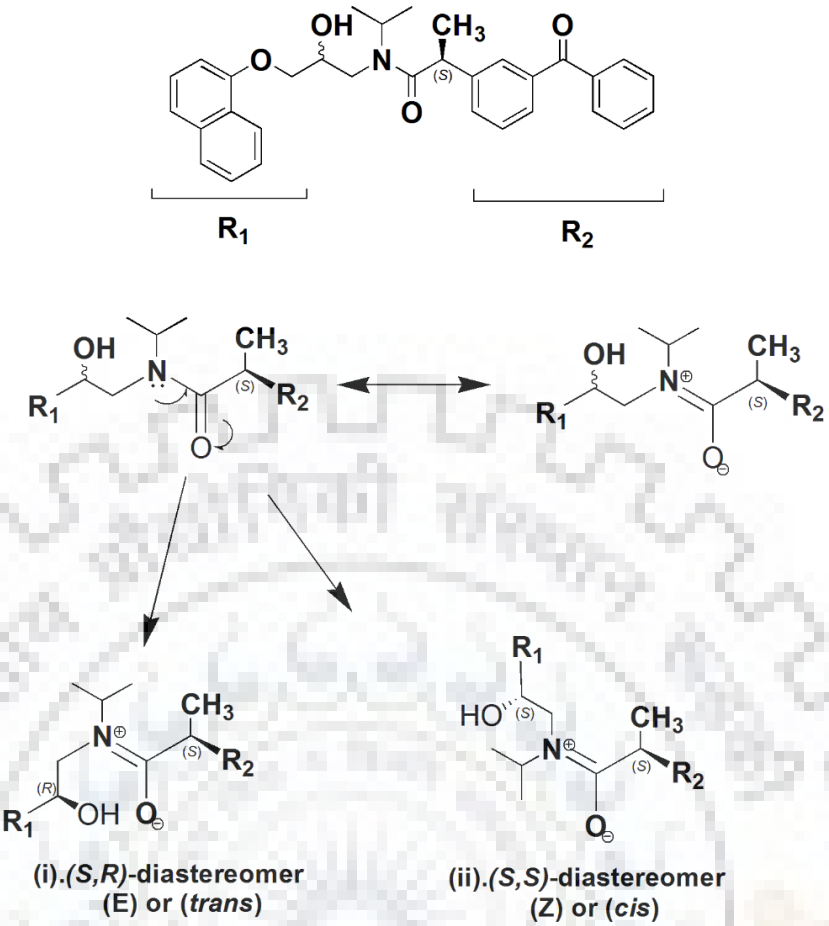


Fig. 5.A3. Scheme showing amide bond formation with partial double bond character providing specific spatial arrangements as (i) '*E*' or (-*trans*), and (ii) '*Z*' or (-*cis*) geometries in the diastereomeric derivatives (DsB-1 and DsB-2, respectively). In the '*E*' orientation the 'O' (of the Kpf) and the proton of –OH of the Prl are in the close proximity favouring formation of H-bond (the (*S*,*R*)-diastereomeric derivative).

H-bond: In the IR spectrum of DsB-1, -OH stretching frequency appears at a value of 3187 cm^{-1} while in DsB-2 it appears at 3329 cm^{-1} which is lower by a value of 142 cm^{-1} as compared to DsB-2; it supports the formation of H-bond in Ds-I [158]. The hydrogen bond arises due to specific spatial arrangements.

There occurs formation of an amide bond between the carbonyl oxygen of the carboxyl group of Kpf and the N of the Prl (Fig. 3.9). Thus, a partial double bond is envisaged in the amide bond in the diastereomeric derivatives due to delocalization of lone pair of N atom. This is evidenced by calculating the C=N bond length from the DFT based software; it comes out as 1.37 A° while the normal C–N bond length (single bond) is known to be 1.47 A°.

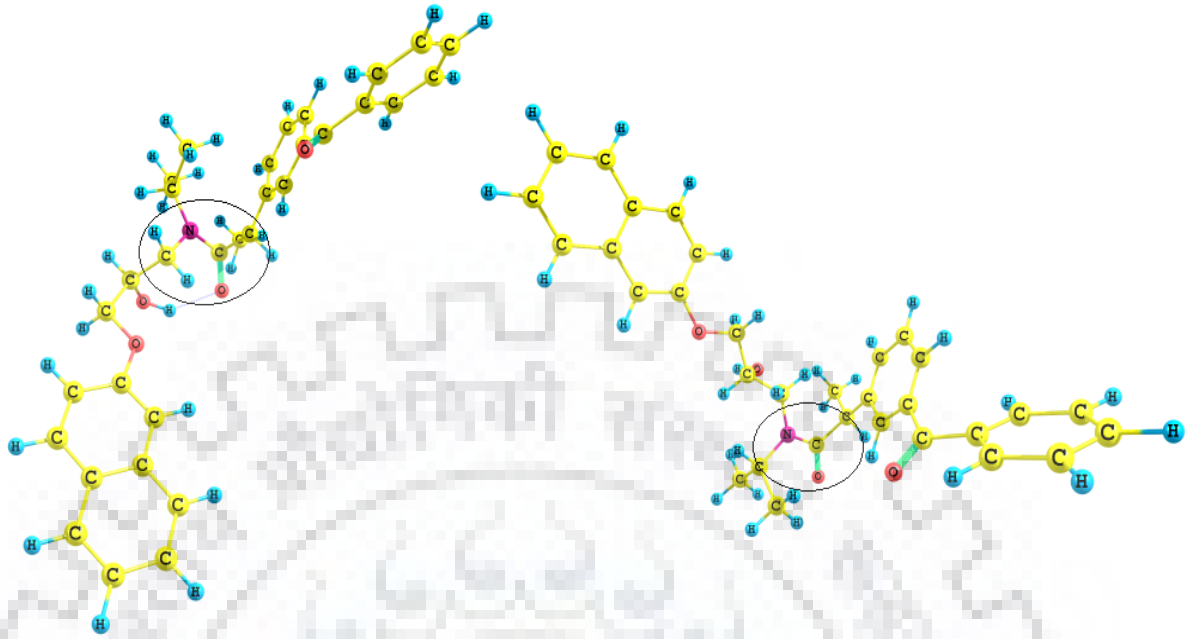
Such a double bond character gives a geometric configuration in which (i) the 'O' (of the Kpf) and the 'isopropyl group' on the N (of the Prl moiety) are oriented as '***E***' or (ii) the 'O' (of the Kpf) and the 'isopropyl group' on the N (of the Prl moiety) are oriented as '***Z***'. These are shown in **Fig. 5.A3**. These orientations were verified by making the molecular models of the '*E*' and '*Z*' geometries using '***orbital molecular building system***' (Cochranes of Oxford Ltd, Leafield, Oxford OX8 5NT, England).

In the '***E***' orientation the 'O' (of the Kpf) and the proton of –OH of the Prl are in the close proximity favouring formation of H-bond (the (*S*,*R*)-diastereomeric derivative). Further, the bond length of O–H bond for the H participating in H-bond formation was calculated as 0.971 A° (in DsB-1) while in the other case (DsB-2) it was 0.960 A°. Thus, the stretched O–H bond supports H-bond formation. It was also noted that the two hydrophobic groups, i.e., naphthyl moiety of the Prl and the benzophenone moiety of the Kpf (marked as R1 and R2, respectively, in **Fig. 5.A3**) are at a maximum spatial distance, appearing to be '*trans*' to each other (DsB-1, i.e, (*S*,*R*)-diastereomeric derivative) with respect to C=N.

On the other hand, when the geometry of the diastereomeric derivative is '**Z**' there is no possibility of H-bond formation as well as the two hydrophobic groups (as mentioned above) become '*cis*'. Thus, in this '**Z**' geometry, –OH group is far away from the carbonyl 'O' to form an H bond. This geometry, therefore, would belong to Ds-II (the (*S*,*S*)-diastereomeric derivative.

4. Optimized lowest energy structures of the diastereomeric derivatives of (*RS*)-Prl

The minimum energy geometry optimized structures of the two diastereomeric derivatives of (*RS*)-Prl, are shown in **Fig. 5.A4**. The present studies are not intended/focused on theoretical calculation of the energies of the different configurations of the isomers for which use of two dihedral angles, as variables of the potential energy surface, and other parameters would be required. The default convergence criteria are implemented in Gaussian software itself; the optimization continues simply on submitting the data. There is no consideration of torsional energy (or rotational energy barrier) to decide the relative stability of conformations. The diastereomeric derivatives under question are quite stable species and the configuration is not changing by rotation at any stage.



(a). (*S*,*R*)-diastereomeric derivative
(DsB-1)
'E' or '*trans*'

(b). (*S*,*S*)-diastereomeric derivative
(DsB-2)
'Z' or '*cis*'

Fig. 5.A4. Lowest energy optimized structures of diastereomeric derivatives developed using the program Gaussian 09 Rev. A.02 and hybrid density function B3LYP with 6-31G*. **(a)**: The naphthyl moiety of the Prl and the benzophenone moiety of the Kpf are at a maximum spatial distance, appearing to be '*trans*' (in DsB-1, (the (*S*,*R*)-diastereomeric derivative) to each other (with respect to C=N) but H-bond formation occurs because of close proximity of the 'O' (of the Kpf) and the proton of –OH of the Prl; **(b)** the two hydrophobic groups (as mentioned above) become '*cis*' ('*Z*' geometry) and make the molecule DsB-2 (the (*S*,*S*)-diastereomeric derivative) more hydrophobic.

