ENANTIOMERIC RESOLUTION OF RACEMIC PHARMACEUTICALS

Ph.D. THESIS

by

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

Submitted in partial fulfilment of the requirement for the award of the degree

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ROORKEE

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"ENANTIOMERIC RESOLUTION OF RACEMIC PHARMACEUTICALS"** 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 December, 2014 to April, 2018 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.

(POONAM)

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

Dated:

(Ravi Bhushan)
Supervisor

Abstract

A brief description of every chapter is described below.

The **first chapter** describes importance of enantiomeric drugs, different methods of enantioseparation, role of liquid chromatography in enantioseparation, ligand exchange chromatography, and comparison between chiral stationary phase and chiral derivatizing reagents. The work presented in this thesis has also been introduced in this chapter that includes selection of racemic analytes, chiral selectors, chiral reagents and methodology adopted.

Second chapter contains details of experimental work done that includes instruments required, all chemicals and reagents used, racemic analytes taken for analysis studies along with the method used for recovery and purification of title compounds from the commercial formulations, preparation of stock solutions and the datails of chiral reagents with their characterization data. The procedure for synthesis of chiral regent (N-hydroxybenzotriazolyl-(*S*)-(+)-Npx) i.e. CR-1 also has been described.

Third chapter deals with studies on enantioseparation of β-adrenolytics (namely, bisoprolol, salbutamol, carvedilol, atenolol and propranolol) by liquid chromatography. It introduces the pharmaceutical importance and literature on enantioseparation of the chosen β-adrenolytics. This chapter is divided into two parts.

Part-I describes enantioseparation and sensitive detection of three β-adrenolytics/β_2-agonists (bisoprolol, salbutamol, and carvedilol) in human plasma with minimal sample clean-up by preparative TLC and RPHPLC via an indirect approach using a single enantiomer reagent [N-hydroxybenzotriazolyl-(*S*)-(+)-Npx, i.e. CR-1] along with determination of absolute configuration of diastereomeric derivatives by recording their ^{1}H NMR spectra. Experimentaldetails with results and discussion and validation of method (as per FDA guidelines) have been described under this part.

Part-II describes direct resolution of the chosen β-adrenolytics and a*β2*-agonist (bisoprolol, salbutamol, carvedilol, atenolol and propranolol) by TLC using bovine serum albumin (BSA) as '*chiral additive in stationary phase*' (CASP) and achiral mobile phase (having no external chiral additive) along with isolation of the native enantiomers. Details of experimental work done with results and discussion have been given.

The **fourth chapter** deals with studies of enantioseparation of nonsteroidal anti-inflammatory drugs (NSAIDs) i.e. ketorolac and etodolac by liquid chromatography. It also introduces the pharmaceutical importance and literature on enantioseparation of the chosen NSAIDs. This chapter is divided into two parts.

Part-I deals with semipreparative HPLC enantioseparation of both the analytes by synthesizing their diastereomeric anhydrides with CR-1. The diastereomeric derivatives were separated on analytical scale by RPHPLC and then these were isolated by preparative HPLC.The diastereomeric derivatives were characterized and their absolute configuration was established. Hydrolysis of the derivatives provided easy recovery of native enantiomers under mild reaction conditions without racemization. Molecular asymmetry was established using ^{1}HNMR and polarometric studies.

Part-II describes direct resolution of (*RS*)-Ket and (*RS*)-Etd by ligand exchange chromatography using three enantiomerically pure amino acids [namely, L-tryptophan (Trp), L-histidine (His) and L-phenylalanine (Phe)] as chiral ligands and Cu(II) as a bivalent complexing ion.Method for isolation of diastereomeric complexes corresponding to each of the enantiomers (of the two racemates) was developed via preparative TLCand their specific rotations were determined. The native enantiomers were obtained from the isolated ligand exchanged diastereomeric complexes.

Complete procedure for enantioseparation of these two title analytes via both techniques (preparative HPLC and LEC) including experimental work, results and discussion and validation (as per ICH guidelines) is described in this chapter.

Chapter five describes enantioseparation of fexofenadine (Fxn) (H2-antihistamine) by liquid chromatography and its enhanced detection with mass spectrometry. This chapter also pharmaceutical importance and literature on enantioseparation of (*RS*)-Fxn. Derivatization of racemic (*RS*)-Fxn was carried out using three chirally pure amines (CR 2-4) and (*S*)-naproxen based CR (CR-1) yielded corresponding diastereomeric amides and anhydrides respectively. The structures and configurations were verified using LC-MS, ^{1}HNMR spectrometry, Chem3D Pro 12.0 software, and the software Gaussian 09 Rev. A. 02 program and hybrid density functional B3LYP with 6-31G basis set supplemented with polarimetry. The structural features and separation characteristics of the two types of diastereomeric derivatives have been compared. The method was also validated in accordance with ICH guidelines.

Sixth chapter is about development of bovine serum albumin bonded silica gel based new chiral stationary phase (CSP). It was characterized by scanning electron microscopy (SEM), CHNS, FTIR, UV-Vis and XRD. Its efficiency of enantioseparation was checked by using three racemates (propranolol, atenolol and phenylalanine) in 30 mg to 3.0 g quantitatively on simple open column. This shows the superiority of new CSP in terms of cost and simplicity and for direct enantioseparation by open column.

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I express my thanks to Chhavi, Ballu, Neha, Mamta, Sheela and Khushbu whose gift of company has made my first time journey to live in hostel, a wonderful and unforgettable memory. My special thanks are due for Ms. Shweta Bajaj for being my close friend and helping me to keep my strains off, for all the fun we had together.

I take this opportunity to extend my deepest sense of indebtedness to my parents for their endless and everlasting affection. I may never repay for the sacrifices of my mother to nourish my career with unflinching dauntless in spite of all the hardships in her life. My silent tears always feel special blessings of my father's soul from heaven. I don't have proper words to express my sentiments to my brother Manish and sisters Priyanka and Priya who always made me cheerful. My special thanks are reserved for Mr. Sunil Kumar for being my best counselor and playing a role of father, mother, brother and friend whenever I needed. His morale boosting behavior and steadfast support will keep safe in my good spirit.

Now peacefully I take a minute simply to thank God & His amazing worthy power, with all my heart and inner spirit, for strengthening me in my purposes, I need You, this day and every day; please be with me in every way and crown me with your unwavering grace and mercy.

Dated: POONAM

Candidate's declaration

Abstract

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References

List of Publications

1. **Poonam Malik**, Ravi Bhushan. Development of liquid chromatographic methods for enantioseparation and sensitive detection of β-adrenolytics/β2-agonists in human plasma using a single enantiomer reagent, *Journal of Chromatography B* 1061–1062 (2017) 117–122. (*cf*: chapter-**3**)

2. **Poonam Malik** and Ravi Bhushan. Synthesis of diastereomeric anhydrides of (*RS*)-ketorolac and (*RS*)-etodolac, semi-preparative HPLC enantioseparation, establishment of molecular asymmetry and recovery of pure enantiomers. *New Journal of Chemistry*41(2017) 13681-91. (*cf*: chapter-**4**)

3. **Poonam Malik** and Ravi Bhushan. Thin Layer Chromatographic Resolution of Some β-adrenolytics and a β2-Agonist Using Bovine Serum Albumin as Chiral Additive in Stationary Phase. *Journal of Chromatographic Science* 56 (2018) 92–98. (*cf*: chapter-**3**)

4. **Poonam Malik** and Ravi Bhushan. Enantioseparation of (*RS*)-fexofenadine and enhanced detection as the diastereomeric amide and anhydride derivatives using liquid chromatography-mass spectrometry. *Biomedical chromatography*32 (2018) pp in press--;DOI: 10.1002/bmc.4217.(*cf*: chapter-**5**)

5. **Poonam Malik** and Ravi Bhushan. Ligand exchange thin layer chromatographic enantioresolution of (*RS*)-Ketorolac and (*RS*)-Etodolac and recovery of native enantiomers. *Journal of Chromatographic Science*, 2018. Manuscript ID: JCS-17-330. (*cf*: chapter-**4**)

6. **Poonam Malik** and Ravi Bhushan. Development of bovine serum albumin bonded silica as chiral stationary phase and its application in quantitative direct enantiomeric resolution.*Organic Process Research & Development*, ms ID: op-2018-000655.(*cf*: chapter-**6**)

7. Manisha Singh, **Poonam Malik**, and Ravi Bhushan. Resolution of Enantiomers of (*RS*)-Baclofen by Ligand-Exchange Thin-Layer Chromatography. *Journal of Chromatographic Science* 54 (2016) 842-846.

8. **Poonam Malik**, Anu Dalal, and Ravi Bhushan. Enantioseparation of (*RS*)-Bupropion and determination of configuration. *Journal of Liquid Chromatography & Related Technologies* 41 (2018) 155-160.

Chapter-1

Introduction

Enantiomeric drugs and related issues

In a thought provoking statement, E. J. Ariëns in 1984 wrote, "It is surprising that many biologists, pharmacokineticists and clinical pharmacologists operate on a pre-Pasteur level with regard to stereochemistry. This implies that unless having been explicitly noticed, they tend to deal with mixtures of isomers as if one compound were involved. Once the mixture is given the name by WHO that name and the brand name stand for the 'drug'…. It is not surprising that a physician, presented with such a drug under a brand name is unaware of isomers. It is understandable but unacceptable that then he makes mistakes"[1].

The enantiomers in the finished drug formulations (marketed as racemates) show major differences in pharmacological properties, pharmacokinetic disposition, metabolic or toxicological activity in stereospecific biological environment because the specific enantiomer of the drug exhibits large bioavailability, high effectiveness and few side effects; it is all due to their different steric molecular architecture.

Since the commonly used drugs were racemates there was triggered a debate among pharmacologists and medicinal chemists, it alerted the drug regulators too. The most infamous example of an enantiomeric drug having unwanted side effects in the background was that of thalidomide [2].The USFDA announced the policy statement in 1992 restricting the marketing of racemic drugs [3]. Later in 1996, European Federation of Pharmaceutical Industries and Associations (EFPIA), US FDA, Pharmaceutical Research and Manufacturers of America (PhRMA) and Japan Pharmaceutical Manufacturers Association (JPMA), under the aegis of International Conference on Harmonization (ICH) produced a comprehensive set of guidelines for registration of new chiral pharmaceuticals [4]. The regulatory agencies across the globe demand, from pharmaceutical industries, detailed investigations of both the enantiomers of a potential drug.

The global chiral technology developments include chiral synthesis, chiral resolution, and chiral analysis. According to "Transparency Market Research" [5], the global market of chiral technology was $4.75 billion in 2010 and generated more than$5.83 billion in 2017.

Methods of resolution

The methods of enantioseparation, particularly by liquid chromatography, are generally classified as direct and indirect.

Direct approach: The direct approach may further have different strategies, e.g., (i) use of a stationary phase which is chiral for its structural feature (commonly termed as CSP), and (ii) use of a chiral additive in the mobile phase with achiral stationary phase. And thirdly, (iii) anotherstrategy under direct approach involves (non-covalent) use of '*chiral additive in achiral stationary phase*' (CAASP) and is thus suitable for TLC, specially. Both in (i) and (iii) the mobile phase remains achiral because there is no external chiral additive in it.

Indirect approach: The indirect approach with a large number of papers appearing in literature on development of novel chiral reagents (CRs) and their application for enantiomeric separations reflect the importance of covalent chiral derivatization of racemates towards a solution to practical problems in pharmaceutical and biomedical analysis. The derivatization leads to formation of diastereomeric derivatives and enhances the detectability by introducing chromophoric or fluorophoric groups into enantiomeric molecules. The CRs are selected or synthesized to make available their property of high molar absorptivity (ε) or high fluorescence quantum yield (φ) to the enantiomeric pair via coupling and to provide ultra violet or visible absorption, fluorescence, or chemiluminescence for highly sensitive on-line detection (of diastereomeric derivatives) commonly done during HPLC separation. UV remains the simplest and the least expensive among different types of detectors available (particularly, in HPLC). The separation is performed on easily available, more rugged and stable achiral phase in a very economical manner. Separation of diastereomeric pair via indirect technique is thus simpler to perform.

Liquid chromatography in enantioseparation: Liquid chromatography (LC) has been extensively employed for chiral separation and detection of the products of organic synthesis, especially enantioselective synthesis, and biological molecules and racemic drugs in the areas of pharmaceutical and biotechnological research & development.

Among the liquid chromatographic (LC) techniques, the advantages of thin-layer chromatography (TLC) include low cost, simplicity of the method requiring little instrumentationand ease of control of experimental strategies and optimization with the advantage that the chromatogram is photographed as a clearly visible evidence of separation. The method holds considerable significance especially in resource limited settings. Separation, detection, isolation, identification, purification, and quantification, of each of the enantiomers of racemic analytes comprise an example of the comprehensive use of analytical TLC. It is a consistent technique and a method of choice in various situations for routine analysis of drugs, pharmaceuticals, products of enantioselective synthesis and detection of counterfeit drugs for regulatory purposes [6]. The compounds separated by TLC can be identified and quantified in many ways, using either off-line or online detection. Sherma [7-8] reviewed literature on application of TLC for enantioresolution in his regular biennial reviews along with advantages of modern TLC in pharmaceutical and drug analysis, comparing to (HPLC) and HPTLC as well.

Fast and efficient separation can be achieved by HPLC method using a fused-core silica particle packing for the analysis of pharmaceutical compounds. Fused-core particles are produced by "fusing" a porous silica layer onto a solid silica particle. The columns packed with 2.7 μm "fused-core" particles (a 0.5 μm porous shell fused to a solid 1.7 μm silica core particle) result in to fast separations with modest operating pressure. The fused-core silica materials providing the shorter diffusional mass transfer path for solutes are less affected in resolving power by increase in mobile-phase velocity than the sub-2 μm porous silica packings resulting in faster separation and higher sample throughput.

Both TLC and HPLC, in practice, supplement each other and are complementary rather than competing, for purposes of analytical research and routine quality control of pharmaceuticals.

Enantioseparation via ligand exchange in LC: Among the various methods and approaches, chiral ligand-exchange chromatography (CLEC) is one of the earliest and the most effective method of direct enantioseparation. It was developed (during 1968-71) when Davankov and Rogozhin modified commercial HPLC columns by coating alkyl derivatives of α-amino acids such as *n*-decyl-L-histidine or *n*-hexadecyl-L-proline, onto C_{18} silica for enantiomeric resolution [9-11].

Based on the work of Davankov, ligand exchange TLC was developed during the period 1984-1986at Degussa AGby Günther, Martens *et al*., [12-15] and resolution of enantiomeric amino acids was achieved. The RP C_{18} silica gel plates were impregnated by sequential immersion in the solutions of Cu(II) acetate and the new chiral selector namely (2*S*,4*R*,2'*RS*)-*N*-(2'-hydroxy dodecyl)-4-hydroxy proline [16]. During the same year (1984) Weinstein [17] reported TLC enantiomeric separation of all the dansyl protein amino acids, except proline, based on ligand exchange using RP plates pre-treated with a copper (II) complex of N,N-di-*n*-propyl-L-alanine. Although the procedure differed from that reported by Günther, Martens *et al*., [12-15] in the choice of chiral selectors and range of applicability, both had a very similar methodology.

CSPs vs CRs: Both direct and indirect approaches for enantioseparation, using CSPs or CRs respectively, have their own advantages and limitations depending upon the situation, particularly, in terms of source and amount of sample availability, chemical structure of the analyte and the ease of availability of laboratory facilities. Of the various types of CSPs, Chiralcel OD or cellulose based CSPs have most commonly been used and recommended by the manufacturers for direct (without resorting to derivatization) enantioresolution of β-adrenolytics and at the same time the '*guide to users*' provided by the manufacturers have clearly advised that one can use only CH_3CN, EtOH or CH_3OH as the mobile phase, use of alcohols causes back pressure, phosphate buffer for pH >8 should

not be used as the basic conditions damage the silica gel matrix and high percentage of organic modifier may precipitate the buffering salt leading to irreversible clogging of column. These or any other types of CSPs (e.g., Lux Amylose or Cellulose, or *α-Burke phase* derived from dimethyl N-3,5-dinitro-benzoylamino-2,2-dimethyl-4-pentenyl phosphonate covalently bound to 5 µm mercaptopropyl silica, or cyclodextrin, or AGP or Ovomucoid based CSPs) are expensive to the extent that they are out of reach of majority of small laboratories and Institutes and have a short life with limited applications and do suffer with one or more such types of limitations (the description is out of the scope of the thesis). Besides, any enantioresolution using such CSPs, in one opinion, is by no means an innovative or research oriented approach because most of the details of experiments for a set of known compounds are provided by the manufacturer in limited boundaries and the experimentalist has no opportunity to apply skills of chemistry in a different or novel manner.

On the other hand, in the indirect approach the experimentalist as a chemist has to look into functional group compatibility of the analyte and the CR and has to establish their reaction conditions. The objective of the research oriented journals is not to add analytical data for an enantioresolution using the commercial CSPs but also to promote some chemistry involved. In case of using commercial CSPs, the chemistry is behind the scene, i.e., in the development of a new successful CSP which is with the manufacturers.

Relevance to present work: The two enantiomers of a pharmaceutically active compound should, in fact, be considered as different drugs because of significant differences in their pharmacodynamics and pharmacokinetic profiles. With such an increasing awareness of these issues among those involved in the drug development (including enantioselective synthesis), marketing and law enforcement the importance of developing simple methods of enantioseparation and control of enantiomeric purity of such pharmaceuticals cannot be overemphasized. The correctness of the enantiomeric excess (*ee*) reported for an enantioselective synthesis can be considered as authentic only if it is determined via enantioseparation soon after the step of synthesis and prior to any purification step by ‘normal’ chromatography [18].

Thus, there continues a strong need to develop rapid and reliable methods that can be used for verification of enantiomeric purity or to monitor stereoselective synthesis.

Preamble to work presented in this thesis

Taking into account various aspects (as a few of these discussed above) of racemic pharmaceuticals and their resolution methods and the current literature in the area it was considered to undertake resolution studies on certain common pharmaceuticals, marketed and administered as racemates.

(a) Selection of racemic analytes

The racemic analytes chosen for these studies are (*RS*)-bisoprolol, (*RS*)-salbutamol, (*RS*)-carvedilol, (*RS*)-propranolol,(*RS*)-atenolol,(*RS*)-ketorolac, (*RS*)-etodolac, (*RS*)-fexofenadine and DL-phenylalanine (**Fig 1.1**). (*RS*)-propranolol, (*RS*)-atenolol and DL-phenylalanine were purchased from Sigma–Aldrich and rest were available commercially, pharmaceutical tablets of which were purchased from the the pharmacist's shop in the local market. (*RS*)-bisoprolol, (*RS*)-propranolol and (*RS*)-atenolol belong to β_1-adrenolytics; (*RS*)-salbutamol belongs to β_2-adrenolytic; (*RS*)-carvedilol belongs to both β_1 and β_2-adrenolytic; (*RS*)-ketorolac and (*RS*)-etodolac are non-steroidal anti-inflammatory drugs; (*RS*)-fexofenadine is H2-antihistamine and DL-phenylalanine is an essential α-amino acid with phenyl group substituted in place of terminal hydrogen of alanine.

The information on the pharmaceutical importance and literature with respect to enantioseparation of the chosen analytes has been given in respective Chapters dealing with present work on their enantioseparation.

(b) Selection of chiral selectors

Different chiral selectors were chosen for enantioseparation investigations depending upon the nature /approach of their application, as briefly mentioned below.

1. Chiral selectorfor synthesis of chiral reagent (CR-1)

(*S*)-(+)-Naproxen (Npx) was selected as the chiral moiety to synthesize CR for the following characteristics. (*S*)-(+)-Npx (an aryl propionic acid, belongs to the class of a non-steroidal anti-inflammatory drugs) was chosen to synthesize **CR-1** for its large conjugated naphthyl ring having higher molar absorptivity (Ɛ >100,000) in comparison to certain other chromophoric molecules, e.g., (*S*)-levofloxacin (Ɛ~24000), *s*-trichlorotriazine (Ɛ~36000), and dinitro phenyl moiety (Ɛ~30000 mol^{-1} cm^{-1}), it is available easily as pure (*S*)-enantiomer and does not require any other chiral auxiliary (as is required by dinitro phenyl or *s*-trichlorotriazine moieties for converting them into chiral reagent), and the carboxylic group of Npx is easily activated by introducing different nucleophilic moieties which serve as good leaving groups in a subsequent nucleophilic substitution reaction for synthesis of diastereomeric derivatives. Higher molar absorptivity facilitates sensitive detection of the derivatized analyte.

2. Chiral selector used as additive and as covalent linker to silica gel

Bovine serum albumin (BSA) was used as a chiral additive in simple silica gel for TLC (the stationary phase, CASP) and as a covalent linker to silica gel for developing a CSP. Both the materials were used for enantioseparation of β-adrenolytics. BSA was selected due to the following characteristics.

BSA is a giant globular protein; it has a molecular weight of about 66000 Dalton (D) and contains about 607 amino acid residues in a single polypeptide chain and no carbohydrates [19]. At pH 5-7 it contains 17 intrachaindisulfide bridges and one sulfhydryl group. The *isoelectric point* of the protein in water at 25 °C is 4.7. BSA is easily available at low costs. BSA is a serum protein that binds mostly acidic and neutral drugs. It behaves as a chiral complexing agent. Direct enantioseparation by liquid chromatography based on enantioselective properties of certain proteins, has been found to be useful with diversity and variety in analytical applications.

It has various biochemical and biotechnological application. It is also not so expensive, since large quantities of it can be readily purified from bovine blood, a

byproduct of the cattle industry. BSA found analytical applications for enantiospecific determinations of chiral drugs. BSA is a serum protein that binds mostly acidic and neutral drugs. Direct enantioseparation by liquid chromatography based on the enantioselective properties of a proteins, has been shown to be a very versatile method in HPLC with the availability of different protein bound CSPs.

3. Chiral selectors used as the chiral dopant

The three essential α-amino acids were chosen for making ligand exchange reagents (LERs) with complexing agent Cu(II) acetate. These are L-tryptophan, L-histidine and L-phenylalanine that contain an α-amino group, an α-carboxylic acid group, and a side chain containing indole, imidazole and phenyl group, respectively.The pI value of tryptophan is 5.9 with $pKa_1$2.4, pKa_2 9.4; pI of His is 7.6 with $pKa_1$1.8 and $pKa_2$9.2 with side chain pKa_3 value 6.1; and pI of Phe is 5.5 with pKa_1 2.1 and $pKa_2$9.1 with side chain pKa_3 value 6.0. These were used as a doping agent for enantioseparation of NSAIDs (ketorolac and etodolac).

(c) The chiral reagents

N-hydroxybenzotriazolyl-(*S*)-(+)-Npx (CR-1), was synthesized by the reaction of chromophoric moiety (*S*)-(+)-Npx with hydroxy-benzotriazole in the presence of DCC.

Besides, chirally pure amines, (i) (*S*)-(−)-1-(1-napthyl) ethylamine, (ii) (*S*)-(+)-1-benzyl-3-aminopyrrolidine, and (iii) (*S*)-(−)-α,4-dimethylbenzylamine were used directly as CR-2, CR-3 and CR-4, respectively; these were obtained from Sigma-Aldrich.

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15. Detection and analysis of diastereomeric derivatives in trace amount

16. Development of bovine serum albumin bonded silica gel as chiral stationary phaseand its application to direct quantitative enantiomeric resolution, by open column.

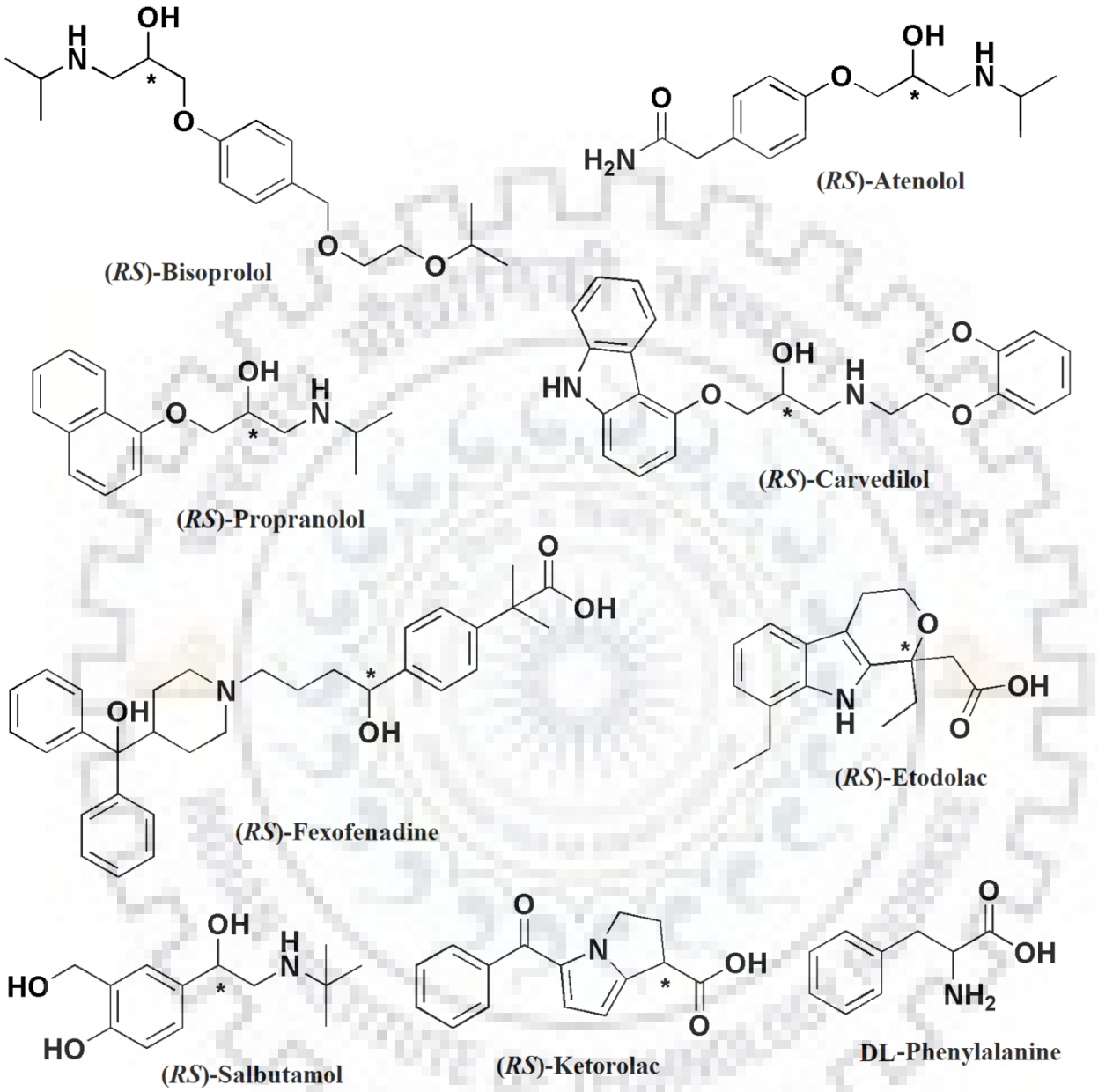


Fig. 1.1: Structures of chosen racemic analytes (β-Adrenolytics, NSAIDs, Antihistamine and Amino-acid).(*Asterics represent the stereogeniccentre in each analyte).

Chapter-2

Experimental

A. Instrumentation

1. HPLC system

The HPLC system (LC-20 AD) was obtained from Shimadzu (Kyoto, Japan), that 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 (PDA) detector, SPD-20A/20AV (UV-VIS Detector), CTO-20AC column oven and LC solution, DAO (data access objects) 3.5 operating software and LiChrospher C_{18} column (length × i.d., 25 cm × 4.6 mm, 5 μm particle size), analytical column (L x i.d. 10 cm x 4 mm, 5 μm particle size, α_1-AGP column was obtained from Chromtech Merck (Darmstadt, Germany).

2. Preparative HPLC system

The preparative HPLC system (LC-20AP Prominence preparative liquid chromatograph) was obtained from Shimadzu (Kyoto, Japan) that consisted of aDGU-20A5R degassing unit, SPD-M20A diode array detector containing D2 lamp, RID-10A referactive index detector, FRC-10A fraction collector containing RACK 2A L, CTO-20 A column oven,CBM-20A communications bus module, SIL-10AP auto sampler, Enable LiChrospher HPLCC_{18} phase preparative column of 30 mm internal diameter and 25 cm length combinations, aswell as having an assortment of 5 μm particle size.

3. LC-MS system

An LC-MS-8030 (Shimadzu Corporation, Kyoto, Japan) fitted with ESI-MS (triple quadrupole) laboratory solution software.

4. Other equipments used

Microwave (Multiwave 3000, 800 W, Perkin-Elmer, Shelton, CT, USA), pH meter (Cyberscan 510, Singapore), CHNS elemental analyzer (Elementar, Vario MICRO cube,

Hanau, Germany), FT-IR spectrometer (Nicolet-6700, Thermo Scientific, USA) with a liquid-nitrogen-cooled MCT detector, an elemental analyzer (Vario EL III, Hanau, Germany), Scanning Spectrophotometer, automatic digital polarimeter (Krüss, model P3001RS, Germany) coupled with a 10 cm tube under sodium lamp irradiation, Scanning Electron Microscope (Hitachi S-700, Tokyo, Japan), centrifuge (REMI-PR-24, Maharashtra, India), commercial TLC (ALUGRAM RP-18W/UV_{254}; Germany), HRMS [Bruker micrOTOF^{TM}-Q II mass spectrometer (ESI-MS)and an NMR spectrometer 400 MHz (Jeol Inc., Peabody, USA) and Bruker 500 MHz instrument using $CDCl_3$, MeOD, and DMSO. UV spectra were recorded in CH_3OH (using Shimadzu, UV-2450 spectrophotometer, Tokyo, Japan).

Structural confirmation of surface was done by collecting patterns of Powder X-ray diffraction studies (Bruker D8 Advance, Billerica, Massachusetts, USA) with Cu Kα radiation (λ = 1.54 A°) on samples taken at regular intervals.

Double distilled water was further purified using a Milli-Q system from Millipore (Bedford, MA, USA) to obtain purified water (18.2 MΩ cm), which was used throughout the experiments.

B. Chemicals and reagents

The solvents employed [ethanol (EtOH), glacial acetic acid (HOAc),ethyl acetate (EtOAc), dichloromethane (CH_2Cl_2), chloroform ($CHCl_3$), acetonitrile (CH_3CN), absolute ethanol (EtOH)and methanol (CH_3OH)] of HPLC grade were purchased from SISCO Research Laboratory (Mumbai, India), E. Merck (Mumbai, India) and BDH (Mumbai, India).

The reagents dimethyl aminopyridine, triethylamine, sodium bicarbonate, phosphoric acid,citric acid, nitric acid, concentrated HCl, sodium bicarbonate ($NaHCO_3$), 1,4-dibromobutane, diethyl malonate and hydrazine hydrate (synthesis grade), dimethylamino pyridine (DMAP), silver nitrate, copper sulphate, benzene, ammonia, sodium hydroxide, dibasic sodium phosphate, ninhydrin and phosphoric acid (H_3PO_4) of

analytical reagent grade were purchased from E. Merck (Mumbai, India). Sodium metal (Spectrochem, Mumbai, India), 2-propanol (Showa, Japan), silica gel G (pH 7.0) having 0.02% impurities of lead,iron and chloride in a 10% aqueous suspension and 13% calcium sulfate as binder (E. Merck, Mumbai, India) and silica gel of particle size 60–120 mesh (125-250 μ) were purchased from (SISCO Research Laboratory, Bombay, India).

Dicyclohexylcarbodiimide (DCC), MW: 206.33 assay 99%; *N*-hydroxybenzotriazole (OH-Btz), MW: 135.12, assay 97%; Bovine serum albumin (BSA) Cohn fraction V, MW: 66,000 Da, assay ≥ 96-99 %; (*S*)-(+)-Naproxen, $[\alpha]_D^{25}$ = (+) 66°, (c =1, $CHCl_3$), MW: 230.26, *assay* ≥ 98.0%, were purchased from Sigma–Aldrich (St. Louis, MO, U.S.A.).

C. The Analytes

The racemic analytes were extracted and isolated from commercially available pharmaceutical tablets; the tabletswere purchased from the the pharmacist's shop in the local market sold by different companies.The common names, names of manufacturer along with brand names and IUPAC names are given below.

1. (*RS*)-Bisoprolol, (*Concor* (Merck Ltd, Waluj, Aurangabad, India), [(1-(4-((2-isopropoxyethoxy)methyl)phenoxy)-3-(isopropylamino)propan-2-ol)]
2. (*RS*)-Salbutamol:(*Asthalin-SA*(Cipla Ltd., Mumbai, India), [(4-(2-(tert-butylamino)-1-hydroxyethyl)-2-(hydroxymethyl)phenol)]
3. (*RS*)-Carvedilol:(*Carca* (IntasPharma, Ahmedabad, India), [(1-((9H-carbazol-4-yl)oxy)-3-((2-(2-methoxyphenoxy)ethyl)amino)propan-2-ol)]
4. (*RS*)-Ketorolac:(Ketorol DT (Dr Reddy Laboratories Ltd, Hyderabad, India)
5. (*RS*)-Etodolac:(Etova (Ipca Laboratories Ltd, Mumbai, India)
6. (*RS*)-Fexofenadine: (Allegra (Sanofi India Ltd., Ankleshwar, India)

Following racemic analyteswere purchased from Sigma–Aldrich (St. Louis, MO, U.S.A.),

7. (*RS*)-Propranolol: MW: 295.80, assay 99%
8. (*RS*)-Atenolol: MW: 266.34 assay 98%
9. DL-Phenylananine: MW: 165.19 assay 99%

Chirally pure (*S*)-propranolol, m.p., 190-192ºC; $[\alpha]_D^{25}$= –25.1º (c =0.01, EtOH); (*S*)-atenolol, m.p., 148-152ºC; $[\alpha]_D^{25}$= –16º (c =1, HCl) and L-phenylalanine = m.p., 275ºC; $[\alpha]_D^{25}$= – 35º (c =2, H_2O) were purchased from Sigma–Aldrich.

For convenience of representation in the thesis the analytes were suitably abbreviated as follows;

(*RS*)-Bisoprolol (Bpl); (*RS*)-Salbutamol (Sbl); (*RS*)-Carvedilol (Cdl); (*RS*)-Ketorolac (Ket); (*RS*)-Etodolac (Etd); (*RS*)-Fexofenadine (Fxn); (*RS*)-Propranolol (Prl); (*RS*)-Atenolol (Atl) and DL-Phenylananine (Phe).

D. Recovery and purification of title compounds from commercial formulations

The method for recovery and purification (*RS*)-Bpl is described below as a representative: The coating of ten *Concor* tablets (each containing 5 mg) was scratched out and these were finely powdered in mortar. The powder was suspended in 20 mL methanol and was sonicated for about 10 min at room temperature. It was filtered and the residue obtained was further extracted with methanol. Both combined filtrates were concentrated and kept in the refrigerator until crystals appeared. The crystals were washed with diethyl ether and dried in a vacuum desiccator.

The active pharmaceutical ingredient (API) of the respective tablets of each remaining analyte/drug was extracted with methanol by sonicating (for nearly 20 min) the coating removed and finely crushed ten tablets; it was isolated and purified as per the above detailed experimental procedure.The sample of ketorolac tromethamine was protected from light throughout the experiments, and was preserved in tight, light-resistant containers at room temperature.The recovery was 98 % against the amount reported on commercial labels in each case. These compounds were characterizedand and were used as standard reference for experiments of enatioresolution.Calibration plots were developed by using the standard references.

The same method was followed for the remaining commercial samples that were used as analytes. The number of tablets used for extraction was different in each case,

depending upon the labelled amount,so that at least 200 mg of the racemate was available.

Separation of tromethamine from ketorolac: (*RS*)-Ket was extracted by sonicating ketorolac tromethamine salt (as obtained above) for about 5 min in dichloromethane (DCM). It was filtered. Though Ket is miscible in DCM while tromethamine is not; the filtrate was concentrated and kept in refrigerator untilcrystals appeared. The crystals were washed with cold DCM and dried in vacuum desiccator. The isolated and purified Ket was characterized by UV (λ_{max}~316 nm) and IR.

E. Stock solutions

- 0.1 M $NaHCO_3$ (prepared by dissolving 0.84 g $NaHCO_3$ in 100 mL distilled water)
- (*RS*)-Bpl (1 mM) in 0.1 M $NaHCO_3$; stock solution was diluted with plasma for preparing required working solutions,
- Stock solutions of (*RS*)-Bpl, Atl, Prl, Sbl, Cdl, Ket and Etd in $CH_3OH$and(*RS*)-Fxn in THF(each 10mM) and (1 mM each) in case of (*RS*)-Ket and (*RS*)-Etd with 0.1 M $NaHCO_3$ were prepared and further diluted with CH_3OH(5×10^{-2} M) for required working solutions.
- Acetate buffer (0.05 M) in purified water with pH adjusted to 4.2
- Triethylammonium phosphate (TEAP) buffer (10 mM) (prepared with 560 μL triethylamine in 400 mL distilled water); solutions of pH 3.7, 3.5 or 7.5 were preparedusing 84% phosphoric acid.
- Solution of CR-1 (Npx-OBtz) (10 mM) was prepared in CH_3CN and of three chirally pure amines i.e. (*S*)-(−)-1-(1-napthyl)ethylamine(CR-2), (*S*)-(+)-1-benzyl-3-aminopyrrolidine (CR-3) and (*S*)-(−)-α,4-dimethylbenzylamine(CR-4) (20 mM each) were prepared in THF.
- Copper(II) acetate (2 mM) was prepared in purified water–methanol (95:5).

All solutions were filtered through a 0.45 μm filter. The solutions were scanned for determination of λ_{max}. Six solutions in the range 1×10^{-4} – 5×10^{-4} M were prepared by dilution. Their absorbance was recorded and a calibration plot was constructed.

F. Chiral Reagents

1. CR-1: N-Hydroxybenzotriazol-(*S*)-(+)-Naproxen

Synthesis of CR-1: Npx ester(Npx-OBtz): The CR was synthesized by the reaction of (*S*)-(+)-Npx (5mL, 0.2 M) with OH-Btz (5mL, 0.2 M) in THF by adding DCC solution (3mL, 0.36 M) dropwise. The reaction mixture was stirred under nitrogen atmosphere for 3 h. Dicyclohexylurea was precipitated and removed by filtration. The product in the filtrate was extracted with ethyl acetate and washed with brine, water and saturated $NaHCO_3$ one by one. Synthesis reaction scheme of Npx-OBtzis shown in **Fig 2.1**. Then it was recrystallized from hot EtOH to give the reagent as 'off white' solid. Following is the characterization data of CR:

Characterization data: Yield= 395 mg (94.7%); $[\alpha]_D^{25}$ = □38.62° (c=0.05, CH_3CN); m.p. 135-137 °C; UV (λ_{max} 231 nm in CH_3OH); IR (KBr): 3328, 2929, 2851, 1626, 1575, 1449, 1392, 1313, 1267, 1228, 1165, 1087, 1028, 855, 816, 741, 648 cm□1; ^1H NMR (400 MHz, $CDCl_3$-d1): δ(ppm) 1.59 (3 H, d, CH_3), 3.72 (1 H, q, -CH-), 3.91 (3 H, s, CH_3), 7.17 (1 H, d, Ar), 7.22 (1 H, d, Ar), 7.36 (2 H, d, Ar), 7.39 (1 H, s, Ar), 7.53 (1 H, s, Ar), 7.78 (1 H, d, Ar), 7.82 (1 H, d, Ar), 7.86 (1 H, d, Ar); 7.92 (2 H, dd, Ar); CHN found for $C_{20}H_{19}N_3O_3$: C, 69.15%; H, 4.93%; N, 12.10%; calculated: C, 69.75%; H, 4.89%; N, 12.07%

Enantiomeric purity of the three chiral amines obtained from Sigma-Aldrich (St Louis, MO, USA) was above 99%, these were used as CRs (2, 3 & 4 respectively) for diastereomeric synthesis (**Fig 2.2**).

