LIQUID CHROMATOGRAPHIC ENANTIORESOLUTION OF PHARMACEUTICALLY IMPORTANT COMPOUNDS

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled, **LIQUID CHROMATOGRAPHIC ENANTIORESOLUTION OF PHARMACEUTICALLY IMPORTANT COMPOUNDS** in partial fulfillment 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 August 2008 to May 2012 under the supervision of Dr. Ravi Bhushan, Professor, Department of Chemistry, Indian Institute of Technology Roorkee, Roorkee.

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

Rituraj Dubey.

(RITURAJ DUBEY)

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

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ABSTRACT

Chiral resolution of enantiomers is one of the emerging areas as the enantiomers of a chiral drug have different pharmacological effects as only one enantiomer of the drug often exhibits the desirable therapeutic activity, while the other shows an antagonistic function, side effects, or even toxic effects. These properties of the enantiomers have created an interest to study the pharmacological and toxicological behaviors of the individual enantiomers of drugs, pharmaceuticals, and agrochemicals. The United States Food and Drug Administration has issued guidelines to pharmaceutical and agrochemical industries to specify the enantiomeric purity of the optically active compounds prior to their marketing and hence demanded a systematic investigation of the biological behavior of their individual enantiomers and significantly encouraged the development of single enantiomer drugs. In view of these facts, the enantiomeric resolution of a variety of compounds is gaining importance continuously.

There exists a multitude of methods and techniques specifically designed for enantiomeric separations, though not all methods are equally applicable for every racemic mixture. Drug development within the pharmaceutical industry focuses heavily on asymmetric synthesis, enzymatic resolution, crystallization methods, chromatographic techniques such as high-performance liquid chromatography (HPLC), thin layer chromatography (TLC), high-performance thin layer chromatography (HPTLC), gas-liquid chromatography, and supercritical fluid chromatography, membrane processes and combinatorial chemistry. Of the possible methods for obtaining enantiomerically pure compounds, chromatographic techniques particularly HPLC is most commonly employed.

Considering the biological importance of chirality and the need for chiral separation from academic, industrial, and biomedical points of view, studies have been carried out on direct and indirect enantiomeric resolution of cinacalcet, penicillamine, cysteine, homocysteine, mexiletine, omeprazole, lansoprasole, rabeprazole, pantoprazole, and amino acid analogues amino alcohols which are of great pharmaceutical and biomedical significance.

First chapter provides the introduction to present studies followed by summary of present work and the brief introduction to the applicable scientific terms. Objective of present work, selection of chiral compounds for enantioseparation purpose, chiral chromatographic separation approaches, chiral stationary phases (CSPs), chiral derivatizing reagents (CDRs) and various scientific terms such as chirality and its biological importance have been discussed in brief. The literature related to the class of compounds chosen has been cited in subsequent chapters.

Second chapter describes the common experimental methods used for present studies. It includes materials, instrumentation, methods for synthesis of two new chiral derivatizing reagents (CDRs) and six others. Six CDRs namely 1-Fluoro-2,4-dinitrophenyl-L-alaninamide (FDNP-L-Ala-NH_2; CDR1),1-Fluoro-2,4-dinitrophenyl-L-phenylalaninamide (FDNP-L-Phe-NH_2; CDR2), 1-Fluoro-2,4-dinitrophenyl-L-valinamide (FDNP-L-Val-NH_2; CDR3), 1-Fluoro-2,4-dinitrophenyl-L-leucinamide (FDNP-L-Leu-NH_2; CDR4), 1-Fluoro-2,4-dinitrophenyl-L-methioninamide (FDNP-L-Met-NH_2; CDR5), and 1-Fluoro-2,4-dinitrophenyl-D-phenylglycinamide (FDNP-D-Phg-NH_2; CDR6) were synthesized by substituting one of the fluorine atoms in 1,5-difluoro-2,4-dinitrobenzene (DFDNB) with six amino acid amides namely L-Ala-NH_2, L-Phe-NH_2, L-Val-NH_2, L-Leu-NH_2, L-Met-NH_2 and D- Phg-NH_2, respectively.
o new CDRs namely (*S*)-naproxen-benzotriazole and (*S*)-naproxen-benzimidazole were thesized by reaction of (*S*)-Nap with 1*H*-benzotriazole and benzimidazole, respectively, ıg coupling reagent dicyclohexyl carbodiimide and 4-dimethylamino pyridine. The racterization data for the new CDRs is also described. The details with respect to synthesis of stereomers and separation by HPLC are described in different chapters along with results and ussions.

Third chapter deals with enantioresolution of (*R,S*)-cinacalcet by using both indirect and direct approaches. For indirect approach, cinacalcet was derivatized with six chiral variants based on DFDNB (i.e. CDRs 1-6) under microwave irradiation (MWI) conditions. The derivatization conditions were optimized with respect to role of the pH of the base, effect of the CDR excess to the cinacalcet and MWI conditions. The synthesized diastereomers were resolved

using reversed-phase high-performance liquid chromatography (RP-HPLC) and ultraviolet (UV) detection at 340 nm using binary mixtures of aqueous trifluoroacetic acid and acetonitrile. Effect of flow rate and various linear gradients was also studied. However using the direct approach the enantiomers of cinacalcet were resolved on thin silica gel layers impregnated with optically pure L-His and L-Arg. Effects of the variations in the concentrations and pH of impregnating reagents and temperature were also studied. Both the resolution approaches were validated.

Fourth chapter deals with enantioresolution of thiol-group containing α-amino acids namely penicillamine, cysteine, and homocysteine by RP-HPLC. These amino acids were derivatized with (*S*)-naproxen-benzotriazole under MWI conditions. The derivatization conditions were optimized with respect to effect of the pH of the base, effect of the CDR excess and MWI conditions. The resultant diastereomers were resolved on a reversed phase column with gradient elution of triethylammonium phosphate (TEAP)-acetonitrile and UV detection at 231 nm. The effects of pH and concentration of TEAP buffer, organic solvent, and flow rate on RP-HPLC separation were studied. The separation mechanism was explored for the three pairs of diastereomers prepared the said CDR. The method was validated for accuracy, precision, and limit of detection.

Fifth chapter explores the HPLC indirect resolution of mexiletine using (*S*)-(-)-(*N*)-trifluoroacetyl-prolyl chloride and (1*S*)-(-)-camphanic chloride. Kinetic resolution method was also applied to yield a diastereomer in excess. The mole ratio of mexiletine to each of the CDRs was investigated to overcome the kinetic resolution. The synthesized diastereomers were subjected to HPLC using reversed phase column and binary composition of aqueous trifluoroacetic acid-acetonitrile as mobile phase and UV detection at 210 nm. This analytical enantioresolution method was optimized, validated, and scaled up to small-scale preparative enantioresolution method only for those resultant diastereomers, which were significantly enriched with one diastereomer, yielded due to kinetic resolution method. The separated diastereomers, which were collected on small-scale preparative enantioresolution method were hydrolyzed using hydrochloric acid and acetic acid to yield (*R*)-mexiletine, the pharmacologically desired native enantiomer.

Sixth chapter explores the direct HPLC enantioresolution of four anti-ulcer drugs namely omeprazole, lansoprazole, rabeprazole, and pantoprazole. The enantioresolution of these anti-ulcer drugs (*i.e.* chiral sulfoxides) was perfomed to investigate the enantioseparation capability of polysaccharide-based Lux cellulose-2 chiral stationary phase under normal and polar-organic phase conditions. The detection was carried out using UV detection at 285 nm. The method was validated for linearity, accuracy, precision, robustness, and limit of detection. The optimized enantioresolution method was compared for both the elution modes. The column was not stultified even after a number of bimodal elutions. The optimized method was further utilized to check the enantiomeric purity of dexrabeprazole drug.

Seventh chapter deals with indirect HPLC resolution of enantiomers of eight β-amino alcohols; DL-alaninol, DL-leucinol, DL-prolinol, DL-phenylalaninol, DL-phenylglycinol, DL-valinol, DL-homophenylalaninol and DL-methioninol. These amino alcohols were derivatized with (*S*)-naproxen-benzimidazole under MWI conditions. The derivatization conditions were optimized with respect to effect of the pH of the base, effect of the CDR excess and MWI conditions. The resultant diastereomers were resolved on a reversed phase column with gradient elution of TEAP-acetonitrile at 231 nm. The effects of pH and concentration of TEAP buffer, organic solvent, and flow rate were studied. The separation mechanism was explained for the diastereomers prepared with the CDR. The method was validated for accuracy, precision, and limit of detection.

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Dated: **(RITURAJ DUBEY)**

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1.	1*H*-Btz	1*H*-Benzotriazole
2.	AGP	α-Acid glycoprotein
3.	Alaol	Alaninol
4.	Anal. Calcd.	Analysis calculated
5.	AU	Absorbance Unit
6.	*aq*	Aqueous
7.	BImz	Benzimidazole
8.	BSA	Bovine serum albumin
9.	CaR	Calcium-sensing receptors
10.	CDR	Chiral derivatizing reagent
11.	$CHCl_3$	Chloroform
12.	CD	Cyclodextrin
13.	$CDCl_3$	Deuterated chloroform
14.	Cin	Cinacalcet
15.	CL	Chemiluminescence
16.	CLC	Centrifugal layer chromatography
17.	CLE	Chiral ligand exchange
18.	cm	Centimetre
19.	CMPA	Chiral mobile phase additive
20.	CSP	Chiral stationary phase
21.	CV	Coefficient of variation
22.	Cys	Cysteine
23.	d	Doublet
24.	dd	Double doublet
25.	DCM	Dichloromethane
26.	DCC	Dicyclohexyl carbodiimide
27.	*de*	Diastereomeric excess
28.	DEA	Diethyl amine
29.	dexRAB	Dexrabeprazole

30.	DFDNB	1,5-Difluoro-2,4-dinitrobenzene
31.	DMAP	4-Dimethyl amino pyridine
32.	DMSO	Dimethyl sulfoxide
33.	DMSO-d_6	Deuterated dimethyl sulfoxide
34.	ECD	Electrochemical detector
35.	*ee*	Enantiomeric excess
36.	ELSD	Evaporative light scattering detector
37.	EtOH	Ethanol
38.	ES	Eight shape
39.	FDNP	1-Fluoro-2,4-dinitrophenyl
40.	FLD	Fluorescence detector
41.	FT	Fourier transform
42.	GLC	Gas liquid chromatography
43.	HCl	Hydrochloric acid
44.	HEX	Hexane
45.	HOAc	Acetic acid
46.	Homocys	Homocysteine
47.	Homopheol	Homophenylalaninol
48.	HPLC	High-performance liquid chromatography
49.	HPT	Hyperparathyroidism
50.	HPTLC	High-performance thin liquid chromatography
51.	HSA	Human serum albumin
52.	h	Hour
53.	ICH	International conference on harmonization
54.	I.D.	Internal diameter
55.	IPA	Isopropanol
56.	IR	Infrared
57.	*k*	Retention/capacity factor
58.	KBr	Potassium bromide
59.	KOH	Potassium hydroxide
60.	KH_2PO_4	Potassium dihydrogen phosphate
61.	LAN	Lansoprazole

62.	Leuol	Leucinol
63.	LIF	Laser induced fluorescence
64.	LOD	Limit of detection
65.	LOQ	Limit of quantification
66.	M	Molar
67.	m	Multiplet
68.	mL	Millilitre
69.	mM	Millimolar
70.	MeOH	Methanol
71.	MeCN	Acetonitrile
72.	$MgSO_4$	Magnesium sulphate
73.	MEX	Mexiletine
74.	MHz	Megahertz
75.	Metol	Methioninol
76.	min	Minute
77.	mg	Milligram
78.	mL	Millilitre
79.	mm	Millimetre
80.	mmol	Millimole
81.	m.p.	Melting point
82.	MR	Marfey's reagent
83.	MS	Mass spectrometry
84.	MWI	Microwave irradiation
85.	N	Normal
86.	Nap	(*S*)-Naproxen
87.	NG	Not given
88.	ng	Nanogram
89.	nm	Nanometre
90.	nmol	Nanomole
91.	NMR	Nuclear magnetic resonance
92.	NR	Not resolved
93.	*o-*	ortho-

94.	ODS	Octadecyl silane
95.	OME	Omeprazole
96.	OPTLC	Over-pressured thin layer chromatography
97.	PAN	Pantoprazole
98.	PDA	Photodiode array
99.	PenA	Penicillamine
100.	Pheol	Phenylalaninol
101.	Phgol	Phenylglycinol
102.	pg	Picogram
103.	pmol	Picomole
104.	P_2O_5	Phosphorus pentaoxide
105.	PPI	Proton pump inhibitors
106.	Prolol	Prolinol
107.	q	Quartet
108.	R^2	Linearity
109.	RID	Refractive index detector
110.	RP	Reversed-phase
111.	R_f	Retention factor
112.	*Rs*	Resolution
113.	RSD	Relative standard deviation
114.	RAB	Rabeprazole
115.	s	Singlet
116.	SD	Standard deviation
117.	SFC	Supercritical fluid chromatography
118.	t	Triplet
119.	*t*	Retention time
120.	TEA	Triethyl amine
121.	TEAP	Triethylammonium phosphate
122.	Temp	Temperature
123.	TFA	Trifluoroacetic acid
124.	TFPC	Trifluoroacetyl-prolyl-chloride
125.	THF	Tetrahydrofuran

126.	TLC	Thin layer chromatography
127.	US FDA	United States Food and drug administration
128.	UV	Ultraviolet
129.	v/v	Volume/volume
130.	Valol	Valinol
131.	Vis	Visible
132.	W	Watt
133.	W	Base width
134.	φ	Fluorescence quantum yield
135.	MΩ	Megaohm
136.	µg	Microgram
137.	µL	Microlitre
138.	µm	Micrometre
139.	µmol	Micromole
140.	ε	Molar absorptivity
141.	α	Separation factor/enantioselectivity
142.	λ	Wavelength
143.	℃	Degree Celsius
144.	%	Percentage

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Analytical and Bioanalytical Chemistry (Communicated), 2012.
(*cf.*, Chapter-7)

Chapter-1

General Introduction

I. Introduction

Chirality is a ubiquitous feature in living systems. It is widely recognized that many biologically important compounds, such as drugs, agrochemicals, food additives, and fragrances, are chiral and their physiological properties usually rely on their chirality due to specific chiral discrimination ability of enzymes and receptors [1-5].

Stereoisomers behave as different chemical entities in chiral environment, like biological systems because the receptor binding sites can discriminate between them. Thus chirality plays an important role both in pharmacodynamics, involving the interaction of bioactive molecules with enzymes and receptors in the target organs, and in pharmacokinetics involving absorption, disribution, metabolic conversion and excretion of the substances.

In general, one enantiomer of most of the racemic drugs shows either significantly higher pharmacological activity or total inactivity and/or adverse side effects. In this case active isomer is called the *eutomer* and inactive isomer the *distomer*. The ratio of activities is termed eudismic ratio and logarithm of this is termed as eudismic index. Pfeiffer states that there is an inverse relationship between effective dose of an active enantiomer and the eudismic ratio [6]. However, interactions between these two enantiomers were also been reported when one's pharmacological effect was altered by the second enantiomer [7]. These differences in pharmacodynamics, pharmacokinetics and toxicological activities of two enantiomers of a drug lead to a variety of effects and also establish the extreme importance of enantioselective analysis in medical, pharmaceutical, agricultural, environmental and food chemistry.

In spite of inherent differences between pairs of enantiomers, a number of synthetic chiral drugs are sold as racemates, instead of a single enantiomer [7-10]. In the policy guidelines of United States Food and Drugs Administration (US FDA) it was stated that it is essential that a stereospecific assay be used from the beginning of the development of chiral drugs. The policy statement of US FDA also requires the submission of a stereochemically specific identity test for a drug product [10]. Good manufacturing practices of chiral drugs require the identity test and the manufacturer must specify that the test is either able to distinguish between enantiomers, or only

between an enantiomer and the racemate or both. European Committee for Proprietary Medicinal Products and other drugs regulatory agencies have also restricted the marketing of racemic drugs unless the clear benefit of such a dosage form is well established. Thus, enantioseparation methods have become essential part of pharmaceutical industry to check whether an inactive/toxic enantiomer is present in an enantiomerically pure drug or not.

Efficient analytical procedures are needed for separation and determination of the two enantiomers. Chromatographic methods are preferred because chromatographic procedures are rapid, sensitive, reliable and easy to use. The development of chromatographic methods, especially those involving liquid chromatography has represented an intensive area of research because it is used for quantification of enantiomers in dosage forms and biological fluids [11]. Besides, chromatographic methods provide high sensitivity and hence found feasible to determine low levels of the enantiomers of a chiral drug in biological systems. Polarimetry, circular dichroism etc., are some classical methods used to check optical purity of enantiomers.

In view of the importance of chromatography and its use on enantioseparation the following plan was devised for present studies.

II. Present Work

1. Objective:

To develop optimized simple, economical, sensitive, and reproducible methods which can be used for enantioseparation and for checking the enantiomeric purity of certain drugs marketed as racemic mixtures along with certain other biologically important compounds.

❖ ***Selection of chiral compounds:***

The racemic chiral compounds were selected based on the following criteria:

- Chiral compounds (racemic or scalemic mixtures) that have low cost are easily available, and have some common functional groups which are present in different

pharmaceuticals or chemicals, and commonly used in organic synthesis.

- Compounds of pharmaceutical importance that are widely used and marketed as racemic mixtures.
- Compounds of biomedical importance with low cost, easy availability and have reactive functional group that could be used for synthetic modifications.

The compounds so selected, included (*R*,*S*)-cinacalcet (first drug of calcimimetics, used for treatment of primary and secondary hyperparathyroidism, chronic kidney disease), DL-penicillamine (since the D-isomer is used for treatment of Wilson's disease, polyarthritis and cystinuria), DL-cysteine (proteinogenic amino acid), DL-homocysteine (non-proteinogenic amino acid), and (*R*, *S*)-mexiletine (MEX) which is a class IB antiarrhythmic, antimyotonic and analgesic agent and all have an amino functional group and a stereogenic center.

Anti-ulcer drugs, (*R*,*S*)-omeprazole (OME), (*R*,*S*)-rabeprazole (RAB), (*R*,*S*)-lansoprazole (LAN) and (*R*,*S*)-pantorazole (PAN) were selected for enantioseparation. Besides, certain α-amino acid analogues β-amino alcohols; DL-alaninol, DL-leucinol, DL-prolinol, DL-phenylalaninol, DL-phenylglycinol, DL-valinol, DL-homophenylalaninol and DL-methioninol were also chosen that have one stereogenic center and have a great biological and pharmaceutical importance since numerous drugs including β-blockers, sympathomimetic agents including ephedrine and pseudoephedrine and hormones including adrenaline and noradrenaline are structurally belong to β-amino alcohols.

2. Approach to Enantioseparation

Normal-phase thin layer chromatography (TLC) and normal-phase, polar-organic and reversed-phase high-performance liquid chromatography (HPLC) were used as separation techniques. Both direct and indirect approaches were adopted.

A. Direct Enantioseparation Approach

Direct TLC enantioseparation of (*R,S*)-cinacalcet was achieved using L-His and L-Arg as chiral impregnating reagents. Experiments were carried out to study the effects of temperature, pH and concentration of chiral selector(s) on enantioresolution.

Besides, HPLC enantioseparation of four anti-ulcer drugs (chiral sulfoxides); (*R,S*)-omeprazole (OME), (*R,S*)-rabeprazole (RAB), (*R,S*)-lansoprazole (LAN) and (*R,S*)-pantoprazole (PAN)) has been attempted using chlorinated cellulose based Lux cellulose-2 chiral column and normal and polar-organic phase elution conditions. The method was validated for linearity, accuracy, precision, robustness, and limit of detection. The optimized enantioresolution method was compared for both the elution modes. The optimized method was further applied to check enantiomeric purity of dexrabeprazole.

B. Indirect Enantioresolution Approach

Indirect approach was adopted for enantioresolution of (*R,S*)-cinacalcet, DL-penicillamine, DL-cysteine, DL-homocysteine, and certain α-amino acid analogues β-amino alcohols; DL-alaninol, DL-leucinol, DL-prolinol, DL-phenylalaninol, DL-phenylglycinol, DL-valinol, DL-homophenylalaninol and DL-methioninol. Several chiral derivatizing reagents (CDRs) were synthesized by microwave irradiation (MWI)/ vigorous stirring and used for the synthesis of diastereomers of the said analytes using MWI. The synthesized diastereomers were separated by reversed phase HPLC.

A reversed-phase HPLC optimized analytical enantioresolution of (*R,S*)-MEX in the form of its diastereomers prepared with (*S*)-(-)-(*N*)-trifluoroacetyl-prolyl chloride ((*S*)-TFPC) and camphanic chloride as CDRs has been achieved and scaled up to small-scale preparative level only for those resultant diastereomers which was significantly enriched with one diastereomer formed due to kinetic resolution. The native enantiomer of (*R*)-MEX were recovered by acidic hydrolysis of the separated diastereomer.

❖ ***Selection of CDR:***

Following criteria were adopted for the selection of the CDRs. These were the CDRs which could be:

- synthesized by using straightforward process in laboratory,
- derived from cost-effective and easily available synthones, and
- used for derivatization of analytes under mild conditions providing stable diastereomers.

In view of these criteria 1,5-difluoro-2,4-dinitrobenzene (DFDNB), and (*S*)-naproxen ((*S*)-Nap) were explored as synthons/structural moieties/platforms to synthesize a wide spectrum of CDRs which were used for enantioresolution of analytes having different functional groups in the form of their diastereomers.

CDRs based on DFDNB

Literature surveyed on various CDRs reported that DFDNB based CDR, 1-fluoro-2, 4-dinitrophenyl-5-L-alanine amide (FDNP-L-Ala-NH_2), commonly known as Marfey's Reagent (MR) has been found to be one of the most successful CDR for resolution of enantiomers of compounds containing primary and secondary amino groups. Six CDRs based on DFDNB were planned to be synthesized by nucleophilic substitution of one fluorine of DFDNB by α-amino acid amides such as L-Ala-NH_2, L-Phe-NH_2, L-Val-NH_2, L-Leu-NH_2, L-Met-NH_2, and D-Phg-NH_2.

CDRs based on (S)-Nap

Two CDRs; (*S*)-naproxen-benzotriazole and (*S*)-naproxen-benzimidazole were synthesized by reaction of (*S*)-Nap with 1*H*-benzotriazole and benzimidazole, respectively, using dicyclohexyl carbodiimide and 4-dimethylamino pyridine (DCC/DMAP) coupling reagent. These CDRs were synthesized by nucleophilic attack of 1*H*-benzotriazole (and benzimidazole) on the carbonyl carbon of the carboxylic acid of (*S*)-Nap followed by the removal of dicyclohexylurea.

Experimental conditions were optimized for synthesis of all the CDRs and diastereomers. The methods developed for enantioseparation were validated. Efforts have been made to explain the mechanism of separation along with the influence of structural features of different CDRs introduced in them in the form of different chiral auxiliaries.

III. Brief Introduction to Applicable Scientific Terms

1. Chirality and Its Importance

Chirality is the property of a molecule which is generated due to asymmetry in its chemical structure. On molecular level, chirality represents an intrinsic property of different molecules.

A molecule that is not superimposable on its mirror image, although the two have identical composition, is termed as chiral. Such non-identical pairs of molecular mirror images are called enantiomers (Fig. 1.1). Enantiomers have identical physical and chemical properties, but differ when they are placed in a chiral environment, e.g., when interacting with other chiral compounds or when subjected to plane-polarized light. A 50/50 mixture of enantiomers is called as racemic mixture.

Easson and Stedman on the basis of three point-attachment model [12] described the differences in the bioaffinity of the enantiomers to a common site on an enzyme or receptor surface, with the receptor or enzyme needing to possess three nonequivalent binding sites to discriminate between the enantiomers. Therefore, metabolic and regulatory processes mediated by biological systems are sensitive to stereochemistry. Due to inherent stereoselectivity exhibited by biological systems, bio-metabolism is highly regulated by chirality. For example only L-amino acids are produced rather than a mixture of D- and L- isomers and during production of glucose a single compound (D-glucose) being metabolized rather than a mixture of sixteen possible hexoses. Enzymes exhibit high stereoselectivity in biological systems in binding and reacting with a particular enantiomer.

2. Chiral Drugs and Importance of Their Enantioseparation

Most biologically active compounds including drugs are chiral and their pharmacologic, toxic and metabolic activities are often different between enantiomers. Therefore, the systematic investigation of their biological properties of individual enantiomers has become indispensable, particularly for the development of new chiral drugs [13-16].

The efficient preparation of both optically pure isomers and the precise determination of the enantiomeric excess of chiral compounds are becoming increasingly important. Today, many top selling drugs around the world have been used as single enantiomers with the desired physiological effect [16]. However, chiral drugs such as morphine, digoxin and penicillin, obtained from natural sources, are produced as single isomers and are usually marketed as such.

There are three possibilities, which may exist in the racemic mixture of drug and are as follows:

- One enantiomer may have all the desired pharmacological activity, in which case the other enantiomer may be regarded as an impurity.
- The enantiomers may have activity that is qualitatively similar but quantitatively different.
- The enantiomers may have qualitatively different pharmacological activity.

Besides, the advantages of using enantiomerically pure drugs are as follows:

- The total dose could be reduced.
- The dose–response relationship would be simpler.
- A source of interobject variability would be removed.
- Toxicity from the inactive stereoisomer would be minimized.

A few examples showing different biological activities of enantiomers of drugs are shown in Fig. 1.2.

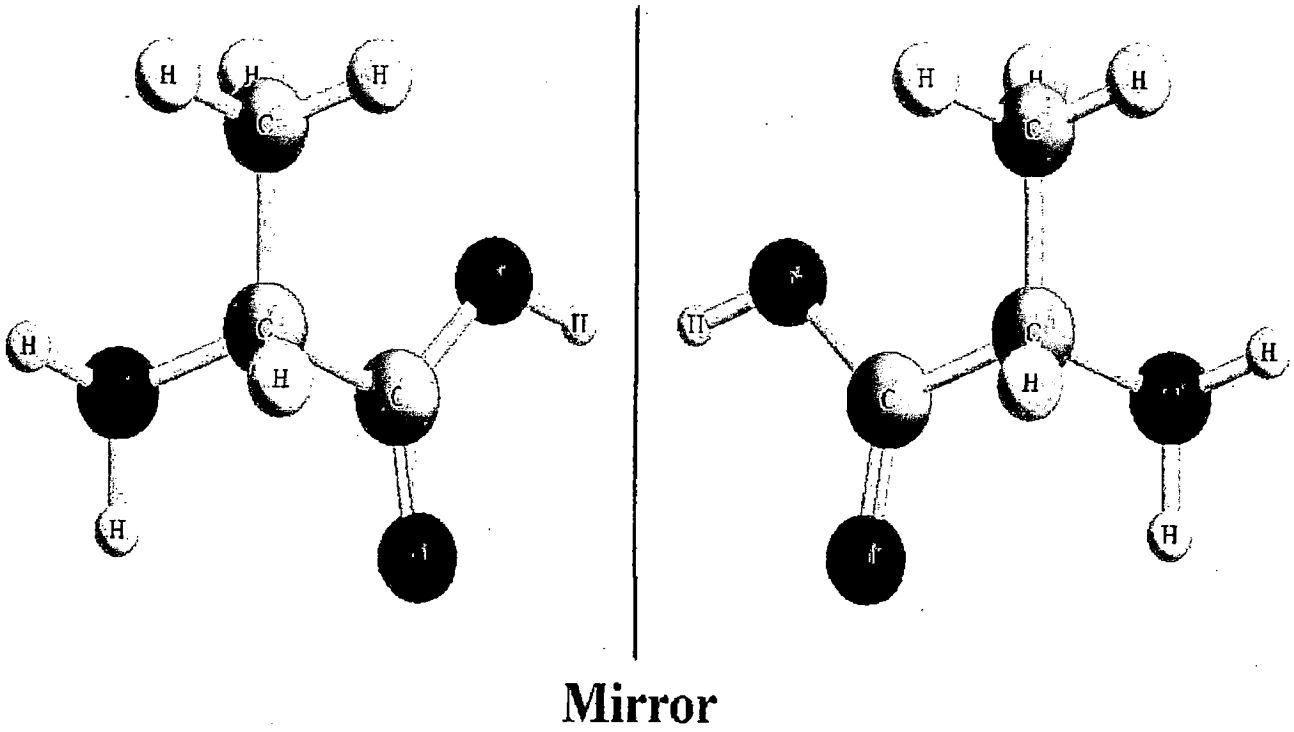


Fig. 1.1: Chirality: Non-superimposable mirror images of alanine, a chiral molecule having a tetrahedral stereocenter

3. Chromatography

Since the time of Russian Botanist Michael Tswett the term chromatography meaning, "color writing" is the separation of the components of a mixture based on the different degrees to which they interact with two separate material phases but in recent times, chromatography has become a group of methods, which involves separation, isolation, identification, and quantification of components both in simple and complex mixtures present in raw materials, and in bulk drugs.

One of the two phases is a moving phase (the mobile phase), while the other does not move (the stationary phase). The principle of chromatography is based upon differential distribution of compounds between stationary and mobile phases.

Based on the type of mobile and stationary phase used, chromatography has been classified as gas-solid, liquid-solid, gas-liquid, liquid-liquid chromatography etc. The forces of interaction involved in determining chromatographic retention and selectivity include hydrogen bonding, charge-charge transfer, ion-ion, ion-dipole and van der Waals interactions.

Different well known chromatographic techniques *viz.* TLC, gas-liquid chromatography (GLC), supercritical fluid chromatography (SFC), and HPLC are widely used in the quality control in pharmaceutical industry. TLC has emerged as a powerful and valuable tool for screening unknown material in bulk drug substance. It provides a relatively high degree of assurance that all possible components of the drug are separated. It is the simplest chromatographic technique in which multiple samples can be handled simultaneously. GLC commands a significant role in the analysis of flavor compounds, pheromones, pharmaceutical compounds, and pesticides, as well as amino acids and carbohydrates. The advent of high molecular weight drug products such as polypeptide or thermally unstable compounds such as the antibiotics limits the scope of the technique.

The principle limitation of GLC for the analysis of drug and pharmaceuticals rests in the relative non-volatility of the substances. Therefore, derivatization is virtually mandatory for analysis of drugs by GLC. SFC, in which mobile phase is a super critical fluid, finds use in industry primarily for separation of chiral molecules. At present HPLC is the most widely used chromatographic technique, especially for non-volatile compounds such as bulk drugs and their formulations. Its features like gradient elution, temperature programming, and wavelength programming provide valuable information regarding the undetected components of a given drug. In all the chromatographic methods, the choice of a proper detection mode is crucial to ensure that all the components are detected.

Thin Layer Chromatography

Some books have appeared which exclusively deals with applications of TLC for analysis of various types of compounds[17-19]. Role of TLC in control of enantiomeric purity purity of pharmaceuticals and amino acids has been reviewed by Martens and Bhushan [20, 21].

Development and introduction of other TLC modified techniques such as HPTLC [22], over-pressured thin layer chromatography (OPTLC) [23] and centrifugal layer chromatography (CLC) [24] have caused renaissance in the field of TLC. Much effort has been devoted to the coupling of TLC and HPTLC with spectrometric methods because of their robustness and simplicity and, the need for detection techniques that provide identification and determination of sample constituents. Infrared (IR) and mass spectrometry (MS) are the main methods that have been coupled with TLC as both have a high potential for elucidation of molecular structures. The TLC-FTIR [25, 26] and TLC-MS [26] coupled methods are used for qualitative and quantitative analysis. The potential and power of these coupled methods are demonstrated by their application in various fields of analysis, such as drug analysis, forensic analysis, food analysis, environmental analysis, biological analysis, etc.

High-performance Liquid Chromatography

The techniques of HPLC also called as high-speed and high-pressure liquid chromatography has already made a significant contribution to pharmaceutical, biochemical, clinical and environmental analysis. The reasons for its popularity are high sensitivity, ready adaptability to accurate quantitative determinations, suitability for separating non-volatile species or thermally fragile ones and, above all, widespread applicability to substances that are of prime interest to industry. In 1941, Martin and Synge, who were subsequently awarded Nobel Prize, described the discovery of liquid-liquid chromatography [27] and in same paper laid the foundation of GLC and HPLC. It was Giddings who showed in 1960s, that the theoretical framework developed for GLC can be applied to liquid chromatography also [28] and this lead to the development of first high-performance liquid chromatography by Kirkland [29], Huber [30]

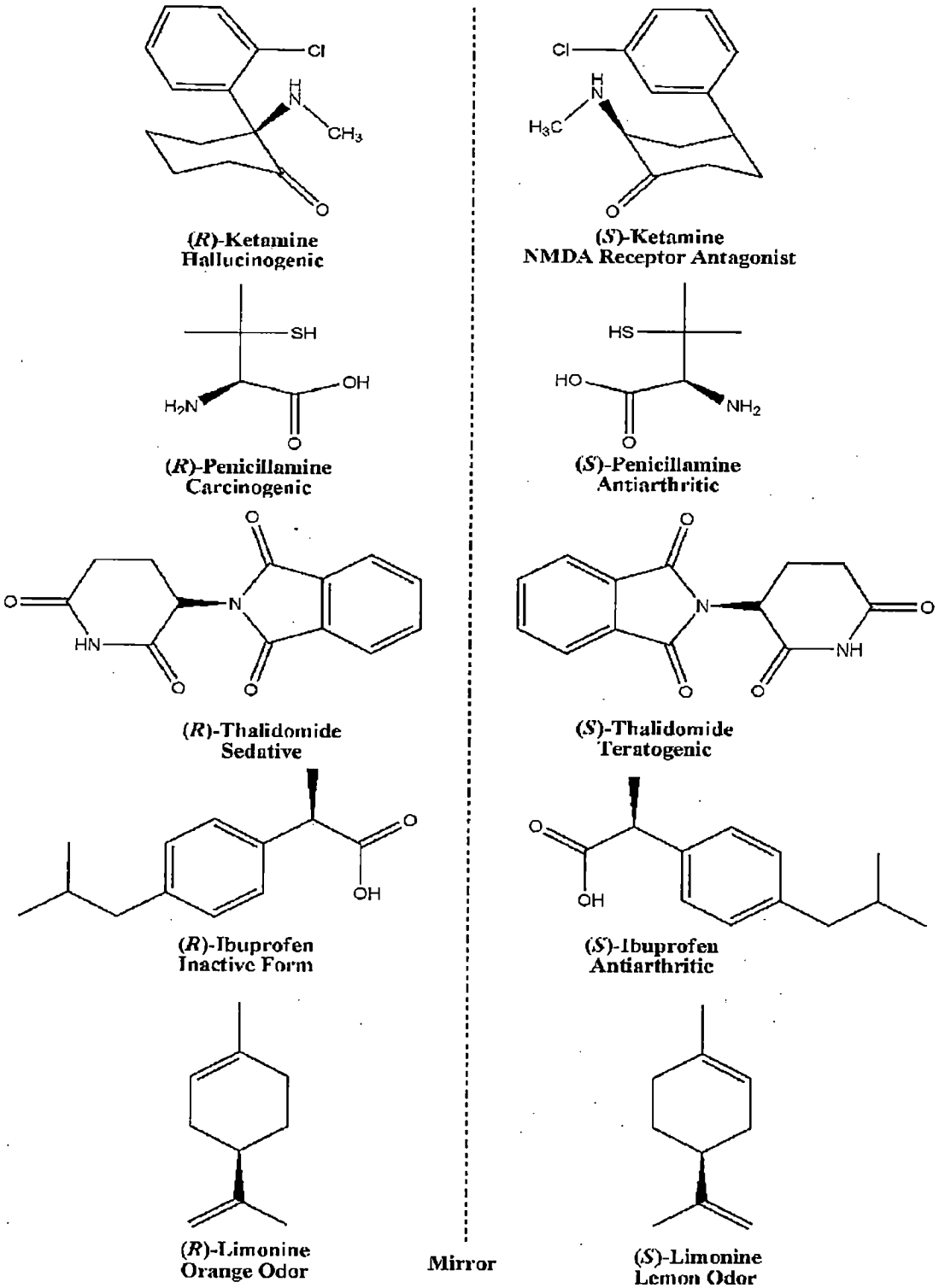


Fig. 1.2: Examples of enantiomers with different biological activity

and Horvath *et al.* [31]. Fast and efficient separation can be achieved by HPLC using a fused-core silica particle packing for the analysis of pharmaceutical compounds. Fused-core particles are produced by "fusing" a porous silica layer onto a solid silica particle. The columns packed with 2.7 μm "fused-core" particles (a 0.5 μm porous shell fused to a solid 1.7 μm silica core particle) result in to fast separations with modest operating pressure. The fused-core silica materials providing the shorter diffusional mass transfer path for solutes are less affected in resolving power by increase in mobile-phase velocity than the sub-2 μm porous silica packings resulting in faster separation and higher sample throughput.