Fig. 5.A4(a) shows formation of an intramolecular hydrogen bond between the carbonyl group of (*S*)-Kpf molecule and the proton of the hydroxyl group of Prl; this is also evidenced and explained in the previous paragraphs. Formation of such an intramolecular hydrogen bond is not shown in the other diastereomeric derivative [**Fig. 5.A4(b)**].

The $\Delta\delta^{RS}$ value obtained for –OH proton has a negative sign (–0.23). If the structures of diastereomeric derivatives are not as represented in Fig. 5.A4, then the $\Delta\delta^{RS}$ value between the diastereomeric pair would not be negative for the –OH proton. The diastereomeric derivative DsB-1 showed a downfield shift for –OH proton while the diastereomeric derivative DsB-2 showed an upfield shift in ^{1}H-NMR spectrum; therefore the diastereomeric

derivative DsB-1 has the (*S,R*)-configuration. Optimized lowest energy structures of diastereomeric derivatives showed that DsB-1 contains (*R*)-Prl and DsB-2 contains (*S*)-Prl.

The spatial orientations shown in Fig. 5.A3(i) are supported by **Fig. 5.A4(a)**, i.e., the naphthyl moiety of the Prl and the benzophenone moiety of the Kpf are at a maximum spatial distance, appearing to be '*trans*' to each other but H-bond formation occurs because of close proximity of the 'O' (of the Kpf) and the proton of –OH of the Prl. These features (in '***E***' geometry) make the molecule (DsB-1, i.e, (*S,R*)-diastereomeric derivative,) less hydrophobic. Similarly, spatial orientations shown in Fig. 5.A3 (ii) are supported by **Fig. 5.A4(b), i.e**., the two hydrophobic groups (as mentioned above) become '*cis*' ('***Z***' geometry) and make the molecule DsB-2 (the (*S,S*)-diastereomeric derivative) more hydrophobic.

5. Elution order

The experimental observation that the (*S,R*)-diastereomeric derivative is eluted first from the column means that it is retained for lesser time in comparison to the (*S,S*)-diastereomeric derivative. As discussed above, it can be contended that the *cis*-, or *trans*-type arrangements of the two diastereomeric derivatives are responsible for difference in their hydrophobicities.

The two hydrophobic groups, i.e., naphthyl moiety of the Prl and the benzophenone moiety of the Kpf (marked as R1 and R2 in **Fig. 5.A3**) contributing to the overall hydrophobicity along with the rheological properties of the mobile phase are responsible for different partition coefficients and different retention times of the (*S,R*)-, and (*S,S*)-diastereomeric derivatives.

Since the hydrophobic groups are at a maximum spatial distance in the (*S,R*)-diastereomeric derivative because of double bond character of the amide bond (i.e., '***E***' geometry) the hydrophobic intensity is diminished. In other words, in the (*S,S*)-diastereomeric derivative the hydrophobic groups come closer spatially and their hydrophobic effect becomes relatively more intense which make (*S,S*)-diastereomeric derivative more hydrophobic, therefore, it interacts more strongly with the C_{18} material of the column and has a longer retention time.

Table-5.A2. Method validation for HPLC separation of diastereomers of (*RS*)-Prl prepared with CDR-4

Linearity		**First eluting diastereomer**				**Second eluting diastereomer**			
Range (ng mL^{-1})		10-50				10-50			
Slope		3.281				3.350			
Intercept		2.2				1.9			
Correlation Coefficient (R^2)		0.999				0.999			
SD intercept		0.306				0.307			
Accuracy and precision									
Actual conc. Of diastereomeric mixture (ng mL^{-1})	**Conc. Of each diastereomers (ng mL^{-1})**	**First eluting diastereomer**				**Second eluting diastereomer**			
		Found conc. Mean± (t*SD/√n)	**Mean recovery (%)**	**Confidence interval (%)**	**RSD (%)**	**Found conc. Mean± SD (ng mL^{-1})**	**Mean recovery (%)**	**Confidence interval (%)**	**RSD (%)**
Intra-day precision									
20	10	9.89 ± 0.20	98.94	98.42-100.59	1.03	9.88 ± 0.25	98.80	97.65-100.75	1.06
40	20	19.75 ± 0.51	98.78	98.25- 99.76	1.21	20.32 ± 0.37	101.64	98.18-101.46	1.24
60	30	30.12 ± 0.35	100.40	97.50-100.33	1.52	29.88 ± 0.31	99.60	98.73-100.15	1.36
80	40	40.48 ± 0.88	101.21	97.55-100.21	1.65	39.73 ± 0.57	99.32	97.85-100.82	1.62
100	50	49.63 ± 0.96	99.26	98.88-99.85	1.78	50.17 ± 0.78	100.35	98.44-101.16	1.88
		Mean	99.72	98.12-100.14	1.48		99.55	98.17-100.86	1.68
Inter-day precision									
20	10	9.81 ± 0.31	98.11	97.69-101.35	1.26	9.56 ± 0.35	95.69	98.81-101.59	1.15
40	20	20.10 ± 0.47	100.05	98.55-101.31	1.38	19.72 ± 0.49	98.61	97.52-100.25	1.26
60	30	29.95 ± 0.52	99.79	98.60-100.21	1.48	29.55 ± 0.74	98.56	98.25-100.73	1.35
80	40	39.37 ± 0.89	98.42	97.81-101.17	1.85	39.72 ± 0.91	99.30	97.85-101.22	1.67
100	50	50.37 ± 1.20	100.75	98.85-100.19	1.67	49.57 ± 1.27	99.15	97.25-101.06	1.76
		Mean	99.42	98.31-101.84	1.52		98.26	97.85-101.05	1.58
Sensitivity									
LOD (ng mL^{-1})	0.308					LOD (ng mL^{-1})	0.302		
LOQ (ng mL^{-1})	0.934					LOQ (ng mL^{-1})	0.915		

['t' represent student 't' value (at 0.05 selected drgree of probability), n (=5) is the number of replicates, SD = standard deviation, RSD = relative standard deviation]

6. Stability of CDRs and recovery of diastereomeric derivatives

The recovery studies of the diastereomeric derivatives (as described above) served as a measure of their yields (greater than 97.0%). Stability of the CDRs was investigated as described in Chapter-4A. The CDRs were found to be stable up to 5 months.

7. Method validation

Validation studies were carried out (as described in Chapter-4A) with respect to linearity, accuracy and precision for HPLC separation of diastereomeric derivatives of *(RS)*-Prl prepared with CDR-4, as a representative (in concentration range between 20-100 ng mL^{-1}). UV absorbing chromophore in the form of Kpf moiety led to sensitive detection with LOD values of 0.308 ng mL^{-1} and 0.302 ng mL^{-1}. Obtained results of method validation data are given in Table-5.A2.

8. Comparison of present work with literature reports

The efficiency of (*S*)-Kpf based CDRs for diastereomeric separation and enantioselectivity was found to be better in terms of low retention times, high *Rs* and low LOD observed in comparison to those obtained with certain other CDRs as reported in literature; the new CDRs were found to be more stable (more than 5 month at 2-5 ^{o}C) as compared to CDRs based on DFDNB [15], and isothiocyanate [19, 20]. Also, the separation factor (α, 1.22-1.92) for the diastereomeric derivatives prepared with the newly synthesized CDRs were found to be better than the diastereomeric derivatives prepared with CDRs based on DFDNB [15], and isothiocyanate [19, 20].

The LOD were found to be 1.790 ng mL^{-1} and 2.132 ng mL^{-1} for diastereomeric derivatives of (*S*)- and (*R*)-Prl, respectively, using (*S*)-(–)-Lfx based CDR [144] while in the present case the LOD values are 0.308 ng mL^{-1} and 0.302 ng mL^{-1} (much lower).

The newly synthesized CDRs were found to provide better resolution (4.54-8.81) in comparison to the resolution reported in literature. The retention times were greatly reduced (5-7 min, at a flow rate of 1 mL min^{-1}) for the diastereomeric derivatives synthesized with CDR-3 and CDR-4 as compared to that reported in literature and this method required 10-15 times less consumption of organic solvents.

B. Enantioseparation of (*RS*)-salbutamol, (*RS*)-carvedilol, (*RS*)-bisoprolol and (*RS*)-betaxolol using CDR-3 and CDR-4 using micellar liquid chromatography

1. Activated esters [CDR-3 and CDR-4] and diastereomeric derivatives

(*S*)-Kpf was selected as the chiral auxiliary for synthesis of CDRs because of its significant characteristics (described in **'Chapter-3')**. The choice of benzotriazole (Btz) and succinimidyl (Suc) moieties as 'auxiliary nucleophiles' and their role in synthesis and conservation of chiral purity of the CDRs along with their role as potent acylating agents to give the desired amide diastereomeric derivatives has been explained in **Chapter-4A**. Reaction for synthesis of (*S*)-Kpf based CDRs, [i.e., CDR-3 and CDR-4], is shown in **Fig. 3.2.** Stability of CDRs were investigated as described in Chapter-5A and it was found that the CDRs were stable up to 6 months.