2. **CR-2: (*S*)-(–)-1-(1-napthyl) ethylamine:**b.p. = 153 °C, density = 1.067 g mL^{-1}, $[\alpha]_D^{25}$ = –59° (c = 5 in CH_3OH), *ee* = 98% (GLC)

3. **CR-3: (*S*)-(+)-1-benzyl-3-aminopyrrolidine:** b.p. = 164-165 °C, density =0.984 g mL^{-1}, $[\alpha]_D^{25}$ = +1.99 (neat), assay 98 %

4. **CR-4: (*S*)-(–)-α,4-dimethylbenzylamine**: b.p. = 205 °C, density = 0.919 g mL^{-1}, $[\alpha]_D^{25}$ = –37° (neat), *ee* = 98% (GLC)

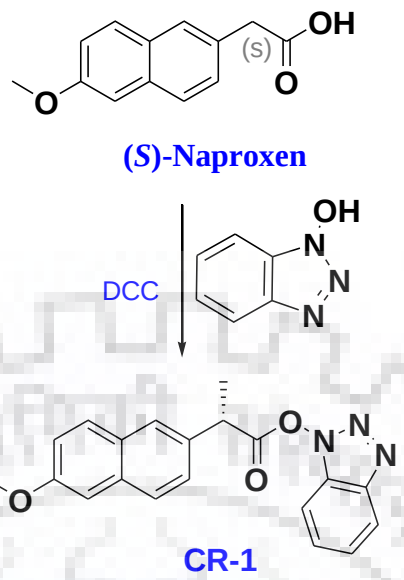


Fig 2.1 Scheme for synthesis of CR-1, i.e. N-hydroxybenzotriazolyl-(*S*)-(+)-Npx

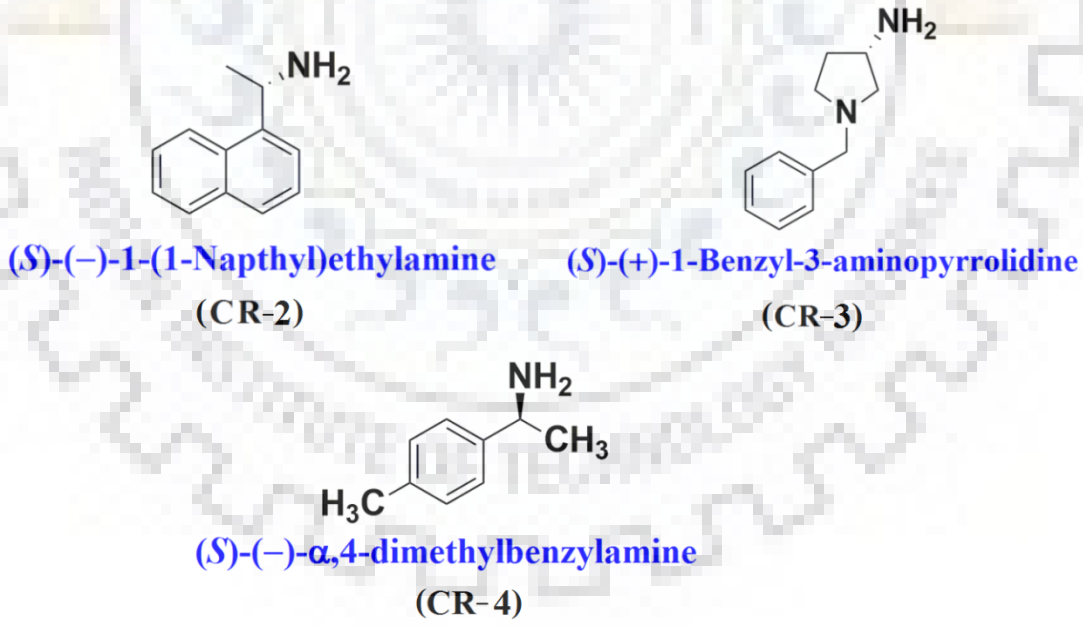
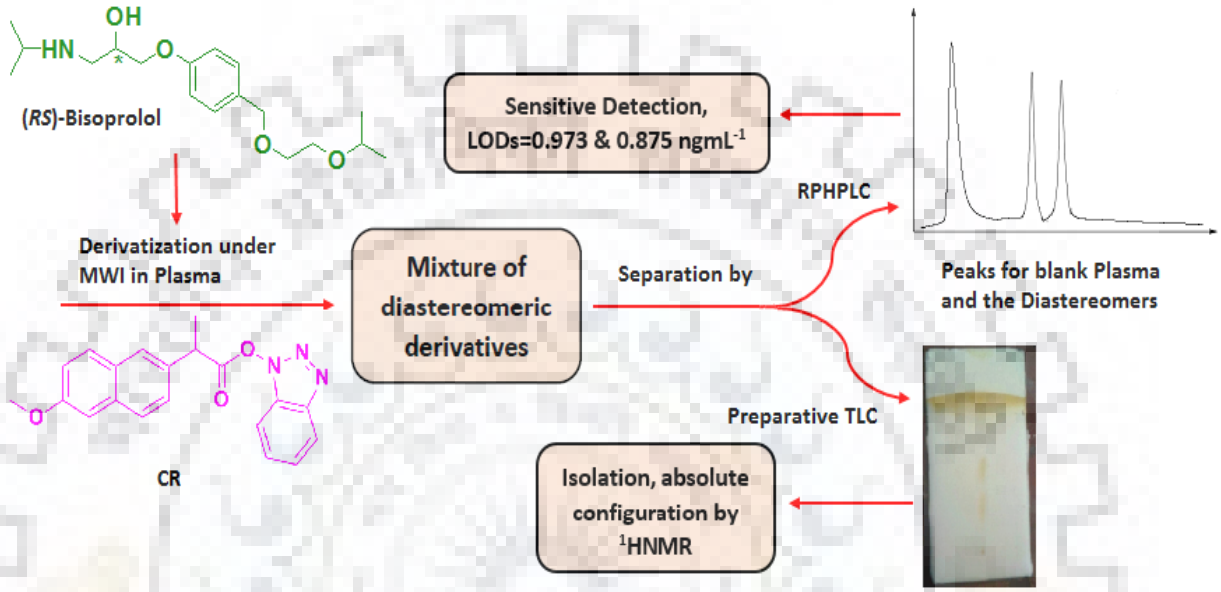


Fig. 2.2: Structures of three chirally pure amines i.e. CR 2-4

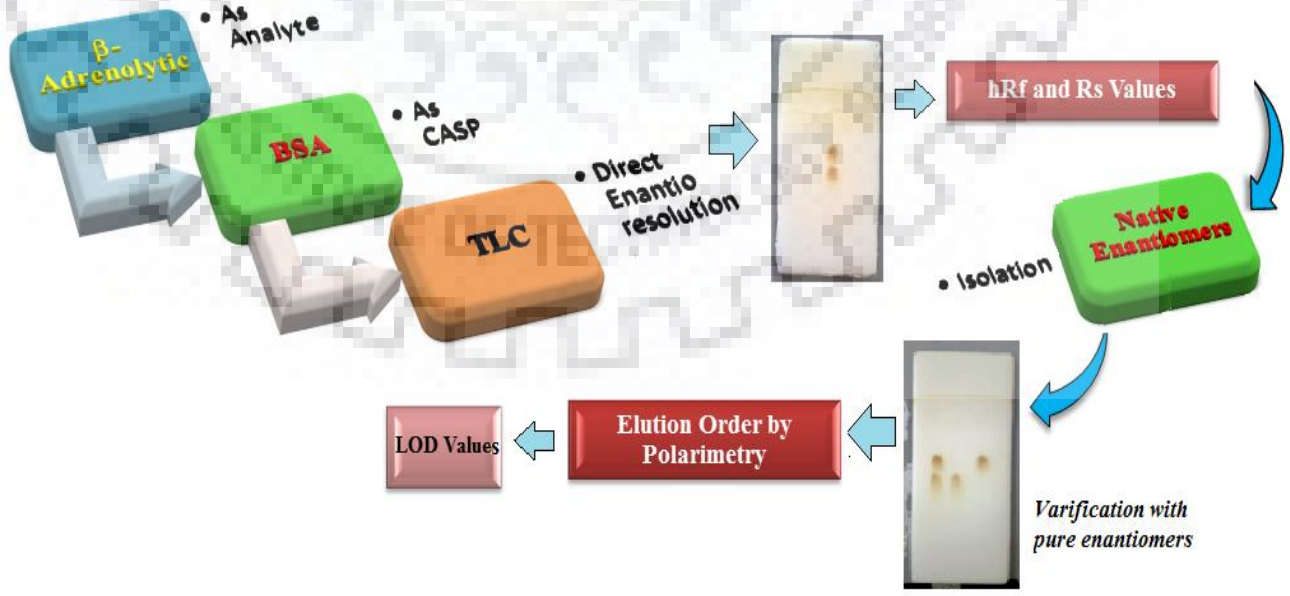
Chapter-3

Enantioseparation of β-Adrenolytics by HPLC and TLC

Part-I



Part-II



A. Introduction

The β-adrenolytics represent a class of medications that are particularly used to manage cardiac arrhythmias and to treat hypertension. Pharmaceutical applications of β-adrenolytics are well documented [20] and it is also well known that the (*S*)-(−)-enantiomer of β-adrenolytics are known to show several hundred times higher therapeutic activity in comparison to (*R*)-isomer. A report based on *Kalorama* Information shows that the worldwide sales of antiarrhythmic drugs increased by more than 100 % in a period of less than two decades.

There are three different prescription drugs that are included in the group β-adrenolytics, for selectively "blocking" the effects of adrenaline for one of the three known types of beta receptors designated β_1, β_2 and β_3 receptors.They belong to a group of synthetic chiral hydroxyl amine-containing compounds. In general, β-adrenolytic drugs are commonly used in the treatment of hypertension and for controlling acute panic symptoms in anxiety-provoking situations, besides in controlling certain other diseases. Use of β-adrenolytics is illegal as a sports enhancing drug.

The structures of the β-adrenolytics chosen [(*RS*)-Bpl, Prl, Atl, Sbl and Cdl] for these studies are given in **Fig. 1.1**. The pharmaceutical importance and the relevant literature on the enantioseparation of these β-adrenolytics are described briefly. The literature search was made through SciFinder, Google scholar, Scopus, web of science and research gate for the last ≈20 years. The relevant references published in English have carefully been included. Thus the literature presented herein is exhaustive but may not be complete.

B. Pharmaceutical importance of the chosen analytes

(i) Bisoprolol

Bisoprolol is a cardioselective β1-adrenergic blocking agent used for secondary prevention of myocardial infarction without membrane stabilizing activity or intrinsic sympathomimetic activity. It is also used for high blood pressure, cardiac ischemia,

congestive heart failure but also showing some side effect like hypotension, bronchospasms, low blood sugar and bradycardia. This drug is administered as a racemic mixture though its desirable properties reside in its (*S*)-isomer. (*S*)-Isomers of bisoprolol is adjuncts to standard ACE inhibitor and diuretic therapy in congestive heart failure.

(ii) Salbutamol

Salbutamol is a β_2 agonist and is marketed and administered as a racemic mixture. It is a synthetic chiral compound, with $pK_a \sim 9.2$, and contains one aromatic ring attached to a side chain of *t*-butyl group having a secondary hydroxyl amine functional group. (*R*)-(−)-Salbutamol causes smooth muscle to relax whereas (*S*)-(+)-salbutamol causes smooth muscle to contract and is also used for the treatment of asthma; the two isomers act on different receptors and thus on different pathways, resulting in the opposing effects. (*R*)-Sbl is the active bronchodilating enantiomers. Its presence in the blood causes a decrease in involuntary muscle spasms and anxiety. Salbutamol may be quantified in blood or plasma; practical needs for this include confirming a diagnosis of poisoning in hospitalized patients, or to aid in a forensic investigation. Besides, abuse of the drug in competitive sports programs may be confirmed by detection of its presence in plasma or urine, typically exceeding 1000 μg/L [21]. Sbl also shows some side effects like anxiety, muscle cramps, arrhythmia, headache, dry mouth and palpitation.

(iii) Carvedilol

Carvedilol is the mixed alpha/beta adrenergic antagonist and a third generation non selective b-adrenolytic which inhibits β_1, β_2 and α_1-receptor. It is used for treating mild to severe congestive heart failure, left ventricular dysfunction, high blood pressure and using for dilating vessels by inhibiting α_1- receptor of blood vessels and improves vascular resistance in liver by release of nitric oxide. (*S*)-carvedilol reduces lipid peroxidation level as evidenced by changes in plasma thiobarbituric reactive substance, and plasma oxidized low-density lipoprotein. It also have some side effect for example dizziness, low blood pressure, diarrhea, slowed heart rate and weight gain etc.

(iv) Atenolol

Atenolol is a selective β_1 receptor antagonist and is used in the treatment of cardiovascular disorders such as hypertension, angina pectoris, arrhythmias, and myocardial infarction coronary heart disease. It is one of the most widely used β-adrenolytics and acts preferentially upon the β-adrenergenic receptors in the heart. Sometime it causes 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.

(v) Propranolol

Propranolol is a cardioselective first generation β1-adrenergic blocking agents used for secondary prevention of myocardial infarction. It is first successful β-blocker developed which is in the list of essential medicines of World Health Organization. It is marketed and administered as racemic mixture, although the desirable action is largely confined to (*S*)-(−)-enantiomer showing about 50–500-fold higher activity. (*S*)-Prl is important in therapy against oxidative stress because it induces the activity of antioxidant and other beneficial enzymes and increase endothelial nitric oxide production, directly protecting cardiovascular cells and tissues against oxidative injury. Besides, (*R*)-Prl is also used for the treatment of hyperthyroidism (by inhibiting the conversion of thyroxin to triiodothyroxine) and under such situation racemic propranolol mixture cannot be administrated as it may cause serious side effects to the patients due the prominent effect of (*S*)-Prl.

C. Literature survey on enantioseparation of the chosen analytes

Till now, none of the reports and the literature cited therein has been deal with enantioseparation of the chosen β-adrenolytics in biological samples like human plasma. Only enantioselective analysis of metoprolol has been done in human plasma or in rat plasma using a Chiralpak® AD column and fluorescence detection [22], or a Chirobiotic T bonded phase [23, 24]. Besides, certain CDRs have been used for enantioseparation of

metoprolol in human urine by GC-MS [25], and that of atenolol in rat plasma by RPHPLC [26]. Protein precipitation method by ultrafilteration for removing protein has been used earlier [27].

BSA has also not earlier been used as chiral additive in stationary phase (CASP) in a non-covalent mode for enantioresolution of racemates of any kind by direct approach. Literature survey on enantioresolution of pharmaceutically important compounds, including β-adrenolytics, by TLC [7, 28-30] and our work published on TLC enantioresolution of the chosen β-adrenolytics [31-33], and the literature cited therein, clearly shows this. Though, there are reports on application of BSA based CSPs for enantioresolution of a variety of chiral compounds by HPLC or CE [34]. Direct and indirect separation of β-blockers by chiral TLC has been reviewed by Agbaba and Ivkovic´ [35].

Enantioseparation of racemic propranolol along with detagging of its diastereomeric amides has been done [36]. Enantioseparation of (*RS*)-Prl has also been achieved using (*R*,*R*)-*O*,*O*'-diacetyltartric acid anhydride (DATAAN) [37],*N*-trifluoroacetyl-L-prolyl chloride [38], (+)-1-(9-fluorenyl)ethyl choroformate (Flec) [39], anhydride of tert. butoxy carbonyl-L-leucine [40], (*S*)-flunoxaprofen isocyanate and (*S*)-naproxen isothiocyanate [41], 2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl isothiocyanate [42] and (1*R*,2*R*)-1,3-diacetoxy-1-(4-nitrophenyl)-2-propyl isothiocyanate [43]; dinitrophenyl-L-Pro-*N*-hydroxysuccinimide ester, *N*-succinimidyl-(*S*)-2-(6-methoxynaphth-2-yl) propionate and difluorodinitrobenzene [44, 45], cyanuric chloride [46] and (*S*)-naproxen anhydride [47] as CDRs.

The direct approach has been achieved by using lactobionic acid/D-(+)-xylose-boric acid complexes as chiral selector for RP-HPLC analysis [48]; chirally pure amino acids [32, 49], chiral Cu-II complexes [50,33,51], macrocylic antibiotics [31] and L-tartaric acid, (*R*)-mandelic acid and (–)-erythromycin [52] as chiral selectors for enantioseparation of (*RS*)-Prl on TLC. Also CSPs based on (+)-(18-crown-6)- 2,3,11,12-tetracarboxylic acid [53], and polyacrylamide and polysaccharide derivatives [54],

cellulose tris-(3,5-dimethylphenylcarbamate) [55], cellulose [56] and ovomucoid [57,58], amylose [59], macrocyclic antibiotic [60] macrocyclic glycopeptide [61], and polysaccharide and Pirkle-type based CSPs [62] have been used for direct separation of enantiomers of (*RS*)-Prl.

Batra and Bhushan presented a review [63] 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.

Enantioseparation of (*RS*)-Cdl has been achieved using (+)-(*R*)-phenyl ethyl isothiocynate [64], (*S*)-naproxen chloride [65], dinitrophenyl-L-Pro-*N*-hydroxysuccinimide ester, *N*-succinimidyl-(*S*)-2-(6-methoxynaphth-2-yl) propionate and difluorodinitrobenzene [44], cyanuric chloride [46] and (*S*)-naproxen anhydride [47] as a chiral derivatising reagents; and chirally pure amino acids [32] as chiral selectors, macrocyclic antibiotic [66,67], macrocyclic glycopeptide [61] and ovomucoid and cellulose [58, 68] as CSPs.

Wang *et al.*, [69] and Nishi and Kuwahara [70] presented reviews on liquid chromatographic methods for enantioseparation, determination and bioassay of enantiomers of (*RS*)-Sbl using both HPLC and capillary electrophoresis by both direct and indirect approaches involving practical applications.

Enantioseparation of (*RS*)-Bpl has been achieved using (−)-menthyl chloroformate (MCF) [71], 2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl isothiocyanate [41] and (1*R*,2*R*)-1,3-diacetoxy-1-(4-nitrophenyl)-2-propyl isothiocyanate [72], dinitrophenyl-L-Pro-*N*-hydroxysuccinimide ester, *N*-succinimidyl-(*S*)-2-(6-methoxynaphth-2-yl) propionate and chiral derivatives of difluorodinitrobenzene [44], and cyanuric chloride [46] as CDRs; lactobionic acid/D-(+)-xylose-boric acid complexes [48]; chirally pure amino acids [32] as chiral selectors; amylose [59], β-cyclodextrine [73], macrocyclic antibiotic [66] and macrocyclic glycopeptide [61] based CSPs.

There are several other amino group containing racemic analytes which have been used for enantioseparation via direct and indirect approach [74].

The present chapter: The **Chapter 3** on investigations related to enantioseparation of β-adrenolytics/β_2-agonists is divided into two parts.

Part-I describes separation of β-adrenolytics in human plasma with minimal sample clean-up by TLC and RPHPLC via an indirect approach along with determination of absolute configuration of diastereomeric derivatives.

Part-II describes direct resolution of β-adrenolytics by TLC using BSA as '*chiral additive in stationary phase*' (CASP) and achiral mobile phase (having no external chiral additive) along with isolation of the native enantiomers.

Part-I. Liquid chromatographic enantioseparation and sensitive detection of β-adrenolytics/β_2-agonists in human plasma using a single enantiomer reagent

1. Present work

Enantioseparation of bisoprolol, salbutamol, and carvedilol, marketed as racemic mixtures has been achieved by both preparative TLC and RPHPLC via an indirect approach. A new chiral reagent, N-hydroxybenzotriazolyl-(*S*)-(+)-Npx, was synthesized and it was characterized by UV, IR, ^{1}HNMR, elemental analysis and polarimetry. (*S*)-(+)-Npx based CR was introduced in human plasma and these samples were spiked separately with each of the four analytes. Synthesis of diastereomeric derivatives was carried out under MWI; these were then separated by TLC and RP HPLC. Diastereomeric derivatives separated by TLC were then isolated by preparative approach; these were characterized and were used as standard reference for determining absolute configuration of diastereomeric derivatives and for establishing validated HPLC method for enantioseparation and sensitive detection of the four β-adrenolytics in human plasma. The configurations of the first and second eluting diastereomeric derivatives (and thus the elution order) were established by recording their ^{1}H NMR spectra. Mobile phase in

gradient mode containing methanol and aqueous triethylaminephosphate (TEAP) was successful for HPLC separation; conditions with respect to pH, flow rate, and buffer concentration were optimized. The method is capable to accurately quantitate β-adrenolytics in human plasma with minimal sample clean-up and rapid separation by TLC and RPHPLC. Method was validated for HPLC separation by using diastereomeric derivatives of (*RS*)-Bpl (as a representative) for linearity, recovery, accuracy and precision according to the acceptance criteria of the FDA guidance for bioanalytical method validation [75].

Literature on enantioseparation of the β-adrenolytics under study: The literature search on enantioseparation of the chosen β-adrenolytics has been described above (Section 2.2). None of these reports and the literature cited therein deal with enantioseparation of the chosen β-adrenolytics in biological samples like human plasma.

Selection of (*S*)-Npx as the chiral moiety

Looking into characteristics of (*S*)-Npx (as described in Chapter-1) as a chiral moiety and the literature on its application in enantioseparation studies [76-78] it was chosen for synthesizing a new chiral reagent (CR). (*S*)-Npx moiety has also been used to synthesize Npx-hydrazine reagent which was used for enantiomeric resolution of certain didactical chiral ketones [79] as their diastereomeric hydrazones; Khurana et. al[80] reported hydrolysis of hydrazones, semicarbazones and phenylhydrazones with sodium hypochlorite. Oxidation of certain ketones and *o*-aminochalcones using hypervalent iodine oxidation for synthesis of substituted benzisoxazoles has also been reported in literature.

2. Experimental

- Details of chemicals and reagents, instrumentation and the preparation of the stock solutions are described inChapter-2.
- The method of synthesis of new CR i.e. CR-1 and its characterization data are given in Chapter-2.

2.1 Synthesis of diastereomeric amides of (*RS*)-Bpl, Sbl and Cdl with CR-1 (Npx-OBtz)

(i) Preparation of plasma samples

Blood (8 mL) was collected, from the healthy volunteers (all male aged between 30 to 40 years from the Institute hospital) in appropriate tubes containing K_2EDTA. Blood samples were centrifuged at 3000 rpm for about 10 min at 4^{o}C to extract plasma and 4mL of the plasma was stored at –20^{o}C in an eppendorf tube. Standard protein precipitation method was used to remove access albumin and other proteins [81-83]. A solution of 80% CH_3CN with 20% *i*-PrOH (12 mL) was taken in a centrifuge vial and plasma (4 mL) was added to it. It was agitated for 1 minute via vortex and was centrifuged for 25 minutes at 25000 rpm. Supernatant was stored at –20^{o}C in an eppendorf tube. The frozen plasma was thawed at room temperature before analysis. Thus the plasma sample was prepared for further experiments pertaining to spiking and derivatization.

Human plasma samples, as prepared above, were spiked with solutions of racemic β-adrenolytics to get standard concentration levels of 0.2, 0.4, 0.6, 0.8 and 1.0 mM each (samples were designated as B1 to B5 for Bpl, S1 to S5 for Sbl, and C1 to C5 for Cdl). Each sample was vortexed for 1 min; it was then centrifuged at 3000 rpm for 4 min. Diastereomeric derivatization reaction was carried out in the supernatant containing the respective analytes. A sample of plasma having no analyte was used as blank for each set of experiments. In all, there were 15 samples (B1 to B5, S1 to S5, and C1 to C5). A combination of *i*-PrOH with CH_3CN was found to be successful to remove matrix components from plasma sample. The HPLC peak obtained for the plasma sample is shown in **Fig 3.1**.

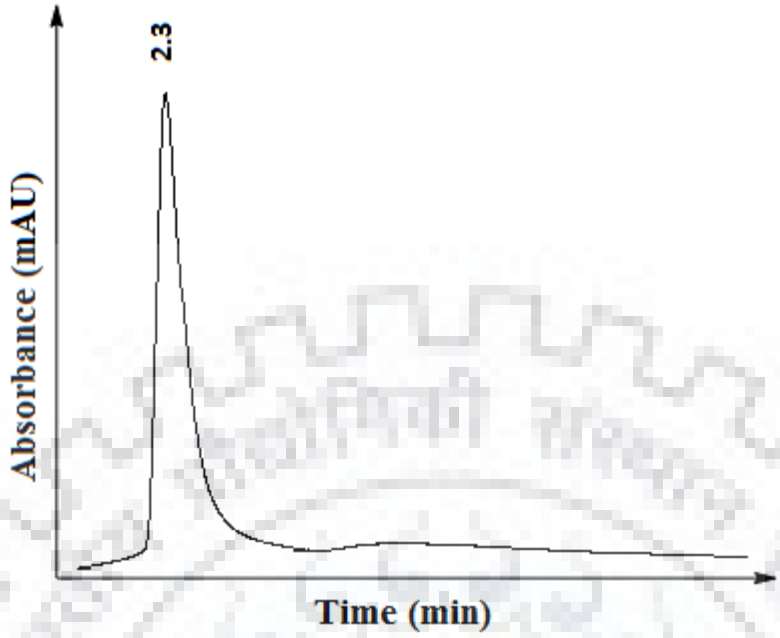


Fig 3.1 Blank plasma chromatogram. Chromatographic conditions: column, LiChrospher C_{18} (250 mm × 4.6 mm I.D., 5 μm particle size); mobile phase: CH_3OH–TEAP buffer (pH 3.7), binary gradient (60 to 40%); flow-rate: 1.0 mL min^{-1}; detection, 230 nm.

(ii) Microwave irradiated synthesis of diastereomeric derivatives (of (*RS*)-Bpl as a representative)

(*RS*)-Bpl (1mM in CH_3CN, 100 μL) solution was mixed with the solution of CR-1 (1mM in CH_3CN, 200 μL) and triethylamine (20 μL). Experimental conditions to synthesize diastereomeric derivatives were optimized by varying pH, ratio of (*RS*)-Bpl:CR (1:1.5, 1:2, 1:2.5, 1:2.8, 1:3) and microwave irradiation (MWI) time (starting from 60 s to go up to 180 s at an interval of 30 s) at 80% power (800W) for separate sets of reaction mixture. The reaction was quenched by adding acetate buffer (50 μl; 0.05 M) after cooling the reaction mixture to room temperature. Synthesis of diastereomeric derivatives was carried out independently in the samples B1-B5, under MWI. Diastereomeric derivatives of (*RS*)-Sbl, and (*RS*)-Cdl using this CR were also synthesized by applying the same procedure and reaction conditions (the reaction samples were designated as S1 to S5 for Sbl, and C1 to C5 for Cdl**)**. The diastereomeric mixture in each case was subjected to HPLC separation.

2.2 TLC separation and isolation of diastereomeric derivatives

TLC plates (10 x 5 cm x 0.5 mm) were prepared by spreading the slurry (prepared by mixing silica gel (30 g) with double amount of distilled water i.e. 60 mL) using a Stahl type applicator and activated overnight at 60 ± 2 °C. Chromatograms were developed in a dried, cleaned, and paper lined rectangular glass chambers by applying a spot of 5 μL solution of diastereomeric mixtures using a 15 μL graduated capillary or 10 μL Hamilton syringe on the TLC plates. Chambers were pre equilibrated every time with those mobile phases which were used for optimization to develop chromatograms. Plates were dried in oven for 10 min., iodine was used for visualize the spots. Based on the successful separation conditions (**Table 3.1**). Further, 10 TLC plates (20 x 20 cm x 1.5 mm thick), were prepared and 10 spots (20 μL per spot) were applied on each TLC plate in parallel. Isolation and purification of the diastereomeric derivatives was performed after extracting with methanol and then their λ_{max} (230 nm) and specific rotations were measured.

2.3 Isolation of diastereomeric derivatives by preparative TLC

Spots corresponding to the two diastereomeric derivatives of (*RS*)-Bpl, for example, were marked on the chromatograms and iodine was allowed to evaporate. Silica gel from each of the spots was scrapped from nearly 40 chromatograms and was extracted with methanol. The combined extracts for each of the diastereomeric derivatives were filtered and concentrated in *vacuo*. The concentration for each isomer was estimated by using 'calibration plots'. Determination of optical rotation was carried out using polarimeter. The isolated diastereomers obtained from TLC were then used as reference standards for comparison with HPLC separation results. Also both diastereomeric derivatives were characterized by m.p., UV, IR, NMR, specific rotation.

2.4 RP HPLC separation of diastereomeric derivatives

20 μL from solution of mixture of diastereomeric derivatives was injected onto RP HPLC column after diluted 10 times with CH_3OH. The tested mobile phases are as follows,

(a) CH_3OH –TEAP buffer (10mM, pH 3.7);

(b) CH_3CN–TEAP buffer (10mM, pH 3.7).

Each mobile phase was used in the gradient elution mode of CH_3OH or CH_3CN with TEAP buffer from low to high eluting strength i.e. 20–80, 30–70, 40–60, 50–50, 60–40, 70–30 and 80–20 ratio with UV detection at 230 nm over 5-25 min using PDA detector. Separation conditions were optimized by investigating the effect of change in flow rate (from 0.5 to 2.0 mL min^{-1} by varying 0.5 mL each time) and concentration of buffer (in the range of 5mM to 15mM). The mobile phase was degassed by sonication and filtered through 0.45 μm filter, before use, for column safety.

3. Results and Discussion

3.1 Formation of CR-1

Reaction of organic amines with esters of carboxylic acid has been the simplest method of acylation in organic synthesis. But, with the use of DCC as coupling reagent both activation and coupling proceed concurrently. The rate of reaction of amine with DCC is much lower in comparison to the rate of addition of carboxylic acid to one of the double bonds of carbodiimide, [84] therefore, the synthesis of the CR1 as ester was a simple straight forward reaction of (*S*)-(+)-Npx with OH-Btz in presence of DCC (**Fig 2.1**). The CR1 was characterized (the relevant data included in the manuscript) and was used for synthesis of diastereomeric derivatives.

The CR presented in this paper is a new one and its characterization data has been provided. The sample of (*S*)-(+)-Npx obtained from Sigma-Aldrich was first purified by recrystallization and its enantiomeric purity was ascertained polarimetrically; since the reaction of achiral OH-Btz was taking place only at the terminal carboxyl group of enantiomerically pure (*S*)-(+)-Npx (and not directly on the stereogenic centre) it was considered that the CR-1 so synthesized was enantiomerically pure (supported with characterization data). Absence of any additional peak in chromatograms (for HPLC separation of the diastereomeric derivatives) further confirmed the absence of any impurity due to the possible presence of (*R*)-(−)-enantiomer of naproxen [at the time of synthesis of CR from (*S*)-Npx] and therefore absence of the corresponding CR.

N-Hydroxybenzotriazole (OH-Btz) is a derivative of benzotriazole and very important moiety as a good leaving group to obtain the good yield. It is an excellent auxiliary and mainly used to suppress the racemization of single-enantiomer chiral molecules in conversion of the acid to its derivative. N-hydroxybenzotriazole derivatives are used with carbodiimide as additives to generate active esters during amide and anhydride bond formation at ambient temperature. It tends to increase the acidity of the α-proton. OH-Btz is believed to work by initially reacting with the O-acylurea to give the OBt active ester, which enhances the reactivity of the ‘‘activated ester’’ by encouraging/stabilising the approach of the amine via hydrogen bonding. However, OH-Btz yields as by-product.

Synthesis of diastereomeric derivatives was found to be completed in 150 s under MWI. To avoid kinetic resolution, conditions for synthesis were optimized starting from molar ratio of 1:1.5 when a 1:2.8 molar ratio of (*RS*)-Bpl to CR-1 was successful.

3.2 Formation of Diastereomeric Derivatives

The synthesis of diastereomeric derivatives under different experimental conditions has been described above. The **Fig 3.2** shows completion of reaction for synthesis of diastereomeric derivatives as inferred from the peak areas obtained at different reaction times when it was found that the peak area becomes constant at 150s starting from a microwave irradiation time of 60 s (and irradiating for 90, 120, 150 and 180 s). Similarly, the reaction was carried out for different β-adrenolytics: CR ratio (1:1, 1:1.5, 1:2, 1:2.5, 1:2.8, and 1:3), at each of the irradiation time; at the ratio 1:2.8 no further increment in the peak area of diastereomeric derivatives was observed. Thus, the reaction was complete and quantitative.

3.3 TLC Separation of diastereomeric derivatives

Diastereomeric derivatives of (*RS*)-Bpl were used as representative for TLC studies. Of the various combinations of solvents tried systematically, 2-propanol-dichloromethane (DCM)-HOAc (7:2:0.5; *v*/*v*) at 28 °C was successful in separating the diastereomeric pair of (*RS*)-Bpl (as shown in **Fig 3.3)**. R_F values for the first and second

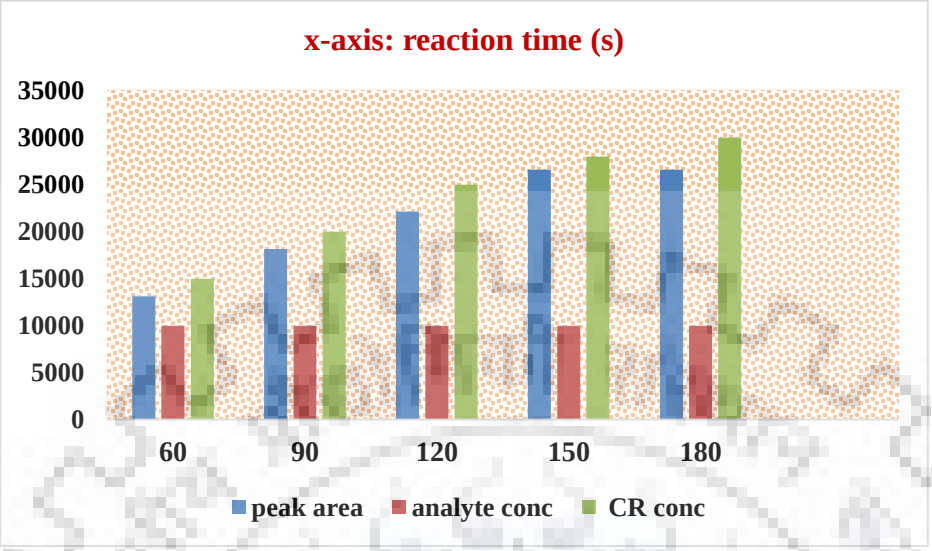


Fig. 3.2 Depiction of completion of reaction for synthesis of diastereomeric derivatives based on peak area vs reaction time and molar ratio of CR:β-blocker. Optimized conditions of derivatization; time, 150 s at 80% power of 800 W under MWI; molar ratio of β-blocker: CR = 1:2.8;mobile phase comprising of CH_3OH–TEAP buffer (pH 3.7) at a flow-rate of 1.0 mL min^{-1}; detection, 230 nm. Only the results for (*RS*)-Bpl are shown.

Fig. 3.3A photograph of an actual chromatogram showing TLC separation of diastereomeric derivatives of (*RS*)-Bpl prepared with Npx-OBtz, as the CR. Solvent system: 2-Propanol- CH_2Cl_2-AcOH (7:2:0.5; *v*/*v*); Temp: 28 °C; solvent front, 9 cm. Lower spot corresponds to the (*S*,*R*)-diastereomeric derivative while the upper spot corresponds to the (*S*,*S*)-diastereomeric derivative of Bpl.

eluting diastereomeric derivatives (i) [(*S*)-Npx-(*S*)-Bpl] and, (ii) [(*S*)-Npx-(*R*)-Bpl] were 0.35, 0.15. The resolution (R_S) values were calculated as per literature [85]. The solvent systems found successful for TLC separation of diastereomeric derivatives, along with respective R_F values, of the remaining (*RS*)-β adrenolytics (Sbl, Lbl and Cdl) under study are summarized in **Table 3.1**.

Table 3.1: Different combinations of solvents showing successful TLC separation of diastereomeric derivatives of β-adrenolytics

Diastreomeric derivatives of -	Solvent system	Solvent ratio (*v*/*v*)	R_f values	
			(*S*,*S*)-derivative	(*S*,*R*)-derivative
(*RS*)-Bisoprolol	2-propanol-DCM-AcOH	7:2:0.5	0.35	0.15
(*RS*)-Carvedilol	CH_3CN-DCM-2-Propanol-AcOH	6:2:2:0.5	0.42	0.19
(*RS*)-Salbutamol	2-propanol-DCM-CH_3OH-AcOH	7:1:3:0.5	0.39	0.20

Solvent front: 9 cm; time: 10-15 min; Detection: iodine vapors;
(*S*,*S*)-derivative and (*S*,*R*)-derivative represent, [(*S*)-Npx–(*S*)-Bpl] and [(*S*)-Npx–(*R*)-Bpl], diastereomeric derivatives (for bisoprolol, for example), respectively.

3.4 Characterization of diastereomeric derivatives

The specific rotation of [(*S*)-Npx-(*S*)-Bpl] was +09.81° and of [(*S*)-Npx-(*R*)-Bpl] was +27.35°. The diastereomeric derivatives so isolated and purified were taken as reference samples for recording ^{1}HNMR and IR spectra and for comparison of retention times during HPLC separation. Characterization data of the two diastereomeric derivatives is given below.

First eluting diastereomer [(*S*)-Npx-(*S*)-Bpl]:yield= 471.4 mg. Recovery: 87.3 %; $[\alpha]_D^{25}$= +09.81° (c, 0.05, CH_3OH); m.p., 146±2 °C; UV (λ_{max} in CH_3OH, 230 nm); IR (KBr): **3178**, 2927, 2852, 2362, 1626, 1572, 1442, 1405, 1313, 1265, 1218, 1162, 1094, 1021, 962, 891, 847, 741 and 648 cm^{-1}; ^{1}H NMR (400MHz, $CDCl_3$-d1): δ1.17 (d, 6 H), 1.62 (d, 6 H), 1.58 (s, 3 H), 3.25-3.51 (s, 2 H), 3.49 (m, 1 H), 3.72 (q, 1 H), 3.87 (t, 4 H), **2.38 (s, 1 H),** 3.91 (s, 3 H), 3.98 (m, 1 H), 3.97-4.48 (d, 2 H), 3.99 (t, 1 H), 4.87 (s, 2 H),

6.80 (dd, 2 H, Ar–H), 6.92 (dd, 2 H, Ar–H), 7.17 (dd, 1 H, Ar–H), 7.22 (dd, 1 H, Ar–H), 7.39 (d, 1 H, Ar–H), 7.53 (d, 1 H, Ar–H); 7.81 (d, 1 H, Ar–H); 7.85 (d, 1 H, Ar–H); calculated for $C_{32}H_{43}NO_6$: C, 71.49%; H, 8.05%; N, 2.61%; CHN found C, 71.83%; H, 8.21%; N, 2.07%.

Second eluting diastereomer[(*S*)-Npx-(*R*)-Bpl]: yield = 504.9 mg. Recovery = 93.5%; $[\alpha]_D^{25}$ = +27.35° (c, 0.05, CH_3OH); m.p., 143±3°C; UV (230 nm, λ_{max} in CH_3OH); IR (KBr): **3328**, 2926, 2851, 2363, 1626, 1572, 1444, 1398, 1309, 1267, 1220, 1162, 1096, 1021, 924, 885, 818, 744 and 649 cm^{-1}; ^1H NMR (400MHz, $CDCl_3$-d1): δ1.16 (d, 6 H), 1.60 (d, 6 H), 1.58 (s, 3 H), 3.25-3.50 (s, 2 H), 3.47 (m, 1 H), 3.73 (q, 1 H), 3.85 (t, 4 H), **2.11 (s, 1 H),** 3.90 (s, 3 H), 3.96 (m, 1 H), 3.95-4.47 (d, 2 H), 3.97 (t, 1 H), 4.87 (s, 2 H), 6.82 (dd, 2 H, Ar–H), 6.95 (dd, 2 H, Ar–H), 7.16 (dd, 1 H, Ar–H), 7.22 (dd, 1 H, Ar–H), 7.38 (d, 1 H, Ar–H), 7.53 (d, 1 H, Ar–H); 7.82 (d, 1 H, Ar–H); 7.87 (d, 1 H, Ar–H); calculated for $C_{32}H_{43}NO_6$: C, 71.49%; H, 8.05%; N, 2.61%; CHN found C, 71.25%; H, 8.41%; N, 2.39%.

3.5 Structure and configuration of diastereomeric derivatives

The chemical structures of the diastereomeric derivatives of (*RS*)-Bpl (as a representative), along with spatial orientation of groups with respect to the amide bond, are shown in **Fig. 3.4**. The '*orbital molecular building system*' (Cochranes of Oxford Ltd, Leafield, Oxford OX8 5NT, England) was used to verify these spatial arrangements.

The two hydrophobic groups, i.e.,'phenoxy group containing moiety' of Bpl and 'napthyl ring of the CR' are oriented on the same side of the amide bond and provide a site for formation of an intramolecular H-bond between the hydroxyl group of Bpl and carbonyl group of Npx molecule in [(*S*)-Npx-(*S*)-Bpl]. Because of steric orientation, H-bond is not possible in the other diastereomeric amide [(*S*)-Npx-(*R*)-Bpl]. The spectral studies carried out with the two amides obtained by preparative TLC confirmed the formation of such an H-bond, e.g., (a) there was a difference in chemical shift (Δδ) value of the –OH signal in ^{1}HNMR spectra of the two amides, (b) IR spectra showed the –OH stretching frequency values at 3178 cm^{-1} and 3328 cm^{-1} in [(*S*)-Npx-(*S*)-Bpl] and [(*S*)-

Npx-(*R*)-Bpl], respectively, and a value lower by 150 cm^{-1} for (i) was a support for the formation of hydrogen bond in [(*S*)-Npx-(*S*)-Bpl], [86], and (c) geometry optimized 'lowest energy' structures, of the two amides, developed with the (density functional theory) DFT based software program (Gaussian 09 Rev. A.02 and hybrid density functional B3LYP with 6-31G* basis set) clearly showed formation of an intramolecular H-bond between the hydroxyl group of Bpl and carbonyl group of Npx molecule in [(*S*)-Npx-(*S*)-Bpl].

Formation of hydrogen bond is not supported for the amide [(*S*)-Npx-(*R*)-Bpl] either by NMR or the IR or the DFT based 'lowest energy' structures and the spatial arrangement in chemical structures (shown in Fig.3.4). Thus the structure, configuration and elution order of diastereomeric derivatives of all racemic analytes was determined, based on IR, and ^{1}HNMR (**Fig. 3.5**) spectra and the lowest energy structures (**Fig. 3.6**) developed through software program Gaussian 09 Rev. A.02 and hybrid density functional B3LYP with 6-31G* basis set.The similar explanation is applicable for all the analytes.

The above mentioned interpretations and the similar interpretations for all other diastereomeric derivatives led to conclude that the diastereomeric amides, e.g., [(*S*)-Npx-(*S*)-Bpl] has the (*S*,*S*)-configuration and [(*S*)-Npx-(*R*)-Bpl] has (*S*,*R*)-configuration (Fig.3.4). The second letter [capital (*R*) or (*S*)] represents the absolute configuration of the enantiomer of the corresponding β-adrenolytic while the first notation represents the absolute configuration of (*S*)-Npx.

3.6 HPLC Separation of diastereomeric mixtures in plasma

The successful HPLC separation conditions along with the sections of chromatograms showing baseline separation of the pair of diastereomeric derivatives of (*RS*)-Bpl are given in **Fig 3.7**. The same conditions were applicable for separation of diastereomeric pairs of each of the three analytes.