➢ HPLC Detectors

An HPLC detector is often a modified spectrophotometer equipped with a small flow cell, which monitors the concentration (or mass) of eluting sample components. A wide variety of detectors such as fluorescence detector (FLD), refractive index detector (RID), evaporative light scattering detector (ELSD), electrochemical detector (ECD), conductivity detector, radioactivity detector, nuclear magnetic resonance and mass spectrometric detectors are used in HPLC. Most applications utilize absorbance detectors such as ultraviolet/visible (UV/Vis) or photodiode array (PDA) detectors.

• UV/Vis Absorbance Detectors

These detectors monitor the absorption of UV or Vis light by analytes in the HPLC eluent. A typical UV/Vis detector consists of a deuterium source and a monochromator (a movable grating controlled by stepper motor to select wavelength through an exit slit) to focus the light through a small flow cell. A dual-beam optical bench is typical for reducing drift. The observed absorbance is controlled by Beer's Law,

$$\text{Absorbance (A)} = \text{molar absorptivity } (\varepsilon) \times \text{path length (b)} \times \text{concentration (c)}$$

Important performance characteristics of UV/Vis detectors are sensitivity, linearity, and band dispersion.

- **PDA Detectors**

PDA detectors provide UV spectra of eluting peaks in addition to monitoring the absorbance of the HPLC eluent. It is the detector of choice for method development and for monitoring impurities particularly for complex biological samples. An entire spectrum from the deuterium source or a selected portion passes through the flow cell and is dispersed onto a diode array element that measures the intensity of light at each wavelength. Most PDAs use a charge-coupled diode array with 512–1024 diodes, capable of spectral resolution of ~1 nm. Sophisticated spectral evaluation software allows the convenient display of both chromatographic and spectral data along three axes (absorbance *versus* wavelength *versus* retention time).

➢ **Resolution**

Resolution (R_S) is the distance between the centers of two peaks divided by the average base width of the peaks.

$$R_S = \frac{t_2 - t_1}{[0.5(W_1 + W_2)]}$$

where t_1 and t_2 are the retention times and W_1 and W_2 are the base widths of peak 1 and 2, respectively, while in TLC W_1 and W_2 are the width of spot 1 and 2, respectively. R_S is the measure of how well two peaks are separated for reliable quantitation. Well-separated peaks are essential for identification and quantitation.

- **Factors Controlling Resolution**

The three major factors that control how the bands can be resolved from each other are the *retention factor, selectivity* and *number of plates.*

- **Retention Factor**

Retention or capacity factor, k', is a measure of the degree of retention of a solute. It is defined as the ratio of the reduced retention time to the dead time and, k' values are obtained from the elution chromatogram by

$$k' = \frac{t - t_0}{t_0}$$

where t_0 is the retention volume of a non-retained peak (or solvent peak).

The R_F coefficient in TLC is used to express the position of solute on the developed chromatogram.

$$Rf = \frac{\text{Distance travelled by the compound}}{\text{Distance travelled by the solvent front}}$$

- **Selectivity**

Selectivity, α, is a ratio of the net retention times of adjacent compounds. It may also be thought of as a ratio of the distribution coefficients. The desire is to get α to be large, so a short column can be used.

$$\alpha = \frac{t_2 - t_o}{t_1 - t_o} = \frac{k_2}{k_1}$$

- **Number of Theoretical Plates**

The efficiency of a chromatographic column is measured by the number of theoretical plates, *N*, to which the column is equivalent. This parameter is calculated from

$$N = 16\left(\frac{t_r}{w}\right)^2 = \frac{L}{H}$$

where t_r is the retention time of the peak and w is the base width of the peak measured in the same units, L is the measure of column efficiency per unit length and H is the height equivalent of a theoretical plate.

Separation of Enantiomers

Two basic approaches have evolved for enantiomeric resolution; a *direct* and an *indirect* approach.

➢ **Direct Approach**

Separation of a pair of enantiomers without a derivatization prior to separation process is termed as direct approach. Separations are based on three point interaction model proposed by Dalgliesh [32], according to which at least three interactions such as electrostatic attraction, hydrogen bonding, π-π interaction, and steric interactions out of which must be stereoselectively required for enantioresolution. Resolution is possible through reversible diastereomeric association

between the chromatographic chiral environment and the solute enantiomers. The enantiomers may interact during the course of chromatographic process with a chiral stationary phase (CSP) or a chiral selector added to the mobile phase or a chiral selector mixed with/immobilized on (especially in TLC) the stationary phase.

- **Chiral Stationary Phase (CSP)**

The different CSPs which are currently in use are either coated or bonded on to silica gel:

- **Polysaccharides:** Polysaccharides such as cellulose and amylose are among the most abundant optically active biopolymers with perfectly defined structures and can resolve enantiomers including amino acid derivatives and atropisomeric biphenyl derivatives, although their chiral recognition ability is not high [33-35]. Hesse and Hagel prepared the first practically useful CSP derived from polysaccharides in 1973 [36], which is microcrystalline cellulose triacetate. Recently, a series of phenylcarbamate derivatives having both an electron-donating methyl group and an electron-withdrawing halogen group on the phenyl moiety were prepared, to enhance their chiral recognition abilities as *meta*-and/or *para*-substituted cellulose phenylcarbamates have improved the resolution ability, but that *ortho*-substituted have decreased the chiral recognition ability[37-40]. In contrast, the *ortho*-substituted amylose phenylcarbamates showed relatively high chiral recognition [38].

- **Brush Type (Pirkle):** In 1976, Mikes *et al.* [41] introduced a new concept by attaching a small chiral molecule to silica gel where the organic groups of the chiral molecule remain directed away from the silica gel, appearing in the form of a brush; hence, it is called a brush-type phase. As a result of pioneering work of Pirkle and House [42] these stationary phases are commonly known as Pirkle-type CSPs. These CSPs are classified into three groups on the basis of *pi*-electron donor or a pi-electron receptor group of chiral selector attached to silica gel [*i.e.*, *pi*-acidic (with *pi*-electron-acceptor groups), *pi*-basic (with *pi*-electron-donor groups), and *pi*-acidic– basic (with *pi*-electron-acceptor and -donor groups)]. The most common π-acceptor phase is *N*-(3,5-dinitrobenzoyl)-phenylglycine bonded to *n*-propylamino silica.

- **Cyclodextrin:** Cyclodextrins are cyclic oligosaccharides containing from six to twelve D-(+)-glucopyranose units bonded through alpha-(1,4) linkages. Interaction of any polar regions of a solute molecule with the surface hydroxyls combined with the hydrophobic interactions in the cavity provides the 3-point interaction required for chiral recognition. Cyclodextrin (CD) stationary phases are formed by bonding the CD molecules to silica gel [43]. Among different forms of CD, β-CD has been found to have the widest application.

- **Macrocyclic Antibiotics:** Application of macrocyclic antibiotic CSPs has first reported by Armstrong *et al.* [44]. Three macrocyclic glycopeptides– vancomycin, teicoplanin and ristocetinA stationary phases are available commercially. These columns provide separation on the basis of π-π interactions, hydrogen bonding, inclusion complexation, ionic interactions and peptide binding.

- **Protein:** The protein stationary phases were the first to be developed in which natural proteins are bonded to a silica matrix. A number of commercially available protein based stationary phases, among them three main protein based stationary phase are human α-acid glycoprotein (AGP), human serum albumin (HSA), bovine serum albumin (BSA) and ovomucoid protein have been used to separate a wide range of chiral substances. A very wide range of molecules have been separated using AGP columns [45]. BSA is a globular protein with iosoelectric point of 4.7. A wide range of compounds has been separated on BSA columns [46].

The direct methods based on CSPs are preferred for preparative separation of enantiomers. The resolving power of the columns and detection sensitivity are not always adequate for sample analysis. The CSP columns are generally very expensive. Depending upon the nature and type of the CSP used, there may be certain critical disadvantages, particularly in HPLC, e.g., protein stationary phases are not durable over time and pH and also have low sample capacity. Stationary phases with crown ethers and cyclodextrins, involving host-guest type complexation, often result in poor band shape and have slow kinetics on a chromatographic time scale.

- **Chiral Selector as Impregnating Reagent**

Incorporation of a suitable reagent with the adsorbent without covalently affecting its inert character, prior to development of chromatogram, is termed as impregnation. The adsorption characteristics are changed without affecting the inert character of adsorbent. Use of chiral selectors as impregnating reagents provides an inexpensive wide choice of separation conditions for direct enantiomeric separation in TLC [47-49].

Various methods are used for impregnation of TLC plates. These are:

- Mixing the chiral selector with the inert support.
- Immersion of plain plates into an appropriate solution of the impregnating reagent.
- Allowing a solution of the impregnating reagent to ascend or descend the plate in a normal manner of development.
- Exposing the plain plates to the vapors of the impregnating reagent.
- Spraying the impregnating reagent (or its solution) on the plain plates.

The reagents or the methods used for impregnation are not to be confused with locating or spray reagents [50].

- **Chiral Ligand Exchange (CLE)**

Chiral Ligand Exchange (CLE) Chromatography, developed by Davankov in year 1969 [51] as the first liquid chromatographic technique successfully applied for complete separation of enantiomers of amino acids. Enantioresolution is based on formation of diastereomeric metal complexes. The presence of metal ions is essential, as it provides the site for the exchange process between the ligands and enantiomers. Racemic compounds are then separated by forming *in situ* diastereomeric metal (e.g. copper) complexes.

Synthesis, characterization and ion exchange properties of a cation exchanger zirconium (IV) tungstophosphate has been studied [52]. Various compounds have been enantioresolved using CLE chromatography using HPLC [53, 54], capillary electrophoresis [55-58] and micellar electrokinetic chromatography [59, 60].

- **Chiral Mobile Phase Additive (CMPA)**

A chiral selector is added to the mobile phase. CMPA is adopted both in TLC and HPLC. The CMPAs, which vary greatly, offer the advantage of using less expensive conventional achiral columns. As compared to the chiral columns, the achiral conventional columns which are more rugged and more efficient and have higher capacities are used in chiral resolution using CMPAs. Generally the reversed-phase columns which are compatible with a variety of mobile phases are used. The ideal CMPAs should have good solubility in the mobile phase and low UV absorbance.

Stereoselective separation obtained in a system with a chiral additive in the mobile phase can be due to one or a combination of the following 'mechanisms':

- A stereoselective complexation in mobile phase
- Adsorption of the chiral selector to the solid phase
- Formation of the labile diastereomeric complexes with different distribution properties between the mobile and stationary phase

Most of the chiral selectors used in CSPs mode of chiral separation are also used in CMPAs approach beside many more chiral selectors which are not used for CSPs.

Table 1.1 lists a few applications of CSPs, CMPAs and chiral impregnating reagents for separation of enantiomers (after year 2000).

Table-1.1: Some CSPs/CMPAs/chiral impregnating reagents used for direct resolution of a variety of enantiomers (after year 2000)

Chiral Stationary Phases	Analytes	References
α_1-Acid glycoprotein column	Propranolol	[66]
	Bupropion	[67]
	Penicillamine and Cysteine	[68]
Cyclodextrin column	Chlorpheniramine	[69]
	β-Lactams	[70]
	Amino alcohols	[71]
Cellulose column	Mexiletine	[72]
	Uniconazole and Imazalil	[73]
	Troglitazone	[74]
D-Penicillamine based ligand exchange column	Unusual secondary amino acids	[75]
	Non-protein amino acids	[76]
Chiral Mobile Phase Additives	**Analytes**	**References**
Cu(II)-L-Pro complex	Thyroxine	[77]
(*R*)-Mandelic acid and/or L-Tartaric acid	Penicillamine	[78]
	Ketamine and Lisinopril	[79]
	Fluoxetine	[80]
Vancomycin	β-Blockers	[81]
Chiral Impregnating Reagents	**Analytes**	**References**
D-Tartaric acid	Metoprolol	[82]
Cu(II)-L-Pro, Cu(II)-L-Phe, Cu(II)-L-His, Cu(II)-L-Trp complexes	β-Blockers	[83]
L-Asp and L-Glu	β-Blockers	[84]
Vancomycin	Verapamil	[85]
L-tartaric acid and L-His	Ephedrine and Atropine	[48]

➢ **Indirect Approach**

Several review articles [61-65] have revealed that the resolution of a pair of enantiomers by reacting them with a CDR, *i.e.* the formation of diastereomers, have different physicochemical properties, followed by their separation by chromatography in an achiral environment, is considered as an indirect approach.

The indirect approach is associated with the following advantages:

- excellent separation and detection possibilities of the resulting diastereomers,
- easy optimization of chromatographic conditions,
- prospect of using relatively inexpensive achiral columns,
- suitable for trace analysis of enantiomers in biological samples, such as blood and urine, because of the option of coupling the enantiomeric mixture with highly sensitive reagents with high molar absorptivity (ε) or high fluorescence quantum yield (φ), and
- sometimes better resolution and suitability for multi-component analysis compared to direct approach.

✸ **Chiral Derivatizing Reagents (CDRs)**

The most crucial aspect of indirect method is selection of suitable CDRs which have significant effect on success of separation, detectability of resulting diastereomer and accuracy of method. Therefore, the chosen CDR should fulfill following criteria:

- CDR should have high optical purity otherwise optical impurity will also react with substrate to form another pair of diastereomers.
- CDR should generally be freely soluble in water or water-miscible solvents, such as alcohol and acetonitrile because many bioactive chiral molecules are in *aq.* solution.
- CDR should possess specificity for the target functional group and should quantitatively label the analyte under mild conditions.
- The resulting diastereomers should have good stability.

- The reaction conditions should ensure a complete derivatization reaction and minimize the chances of racemization or degradation of the analyte.

Derivatization of analytes with reagents that afford structures absorbing in UV or Vis regions is the most popular means of derivatization. The reagents absorbing in Vis region are preferred in terms of selectivity because impurities present in sample generally absorb in UV region. Various types of FL labels have been developed for trace analysis in real samples. The fluorescence properties of the substances are greatly affected by temperature, viscosity of solvent, pH of medium, and contamination with halide ions such as Cl^- and Br^-. Detection limits with laser induced fluorescence (LIF) detection are typically one to five orders of magnitude lower than these are with FL and UV detection. Chemiluminescence (CL) reagent like luminol has provided the detection limits of 500 attomole during HPLC analysis [86].

✷ CDRs for Amino Compounds

Many biochemically important compounds, such as amino acids, biogenic amines and drugs, have at least one amino functional group in their structure. The reactions involving diastereomeric synthesis of primary and secondary amines with different CDRs have been extensively investigated. The major types of reactions for chiral amines, involving amino acids, are based on the formation of amides, carbamates, ureas and thioureas. The diastereomers so obtained have been generally separated by HPLC. The formation reactions of diastereomeric amides are widely used for the resolution of enantiomers of various amines. The reactions with acid halides and chloroformate reagents proceed rapidly to furnish the corresponding amides and carbamates. The acid halides are good labels because the reactions proceed under mild conditions. Since the reagent readily undergoes hydrolysis with water in the sample solution, contamination with water in the medium should be avoided. Another mode of amide formation is the reaction of amines with *N*-succinimidyl ester. These types of reagents are stable and can be used in *aq* media. Chiral isocyanates and mainly isothiocyanates are good labels to produce stable ureas and thioureas. *o*-Phthalaldehydes with chiral thiols give highly fluorescent adducts with the primary amino groups of amino acids, ensuring a very low detection limit in enantiomer analysis.

Amine has been converted to carbamate under mild and convenient synthesis conditions [87] and to amide under controlled microwave irradiation conditions [88]. The amino acid, DL-methionine is oxidized to its sulfoxide. The kinetics and mechanism of its oxidation by pyridinium fluorochromate has been studied [89]. The analogy between the syntheses of heterocyclic aromatic organic compounds indolizine and phosphaindolizines using cyclocondensation, cycloaddition, and electrocyclization method has been reviewed [90].

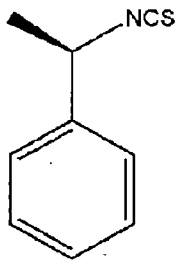
The CDRs based on DFDNB, and (*S*)-Nap have also been used for formation of diastereomers of compounds containing amino group. These are discussed in the respective paragraphs since such CDRs have been employed in the present studies.

The structures of a few representative CDRs used for amino compounds are shown in Fig. 1.3. Table 1.2 lists a few applications of CDRs for enantioseparation of amino compounds (after year 2002).

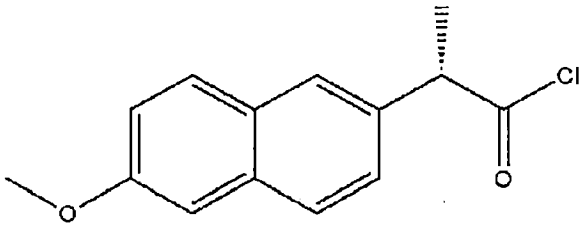
IV. Method Validation

After development of a method it is important to validate it to confirm that it is suitable for its intended purpose. The method validation is an essential concern in the activity of analytical chemical laboratories and is well implemented in pharmaceutical industry. The USFDA has edited draft guidelines with detailed recommendations for method validation of bioanalytical methods in the pharmaceutical industry. The International Conference on Harmonization (ICH) has provided definitions of validation issues and included them in "analytical procedures" for the field of bioanalytical methodology [91-93].

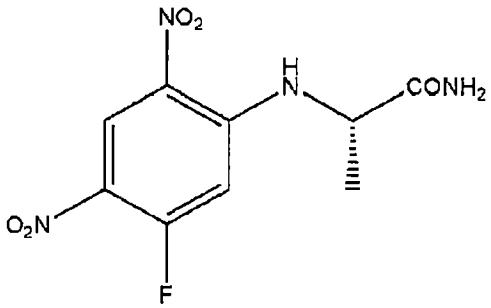
Validation of a method is the process by which a method is tested by the developer or user for reliability, accuracy and precision of its intended purpose. Data thus generated become part of the method validation package submitted to Centre for Drug Evaluation and Research. Method validation should not be a one-time situation to fulfill agency-filing requirement, but the method should be validated and also designed by the developer or user to ensure ruggedness or robustness.



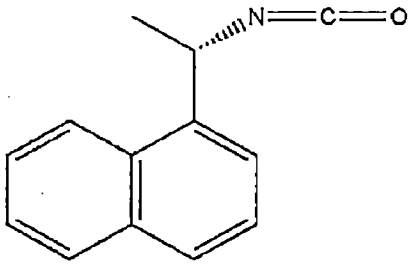
(*R*)-Methyl benzyl isothiocyanate



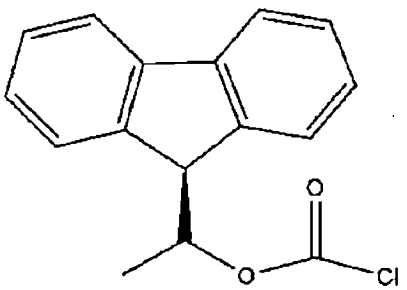
(*S*)-Naproxen chloride



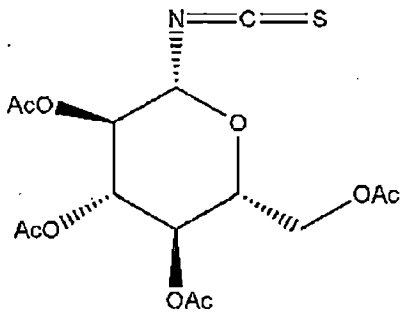
Marfey's reagent (MR)
FDNP-L-Ala-NH_2



(*S*)-1-(1-Napthyl) ethyl isocyanate



(+)-1-(9-Fluorenyl)ethyl chloroformate



2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate

Fig. 1.3: Structures of certain CDRs used for derivatization of amino compounds

Table-1.2: Some CDRs used for indirect HPLC resolution of enantiomers of different amino compounds (after year 2002)

S.No.	Chiral derivatizing reagents (CDRs)	Analytes	Source	Ref. No.
1	*o*-Phthalaldehyde/ *N*-(*R*)-mandelyl-L-cysteine	α-Amino acids	Beer	[94]
2	*o*-Phthalaldehyde/ *N*-acetyl-L-cysteine	α-Aminonitriles and α-alkyl-α-aminonitriles Selenomethionine	Standard compounds Antarctic krill	[95] [96]
3	*o*-Phthaldialdehyde/ *N*-isobutyryl-L-cysteine or *N-tert*-butyloxycarbonyl-L-cysteine	D-Serine and related neuroactive amino acids	Human plasma	[97]
4	*o*-Phenylenediamine and 2-mercaptoethanol	D-Amino acids	Amino acid oxidase	[98]
5	2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC)	Carvedilol Esmolol and its acid metabolite Spin-labeled, cyclic and β-amino acids	Human plasma Human plasma Standard compounds	[99] [100] [101]
6	2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl isothiocyanate (GATC)	β-Blockers	Standard compounds	[102]
7	(+)-1-(9-Fluorenyl)ethyl chloroformate (FLEC)	Glufosinate 3,4-Methylenedioxymethamphetamine Amino acids *N*-Methyl-D-glutamate and *N*-methyl-L-glutamate	Human serum Plasma and urine Biological tissues *Scapharca Broughtonii*(*Mollusca*)	[103] [104] [105] [106]

8	(*R*)-1-(1-Naphthyl)ethyl isocyanate (NEIC)	Fluoxetine and Norfluoxetine	Rat plasma and brain	[107]
9	(*S*)-Naproxen acyl chloride	Vigabatrin	Human Serum	[108]
10	(*R/S*)-4-(3-Isothiocyanatopyrrolidin-1-yl)-7-(*N,N*-dimethylaminosulfonyl-2,1,3-benzoxadiazole (DBD-PyNCS)	Thyroxine	Standard componds Human serum	[109] [110]
11	(1*S*,2*S*)-l,3-diacetoxy-l-(4-nitrophenyl)-2-propyl isothiocyanate (DANI)	Amino acids and β-blockers	Standard compounds	[111]
12	(*S*)-*N*-(4-Nitrophenoxycarbonyl)phenylalanine methoxyethyl ester (NIFE)	β-Alkyl-substituted amino acids α-Substituted proline analogues β-Amino acids	Standard compounds	[112] [113] [114]
13	*Trans*-2-(2,3-anthracenedicarboximido)cyclohexanecarbonyl chloride	Primary amines	Standard compounds	[115]
14	*N*-Fluorenylmethoxycarbonyl-L-alanyl *N*-carboxyanhydride	Amino acids	Standard compounds	[116]
15	DBD-*trans*-4-hydroxy-L-Pro, DBD-*cis*-4-hydroxy-L-Pro, DBD-*cis*-4-hydroxy-D-Pro, DBD-*trans*-3-hydroxy-L-Pro	Phenylethylamine, phenylalanine methylester, 1-(1-naphtyl) ethylamine	Rat plasma	[117]
16	4-(*N*-Chloroformylmethyl-*N*-methyl)amino-7-nitro-2,1,3-benzoxadiazole and 4-(*N*-chloroformylmethyl-*N*-methyl)amino-7-*N,N*-dimethylaminosulfonyl-2,1,3 benzoxadiazole	Fluoxetine	Rat plasma	[118]

The most common validation parameters are briefly described as follows:

Linearity

Beer's law is dependent on the linear range of detectability of compound analyzed and detector used. The working sample concentration and sample tested for accuracy should be in the linear range. The calibration curves should be linear in the studied range. The calibration curve equation is $y = bx+c$, where y is the absorbance and x represents the concentration of the drug.

Accuracy

Accuracy is the degree of closeness of a measured value to its true value. Accuracy studies for drug substance and drug product are recommended to be performed at the 60, 80, 100, 120, 140% levels of label claim as stated in the guidelines for submitting samples and analytical data for method validation.

Precision

The degree of agreement between two or more replicate measurements made on a sample in an identical manner, i.e., exactly in the same fashion, is known as the precision of the measurement. ICH has defined precision to contain the three components: repeatability, intermediate precision and reproducibility.

- **Repeatability (Intra-day Precision)**

The sensitivity or precision as measured by multiple injections of a homogenous sample indicates performance of the HPLC instrument under the chromatographic condition and the day tested.

- **Intermediate Precision (Inter-day Precision)**

The attribute valuates the reliability of the method in a different environment other than

that used during development of the method. The objective is to ensure that the method will provide the same result when similar samples are analyzed once the method development phase is over.

As defined by ICH, reproducibility expresses the precision between laboratories as in collaborative studies.

Limit of Detection and Limit of Quantitation

Limit of detection (LOD) is the lowest concentration of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. Limit of quantitation (LOQ) is the lowest concentration of analyte in a sample which can be determined with acceptable precision and accuracy under the stated experimental conditions.

Recovery

Recovery is expressed as the amount/weight of compound of interest analyzed as percentage to the theoretical amount present in the medium. The recovery should be determined by a minimum of six determinations for at least three concentrations in the range of expected concentrations.

Robustness

ICH defines robustness as a measure of the method capability to remain unaffected by small, but deliberate variations in method parameters. Robustness can be partly assured by good system suitability specifications. Thus, it is important to set tight, but realistic, system suitability specifications. Testing by varying some or all conditions, e.g., age of columns, column type, column temperature and pH of buffer in mobile phase is normally performed.

Sample Solution Stability

Solution stability of the drug substance or drug product after preparation according to the test method should be evaluated. Most of the laboratories utilize auto samplers with overnight runs and the sample will be in solution for hours in the laboratory environment before the test procedure is completed. This is of concern especially for drugs that can undergo degradation by hydrolysis, photolysis or adhesion to glassware.

Specificity/ Selectivity

The analyte should have no interferences from other extraneous components and should be well resolved from them. A representative HPLC chromatogram or profile should be generated and submitted to show that the extraneous peaks either by addition of known compounds or samples from stress testing are baselines resolved from the parent analyte.

Chapter-2

Experimental

I. Materials

All racemic and enantiomerically pure β-amino alcohols, 1,5-difluoro-2,4-dinitrobenzene (DFDNB), L-alaninamide hydrochloride (L-Ala-NH_2.HCl), L-phenylalaninamide hydrochloride (L-Phe-NH_2.HCl), L-valinamide hydrochloride (L-Val-NH_2.HCl), L-leucinamide hydrochloride (L-Leu-NH_2.HCl), L-methioninamide hydrochloride (L-Met-NH_2.HCl), DL-cysteine, D-cysteine, (*S*)-naproxen ((*S*)-Nap), 1*H*-benzotriazole, benzimidazole, dicyclohexyl carbodiimide (DCC), 4-dimethylamino pyridine (DMAP), D-penicillamine, DL-penicillamine, DL-homocysteine, (*R*, *S*)-mexiletine, (*S*)-(-)-(*N*)-trifluoro acetyl prolyl chlotide (TFPC), (1*S*)-(-)-camphanic chloride, racemic anti-ulcer drugs; omeprazole (OME), rabeprazole (RAB), lansoprazole (LAN), and pantoprazole (PAN) and optically pure (*S*)-omeprazole were obtained from Sigma-Aldrich (St. Louis, MO, USA). (*S*)-Lansoprazole and (*S*)-pantoprazole were obtained from Chemsky International Co., Ltd. (Shanghai, China) and Nanjing Linlead Pharmaceutical & Chemical Co., Ltd. (Jiangsu Province, China), respectively. (*S*)-Rabeprazole, (*R*,*S*)-cinacalcet and (*R*)-cinacalcet were obtained from Manus Aktteva (Ahmedabad, India). D-Phenylglycinamide (D-Phg-NH_2) was obtained from Pure Chemistry Scientific Inc. (TX, USA). L-Homocysteine was purchased from AK Scientific Inc. (Ahern Ave Union City, CA, USA). Tablets of dexrabeprazole (Emcure Pharmaceuticals Ltd., Pune, India) were obtained from the local drugstore.

Trifluoroacetic acid (TFA), glacial acetic acid, phosphoric acid, concentrated hydrochloric acid, sodium acetate, potassium hydroxide, sodium hydrogen carbonate, sodium sulphate, phosphorus pentaoxide, dimethyl sulfoxide (DMSO), acetone, chloroform, pyridine and, HPLC grade acetonitrile (MeCN), methanol (MeOH), ethanol (EtOH), hexane (HEX), isopropanol (IPA), dichloromethane (DCM), triethylamine (TEA) diethylamine (DEA) and tetrahydrofuran (THF) were purchased from E. Merck (Darmstadt, Germany) and E. Merck (Mumbai, India). All other chemicals were of analytical grade and were obtained from 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% aq suspension was from E. Merck (Mumbai, India). The names and abbreviations of amino acids, β-amino alcohols, and chiral compounds used in present studies are given in Table 2.1.

Table 2.1: Compounds used in present study

Sr. No.	Amino Alcohols	Abbreviations	Sr. No.	Other Compounds	Abbreviations
1	Alaninol	Alaol	9	Cinacalcet	Cin
2	Valinol	Valol	10	Mexiletine	MEX
3	Prolinol	Prool	11	Omeprazole	OME
4	Methioninol	Metol	12	Rabeprazole	RAB
5	Leucinol	Leuol	13	Lansoprazole	LAN
6	Phenylalaninol	Pheol	14	Pantoprazole	PAN
7	Homophenylalaninol	Homopheol	15	Cysteine	Cys
8	Phenylglycinol	Phgol	16	Penicillamine	PenA
			17	Homocysteine	Homocys

II. Equipment

✷ HPLC

The HPLC system consisting of a 10 mL pump head 1000, manager 5000 degasser, UV detector 2500, photodiode array (PDA) detector 2600, manual injection valve and Eurochrom operating software was from Knauer (Berlin, Germany).

Another HPLC system consisting of 515 pump, pump control module II, 2489 UV/Visible detector, and Empower operating software was from Waters Corporation, Milford, USA.

✷ HPLC Columns

Reversed-phase column, LiChrospher C_{18} (250 mm × 4.6 mm, I.D., 5 μm) was from Merck (Darmstadt, Germany).

Spherisorb ODS2 (250 × 4.6 mm I.D., 5 μm) column was from Waters Corporation, Milford, USA.

Lux cellulose-2 (250 × 4.6 mm I.D., 5 μm) column was from Phenomenex, CA, USA.

✸ **Other Instruments**

^{1}H and ^{13}C NMR spectra were recorded on a Brüker 500 MHz and 125 MHz instruments, respectively, using DMSO-d_6 and $CDCl_3$ as solvents. Perkin Elmer 1600 Fourier Transform Infrared (FT-IR) Spectrophotometer was used to record IR spectra in KBr pellets. CHN analysis was performed using Elementar Analysensysteme GmbH VarioEL III. Optical rotation was measured with the help of a digital polarimeter from Krüss Optronic instrument (P3002). UV spectra were recorded on UV-spectrophotometer model Shimadzu UV-1601 or Hitachi U 2001.

The pH of the solutions/buffers was maintained by Cyberscan 510 pH meter. It was calibrated by using buffer solutions of pH 4.0, pH 7.0 and 10.0 obtained from E. Merck (Mumbai, India). The Milli-Q system of Millipore (Bedford, USA) was used to purify double distilled water to deionised water (18.2 MΩ cm^3). The microwave used was Multiwave 3000 (800 W, Perkin-Elmer, Shelton, CT, USA).

III. Preparation of Stock Solutions

The following stock solutions were prepared:

- $NaHCO_3$ (1 M), triethyl amine (TEA, 6%), HOAc (5% and 1M), TEAP (10 mM), KH_2PO_4 (10 mM), borate buffer (0.1 M) and all the racemic and optically pure amino alcohols (0.4 mM) in purified water.
- (*R*,*S*)-Cin (11 mM) in 1 M $NaHCO_3$.
- DL-PenA, DL-Cys, DL-Homocys, D-PenA, D-Cys, and L-Homocys (0.5 mM) in 1 M $NaHCO_3$.

- (*R,S*)-MEX (12 mM and 20 mM), camphanic chloride (10 mM and 13 mM), and TFPC (10 mM and 13 mM) in DCM.
- All the racemic and optically pure chiral sulfoxides (0.1 g/L) in EtOH.

All solutions and samples were filtered through a 0.45 μm filter.

IV. Synthesis of Chiral Derivatizing Reagents (CDRs)

On the basis of experimental strategy as discussed in "present studies" following types of total eight CDRs were synthesised and characterized:

1. DFDNB based CDRs

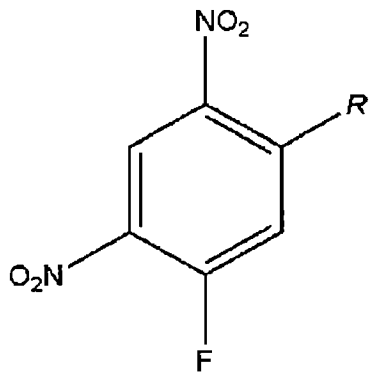
Six DFDNB based CDRs namely 1-Fluoro-2,4-dinitrophenyl-L-alaninamide (FDNP-L-Ala-NH_2; CDR1, Marfey's reagent, MR),1-Fluoro-2,4-dinitrophenyl-L-phenylalaninamide (FDNP-L-Phe-NH_2; CDR2), 1-Fluoro-2,4-dinitrophenyl-L-valinamide (FDNP-L-Val-NH_2; CDR3), 1-Fluoro-2,4-dinitrophenyl-L-leucinamide (FDNP-L-Leu-NH_2; CDR4), 1-Fluoro-2,4-dinitrophenyl-L-methioninamide (FDNP-L-Met-NH_2; CDR5), and 1-Fluoro-2,4-dinitrophenyl-D-phenylglycinamide (FDNP-D-Phg-NH_2; CDR6) were synthesized by substituting one of the fluorine atoms in DFDNB with six amino acid amides i.e. L-Ala-NH_2, L-Phe-NH_2, L-Val-NH_2, L-Leu-NH_2, L-Met-NH_2 and D- Phg-NH_2, respectively.