The structures of the diastereomeric derivatives of (*RS*)-Sbl, (*RS*)-Cdl, (*RS*)-Bpl and (*RS*)-Bxl prepared with CDR-4 are shown in **Fig. 3.10;** these are designated as DsB-7, DsB-8 for (*RS*)-**Sbl**; DsB-9, DsB-10 for (*RS*)-**Cdl**; DsB-11, DsB-12 for (*RS*)-**Bpl**, and DsB-13, DsB-14 for (*RS*)-**Bxl**. Since the Btz and Suc moieties are removed during nucleophilic substitution by the amino group of the analyte the structures of the diastereomeric derivatives obtained by using either CDR-3 or CDR-4 remain the same. Chemical structures of diastereomeric derivatives of racemic β-adrenolytics shown in Fig. 3.10 are thus represented as (*S,R*)- and (*S,S*)-configurations, where the first (*S*) corresponds to the configuration of (*S*)-Kpf.

2. Water micellar mobile phase system

As an important property, surfactants form micelles at a concentration above CMC. For SDS, CMC is 8.2 mM and the aggregation number (N_A) is about 70 monomers of surfactant per micelle at 25 °C. Therefore, SDS, an anionic surfactant, belonging to the alkyl sulfate group is the most widely used surfactant in MLC [146]. Though the application of the mixed surfactant systems is less common in liquid chromatography but a combination of the two makes a difference in chromatographic properties for separation enhancement because there is an effect of negatively charged micelles (while using SDS) and non-charged micelles (when Brij-35 is used) when they are dissolved in water.

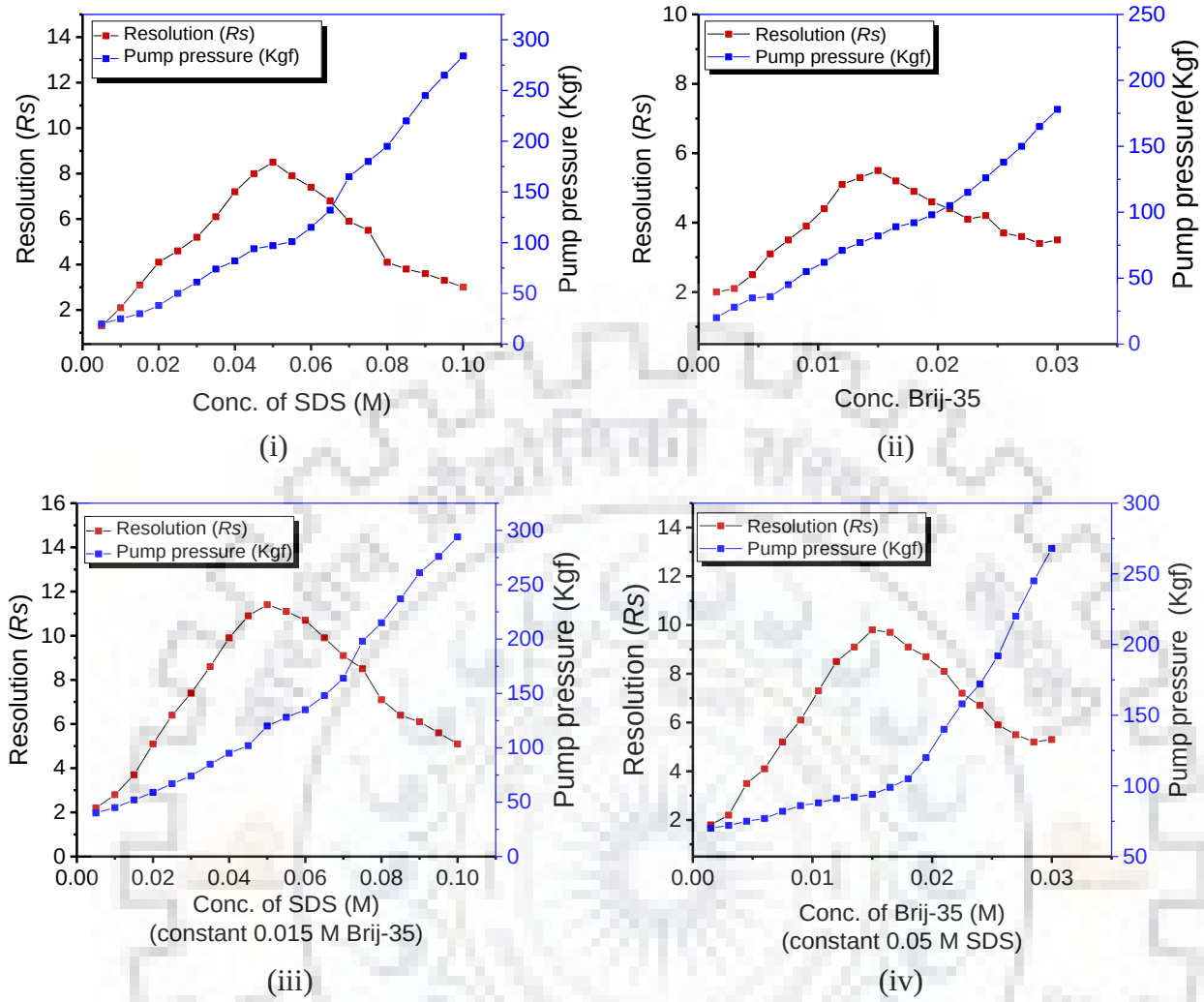


Fig. 5.B1. Showing effect of concentration of SDS and Brij-35 on resolution of analytes and pressure of HPLC pump. (i) showing effect of increasing SDS concentration, (ii) showing effect of increasing Brij-35 concentration, (iii) showing effect of increasing SDS concentration in 0.015 M Brij-35 solution and (iv) showing effect of increasing Brij-35 concentration in 0.05 M SDS solution.

Such a water micellar mobile phase system increases overall polarity of the stationary phase of the C_{18} column in the following manner. *SDS formed micelles* contain a dodecyl apolar core and *Brij-35 formed micelles* have relatively polar surface due to oxyethylene chains in micelles. There occurs 'on surface' interaction between C_{18} material and carbon chain of Brij-35. The polyoxyethylene carbon chain of Brij-35 increases polarity of the stationary phase (as it is significantly more polar in comparison to the C_{18} material). Presence of SDS in the system also helps increasing polarity of the stationary phase because of the orientation of its negatively charged sulphate groups. At the same time, the surfactants decrease surface tension and viscosity of the mobile phase.

Such an *in-situ* modification of the stationary phase reduces the retention time (of the compounds undergoing separation) and also indicates that there are no specific interactions such as hydrogen bonding between the surfactant and the analytes [143] and the formation of micelles of diastereomeric derivatives is considered responsible for good chromatographic separation along with faster elution.

In presence of ionic or non-ionic surfactants the association of a solute takes place in (a) bulk water with binding sites of the stationary phase and (b) with the surfactant monomers in the micelles dissolved in the mobile phase and there occur association equilibria between stationary phase and solute, and between micelle and the solute. Experiments were carried out (i) by increasing concentration of SDS (0.005 M to 0.05 M) in constant concentration solution of Brij-35 (i.e., 0.015 M), and (ii) by increasing the concentration of Brij-35 (0.0015 M to 0.015 M) in the constant concentration solution of SDS (i.e., 0.05 M). It was observed that in case (i) the retention time of the analytes decreased but peak heights increased while in case (ii) the retention time decreased. Thus, the mixed system provided very good resolution with lower retention times in comparison to the mobile phase having single surfactant (Fig. 5.B1 and Fig. 5.B2).

3. Separation of diastereomeric derivatives by MLC

The C_8 columns are reported [146] to offer lower retention times, poor peak efficiencies and increasing asymmetry factors, therefore, C_{18} column was preferred for the present studies.

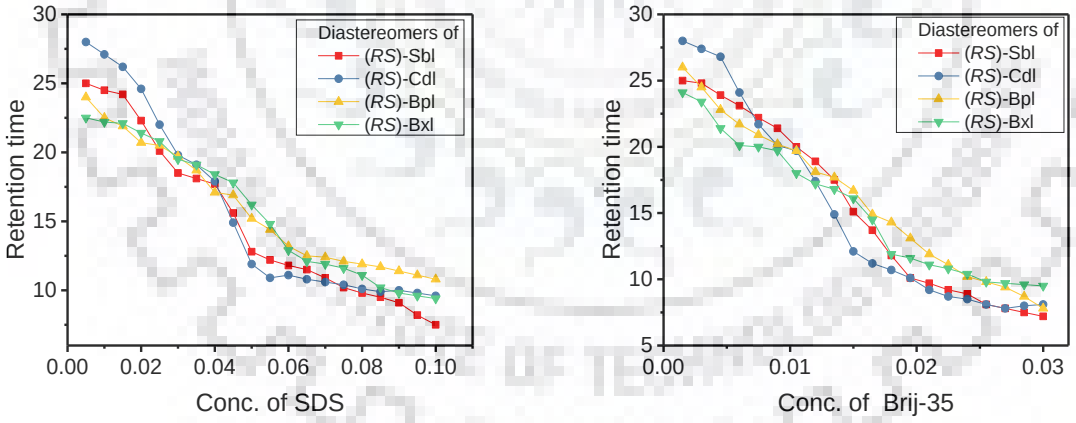
Fig. 5.B3 shows chromatographic separation of diastereomeric derivatives of (*RS*)-Sbl, (*RS*)-Cdl, (*RS*)-Bpl and (*RS*)-Bxl prepared with CDR-3, and Fig. 5.B4 shows the separation peaks for diastereomeric derivatives prepared with CDR-4. The optimized conditions for successful separation of the diastereomeric mixtures of all the four β-adrenolytics, in terms of reproducibility and selectivity were, WMP-TEAP (pH 3.5) in 80:20 ratio in isocratic mode as mobile phase at a flow rate of 0.70 mL min^{-1} in 60 min run time.