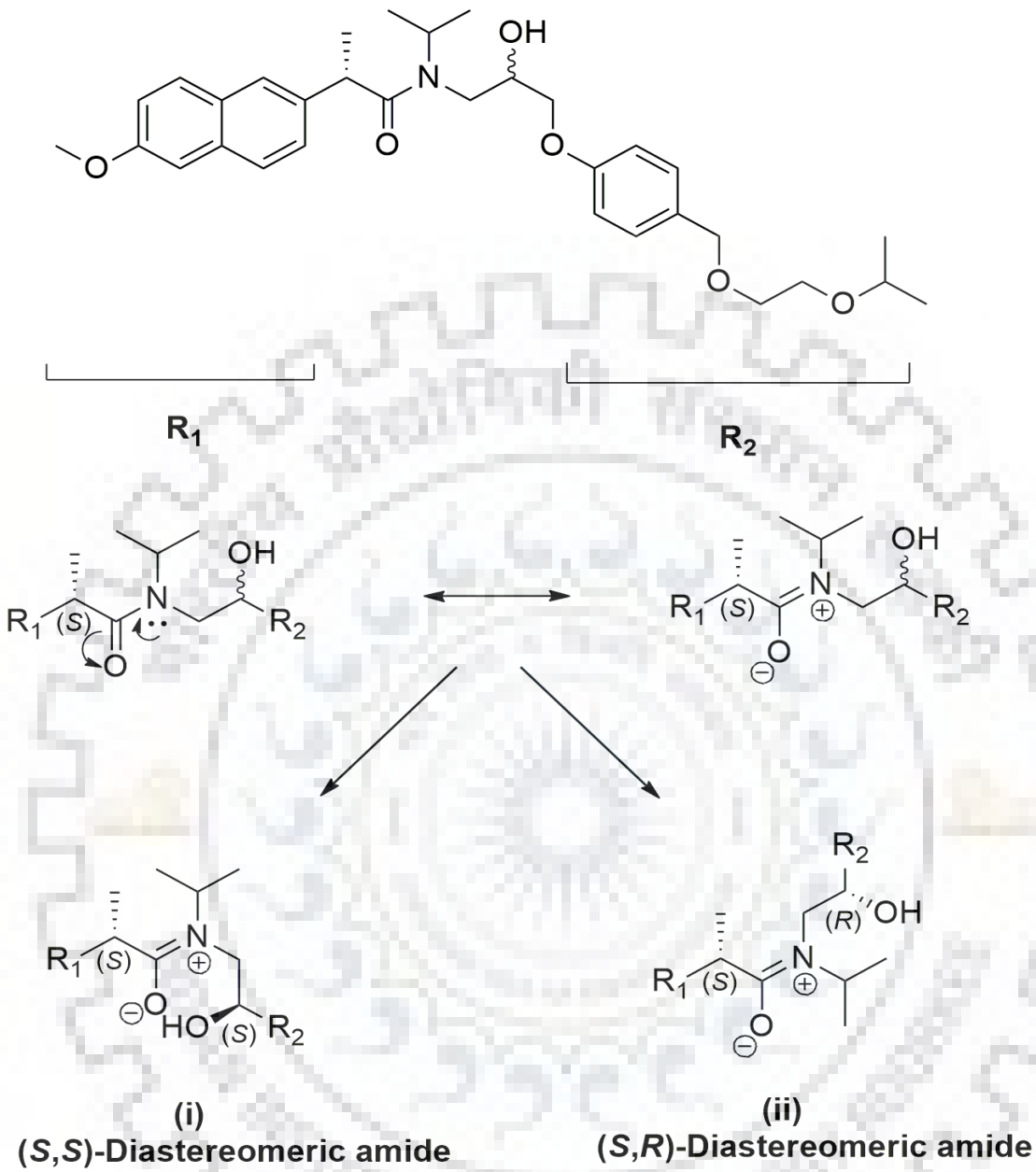


Fig. 3.4 Structures of diastereomeric derivatives **(**i and ii) along with spatial arrangements of the groups of (*S*)-naproxen moiety and the enantiomers of Bpl moiety leading to (*S*,*S*)-, and (*S*,*R*)-diastereomeric amides. R_1 represents the (*S*)-naproxen moiety and R_2 represents the Bpl moiety.

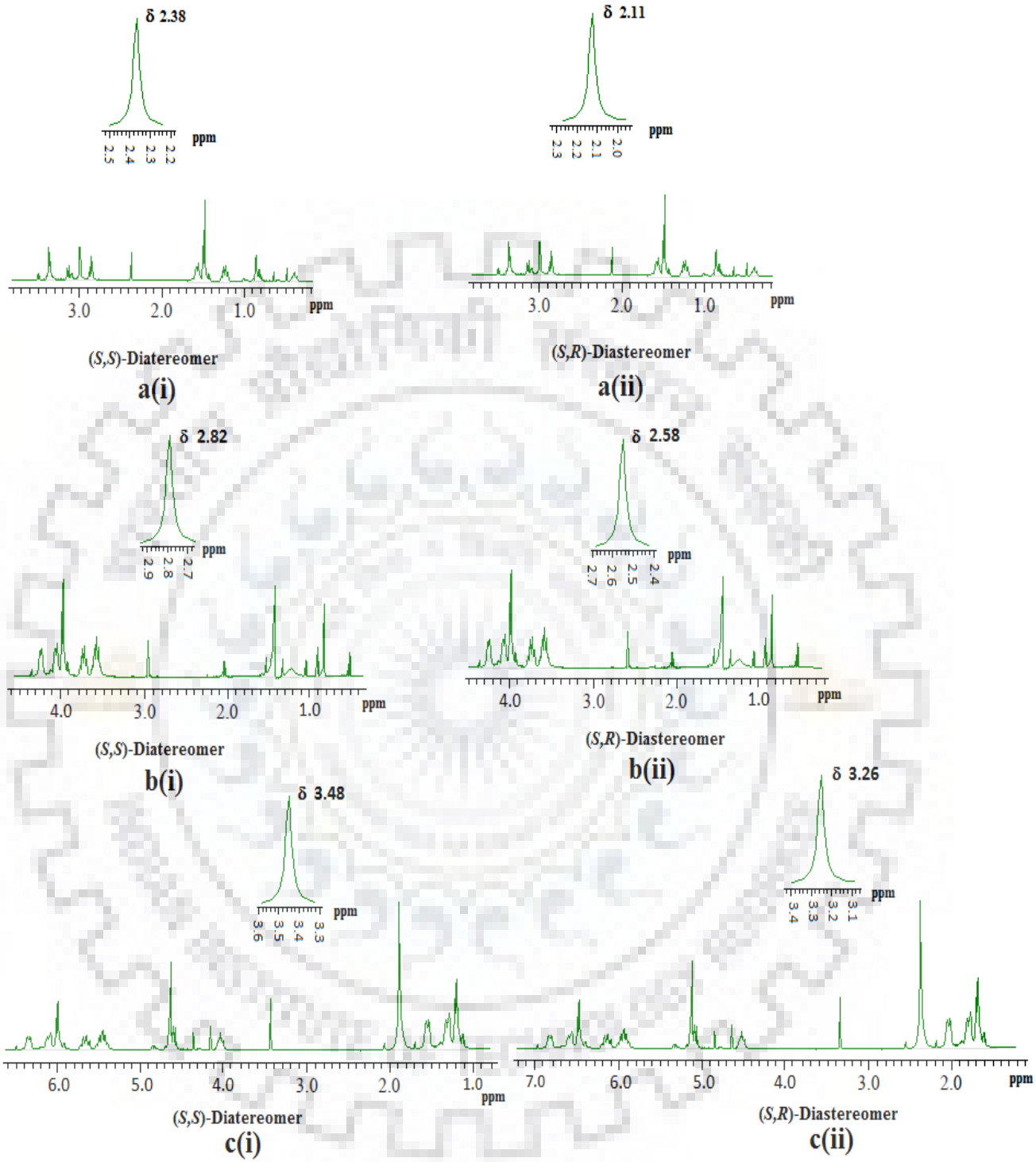


Fig 3.5 Sections of ^{1}HNMR spectra of (*S,S*)-, and (*S,R*)-diastereomeric derivatives of β-adrenolytics under study illustrating chemical shift values for the peaks of -OH (at the stereogenic center). a(i) a(ii); b(i), b(ii); and c(i), c(ii) represent, respectively, diastereomeric derivatives of Bpl, Sbl, and Cdl.

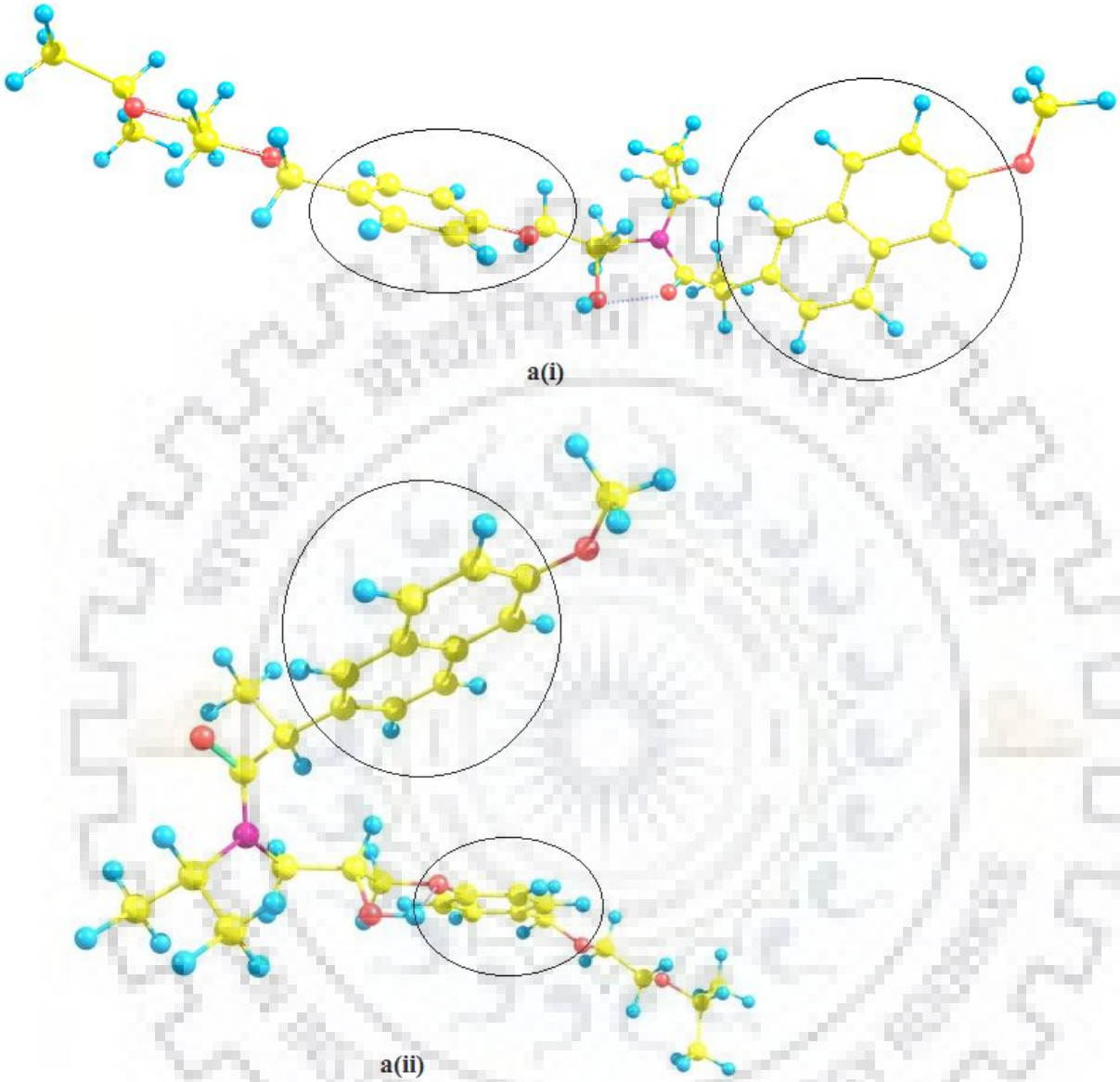


Fig 3.6 Geometry optimized 'lowest energy' structures of various diastereomeric derivatives developed using the software program Gaussian 09 Rev. A.02 and hybrid density functional B3LYP with 6-31G* basis set; a(i) a(ii) represent, respectively, diastereomeric derivatives of Bpl.

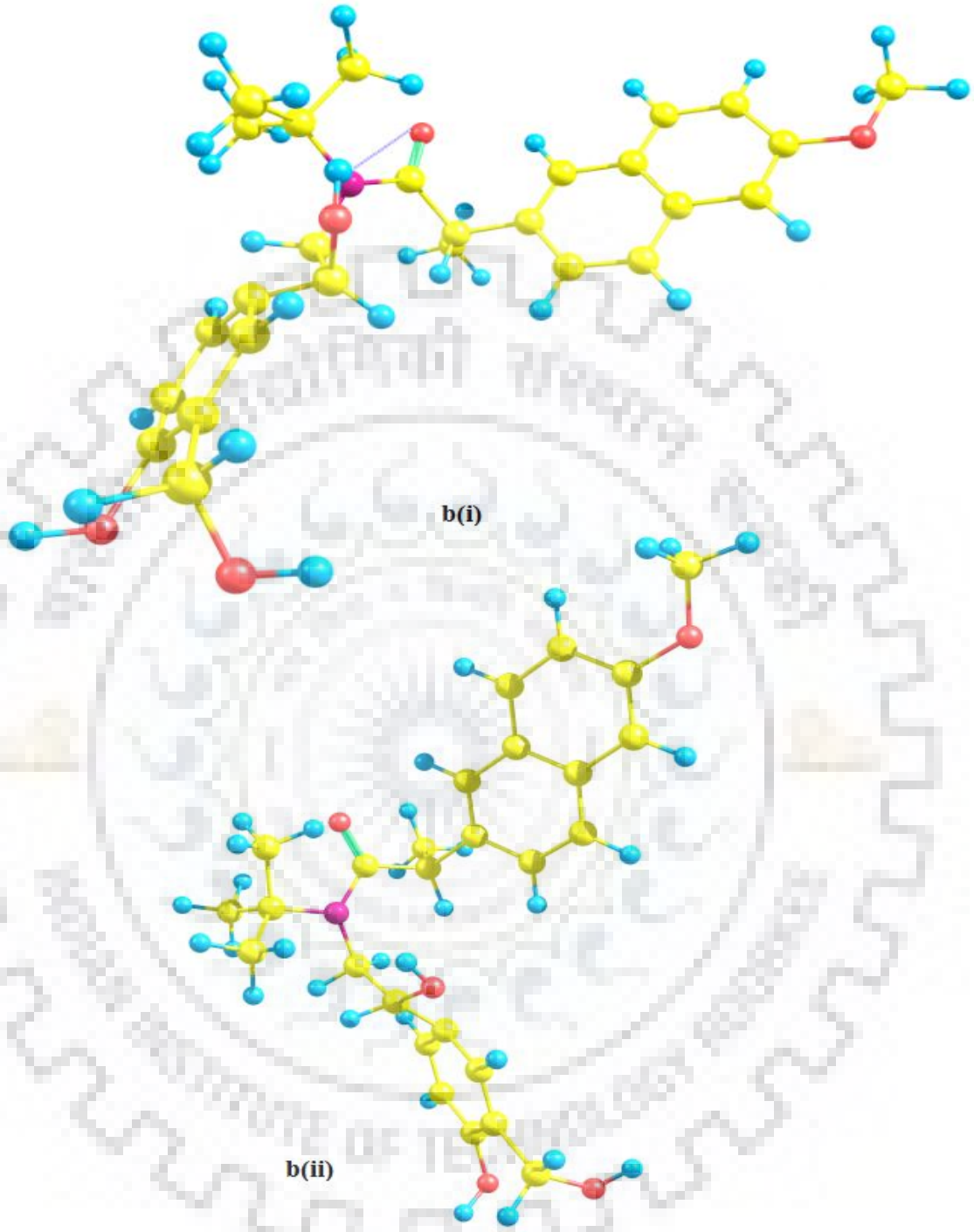


Fig 3.6 continued...b(i), b(ii) represent, respectively, diastereomeric derivatives of Sbl.

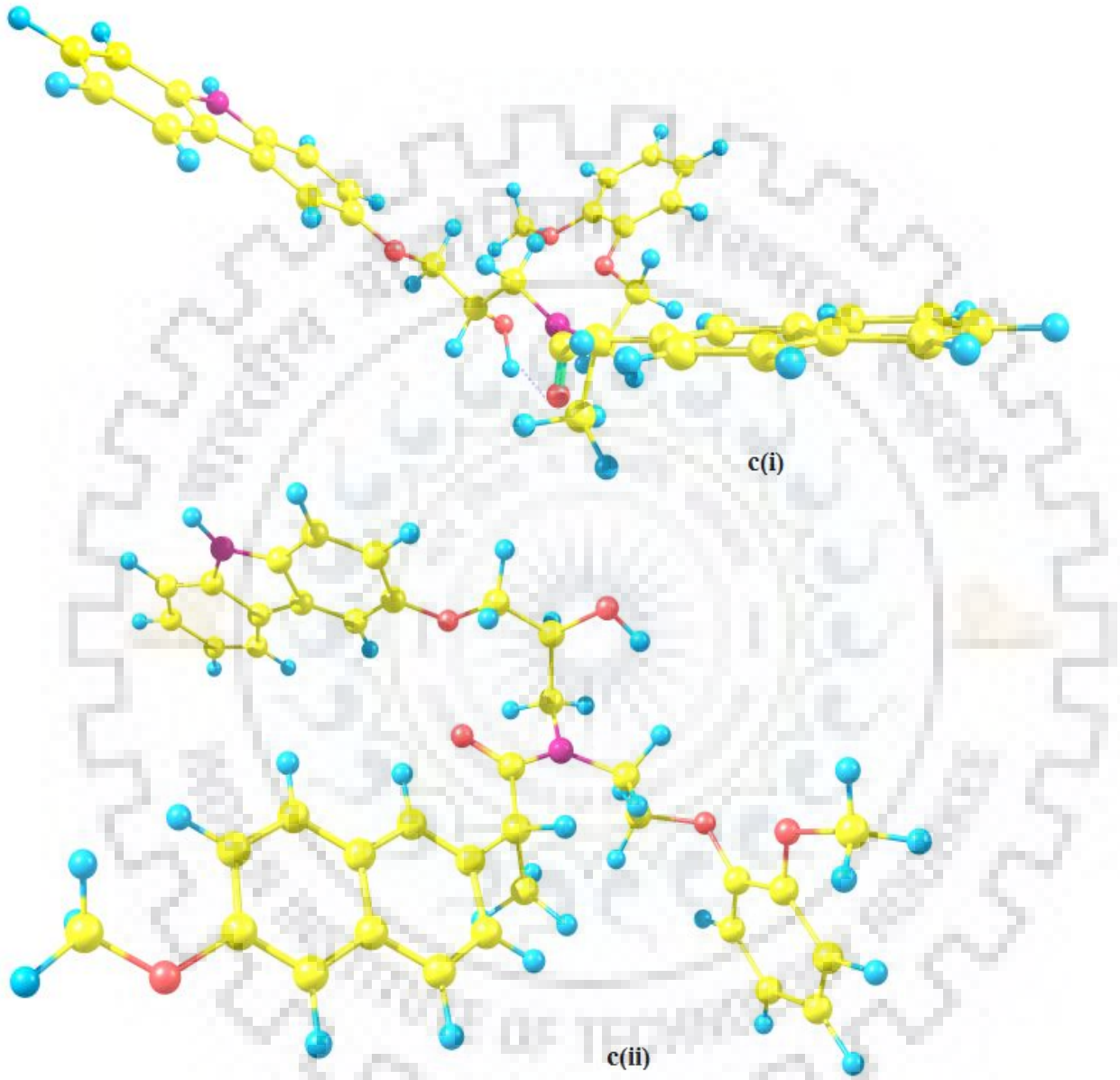


Fig 3.6 Continued...c(i), c(ii) represent, respectively, diastereomeric derivatives of Cdl. Structures at (**i**) in all cases show formation of H-bond (*trans*-type arrangement) and have the (*S*,*S*)-configuration, while (**ii**) in all cases (*cis*-type arrangement) have (*S*,*R*)-configuration.

The chromatogram for the blank plasma showed no endogenous interference as there were no additional peaks at the retention times of diastereomeric derivatives of (*RS*)-Bpl; it indicated that the method was selective for enantiomeric resolution of (*RS*)-Bpl (and other test compounds) in human plasma. **Table 3.2** summarizes the peak areas and retention times of both the diastereomeric derivatives of all β-adrenolytics. The diastereomeric derivatives **[**(*S*)-Npx−(*S*)-Bpl**]** and [(*S*)-Npx−(*R*)-Bpl] separated and purified through TLC were run independently and as an equimolar mixture under HPLC conditions optimized for separation of diastereomeric derivatives obtained by synthesis and the separation results of plasma samples were compared. The values of retention factors were in agreement. The diastereomeric derivatives of all the three β-adrenolytics present in the plasma samples were very well separated.

A comparison of the present results has been made with literature reports (described separately in another section below) which show that the present indirect approach is successful in selectivity, sensitivity and easier experimental optimization with lower costs.

3.7 Elution order of diastereomeric derivatives

Since the therapeutic behavior of the two enantiomers of β-adrenolytics is different it becomes important to know about the elution order. The isolation of the two diastereomeric amides by preparative TLC followed by recording of ^{1}HNMR and IR for each established the absolute configuration which was a verification of the elution order that [(*S*)-Npx−(*S*)-Bpl] was eluted before [(*S*)-Npx−(*R*)-Bpl], with the mobile phase. The elution results are the same in HPLC separation too. It may also be argued that the presence of hydrogen bond in (*S*,*S*)-diastereomeric derivative makes it slightly more polar and causes a greater affinity with the mobile phase and thus it eluted first. The same explanation is applicable for the diastereomeric derivatives of all the other β-adrenolytics under study.

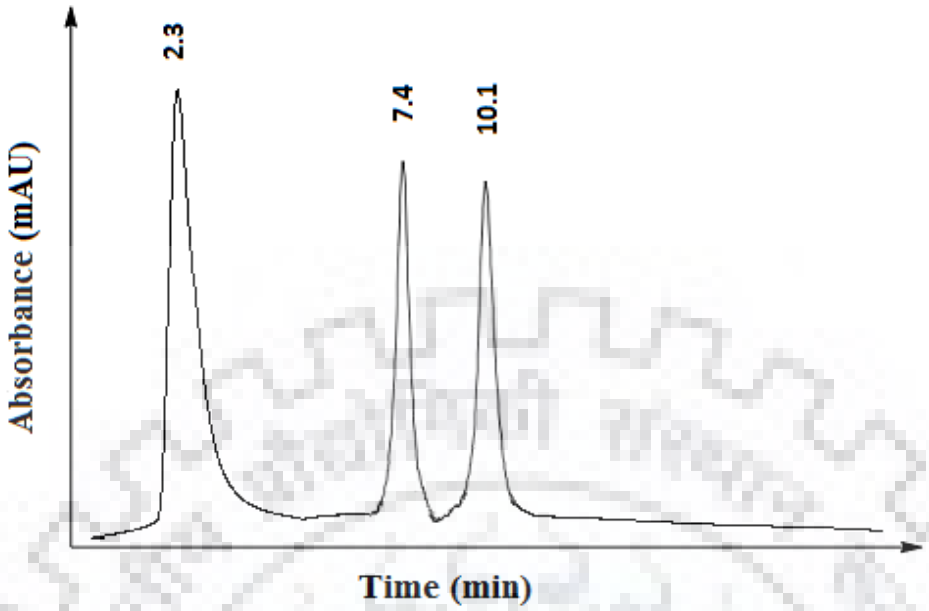


Fig.3.7 Chromatogram showing separation of mixture of diastereomeric derivatives of (*RS*)-Bpl in plasma sample. The successful separation conditions included mobile phase comprising of CH_3OH–TEAP buffer (pH 3.7) at a flow-rate of 1.0 mL min^{-1} in a gradient mode (60 to 40%) using the C_{18} column (250 mm × 4.6 mm I.D., particle size 5 μm) and detection at 230 nm. First peak (at 2.3 min) corresponds to blank plasma and after this two peaks represent the separated diastereomeric derivatives.

Table 3.2 HPLC separation data for diastereomeric derivatives of β-adrenolyticsin plasma samples

β-adrenolytics	Characteristics of separated diastereomeric derivatives			
	(*S*,*S*)-derivative		(*S*,*R*)-derivative	
	R_{t1}	*Peak area (mAU x sec.)	R_{t2}	*Peak area (mAU x sec.)
(*RS*)-Bisoprolol	7.4	26620	10.1	26369
(*RS*)-Salbutamol	5.8	27845	7.9	27749
(*RS*)-Carvedilol	7.4	26258	10.2	26589

Mobile phase, CH_3OH and TEAP buffer of pH 3.7 (60: 40, *v*/*v*) in gradient mode at a flow rate of 1 mL min^{-1}, R_{t1} and R_{t2} = retention time of the (*S*,*S*) and (*S*,*R*) diastereomeric derivatives respectively. *Peak areaas given by the system software; mAU = mili absorbance unit.

4. Method validation

Method was validated for HPLC separation, using diastereomeric derivatives of (*RS*)-Bpl (as a representative) for linearity, recovery, accuracy and precision according to the acceptance criteria of the FDA guidance for bioanalytical method validation [75].

4.1 Linearity, accuracy and precision

Samples of both diastereomeric derivatives of Bpl was prepared in the concentration range of 30 to 3000 ng mL^{-1} and plotted as concentration of diastereomeric derivative (x) vs. peak area (y). Linearity, slope and intercept were determined using developed regression equations $y = 4.02x + 99.3$ ($r^2 = 0.9999$) and $y = 3.98x + 87.8$ ($r^2 = 0.9998$) for the first and second eluted diastereomeric derivative, respectively. A linear correlation was found over this range.

The accuracy and precision data of the method was determined by analyzing samples in replicate (n=5) using different concentration (expected to contain 30, 250, 650, 1200 and 3000 ng $mL^{-1}$of individual diastereomeric derivatives) of (*RS*)-Bpl prepared with the CR, on HPLC (**Table 3.3**). As per FDA guidelines, the acceptance criterion for accuracy is ±15% of the reference value and the found mean value is within 15% of the actual value taken. Also the precision determined at each concentration is within 15% of the coefficient of variation (CV). The calculated relative standard deviation (RSD) for (*S,S*)-, and (*S,R*)-diastereomeric derivatives, respectively, was 0.62–0.85 and 0.55–0.75% for inter-day assay precision, and 0.21–0.51 and 0.19–0.31 % for intra-day assay precision. The solutions were considered stable if the deviation from nominal value was within ±10.0%. The calculated recovery for the first and second eluting diastereomeric derivatives was 99.78–100.05 and 99.89–102.22 % for inter-day assay and 99.33–107.04 and 99.09–103.34 % for intra-day assay, respectively. The method was inferred to be accurate due to very low RSD values and high percentage recovery.

4.2 LOD and LOQ

The LOQs were found to be 2.89 and 2.60 ng mL^{-1} for diastereomeric derivatives of (*S*)- and (*R*)-Bpl, respectively. Peak areas (as obtained by the system software) were used for validation studies like quantitation, recovery and stability.

UV absorbing chromophore in the form of (*S*)-(+)-Npx moiety led to sensitive detection with LOD values of 0.97 and 0.87 ng mL-1 for diastereomeric derivatives of (*S*)- and (*R*)-Bpl, respectively. The LODs for (*S*,*S*)- and (*S*,*R*)-diastereomeric derivatives are much lower than the prescribed LOD (of 1%) for pharmaceutical industries and the acceptable limit for abuse of the drug.

5. Comparison of present work with literature reports

The LOD values are 0.97 and 0.87 ng mL^{-1} for diastereomeric derivatives of (*S*)- and (*R*)-Bpl, respectively which are very low in comparison to earlier reports [85, 87, 88].There occurred easy and successful enantioseparation of (*RS*)-carvedilol and (*RS*)-salbutamol as their diastereomeric derivatives prepared with this CR while the enantioseparation of these two β-adrenolytics, in particular, was not successful by using CRs based on DFDNB [89, 44].

Table 3.3: Method validation for HPLC separation of diastereomeric derivatives of Bpl in plasma sample: Linearity, Accuracy and Precision

	First eluting diastereomer			Second eluting diastereomer		
Range (ng mL^{-1})	30-3000			30-3000		
Slope	4.02			3.98		
Intercept	99.3			87.8		
Determination coefficient (r^2)	0.9999			0.9998		
SD intercept	1.46			1.13		
SE intercept	0.83			0.68		
LOD	0.965			0.868		
LLOQ	2.89			2.60		
Actual concentration (ng mL^{-1})	Measured concentration mean± SD(ng mL^{-1})	Mean recovery (%)	RSD (%)	Measured concentration mean± SD (ng mL^{-1})	Mean recovery (%)	RSD (%)
Intra-day precision						
3000	2997.05±1.05	99.90	0.21	2996.52± 1.16	99.88	0.21
1200	1210.79±0.86	100.89	0.27	1214.23± 0.14	101.18	0.19
650	645.68±0.13	99.33	0.41	642.55 ± 0.70	99.85	0.19
250	244.19±0.90	99.67	0.50	242.75 ± 0.52	99.09	0.31
30	32.24± 0.74	107.04	0.35	34.01 ± 0.65	103.34	0.28
Inter-day precision						
3000	2999.34± 0.52	99.99	0.85	3002.39±1.85	100.08	0.62
1200	1192.42± 0.17	99.99	0.74	1186.78±0.63	98.89	0.59
650	672.18± 0.90	99.96	0.77	664.44± 0.96	102.22	0.75
250	237.13± 0.77	99.78	0.69	247.27± 1.03	98.91	0.62
30	29.01± 1.14	100.05	0.62	29.12 ± 0.99	99.07	0.55

Part II. Thin layer chromatographic resolution of some *β*-adrenolytics and a *β2*-agonist using BSA as chiral additive in stationary phase

1. Present work

Literature survey on enantioresolution of pharmaceutically important compounds, including β-adrenolytics, by TLC and the work published on TLC enantioresolution of the chosen β-adrenolytics (31-33), and the literature cited therein, clearly shows that BSA has not been used as chiral additive in stationary phase (CASP) in a non-covalent mode for enantioresolution of racemates of any kind by direct approach. Though, there are reports on application of BSA based CSPs for enantioresolution of a variety of chiral compounds by HPLC or CE (34). Therefore, we were prompted to develop simple sensitive TLC method for direct enantioresolution of certain commonly used β-adrenolytics using BSA. To the best of authors' awareness, the novelty of the present paper is the first time application of BSA as CASP in planar chromatography to achieve direct enantioresolution of certain racemic β-adrenolytics.

Selection of BSAas chiral selector

Looking into characteristics of BSA (as described in Chapter-1) it was selected as a chiral additive in stationary phase (CASP).

BSA has been used as a chiral mobile phase additive for TLC separation of a variety of enantiomers such as amino acids and their derivatives, specific drugs, uncharged compounds likebenzoin, 2-hydroxy-flavanone, homoeriodictyol, and oxazolidinones [90-92], warfarin and *p*-chlorowarfarin [28]. Twelve dansyl amino acids have been enantioseparated on RPTLC plates using BSA as chiral complexing agent in mobile phase [93]. BSA bonded chiral stationary phase (CSP) was used for HPLC separation of nineteen racemic dansyl α-amino acids [94]. BSA modified silica nanoparticles were prepared and used as a chiral adsorbent for enantioseparation of propranolol and tryptophan [95]. There are a few reports on application of BSA based CSPs for enantioresolution of certain chiral compounds by HPLC or CE [34].

2. Experimental

Details of chemicals and reagents, instrumentation and the preparation of the stock solutions are described inChapter-2.Isolation and purification of racemic analytes from commercial formulations has also been described in Chapter-2**.**

2.1 Preparation of TLC plates

The TLC plates were prepared in the laboratory as described earlier (in section I) except that BSA was used as chiral additive in the stationary phase and the concentration of BSA was varied in the silica gel slurry from 0.2 to 0.5 mM, at an interval of 0.1 mM. For this purpose, at first solutions of different molarities of BSA were prepared in water containing 0.20 % glacial acetic acid and the slurry of silica gel (25 g) was prepared in these solutions. The slurry was adjusted at four different pH, i.e, 3, 4, 5, and 6 for each concentration of BSA. The slurry was applied on glass plates (10 × 5 cm × 0.5 mm) with a Stahl-type applicator. Thus, 16 sets of TLC plates were prepared. The thin silica gel plates were kept overnight in oven (at 50 ± 2 °C). 10 µL solution of each of the racemic analytes were spotted on TLC plates with a 25 µL Hamilton syringe.

2.2 Development of chromatograms and isolation of enantiomers

Chromatograms were developed in a completely dried, pre-equilibrated, paper-lined rectangular glass chamber and then dried in an oven. Experiments were performed with binary, ternary, and quaternary mixtures of solvents such as chloroform, acetic acid, ethanol, methanol, dichloromethane and acetonitrile to achieve enantiomer separation. The chromatographic chambers were placed inside an incubator to maintain each specific temperature (15, 20, 25 or 30 °C) before development. The chamber was pre-equilibrated for nearly 15 min at each temperature.

Ten TLC plates were run by applying four spots in parallel on a single plate for one racemic analyte using final optimized mobile phase. Chromatograms were dried at 40 °C in an oven for 10 min and cooled to room temperature. The spots were located in an iodine chamber. The spots were marked and left at room temperature for iodine to evaporate from the TLC plates. The silica gel of each marked spot was scraped and extracted with ethanol.

The combined extracts, pertaining to each of the enantiomers, were centrifuged at 2500 rpm for 5 minutes and the supernatant was concentrated in vacuum. The same procedure was followed for all the analytes. Each of these solutions was examined by UV spectrophotometer and polarimeter to ascertain their concentration (using the standard plot as described above) and to calculate specific rotation.

3. Results

Recovery of the active pharmaceutical ingredients obtained from the commercial formulations was of the order of 96-99 % and the purity of the crystals was confirmed by recording melting point, λ_{max} and IR spectra; the m.p. data was found in agreement with the literature values [96]. Since the focus is on enantioresolution by direct approach using BSA the characteristic IR peaks or the λ_{max} values are not being included.

3.1 TLC Enantioresolution

Only the solvent systems, i.e. the combinations of different solvents, enabling successful resolution are reported in **Table 3.4** along with the hR_F (R_F x100) values. hR_F values are averages of at least five runs on different plates under identical conditions on the same day and on different days. The resolution was calculated by dividing the distance between two spots by the sum of the two spot radii. The resolution (R_S) varied from the lowest 1.3 for (*RS*)-Bpl to 2.6 for (*RS*)-Atl. Representative photographs of actual chromatograms are shown in **Fig 3.8**. Specific rotation values were calculated and were found to be $[\alpha]_D^{25}$ = +22.9° (c=0.7, CH_3OH), +11.0°, +26.1°(c=1.0, CH_3OH), +24.0°(c=0.5, CH_3OH), +14.1°(c=1.0, CH_3OH) for the upper spots of Bpl, Atl, Prl, Sbl and Cdl, respectively.

3.2 Effect of pH, temperature and amount of chiral additive on enantioseparation

The effect of varying pH, temperature and the concentration of the impregnating reagent were studied for a large number of solvent systems tried for enantioresolution.

pH: As mentioned before, resolution studies were conducted on plates with four different pH. Two clear spots were observed on the plates prepared at pH 4.0

(approximately). The increase in pH caused a loss of resolution. The results clearly indicated that very good enantioresolution of all the racemates was obtained at acidic pH close to the isoelectric point of BSA.

Temperature: As given in **Table 3.4**, it was observed that (*RS*)-Bpl, Atl, and Prl got resolved at 28°C, and Sbl was resolved at 25 °C while Cdl resolved at 22°C. There was poor resolution or no resolution as indicated by the observation of tailing or elongated spots outside this temperature range.

Amount of chiral additive: The best separation for all the analytes was obtained at 0.3 mM of BSA (**Table 3.4**). At lower concentration there was no resolution and at concentration higher than 0.3mM there was observed long tailing of spots.

Table 3.4: TLC experimental conditions for successful resolution of the five (*RS*)-β–adrenolytics using BSA as chiral additive in the silica gel along with hR_Fvalues and resolution data

(*RS*)-Analyte	Mobile phase	solvent ratio*	Temp (°C)	hR_F values		R_S
				(i)	(ii)	
Bisoprolol	CH_3CN-$CHCl_3$-EtOH	2.5:2:3	28	65	52	1.3
Atenolol	CH_3CN-CH_2Cl_2-CH_3OH	3:2:3	28	37	21	2.6
Propranolol	CH_3CN-CH_2Cl_2-$CHCl_3$-CH_3OH	2:2:2:1.5	28	57	44	2.4
Salbutamol	CH_3CN-CH_2Cl_2-CH_3OH	5:2:1.5	25	63	48	2.3
Carvedilol	CH_3CN-CH_2Cl_2-EtOH	3:1:3	22	65	44	2.0

*R*s: resolution; hR_F: retardation factor x 100 (R_F x 100); Development time: 10-15 min;
Detection: iodine vapors; Temp: temperature; BSA concentration: 0.3 mM; *Solvent ratio (*v*/*v*)

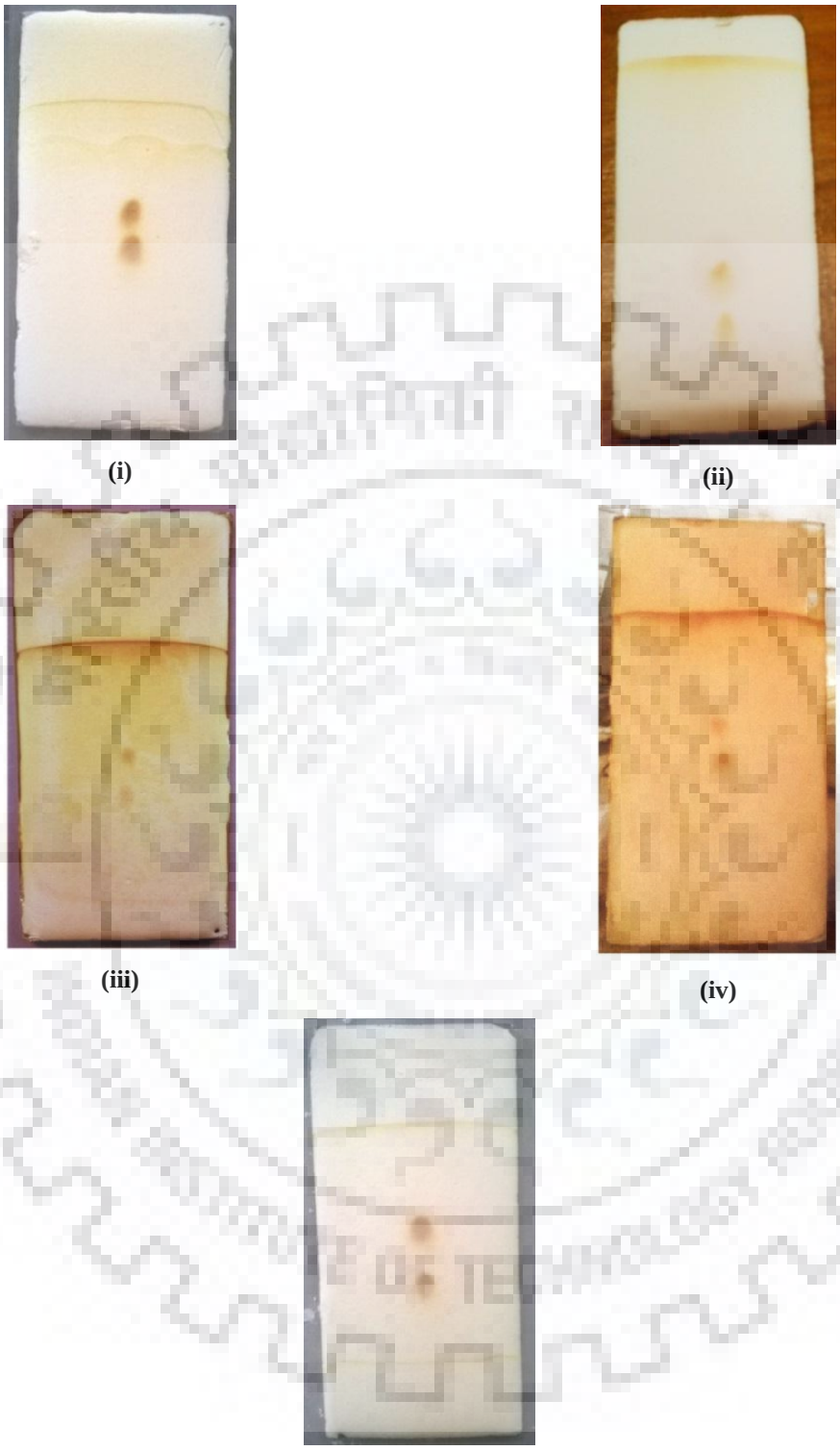


Fig 3.8 Actual photographs representing separation of racemic β-adrenolytics using BSA as chiral additive in stationary phase, (i) bisoprolol, (ii) atenolol, (iii) propranolol, (iv) salbutamol, and (v) carvedilol. TLC conditions are as per Table 3.4. Upper spot in each case belongs to (+)-isomer.

4. Discussion

4.1 Chiral additive and native enantiomers

The TLC plates with 'chiral additive in stationary phase' (CASP) were successful in resolving the racemic analytes. When the TLC plates having no CASP were spotted with the racemic analytes and developed under the identical experimental conditions each of the racemates gave a single spot. This confirmed that presence of BSA was necessary for resolution of the enantiomers.

Since BSA is insoluble in ethanol the (+)- or (–)-isomer of the corresponding racemate present in the scrapped silica gel, pertaining to each spot, went into ethanol when the said silica gel was extracted. Results from polarimetric experiments and spectrophotometric determination of concentration of the isolated enantiomers of all the analytes were used to calculate specific rotation. The polarimetric measurements also showed that the two isomers were in the ratio of 1:1 and the (+)-isomer had R_F higher than the (–)-isomer and thus eluted first. These results also confirmed the elution order. The specific rotation values so determined were in agreement with literature values [90, 96]. The enantiomers isolated in this manner were taken as reference samples for all fiveβ-adrenolytics and were used in a second set of TLC experiments in which they were applied to the plate adjacent to the racemic mixture, for comparison of R_F values with those separated from the mixture (**Fig 3.9**). Thus the isolation of native enantiomers characterized by their specific rotation values confirms direct resolution of all the β-adrenolytics. Though there was a very good resolution of all the β-adrenolytics; resolution of atenolol was better in comparison to other analytes. The five β-adrenolytics can be arranged as Atl>Prl>Sbl>Cdl>Bpl in decreasing resolution (Rs) order.

Effect of temperature: Experiments were performed in a range of temperature systematically until its effect was noted in terms of either tailing or figure-of-eight shaped spots or clear resolution. The racemates, under study, resolved well into their enantiomers in a temperature range between 22-28 °C (**Table 3.4**) when BSA was used as a chiral additive in the silica gel used for making TLC plates. A change in temperature might be

affecting the formation and/or mobility of the transient diastereomers, resulting into poor resolution or no resolution.

Mobile phase: Addition of CH_3OH in different combinations of CH_3CN-CH_2Cl_2 was successful in resolving (*RS*)-Atl and (*RS*)-Sbl while addition of EtOH was required in different combinations of the same two solvents [CH_3CN-CH_2Cl_2] for successful resolution of (*RS*)-Bpl and (*RS*)-Cdl (**Table 3.4**). Though all the racemates resolved well into their enantiomers the resolution of enantiomeric pair (*RS*)-Atl was the best among the analytes investigated.

