CHIRAL SEPARATION OF PHARMACEUTICAL COMPOUNDS USING LIQUID CHROMATOGRAPHY

Ph.D. THESIS

by

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A THESIS
Submitted in partial fulfilment of the requirements for the award of the degree of
DOCTOR OF PHILOSOPHY
in
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ROORKEE

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"CHIRAL SEPARATION OF PHARMACEUTICAL COMPOUNDS USING LIQUID CHROMATOGRAPHY"** in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Chemistry of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period from January 2013 to June 2016 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 Institute.

(Manisha Singh)

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

Signature of Supervisor

The Ph.D. Viva-Voce Examination of Manisha Singh, Research Scholar, has been held on 09th September 2016.

Chairman, SRC **Signature of External Examiner**

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

(Ravi Bhushan)
Supervisor **Head of the Department**
Dated**:**

CONTENTS **Page No.**

CHAPTER 5: Resolution of (*RS*)-Baclofen by Ligand Exchange TLC

CHAPTER 6: Enantioseparation of pharmaceutical drugs using both achiral phases

Section A: HPLC Enantioseparation of (*RS*)-Bupropion, (*RS*)-Baclofen and (*RS*)-Etodolac: Modification of Conventional Ligand Exchange Approach

Section B: TLC Enantioseparation of (*RS*)-Etodolac using both achiral phases

CHAPTER 7: Enantioresolution of chiral carbonyl compounds

ABSTRACT

In chiral drugs, “often only one of the enantiomers is responsible for the desired physiological effects while the other enantiomer is less active, inactive, or sometimes even causing adverse effects. Drugs composed of only one enantiomer can be developed to enhance the pharmacological efficacy” and eliminate some side effects. The regulatory agencies in many countries, “involved in the registration of new active ingredients, insist on registration of single enantiomer of a new drug and ask the pharmacologists to present full information on the stereochemistry and stereoselectivity of both the enantiomers including the necessary stereoselective analytical methods”. Therefore, efficient methods of enantioseparation are required to control the enantiomeric purity, or to separate the target molecule or one of its chemical precursors (obtained from conventional synthetic procedures), or for monitoring the completion of enantioselective reaction process (since the production of single enantiomer is a real difficult task). “Liquid chromatographic techniques especially thin layer chromatography and high performance liquid chromatography are commonly used”.

Present thesis deals with the studies on direct and indirect enantioseparation of certain racemic compounds; these include some commonly administered and marketed drugs like (*RS*)-Atenolol, (*RS*)-Propranolol, (*RS*)-Baclofen, (*RS*)-Etodolac, (±)-Bupropion, (*RS*)-Metoprolol, (*RS*)-Carvedilol, and certain didactic chiral aldehydes and ketones. A chapter wise brief account is given below.

The **first chapter** deals with preamble to present studies including introduction to chirality, enantiomers and their separation. The chapter also includes discussion about liquid chromatography, HPLC and TLC. The CDRs, CSP and chiral selectors for TLC used in the present studies have been discussed in brief.

The **second chapter** presents description of materials, equipments, preparation of stock solutions and extraction of active pharmaceutical ingredients from the commercial tablets.

The **third chapter** describes formation of diastereomers of (*RS*)-Etodolac. Three pairs of diastereomers were synthesized using enantiomerically pure amines, namely, (*R*)-(+)-α-methyl benzyl amine, (*S)*-(–)-α,4-dimethylbenzylamine, (*R*)-(–)-1-cyclohexylethylamine. Separation of diastereomers was successful using C_{18} column and a binary mixture of methanol and triethyl ammonium phosphate buffer (TEAP) of pH 4.5 (80:20 *v/v*) as “mobile phase at a flow rate of 1 mL/min and UV detection at 223nm. Separation method was

validated as per ICH guidelines. Derivatization reactions were carried out under conditions of stirring at room temperature (30 °C for 2 h) as well as under microwave irradiation (MWI) and the two types of diastereomers were compared". Reaction conditions for derivatization were optimized with respect to mole ratio of CDR and (*RS*)-etodolac and MWI time. "Formation of diastereomers of (*RS*)-Etodolac was confirmed using LC-MS when $[M+H]^+$ or $[M]^+$ were recorded for the diastereomers. Lowest energy optimized structures of two diastereomers were drawn which confirmed three dimensional geometry of the diastereomers".

The **fourth chapter** describes the "synthesis of a new chiral derivatizing reagent from (*S*)-(+)-Naproxen and its application" for C–N bond formation to prepare diastereomeric amides of (*RS*)-Propranolol, (*RS*)-Atenolol, (*RS*)-Carvedilol and (*RS*)-Metoprolol. Derivatization reactions were done at room temperature (30 °C for 30 min) under stirring conditions as well as under microwave irradiation (MWI). "Separation of diastereomers was achieved by open column chromatography". ^{1}H NMR spectra of the isolated and purified "diastereomers were recorded to establish the configurations of the first and second eluting diastereomers (and thus the elution order) and" to compare the chromatographic separation characteristics when the diastereomeric mixture was separated by "RP-HPLC (using C_{18} column and a binary mixture of MeCN and triethyl ammonium phosphate buffer of pH 3.5 (60:40 *v*/*v*) as mobile phase at a flow rate of 1 mL/min and UV detection at 230 nm). No racemization was observed throughout the study". Test samples of β-blockers were "isolated from commercial tablets and then were purified and characterized to be used as racemic standard. The conditions for derivatization and separation were optimized. Lowest energy optimized structures of the two diastereomers were developed using the Gaussian 09 Rev. A.02 program and hybrid density functional B3LYP with 6-31G basis set which supplemented ^{1}H NMR interpretations and confirmed three dimensional geometry of the diastereomers. Separation method was validated as per ICH guidelines. The limit of detection and limit of quantification for each isomer were 0.4 ng/mL and 1.2 ng/mL, respectively".

The **fifth chapter** presents direct enantioresolution of (*RS*)-baclofen by ligand exchange TLC adopting two different approaches; (**A**) "TLC plates were prepared by mixing the ligand exchange reagent (LER) with silica gel slurry" and the chromatograms were developed with different achiral solvents or solvents having no chiral additive, and (**B**) the LER consisting of "Cu(II)-L-amino acid complex was used as chiral mobile phase additive and the plain plates

of silica gel having no chiral selector were used. Cu(II) acetate and four L-amino acids (namely, L-tryptophan, L-histidine, L-proline and L-phenylalanine were used for the preparation of ligand exchange reagents. Spots were located by use of iodine vapour. Effect of temperature and mole ratio of Cu(II) to amino acid on enantioresolution was also studied. The results for the two methods have been compared and the issue of involvement of the Cu(II) cation for the best performance of the two methods has been discussed with respect to the same mobile phase".

The **sixth chapter** deals with enantioseparation of the chosen racemic drugs with a concept of using both achiral phases in TLC and HPLC. It has been divided into two sections.

Section A: It deals with the enantioseparation of (*RS*)-Bupropion, (*RS*)-Baclofen and (*RS*)-Etodolac. It has been achieved by modifying the conventional ligand exchange approach. The Cu(II) complexes were first prepared with a few "L-amino acids, namely, L-proline, L-histidine, L-phenylalanine, and L-tryptophan" and to these was introduced mixture of enantiomer pair of (*RS*)-Bupropion, or (*RS*)-Baclofen or (*RS*)-Etodolac. As a result, formation of a pair of *diastereomeric complexes* occurred by '*chiral ligand exchange*' via the competition between the chelating L-amino acid and each of the two enantiomers from a given pair. The diastereomeric mixture formed in the pre-column process was loaded onto HPLC column. Thus, both the phases during chromatographic separation process were *achiral* (i.e., neither the stationary phase had any chiral structural feature of its own nor the mobile phase had any chiral additive). Separation of diastereomers was successful "using C_{18} column and a binary mixture of acetonitrile and TEAP buffer of pH 4.0 (60:40, *v/v*) as mobile phase at a flow rate of 1 mL/min and UV detection at 230 nm for (*RS*)-Bupropion, 220 nm for (*RS*)-Baclofen, and 223 nm for (*RS*)-Etodolac. Baseline separation of the two enantiomers was obtained with a resolution of 6.63 in less than 15 min.

Section B: It deals with direct enantioresolution of (*RS*)-Etodolac involving both achiral phases in TLC. Enantiomerically pure "L-tryptophan, L-phenyl alanine, L-histidine, and L-arginine, were used as *chiral inducing reagents"* (CIR); any of these was neither impregnated with silica gel (while making TLC plates) nor mixed with the mobile phase. The solvent system acetonitrile-dichloromethane-methanol, in different proportions, was found to be successful for enantioresolution. "Spots were located in iodine chamber. Effect of concentration of chiral inducing reagent and temperature on enantioresolution was studied".

The **seventh chapter** deals with a new method which has been developed involving, solid phase microwave-assisted conditions for synthesis of 2,4-dinitrophenyl hydrazone(s) of racemic carbonyl compounds wherein there occurred no inversion of configuration. The method provided high yields (91–95%) in short reaction time (4-6 min). The method proposed clearly has synthetic advantages over current practices. "The hydrazones were characterized by IR, ^{1}H NMR and CHN analysis". The hydrazones represent enantiomeric pairs tagged with a strong chromophore rather than diastereomers. The enantiomeric pairs were separated by HPLC using α_1-acid glycoprotein column and the "best resolution of all the analytes was achieved with mobile phase" containing 0.5% 2-propanol in 10 mM citrate phosphate buffer at pH 6.5. The chromatographic peaks clearly showed base line separation with comparable peak areas and thus the results confirmed that there was no spontaneous inversion of configuration during derivatization.

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"I am indebted to my father for living, but to my teacher for living well".

- Alexander The Great.

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And finally to the Almighty, the Omnipresent, from all blessings flow now and forever.

Dated: (Manisha Singh)

LIST OF PUBLICATIONS

1. **Manisha Singh** and Ravi Bhushan, A novel approach for enantioseparation as applied to (*RS*)-etodolac from pharmaceutical formulations: LC MS and density functional theory support for confirmation of diastereomers so separated. *Biomedical Chromatography*, **29** (2015) 1330–1337. **(Chapter 3)**

2. **Manisha Singh** and Ravi Bhushan, (*S*)-Naproxen based novel chiral reagent for C-N bond formation: enantioseparation of some β-blockers, determination of absolute configuration and elution order of diastereomers. *RSC Advances*, **5** (2015) 70255-70264. **(Chapter 4)**

3. **Manisha Singh** and Ravi Bhushan, A modification of conventional technique for synthesis of hydrazones of racemic carbonyls: prevention of spontaneous chiral inversion. *RSC Advances*, **5** (2015) 105719-105726. **(Chapter 7)**

4. **Manisha Singh**, Poonam Malik and Ravi Bhushan, Resolution of enantiomers of (*RS*)-Baclofen by Ligand Exchange Thin-Layer Chromatography. *Journal of Chromatographic Sciences*, **54** (2016) 842-846. **(Chapter 5)**

5. **Manisha Singh** and Ravi Bhushan, Enantiomeric resolution of (±)-Etodolac by direct approach using both achiral phases in Thin Layer Chromatography: A conceptual approach. *Journal of Planar Chromatography*, **29** (2016) 184-189. **(Chapter 6)**

6. **Manisha Singh** and Ravi Bhushan, HPLC enantioseparation of racemic Bupropion, Baclofen and Etodolac: Modification of conventional ligand exchange approach by pre-column formation of 'chiral ligand exchange complexes. *Biomedical Chromatography*, **30** (2016) pp in press; DOI 10.1002/BMC.3746 **(Chapter 6)**

7. Sonika Batra, **Manisha Singh** and Ravi Bhushan, L-Amino Acids as Chiral Selectors for the Enantioseparation of (±)-Bupropion by Ligand Exchange Thin-Layer Chromatography Using Cu(II) Complex *via* Four Different Approaches. *Journal of Planar Chromatography*, **27** (2014) 367–371.

LIST OF ABBREVIATIONS

1.	AAs	Amino acid
2.	API	Active Pharmaceutical Ingredient
3.	anal. calcd	Analytically calculated
4.	Bup	Bupropion
5.	CC	Cyanuric chloride
6.	CDR	Chiral Derivatization Reagent
7.	CMPA	Chiral Mobile Phase Additive
8.	CSP	Chiral Stationary Phase
9.	DCC	Dicyclohexylcarbodiimide
10.	DCM	Dichloromethane
11.	d	Doublet
12.	dd	Double Doublet
13.	DFDNB	1,5- Difluoro-2,4-dinitrobenzene
14.	DMSO	4-Dimethyl sulphoxide
15.	DCU	Dicyclohexyl Urea
16.	DMAP	4-Dimethyl Amino Pyridine
17.	Etd	Etodolac
18.	EtOH	Ethanol
19.	HPLC	High Performance Liquid Chromatography
20.	HOBt	Hydroxybenzotriazole
21.	ICH	International Conference on Harmonization
22.	IR	Infrared
23.	k	Retention factor
24.	LEC	Ligand Exchange Chromatography
25.	LC	Liquid Chromatography
26.	LOQ	Limit of Quantitation
27.	LOD	Limit of Detection
28.	min	Minute
29.	MWI	Microwave Irradiation
30.	MeOH	Methanol

31. MeCN	Acetonitrile
32. mM	Milimolar
33. MP	Mobile Phase
34. NP	Normal Phase
35. NMR	Nuclear Magnetic Resonance
36. nmol	Nanomole
37. R_f	Retention factor
38. RP	Reversed Phase
39. R^2	Correlation coefficient
40. TEA	Triethylamine
41. TLC	Thin Layer Chromatography
42. TFA	Trifluoroacetic acid
43. THF	Tetrahydrofuran
44. UV	Ultraviolet
45. USFDA	United States Food and Drug Administration
46. μL	Microlitre

Chapter- 1

General Introduction

I. Introduction

Chirality and Enantiomers

Kelvin was the first who used the term chirality [1] and according to him it is a property of a molecule which is produced due to the asymmetry in its chemical structure arising from different spatial arrangements of groups around the asymmetric center. Chiral molecule is non-superimposable on its mirror image and such non-superimposable pairs of molecular mirror images are called enantiomers (**Fig. 1.1**). They have same physical and chemical properties in achiral environment but in chiral environment they behave differently.

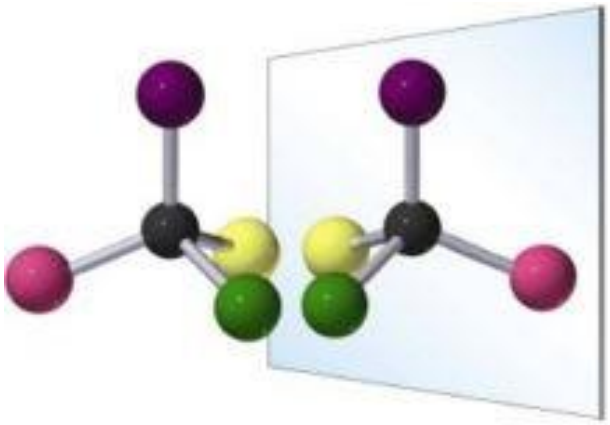


Fig. 1.1 Non-superimposable mirror image isomers of a chiral molecule

Nearly 56% of the pharmaceuticals marketed and in use are chiral compounds, and, amongst them, 88% are administrated as racemates. The chiral nature of living systems with their inherent chiral selectivity to enzyme and receptor causes the two enantiomers of a drug molecule to have different toxicities, pharmacokinetics and pharmacodynamics [2-7]. One enantiomer may have all the desired pharmacological activity while presence of undesirable enantiomer in a racemic drug is regarded as impurity. There are many cases in which two enantiomers exhibit equal

pharmacokinetics and pharmacodynamics profiles. Moreover, other situations occur in which a synergistic action involves the two enantiomers with the distomer being able to antagonize the unwanted side-effect of the eutomer. Thalidomide case is the most notorious example of an enantiomeric drug having unwanted side effects [8]. In the 1960s thalidomide was prescribed to relieve pregnant women of indication associated with morning sickness. The (*S*)-thalidomide caused serious birth defects in children while the (*R*)-thalidomide was effective in reducing morning sickness.

Relevance and importance of enantioseparation

The chiral nature of living systems with their inherent chiral selectivity to enzyme and receptor causes the two enantiomers of a drug molecule to have different toxicities, pharmacokinetics and pharmacodynamics. In conventional analysis, a pair of enantiomers often behaves as one compound. The degree of enantioselectivity in pharmacokinetics is markedly species dependent and the data cannot be transposed between species since enantiomers of racemate drugs exhibit pronounced pharmacokinetic differences, resulting from stereoselective pharmacokinetic processes [9]. In view of these differences, studies of the pharmacokinetics of chiral drugs should measure and quantify the separate enantiomers and not "total drug" as these enantiomers are indeed different drugs. U.S. Food and Drug Administration (USFDA) and other regulatory agencies in Japan and EC have produced guidelines for registration of new chiral pharmaceuticals which require investigations of stereospecific fate of drugs in the body and enantiomeric purity determination of chiral drugs before their introduction into the market and during industrial manufacture [10]. Therefore, it is of great significance to develop simple methods for enantioseparation and control of enantiomeric purity for chiral drugs to facilitate an accurate evaluation of the risks posed by them to human health.

Methods of Separation

Chromatography

It is the separation technique which finds widespread applications in research and industry. Enantiomeric separations in chromatography can be achieved either by direct or indirect methods.

The term Thin layer chromatography was coined by Egon Stahl [11] in Germany. From 1950 to 1954, Kirchner and colleagues at the US Department of Agriculture developed TLC essentially as we know it today" [12, 13]. TLC has been reviewed by Heftmann [14], Stahl [15], Kirchner [16] and Pelick et al., [17]. A comprehensive study on TLC separations has been reviewed by Sherma [18]. Development and introduction of high performance thin layer chromatography (HPTLC), over-pressured thin layer chromatography (OPTLC) and centrifugal layer chromatography (CLC) has caused a great advancement in the field of TLC and has been described by Mincsovics *et al.*, [19]. The TLC/FTIR [20] and TLC/MS [21] coupled method has also been used in modern laboratories for qualitative and quantitative analysis. With the discovery of liquid-liquid chromatography Martin and Synge [22] also "laid the foundation of 'high-performance liquid chromatography' (HPLC) and 'gas-liquid chromatography' (GLC) in the same paper. In the year 1965, Giddings showed that the theoretical framework developed for GLC applied equally well to liquid chromatography [23] and in 1969 Kirkland [24] and Huber [25] described the first high performance liquid chromatography.

Various terms used for representing the chromatographic separation data are, Resolution (R_S), Retention Factor, Selectivity, Number of Theoretical Plates. These are well described in several texts on chromatography, cited above, and are frequently used in the present thesis and the papers already published based on the results presented herein.

Literature on Enantioseparation

Direct approach: Separation of a pair of enantiomers without resorting to derivatization (i.e., without formation of a covalent bond between the analyte enantiomers and the chiral reagent) prior to separation process is termed as direct approach. Since there does not occur formation of any covalent bond, at any stage, the native identity of the enantiomers is preserved and the two enantiomers (after resolution) can be isolated and used for further studies. A three point interaction

(electrostatic attraction, hydrogen bonding, π-π interaction, steric repulsion) between the chiral stationary phases and at least one of the three interactions must be stereochemically dependent [26, 27].

Nevertheless, achiral derivatization is sometimes necessary in order to (i) introduce a suitable achiral chromophore for the purpose of on-line detection, and (ii) to introduce functional groups into enantiomeric molecules that enhance the possibilities for chiral interactions (when separation is achieved using chiral stationary phases) or block functional groups to avoid non-specific interactions. In principle, it remains direct approach to enantioseparation.

Indirect approach: Indirect method involves the use of a chiral derivatizing reagent to convert a pair of enantiomers into diastereomers. Because of the coupling with highly sensitive reagents having high molar absorptivity (ε) or high fluorescence quantum yield (φ) the indirect methods improve detectability and chromatographic conditions are much easily optimized [28-30]. To bring in such properties, dinitrophenyl (DNP) moiety has been introduced in various chiral derivatizing reagents which were synthesized from "1,5-difluoro-2,4-dinitrobenzene (DFDNB)" as starting material; a variety of enantiomerically pure compounds such as L-amino acids have been substituted as the chiral auxiliaries in the DFDNB molecule [31-33]. These chiral derivatizing reagents have successfully been used for "enantioseparation of a variety of amino group containing compounds including several pharmaceuticals, used for enantioseparation of a variety of amino group containing pharmaceuticals" [34-38].

II. Preamble to present studies

1. Objectives

a) To separate enantiomers of certain pharmaceutically important compounds,

b) To develop simple, inexpensive, sensitive and reproducible methods for enantiomeric separation and control of enantiomeric purity in different situations,

c) To optimize and validate the methods so developed,

d) To establish mechanism of chiral recognition and to determine elution order.

2. Selection of chiral compounds for enantioseparation

At the first instance, different categories of pharmaceuticals (like muscle relaxants, NSAIDs, anti-depressant and β-adrenergic blocking agents) were chosen for enantioseparation; these included, (*RS*)-Etodolac, (*RS*)-Baclofen, (±)-Bupropion, β-blockers (namely, (*RS*)-Propranolol, (*RS*)-Atenolol, (*RS*)-Carvedilol, (*RS*)-Metoprolol (**Fig. 1.2**). These drugs are easily available as racemates in the market and have low cost and they have a particular functional group (like amino or carboxylic) for appropriate reaction and synthetic alterations.

In order to investigate direct resolution by introducing a suitable achiral chromophore, for the purpose of on-line detection, certain chiral aldehydes and ketones (namely, (±)-2-phenylpropionaldehyde, (±)-2-methylbutyraldehyde, (±)-2-methylcyclohexanone, (±)-3-methyl-2-pentanone, (±)-2-Methylcyclopentanone, (±)-3-methylcyclohexanone were chosen for the present studies. It may not be an exaggeration to state that the carbonyl group is the centrepiece of organic chemistry. It is not only present itself in most of the main functional groups with multiple bond from carbon to heteroatom but it also serves as a model for reactions of all functions with π-bonds between dissimilar atoms. Besides, its modes of reaction are simple and are very versatile in terms of synthetic applications.

3. Selection of Chiral Derivatizing Reagents (CDR) for enantioseparation

The CDRs (chosen or synthesized for preparation of diastereomers) have the following characteristics:

- CDRs should be enantiomerically pure.
- CDRs should be synthesized easily in laboratory.
- CDRs should have good chromophoric moiety.
- For the reaction between the CDR and that of the functional group of analyte the reaction conditions should be mild.
- CDRs should not racemize during derivatization or under storage condition and should be stable.

By proper experimental method the chiral purity of CDRs is ascertained.

In light of these points (*S*)-Naproxen was explored as chiral structural moiety to synthesize CDR which was used for enantioresolution of the chosen analytes.

A. Enantiomerically pure amines as CDRs

Certain enantiomerically pure amines were chosen as CDRs for enantioseparation of (*RS*)-Etodolac; these are:

- (*R*)-(+)-α-methylbenzylamine, (**CDR 1**)
- (*S)*-(−)-α,4-dimethylbenzylamine, (**CDR 2**)
- (*R*)-(−)-1-cyclohexylethylamine, (**CDR 3**)

B. CDRs based on (*S*)-Naproxen

Literature reveals that (*S*)-Naproxen has been used as a CDR or as a synthon for preparing CDRs. It was economical and stable. It was used to synthesize a chiral derivatizing reagent, (*S*)-Naproxen anhydride (**CDR 4**), and that CDR was utilized for the preparation of diastereomers of four β-blockers.

4. Selection of chiral reagents for TLC and HPLC

Cu(II) acetate and four L-amino acids (namely, L-tryptophan, L-histidine, L-proline and L-phenylalanine) were used for the preparation of Ligand Exchange Reagents.

Enantioresolution of (*RS*)-baclofen was carried out by using four LERs. Using the same LERs enantioseparation of (±)-Bupropion, (*RS*)-Baclofen and (*RS*)-Etodolac were done on HPLC.

Direct enantioresolution of (*RS*)-Etodolac was achieved by adopting a new conceptual approach involving both achiral phases in thin TLC. Enantiomerically pure L-tryptophan, L-phenyl alanine, L-arginine, and, L-histidine were used as *chiral inducing reagents* (CIR).

5. Methodology adopted

For enantiomeric resolution two basic strategies have evolved over the past years; direct and an indirect method. Both indirect and direct methods of enantioseparation were applied using HPLC and TLC as separation techniques.

A. Direct enantiomeric resolution

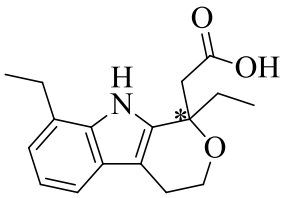
Direct resolution of (*RS*)-Etodolac was achieved, along with isolation of native enantiomers by TLC on silica gel plates using optically pure L-tryptophan, L-phenyl alanine, L-histidine, and L-arginine, as *chiral inducing reagents* (CIR). Direct

enantioseparation of (*RS*)-Baclofen was also done by using Ligand Exchange Chromatography (LEC).

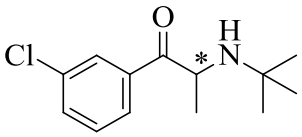
Since direct enantioresolution based on reversible diastereomeric association between solute enantiomers and chiral stationary phases (CSPs) offers the advantages of simple chromatographic runs and absence of kinetic resolution and racemization over indirect method of chiral separation [39,40] α_1-acid glycoprotein column was chosen for the enantioresolution of these racemic aldehydes and ketones as their DNP derivatives. The structures of pharmaceuticals used in the present study are shown in (**Fig. 1.2**).

B. Indirect method of enantioresolution

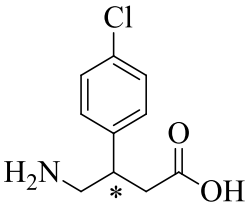
(*RS*)-Etodolac, (*RS*)-Propranolol, (*RS*)-Atenolol, (*RS*)-Carvedilol and (*RS*)-Metoprolol were separated by RP-HPLC on C_{18} column after derivatization with either synthesized CDR or commercially available CDRs. The derivatization process was carried out using conventional and microwave irradiation (MWI) method.



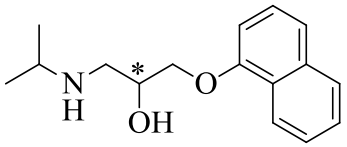
(*RS*)-Etodolac



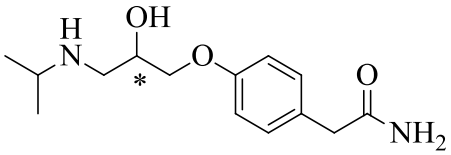
(±)-Bupropion



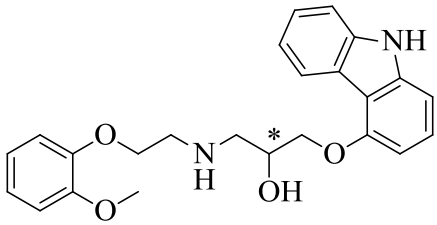
(*RS*)-Baclofen



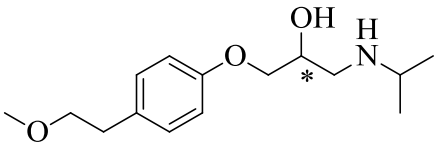
(*RS*)-Propranolol



(*RS*)-Atenolol



(*RS*)-Carvedilol



(*RS*)-Metoprolol

Fig. 1.2 Structures of the pharmaceuticals used for their LC enantioseparation studies

Chapter- 2

Experimental

I. Materials

Enantiomerically pure amino acids *viz*. L-tryptophan (L-Trp), L-histidine (L-His), L-proline (L-Pro), L-arginine (L-Arg) and L-phenylalanine (L-Phe), (*S*)- Naproxen (Npx), (*R*)-(+)-α-methylbenzylamine, (*S)*-(-)-α,4-dimethylbenzylamine, (*R*)-(–)-1-cyclohexylethylamine, dicyclohexyl carbodiimide (DCC), 1H-benzotriazole (HOBT), Dimethylaminopyridine (DMAP), (*RS*)-Atenolol (Atl), (*RS*)-Propranolol (Prl), (*RS*)-Baclofen (Bac), (*S*)-Baclofen, (±)-2-phenylpropionaldehyde, (±)-2-methylbutyraldehyde, (±)-2-methylcyclohexanone, (±)-2-Methylcyclopentanone, (±)-3-methylcyclohexanone, (±)-3-methyl-2-pentanone, and 2,4-dinitrophenyl hydrazine (2,4-DNPH) were obtained from Sigma-Aldrich (St. Louis, MO, USA). (*RS*)-Etodolac (Etd) marketed as Etova (Ipca Laboratories Ltd.), Mumbai, India), (±)-Bupropion (Bup) marketed as Bupron SR150 (Sun Pharma Sikkim, India), (*RS*)-Metoprolol (Mel) marketed as Betaloc (AstraZeneca, Bangalore, India), (*RS*)-Carvedilol (Cal) marketed as Carca (Intas, Dehradun, India) were purchased from local drug store.

Trifluoroacetic acid (TFA), triethylamine (TEA), ammonium formate, formic acid, phosphoric acid (H_3PO_4), acetic acid (CH_3COOH), concentrated hydrochloric acid (HCl), sodium hydrogen carbonate ($NaHCO_3$), and HPLC grade methanol (MeOH), acetonitrile (MeCN), 2-propanol (2-Pr-OH), chloroform ($CHCl_3$) and dichloromethane (DCM) were obtained from E. Merck (Mumbai, India). Other chemicals and reagents, of analytical grade, were obtained from BDH (Mumbai, India), Merck (Mumbai, India) and Sisco Research Laboratory (Mumbai, India). Silica gel G with 13% calcium sulphate as binder, having chloride, iron, and lead impurities up to 0.02% and with pH 7.0 in a 10% aqueous suspension was from Merck (Mumbai, India). The names and abbreviations of α-amino acids and details of pharmaceutical formulations are given in **Table 2.1.**

II. Equipments

HPLC System

• HPLC system of Waters (Milford, MA, USA) was used that consisted of a 515 HPLC pump, a Waters 2489 UV-vis dual wavelength detector, high pressure binary gradient pump control module II, a manual injection valve, an empower2 operating software (build number, 2154).