The flow rate was optimized by varying it as 0.50, 0.70, and 1.0, mL min^{-1}. It was observed that sharpness of peaks increased and retention time and Δt (difference in retention times) decreased when the flow rate was increased; the pressure of the system also increased with increasing flow rate and it went around 80 and 120 respectively at the flow rates of 0.70, and 1.0, mL min^{-1}. Therefore, a flow rate of 0.70 mL min^{-1} which provided a very good resolution (Fig. 5.B3 and Fig. 5.B4) at low HPLC pressure condition was taken as the successful and optimised condition. The mobile phase composition was optimized which

resulted into low retention time with high resolution at suitable low HPLC pump pressure and there is a less chance to damage column material and reduce unnecessary pressure on HPLC system.

The values for retention factor (k), separation factor (α), and resolution (R_S) obtained under the optimized conditions of separation are given in Table-5.B1 for the diastereomeric derivatives prepared with CDR-3 and CDR-4. The values of (k), (α) and (R_S) were calculated using the equations (i) $k = (t_x - t_0)/t_0$, (ii) $\alpha = k_2 / k_1 = (t_{x2} - t_0)/ (t_{x1} - t_0)$ and (iii) $R_S = 2 (t_{x2} - t_{x1})/ (W_1 + W_2)$, respectively where t_0 is the dead time, t_{x1} and t_{x2} are retention times of first and second eluted peaks, respectively; W_1 and W_2 are the corresponding base peak widths.

The data in **Table-5.B1** clearly shows a very good separation of all the diastereomeric pairs using *aq* mobile phase which had no organic modifier. Therefore, the use of organic modifiers is not recommended. The presence of the two surfactants in the mobile phase does not interfere with detection as both have low molar absorptivity as compared to (*S*)-Kpf. As a representative, the UV spectrum was captured for diastereomeric derivatives of (*RS*)-Sbl at 4.511 and 6.302 min using PDA detector; these were found to be identical.



(i) (ii)

Fig. 5.B2. Showing (i) effect on difference of the retention times of diastereomeric derivatives of (*RS*)-Sbl, (*RS*)-Cdl, (*RS*)-Bpl and (*RS*)-Bxl when increasing concentration of SDS, (ii) effect on difference of the retention times of diastereomeric derivatives of (*RS*)-Sbl, (*RS*)-Cdl, (*RS*)-Bpl and (*RS*)-Bxl when increasing concentration of Brij-35.

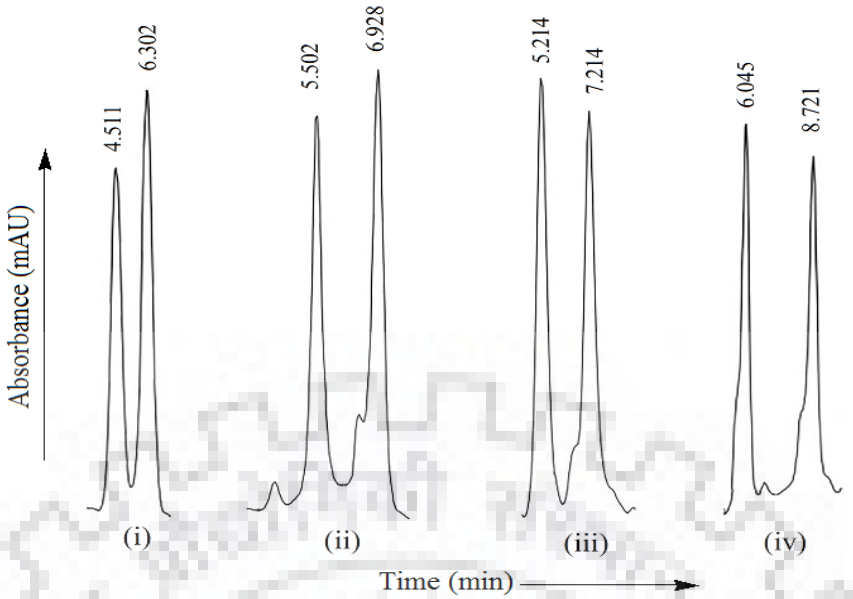


Fig. 5.B3. Sections of chromatograms showing separation of diastereomeric derivatives by MLC; (i), (ii), (iii) and (iv) represent separation of diastereomeric derivatives of (*RS*)-Sbl, (*RS*)-Cdl, (*RS*)-Bpl and (*RS*)-Bxl**,** respectively, synthesized with CDR-3. The data provided by the system software showed identical peak areas for a particular pair.

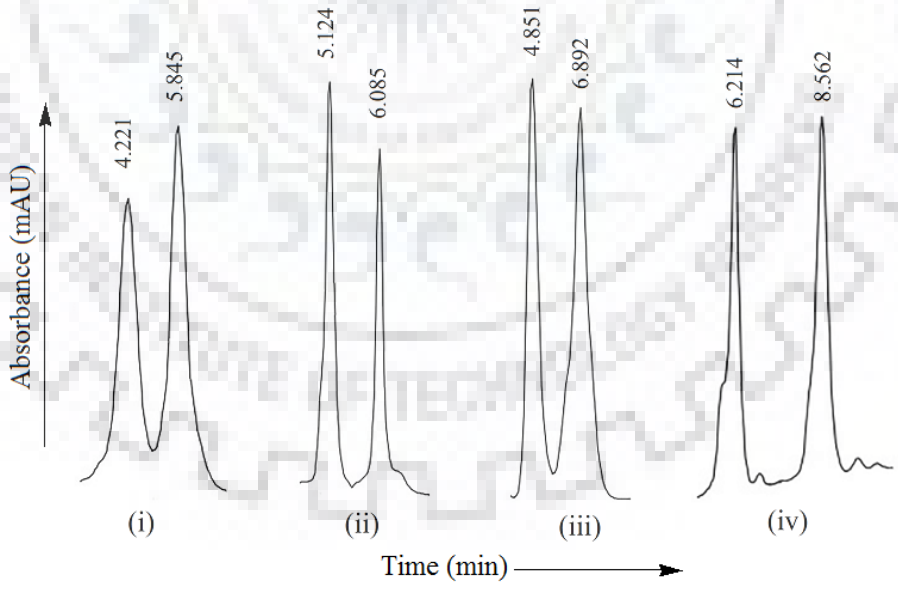


Fig. 5.B4. Sections of chromatograms showing separation of diastereomeric derivatives by MLC; (i), (ii), (iii) and (iv) represent separation of diastereomeric derivatives of (*RS*)-Sbl, (*RS*)-Cdl, (*RS*)-Bpl and (*RS*)-Bxl**,** respectively, synthesized with CDR-4. The data provided by the system software showed identical peak areas for a particular pair.

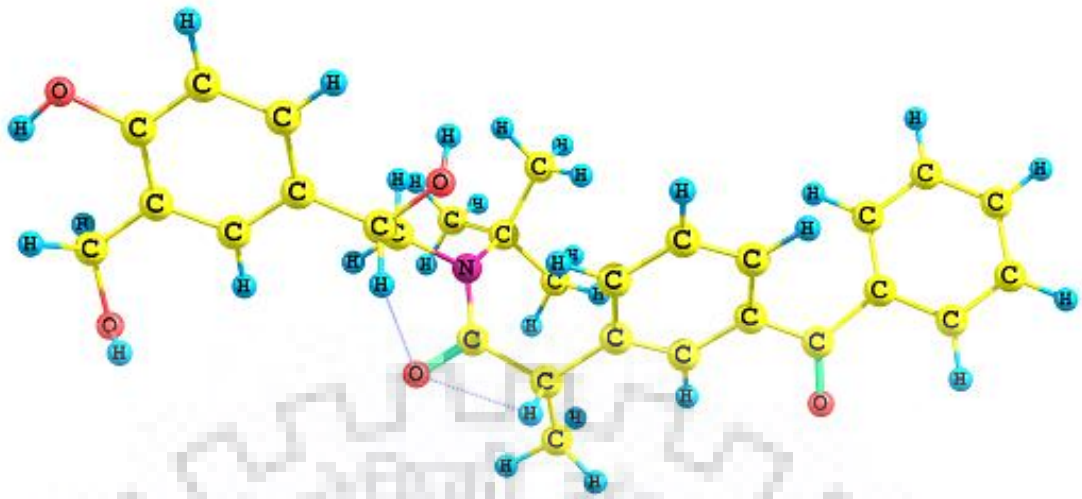
Table-5.B1. Chromatographic separation data of diastereomeric derivatives prepared with CDR-3 and CDR-4.