A summary of DFDNB based CDRs has been given in Fig. 2.1.

These six CDRs were synthesized as per procedure reported [119-121]. The reaction was carried out under MWI. The CDRs were characterized with the help of UV, IR, CHN, and ^{1}H NMR, and the characterization data is given below:

CDR1: FDNP-L-Ala-NH_2 (1-Fluoro-2,4-dinitrophenyl-L-alaninamide)

Color: yellow; m.p.: 221 °C (literature mp: 222 °C); Yield: 88%; UV (nm, in MeCN): 332 (λ_{max}), 263; IR (KBr): 3437, 3338, 2843, 1687, 1672, 1638, 1585, 1500, 1425, 1366, 1335, 1295, 1123, 1058, 841, 739, 605 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 1.44-1.46 (d, 3H, -CH_3), 4.37-4.42 (m, 1H, -CH), 6.95-6.98 (d, 1H, Ar-H), 7.51 (s, 1H, -$CONH_2$), 7.74 (s, 1H, -$CONH_2$), 8.90-8.91 (d, 1H, Ar-H), 9.10-9.12 (d, 1H, -NH); Anal. Calcd. for $C_9H_9FN_4O_5$: C, 39.71%; H, 3.33%; N, 20.58%. Found: C, 39.68%; H, 3.32%; N, 20.44%.



CDR No.	Chiral Auxiliary (-*R*)	CDR's Name*
1	L-Ala-NH_2	FDNP- L-Ala-NH_2
2	L-Phe-NH_2	FDNP- L-Phe-NH_2
3	L-Val-NH_2	FDNP-L-Val-NH_2
4	L-Leu-NH_2	FDNP- L-Leu-NH_2
5	L-Met-NH_2	FDNP- L-Met-NH_2
6	D-Phg-NH_2	FDNP- D-Phg-NH_2

*FDNP is abbreviated for fluoro dinitro phenyl moiety

Fig. 2.1: Summary of DFDNB based CDRs

CDR2: FDNP-L-Phe-NH_2 (1-Fluoro-2,4-dinitrophenyl-L-phenylalaninamide)

Color: yellow; m.p.: 192 °C (literature m.p.: 191 °C); Yield: 76%; UV (nm, in MeCN): 331 (λ_{max}), 262; IR (KBr): 3435, 3336, 2916, 1683, 1635, 1582, 1544, 1519, 1425, 1365, 1334, 1290, 1275, 1209, 1051, 918, 836, 739, 701, 588 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6): 3.12-3.16 (m, 1H, -CH_2), 3.24 (m, 1H, -CH_2), 4.67-4.71 (m, 1H, -CH), 6.80-6.83 (d, 1H, Ar-H), 7.20-7.29 (m, 5H, Ar-H (side chain)), 7.54 (s, 1H, -$CONH_2$), 7.87 (s, 1H, -$CONH_2$), 8.84-8.86 (d, 1H, Ar-H), 9.01-9.03 (d, 1H, -NH); Anal. Calcd. for $C_{15}H_{13}FN_4O_5$: C, 51.73%; H, 3.76%; N, 16.09%. Found: C, 51.58%; H, 3.65%; N, 16.03%.

CDR3: FDNP-Val-NH_2 (1-Fluoro-2,4-dinitrophenyl-L-valinamide)

Color: yellow; m.p.: 173 °C (literature m.p.: 174 °C); Yield: 87%; UV(nm, in MeCN): 329 (λ_{max}), 263, 215; IR (KBr): 3436, 3340, 2970, 2855, 1666, 1636, 1584, 1524, 1462, 1334, 1288, 1123, 1086, 923, 833, 747, 665 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6): 0.99-1.10 (dd, 6H, -$2CH_3$), 2.22-2.28 (m, 1H, -CH (side chain)), 4.22-4.25 (m, 1H, -CH), 7.01-7.04 (d, 1H, Ar-H), 7.40 (s, 1H, -$CONH_2$), 7.66 (s, 1H, -$CONH_2$), 8.85-8.86 (d, 1H, Ar-H), 8.90-8.91 (d, 1H, -NH); Anal. Calcd. for $C_{11}H_{13}FN_4O_5$: C, 44.00%; H, 4.36%; N, 18.66%. Found: C, 43.78%; H, 4.43%; N, 18.54%.

CDR4 : FDNP-Leu-NH_2 (1-Fluoro-2,4-dinitrophenyl-L-leucinamide)

Color: yellow; m.p.: 171 °C (literature m.p.: 171-173 °C); Yield: 91%; UV (nm, in MeCN): 328 (λ_{max}), 261; IR (KBr): 3412, 2957, 1662, 1618, 1571, 1408, 1302, 1214, 612 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6): 0.88-0.96 (dd, 6H, $2CH_3$), 1.73-1.76 (m, 3H, -CH_2CH), 4.31-4.34 (m, 1H, -CH), 6.97-7.00 (d, 1H, Ar-H), 7.43 (s, 1H, -$CONH_2$), 7.72 (s, 1H, -$CONH_2$), 8.70-8.72 (d, 1H, Ar-H), 8.89-8.91 (d, 1H, -NH); Anal. Calcd. for $C_{12}H_{15}FN_4O_5$: C, 45.86%; H, 4.81%; N, 17.83%. Found: C, 45.67%; H, 4.57%; N, 17.66%.

CDR5: FDNP-Met-NH_2 (1-Fluoro-2,4-dinitrophenyl-L-methioninamide)

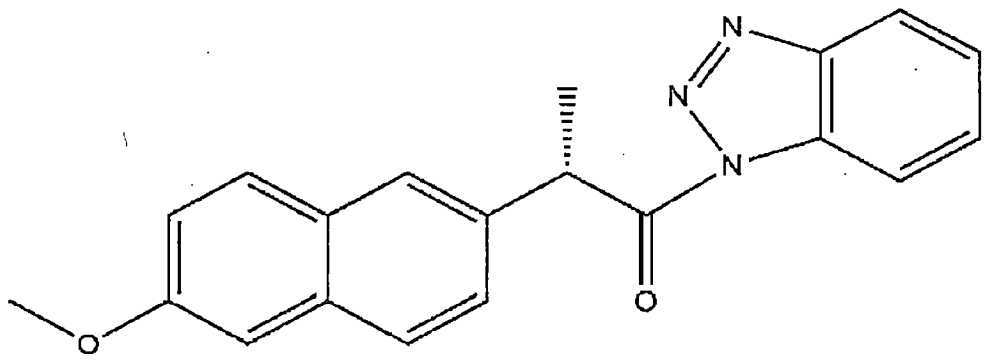
Color: yellow; m.p.: 135 °C; Yield: 84%; UV (nm, in MeCN): 331 (λ_{max}), 262; IR (KBr): 3436, 3342, 3174, 2919, 2872, 1692, 1656, 1633, 1587, 1518, 1466, 1422, 1376, 1326, 1282, 1122, 1057, 933, 855, 833, 739, 703, 586 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6, Fig. 2.1): 2.04 (s, 3H, -S-CH_3), 2.10-2.16 (m, 2H, -CH_2-), 2.46-2.50 (t, 2H, -CH_2-S), 4.46-4.50 (m, 1H, -CH), 7.05-7.08 (d, 1H, Ar-H), 7.55 (s, 1H, -$CONH_2$), 7.80 (s, 1H, -$CONH_2$), 8.89-8.90 (d, 1H, Ar-H), 8.99-9.00 (d, 1H, -NH); Anal. Calcd. for $C_{11}H_{13}FN_4O_5$: C, 39.76% ; H, 3.94%; N, 16.86%. Found: C, 39.44%; H, 3.77%; N, 16.47%.

CDR6: FDNP-Phg-NH_2 (1-Fluoro-2,4-dinitrophenyl-D-phenylglycinamide)

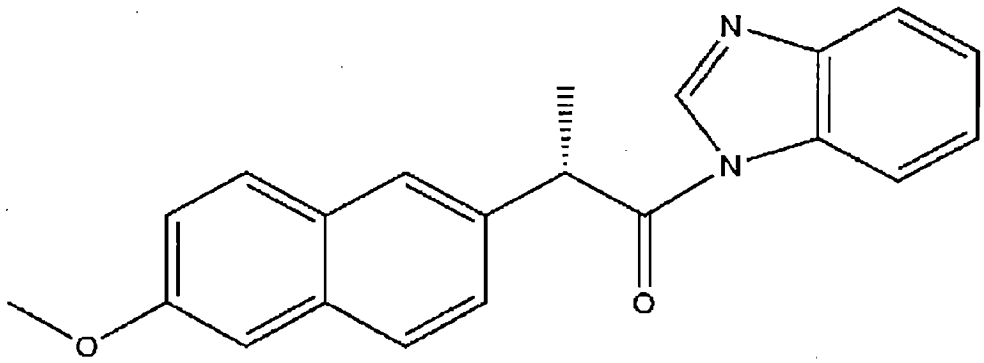
Color: yellow; m.p.: 188 °C; Yield: 88%; UV (nm, in MeCN): 330 (λ_{max}), 261, 206; IR (KBr): 3436, 3339, 3095, 1682, 1636, 1613, 1578, 1525, 1501, 1453, 1413, 1369, 1328, 1281, 1232, 1114, 1050, 933, 853, 828, 741, 720, 695, 655, 605 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6, Fig. 2.2): 5.43-5.44 (m, 1H, -CH), 6.60-6.63 (d, 1H, Ar-H), 7.34-7.41(m, 3H, Ar-H), 7.55-7.57 (d, 2H, Ar-H), 7.64 (s, 1H, -$CONH_2$), 7.83 (s, 1H, -$CONH_2$), 8.88-8.90 (d, 1H, Ar-H), 9.74-9.75 (d, 1H, -NH); Anal. Calcd. for $C_{14}H_{11}FN_4O_5$: C, 50.31%; H, 3.32%; N, 16.76%. Found: C, 50.22%; H, 3.23%; N, 16.65%.

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

Two CDRs based upon (*S*)-naproxen; (*S*)-naproxen-benzotriazole and (*S*)-naproxen-benzimidazole were synthesized for the first time and characterized with the help of UV, IR, CHN, and ^1H NMR and ^{13}C NMR. The two CDRs are shown in Fig. 2.2.



(a)



(b)

Fig. 2.2: Structures of (a) (*S*)-Naproxen-benzotriazole (b) (*S*)-Naproxen-benzimidazole

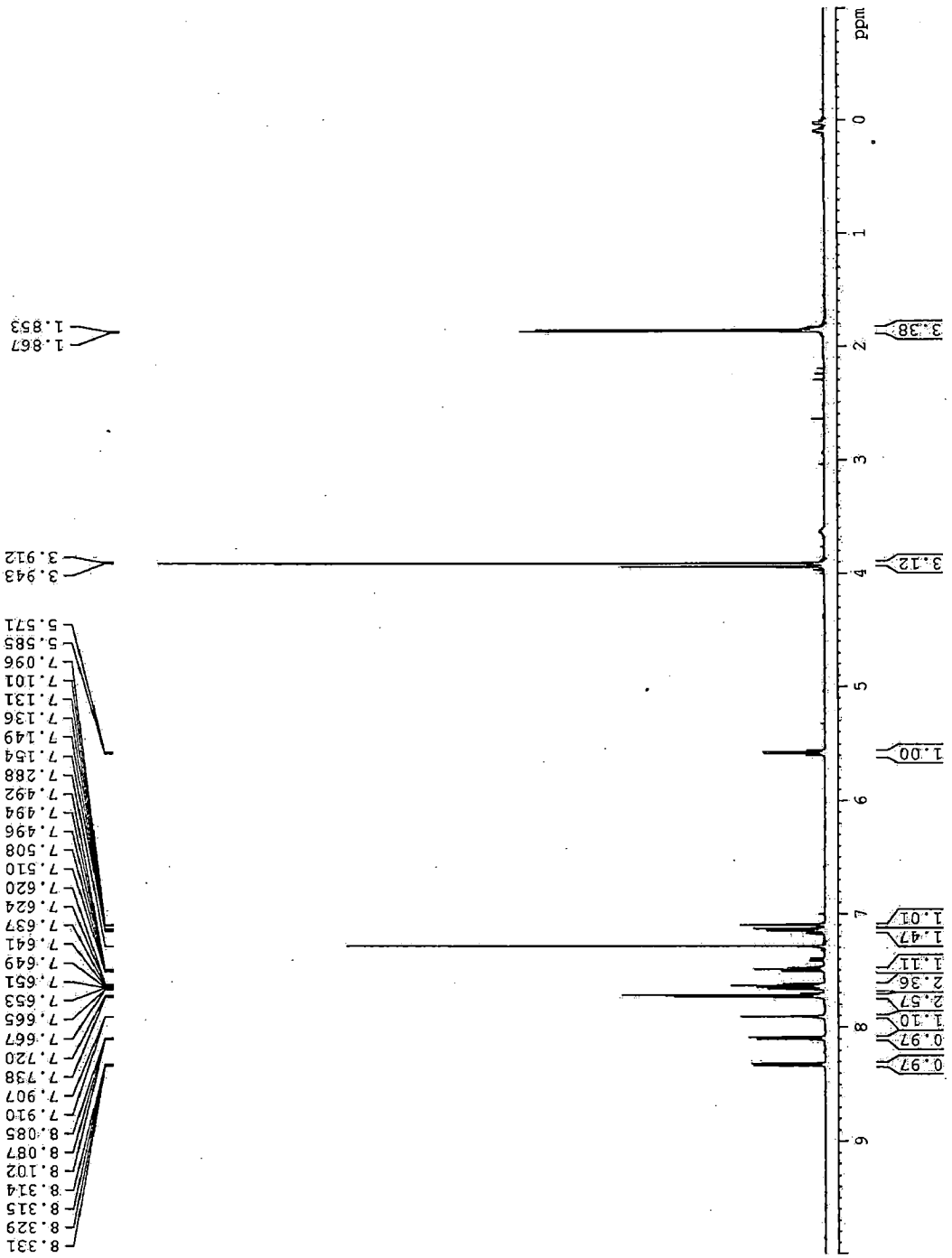


Fig. 2.3: ^{1}H NMR spectrum of (*S*)-Nap-Btz

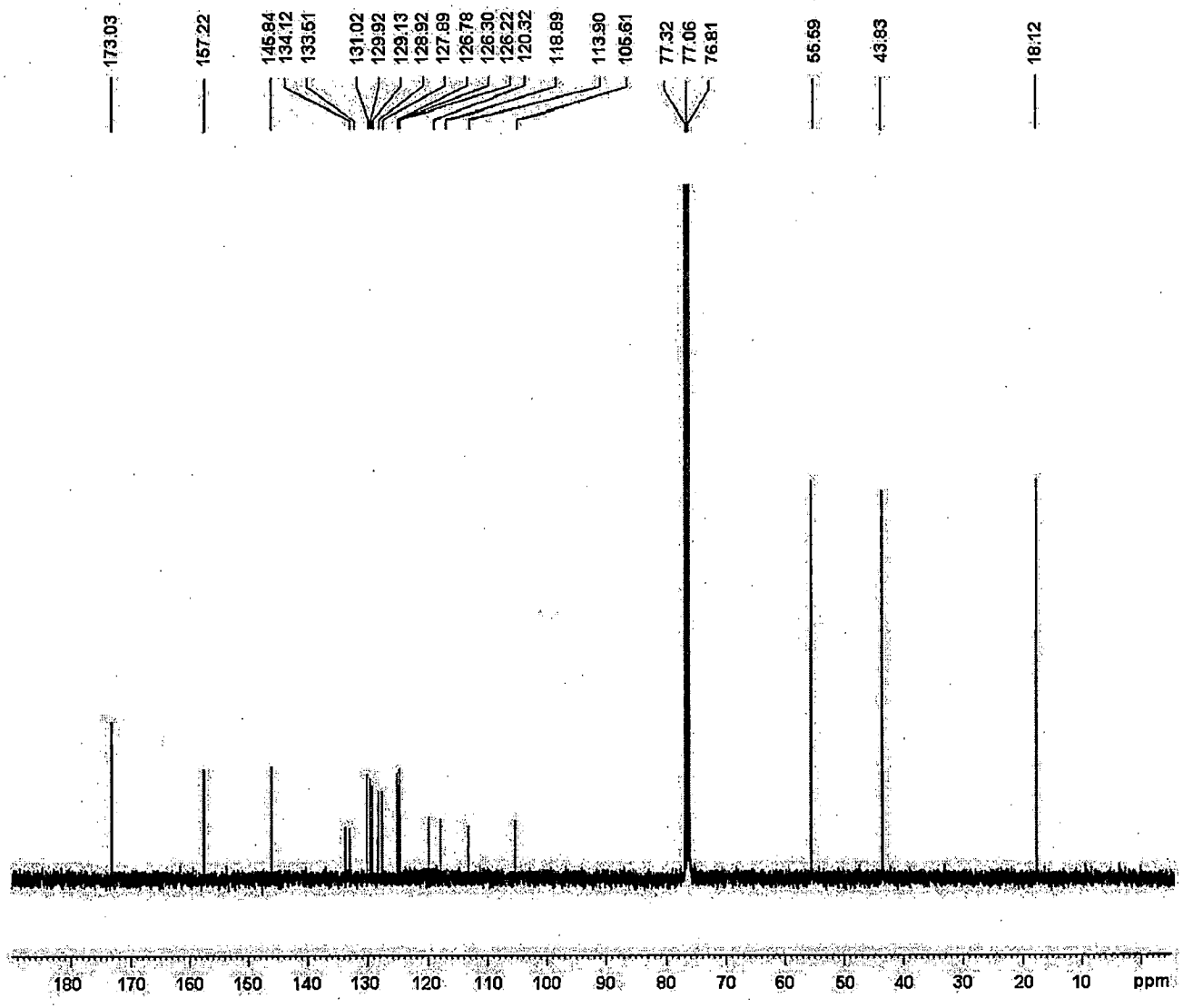


Fig. 2.4: ^{13}C NMR spectrum of (*S*)-Nap-Btz

Synthesis of (*S*)-Nap-Btz ((*S*)-1-(1*H*-benzo[d][1,2,3]triazol-1-yl)-2-(6-methoxynaphthalen -2-yl) propan-1-one)

4-dimethyl amino pyridine (DMAP, 16 mg; 0.13 mmol) and dicyclohexyl carbodiimide (DCC, 540 mg; 2.60 mmol) were added to a stirred solution of 1*H*-benzotriazole (1*H*-Btz, 155 mg; 1.30 mmol) and (*S*)-naproxen (Nap, 300 mg; 1.30 mmol) in dichloromethane (10 mL). The reaction mixture was stirred vigorously for 2.5 h at room temperature. The reaction mixture was filtered and the residue (dicyclohexyl urea) was rejected. The filtrate was concentrated in vacuuo and the residue was re-dissolved in ethyl acetate (EtOAc, 15 mL). The extract was washed five times with water, five times with brine solution, five times with concentrated $NaHCO_3$ and finally ten times with 1N HCl. The purified extract was dried over $MgSO_4$ and the solvent was removed under reduced pressure. The residue (as the desired CDR) was recrystallized from hexane.

Colour: white; m.p. 181.0-182.0 °C; Yield: 94.5%; $[\alpha]_D^{25}$ (c = 1.880, DMF) = +249.42°; UV (nm, in DMF): 231 (λ_{max}); IR (KBr): 3327, 3125, 3037, 2926, 2851, 2659, 2345, 1625, 1572, 1441, 1311, 1239, 1184, 1086, 1054, 947, 894, 796 and 652 cm^{-1}; ^1H NMR (500MHz, $CDCl_3$, Fig. 2.3) δ 1.85 (d, 3H, CH_3–CH<), 3.92 (s, 3H, CH_3–O–), 5.57 (q, 1H, >CH–CH_3), 7.09 (d, 1H, Nap–5–H), 7.13 (dd, 1H, Nap–3–H), 7.28 (d, 1H, Nap–7–H), 7.49-7.51 (m, 1H, Btz–5–H), 7.62-7.67 (m, 1H, Btz–6–H), 7.72 (d, 2H, Nap–4,8–H), 7.91 (d, 1H, Nap–1–H), 8.08 (d, 1H, Btz–4–H), 8.32 (d, 1H, Btz–7–H); ^{13}C NMR (125 MHz, $CDCl_3$, Fig. 2.4) δ 18.1, 43.8, 55.6, 105.6, 113.9, 118.9, 120.3, 126.2, 126.3, 126.8, 127.9, 128.9, 129.1, 129.9, 131.0, 133.5, 134.1, 145.8, 157.2, 173.0; Anal. calcd. for $C_{20}H_{17}N_3O_2$: C, 72.49%; H, 5.17%; N, 12.68%. Found: C, 72.42%; H, 5.20%; N, 12.61%.

Synthesis of (*S*)-Nap-BImz ((*S*)-1-(1H-benzo[d]imidazol-1-yl)-2-(6-methoxynaphthalen-2-yl) propan-1-one)

To a solution of (*S*)-naproxen (Nap, 250 mg; 1.08 mmol) and benzimidazole (BImz, 128 mg; 1.08 mol) in dichloromethane (10 mL), dicyclohexyl carbodiimide (DCC, 450 mg; 2.16 mmol) and 4-dimethyl amino pyridine (DMAP, 14 mg; 0.11 mmol) were added and stirred

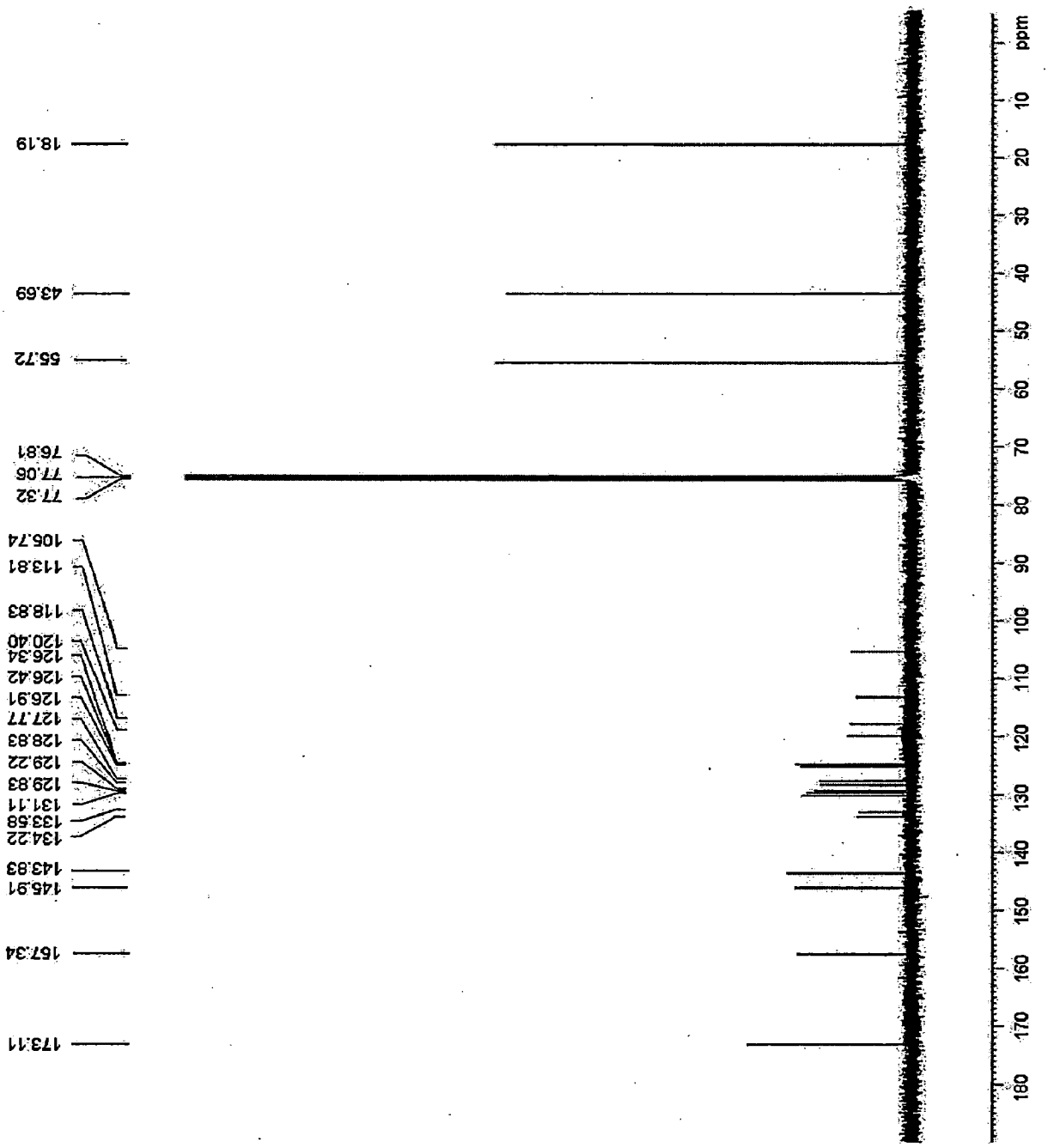


Fig. 2.5: ^{13}C NMR spectrum of (*S*)-Nap-BImz

vigorously for 4 h at room temperature. A white precipitate of dicyclohexyl urea appeared on completion of reaction and was removed after filtration. The filtrate was concentrated in vacuuo and the residue was re-dissolved in ethyl acetate (EtOAc, 10 mL). The extract was washed five times with water, five times with brine solution, five times with concentrated $NaHCO_3$ and finally ten times with 1N HCl before drying over $MgSO_4$ and the solvent was removed from the washed extract under reduced pressure. The residue was recrystallized from hexane to obtain the desired CDR as crystals.

Colour: white; m.p. 189.0-190.0 °C; Yield: 92.5%; $[\alpha]_D^{25}$ (c = 1.910, DMF) = +236.14°; UV (nm, in DMF): 231 (λ_{max}); IR (KBr): 3327, 2926, 2855, 2655, 2357, 1625, 1572, 1437, 1313, 1241, 1090, 894, 681 and 645 cm-1; ^{1}H NMR (500MHz, $CDCl_3$) δ 1.84 (d, 3H, CH_3– CH<), 3.93 (s, 3H, CH_3–O–), 5.58 (q, 1H, >CH–CH_3), 7.08 (d, 1H, Nap–5–H), 7.14 (dd, 1H, Nap–3–H), 7.29 (d, 1H, Nap–7–H), 7.49-7.51 (m, 1H, BImz–5–H), 7.62-7.67 (m, 1H, BImz–6–H), 7.71 (d, 2H, Nap–4,8–H), 7.92 (d, 1H, Nap–1–H), 8.07 (d, 1H, BImz–4–H), 8.15 (s, 1H, BImz–2–H), 8.31 (d, 1H, BImz–7–H); ^{13}C NMR (125 MHz, $CDCl_3$, Fig. 2.5) δ 18.2, 43.7, 55.7, 105.7, 113.8, 118.8, 120.4, 126.3, 126.4, 126.9, 127.8, 128.8, 129.2, 129.8, 131.1, 133.6, 134.2, 143.8, 145.9, 157.3, 173.1; Anal. calcd. for $C_{21}H_{18}N_2O_2$: C, 76.34%; H, 5.49%; N, 8.48%. Found: C, 76.32%; H, 5.50%; N, 8.49%.

3. Enantiomeric purity of CDRs

The chiral purity of the CDRs1-6 is based on the chiral purity of the amino acid amides used for synthesis. The amino acid amides were considered to be chirally pure as the Sigma-Aldrich catalogue [122] has mentioned their specific rotation values which can be considered as the literature values for chirally pure samples. Nevertheless, the chiral purity of these amides was further verified by the following method. In all, six amides were used to prepare the CDRs. Each of these CDRs was reacted with chirally pure L-Leu, $[\alpha]_D^{25}$ = +15.5° (c=5 % in 5 M HCl) [123], when the corresponding diastereomers of only L-Leu were obtained. A single HPLC peak using mobile phase mentioned in literature [119] corresponding to the diastereomer of optically pure L-Leu prepared with each of the six CDRs confirmed the chiral purity of amides. Any impurity of the

other antipode in the amide (i.e. of the corresponding D-amino acid) would have provided a mixture of diastereomers of L-Leu with each of the CDR, and thus two peaks in HPLC.

When the racemic analyte, say (*R,S*)-cinacalcet, was reacted with the CDR1 and the product (i.e. the diastereomeric mixture) so obtained was investigated on RP-column using different mobile phases, appearance of a pair of diastereomeric peak detected by PDA detector confirmed the enantiomeric purity of the CDR (and the separation of the two diastereomers of the analyte). Thus, the CDR1 was enantiomerically pure. The presence of only two diastereomeric peaks corresponding to (*R,S*)-cinacalcet in itself is a confirmation of the enantiomeric purity of the CDR. Any impurity of the other antipode in the amide would have provided another pair of corresponding diastereomeric peaks in the chromatogram. The same procedure was applied to check the chiral purity of the rest of the CDRs2-6.

V. Extraction of Dexrabrprazole from Commercial Tablet

Ten commercial tablets of 10 mg of dexRAB pulverized to powder form with a pestle. Methanol was added to the powder and the mixture was sonicated for 30 min and filtered. The filtrate was evaporated to dryness under reduced pressure, and the residue was dissolved in 10 mM KH_2PO_4 buffer (pH 5), and the free form of dexRAB was extracted with dichloromethane. The upper aqueous phase was removed and the dichloromethane phase, containing the free form of dexRAB, was evaporated under vacuum.

Chapter-3

Enantioseparation of Cinacalcet

I. Introduction

Cinacalcet (Cin) is the first drug of a new class of therapeutic agents known as calcimimetics. It is a principal negative regulator of parathyroid hormone release. It is chemically named as [1-(*R*,*S*)-(-)-(1-naphthyl) ethyl]-3-[3-(trifluoromethyl) phenyl]-1- aminopropane (Fig. 3.1). Cinacalcet is currently used for primary and secondary hyperparathyroidism (HPT) [124]. It binds to the calcium-sensing receptors (CaR) of the parathyroid glands, lowers the sensitivity for receptor activation by extracellular calcium, and thus parathyroid hormone release is diminished [125]. It is also proved effective in a broad range of chronic kidney disease patients on dialysis with uncontrolled HPT [126]. The active part of cinacalcet is its (*R*)-enantiomer.

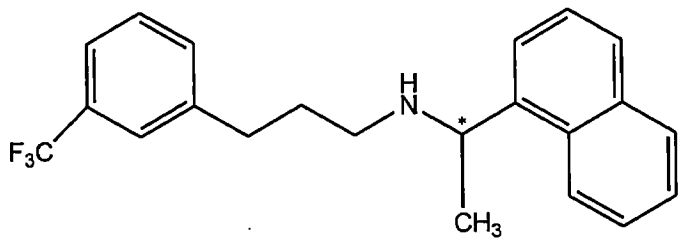


Fig. 3.1: Structure of (*R*, *S*)- Cinacalcet

II. Literature Review

Literature reveals only one report on the separation of (*S*)-Cin from (*R*)-Cin using a Chiralpak-IA column [127].

✷ **Selection of Appropriate CDR for the Synthesis of Diastereomers of (*R*,*S*)-Cin**

Since Cin has secondary amine functional group it was considered to develop/ synthesize by reaction of secondary amine functional group with the suitable CDR providing appropriate reaction site. Therefore, Marfey's reagent and its chiral variants were considered to be possible useful CDRs.

Based on scope of indirect approach for enantioseparation (see Chapter-1) applications of Marfey's reagent and its various chiral variants have been reviewed as under:

➢ **Application of Marfey's Reagent and its Chiral Variants as CDR**

Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide, FDNP-L-Ala-NH_2, MR) was first prepared by substituting one of the fluorine atoms in DFDNB by L-Ala-NH_2 [128]. It has been reported to be one of the most successful ones [129, 130] for indirect enantioseparation of a variety of compounds containing 1° or 2° amino group. The diastereomers of different racemate prepared with MR show strong absorbance at 340 nm. The DFDNB moiety has been used to prepare a variety of CDRs by substituting one of the fluorine atoms with different amino acid amides, amino acids and other chiral amines as chiral auxiliaries. In the recent years several such CDRs have been used for reversed-phase high-performance liquid chromatographic enantioresolution of penicillamine [131], baclofen [132], protein and non-protein amino acids [119, 133, 134], β-blockers [135, 136] and mexiletine [137] (all containing an amino group and capable of substituting the fluorine atom in MR (and its chiral variants) giving rise to corresponding diastereomers).

On the other hand, because of versatile applicability of TLC (see Chapter-1) the impregnated TLC has been reviewed and summarized as below:

✷ **Impregnated Thin Layer Chromatography**

We know TLC is one of the most simple, economical and versatile technique for direct enantioseparation. Literature reveals that TLC has been successfully applied for direct enantioseparation of DL-amino acids [47, 50, 138, 139] and non-steroidal anti-inflammatory drugs [140]. Application of optically pure amino acids as suitable chiral impregnating reagents in TLC for direct enantiomeric separation and determination of enantiomeric purity of certain pharmaceutically important compounds has been discussed and reviewed [50]. L-Arg [141, 142] and other basic amino acids were the most investigated ones as chiral selectors.

III. Present Work

The calcimimetic drug (*R,S*)-Cin was enantioresolved using both indirect and direct approaches. Six CDRs based on DFDNB having L-Ala-NH_2, L-Phe-NH_2, L-Val-NH_2, L-Leu-NH_2, L-Met-NH_2 and D-Phg-NH_2 as chiral auxiliaries were synthesized employing microwave irradiation and were characterized (see Chapter-2). These were used as chiral derivatizing reagents for derivatization for (*R,S*)-Cin under microwave irradiation conditions and the derivatization conditions were optimized. RP-HPLC was applied using binary mixtures of aqueous trifluoroacetic acid and acetonitrile for separation of diastereomeric pairs with ultraviolet detection at 340 nm. Thin silica gel layers impregnated with optically pure L-His and L-Arg were used for direct resolution of enantiomers of (*R,S*)-Cin.

(A) Indirect Resolution Using CDRs Based on DFDNB

1. Synthesis of CDRs Based on DFDNB

Six CDRs having amino acid amides, viz., L-Ala-NH_2, L-Phe-NH_2, L-Val-NH_2, L-Leu-NH_2, L-Met-NH_2 and D-Phg-NH_2, as chiral auxiliaries namely CDRs1-6, respectively, were synthesized as per procedure reported [119-121]. The reaction was carried out under MW

irradiation. The CDRs were characterized with the help of UV, IR, CHN, and ^{1}H NMR. The synthesis and characterization data of these CDRs have been described in Chapter-2.