Table 2.1: Names and abbreviations of α-amino acids and pharmaceutical drugs

S.No.	Amino acids	Abbreviation
1.	L-proline	L-Pro
2.	L-phenylalanine	L-Phe
3.	L-histidine	L-His
4.	L-tryptophan	L-Trp
5.	L-arginine	L-Arg
	Pharmaceutical drugs	**Abbreviation**
1.	(*RS*)-Etodolac	(*RS*)-Etd
2.	(*RS*)-Baclofen	(*RS*)-Bac
3.	(±)-Bupropion	(±)-Bup
4.	(*RS*)-Atenolol	(*RS*)-Atl
5.	(*RS*)-Propranolol	(*RS*)-Prl
6.	(*RS*)-Carvedilol	(*RS*)-Cal
7.	(*RS*)-Metoprolol	(*RS*)-Mel

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

- HPLC column: α_1-AGP (L x I.D., 10 cm x 4 mm, 5 μm particle size) column from Chromtech Merck (Darmstadt, Germany),Waters Spherisorb C_{18} (L x I.D., 25cm × 4.6 mm, 5 μm particle size) column from Parker Style (Fittings, Ireland), LiChrospher C_{18} column (L x I.D., 25cm × 4.6 mm, 5 μm particle size) from Merck (Darmstadt, Germany).

Other equipments:

Microwave Multiwave 3000 (800 W) was from Perkin-Elmer (Shelton, CT, USA). Double distilled water was purified (18.2 MΩ cm) with a Milli-Q-system of Millipore (Bedford, MA, USA), UV-1800 spectrophotometer (Shimadzu), FT-IR Spectrometer 1600 (Boardman, OH,USA), pH meter Cyberscan 510 (Singapore), LC-MS-8030 (Shimadzu Corporation, Kyoto, Japan) fitted with an ESI-MS (triple quadrupole) lab solution software, ^{1}H NMR spectra were recorded on 400 MHz (JEOL Inc., Peabody, USA), ^{1}H NMR spectra were recorded on a Bruker 500 MHz instrument using $CDCl_3$ as solvent, elemental analyzer (Vario EL III, Hanau, Germany), and Polarimeter (P3001RS Krüss 140, Hamburg, Germany).

III. Preparation of stock solutions

- (*RS*)-Etd (10 mM) was prepared by dissolving calculated amount in DCM.
- Solutions of all CDRs (20 mM) were prepared in DCM.
- Racemic β-blockers (10 mM) in MeCN.
- Npx anhydride, the CDR, (15 mM) in MeCN.
- Triethylammonium phosphate (TEAP) buffer solution by dissolving triethylamine (10 mM) in purified water; pH was adjusted to 3.5 by adding phosphoric acid.
- 0.1% TFA in purified water.
- Acetate buffer (0.05 M, pH 4.0) in purified water.
- Borate buffer (0.2 M, pH 9.5) in purified water.
- Solutions of 2,4-Dinitrophenyl hydrazone of each of the six carbonyl compounds were prepared in 2-propanol at a concentration of 10 mM and then diluted to a final concentration of 0.1 mM.
- Citrate phosphate buffer was prepared using 0.1 M solution of citric acid and 0.2 M solution of dibasic sodium phosphate.

- Solution of Cu (II) acetate (10 mM) and chiral ligands (20 mM) such as L-proline, L-phenylalanine, L-histidine, and L-tryptophan were prepared in water-MeOH (95:5).

IV. Extraction of active pharmaceutical ingredients from commercial tablets

A. (*RS*)-Etd: Etova tablets (5 numbers, each containing 400 mg of (*RS*)-Etd were ground to a fine powder. The powder was extracted with MeOH (20 mL) by sonication for 15 to 20 min. The solution was centrifuged; the residue was further treated with MeOH and centrifuged. The combined extract was concentrated in vacuum and left to cool until crystals appeared. The mother liquor was decanted and the crystals were dried. The sample was further purified by recrystallization with MeOH. Melting point, yield, λ_{max}, specific rotation and IR spectra (in KBr) of the purified compound were recorded. It was used as a racemic standard.

B. β-Blockers: Ten tablets of (*RS*)-Mel (each labelled to contain 100 mg) was finely pulverized, and was extracted with 100 mL MeOH at 25 °C using sonicator for 15 min. The solution was filtered through Whatman filter paper grade number-1 (particle retention 11 μm) and the residue was further treated thrice with MeOH and filtered. The combined filtrate was concentrated in vacuum and water was added drop by drop till turbidity appeared and the solution was then left for crystals to grow. The mother liquor was decanted and the crystals were washed with diethyl ether and dried in air. The same procedure was repeated for extraction, isolation and purification of (*RS*)-Cal from its tablets.

V. TLC

TLC plates (10 × 5 cm with 0.5 mm thickness) were prepared by spreading the slurry of silica gel G (25 g in 50 mL water) by a Stahl type applicator. The plates were activated by keeping overnight in oven (at 60 ± 2 °C). Besides, plain plates were prepared by adjusting the slurry at pH 5, 8 and 9 by using a few drops of acetic acid or ammonia.

Development of chromatograms

Solution of analyte was spotted on the TLC plates with the help of 25 µL Hamilton syringe. The clean, dry, and paper-lined rectangular glass chamber was pre-equilibrated for 15 min at each temperature fixed for chromatographic development inside an incubator and allowed to reach the specific temperature before development, for each experiment. The chromatograms were developed for 25-30 min. The spots were located using iodine vapours. Experimental conditions are described in respective chapters.

VI. HPLC

For optimization of separation conditions the present work required a lot of experimental work using HPLC. Details are described in respective chapters.

VII. Method Validation

The method was validated in terms of linearity, accuracy, precisions, limit of detection (LOD), and limit of quantitation (LOQ) according to ICH guidelines [41]. Calibration graphs [peak area (y) vs concentration of diastereomer (x)] were ploted and linear regression equations were used to determine slopes and correlation coefficients. The LOD and LOQ were taken as concentration of the analytes where signal-to-noise ratio (S/N) of 3 and 10 (n=5), respectively. The precision and accuracy of the method were determined at three concentration levels (low, medium and high), in five replicates on consecutive days (inter-day), and on the same day (intra-day).

Chapter-3

Enantioseparation of (*RS*)-Etodolac using enantiomerically pure amines as CDRs by HPLC and LC-MS

I. Introduction to Etdolac and Literature Survey on its Enantioseparation

The systematic chemical name of Etodolac is (*RS*)-1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-*b*]-indole-1-acetic acid; **Fig. 3.1**. It is a non-steroidal anti-inflammatory drug (NSAID) and is marketed and administered as racemate and used as analgesic and for the treatment of rheumatoid arthritis and osteoarthritis; it produces less gastrointestinal toxicity compared to other NSAIDs [42]. The biochemical and pharmacological effects reside in the (*S*)-isomer, primarily owing to its high receptor affinity and better stereospecificity as compared to the (*R*)-isomer [43, 44].

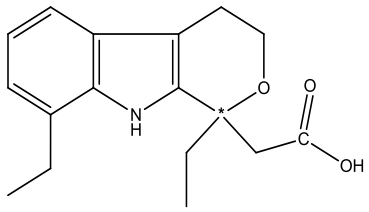


Fig. 3.1 Structure of (*RS)*-Etd

Determination of enantiomeric ratio of Etd was achieved using different protein-bonded chiral stationary phases and the enantiomers of Etd were also resolved by fractional crystallization of its diastereomeric salts prepared with optically active 1-phenylethylamine [45]. Direct separation of enantiomers of (*RS*)-Etd has been accomplished using chiral stationary phase consisting of cellulose [46]; (*R*)-DNBPG [47]; (*R*,*R*)-N-3,5-dinitrobenzoyl-l,2-diphenyl-ethane-1,2-diamine, [(*R*,*R*)-DNB-DPEDA], and tartardiamide-DMB (CHI-DMB) [48]; CHI-DMB is based on tartaric acid derivatives which are polymerized, cross linked, and covalently bound to a functionalized Kromasil® silica. Hewala et. *al*. [49] developed and validated an enantioselective HPLC method with diode array detection for determination of Etd enantiomers in tablets and human plasma using a Kromasil® CelluCoat® chiral column

(consisting of cellulose derivative for enantioselectivity). Nevertheless, chiral HPLC columns, for direct analysis, are found to have high cost and relatively poor selectivity [**50]**.

Application of (*S*)-(–)-α-(1-naphthyl)ethylamine (NEA) as a chiral derivatizing agent (CDR) is the only available report on enantioseparation of (*RS*)-Etd by indirect approach as the amino group of the reagent reacted with the carboxylic group of Etd and the diastereoisomeric amides were resolved on C_{18} column with detection at 278 nm [51].

Literature reveals that enantiomerically pure amines, viz., (–)-*cis*-myrtanylamine, (+)-dehydroabietylamine, (*R*)-(–)-1-cyclohexylethylamine, and (*S*)-(–)-α,4-dimethylbenzylamine were incorporated as chiral auxiliaries in (DFDNB) moiety to prepare chiral variants of Marfey's reagent which were used as CDRs for enantioseparation of (*RS*)-mexilitine [52]. Besides, several amine moieties have been incorporated in molecular structure of DFDNB acting as powerful CDRs [32-34].

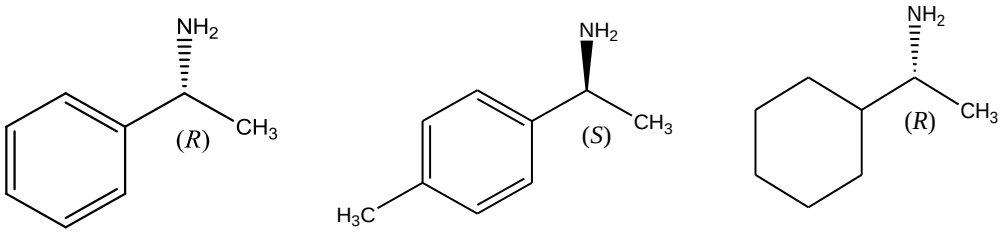
Each amine acted as a nucleophile and substituted one of the F atoms of DFDNB and got bonded to phenyl ring via their amino nitrogen-thus the primary amino group was no more available. The remaining F in these CDRs undergo nucleophilic substitution to react quantitatively with (racemic) analyte containing primary or secondary amino group and yield a pair of diastereomers which absorb at 340 nm; these DFDNB based CDRs have limitations that they are light sensitive and are applicable to analytes containing amino group.

II. Present work

The importance and potential of indirect approach for enantioseparation, along with the selection of CDRs, has been described in Chapter-1. Besides, the present work and its result lead to overcome the above mentioned limitations shown by the literature search on enantioseparation of (*RS*)-Etd.

Accordingly, (*R*)-(+)-α-methyl benzyl amine, (*S)*-(–)-α,4-dimethylbenzylamine, (*R*)-(–)-1-cyclohexylethylamine (structures are shown in **Fig. 3.2)** were chosen as CDRs, and numbered as 1, 2 and 3, respectively. The chosen CDRs are enantiomerically pure amines which have an appropriate functional group to react with the carboxyl group of the analyte.

Most of the methods reported on enantioseparation are limited to formation of diastereomers followed by their LC separation while the present method is an improvement over such existing reports because formation of diastereomers (and their structures), so separated by RPHPLC, was confirmed using LC-MS when $[M+H]^+$ or $[M]^+$ were recorded for the diastereomers. Additionally, 3D-geometry of the diastereomers was confirmed by developing lowest energy optimized structures using the Gaussian 09 Rev. A.02 program and hybrid density functional B3LYP with 6-31G basis set. Separation of three pairs of diastereomers was achieved using C_{18} column and UV detection at 223 nm; HPLC conditions were optimized with respect to mobile phase and flow rate, for separation. The separation method was validated for linearity, limit of detection and limit of quantitation.



CDR1 CDR2 CDR3

Fig. 3.2 Structures of CDRs:
CDR1 = (*R*)-(+)-α-methyl benzyl amine;
CDR2=(*S*)-(−)-α,4-dimethylbenzylamine;
CDR3=(*R*)-(−)-1-cyclohexylethylamine

1. Experimental

The details of chemicals, reagents, solvents and the equipment used have been described in **Chapter-2**. Extraction, isolation and purification of the active pharmaceutical ingredient (Etd in the present case) have also been given in Chapter-2.

Synthesis of Diastereomers

The physical characteristics of the optically pure amines used as CDRs (**Fig. 3.2)** are given below:

CDR1: (*R*)-(+)-α-methyl benzyl amine, (*ee,* 99.0%, $[\alpha]_D^{25}$ = (+)30±1°, c = 10% in ethanol); CDR2: (*S)*-(−)-α,4-dimethylbenzylamine (*ee*, 98.5%, b.p., 205 °C, $[\alpha]_D^{25}$ = (−)37°, neat); CDR3: (*R*)-(−)-1-cyclohexylethylamine (*ee*, 98.0 %), b.p. 177-178 °C, $[\alpha]_D^{25}$ = (−)4°, neat). Considering enantiomeric purity of the chiral amines above 99%, these were used for diastereomeric synthesis.

Three pairs of diastereomers were synthesized using the above mentioned enantiomerically pure amines as CDRs as per following procedure, representative for CDR1, 100 µL of HOBT (1.53 mg; 10 mM) in 1mL DCM, 100 µL of DCC (2.06 mg; 10 mM) in 1 mL DCM, 60 µL of DMAP and 100 µL of CDR1 (2.42 mg; 20 mM) in 1 mL DCM) were added to 100 µL of Etd (2.8 mg; 10 mM) in 1 mL DCM). The reaction mixture was stirred at room temperature (30 °C) for 2 h.

Separate sets of reaction mixture were subjected to MWI for 80, 100, 120, 130, 140, 150 and 160 s at 80% power of 800W. The reaction was monitored to check the completion of synthesis by injecting the reaction mixture for HPLC analysis for each set of synthesis after each irradiation time.

The two approaches of synthesis were applied in the similar manner for synthesis of diastereomers using CDR2 and CDR3. The corresponding diastereomers were preserved under refrigerated condition (3-5 °C).

HPLC

Aliquots from the solutions of the three diastereomeric mixtures were diluted 10 times with MeOH and 20 µL of one of the diastereomeric mixtures was injected onto the column (as one set of experiment independently). Following mobile phases were used,

(i), MeOH-TEAP buffer;

(ii), MeCN-TEAP buffer;

(iii), MeCN-TFA;

(iv), MeOH-TFA

Each mobile phase was used in isocratic mode with the ratio of, 70:30, 80:20, and 90:10.

HPLC conditions were optimized for separation of all the three pairs of diastereomers by using TEAP buffer of three varying concentrations, i.e., 5, 10 and 15 mM; for each molarity, pH was adjusted to 3.5, 4.5 and 5.5, by addition of phosphoric acid. Thus, there were nine combinations of each of the mobile phase (i) and (ii). For the mobile phase (iii) and (iv) three concentrations of TFA (0.05, 0.1, and 0.15 %) were tried.

A flow rate of 0.5, 1.0 and 1.5 mL/min of each of these mobile phases was applied. Mobile phase was filtered through a 0.45 mm filter and degassed by sonication and passing nitrogen before use. Detection was at 223 nm because it is the λ_{max} of Etd.

LC-MS Analysis

Mobile phase consisted of MeCN-ammonium formate buffer (10 mM) pH 3.7, (70:30, *v/v*). Other conditions for sample analysis were, flow rate: 0.75 mL /min; temperature of column oven: 40 ºC; nebulizing gas flow: 3L /min; drying gas flow: 10 L /min, and heat block temperature: 400 ºC; C_{18} column. The diastereomers of Etd formed with CDR1, exhibited ion peaks at m/z 391.15 ($[M+H]^+$), m/z 172.1, m/z 100.1, m/z 59. The ion peaks exhibited by the diastereomer formed with and CDR3 are, m/z 397.25 ($[M+H]^+$), m/z 100.1, m/z 59.0. The diastereomer prepared with CDR2 gave $[M]^+$ at m/z 404.35, m/z 100.1, and m/z 59.0.

Method Validation

Method validation was done according to ICH guidelines (1996) [**41**] using diastereomers of (*RS*)-Etd prepared with all the three CDRs; however, details and data is presented for the diastereomers prepared with CDR1, as a representative. Slopes and regression equations were determined by drawing linearity plots for peak area vs concentration and using linear regression equations. Recovery studies were carried out by derivatizing standard solutions of different known concentrations and mean recovered values (four replicate runs) were represented as a percentage of calculated values. To determine precision, inter-day and intra-day stability studies were carried out and the results are represented as relative standard deviation (RSD). Limit of detection (LOD) and limit of quantification (LOQ) were also evaluated.

For the studies of stabilities and recoveries, peak areas (as provided by the system software) were used for quantitation referring to the calibration curve.

2. Results and Discussion

(*RS*)-Etd was isolated from commercial preparations available in the market using MeOH. It was purified by recrystallization. Melting point (145-148 °C), and λ_{max} (223 nm) value were in agreement with the reported values. Characteristic IR (KBr) peaks correspond to: 1705(-C=O), 1593, 1570 (aromatic C=C), 3469 (-NH) cm^{-1}. Recovery of the sample was 92% against the labelled amount on commercial tablets. Since the efforts have been made to draw attention on the aspect that the majority of chiral drugs (Etd in present case) are marketed and administered as racemic mixtures, it was considered worthwhile to examine the commercial sample. Moreover, the sample supplied by the standard 'reagent suppliers' are extremely expensive.

Diastereomers

The enantiomerically pure amines have directly been used as CDRs in the present study. These are capable of providing stable derivatives with enhanced detectability and quantitative yield of the reaction [52]. The amino group of the CDR reacts with the carboxylic group of (*RS*)-Etd to form a pair of diastereoisomeric amides. A representative reaction of Etd with CDR1 is shown in (**Fig. 3.3)**. Designating the first letter to the configuration of Etd and second to that of the chiral auxiliary in the diastereomers, the diastereomers are represented as (*R*,*S*)-, and (*S*,*S*)-diastereomers when the chiral auxiliary is (*S*)-(−)-α,4-dimethyl benzyl amine (CDR2), while the diastereomers would be represented as (*R*,*R*)-, and (*S*,*R*)-diastereomers when the chiral auxiliary is (*R*)-(+)-α-methyl benzyl amine (CDR1) or (*R*)-(−)-1-cyclohexyl ethyl amine (CDR3).

The two enantiomers in the (*RS*)-mixture may react at different rates with the CDR since CDR is a chiral molecule and the rate of reaction of enantiomers with chiral molecules is generally different. Therefore, in order to compensate for the reagent consumed by one of the enantiomers, due to its faster rate, a large excess of CDR was used to force the reaction to completion and method optimization was carried out as described below. All the three CDRs were used in 1-3 fold molar ratio to find the optimum reagent concentration for derivatization of (*RS*)-Etd, i.e. ratio of 1:1, 1:1.5, 1:2, 1:2.5 and 1:3 (Etd: CDR) were tried. A slight kinetic resolution was observed at a

ratio of 1:1.5 (Etd: CDR) due to different reaction rates of enantiomers. The derivatization was complete when 2 fold molar excess was used. An
increase in reagent ratio had very little effect on derivatization with respect to reaction time and yield.

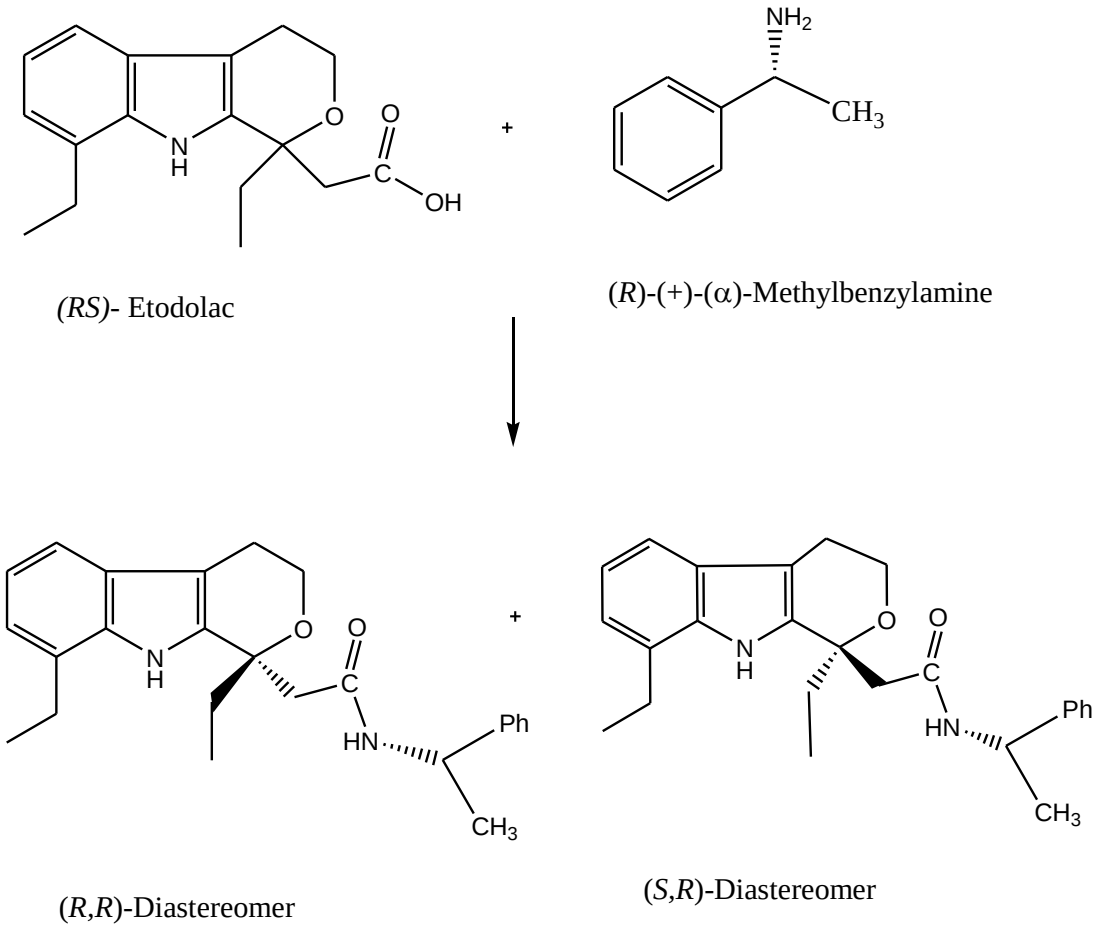


Fig. 3.3 Synthesis of diastereomers of (*RS*)-Etd using CDR1

Thus, the optimized conditions for derivatization were two fold molar excess of CDRs under conditions of stirring at room temperature (30 °C for 2 h) or microwave irradiation; it was found that the reaction with CDR1 and CDR2 was completed in 120 sec and reaction with CDR3 was completed in 150 sec (at 80% of 800W) because no

change in peak areas was observed for the reaction after the said duration of irradiation. Effect of MWI time on completion of derivatization reaction is shown in (**Fig. 3.4).**

In the indirect approach, the analyte must contain a suitable reactive group (preferably only one function in close proximity to the stereogenic center) which is prone to a quantitative transformation with the CDR (or the chiral auxiliary introduced in a chromophoric moiety) and no side-products should be formed. This condition is met with in the present work. Besides, there must be no racemization of the sample and of the CDR in the reaction which transforms the mixture of enantiomers into diastereomers. Since the derivatization reaction with these chiral amines (used as CDRs) did not involve a direct attack on stereogenic center no racemization of the sample and of the chiral auxiliary compound was detected.

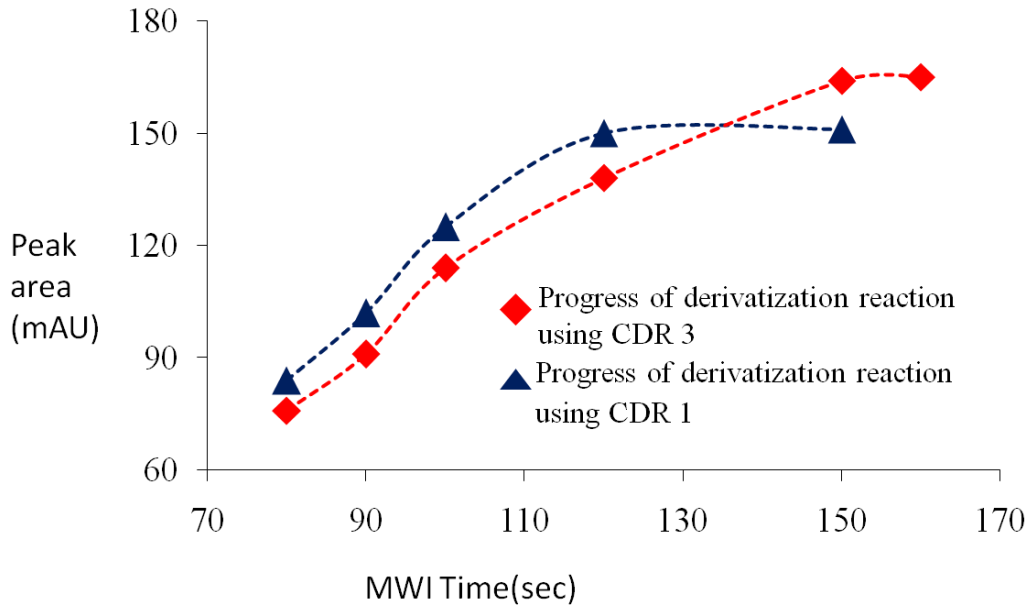


Fig. 3.4 Effect of MWI time on completion of derivatization reaction

CDR1 has *ee*, 99.0%, CDR2 has *ee* of 98.5%, and CDR3 has *ee*, 98.0%; therefore, these were taken/considered as enantiomerically pure and were used as such for chiral

derivatization. Under the optimized separation conditions of HPLC, each diastereomeric mixture showed only two peaks corresponding to two expected diastereomers; this in itself is a confirmation of the enantiomeric purity of the CDR. Moreover, it can be inferred that the impurity with respect to the presence of other enantiomer in the three CDRs is too small to initiate any derivatization reaction with (*RS*)-Etd.

Separation by HPLC

The resolution (R_S), retention factor (k), and separation factor (α) for diastereomers of (*RS*)-Etd separated under optimized condition of HPLC are given in **Table 3.1**. The three pairs of diastereomers were well separated on C_{18} column. Sections of chromatograms showing resolution of three pairs of diastereomers of (*RS*)-Etd are shown in (**Fig. 3.5**). The integration data for % peak area for the separation of each pair of diastereomers, as recorded by the system software, verifies that the two diastereomers were in the ratio of 1:1. Replicate HPLC analysis (n=4) was carried out (as mentioned under 'Accuracy and Precision') and for each analysis $\boldsymbol{R_S}$ values (as provided by the system software with the chromatograms) were found to be the same (**Table 3.1**). 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; $k_1 = t_1\text{-}t_0 / t_0$. The void volume peak or Column Dead Volume or Time (t_0,"Tee Zero"), sometimes called the solvent front, is a peak which 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. In present case void volume peak was observed at around 2.5 min; methanol and TEAP buffer were used as solvent. It was also confirmed by injecting the mobile phase only (without sample) under experimental conditions. HPLC conditions were optimized for separation of all the three pairs of diastereomers by varying the concentration of TEAP buffer, pH and flow rate as described under 'HPLC' above. With the presence of trifluoroacetic acid in mobile phase, decrease in resolution was observed in comparison to the use of TEAP buffer. The best resolution was observed using mobile phase (**i**) consisting of binary mixture of MeOH and TEAP buffer of pH 4.5 (80:20 *v/v*) in

isocratic mode at a flow rate of 1.0 mL/min and detection at 223 nm. Mobile phase containing MeOH provided sharper peaks, enhanced resolution and shorter retention time under the same HPLC conditions when mobile phase contained MeCN.

Among all the 3 pairs of diastereomers the highest and the lowest $\boldsymbol{R_S}$ was observed for the separation of diastereomeric pair prepared with CDR3 and CDR1, respectively. Thus, the diastereomers prepared with CDR3 were better resolved than the diastereomers prepared with other two chiral amines. The CDRs can be arranged as 3>2>1 for the decreasing order of $\boldsymbol{R_S}$ obtained for the corresponding diastereomeric pair.

Confirmation of diastereomers by LC-MS

In this study, using LC-MS we confirmed the formation of diastereomers (product) formed by the reaction of (*RS*)-Etd with three different CDRs. The ESI source was operated in positive mode. Full scan product ion mass spectra were obtained by direct infusion of samples, diluted in mobile phase, into the mass spectrometer. The diastereomers of Etd formed by CDR1, and CDR3 exhibited mass ion peak $[M+H]^+$ at m/z 391.15, and m/z 397.25, respectively, while the $[M]^+$ at m/z 404.35 was obtained for the diastereomer prepared with CDR2. Full scan product ion mass spectra of all the three diastereomers are displayed in (**Fig. 3.6 a, b, and c**). MS fragmentations of diastereomers (as per m/z and relative intensities of the fragments) are given in **Table 3.2.**

Yields and Stability of Diastereomers

The recovery studies of all the three diastereomers (as described in "Accuracy and Precision"), served as a measure of their yields since recovery of all eluted diastereomers was found to be more than 96.1%.

Stability of the diastereomers was investigated after long-term (refrigerated at a temperature of 3-5 °C) and short-term (room temperature) storage. The stability was also evaluated for the analyte in stock solution and experimental situations of actual sample handling and analysis. Based on HPLC experiments carried out at an interval of 10 days up to 100 days from the day of synthesis the diastereomers were found to be stable for 90 days under the refrigeration conditions (3-5 ^{o}C).

Table 3.1: HPLC separation data for diastereomeric derivatives of (*RS*)-Etd prepared with different CDRs

diastereomeric derivatives prepared with	Characteristics of the separated diastereomeric derivatives			
	k_1	k_2	α	R_S
CDR 1	1.80	1.88	1.04	1.95
CDR 2	5.00	4.56	1.09	2.12
CDR 3	2.70	2.99	1.10	2.19

C_{18} column; mobile phase: binary mixture of MeOH and TEAP buffer of pH 4.5 (80:20 *v/v*) in isocratic mode at a flow rate of 1.0 mL/min and detection at 223 nm. k_1= retention factor of first eluted diastereomer; k_2= retention factor of second eluted diastereomer; α = separation factor; R_S = resolution.

Separation Mechanism

Optimized structures of two diastereomers were drawn using the Gaussian 09 Rev. A.02 program and hybrid density functional B3LYP with 6-31G basis set (**Fig. 3.7 a and b).** In Fig. 3.7, in (*S,R*)-diastereomer, the hydrophobic group $–CH_3$ and –Ph on the stereogenic center of CDR (C1 in **Fig. 3.7a**) and $–C_2H_5$ on stereogenic center of Etd (C2 in Fig. 3.7a), are oriented on the same side with respect to the amide bond and are considered to be *cis* to each other. The *cis*-type arrangement arises since there is a partial double bond character in the amide bond with restricted rotation around C–N. On the other hand, in the (*R,R*)-diastereomer, the $-CH_3$,-Ph and $–C_2H_5$ groups are oriented in space below and above the amide bond and thus have a *trans*-type arrangement (Fig. 3.7b). The *cis*-, or *trans*- type arrangements of the two diastereomers are responsible for difference in their hydrophobicities. It can be stated that the (*S,R*)-diastereomers (having *cis* type orientation) interact more strongly with the reversed phase material of the column and are retained for a longer time in comparison to the (*R,R*)-diastereomers. Thus, it can be inferred that the hydrophobic interaction of the two diastereomers with the reversed phase material of the column along with the influence of rheological properties of the mobile phase are responsible for their different partition coefficients

and different retention times and for these different physical properties they elute one after another.