Fig 3.9 Photograph of chromatogram showing resolution of (*RS*)-Bpl by use of BSA as chiral additive in stationary phase. From left to right: Spot 1: lower spot for(*R*)-enantiomer and the upper spot for (*S*)-enantiomer resolved from the racemate; Spot 2: pure (*R*)-isomer and Spot 3: pure (*S*)-isomer (both were isolated and characterized during this experiment, as described in the text).

4.2 Enantioselective recognition using BSA

Proteins are chiral in nature due to their chemical composition and three-dimensional shape/structure and different spatial arrangements of the functional groups, and thus show stereoselective binding to chiral molecules. Although the mechanism of chiral recognition by proteins, e.g., BSA, is largely unknown some empirically found correlations between retention behavior and mobile phase composition give a general idea

of the main types of solute-protein interactions involved [97, 98]. The three dimensional structure of proteins can have various kinds of interactions, e.g., electrostatic interaction, dipole interaction, hydrophobic interaction, π-π interaction, steric interaction, complex formation, and cavity inclusion between protein and analytes [99, 100];such interactions including hydrogen bonding between –OH of the β-adrenolytics and $–NH_2$ of the chiral selectors may be held responsible for enantioresolution using BSA in the present case in accordance with the three point interaction as explained by Dalgliesh [101].These interactions favored formation of *transient* diastereomers *in situ* and, hence, enantiomer resolution, as evidenced by the isolation of individual enantiomer(s) from the two spots on the TLC plate.

BSA has a pI of *ca* 5.4 and good resolution was obtained at pH 4.0and the selectivity of BSA is due to the presence of a large number of amino and carboxylic groups. BSA contains nearly 60 lysine residues [92] which exist in the hydrophobic regions of the macromolecular moiety. The primary interaction between the chiral selector (BSA) and the analytes for enantioseparation seems to involve steric and hydrophobic interactions for the large size of BSA. It is proposed that separation of the enantiomers could be a result of the formation of a hydrophobic pocket resulting in a selective interaction for inclusion of enantiomeric molecules from the racemic mixtures of β-adrenolytics.

Literature [102, 103] reveals that high polarity or high ionic strength of the mobile phase is not favorable for enantioresolution because it reduces the electrostatic interaction or hydrogen bonding between the BSA-silica stationary phase and analyte and thus affects stereoselectivity and retention; the success of the mobile phase in resolution of the analytes, in the present studies is in agreement with literature explanation it is not a very polar system and also does not contain any buffer to provide any kind of ions for interactions during resolution.

In a study of BSA adsorption at the hydrophilic silica/water interface Su *et al*., [104] reported that BSA had a high surface affinity since adsorption reached a plateau at a

very low BSA bulk concentration at pH 5, close to its pI. Adsorption was found to be irreversible with respect to changes in BSA concentration but reversible with respect to solution pH at low BSA concentrations and BSA formed a uniform layer between 30 and 40 Å thick [105]. These findings suggest that there was a uniform irreversible layer of BSA on the silica gel surface on the TLC plate and in this manner there was available a very good CSP without any covalent linkage in comparison to the reports where covalently bonded BSA-silica CSP was synthesized [106] and was used for resolution of tryptophan enantiomers [34] and enantioresolution of different chiral compounds at small scale HPLC [103].

5. Method validation

Different solutions of known concentration (300, 500 and 1000 μg mL^{-1}) of each of the racemic analytes were applied three times on the TLC plates having BSA as a chiral additive and determined repeatability of the method. Relative standard deviation (RSD) was found to be 0.95 %. Recovery of the enantiomers was in the range 96‑99 %. The results indicate that TLC with BSA as chiral additive can be used for detection of very small amounts of each enantiomer as per the detection limits of 0.7, 1.2, 0.84, 1.6 and 0 .9 μg (per spot) for each enantiomer of Bpl, Atl, Prl, Sbl and Cdl, respectively.

6. Comparison of *Rs* and LOD with literature reports

A comparison of the present results with those reported in literature on chromatographic separation parameters (*Rs* and LOD) using different CSPs in HPLC or chiral selectors in TLC with respect to the β-blockers under study has been given in **Table 3.5**. It shows that the present results are superior in terms of *Rs* and LOD values. It has been observed that higher *Rs* (as mentioned in **Table 3.4**) and lower LOD (as shown under 'Method Validation') for TLC resolution of enantiomers of racemic β-adrenolytics are obtained than early literature reports whether the enantioseparation was done using different types of CSPs or by using expensive instrumental techniques like RPHPLC or gas chromatography.

Table 3.5 Comparison of literature reports with present study on TLC separation (in terms of *R*s and LOD) of enantiomers of (*RS*)-β-adrenolytics using different CSPs/chiral selectors/CRs/CIR.

Sr. No.	CSP/Chiral Selector/CR/ CIR	Technique used	LOD	*Rs*	Reference
1.	ChirobioticVvancomycin	i	NA	0.8	18
2.	Chirobiotic T (Teicoplanin)	i	15 μg L^{-1}	1.3	7
3.	Cellulose tris(3,5-dimethylphenyl-carbamate) derivative	i	NA	0.6	8
4.	Amylose tris(3,5-dimethoxyphenyl carbamate)	i	10 μg mL^{-1}	1.80	19
5.	(–)–α-methoxy-α-(trifluoromethyl)-phenylacetyl chloride	ii	NA	1.44	91
6.	1-Fluoro-2,4-dinitrophenyl-L-alanine	ii	NA	1.11	91
7.	(–)–α-methoxy-α-(trifluoromethyl) phenylacetyl chloride	ii	NA	1.30	91
8.	(1-Fluoro-2,4-dinitrophenyl)-(R)-(−)-1-cyclohexylethylamine	ii	NA	1.05	91
9.	Cu(II)-L-Phenylalanine	iii	NA	1.7	92
10.	Cu (II)–L-Arginine complex	iii	NA	1.20	93
11.	L-Lysine	iii	2.6 μg	NA	28
12.	Vancomycin	iii	1.3 and 1.5 μg	NA	94
13.	Cellulose tris (4-chlorophenylcarbamate	i	NA	2.18	96
14.	Chiralpak AD-H	i	NA	0.7	96
15.	Chirobiotic V2	i	NA	1.48 & 2.21	29
16.	Chirobiotic V	i	NA	1.11 & 2.10	30
17.	CHIRALPAK IA; tris(3,5-dimethylphenylcarbamate)	i	NA	1.97	30
18.	Amylose tris(2-chloro-5-methylphenylcarbamate)	i	NA	2.2	31
19.	Bovine Serum Albumin	iii	0.7-1.6 μg mL^{-1}	1.3-2.6	Present Work

CSP, chiral stationary phase; CR, chiral reagent; CIR, chiral inducing reagent;
i, ii and iii represent the techniques used as direct HPLC, indirect HPLC and direct TLC separation, respectively; NA= Not available in the paper cited.

D. Novelty of the present work

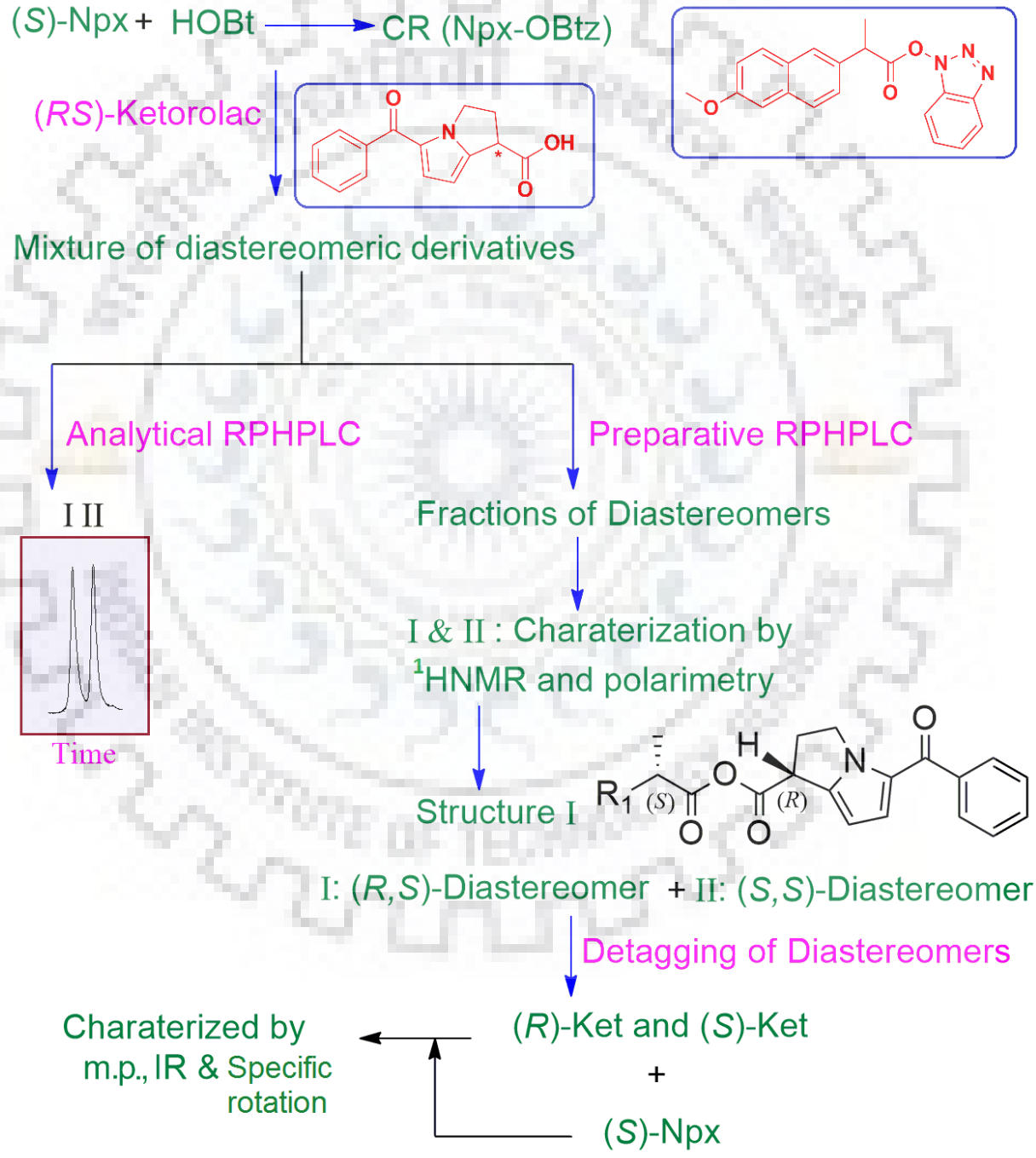
The method of enantioseparation, by indirect approach, of three β-adrenolytics was successful using both TLC and RPHPLC as their derivatives prepared with (*S*)-Npx based chiral reagent. HPLC was supplemented with TLC and, ^{1}HNMR and IR verified the structure and configuration of the diastereomeric derivatives and the enantiomeric composition of the commercial preparations. An advantage of the method, presented herein, is that it provides chirality recognition even in the absence of pure enantiomers. This work also shows a potential in the area of developing new CRs.

The method of direct approach, presented herein, provided a novel approach to use BSA as chiral additive in stationary phase for direct enantioresolution of β-adrenolytics by TLC in contrast to the literature reports on application of various synthetic resin matrices and covalently bonded BSA-silica CSP for resolution of chiral compounds by HPLC or CE. The method is very simple, direct, fast, sensitive (with very low LODs) and economical for the resolution of enantiomers of all the selected pharmaceutical analytes. The method is successful in obtaining native enantiomers for further use. The method may be worked out for successful resolution of a variety of pharmaceuticals and other organic racemic mixtures along with isolation of native enantiomers which is otherwise not feasible in other approaches or becomes very expansive using preparative chiral HPLC. Use of BSA in very small amount as chiral additive with simple silica gel provides a very good CSP for application in planar mode. Nevertheless, selection of the appropriate matrix or CSP or chiral additive in stationary or mobile phase required for resolution of a given pair of enantiomers is difficult and usually empirical.

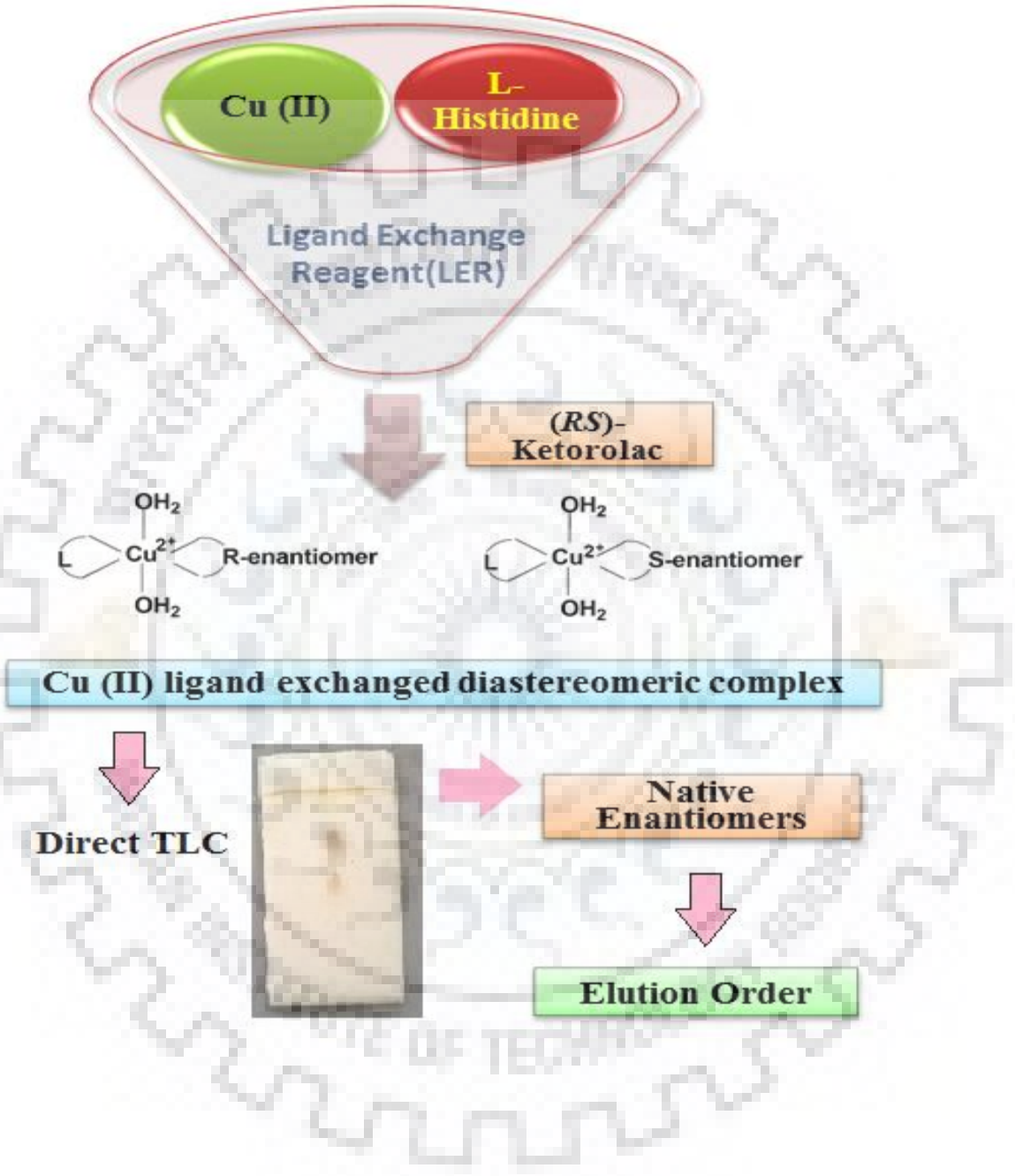
Chapter-4

Enantioseparation of NSAIDs by HPLC and TLC

Part-I



Part-II


Cu (II)
L-Histidine
Ligand Exchange Reagent(LER)
(RS)-Ketorolac
R-enantiomer
S-enantiomer
Cu (II) ligand exchanged diastereomeric complex
Direct TLC
Native Enantiomers
Elution Order

A. Introduction

Both ketorolac (Ket) and etodolac (Etd) are widely used as non-steroidal anti-inflammatory drugs (NSAIDs). The non-steroidal anti-inflammatory drugs (NSAIDs) are the most often used ones in human health care since they are available without prescription. It is a group of drugs which reduce pain, decrease fever, and, in higher doses, decrease inflammation; side effects include an increased risk of stomach ulcers. Every day more than 30 million Americans use them to soothe headaches, sprains, arthritis symptoms, and other daily discomforts. The physiological activity of both resides almost exclusively with the (*S*)-(−)-enantiomer while the drugs are marketed and administered as racemic mixtures.

The structures of the NSAIDs chosen [(*RS*)-Ket and (*RS*)-Etd] for these studies are given in **Fig. 1.1.** The pharmaceutical importance and the relevant literature on the enantioseparation of these analytes are described briefly.

B. Pharmaceutical importanceof the chosen analytes

(i) Ketorolac

Ketorolac (Ket) was introduced as a safer intravenous alternative to opioid analgesics. It has a stereogenic centre located within the pyrrolidine ring. Ket produces analgesia and decreases inflammation by inhibiting the enzyme cyclooxygenase, resulting in the decrease in formation of prostaglandins and sensitization to pain at sites of inflammation. It is more effective than other NSAIDs in reducing pain from both inflammatory and non-inflammatory causes. Its rapid onset of action, effectiveness, lack of opiate action, and safety makes it an attractive agent for general-purpose analgesia. It also has antipyretic properties 20 times more potent than those of aspirin.

(ii) Etodolac

Etodolac is used as analgesic and for the treatment of rheumatoid arthritis and osteoarthritis; it is considered relatively better as a therapeutic agent as it produces less gastrointestinal toxicity in comparison to certain other NSAIDs. It also decreases synthesis of peripheral prostaglandins involved in mediating inflammation by inhibition of

cyclooxygenase (COX) enzyme. As compared to other NSAIDs, it produces less gastrointestinal toxicity. The biochemical and pharmacological effects of (*RS*)-Etd are due to (*S*)-enantiomer, while the (*R*)-enantiomer lacks COX-inhibitory activity. The (*R*)- and (*S*)-enantiomers of Etd are not metabolically interconvertible.

C. Literature survey on enantioseparation of the chosen analytes

The method for determination of enantiomers of Ket has been described by Ing-Lorenzini*et al*., [107] but it involved tedious and time consuming extraction procedure using liquid liquid extraction. HPLC separation of diastereomeric amides [108] of (*RS*)-Ket was reported with thionyl chloride/(*S*)-1-phenyl ethyl amine but the use of thionyl chloride has been discouraged in chemical methodology since a long time. Vakily *et al*., 1995 reported pre-column derivatization of (*RS*)-Ket [109] but it also had a drawback of rapid and complete racemization due to the strong basic conditions required for derivatization.Separation of (*RS*)-Ket has been carried out using α-acid glycoprotein chiral column and (*R*)-(+)-l-(l-naphthyl)ethylamine as the derivatizing reagent [110] but the experimental procedure of sample preparation and analysis was very cumbersome, thus, there seems to be no reasonable justification for using a chiral derivatizing reagent (CDR) and then performing separation on chiral column.

Radio-chemical purification of (the synthesized) tritium-labelled *rac*-ketorolac by RPHPLC followed by direct resolution using a chiral α1-acid glycoprotein HPLC column afforded labelled enantiomers of high specific activity [111].

Analytical and semi-preparative HPLC enantioresolution of (*RS*)-Ket from pharmaceutical formulation and in human plasma using chiral columnsof monochloro-methylated derivatives of cellulose and amylose [112, 113], polysaccharide based CSP [114, 115] and teicoplanin-based stationary phase [116] has been reported.

Enantioseparation of Etd has been achieved on chiral-AGP (α1-acid glycoprotein) column [117] and on chiral stationary phases such as CHI-DMB [chemically known as *O*,*O*'-di(3,5-dimethylbenzoyl)-(2*R*,3*R*)-diallyltartardiamide] and (*R*,*R*)-DNB-DPEDA [i.e.,

(*R*,*R*)-*N*-3,5-dinitrobenzoyl-1,2-diphenylethane-1,2-diamine] [118] which are π-electron acceptor/donor stationary phases (of broad applicability).

The indirect HPLC enantioseparation of (*RS*)-Etd using (*S*)-(−)-α-(1-naphthyl)ethylamine as the derivatizing reagent [119] involved a very cumbersome derivatization reaction and multistep sample preparation till loading onto column though it was claimed as a simplified method with shortened derivatizing time.

Enantioseparation of (*RS*)-Etd has been reported from our laboratory by different methods, these include (i) HPLC enantioseparation by 'pre-column formation of chiral ligand exchange complexes', [120] (ii) direct enantioresolution involving both achiral phases in TLC where a few L-amino acids were used as *chiral inducing reagents*,[121] (iii) LC MS separation of diastereomeric derivatives that were synthesized using three different enantiomerically pure amines, [122] ($[M+H]^+$ or $[M]^+$ were recorded for confirmation of formation and structures of diastereomers), and (iv) TLC enantioseparation of diastereomeric amide derivatives synthesized with enantiomerically pure (*R*)-(−)-1-cyclohexylethylamine [123] but there was no recovery of native enantiomers.

Starek *et al*., (2010) [124] reviewed TLC methods and new TLC techniques, along with patent developments, which were developed and used for determination of NSAIDs (including Ket and Etd) in bulk drugs, formulations and biological fluids for the period from 1990 to 2008.

The present chapter: The **Chapter 4** on investigations related to enantioseparation of (*RS*)-Ket and (*RS*)-Etd is divided into two parts.

Part-I deals with semipreparative HPLC enantioseparation of the two analytes(as their diastereomeric derivatives) with easy recovery of native enantiomers without racemization and establishment of molecular asymmetry using ^{1}HNMR and polarometric studies.

Part-II describes direct resolution of (*RS*)-Ket and (*RS*)-Etd by ligand exchange TLC along with the development of method for isolation of diastereomeric complexes corresponding to each of the enantiomers (of the two racemates) via preparative TLC and obtaining the native enantiomers.

Part-I: Synthesis of diastereomeric anhydrides of (*RS*)-Ket and (*RS*)-Etd, semipreparative HPLC enantioseparation, establishment of molecular asymmetry and recovery of pure enantiomers

1. Present work

Enantioseparation of (*RS*)-Ket and (*RS*)-Etd has been achieved by RPHPLC. (*S*)-Naproxen benzotriazole ester was used as the chiral reagent (CR); it was synthesized and was characterized by UV, IR, ^{1}HNMR, elemental analysis and polarimetry. The diastereomeric derivatives were synthesized under microwave irradiation. The CR provided a pair of diastereomeric anhydrides for each of the two analytes. The diastereomeric derivatives were separated on analytical scale by RPHPLC and then these were isolated by preparative HPLC; mobile phase in isocratic mode containing methanol and aqueous TEAP was successful and separation conditions with respect to pH, flow rate, and buffer concentration were optimized. The diastereomeric derivatives were characterized and their absolute configuration was established. Hydrolysis of the derivatives provided native enantiomers under mild reaction conditions. The method provided very low limits of detection values. The present report is novel and different from existing literature being asemipreparative HPLC enantioseparation of the two analytes with easy recovery of native enantiomers without racemization and establishment of molecular asymmetry using ^{1}HNMR and polarometric studies.

2. Experimental

- Details of chemicals, and equipment and isolation and purification of the title compounds, as reference standards, along with preparation of stock solutions has been described in Chapter-2.

- Synthesis and characterization of CR-1, has been described in Chapter-2**.**

2.1 Microwave irradiated synthesis of diastereomeric derivatives of (*RS*)-Ket or (*RS*)-Etdwith CR-1, i.e. Npx-OBtz

Separate sets of reaction mixture were prepared by taking solutions of (*RS*)-Ket or (*RS*)-Etd(1mM, 100 μL) and to each was added solution of the CR-1 (1mM, 200 μL) and triethylamine (20 μL). Synthesis of diastereomeric derivatives was carried out independently.Experimental conditions were optimized for best separation by varying pH, ratio of racemic drug: CR-1 (1:1, 1:1.5, 1:2, 1:2.5)andmicrowave irradiation (MWI) time (in a range from 60 s to 150 s at an interval of 30 s) at 80% power (800 W). The diastereomeric mixture in each case was subjected to HPLC separation.

2.2 Preparative synthesis of diastereomeric derivatives

Diastereomeric derivatives were synthesized on a preparative level by scaling up the conditions optimized for synthesis. (*RS*)-Ket (150 mg, 59 mM) or (*RS*)-Etd (150 mg, 52 mM) was dissolved in 10 mL of 0.1 M $NaHCO_3$, and to each, a solution of CR (200 mg, 58 mM) in 20 mL acetonitrile was added. The yield was 320 mg (92%).

2.3 RPHPLC of diastereomeric derivatives

The diastereomeric mixture from the reaction vial was diluted 10 times using solvent system consisting of CH_3OH and TEAP buffer (10 mM, pH 3.5) and 20 μL of the same was injected onto the simple C_{18} column. The solvent system was degassed, sonicated and filtered through 0.45 μm filters before use.

To optimize the separation conditions following variations were carried out, (i) mobile phase in the isocratic elution mode with varying strength of TEAP buffer (i.e., 25 to 75 %, with a difference of 10% each time) in a period of 5-25 minutes, (ii) buffer concentrations of 5mM, 10 mM and 15mM, and (iii) flow rates of 0.5 and 1.0 mL min^{-1}, at each stage of the experiment. Oven temperature was fixed at 40 °C. The detection was done at 320 nm using PDA detector.

2.4 Isolation of diastereomeric derivatives by preparative HPLC

Separation conditions were optimized even on preparative HPLC by applying similar separation conditions as for analytical RP-HPLC. Experiments were carried out using both isocratic and gradient modes to achieve preparative enantioseparation using CH_3OH and TEAP (3.5 pH) as solvent system. Elution strength was changed from 25 to 75 % of TEAP buffer in a gradient over 30 min. Flow rate was used 5 mL min^{-1}. The mixture of diastereomers (as obtained from preparative synthesis) was diluted with CH_3OH and 500 μL of it was injected on C_{18} achiral column. Two vials were placed in rack 1 in sample collector block in preparative HPLC. Method was set for the time-interval at which two peaks were obtained for collection of separated fractions from fraction collector. 2 mL per fraction were collected in each injection. Further injections were made for twenty times and nearly 40 mL per fraction were collected in total runs. The fractions containing single and identical diastereomers were combined and concentrated in vacuum. The diastereomeric derivatives so separated were recovered and their purity was checked. These were designated as Ds-I and Ds-II as the first and second eluting diastereomeric derivatives (of Ket) and Ds-III and Ds-IV for diastereomeric derivatives (of Etd). The characterization data of the two separated diastereomeric anhydrides is given in 'results and discussion' section.

2.5 Detagging of diastereomeric derivatives and isolation of enantiomers

Ds-I and Ds-II (100 mg each) were first dissolved individually in a little amount of CH_3OH and then H_2SO_4 (10mL, 2N) was added. The solution(s) was irradiated in sealed vessel conditions under MW for hydrolysis. The MWI system operates at a frequency of 2.45 GHz with 80% continuous microwave irradiation power at 800 W. The experiment was performed in the following manner.

(i) At each change (60, 90, 120, 150 and 180 s) of MWI time, samples were analyzed by HPLC as follows; 10 μL of each of these solutions was diluted ten times with the mobile phase and 20 μL of the same was subjected to simple C_{18} column; since the retention times of the diastereomeric derivatives (of Ket) and Npx were different, there were observed corresponding peaks, till there occurred complete

hydrolysis. Based on peak areas (as given by the system software) it was observed that after a few optimizations runs complete hydrolysis or conversion of individual diastereomeric anhydrides to their respective acids was achieved within 6 min. (it was a simple achiral RPHPLC, therefore, one peak for an enantiomeric pair was obtained). Similarly, Ds-III and Ds-IV were hydrolyzed.

(ii) The hydrolysates corresponding to Ds-I to Ds-IV were freeze dried and the residue was extracted with water; it was expected that only (*R*)- and (*S*)-Ket would go into solution since (*S*)-Npx is insoluble in water. The solution was centrifuged and (*S*)-Npx 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 CH_3OH.

The methanolic solutions corresponding to the four enantiomers were concentrated and were allowed to crystallize after adding a few drops of water. The crystals were obtained by decantaion; these were air dried and were characterized.

3. Results and Discussion

The recovery of native enantiomers from diastereomeric derivatives constitutes a difficult proposition because the experimental conditions of hydrolysis may lead to either decomposition of enantiomers or racemization. Most of the time pure enantiomer (and thus the corresponding diastereomer) of the analyte is not available, therefore, additional supporting investigations such as isolation of diastereomers and recovery of enantiomers (ensuring no racemization) would be required for determination of absolute configuration and confirmation of elution order which is achieved, in the present case, by recording of ^{1}HNMR spectrum and specific rotation. The success of synthesis of diastereomeric derivatives and their separation is, however, subject to recovery of native enantiomers and verification of their configuration.

3.1 The Chiral Reagent

The synthesized CR-1 was considered to be enantiomerically pure (as described in Chapter-3 section 3.1). Since no additional peaks were observed in HPLC separation

chromatograms (for the diastereomeric derivatives) it was further inferred that the CR-1 used for synthesis of diastereomeric derivatives was enantiomerically pure. Had there been any impurity in the CR-1 corresponding to (*R*)-(−)-isomer of naproxen there would have obtained additional peaks in the HPLC separation chromatograms for the diastereomeric derivatives that would have formed with such a small amount of the (*R*)-(−)-isomer of the CR-1.

3.2 The Diastereomeric Derivatives

The optimized reaction conditions for completion of reaction for the synthesis of diastereomeric derivatives were 1:2 molar ratio of analytes CR-1 and a MWI time of 150 s. It is known that the rate of reaction of enantiomers with chiral molecules is different. The two enantiomers in the (*RS*)-analyte may react at different rates with the CR-1 which is a chiral molecule. The derivatization reaction of (*RS*)-Ket or (*RS*)-Etd with the CR-1 (from among different mole ratios) showed a slight kinetic resolution at 1:1 ratio of (*RS*)-Etd: CR-1. Completion of derivatization reaction was inferred from the identical peak areas (as provided by the system software) and the 94 % recovery of eluted diastereomers provided an estimate of yields**.** Effect of MWI time on completion of derivatization reaction of (*RS*)-Ket is shown in **Fig. 4.1.**

A representative scheme for synthesis of CR-1 and diastereomeric derivatives of (*RS*)-Ket is shown in **Fig. 4.2**. Since the derivatization involved a reaction between the activated terminal of (*S*)-(+)-Npx and the carboxyl terminal of the analyte [say (*RS*)-Etd] and in none of the two reactants direct attack on stereogenic centre was involved and there was detected no racemization throughout the study. Thus, formation of the following types of diastereomeric derivatives were considered to have been formed, (a) [(*R*)-Ket-(*S*)-Npx] and (b) [(*S*)-Ket-(*S*)-Npx], and (c) [(*R*)-Etd-(*S*)-Npx] and (d) [(*S*)-Etd-(*S*)-Npx]. The first letter [capital (*R*) or (*S*)] represents the absolute configuration of the enantiomer of the corresponding analyte while the second notation represents the absolute configuration of (*S*)-Npx.

The earlier reports in literature [76, 126, 127] have shown that a chiral amide prepared from (*S*)-(+)-Npx and 1H-benzotriazole acts as a CR-1 for the formation of diastereoisomeric derivatives of various pharmaceuticals and other compounds containing an amino group; the amino group acts as a nucleophile, attacks the CR-1 and substitutes the benzotriazole moiety and the overall reaction results into formation of the pair of diastereomeric derivatives having an amide bond. But in none of these reports native enantiomers were obtained.

Besides, there is no report on the formation of diastereoisomeric derivatives for any of the chosen analytes [(*RS*)-Ket or (*RS*)-Etd] with a CR-1 based on (*S*)-(+)-naproxen and 1-hydroxybenzotriazole. It may be argued that the present CR, being an ester with (*S*)-(+)-Npx as the chiral moiety, is more reactive (in comparison to an amide) to form the diastereomeric derivatives of the chosen analytes under mild reaction conditions, and because of mild conditions the formation of diastereomeric derivatives is faster and chances of racemization are reduced.

The possible mode of bond formation leading to diastereomeric derivatives, as the anhydrides in nature (as shown in **Fig. 4.3**), is discussed below. Generally anhydrides and esters have intermediate reactivity.

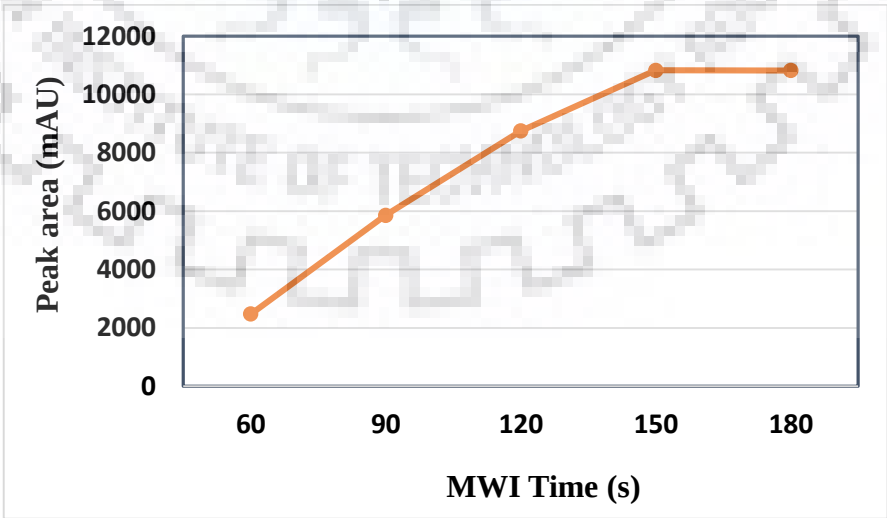


Fig. 4.1 Effect of MWI time on completion of derivatization reaction of (*RS*)-Ket

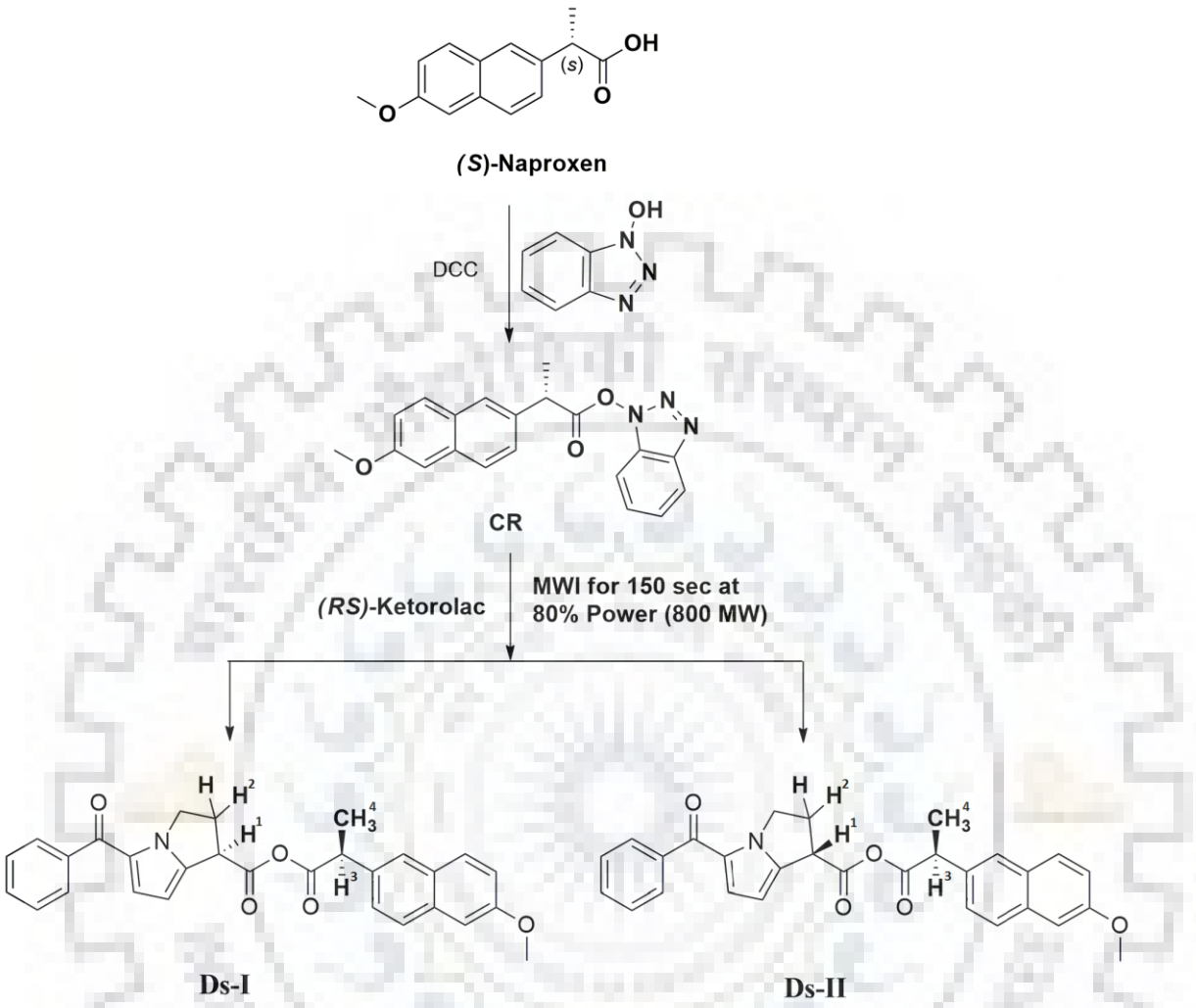


Fig.4.2. A representative scheme for synthesis of CR-1 and diastereomeric derivatives of (*RS*)-Ket.

The electronegativity values for N, C and O atoms are 3.04, 2.55 and 3.44, respectively, on the scale developed by Linus Pauling [128, 129]. Besides, the calculated electronegativity of carbonyl group (>C=O) by revised Lewis-Langmuir equation is 2.706 ± 0.01 [130]. The difference between the electronegativities of carbon and oxygen is large enough to make the C=O bond moderately polar. In the CR, there is a N atom (of the benzotriazole moiety) at the position of hydroxyl hydrogen of the carboxyl group (of the parent naproxen moiety). Thus, the carbonyl carbon (>C=O) becomes more electron deficient due to the presence of highly electronegative elements (O and N) in its close

vicinity (**II** in **Fig. 4.3**). Literature suggests that the electronegativity depends on the environment of a particular atom within a molecule as well as on its degree of hybridization [131-133].

In the present situation of synthesis of diastereomeric derivatives, the COO^- group of analyte (acts as a nucleophile and) attacks on the electrophilic carbon of CR-1, and the *pi*-bond electrons are resonated to the more electronegative oxygen forming a tetrahedral adduct via carbon-nucleophilic bond with a change in hybridization from sp^2 to sp^3 (as transitional intermediate, **III** in **Fig. 4.3**). The consequence of such inductive and resonance effects results into the carbonyl carbon having large δ^+ charge and its high reactivity; it thus gets easily attacked by nucleophile (the COO^- group from the (*R*)-, or (*S*)-enantiomers of Ket or Etd in the racemic analyte). At the same time, the OH-benzotriazole moiety acts as a good leaving group and is knocked out very easily.

Thus, the overall reaction leads to the formation of the product (**IVa** and **IVb** in **Fig. 4.3**) as a pair of diastereomeric derivatives which are structurally anhydrides. Such a structure is more stable due to greater resonance or greater delocalization (with two oxygen atoms) and therefore its formation is favored. The presence of a weak base (triethylamine) during the derivatization reaction would facilitate the formation of the said diastereomeric derivatives by making the analyte more nucleophilic in nature and the excess of substrate would not react with its own molecule to form naproxen anhydride.

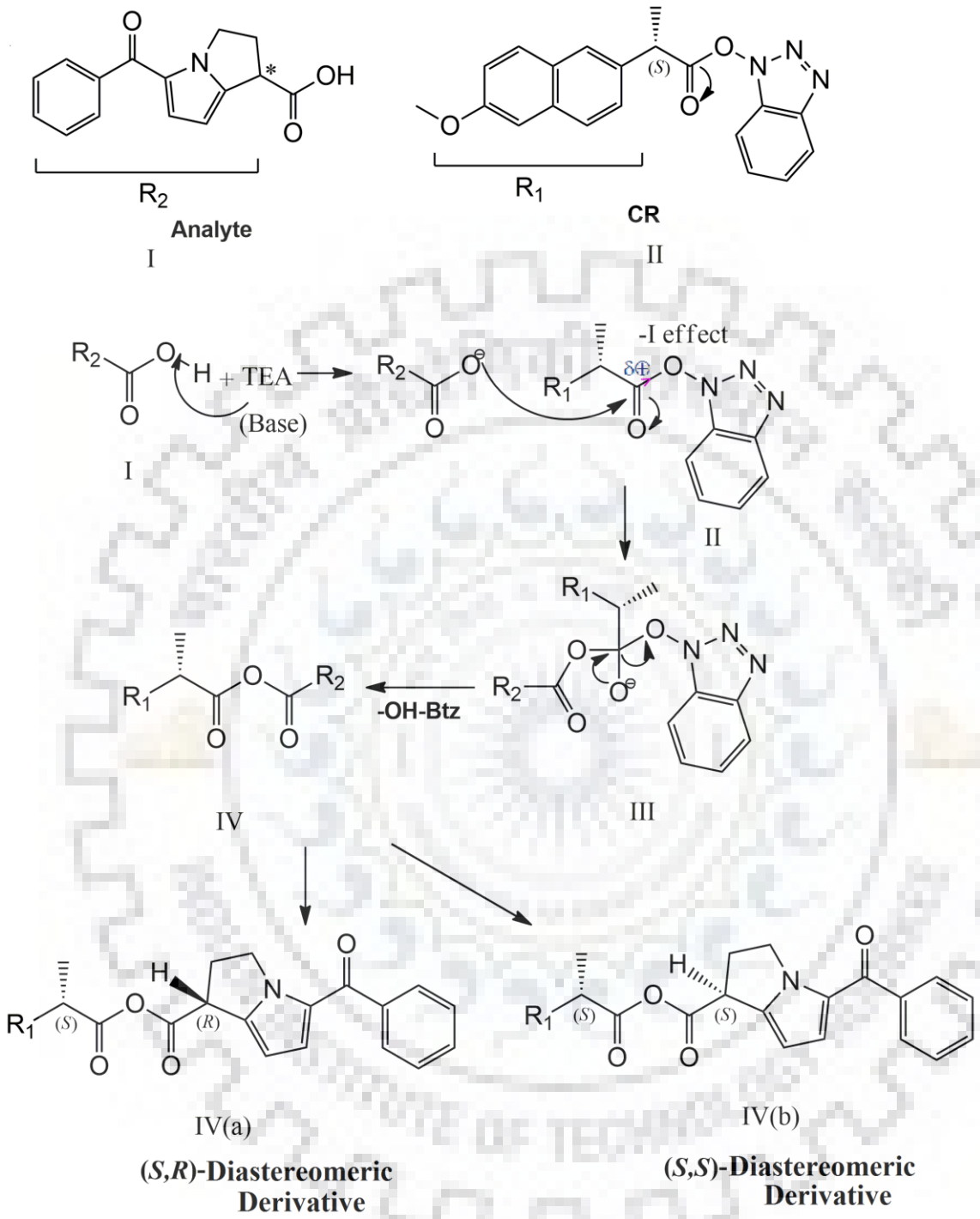


Fig. 4.3 Plausible mechanistic way of anhydride synthesis

3.3 HPLC Separation of diastereomeric mixtures

It was observed that the same chromatographic conditions were successful for separation of diastereomeric pairs of both the analytes; these included mobile phase comprising of CH_3OH-TEAP buffer (pH 3.5) at a flow-rate of 1.0 mL min^{-1} in the isocratic mode (75 to 25 %) using C_{18} column and detection at 320 nm.