The analyte	Separation data for diastereomeric derivatives prepared with CDR-3				Separation data for diastereomeric derivatives prepared with CDR-4			
	k_1	k_2	α	*Rs*	k_1	k_2	α	*Rs*
(*RS*)-Sbl	2.446	3.367	1.376	6.41	2.202	3.251	1.478	6.72
(*RS*)-Cdl	1.550	2.543	1.637	7.62	1.345	2.223	1.670	6.49
(*RS*)-Bpl	2.258	3.508	1.554	8.69	2.031	3.306	1.627	8.89
(*RS*)-Bxl	2.358	3.845	1.630	11.63	2.452	3.756	1.532	10.21

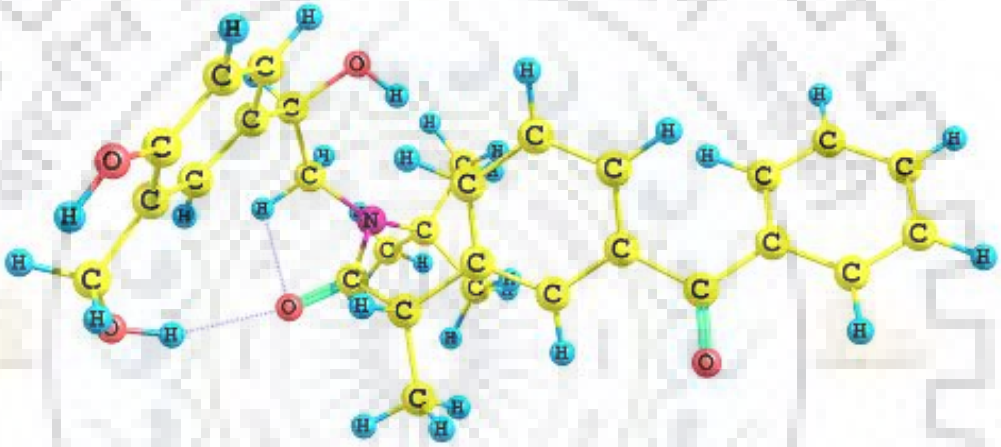
4. Lowest energy optimized structures and elution order of the diastereomeric derivatives

The structures of synthesized diastereomeric derivatives are shown in **Fig. 3.10**. These were verified by developing molecular models, 'orbital molecular building system' (Cochranes of Oxford Ltd., Leafield, Oxford, UK). To give additional support for the geometry of diastereomeric derivatives of (*RS*)-Sbl, the optimized lowest energy structures were also developed (Fig. 5.B5) using Gaussian 09 Rev. A.02 program and hybrid density functional B3LYP with 6-31G* basis set (based on density functional theory).

The structure in Fig. 3.10 and Fig. 5.B5 show a spatial nearness, due to rigid amide bond, between the benzyl of (*RS*)-Sbl and benzophenone of CDR-4 in **DsB-8**; the two hydrophobic groups, thus, cause a relatively more compact situation and the diastereomeric derivative DsB-8 interacts more strongly with hydrophobic ODS material of C_{18} column [87], resulting into higher retention time and thus elutes after the diastereomeric derivative **DsB-7**, with the micellar based mobile phase. Therefore, it can be contended that the steric arrangement of the bulky groups at the stereogenic center of diastereomeric derivatives causes a difference in hydrophobicity of the two isomers which results in the differential interaction of the diastereomeric derivatives with the C_{18} material of the column and their separation.



(i). DsB-7



(ii). DsB-8

Fig. 5.B5. Structures of diastereomeric derivatives, optimized for lowest energy, developed by using the program Gaussian 09 Rev. A.02 and hybrid density function B3LYP with 6-31G*: (i) the (*S*,S)-diastereomeric derivative (DsB-7) and (ii) the (*S*,*R*)-diastereomeric derivative (DsB-8).

5. Method validation

The method validation for accuracy and precision was carried out for diastereomeric derivatives of (*RS*)-Sbl prepared with CDR-4, as a representative, and the data is given in **Table-5.B2**. The calculated values of recovery for the first and second eluting diastereomeric derivatives, respectively, are 100.04 and 99.91% for intra-day assay and 99.95 and 99.73% for inter-day assay**.** The LOD and LOQ were found, respectively, 0.306 ng mL^{-1} and 0.930 ng mL^{-1} for diastereomeric derivatives of (*RS*)-Sbl.

Table-5.B2. Method validation for HPLC separation of diastereomeric derivatives of (*RS*)-Sbl prepared with CDR-4.

Linearity	**First eluting diastereomeric derivative**			**Second eluting diastereomeric derivative**		
Range (ng mL^{-1})	40-4000			40-4000		
Slope	3.296			3.360		
Intercept	11.28			6.473		
Correlation Coefficient (R^2)	0.999			0.999		
Accuracy and precision						
Conc. Of each diastereomers (ng mL^{-1})	**First eluting diastereomeric derivative**			**Second eluting diastereomeric derivative**		
	Found conc. Mean± SD (ng mL^{-1})	Recovery (%)	RSD (%)	Found conc. Mean± SD (ng mL^{-1})	Recovery (%)	RSD (%)
Intra-day precision						
20	20. 62 ± 0.47	100.61	0.91	20.30 ± 0.39	101.25	1.02
100	99.72 ± 0.65	99.72	1.12	100.13 ± 0.51	100.13	1.21
200	198.83 ± 3.14	99.41	1.31	202.30 ± 3.98	101.15	1.16
600	604.11 ± 8.74	100.68	1.72	593.25 ± 9.04	98.89	1.42
2000	1998.94 ± 12.05	99.94	1.68	2001.60 ± 11.19	100.07	1.54
	Mean	100.07	1.34		99.91	1.27
Inter-day precision						
20	19.74 ± 0.31	98.74	1.05	20.24 ± 0.42	101.21	0.95
100	99.54 ± 0.62	99.54	1.09	100.45 ± 0.69	100.45	1.12
200	199.31 ± 2.05	99.65	1.27	199.96 ± 1.99	99.98	1.35
600	588.52 ± 6.11	98.08	1.58	590.40 ± 4.18	98.14	1.75
2000	2000.39 ± 10.91	100.01	1.71	2002.42 ± 8.56	100.12	1.92
	Mean	99.20	1.34		99.98	1.41
Sensitivity LOD (ng mL^{-1}) 0.306; LOQ (ng mL^{-1}) 0.930 [n (=5) is the number of replicates, SD = standard deviation, RSD = relative standard deviation]						

6. Comparison with literature reports

Present work shows very good enantioseparation of the β-adrenolytics (under study) in terms of resolution, separation factor and retention times of their diastereomeric derivatives. The existing literature shows only a few reports on enantioseparation of (*RS*)-Sbl and (*RS*)-Cdl by both direct and indirect approaches, for example, enantiomers of (*RS*)-Sbl and (*RS*)-Cdl were not resolved neither using indirect approach [14, 16] nor direct approach of enantioseparation [152, 153] and in a few cases enantiomers were resolved with very low

resolution [153-157]. Similarly, (*RS*)-Bpl and (*RS*)-Bxl were better resolved in the present case as compared to previous literature [16, 157]. The CDRs (activated esters of (*S*)-Kpf), reported herein, were found to be more stable (> 6 months at 2–5 °C) as compared to CDRs based on DFDNB [14, 15] and isothiocyanate [18-20]. In comparison to literature reports, the method was successful in providing very low LOD and LOQ values, better resolution [14, 16, 18-21, 125, 126] and low retention times resulting into lesser consumption of mobile phase as well. The separation factors (α) for the diastereomeric derivatives prepared with the CDR-3 and CDR-4 were found to be better than those for the diastereomeric derivatives prepared with CDRs based on (*S*)-naproxen [87, 126], and CDRs having L-amino acids as chiral auxiliary in cyanuric chloride, DFDNB [14, 15] and isothiocyanate [18, 20].

Chapter-6

Result and discussion for enantiomeric separation of sulphur amino acids and selenomethionine using micellar liquid chromatography and activated esters of (*S*)-levofloxacin, (*S*)-ketoprofen and (*S*)-ibuprofen (CDR-2, CDR-4 and CDR-5)

Diastereomeric derivatives of (*RS*)-SeMet, (*RS*)-Met, (*RS*)-Cys and (*RS*)-PenA were synthesized with CDR-2, CDR-4 and CDR-5. The separation of these diastereomeric pairs was achieved using micellar liquid chromatography (MLC) wherein the mobile phase contained simple surfactants in the aq. phase and without any organic solvent. The absolute configuration for each was established. Additionally the lowest energy structures of diastereomeric derivatives of (*RS*)-SeMet were developed using the program Gaussian 09 Rev. A.02 and hybrid density function B3LYP with 6-31G*.

The experimental details for the synthesis of CDRs and 12 pairs of diastereomeric derivatives are already described in **Chapter-3**.

1. CDRs and diastereomeric derivatives

The explanation for synthesis of CDRs (2, 4 and 5) and diastereomeric derivatives of AAs was same as described in Chapter-4A. The structures of the analytes, under study, are shown in Fig. 2.2; reaction for synthesis of (*S*)-Ibf based CDR (CDR-5) is shown in Fig. 3.3, and synthesis of CDR-2 and CDR-4 are given in Fig.3.1 and Fig.3.2, respectively.