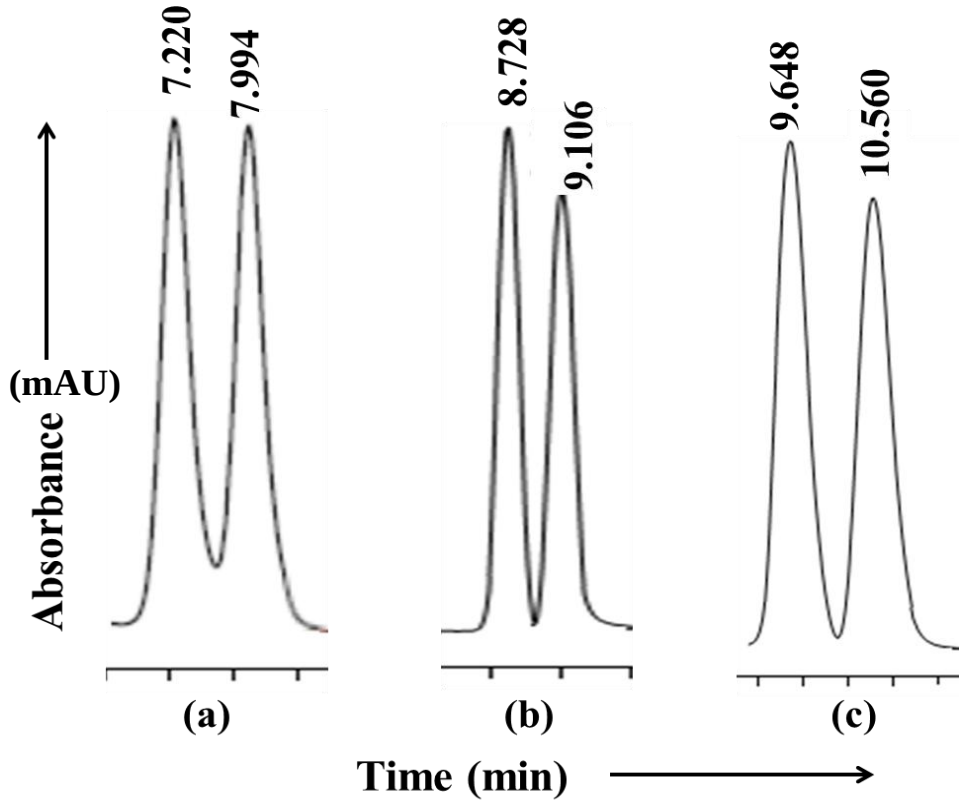
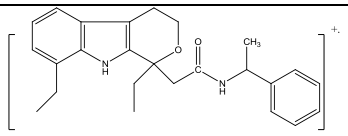
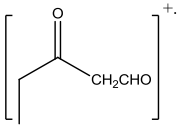
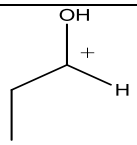
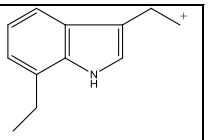
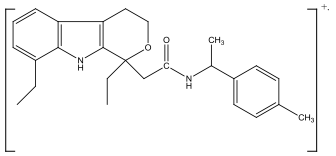
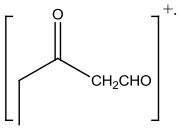
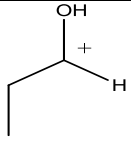
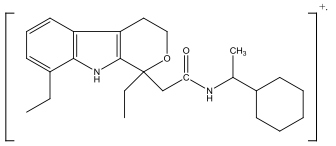
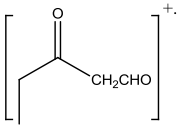
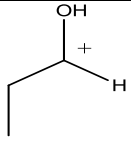


Fig 3.5 Sections of Chromatograms showing separation of diastereomers of (*RS*)-Etd prepared with CDR1 (a); CDR 2 (b); CDR 3 (c). Mobile phase, binary mixture of MeOH and TEAP buffer pH 4.5 (80:20 *v/v*) in isocratic mode at a flow rate of 1.0 mL/min; Column, Waters Spherisorb C_{18} (L x I.D., 25 cm × 4.6 mm, 5 µm particle size).

Table 3.2: MS fragmentation of diastereomers (as per m/z and relative intensities of the fragments)

Diastereomer prepared with CDR 1	 $[M+H]^+$ m/z =391.1 (98%)	 m/z =100.1 (100%)	 m/z =59.0 (88%)	 m/z =172.1 (12%)
Diastereomer prepared with CDR 2	 $[M]^+$ m/z =404.3 (11%)	 m/z =100.1 (100%)	 m/z =59.0 (95%)	
Diastereomer prepared with CDR 3	 $[M+H]^+$ m/z =397.2 (86%)	 m/z =100.1 (100%)	 m/z =59.0 (97%)	

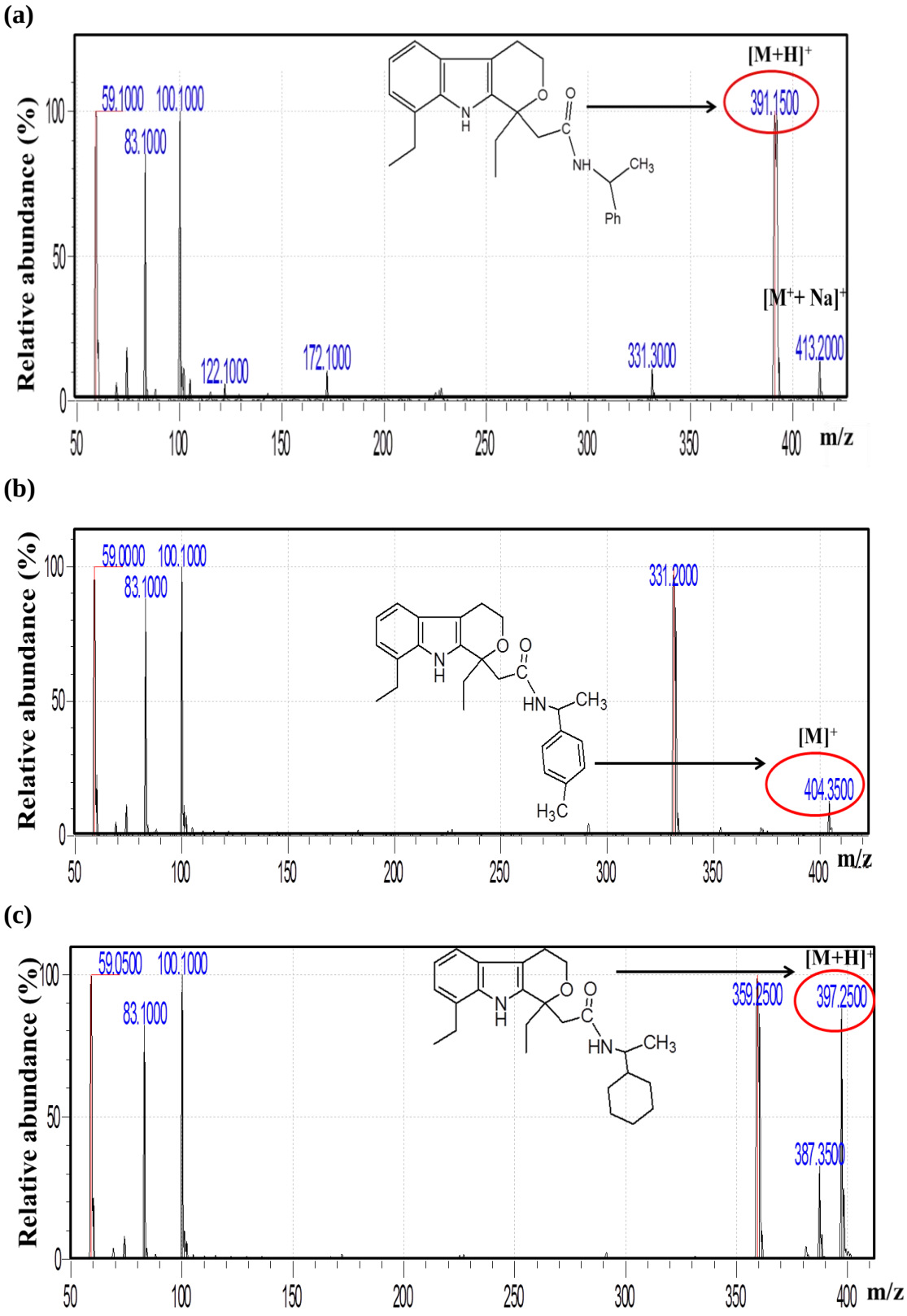


Fig. 3.6 Mass spectra of the diasteromers formed by the reaction of (*RS*)-Etd with (a) CDR1, (b) CDR2 and (c) CDR3

Comparison with literature reports

Hewala et. *al*., [49] using Kromasil® CelluCoat® chiral column reported that resolution between the two enantiomers was 4.25 with LOQ of (*R*)-Etd and (*S*)-Etd being 0.20 and 0.19 μg/mL, respectively. LOQ by HPLC (Zorbax C_{18} column) was 0.5 μg/mL for the diastereomers of (*RS*)-Etd prepared with (*S*)-(−)-α-(1-naphthyl)ethylamine, (*S*-NEA), [51].

In the present studies the resolution ranges between 1.05 and 2.19 for the diastereomeric pairs prepared with different CDRs along with LOQ of 0.024 μg/mLfor each of the diastereomers prepared with CDR1; thus LOQ is much lower than that obtained by direct approach using chiral column as well as in comparison to the LOQ obtained by using (*S*)-NEA) as CDR.

Method Validation

All validation procedures were performed according to ICH guidelines (ICH, 1996) for the diastereomers of (*RS*)-Etd prepared with all the CDRs; however, details and data is presented for the diastereomers of (*RS*)-Etd prepared with CDR1, as a representative. The experimental method was validated with respect to linearity, accuracy and precision and the data have been compiled in **Table 3.3**.

Linearity

A range of 4-10 μg/mL was chosen for drawing linearity plot (peak area vs. concentration of diastereomer, μg/mL) and the slope and intercept were determined using linear regression equations. 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 (less than 1.5%). The regression equations were, $y = 107.6x+461.9$ ($r^2 = 0.9964$, ± SD value for intercept and slope are 0.052 and 0.034) and $y = 105.3x+431.6$ ($r^2=0.9962$, ± SD value for intercept and slope are 0.024 and 0.067) for the first and second eluted diastereomer, respectively.

Accuracy and precision

The accuracy and precision studies were carried out by replicate HPLC analysis (n=4) of each of the four standard solutions (4, 6, 8, 10 μg/mL) of mixture of diastereomers of

(*RS*)-Etd, prepared with all the three CDRs, while the data corresponding to diastereomers prepared with CDR1 is given, as a representative; for each concentration the reproducibility of retention time was up to third place of decimal. On the basis of peak areas of first and second eluting diastereomer the recovery and RSD for each of the diastereomers were calculated. These are shown in **Table 3.3**. The calculated RSD for intra-day assay precision varied from 0.98 to 1.51% and from 0.94 to 1.86% for the first and second diastereomers, respectively; for inter-day assay precision varied from 0.96 to 1.38% and from 0.89 to 1.96% for the first and second diastereomer, respectively. The calculated recovery for the first and second eluting diastereomer, respectively varied from 97.6 to 102.5% and from 97.2 to102.0 % for intra-day assay and from 96.5 to 100.8 % and from 96.0 to 100.2% for inter day assay (**Table 3.3**).

Limit of detection and limit of quantitations

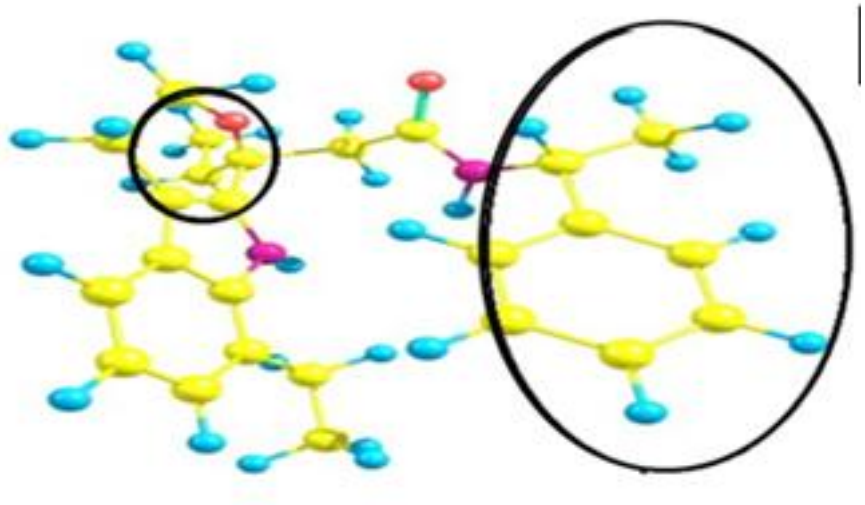
LOD was found to be 0.008 µg/mL and LOQ was found to be 0.024 µg/mL for each of the diastereomers prepared with CDR1.

The LOD and LOQ of both the diastereomers were determined by signal to-noise (S/N) ratio method by injecting a series of diluted solutions of mixtures of diastereomers. For LOD and LOQ the S/N ratios were 3:1 and 10:1, respectively.

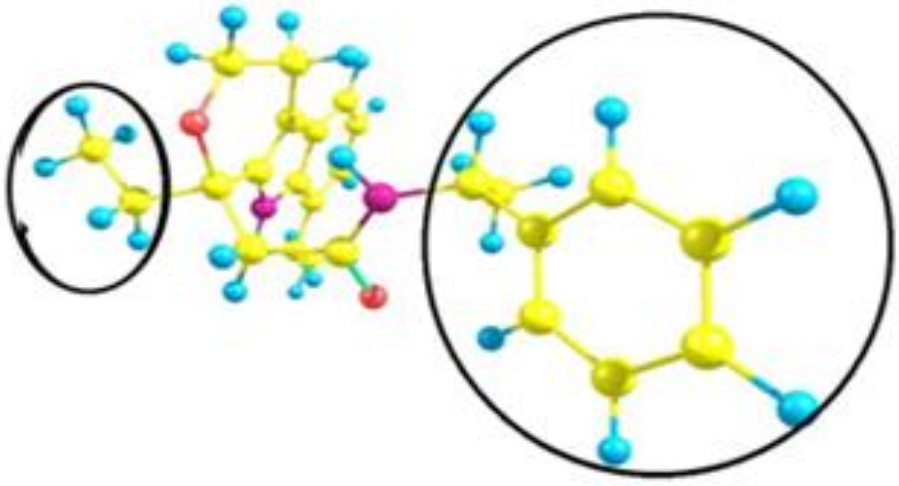
III. Conclusion

The optimized experimental conditions for synthesis and analytical separation of diastereomers are supported by confirming the formation and structures of diastereomers via recording full scan product ion mass spectra using LC-MS and by developing the lowest energy optimized configurations of the two diastereomers which confirmed 3D geometry of the diastereomers. The computational analysis can be of aid for hypothesizing and rationalizing the elution order. Since the structures and formation of diastereomers are established and the method is optimised and validated the RPHPLC separation conditions can be successfully applied for determination and control of enantiomeric purity of Etd routinely in industries and R&D laboratories (even without resorting to LC-MS each time). Further advantages of the method are (a) the diastereomers are synthesized in a simple straight forward one step using enantiomerically pure amines as CDRs, (b) the CDRs and the diastereomers are not

light sensitive, (c) improved LOQ (0.024 µg/mL) in comparison to any literature report (including the one by direct approach too) is obtained, and (d) no racemization is observed while the kinetic resolution is overcome by taking excess of CDR for derivatization, under optimized conditions.



(*S*,*R*)-diastereomer (a)



(*R*,*R*)-diastereomer (b)

Fig. 3.7 Optimized structures of diastereomers of (*RS*)-etodolac drawn using the program Gaussian 09 Rev. A.02 and hybrid density functional B3LYP with 6-31G basis set. (a), the (*S*,*R*)-diastereomer (b), the (*R*,*R*)-diastereomer

Table 3.3: Summary of validation data obtained for separation of diastereomers of (*RS*)-Etd prepared with CDR 1

Linearity		first eluting diastereomer			second eluting diastereomer	
Range		4-10µg mL^{-1}			4-10µg mL^{-1}	
Slope		107.6			105.3	
Intercept		461.9			431.6	
Correlation coefficient (r^2)		0.9964			0.9962	
Accuracy and precision						
Concentration µg/mL (actual)	Mean±SD	RSD (%)	Recovery (%)	Mean±SD	RSD (%)	Recovery (%)
Intra-day precision(n=4)						
4	2.05±0.025	1.24	102.5	2.04±0.019	0.94	102.0
6	2.97±0.034	1.14	99.0	2.93±0.045	1.56	97.66
8	3.90±0.059	1.51	97.62	3.89±0.057	1.47	97.2
10	5.07±0.050	0.98	101.5	5.07±0.094	1.86	101.5
Inter day precision(n=4)						
4	2.01±0.027	1.38	100.5	2.02±0.017	0.89	101.1
6	2.93±0.039	1.36	98.0	2.91±0.035	1.21	97.0
8	3.86±0.037	0.96	96.5	3.84±0.075	1.96	96.1
10	5.04±0.055	1.10	100.8	5.01±0.059	1.18	100.2

The concentration values listed in columns 2 and 5 refer to the racemate.

Chapter- 4

Synthesis of (*S*)-Naproxen based new CDR and enantioseparation of some β-blockers as their diastereomeric amide

I. Introduction to the Analytes and Literature Survey on their Enantioseparation

Beta blockers are synthetic chiral hydroxyl amine-containing compounds used clinically to treat cardiovascular diseases such as hypertension, coronary heart disease, arrhythmias, sinus tachycardia and myocardial infarction, where they act preferentially upon the β-adrenergic receptors in heart [53]. They cause fewer bronchospastic reactions and significantly fewer side effects on central nervous system (e.g., depression and nightmares). The sales of antiarrhythmic drugs increased from U.S. $1.8 billion in1999 to U.S. $2.6 billion in 2009; it is anticipated to reach U.S. $3.5 billion in 2015 (*The Worldwide Market for Prescription Cardiovascular Drugs Reports*" from *Kalorama* Information [54]). So far, most of the β-blockers are being used as a racemic mixture despite the fact that pharmacological action is largely confined to (*S*)-(−)-enantiomers showing about 50–500 fold higher activities [55]. The bioavailability of both (*S*)-(−)-propranolol and (*S*)-(−)-metoprolol, in humans, exceeds that of the (*R*)-isomer. (*S*)-(−)-Propranolol is important in therapy against oxidative stress and its beta blocking potency is *ca*. 40 times greater than that of the (*R*)-(+)-enantiomer; metoprolol has been shown to protect red blood cells against phenazine methosulfate (PMS)-induced toxicity. Thus, there is a need to review current prescription of racemic β-adrenergic blockers from the clinical, medical and health point of views.

CDRs for β-blockers: There have been a few reports from this laboratory [56-58] on successful enantioseparation of certain β-blockers as their diastereomers prepared with newly synthesized CDRs based on difluorodinitrobenzene (DFDNB) and cyanuric chloride (CC) moieties using RPHPLC. These moieties added a very strong chromophore for UV absorption and led to sensitive detection of the derivative. The diastereomers prepared with DFDNB based CDRs had a very similar absorption spectrum characterized by a λ_{max} at 340 nm; the spectrum are stable if the solutions are kept in dark, otherwise, a gradual change occurs as a result of a photochemical decomposition of the absorbing chromophore (for DFDNB based derivatives). Many

other reagents used for pre-derivatization suffer inherent problems, *e.g.*, unstable derivatives, poor detection of certain derivatives, or lack of quantitative yield of the reaction.

Chiral separation of β-adrenergic antagonists by TLC using both direct and indirect modes has been critically reviewed [59]. CDRs, such as, (–)-menthyl chloroformate [60]; (+)-1-(9-fluorenyl) ethyl chloroformate [61]; (1*R*,2*R*)-1,3-diacetoxy-1-(4-nitrophenyl)-2-propyl isothiocynate [62]; *N*-trifluoroacetyl-l-prolyl chloride [63]; optically active anhydrides derived from (*R*,*R*)- and (*S*,*S*)-tartaric acid such as, *O*,*O*-dibenzoyl derivative [64,65]; *O*,*O*-diacetyl derivative [66] and *O*,*O*-di-*p*-toluoyl derivative [67] have been used for hplc separation of diastereomers of pharmaceutically active compounds containing amino or hydroxyl group, e.g., atenolol and other β-blockers.

All the reports as cited above and the literature cited therein, show that there have been no attempts to verify the configuration of diastereomers so separated. In practice, most of the time diastereomer corresponding to pure enantiomer of the analyte is not available. Therefore, establishment of absolute configuration (or the molecular dissymmetry) of the diastereomers becomes desirable to ensure the success of diastereomeric synthesis and reliability of enantioseparation.

II. (*S*)-Naproxen for synthesis of new CDR for C-N bond formation

(*S*)-(+)-Naproxen (Npx) is a non-steroidal anti-inflammatory drug and is available as a pure enantiomer. Its systematic chemical name is, (*S*)-2-(6-methoxynaphthalen-2-yl)propanoic acid. The literature reveals synthesis of Npx based chiral derivatizing reagents (CDRs) and their application in enantioresolution of certain pharmaceuticals [68-70] the carboxylic group of Npx was activated by introducing different nucleophilic moieties. These served as good leaving groups in a subsequent nucleophilic substitution reaction when the said CDR was reacted with a racemic drug containing amino group. Corresponding diastereomers having amide bond were formed. The large conjugated naphthyl ring having high molar absorptivity (ε >100,000) facilitated detection of diastereomers.

From the literature cited above, and the literature cited therein, on synthesis and application of (*S*)-(+)-Npx based CDRs and considering the application and importance

of anhydride type of reagents, it occurred to us that a new CDR could be synthesized from (*S*)-(+)-Npx as its anhydride, due to the presence of carboxylic acid group in Npx, which could be used as a versatile reagent. The strategy proposed in the study regards the use of the symmetrical anhydride of Npx which is the real CDR used for the indirect enantioseparation of the group of selected β-blockers. (*S*)-(+)-Npx based CDRs have not been used for enantioseparation of β-blockers.

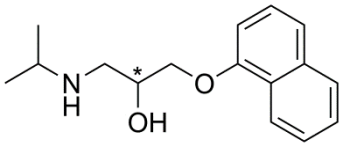
III. Present work: A new CDR was synthesized from (*S*)-(+)-Npx as its anhydride. It was used for synthesis of diastereomers of (*RS*)- (Prl), (*RS*)- (Atl), (*RS*)- (Cal), and (*RS*)- (Mel) (**Fig. 4.1**). The β-blockers were chosen for the present studies based on their high prescription rate and ease of availability and to test the efficiency of the new anhydride reagent for forming carbon-nitrogen bond appearing in the diastereomeric amide products. The diastereomers so synthesized (four pairs) were separated and isolated by open column chromatography. The isolated and purified diastereomers were used to (i) establish the configurations of the first and second eluting diastereomers (and thus the elution order) by recording their ^{1}H NMR spectra, and (ii) to correlate the configurations of the two diastereomric derivatives (so established by ^{1}HNMR) with the data obtained from the DFT based Gaussian software for the two structures developed for lowest energy and to compare the chromatographic separation conditions (and other characteristics of the two diastereomers) and to complete the validation studies for establishing linearity, limit of detection and limit of quantitation. The above mentioned aspects constitute the novelty of work.

1. Experimental

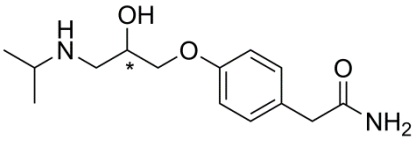
The details of chemicals, reagents, solvents and the equipment used have been described in **Chapter-2**.

Synthesis and characterization of CDR

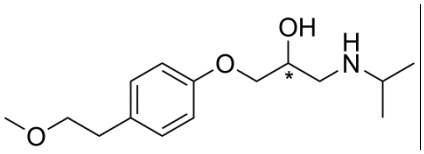
DCC (206 mg, 1 mmol) and DMAP (18.5 mg, 0.15 mmol) were added to a stirred solution of (*S*)-(+)-Npx (460 mg, 2 mmol) in 30mL of dry tetrahydrofuran (THF). The reaction mixture was stirred for 2 h at room temperature under nitrogen atmosphere. It



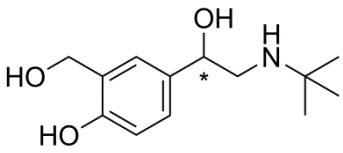
(a) (*RS)*-Prl



(b) (*RS)*-Atl



(c) (*RS)*-Cal



(d) (*RS)*-Mel

Fig. 4.1 Structures of β-blockers

was then filtered to remove the N,N'-dicyclohexylurea formed during the reaction and then the filtrate was washed five times with brine solution, five times with water, five times with $NaHCO_3$ and finally with 1 N HCl. It was then extracted with 50 mL of ethyl acetate. The ethyl acetate extract was dried over $MgSO_4$. The solvent was evaporated to dryness in vacuum to give symmetrical anhydride of (*S*)-(+)-Npx as white solid. It was recrystallized with MeOH. The CDR was stored in a tightly closed container at 4 °C. The characterization data are given below. Yield: 95%; mp: 115 °C; UV (λ_{max}, 230 nm, DCM); IR (KBr): 3413, 1692, 1633, 1617, 1556, 1331, 1241 cm^{-1}; ^1H NMR (500MHz, $CDCl_3$): d (ppm) 1.57–1.59 (d, 6H, CH_3); 3.90 (s, 6H, OCH_3); 7.10–7.14 (m, 4H, Ar); 7.40–7.14 (dd, 2H, Ar); 7.68–7.70 (d, 6H, Ar); anal. calcd for $(C_{14}H_{13}O_2)_2O$: C, 76.00%; H, 5.92%. Found: C, 75.88%; H, 5.75%. It was observed that in the absence of DMAP reaction took 4h for completion and in presence of DMAP the reaction was completed in 2 h.

Synthesis of diastereomers via conventional heating and microwave irradiation

To a solution of (*RS*)-Prl (1000 µL, 10 mM), the CDR (Npx anhydride) solution (1000 µL, 15 mM) and borate buffer (500 µL, 0.2 M, pH 9.5) were added. Separate sets of

reaction mixture were stirred for 15, 30 and 45 min, each at a temperature of 15, 30 and 45 ^{o}C; acetate buffer (500 μL, 0.05 M, pH 4) was then added to quench the reaction. Effect of ratio of analyte to CDR (mole ratio of 1:1, 1:1.5, 1:2, and 1:2.5) was investigated for each set of conditions. Stirring for 30 min at 30 ^{o}C with a mole ratio of 1:1.5 was found successful

For microwave irradiation, the reaction mixture (reactants taken in the same molar ratio, as mentioned above) was taken in a vial; the reaction mixture was then exposed to MWI for 190 s at 80% of 800 W powers. It was then cooled and acetate buffer was added to quench the reaction. Separate sets of reaction mixture were subjected to MWI for 80, 110, 130, 150, 170 and 190 s at 80% power of 800W.

The solvent (from each set of the reaction mixture from the two methods) was evaporated with nitrogen and re-dissolved in 500 μL of MeCN. It was filtered through 0.45 mm filter and 10 μL of this solution was diluted with 100 μL of MeCN; 20 μL of this solution was loaded onto the HPLC column. The reaction was monitored to check the completion of synthesis by injecting the reaction mixture for HPLC analysis for each set of synthesis. There are a few literature reports on conversion of amines to amides under MWI conditions [71] and their conversion to carbamates under mild and convenient synthesis conditions [72].

The diastereomers synthesized by the two approaches were compared for their separation parameters.

Preparative synthesis of diastereomers: The optimized conditions of synthesis of diastereomers were scaled up to preparative level; the solutions used were, (*RS*)-Prl (155 mg, 60 mM), solution of CDR (397 mg, 90 mM) and borate buffer (5 mL, 0.2 M, pH 9.5).

Open column chromatography for separation of diastereomers

A glass column (2.5 x 35 cm) was packed with silica gel in *n*-hexane. The mixture of diastereomers (as obtained from preparative synthesis) was loaded. Solvent system consisting of MeOH-DCM (9:1, *v*/*v*) was found successful for adequate separation of the two diastereomers; fractions collected from the column were examined by RP HPLC for verification of purity of each diastereomer. The fractions containing single

and identical diastereomer were combined and were concentrated in vacuum. The characterization data of the two separated diastereomers of (*RS*)-Prl is presented below.
First eluting diastereomer (Ds-I): Yield: (85.08%); Color: colourless; ^{1}H NMR ($CDCl_3$, 500 MHz): δ= 1.24 (d, 6H), 1.32 (d, 3H), 2.54 (s, 1H), 3.34 (dd, 2H), 3.67 (m, 1H), 3.98 (s, 3H), 4.19 (m, 1H), 4.61 (m,1H), 4.47 (m, 2H), 6.73 (dd, 1H, Ar-H),7.23(m, 2H, Ar-H) 7.46 (m, 3H, Ar-H), 7.59 (t, 2H, Ar-H), 7.79 (d, 1H, Ar-H), 7.87 (m, 2H, Ar-H), 8.43 (m, 2H, Ar-H); Anal. calcd for $C_{30}H_{33}NO_4$: C, 76.41; H, 7.05; O, 13.57; N, 2.97; found: C, 76.29; H, 6.93; O, 13.36; N, 2.75.

Second eluting diastereomer (Ds-II): Yield: (83.14%); Color: colourless; ^{1}H NMR ($CDCl_3$, 500 MHz): δ= 1.24 (d, 6H), 1.33 (d, 3H), 2.26 (s, 1H), 3.34 (dd, 2H), 3.67 (m, 1H), 4.01 (s, 3H), 4.19 (m, 1H), 4.64 (m, 1H), 4.36 (m, 2H), 6.74 (dd,1H, Ar-H),7.21 (m, 2H, Ar-H), 7.45 (m, 3H, Ar-H), 7.59 (t,2H, Ar-H),7.78 (d, 1H, Ar-H), 7.88 (m,2H, Ar-H), 8.44 (m, 2H, Ar-H); Anal. calcd for $C_{30}H_{33}NO_4$: C, 76.41; H, 7.05; O, 13.57; N, 2.97; found: C, 76.19; H, 6.81; O, 13.14; N, 2.56.