2. Microwave Irradiated Synthesis of Diastereomers of (*R*,*S*)-Cin

Solution of Cin (11 mM) was prepared in $NaHCO_3$ (1 M)-MeCN (90:10, v/v). To a solution of (*R*,*S*)-Cin (100 mL, 1.1 mmol) in a Teflon tube were added the solutions of CDR1 (110 mL, 1% in acetone, 1.7 mmol) and TEA (60 mL, 6%). The resulting mixture was irradiated under MW for 3 min using 75% power. After cooling to room temperature, HOAc (60 mL, 5 %) was added to quench the reaction. Ten microlitres of the resulting solution, containing diastereomers, was diluted ten times with MeCN, and 20 μL of it was injected onto column.

The above described method was followed for the synthesis of diastereomers of (*R*,*S*)-Cin using the rest five CDRs. The reaction conditions for derivatization were optimized by derivatizing (*R*,*S*)-Cin with CDR5. Solutions of chiral derivatizing reagents and synthesized diastereomers of Cin were found to be quite stable up to 1 week when they were kept in dark at 4°C.

3. HPLC Operating Conditions for Separation of Diastereomers

RP-HPLC was performed on a Waters Spherisorb ODS2 (250 × 4.6 mm I.D., 5 μm) column with the mobile phase consisting of aq TFA (0.01 M)-MeCN in a linear gradient of MeCN from 30 to 65% in 45 min at a flow rate of 1.0 mL/min and UV detection at 340 nm.

4. Method Validation

The experimental method was validated with respect to linearity, accuracy and precision for the diastereomers of (*R*,*S*)-Cin prepared with CDR5 according to ICH guidelines [92] and summarized here:

Linearity

The peak area response of (*R*)-Cin and (*S*)-Cin, the first and the second eluting diastereomers, prepared with CDR5 was plotted against the corresponding concentration (100-500 pmol) and the linear regression was computed by the least square method using Microsoft Excel program.

Accuracy and Precision

The intra-day assay and inter-day assay studies for accuracy and precision were carried out by replicate HPLC analysis (n=5) of diastereomers of (*R,S*)-Cin at five concentrations (25, 30, 35, 40, 50 $ngmL^{-1}$).

(B) Direct Enantioresolution Using L-Histidine and L-Arginine as Chiral Impregnating Reagents

1. Preparation of Stock Solutions and Impregnated Plates

Stock solutions (25 mg ml^{-1}) of (*R,S*)-Cin and (*S*)-Cin were prepared in dilute HCl (pH 4). Solutions of L-His and L-Arg (1.0%) were prepared in distilled water (50 mL) and a few drops of ammonia were added to bring the pH to 9, 10, 11, 11.5 and 12. Slurry of silica gel G (25 g) was prepared in these solutions. The TLC plates (10 cm × 5 cm × 0.5 mm) were prepared by spreading the slurry with Stahl type applicator; the plates were activated overnight at 60 ± 2 °C.

2. TLC Resolution

Solutions of racemic and pure isomers of Cin were applied side by side on the plates with a 25 µL Hamilton syringe. Cleaned, dried and paper-lined rectangular glass chambers were used for developing the chromatograms. These were pre-equilibrated with mobile phase MeCN-MeOH-H_2O in different proportions at 18 ± 2°C for 10–15 min. Chromatograms were dried at 45 °C in an

oven for 10–15 min and cooled to room temperature; spots were located in an iodine chamber.

3. Method Validation

The developed method was validated on the basis of correlation between absorbance and concentration of enantiomers recovered from the plates impregnated with L-His and L-Arg.

IV. Results and Discussion

(A) Indirect Resolution Using CDRs Based on DFDNB

1. Optimization of Conditions for Derivatization

✷ Role of pH

Use of triethyl amine (60 μL, 6%) at a pH around 10.0 was found to be optimum to obtain the best yield for derivatization of (*R,S*)-Cin with CDR5. No derivatization was observed in the absence of triethyl amine. These conditions were, therefore, used for all the derivatization reactions for quantitative yields.

✷ Effect of Excess of CDR

CDR5 was used in 1-3 fold molar ratio to find the optimum reagent concentration for derivatization of (*R,S*)-Cin. The derivatization was complete when 1.5 fold molar excess of the reagent was used for 3 min using MW (at 75% power). Further increase in reagent concentration (2 to 3 fold) had little effect on reaction time and yield of derivatization. Therefore, all the CDRs were used in 1.5 molar excess for quantitative derivatization and to overcome kinetic resolution.

✷ **Microwave Irradiation**

(*R,S*)-Cin was irradiated for 2-4 min with MW (at 75% power); a time of 3 min was found successful. According to the literature [120] derivatization of β-amino alcohols took place within a minute using MR and its chiral variants as the CDRs but in the present case reaction time is relatively high which may be because Cin is overcrowded at just next to the stereogenic centre.

2. HPLC

The chromatographic data for separation of diastereomers is given in Table-3.1. It shows that the diastereomeric pairs prepared with all the six CDRs (having amino acid amides as the chiral auxiliaries) were well separated. The R_S value for the diastereomeric pair prepared with FDNP-L-Met-NH_2 (CDR5) is 2.27 and is thus better resolved than the remaining five diastereomeric pairs. The R_S value for the diastereomeric pair prepared with FDNP-D-Phg-NH_2 (CDR6) is 1.61, the lowest among the six diastereomeric pairs.

Six pairs of diastereomers of Cin were synthesized as mentioned above. (*R*)-Isomer was found to elute before the (*S*)-isomer in the case of diastereomers prepared with CDRs1–5. The elution order was reversed for the diastereomers prepared with CDR6 as the chiral auxiliary has D-configuration in this reagent. Sharp peaks showing base line separation for the separation of diastereomers prepared with CDRs3-6 and slightly broader peaks for those prepared with CDRs1-2 were obtained. The chromatograms showing the separation of respective pairs are given in Fig. 3.2.

Table 3.1: High performance liquid chromatographic data for enantioseparation of (*R,S*)-Cin

Para meter	Diastereomers prepared with					
	FDNP-L-Ala-NH_2 (CDR1)	FDNP-L-Phe-NH_2 (CDR2)	FDNP-L-Val-NH_2 (CDR3)	FDNP-L-Leu-NH_2 (CDR4)	FDNP-L-Met-NH_2 (CDR5)	FDNP-D-Phg-NH_2 (CDR6)
k_R	4.33	4.36	4.33	4.71	3.91	7.19
k_S	5.32	5.36	5.43	5.66	4.92	8.11
α	1.23	1.22	1.25	1.20	1.26	1.23
R_s	1.93	1.77	2.11	1.75	2.27	1.61

k_R and k_S are retention factor of the diastereomers of (*R*)-Cin and (*S*)-Cin respectively;
α is stereoselective factor;
R_S is the resolution of diastereomer of (*S*)-Cin

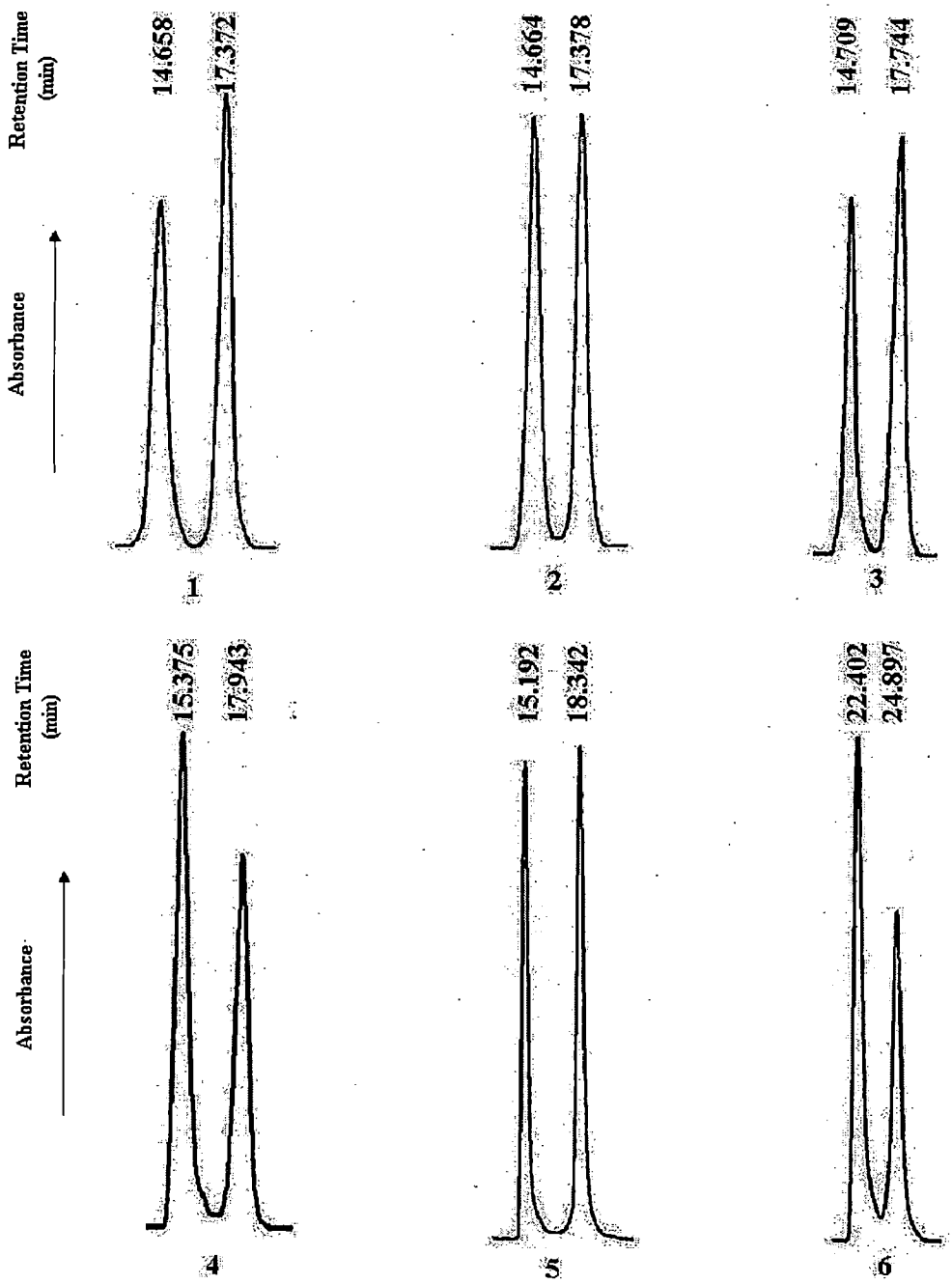


Fig. 3.2: Sections of chromatograms showing resolution of (*R*,*S*)-Cin as their diastereomers prepared with CDRs1–6; Waters Spherisorb ODS2 (250 × 4.6 mm I.D., 5µm); detection at 340 nm for all the diastereomers; (*R*)-enantiomer elutes first prior to (*S*)-enantiomer for CDRs1-5 while elution order reverses for CDR6; mobile phase was *aq* TFA (0.01 M) - MeCN in a linear gradient of MeCN from 30 to 65% in 45 min

The CDRs are as mentioned in Table 3.1.

Optimization of HPLC Conditions

Acetonitrile was found to be a better organic solvent in comparison to methanol as broader peaks (and no separation in a few cases) were obtained with the later. Linear gradients of MeCN from 20-65%, 25-65%, 30-65%, 35-65%, 40-65%, 45-65% and 50-60% in 45 min were applied. The experiments showed that both the retention times and R_S decreased with the increasing amount of MeCN. Thus, a linear gradient of MeCN from 30 to 65% in 45 min was found to be successful. Effect of change of flow rate revealed that the retention times increased with broadening of peaks as the flow rate was decreased from 1.0 to 0.5 mL/min. On the other hand, an increase in flow rate from 1.0 to 2.0 mL/min resulted into a decrease in both the retention times and R_S. Thus the optimized flow rate of 1.0 mL/min was used throughout the run.

3. Method Validation

Linearity

A good linear relationship was obtained over the range mentioned in "Present Work" section. The regression equations were y=0.679x+1.213 (R^2=0.999) and y=0.647x-2.43 (R^2 = 0.998) for the diastereomers of (*R*)-Cin and (*S*)-Cin prepared with CDR5, respectively.

Accuracy and Precision

The coefficient of variation (%) for (*R*)-and (*S*)-Cin varied from 0.10 to 1.39 and 0.40 to 1.28 for intra-day assay precision and 0.44 to 1.38 and 0.40 to 1.43 for inter-day assay precision. The percentage recovery for (*R*)- and (*S*)-Cin varied from 99.0 to 99.7 and 98.6 to 99.1 for intra-day assay and 97.8 to 98.9 and 97.6 to 97.9 for inter-day assay (Table-3.2).

For indirect approach, using CDR5, LOD was found to be 60 pmol and LOQ was found to be 180 pmol, for each diastereomer of the diastereomeric pair.

Table 3.2: Intra-day assay and inter-day assay precision of diastereomers of (*R,S*)-Cin prepared with CDR5

Concentration ($ngmL^{-1}$)	First Eluting Diastereomer			Second Eluting Diastereomer		
	Mean±SD	Recovery*	CV*	Mean±SD	Recovery*	CV*
Intra-day						
25	24.92±0.06	99.6	0.24	24.65±0.16	98.6	0.64
30	29.90±0.03	99.6	0.10	29.69±0.12	98.9	0.40
35	34.89±0.08	99.7	0.23	34.63±0.38	98.9	1.09
40	39.61±0.34	99.0	0.86	39.64±0.51	99.1	1.28
50	49.55±0.69	99.1	1.39	49.34±0.54	98.7	1.09
Inter-day						
25	24.71±0.11	98.8	0.44	24.39±0.12	97.6	0.49
30	29.63±0.32	98.7	1.07	29.31±0.39	97.7	1.33
35	34.62±0.26	98.9	0.75	34.22±0.49	97.7	1.43
40	39.52±0.40	98.8	1.02	39.17±0.16	97.9	0.40
50	48.94±0.68	97.8	1.38	48.91±0.57	97.8	1.16

*** represents percentage values;**
CV coefficient of variation;
SD standard deviation

(B) Direct Enantioresolution Using L-Histidine and L-Arginine as Chiral Impregnating Reagents

1. Optimization of TLC Resolution Conditions

Several experiments were carried out using different combinations of MeCN, chloroform, dichloro methane, methanol, ethanol, and water to work out successful solvent system for enantioseparation of (*R,S*)-Cin. The summary of solvent systems used is shown in Table 3.3.

Table-3.3: Observation of variations in the compositions of solvent systems on the resolution of (*R,S*)-Cin Using L-His and L-Arg impregnated TLC

Using L-His Impregnated TLC		
Solvent Systems	**Composition**	**Observation**
MeCN-MeOH-DCM	4:2:0.5	NR
MeCN-MeOH-DCM	5:0.5:1.5	NR
MeCN-MeOH-$CHCl_3$	4:1:1	NR
MeCN-EtOH-H_2O	4:1:1.5	NR
MeCN-EtOH-H_2O	5:1.5:1	NR
MeCN-MeOH-H_2O	5:2.5:1	ES
Using L-His Impregnated TLC		
Solvent Systems	**Composition**	**Observation**
MeCN-MeOH-DCM	5:1:1	NR
MeCN-MeOH-$CHCl_3$	5:4:1.5	NR
MeCN-MeOH-$CHCl_3$	5:3:2	NR
MeCN-EtOH-H_2O	4:3:1.5	NR
MeCN-EtOH-H_2O	5:2:0.5	NR
MeCN-MeOH-H_2O	5.5:3:1.5	ES

Temperature: 16 ± 2 °C; NR: Not resolved; ES: Eight shape

Successful solvent systems were obtained for direct enantiomeric resolution of (*R,S*)-Cin on plates impregnated with L-His and L-Arg, after trying a number of solvent systems. These

were, 4:3.5:1 and 5:4:1 combinations of acetonitrile–methanol–water for plates impregnated with L-His and L-Arg, respectively. The spots of the two enantiomers were visualized by exposure to iodine vapors. The R_f values obtained with these solvent combinations are given in Table 3.4. The resolution was calculated by dividing the distance between two spots by the sum of two spot radii. The results are the averages of at least five runs under identical conditions. It was observed that the (*S*)-Cin eluted before the (*R*)-Cin, in both the cases. Actual photographs of chromatograms are shown in Fig. 3.3 and Fig. 3.4.

✷ **Effect of pH and Temperature**

Studies with respect to effect of change of concentration of the impregnating reagents, pH and temperature were made for the successful solvent systems only. The optimized concentration was 1.0% for each of the selectors. There was no resolution below this concentration (1.0%).

Resolution studies were carried out on plates prepared in the solution of pH 9, 10, 11, 11.5 and 12. This ensured that the racemic analyte (*R,S*)-Cin at the time of spotting on each of the impregnated plates was in the cationic form and the chiral selector was in the anionic form. Resolution of enantiomers was observed at pH 11(using L-His) and at pH 11.5 (using L-Arg). With the variation of these pH values, poor resolution with tailing or even no resolution was obtained.

Additional experiments were carried out with successful solvent system within the temperature range 10-30 °C within 10 min. For this purpose the chromatographic chambers were placed inside an incubator to attain the specific temperature. The best resolution was obtained at 18 ± 2°C with both the impregnating reagents. Increase of temperature to 25 or 30 °C resulted in tailing of spots and a decrease in temperature to 10 °C showed no resolution.

2. Separation Mechanism

According to Dalgliesh [32] a *three point interaction* between the CSP and at least one of

the enantiomers is must for chiral resolution of the enantiomers. Since the pI values of L-His and L-Arg are 7.5 and 10.8, respectively, and thus both the plates impregnated with L-His (at pH 11) and L-Arg (at pH 11.5) exist in the anionic form under the experimental conditions while the enantiomers in the (*R*,*S*)-Cin exist as protonated cations. Consequently, resolution can be regarded as based on the mechanism involving formation of *in situ* diastereomeric ion-pairs of the type [(*R*)-Cin-L-His] and [(*S*)-Cin-L-His].

Each of the *three interactions* can be hydrogen-bonding, pi–pi, steric, hydrophobic or dipole–dipole. Thus with the use of L-His and L-Arg, columbic/charge–charge interaction, pi–pi, steric and hydrophobic interactions occurred that favored formation of diastereomers *in situ* and hence enantioresolution.

Table 3.4: Enantioseparation of (*R*,*S*)-Cin on L-His and L- Arg impregnated plates

Impregnating Reagents	Solvent ratio	R_f from racemic (*R*,*S*)-Cin		R_f of pure (*S*)-Cin	R_S
		(*S*)-Cin	(*R*)-Cin		
L-His	4:3.5:1	0.62	0.26	0.62	2.18
L-Arg	5:4:1	0.87	0.63	0.87	1.70

Solvent: MeCN-MeOH-H_2O (v/v);
R_f: Retention factor;
R_S: resolution factor;
Development time: 10 min;
Temperature, 16 ± 2°C;
Detection: iodine vapors

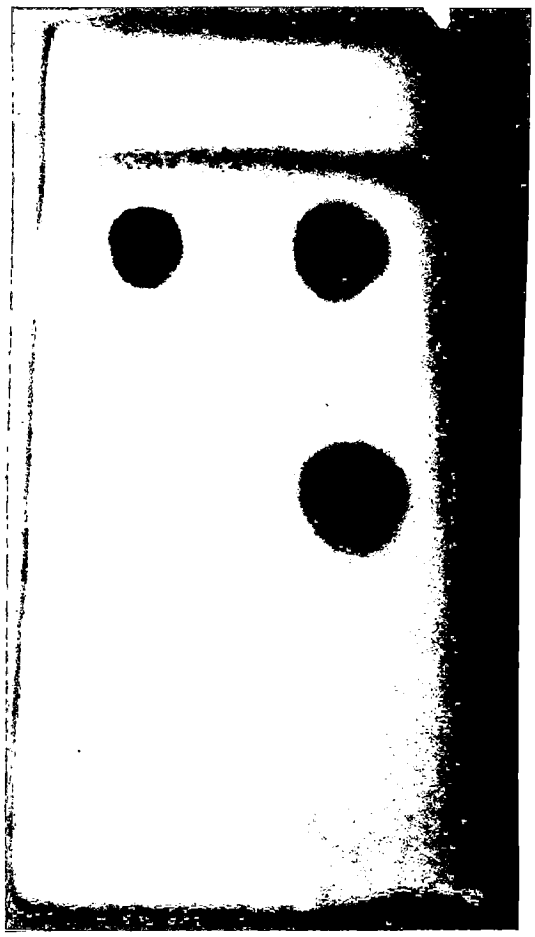


Fig. 3.3: Photograph of chromatogram showing resolution of (*R*,*S*)-Cin by using L-His impregnated plates. From left to right: spot 1, pure (*S*)-Cin; spot 2, l ower spot for (*R*)-Cin and upper spot for (*S*)-Cin resolved from (*R*, *S*)-Cin. Development time, 10 min; temperature, 18 ± 2°C; Detection, iodine vapors

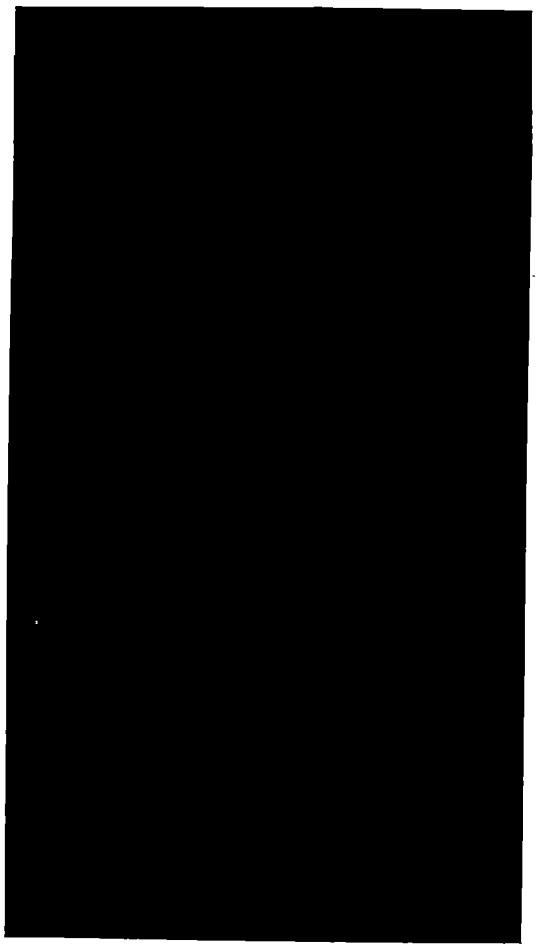


Fig. 3.4: Photograph of chromatogram showing resolution of (*R*,*S*)-Cin by using L-Arg impregnated plates. From left to right: spot 1, pure (*S*)-Cin; spot 2, lower spot for (*R*)-Cin and upper spot for (*S*)-Cin resolved from (*R*, *S*)-Cin. Development time, 10 min; temperature, 16 ± 2°C; Detection, iodine vapors

3. Isolation of Enantiomers

By the exposure to iodine vapors the spots of the two enantiomers of (*R,S*)-Cin on L-His impregnated plates were located. These located spots were marked and the iodine was allowed to evaporate off. The silica gel was scrapped (from 60 chromatograms) and extracted with methanol. The extracts, pertaining to each of the enantiomers, were filtered and concentrated *in vacuo*. It was expected that only (*S*)- or (*R*)-Cin went into the solution since amino acids are insoluble in methanol. Each extract was concentrated *in vacuo* and left to cool until crystals appeared. The crystals were washed with diethyl ether and dried in air. Melting point and UV absorption (λ_{max}) were recorded. The specific rotations were also observed and were found to be in agreement with literature [143], i.e., $[\alpha]_D^{20}$ = - 29.1° (c=2, MeOH) for (*R*)-Cin.

Similarly, L-Arg impregnated plates were used for resolution of enantiomers of (*R,S*)-Cin followed by their isolation.

4. Method Validation

Calibration data of enantiomers of (*R,S*)-Cin recovered from the impregnated plates is shown in Table 3.5.

The repeatability of the developed method was determined by applying the solution of known concentration of (*R,S*)-Cin for six times. The mean values for precision (RSD) were in the range 0.46%-0.81% for separation on L-His impregnated plates while that for separation on L-Arg impregnated plates was in the range 0.39%-0.77%. The accuracy was determined by investigating the recoveries of single enantiomers from the racemic mixture. The recovery of the enantiomers was found in the range of 96-97%. LODs were found to be 0.28 µg and 0.26 µg using L-His and L-Arg, as chiral impregnating reagents, respectively.

The direct enantioresolution developed method provides a simple, sensitive, rapid and economical approach to control the chiral purity of (*R,S*)-Cin which can be applied even in a small laboratory. The amino acids used as chiral impregnating reagent have provided very good

separation and are easily available at low cost. The method is useful for recovery and quantification of native enantiomers and, has potential application in pharmaceutical field.

Table-3.5: Calibration data of enantiomers of (*R*,*S*)-Cin recovered from L-His and L-Arg impregnated plates

Chiral impregnating reagent	Compound	Calibration curve*	Range (µg/mL)	Linearity (R^2)	Detection limit (µg/mL)
L-His	(*R*)-Cin	y=0.112x+0.510	10-30	0.998	0.28
	(*S*)- Cin	y=0.129x+0.311	10-30	0.999	0.28
L-Arg	(*R*)- Cin	y=0.234x+0.167	10-30	0.998	0.26
	(*S*)- Cin	y=0.241x+0.193	10-30	0.998	0.26

***x: sample concentration (µg/mL);**
y: absorbance;
R^2=correlation coefficient

Chapter-4

Indirect Enantioseparation of Thiol Group Containing Amino Acids

I. Introduction

Penicillamine (PenA), cysteine (Cys) and homocysteine (Homocys) are thiol group containing amino acids and are easily available pharmaceutically important compounds (Fig. 4.1). D-isomer of PenA is pharmacologically active while L-isomer, found naturally, is toxic [144] and hence use of DL-PenA is restricted [145]. Certain pharmaceutical applications and aspects of biological importance of PenA [146-150], Cys [151, 152], and Homocys [153] are described in literature.

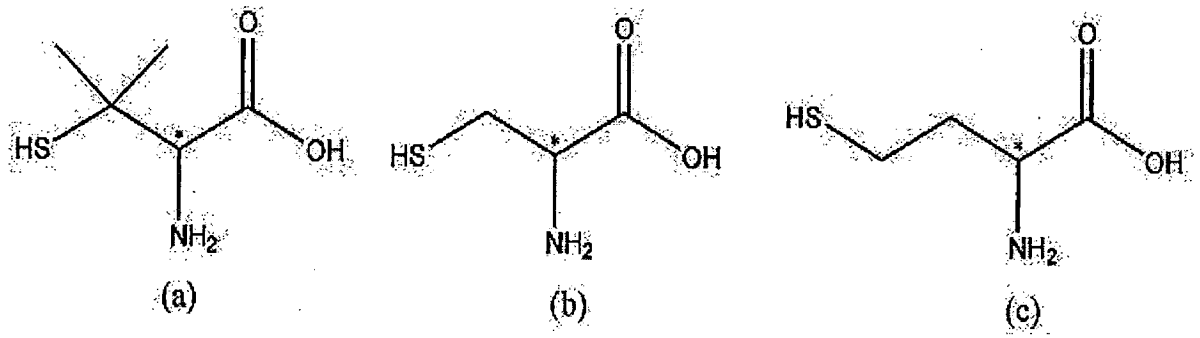


Fig. 4.1: Structures of (a) DL-Penicillamine (b) DL-Cysteine (c) DL-Homocysteine

II. Literature Review

✱ Enantioseparation of Penicillamine, Cysteine and Homocysteine

Different CDRs have been used for enantioseparation of PenA [131, 154-158], Cys [133, 156, 159-162] and Homocys [156, 162]. Enantiomeric mixture of derivatives of DL-PenA,

prepared by cyclization with HCHO, was separated by ligand exchange approach both in TLC (using ChiralPlate®) [163] and HPLC [164]. Separation of spiro-derivatives of DL-PenA, prepared by cyclization with ninhydrin, was made using Cu(II)-L-proline complex as chiral mobile phase additive in HPLC [165], while β-cyclodextrin and α-glycoprotein columns provided analytical and preparative enantioseparation [68] of spiro derivatives of DL-PenA and DL-Cys.

Enantioresolution of Homocys has been achieved by HPLC as homocysteine thiolactone using different CSPs based on L-valine-3,5-dimethylanilide attached to monodisperse poly(glycidyl methacrylate-co-ethylene dimethacrylate) beads [166], isopropyl-carbamate functionalized cyclofructan6 bonded to silica gel [167] and diphenyl substituted 1,1'-binaphthyl crown ether [168].

✷ ***N*-Acylbenzotriazoles**

Literature reveals that different *N*-acylbenzotriazoles have been synthesized via reaction of benzotriazole (Btz) with different unsaturated carboxylic acids [169] and reaction of 1-(1-methanesulfonyl) benzotriazole with different carboxylic acids [170] and *N*-Boc-α-amino acids [171]. These *N*-acylbenzotraizoles have been further utilized for *N*-acylation of different amino compounds [172-174]. (*S*)-naproxen (Nap) provides a chiral platform having a UV absorbing chromophore to synthesize CDRs since it has a carboxylic acid group. *N*-succinimidyl-(*S*)-2-(6-methoxynaphth-2-yl) propionate was synthesized by the reaction of (*S*)-naproxen with *N*-hydroxysuccinimide and was used as a CDR for enantioseparation of DL-PenA [175].

III. Present Work

On account of the above cited literature and to search new CDR for enantioseparation of pharmaceutically important amino compounds, a Btz derivative of Nap, (*S*)-1-(1*H*-benzo[d][1,2,3]triazol-1-yl)-2-(6-methoxynaphthalen-2-yl)propan-1-one (Nap-Btz), was

synthesized and was used as a CDR for enantioseparation of PenA, Cys and Homocys. The novelty introduced here is the synthesis of a new CDR and its application for microwave assisted synthesis, and reversed phase HPLC separation, of the diastereomers of penicillamine, cysteine and homocysteine.

1. Synthesis of (*S*)-Naproxen-benzotriazole

A Btz derivative of Nap, (*S*)-Nap-Btz, was synthesized. It was characterized with the help of UV, IR, CHN, ^{1}H NMR and ^{13}C NMR spectra. The synthesis and characterization data of the CDR has been described in Chapter-2.

2. Microwave Irradiated Synthesis of Diastereomers of Penicillamine, Cysteine and Homocysteine

To the solutions of DL-Cys (100 µL, 50 nmol, in $NaHCO_3$ (1 M)) and Nap-Btz (150 µL, 85 nmol, in MeCN) in mole ratio of 1:1.7 in a Teflon tube of 1.5 mL, borate buffer (0.1 M, pH 9.5, 60 µL) was added and the reaction mixture was then exposed to microwave irradiation (MWI) for 30 s at 80% (of 800 W) and cooled to room temperature. The reaction was quenched by addition of HOAc (1 M, 60 µL). A 10 µL volume of resulting solution, containing the diastereomers, was diluted 10 fold with MeCN, and 20 µL of it was injected onto the column. The chiral derivatizing reagent Nap-Btz was quite stable for one month while the solution of diastereomers of cysteine was quite stable up to one week under refrigerated condition (4 °C).

The same procedure was followed to derivatize DL-PenA, DL-Homocys, D-Cys, D-PenA and L-Homocys.

The experiments related to synthesis of diastereomers followed by their HPLC resolution under the optimized condition were repeated after the interval of 10, 20, 30 and 40 days, to check the stability of CDR. The results (in terms of percentage peak area of diastereomers and their resolution) showed that the CDR was successful when used within 30 days.

3. HPLC Operating Conditions for Separation of Diastereomers

RP-HPLC was performed on a Waters Spherisorb ODS2 (250 × 4.6 mm I.D., 5 μm) column with the mobile phase consisting of aq TEAP (10 mM, pH 3.5) - MeCN in a linear gradient of MeCN from 30 to 65% in 30 min at a flow rate of 1.0 mL/min and UV detection at 231 nm. Triethylammonium phosphate (TEAP) buffer solution was prepared by diluting triethyl amine to 10 mM with ultra-purified water and adjusting to pH 3.5 by addition of phosphoric acid.

4. Method Validation

The experimental method was validated with respect to linearity, accuracy and precision for the diastereomers of DL-Cys prepared with Nap-Btz according to ICH guidelines [92] and summarized here:

Linearity

The linear regression was computed by the least square method using Microsoft Excel program to determine the slopes and correlation coefficients for the calibration graphs between the peak area (in AU; absorbance unit) responses of ((*S*), D)- diastereomer and ((*S*), L)- diastereomer and the corresponding concentration (0.025-0.100 nmol).

Accuracy and Precision

The intra-day assay and inter-day assay studies for accuracy and precision were carried out by replicate HPLC analysis (n=6) of diastereomers of DL-Cys at three concentrations (0.025, 0.050, 0.100 nmol).

To determine LOD, corresponding to the signal-to-noise ratio of 3, the recoveries of L-Cys from the solution containing excess of D-Cys was investigated. Solution of D-Cys was spiked with fixed amount of L-Cys within the range of 0.01% to 0.10%.

IV. Results and Discussion

1. Synthesis and Characterization of (*S*)-Naproxen-benzotriazole

The reaction for the synthesis of CDR, Nap-Btz and diastereomers by its reaction with the representative analyte, cysteine which is one of the chosen three thiol group containing amino acids is shown in Fig. 4.2. Nap-Btz was formed by nucleophilic attack of 1*H*-benzotriazole on the carbonyl carbon of the carboxylic acid of Nap followed by the removal of dicyclohexylurea. An excellent yield (>94%) of Nap-Btz was obtained by the reaction of (*S*)-naproxen with 1*H*-benzotriazole in the presence of coupling reagent DCC/DMAP under mild conditions. The CDR, Nap-Btz was characterized by IR, UV, CHN, ^{1}H NMR and ^{13}C NMR. The characterization data related to Nap-Btz is given in Chapter 2. The chiral purity of Nap-Btz was established according to the earlier reports on determination of chiral purity of different CDRs [155].

Katritzky et al. [176] synthesized benzotriazole activated naproxen i.e. the same (*S*)-naproxen-benzotriazole in 4 h using hazardous thionyl chloride for activation of carboxylic group of (*S*)-Nap while in the present work Nap-Btz has been synthesized under mild conditions using DCC/DMAP coupling reagent (used for activation purpose). The effect of presence and absence of DMAP on the synthesis of Nap-Btz was also investigated. In the absence of DMAP the reaction took 4 hr for completion. Besides, the newly synthesized CDR (Nap-Btz) is an amide and is more stable than *N*-succinimidyl-(*S*)-2-(6-methoxynaphth-2-yl) propionate (SINP) [175], an ester, due to higher thermodynamic stability of amides over esters.