The structures of the diastereomeric derivatives of (*RS*)-SeMet, (*RS*)-Met, (*RS*)-Cys and (*RS*)-PenA prepared with CDR-**2** are shown in **Fig. 3.11** (these are designated as DsA-15, DsA-16 for SeMet; DsA-17, DsA-18 for Met; DsA-19, DsA-20 for Cys, and DsA-21, DsA-22 for PenA). In **Fig. 3.11**, the chemical structures of the diastereomeric derivatives are represented to show (*S,S*)- and (*S,R*)-configurations, respectively, where the first letter (*S*) corresponds to the configuration of chiral auxiliary and the other letter (*S*)/(*R*) corresponds to that of (*S*) or (*R*) AA. The structures of the diastereomeric derivatives of the rest of the AAs prepared with CDR-4 and CDR-5 are shown in **Fig. 3.12** and **Fig. 3.13**, respectively**.** The diastereomeric derivatives of (*RS*)-SeMet, (*RS*)-Met, (*RS*)-Cys and (*RS*)-PenA prepared with CDR-**4** are shown in **Fig. 3.12** (these are designated as DsB-15, DsB-16 for SeMet; DsB-17, DsB-18 for Met; DsB-19, DsB-20 for Cys, and DsB-21, DsB-22 for PenA) and the diastereomeric derivatives of (*RS*)-SeMet, (*RS*)-Met, (*RS*)-Cys and (*RS*)-PenA prepared with

CDR-5 are shown in **Fig. 3.13** (these are designated as DsC-1, DsC-2 for SeMet; DsC-3, DsC-4 for Met; DsC-5, DsC-6 for Cys, and DsC-7, DsC-8 for PenA).

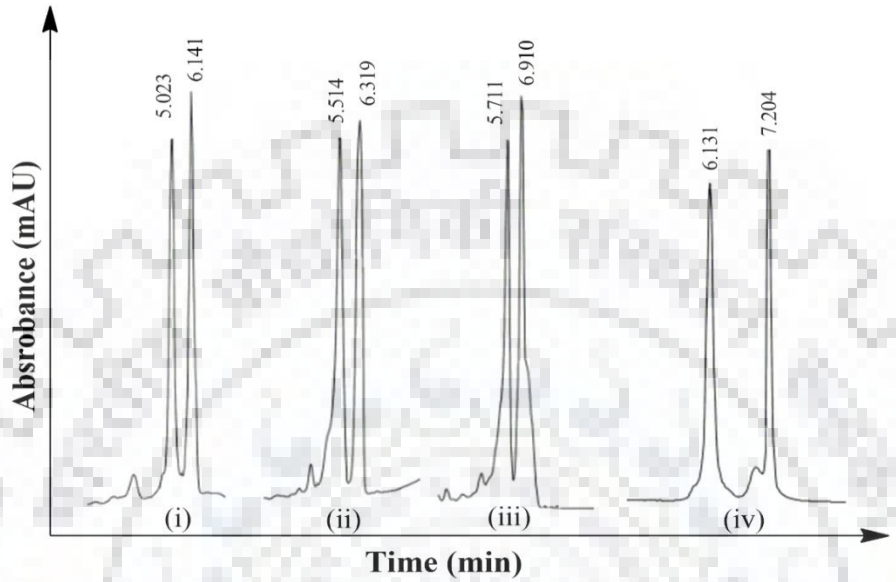


Fig. 6.1. Sections of chromatograms showing separation of diastereomeric derivatives by MLC; (i), (ii), (iii), and (iv) represent separation of diastereomeric derivatives of (*RS*)-SeMet, (*RS*)-Met, (*RS*)-Cys and (*RS*)-PenA, respectively, synthesized with CDR-5.

2. RP-HPLC of diastereomeric derivatives of racemic AAs

The UV-visible spectrum of the first and second eluting diastereomeric derivatives in all the four cases were captured using PDA detector. A mixed aqueous surfactant system as a mobile phase, as used in the present studies, completely eliminated the need of organic solvents.

WMP-TEAP (pH 5.0, 80:20) as mobile phase was used on RP-HPLC in isocratic mode. A flow rate of 0.70 mL min^{-1} for 60 min run time was found successful for separation of diastereomeric derivatives of racemic AAs in terms of reproducibility and selectivity. The chromatographic separation values (retention factor, separation factor and resolution) under the optimized HPLC conditions were calculated and are given in **Table-6.1** for the diastereomeric derivatives prepared with CDR-2, CDR-4 and CDR-5. The chromatographic separation data of all the twenty four diastereomeric derivatives, given in Table-6.1, shows

that micellar based mobile phase is sufficient to achieve good separation. **Fig. 6.1** shows chromatographic separation of diastereomeric derivatives of SeMet, Met, Cys and PenA prepared with CDR-5 (as a representative), and **Fig. 6.2** shows chromatographic separation of diastereomeric derivatives of SeMet, Met, Cys and PenA prepared with CDR-2 and CDR-4.

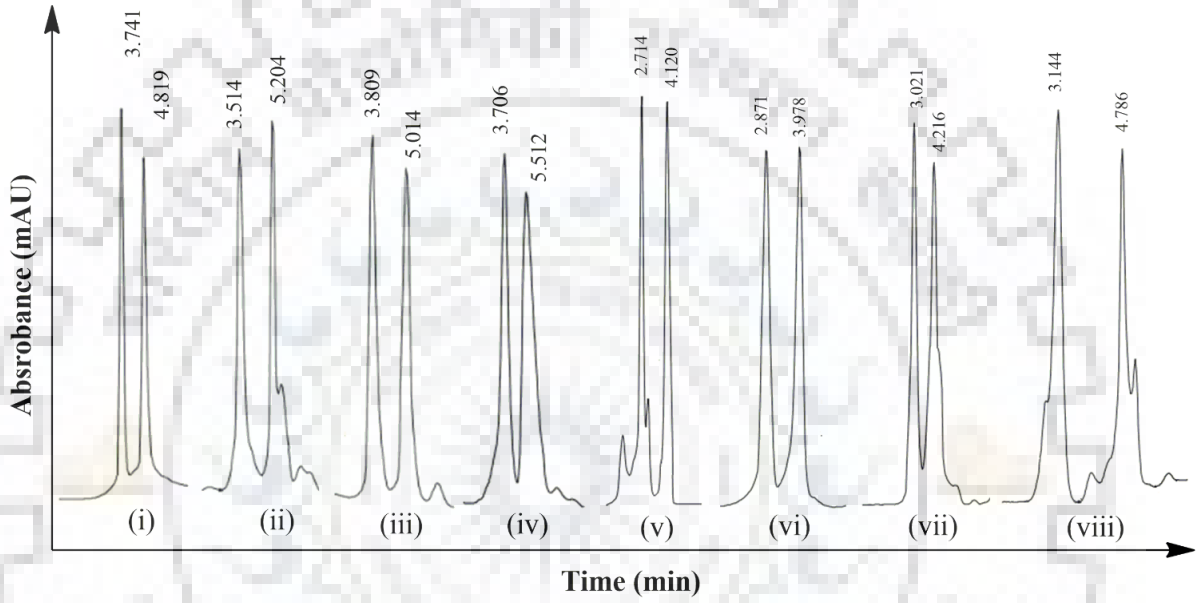


Fig. 6.2. Sections of chromatograms showing separation of diastereomeric derivatives by MLC; (i), (ii), (iii), and (iv) represent separation of diastereomeric derivatives of (*RS*)-SeMet, (*RS*)-Met, (*RS*)-Cys and (*RS*)-PenA, respectively, synthesized with CDR-4. (v), (vi), (vii) and (viii) represent separation of diastereomeric derivatives of (*RS*)-SeMet, (*RS*)-Met, (*RS*)-Cys and (*RS*)-PenA, respectively, synthesized with CDR-2.

3. Water micellar mobile phase (WMP)

The explanation for water micellar phase system was same as described in Chapter-5B.

Effect of concentration and ratio of surfactants in the mobile phase: Taking into account the literature reports [159], a reference aqueous mobile phase was prepared containing 0.05 M SDS and 0.015 M Brij-35 in aqueous phase. In the present studies, it was observed that *Rs* increased (Fig. 6.3) by increasing the concentration of SDS (0.005 to 0.05 M) or Brij-35

(0.0015 to 0.015 M). The retention time of analyte decreased on increasing the concentration of the SDS and Brij-35 in the mobile phase (Fig. 6.4). In the mixed surfactant system, the increasing concentration of SDS (0.005 M to 0.05 M) in the solution of 0.015 M Brij-35 or increasing concentration of Brij-35 (0.0015 M to 0.015 M) in the solution of 0.05 M SDS resulted into decrease in retention time with increase in peak height.

Thus, the mixed system provided good resolution with lower retention times in comparison to the mobile phase having single surfactant. Successive additions of the non-ionic surfactant gradually reduced the retention time, though to a smaller extent, in comparison to the addition of SDS to a mobile phase containing a fixed amount of Brij-35.

Table-6.1. Chromatographic separation data of diastereomeric derivatives.