HPLC conditions for separation of diastereomers

The following mobile phases were used for separation experiments:

(i) MeCN-10 mM TEAP buffer, (ii) MeOH-10 mM TEAP buffer,
(iii) MeCN-0.1% aq TFA, (iv) MeOH-0.1% aq TFA

These mobile phases were used in an isocratic mode (with the ratio of 90:10, 80:20, 70:30 and 60:40) in 30 min run and by varying flow rate from 0.5 mL/min to 2 mL/min (with a difference of 0.5 mL) for each mobile phase. Chromatographic conditions were also optimized by changing the pH (in the range 2.0–6.0) and TEAP buffer concentration (5, 10, 15 and 20 mM) and TFA (0.05, 0.1, 0.15 and 0.2%). The mobile phases were filtered through a 0.45 mm filter.

Method validation

Validation of HPLC separation method was performed according to International Conference on Harmonization (ICH) guidelines for the proposed method, using diastereomers of (*RS*)-Prl prepared with Npx anhydride as the CDR. Peak areas (as

provided by the system software) were used for quantitation referring to the calibration curve for the studies related to stabilities and recoveries.

2. Results and discussion

Application of symmetrical anhydride of Npx (as CDR)

The anhydride of (*S*)-(+)-Npx is a symmetrical molecule. It was obtained when two molecules of (*S*)-(+)-Npx reacted in presence of DCC/DMAP in THF (**Fig. 4.2**). In organic synthesis one of the simplest and most efficient methods of acylation is the treatment of amines (or other nucleophile) with anhydrides of carboxylic acid. The introduction of DCC as coupling reagent had a novel feature that it could be added with carboxyl component and amine component; thus, activation and coupling proceed concurrently. However, the rate of reaction of amine with DCC is much lower in comparison to the rapid rate observed in the addition of carboxylic acid to one of the double bonds of carbodiimide [73].

In an attempt to achieve formation of diastereomers of (*RS*)-Prl (for example) directly with (*S*)-(+)-Npx in presence of DCC there were obtained certain co-eluting and unidentified peaks in HPLC. These may be due to formation of a symmetrical coupling product of (*S*)-(+)-Npx since the N=C group of the intermediate formed by the addition of carboxylic acid, i.e., (*S*)-(+)-Npx, to carbodiimide provided powerful activation leading to coupling. This condition is met with in the present work.

In view of the above, experiments were planned to prepare a symmetrical anhydride of (*S*)-(+)-Npx using DCC. The application of such an anhydride was helpful in preventing the formation of unreactive byproduct such as N-acyl urea if the amine component (i.e., (*RS*)-Prl, the amine group containing analyte) was allowed to react directly with (*S*)-(+)-Npx in presence of DCC because the nucleophilic centre on O-acylisourea (the intermediate formed by addition of (*S*)-(+)-Npx, being the carboxylic acid) competes with the amine component for acyl residue. Thus, it can be concluded that the application of DCC is justified in activating more for the formation of symmetrical anhydride which in turn was applied for the desired N-acylation.

Enantiomeric purity of CDR

The enantiomeric purity is a parameter different than the chemical purity of the sample. Since (*S*)-(+)-Npx had *ee* of 98%; it was considered that the CDR corresponding to 1%

of the other isomer of Npx (if any) was eliminated during synthesis and purification process (of crystallization and recrystallization) of the desired CDR. When (*RS*)-Prl was

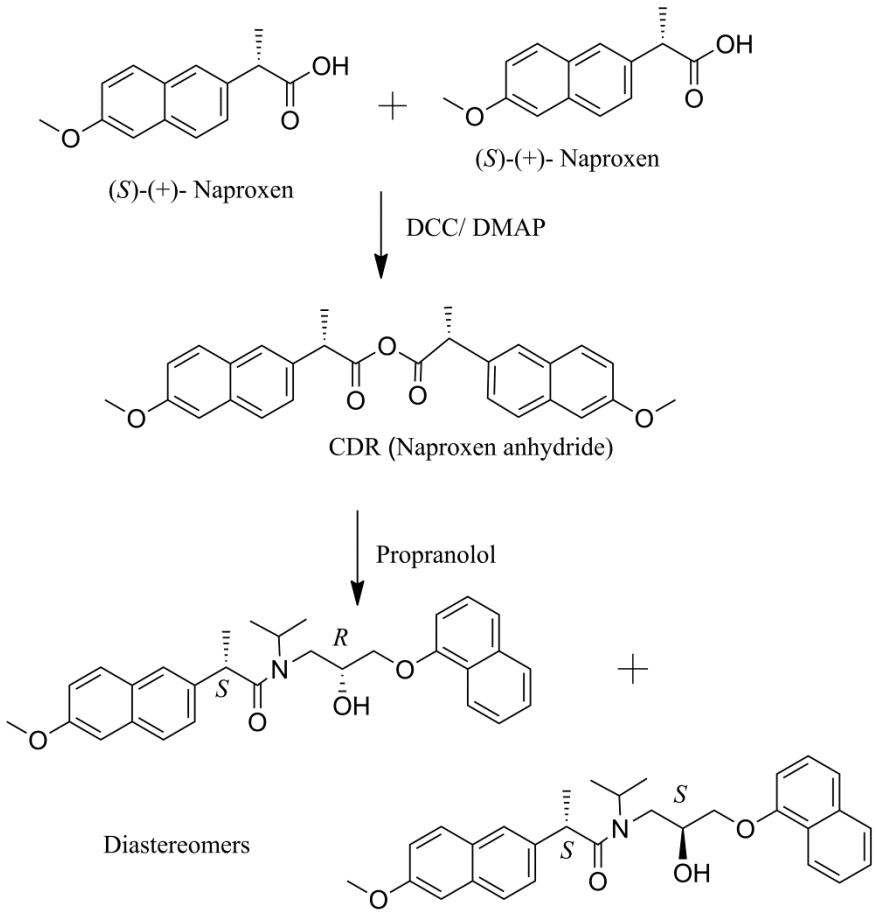


Fig. 4.2 The general scheme for synthesis of naproxen anhydride (the CDR) and synthesis of diastereomers of propranolol (as a representative β-blocker)

reacted with the CDR and the product so obtained was investigated on an RP-column using different mobile phases there appeared only two diastereomeric peaks. Had there been the CDR arising from the (*R*)-isomer of Npx there would have resulted corresponding diastereomeric peaks in the chromatogram. Since the PDA detector showed only diastereomeric peaks and confirmed the absence of impurities in the same retention time of the peaks of interest the absence of racemization as well as enantiomeric purity of the recrystallized CDR was confirmed. The continued experiments involving LC-MS clearly confirm the absence of any such impurity.

Diastereomeric derivatives of β-blockers

Diastereomers of all the four title compounds [(*RS*)-Prl, (*RS*)-Atl, (*RS*)-Cal, and (*RS*)-Mel] were prepared by their reaction with the symmetrical anhydride of (*S*)-(+)-Npx in borate buffer (0.2 M, pH 9.5), as the catalyzing agent. Reaction scheme for synthesis of the CDR and synthesis of diastereomers of (*RS*)-Prl (as a representative β-blocker) is shown in **Fig. 4.2.** Since the derivatization reaction with the CDR did not involve a direct attack on stereogenic center there was no racemization detected throughout the study. The derivatives are designated as the (*S,R*)- and (*S,S*)-diastereomers, the first letter refers to the configuration of the CDR and the second to that of the Prl.

The optimized experimental conditions for synthesis of diastereomeric pair of (*RS*)-Prl were: '30 min at 30 ^{o}C by stirring' and '190 s at 80% of 800 W power under MWI', with a 1.5 fold molar excess of CDR. Effect of MWI time on completion of derivatization reaction of (*RS*)-Prl is shown in **Fig. 4.3**. Synthesis of diastereomers of (*RS*)-Atl, (*RS*)-Cal, and (*RS*)-Mel was carried out under the optimized reaction conditions.

Generally, the rate of reaction of enantiomers with chiral molecules is different. The CDR is a chiral molecule and, therefore, the two enantiomers in the (*RS*)-mixture may react at different rates. The derivatization reaction was performed using (*RS*)-β-blocker(s) and the CDR in different mole ratio; slight kinetic resolution was observed when lower ratio of (*RS*)-Prl: CDR (1:1) was applied. The recovery studies of the four eluted diastereomers (as described in "Accuracy and Precision") served as a measure of their yields. Since the recovery of the eluted diastereomers of Prl, Atl and Mpl was found to be more than 94% it was contended that the steric effects due to *tert*-butyl group in these molecules did not adversely affect the derivatization reaction.

The reaction conditions for synthesis of diastereomers were investigated and established keeping in view that β–blockers possess a secondary hydroxyl group besides the secondary amino group. Basic medium (borate buffer, 0.2 M, pH 9.5) made the reaction to occur at amino group. Excess of the reagent would not react with hydroxyl function (even after quenching the reaction by adding acetate buffer) since the medium has been basic (pH 8.5).

In the indirect approach, a suitable reactive group (preferably only one function in close proximity to the stereogenic center) should be present in the sample to be

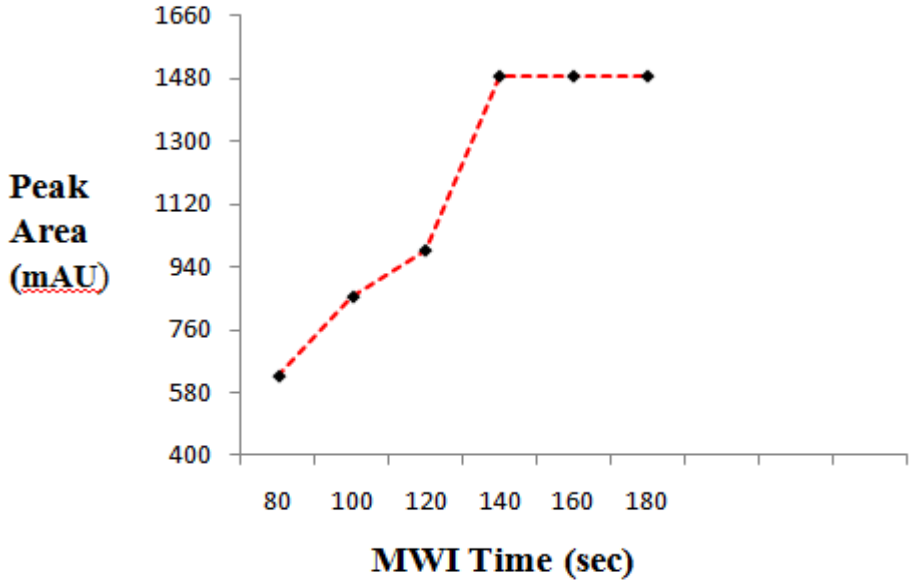


Figure 4.3 Effect of MWI time on completion of derivatization reaction of propranolol with the CDR

analyzed which is prone to a quantitative transformation with the chiral reagent; this condition is met with in the present work.

Stability of Diastereomers: Stability of diastereomers was investigated after short-term (room temperature) and long term (refrigerated at a temperature of 3–5 °C) storage as a function of storage conditions including the container system and their chemical properties. The evaluation also included the stability of the analyte in stock solution and situations encountered during actual sample handling and analysis. HPLC experiments were carried out at an interval of 10 days up to 130 days from the day of synthesis of the diastereomers and it was found that the diastereomers were stable for 120 days under

refrigeration conditions (3–5 °C). The chemical stability remains unaffected in the solvents used as mobile phase in HPLC.

Configuration of the diastereomers

Because of the reaction of the carbonyl group of CDR with the amino group of Prl there occurred formation of diastereomers having amide bond. ^{1}H NMR spectra of the purified diastereomers were recorded (**Fig. 4.4**). Difference in chemical shifts (Δδ) formed the basis for establishing the absolute configuration and discrimination among the chiral diastereomers. In the two diastereomers, the chemical shift values for the peaks of –OH signal are δ 2.54 and δ 2.26. The difference in chemical shift (*i.e.*, $\Delta\delta^{RS}$ value of -0.28) is large enough for a diagnostic –OH signal which can be represented as, [$\Delta\delta^{RS}$ (OH) = δ^{R}(OH)– δ^{S}(OH)], where (*R*) and (*S*) descriptors refer to the configuration at the stereogenic centre of the two enantiomeric Prl molecules. The difference in the chemical shift of the –OH signal may be attributed to the formation of H-bonding in one of the diastereomers whereas in the other diastereomer H-bonding is not possible. The δ values (in ppm) of –OH proton corresponding to the asymmetric centre in Prl in the first eluting (DsI) and second eluting (DsII) diastereomers are shown in (**Fig. 4.4**) and these correspond to (*S*,*S*)- and (*S*,*R*)-diastereomers, respectively.

Further evidence for the formation of H-bond was provided by the DFT based lowest energy structures of the two diastereomers (**Fig. 4.5(i) and 4.5(ii)** corresponding to DsII and DsI, respectively) developed (by using the Gaussian 09 Rev A. 02 program and hybrid density functional B3LYP with 6-31G (d, p) basis set). **Fig. 4.5(ii)** showed that the naphthyl ring of CDR and the naphthyl ring of Prl are in the same plane; this steric situation favours the formation of H-bond which in turn causes a downfield shift and there is observed a difference in chemical shift of –OH protons in the two diastereomers. The chemical shift for the –OH proton in the ^{1}H NMR spectrum of the diastereomers is greatly affected by the presence of the H-bond which arises due to specific spatial arrangement. The $\Delta\delta^{RS}$ value obtained for –OH proton has a negative sign. The $\Delta\delta^{RS}$ value between the diastereomeric pair would not be negative for the –OH proton if the structure of the diastereomer is not as represented in **Fig. 4.5**. The first eluted diastereomer showed a downfield shift for –OH proton while the second eluted diastereomer showed an upfield shift in ^{1}H NMR spectrum; therefore, the first eluted

diastereomer has (*S*,*S*) configuration. The ^{1}H NMR spectrum (**Fig. 4.4**) and **Fig. 4.5(ii)** confirmed that hydrogen bond between –OH proton (in Prl moiety) and the carbonyl oxygen (of Npx moiety) is in one of the diastereomers only and thus it would be the (*S*,*S*) diastereomer. Such a hydrogen bond formation does not occur in (*S*,*R*) diastereomer due to the specific spatial arrangement.

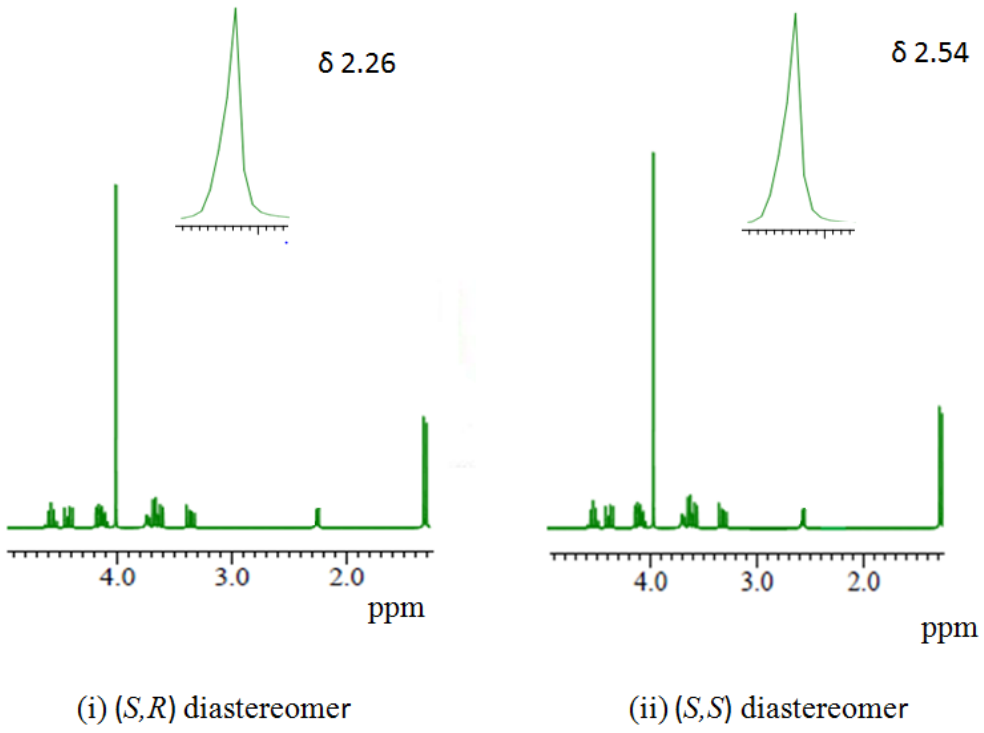


Fig. 4.4 Sections of ^{1}H NMR spectra of diastereomers illustrating chemical shift values for the peaks of –OH (at the stereogenic center) signal at d 2.26 and d 2.54, respectively, in (i) the (*S*,*R*)-diastereomer, and in (ii) the (*S*,*S*)-diastereomer. This difference in chemical shift of –OH signal is attributed to the formation of H-bonding in (*S*,*S*)-diastereomer only

Support to configurations of the diastereomers

The present studies are neither intended nor focused on theoretical calculation of the energies of the different configurations of the isomers for which use of different parameters in terms of two dihedral angles, as variables of the potential energy surface,

would be required. The default convergence criteria are implemented in Gaussian software itself; the optimization continues simply on submitting the data. Moreover, the diastereomeric molecules are not large and have no metal atom and there was no situation of running out of cycles. Software program routinely provides results for molecular geometries and energies at a very good level of accuracy. Therefore, the phrase "lowest energy structures" has been used for the structures of the two diastereomers (**Fig. 4.5**) developed (by using the above said Gaussian program)[74,75].

The diastereomers have been obtained by a synthesis process using a new chiral anhydride type reagent for facile C-N bond formation. The diastereomers were isolated by open column chromatography and the absolute configuration of the diastereomers so isolated was determined by ^{1}H NMR. The stability aspect is related to the chemical or composition stability in terms of time of storage and temperature. The chemical stability remains unaffected in the solvents used as mobile phase in HPLC. The structures shown in **Fig. 4.5** belong to the 'stable diastereomers'. As a result, there is no consideration of torsional energy (or rotational energy barrier) to decide the relative stability of conformations (the diastereomers are not a kind of atropisomers). The diastereomers under question are quite stable species. And the two structures belong to the lowest energy structures which are supplementing the information on configuration (derived from ^{1}H NMR) and the configuration is not changing by rotation at any stage.

The **Fig. 4.5(ii)** showing the structure for the (*S*,*S*)-diastereomer, for example, corresponds to the "lowest energy". There may be different conformations because of rotations at the bond (i) between carbonyl C and the stereogenic centre bearing methyl group on one side and (ii) the bond between N (of the amide bond) and the C of the $-CH_2$ group in the close proximity of the stereogenic centre bearing –OH group (the two dihedral angles to be considered) on the other side. But, these rotations are not affecting the (absolute) configuration at the two stereogenic centres. The configurations at the two stereogenic centres have been established by ^{1}H NMR separately, as discussed above. Efforts have been made to correlate the establishment of configuration.

HPLC

Retention factor (k), separation factor (α) and resolution (R_s) for the resolved diastereomers of each of the four β-blockers are given in **Table 4.1**. Selectivity or

separation factor (α) is a measure of relative retention of the two adjacent sample components, $\alpha = k_2 / k_1$. The retention factor or capacity factor (k) was calculated as $k_1 = t_1\text{-}t_0 / t_0$; t_0 is the retention time of a non-retained component (or the first baseline disturbance peak) which is the solvent as it is essentially unretained by the column. In present case void volume peak was observed at around 2.5 min. R_s values (as provided by the system software with the chromatograms) were found to be the same (**Table 4.1**) for replicate HPLC analysis (n=4). The diastereomers synthesized by MWI and by conventional heating were found to be in agreement in terms of R_s and elution order. Further, the integration data for % peak area for the separation of each pair of diastereomers, as recorded by the system software, verified that the two diastereomers were in the ratio of 1:1. **Table 4.1** shows that the highest R_s was for the diastereomeric pair of Prl, and in terms of decreasing order of R_s the four β-blockers can be arranged as Prl>Atl>Mel>Cal. The diastereomers of Mel were found to have greater retention time in comparison with the others. HPLC conditions were optimized for separation of all the three pairs of diastereomers by varying the concentration of TEAP buffer, pH and flow rate as described above. The mobile phase consisting of MeCN and triethyl ammonium phosphate buffer of 10 mM pH 3.5 (60:40 *v*/*v*) in isocratic mode at a flow rate of 1.0 mL /min (and detection at 230 nm) was successful in providing baseline separation of the diastereomeric amide derivatives on the C_{18} column except for pair of diastereomeric derivatives of metoprolol.

Sections of chromatograms showing baseline resolution of diastereomeric pairs (of racemic β-blockers) are shown in **Fig. 4.6**.

MeCN was found to be better organic modifier in comparison to MeOH as larger retention time and broader peaks were observed with MeOH. The reason for broader peak shapes and higher retention times with MeOH is because of its lower dielectric constant (33 D) and higher viscosity (0.59 cP at 25 ^{o}C) in comparison to MeCN which has a higher dielectric constant (37.5 D) and lower viscosity (0.343 cP at 25 ^{o}C).

Fig. 4.6 shows that the retention time for the two diastereomers of each of the four analytes was between 3.91 and 7.72 min. There was observed a peak at 6.7 min corresponding to (*S*)-Npx as the sample injected was expected to contain (*S*)-Npx (the leaving group from the anhydride CDR) because the CDR, being an acid anhydride, was a source of reactive acyl group and the reaction afforded equal amounts of the acylated

product (the diastereomer) and the carboxylic acid (Npx); there was no interference in the diastereomeric separation as it appeared much later from the diastereomeric mixtures of (*RS*)-Prl, (*RS*)-Atl, and (*RS*)-Cal while it eluted much earlier than the diastereomers of (*RS*)-Mel. Retention time for (*S*)-Npx was found to be 6.4 min when the sample of pure isomer was subjected to hplc under identical conditions.

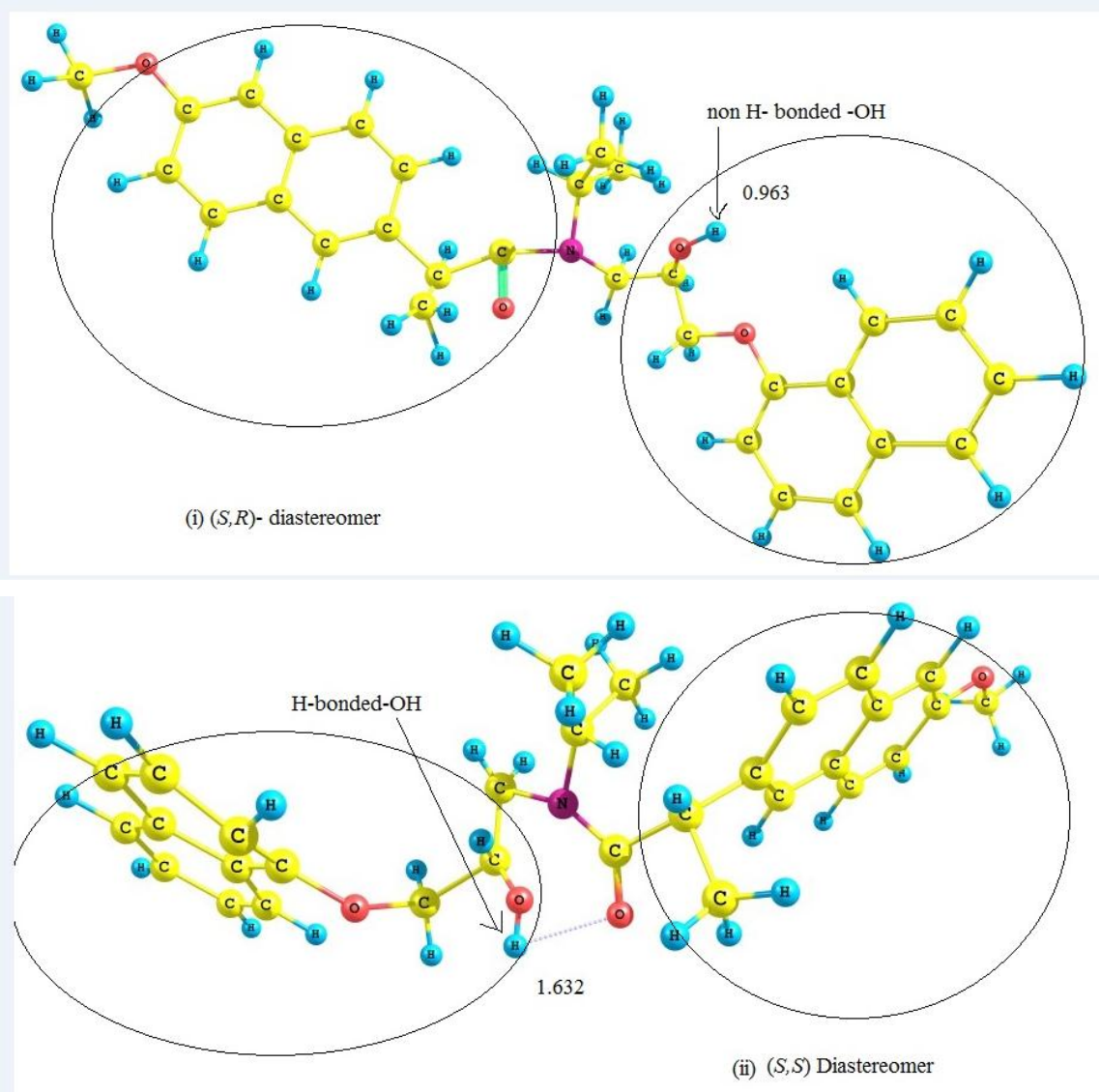


Figure 4.5 Lowest energy structures of diastereomers of (*RS*)-propranolol drawn using the program Gaussian 09 Rev. A.02 and hybrid density functional B3LYP with 6-31G basis set; (i) and (ii) correspond to (*S*,*R*)-diastereomer (DsII) and (*S*,*S*)-diastereomer (DsI), respectively.

Table 4.1: Chromatographic separation data for diastereomers of β-blockers prepared with the CDR (Npx-anhydride)

β-blockers for which diastereomers were prepared with the CDR	**Characteristics of separated diastereomers**			
	k_1	**k_2**	**α**	**R_s**
Propranolol	1.77	2.57	1.45	8.26
Atenolol	1.64	2.24	1.36	6.35
Carvedilol	1.57	1.65	1.05	5.98
Metoprolol	2.98	3.21	1.07	6.14

Mobile phase, binary mixture of MeCN and triethyl ammonium phosphate buffer of pH 3.5 (60:40, *v*/*v*) in isocratic mode at a flow rate of 1.0 mL /min; k_1= retention factor of first eluted diastereomer, k_2= retention factor of second eluted diastereomer, Column temperature = 30 °C, the first eluting peak corresponds to (*S*,*S*)-diastereomer in all the four cases, α = separation factor, R_s = resolution.

The values of retention factors for the first eluting and second eluting diastereomers (**Table 4.1**) were in agreement with the values of retention factors obtained for DsI and DsII which were run under optimized hplc conditions. Therefore, it can be contended that DsI has (*S*,*S*)- configuration (and elutes at first) while DsII has (*S*,*R*)- configuration (and elutes later, with the mobile phase).

Separation and elution order of diastereomers

Separation of diastereomers is considered to be influenced by the distance between the two asymmetric centers in the substrate and the reagent and the distance should be minimized for the best separation. The conformational rigidity around the chiral centers is another important factor for separation. The structures of the diastereomers in the present case satisfy this condition as there is no free rotation near the asymmetric center of the substrate due to partial double bond character of the amide bond. It remains

difficult to distinguish the diastereomers at the time of elution unless the elution order is compared with the standard samples or the configuration of the diastereomers is confirmed and correlated with structures developed by another method.

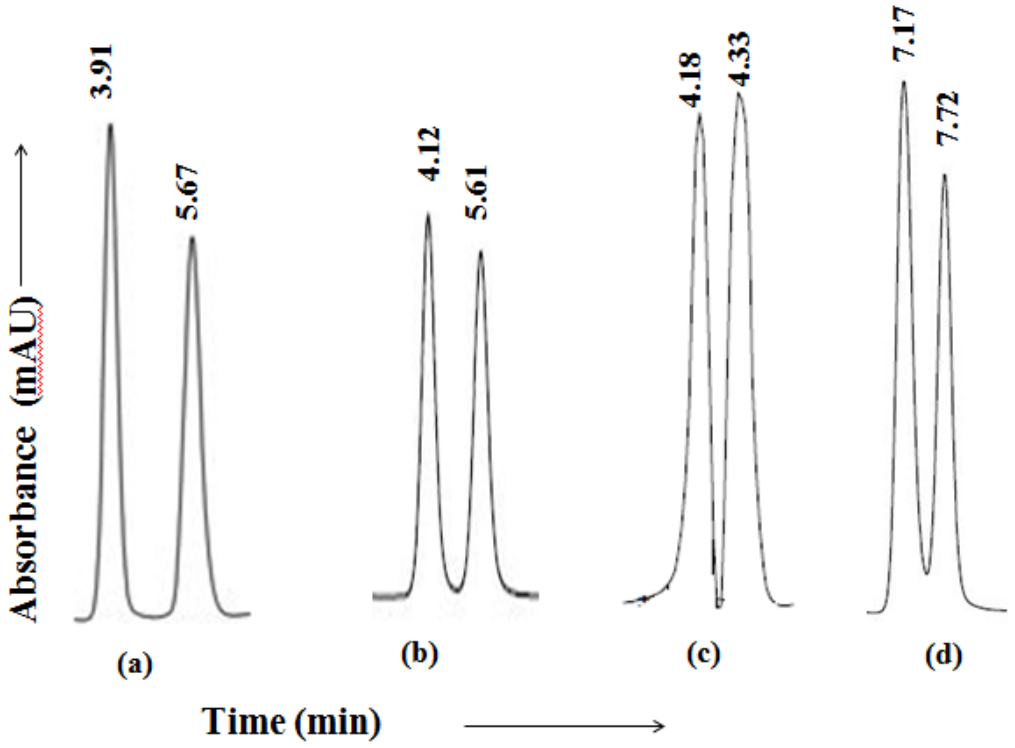


Figure 4.6 A section of the chromatogram showing separation of diastereomers of (a) (*RS*)-propranolol, (b) (*RS*)-atenolol, (c) (*RS*)-carvedilol, (d) (*RS*)-metoprolol; Mobile phase: MeCN-TEAP buffer (60:40, *v*/*v*); flow-rate 1.0 mL/ min; injection volume 20µL; the first eluting peak corresponds to (*S*,*S*)-diastereomer in all the four cases.