2. Optimization of Derivatization Conditions

The experimental conditions for MWI synthesis of diastereomeric pairs of PenA, Cys, and Homocys with Nap-Btz were optimized to ascertain the best and successful conditions; effect of buffer pH, reagent excess, and reaction time and MW power was systematically investigated. The mixture of the two diastereomers so formed under each change of experimental conditions was subjected to HPLC resolution and the peak areas corresponding to the two resolved diastereomers, calculated by system software, were taken as a measure of complete derivatization.

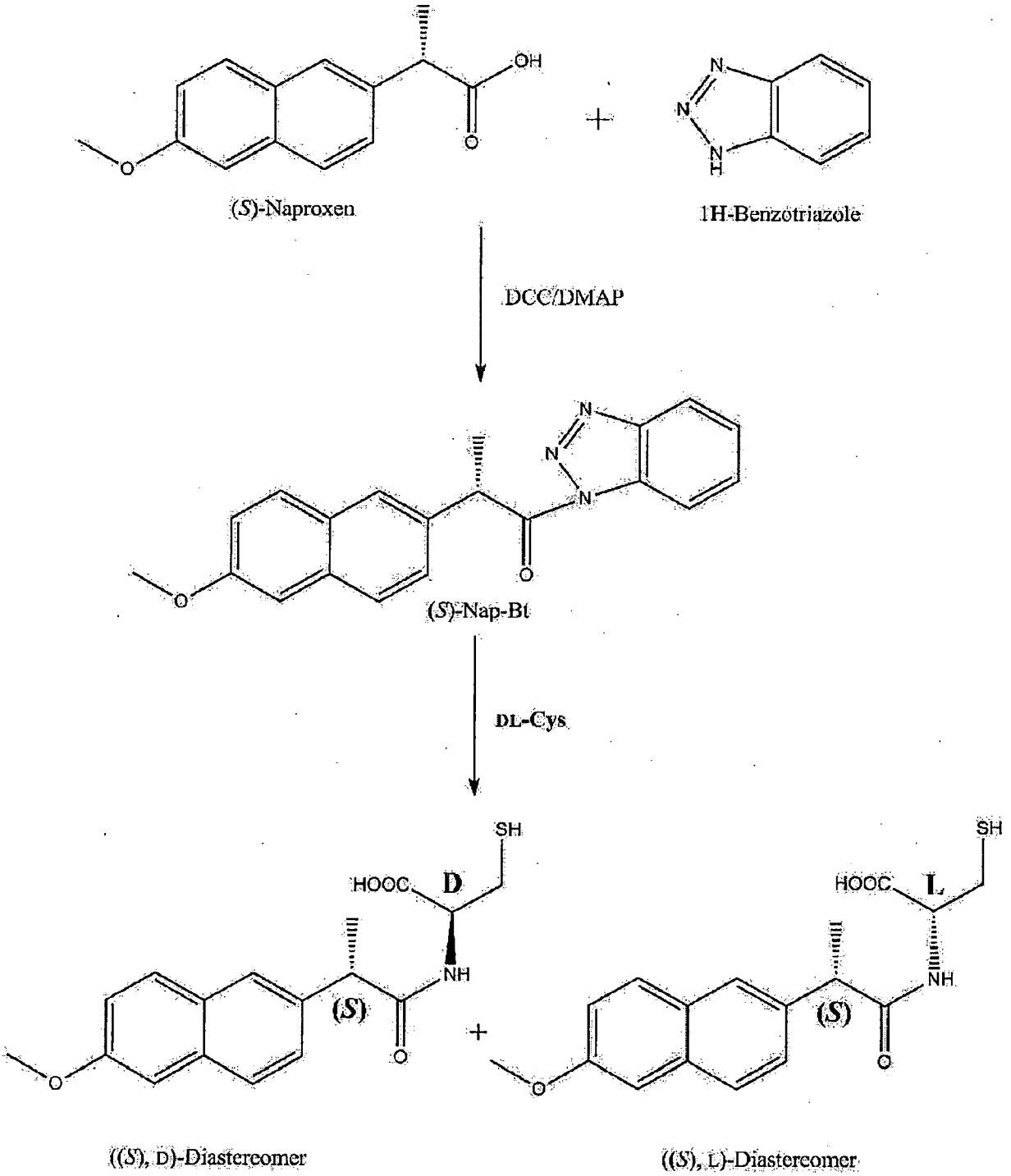


Fig. 4.2: Synthesis of (*S*)-Nap-Btz chiral derivatizing reagent and diastereomers of representative analyte DL-Cys

✱ Effect of pH of Buffer

Since reaction of Cys (and other analytes) with Nap-Btz follows a nucleophilic substitution, the reaction requires basic medium. Borate buffer was used to facilitate the derivatization. Effect of pH of buffer was investigated within the range of 6.5-10. Borate buffer at pH 9.5 (0.1 M, 60 µL) was found to be optimum to obtain the best yield for derivatization of DL-Cys with Nap-Btz. The derivatization (in terms of peak area) increased with increase in pH from 6.5-9.5 and increment in pH up to 10 showed no significant change in derivatization yield (Fig. 4.3). Therefore, a buffer of pH 9.5 was chosen for further experiments on derivatization. No derivatization was observed in the absence of buffer.

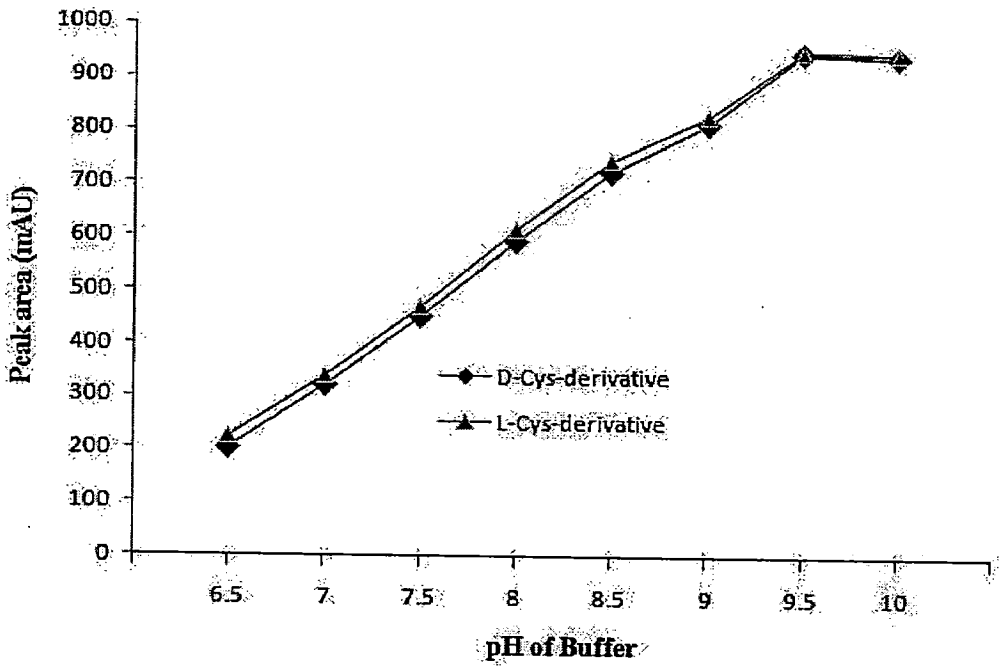


Fig. 4.3: Effect of pH of the buffer on derivatization of DL-Cys with (*S*)-Nap-Btz

✱ **Effect of Reagent Excess**

Nap-Btz was used in 1-5 fold molar excess to ascertain complete derivatization. Derivatization of Cys was found optimum at a molar ratio of 1:1.7 (Cys: CDR) using MWI (30 s at 80% power). Slight kinetic resolution was observed at lower ratios of Cys: CDR. Increase in reagent concentration up to 5 fold had no significant effect on yield of derivatization and reaction time. Therefore, the CDR was used in the same ratio for all the three analytes.

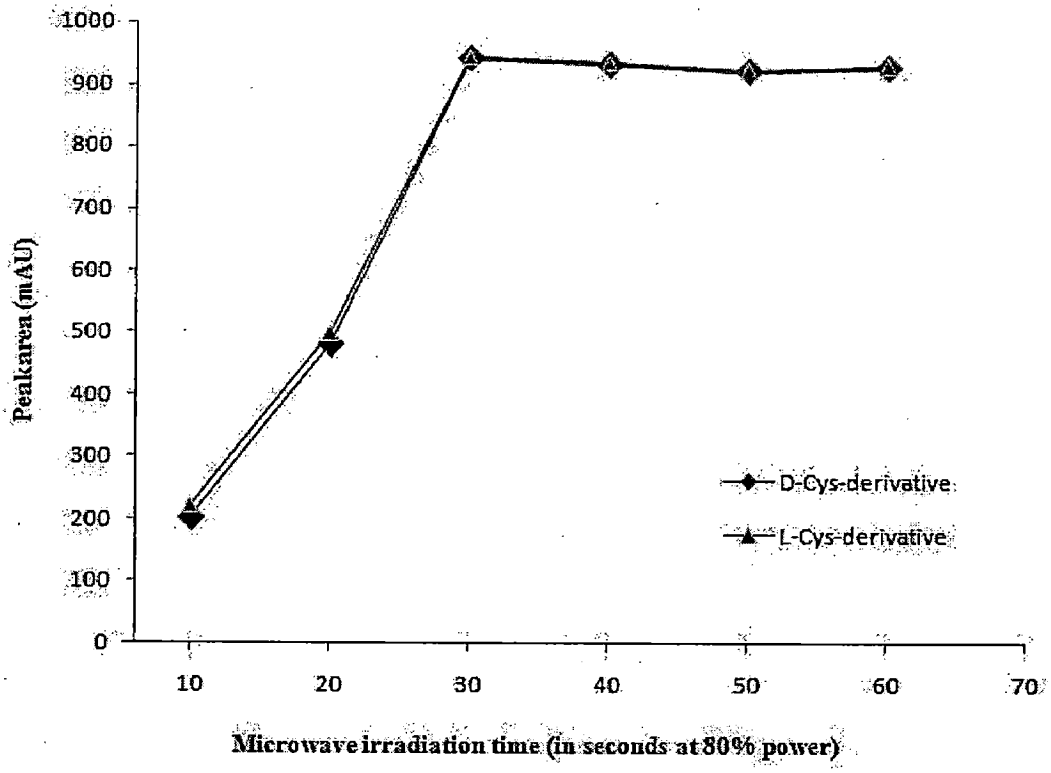


Fig. 4.4: Effect of microwave irradiation time on completion of synthesis of diastereomers of DL-Cys with (*S*)-Nap-Btz at 80% power (of 800 W)

✷ **Microwave Irradiation**

Separate sets of reaction mixture were irradiated in the microwave oven for 10, 20, 30, 40, 50, and 60 s at 75-90% power (800 W). A derivatization time of 30 s (at 80% power) was found successful for complete derivatization of DL-Cys as the area of diastereomeric peaks became constant (Fig. 4.4). The MWI time and power corresponding to maximum diastereomeric peak areas (representing the completion of reaction) were taken as optimized derivatization conditions and were used for the three analytes.

3. HPLC

The chromatographic parameters: retention factor (k), separation factor (α) and resolution (R_S) for the resolved diastereomers of PenA, Cys and Homocys prepared with Nap-Btz are given in Table-4.1. The three pairs of diastereomers were well separated under the reversed-phase HPLC conditions. D-Isomer was eluted prior to L-isomer for all the three cases. Sections of chromatograms showing resolution of diastereomers of PenA, Cys and Homocys prepared with Nap-Btz are shown in Fig. 4.5.

Optimization of HPLC Conditions

✷ **Effect of pH and Concentration of Buffer Used in Mobile Phase**

The effect of pH of buffer used in the mobile phase on the separation of diastereomers of DL-Cys prepared with Nap-Btz was studied within the pH range of 2.5-6.0 keeping the concentration of buffer at 10 mM. TEAP buffer was used to investigate within the range of pH 2.5-4.0 and the best resolution was observed at pH 3.5. To study the effect of pH within the range 4.0-6.0, triethylammonium acetete buffer was used and it was found that the diastereomers were not resolved in this pH range. Thus using TEAP buffer of pH 3.5, effect of varying buffer concentration was studied within the range of 05-40 mM. Peak broadening was found with

decrease in concentration of TEAP buffer below 10 mM and little difference in resolution was found when buffer concentration was above 10 mM (upto 40 mM; with a change of 5 mM at a time). Since high buffer concentration may harm the column hence 10 mM buffer concentration was taken as optimized concentration.

✷ **Effect of Organic Solvent**

A binary mobile phase consisting of MeCN and TEAP buffer was found to be the best. Both the gradient and isocratic elution modes were investigated. Sharp peaks were obtained under gradient elution. MeCN was found to be a better organic modifier in comparison to methanol as broader peaks (and even no separation in a few cases) and larger retention times were observed with methanol.

Table 4.1: Chromatographic data for resolution of diastereomers of penicillamine, cysteine, and homocysteine prepared with (*S*)-Naproxen-benzotriazole

Amino Acids	Chromatographic parameters			
	k_D	k_L	α	R_S
DL-Penicillamine	9.19	11.86	1.29	24.09
DL-Cysteine	8.76	10.25	1.17	20.71
DL-Homocysteine	8.98	10.87	1.21	21.23

k_D and k_L: retention fators of D- and L-enantiomers respectively;
α: stereoselective factor;
R_S: resolution of the diastereomers of corresponding amino acids;
Mobile phase: *aq* TEAP (10mM, pH 3.5) - MeCN in a linear gradient of MeCN from 30 to 65 % in 30 min at a flow rate of 1.0 mL/min;
UV detection: 231 nm

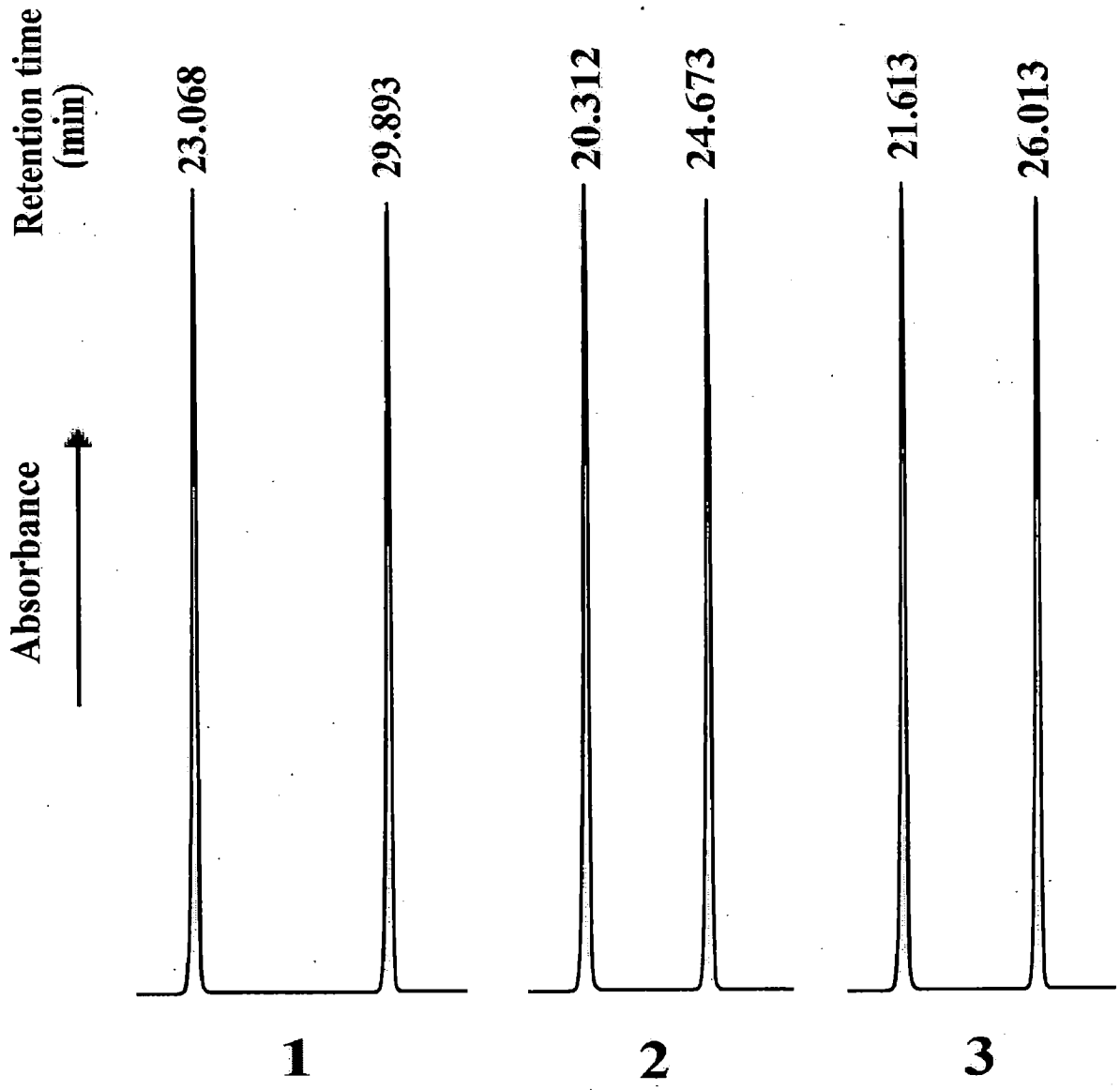


Fig. 4.5: Sections of chromatograms showing resolution of DL-Penicillamine (1), DL-Cysteine (2), and DL-Homocysteine (3) as their diastereomers prepared with Nap-Btz; Waters Spherisorb ODS2 (250 × 4.6 mm I.D., 5µm); diastereomeric peak corresponding to D-enantiomer was eluted prior to that of L-enantiomer; mobile phase was *aq* TEAP (10mM, pH 3.5) - MeCN in a linear gradient of MeCN from 30 to 65% in 30 min at a flow rate of 1.0 mL/min and detection at 231 nm for all the diastereomers

✷ **Effect of Flow Rate**

The effect of change of flow rate on the separation of diastereomers was examined by varying the flow rate in portions of 0.5 mL/min within the range of 0.5-1.5 mL/min. There was observed an increase in retention time along with broadening of the diastereomeric peaks with the decrease in the flow rate from 1.0 to 0.5 mL/min. The retention times and resolution values decreased when flow rate was increased from 1.0 to 1.5 mL/min. Hence flow rate of 1.0 mL/min was used throughout the experiment.

4. Separation Mechanism

Since the pH of the mobile phase is around 3.5 (pH of TEAP buffer, used in the mobile phase) and is considerably acidic the carboxylic group of the analyte will not be ionized, therefore, ionic interaction is likely to play the least role. The factors contributing to the hydrophobicity of diastereomers are then required to be considered. The following factors may contribute to the hydrophobicity of diastereomers, (i) presence of naphthyl group in the reagent platform of the diastereomers, (ii) the sulphur atom in the sulfydryl group with its less electronegativity and large size by its position in periodic table, (iii) the partial double bond character of the amide bond because of lone pair of the amino nitrogen of the analyte that may be undergoing delocalization with the neighboring carbonyl group. It can be interpreted that the hydrophobic interactions of the two diastereomers with the reversed phase material of the column are responsible for their different partition coefficients resulting into different retention times and thus separation. The characteristic feature of diastereomers is that they have different physical properties. Based on the chemical structures, the analytes may also be arranged in their decreasing order of hydrophobic character as PenA> Homocys> Cys because PenA contains two $-CH_3$ groups and Homocys contains an additional $-CH_2$ in comparison to Cys. This is reflected in the observed resolution values of the three sets of analytes, PenA (*Rs*, 24.09)> Homocys (*Rs*, 21.23) > Cys (*Rs*, 20.71).

A comparison of present work to the earlier reports with respect to time required for derivatization of penicillamine, cysteine and homocysteine using different CDRs/tagging reagents and their enantioseparation (in terms of resolution) using different HPLC resolution methods is shown in Table-4.2. It clearly establishes the novelty and superiority of the present report.

Table 4.2: Comparison of HPLC enantioseparation of penicillamine, cysteine, and homocysteine using different CDRs/tagging reagents

Basis of separation	CDRs/tagging reagents used for derivatization of	Derivatization time	Resolution of the derivatives (*Rs*)	References
	DL-Penicillamine			
Diastereomers	Nap-Btz	30 s	24.09	**Present Work**
	FDNP-L-Val-NH_2	1hr	0.815	[131]
	SINP	15-18 min	23.19	[175]
	DBD-PyNCS	60 min	3.33	[156]
Ligand Exchange	HCHO	2hr	NG	[164]
CMPA	Ninhydrin	NG	1.31	[165]
CSPs[a,b]	Ninhydrin	5 min	6.92[a], 2.38[b]	[68]
CSPs[a,b]	DNFB	45 min	7.60[a], 1.75[b]	[68]
	DL-Cysteine			
Diastereomers	Nap-Btz	30 s	20.71	**Present Work**
	OPA and L-Val	1 min	3.44	[162]
	(*S*)-NIFE	20 min	1.15	[161]
	DBD-PyNCS	60 min	1.15	[156]
	FDNP-L-Val	50 s	6.83	[133]
CSPs[a,b]	Ninhydrin	5 min	5.95[a], 2.34[b]	[68]
CSPs[a,b]	DNFB	45 min	6.71[a], 1.27[b]	[68]
	DL-Homocysteine			
Diastereomers	Nap-Btz	30 s	21.23	**Present Work**
	OPA and L-Val	1 min	1.13	[162]
	DBD-PyNCS	60 min	NR	[156]
CSPs[c]	Enantiomeric mixture of Homocys thiolactone was used as the analyte	NG	NG	[166]
CSPs[d]		NG	1.1	[167]
CSPs[e]		NG	2.14	[168]

CMPA, Chiral Mobile Phase Additive; CSPs, Chiral Stationary Phases; NG, not given; NR, not resolved; FDNP, Fluoro Dinitro Phenyl; SINP, *N*-succinimidyl-(S)-2-(6-methoxynaphth-2-yl) propionate; DBD-PyNCS, 4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N*, *N*-dimethylaminosulfonyl)-2, 1, 3-benzoxadiazole; DNFB, Dinitro fluoro benzene; OPA, *o*-Phthalaldehyde and;
[a]α-Acid Glycoprotien Column;
[b]β-Cyclodextrin Column;
[c]based on L-valine-3,5-dimethylanilide attached to monodisperse poly(glycidyl methacrylate-co-ethylene dimethacrylate) beads;
[d]isopropyl-carbamate functionalized cyclofructan6 bonded to the silica gel;
[e]diphenyl-substituted 1,1'-binaphthyl crown ether

5. Method Validation

Linearity

A good linear relationship was obtained over the range mentioned in "Present Work" section. The regression equations were y=1.085x+1.447 (R^2=0.999) and y=1.132x+1.398 (R^2 = 0.998) for the ((*S*), D) - diastereomer and ((*S*), L) - diastereomer, respectively.

Accuracy and Precision

The relative standard deviation (%) for D- and L-Cys varied from 0.46 to 0.80 and 0.39 to 1.25 for intra-day assay precision and 0.62 to 1.37 and 0.72 to 1.21 for inter-day assay precision. The percentage recovery for D- and L-Cys varied from 98.1 to 99.6 and 98.2 to 99.0 for intra-day assay and 96.0 to 99.1 and 95.9 to 99.0 for inter-day assay (Table-4.3).

The result of spiking indicate that this method can be applied for detection of L-Cys in D-Cys up to 0.04% by HPLC.

Table 4.3: Intra-day assay and inter-day assay precision of diastereomers of DL-Cys prepared with Nap-Btz

Concentration ($pgmL^{-1}$)	First Eluting Diastereomer			Second Eluting Diastereomer		
	Mean±SD	Recovery*	RSD*	Mean±SD	Recovery*	RSD*
Intra-day						
25	24.87±0.21	99.5	0.80	24.76±0.31	99.0	1.25
50	49.06±0.32	98.1	0.65	49.12±0.28	98.2	0.57
100	99.60±0.46	99.6	0.46	99.68±0.39	99.7	0.39
Inter-day						
25	24.01±0.33	96.0	1.37	23.98±0.29	95.9	1.21
50	48.98±0.53	97.8	1.08	48.99±0.47	97.9	0.96
100	99.10±0.62	99.1	0.62	99.02±0.72	99.0	0.72

*** represents percentage values;**
CV coefficient of variation;
SD standard deviation

Chapter-5

Enantioseparation of Mexiletine

I. Introduction

Mexiletine [1-(2,6-dimethylphenoxy)-2-amino-propane, Fig. 5.1] (MEX) is a Class IB antiarrhythmic, antimyotonic, and analgesic agent in its racemic form [177] and used in the treatment of ventricular arrhythmia [178]. The enantiomers of (*R,S*)-MEX have different pharmacokinetic, pharmacodynamic and receptor binding properties [179, 180]. Its (*R*)-enantiomer has more potential for protein binding [181] than its (*S*)-enantiomer.

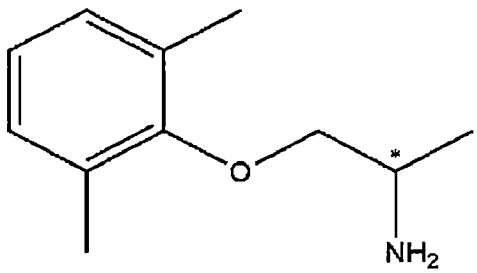


Fig. 5.1: Structure of (*R,S*)-mexiletine

II. Literature Review

✸ Enantioseparation of Mexiletine

CSPs based on diphenyl-substituted 1,19-binaphthyl crown ether [168], amylose tris(3,5-dimethylphenylcarbamate) [182], Crownpak CR(+) [183] and (18-crown-6)-2, 3, 11, 12-tetracarboxylic acid [184, 185] were used for direct resolution of (*R,S*)-MEX while its derivatives such as *N*-anthroyl [186] and 2-naphthoyl [180] were resolved using Pirkle type CSP and *o*-phthaldialdehyde. Chiralpak AD CSP was used for resolution of 2-mercaptoethanol [187] derivatized enantiomers of MEX and its four major metabolites, in plasma and in urine. The direct enantioseparation methods, however, employ costly and less durable chiral columns.

Different CDRs like (-)-4-(6-methoxy-2-naphthyl)-2-butyl chloroformate (NAB-C) [188], *S*-(+)-1-(1-naphthyl) ethyl isocyanate (NEIC) [189], *o*-phthaldialdehyde combined with *N*-acetyl-L-cysteine [190], (1*S*, 2*S*)-*N*-[(2-isothiocyanato)-cyclohexyl]-pivalinoyl amide (PDITC) and 2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl isothiocyanate (GITC) [191], variants of MR; Nap, (*S*, *S*)-*O*, *O*'-di-*p*-toluoyl tartaric acid [192], *N*-succinimidyl-(*S*)-2-(6-methoxynaphth-2-yl)propionate, dinitrophenyl-L-Pro-*N*-hydroxysuccinimide ester [137] and chiral trichloro-*s*-triazines [193] have been used for enantioresolution of MEX.

These aforementioned CDRs have shown excellent reactivity to MEX under mild conditions and have been used as either UV [137, 188, 191-193] or FL [189, 190] labels for MEX. The synthesized diastereomers were readily separated on reversed phase columns. These CDRs have various limitations. The UV labels were not applicable to some real samples due to lack of enough sensitivity of UV labeled diastereomers. Due to light sensitivity of MR based reagents, the whole method of synthesis i.e. the synthesis of the CDRs and synthesis of diastereomers of (*R*, *S*)-MEX was protected from light.

✱ Kinetic Resolution

The method of kinetic resolution of a racemic mixture may be applied to obtain chirally pure biologically active substances [194, 195]. It is based on different rates of reaction of enantiomers with certain chiral derivatizing reagent (CDR) and/or catalyst. Kinetic resolution of pharmecutically active compounds, containing amino or alcoholic group, by reaction with anhydride of optically active acids has been reported in literature [195]. Literature showed that acid chloride based chiral derivatizing reagents such as (*S*)-naproxen acyl chloride [196] and *N*-tosyl-(*S*)-prolyl chloride [197] have been used for kinetic resolution of secondary amino group containing pharmaceuticals of fluoroquinolone series. The use of such enantiomerically pure acylating reagents for the resolution of alcohols and amines provides an alternative to the methods involving enzymes and chiral catalysts.

✷ **(*S*)-(−)-*N*-Trifluoro acetyl prolyl chloride and camphanic chloride**

The chiral reagents, (*S*)-(−)-*N*-trifluoro acetyl prolyl chloride ((*S*)-TFPC) and camphanic chloride are volatile in nature and thermally stable. (*S*)-TFPC has been utilized as CDR for enantiomeric resolution of amphetamine and methamphetamine in biological fluids such as urine and blood [197-199], and hair [200] by gas chromatography/ mass spectroscopy. Camphanic chloride has been utilized to synthesize diastereomers of different alcohols [201-204]. The diastereomers, so formed, were separated by column chromatography and were hydrolyzed to individual enantiomers. Recovery of enantiomers from such derivatives was successful because the said CDRs viz. (*S*)-TFPC and camphanic chloride could be easily detached from the product.

III. Present Work

On account of the above cited literature (*R,S*)-MEX was resolved using (*S*)-TFPC and camphanic chloride as CDRs and RP-HPLC. The analytical separation was scaled up to small-scale preparative level using the same mobile phase and the same reversed phase column; the sample so injected were drawn from diastereoselective synthesis set up. The native enantiomers were recovered by detagging the chiral reagent under mild reaction condition with enhanced yield of (*R*)-MEX.

1. Synthesis of Diastereomers of (*R,S*)-MEX

Synthesis of diastereomers (*R,S*)-MEX was carried out under the following reaction conditions (**A1-A4**):

Solutions of (*R,S*)-MEX (0.1 mmol in dichloromethane, 5 mL) and (*S*)-TFPC (0.05 mmol in dichloromethane, 2 mL) were mixed in a molar ratio of 2:1 (**A1**). The reaction mixture was vortexed for 1 hr at room temperature.

At the same time solutions of (*R,S*)-MEX (1.2 μmol in dichloromethane, 100 μL) and (*S*)-TFPC (1.3 μmol in dichloromethane, 130 μL) were also mixed in a mole ratio of 1:1.1(**A2**) in Teflon tube of 1.5 mL and pyridine (60 μL) was added. The reaction mixture was vortexed for 20 min at room temperature. The reaction was quenched by addition of HOAc (1 M, 60 μL). Similarly the reaction of (*R, S*)-MEX and (*S*)-TFPC was also carried out at a mole ratio of 1:1.5 (**A3**) and 1:2 (**A4**).

Similar sets of experiments were carried out for the synthesis of diastereomers of (*R,S*)-MEX using (1*S*)-(-)-camphanic chloride and denoted as **B1**, **B2**, **B3** and **B4**, respectively. A 10 μL volume of each of the resulting solutions (of **A1-A4** and **B1-B4**), containing the diastereomers, was diluted 10 fold with MeCN, and 20 μL of it was injected onto the column.

2. HPLC Operating Conditions for Separation of Diastereomers

Reversed phase HPLC separation was performed for all the samples of the diastereomers synthesized under different conditions (i.e. **A1-A4** and **B1-B4**) on a Waters Spherisorb ODS2 (250 × 4.6 mm I.D., 5 μm) column with the same mobile phase consisting of aqueous trifluoroacetic acid (TFA) (0.1%)–acetonitrile (MeCN) in a linear gradient of MeCN from 30 to 70 % in 45 min at a flow rate of 1.0 mL/min and UV detection at 210 nm.

3. Method Validation

The experimental method for analytical enantioresolution was validated with respect to linearity, accuracy and precision for the diastereomers of (*R,S*)-MEX synthesized with both the CDRs following ICH guidelines [92] and summarized here:

Linearity

The linear regression was computed by the least square method using Microsoft Excel program to determine the slopes and correlation coefficients for the calibration graphs between the peak area (in AU; absorbance unit) responses of (*R,S*)-diastereomer and (*S,S*)-diastereomer and the

corresponding concentration (50-150 $ngmL^{-1}$).

Accuracy and Precision

The intra-day assay and inter-day assay studies for accuracy and precision were carried out by replicate HPLC analysis (n=6) of diastereomers of (*R,S*)-MEX synthesized with the two CDRs (i.e. (*S*)-TFPC and camphanic chloride) at three concentrations (50, 100, 150 $ngmL^{-1}$).

4. Small-scale Preparative HPLC for Recovery of Pure Enantiomer

Small-scale preparative HPLC was performed after the results and conditions for analytical enantioresolution method were optimized. For small-scale preparative HPLC the solutions containing diastereomeric excess (**A1** and **B1**) were injected onto the column (without dilution) using 200 µL injection loops. This process was repeated for 10 times. The mobile phase was the same as described in HPLC experiments.

After 10 repeated injections, approximately 25 mL of first eluting diastereomer was collected for sample **A1**. The collected fraction was concentrated in vacuuo. The concentrate was refluxed in a mixture of concentrated HCl and glacial AcOH (2 mL each) for 4 h. The reaction mixture was then evaporated to dryness under vacuum. The residue was dissolved in water (5 mL). It was cooled in an ice bath and then treated with sodium carbonate (1 M, 5 mL) and precipitate of the corresponding enantiomer i.e. (*R*)-MEX was obtained.

Similarly after same number of repeated injections approximately 40 mL of first eluting diastereomer was collected for sample **B1**. The collected fraction was subjected to the aforementioned set of experiments and yielded (*R*)-enantiomer of MEX.

IV. Results and Discussion

Total eight samples (**A1-A4** and **B1-B4**), containing diastereomers of (*R,S*)-MEX synthesized from each of the two CDRs, (*S*)-TFPC and camphanic chloride were subjected to

HPLC experiments, described herein. The reaction of (*R,S*)-MEX and (*S*)-TFPC, as the CDR, for the synthesis of diastereomers is shown in Fig. 5.2. The synthesized diastereomeric mixture of amides were designated as (*R,S*)-diastereomer and (*S,S*)-diastereomer (first letter represents the absolute configuration of MEX and the second letter represents the same of (*S*)-TFPC).