The analyte	**Separation data for diastereomeric derivatives prepared with CDR-2**			
	k_1	k_2	α	***Rs***
(*RS*)-SeMet	2.015	3.577	1.77	11.25
(*RS*)-Mel	1.610	2.611	1.62	8.89
(*RS*)-Cys	1.517	2.513	1.65	8.85
(*RS*)-PenA	1.245	2.418	1.94	13.13
The analyte	**Separation data for diastereomeric derivatives prepared with CDR-4**			
	k_1	k_2	α	***Rs***
(*RS*)-SeMet	2.401	3.015	1.25	7.186
(*RS*)-Mel	2.194	3.345	1.52	11.33
(*RS*)-Cys	2.174	2.856	1.31	9.64
(*RS*)-PenA	2.088	2.937	1.40	14.45
The analyte	**Separation data for diastereomeric derivatives prepared with CDR-5**			
	k_1	k_2	α	***Rs***
(*RS*)-SeMet	4.581	5.820	1.27	7.453
(*RS*)-Mel	4.012	4.744	1.18	7.366
(*RS*)-Cys	3.759	5.003	1.33	9.393
(*RS*)-PenA	3.716	4.541	1.22	7.162

WMP-TEAP (pH 5.0, 80:20) as mobile phase was used on RP-HPLC in isocratic mode for 60 min at a flow rate of 0.70 mL min^{-1}.

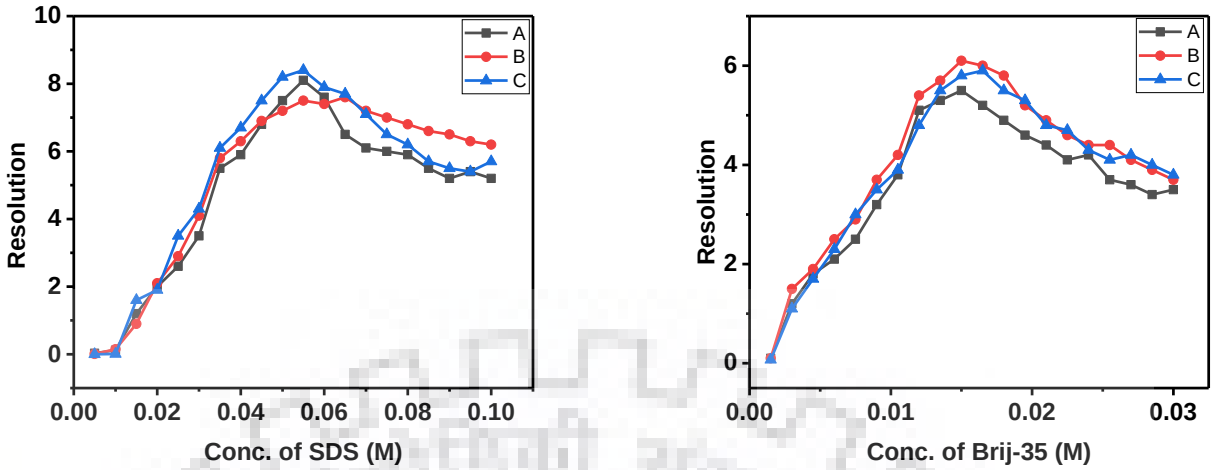


Fig. 6.3. Effect of the concentration of surfactant on separation of diastereomeric derivatives of (*RS*)-SeMet prepared with CDR-2, -4 and -5.

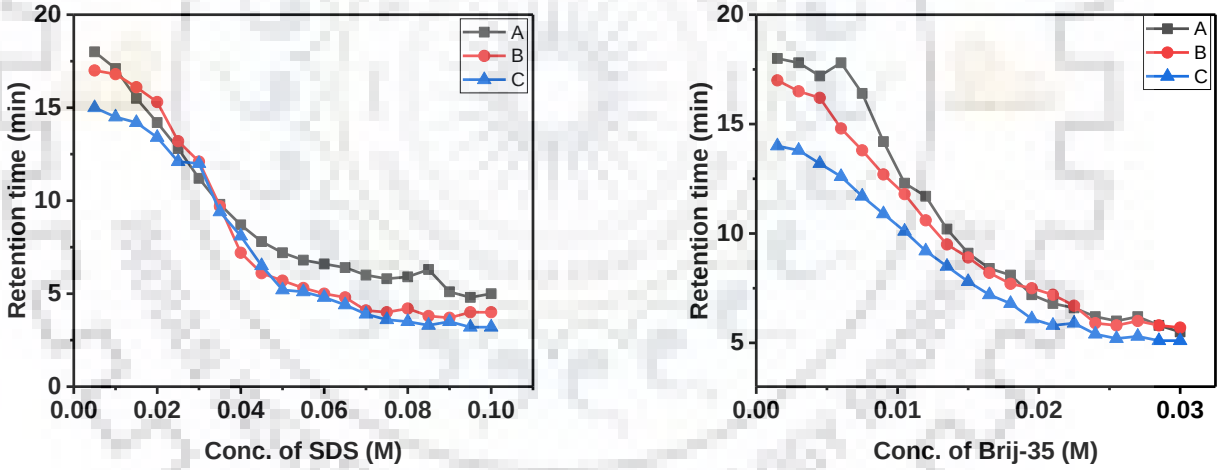
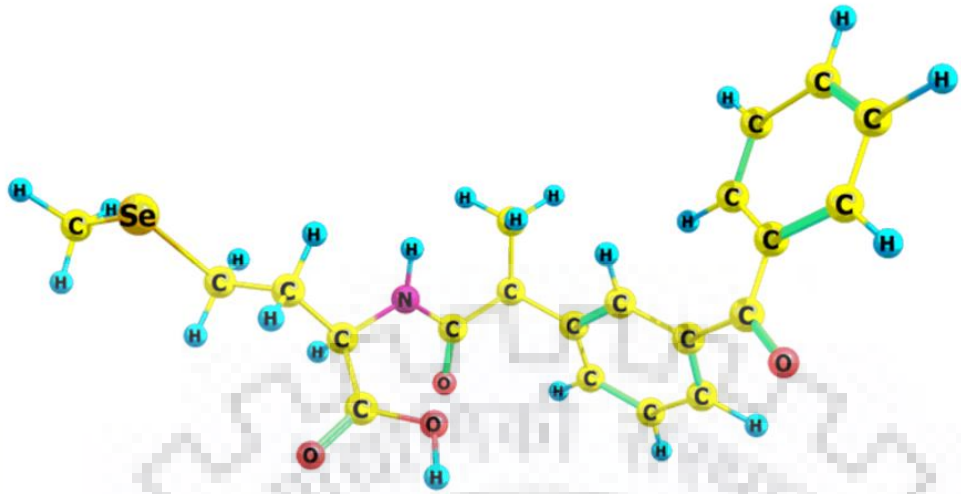
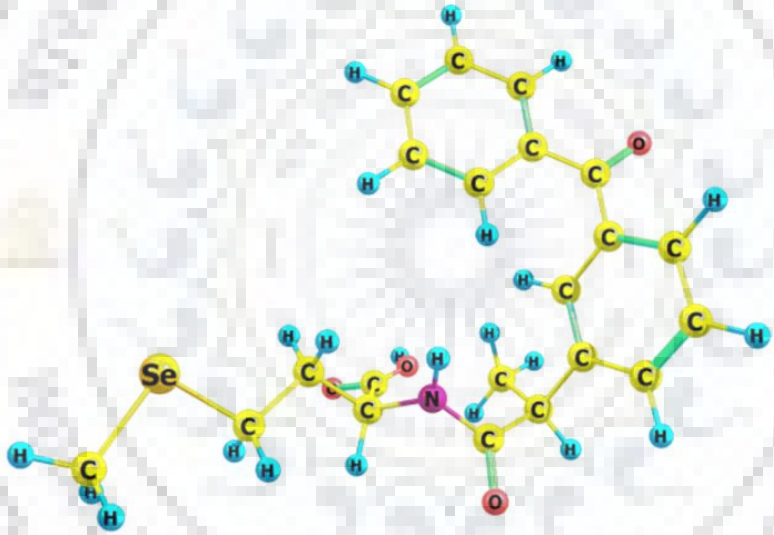


Fig. 6.4. Effect of the concentration of surfactant on retention time of prepared diastereomeric derivatives of (*RS*)-SeMet with CDR-2, -4 and -5.

(In fig. 6.3 and 6.4; A- diastereomeric derivatives of (*RS*)-SeMet prepared with CDR-2; **B-** diastereomeric derivatives of (*RS*)-SeMet prepared with CDR-4, **C-** diastereomeric derivatives of (*RS*)-SeMet prepared with CDR-5.



(i). DsB-15 or (*S*,*R*)-diastereomeric derivative



(ii). DsB-16 or (*S*,*S*)-diastereomeric derivative

Fig. 6.5. Structures of diastereomeric derivatives of (*RS*)-SeMet synthesized with CDR-4, optimized for lowest energy, developed using the program Gaussian 09 Rev. A.02 and hybrid density function B3LYP with 6-31G*: (i) DsB-15 or (*S*,*R*)-diastereomeric derivative (ii) DsB-16 or (*S*,*S*)-diastereomeric derivative.

4. Elution order and the lowest energy optimized structures of the diastereomeric derivatives

Elution order of diastereomeric derivatives (of all the analytes separated under optimized conditions) was determined by comparing the chromatographic behaviour with diastereomeric derivatives prepared from the pure enantiomers, i.e., (*S*)-SeMet, (*R*)-Met, (*S*)-Cys and (*S*)-PenA. It was observed that the derivative of (*R*)-enantiomer of SeMet and Met and the derivatives of (*S*)-enantiomer of Cys and PenA eluted first.

The lowest energy 3D structures of diastereomeric derivatives (DsB-15 and DsB-16) of (*RS*)-SeMet (Fig. 6.5) were developed using Gaussian 09 rev. A.02 and hybrid density functional B3LYP with 6-31G* basis set program to support elution order. The explanation for optimized lowest energy structure of diastereomeric derivatives of (*RS*)-SeMet was same as described in Chapter-5B.