The diastereomers, in the present case, were isolated by open column chromatography and the absolute configuration of the diastereomers so isolated was determined by ^{1}H NMR. The results have been correlated with DFT based lowest energy structures to explain the elution order.

Since there is no ionic group in the diastereomers and the mobile phase causing separation has pH around 3.5 it was inferred that ionic interactions were not playing a role. Thus, the factors contributing to the hydrophobicity of diastereomers are affecting separation process and elution order. The overall hydrophobicity due to (i) the presence

of naphthyl group in propranolol and in the CDR, (ii) the partial double bond character of the amide bond in the diastereomers, and (iii) the rheological properties of the mobile phase are responsible for different retention times and different partition coefficients of the (*S*,*R*)- and (*S*,*S*)-diastereomers. Therefore, for these different physical properties the diastereomers elute one after another. (*S*,*R*)-diastereomer is slightly less polar than (*S*,*S*)-diastereomer. The presence of H-bonding in the (*S*,*S*)-diastereomer makes it more polar and causes a greater affinity with the mobile phase and thus the same is eluted first. The same explanation is applicable for the diastereomers of all the other β-blockers under study.

Method Validation

To evaluate the validity of method, samples of drug of different concentration in the range of 40 to 200 ng/mL were analyzed. Inter-day and intra-day assay studies were carried out to determine accuracy and precision, and the results are represented as relative standard deviation (RSD). The RSD is a statistical parameter generally used to describe the precision of the method; differently, the recovery % allows to evaluate the accuracy of the method. The limit of detection (LOD) and limit of quantification (LOQ) were also evaluated

Linearity, limit of detection and limit of quantitation

Concentration of diastereomer (x) versus peak area (y) were plotted for both diastereomers of Prl prepared with CDR in the range of 40 to 200 ng/mL and linear regression equations were used to determine the slopes and correlation coefficients. A good linear relationship was obtained over this range.

LOD and LOQ represent the concentration of the analyte that would yield a signal to noise ratio (S/N) of 3 and 10, respectively. They were determined by injecting a series of diluted solutions of mixture of diastereomers. LOD was found to be 0.4 ng/mL and LOQ was found to be 1.2 ng/mL for each of the diastereomers prepared with CDR.

Accuracy and Precision

The intra-day precision was carried out by the replicate analysis of five different concentrations (40, 80, 120, 160, 200 ng/mL) of the diastereomers of (*RS*)-Prl on three

successive times. The inter-day precision was evaluated through a replicate analysis of five concentrations for a period of three successive days. This was done for all the β-blockers while the data corresponding to diastereomers of Prl prepared with CDR is given as representative. The recovery and RSD for each of the diastereomers were calculated. Small values of RSD and high percentage recoveries indicate the accuracy of the proposed method. The calculated values of RSD varied from 0.92–1.98% and recovery was >94.3%. The results of intraday and inter-day precision are summarized in **Table 4.2**.

IV. Conclusion

Commercial availability of Npx as the pure (*S*)-enantiomer allowed easy synthesis of a symmetrical anhydride of (*S*)-(+)-Npx using DCC under MWI; the acid anhydride is highly reactive and stable at room temperature. Under basic conditions it enabled convenient preparation of diastereomeric amide of certain β-blockers. The method provides an efficient novel carbon-N bond forming reaction by using "aromatic carboxylic anhydride" (i.e., the (*S*)-(+)-Npx anhydride). Besides, presence of hydrophobic naphthyl ring helped RP-HPLC separation of the diastereomers along with facilitation of their on-line detection due to high molar absorptivity related to conjugated system.

In the present case structures of the diastereomers have been confirmed by isolating the diastereomers using open column chromatography and recording their ^{1}H NMR spectrum while literature reports on enantioresolution by indirect approach are limited to synthesis of diastereomers and their HPLC separation only. DFT based lowest energy optimized structures of the diastereomers supplemented confirmation of absolute configuration of the diastereomers. LOD and LOQ were found to be 0.4 ng/mL and 1.2 ng/mL, respectively, for each of the diastereomers under simple reversed-phase HPLC conditions. Optimised and validated RP-HPLC separation conditions can be successfully applied for determination and control of enantiomeric purity routinely in laboratories associated with regulatory agencies and industries, even without resorting to DFT calculations, each time.

Table 4.2: Validation data for separation of diastereomers of (*RS*)-propranolol prepared with the CDR

	First eluting diastereomer			Second eluting diastereomer		
Concentration (actual) (ng/mL)	Mean±S.D (observed) (ng/mL)	Recovery (%)	RSD (%)	Mean±S.D (observed) (ng/mL)	Recovery (%)	RSD (%)
Intra-day precision						
40	19.92±0.374	99.6	1.88	19.8±0.251	99.3	1.27
80	39.40±0.362	98.5	0.92	39.1±0.738	97.9	1.89
120	60.36±1.092	100.6	1.81	58.10±0.85	96.8	1.46
160	77.76±0.995	97.2	1.28	77.42±1.153	96.7	1.49
200	99.2±1.944	99.2	1.96	100.2±1.352	100.2	1.35
Inter-day precision						
40	19.44±0.258	97.2	1.33	19.72±0.303	98.6	1.54
80	38.68±0.765	96.7	1.98	38.24±0.412	95.6	1.08
120	59.64±0.697	99.4	1.17	57.69±1.03	96.2	1.79
160	76.10±0.981	95.1	1.29	76.72±1.104	95.9	1.44
200	96.20±1.69	96.1	1.77	94.3±1.593	94.3	1.69

The concentration values listed in columns 2 and 5 refer to the racemate.

Chapter 5

Resolution of (*RS*)-Baclofen by Ligand Exchange TLC

I. Introduction to (*RS*)-Baclofen and Literature Survey on its Enantioseparation

(*RS*)-Baclofen

It is a γ-aminobutyric acid analog and has the systematic chemical name as, 4-amino-3-(4-chlorophenyl) butyric acid (**Fig. 5.1**). It is extensively used as a stereoselective agonist for $GABA_B$ receptor [**76**] and [**77**]. It has also been used as a muscle relaxant, and to treat spasticity due to multiple sclerosis, cerebral and spinal cord injury, cerebral palsy, and complex region pain syndrome [**78**] and [**79].** Bac is marketed and used as a racemic mixture. However, it is claimed that only the (*R*)-enantiomer is *stereoselectively* active on $GABA_B$-receptors and is more active than the (*S*)-enantiomer [**80**] and [**81**].

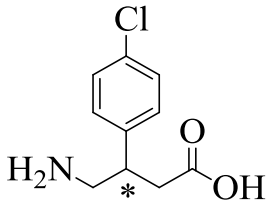


Fig. 5.1 The structure of (*RS*)-Baclofen

Enantioseparation via Ligand Exchange

Ligand-exchange enantioseparation, suggested by Davankov and Rogozhin, is a typical case of complexation chromatography and has been shown to be a powerful tool for chiral separation of amino acids and other chelate complex forming compounds [82, 83]. Davankov observed that the stability of the diastereomeric complexes formed in ligand exchange chromatography (LEC) is higher than the stability of the diastereomeric adducts formed by other chiral selectors [84]. Based on the work of Davankov *et al.* [85,86] who modified commercial HPLC columns by grafting alkyl derivatives of α-amino acids such as *n*-decyl-L-histidine or *n*-hexa decyl-L-proline,

onto the resin for enantiomeric resolution (explained as ligand exchange chromatography), a new chiral selector (2*S*, 4*R*, 2'*RS*)-N-(2'-hydroxy dodecyl)-4-hydroxy proline was developed at Degussa AG, Germany [87] and was used for ligand exchange TLC resolution of enantiomeric amino acids [88-91].

Amino acids as chiral selectors: Amino acids have been used as chiral auxiliaries to prepare derivatizing reagents [92]. L-AAs have been and are still largely used in chiral ligand exchange chromatography (CLEC) both for TLC and HPLC applications. In the former case, L-AAs have been employed both as impregnating reagents for the adsorbing material and as chiral additives to the developing solvent. Instead, in HPLC settings, L-AAs have been used either as a part of the CSP (via covalent grafting of a suitable function of the stationary phase or through hydrophobic adsorption on the achiral RP material) or as chiral mobile phase additives (CMPA) to the mobile phase. Such an application of L-amino acids has been reviewed for direct enantioresolution of a variety of racemic compounds [93].

Literature Survey on Enantioseparation of (*RS*)-Bac

Literature reports on HPLC separation of enantiomers of (*RS*)-Bac by direct approach include use of chiral stationary phase consisting of Crownpak® CR [**94**], macrocyclic antibiotic teicoplanin [**95**], and use of chiral mobile phase consisting of aqueous copper(II) acetate and *N*,*N*-di-*n*-propyl-L-alanine [**96**] and D-penicillamine based ligand exchange chiral column [**97**]. Enantioseparation of (*RS*)-Bac by indirect approach has been reported using CDRs like (*S*)-Npx chloride [**98**], *o*-phthaldialdehyde combined with *N*-acetyl-L-cysteine [**99**], 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide [**100**], and N-(4-Chloro-6-piperidinyl-[1,3,5]-triazine-2-yl)-L-phenylalanine [**101**]. Besides, dichloro-*s*-triazine and monochloro-*s*-triazine [**102**] reagents have also been used as CDRs for enantioseparation of (*RS*)-Bac by using reversed-phase HPLC.

The literature dealing with TLC analysis/separation of certain important enantiomeric drugs [**10**], along with the literature cited above and the references cited therein, revealed a scope for developing new sensitive, simple TLC methods of enantioseparation of (*RS*)-Bac though it has a high prescription rate and is easily available. Literature search also revealed that there were no reports available on enantioseparation of (*RS*)-Bac by ligand exchange TLC. Keeping in view lack of

literature reports (particularly by ligand exchange TLC) on enantioseparation of (*RS*)-Bac it was considered to establish validated experimental protocols for analytical enantioseparation of (*RS*)-Bac. Considering these aspects and structure of Bac following experiments were planned and carried out.

II. Present Work: L-Trp, L-His, L-Pro and L-Phe were chosen for preparing chiral ligand exchange reagents (LER) (considering their success in enantioresolution of certain β-blockers [**103**] by ligand exchange TLC). TLC was performed by the following two different approaches, (**A**): TLC plates were prepared by mixing the LER with silica gel slurry and the chromatograms were developed with different achiral solvents or solvents having no chiral additive, and (**B**) the LER consisting of Cu(II)-L-amino acid complex was used as chiral mobile phase additive and the plain plates of silica gel having no chiral selector were used. By choosing one mobile phase the performance of the two methods was compared to discuss the issue of involvement of Cu(II) for the best resolution.

1. Experimental

The details of chemicals, reagents, solvents and the equipment used have been described in **Chapter-2**.

Preparation of Cu(II)–L-Amino Acid complex (the Ligand Exchange Reagents)

L-Trp, L-His, L-Pro and L-Phe were chosen as chiral selectors. The solutions of Cu(II) and each of the chiral selectors were mixed in a ratio of 1:2 and pH 7 of the solution was maintained by addition of a few drops of ammonia. In all, four ligand exchange reagents were prepared. The UV spectra for the complexes were different from the UV spectrum of the Cu (II) solution. The UV spectrum for the complexe is shown in Fig. 5.2.

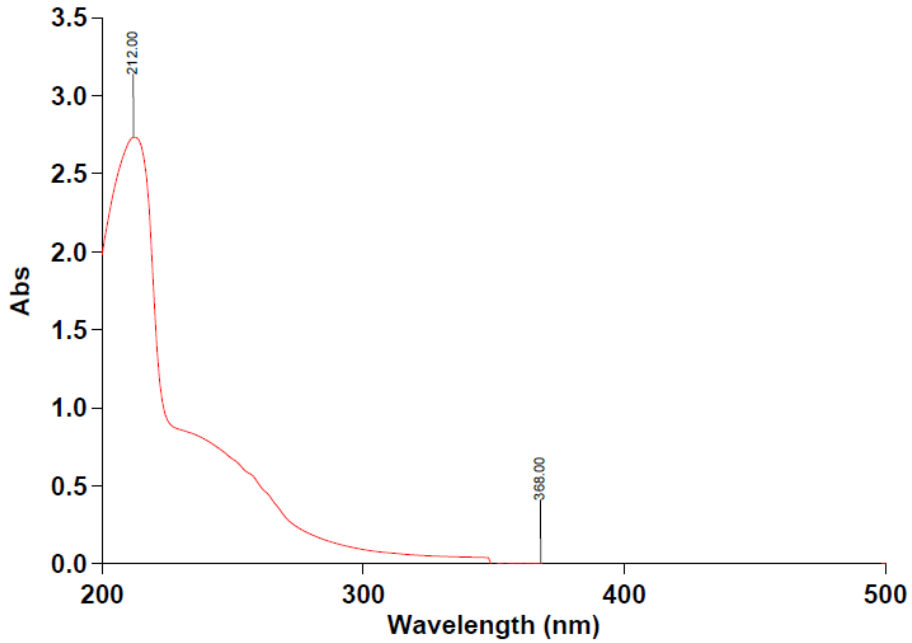


Fig. 5.2 The UV spectra for the complex of L-Phe and Cu(II) acteate.

Preparation of TLC plates

The details of preparation of TLC plates have already been described in **Chapter-2**.

Use of LER in TLC

LER were used in the following manner.

A. Impregnation of plain plates by mixing the LER with slurry. Slurry of silica gel G (25 g) was prepared in the solution of each of the four LERs (50 mL) and the plates were prepared as described above. The chromatograms were developed with different solvents.

B. LER as mobile phase additive. The LER consisting of Cu(II)-L-amino acid complex was used as mobile phase additive for developing the chromatograms and the plain plates of silica gel having no chiral selector were used.

Development of chromatograms

Solution of (*RS*)-Bac (10 µL, 10^{-2} mM, in 0.1 M $NaHCO_3$) was spotted on the TLC plates with the help of 25 µL Hamilton syringe. The chromatograms were developed,

under approach (A) and (B), as described in Chapter-2. Binary and ternary mixtures of MeOH, MeCN, acetone, $CHCl_3$, water and DCM were tried as mobile phase to achieve enantiomeric resolution. Chromatograms were dried in an oven at 40 °C and then cooled to room temperature; spots were located in an iodine chamber. For separation of enantiomers of (*RS*)-Bac optimization of conditions with respect to temperature and concentration of the chiral selectors were carried out. To determine repeatability, solutions of known concentration of (*RS*)-Bac (10^{-2} mM) were applied on TLC plates six times.

Mole Ratio of Cu(II) to Amino Acid

Experiments were performed to optimize the ratio of L-amino acid to Cu(II) by using each of the amino acids in four different concentrations. The plates were prepared by using 1 mM, 2 mM, 4 mM, and 6 mM concentrations of each of the amino acids with 2 mM of Cu(II) acetate; this provided L-amino acid-Cu(II) ratio of 1:2, 1:1, 2:1, and 3:1. Further experiments were carried out by using a fixed concentration of amino acid (4 mM) and varying the concentrations of Cu(II) acetate (2 mM, 4 mM, and 6 mM); in this case the ratio of L-amino acid-Cu(II) was 2:1, 1:1, and 2:3, respectively. While keeping the ratio of L-amino acid: Cu(II) as 2:1, 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).

Effect of Temperature

The chamber was pre-equilibrated for 20-30 min with mobile phases at different temperatures, 16, 20, 24, 28 and 32 °C (when the room temperature was about 28 °C). Each temperature was maintained/controlled using an incubator. The chromatograms so developed were processed as described above.

2. Results and Discussion

In the present chapter, resolution of enantiomers of (*RS*)-Bac was achieved by two different LEC approaches [(A) and (B)] and different mobile phases were tried for each case. The two approaches were found successful for enantioseparation of (*RS*)-Bac via ligand exchange mechanism.

Mobile phases and hR_F (R_F x100) values

The successful mobile phases and hR_F (R_F x100) values are listed in **Table 5.1.** The R_F values are averages from at least five experiments performed on the same day and on different days. The (*S*)-isomer was found to have a higher R_F value than the (*R*)-isomer. Resolution (R_S) of two adjacent spots was calculated by dividing the distance between the two spot centres with the sum of their radii; the two spots were considered to have reasonably separated when R_S > 1.2 [**104**]. **Table 5.1** shows that the two impregnation modes can be arranged in decreasing order of resolution as (A) > (B). In terms of resolution approach A was found to be a better method than approach B that is shown in **Table 5.1**.

The performance of the two methods was compared for the best resolution by choosing one mobile phase. It was found that one particular ratio of the three solvents (MeCN-$CHCl_3$-MeOH) was hardly successful in all the situations; a variation in the ratio of the three solvents was to be made to achieve resolution. Representative photographs of chromatograms showing resolution of (*RS*)-Bac by use of Cu(II)‑L-Trp complex are shown in **Fig. 5.3**.

Effect of temperature

To study the effect of temperature on resolution of enantiomers it was varied systematically and the effect was noted by observing eight-shaped spots, tailing, or clear resolution. Experiments carried out at 16, 20, 24, 28 and 32 °C using the successful solvent systems (MeCN-$CHCl_3$-MeOH) showed that the best enantioresolution of (*RS*)-Bac was obtained at 28 °C under two approaches with any of the chiral selectors used in these studies.

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

The ratio of L-amino acid to Cu(II) was optimized for (*RS*)-Bac. It was found that the best resolution (R_S) was achieved when (4 mM) L-amino acid and (2 mM) Cu(II) were used, i.e., a ratio of 2:1.

Table 5.1: The successful mobile phases and hR_F (R_F x 100) values for enatioresolution of (*RS*)-Bac under approach (A) and (B). Ratios are for the solvent, MeCN-$CHCl_3$-MeOH

Chiral Selector	Approach (A)					Approach (B)				
	Solvent ratio	hR_F				Solvent ratio	hR_F			
		(*R*)	(*S*)	Pure (*S*)	*R*s		(*R*)	(*S*)	Pure (*S*)	*R*s
L-Trp	6: 2: 2	54	82	82	3.2	6: 3: 1	51	68	68	2.1
L-His	5: 1: 4	48	61	61	2.9	5: 2: 3	47	53	53	1.8
L-Pro	6: 1: 3	58	79	79	3.0	7: 2: 1	43	56	56	1.9
L-Phe	4: 2: 4	41	54	54	2.7	4: 2: 4	34	27	27	1.2

Precision and Limit of Detection (LOD)

The relative standard deviation (RSD) was between 1.35 and 1.75%. Different concentrations of (+)-enantiomer were spiked into fixed concentration of (–)-enantiomer in the range of 0.1‑2% using standard solutions of the two isomers, to establish detection limits. Using approach (A), the chromatograms were developed followed by visualization with iodine vapors. L-Trp as chiral selector and the mobile phase MeCN-$CHCl_3$-MeOH (6:2:2, *v*/*v*) was used. Detection was successful up to 0.5%.

Ligand exchange TLC resolution of (*RS*)-Bac

Davankov and Rogozhin [**82**] introduced chiral ligand-exchange chromatography (CLEC). Separations by means of CLEC are based on the formation of labile ternary metallic complexes in the mobile and/or in the stationary phase. TLC enantiomeric separations based on ligand exchange were published by Günther et al. [**88,89**] and Weinstein [**105**] in 1984, for the first time. Though the procedures differed in their

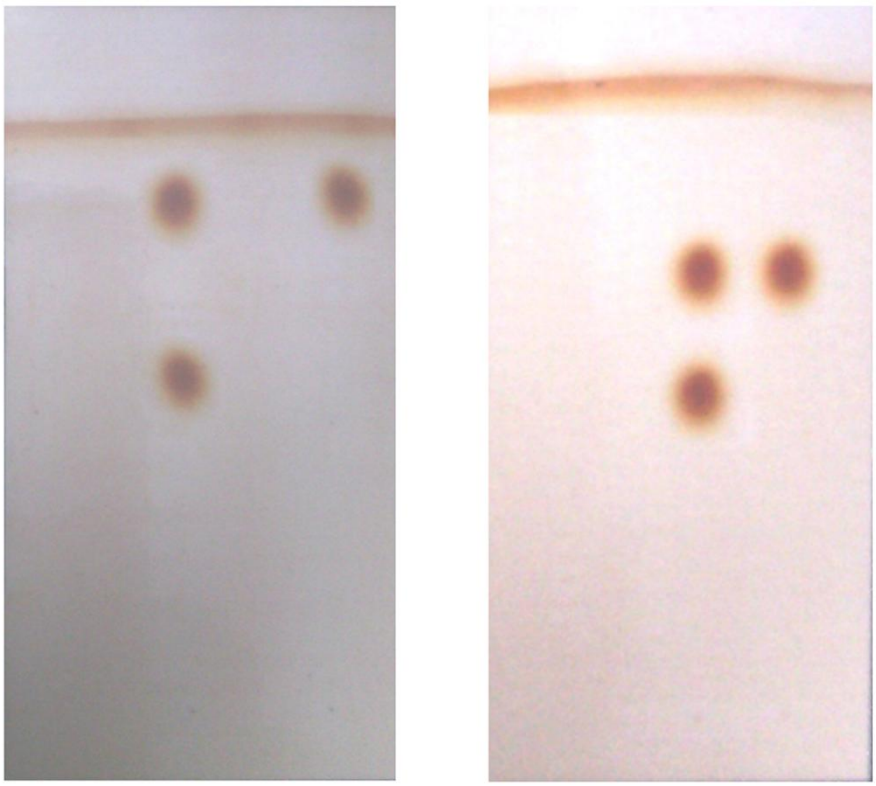


Fig. 5.3 Photographs of chromatograms showing resolution of (*RS*)-Bac by use of Cu(II)–L-Trp complex by approach A and approach B. Experimental details are given in Table 5.1. From left to right: Spot 1: lower spot for (*R*)-enantiomer and the upper spot for (*S*)-enantiomer resolved from the racemate; Spot 2: pure (*S*)-isomer.

choice of chiral selector and range of applicability, they had a very similar methodology. Separation models developed for ligand exchange HPLC [**106,107]** are also valid for TLC. The chosen chiral selector (the L-amino acid, in the present studies too) has carboxylic and amino functional groups which are capable of interacting with the metal ion, Cu (II). The L-amino acid in the Cu(II) complex (which is immobilized in a stationary phase or loaded on the solid support) is one of the ligands and acts as the chiral selector while the second ligand is one of the components of the sample (racemic) mixture. The complex undergoes very specific interaction with the enantiomer (i.e., the

ligand, donating a lone pair of electrons). The metal ion thus coordinates simultaneously to the chiral selector and the enantiomer to be separated. In other words, the interaction between the chiral selector and the enantiomer is mediated by the metal ion, Cu^{2+}, for example, in this case. This results in the formation of diastereomeric complexes of different stability, during the separation process. The different stability is only one of the aspects governing the enantiorecognition process in CLEC. In many instances, even more relevant is the different affinity of the two diastereomeric ternary complexes for the stationary phase. This explanation has been described in literature for the separation of enantiomers of α-amino acids and α-hydroxy acids and is well satisfactory for the separation of enantiomers of the (*RS*)-Bac, under study, as suggested by its structure. On the other hand, the mechanism of separation involves a series of complexation equilibria in the mobile and in the stationary phase [**108**] when the LER is used as mobile phase additive; it has been reported that the enantioseparation takes place because of different affinities of the diastereomeric complexes for the stationary phase rather than the stereoselectivity observed in solution [**109**].

Thus it can be contended that the mechanism of enantioselectivity in the LEC depends on whether the chiral ligand is linked on the stationary phase or it is added in the mobile phase. The stability of the diastereomeric complexes formed in ligand exchange chromatography (LEC) is higher than the stability of the diastereomeric adducts formed by other chiral selectors [**84**]. Schmid *et al*. [**110**] proposed formation of ternary mixed metal complexes between the selector and the analyte in ligand exchange chromatography.

Many interdependent factors are considered to affect the formation of complexes. The better results with approach (A) are in agreement with the previous explanations for enantioresolution of certain β-blockers by ligand exchange TLC (28). In approach (A) the formation of ternary complex is probably taking place during the chromatographic development, as the Cu(II) is available at the same time to the L-amino acid (present in the impregnated form) and to the analyte molecule spotted on the plate. The two approaches are successful for enantioseparation via the same ligand exchange mechanism for which the method of impregnation and successive formation of ternary complex is different.

III. Conclusion

The results indicate that the TLC method using ligand exchange approach via impregnation or by adding LER to the mobile phase can be applied for the detection of each enantiomer in low amounts up to 0.5%. The use of home-made plates is quite common for successful and less expensive routine works. The method presented herein provides a rapid and effective approach in planar mode for control of enantiomeric purity of (*RS*)-Bac (and other structurally similar pharmaceuticals) which can be realized even in a small laboratory. The method is less expensive and simple in comparison to existing methods requiring either chiral hplc columns or derivatization reactions for indirect mode of enantioseparation. Both the approaches presented herein have additional advantage that many other different chiral selectors (e.g., L-, or D-amino acids) can be easily tested to resolve the analyte and only a small amount of ligand is required which results in increased rate of ligand exchange and enhanced separation efficiency. Chromatographic separation and detection takes place separately in TLC which enables carrying out the analysis at a different time and making full use of detection techniques for the analysis of constituents. An advantage of TLC is the possibility of localization of all constituents on chromatograms contrary to HPLC where lack of detection may be observed in case of the retention of a constituent on the column.

Chapter- 6

Enantioseparation of pharmaceutical drugs using both achiral phases

I. Background for using both achiral phases in liquid chromatography

Liquid chromatographic resolution (of racemic compounds into their enantiomers) by direct approach has largely been dependent on the chiral environment in either of the two phases of chromatography. With respect to the stationary phase the approach could either be (a) preparing the column / the TLC plate with the material which is chiral due to its own structure (e.g., cellulose), or (b) immobilising the *chiral selector* onto the achiral inert support (or the stationary phase), or (c) bonding certain chiral moieties of interest to reactive groups of the achiral inert support making it chiral. When a *chiral selector* is added to the mobile phase the chiral environment is generated with the flow of the mobile phase containing the chiral selector. In all the cases resolution relies on the formation of transient diastereomers.

Resolution of racemic mixture with both achiral phases is not normally brought into consideration in chromatography. There are certain reports in literature on separation of enantiomers from non racemic mixture (i.e., the excess enantiomer) under achiral conditions. Martens and Bhushan presented a review on such separations by liquid chromatography with both achiral phases, in 1992 [111] and later in 2014 [112]. They addressed basic question 'why separation of one particular enantiomer from the non-racemic mixture should take place under achiral phase chromatography from the non-racemic mixture' and discussed the concept of self segregation of the enantiomers of a molecule in the absence of external chiral auxiliary.

Resolution of a *racemic mixture* with both achiral phases in chromatography needs to be looked into with an approach and concept different than the one described and explained for separation of enantiomers from *non-racemic mixture* (under achiral phases) because the latter represents enantiomeric enrichment of a non-racemic mixture. Tateishi *et al*., (2013) mixed optically pure (*S*)-CH C_6H_5 CH_3 NHCHO as '*chiral inducing reagent*' (CIR) and different derivatives of *rac*-1-(3-methoxyphenyl)-ethyl amine (as the analyte) [113], in a mole ratio of 1:1, and subsequently subjected (this premixed sample) to medium-pressure liquid chromatography using an achiral column

and an achiral mobile phase. There was no base line separation of enantiomers and *enantiomeric composition (of the sample so separated) was determined using chiral HPLC*; there was obtained only 21% or 26% of enantiomerically pure (*S*)-enantiomer from the starting racemic mixture in terms of isolated yield. Since the enantiomeric composition was determined using chiral HPLC the approach or method looses the usefulness of resolution of racemate under achiral phase. This may better be termed as another approach to *enantiomeric enrichment*.

In an approach of pre-mixing the racemic mixture with the chiral selector, before applying to the TLC plate (i.e. without resorting to any covalent linkage between any of the enantiomers of the racemate and the chiral selector), resolution of (±)-ibuprofen using (–)-brucine [114], and resolution of DL-(±)-SeMet (using (−)-quinine) and resolution of racemic samples of atenolol, betaxolol and orciprenaline by application of L-glutamic acid as *chiral inducing reagents* (CIR) has been achieved [115].

The concept of resolution of racemic mixture using both achiral phases was inventively adapted for non-chiral HPLC (and presented in this Chapter) which is in accordance with the concept reported in recent literature on (i) separation of enantiomers from *non-racemic mixture* under achiral phases [112], and (ii) resolution of *racemic mixtures* with both achiral phases in TLC [115].

Accordingly, the present chapter is divided in two sections. One deals with enantioseparation of (*RS*)-Bup, (*RS*)-Bac and (*RS*)-Etd by modifying the conventional ligand exchange approach, though using Cu(II)-L-amino acid complexes as ligand exchange reagents. At the same time, the concept of resolution of racemic mixture with both achiral phases was put to experimental investigation by studying TLC resolution of (*RS*)-Etd using certain pure L-amino acids as *chiral inducing reagents* (CIR) and the same has been described in second section of this Chapter. In both the cases the two phases at the time of chromatographic separation were achiral. Thus, a potential scientific issue for direct enantioresolution of a racemic mixture using both achiral phases in chromatography has been addressed.

Amino acids as chiral selectors: Application of L-amino acids and complex of L-amino acids with a metal ion, particularly Cu(II), as impregnating reagent in thin layer chromatography (TLC) or as chiral ligand exchange reagent or as chiral mobile phase

additive both in TLC and high performance liquid chromatography (HPLC) has been reviewed for direct enantioresolution of a variety of racemic compounds [93].

Section A: HPLC Enantioseparation of (*RS*)-Bupropion, (*RS*)-Baclofen and (*RS*)-Etodolac: Modification of Conventional Ligand Exchange Approach

II. Introduction to analytes and Literature Survey on their Enantioseparation

Introduction to (*RS*)-Etd and (*RS*)-Bac with respect to their pharmaceutical importance and the literature survey on their enantioseparation by both direct and indirect approaches has already been discussed in Chapter-3 and Chapter-5, respectively. Therefore, a discussion on pharmaceutical importance and the literature survey on enantioseparation of (*RS*)-Bup is given below.