Actual chromatograms (obtained from the system) showing baseline separation of pairs of diastereomeric derivatives of (*RS*)-Ket and (*RS*)-Etd are given in **Fig. 4.4** (i), and (ii), respectively. The diastereomeric derivatives of both racemic analytes showed a very good separation. **Table 4.1** summarizes the values for retentionfactor (*k*), separation factor (α) and resolution (*Rs*) for the diastereomeric derivatives separated under the optimized HPLC conditions. It was observed that resolution for separation of diastereomeric derivatives of Etd was higher than that for diastereomeric derivatives of Ket while the retention time of diastereomeric derivatives of Ket was higher.

The peak areas as obtained from the system software are the same i.e., 236390 and 236274 for each peak of the two diastereomeric derivatives of Ket and, 288726 and 288364 for each peak of the two diastereomeric derivatives of Etd, respectively.An equimolar mixture of the diastereomers, [(*R*)-Ket-(*S*)-Npx] and [(*S*)-Ket-(*S*)-Npx], separated and isolated through preparative HPLC was run independently under the same optimized HPLC separation conditions of diastereomeric derivatives. The results were in agreement in terms of retention time and peak area.

A comparison of the present results with the literature shows that the indirect approach is better in selectivity, sensitivity, lower costs, and easier experimental optimization while CSPs are very expensive and optimization of separation conditions is cumbersome and time consuming and the UV detection of the underivatized enantiomers is less sensitive in the absence of a good chromophore.

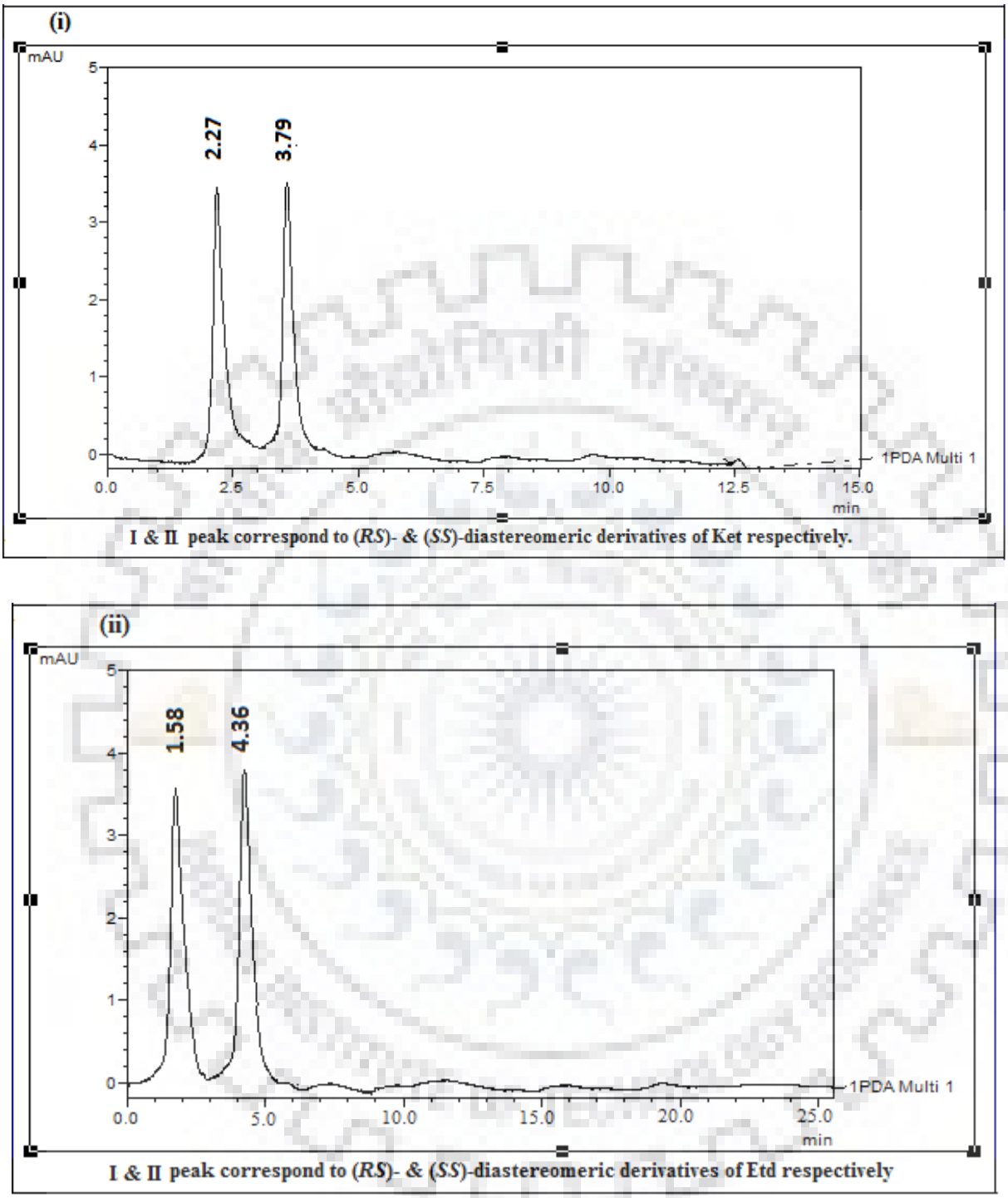


Fig. 4.4 Actual chromatograms (obtained from the system) showing separation of diastereomeric derivatives of (i) (*RS*)-Ket, (ii) (*RS*)-Etd. Chromatographic conditions: column, LiChrospher C_{18} (250 mm × 4.6 mm I.D., 5 μm particle size); mobile phase: CH_3OH–TEAP buffer (pH 3.5), binary gradient (75 to 25%); flow-rate: 1mL min^{-1}; detection, 320 nm; injection volume 10 μL; the first eluting peak corresponds to (*R*,*S*)-diastereomeric derivative in both cases.

Table 4.1: HPLC separation data for diastereomeric derivatives of racemic analytes

Racemic Analytes	Characteristics of separated diastereomers			
	k_1	k_2	α	*Rs*
(*RS*)-Ketorolac	0.89	2.16	2.42	6.1
(*RS*)-Etodolac	0.41	1.90	4.63	7.2
Mobile phase, CH_3OH and TEAP buffer of pH 3.5 (75: 25, *v*/*v*) in isocratic mode at a flow rate of 1mL min^{-1}; k_1 = retention factor of the diastereomeric derivative (Ds-I); k_2 = retention factor of the diastereomeric derivative (Ds-II); α = separation factor; *Rs* = resolution.				

Formula used for calculation of resolution: $\boldsymbol{Rs} = \frac{\boldsymbol{t_2 - t_1}}{\boldsymbol{0.5\, w_1 + w_2}}$

where,

Rs = resolution

t_1 and t_2= retention times of first and second peak, respectively

$w_1$and w_2= base widths of first and second peak, respectively;

The values for t_1, t_2, w_1 and w_2 have been provided by the system software.

here,

(i) for analyte Ket: t_1 = 2.27, t_2 = 3.79; w_1 = 0.2, w_2 = 0.3; so calculated *Rs* value = 6.1

(ii) for analyte Etd: t_1 = 1.58, t_2 = 4.36; w_1 = 0.5, w_2 = 0.3; so calculated *Rs* value = 7.2

The retention times were compared with the diastereomers Ds-I and Ds-II that were separated through preparative HPLC and were run independently and as an equimolar mixture. Based on the data obtained through system software, resolution (*Rs*) of diastereomeric derivatives of racemic analytes (Ket and Etd, respectively) for HPLC separation has been calculated as per standard formula.

3.4 Isolation by preparative HPLC and characterization of diastereomeric derivatives

The chromatographic separation conditions optimized for analytical separation were applied for preparative scale separations and adjusted suitably. Use of mobile phase CH_3OH-TEAP buffer in 75:25 ratio (in isocratic mode) at a flow rate of 5 mL min^{-1} was found as the optimized successful separation condition for isolation of derivatives of (*RS*)-Ket.

The diastereomeric derivatives so isolated by preparative HPLC were characterized by recording ^{1}HNMR, UV and IR spectra, specific rotation, melting point, and CHN analysis. The characterization data of diastereomeric anhydrides of (*RS*)-Ket is given below.

First eluting diastereomer (Ds-I):

Yield: 165 mg (94.3 %); Recovery: 91.3 %; $[\alpha]_D^{25}$ = –66.7° (c, 1.0, CH_3OH); m.p., 132±4°C; UV (λ_{max} in CH_3OH, 231 nm); IR (KBr): 3767, 3411, 2993, 2926, 2849, 2360, 2325, 1828, 1754, 1611, 1459, 1389, 1270, 1203, 1168, 1038, 944, 891, 806, 752, 691, 606, 562, 524, 477 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$-d1): δ7.89 (d, 1 H, Ar), 7.86 (d, 2 H, Ar), 7.84 (d, 1 H, Ar), 7.7 (t, 1 H, Ar), 7.67 (dd, 2 H, Ar), 7.47 (d, 1 H, Ar), 7.42 (s, 1 H, Ar), 7.40 (s, 1 H, Ar), 7.30 (d, 1 H, Ar), 7.2 (d, 1 H, Ar), 7.09 (d, 1 H, Ar), 3.96 (t, 1 H), 3.93-3.85 (t, 2 H), 3.89 (s, 3 H), 3.87 (q, 1 H), 2.31-2.17 (m, 2 H), 1.43 (d, 3 H); Calculated for $C_{29}H_{25}NO_5$: C, 74.50 %; H, 5.39 %; N, 3.00 %; CHN found C, 74.46 %; H, 5.41 %; N, 2.87 %.

Second eluting diastereomer (Ds-II):

Yield: 160 mg (91 %); Recovery = 96.5%; $[\alpha]_D^{25}$ = +102.3° (c, 1.0, CH_3OH); m.p., 133±2 °C; UV (231 nm, λ_{max} in CH_3OH); IR (KBr): 3761, 3414, 2927, 2849, 2383, 2293, 1768, 1836 1614, 1459, 1389, 1303, 1270, 1203, 1174, 1124, 1037, 945, 886, 812, 752, 688, 603, 553, 473 cm^{-1};^{1}H NMR (400MHz, $CDCl_3$-d1): δ7.89 (d, 1 H, Ar), 7.87 (d, 2 H, Ar), 7.85 (d, 1 H, Ar), 7.69 (t, 1 H, Ar), 7.65 (dd, 2 H, Ar), 7.48 (d, 1 H, Ar), 7.40 (s, 1 H, Ar), 7.39 (s, 1 H, Ar), 7.29 (d, 1 H, Ar), 7.2 (d, 1 H, Ar), 7.1 (d, 1 H, Ar), 3.89 (s, 3 H), 3.82-

3.84 (t, 2 H), 3.81 (t, 1 H), 3.79 (q, 1 H), 2.30-2.01 (m, 2 H), 1.57 (d, 3 H); Calculated for $C_{29}H_{25}NO_5$:C, 74.5 %; H, 5.39%; N, 3.00%; CHN found C, 74.28%; H, 5.43%; N, 2.96%.

3.5 Structure and configuration of diastereomeric derivatives

The three dimensional chemical structures and configurations of the diastereomeric derivatives of (*RS*)-Ket (as a representative), along with spatial orientation of groups with respect to the anhydride bond, (as shown in Fig. 4.3) were examined and verified by developing the molecular models with the '*orbital molecular building system*' (Cochranes of Oxford Ltd, Leafield, Oxford OX8 5NT, England).

^{1}HNMR spectra of the diastereomric derivatives: The chemical shifts for four (α and β) protons, i.e. H^1, H^2, H^3 and H^4 in the two diastereomeric derivatives (as marked in Fig. 4.2) are observed in the ^{1}HNMR spectrum (Fig. 4.5). The relative orientation of the nucleus with respect to the external field changes its chemical shift (δ) and the ring current induced due to the delocalized π-electrons of the aromatic rings; it is clearly seen that in the first eluting diastereomer (Ds-I) H^1, H^2, and H^3 protons (shown in Fig. 4.2) are more deshielded and H^4 proton is more shielded as evidenced by chemical shift observed for the second diastereomer (Ds-II). The protons that lie outside the ring experience deshielding because the induced magnetic field has the same direction outside the ring as the external field. In contrast, the protons lying in the vicinity of the aromatic ring, experiences shielding because both fields are in opposite direction.

By observing their shielding and deshielding chemical shift values, conformations were drawn in 3D view by using software '*Chem3D Pro 12.0*'. By these structures, all the δ-values could be explained well. **Fig. 4.6** shows 3D structures of different conformations for the (*R,S*)-diastereomeric derivative of Ket prepared with CR-1; in4.6a the H^1 (as already marked in Fig. 4.2 and correlated with NMR spectrum shown in Fig. 4.5) lies in the plane of π-electron containing rings (but outside the rings), and thus experiences deshielding effect while H^2 proton of Ket (in 4.6b) and H^4 of CR-1 (in 4.6d) are pointing to the ring current of the pyrrole moiety of Ket. The proton H^3 at asymmetric carbon of CR (Fig. 4.6c) points away from both the π-electron containing rings.

On the other hand, 3D structures of different conformations drawn for the (*S*,*S*)-diastereomeric derivative are shown in **Fig. 4.7**. The proton H^2 (Fig. 4.7b) lies in the ring current of the naphthalene ring while H^3 (Fig. 4.7c) points to the ring current of the pyrrole ring. The protons H^1 and H^4 (Fig. 4.7a and 4.7d, respectively) point away from any of the rings.

Both pyrrole and naphthalene rings have strong anisotropic shielding effect in the presence of external magnetic field resulting into a chemical shift difference of 0.15 δ for H^1 protons between the two diastereomeric derivatives; similarly the difference for H^2, H^3 and H^4 was observed to be 0.16, 0.08, and -0.14 δ, respectively (Fig. 4.5). Hence pyrrole ring shields the H^2 and H^4 protons (shown in Fig. 4.6b and 4.6d) and H^3 proton (in Fig. 4.7c) while H^2 proton (Fig. 4.7b) experiences shielding effect due to naphthalene ring. As naphthalene ring has more number of π-electrons its anisotropic effect will be more and will shield even more in Fig. 4.7b than Fig. 4.6b.

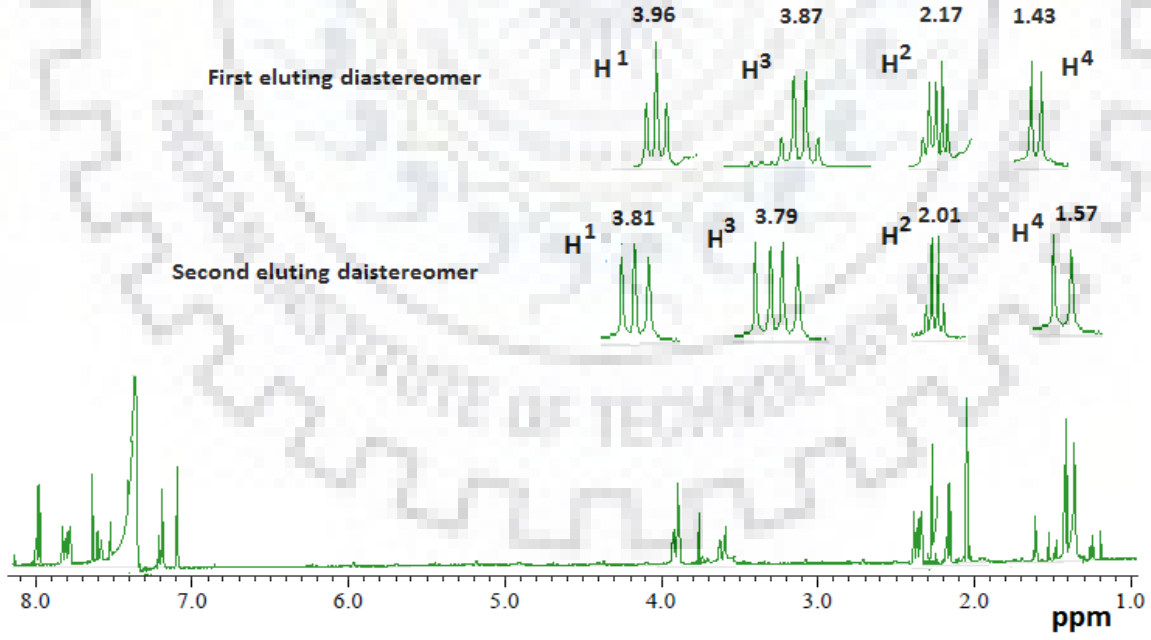


Fig. 4.5 Sections of ^{1}H NMR (400 MHz using $CDCl_3$) spectra of diastereomeric derivatives, illustrating the difference in δ for (α and β) protons corresponding to asymmetric carbon.

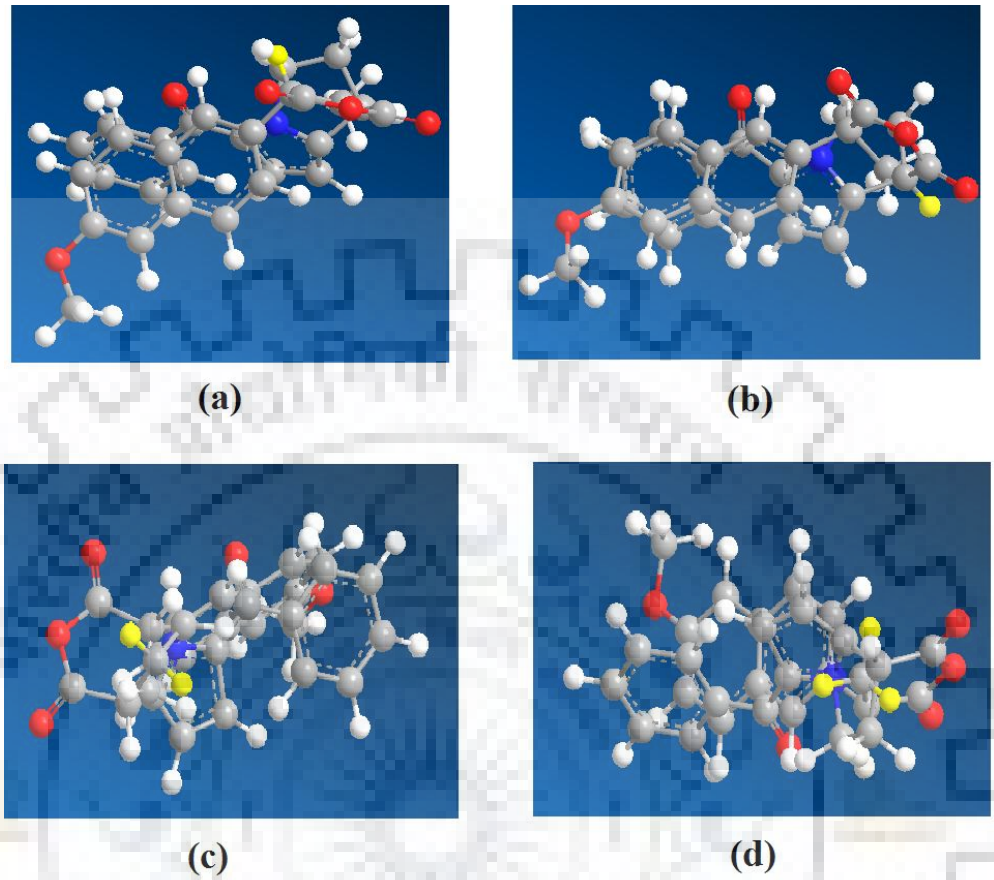


Fig. 4.6 3D structures of different conformations (drawn by using software chem3D Pro 12.0) for the (*R*,*S*)-diastereomeric derivative of Ket prepared with CR; in **4.6a** the H^1 (as already marked in **Fig. 4.2** and correlated with NMR spectrum shown in **Fig. 4.5**) lies in the plane of π-electron containing rings (but outside the rings), and thus experiences deshielding effect while H^2 proton of Ket (in **4.6b**) and H^4 of CR (in **4.6d**) are pointing to the ring current of the pyrrole moiety of Ket. H^3 proton at asymmetric carbon of CR (**Fig. 4.6c**) points away from both the π-electron containing rings.

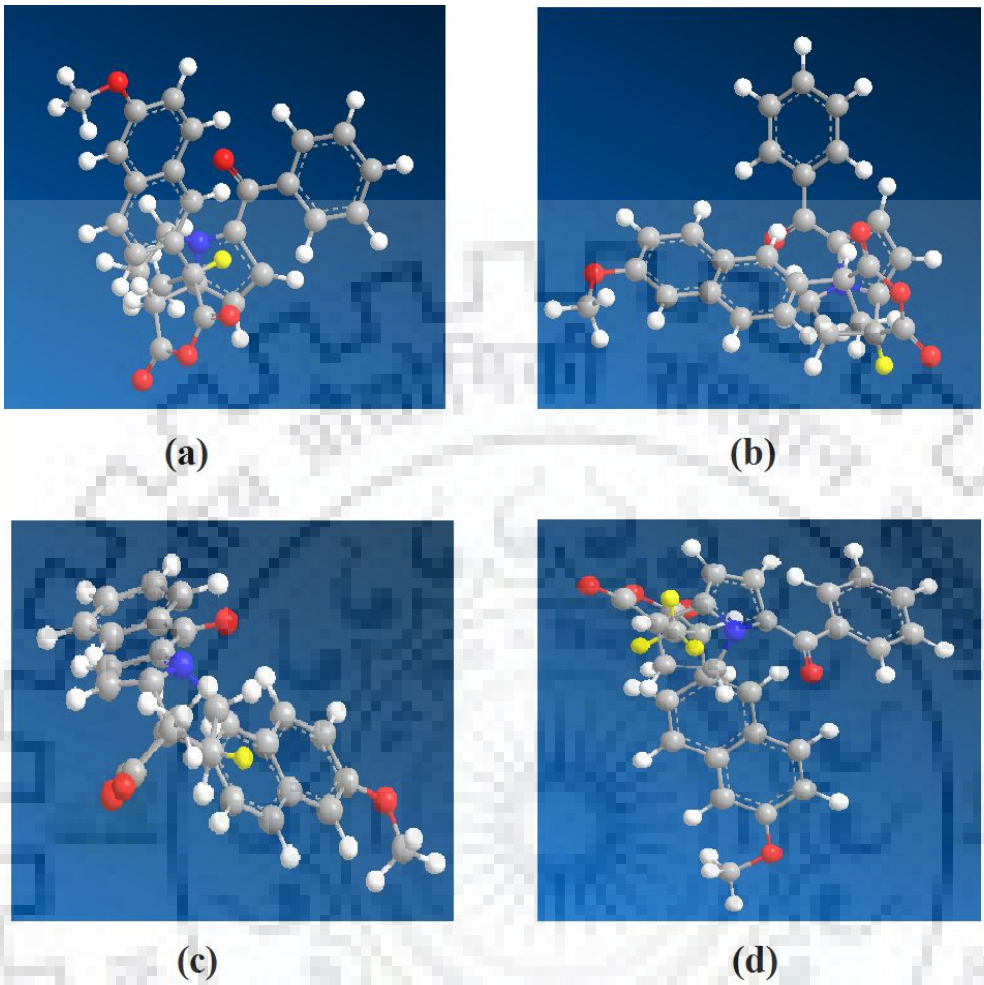


Fig. 4.7 3D structures of different conformations of diastereomeric derivative, having (*S*,*S*)-configuration, of Ket prepared with CR, using software chem3D Pro 12.0. The proton H^2 (**Fig 4.7b**) lies in the ring current of the naphthalene ring while H^3 (**Fig. 4.7c**) points to the ring current of the pyrrole ring. The protons H^1 and H^4 (**Fig 4.7a and 4.7d**, respectively) point away from any of the rings.

Based on these figures and values, it is inferred that the first eluting diastereomeric derivative corresponds to [(*R*)-Ket-(*S*)-Npx] and the second diastereomeric derivative corresponds to [(*S*)-Ket-(*S*)-Npx]; the results are in agreement with the calculated values of the specific rotation of the pure enantiomers obtained by detagging from the corresponding diastereomeric derivatives.

3.6 Detagging of diastereomeric derivatives

One of the objectives of the present investigations has been to obtain native enantiomers from the diastereomeric derivatives after their HPLC separation. Therefore, the experiments were designed to obtain such derivatives that could be detagged under relatively mild conditions and thus anhydrides; these are reactive and undergo easy hydrolysis. The acid hydrolysis did not involve any reaction at the stereogenic centre, therefore, racemization was neither theoretically expected nor experimentally observed. Recovery of enantiomers of both the analytes [(*RS*)-Ket and (*RS*)-Etd] and that of (*S*)-(+)-Npx were in appropriate molar ratio and in good yields.

All the native enantiomers and (*S*)-(+)-Npx so isolated and purified were characterized by recording specific rotation and by spectroscopic techniques like ^{1}HNMR, HRMS, and IR. The characterization data of the four purified enantiomers and (*S*)-Npx are presented below.

(*S*)-Ketorolac: Yield, 38.4 mg (96 %); color: beige; solid; m.p., 163-166 °C; $[\alpha]_D^{25}$ = –165° (c=1, CH_3OH); analyzed calculated for $C_{15}H_{13}NO_3$: C, 70.58 %; H, 5.13 %; N, 5.49 %; found C, 70.42%; H, 5.18%; N, 5.41%.

(*R*)-Ketorolac: Yield, 37.6 mg (94 %); color: beige; m.p., 163-166 °C; $[\alpha]_D^{25}$ = +164° (c=1, CH_3OH); analyzed calculated for $C_{15}H_{13}NO_3$: C, 70.58%; H, 5.13 %; N, 5.49 %; found C, 70.45 %; H, 5.20%; N, 5.32 %.

Some of the common characterization data for both the enantiomers of Ket is as follows:

UV (λ_{max} 320 nm in CH_3OH,); IR (KBr) 3423, 3055, 2858, 1666, 1614, 1519, 1462, 1393, 1336, 1272, 1209, 1150, 1056, 939, 891, 770, 724, 665, 593 and 447 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$-d1): δ 11.4 (s, 1 H), 7.88 (d, 2 H, Ar), 7.71 (t, 1 H, Ar), 7.62 (dd, 2 H, Ar), 7.40 (d, 1 H, Ar), 6.12 (d, 1 H, Ar), 3.81 & 3.72 (t, 2 H), 3.68 (t, 1 H), 2.18-2.38 (m, 2 H).

(*S*)-Etodolac: Yield, 35.6 mg (95.3 %); color: whitish; m.p., 146-149 °C; $[\alpha]_D^{25}$ = –23.98° (c=1, EtOH); analyzed calculated for $C_{17}H_{21}NO_3$: C, 71.06 %; H, 7.37 %; N, 4.87 %; found C, 71.18 %; H, 7.23 %; N, 4.92 %.

(*R*)-Etodolac: Yield, 36.8 mg (94 %); color: whitish; solid; m.p., 146-149°C; $[\alpha]_D^{25}$ = +24° (c=1, EtOH); analyzed calculated for $C_{17}H_{21}NO_3$: C, 71.06 %; H, 7.37 %; N, 4.87 %; found C, 71.09 %; H, 7.19 %; N, 4.82 %.

Some of the common characterization data for both the enantiomers of Etd is as follows:

UV (λ_{max} 223 nm in CH_3OH,); IR (KBr) 3467, 3306, 2991, 2818, 2344, 1706, 1622, 1590, 1570, 1510, 1451, 1382, 1227, 1178, 1025, 1060, 899, 728, 662, 595 and 535 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$-d1): δ 12.38 (s, 1 H), 11.8 (s, 1 H), 7.14 (dd, 1 H, Ar), 7.10 (d, 1 H, Ar), 6.95 (d, 1 H, Ar), 3.53 & 3.81 (t, 2 H), 2.63 & 2.57 (t, 2 H), 2.70 & 2.51 (s, 2 H), 0.85 (s, 3 H), 1.72 (q, 2 H), 2.71 (q, 2 H), 1.31(t, 3 H);

(*S*)-Npx. Yield, 57.2 mg (95.3%) and 56.5 mg (94.2%) respectively from Ds-I and Ds-II;Color, off white, crystalline; 224 °C; $[\alpha]_D^{25}$ = –104° (c = 0.5, $CHCl_3$); UV (λ_{max} in CH_3OH, 294 nm); IR (KBr) 3265, 2931, 1724, 1294 and 1085 cm^{-1}. HRMS: calculated for $C_{16}H_{21}NO_2$, 362.1471 ($M^{++}H$), found 362.1556; calculated 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 %.

3.7 Elution Order

Determination of elution order of the species getting separated in a chromatographic experiment is an important criterion to establish the success of the

analytical procedure. The diastereomeric derivatives were obtained by preparative HPLC as first eluting and second eluting species. Further, the native enantiomers were isolated via hydrolysis of each of these derivatives and were characterized, and thus the elution order is very clearly established.

4. Method Validation

Validation studies were done for recovery, linearity, accuracy and precision using diastereomeric derivatives of (*RS*)-Ket (as a representative) by following International Council for Harmonisation (ICH) guidelines [4]. Peak areas (given by system software) were used to establish stability and recovery.

Validation studies were carried out by taking ketorolac as a representative. Sample solutions of both the diastereomeric derivatives of Ket were prepared in 45 to 5000 ng mL^{-1} concentration range. Samples were analyzed in replicate (n=5) using concentrations (45, 500, 2000, 3500 and 5000 ng mL^{-1} of individual diastereomeric derivatives) of (*RS*)-Ket prepared with the CR-1, on HPLC.

The acceptance criterion for accuracy is ±15% of the reference value and the found mean value is within 15% of the actual value taken. Also the precision determined at each concentration is within 15% of the coefficient of variation (CV). Regression equations were developed by plotting graphs between concentration of diastereomeric derivative (x) and peak area (y) and found $y = 4.97x + 4721.4$ ($r^2 = 0.99$) and $y = 4.96x + 4723$ ($r^2 = 0.99$) for the first and second eluted diastereomeric derivative, respectively. Slope, intercept, relative standard deviation (RSD), recovery and linearity were determined using these regression equations.

The calculated RSD for (*R,S*)-, and (*S,S*)-diastereomeric derivatives, respectively, was 0.25-0.33 and 0.17-0.25% for inter-day assay precision, and 0.24-0.41 and 0.22-0.48 % for intra-day assay precision. The solutions were considered stable if the deviation from nominal value was within ±10.0%. The calculated recovery for the first and second eluting diastereomeric derivatives was 99.4-100.6 and 99.2-100.1 % for inter-day assay and 99.4-

102.3 and 99.3-102.7 % for intra-day assay, respectively (**Table 4.2**). The method was inferred to be accurate due to very low RSD values and high percentage recovery.

Limit of sensitive detection and quantification i.e. LOD and LOQ values were also determined for doing validation studies. 11.19 and 9.15 ng mL^{-1} LOQs and 3.69 and 3.02 ng/mL LODs were found for the diastereomeric derivatives of (*R*)- and (*S*)-Ket respectively. Here LODs are much lower as the prescribed LOD (1 %) for pharmaceutical industries.

Table 4.2: Method validation for HPLC separation of diastereomeric derivatives of Ket: linearity, accuracy and precision

	First eluting diastereomer			Second eluting diastereomer		
Range ng mL^{-1}	45-5000			45-5000		
Slope	4.97			4.96		
Intercept	4721.4			4723.0		
Determination coefficient (r^2)	0.99			0.99		
SD intercept	1.11			0.91		
SE intercept	0.25			0.26		
LOD	3.69			3.02		
LOQ	11.19			9.15		
Actual concentration ng mL^{-1}	Measured concentration mean± SD (ng mL^{-1})	Mean recovery (%)	RSD (%)	Measured concentration mean± SD (ng mL^{-1})	Mean recovery (%)	RSD (%)
Intra-day precision						
5000	5010.39±1.3	100.2	0.25	5022.39±0.8	101.2	0.24
3500	3479.80±0.8	99.4	0.24	3597.50±0.9	100.6	0.25
2000	2009.63±1.1	100.4	0.34	2005.83±1.4	100.1	0.37
500	499.17±0.9	99.8	0.27	497.09±0.5	99.3	0.22
45	46.04± 0.6	102.3	0.41	47.24± 0.2	102.7	0.48
Inter-day precision						
5000	5007.66±1.1	100.2	0.26	5006.66±1.4	100.1	0.25
3500	3482.61±0.9	99.5	0.25	3473.61±0.6	99.2	0.24
2000	2011.97±0.5	100.6	0.27	2006.97±0.5	100.1	0.23
500	496.99±0.9	99.4	0.33	499.94±1.3	99.9	0.17
45	45.73± 0.7	101.6	0.38	45.91± 0.9	101.7	0.38

Part II: Ligand exchange thin layer chromatographic enantioresolution of (*RS*)-Ket and (*RS*)-Etd using Cu(II) as a complexing ion and recovery of native enantiomers

1. Present work

The literature (cited as above and the cross references therein) on enantiomeric resolution of (*RS*)-Ket and (*RS*)-Etdclearly suggest that (i) there is no report on TLC resolution of (*RS*)-Etd or (*RS*)-Ket by using CLEC, and (ii) CLEC (as described above) is an important and successful method for enantioresolution. Taking into account (i) and (ii), experiments were designed and performed to achieve enantioresolution of the two title analytes by TLC using chiral ligand exchange reagent (LER) as '*chiral additive in achiral stationary phase*' and the chromatograms were developed using mobile phase having no chiral additive. LER was mixed with the slurry of silica gel while preparing the TLC plates. Three enantiomerically pure amino acids [namely, L-Trp, L-His and L-Phe] were used as chiral ligands with Cu(II) as a bivalent complexing ion. Chromatograms were developed using different combinations of solvent systems in different ratio having no chiral additive. Different experimental factors i.e. effect of temperature, mole ratio of Cu(II) to L-amino acids and solvent ratio were optimized in order to improve the separation efficiency. The enantiomers of each of the two analytes, so separated, were located with iodine vapors. Results (for R_F and *Rs*) have been compared.

The present work is different from existing reports on enantiomeric resolution of (*RS*)-Etd or (*RS*)-Ket in terms of the following aspects (a) it is a direct approach using CLEC, (b) the ligand exchanged diastereomeric complexes corresponding to each of the enantiomers (of the two racemates) were isolated via preparative TLC and their specific rotations were determined, and (c) the native enantiomers were obtained from the isolated ligand exchanged diastereomeric complexes.

Ligand exchange TLC: Various aspects of ligand-exchange chromatography have been reviewed [134-136]. Application of ligand exchange and use of different chiral selectors as impregnating reagents has been described for TLC resolution of enantiomers of a variety of compounds [137]. Chiral ligand exchange chromatography has been used

for enantioseparation of various compounds using HPLC [138, 139], and capillary electrophoresis [140,141]. Bhushan *et al*., resolved a few racemic amino acids [142], certain β-blockers [50, 33], and bupropion and baclofen [143-145] via ligand exchange on silica plates using a complex of copper (II)-L-proline or other L-amino acids. Molecular interactions studies of volumetric investigations of peptides and amino-acids with other molecules like sorbitol, glycine betaine have been carried out [146].

The method holds considerable significance especially in resource limited settings. The overall methodology of CLEC used both in TLC or HPLC remains advantageous and efficient and a technique of choice for serial determinations of enantiomeric compositions as it neither requires expensive chiral columns nor requires any pre-column derivatization with chiral reagent.

2. Experimental

- Details of chemicals, and equipment and isolation and purification of the title compounds, as reference standards, along with preparation of stock solutions has been described in Chapter-2.

2.1 Preparation of Cu (II)–L-amino acid complex (the LER) and TLC plates[143]

As already mentioned, L-Trp, L-His and L-Phe were chosen as chiral selectors. Following combinations of varying molar concentrations of L-amino acid(s) and Cu(II) were used to prepare TLC plates by preparing the slurry of silica gel in these solutions. The plates were prepared by, (i) using 1, 2, 4 and 6 mM concentrations of each of the amino acids with 2 mM of Cu (II) acetate; this provided the L-amino acid-Cu (II) ratio of 1:2, 1:1, 2:1 and 3:1, (ii) using a fixed concentration of amino acid (4 mM) and varying the concentrations of Cu(II) acetate (2, 4 and 6 mM); in this case, the ratio of L-amino acid-Cu(II) was 2:1, 1:1 and 2:3, respectively, (iii) keeping the ratio of L-amino acid-Cu(II) as 2:1, the following combinations were also tried, 2 mM L-amino acid-1 mM Cu(II), 4 mM L-amino acid–2 mM Cu(II), 6 mM L-amino acid-3 mM Cu(II) and 8 mM L-amino acid–4 mM Cu(II). In this manner experiments were performed using all three AAs, i.e., L-Trp, L-His and L-Phe.

The UV spectra and pH for each of the solutions mentioned at (i), (ii) and (iii), above, were recorded. TLC plates (10x20 cm x 0.5 mm thick) were prepared in the laboratory by spreading the slurry of silica gel G (25 g), with a Stahl-type applicator. The plates were allowed to set at room temperature and then were activated for 8–10 h at 60 ± 2 °C. These were considered to be impregnated plates.

2.2 Development of chromatograms and isolation of native enantiomers

10 µL solution of each of the racemic analytes [(*RS*)-Ket or (*RS*)-Etd] were spotted on TLC plates with a 25 µL Hamilton syringe. The chromatograms were developed in a completely dried, paper-lined, pre equilibrated rectangular glass chamber at different temperatures. The chamber was pre-equilibrated for 15 min with mobile phase at different temperatures, 16, 20, 24, 28 and 32 °C (when the room temperature (RT) was ~ 28 °C). Each temperature was maintained/controlled using an incubator.

Experiments were performed with binary, ternary, and quaternary mixtures of solvents such as ethyl acetate, ethanol, methanol, water, dichloromethane and acetonitrile to achieve enantiomer separation. Chromatograms were dried at 40 °C in an oven for 10 min and cooled to room temperature. The spots were located in an iodine chamber and R_F values were measured. The effect of *copper ion concentration to the concentration of the L-amino acids* and temperature on chiral separation was investigated and evaluated.

2.3 Preparative TLC

For preparative work, TLC plates (10 x 20 cm x 2.0 mm thick) were prepared using the molar concentrations of L-amino acid(s) and Cu(II) that was found successful (**Table 4.3**) for enantiomeric separation. On each plate, 10 spots in parallel were applied (50 µL of each solution of each racemate per spot). The silica gel corresponding to two separated spots of the ligand exchanged complex was scrapped from the plates. It was extracted with 90% ethanol by sonication for a few minutes and the insoluble silica was removed by filtration. The filtrates were dried under vacuum. The residues were dissolved individually in a little amount of DCM.

Following experiments were performed further.

(**A**) Each of the solutions was examined by UV spectrophotometer and polarimeter to ascertain concentration (using the standard plot as described above) and to calculate specific rotation (of both eluted complexes, e.g., [L-AA-(*R*)-Ket-Cu] and [L-AA-(*S*)-Ket-Cu]. The same approach was adopted for the separated spots of complexes of (*RS*)-Etd. Thus there were six samples for each of the analytes corresponding to the complex species obtained from the chromatograms.

(**B**) One drop of *conc.* HCl was added to each of the solutions. The solution(s) were irradiated under microwave (80% at 800 W) for one minute. The resulting solutions were lyophilized. Each residue was extracted with DCM. The solutions corresponding to the both enantiomers were concentrated. UV spectrum and optical rotations were measured for each of the solutions. There were six samples corresponding to native enantiomer dissociated from the complex, for each of the racemates.

3. Results

The purity of the racemic analytes isolated from commercial formulations was confirmed by determining the melting point and UV absorption (λ_{max}), and IR spectra which were in agreement with literature reports [147, 148]. In both the cases the recovery was of the order of 98% of the quantity reported on the commercial label. Both the purified compounds were used as racemic standards.

The λ_{max} for Cu(II) solution was 670 nm i.e. in the visible region [149]. The λ_{max} for L-Trp, L-His and L-Phe were found to be 280 nm, 213 nm and 260 nm, respectively. These values are in agreement with the literature values [150] for their λ_{max}. The UV spectrum for each of the solution by Cu(II) mixed with the L-AA(s) was also recorded and the λ_{max} was found to be different than that of the Cu(II) solution and any of the AAs individually. This confirmed /indicated the formation of a Cu (II) complex with the respective amino acid i.e. LER.

Table 4.3 The *h*R_F (R_F× 100), resolution (*Rs*) values and solvent systems for successful resolution of (*RS*)-Ket and Etd under LEC approach

	Mobile phase	Solvent ratio (*v/v*)	*h*R_F values		*Rs*	Specific rotation values $[\alpha]_D^{25}$ =(c=1, CH_3OH)	
Ketorolac			(*R*)	(*S*)		Upper spot	Lower spot
Tryptophan	CH_3OH-CH_3CN-DCM-H_2O	3:6:1:0.5	52	75	2.2	+63	–101
Histidine	CH_3OH-CH_3CN-EtOAc-H_2O	2:4:3:1	53	75	2.0	+60	-104
Phenylalanine	CH_3OH - CH_3CN-DCM	2:3:3	43	58	1.4	+62	-102
Etodolac							
Tryptophan	EtOH- CH_3CN-DCM	3:4:2	47	74	2.3	–7	–31
Histidine	EtOH- CH_3CN-EtOAc-DCM	4:1:4:3	56	79	2.2	–10	–34
Phenylalanine	CH_3OH- CH_3CN-DCM	1:5:2	42	57	1.5	–8	–32

Rs = resolution; *h*R_F = R_F × 100; detection, by iodine vapors, Solvent front, 8.0 cm; development time, 15-20 min, temperature, 28 °C (RT). The spots correspond to the diastereomeric complex species.