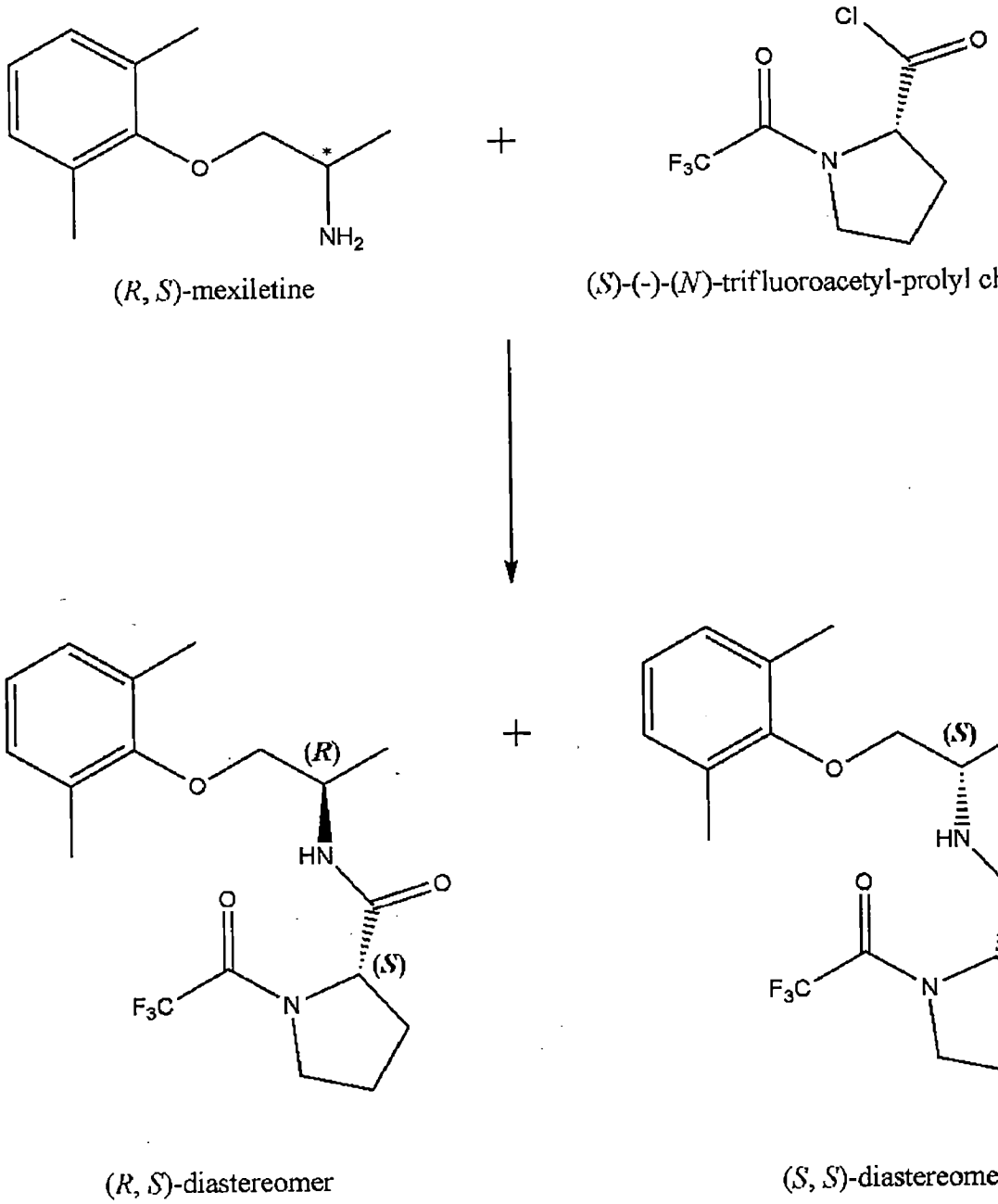
1. Kinetic Resolution of (*R,S*)-MEX

The HPLC experiments concluded that when mole ratio of (*R,S*)-MEX to (*S*)-TFPC was 2:1, in the absence of pyridine (in case of **A1**), a diastereoselective reaction occurred and the resulting product was found to be significantly enriched with (*R,S*)-diastereomer with diastereomeric excess (*de*) of 71 %. It means there occurred a kinetic resolution.

In the similar way same sets of experiments were carried out using camphanic chloride as CDR (in case of **B1**) and obtained same type of diastereoselective product (i.e. (*R,S*)-diastereomer) with slightly increased de upto 74 %. The diastereoselective yields of the reactions were estimated by HPLC experiments.

2. Effect of Molar Ratio of (*R,S*)-MEX: CDR on Enantioresolution

(*S*)-TFPC was used in 1-2 fold molar ratio(s) (with respect to 1 mole of (*R,S*)-MEX) to find out the optimum (*R,S*)-MEX: CDR ratio required (in the presence of pyridine) to yield equimolar mixture of (*R,S*)- and (*S,S*)-diastereomeric amides (using **A2-A4**). The sample (**A2**), containing 1.1 molar excess of (*S*)-TFPC was found to be the required optimum concentration to yield both the diastereomers in equal ratio. Further increase in the MEX:CDR ratio (1:1.5, 1:2 i.e. for **A3** and **A4**) also resulted into equimolar mixture of diastereomeric products. Similar set of experiments were carried out using camphanic chloride as CDR (using **B2-B4**) and concluded that the of ratio of 1:1.1 of (*R,S*)-MEX: CDR (i.e. using **B2**) was the optimum ratio to yield the two diastereomers in equal amounts.



(*R*, *S*)-mexiletine

(*S*)-(-)-(*N*)-trifluoroacetyl-prolyl chloride

(*R*, *S*)-diastereomer

(*S*, *S*)-diastereomer

Fig. 5.2: Synthesis of diastereomers of (*R*,*S*)-MEX synthesized with a representative CDR; (*S*)-(-)-(*N*)-trifluoro acetyl prolyl chloride

3. HPLC

(i) Analytical Separation

The chromatographic parameters: retention factor (k), separation factor (α) and resolution (R_S) for the diastereomers of (*R*,*S*)-MEX synthesized with (*S*)-TFPC (using **A2**) and camphanic chloride (using **B2**) are given in Table-5.1. The two pairs of diastereomers were well separated under the RP- HPLC conditions (as described in experimental section). Sections of chromatograms showing resolution of diastereomers are shown in Fig. 5.3. (*R*)-(–)-MEX was eluted prior to that of (*S*)-(+)-MEX in both the cases of diastereomers prepared with each of the two CDRs. Sharp peaks and good separation factor values ($\alpha > 1.20$) and resolution values ($R_S > 2$) were obtained for diastereomers of (*R*,*S*)-MEX synthesized with the two CDRs (Table-5.1). The values of separation factor (α) and resolution (R_S) for the HPLC separation of the diastereomers of (*R*,*S*)-MEX synthesized with camphanic chloride are higher than those synthesized with (*S*)-TFPC (Table-5.1). Hence it can be said that camphanic chloride was found better than (S)-TFPC as a CDR for HPLC enantioseparation of (*R*,*S*)-MEX.

Optimization of HPLC Conditions

The mobile phase consisting of aq. TFA (0.1%)-MeCN in a linear gradient of MeCN from 30 to 70% in 45 min at a flow rate of 1.0 mL/min was used for enantioseparation (*R*,*S*)-MEX using (*S*)-TFPC and camphanic chloride as CDRs. This mobile phase was established after a set of experimental work out. The description of the experiments carried out for this purpose is given here.

A binary mobile phase consisting of MeCN and TFA (0.1%, pH~2) was used for gradient and isocratic elution of the diastereomers of (*R*,*S*)-MEX synthesized with (*S*)-TFPC and camphanic chloride. Sharp peaks were obtained under gradient elution. MeCN was found to be a better organic modifier in comparison to methanol as broader peaks were observed with methanol. The concentration of TFA was established after studying its effect on resolution of diastereomers within the concentration range of 0.01-0.2%. Increase in TFA concentration from 0.01 % to 0.1% resulted to increment in separation factor, while further enhancement in its concentration resulted

to a little effect. Since high concentration of buffer may harm the column 0.1% TFA concentration was considered as optimized buffer concentration.

The effect of change of flow rate on the separation of diastereomers (*R*,*S*)-MEX synthesized with (*S*)-TFPC was also examined by varying the flow rate in portions of 0.5 mL/min within the range of 0.5-1.5 mL/min. Decrease in retention time along with sharpening of the diastereomeric peaks was observed with the increase in the flow rate from 0.5 to 1.0 mL/min. Further increase in flow rate resulted to decrease in both the retention times and resolution values. Hence flow rate of 1.0 mL/min was used throughout the experiment.

Similar experiments have been carried out to achieve the mobile phase which was considered as best optimized one used for the HPLC separation of diastereomers of (*R*,*S*)-MEX synthesized with camphanic chloride. The separation of these diastereomers were followed the similar trends along with the variations in elution modes, constitution (viz. organic modifier and concentration of TFA buffer) and flow rate of the mobile phase. Thus the aforementioned mobile phase was used for the HPLC resolution of the diastereomers of (*R*,*S*)-MEX synthesized with each of two CDRs (i.e. (*S*)-TFPC and camphanic chloride).

Table 5.1: Chromatographic data for resolution of diastereomers of (*R*,*S*)-MEX synthesized with CDRs

CDRs	Resolution of diastereomers		
	k_1	α	R_S
(*S*)-(-)-(*N*)-Trifluoro acetyl prolyl chloride	4.50	1.22	3.59
(1*S*)-(-)-Camphanic chloride	7.41	1.32	4.82

k_1: retention factor of first eluted diastereomer;
α: stereoselective factor;
R_S: resolution of the diastereomers of (*R*,*S*)-MEX synthesized with (*S*)-(-)-(*N*)-trifluoro acetyl prolyl chloride and (1*S*)-(-)-camphanic chloride

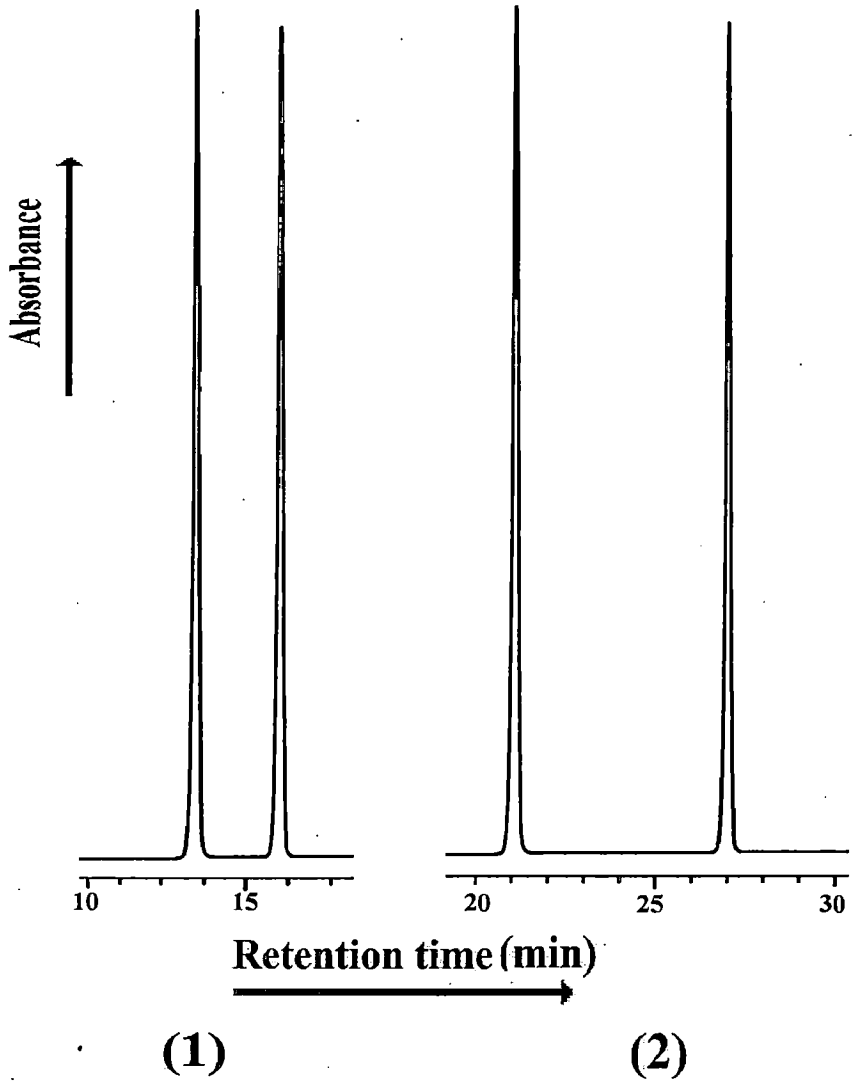


Fig. 5.3: Sections of chromatograms showing HPLC resolution of (*R*,*S*)-MEX as their diastereomers synthesized with (*S*)-TFPC (using A2) (1) and Camphanic chloride (using B2) (2); Waters Spherisorb ODS2 column (250 × 4.6 mm I.D., 5μm); diastereomeric peak corresponding to (*R*)-MEX was eluted prior to that of (*S*)-MEX; mobile phase was aq TFA (0.1%)-MeCN in a linear gradient of MeCN from 30 to 70% in 45 min at a flow rate of 1.0 mL/min and UV detection at 210 nm

The peak areas of the first and second eluting diastereomer are same in each of the two cases ((1) and (2)) (see section "2. Effect of molar ratio of (*R*, *S*)-MEX:CDR on enantioresolution")

(ii) Small-scale Preparative Separation

The chromatographic results for analytical enantioresolution method: $k_1 < 10$; $\alpha > 1.20$ and $R_S > 2$ (Table-5.1) have inspired us for scaling of analytical enantioresolution to small-scale preparative level. Sections of chromatograms showing HPLC resolution of diastereoselective enriched diastereomers (formed via kinetic resolution method) on small-scale preparative level (for samples **A1** and **B1**) are shown in Fig. 5.4. On account of the fact that an optimized scale-up strategy should be employed to small-scale preparative separation for avoiding time-consumption and wastage of materials [205], the optimized analytical HPLC enantioresolution of the diastereomers of (*R*,*S*)-MEX synthesized with each of the two CDRs (i.e. (*S*)-TFPC and camphanic chloride) was scaled up to the small-scale preparative separation of (*R*,*S*)-MEX using a reversed phase column (250 × 4.6 mm I.D., 5 μm) having the same line of packing material. The particle size of the column used for small-scale preparative separation is of 5-μm average diameter which is cost effective in comparison to the smaller and more expensive particles of 1.8- and 3.5-μm average diameters [206].

The binary mobile phase consisting of TFA and MeCN used for analytical enantioresolution method (mentioned in experimental section) is also used for small-scale preparative enantioresolution of the diastereomers of (*R*,*S*)-MEX synthesized with each of the two CDRs (i.e. (*S*)-TFPC and camphanic chloride). TFA, used as buffer in the mobile phase, was easily removed from the collected fractions of the diastereomers of (*R*,*S*)-MEX synthesized with (*S*)-TFPC and camphanic chloride. The pure enantiomers of (*R*)-MEX were obtained via acidic hydrolysis of collected concentrated fractions of the individual first eluted diastereomers. The two CDRs which have been used in the present work for indirect analytical enantioseparation are volatile and thermally stable in nature. These are the additional advantages of the CDRs and were utilized during the acidic hydrolysis of individual diastereomers, as these are easily removed as waste and hence yielded individual enantiomer i.e. (*R*)-MEX.

Recovery of the diastreoselective product [(*R*)-MEX-CDR], yield and enantiomeric excess of (*R*)-MEX enantiomer obtained after acidic hydrolysis of (*R*)-MEX-CDR have been summarized in Table-5.2. The amounts of diastereoselective product [(*R*)-MEX-CDR] recovered were 4.09 mg

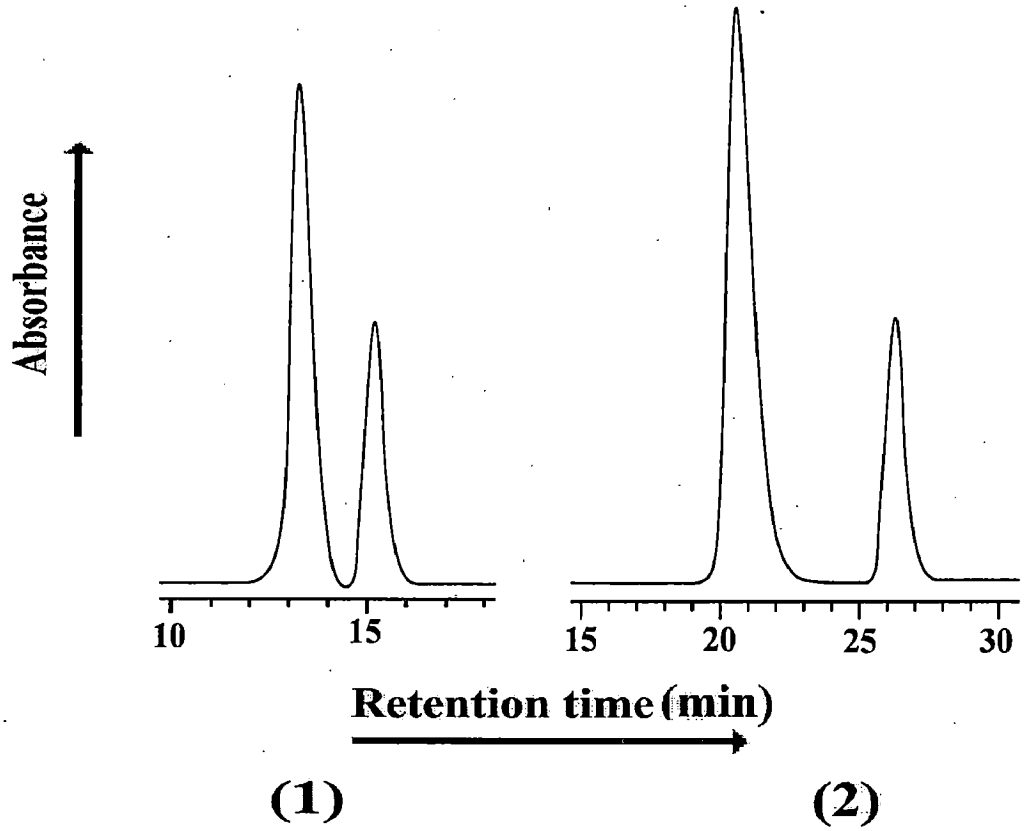


Fig. 5.4: Sections of chromatograms showing HPLC small-scale preparative resolution of diastereoselective products of (*R*,*S*)-MEX as synthesized with (1) (*S*)-TFPC (using A1) and (2) Camphanic chloride (using B1), as a result of kinetic resolution; Waters Spherisorb ODS2 (250 × 4.6 mm I.D., 5μm); diastereomeric peak corresponding to (*R*)-MEX was eluted prior to that of (*S*)-MEX; mobile phase was *aq* TFA (0.1%)-MeCN in a linear gradient of MeCN from 30 to 70% in 45 min at a flow rate of 1.0 mL/min and UV detection at 210 nm

The peak area of the first eluting diastereomer corresponds to 71% (*de*) and to 74% (*de*) for samples **(1)** and **(2)**, respectively (in terms of *de*; see section "1. Kinetic resolution of (*R*, *S*)-MEX")

and 4.22 mg from samples **A1** and **B1**, respectively. The yields of (*R*)-MEX obtained from the acidic hydrolysis of the respective diastreoselective product [(*R*)-MEX-CDR] 1.27 mg and 1.50 mg for **A1** and **B1** samples, respectively.

4. Reaction Enantioselectivity

The reaction of CDR *viz.* (*S*)-TFPC or camphanic chloride (the acyl chlorides) with (*R,S*)-MEX (the primary amine) leading to the formation of diastereomers (amides) is accompanied by production of one equivalent of HCl which needs to be neutralized by a second equivalent of amine. Alternatively, the reaction could be carried out in the presence of another base such as NaOH, which would neutralize HCl, but at the same time it would also attack the acyl chloride to give corresponding carboxylic acid. Therefore, in the present case the (*R,S*)-MEX and CDR were taken in the ratio of 2:1 (in case of **A1** and **B1**) wherein the second mole of MEX does the job of neutralizing HCl i.e. the diastereomeric amides are yielded *via* acylammonium intermediate salt (**(1)**, Fig. 5.5, Path A) following a nucleophilic mechanism. The HPLC analysis reveals the presence of unreacted MEX which was eluted prior to the diastereomeric peaks.

On the other hand, the reaction was also carried out in the presence of pyridine (in the cases **A2-A4** and **B2-B4**). The reaction proceeds via an acyl ammonium intermediate (**(2)**, Fig. 5.5, Path B) which is converted to the amides (the diastereomers) with the elimination of HCl. The HCl gets neutralized with pyridine and the reaction was successful in 1:1.1 molar ratio of (*R,S*)-MEX and CDR in the presence of pyridine.

Schotten-Baumann reported a method for synthesis of an amide using dichloromethane or chloroform as the common organic solvents, which are heavier than water, when the acyl chloride in the lower dichloromethane layer reacted with the amine and HCl produced dissolved in aqueous layer and neutralized by NaOH present in it [207].

In the present case, the problem of formation of HCl has been tackled firstly by taking additional mole of amine and secondly by taking pyridine as base.

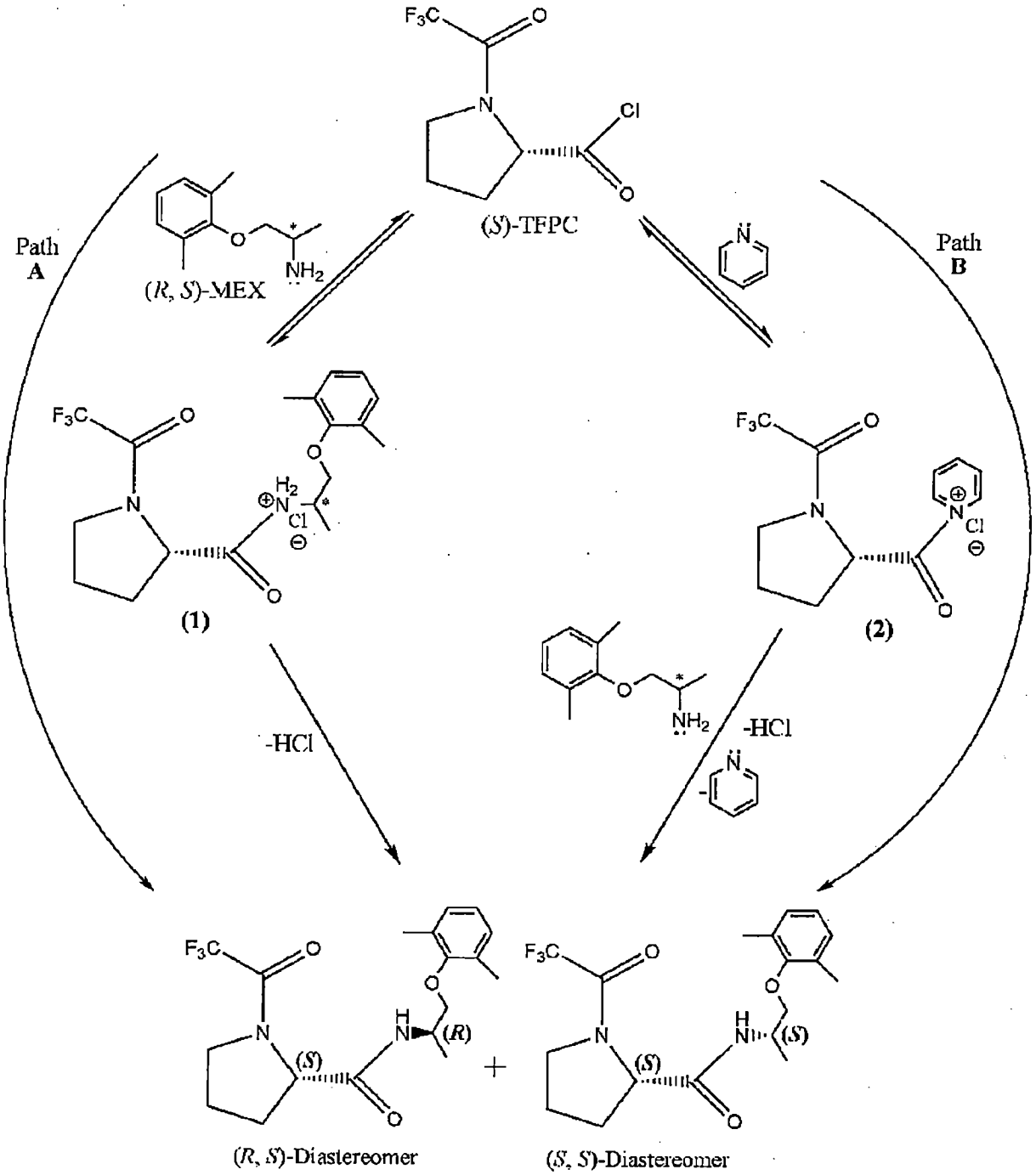


Fig. 5.5: Schematic diagram to represent the effect of the presence and absence of pyridine on the reaction of (*R*,*S*)-MEX to a representative CDR, (*S*)-TFPC. Path A: a nucleophilic mechanism *via* acylammonium intermediate salt (1); Path B: a base-catalyzed mechanism *via* acylammonium intermediate salt (2)

Table 5.2: Recovery of diastereoselective (*R*)-MEX-CDR diastereomer, yield, and enantiomeric excess (*ee*) of (*R*)-MEX

CDRs	Recovery#[a]	Yield*[b]	Enantiomeric excess*[b]
(*S*)-TFPC	92.91	65	97.6
Camphanic Chloride	93.74	69	97.8

 # value given in percentage
[a] of (*R*)-MEX-CDR diastereomer
[b] of (*R*)-MEX enantiomer

5. Method Validation

Linearity

A good linear relationship was obtained over the range mentioned in "Present Work" section. The regression equations, LOD and LOQ for each of the diastereomers were summarized in Table- 5.3.

Accuracy and Precision

The data related to intra-day assay precision and inter-day assay precision were summarized in Table 5.4.Validation studies results have also indicated that the solutions of diastereomers of MEX were quite stable up to one week under refrigerated condition (4 °C).

Table-5.3: Linear regression equations of the HPLC enantioresolution of diastereomers of (*R*,*S*)-mexiletine synthesized with two CDRs within the range of 50-150 ngmL^{-1}

Chiral derivatizing reagent	Compound	Calibration curve*	Linearity (R^2)	LOD (ng/mL)	LOQ (ng/mL)
(*S*)-TFPC	(*R*,*S*)-Diastereomer	y=0.0085x–0.904	0.997	45	150
	(*S*,*S*)-Diastereomer	y=0.0081x+0.981	0.998	45	150
Camphanic chloride	(*R*,*S*)-Diastereomer	y=0.0107x+0.192	0.999	80	266
	(*S*,*S*)-Diastereomer	y=0.0122x+0.185	0.998	80	266

*x: sample concentration (ng/mL);
y: absorbance;
R^2=correlation coefficient

Table-5.4: Intra-day assay and inter-day assay precision of the HPLC enantioresolution of diastereomers of (*R*,*S*)-mexiletine synthesized with two CDRs within the range of 50-150 ngmL^{-1}

	Conc. (ng/mL)	(*S*)-TFPC				Camphanic chloride			
		(*R*,*S*)-Diastereomer		(*S*,*S*)-Diastereomer		(*R*,*S*)-Diastereomer		(*S*,*S*)-Diastereomer	
		Mean±SD	Recovery ±RSD*	Mean ± SD	Recovery ±RSD*	Mean ± SD	Recovery ±RSD*	Mean±SD	Recovery ±RSD*
Intra-day	50	24.58±0.27	98.32±1.08	24.70±0.19	98.80±0.76	24.63±0.18	98.52±0.72	24.89±0.21	99.56±0.84
	100	49.45±0.44	98.90±0.88	49.85±0.20	99.70±0.40	49.57±0.58	99.14±1.16	49.61±0.45	99.22±0.90
	150	75.83±0.49	101.11±0.65	76.20±0.43	101.60±0.57	75.91±0.88	101.21±1.73	75.77±0.78	101.03±1.04
Inter-day	50	24.40±0.36	97.60±1.44	24.34±0.24	97.36±0.96	24.42±0.21	97.68±0.84	24.66±0.15	98.64±0.60
	100	48.95±0.48	97.90±0.96	49.15±0.46	98.30±0.92	49.50±0.49	99.00±0.98	49.55±0.47	99.10±0.94
	150	73.58±0.60	98.11±0.80	73.67±0.62	99.23±0.83	75.89±0.76	101.19±1.01	75.54±0.71	100.72±0.95

* represents percentage values;
SD standard deviation;
RSD relative standard deviation

Chapter-6

Direct Enantioseparation of Anti-ulcer Drugs

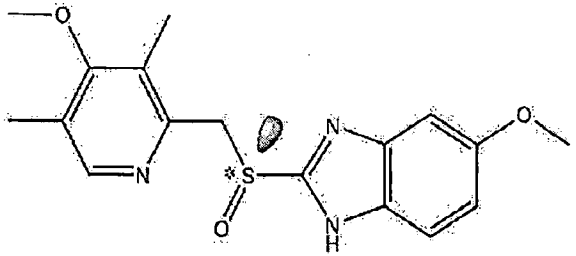
I. Introduction

(*R,S*)-Omeprazole (OME; 5-methoxy-2-((4-methoxy-3,5-dimethylpyridin-2-yl)methyl sulfinyl)-1*H*-benzo[d]imidazole), (*R,S*)-rabeprazole (RAB; 2-((4-(3-methoxypropoxy)-3-methyl pyridin-2-yl)methylsulfinyl)-1*H*-benzo[d]imidazole), (*R,S*)-lansoprazole (LAN; 2-((3-methyl-4-(2,2,2-trifluoroethoxy)pyridin-2-yl)methylsulfinyl)-1*H*-benzo[d]imidazole) and (*R,S*)-pantoprazole (PAN;5-(2,2-difluoroethyl)-2-((3,4-dimethoxypyridin-2-yl)methylsulfinyl)-1*H*-benzo[d]imidazole) are some of the commonly used anti-ulcer drugs. Anti-ulcer drugs block the H^+/K^+ ATPase enzyme system of gastric proton pump, and reduce the gastric-acid production and are termed as proton pump inhibitors (PPIs); these are structurally chiral sulfoxides. These drugs induce sensitivity of tumor cells to anti-tumoral agents [208]. These chiral sulfoxides have an asymmetric sulfoxide centre, have various substituted pyridine and benzimidazole moieties (Fig. 6.1), and have pyramidal structure due to the lone pair available at the sulfur of sulfoxide group. These are racemic compounds and have different pharmacodynamic and pharmacokinetic properties. The difference in clinical efficacy between pure enantiomer and racemic PPIs is attributed to their stereoselective pharmacokinetics instead of pharmacological activities [209].

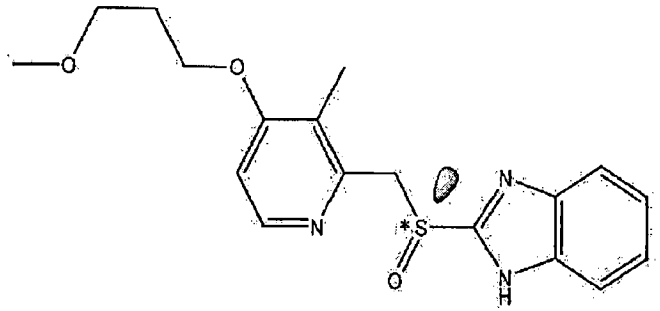
II. Literature Review

✱ OME, LAN, PAN and RAB

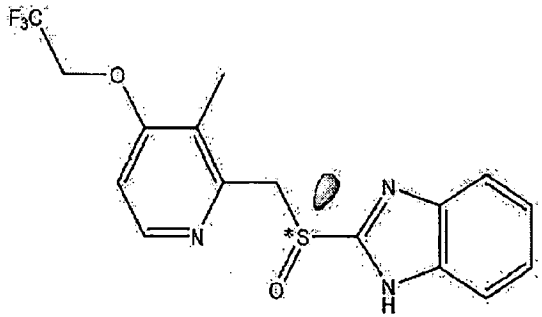
OME [210] is the first registered drug of PPIs class and is one of the most selling drugs. (*S*)-Enantiomer of OME has more potent effect on gastric and duodenal ulcers in comparison to the racemic OME [211, 212]. Pharmacological studies have shown that (*R*)-LAN and (*S*)-LAN have identical effects as inhibitor of acidic secretion. Nevertheless, the (*S*)-LAN is easily metabolized to pharmacologically inactive 5-hydroxy and sulphone metabolites [213, 214]. (*R*)-LAN is more valuable for clinical usage than the (*S*)-LAN due to its greater binding to human serum proteins [209, 215]. (*S*)-PAN is more potent than (*R*)-PAN in the reduction of the duodenal and gastric ulcers [216].



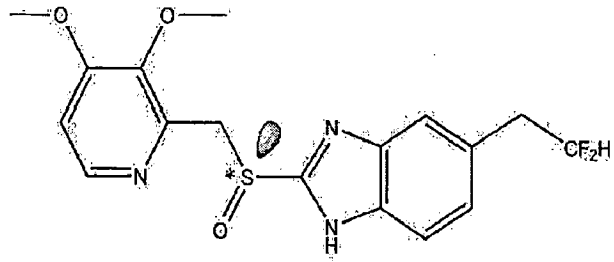
Omeprazole (OME; 5-methoxy-2-((4-methoxy-3,5-dimethylpyridin-2-yl)methylsulfinyl)-1*H*-benzo[*d*]imidazole)



Rabeprazole (RAB; 5-methoxy-2-((4-methoxy-3,5-dimethylpyridin-2-yl)methylsulfinyl)-1*H*-benzo[*d*]imidazole)



Lansoprazole (LAN; 2-((3-methyl-4-(2,2,2-trifluoroethoxy)pyridin-2-yl)methylsulfinyl)-1*H*-benzo[*d*]imidazole)



Pantoprazole (PAN; 5-(2,2-difluoroethyl)-2-((3,4-dimethoxypyridin-2-yl)methylsulfinyl)-1*H*-benzo[*d*]imidazole)

Fig. 6.1: Structures along with IUPAC names of chiral sulfoxides: omeprazole (OME; 5-methoxy-2-((4-methoxy-3,5-dimethylpyridin-2-yl)methylsulfinyl)-1*H*-benzo[d]imidazole), rabeprazole (RAB; 2-((4-(3-methoxypropoxy)-3-methylpyridin-2-yl)methylsulfinyl)-1*H*-benzo[d]imidazole), lansoprazole (LAN; 2-((3-methyl-4-(2,2,2-trifluoroethoxy)pyridin-2-yl)methylsulfinyl)-1*H*-benzo[d]imidazole) and pantoprazole (PAN; 5-(2,2-difluoroethyl)-2-((3,4-dimethoxypyridin-2-yl)methylsulfinyl)-1*H*-benzo[d]imidazole)

Dexrabeprazole (dexRAB) is (*R*)-(+)-isomer of RAB (Fig. 6.1). RAB causes dose-dependent inhibition of acid secretion and has more rapid onset of action than OME [217]. Recently a correlation between dissolution and thermal rate constants of Rabeprazole sodium drug and their tablets has been studied [218]. A 10 mg dose of dexRAB has been found better than 20 mg dose of RAB in the treatment of gastroesophageal reflux diseases in improving/healing of endoscopic lesions and relief from symptoms of regurgitation [219]. The anti-ulcer activity of (*R*)-(+)-RAB is more effective than (*S*)-(–)-RAB in aspirin- and histamine-induced ulcers in rats [220]. (*R*)-RAB disposition in liver is influenced to a greater extent by CYP2C19 genetic polymorphisms than that of (*S*)-RAB [214].