Pharmaceutical Importance and Literature Survey on Enantioseparation of (*RS*)-Bupropion

Bup (2-(*t*-butylamino)-3′-chloropropiophenone) (Fig. 6.1), also known as amfebutamone, is a "second generation" antidepressant. It is a monocyclic aminoketone and a weak inhibitor of dopamine reuptake or of monoamine oxidase activity [116]. It is formulated and used clinically as a racemate, but there is a paucity of data available on the properties of the individual enantiomers [117]. The racemic mixture of Bup is associated with several disadvantages, like seizures, excitement, agitation, blurred vision, nausea, vomiting, restlessness, postural tremor, and some hallucinations/confusional states with the potential for abuse [118]. Pure (−)-isomer of Bup is more effective than the racemic mixture to cure depression, obesity, and Parkinson's disease

Literature reports on hplc separation of enantiomers of (*RS*)-Bup by direct approach using an ovomucoid column and α_1-acid glycoprotein column [119, 120] and chlorinated methylated cellulose-based stationary phases [121]. Direct enantioresolution of Bup using thin layer chromatography has also been done [122]. By using cyanuric

chloride and isothiocyanate- based chiral derivatizing reagents [123,124] for the enantioseparation of Bup as an indirect approach by HPLC have also been reported.

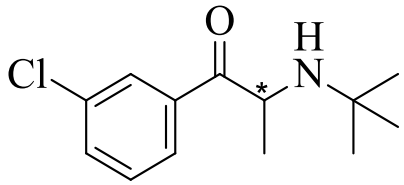


Fig. 6.1 Structure of (*RS*)- Bupropion

III. Present work

Separation of racemic mixture of (*RS*)-Bup, (*RS*)-Bac and (*RS*)-Etd has been achieved by modifying the conventional ligand exchange approach. To the pre-formed Cu(II) complexes of a few L-amino acids (namely, L-Pro, L-His, L-Phe, and L-Trp) was added the mixture of enantiomer pair of (*RS*)-Bup, or (*RS*)-Bac or (*RS*)-Etd. As a result, formation of a pair of *diastereomeric complexes* occurred by '*chiral ligand exchange*' via the competition between the chelating L-amino acid and each of the two enantiomers from a given pair. The diastereomeric mixture formed in the pre-column process was loaded onto HPLC column. Thus, both the phases during chromatographic separation process were *achiral* (i.e., neither the stationary phase had any chiral structural feature of its own nor the mobile phase had any chiral additive).

Due to this, the "ready-made" diastereoisomers enter the column and enantioseparation occurs. The separation method was validated for linearity, accuracy, limit of detection (LOD) and limit of quantification (LOQ) using diastereomeric complexes of (*RS*)-Bup prepared with Cu(II)-L-Phe.

1. Experimental

The details of chemicals, reagents, solvents and the equipment used have been described in **Chapter-2**.

Preparation of Cu (II)-L-amino acid complex

Preparation of stock solutions of Cu(II) acetate and chiral ligands have already been described in **Chapter-2.** The solutions of Cu(II) and each of the chiral ligands were mixed in a ratio of 1:2 for formation of Cu(II)-L-chiral ligand complex. The UV spectrum of each of the complexes was different from that of Cu(II) solution.

Preparation of diastereomeric 'ligand exchanged complexes'

To 1000 μL (10 mM) of (*RS*)-Bup, 2000 μL (20 mM) of chiral ligand complex solution was added. Thus, the concentration values for Bup and chiral ligand complex solution were allowed to react at a ratio of 1:2. At ambient temperature the combined solution was vortex mixed for five minute. It was kept in refrigerator until further analysis. The chiral ligand exchanged diastereomeric complexes were prepared in the same way with other two racemic analytes. It was observed that the UV spectrum of diastereomeric ligand exchanged complex of each of the racemic analyte was different from UV spectrum of the Cu(II)-L-chiral ligand complex. 100 μL of diastereomeric sample was diluted ten times with mobile phase and 20 μL of this solution was injected for HPLC analysis.

HPLC conditions

HPLC conditions were optimized for separation of all the twelve pairs of diastereomeric ligand exchanged complexes by using TEAP buffer concentrations (5, 10 and 15 mM) and TFA (0.05%, 0.1, and 0.15%) were tried. Following mobile phases were used:

(i) MeOH-TEAP buffer,

(ii) MeCN-TEAP buffer,

(iii) MeCN-TFA,

(iv) MeOH-TFA,

Each of the mobile phases **(i) to (iv)** was used in isocratic mode with the ratio of, 90:10, 80:20, 70:30, and 60:40 and at a flow rate of 0.5, 1.0 and 1.5 mL/min. Mobile phase was filtered through a 0.45 μm filter and degassed by sonication and passing nitrogen before use (sonication and nitrogen atmosphere ensure removal of air and oxygen). Detection was carried out at 230 nm for (*RS*)-Bup, 220 nm for (*RS*)-Bac, and 223 nm for (*RS*)-Etd. HPLC was performed on a Spherisorb C_{18} (L x I.D., 25cm × 4.6 mm, 5 μm particle size) column.

Method Validation

Method validation was done using diastereomeric complexes of (*RS*)-Bup prepared with Cu(II)-L-Phe in accordance to ICH guidelines (1996). The calibration curves were obtained by plotting peak areas against concentrations and linear regression equations were used to determine slopes and correlation coefficients.

2. Results and Discussion

Literature [122, 125] shows that Cu(II)-L-amino acid complex is formed when the two species are allowed to react at a ratio of 1:2. The results of the experiments in the present case, as described above, are in agreement.

Formation of diastereomeric 'chiral ligand exchange complexes'

The experiment was so designed that the 'chiral ligand exchanged complexes' of racemic analytes (diastereomeric in nature) were formed prior to loading onto column so that the stereorecognition process of already formed 'ligand exchanged' diastereomers takes place based on the different partition of the mixed ternary diastereomeric complexes between the two chromatographic phases. Two chiral selector amino acid molecules act as chelating ligands for the Cu(II) ion, one chelating amino acid molecule is replaced by the competing enantiomer molecule from the racemic mixture. This results in the formation of diastereomeric complexes (of the two enantiomers undergoing separation) having different stability and thus the enantioseparation is observed. In other words, the formation of labile ternary metallic complexes is not allowed to be formed in the mobile and/or in the stationary phase during the process of separation. In all there were twelve pairs of diastereomeric 'ligand exchanged complexes'; their UV spectra were recorded to verify the formation of such diastereomeric complexes prior to HPLC analysis. The presence of Cu(II) was tested in the HPLC effluent and was found to be absent. The absence of Cu(II) proves that the stereorecognition process is essentially based on a different partition of the mixed ternary diastereomeric complexes between the two chromatographic phases.

The principle and application of chiral ligand exchange chromatography (CLEC) have been well documented in literature [82, 126-129] and the technique has been found to

be successful for direct enantiomeric (HPLC) resolution of amino acids, beta-blockers and hydroxy acids by HPLC [130-135].

The enantioseparation of the chosen racemates by forming the 'chiral ligand exchanged complexes' prior to chromatographic process gets support with the literature [110,125] where the chiral ligand exchange process occurs in the mobile/ or in the stationary phase during the development of chromatogram and the separation process. The present results are also in conformity with the literature in the sense that (i) the chiral ligand exchanged diastereomeric complexes are formed by using the chiral complex and the racemate in a ratio of 2:1, and (ii) they are formed easily at room temperature and are stable to sustain the separation process on loading onto the column.

HPLC separation of 'chiral ligand exchanged complexes'

Data representing resolution (R_S), and separation factor (α) for diastereomers of (*RS*)-Bup, (*RS*)-Bac and (*RS*)-Etd separated under optimized conditions of HPLC are given in **Table 6.1**. Chromatograms showing base line separation of the diastereomers of the three racemates under study are given in (**Fig. 6.2, 6.3, and 6.4**) respectively for (*RS*)-Bup, (*RS*)-Bac and (*RS*)-Etd. Interestingly, the mobile phase (**ii**) consisting of binary mixture of MeCN and TEAP buffer of pH 4.0 (60:40, *v/v*) in isocratic mode at a flow rate of 1.0 mL/min was found successful for separation of all the twelve pairs of diastereomers.

Separation Mechanism

The present method of separation, described here, differs from conventional ligand exchange chromatography as the formation of diastereomeric complex is not involved in the mobile and/or in the stationary phase during the process of separation (and thus it may be termed as indirect ligand exchange chromatography). Bull and Breese (1974) [136] produced a hydrophobicity scale of amino acids by calculating their apparent partial specific volume. Among the chiral selectors the largest retention factor was obtained for the diastereomeric complex formed with L-Phe. The resolution order of diastereomers formed with Cu(II) complexes of L-Trp and L-Pro falls in between and the lowest resolution was shown by diastereomers formed with Cu(II) complexes of L-

His. This behaviour is in accordance with the hydrophobicity scale for amino acids proposed by Bull and Breese (1974).

Since the amino acids (Pro, His, Phe, Trp), used as ligands, have L-configuration the diastereomers can be represented as (*R*,L)-, and (*S*,L)-diastereomers, where (*R*)-, or (*S*)-, denotes the absolute configuration of the enantiomers present in the racemates of the three analytes (**Fig. 6.5**). Schmid *et al*., (2000) [110], proposed the possible model structures of the mixed Cu(II) complexes between the chiral selector and the analyte in ligand exchange chromatography. Considering the configuration of the amino acid (the ligand) as L-, the structure of complex suggests that the alkyl/ aryl side chain 'R' of the amino acid (at its stereogenic centre) is *cis* to methyl group (**Fig. 6.5a**) at the stereogenic centre of the (*R*)-enantiomer of the drug molecule (say, Bup). Accordingly, it would be *trans* for (*S*)-enantiomer of Bup (**Fig. 6.5b**). Since in the (*R*,L)-diastereomer the hydrophobic groups at the two stereogenic centre are *cis* to each other. They may have stronger interaction with the C_{18} material of the column and may have longer retention time and elute later in comparison to the (*S*,L)-diastereomer in which the hydrophobic groups are *trans* to each other and thus, have a relatively weaker interaction with C_{18} material of the column. Therefore, (*S*,L)-diastereomer elutes before the (*R*,L)-diastereomer.

The present explanation is in line with the literature reports where a *cis* type arrangement of certain groups in different diastereomers has been considered responsible for greater hydrophobicity [33, 137]. Thus, it can be concluded that in ligand exchange chromatography also the elution order depends on hydrophobicity of diastereomeric ligand exchange complexes.

Table 6.1 Chromatographic data for resolution of the three racemates under study

Chiral Selector*	(*RS*)-Bup				(*RS*)-Bac				(*RS*)-Etd			
	α	**k_1**	**k_2**	**R_S**	**α**	**k_1**	**k_2**	**R_S**	**α**	**k_1**	**k_2**	**R_S**
L-Phe	1.05	4.97	5.22	5.92	1.18	5.83	6.89	6.63	1.12	5.54	6.21	5.12
L-Pro	1.08	7.62	8.24	4.61	1.45	3.37	4.89	3.82	1.45	3.77	5.48	4.56
L-Trp	1.25	3.18	3.98	2.98	1.95	3.52	6.87	2.53	1.64	3.63	5.96	3.87
L-His	1.02	4.47	4.56	2.76	1.31	3.19	4.18	2.06	1.56	2.96	4.63	1.93

Chiral selector*: Diasteromer prepared with Cu(II) complex of L-amino acid via ligand exchange in a pre column step. Mobile phase: binary mixture of MeCN and TEAP buffer of pH 4.0 (60:40, *v/v*) in isocratic mode at a flow rate of 1.0 mL/min; Column: Waters Spherisorb C_{18} (L x I.D., 25cm × 4.6 mm, 5 μm particle size); Column temperature: 30 °C. α = separation factor; R_S = resolution. The integration data for % peak area for the separation of each pair of diastereomers, as recorded by the system software has been given and used to calculate resolution factor values.

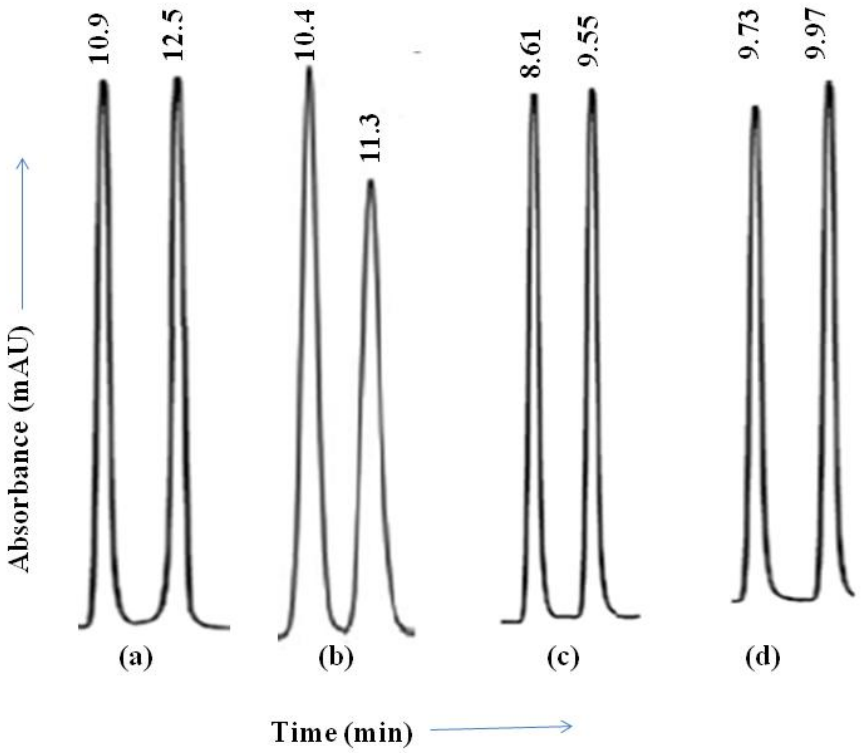


Fig. 6.2 Sections of chromatograms showing resolution of (*RS*)-Bup. (a)-(d) represent the diastereomers of (*RS*)-Bup prepared with chiral ligand exchnage Cu(II) complex of L-Phe, L-Pro, L-Trp, L-His, respectively. Mobile phase (ii) consisting of binary mixture of MeCN and TEAP buffer of pH 4.0 (60:40 *v/v*) in isocratic mode at a flow rate of 1.0 mL/min; column, Spherisorb C_{18} (L x I.D., 25cm × 4.6 mm, 5 µm particle size).

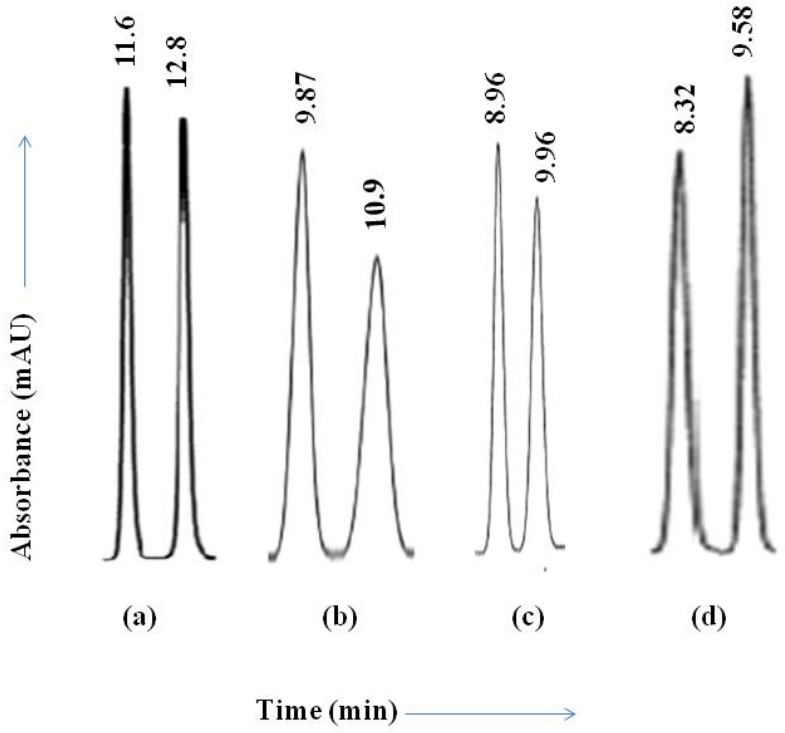


Fig. 6.3 Sections of chromatograms showing resolution of (*RS*)-Bac. (a)-(d) represent the diastereomers of (*RS*)-Bac prepared with chiral ligand exchnage Cu(II) complex of L-Phe, L-Pro, L-Trp, L-His, respectively. Mobile phase (ii) consisting of binary mixture of MeCN and TEAP buffer of pH 4.0 (60:40 *v/v*) in isocratic mode at a flow rate of 1.0 mL/min; column, Spherisorb C_{18} (L x I.D., 25 cm × 4.6 mm, 5 µm particle size).

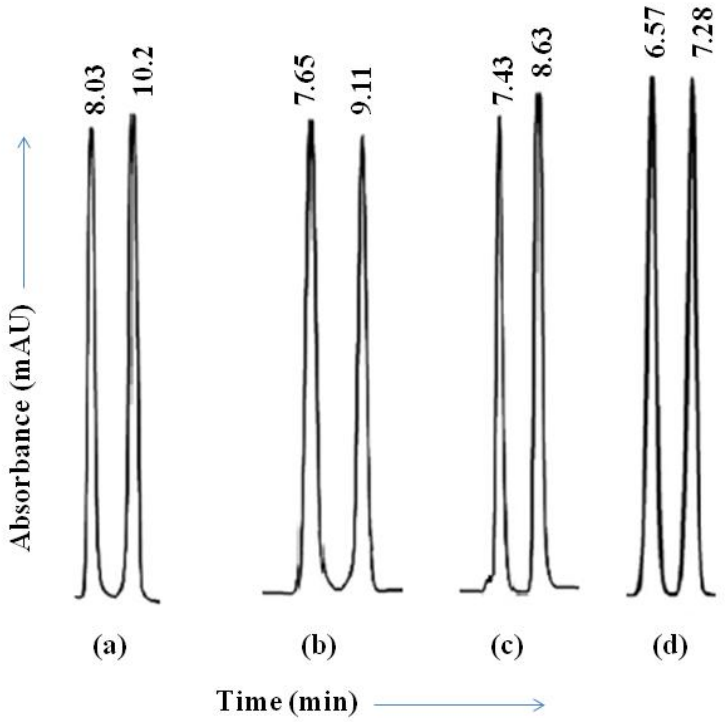


Fig. 6.4 Sections of chromatograms showing resolution of (*RS*)-Etd, (a)-(d) represent the diastereomers of (*RS*)-Etd prepared with chiral ligand exchnage Cu(II) complex of L-Phe, L-Pro, L-Trp, L-His, respectively. Mobile phase (ii) consisting of binary mixture of MeCN and TEAP buffer of pH 4.0 (60:40 *v/v*) in isocratic mode at a flow rate of 1.0 mL/min; column, Spherisorb C_{18} (L x I.D., 25 cm × 4.6 mm, 5 µm particle size).

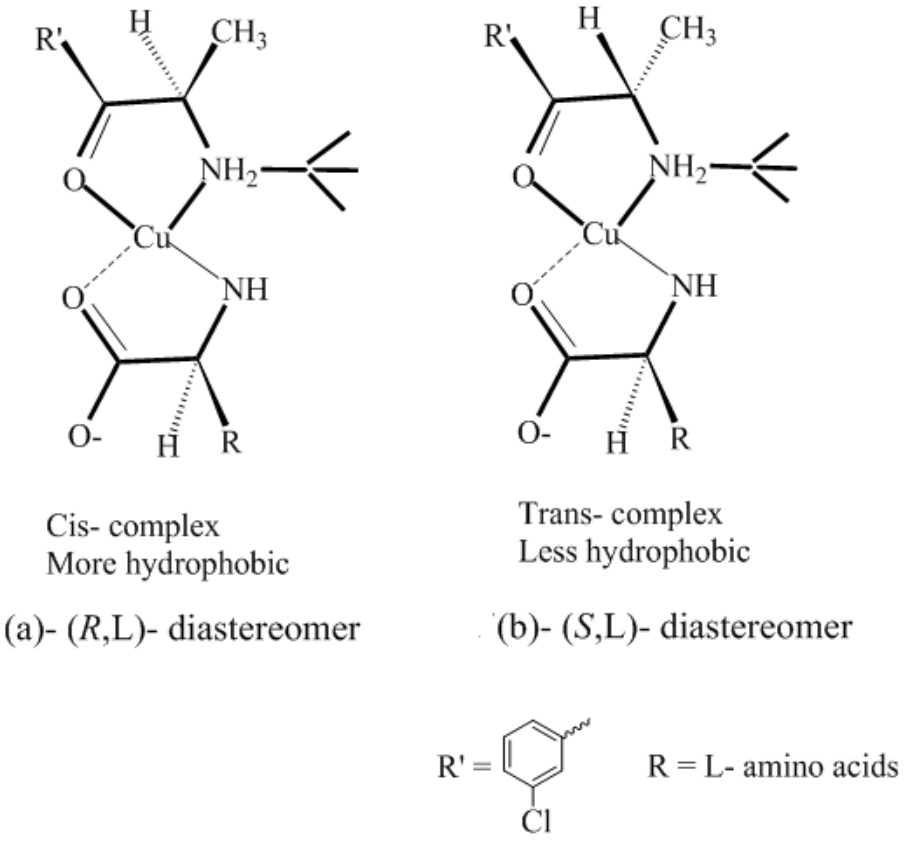


Fig. 6.5 Graphical representation of *cis* and *trans* Ligand Exchange Complexes

Validation studies

Linearity

A range of 40-100 ng/mL was chosen for drawing linearity plot (peak area vs. concentration of diastereomer, ng/mL) and the slope and intercept were determined using linear regression equations. 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. The regression equations were, $y = 10.72x + 198$ ($r^2 = 0.990$) and $y = 10.54x + 192$ ($r^2 = 0.994$) for the first and second eluted diastereomer, respectively.

Accuracy and precision

The accuracy and precision studies were carried out by replicate HPLC analysis (n=3) of diastereomers of (*RS*)-Bup at four different concentrations (40, 60, 80 and 100 ng/mL). Based on the peak areas of first and second eluting diastereomer the recovery and mean standard deviation (SD) for each of the diastereomer was calculated. These are shown in **Table-6.2**. The calculated relative standard deviation for first and second eluting diastereomer varied from 1.02 to 1.14% and 0.98 to 1.12% for intra-day precision and 0.97 to 1.81% and 1.0 to 1.68% for inter-day precision. The calculated recovery for first and second eluting diastereomer, respectively, varied from 97.6 to 99.5% and 97.9 to 98.8% for intra-day and 97 to 98.9% and 97.1 to 98.2% for inter-day.

Limit of detection and limit of quantitations

The LOD and LOQ of the method were found to be 0.2ng/mL and 0.6ng/mL respectively. LOD and LOQ are terms used to describe the smallest concentration of an analyte that can be reliably measured by an analytical procedure. The LOD and LOQ of both the diastereomers were determined by signal to-noise (S/N) ratio method by injecting a series of diluted solutions of mixtures of diastereomers. For LOD and LOQ the S/N ratios were 3:1 and 10:1, respectively.

Conclusion

Since the formation of labile ternary metallic complexes is not allowed to be formed in the mobile and/or in the stationary phase during the process of separation the method may be designated as indirect ligand exchange chromatographic (ILEC) separation. The method demonstrates the use of copper(II) as a central complexing ion source for enantioseparation of (*RS*)-Bup, (*RS*)-Bac and (*RS*)-Etd in silica-based reversed phase HPLC columns. The developed ILEC technique showed significant improvement over conventional ligand exchange chromatography in terms of reduction in amount of chiral selector and concentration and thereby overcoming the detection and sensitivity problems which are serious limitations with CLEC due to larger background noise encountered when Cu(II) is used in the mobile phase. This HPLC enantioseparation represents an attractive, simple and easy to perform option.

Table 6.2: Summary of HPLC method validation data obtained for (*RS*)-Bup with Cu(II)-L-Phe complex.

Concentration (ng/mL)	First eluting diastereomer*			Second eluting diastereomer*		
	Mean ± SD (ng/mL)	RSD (%)	Recovery (%)	Mean ± SD (ng/mL)	RSD (%)	Recovery (%)
Intra-day precision (n=3)						
40	19.7 ± 0.21	1.10	98.7	19.7 ± 0.20	1.04	98.6
60	29.2 ± 0.31	1.08	97.6	29.4 ± 0.32	1.12	98.1
80	39.6 ± 0.45	1.14	99.2	39.5 ± 0.42	1.08	98.8
100	49.7 ± 0.50	1.02	99.5	48.9 ± 0.48	0.98	97.9
Inter-day precision (n=3)						
40	19.6 ± 0.35	1.81	98.1	19.5 ± 0.19	1.00	97.7
60	29.1 ±0.45	1.58	97.0	29.4 ± 0.49	1.68	98.0
80	39.5 ± 0.38	0.97	98.8	39.2 ± 0.59	1.52	98.2
100	49.4 ± 0.68	1.38	98.9	48.5 ± 0.68	1.41	97.1

- **First eluting diastereomer*(*R*,L) and Second eluting diastereomer* (*S*,L)**

The concentration values listed in column 1 refer to the racemate.

Section B: TLC Enantioseparation of (*RS*)-etodolac using both achiral phases

Introduction to (*RS*)-Etd (**Fig.3.1**) with respect to its pharmaceutical importance and the literature survey on its enantioseparation by both direct and indirect approaches has already been discussed in Chapter-3. Nevertheless, literature reveals that there is no previous report on TLC separation of (*RS*)-Etd by using either a chiral stationary phase or using chiral additive to mobile phase or attempting TLC enantioseparation of diastereomers of (*RS*)-Etd.

II. Present Work

The sample of (*RS*)-Etd to be resolved was prepared by mixing the 'racemic analyte' and the '*chiral inducing reagent*' prior to application on plain silica gel plates (without any chiral selector as an impregnating reagent) and the chromatograms were developed in solvent systems having no chiral additive. Thus, both the phases at the time of chromatographic separation were achiral. L-amino acids (namely, L-Trp, L-Phe, L-His, and L-Arg) were used as '*chiral inducing reagents*'. Spots were located in iodine chamber. Effect of concentration of chiral inducing reagent and temperature on enantioresolution was studied.

1. Experimental

The details of chemicals, reagents, solvents and the equipment used have been described in **Chapter-2**.

Preparation of thin layer plates and solutions

The details of preparation of TLC plates have already been described in **Chapter-2**. Solution (10^{-2} M) of (*RS*)-Etd was prepared by dissolving it in MeOH, and solution of L-Trp, L-Phe, L-His, and L-Arg (0.5%) were prepared by dissolving them in purified water. All solutions were filtered through a 0.45 µm filter. The stock solution of (*RS*)-Etd was diluted to 10^{-3} M and was scanned by UV–visible spectrometer to determine λ_{max}. Solutions in the range 0.5×10^{-4} to 5×10^{-4} M were prepared by dilution. Their absorbance was recorded and calibration plot was constructed.

TLC on Plain Plates without Chiral Mobile Phase Additive

Solutions of L-Trp and (*RS*)-Etd were mixed in a mole ratio of (1:1) to be used as the sample for analysis. Similarly, other three sets of samples were prepared by using the rest of the CIRs. These were spotted (5 μL) on plain plates (having no chiral impregnating reagent in the slurry) using Hamilton syringe, 2 cm above the margin. Chromatograms were developed, with solvent systems having no chiral additive, in cleaned, dried and paper-lined rectangular glass chambers that were pre-equilibrated with the same solvent for 15 min at different temperatures (16, 20, 24, 28 and 32 °C). After development, chromatograms were dried at 40 °C in an oven for a few minutes. The spots were located by using iodine vapor.

Isolation of the Resolved Enantiomers

The two spots representing (*R*)-, and (*S*)-enantiomers of Etd were marked and the iodine was then allowed to evaporate off. Silica gel of each of the spots was scraped (from nearly 30 chromatograms); the combined silica gel for each spot was extracted with MeOH. The combined extracts for each of the enantiomers of Etd were filtered and concentrated under vacuum. These were then dried by lyophilization and the residues were dissolved in MeOH. Similarly, the enantiomers so separated by using the other three CIRs were extracted and isolated. Concentration for each of the extracts was determined using calibration plot. These solutions were examined by polarimeter and their specific rotation was recorded/calculated using the concentration determined, as described above.

Effect of temperature

To study the effect of temperature on resolution of (*RS*)-Etd, the chromatographic chamber was pre-equilibrated with mobile phase at different temperatures, 16, 20, 24, 28 and 32 °C for 10‑15 min, considering 28 °C to be the room temperature. Each temperature was maintained inside an incubator. The developed chromatograms were dried at 40 °C in an oven for a few minutes and then cooled to room temperature; spots were located in an iodine chamber. The effect of temperature was noted in terms of figure of spots as eight shaped, or tailing or clear resolution.

2. Results and Discussion

Since Etd is marketed and administered as racemic mixture it was considered worthwhile to examine the commercial sample. Moreover, the samples supplied by the standard reagent suppliers are extremely expensive. Therefore, (*RS*)-Etd was isolated, by extracting with MeOH, from commercial preparations available in the market in the racemic form. It was purified by recrystallization, and characterized by determining melting point (145–148 °C), and λ_{max} (223 nm) and recording IR spectrum (KBr) showing peaks corresponding to 1705 (-C = O), 1593, 1570 (aromatic C= C) and 3469 (-NH) cm^{-1}. Recovery of the sample was 92% against the labeled amount on commercial tablets. The characterization data was in agreement with the literature report.

Resolution of enantiomers of (*RS*)-Etd

This is the first TLC separation of (*RS*)-etodolac (using direct approach). The direct approach has been adopted in a manner different than the earlier reported methods of impregnating the TLC plate with chiral selector [138,139] or using the chiral selector as mobile phase additive [140,141]. The different approach presented in this chapter has been looked into in terms of '*a conceptual approach with both achiral phases*'. It is because both the phases at the time of chromatographic separation were achiral and the 'chiral selector' was used prior to development of chromatogram and that too without formation of a covalent bond between Etd and one of the chiral amino acids.

This approach makes the overall process simpler in comparison, particularly, to a process of impregnation [138,139] which is carried out by different methods to make chiral environment in one of the chromatographic phases (particularly, the stationary phase).

hR_F (R_F x 100) values of the two enantiomers resolved with the help of different CIRs are shown in **Table 6.3**. R_F values are average of at least five experiments performed on the same day and on different days. The resolution was calculated by dividing the distance between two spots by the sum of two spot radii; a value of 1.50 was taken as an indication of complete resolution and the two spots were considered to have reasonably separated when R_S =1.2 [**104**].

Experiments were carried out with binary, ternary and quaternary mixtures of a variety of solvents (in different ratios) such as MeCN, MeOH, H_2O, DCM, and $CHCl_3$ to achieve enantiomeric separation. Only the finally successful solvent combinations showing the best resolution are reported. The solvent system MeCN-DCM-MeOH in different ratio (**Table 6.3**) was found to be successful for enantioresolution of (*RS*)-Etd. Additional experiments to investigate the effect of change of pH and temperature on enantiomeric resolution were carried out for the successful solvent systems only. The highest R*s* value was obtained when MeCN-DCM-MeOH were in the ratio of 6:2:4, (*v*/*v*) and L-Trp was the CIR.