3.1 Mobile phase and *h*R_F (R_F ×100) values

Various solvent systems, i.e. the combinations of different solvents, enabling successful resolution along with the *h*R_F (R_F x100) and the resolution (*Rs*) values are reported in **Table 4.3**.Though both the racemates resolved well into their enantiomers, a different combination was found successful for resolution of (*RS*)-Ket and (*RS*)-Etd when there was a different amino acid in the LER as the chiral ligand. The pH of the solvent system (the mobile phase) in each case was measured and found to be between 5.8 to7.5. Application of L-Trp as the chiral ligand in the LER provided the best resolution (*Rs*) for (*RS*)-Etd among the various situations mentioned in **Table-4.3**.

hR_F values are averages of at least five runs on different plates under identical conditions on the same day and on different days (to evaluate reproducibility).

The resolution was calculated by dividing the distance between the centre of two spots by the sum of the radii of both the spots. The resolution (*Rs*) varied from the lowest 1.4 for (*RS*)-Ket with L-Phe (as the chiral ligand in the LER) to 2.3 for (*RS*)-Etd with L-Trp (as the chiral ligand in the LER). Representative photographs of actual chromatograms showing resolution of the two racemates are shown in **Fig 4.8**.

The racemates under study resolved well into their enantiomers at 28 °C using LERs having any of the three L-amino acids as chiral ligand. Experiments were performed in a range of temperature systematically until its effect was noted in terms of either tailing or figure-of-eight shaped spots or clear resolution. Experiments were carried out at 16, 20, 24, 28 and 32 °C using the successful solvent systems. The decrease in temperature to 16 °C showed eight shaped structures or no resolution (with a change of solvent system) and the increase in temperature to 32 °C resulted in tailing of spots.

3.2 Effect of mole ratio of Cu(II) to amino acid

The TLC experiments were carried out using varying molar concentration of Cu(II) and L-AAs (as described under experimental section) which showed that the best resolution of the each of the two racemates was achieved when Cu(II) (2mM) and L-amino acid (4mM) were in 1:2 molar ratio.

3.3 Polarimetry

Specific rotation values for all the corresponding complexes of upper spots of Ket and Etd (obtained by preparative TLC and as described under (**A**), above) were calculated which are summarized in **Table 4.3**. Specific rotation values for enantiomers recovered from the complex species (via acid hydrolysis, as described under (**B**), above) were found to be $[\alpha]_D^{25} = +163°$ (c=1,CH_3OH) for pure enantiomer of Ket (recovered from the upper spots) and $[\alpha]_D^{25} = +24°$ (c=1, CH_3OH) for Etd (recovered from the upper spot of the corresponding complex). The specific rotation values for the enantiomer of Ket and Etd recovered from the complex corresponding to lower spot were identical in magnitude with an opposite rotation.

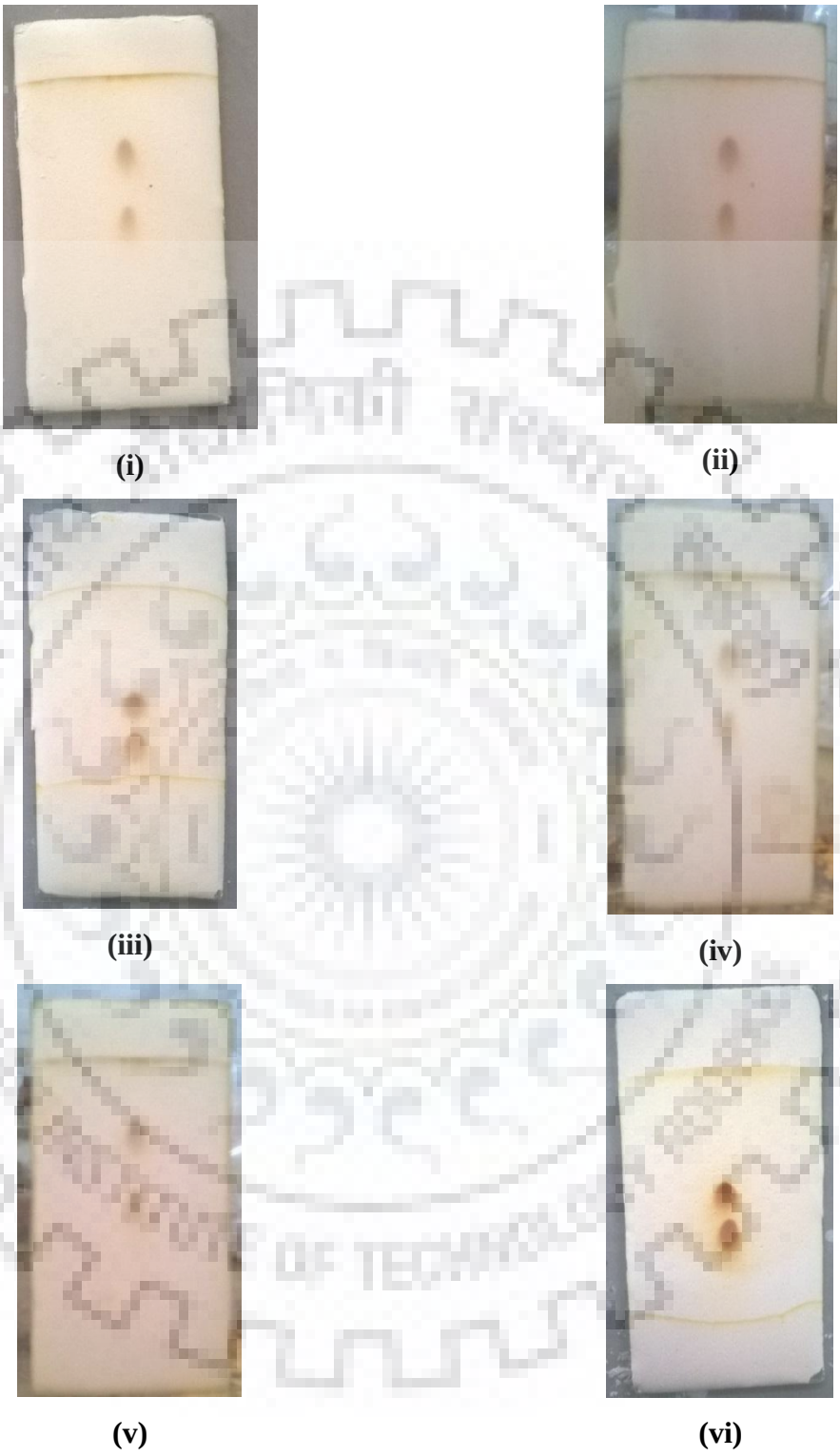


Fig. 4.8 Actual photographs of chromatograms showing resolution of (*RS*)-Ket and (*RS*)- Etd by use of Cu(II)–L-amino acids complex by impregnation method. The plates at (i), (ii) and (iii) show resolution of (*RS*)-Ket using (i) L-Trp, (ii) L-His, and (iii) L-Phe, respectively, as the chiral agents for ligand exchange. The plates at (iv), (v) and (vi) show resolution of (*RS*)-Etd using the same set of L-amino acids. Chromatographic conditions as given in table 4.3; the upper spot is (+)-isomer in each case.

By observing specific rotation values, it is concluded that the (*R*)-isomer was found to have a higher R_F value than the (*S*)-isomer. The polarimetry measurements also showed that the two isomers were in the ratio of 1:1. These results also confirmed the elution order.

4. Discussion

4.1 Ligand exchange TLC resolution of (*RS*)-Ket and (*RS*)-Etd

Separations by means of CLEC are based on the formation of labile ternary metallic complexes in the mobile phase and/or in the stationary phase. Separation models developed for ligand exchange HPLC [151, 152] are also valid for TLC.

The mechanism of enantioselectivity in the CLEC depends on whether the chiral ligand is linked on the stationary phase or it is added in the mobile phase since there is involved a series of complexation equilibria in the mobile phase and in the stationary phase. Thermodynamic enantioselectivity of the system, i.e. the difference in the stability of the above two diastereomeric ternary complexes is considered to be responsible for the observed enantioresolution. The stability of the diastereomeric complexes formed in LEC is higher than the stability of the diastereomeric adducts formed by other chiral selectors [153].

Schmid *et al*., [154] proposed formation of ternary mixed metal complexes in CLEC between the chiral selector, the complexing metal ion, and the enantiomer to be recognized. In general, two chiral selector amino acid molecules act as chelating ligands for the Cu (II) ion. In the course of the enantioseparation, one chelating amino acid molecule is replaced by the competing enantiomer molecule of the separated enantiomeric mixture (as depicted in **Fig. 4.9**). As a result, there occurs formation of diastereomeric complexes of the two enantiomers, so separated, having different stability and different retention through the interaction with the normal silica gel in TLC (or column packing in HPLC).

The denting strength (arising from the functional group causing chelation) of the ligand strongly influences retention and hence separation. The amino acids (i,e., L-Trp, L-His and L-Phe) used in the present studies are the chiral selectors for developing chiral LERs have bidentate chelating properties due to their carboxylic and amino functional groups which are sufficiently strong for complexation with the Cu(II) metal ion and are thus successful for direct enantioseparation of the two racemic analytes. The explanation described in the literature is well satisfactory for enantioseparation of (*RS*)-Ket and (*RS*)-Etd under study.

It was expected that the complex broke down into constituent units and only the (*R*)- and (*S*)-Ket would go into solution since copper ion and L-amino acid are insoluble in DCM. The diastereomeric complexes separated by TLC were isolated and were hydrolyzed. It was expected that the complex broke down into constituent units and only the (*R*)- and (*S*)-Ket (and also (*R*)- and (*S*)-Etd) would go into solution since copper ion and L-amino acid are insoluble in DCM. Specific rotation values for enantiomers (of each of the two analyte racemates) obtained from the complex species confirmed formation of diastereomeric ligand exchanged complexes and recovery of the native enantiomers. Thus the isolation of native enantiomers characterized by their specific rotation values confirms direct resolution of both the racemic analytes, i.e., (*RS*)-Ket and (*RS*)-Etd.

5. Method validation

Different solutions of known concentration (200, 600 and 900 μg mL^{-1}) of both racemic analytes were applied three times on impregnated TLC plates and determined repeatability of the method. Relative standard deviation (RSD) was found to be 1.15 %. The sensitivity of the method was established in terms of limit of detection (LOD). LOD were 0.6 and 0.8 μg (per spot) for each enantiomer of Ket and Etd, respectively.

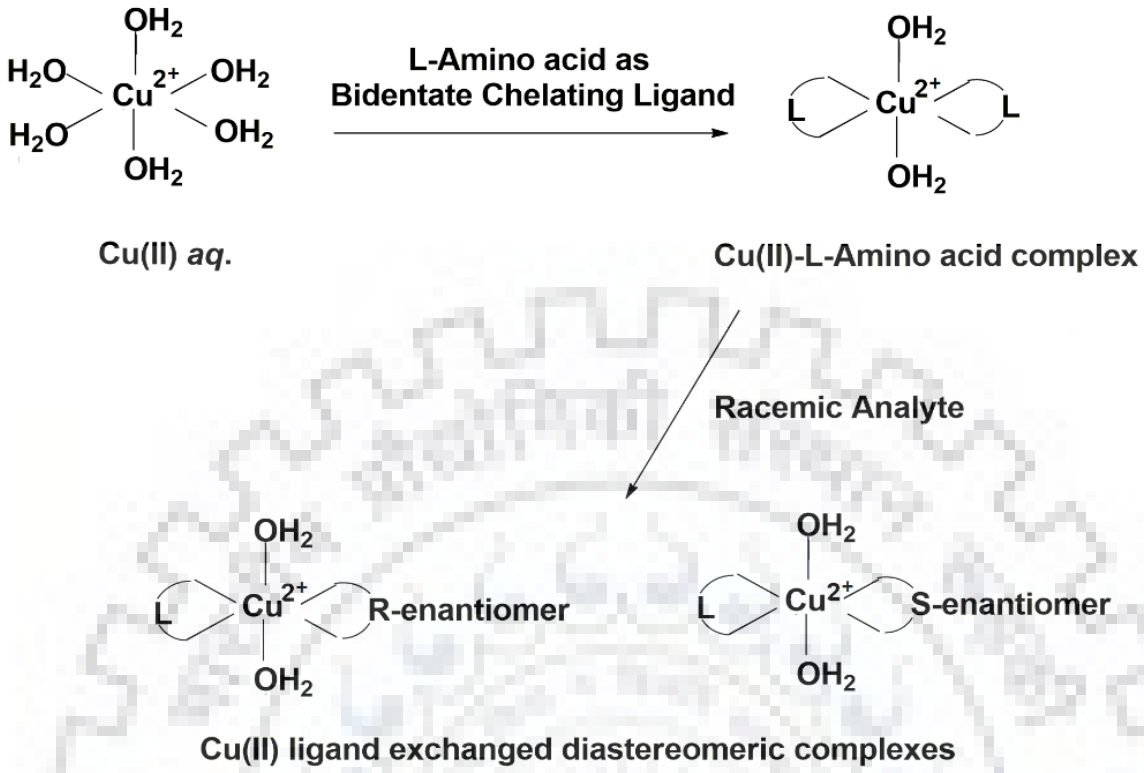


Fig. 4.9 Scheme showing formation and structure of ternary complex and the ligand exchanged complex on resolution of racemic analytes. Cu(II) *aq*. represents complexed state of Cu(II) in *aq* medium.

D. Novelty of present work

Enantioseparation of two commonly used NSAIDs, i.e., (*RS*)-Ket and (*RS*)-Etd has been achieved in the form of their anhydride type diastereomeric derivatives by both analytical and preparative HPLC. The reported indirect approach for enantioseparation is simpler as separation is possible on an achiral C_{18} column with easy optimization of chromatographic conditions with the CR providing highly sensitive detector response as compared to chiral columns which are highly expensive and less durable. The method is successful for enantioseparation and easy recovery of native enantiomers of (*RS*)-Ket and (*RS*)-Etd via hydrolysis, under mild conditions. It does not require a reference standard for comparison of retention time and is capable of establishing the true elution order. The confirmation of molecular asymmetry of diastereomeric derivatives followed by verification of the configuration of the enantiomers recovered from them (via hydrolysis, under mild conditions) make the method ahead of the literature reports in the area of

enantioseparation where only the application of CR remained limited to derivatization followed by separation of diastereomeric derivatives.

On the other hand, enantioseparation of the same two NSAIDs, i.e., (*RS*)-Ket and (*RS*)-Etdhas also been achieved by TLC using ligand-exchange approach. Though, obtaining native enantiomers from the ligand exchanged complexes may not always be required. But, the experiments so described are clearly an evidence for the formation of such complexes and could be an approach for obtaining native enantiomers. It is the first report to isolate pure enantiomers after doing ligand exchange chromatography. The approach presented herein has additional advantage that the commonly available amino acids, as a pool of chiral selectors, containing opposite D-configuration can be easily used as chiral ligands; this extends the scope of the investigations to understand the chiral mechanism by using the same chiral ligand in different configurations; only a small amount of ligand is required.

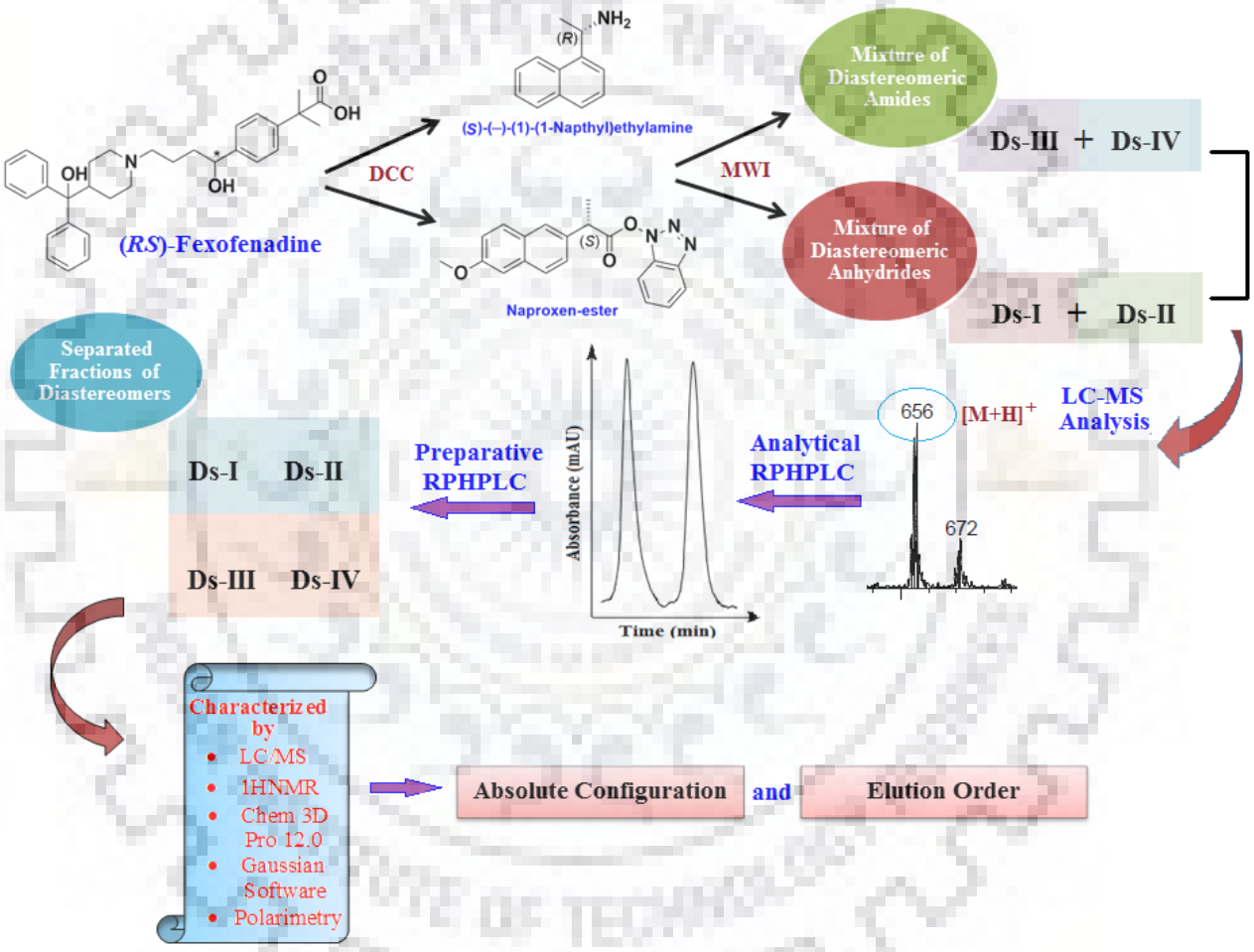
Isolation of native enantiomers is otherwise not feasible in other approaches or becomes very expansive using preparative chiral HPLC. The ligand exchange TLC method is less expensive and does not require any spray reagent as spots are visible with iodine vapors; it is very simple, direct, fast, and sensitive and can be applied for resolution, detection and control of enantiomeric purity with low LODs in planar mode. The use of home-made plates is quite easy and simple for successful and economical resolution.

Chromatographic separation and detection take place separately in TLC which enables us to carry out the analysis at different times and to make full use of detection techniques for the analysis of constituents. The method is self-sustained and can be realized even in a small laboratory. It opens up an area to control enantiomeric purity of chiral drugs in industry and analytical laboratories (especially associated with regulatory agencies).

The present study highlights the significance of liquid chromatography as a tool for qualitative and quantitative assessment or for simple separations at the analytical or preparative scale.

Chapter-5

Enantioseparation and detection of Fexofenadine by LC-MS as the diastereomeric amide and anhydride derivatives



A. Introduction

Usually antihistamines are first to try to stop allergy, cold and flu symptoms instead of other medications like steroids and allergy shots. These reduce the activity of histamine receptors on smooth muscles, mast cells and nerves or they block the binding of histamine to its receptor sites. These are used in different types of allergies, sneezing, nasal congestion, insect bites, hay fever,animal and food allergies. These are inexpensive, generic and have very few side effects. Most of these drugs could be taken over the counter (OTC) from pharmacist's shop like fexofenadine, cetirizine, chlorpheniramine, brompheniramine *etc* under different trade names. These are classified into two majorgroups,i.e. H1-antihistamines (first-generation) and H2-antihistamines (second generation), according to the histamine receptor that they act upon. H1-antihistamines were developed around seventy years ago and have action on brain, spinal cord and serotonin receptors. These cause side effects like rapid heartbeat, sedation, low BP, and dizziness. H2-antihistamines were developed around twenty years ago having action on histamine-1 receptors in the periphery instead to penetrate the brain, so are less likely to cause side effects.

Considering these aspects, (*RS*)-fexofenadine (**Fxn**) an H2-antihistamine (easily available in Indian market under the commercial name 'Allegra')was chosen for study.

The structure (**Fig 1.1**), pharmaceutical importance and the relevant literature on the enantioseparation of Fxn is described briefly below.

B. Pharmaceutical importance of the chosen drug i.e. (*RS*)-Fxn

Fxnis administered clinically as a racemic mixture or is used as a P-glycoprotein probe. Fexofenadine hydrochloride (λ_{max}= 225 nm; Mol. Wt. 538.13), the active ingredient of *Allegra* (the brand name), is a cost saving antihistamine with highly selective second-generation H_1-receptor antagonist activity. It exists as a zwitterion in aqueous media (pI=6.9, pK_1 = 4.25 and pK_2 = 9.53). It is free from adverse cardiac, other central nervous system or cognitive/psychomotor effects occasionally associated with its parent compound, *i.e.*, terfenadine. It is used in the treatment of allergy symptoms, hay fever,

nasal congestion, and urticaria. It does not cross the blood-brain barrier and causes less drowsiness than first-generation histamine-receptor antagonistsproviding much relief from repeated sneezing, runny nose, itchy eyes and general body fatigue.

C. Literature survey on enantioseparation of (*RS*)-Fxn

The few reports related to determination of Fxn or separations of its enantiomers are as follows. Determination of Fxn in human plasma has been reported, without any considerations for separation of its enantiomers, by LC coupled with fluorescence detection [155], or UV detection [156], with LC–MS [157], with ionspray tandem mass spectrometry detection [158] and with electrospray ionization mass spectrometry (LC–ESI–MS–MS) [159].

Robbins *et al*., (1998) [160] reported clinical study of the pharmacokinetics of Fxn enantiomers but did not describe the method for analysis of its enantiomers in human plasma. The first report on HPLC separation of enantiomers of Fxn in human plasma was from Miura *et al*., (2007) [161] wherein they used solid-phase extraction and β-cyclodextrin phenylcarbamate chiral column. The method involved a cumbersome extraction method involving several extraction and evaporation steps for sample preparation and the mobile phase had 0.5% KH_2PO_4 in CH_3CN and the HPLC run time for one sample was about 60 min while the chiral column was required to be washed for 1 h after a few samples run; moreover the chromatograms did not show baseline separation. The diastereomeric derivatives of (*RS*)-Fxn prepared with (*R*)-(+)-1-phenylethylisocyanate were separated by using C_{18} column [162] and the corresponding eluates were collected which were evaporated to dryness and subjected to off-line mass spectrometric analysis; in the mass spectra neither the $[M]^+$ peak nor other significant fragments were detected. Amide bond was synthesized from amines by irradiated with microwaves and further amides bond has been converted to carbamates under very mild reaction conditions [163]. Studies of synthesis of substituted benzisoxalones have been carried out using hypervalent iodine of o-aminochalcones and certain other ketones [164]. Like fexofenadine, some

other carboxylic group containing drugs i.e. ibuprofen and ketoprofen has been studied for chiral conversion in vitro spontaneous oscillatory [165, 166].

The literature search showed that there are no studies related to pharmaceutical efficiency of individual (*R*)- or (*S*)-enantiomers of Fxn. The US and other pharmacopeia do not describe data on enantiomers of Fxn, including that on pharmacokinetics and pharmacodynamics studies. Thus, it becomes important to have simple methods for enantioselective analysis of (*RS*)-Fxn and to obtain its native enantiomers.

1. Present work

The **Chapter-5** describes enantioseparation of (*RS*)-Fxn and enhanced detection as itsdiastereomeric amide and anhydride derivatives using liquid chromatography-mass spectrometry (LC-MS).

Derivatization of racemic (*RS*)-Fxn was carried out using three chirally pure amines as the CRs. These are,

CR-2:(*S*)-(−)-1-(1-napthyl)-ethylamine;

CR-3: (*S*)-(+)-1-benzyl-3-aminopyrrolidine, and

CR-4: (*S*)-(−)-α,4-dimethylbenzylamine.

The structures of **CR 2-4** are shown in **Fig.2.2**. The **CR 2-4** yielded corresponding amide diastereomeric pair.

Besides, diastereomeric derivatives of (*RS*)-Fxn were synthesized using **CR-1**, i.e., N-Hydroxy-benzotriazolyl-(*S*)-naproxen ester (Npx-ester)as mentioned in Chapter-2. It was synthesized and characterized andwas used as the CR for characteristics properties of (*S*)-naproxen (**CR-1, Fig. 2.1**).

So, there were synthesized two types of diastereomeric derivatives of (*RS*)-Fxn, (i) diastereomeric amides using the three chiral amines and (ii) diastereomeric anhydrides using the (*S*)-naproxen based CR under microwave irradiation.

Semipreparative HPLC enantioseparation provided the two independent diastereomeric derivatives which were used for determination of configuration.The structures and configurations were verified using LC-MS, ^{1}HNMR spectrometry, Chem3D Pro 12.0 software, and the software Gaussian 09 Rev.A.02 program and hybrid density functional B3LYP with 6-31G basis set supplemented withpolarimetry.The structural features and separation characteristics of the two types of diastereomeric derivatives have been compared. The method was validated in accordance with ICH guidelines.

2. Experimental work

- Details of chemicals, and equipment and isolation and purification of the title compounds, as reference standards, along with preparation of stock solutions has been described in Chapter-2.
- Synthesis and characterization of CR-1, has been described in Chapter-2.
- The **CR 2-4** are chirally pure organic compounds having one asymmetric carbon (that contain napthyl, benzyl-pyrrolidine and toluene moiety respectively) and used as chiral reagents (as such) for the synthesis of diastereomeric derivatives via amide bond in the presence of DCC. Out of these, (*S*)-(−)-α-(1-naphthyl)ethylamine has been used as a derivatizing reagent for HPLC enantioseparation of (*RS*)-Etd by indirect approach [167].

2.1 Microwave-assisted synthesis of diastereomeric derivatives

Three pairs of diastereomeric derivatives were synthesized as per the following procedure described asarepresentative using CR-2; 200 µL of each of **(*RS*)-Fxn** (10.0 mg in 2 mL THF, 0.02 mmol), **DCC** (4.1 mg in 2 mL THF, 0.02 mmol), and OH-Btz (2.7 mg in 2 mL THF, 0.02 mmol) were taken in a 2 mL micro centrifuge vial, followed by addition of one drop of dimethylamino pyridine; **CR-2** (6.8 mL in 2 mL THF, 0.04 mmol) was added up to a molar ratio of 2:1 compared to the analyte. Similar sets of reaction mixture were prepared using CR-3 and CR-4.Diastereomeric derivatives with CR-1 were prepared by taking 100 µL CR-1 (13.8 mg in 2 mL THF, 0.04 mmol) having two drops of triethylamine with 100 µL(*RS*)-Fxn (10.0 mg in 2 mL THF, 0.02 mmol).

To optimize the reaction conditions, Fxn: CR were taken in the ratio of 1:1, 1:1.5, 1:2 and 1:2.5, and each of these reaction mixtures was then subjected to microwave irradiation (MWI) at power 8 (800 W) for 30 to180 s at an interval of 30 s. Completion of synthesis, for each set, was checked by TLC and by injecting the reaction mixture to RPHPLC after each irradiation time. The synthesized diastereomeric derivatives were stored under refrigerated conditions (3–5 °C). Each set of mixture of diastereomeric derivatives was subjected to HPLC separation.

Diastereomeric derivatives were also synthesized on a preparative level by scaling up the optimized synthesized conditions. Derivatization reaction were carried out using (*RS*)-Fxn (300 mg, 0.6 mmol) with CR-1 (600 mg, 1.8 mmol) and CR-4 (600 mg, 4.5 mmol) in a similar manner as described above. The yield was 892 mg (99.1 %) and 885 mg (98.3 %) with CR-1 and CR-4, respectively.

2.2 RPHPLC separation

A 10 µL volume of each set of mixture of diastereomeric derivatives was diluted 10 fold with CH_3OH and TEAP buffer (10 mM, pH 7.5), and 20 µL of it was injected onto the analytical C_{18} column.

Mobile phase was optimized for successful separation of each set of diastereomeric mixture using various combinations (TEAP buffer with CH_3OH or CH_3CN) in isocratic mode. 10 mM and 15mM buffer concentrations were used by adjusting different pH6.0, 6.5, 7.0, 7.5 and 8.0 using phosphoric acid for each concentration. So, there were 10 combinations of each mobile phase having CH_3OH and 10 combinations having CH_3CN. In total, 20 numbers of mobile phases were tried for each set of diastereomeric mixture. For each mobile phase, flow rate was tried at 1 mL min^{-1} and then at 0.7 mL min^{-1}. Each mobile phase was degassed, sonicated, and filtered through 0.45 µm filter before use. Detection was carried out at 225 nm using a PDA detector.

2.3 LC-MS analysis

Experiments were carried out using CH_3CN as mobile phase with distilled water as autosampler, after it was sonicated and filtered through 0.45 μm filter, at a flow rate of 0.5 mL min^{-1} at C_8 analytical column.

User-specified parameters were as follows, temperature of the column oven: 40 °C; heat block temperature 250 °C; drying gas flow 10 L min^{-1}; nebulizing gas flow: 1.5 L min^{-1}; event time (determines the scan speed): 0.5 msec; λ_{max} value was set at 225 nm and mass range was selected randomly in the range of 200-1000. The electron spray ionization (ESI) source was operated as Q_1 scan in positive mode at 1 atm pressure.

The concentration of diastereomeric mixtures was in ppm range by diluting with CH_3OH and 0.5 μL of these were injected onto the C_8 achiral columnby placing vials of diastereomeric mixtures in a tray manually.

2.4 Preparative HPLC

Separation conditions for diastereomeric pairs were found similar on preparative HPLC; the flow rate was 5 mL min^{-1}. The mixture of derivatives was diluted with CH_3OH and 500 μL of it was injected on C_{18} achiral column. The fraction corresponding to each peak representing the separated isomers was collected (at the set time-interval) in separate vials placed suitably in the given rack. There was collected a total volume of 40 mL per fraction (@ 2 mL per fraction for each injection) for injections made twenty times. The combined fractions corresponding to individual isomeric peak were concentrated in vacuum. Based on the elution order, these samples were marked as Ds-I and Ds-II for diastereomeric anhydrides (prepared with CR-1) and Ds-III and Ds-IV for diastereomeric amides of Fxn prepared with CR-4. The characterization data of the diastereomers so separated is given under results and discussion.

3. Results and Discussion

3.1 The CRs

Reaction schemes for synthesis of CR-1 is illustrated in **Fig. 2.1**; the reaction of achiral OH-Btz occurs only at the terminal carboxyl group of (*S*)-(+)-Npx while its stereogeniccentre remains unaffected; the CR so synthesized and characterized was enantiomerically pure [168]. Optical rotation of CRs 2-4 was verified before derivatizationreaction.Under the optimized separation conditions of HPLC, each diastereomeric mixture showed only two peaks corresponding to two expected diastereomers; these results also confirmed the enantiomeric purity of the CRs.

3.2 Synthesis of diastereomeric derivatives

A representative scheme showing derivatization reaction of **Fxn** with CR-1 and CR-2 is illustrated in **Fig. 5.1**. At pH 7.5 (greater than its pI) Fxn exists as anion and derivatization reaction occurs fast in presence of DCC. Since the derivatization reaction did not involve a direct attack on stereogenic centre, no racemization of the sample and of the chiral auxiliary compound was detected.

A large excess of CR was used to force the derivatization reaction to completion considering that the enantiomers may react at different rates with the chiral reagent. Kinetic resolution was observed at 1:1.5 of Fxn:CR. The successful ratio of Fxn:CR for derivatization reaction was 1:2. There was no effect on reaction time and yield by further increasing the ratio to 1:2.5 of Fxn: CR. The MWI reaction was completed in 90 s with CR-1,120 s with CR-2, and in 150 s with CR-3 and 4 (at 80% of 800 W). The effect of MWI time on completion of derivatization reaction is shown in **Fig. 5.2**.

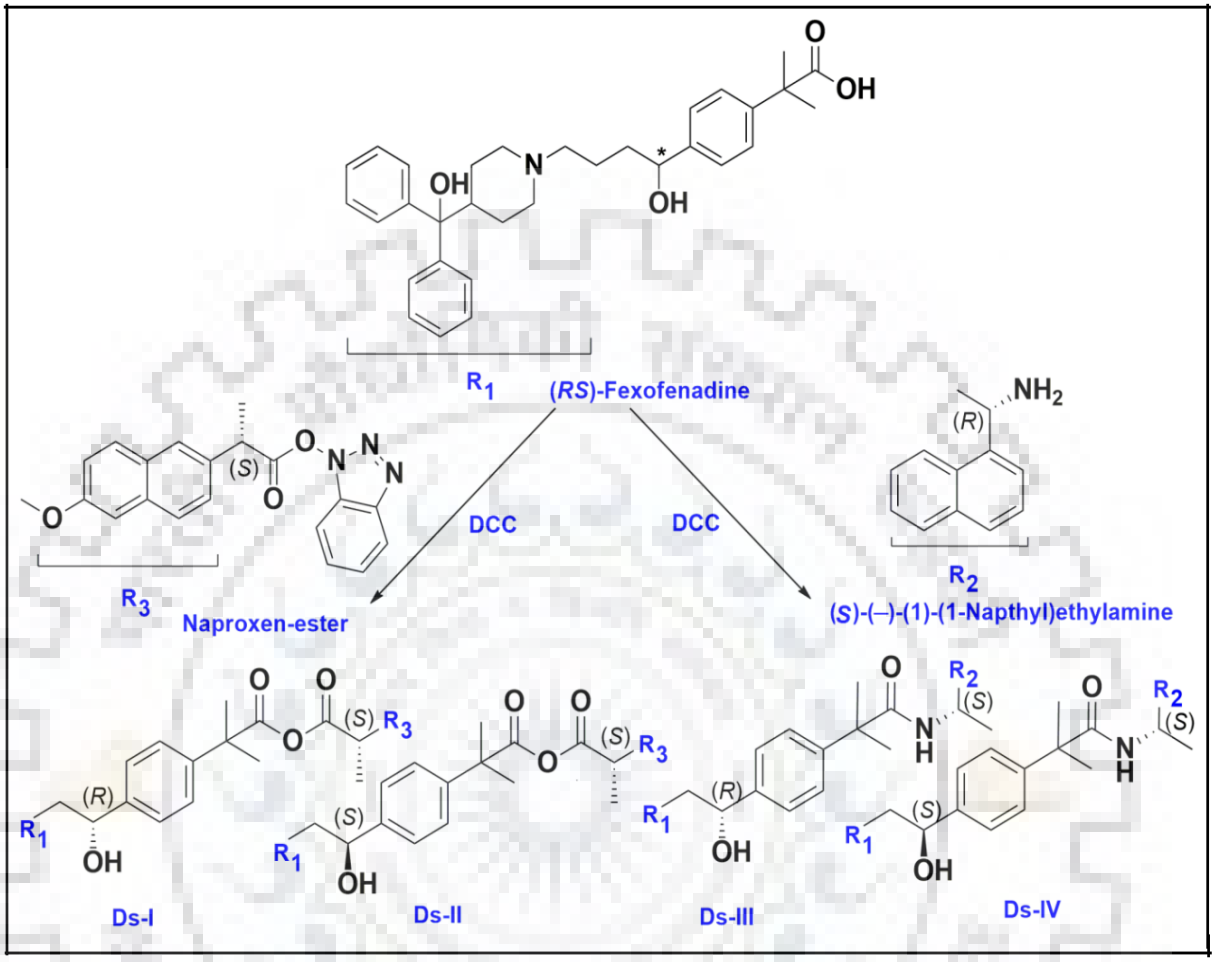


Fig 5.1 Derivatization reaction scheme for the synthesis of diastereomeric derivatives of (*RS*)-Fxn using CR-1 and CR-2; Ds-I and Ds-II represent (*R,S*)- and (*S,S*)-diastereomeric derivatives (the anhydrides) of Fxn corresponding to CR-1 and Ds-III and Ds-IV represent (*R,S*)- and (*S,S*)-diastereomeric derivatives (the amides) corresponding to CR-2, respectively.

3.3 Structure of diastereomeric derivatives

The derivatives are represented as (*R*,*S*)- and (*S*,*S*)-diastereomeric derivative, where the first letter corresponds to the configuration of analyte and second letter to that of CR.Standard models were developed using the '*orbital molecular building system*' (Cochranes of Oxford Ltd, Leafield, Oxford OX8 5NT, England) to verify the spatial orientation of groups along with the three dimensional chemical structures and configurations of the diastereomeric derivatives (as shown in **Fig.5.1**). Besides, the **Fig.5.3** shows the lowest energy optimized structures of diastereomeric derivatives (designated as Ds-III and Ds-IV) of (*RS*)-Fxn prepared with (*S*)-(−)-α,4-dimethylbenzylamine (i.e. CR-4) drawn by using the Gaussian 09 Rev.A.02 program and hybrid density functional B3LYP with 6-31G basis set.

In the (*S*,*S*)-diastereomeric derivative, the toluene moiety of CR-4 and diphenylpiperidinyl moiety of Fxn are spatially oriented on the same side corresponding to amide bond; it is in agreement with the structures shown in **Fig.5.1** as verified with the standard models and discussed above.

The configuration of diastereomeric derivatives prepared with CR-1 were confirmed by drawing their 3D structures by using Chem3D Pro 12.0 software; further confirmation of configuration was obtained by recording ^{1}HNMR spectra and the specific rotation values of respective diastereomeric derivatives [168].

3.4 Comparison of formation of amide and anhydride types of diastereomeric derivatives

There were obtained two types of diastereomeric derivatives in terms of their functional group. The amino group of first three CRs reacts with the carboxylic group of (*RS*)-Fxn to form a pair of diastereoisomeric amides. The reaction of organic amines (the CRs 2-4) with carboxylic acid (the Fxn as the analyte in the present case), with the use of DCC as a coupling reagent, has been the simplest method of amide bond formation in organic synthesis; both activation and coupling proceed concurrently with DCC. Though

Fxn has two hydroxyl groups there is no ester formation by its reaction with the CRs 2-4 since the latter do not have any carboxyl group.

The rate of reaction of an amine with DCC is much lower in comparison with the rate of addition of a carboxylic acid to one of the double bonds of carbodiimide [84]. Therefore, the synthesis of the CR-1 as an ester comprised the simple straight forward reaction of (*S*)-(+)-Npx with OH-Btz in presence of DCC. It may be argued that the CR-1, being an ester with (*S*)-(+)-Npx as the chiral moiety, is more reactive (in comparison to an amino group containing reagent) in forming the diastereomeric derivatives of the chosen analytes under mild reaction conditions.