✸ Enantioseparation of (*R,S*)-OME, (*R,S*)-RAB, (*R,S*)-LAN and (*R,S*)-PAN

The structures of chiral sulfoxides (Fig. 6.1) indicate that indirect approach for their enantioseparation would not be quite effective due to absence of reactive functional group. Enantioseparation of these chiral sulfoxides has been reported in literature using CSPs. Polysaccharide based CSPs viz. Chiralcel-OJ, Chiralpak-AD, cellulose tris (3,5-dichlorophenylcarbamate) [221] and Chiralpak-IC [222] have been used for HPLC enantioseparation of (*R,S*)-OME. (*R,S*)-RAB has been enantioresolved using Chiralpak AD-H column and photo diode array and polarimetric detectors, connected in series [223]. (*R,S*)-LAN has been enantioseparated using HPLC on Chiralpak-IB as well as on Chiralcel OD-H [224] columns.

Amylose tris(3,5-dimethylphenylcarbamate), amylose tris[(*S*)-1-phenylethylcarbamate], and amylose tris(3,5-dimethoxyphenylcarbamate) based CSPs have been used for HPLC enantioseparation of (*R,S*)-OME, (*R,S*)-LAN and (*R,S*)-PAN [225]. Two polysaccharide based columns viz. Chiralpak-AD column [226] and Chiralpak-IA column [227] have been used for the HPLC chiral separation of all the four anti-ulcer drugs; (*R,S*)-OME, (*R,S*)-RAB, (*R,S*)-LAN and (*R,S*)-PAN. The enantiomeric purities of OME [228] and LAN [229] have been checked by HPLC enantioresolution method using polysaccharide based CSP viz. Chiralpak-IA column.

The aforementioned polysaccharide based CSPs which have been used for enantioseparation of different anti-ulcer drugs are either of coated type or immobilized type CSPs. Both have their advantages and limitations. The usage of coated type CSPs for enantioseparation has been limited to standard mobile phase conditions viz. (i) different compositions of hydrocarbon/alcohol and (ii) alcohols. On the other hand, in spite of versatile and robust nature, the immobilized type CSPs are specially limited to analytical purpose. Nevertheless the coated type CSPs are fruitful for the preparative enantioseparation of these anti-ulcer drugs.

III. Present Work

In view of the literature reports, as above, enantioseparation of four anti-ulcer drugs ((*R,S*)-OME, (*R,S*)-RAB, (*R,S*)-LAN and (*R,S*)-PAN) has been attempted using a chlorinated cellulose based Lux cellulose-2 chiral column (Fig. 6.2) and under normal and polar-phase elution conditions. To the best of authors' knowledge there were no reports on enantioseparation of any of the aforementioned anti-ulcer drugs of this kind. The method so developed was applied for investigating the chiral purity of dexRAB.

1. Extraction of DexRabeprazole Drug from Commercial Tablet

The extraction of dexRabeprazole (dexRAB) from commercial tablet has been described in Chapter-2.

2. HPLC of Anti-Ulcer Drugs (Chiral Sulfoxides)

Lux cellulose-2 chiral column [230] used in present studies is manufactured by Phenomenex (CA, USA) and is a CSP made with inert and high pressure stable silica, based on a specialized coating and packing of cellulose tris(3-chloro-4-methylphenylcarbamate) and is

susceptible to both the normal (e.g. hydrocarbon/ alcohol) and polar-organic eluents (e.g. *n*-HEX, *n*-heptane, MeOH, EtOH, IPA, MeCN and combinations of MeCN/ alcohol).

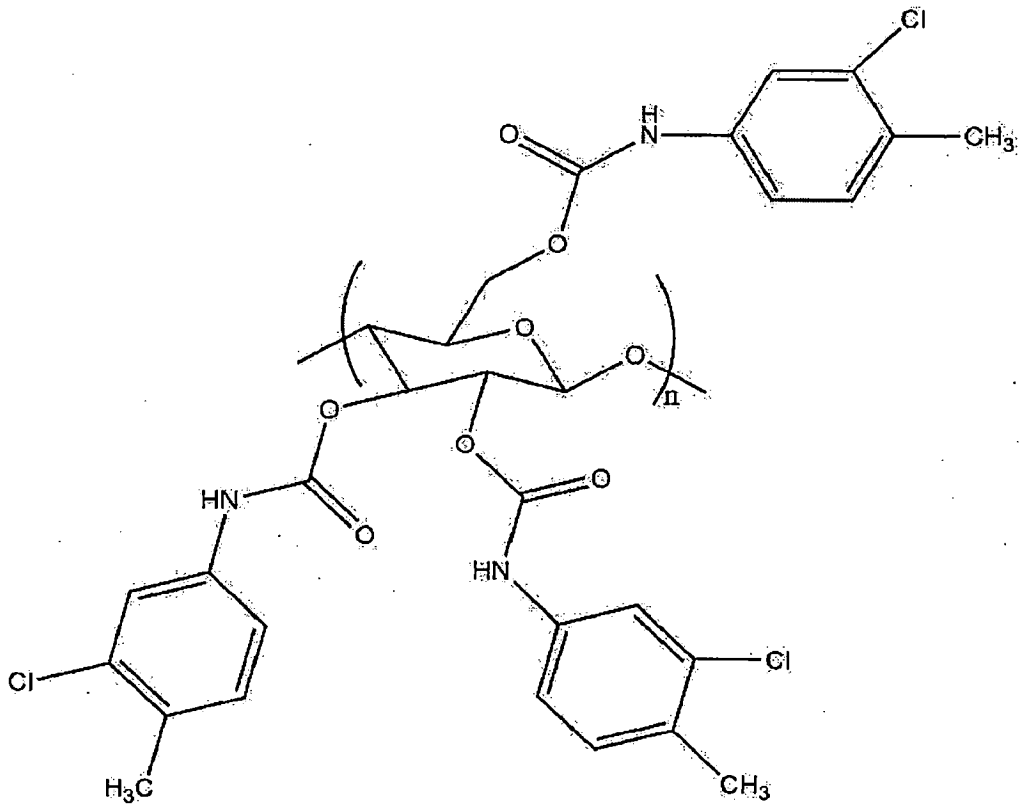


Fig. 6.2: Structure of chiral selector, 3-chloro-4-methyl phenylcarbamate present in Lux cellulose-2 *i.e.* cellulose tris(3-chloro-4-methyl phenylcarbamate) column

Using Normal and Polar-organic Mobile Phases

Standard solutions of the chiral sulfoxides were prepared by dissolving 1 mg of each chiral sulfoxide into 10 mL of ethanol for enantioseparation and 20 µL of it was injected onto the column. Different compositions of IPA and EtOH (as organic modifiers) with HEX were used as eluents in normal-phase mode. On the other hand, 100% MeOH, EtOH, IPA, and MeCN were used as eluents in polar-organic mode. Diethyl amine (DEA) was used as modifier additive.

3. Method Validation

The experimental method was validated with respect to linearity, accuracy, precision and robustness for the elution of all the four analytes using the mobile phases resulted the best enantioresolution (in terms of R_S) on Lux cellulose-2 column according to ICH guidelines [92] and summarized here:

Linearity

The least square method using Microsoft Excel program to determine the slopes and correlation coefficients for the calibration graphs computed the linear regression equations. The calibration graphs were drawn between the peak area responses of first eluting enantiomer and second eluting enantiomer and the corresponding nominal concentration of the enantiomers in the range of 20-100 μgmL^{-1} of analytes' racemic solutions.

The LOD and LOQ were determined by measuring signal/noise (S/N) ratios. LOD was taken as the concentration of the analyte where S/N was 3 [231].

Accuracy and Precision

The accuracy of the method was tested by the analysis of the standard solutions of analytes at five various concentration levels in the range of 20-100 μgmL^{-1}.

The precision of the method was determined by intra-day assay (the repeatability) and inter-day assay (the intermediate precision) studies of peak areas of enantiomers on replicate HPLC analysis (n=6) of solutions of racemic analytes for three days. Precision was reported as percentage of relative standard deviation (RSD) at three concentration levels 20-200% of a specific concentration 20 μgmL^{-1}.

Robustness

By making small deliberate changes in the contents of organic modifier and modifier additive (i.e. DEA) in the eluents robustness of the method was checked. Effect of flow rate was also investigated by applying flow rate 1.0 ± 0.1 $mLmin^{-1}$ for the samples.

IV. Results and Discussion

1. Enantioseparation in Normal-phase Mode

For this purpose, eluents containing different compositions of IPA and EtOH (as organic modifiers) with *n*-HEX were used. The chromatographic data related to the enantioseparation has been summarized in Table 6.1. The flow rate of the mobile phase was 1.0 mL/min. Chiral sulfoxides showed increment in their retention (k_1) when the eluent containing EtOH as organic modifier switched to that containing IPA as organic modifier (Table 6.1). This is because of the stronger elution strength of EtOH than IPA on polysaccharide derived stationary phases. The enantiomers of (*R,S*)-RAB, (*R,S*)-LAN, and (*R,S*)-PAN were well separated with good peak shapes and have highest resolution, respectively, with eluent at entries 9, 15 and 19 among the aforementioned sets of eluents. In order to seek the enantioseparation of (*R,S*)-OME, diethyl amine (DEA) was used as modifier additive. Since this has been reported [224, 228, 229, 232, 233] to enhance the enantioseparation and peak shapes. Systematic studies using different percentage ratios of DEA showed that a 0.2% concentration was found successful for

Table 6.1 HPLC enantioseparation of chiral sulfoxides in normal phase elution conditions using Lux cellulose-2 column

Entry	*Chiral Sulfoxide*	*Eluent*	k_1	α	R_S
	(*R*,*S*)-OME				
1.		*n*-HEX–EtOH 70:30	2.44	1.00	NR
2.		*n*-HEX–EtOH 65:35	2.29	1.09	NR
3.		*n*-HEX–IPA 70:30	2.51	1.00	NR
4.		*n*-HEX–IPA 65:35	2.32	1.10	NR
5.		*n*-HEX–EtOH–DEA 65:35:0.2	2.21	1.91	8.32
6.		*n*-HEX–IPA–DEA 65:35:0.2	2.37	2.08	9.57
	(*R*,*S*)-RAB				
7.		*n*-HEX–EtOH 80:20	3.31	1.08	0.91
8.		*n*-HEX–IPA 80:20	3.42	1.11	0.85
9.		*n*-HEX–EtOH 90:10	3.72	1.28	3.41
10.		*n*-HEX–IPA 90:10	3.91	1.12	1.16
11.		*n*-HEX–EtOH–DEA 90:10:0.4	3.81	1.43	4.52
	(*R*,*S*)-LAN				
12.		*n*-HEX–EtOH 80:20	1.83	1.12	0.96
13.		*n*-HEX–IPA 80:20	1.95	1.11	0.89
14.		*n*-HEX–EtOH 65:35	1.32	1.10	0.87
15.		*n*-HEX–EtOH 70:30	1.67	1.52	3.51
16.		*n*-HEX–IPA 70:30	1.72	1.41	2.11
17.		*n*-HEX–EtOH–DEA 70:30:0.2	1.68	1.56	3.48
18.		*n*-HEX–EtOH–DEA 70:30:0.4	1.66	1.55	3.49
	(*R*,*S*)-PAN				
19.		*n*-HEX–IPA 80:20	1.48	1.42	2.60
20.		*n*-HEX–IPA 75:25	1.92	1.32	1.81
21.		*n*-HEX–IPA 85:15	1.31	1.41	1.97
22.		*n*-HEX–IPA 90:10	1.28	1.29	1.87
23.		*n*-HEX–EtOH 80:20	1.39	1.18	1.10
24.		*n*-HEX–IPA–DEA 80:20:0.2	1.50	1.45	2.58
25.		*n*-HEX–IPA–DEA 80:20:0.4	1.52	1.55	2.57

Lux Cellulose-2 column (250 × 4.6 mm I.D., 5 µm); Column: Flow rate: 1.0 ml/min; Detection: 285 nm; NR: Not resolved;

enantioseparation of (*R,S*)-OME (entries 5 and 6). Besides, a better resolution was obtained for (*R,S*)-RAB (entry 11) when 0.4% concentration of DEA was used. There was no significant effect of DEA on resolution of (*R,S*)-LAN and (*R,S*)-PAN (entries 17, 18, 24 and 25).

Section of chromatograms showing best enantioresolution (in terms of R_S, Table-6.1) of the four anti-ulcer drugs using normal-phase eluents are shown in Fig. 6.3.

2. Enantioseparation in Polar-organic Phase Mode

Literature [207] reveals that pure polar-organic elution mode has following advantages: (i) it provides higher solubility, (ii) it follows alternative chiral recognition mechanisms, and (iii) it is easily removed from analytes in preparative scale applications. Nevertheless, use of 100 % MeOH, EtOH, IPA, and MeCN resulted into enantioresolution in the present studies. There was observed a baseline resolution of each of the analytes in at least one of the polar-organic eluents (Table-6.2). The flow rate of the mobile phase was 1.0 mL/min except some cases where flow rate was 0.7 mL/min. The enantioseparation of (*R,S*)-LAN and (*R,S*)-RAB with MeCN (entries 8 and 12) was better in comparison to their resolution with the protic eluents (Table-6.2). Section of chromatograms showing best enantioresolution (in terms of R_S, Table-6.1) of the four anti-ulcer drugs using polar organic eluents are shown in Fig. 6.4.

3. Enantiomeric Elution Order

To evaluate the enantiomeric excess in a non-racemic mixture, prior elution of the minor enantiomer to the major enantiomer is required to avoid the probable errors generated due to the tailing of first eluting enantiomer. The determination of elution order of enantiomers is an important concern in pharmaceutical chemistry, quality control analysis, and enantiomeric recognition studies as well as in asymmetric synthesis [227]. It may mainly be because the elution order of enantiomer changes on polysaccharide based CSPs by changing the polymeric type i.e. from cellulose based CSP to amylose based CSP [234] or by changing the eluent composition [235]. In the present studies the standard solution of racemic chiral sulfoxides was

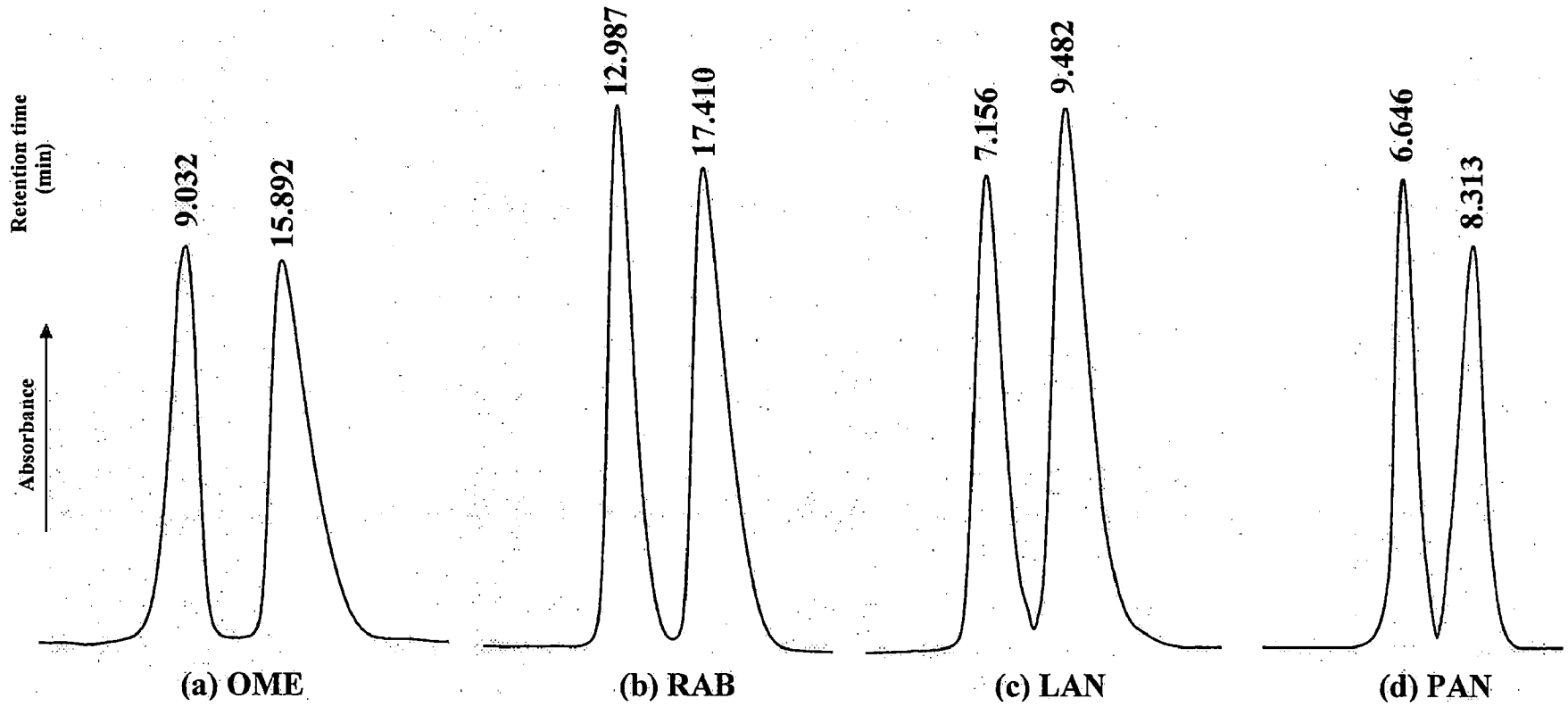


Figure 6.3: Sections of chromatograms showing best enantioresolution of (a) OME (b) RAB (c) LAN and (d) PAN using normal-phase eluents in the entries 6, 11, 14 and 20 (Table-6.1), respectively; Lux Cellulose-2 column (250 × 4.6 mm I.D., 5 µm); Column: Flow rate: 1.0 ml/min; Detection: 285 nm

Table 6.2 HPLC enantioseparation of chiral sulfoxides in polar-organic phase elution conditions using Lux cellulose-2 column

Entry	*Chiral Sulfoxide*	*Eluent*	k_1	α	R_S
	(*R*,*S*)-OME				
1.		MeOH	1.28	2.10	3.82
2.		EtOH[a]	1.68	2.18	4.64
3.		IPA[a]	2.19	2.21	5.17
4.		MeCN	4.82	1.00	NR
	(*R*,*S*)-RAB				
5.		MeOH	1.31	1.08	0.56
6.		EtOH[a]	1.76	1.00	NR
7.		IPA[a]	2.27	1.10	0.61
8.		MeCN	2.91	1.85	1.72
	(*R*,*S*)-LAN				
9.		MeOH	0.48	1.11	0.98
10.		EtOH[a]	0.51	1.18	1.16
11.		IPA[a]	0.68	1.22	1.73
12.		MeCN	1.51	1.71	1.81
	(*R*,*S*)-PAN				
13.		MeOH	0.51	1.06	0.91
14.		EtOH[a]	0.56	1.17	1.11
15.		IPA[a]	0.94	1.18	1.71
16.		MeCN	1.28	1.10	0.87

Lux Cellulose-2 column (250 × 4.6 mm I.D., 5 µm); Column: Flow rate: 1.0 ml/min; Detection: 285 nm; NR: Not resolved;
[a]Flow rate: 0.7 ml/min

spiked with the corresponding available pure form of enantiomer. These enriched mixtures were injected onto the column using the experimental conditions summarized in Table-6.1 and Table 6.2 to know the elution order of enantiomers.

In both the normal and the polar-organic phase elution conditions prior elution of (*S*)- to the (*R*)-enantiomer was observed for (*R,S*)-RAB, (*R,S*)-LAN, and (*R,S*)-PAN. The enantiomers of (*R,S*)-OME showed reverse order with all the eluents except the eluents in which IPA was used (Table-6.1:entry 6 and Table 6.2: entry 3). (*R,S*)-LAN also showed reverse enantiomeric affinity to CSP when IPA was used as organic modifier in normal-phase eluents (Table 6.1: entries 13 and 16).

4. Enantioselectivity of the CSP

Literature [232, 236] reveals that the helical structure of polysaccharide based CSPs having phenylcarbamate moieties is owing to the N–H and C=O groups which are involved in intramolecular hydrogen-bonding. The remaining free N–H and C=O groups and the substituents on the phenyl group of phenyl carbamate moiety are responsible for enantioseparation interaction between the CSP and analytes. The introduction of an alkyl substituent onto the phenyl moiety the fraction of carbamate groups involved in intramolecular hydrogen-bonding increases while with the introduction of halogen substituent the fraction of free carbamate groups increases [232, 236]. Therefore introduction of both the halogen (-Cl) and alkyl (-CH_3) substituents on the phenyl group of phenyl carbamate moiety of the CSP (used in the present case, as described by the manufacturer of the Lux cellulose-2 chiral column) is responsible for efficient enantiorecognition ability of the mentioned column. The presence of chlorine atom in the backbone of the said CSP can easily polarize the racemic mixtures and hence found better for preferential interaction with one enantiomer.

The chiral sulfoxides (molecular formula are shown in parenthesis); OME ($C_{17}H_{19}N_3O_3S$), RAB ($C_{18}H_{21}N_3O_3S$), LAN ($C_{16}H_{14}F_3N_3O_2S$) and PAN ($C_{17}H_{17}F_2N_3O_3S$) have the same backbone

in the form of substituted pyridine and benzimidazole moieties and have different electronegative atoms (electronegativity on Pauling Scale are shown in parenthesis); F (3.98), O (3.44), N (3.04) and S (2.58). Based on these structural features the aforementioned chiral sulfoxides may be arranged in the following hydrophobicity sequence: RAB> OME> PAN> LAN and the hydrogen-bonding affinity sequence: LAN> PAN> OME~RAB. Thus, hydrogen-bonding interactions between the analytes and the CSP used in the present studies played a vital role in the enantioresolution of the chiral sulfoxides.

The availability of fraction of free carbamate fragments (i.e. fraction of free N–H and C=O groups) is higher in those CSPs which are based on chloromethyl–phenyl carbamate derivatives of cellulose in comparison to those based on dimethyl–phenyl carbamate derivatives of cellulose [37]. In contrast to the solubility of dichloro–phenyl carbamate derivatives of cellulose in hydrocarbon-alcohol mixtures the chloromethyl–phenyl carbamate derivatives of cellulose is insoluble in both the hydrocarbon-alcohol and polar eluents. Thus the use of the CSP containing both an alkyl and halogen substituent on the phenyl group of phenyl carbamate moiety in the present study proved to be successful due to its bimodal (i.e. normal-phase and polar organic) elution characteristic.

5. Elution Mode Selectivity

(i) Normal-phase Elution Mode

The enantioresolution on polysaccharide based CSPs are attributed to hydrogen bonding, π-π, and dipole–dipole induced interactions [232, 236]. During elution of the chiral sulfoxides through the Lux cellulose-2 CSP there is competition between organic modifier (e.g. EtOH or IPA) and the chiral sulfoxide enantiomers for the active sites of CSP. Less bulky alcohol (i.e. EtOH) gets easily inserted into the cavity of the CSP in comparison to the more bulky alcohol (i.e. IPA). This insertion of the alcohols into the chiral cavities of the Lux cellulose-2 CSP induces changes in the dominant chiral recognition sites and hence leads to the formation of more stable

diastereomeric complexes with the two enantiomers. So in the presence of EtOH chiral sulfoxide enantiomers might interact strongly with the Lux cellulose-2 CSP, leading to higher resolution on the column (Table 6.1: entries 7 *vs* 8, 9 *vs* 10, 12 *vs* 13, 15 *vs* 16).

On the other hand, (*R,S*)-OME and (*R,S*)-PAN have shown high resolution with IPA (Table 6.1). The probable reason for this could be a competition for hydrophobic interaction between methyl group present on phenyl moiety of the CSP and the hydrophobic chain of the organic modifier.

(ii) Polar-organic Phase Elution Mode

Table-6.2 showed that the retention of LAN was shortest among the four chiral sulfoxides. The result is in agreement with the expectations because LAN has the least hydrophobicity among all the chiral sulfoxides (as mentioned in section "4. Enantioselectivity of the CSP") and hence it has the least interaction with CSP.

The enantioseparation data shown in Table-6.2 has revealed that the interaction between the chiral sulfoxide and the polysaccharide chloromethylphenyl carbamate increases with the increase in hydrophobicity of the eluents (MeOH< EtOH< IPA). Exceptionally (*R,S*)-RAB was not resolved in the presence of 100% EtOH. These observations were supported by the fact that the hydrophobic interactions might be less favoured in higher alcohols or in other words, we can say that the hydrogen-bonding interactions might be more favoured in higher alcohols. Thus, the combined contribution of the hydrogen bonding interactions in between the chiral sulfoxide (i.e. RAB, LAN and PAN) and Lux cellulose-2 CSP would be increased at the expense of hydrophobic interactions with the increase in hydrophobicity of the polar-organic eluents (Table-6.2). OME did not follow this trend and show highest resolution among the four chosen chiral sulfoxides. Complementary enantioseparation results were observed with aprotic polar organic eluent (MeCN) in comparison to protic polar organic eluent (MeOH) (Table-6.2).

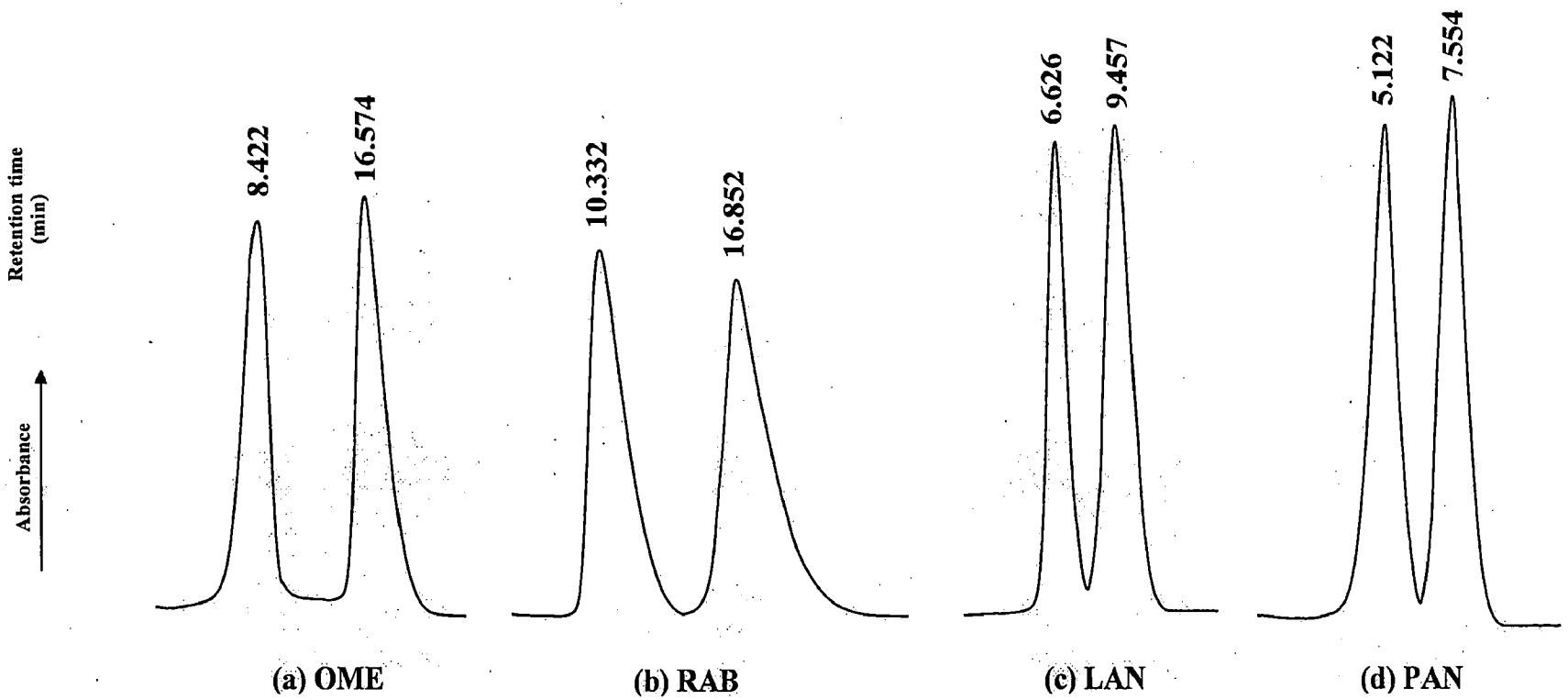


Fig. 6.4: Sections of chromatograms showing best enantioresolution of (a) OME (b) RAB (c) LAN and (d) PAN using polar organic eluents in the entries 3, 8, 12 and 15 (Table-6.2), respectively; Lux Cellulose-2 column (250 × 4.6 mm I.D., 5 µm); Column: Flow rate: 0.7 mL/min for (a) OME and (d)PAN while 1.0 ml/min for (b) RAB and (c) LAN; Detection: 285 nm

The whole description in bimodal elution has revealed that the intermolecular forces are responsible for the retention of chiral sulfoxides but their enantioseparation are multivariate and hence enantioseparation of the chiral sulfoxides is based not only on the mobile and stationary phase but also on the nature of the chiral sulfoxides.

A comparison of present work to the earlier reports on enantioresolution of (*R*,*S*)-OME, (*R*,*S*)-RAB, (*R*,*S*)-LAN and (*R*,*S*)-PAN using coated type polysaccharide based different (in terms of chiral selector) CSPs is shown in Table-6.3. It clearly establishes the novelty and superiority of the present report (in terms of resolution).

6. Enantiomeric Purity of Dexrabeprazole

The standard solution of dexRAB was injected onto Lux cellulose-2 column using both the normal phase (Table 6.1) and polar-organic phase (Table 6.2) elution modes. A typical chromatogram corresponding to a pharmaceutical formulation of dexRAB using *n*-HEX–EtOH–DEA 90:10:0.4 (v/v/v) is shown in Fig. 6.5. The validation studies have revealed that there was no interference in determination of enantiomeric excess of (*R*)-RAB. Thus the developed method was found reliable for checking the enantiomeric purity of dexRAB and thus helped to determine the enantiomeric excess of dexRAB in bulk pharmaceuticals.

7. Method Validation

Linearity

A good linear relationship was obtained over the range mentioned in "Present Work" section. The regression equations, LOD and LOQ for each enantiomer were summarized in Table 6.4.

Table 6.3: Comparison of HPLC enantioseparation of OME, RAB, LAN, and PAN using coated type polysaccharide based different CSPs

Analyte	Chiral Column	Chiral Selector	*Rs*	Reference
(*R,S*)-OME	***Lux cellulose-2***[a]	***Cellulose tris(3-chloro-4-methylphenylcarbamate)***	***9.57***	***Present Work***
	Chiralpak AD[b]	Amylose tris(3,5-dimethylphenyl carbamate)	4.35	[226]
	Chiralcel OJ[a]	Cellulose tris(4-methyl benzoate)	NR	[221]
	Chiralpak AD[a]	Amylose tris(3,5-dimethylphenyl carbamate)	5.0	[221]
	CDCPC[c]	Cellulose tris(3,5-dichlorophenylcarbamate)	2.5	[221]
	CSP 1[d]	Amylose tris(3,5-dimethylphenyl carbamate)	NG	[225]
	CSP 2[d]	Amylose tris[(*S*)-1-phenylethyl carbamate]	NG	[225]
	CSP 3[d]	Amylose tris(3,5-dimethoxyphenylcarbamate)	NG	[225]
(*R,S*)-RAB	***Lux cellulose-2***[a]	***Cellulose tris(3-chloro-4-methylphenylcarbamate)***	***4.52***	***Present Work***
	Chiralpak AD[b]	Amylose tris(3,5-dimethylphenyl carbamate)	0.84	[226]
	Chiralpak AD-H[a]	Amylose tris(3,5-dimethylphenyl carbamate)	3.24	[223]
(*R,S*)-LAN	***Lux cellulose-2***[a]	***Cellulose tris(3-chloro-4-methylphenylcarbamate)***	***3.51***	***Present Work***
	Chiralpak AD[b]	Amylose tris(3,5-dimethylphenyl carbamate)	0.72	[226]
	CSP 1[d]	Amylose tris (3,5-dimethylphenyl carbamate)	NG	[225]
	CSP 2[d]	Amylose tris[(S)-1-phenylethyl carbamate]	NG	[225]
	CSP 3[d]	Amylose tris(3,5-dimethoxyphenylcarbamate)	NG	[225]
	Chiralcel OD-H[a]	Cellulose tris(3,5-dimethylphenylcarbamate)	2.61	[224]
(*R,S*)-PAN	***Lux cellulose-2***[a]	***Cellulose tris(3-chloro-4-methylphenylcarbamate)***	***2.60***	***Present Work***
	Chiralpak AD[b]	Amylose tris(3,5-dimethylphenyl carbamate)	1.92	[226]

R_S, resolution; NR, not resolved; NG, not given; [a]on 5 µm silica-gel; [b]on 10 µm silica-gel; [c]Macroporous silica gel Daisogel SP- 2000 with the pore size of 200 nm and particle diameter 7 µm; [d]on APS-Nucleosil, 7 µm particle and 500 Å pore sizes (20%, w/w)

Accuracy and Precision

The recoveries of the HPLC enantioresolution of (*R,S*)-OME and (*R,S*)-RAB within the range of 20-100 µg/mL were shown in Table 6.5 [A] while that of (*R,S*)-LAN and (*R,S*)- PAN within the same range were shown in Table 6.5 [B].

Detailed precision data for (*R,S*)-OME and (*R,S*)-RAB at three concentration levels 20-200% of a specific concentration 20 μgmL^{-1} were presented in Table 6.6 [A] while that of (*R,S*)-LAN and (*R,S*)-PAN within the same range were shown in Table 6.6 [B]. The results from the precision studies have revealed that there was a RSD below 1% ($\leq$ 0.89%) which comply within the proposed acceptance criteria (% RSD: not more than 2.0%) [237].

Robustness

A small change in retention times but no significant change in the resolution was observed.

These results also suggest that the method is quite fruitful to detect the enantiomeric impurity present in dexRAB.