Photographs of the actual chromatogram showing TLC resolution of (*RS*)-Etd using L-Trp and L-Phe, as CIR are given as **Fig. 6.6. Fig. 6.7** shows the photographs of the chromatograms for resolution of (*RS*)-Etd using L-His and L-Arg as CIR.

The silica gel belonging to each of the two spots (representing the two enantiomers of Etd) was scrapped and extracted with MeOH. It was expected that only (*R*)-, or (*S*)-Etd would go into the solution because L-amino acids are insoluble in MeOH. For each extract, optical density was measured at the λ_{max} and the concentration was estimated by use of calibration plots (developed by using the standard reference as the UV (λ_{max}); the racemate and any of the single enantiomers have the same UV absorbance. Concentration (and thus the recovery of the enantiomers from the chromatograms) was estimated with the help of linearity plot based on absorbance and concentration (as described above). Recovery for enantiomers was within the range of 93 to 96 %. These solutions were examined by polarimeter, and their specific rotations were calculated using the concentration determined, as above. The specific rotation of (*S*)-Etd so isolated was found to be, $[\alpha]_D^{25}$ = + 23.96° (c= 3, C_2H_5OH); (*R*)-Etd isolated was found have, $[\alpha]_D^{25}$ = –23.87° (c= 3, C_2H_5OH). The values were in agreement with literature. The polarimetric experiments suggested that the (*S*)-isomer had higher R_F than the (*R*)-isomer and the two isomers were in 1:1 ratio. Thus the resolution of racemic mixture gets confirmed from the isolation experiments (as described above). The enantiomers so isolated were further used as reference samples in a second set of TLC experiments in which they were applied in parallel to the racemic mixture (treated with CIR, and

applying the same chromatographic conditions described above); the R_F values were found in agreement with those separated from the mixture.

Based on the above mentioned results and the earlier explanation available in literature [112, 113], following explanation can be contended for resolution of racemic Etd. Instead of '*mixed homo-/ heterochiral high-order species*' with different retention times there occurred '*preferential formation of high-order (enantiomeric) associates*', of the type [(*S*)-Etd–(L)-Trp] and [(*R*)-Etd–(L)-Trp]; thus initial racemate transformed into mixture of (transient) diastereomers with different chromatographic mobilities in achiral phases and hence resolution. The formation of *diastereomeric associates* has been considered. The native enantiomer has been isolated it can be concluded that the retention factor for the additive is the same as it is for the enantiomer of the analyte only the difference in the retention factors is for the diastereomers.

Table 6.3: Successful solvent systems for resolution of (*RS*)-Etd and separation data using L-amino acids as CIR.

CIR	SolventSystem (*v/v*)	hR_F separated from (*RS*)-Etd		Rs
		(*R*)	(*S*)	
L-Trp	MeCN-DCM-MeOH (6:2:4)	33	62	2.17
L-Phe	MeCN-DCM-MeOH (6:1:3)	61	77	1.29
L-His	MeCN-DCM-MeOH (5.5:1.5:3)	67	88	1.38
L-Arg	MeCN-DCM-MeOH (6: 3:3)	39	52	1.75

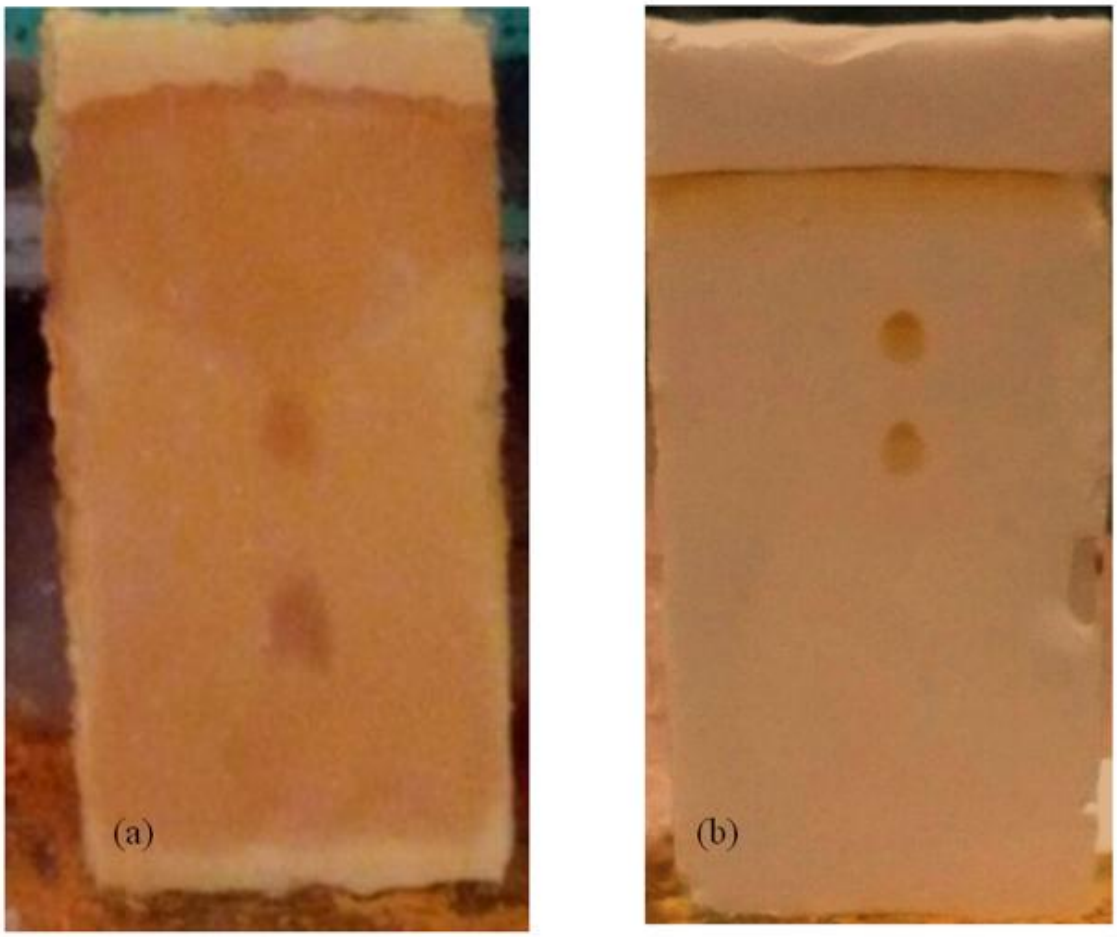


Fig. 6.6 Photographs of actual chromatograms showing resolution of (*RS*)-Etd using (a) L-Trp, and (b) L-Phe, as CIR. Successful solvent systems and separation data are given in Table 1. Lower spot is of (*R*)-enantiomer and upper spot is of (*S*)-enantiomer.

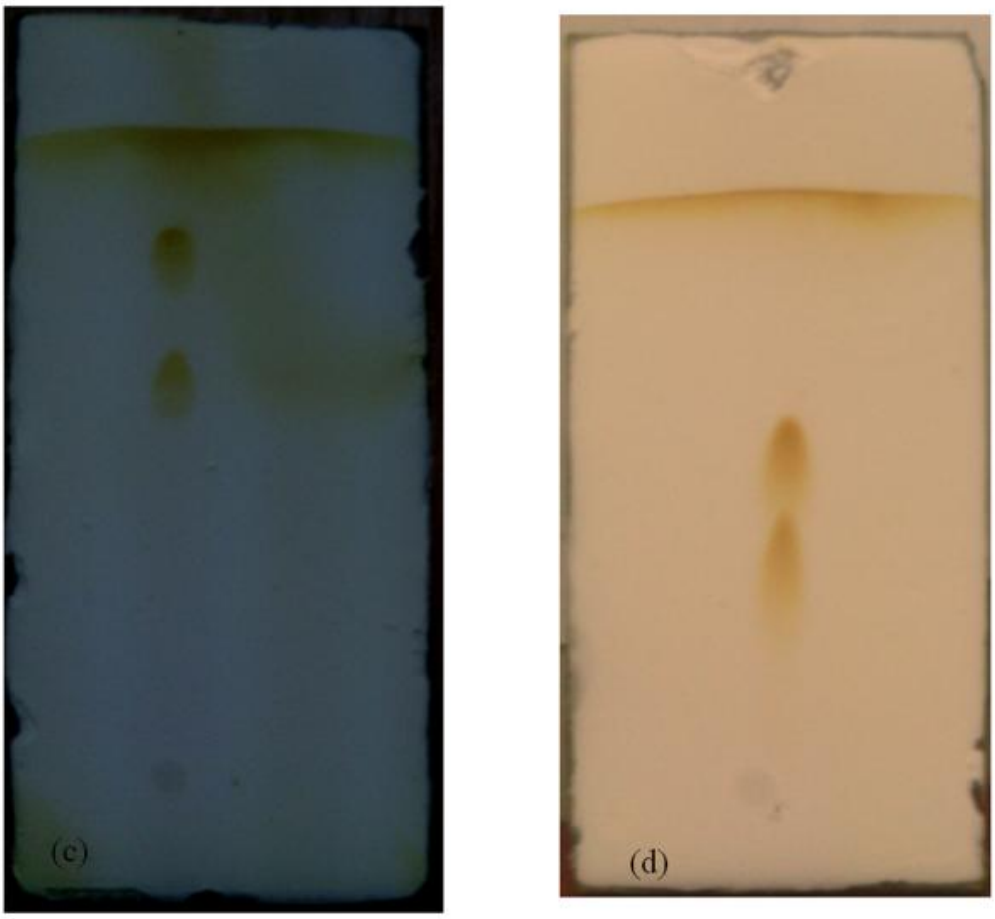


Fig. 6.7 Photographs of actual chromatograms showing resolution of (*RS*)-Etd using (c) L-His, and (d) L-Arg, as CIR. Successful solvent systems and separation data are given in Table 1. Lower spot is of (*R*)-enantiomer and upper spot is of (*S*)-enantiomer.

The formation of diastereomers via non-covalent reversible interactions is thus confirmed by the following results (i) isolation of two enantiomers from TLC plates, (ii) a positive test for the presence of amino acid (used as CIR) in both the spots (resolved under achiral phase chromatographic conditions, (iii) when one set of the chromatogram was sprayed with ninhydrin, each resolved spot was visible in characteristic pink-purple colour with appropriate intensity and sharpness for the presence of amino acid (used as CIR), and (iv) when TLC was performed using the racemic analyte without any CIR on plain plates and the chromatograms were developed under the identical experimental conditions (i.e., there was no chiral selector in both the stationary and the mobile phase), there was obtained a single spot. These confirmed that the presence of CIR was playing a role as a necessary requirement for enantiomeric resolution. The effect of pH and temperature was investigated on the formation of diastereomers (formed through noncovalent interactions); the best results for enantioresolution of (*RS*)-Etd were obtained at pH 8 and 28± 2 °C.

Several laboratories use commercial precoated TLC plates. However, such precoated plates are several times (may be twenty times) more expensive than the plates prepared in the laboratory; everybody cannot afford. Moreover, mixing of chiral selector, with the silica gel, as the impregnating reagent is not possible with the precoated plates for direct enantioseparation, if required. The method presented herein provides a simple, rapid and effective approach in planar mode for separation and control of enantiomeric purity of Etd and certain other pharmaceuticals (taking into account the functional group compatibility) which can be realized even in a small laboratory. Certain previously published results suggest that there is no significant difference in the separation reproducibility of home-made plates and commercial pre-coated plates.

Effect of mole ratio of (*RS*)-Etd to amino acids

The ratio of (*RS*)-Etd to CIR was optimized by using different ratios and it was found that among the several possible combinations a ratio of 1:1 gave the best result because this proved to be the required ratio for the formation of diastereomers.

The method presented herein was found to be successful for direct and sensitive resolution of (*RS*)-Etd from racemic mixture under achiral phases of chromatography and for detection of their enantiomers in a range lower than the limits prescribed (1%)

for pharmaceuticals in industry. This approach was also found successful to separate non-racemic mixture of (*R*)-, and (*S*)-enantiomers of Etd in the ratio of 1:99.

Support to the proposed mechanism

In the present studies two 'diastereomeric associates' of the type [(*S*)-Etd–(L)-Trp] and [(*R*)-Etd–(L)-Trp], (for example, with L-Trp used as CIR) are formed which undergo a different adsorption process on the silica gel; these diastereomers are formed via non-covalent reversible interactions. The polarimetric studies showed that (*S*)-isomer had higher R_F than the (*R*)-isomer, it means that R_F of [(*S*)-(L)]-diastereomer is greater than the R_F of corresponding [(*R*)-(L)]-diastereomer. It can thus be argued that [(*R*)-(L)]-diastereomer represents *hetero-chiral enantiomeric associate* [112, 142, 143] which is preferentially formed and is more stable and has stronger interactions with the column material and is retained for longer time in comparison to [(*S*)-(L)]-diastereomer (representing *homo-chiral enantiomeric associate*).

Most of the reports on separation of non racemic mixtures under achiral conditions have been covered and discussed in the reviews mentioned above [112, 113]; among various explanations based on experimental studies there have been included theoretical models which suggested that such enantioresolution occurs when *associations* of the *dimer type* takes place between the molecules to be resolved [144, 145]. Considering these aspects, in the present case too, it can be contented and explained that *self-association* occurring on the stationary phase with consequent stereoselective interactions result into an excess of one enantiomer along with dissymmetry in both phases and, therefore, the stabilities of *hetero-* and *homoenantiomeric associates* (say, dimers) were unequal. The separation starts because of the creation of an anisometric medium and dissymmetric system by the presence of existing enantiomeric excess.

III. Conclusion

The prevailing concept of stereochemistry does not consider 'resolution' or 'enantioseparation' to be practically possible in achiral environment. Use of both achiral phases (in chromatography) for enantioresolution is itself a 'conceptually different approach'. Nevertheless, it required chiral reagent to induce the formation of

diastereomers but at a different stage and in a different manner. Since TLC provides documented evidence in the form of photographs the concept of direct enantioresolution with both achiral phases gets support. And still it is different from the concept of enantioseparation from non racemic mixture under achiral conditions as reported in literature [112]. The advantages of the reported method are, (i) it is successful in resolving racemic mixture under achiral phases of chromatography and can be easily applied for small scale preparative separation too, (ii) it is direct approach of separation and prior formation of a covalent bond between any of the enantiomers of etodolac and one of the chiral amino acids is not required, (iii) it does not require verification of separation by chiral HPLC (or any other supplementary method) in contrast to literature report on '*conceptually new approach*' for '*resolution of racemic mixture'* by *'achiral chromatography'* [113], (iv) it is very simple and inexpensive and is capable of isolating native enantiomers, and (v) it may be helpful in quality control of pharmaceutical formulations and could be practiced both in analytical laboratories and industry for routine analysis and R&D activities.

Chapter-7
Enantioresolution of chiral carbonyl compounds

I. Introduction

It can be observed that many biologically active compounds such as drugs, steroids agrochemicals contain carbonyl group and are chiral in nature. Chiral ketones are the important building blocks, intermediate and play important role in asymmetric synthesis [146]. Compounds having a primary amino group cause a nucleophilic addition at the electron deficient carbonyl carbon of aldehydes and ketones (with a subsequent condensation step to replace the carbonyl oxygen by nitrogen (>C=O ⟶ >C=N + H_2O). Notable among such reagents are phenyl-, *p*-nitrophenyl, and 2,4-dintrophenyl-hydrazines which are used much more often and give the corresponding hydrazones with most aldehydes and ketones [147]. These make excellent derivatives as sharp melting solids (and having characteristic IR spectra), useful for characterization of the parent aldehydes or ketones.

Such derivatizations (including D-exchange and bromination) proceed via enol formation which is rate determining (and are generally acid catalyzed). Ketones offer a choice of enolization in two directions. In saturated molecules, the enolate from the less substituted side is favoured in base catalysed reaction while in acid the more substituted enol is preferred. It is the more resonance-stabilized enol or enolate that is always preferred.

Enolization causes inversion of configuration at the asymmetric α-carbon to carbonyl since the enol has no asymmetry. The most stable configuration at the α-carbon will result from equilibration by enolization. In addition reactions to carbonyl carbon, the asymmetry present on the carbon α- to carbonyl makes the less hindered side of the carbonyl π-orbital more accessible and may cause spontaneous configurational inversion resulting into enantiomeric mixtures (which are often not predictable). Oxidative hydrolysis of hydrazones, semicarbazones and phenylhydrazones with sodium hypochlorite has been reported [148].

Enantioresolution of chiral aliphatic or alicyclic aldehydes and ketones by direct approach using liquid chromatography would require the presence of a suitable chromophore for on-line detection in UV-visible region. Among the various

nucleophilic reagents containing amino group 2,4-dinitrophenyl hydrazine is the one that could act as an achiral strong chromophore for carbonyl compounds for its DNP moiety.

DNP-derivatives: Since the pioneering work of Sanger [149] on the preparation of dinitrophenyl (DNP) derivatives of amino acids their use for sequence analysis has declined in the modern times. Nevertheless, DNP moiety attracted attention for its application, as a strong chromophore, for synthesis of several chiral derivatizing reagents (CDRs) which have been used for separation and detection of diastereomers of a variety of pharmaceutically important racemic compounds [33,34]. Derivatization reaction of acetaldehyde with 2,4-dinitrophenylhydrazine (DNP) has been used for assessment of acetaldehyde levels in clinical and experimental studies of ethanol metabolism; the samples from blood and tissue were reacted with a methanolic solution of 2,4-dinitrophenylhydrazine (DNP) and DNP-[^{14}C]formaldehyde was added as internal standard and the acetaldehyde adduct (after extraction and purification) was identified by co-chromatography with the authentic derivative and by mass spectrometry [150].

Enantioresolution of chiral carbonyl compounds: Literature reveals sporadic reports on enantioresolution of chiral carbonyl compounds involving application of DNP moiety or hydrazone derivative. These include indirect enantioresolution using CDRs developed from 1,5-difluoro-2,4-dinitrobenzene [151] wherein the DNP moiety serves as a chromophore for on-line detection of the corresponding diastereomers. CSP derived from (*S*)-1-(6, 7-dimethyl-1-naphthyl) isobutylamine was used for resolution of cyclic and acyclic chiral ketones as their oxime 3,5-dinitrophenyl carbamates [152]. A chiral phosphorylhydrazine reagent was used to prepare hydrazone diastereomers of chiral ketones which were analyzed by ^{31}P NMR and HPLC [153].

Some other reports include resolution of chiral cyclic ketones by direct approach using CSPs based on amylose *tris*(3,5-dimethylphenyl carbamates) and cellulose *tris*(3,5-dimethylphenyl carbamates) [154], cellulose tribenzoate [155], and β-cyclodextrin [156, 157]. Enantioresolution of Wieland-Miescher ketones, their C(5) homologue, and their C(1) dioxolane derivatives has been reported [158] using commercially available CSPs

like cellulose *tris*-(3,5-dimethyl-phenylcarbamate), native β-cyclodextrin, and acetylated, carboxymethylated and permethylated β-cyclodextrins. Direct enantioresolution of Mannich ketones has been achieved by using aqueous copper (II) acetate and L-aspartame [159] and, cellulose and cyclodextrin derivatives [160] as chiral mobile phase additives.

Thus, it is evident that till now the scientific issue with respect to spontaneous configurational inversion of any chiral carbonyl compound (used either as an enantiomerically pure sample or a racemic mixture) undergoing derivatization with an amino group containing nucleophilic reagent has not been investigated.

II. Present work: The objective of the present work has been to develop method of synthesis of derivatives (DNP-hydrazones in the present case) of certain didactic racemic carbonyl compounds (four ketones and two aldehydes (**I-VI**, **Fig. 7.1**) which would proceed without spontaneous chiral inversion, and to establish 'racemic' and 'non-racemic' composition of the products obtained under newly developed method, and the products obtained by derivatization of the same racemic carbonyl compound under conventional acidic conditions. To achieve the objective, (a) solid phase microwave-assisted conditions were developed for synthesis of 2,4-dinitrophenyl hydrazone(s) (DNPHz) of certain didactic chiral carbonyl compounds, and (b) the DNPHz derivatives of racemic carbonyl compounds were then resolved by chiral HPLC into enantiomers using α_1-AGP column. The emphasis was not on developing a method for enantioseparation but it was to verify the 'racemic' and 'non-racemic' nature of the product. And the novelty of the present work lies in the above said two aspects.

1. Experimental

The details of chemicals, reagents, solvents and the equipment used have been described in **Chapter-2**.

Synthesis and characterization of dinitrophenyl hydrazones (2,4-DNPHz)

Solid phase microwave-assisted approach

Representative synthesis of 2,4-DNPHz of (±)-*3-methyl-2-pentanone* and characterization data of all the resulting six hydrazones is given below.

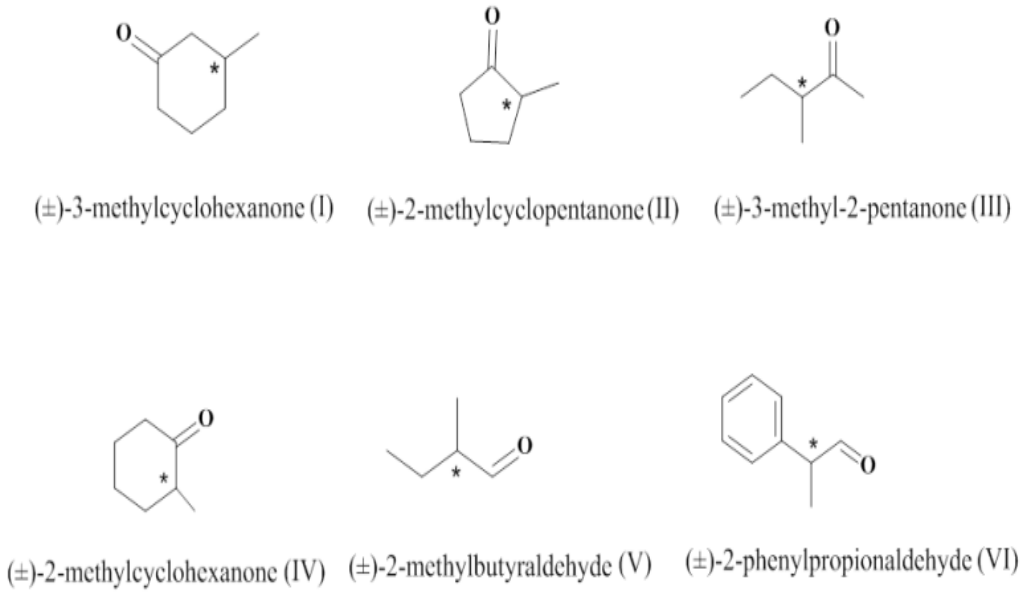


Fig. 7.1 Structures of chiral aldehydes and ketones

Synthesis of 2,4-DNPHz *of* (±)-*3-methyl-2-pentanone* **(3)**:

2,4-DNPH (0.019 g; 10 mmol) and (±)-*3-methyl-2-pentanone* (0.010 g; 10 mmol) were dissolved in MeOH (10 mL) followed by addition of silica gel (6 g) to this solution. After about 20 minutes, the solvent was evaporated and the silica gel (on which the two reactants were adsorbed) was irradiated with MW in an oven at 500 W for 4 min (with 1 min interval). The MW irradiated silica gel was then stirred in ethyl acetate (10 mL) for 10 min and then filtered. The residual silica gel was washed twice with 5 mL ethyl acetate; the combined extract was concentrated under the stream of nitrogen and was left for crystallization. The yields were in the range of 91 – 95%. The hydrazones obtained, as the product, under experimental conditions (i) were designated as (**1**-**6**). These derivatives were analysed by chiral HPLC.

Conventional acid catalyzed synthesis

2,4-DNPH (0.019 g; 10 mmol) was dissolved in 10 mL MeOH, in a small conical flask; as a representative, solution of (±)-*3-methyl-2-pentanone* (0.010 g; 10 mmol) in 10 mL MeOH was added to it. Concentrated sulphuric acid was then added drop by drop with constant stirring till pH 4 was obtained. The reaction mixture was allowed to stand for 10 min. Formation of corresponding derivative as 2,4-DNPHz occurred during this time. It was filtered and recrystallized from MeOH. The yields were in the range of 78 – 82%. The hydrazones obtained, as the product, under experimental condition (ii) were analysed by chiral HPLC.

Characterization of the hydrazones

Melting points were determined in open ended glass capillaries and were uncorrected. The hydrazones were characterized by IR, ^{1}H NMR and CHN analysis; the data is given below.

- 2,4-DNPHz *of* (±)-*3-methylcyclohexanone* **(1)**: Color: yellow; mp 103±2 ^{o}C; UV ($\lambda_{max,}$ 365 nm, MeOH): 365; IR (KBr): 3308 (NH), 1617 (C=N), 1590 (Ar); ^{1}H NMR: δ 9.15 (1H, s, ArH), δ 8.31-8.30 (1H, d, ArH), δ 8.00-7.98 (1H, d, ArH), δ 1.07 (3H, d), δ 2.07 (1H, m), δ 1.36 (2H, m), δ 1.98 (2H, m), δ 1.58 (2H, m), δ 11.2 (1H, s); Anal. Calcd for $C_{13}H_{16}N_4O_4$: C, 53.42%; H, 5.52%; N, 19.17%. Found: C, 53.22%; H, 5.30%; N, 19.08%.
- 2,4-DNPHz *of* (±)-*2-methylcyclopentanone* **(2)**

Color: yellow; mp 105±2^{o}C; UV ($\lambda_{max,}$ 363 nm, MeOH); IR (KBr): 3416 (NH), 1619 (C=N), 1511 (Ar); ^{1}H NMR: δ 10.8 (1H, s), δ 9.15 (1H, s, ArH), δ 8.31 (1H, d, ArH), δ 8.01-7.99 (1H, d, ArH), δ 1.28 (3H, d), δ 2.76 (2H, m), δ 2.10 (2H, m), δ 2.24 (2H, m), δ 2.41 (1H, m); Anal. Calcd for $C_{12}H_{14}N_4O_7$: C, 51.80%; H, 5.07%; N, 20.13%. Found: C, 51.62%; H, 4.95%; N, 19.99%.

- 2,4-DNPHz *of* (±)-*3-methyl-2-pentanone* **(3)**

Color: orange; mp 98±2^{o}C; UV ($\lambda_{max,}$ 361 nm, MeOH); IR (KBr): 3290 (NH), 1617 (C=N), 1516 (Ar); ^{1}H NMR: δ11.04 (1H, s), δ 9.13 (1H, s, ArH), δ 8.31- 8.29 (1H, d, ArH), δ 7.98 (1H, d,ArH), δ 2.51(1H, q), δ 1.98(3H, s), δ 1.66(2H, m), δ 1.18(3H, d), δ 0.93(3H, t); Anal. Calcd for $C_{12}H_{16}N_4O_4$: C, 51.42%; H, 5.75%; N, 19.99%. Found: C, 51.11%; H, 5.52%; N, 19.46%.

- 2,4-DNPHz *of* (±)-*2-methylcyclohexanone* **(4)**

Color: yellow-brown; mp 112±2^{o}C; UV ($\lambda_{max,}$ 364 nm, MeOH); IR (KBr): 3320 (NH), 1621 (C=N), 1586 (Ar); ^{1}H NMR: δ 9.14 (1H, s, ArH), δ 8.30-8.29 (1H, d, ArH), δ 8.00-7.98 (1H, d, ArH), δ 2.00 (1H, m), δ 1.06 (3H, d), δ 1.37 (2H, m), δ 1.86 (2H, m), δ 1.24 (3H, d), δ 1.58 (2H, m), δ 11.2 (1H, s) ; Anal. Calcd for $C_{13}H_{16}N_4O_4$: C, 53.42%; H, 5.52%; N, 19.17%. Found: C, 53.2%; H, 5.23%; N, 19.01%.

- 2,4-DNPHz *of* (±)-*2-methylbutyraldehyde* **(5)**

Color: yellow-brown; mp 95±2^{o}C; UV ($\lambda_{max,}$ 361 nm, MeOH); IR (KBr): 3287 (NH), 1621 (C=N), 1516 (Ar); ^{1}H NMR: δ 9.17 (1H, s, ArH), δ 8.30(1H, d, ArH), δ 8.00-7.98 (1H, d, ArH), δ 10.9 (1H, s), δ 1.21 (6H, d), δ 1.48 (2H, m), δ 9.87 (1H, d); Anal. Calcd for $C_{11}H_{14}N_4O_4$: C, 49.62%; H, 5.30%; N, 21.04%. Found: C, 49.43%; H, 5.21%; N, 20.98%.

- 2,4-DNPHz *of* (±)-*2-phenylpropionaldehyde* **(6)**

Color: yellow; mp 98±2^{o}C; UV ($\lambda_{max,}$ 362 nm, MeOH); IR (KBr): 3290 (NH), 1617 (C=N), 1518 (Ar); ^{1}H NMR: δ 9.30 (1H, s, ArH)), δ 8.31(1H, d, ArH), δ 8.00 (1H, d, ArH), δ 10.8 (1H, s), δ 7.28 (2H, m), δ 7.98 (2H, m), δ 7.26 (1H, m), δ 1.24 (3H, d), δ 2.59 (1H, m), δ 10.1 (1H, m); Anal. Calcd for $C_{15}H_{14}N_4O_4$: C, 57.32%; H, 4.49%; N, 17.83%. Found: C, 57.17%; H, 4.32%; N, 17.44%.

Chiral HPLC of reaction products

The composition of mobile phase for achieving enantioresolution was optimized by using binary mobile phase system consisting of citrate phosphate buffer (in the concentration range 5-25 mM, and pH 3.5-6.5) and 2-propanol (in the range 0.5% to 3.0%) or MeCN (in the range 1 to 5%) at a flow rate of 1mL/min. Mobile phase was filtered through a 0.45 μm filter and degassed by sonication and passing nitrogen before use. 20 μL of the sample was injected onto the column. Detection was at 365 nm.

2. Results and Discussion

The reaction of racemic carbonyl compounds (**I**) to (**VI**) with the reagent (2,4-DNPH) does not lead to formation of diastereomers as the reagent is achiral. There occurs '*tagging*' of enantiomers with a strong chromophore in the form of DNP moiety from 2,4-DNPH. The scheme showing synthesis of derivatives is given in (**Fig. 7.2**). The

hydrazones obtained, as the product, under experimental condition (i) were designated as (**1**) **to** (**6**) and are also shown in (**Fig. 7.2**). The reaction under condition (ii) requires nearly pH 4 for maximum rate while basic or highly acidic conditions lower the rate. In more strongly acid solution (pH < 3.5) the unshared pair of electrons (the nucleophilic site) of N is protonated and is no more a nucleophile [161]. It was interesting to observe that addition of sulphuric acid (till pH 4 is obtained) to the mixture of 2,4-DNPH and the carbonyl compound resulted into higher yield of the product hydrazone in comparison to an approach in which sulphuric acid was added at first to the solution of 2,4-DNPH followed by addition of the solution of carbonyl compound [162].