It was observed that the presence of Ibf or Kpf or Lfx moiety in diastereomeric derivatives not only enhances UV-visible sensitivity but also plays a major role in chromatographic separation. The initial configuration of these enantiomerically pure chiral moieties is helpful in determining/establishing the configuration of the two diastereomeric derivatives in each case. In the present studies, diastereomeric derivatives prepared with CDR-2 had the lowest retention time (2.714-4.786 min) as compared to diastereomeric derivatives prepared with CDR-4 (3.741-5.512 min) and CDR-5 (5.023-7.024 min).

5. Stability of CDRs

Stability of the CDRs (2, 4 and 5) was investigated as described in Chapter-4A. The CDRs were found to be stable up to 10 months.

6. Method validation

The validation study was carried out for accuracy and precision for diastereomeric derivatives DsB-15 and DsB-16, as representative, in a concentration range between 30-3000 ng mL^{-1}. The validation data is given in Table-6.2. The calculated recovery values for the first and second eluting diastereomeric derivative, respectively, are 100.24 and 100.01% for intra-day assay and 99.14 and 99.59% for inter-day assay. The LOD and LOQ were found, respectively, 0.295 ng mL^{-1} and 0.896 ng mL^{-1}.

Table-6.2. Method validation for HPLC separation of diastereomeric derivatives of (*RS*)-SeMet prepared with CDR-4.

Linearity	**First eluting diastereomeric derivative (DsB-15)**			**Second eluting diastereomeric derivative (DsB-16)**		
Range (ng mL^{-1})	30-3000			30-3000		
Slope	3.296			3.360		
Intercept	11.28			6.473		
Correlation Coefficient (R^2)	0.999			0.999		
Accuracy and precision						
Conc. Of each diastereomers (ng mL^{-1})	**First eluting diastereomeric derivative**			**Second eluting diastereomeric derivative**		
	Found conc. Mean± SD (ng mL^{-1})	Recovery (%)	RSD (%)	Found conc. Mean± SD (ng mL^{-1})	Recovery (%)	RSD (%)
Intra-day precision						
15	15.16 ± 0.35	101.06	0.89	15.08 ± 0.29	100.53	0.98
75	74.79 ± 0.48	99.62	1.08	75.09 ± 0.38	100.12	1.11
150	149.12 ± 2.35	99.41	1.29	150.65 ± 2.98	100.41	1.06
450	453.01 ± 6.55	100.66	1.64	445.1 ± 6.78	98.99	1.38
1500	1499.2 ± 9.03	99.92	1.72	1500.7 ± 8.32	100.04	1.52
	Mean	100.24	1.32		100.01	1.21
Inter-day precision						
15	14.80 ± 0.23	98.66	1.02	15.18 ± 0.31	101.20	0.92
75	74.65 ± 0.46	99.36	1.06	75.31 ± 0.51	100.41	1.15
150	149.4 ± 1.53	99.60	1.31	144.9 ± 1.46	97.98	1.41
450	441.1 ± 4.58	98.17	1.54	442.1 ± 3.13	98.24	1.68
1500	1498.7 ± 8.12	99.91	1.69	1501.8 ± 6.44	100.12	1.89
	Mean	99.14	1.32		99.59	1.41
Sensitivity						
LOD (ng mL^{-1})	0.295					
LOQ (ng mL^{-1})	0.896					

[n (=5) is the number of replicates, SD = standard deviation, RSD = relative standard deviation]

7. Comparison of present work with literature reports

As compared to previous literature current method is considered green analytical method; because only aqueous micellar mobile phase was used instead of hazardous organic mobile phase which is commonly used in traditional chromatographic methods. The synthesis of CDRs was taking less time as compared to previously synthesized CDRs. This method is advantageous over pervious literature in terms of its requiring less time without organic solvents [84, 85, 89, 125, 160].

As compared to literature, present work shows very good enantioseparation of the diastereomeric derivatives of the AAs under study in terms of resolution (7.162-14.45), separation factor (1.18-1.94), and retention times (2.714-6.910 min). It was observed that the calculated values of resolution (*Rs*), separation factor (α) and retention time were better than previously used CDRs, e.g., DFDNB [85], cyanuric chloride [85], isothiocyanate [84] and (*S*)-naproxen [89, 125], *ortho*-Phthalaldehyde and chiral thiols [154] based reagents; and also the results were found better than direct methods in which β-cyclodextrine [117], and carbamoylated quinidine functionalized monolithic [153] based chiral stationary phase were used. The current method provided very low values of LOD (0.295 ng mL^{-1}) and LOQ (0.896 ng mL^{-1}) as compared to literature [85, 89, 117, 160, 161].

Chapter-7: Conclusion

The work presented herein ensures the success of diastereomeric synthesis and thus establishes reliability of enantioseparation. The confirmation and establishment of absolute configuration of diastereomeric derivatives (so separated) becomes important particularly because most of the time diastereomeric derivative corresponding to pure enantiomer of the analyte is not available. In spite of certain literature reports on enantioresolution of (*RS*)-Prl the thesis presents the first report on recovery of native enantiomers (and the chiral auxiliary) in good yield from the diastereomeric derivatives. This recovery cycle (Fig.4.B6) for chiral auxiliary and native enantiomers opens up new opportunities to develop processes for enantioseparation and for obtaining native enantiomers from their mixtures via application of CDRs.

The work is significant and is of interest to people working in the area of enantioseparation of widely prescribed racemic drugs, synthesis of new CDRs, and determination of absolute configuration of the diastereomeric derivatives; it is because all the reports in literature are limited to synthesis of diastereomeric derivatives and their separation by liquid chromatography and there have been no attempts to verify the configuration of diastereomeric derivatives so separated. The method becomes further significant in view of the fact that (i) direct approach requires expensive HPLC chiral columns which have their own limitations, and (ii) majority of synthetic organic chemists and analytical chemists are not aware that there may be a change in enantiomeric ratio (*er*) value while employing standard chromatography as a purification step before employing their sample to chiral column [162]. High molar absorptivity (ε) due to the presence of (*S*)-Lfx in CDR provided high sensitivity for on-line detection (with LOD values of 1.790 ng mL^{-1} and 2.132 ng mL^{-1}) during HPLC separation and trace analysis of enantiomeric mixture which can be applied for biological samples and in pharmaceutical industry.

The work presented here is a successful and simple method for enantioseparation, by derivatization approach, not only on an analytical scale but also on a preparative level. (*S*)-Lfx based CDRs are esters in terms of nature of chemical bonding introduced during their synthesis by activating carboxyl group by its reaction with *N*-hydroxysuccinimide or *N*-hydroxybenzotriazole in presence of DCC as the coupling reagent.

This method is focussed on analytical separation of diastereomeric derivatives of (*RS*)-Mel and (*RS*)-Atl by RP-HPLC and preparative separation and isolation of diastereomeric derivatives by open column chromatography. For this purpose, two new CDRs were synthesized using (*S*)-Lfx as the chiral moiety and microwave assisted synthesis of diastereomeric derivatives of (*RS*)-Mel and (*RS*)-Atl was carried out. Absolute configuration of the diastereomeric derivatives was established with the help of ^{1}H-NMR (supported by developing the lowest energy optimized structures of the diastereomeric derivatives) to ensure the success of diastereomeric synthesis and to establish the reliability of enantioseparation. It becomes desirable because most of the time diastereomeric derivative corresponding to pure enantiomer of the analyte is not available while for the enantioseparation investigation to be reliable and to apply the said separation method conveniently to determine enantiomeric purity of the pharmaceutical sample it is desirable to confirm the absolute configuration of diastereomeric derivatives so separated at the time of optimizing the separation conditions. The method can be successfully applied for determination and control of enantiomeric purity of (*RS*)-Mel routinely in industries and R&D laboratories (even without resorting to ^{1}H-NMR, and DFT, each time). The method can also be applied for detection of trace amount of amino group containing pharmaceuticals which are marketed and administered as racemic mixture. In comparison to literature reports, the method was successful in providing very low LOD (1.85 ng mL^{-1}) and LOQ (5.62 ng mL^{-1}) values, better resolution (*Rs*), low retention times, and better separation factors (α) for the diastereomeric derivatives.

There has been developed an efficient and 'green' RP-HPLC method for enantioseparation of certain commonly used β-adrenolytics. Application of ultrasonic and microwave irradiation, and replacement of hazardous reagents and organic solvents in the synthesis (of CDRs and diastereomeric derivatives) and use of C_{18} column and water micellar mobile phase without any organic solvent has the advantages of waste reduction, process economy and use of non-toxic and environment friendly solvents with low cost, low toxicity and possibility of simultaneous separation of ionic and non-ionic compounds. It can be successfully applied for determination of trace amount and control of enantiomeric purity of amino group containing compounds from organic syntheses or the pharmaceutical industries which are marketed and administered as racemic mixture. Present method provides chirality recognition even in the absence of pure enantiomers, as supported by the lowest energy optimized structures obtained by the DFT based software.

In comparison to literature reports, the method was successful in providing very low LOD and LOQ values, better resolution, low retention times, and better separation factors (α) for the diastereomeric derivatives. The method leads to a new opportunity and scope to modify liquid chromatographic chiral separations in *green* ways.

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