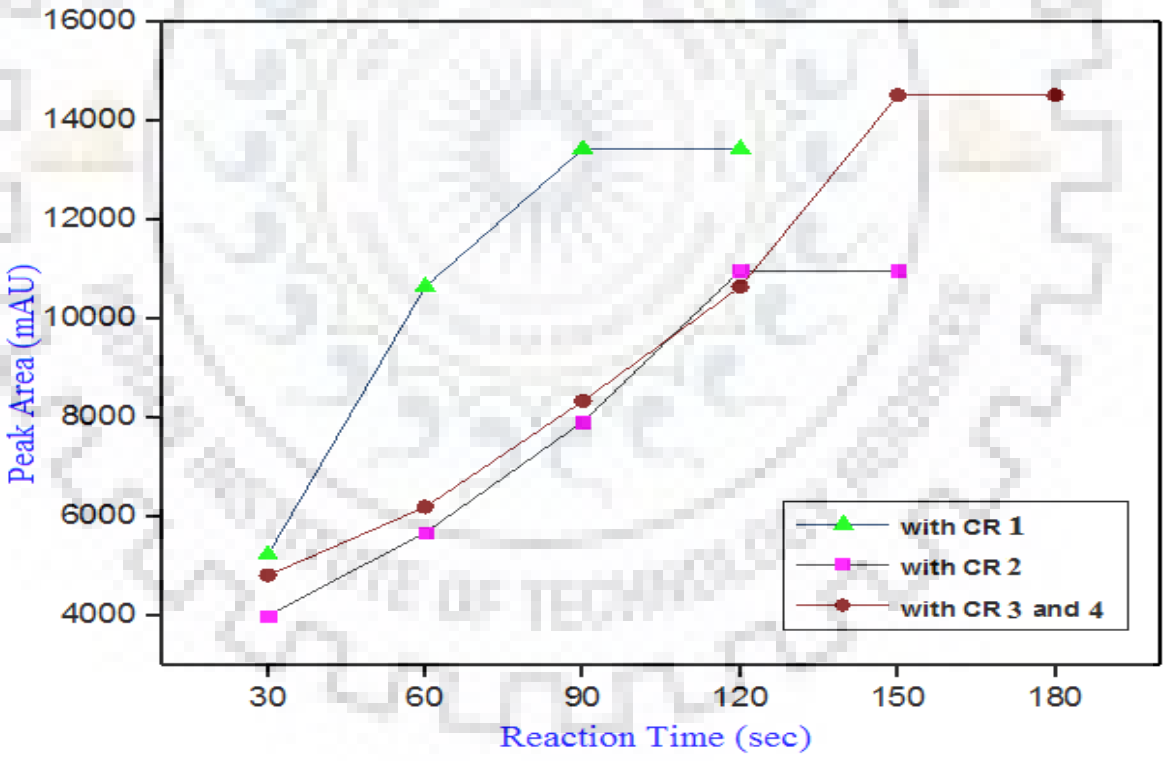


Fig 5.2 Representation of progress of derivatization reaction using each CR by microwave irradiation for completion of reaction

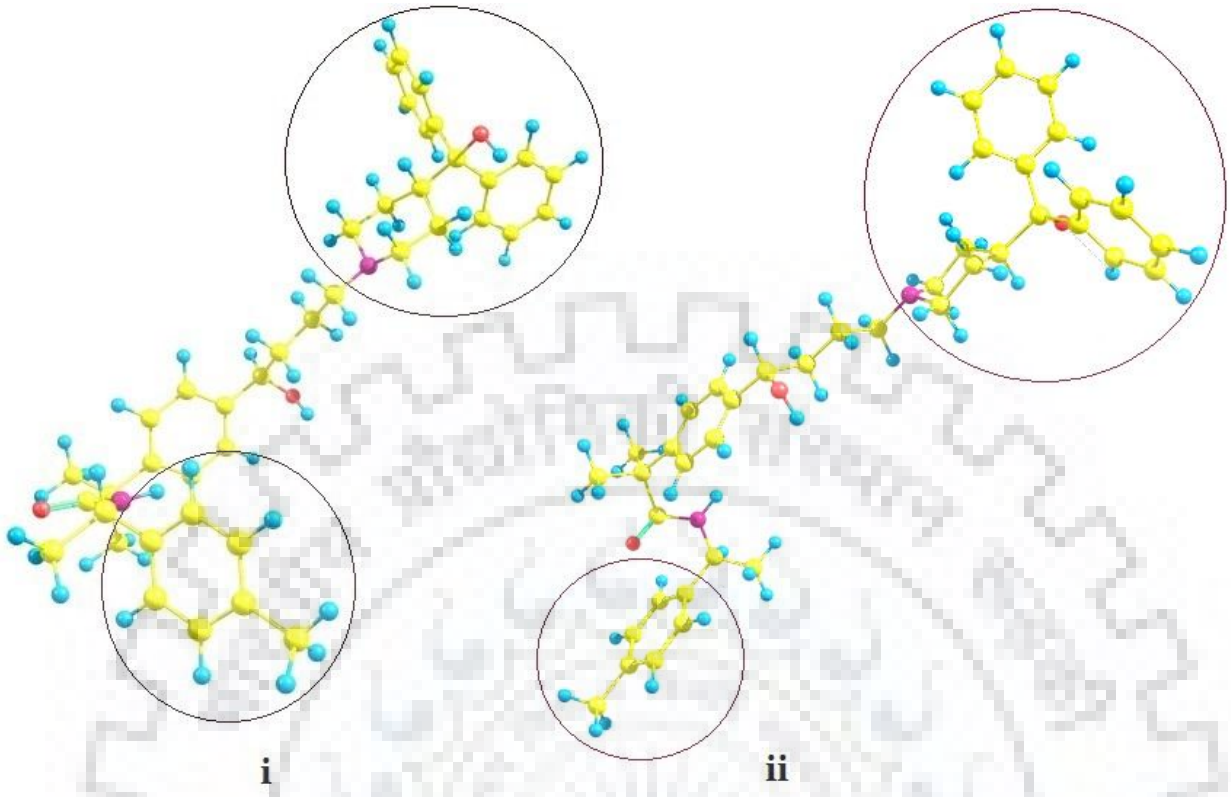


Fig 5.3 The lowest energy optimized structures of diastereomeric derivatives of (*RS*)-Fxn prepared with CR-4 drawn by using the program Gaussian 09 Rev. A.02 and hybrid density functional B3LYPwith 6-31G basis set. (i) represents (*R,S*)-diastereomeric derivative and (ii) represents (*S,S*)-diastereomeric derivative.

As a consequence of combined inductive and resonance effects, the electrophilic carbonyl carbon of the CR-1 gets large δ^+ charge and high reactivity (due to the presence of highly electronegative elements (O and N) in its close vicinity, **Fig. 5.1**) and gets easily attacked by the nucleophile (the COO^- group from the (*R*)-, or (*S*)-enantiomers ofFxn in the racemic analyte), and, for anchimeric assistance, the OH-benzotriazole moiety is knocked out very easily acting as a good leaving group.

Because of the mild conditions, the formation of diastereomeric anhydrides is faster and the chances of racemization are reduced. The MWI time for the derivatization reaction was also found very less (only 90 s) as compared to other CRs. The diastereomeric anhydride is more stable due to greater delocalization (with two oxygen atoms) and, therefore, its formation is favoured. The presence of weak base during derivatization is

important to promote the reaction, as confirmed by analysing the reaction mixture without any base. Base facilitates the formation ofdiastereomeric derivatives by making the analyte more nucleophilic in nature.

3.5 Separation of diastereomeric derivatives

Analytical HPLC

Actual chromatograms (obtained from the system) showing the baseline separation of four pairs of diastereomeric derivatives of (*RS*)-Fxn are given in **Fig. 5.4.** The retention factors (k_1 and k_2), separation factor (α) and resolution (*Rs*) values of diastereomeric derivatives separated under the optimized HPLC conditions are calculated as per the standard formulae and are summarized in **Table 5.1**. All the diastereomeric mixtures were well resolved on achiral analytical column.

Selectivity (α) was calculated as $\alpha = k_2 / k_1$; the retention factor (*k*) is the degree of retention of the sample component in the column and was calculated as $k_1 = t_1\text{-}t_0 / t_0$,where t_1 is the retention time of the first eluted diastereomer and, t_0 ("Tee Zero") is the column dead volume or time or void volume peak or sometimes called the solvent front is a peak (used to calculate retention factor) that arises when the solvent, which was used to dissolve the sample, arrives unretained at the detector flow cell; since the solvent is essentially unretained by the column, it takes the fastest way through the column, and its elution time is a measure of the volume between the injector and the detector, through the column. This volume is fixed, unless the tubing, the loop or the column are changed.The void volume peak was observed at 1.4 min for the diastereomeric pair prepared with CR-1 and CR-2, at 0.8 min for the diastereomeric pair obtained with CR-3 and 1.0 min for the diastereomeric pair synthesized with CR-4.

The evidence for the formation of diastereomeric derivatives and their successful separation in the column was obtained when the UV spectra (from the PDA detector) corresponding to the two peaks for the diastereomeric derivatives were found identical within each pair of diastereomeric derivatives.

The optimized chromatographic separation conditions were found to be different for each pair of diastereomeric derivatives, these are shown in **Table 5.1**. However, the flow rate was 0.7 mL min^{-1}, and pH was 7.5 in each case. The pH of the mobile phase was found to affect the retention and selectivity of diastereomeric compounds. Among the four sets of diastereomeric pairs, the highest resolution (i.e., 12.8) was obtained for the diastereomeric anhydride derivatives prepared with CR-1. Both the separation factor (2.77) and resolution (12.8) were higher for the diastereomeric anhydrides prepared with CR-1 as compared to the values for diastereomeric amides obtained by reaction with the other three CRs.

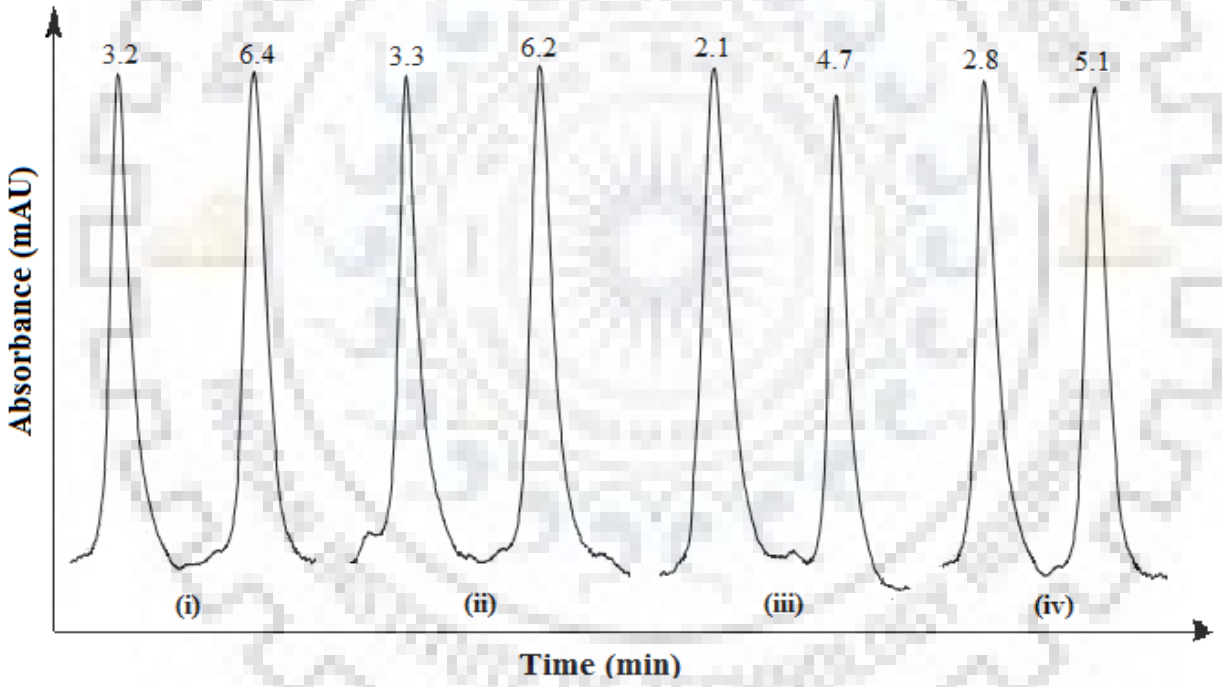


Fig.5.4 Actual chromatograms (obtained from the system) showing the separation of the diastereomeric derivatives of (*RS*)-Fxn with CR 1, 2, 3 and 4 respectively, along with their corresponding retention time. Chromatographic conditions: column: LiChrospher C_{18} (250 mm x 4.6 mm I.D., particle size 5 μm); mobile phases: CH_3OH–TEAP buffer with CR-1 & 2 and CH_3CN-TEAP buffer with CR-3 and 4 (pH 7.5), isocratic condition; flow rate: 0.7 mL min^{-1}; detection: 225 nm; and injection volume: 20 μL. The first elution peak corresponds to the (*R,S*)-diastereomeric derivative in all the cases.

Table 5.1 Chromatographic separation data for diastereomeric derivatives of (*RS*)-Fxn prepared with different chiral derivatizing agents (CRs)

Synthesis of diastereomeric derivatives using	Successful mobile phase	Chromatographic characteristics			
		k_2	k_1	α	*Rs*
CR-1	CH_3OH-TEAP (75:25, *v*/*v*)	3.57	1.29	2.77	12.8
CR-2	CH_3OH-TEAP (70:30, *v*/*v*)	3.43	1.36	2.52	9.67
CR-3	CH_3CN-TEAP (80:20, *v*/*v*)	4.88	1.63	2.99	8.67
CR-4	CH_3CN-TEAP (80:20, *v*/*v*)	4.10	1.80	2.28	6.57

The decreasing resolution order of diastereomeric derivatives prepared with CR-1 to 4 is as follows: 1>2>3>4. Diastereomeric derivatives prepared with CR-4 had the lowest resolution values among all others whereas retention time for diastereomeric derivatives prepared with CR-2 was higher. This may depend on difference in the type of interactions. The existing literature [the only one report by Rustichelli *et al*., [162] shows resolution (*Rs*) value 1.42 using Chiralcel OD column and 5.37 for the diastereomeric derivatives prepared with (*R*)-(+)-1-phenylethylisocyanate while in the present case *Rs* was 6.57 to 12.8, which is better.

Stability

The solutions were considered stable when the deviation from the nominal value was within ±10.0%. Stability of the diastereomeric derivatives was investigated after long-term under refrigeration conditions at a temperature of 3–5 °C. Reaction was found to be stable for 6 months since the day of derivatization by checking them on HPLC with interval of 15 days.

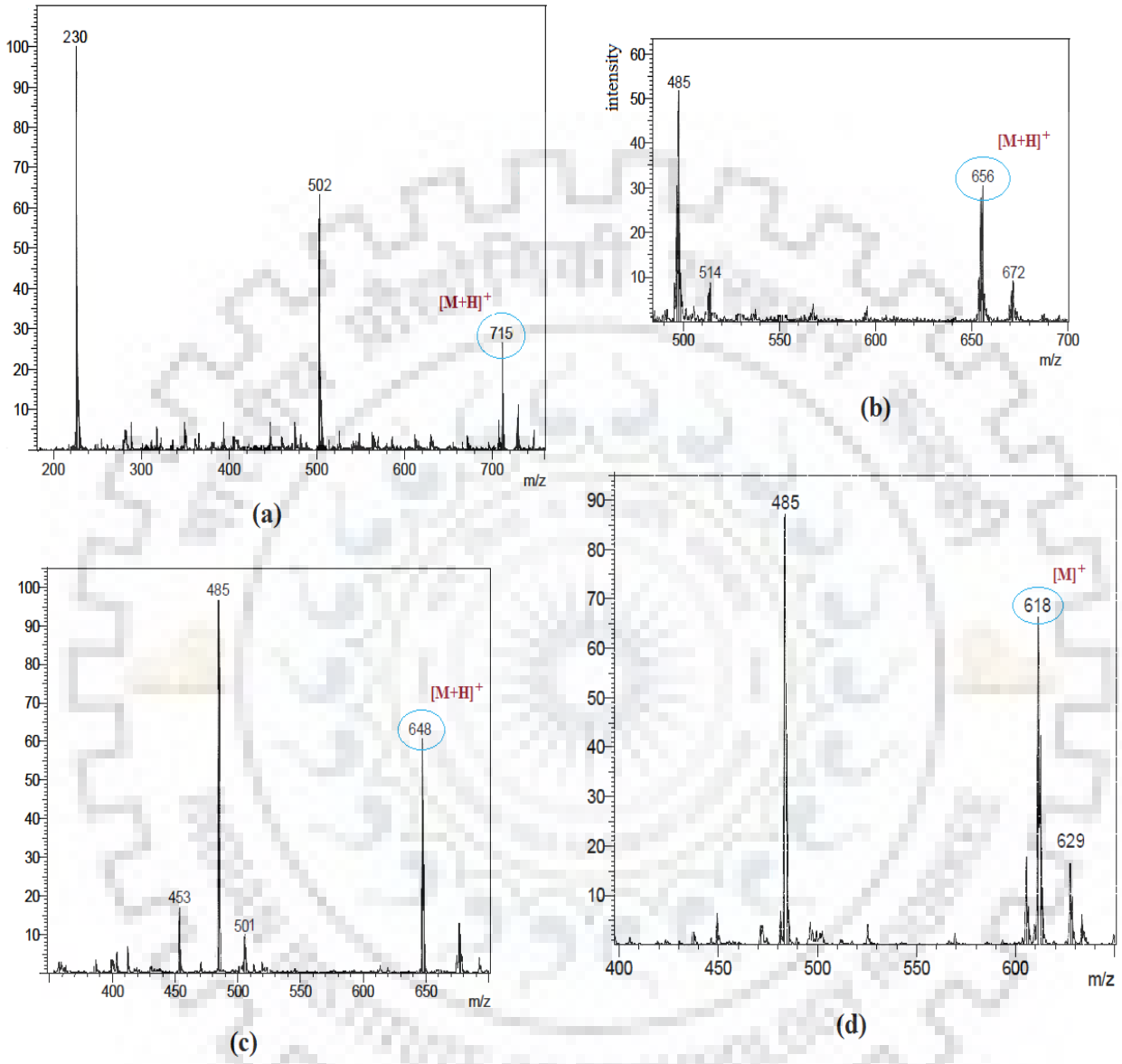
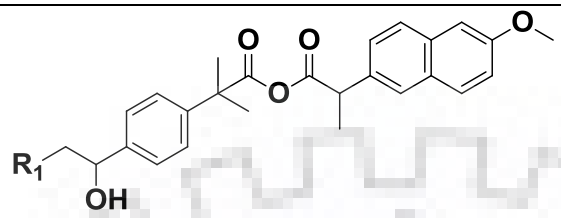
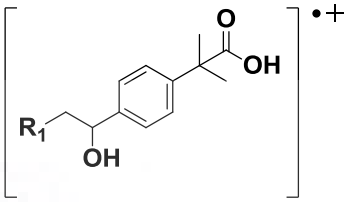
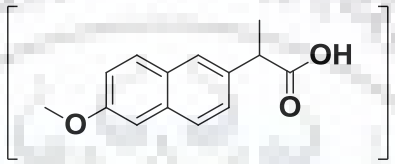
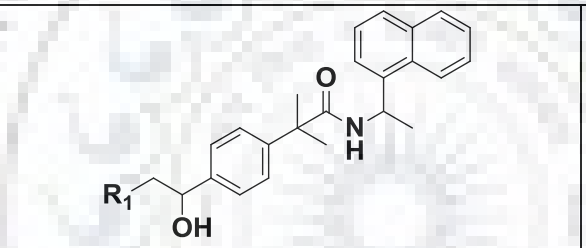
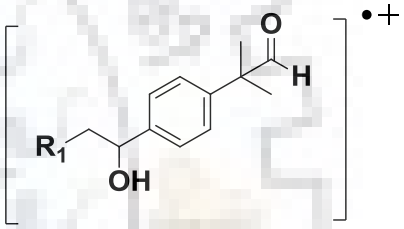
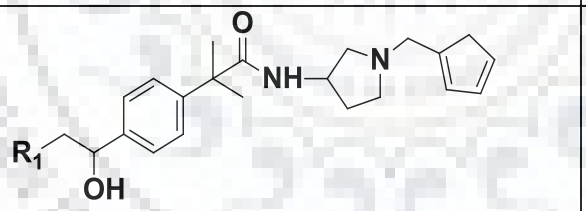
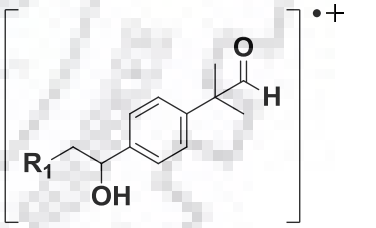
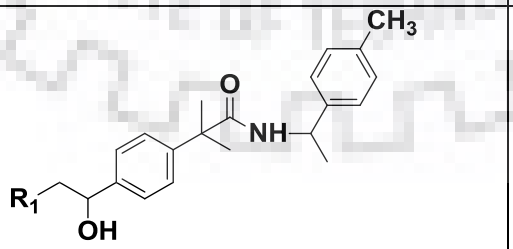
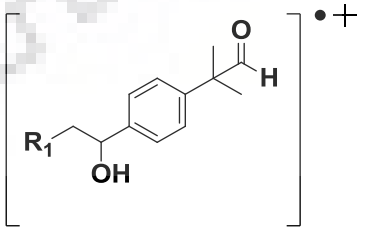


Fig 5.5 Mass spectra of the diastereomeric derivatives formed by the reaction of (*RS*)-Fxn with (a) CR-1, (b) CR-2 and (c) CR-3 and (d) CR-4; base peaks and molecular ion peaks are shown.

Table 5.2 The structures corresponding to mass ion peaks of diastereomeric derivatives of Fxn formed with CR-1, 2,3 and 4 along with ion peaks of some other fragments

With CR	Structures corresponding to mass ion peaks of diastereomeric derivatives and m/z value	
1	 **[M+H]⁺ 715**	 **[M]⁺ 502**
	 **Base peak 230**	
2	 **[M+H]⁺ 656**	 **Base peak 485**
3	 **[M+H]⁺ 648**	 **Base peak 485**
4	 **[M]⁺ 618**	 **Base peak 485**

3.6 LC-MS and verification of structure of diastereomeric derivatives

By observing $[M+H]^+$ or $[M]^+$ peak using LC-MS, the formation of diastereomeric derivatives of (*RS*)-Fxn with four different CRs was confirmed. The mass spectra of both the enantiomers derivatized with the CRs are in accordance with theoretical considerations as only the carboxyl group of Fxn is involved in derivatization reactions with CRs.

Full-scan product ion mass spectra were obtained by direct infusion of samples into the mass spectrometer. The mass spectrum of corresponding diastereomeric mixtures is shown in **Fig. 5.5** along with the structures of the identified peaks (**Table 5.2**). The mass ion $[M+H]^+$ peaks exhibited for pairs of diastereomers prepared from different CRs were as follows: m/z 715 for diastereomeric derivatives prepared with CR-1, m/z 656 for diastereomeric derivatives prepared with CR-2, and m/z 648 for diastereomeric derivatives prepared with CR-3, while $[M]^+$ 618 for diastereomeric derivatives prepared with CR-4.

Preparative HPLC

The chromatographic separation conditions optimized for analytical separation were found suitable for preparative scale separations. The details of the characterization data of the isolated diastereomeric derivatives of (*RS*)-Fxn are presented below.

Ds-I: Yield/recovery: 140 mg (96.5 %); $[\alpha]_D^{25}$= +44.1° (c, 1.0, $CHCl_3$); m.p.= 127±2 °C; UV (λ_{max} in CH_3OH, 230 nm); IR (KBr): 3761, 3412, 3367, 3055, 2936, 2861, 2354, **1826, 1746**, 1518, 1488, 1447, 1386, 1261, 1238, 1172, 1149, 1078, 1009, 967, 884, 789, 744, 694, 641 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$-d1): δ 7.90 (s, 1H, Ar), 7.87 (s, 1H, Ar), 7.48 (d, 4H, Ar), 7.43 (s,1 H, Ar), 7.42 (d, 6H, Ar), 7.39 (s, 1 H, Ar), 7.26 (d, 2H, Ar), 7.21 (d, 2H, Ar), 7.2 (s, 1H, Ar), 7.12 (s, 1H, Ar), 4.5(t, 1H), 3.89 (s, 3 H), 3.81 (q, 1H),3.45 (s, 2H), 2.52-2.44 (t, 2 H), 2.49 (t, 2 H), 2.38 (d, IH), 1.77 (s, 6H), 1.69-1.47 (t, 2 H), 1.67 (d, 3 H), 1.54 (q, 2H), 1.41 (m, 2 H); Calculated for $C_{46}H_{51}NO_6$: C, 77.42 %; H, 7.19 %; N, 1.95 %; CHN found C, 77.39 %; H, 7.20 %; N, 1.96 %.

Ds-II: Yield/recovery: 142 mg (98 %); $[\alpha]_D^{25}$ = –4.8° (c, 1.0, $CHCl_3$); m.p.= 128±2 °C; UV (λ_{max} in CH_3OH, 230 nm); IR (KBr): 3762, 3412, 3366, 3057, 2935, 2862, 2355, **1824, 1747**, 1517, 1489, 1449, 1386, 1263, 1241, 1173, 1144, 1075, 1009, 969, 884, 791, 744, 697, 642 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$-d1): δ 7.90 (s, 1H, Ar), 7.85 (s, 1H, Ar), 7.46 (d, 4H, Ar), 7.41 (s,1 H, Ar), 7.39 (d, 6H, Ar), 7.33 (s, 1 H, Ar), 7.26 (d, 2H, Ar), 7.21 (d, 2H, Ar), 7.16 (s, 1H, Ar), 7.1 (s, 1H, Ar), 4.4 (t, 1H), 3.87 (s, 3 H), 3.79 (q, 1H), 3.41 (s, 2H), 2.51-2.43 (t, 2 H), 2.44 (t, 2 H), 2.36 (d, IH), 1.77 (s, 6H), 1.69-1.47 (t, 2 H), 1.65 (d, 3 H), 1.55 (q, 2H), 1.40 (m, 2 H); Calculated for $C_{46}H_{51}NO_6$: C, 77.45 %; H, 7.23 %; N, 1.93 %; CHN found C, 77.39 %; H, 7.20 %; N, 1.96 %.

Ds-III: Yield/recovery: 137 mg (94.5 %); $[\alpha]_D^{25}$ = –42.3° (c, 1.0, $CHCl_3$); m.p.= 181±2 °C; UV (λ_{max} in CH_3OH, 226 nm); IR (KBr): 3765, 3492**, 3358,** 3007, 2884, 2736, 1713, **1668, 1589,** 1498, 1446, 1389, 1271, 1248, 1166, 1098, 1092, 1006, 965, 841, 754, 704, 663, 632 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$-d1): δ 7.89 (d, 1 H), 7.48 (d, 4H, Ar), 7.42 (d, 6H, Ar), 7.26 (d, 2H, Ar), 7.25 (d, 2 H, Ar), 7.21 (d, 2H, Ar), 7.2 (d, 2 H, Ar), 4.76 (m, 1H), 4.5(t, 1H), 3.67 (s, 1H), 2.52-2.44 (t, 2 H), 2.49 (t, 2 H), 2.38 (d, IH), 2.32 (s, 3 H), **2.30 (s, 1 H),**1.82 (d, 3H), 1.77 (s, 6H), 1.69-1.47 (t, 2 H), 1.54 (q, 2H), 1.41 (m, 2 H); Calculated for $C_{41}H_{50}N_2O_3$: C, 79.51 %; H, 8.19 %; N, 4.55%; CHN found C, 79.57 %; H, 8.14 %; N, 4.53 %.

Ds-IV: Yield/recovery: 135 mg (93.1 %); $[\alpha]_D^{25}$ = +6.2° (c, 1.0, $CHCl_3$); m.p.= 183±2 °C; UV (λ_{max} in CH_3OH, 231 nm); IR (KBr): 3766, 3491**, 3356,** 3007, 2882, 2737, 1713, **1668, 1585,** 1485, 1451, 1388, 1271, 1245, 1167, 1099, 1091, 1007, 966, 841, 755, 704, 663, 631 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$-d1): δ 7.89 (d, 1 H), 7.47 (d, 4H, Ar), 7.39 (d, 6H, Ar), 7.26 (d, 2H, Ar), 7.25 (d, 2 H, Ar), 7.23 (d, 2H, Ar), 7.2 (d, 2 H, Ar), 4.74 (m, 1H), 4.5 (t, 1H), 3.65 (s, 1H), **2.54 (s, 1H),** 2.51-2.44 (t, 2 H), 2.46 (t, 2 H), 2.37 (d, IH), 2.32 (s, 3 H), 1.83 (d, 3H), 1.79 (s, 6H), 1.69-1.45 (t, 2 H), 1.55 (q, 2H), 1.43 (m, 2 H); Calculated for $C_{41}H_{50}N_2O_3$: C, 79.53%; H, 8.17%; N, 4.59%; CHN found C, 79.57 %; H, 8.14 %; N, 4.53 %.

3.7 Elution order of diastereomeric derivatives and separation mechanism

The determination of elution order of diastereomeric derivatives being separated by observing their configuration in a chromatographic experiment is very important criterion for establishing the success of any analytical procedure.

The spatial orientation of the toluene moiety of CR-4 and diphenylpiperidinyl moiety of Fxn being on the same side corresponding to amide bond, in the (*S*,*S*)-diastereomeric derivative (as shown in **Fig. 5.1 and Fig. 5.3**),may result into more hydrophobic interactions with the C_{18}material of the column and, therefore, the (*S*,*S*)-diastereomeric derivative is retained for a longer time. In the (*R*,*S*)-diastereomeric derivative, these two moieties are oriented away from each other with respect to amide bond; the molecule is thus relatively less hydrophobic and has weak interactions with the hydrocarbon chain of reversed-phase column and is eluted first. Thus, the first eluted peak was interpreted to belong to the diastereomer corresponding to (*R*)-Fxn, and the second one was for its (*S*)-counterpart.

It may thus be inferred that thedifference in hydrophobic nature of the two diastereomeric derivatives (and the corresponding difference in terms of stronger or weaker interaction with the C_{18} material of column) along with the influence of rheological properties of the mobile phase is responsible for their different partition coefficients and different retention times.

Since the configurations of (*S*,*S*)-, and (*R*,*S*)-diastereomeric derivatives prepared with CR-1 were also verified with the help of 3D figures (using Chem3D Pro 12.0 software) and NMR spectra it may be inferred that the first eluting diastereomeric derivative had (*R*,*S*)-configuration and the second eluting derivative corresponded to (*S*,*S*)-isomer. The chem3D structures of diastereomeric derivatives of Fxn prepared with CR-1 are shown in **Fig. 5.6**.

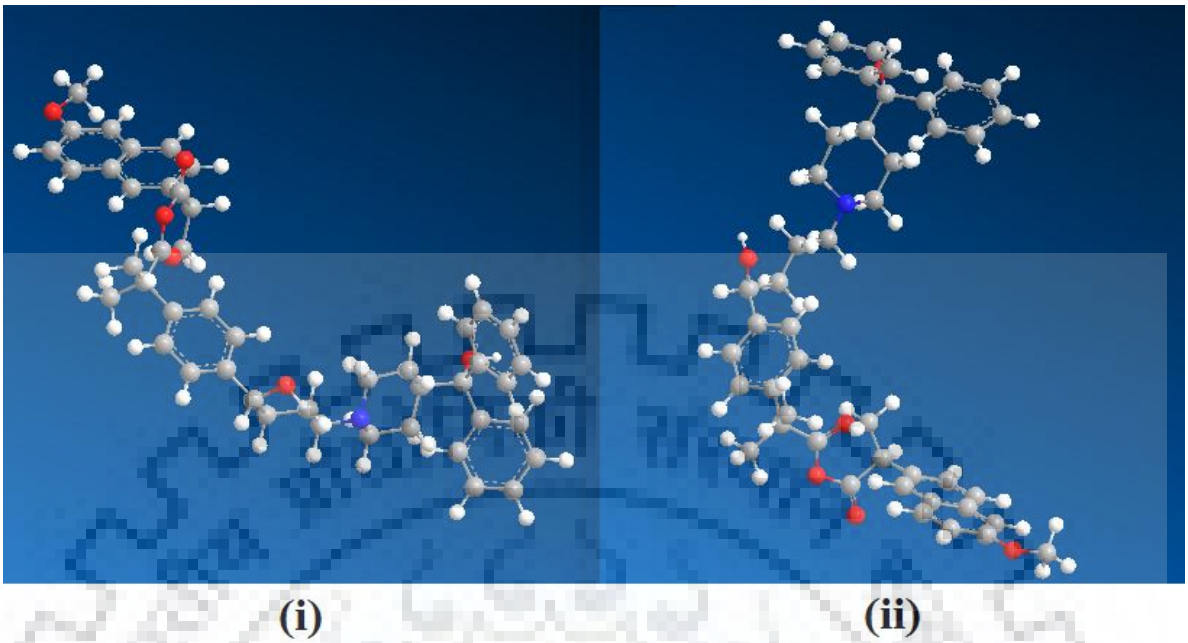


Fig. 5.6 3D structures drawn for diastereomeric derivatives of Fxn with CR-1 using chem3D Pro 12.0 software. (i) and (ii) represent the (*R*,*S*)- and (*S*,*S*)-diastereomeric derivatives respectively.

3.8 Detection and analysis of diastereomeric derivatives in trace amount

Diastereomeric derivatives were analyzed in trace amounts by preparing a mixture of solutions of Ds-III and Ds-IV in the ratio of 1:99; the samples were prepared by appropriate dilution of stock solutions in μg mL^{-1} and in ng mL^{-1}; the peaks obtained from each sample were in agreement with those shown in **Fig 5.4**. There was observed a small detectable peak for Ds-III in second sample. The experiment clearly demonstrated and ensured the sensitive analysis and detection of samples present in trace amounts.

4. Validation of the method

Validation studies were performed in accordance with the ICH guidelines (4). Stability and recovery were determined by observing peak areas (from chromatograms obtained by HPLC). Linear plots of peak area *vs* concentration were drawn, and the linear regression equation was used to determine slopes and intercepts.

Sample solutions of both diastereomeric derivatives of Fxn (prepared with CR-2) were prepared. Replicate samples were analyzed (n = 5) by HPLC using concentrations

(100, 2000, 4000, 6000, and 8000 ng mL^{-1}) of the individual diastereomeric derivatives to determine the repeatability of the proposed method.The results were averages of at least five identical runs on different days and at different time.The standard curve was constructed in a very broad range of concentration (100-8000 ng mL^{-1}) to show reliability, reproducibility and robustness of the method.

A good linear relationship was obtained over this range, which is evident from the relative standard deviation (RSD) values of slope, intercept and correlation coefficient (<1.5 %).The mean value was found below 15 % of acceptance criterion for accuracy. The precision determined at each concentration was within 15 % of the coefficient of variation (CV).

The linear plots of concentration (x) *vs* peak area (y) of first and second eluted diastereomeric derivatives were drawn and mean regression equations were obtained as y = 3.85 x + 7193.2 (r^2 = 0.99) and y = 3.98 x + 6365.6 (r^2 = 0.99), respectively. The high value of correlation coefficient (r^2 = 0.99) for both diastereomeric derivatives indicates excellent linearity in the concentration range studied.

Using the following formula,

$\boldsymbol{R.S.D.} = \mathbf{100} * \frac{\boldsymbol{S.D.}}{\boldsymbol{\mu}}$ the RSD were calculated and were found 1.20–1.98 % and 0.98–1.99 %, for inter-day assay, and 1.02–1.84 % and 1.08–2.06 %, for intra-day assay precision for (*R*,*S*)- and (*S*,*S*)-diastereomeric derivatives, respectively. Here '*S.D.*' stands for standard deviation and 'μ' is the value of each measured concentrations against actual concentration of each of the diastereomeric derivatives (**Table 5.2**). Standard deviation was calculatedby the formula,

$\boldsymbol{S.D.} = \sqrt{\frac{\sum|\boldsymbol{\mu}-\boldsymbol{\theta}|^2}{\boldsymbol{N}}}$ where 'N' is the number of replicate samples used at different concentration and 'θ' is the average value of the measured concentrations of the diastereomeric derivatives; S.D. were found to be1.46 and 1.89 for the first and second eluting derivatives, respectively.

Table 5.3 Method validation for HPLC separation of diastereomeric derivatives of Fxnprepared with CR-2 (Linearity, Accuracy and Precision)

	First eluting diastereomer			Second eluting diastereomer		
Range (ng mL^{-1})	100-8000			100-8000		
Slope	3.85			3.98		
Intercept	7193.2			6365.6		
Determination coefficient (r^2)	0.99			0.99		
SD intercept	1.46			1.89		
SE intercept	3.26			4.24		
LOD	6.25			7.87		
LOQ	18.94			23.84		
Actual concentration (ng mL^{-1})	Measured concentration mean± SD (ng mL^{-1})	Mean recovery (%)	RSD (%)	Measured concentration mean± SD (ng mL^{-1})	Mean recovery (%)	RSD (%)
Intra-day precision						
8000	7965.23±2.4	99.6	1.32	7950.79±1.7	99.4	1.68
6000	6053.50±1.7	100.9	1.98	6068.61±2.5	101.1	1.75
4000	4000.10±2.3	100.0	1.46	4006.74±3.0	100.2	1.89
2000	1978.62±0.6	99.9	1.20	1977.78±2.1	98.9	1.99
100	102.69± 1.3	102.7	1.66	96.11± 1.9	96.1	0.98
Inter-day precision						
8000	7980.66±3.1	99.8	1.72	7954.84±2.3	99.7	0.76
6000	6014.22±1.9	100.8	1.56	6071.25±1.5	101.3	1.08
4000	4011.52±2.8	100.1	1.06	4018.71±2.7	100.4	2.06
2000	1981.86±0.8	99.9	1.84	1989.08±1.6	99.1	1.42
100	101.87± 1.4	101.9	1.02	98.81± 0.8	98.8	1.96

Such a low value of RSD is clearly showing how precise are the obtained results over such a large range of concentration taken (i.e. 100-8000 ng mL^{-1}). In other words the results are tightly clustered around the average value of concentrations showing robustness of the method.

The recoveries for each diastereomeric derivatives were calculated as 99.8–101.9% and 98.8–101.3%, for the inter-day and 99.6–102.7% and 96.1–101.1%, for the intra-day assay, respectively (**Table 5.3**). The sensitivity of the method was established in terms of limit of detection (LOD); detection limits were established by injecting the different known concentrations and known volume of each diastereomeric derivative to develop the chromatogram under the established conditions (i.e. experimentally) as well as comparing the estimated values using formula, $\boldsymbol{LOD = 3.3 * \frac{S.D.}{m}}$, (theoretically, using calibration curve of each diastereomeric derivative). Here 'm' is slope of the calibration curve. Dilution of both samples of diastereomericderivativeswas done until the peak was detected in chromatogram. The detection was successful up to 6.25 and 7.87 ng mL^{-1} for the diastereomeric derivatives of (*R*)- and (*S*)-Fxn with CR-2, respectively. Similarly, LOQ was calculated using equation, $\boldsymbol{LOQ = 10 * \frac{S.D.}{m}}$, and found 18.94 and 23.84 ng mL^{-1} corresponding to first and second diastereomeric derivatives.

D. Novelty of the present work

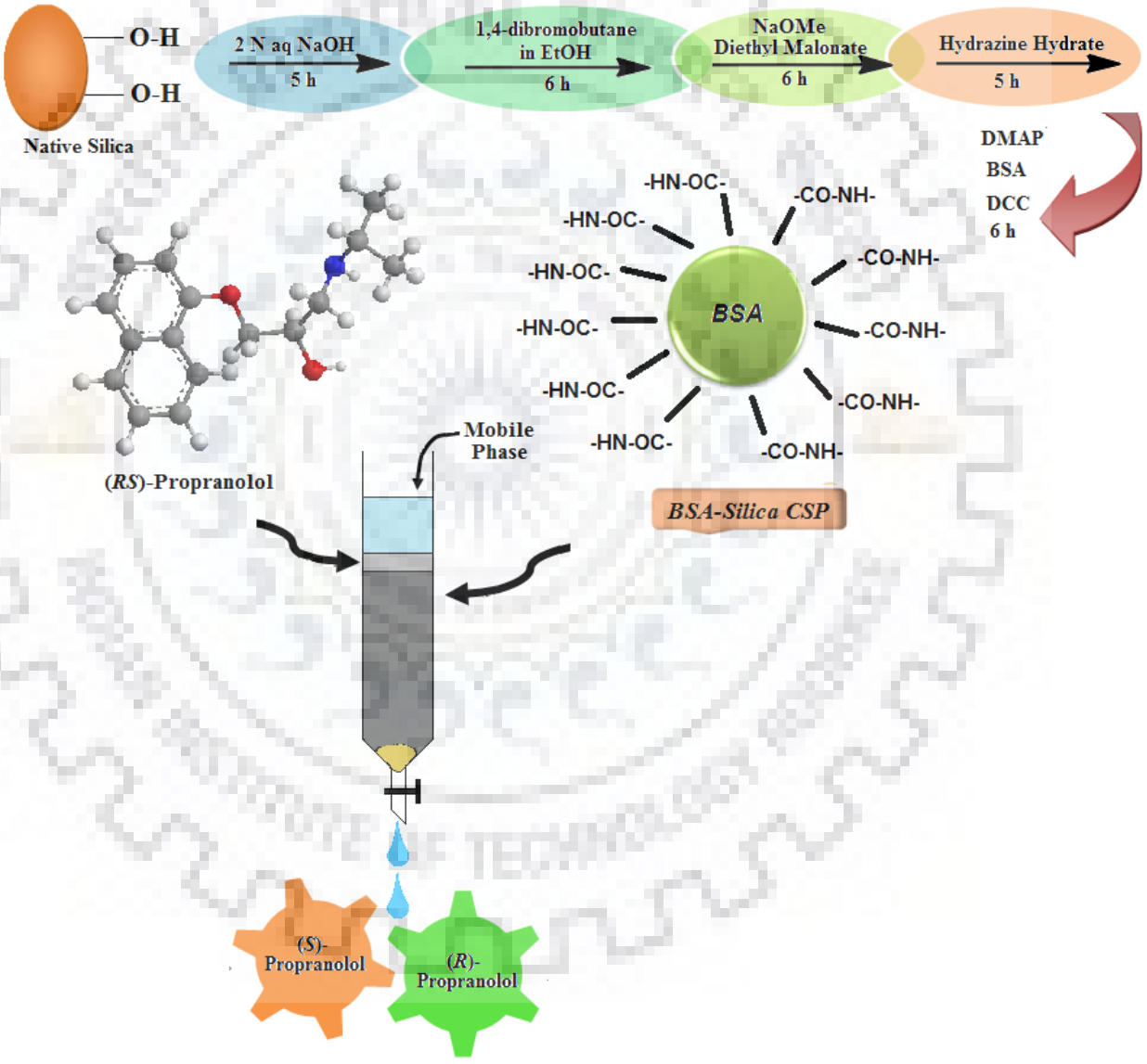
This report is novel for HPLC enantioseparation of (*RS*)-Fxn in terms of LC isolation of diastereomeric derivatives and verification of their configurations via recording full scan product ion mass spectra using LC-MS, and ^{1}HNMR spectra supplemented with polarimetry, and Chem3D Pro 12.0 software (including establishment of elution order); these results are also a measure of the success of synthesis of diastereomeric derivatives and their separation. The synthesis of diastereomeric pair of anhydride required only 90 s under MWI. There was no racemization at any stage and the reactions were not light sensitive.

The method is self-sustained and opens up an area that would yield diastereomeric anhydrides which could be easily hydrolysed to native enantiomers of Fxn. The availability of pure single enantiomers would be helpful in establishing their pharmacokinetics and pharmacodynamics data individually.Hydrolysis of diastereomeric anhydride derivatives of (*RS*)-ketorolac [168] and amide derivatives of (*RS*)-propranolol [36]has earlier been reported from this laboratory and hydrolysis of diasteomeric derivatives of (*RS*)-ketorolac has been described in this thesis in Chapter-4 [168].

The method is useful in academia and industry for controlling enantiomeric purity and determining the *ee* of a sample from enantioselective synthesis (prior to purification step involving work up by normal chromatography because such a step generally leads to un-noticed enantiomeric enrichment) of chiral drugs containing carboxylic functional group, in particular. The method was inferred to be precise owing to the high percentage recoveries and very low RSD values.

Chapter-6

Development of bovine serum albumin bonded silica as chiral stationary phase and direct quantitative enantiomeric resolution



1. Introduction

Methods reported in literature for enantiomeric resolution involving chiral stationary phases (CSPs), capillary electrophoresis and preparative HPLC, though useful in determining enantiomeric purity, are expensive and the amount of enantiomers obtained cannot be considered as quantitative in practical sense.