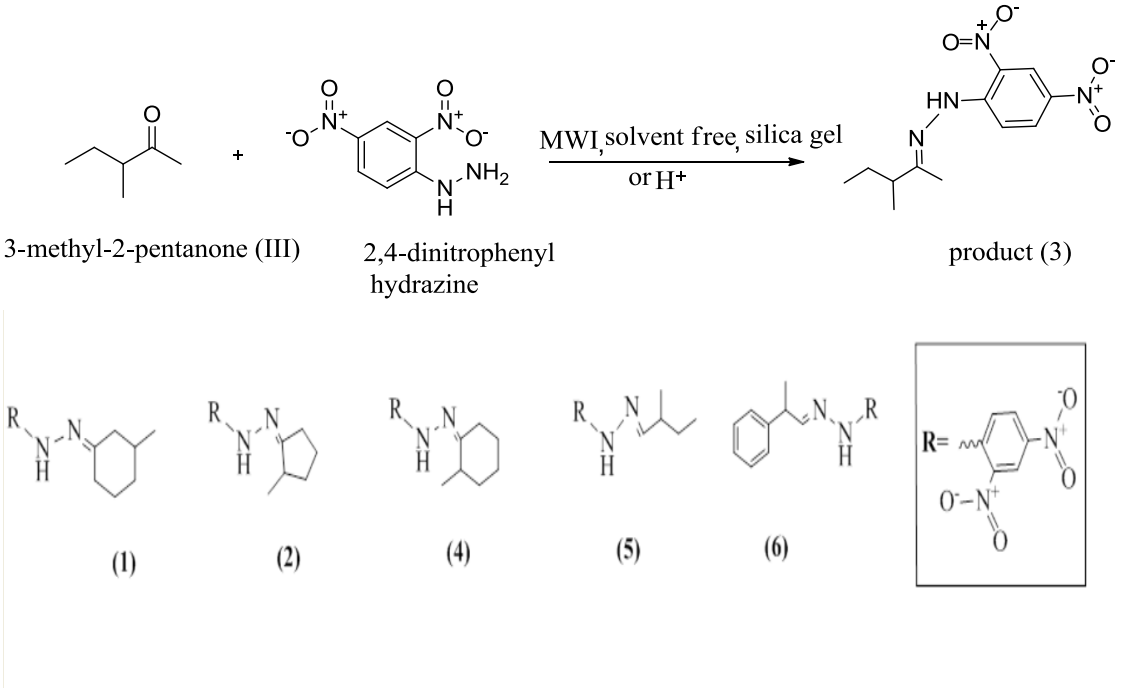


Fig. 7.2 Scheme showing synthesis of hydrazones

The problem or the question of spontaneous configurational inversion in each of the enantiomers (present in the racemic mixture of the carbonyls) cannot be overruled when the synthesis of hydrazones was taking place in acidic liquid medium (ii). As a result the ratio of the two enantiomers is expected to get disturbed resulting into possibly a non-racemic mixture of hydrazones of the chiral carbonyls under study. Configurational inversion would occur only when the asymmetric carbon (α- to carbonyl function) is involved in the formation of enol. The possible mechanism for enol formation and configurational inversion at the asymmetric α-carbon to carbonyl and the formation of

hydrazone in the subsequent condensation step (replacing the carbonyl oxygen by nitrogen) is shown in (**Fig. 7.3**).

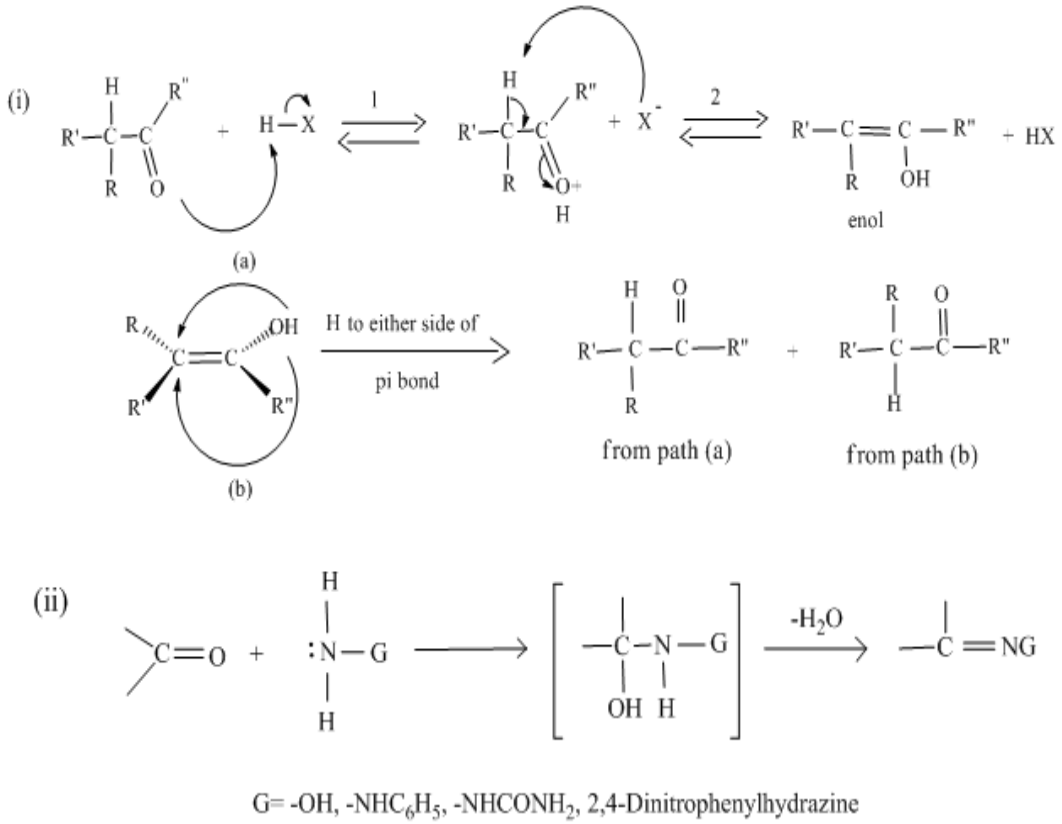


Fig. 7.3 The possible mechanism for the formation of hydrazones via enol formation

The characterization data based on IR, ^{1}H NMR and CHN analysis does not differentiate in the enantiomeric ratio of the products obtained from the two approaches, as the products are structurally and chemically the same. In order to investigate the issue of spontaneous configurational inversion chiral HPLC of the products obtained under conditions (i) and (ii) was performed; racemic and non-racemic nature of products (enantiomeric composition) was a decisive factor.

Determination of racemic and non-racemic nature of products by chiral HPLC

HPLC analysis of the products obtained by approach (ii) showed peaks with unequal areas and this observation led to inference that the product was non-racemic though the

reactant carbonyl compound was racemic in nature. Since the products are formed via enol and the enol (formed in rate determining step involving asymmetric carbon α- to carbonyl group) is a planar moiety it receives H from either side (side 'a' or side 'b' shown in (**Fig. 7.3)**, depicting mechanism) configurational inversion occurs and the product hydrazone is a non racemic mixture. It was, therefore, contended that spontaneous inversion of configuration was taking place during derivatization under acidic conditions. A representative chromatogram with unequal areas corresponding to the products of the analyte (**III**) is shown in **Fig. (7.4**). The peak areas were 386 and 691 mAU at retention time 9.09 and 11.32 min, respectively. Peaks with unequal areas indicate non-racemic nature.

It was further confirmed by the observation that the products corresponding to analyte (**I**), i.e., (±)-*3-methylcyclohexanone* did not show peaks with unequal areas. It was because in the molecule (**I, Fig. 7.1**) the carbon α- to carbonyl is not asymmetric and it is not involved in the formation of enol.

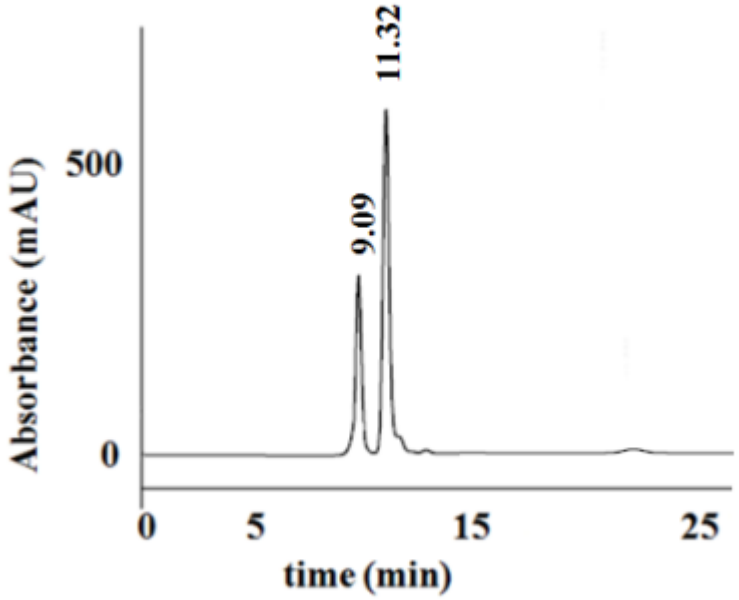


Fig. 7.4 Full chromatogram (as representative) showing resolution of product (3) obtained by approach (ii). The peak areas were 386 and 691 mAU at retention time 9.09 and 11.32 min, respectively. Peaks with unequal areas indicate non-racemic nature

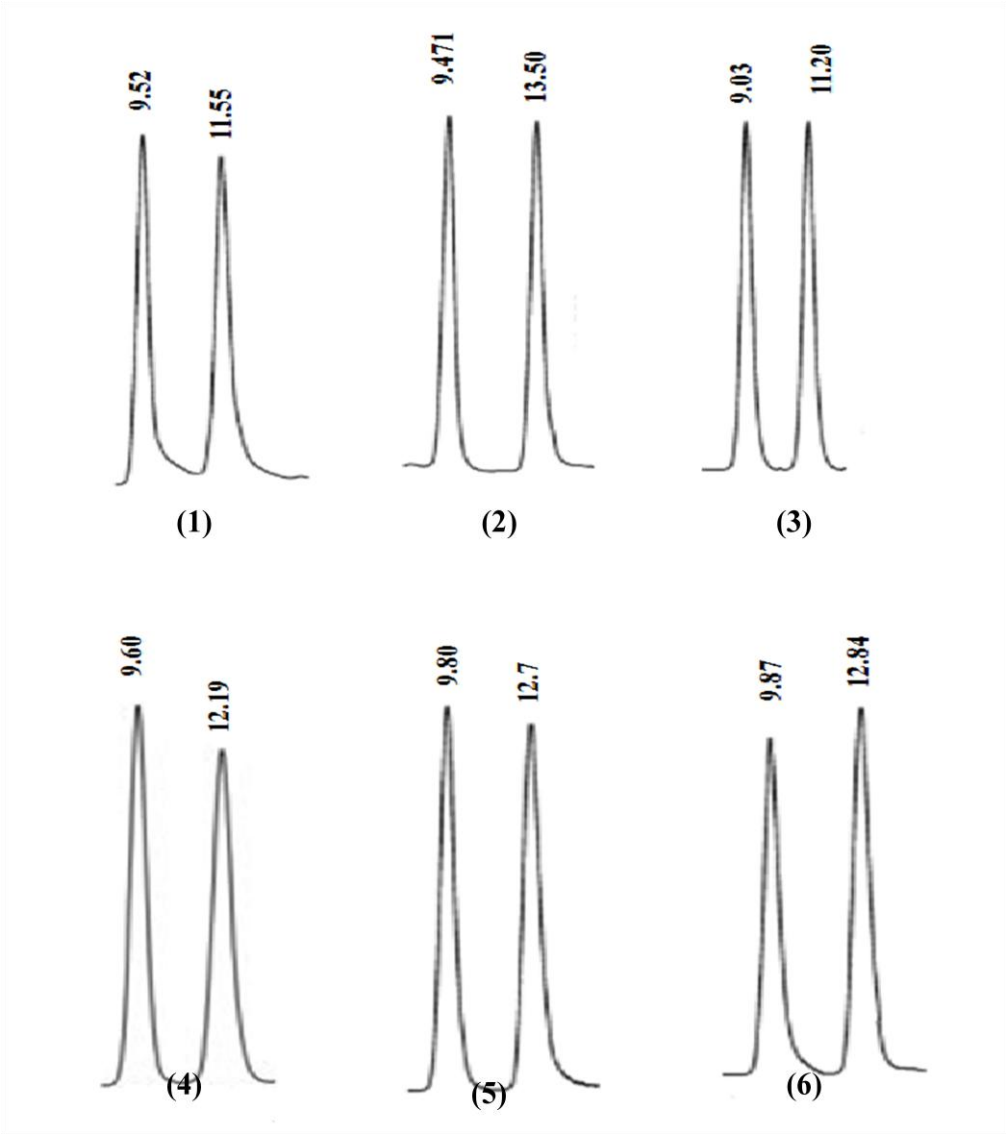


Fig. 7.5 Sections of chromatograms showing resolution of enantiomeric pairs of six 2,4-DNPHz (retention times are in minutes). Column, α_1-AGP (L x I.D, 10 cm x 4 mm, 5 µm particle size); mobile phase, 0.5% 2-propanol in 10 mM citrate phosphate buffer at pH 6.5; flow rate, 1.0 mL/min; detection, 365 nm.

On the other hand, the chromatograms corresponding to the products obtained under solid state microwave-assisted conditions, approach (i), clearly showed base line separation with comparable peak areas (as provided by the system software). Sections of chromatograms showing baseline resolution of all the six enantiomeric pairs of hydrazones, corresponding to approach (i), are shown in Fig. 7.5; a full chromatogram

as a representative is given as **Fig. 7.6**. In approach (i) silica gel allowed convenient workup. It served as a very efficient adsorbent with a large surface area for homogeneous heating and thus facilitated faster reactions with short reaction times and higher yields. The catalytic amount of acid could probably have been provided by the silica gel (having adsorbed water) and the MWI triggered reaction being very fast provided no opportunity for spontaneous chiral inversion during derivatization.

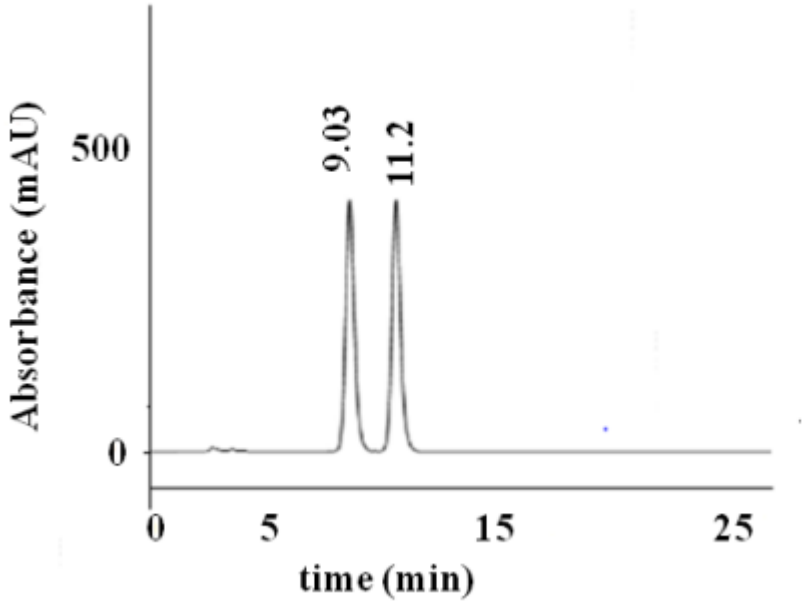


Fig. 7.6 Full chromatogram (as representative) showing resolution of product (3) obtained by approach (i). The peak areas were 402 and 403 mAU at retention time 9.03 and 11.2 min, respectively. Peaks with equal areas indicate racemic nature

It can thus be concluded that each of the products (**1**-**6**) was a racemic mixture of hydrazones (i.e., the tagged enantiomers) of the corresponding racemic carbonyl compound. Thus, the chiral HPLC results clearly verified that there was no configurational inversion of the chiral carbonyl compounds when they were derivatized with 2,4-DNPH under the solid state conditions using MWI. The DNP moiety of 2,4-dinitrophenyl hydrazine serves as a strong chromophore and a suitable substrate for *inclusion phenomenon* with the chiral material of the α_1-acid glycoprotein (α_1-AGP) column for enantiomeric resolution.

Chromatographic separation data for resolution of the six pairs of enantiomers (**1**-**6**) in the form of 2,4-DNPHz is given in Table- 7.1 while Table- 7.1 shows the values of enantioselectivity, resolution and retention time (in terms of α, *R*s and k_1) obtained by using mobile phase, 0.5% 2-propanol in citrate phosphate buffer (10 mM, pH 6.5).Varying buffer concentration above or below 10 mM (pH 6.5) resulted in decrease in enantioselectivity **(Fig. 7.7**). The enantiomers were not resolved using only the citrate phosphate buffer (10 mM, pH 6.5) as the mobile phase; a base line resolution of all the six analytes was observed after addition of 2-propanol to it at a level of 0.5%. A further increment (by a value of 0.5% at a time) in the concentration of 2-propanol up to 3% caused a decrease in resolution. 2-Propanol was found to be a better organic modifier in comparison to MeCN as lower enantioselectivity and resolution (α and *R*s) and higher retention time were obtained by using MeCN in the mobile phase. Increment in the pH of mobile phase (by a value of 0.5 at a time in the range of 3.5 to 6.5) resulted in increase of α, *R*s and k_1 for all the six pairs of enantiomeric hydrazones; thus finally pH 6.5 was found to be the best **(Fig. 7.8).**

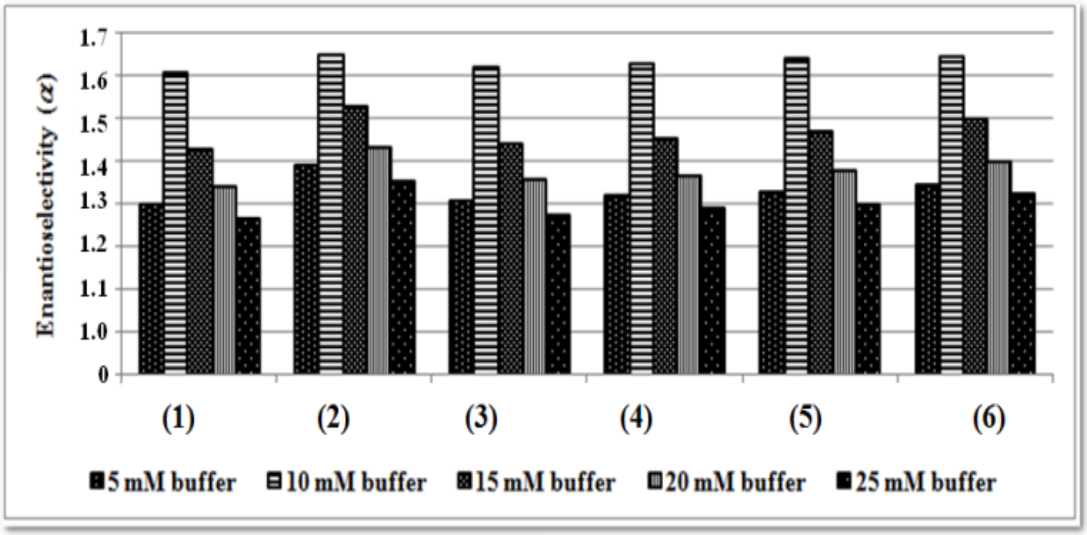


Fig. 7.7 Effect of buffer concentration (in the range 5-25 mM) in mobile phase consisting 0.5% 2-propanol at pH 6.5, on enantioselectivity

Table 7.1: Chromatographic data for direct resolution of six pairs of enantiomers in the form of 2,4-DNPHz of racemic aldehydes and ketones

2,4-DNPHz of racemic aldehydes and ketones																	
(1)			(2)			(3)			(4)			(5)			(6)		
k_1	α	R_s	k_1	α	R_s	k_1	α	R_s	k_1	α	R_s	k_1	α	R_s	k_1	α	R_s
5.60	1.42	4.31	5.56	1.56	5.82	5.45	1.44	4.43	5.66	1.46	4.84	5.80	1.48	5.10	5.84	1.49	5.21

Chromatographic conditions: Column, α_1-AGP (L×I.D. 10cm × 4mm, 5 µm particle size), Mobile phase, 0.5% 2-propanol in citrate phosphate buffer (10mM, pH 6.5); Flow rate, 1.0 mL/ min; Detection at 365 nm; Column temperature: 30 °C ; k_1, retention factor of first eluting enantiomer; α, separation factor; *R*s, resolution. (**1**) to (**6**) represent 2,4-DNPHz of chiral aldehydes and ketones as mentioned in experimental (section -2.3). The data presented in the table are the mean values of three independent experiments. 2,4-DNPHz synthesized by approach (i).

Separation mechanism

Over the pH range 3.5 to 6.5, AGP bears a net negative charge. Electrostatic interactions along with hydrogen bonding play important role in the chiral discrimination on an AGP column [163]. Effect of change of pH on enantioresolution of the analytes using AGP column (as noted above) can be attributed to the involvement of coulombic interactions between the analytes and the immobilized protein as the overall charge of the protein and potential conformational changes are pH dependent [164, 165]. Lowering of pH from 6.5 to 3.5 caused a decrease in the net negative charge of the protein that resulted in a reduced electrostatic attraction of cationic dinitrophenyl hydrazones with the immobilized protein resulting in decrease of retention time, enantioselectivity and resolution (**Fig. 7.8**). AGP is also able to bind a variety of hydrophobic compounds due to interactions with an apolar cavity formed by the folding of the secondary structure of AGP [166]. DNP moiety serves as a strong chromophore and is also a suitable substrate for inclusion phenomenon with the chiral material of the AGP column for enantiomeric resolution. The baseline resolution achieved in presence of 2-propanol at a concentration of 0.5% can be attributed to the reversible changes in

the secondary structure of immobilized protein; further increment of 2-propanol makes the mobile phase less polar and may cause reduction of hydrophobic interactions between the enantiomers and protein-based CSP followed by lowering of retention times and enantioselectivity [167]. In conclusion, hydrogen bonding, inclusion phenomenon and ionic interactions and/or reversible changes in the protein conformation are held responsible as the main factors for enantiomeric separation of the said dinitrophenyl hydrazones on AGP column.

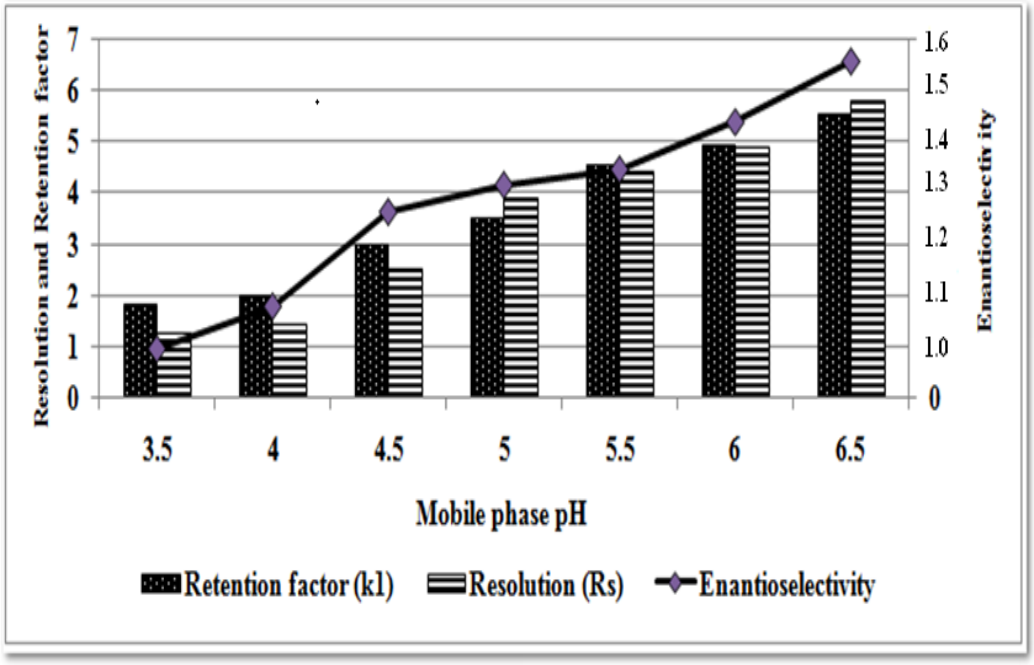


Fig. 7.8 Effect of pH of mobile phase (in the range 3.5-6.5) on enantioselectivity, resolution and retention times (in terms of *α*, *R*s and k_1, respectively) for product (2) using mobile phase 0.5% 2-propanol in 10 mM citrate phosphate buffer at pH 6.5

III. Conclusion

This chapter presents an efficient methodology for synthesis of 2,4-DNPHz of racemic (or enantiomerically pure) carbonyls under solid phase MWI conditions without spontaneous inversion of configuration. The method provided high yields (91–95%) in short reaction time (4-6 min). The method is successful in introducing a chromophore for on-line detection. The experimental results confirmed that there was no

configurational inversion of any of the chiral carbonyl compounds. The study is an important step not only for derivatization of enantiomeric carbonyls without spontaneous inversion of configuration (during synthesis) but also for direct enantioresolution of several chiral carbonyl compounds via introducing an achiral chromophore.

CONCLUSION

The chiral nature of living systems with their inherent chiral selectivity to enzyme and receptor causes the two enantiomers of a drug molecule to have different toxicities, pharmacokinetics and pharmacodynamics. Present work has resulted in development of less expensive, sensitive, flexible and reproducible methods using TLC and HPLC for enantioseparation of different categories of pharmaceuticals which are administered as racemic mixtures namely, (*RS*)-Etd, (*RS*)-Bac, (±)-Bup, and β-blockers along with certain didactic racemic carbonyl compounds. Some of the salient features and conclusions are given below.

1. Commercial samples (of the chosen drugs) were obtained and the active pharmaceutical ingredient was isolated, purified and characterized for further use as standard racemic sample since these and many other chiral drugs are marketed and administered as racemic mixtures. Experiments were successful for enantioseparation of these drugs with low limits of detection using both direct and indirect methods. Separation mechanisms have been discussed and correlated with the experimental results.

2. In the present studies, enantioseparation of (*RS*)-Etd was achieved by three different methods involving direct as well as indirect approaches. In one case, besides the separation of three pairs of diastereomers of (*RS*)-Etd (prepared by reaction with three enantiomerically pure amines under MWI) by RP HPLC, formation of diastereomers was confirmed using LC-MS when $[M+H]^+$ or $[M]^+$ were recorded and the ion peaks of the fragments were correlated with the structures of the diastereomers while three dimensional geometry of the diastereomers was verified by developing the lowest energy optimized structures of two diastereomers. These studies provided a new approach and insight in the area of enantioseparation (by indirect approach) in view of the existing reports in literature that are limited only to synthesis and enantioseparation of diastereomers with no attempts to verify the structures of the diastereomers.

3. Enantioresolution of (*RS*)-Etd was also achieved under both achiral phases in TLC and in HPLC (i.e., neither the mobile phase nor the stationary phase was having any chiral character); in TLC L-amino acids were used as '*chiral inducing reagents*' while in HPLC formation of a pair of *diastereomeric complexes* occurred by '*chiral*

ligand exchange'. This addressed the potential scientific issue for direct enantioresolution of a racemic mixture using both achiral phases in chromatography and provided an evidence for a 'conceptually different approach'. The method, in fact, did not require any CSP or any chiral reagent in the mobile phase during separation or any further verification of the results of separation by chiral HPLC etc and thus enhanced the usefulness of resolution of a racemate under achiral phases and makes it different from the methods reported in literature that are classified as *enantiomeric enrichment*.

4. High molecular absorptivity of the newly synthesized (*S*)-Npx based CDR allowed its application for enantioseparation of the β-blockers. (*S*)-Npx allowed easy synthesis of a symmetrical anhydride using DCC under MWI. The new reagent is highly reactive and stable at room temperature and provided an efficient novel C–N bond forming reaction. Recording of the ^{1}H NMR spectrum and the development of DFT based lowest energy optimized structures of the diastereomers helped in confirming the absolute configuration of diastereomers of the chosen β-blockers (prepared with this (*S*)-Npx based CDR). Thus, the method gains advantage and become superior to the literature reports (as cited in Chapter-4) on enantioseparation of β-blockers which show that there have been no attempts to verify the configuration of diastereomers so separated. In practice, most of the time diastereomer corresponding to pure enantiomer of the analyte is not available while the establishment of absolute configuration of the diastereomers becomes desirable to ensure the success of diastereomeric synthesis and reliability of enantioseparation. So the method is applicable for sensitive and easy detection, in the absence of pure enantiomer, of these analytes as well as certain other molecules having amino group but having a poor chromophore .

5. The 'conceptually different approach' of formation of a pair of *diastereomeric complexes* by '*chiral ligand exchange*' was also successful for enantioseparation of (*RS*)-Bac, and (±)-Bupropion using C_{18} column, with high resolution in less than 15 min.

The method, reported herein, provided a rapid, less expensive and effective approach in planar mode for direct separation and control of enantiomeric purity of (*RS*)-Bac (and other structurally similar pharmaceuticals) by using 'ligand exchange reagent' as the

mobile phase additive or as the impregnating reagent with the homemade plates for routine works which can be realized even in a small laboratory. The method is simple in comparison to existing methods requiring either chiral hplc columns or derivatization reactions for indirect mode of enantioseparation.

6. Achiral derivatization of certain chiral aldehydes and ketones (Chapter-7) was carried out for direct separation of their enantiomers using chiral columns. The method developed for synthesis of 2,4-dinitrophenyl hydrazones of racemic carbonyls under solid phase MWI conditions was without spontaneous inversion of configuration and proved to be an efficient methodology. The achiral derivatization (i) introduced a suitable achiral chromophore for the purpose of on-line detection, and (ii) added functional groups into enantiomeric molecules that enhanced the possibilities for chiral interactions (during separation on the chiral stationary phase) or blocked functional groups to avoid non-specific interactions. In principle, it remained direct approach to enantioseparation.

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