Nowadays, the research hotspot is the preparation of new chiral materials for enantioseparation via immobilization of simple chiral selectors on the surface of nanomaterials. Li *et al.*, [169] grafted bovine serum albumin (BSA) onto the polydopamine-coated silica nanoparticles (SiNPs) surface via Michael addition or Schiff base reactions and the material (SiO_2@PDA@BSA NPs) was used for enantiomeric enrichment of propranolol (LOD, 0.58 mg mL^{-1}) and tryptophan (LOD, 0.1 mg mL^{-1}) by multistep adsorption strategy followed by chiral CE using CM-β-CD and α-CD; the method is overall cumbersome and expensive in terms of running cost and cost of equipment. Fu *et al.*, [170] reported immobilization of BSA on the magnetic Fe_3O_4 nanoparticles through electrostatic adsorption interaction; though the chiral material was successful for separation of chiral drug enantiomers the new material was susceptive to recycle use due to the unstable electrostatic interactions between BSA and NPs. A composite chiral stationary phase (CSP) derived from BSA and β-CD-bonded silica was prepared using 2,4,6-trichloro-1,3,5-triazine as a cross linker and was applied for enantioseparation of tryptophan and hydrobenzoin [171]; the material was packed into a stainless-steel tube (250 x 4.6 mm, i.d.) by a conventional slurry packing technique; the sample loading was in the range of 0.01–4 mM. These and other similar methods (as cited therein) were expensive, cumbersome and were not found suitable for large scale enantioseparation. Besides, there has been an upsurge and increasing interest, in recent years, in liquid chromatographic separation of non-racemic mixtures under both achiral phases [18, 172] but all these reports are limited to laboratory level and that too in terms of enantiomeric enrichment. Many of the other nanoparticles have been synthesized and characterized their structural, magnetic and catalytic properties but have not been used for enantioseparation of any racemic compound [173-176].

Even after 165 years, the methods originated by Pasteur, enzymatic resolution (very selective and one of the two enantiomers would be totally lost), manual sorting of left and right hemihedral crystals (very cumbersome), and separation through (fractional crystallization) formation of ionic diastereomeric pairs [177] still form the basis of modern industry for resolution of racemates of chiral drugs and for obtaining enantiomerically pure materials [178].

There seems to have reached a point of saturation in terms of development of CSPs based on cellulose, amylose and cyclodextrins and nanoparticle based materials for resolution. The overall present scenario of methods leading to separation of enantiomeric mixtures (or resolution) into pure enantiomers remains in the confines of very small amounts which may, of course, be useful in determination or control of enantiomeric purity but not for separations at large scale, in real terms.

Our focus is to draw attention towards the facts that (i) over 200 CSPs have been commercialized and none of these has been used for *large scale separations in real terms*, (ii) these are expensive and are out of reach of majority of small laboratories and Institutes, and have a short life with limited applications and do suffer with one or more limitations (the '*guide to users*' provided by the manufacturers clearly advise about the limitations in the name of 'precautions'), (iii) the preparative HPLC is also not a large scale separation in real terms, [168] and (iv) there is no report or attempt to develop a CSP that would be stable, less expensive and could be used by open column chromatography for large scale separation of enantiomeric mixtures from enantioselective synthesis or in pharmaceutical industries.

2. Present work

This chapter reports synthesis (illustrated in Fig. 6.1) and characterization of a simple chiral material by binding of BSA on the surface of normal silica gel (particle size of 125-250μ); it was characterized by scanning electron microscopy (SEM), CHNS, FTIR, UV-Vis and XRD. The CSP was successful in providing direct resolution (using simple open column chromatography) of organic racemates [(*RS*)-propranolol, (*RS*)-atenolol and

DL-phenylalanine] at a scale of 30 mg to 3.0 g with very good separation efficiency on open column. The strategy developed here can easily be applied to other types of racemates and for large scale quantitative separations. The method presented is preparative in real terms and is superior for its simplicity and cost over the existing 'preparative methods' based on HPLC etc techniques which are really not preparative and have high running and equipment costs. Both BSA and silica gel are easily available at low costs. The material and the method have practical potential for pilot scale separation of enantiomeric mixtures.

3. Experimental work

- Equipments, materials and reagents are described in Chapter-2.

3.1 Preparation of carboxyhydrazide silica by its chemical modification

Chemical modification of silica gel to get carboxyhydrazide silica via its step wise reaction with 1,4-dibromobutane (for the attachment of a hydrocarbon chain as an anchor on the surface of silica particles), diethylmalonate, and hydrazine hydrate was carried out; though the steps were based on the report by Jal *et al*., [179] but each step was suitably modified so that surface area and particle size of the silica gel would not change.

Step 1: Silica gel of mesh size 60-120 (200 g) was washed thrice with boiled water and then with 6N HCl to remove impurities of Fe^{2+} ions (reported to be present as per specifications given by the supplier). The conditioned silica gel was stirred on rotor with *aq*NaOH (0.5N, 150 mL) for 5h at room temperature and was washed several times with distilled water to remove NaOH. **Step 2:** The alkali treated silica gel was stirred on rotor with 1,4-dibromobutane (5 mL) in EtOH (150 mL, dehydrated with Na_2SO_4) for 6h and then it was filtered; it was then washed first with 70% ethanol and then with purified water and dried. It was inferred that a robust anchor was formed on the surface of silica particles via a 4-membered hydrocarbon chain (**Fig.6.1**). The (bromobutyloxy-) silica gel (**2**) so obtained showed a positive test for bonding of Br (through a four carbon chain). **Step 3**: Diethylmalonate (4 mL) in presence of sodium methoxide (freshly prepared by dissolving 1 g of Na metal in 50 mL of CH_3OH) was added to (**2**) and was stirred on rotor for 6h.

The product (**3**) was filtered and washed with purified water three to four times. Here, a test for Br was negative. **Step 4**: (**3**) was refluxed in hydrazine hydrate (150 mL) for 5h with constant stirring. The carboxyhydrazide silica (**4**) was filtered, washed with ethanol and dried for 4h at 80 °C. A test for hydrazine was positive. The weight of modified silica gel (**4**) was 179 g (appx. yield 90%).

Covalent bonding of Br in step 2 was confirmed by testing for Br by qualitative analysis as follows; to a small sample of the (bromo alkylated) silica gel was added a small volume of 1N NaOH, it was then irradiated under microwave for 10 min at (800W); the filtrate was acidified with *dil* HNO_3 followed by addition of a few drops of $AgNO_3$ solution; there was obtained a yellow precipitate, slightly soluble in NH_4OH, confirming the presence of bromine.

3.2 **Binding of BSA on carboxyhydrazide silica** (Preparation of BSA-Silica CSP)

The BSA-silica CSP was developed from the carboxyhydrazide silica by its reaction with BSA in presence of DMAP and DCC; the covalent binding of BSA was further verified as illustrated in **Fig. 6.1**.

The dried (**4**) (150 g) was taken in benzene (250 mL) and to it were added solutions (prepared in benzene) of BSA (1000 mg) and DMAP (300 mg). DCC (500 mg in benzene) was added drop by drop with constant stirring for 6h. The resulting mixture was filtered under vacuum and washed several times with saline solution (to remove any free BSA). It was dried in an oven at <50 °C. The complete scheme showing modification of silica surface leading to formation of the new CSP **(5)** is illustrated in **Fig.6.1**. Following tests were performed on **(5)** (the new BSA-Silica CSP).

(i) A small amount (1.0 g) of **(5)** was suspended, shaken and heated in water; the filtrate was concentrated under vacuum, and then it was microwave irradiated for 12 min at 800W in presence of 6M HCl; the solution tested negative to ninhydrin. This confirmed that there was no release of BSA simply by suspending/shaking/heating the CSP in water.

(ii) The residue from (i) was microwave irradiated for 12 min at 800W in presence of 6M HCl followed by neutralization with *dil aq NaOH* and addition of a few drops of ninhydrin solution; on heating there was observed typical color of Ruhemann's purple complex. It was inferred that there were available amino acids due to hydrolysis of BSA and that BSA was covalently bound to silica as a result of reaction of carboxyhydrazide silica gel with BSA in presence of DCC and DMAP.

(iii) The ninhydrin test was repeated for quantitative determination of BSA binding concentration. The optical density (UV absorbance) of the hydrolysate obtained by ninhydrin test (as explained above) was compared with that of hydrolysates of five different concentrations of native BSA at 570 nm (as a standard plot); it was found that about 90 mg of BSA was bound per g of **(5)**. It was concluded that a new chiral stationary phase (CSP) was synthesized.

3.3 Surface characterization

The surface characterization of (**5**) was carried out with the help of FTIR, FESEM, CHNS, and X-ray powder diffraction (XRD) and a comparison between pure silica gel and the BSA-Silica CSP was done.

The % of elements found by CHNS analytical testing is as follows, for native silica: H, 4.05; (**2**): C, 8.8; H, 4.2; (**3**): C, 14.2; H, 4.8; (**4**): C, 12.4; N, 9.77; H, 6.42; (**5**): C, 14.8; H, 6.80; N, 12.41; S, 1.08. The FTIR spectra with characteristic peaks are shown in Fig. 6.2a (pure silica gel), Fig. 6.2b (pure BSA) and Fig. 6.2c (the BSA-silica CSP).

Table 6.1 shows summary of FESEM edx data of elemental analysis (weight % of elements) of surface of pure silica and of the products from each step.

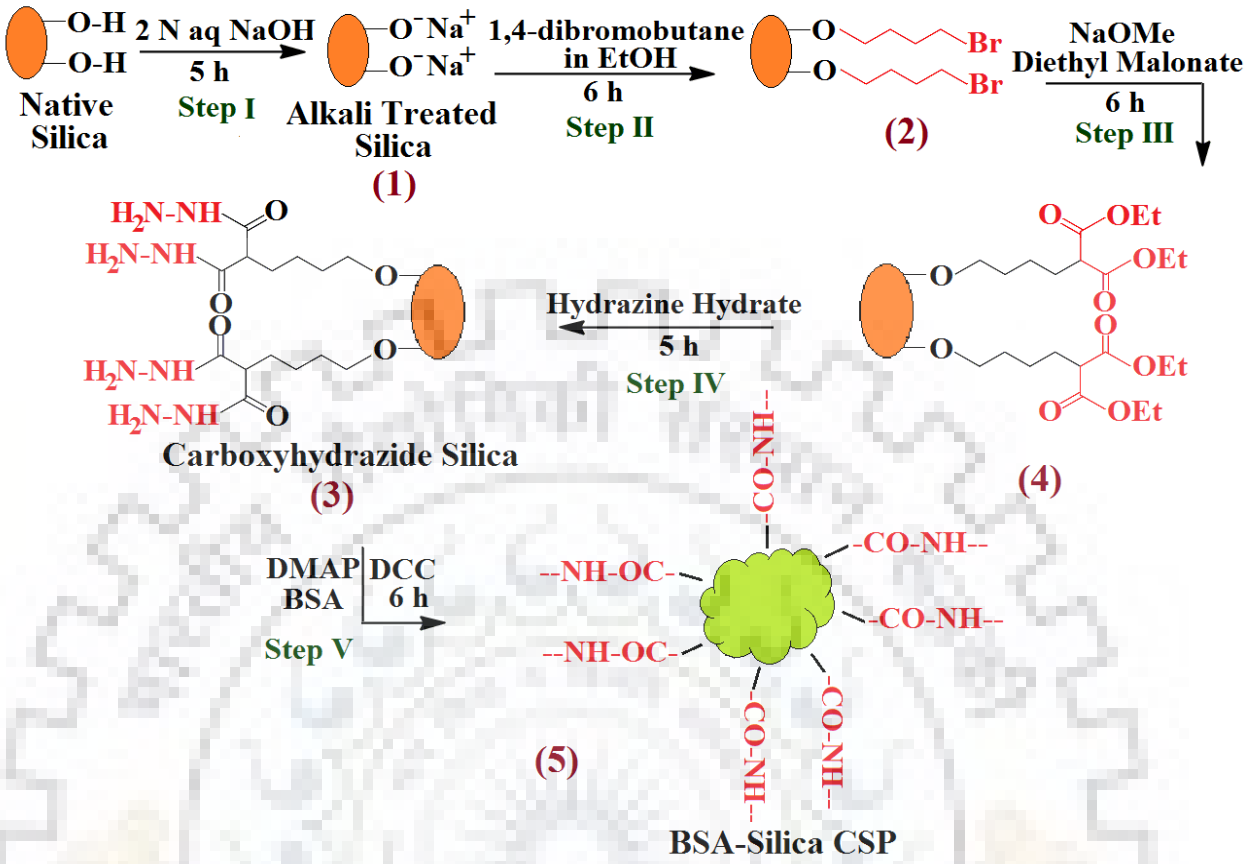


Fig 6.1 Step by step modification of silica surface and preparation of BSA-Silica CSP.

Table 6.1: FESEM edx data showing elemental analysis of modified silica surface (step by step observation data according to scheme shown in Fig. 6.1)

Wt. % of Elements	(1) Bare silica surface	(2)	(3)	(4)	(5) BSA-CSP surface
Si	43.4	33.5	32.65	32.04	28.31
Fe	1.52	0.03	–	–	–
O	55.3	47.8	53.12	48.37	43.03
C	–	10.86	14.76	10.26	12.2
Br	–	7.79	–	–	–
N	–	–	–	9.77	11.41
S	–	–	–	–	5.7

Note: *dash* means 'Not Found'.

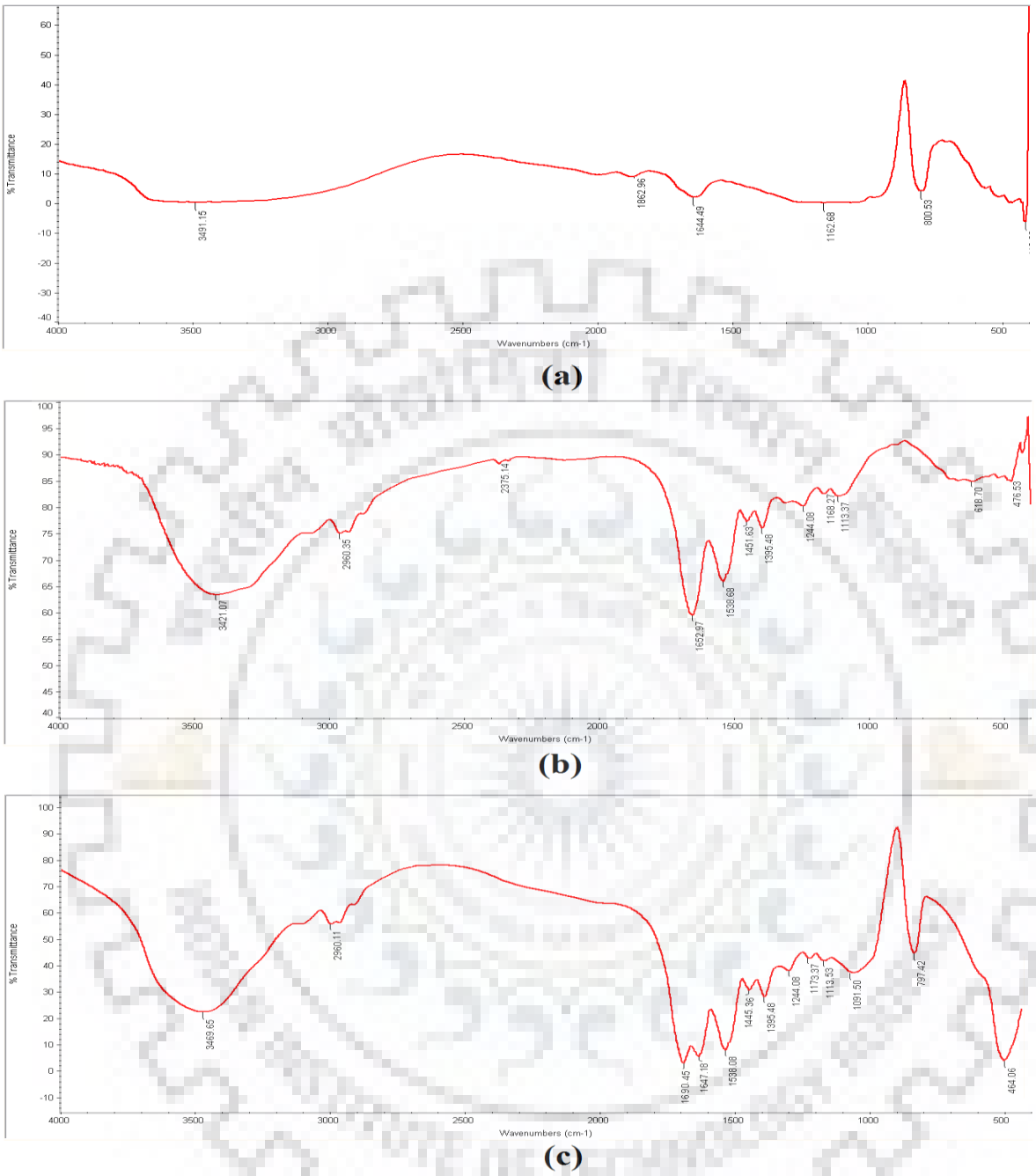


Fig 6.2: FTIR spectra showing modification of silica surface: (a) Pure silica, (b) Pure BSA, and (c) the BSA-silica CSP.

A characteristics difference can be observed in (c) as compared to spectra of pure silica or pure BSA. A broad band at around 3491 cm^{-1} due to hydrogen-bonded silanol showed features originating from surface of prepared BSA-Silica CSP. A vibration hump of a hydroxyl group (υ O-H, a broad hump peak at 3469 cm^{-1}) was also observed which verified attachment of the protein onto silica surface.

3.4 Separation of racemates by open column chromatography

A glass column with a height of 50 cm and a diameter of 1.0 cm was packed with slurry of **(5**) in *n*-hexane. Samples of 30 mg of (*RS*)-Prl and (*RS*)-Atl and 3.0 g of DL-Phe were loaded systematically after eluting the individual enantiomers for each racemate and careful washing with methanol before loading the next sample. Resolution of (*RS*)-propranolol is described here as a representative. Different concentrations of properly degassed and sonicated, isopropanol-hexane (20+80, 30+70, 40+60, 50+50, 60+40, 70+30, 80+20*v*/*v*) with CH_3OH (2 mL added in each combination)was tried as mobile phase.

A combination of isopropanol-hexane-CH_3OH (30+70+2 mL, *v*/*v*) was found successful as mobile phase for resolving the racemates of Prl and Atl, and isopropanol-hexane-CH_3OH (40+60+2 mL, *v*/*v*) was found successful for resolving DL-Phe. By decreasing the concentration of isopropanol (from 30 to 20) in the mobile phase, the retention time was greatly increased; on increasing the concentration of isopropanol by a proportion of 10 systematically up to a ratio of 80+20 (with 2 mL of CH_3OH in each set, *v*/*v*), elution became faster without resolution of the enantiomers as revealed by polarimetric measurements. The mobile phase had pH 6.7 (in the range of 6-7), during the experiment. Fractions (of 10 mL) were collected manually, solvent was evaporated from each fraction on water bath and then made up to 2 mL by adding CH_3OH; UV spectrum of each sample was recorded and was compared with UV spectra of a standard solution of propranolol as a reference. λ_{max} and absorbance of each sample was recorded. The UV visible spectrum of Prl displayed a characteristic λ_{max} at 290 nm, Atl at 226 nm and Phe at 258 nm in CH_3OH.

By taking Prl as a representative compound, total 110 fractions were collected from the column. The time taken in collecting every 10 mL fraction was nearly 9 min. Every fraction was also examined on polarimeter. Fractions from 14 to 40 were found to be dextrorotatory while fractions from 53 to 80 were found to be levorotatory. Accordingly, the fractions were combined and the solvent was evaporated. Using the UV absorbance data, the concentration of each of the enantiomer was calculated through a calibration plot.

The recovery and the specific rotation values are summarized in **Table 6.2**. These are in agreement with the standard values and thus confirmed separation of enantiomers [180**]**. It was found that (*R*)-isomer of Prl and Atl and D-isomer of Phe eluted first.

After obtaining resolution of (*RS*)-Atl, the column was run washed with pure methanol and the CSP was reused for resolution of (*RS*)-Prl, followed by washing and resolution of DL-Phe. Thus, the material was successful for reuse which established conventional open-column chromatography as universally practiced method for simplicity of its operation and success.

Table 6.2: Recovery data and specific rotation values of the enantiomers obtained from the open column using BSA-Silica CSP

Analytes	Recovery		Specific rotation values of first eluted isomers, $[\alpha]_D^{25}$
	(*R*)-enantiomer	(*S*)-enantiomer	
Prl	99.5 % (14.93 mg)	97.3 % (14.6mg)	+26°, (c =1.0, CH_3OH)
Atl	98.0 % (14.70 mg)	99.3 % (14.9 mg)	+12°, (c =1.0, CH_3OH)
Phe	99.40 % (1.49 g)	98.7 % (1.48 g)	+34°, (c =2, H_2O)

3.5 Purity test of separated enantiomers by chiral HPLC

Further, purity of individual enantiomers (of Prl and Atl) so separated from the BSA-Silica CSP column was verified by chiral HPLC using the optimized conditions of resolution (α_1-AGP column with mobile phase consisting of isopropanol having 0.5 % CH_3OH-citrate phosphate buffer (pH 3.5) in the ratio of 80: 20, *v*/*v* at a flow rate of 0.7 mL min^{-1}, under isocratic conditions using PDA detector at 290 and 226 nm for Prl and Atl respectively; the chromatograms are shown in **Fig 6.3.**

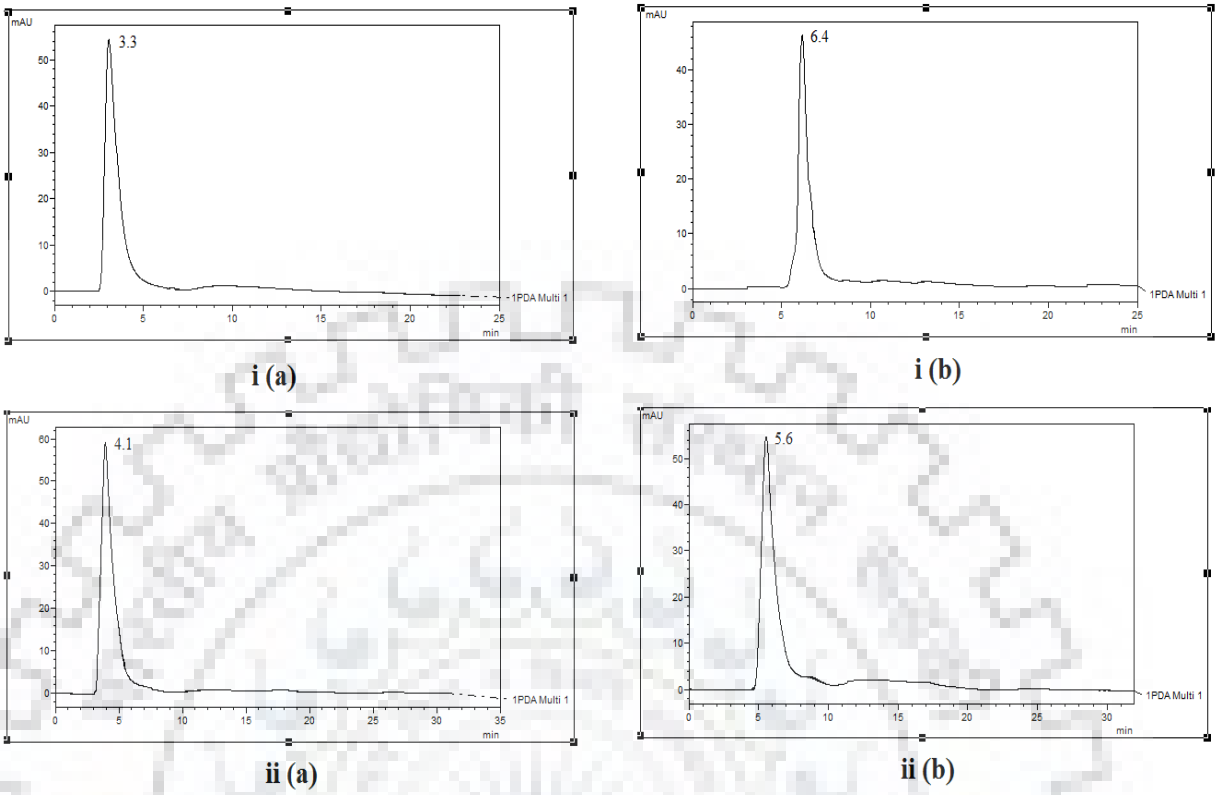


Fig 6.3 Chromatograms showing chiral HPLC results of (*R*)-Prl, (*S*)-Prl and (*R*)-Atl, (*S*)-Atl isolated through open column as, i(a), i(b) and ii(a), ii(b), respectively.

4. Results and Discussion

The surface structure of amorphous silica is known to be fully hydroxylated, covered by isolated and vicinal hydroxyl groups, and is highly disordered in terms of arrangement of hydroxyl groups. At the surface, the structure generally terminates in one of several forms of silanol groups (≡Si-OH).The silanol groups on silica gel are weakly acidic.

In the studies reported herein, the alkali treatment of silica generated silanoxy group at the surface. The treatment of alkali treated silica gel with 1,4-dibromobutane yielded 4-bromobutyloxysilica (**2**); this robust anchor of alkyl chain present onto silica surface acted as a spacer to reduce the steric effect at the silanol site and decreased the conformational disorder, most probably due to better chain packing. The silica surface was

further modified by treatment with diethylmalonate in presence of sodium methoxide (**3**) which on reaction with hydrazine hydrate produced carboxyhydrazide silica (**4**).

In the last step, carboxyhydrazide functional group provided amino terminal and the addition of DCC to the reaction mixture (containing both carboxyl component and the amine component) caused activation and coupling concurrently; there occurred coupling of BSA as the chiral selector via amide bond. Thus, a BSA-Silica CSP came into existence. At first, the synthesis of BSA-Silica CSP was carried out at small scale (starting from 15 g silica gel) and then repeated with bulk quantity (ten times). It was equally easy to handle it during experiments and to show real application at a larger scale with a potential at industrial scale.

The presence of Br in (**1**), absence of Br in (**2**), absence of free BSA and its covalent attachment to silica (**5**) was verified by different chemical tests at each step. The result of elemental analysis (C, H, N) of the products of synthesis (**2**-**4**) showed a corresponding increase in comparison with pure silica gel which verified that alkyl and amino groups had been successfully bonded onto silica surface. The presence of element S found in (**5**) inferred that BSA molecules were successfully bonded on to the silica surface.

FTIR spectra of BSA-Silica CSP showed υOH stretching for silanols [181, 182] in the region of 3200–3800 cm^{-1}. A broad band in FTIR (**Fig 6.2**) at around 3491 cm^{-1} due to hydrogen-bonded silanol showed features originating from surface of the so prepared BSA-Silica CSP. The FTIR spectrums showing characteristic peaks are given as **Fig.6.2** a, b, and c, for pure silica gel, pure BSA and the BSA-Silica CSP, respectively. A vibration hump of a hydroxyl group (υO-H, a broad hump peak at 3469 cm^{-1}) was also observed which verified attachment of the protein onto silica surface [183]. It was interesting to note that peaks of hydroxyl groups of BSA-Silica CSP shifted towards lower wave numbers without much attenuation as compared to native silica gel.

The phase determination was done using XRD and the image obtained by it clearly revealed the highly amorphous structure of the silica surface corresponding to every step

because a very broad and single diffraction hump from 15 to 29° (2θ) on XRD was displayed. The nature of the surfaces (i.e. amorphous) remain saturated (not changed to other form while going from one to other steps during modification), as evident by typical XRD patterns shown in **Fig 6.4**.

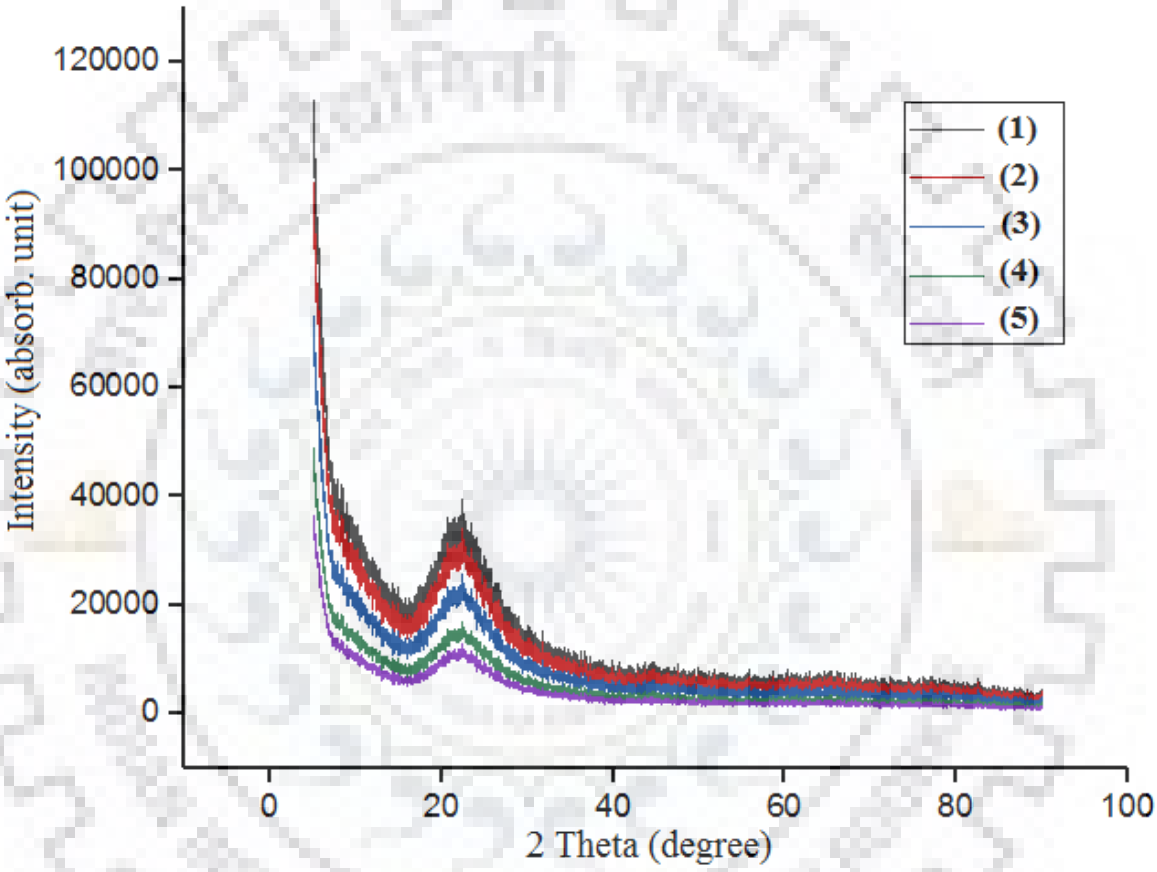


Fig 6.4 Powder XRD graph of each step where step **I-IV** represent the chemically modified states (**1**)–(**4**) of silica as per Fig. 1; (**5**) represents the BSA-Silica CSP. All are showing a single broad hump i.e. amorphous nature of CSP surface having high concentration of Si.

With all characterization data in hand, we compared the untreated silica surface and modified surface of silica. **Fig 6.5** illustrates typical field emission scanning electron microscopic (FESEM) images of the pure silica and the product obtained at the end of each step of chemical modification of the surface. The images and *edx* data clearly suggest the differences in their surface characteristics and elemental composition. It was observed

that the particles at the surface of product of every step had different functional groups and random shape without any detectable change in surface morphology. SEM *edx* data (**Table 6.1)** clearly showed a noticeable difference in elemental composition indicating the bonding of respective functional group at each step.

Millot [184] reviewed various methods of immobilization of different proteins (including BSA) on matrices like silica and zirconia along with stability and enantioselectivity aspects of CSPs so prepared; all of these have their limitations depending upon whether the immobilization was through physical adsorption or via intermediate reactive polymer or by using an additional cross linking reaction or by involving amino-, diol- and epoxide-derivatized silica materials. These chiral materials were used for enantioseparation either by HPLC or capillary electrophoresis; none of these was used in an open column for resolution of larger quantity of the racemate.

In the present case, BSA is covalently bound (and not through physical adsorption) to silica gel. BSA has a number of different types of binding sites and it is relatively nonselective in its binding ability; upon being irreversibly bound to a surface such as silica it provides a CSP that is capable of separating the enantiomers of a variety of chiral amines and carboxylic acids.

BSA is a giant globular protein; it has a molecular weight of about 66000 D and contains about 607 amino acid residues in a single polypeptide chain and no carbohydrates [19]. It is a serum protein that binds mostly acidic and neutral drugs. BSA contains nearly 60 lysine residues [92] which exist in the hydrophobic regions of the macromolecular moiety causing a possibility of stericcavity type inclusion of the enantiomers of analytes.

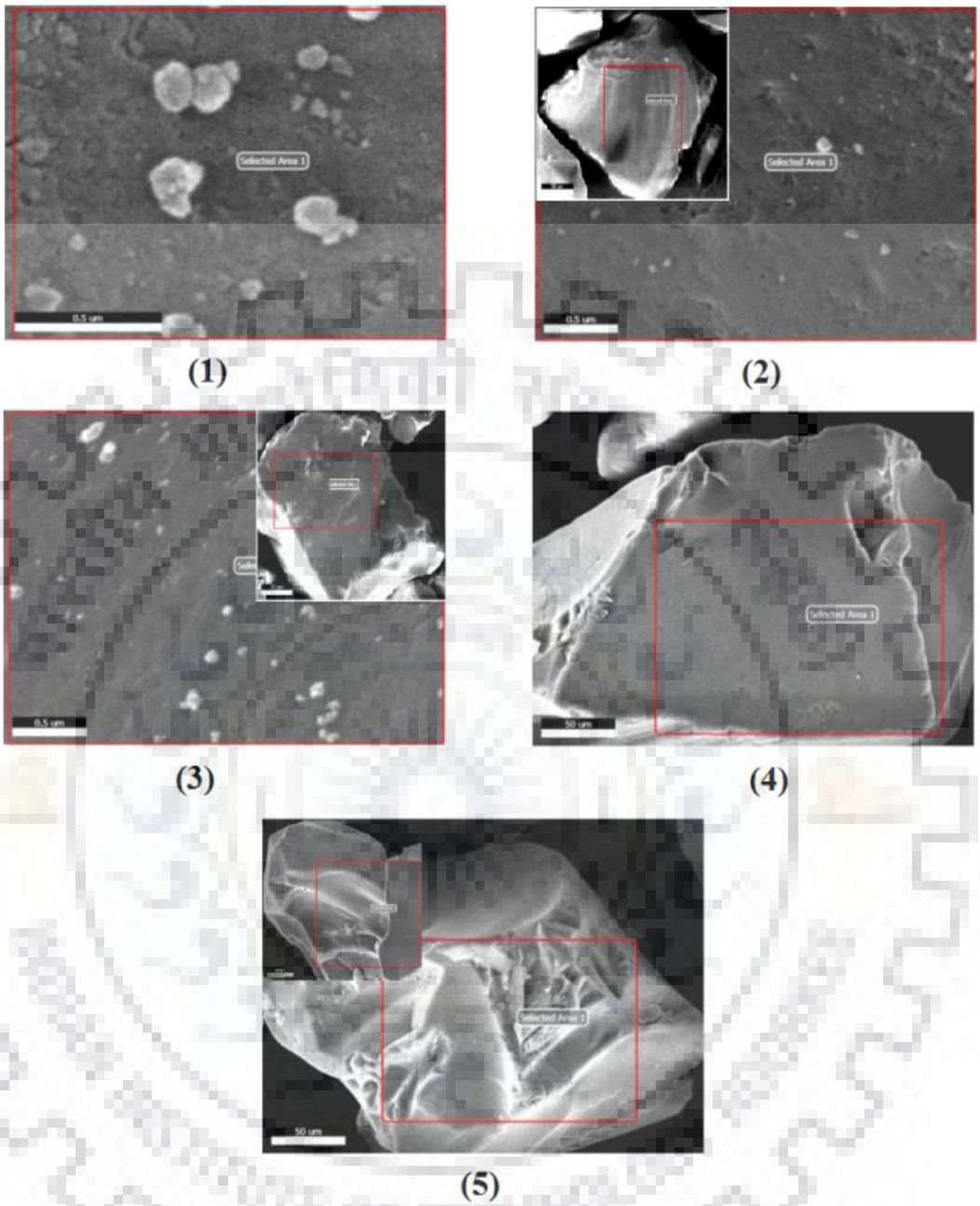


Fig 6.5 Typical FESEM images of chemically modified states of silica as per **Fig. 6.1**. Scale bar is 50 μm and 0.5 μm.

Direct resolution of enantiomers on a CSP (or in presence of a chiral selector or in chiral environment) requires a minimum of three simultaneous intermolecular interactions [92], i.e., the CSP and at least one of the enantiomers, with at least one of these interactions being stereochemically dependent. And, at least, one of the interactions will be absent or significantly altered by replacing one enantiomer with the other without conformational change in any component. The selector distinguishes between the two enantiomers by the presence or absence of a third interaction which must not be collinear with the first two.

BSA as the chiral selector would display chiral discrimination properties owing to multiple possibilities of intermolecular interactions with chiral compounds, such as electrostatic, dipole-dipole, hydrophobic, π–π, and steric interaction [101] or complex formation, and cavity inclusion between protein and analytes [99, 100]. Besides, enantioseparation seems to involve steric and hydrophobic interactions for the large size of BSA (present on the CSP). Binding of BSA has been stable enough for repeated application which is essential for chiral discrimination.

The mobile phase that was successful in resolving the three racemates was not a very polar system and also did not contain any buffer to provide any kind of ions for interactions during resolution. Literature [102, 103] reveals that high polarity or high ionic strength of the mobile phase is not favorable for enantioresolution because it reduces the electrostatic interaction or hydrogen bonding between the BSA-Silica CSP and analyte and thus affects stereoselectivity and retention; the success of the mobile phase in resolution of the analytes, in the present studies, is in agreement with literature explanation.

The selectivity and retention characteristics of a CSP get influenced by an organic modifier in the mobile phase. The structure of BSA-Silica CSP suggests that the polar character of the silica gel is altered to a greater extent due to covalent binding of BSA, yet, the CSP is not a reversed phase in the strict sense. As the enantiomers (Atl and Prl having aromatic moieties) are hydrophobic in nature, there is required an additive like methanol in the mobile phase which would vary the retention of analytes by changing the polarity of

the mobile phase which is isopropanol-hexane (30+70, *v*/*v*). So, CH_3OH (2 mL) was added.

The results reported herein showed that the BSA-Silica CSP had much greater affinity for separation of racemic pharmaceuticals and was highly stable for multiple applications. To examine the long term stability of the BSA-Silica CSP, separation of propranolol enantiomers was repeated during 4 months under identical conditions at an interval of 15 days and the results were in agreement each time.

5. Novelty of the present work

The novelty is embedded in this present work because obtained results demonstrate that the new BSA-Silica CSP was easily synthesized using less expensive materials. The CSP was used in an open column for direct resolution of different racemates in real terms of quantitative/preparative manner. The BSA-Silica CSP has widespread applicability and is not functional group specific. The CSP is quite stable, can be reused several times simply by washing the column with methanol and has the potential for pilot scale application. We anticipate that this CSP will be applicable for a wide range of racemic pharmaceuticals through an open column of a large size.

Chapter-7

Conclusion

There is an enormous scope to develop the chiral technology for obtaining the single enantiomer of pharmaceuticals such as β-adrenolytics, NSAIDs, and antihistamines (as described in Chapters 3, 4 and 5) which would then change the prescription approach for such racemic pharmaceuticals for medical and clinical purposes. Complete enantiomeric profile with correct pharmacokinetic data on these pharmaceuticals (being marketed or to be marketed) would be available through studies related to enantioseparation in biological samples. It would also deal with various possible phenomena such as enantiomer/enantiomer interactions or *in vivo* racemizations. The work presented in this thesis based on systematic simple scientific approach provides chirality recognition even in the absence of pure enantiomers.

The approach and the method, so developed, are useful for both academic and industrial scientists in all areas of the chemical science. Both direct and indirect approaches so adopted with development of novel methods (as describred in the thesis) are self-sustained and complete in totality and support the future usefulness of the chiral reagent as well. It opens up an area (i) for development of new CRs which would yield such diastereomeric derivatives that could be easily hydrolysed to native enantiomers without racemization, (ii) to measure the success of enantioseparation method and synthesis of diastereomers, (iii) to control enantiomeric purity of chiral drugs in industry and analytical laboratories (especially associated with regulatory agencies), and (iv) to be used in different stages of enantioselective synthesis since there generally exists a chance of error in establishing *ee* of a sample because a purification step employing normal chromatography prior to loading it onto chiral column may lead to un-noticed enantiomeric enrichment.

Application of BSA as '*chiral additive in achiral stationary phase*' and especially as a covalent binder to simple silica gel resulting into a novel CSP provides (i) a fundamental insight in developing a very low cost CSP that can be used at industrial level

for enantiomeric separation in real preparative manner, (ii) application of normal column chromatography, and (iii) a simple direct and truly preparative approach over the practiced 'preparative methods' based on HPLC etc techniques (the latter are in real terms not preparative and are very expensive in terms of the cost of the equipment and running costs).

The methods may be applied for successful resolution of a variety of pharmaceuticals and other organic racemic mixtures along with isolation of native enantiomers. Besides, it shows a potential in the area of developing new CRs and CSPs (for preparative resolutions).

As a common/routine laboratory practice in the area of enantioselective synthesis the sample so synthesized is first purified by normal column chromatography prior to establishing the *ee* on chiral column; there occurs an un-noticed enantiomeric enrichment [172] and the *ee* does not represent the true result. To establish the success of synthesis methodology and reliability of enantioseparation it may be useful to derivatize a sample of the racemic or the non-racemic product with a chiral reagent (as per functional group compatibility) followed by separation of diastereomeric derivatives. To ensure the success of diastereomeric synthesis and their enantioseparation it becomes desirable to understand the molecular dissymmetry (absolute configuration) of the separated species.

Thus (i) development of new CR for indirect enantioseparation and sensitive detection, via HPLC and TLC both, (ii) application of different chiral selectors for direct resolution using TLC, especially, and (iii) development of new CSP, to be used for open column chromatography (in contrast to the existing CSPs for HPLC) for real preparative resolution and recovery of enantiomers are the need of the hour and suggest a wide scope in this area.

The establishment of reliable analytical methods for determination of enantiomers shall continue to be challenging and important and in an increasing mode in the fields of chromatography, pharmacology, medicine, asymmetric synthesis, mechanistic studies, extra-terrestrial chemistry, life sciences *etc*.

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