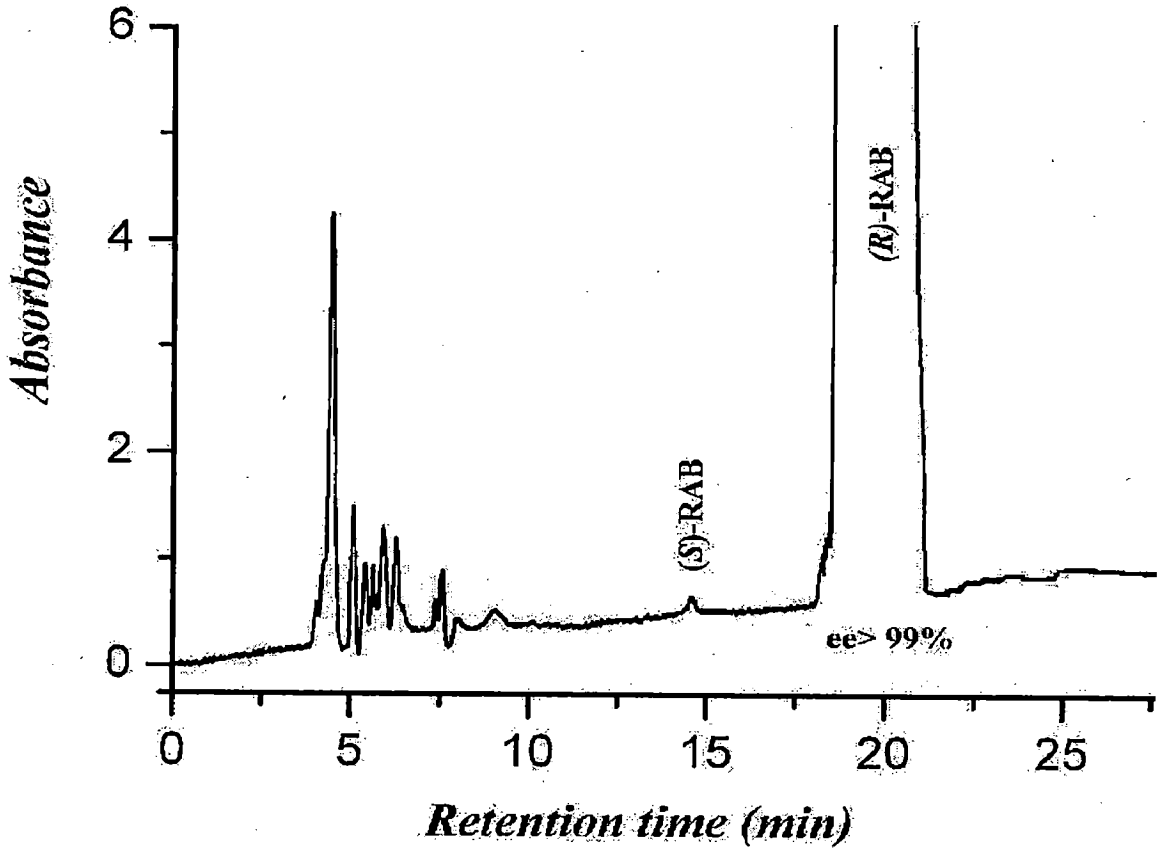


Fig. 6.5: Checking of the enantiomeric purity of dexRAB. Lux cellulose-2 column (250 × 4.6 mm I.D., 5 μm); mobile phase *n*-HEX–EtOH–DEA 90:10:0.4 (v/v/v); UV detection at 285 nm; flow rate 1.0 mL/min

Table-6.4: Linear regression equations of the HPLC enantioresolution of (*R,S*)-chiral sulfoxides within the range of 20-100 µg/mL

Chiral Sulfoxide	First and second eluting enantiomer	Calibration Graph*	Linearity (R^2)	LOD (ng/mL)	LOQ (ng/mL)	Mobile Phase
(*R,S*)-OME	(*S*)-OME	y=49612x+451	0.998	20	67	1
	(*R*)-OME	y=49589x+484	0.998	20	67	
(*R,S*)-RAB	(S)-RAB	y=49885x-904	0.997	18	61	2
	(*R*)-RAB	y=47981x+898	0.998	18	61	
(*R,S*)-LAN	(*S*)-LAN	y=49703x-633	0.999	22	74	3
	(*R*)-LAN	y=48881x+210	0.998	22	74	
(*R,S*)-PAN	(*S*)-PAN	y=48381x+970	0.999	23	77	4
	(*R*)-PAN	y=48549x+790	0.999	23	77	

1 *n*-HEX–IPA–DEA 65:35:0.2 (v/v/v/) at the flow rate of 1.0 mL/min;
2 *n*-HEX–EtOH–DEA 90:10:0.4 (v/v/v/) at the flow rate of 1.0 mL/min;
3 *n*-HEX–EtOH 70:30 (v/v/v/) at the flow rate of 1.0 mL/min;
4 *n*-HEX–IPA 80:20 (v/v/v/) at the flow rate of 1.0 mL/min;
x = sample concentration (µg/mL);
y = peak area;
R^2 = correlation coefficient

Table-6.5 [A]: Recovery of the HPLC enantioresolution of (*R*,*S*)-OME and (*R*,*S*)-RAB within the range of 20-100 µg/mL

Amt. $	Std. value #	(*R*,*S*)-OME[1]				(*R*,*S*)-RAB[2]			
		(*S*)-OME		(*R*)-OME		(*S*)-RAB		(*R*)-RAB	
		Found	Recovery ±RSD*	Found	Recovery ±RSD*	Found	Recovery ±RSD*	Found	Recovery ±RSD*
20	10	9.98	99.80±0.37	9.99	99.90±0.52	9.93	99.30±0.29	10.02	100.20±0.39
40	20	20.02	100.01±0.41	20.03	100.15±0.69	20.04	100.21±0.81	19.89	99.45±0.53
60	30	29.91	99.70±0.62	30.04	100.13±0.53	29.76	99.21±0.37	30.16	100.53±0.81
80	40	40.10	100.25±0.67	39.89	99.73±0.37	40.13	100.33±0.33	40.22	100.55±0.60
100	50	50.07	100.14±0.52	49.95	99.90±0.19	50.18	100.36±0.45	50.25	100.50±0.77

[1] **Mobile phase used: *n*-HEX–IPA–DEA 65:35:0.2 (v/v/v/) at the flow rate of 1.0 mL/min;**

[2] **Mobile phase used: *n*-HEX–EtOH–DEA 90:10:0.4 (v/v/v/) at the flow rate of 1.0 mL/min;**

$ Amount injected;

 # Standard value of amount per enantiomer injected

*** In percentage**

Table-6.5 [B]: Recovery of the HPLC enantioresolution of (*R*,*S*)-LAN and (*R*,*S*)-PAN within the range of 20-100 µg/mL

Amt. $	Std. value #	LAN[3]				PAN[4]			
		(*S*)- LAN		(*R*)- LAN		(*S*)- PAN		(*R*)- PAN	
		Found	Recovery ±RSD*	Found	Recovery ±RSD*	Found	Recovery ±RSD*	Found	Recovery ±RSD*
20	10	10.04	100.40±0.28	9.95	99.50±0.65	10.06	100.60±0.83	9.97	99.70±0.71
40	20	20.09	100.45±0.38	19.85	99.25±0.89	19.90	99.50±0.63	20.11	100.55±0.80
60	30	29.89	99.63±0.53	29.93	99.77±0.64	30.11	100.37±0.29	29.91	99.70±0.57
80	40	40.08	100.20±0.86	40.11	100.28±0.41	39.81	99.53±0.71	39.79	99.46±0.55
100	50	50.12	100.24±0.44	50.06	100.12±0.47	49.98	99.96±0.81	50.39	100.78±0.45

[3] **Mobile phase used: *n*-HEX–EtOH 70:30 (v/v/v/) at the flow rate of 1.0 mL/min;**

[4] **Mobile phase used: *n*-HEX–IPA 80:20 (v/v/v/) at the flow rate of 1.0 mL/min;**

$ Amount injected;

 # Standard value of amount per enantiomer injected

*** In percentage**

Table-6.6 [A]: Intra-day assay and inter-day assay precision of the HPLC enantioresolution of (*R*,*S*)-OME and (*R*,*S*)-RAB at three concentration levels 20-200% of a specific concentration 20 μgmL^{-1}

	Amt. $	Std. value #	OME[1]				RAB[2]			
			(*S*)-OME		(*R*)-OME		(*S*)-RAB		(*R*)-RAB	
			Found	Recovery ±RSD*	Found	Recovery ±RSD*	Found	Recovery ±RSD*	Found	Recovery ±RSD*
Intra-day	4	2	2.01	100.50±0.12	1.98	99.00±0.87	1.99	99.50±0.87	1.98	99.00±0.65
	20	10	9.98	99.80±0.37	9.99	99.90±0.52	9.93	99.30±0.29	10.02	100.20±0.39
	40	20	20.02	100.01±0.41	20.03	100.15±0.69	20.04	100.21±0.81	19.89	99.45±0.53
Inter-day	4	2	2.02	101.00±0.66	1.99	99.50±0.58	1.98	99.00±0.62	2.01	100.50±0.44
	20	10	9.99	99.90±0.65	9.97	99.70±0.88	9.94	99.40±0.55	10.06	100.60±0.61
	40	20	20.03	100.15±0.73	20.09	100.45±0.74	19.94	99.70±0.73	19.98	99.90±0.79

[1] **Mobile phase used: *n*-HEX–IPA–DEA 65:35:0.2 (v/v/v/) at the flow rate of 1.0 mL/min;**

[2] **Mobile phase used: *n*-HEX–EtOH–DEA 90:10:0.4 (v/v/v/) at the flow rate of 1.0 mL/min;**

$ Amount injected;

 # Standard value of amount per enantiomer injected

*** In percentage**

Table-6.6 [B]: Intra-day assay and inter-day assay precision of the HPLC enantioresolution of (*R*,*S*)-LAN and (*R*,*S*)-PAN at three concentration levels 20-200% of a specific concentration 20 μgmL^{-1}

	Amt. $	Std. value #	LAN[3]				PAN[4]			
			(*S*)- LAN		(*R*)- LAN		(*S*)- LAN		(*R*)- LAN	
			Found	Recovery ±RSD*	Found	Recovery ±RSD*	Found	Recovery ±RSD*	Found	Recovery ±RSD*
Intra-day	**4**	2	1.98	99.00±0.76	1.99	99.50±0.64	2.01	100.50±0.53	1.99	99.50±0.49
	20	10	10.04	100.40±0.28	9.95	99.50±0.65	10.06	100.60±0.83	9.97	99.70±0.71
	40	20	20.09	100.45±0.38	19.85	99.25±0.89	19.90	99.50±0.63	20.11	100.55±0.80
Inter-day	**4**	2	2.02	101.00±0.42	2.01	100.50±0.32	1.99	99.50±0.62	1.98	99.00±0.60
	20	10	10.07	100.70±0.31	9.99	99.90±0.22	10.03	100.30±0.11	9.92	99.20±0.26
	40	20	20.01	100.05±0.54	19.97	99.85±0.51	20.09	100.45±0.57	20.06	100.30±0.74

[3] **Mobile phase used: *n*-HEX–EtOH 70:30 (v/v/v/) at the flow rate of 1.0 mL/min;**

[4] **Mobile phase used: *n*-HEX–IPA 80:20 (v/v/v/) at the flow rate of 1.0 mL/min;**

$ Amount injected;

 # Standard value of amount per enantiomer injected

*** in percentage**

Chapter-7

Indirect Enantioseparation of Amino Alcohols

I. Introduction

The β-amino alcohols are structural analogs of α-amino acids (Fig. 7.1). β-Amino alcohols are present in a Papua-New Guinea sponge, Xestospongia sp [238]. 1-Amino-2-propanol forms an indispensable part of vitamin B12 [239]. (*S*)-(+)-2-Amino-1-butanol is used to derive a semi synthetic ergot alkaloid viz. methylergometrin [240]. Besides, β-amino alcohols *viz.* phenylalaninol and leucinol are important constituents of certain polypeptide antibiotics (mycotoxins) which are also called as peptaibols due to the presence of α–aminoisobutyric acid (Aib) [241]. In fact, Fungi of the genus Trichoderma with teleomorphs in Hypocrea are abundant producers of a group of amphiphilic and non-ribosomal peptide antibiotics, and are referred as peptaibols, only if a 1,2-amino alcohol is present at the C-terminus [242]. Since numerous drugs including β-blockers, sympathomimetic agents including ephedrine and pseudoephedrine and hormones including adrenaline and noradrenaline structurally belong to β-amino alcohols and thus its enantioresolution has a great biological and pharmaceutical importance.

Literature survey [50, 62] has revealed that the indirect method has been found advantageous than the direct method of enantioresolution due to availability of a number of CDRs, excellent detection senstivity of the CDRs and flexibility within the chromatographic conditions required for resolution of diastereomers on inexpensive reversed-phase achiral columns. The chosen β-amino alcohols in the present study (Fig. 7.1) do not have a strong ultraviolet/visible (UV/Vis) absorption property that is why their derivatization (either achiral or chiral) is required prior to HPLC separation.

II. Literature Review

✱ Enantioseparation of Amino Alcohols

Direct enantioresolution of amino alcohols has been achieved for their *N*-(3,5-dinitrobenzoyl) derivative on chiral stationary phases derived from α-amino acids and pyrrolidinyl-

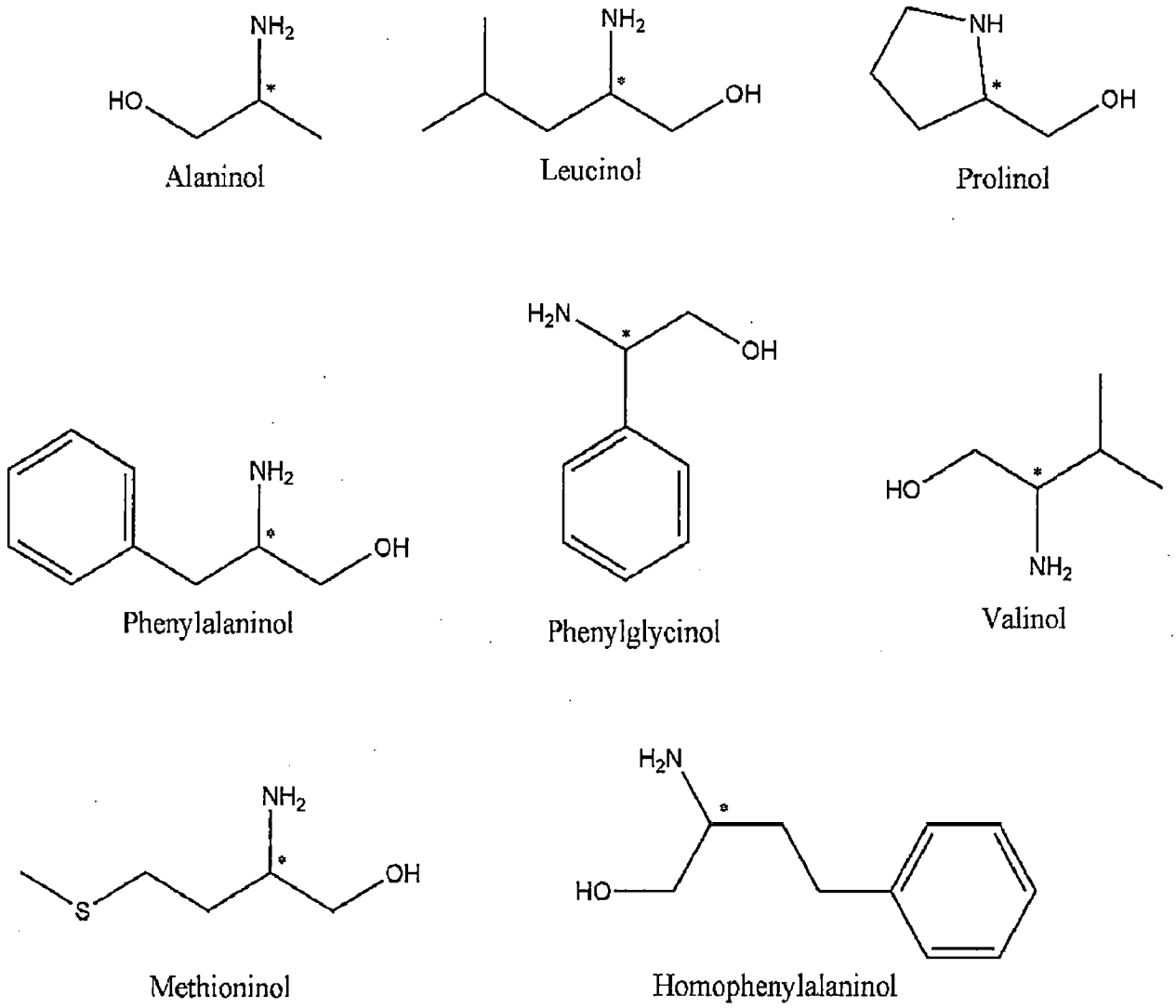


Fig. 7.1: Structures of eight *β*-amino alcohols enantioseparated in present study

disubstituted cyanuric chloride [243]. However, UV detectable secondary amino alcohols have been enantioresolved on (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid based chiral stationary phase [244] using direct approach. The mobile phase additive like helically distorted nickel (II) Schiff base chelate was used for RP-HPLC enantioresolution of some β-amino alcohols [245]. Indirect RP-HPLC enantioresolution of β-amino alcohols has been performed using different CDRs based on MR [107] and using (*S*)-*N*-(4-Nitrophenoxycarbonyl) phenylalanine methoxyethyl ester ((*S*)-NIFE) CDR [246], in this laboratory. Alaninol and valinol were enantioseparated by using MR and liquid chromatography-mass spectrometry [247]. The retention times and resolution power of the diastereomers of these β-amino alcohols derivatized with MR were almost the same as those of their structural analogues α-amino acids. These results indicated that the α-carboxyl group of an amino acid was not always essential for their resolution. Some *N*-(*R*)-mandelyl-(*S*)-cysteine derivatives of β-amino alcohols were also resolved using RP-HPLC [248].

MR and its chiral variants require special protection from light during all steps of synthesis of CDRs and diastereomers of β-amino alcohols. On the other hand, the use of (*S*)-NIFE as CDR is limited due to formation of several by products after derivatization of amino compounds.

✸ **(*S*)-Naproxen Derivatives**

Nap is a UV absorbing chromophoric chiral molecule. It has carboxyl functional group, which is less reactive in comparison to its acid chloride or an ester analogue. Literature survey reveals that its reactivity has been enhanced by synthesizing its ester derivatives; *N*-succinimidyl-2-(*S*)-methoxy-2-phenylacetic acid ester [249], *N*-succinimidyl-(*S*)-2-(6-methoxynaphth-2-yl)propionate [154] which have been further used as CDRs for enantioresolution of different amino group containing compounds.

III. Present Work

On account of above cited literature, and to search a new UV absorbing chromophoric

chiral platform for enantioresolution of pharmaceutically and biologically important amino alcohols, benzoimidazole-(*S*)-naproxen amide, (*S*)-1-(1*H*-benzo[d]imidazol-1-yl)-2-(6-methoxynaphthalen-2-yl)propan-1-one ((*S*)-Nap-BImz) has been synthesized and utilized as a CDR for HPLC enantioresolution of eight *β*-amino alcohols *viz.* DL-alaninol (Alaol), DL-leucinol (Leuol), DL-prolinol (Prool), DL-phenylalaninol (Pheol), DL-phenylglycinol (Phgol), DL-valinol (Valol), DL-homophenylalaninol (Hphol) and DL-methioninol (Metol).

The scientific novelty of the work is the synthesis of a new CDR ((*S*)-Nap-BImz) which provides a UV detectable chiral platform, applied for microwave-irradiated synthesis, and reversed phase HPLC resolution of the diastereomers of eight *β*-amino alcohols.

1. Synthesis of (*S*)-Naproxen-benzimidazole

A benzimidazole (BImz) derivative of Nap, (*S*)-Nap-BImz, was synthesized. It was characterized with the help of UV, IR, CHN, ^{1}H NMR and ^{13}C NMR spectra. The synthesis and characterization data of the CDR has been described in Chapter-2.

2. Microwave Irradiated Synthesis of Diastereomers of Amino Alcohols

To the solutions of DL-Leuol (100 µL, 40 nmol, in H_2O) and (*S*)-Nap-BImz (150 µL, 65 nmol, in acetonitrile; molar ratio of DL-Leuol to (*S*)-Nap-BImz 1:1.6) in a Teflon tube of 1.5 mL, triethylamine (60 µL, 6%) was added and reaction mixture was then irradiated under microwave for 35 s at 75% (of 800 W) and cooled to room temperature. The reaction was quenched by addition of HOAc (1 M, 60 µL). A 10 µL volume of resulting solution, containing the diastereomers, was diluted 10 fold with MeCN, and 20 µL of it was injected onto the column.

The aforementioned conditions for synthesis of diastereomers were optimized by performing various experiments with different mole ratio of DL-Leuol *vs* (*S*)-Nap-BImz (in the range of 1-4 fold molar excess of (*S*)-Nap-BImz), and microwave irradiation (MWI) conditions (for 15-65 s; at 75% power). The optimized method was applied to the synthesis of the

diastereomers of rest of the amino alcohols with Nap-BImz.

3. HPLC Operating Conditions for Separation of Diastereomers

Reversed-phase HPLC was performed on a Waters Spherisorb ODS2 (250 × 4.6 mm I.D., 5 μm) column with the mobile phase consisting of triethylammonium phosphate (10 mM, pH 4.0)-acetonitrile (MeCN) in a linear gradient of MeCN from 35 to 65% within 45 min at a flow rate of 1.0 mL/min and UV detection at 231 nm. TEAP buffer solution was prepared by diluting TEA to 10 mM with ultra-purified water and adjusting to pH 4.0 by addition of phosphoric acid.

4. Method Validation

The experimental method was validated with respect to linearity, accuracy and precision for the diastereomers of of DL-Leuol prepared with (*S*)-Nap-BImz according to ICH guidelines [92] and summarized here:

Linearity

The linear regression was computed by the least square method using Microsoft Excel program to determine the slopes and correlation coefficients for the calibration graphs between the peak area (in AU; absorbance unit) responses of ((*S*), D)- diastereomer and ((*S*), L)- diastereomer and the corresponding concentration (50-150 pmol).

Accuracy and Precision

The intra-day assay and inter-day assay studies for accuracy and precision were carried out by replicate HPLC analysis (n=6) of diastereomers of DL-Leuol at three concentrations (50, 100, 150 pmol).

To determine LOD, corresponding to the signal-to-noise ratio of 3, the recoveries of the

recoveries of L-Leuol from the solution containing excess of D-Leuol was investigated. Solution of D-Leuol was spiked with fixed amount of L-Leuol within the range of 0.01 % to 0.20 %.

IV. Results and Discussion

1. Synthesis and Characterization of (*S*)-Naproxen-benzimidazole

The chiral derivatizing reagent; (*S*)-Nap-BImz was synthesized by nucleophilic attack of BImz on the carbonyl carbon of the carboxylic acid of (*S*)-Nap followed by the removal of dicyclohexylurea. This reaction of (*S*)-Nap with BImz in the presence of DMAP and DCC under mild conditions yielded (*S*)-Nap-BImz in an excellent amount (>92%). The CDR; (*S*)-Nap-BImz was characterized by IR, UV, CHN, ^{1}H NMR and ^{13}C NMR. The characterization data related to (*S*)-Nap-BImz is given in Chapter-2. The chiral purity of (*S*)-Nap-BImz was established according to the earlier reports on determination of chiral purity of different CDRs [50].

The reaction for the synthesis of the diastereomers by reaction of (*S*)-Nap-BImz and with amino alcohols (*R*–$CH(NH_2)CH_2OH$) is shown in Fig. 7.2.

2. Optimization of Derivatization Conditions

The experimental conditions for microwave-assisted synthesis of diastereomeric pairs of all the eight amino alcohols with (*S*)-Nap-BImz were optimized with respect to the effect of the reagent excess, effect of base, and microwave irradiation conditions; to ascertain the best and successful conditions for the abovementioned synthesis of diastereomers. The mixture of the two diastereomers so formed under each change of experimental conditions was subjected to HPLC resolution and the peak areas corresponding to the two resolved diastereomers, calculated by system software, were taken as a measure of complete derivatization.

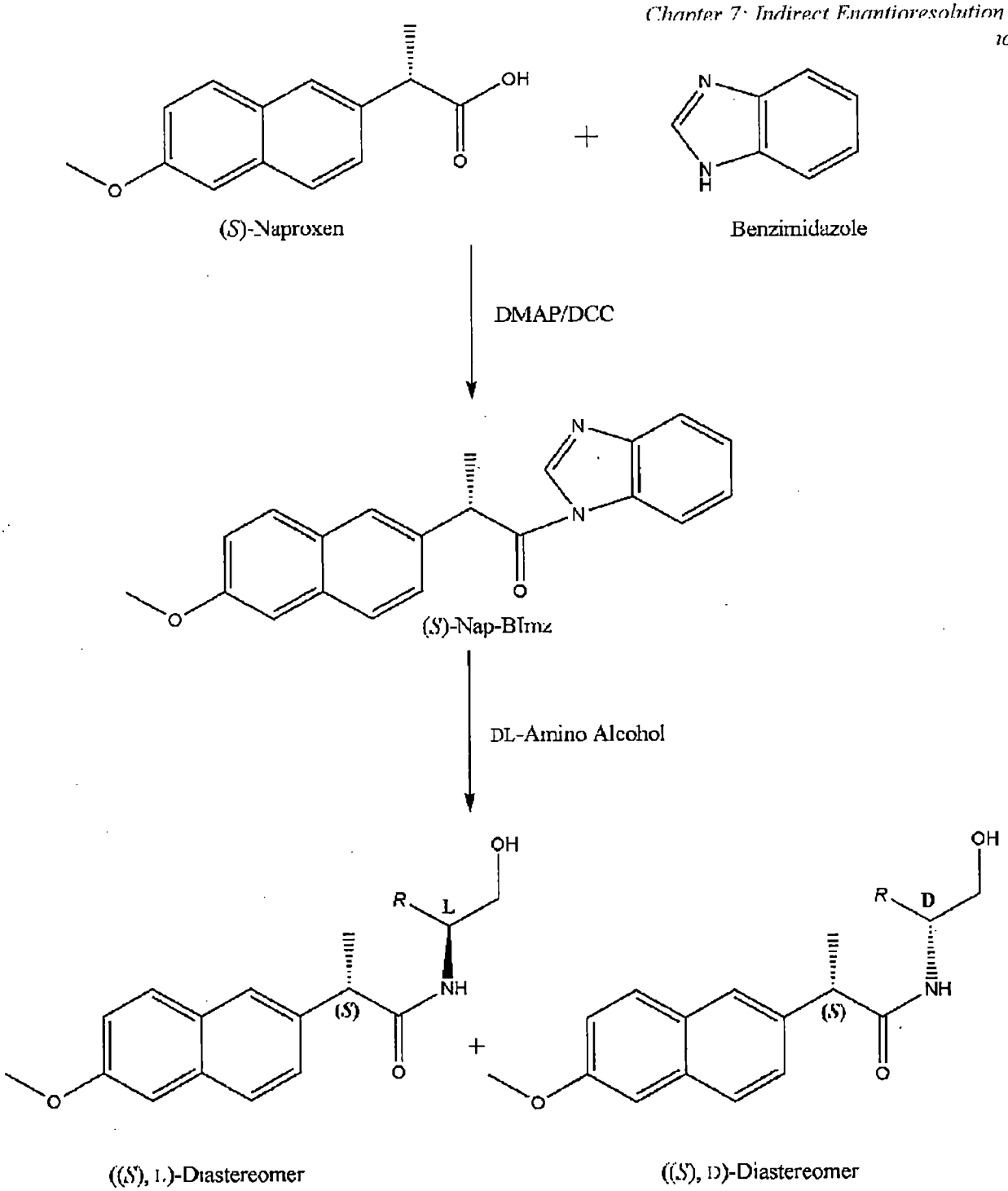


Fig. 7.2: Synthesis of chiral derivatizing reagent ((*S*)-Nap-BImz) and diastereomers of *β*-amino alcohols

R represents hydrophobic chain attached to amino alcohols

✷ **Effect of Reagent Excess**

(*S*)-Nap-BImz was used in 1-4 fold molar excess to ascertain complete derivatization without kinetic resolution. Derivatization of Leuol was found optimum at a molar ratio of 1:1.6 (DL-Leuol : (*S*)-Nap-BImz) using MWI (35 s at 75% of 800 W). Slight kinetic resolution was observed at lower ratios of Leuol : (*S*)-Nap-BImz. Increase in reagent concentration up to 4 fold had no significant effect on yield of derivatization and reaction time. Therefore, (*S*)-Nap-BImz was used in the same ratio for all the eight amino alcohols.

✷ **Effect of Base**

Since reaction of Leuol (and other analytes) with (*S*)-Nap-BImz follows a nucleophilic substitution, the reaction requires basic medium. Triethyl amine (60 µL, 6%) at a pH around 10.0 was used to facilitate the derivatization. No derivatization was observed in the absence of triethyl amine. These conditions were, therefore, used for all the derivatization reactions for quantitative yields.

✷ **Microwave Irradiation Conditions: Reaction Time and Power**

The effect of the parameters viz. microwave power and time of microwave irradiation on derivatization yields (in terms of peak area) were investigated. A number of reaction mixture sets were irradiated separately in the microwave oven for 15, 25, 35, 45, and 55 s (at 70-90% power of 800 W). A derivatization time of 35 s (at 75% power of 800 W) was found successful for complete derivatization of Leuol. The MWI reaction time and power corresponding to maximum diastereomeric peak areas (representing the completion of reaction) were taken as optimized derivatization conditions and were used for the eight amino alcohols.

3. HPLC

The chromatographic parameters; retention factor (k), resolution factor (α) and resolution (R_S) for the resolved diastereomers of β-amino alcohols prepared with (*S*)-Nap-BImz are given in Table-7.1. Total eight pairs of diastereomers were well separated under the reversed-phase HPLC

conditions. The synthesized diastereomers of DL-Leuol were best resolved (R_S, 23.16) with highest selectivity (α, 1.22) among the chosen eight amino alcohols. L-Isomer was eluted prior to D-isomer in each case. Sections of chromatograms showing resolution of diastereomers of β-amino alcohols prepared with (*S*)-Nap-BImz are shown in Fig. 7.3.

Table 7.1: Chromatographic data for RP-HPLC resolution of diastereomers of DL-amino alcohols prepared with (*S*)-Nap-BImz

Entries	DL-Amino alcohol	Chromatographic parameters			
		Δt	k_L	α	R_S
1	Alaol	3.20	8.67	1.15	15.11
2	Leuol	4.93	9.31	1.22	23.16
3	Prool	3.42	8.80	1.16	17.74
4	Pheol	3.64	9.10	1.16	19.79
5	Phgol	3.60	8.99	1.16	18.19
6	Valol	4.47	9.20	1.20	21.83
7	Hphol	3.98	9.14	1.18	21.08
8	Metol	3.38	8.70	1.16	16.12

Δt: difference between the retention times of the separated diastereomers;
k_L: retention fators of L-enantiomer;
α: stereoselective factor;
R_S: resolution of the diastereomers of corresponding amino alcohols;
Mobile phase: *aq* TEAP (10 mM, pH 4.0)- MeCN in a linear gradient of MeCN from 35 to 65% within 45 min at a flow rate of 1.0 mL/min;
UV detection: 231 nm

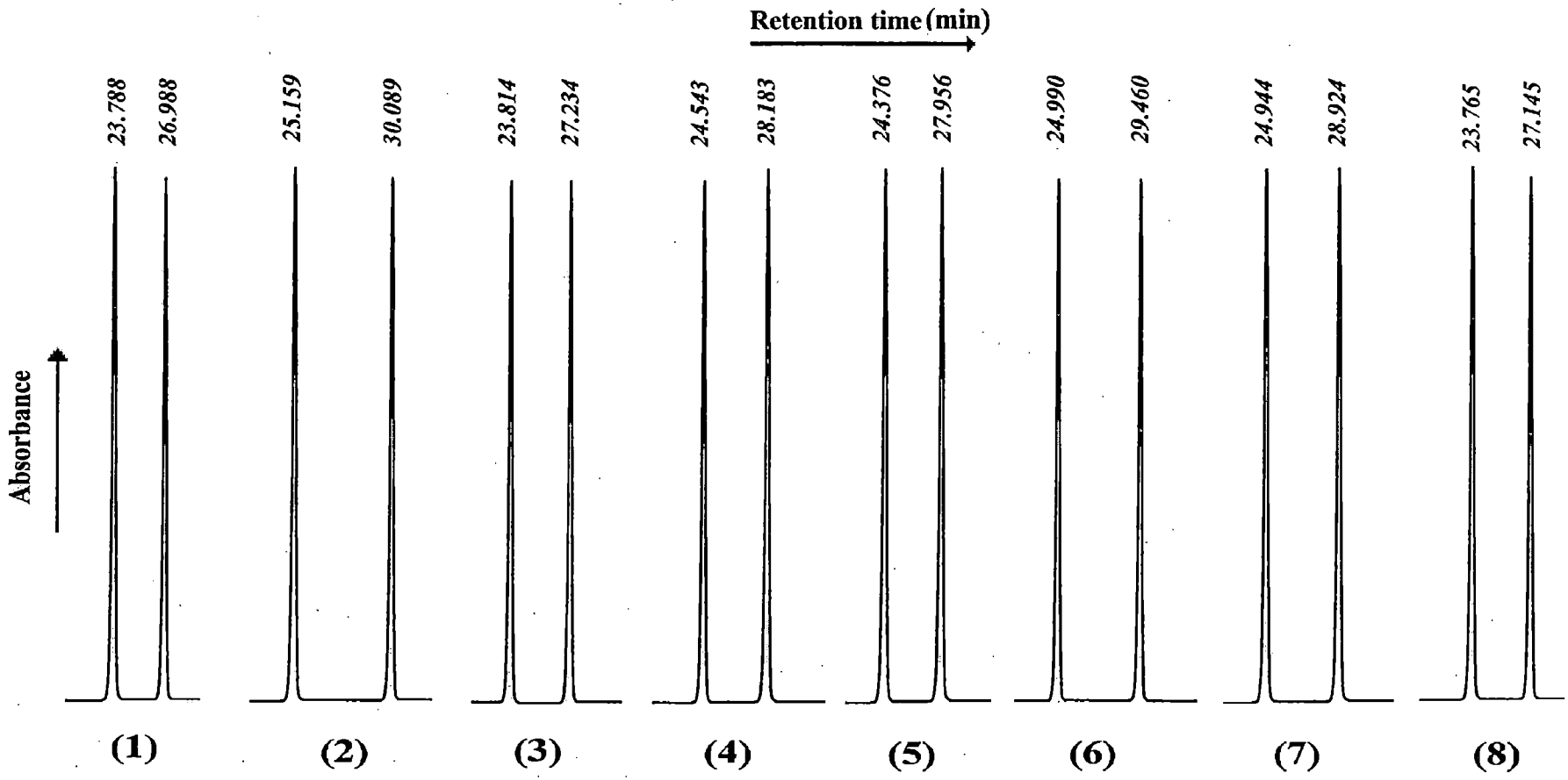


Fig. 7.3: Sections of chromatograms showing resolution of DL-Alaol (1), DL-Leuol (2), DL-Prool (3), DL-Pheol (4), DL-Phgol (5), DL-Valol (6) DL-Hphol (7) and DL-Metol (8) as their diastereomers prepared with (*S*)-Nap-BImz; Waters Spherisorb ODS2 (250 × 4.6 mm I.D., 5μm); diastereomeric peak corresponding to L-enantiomer was eluted prior to that of D-enantiomer; mobile phase consisting of *aq.* TEAP (10 mM, pH 4.0)-MeCN in a linear gradient of MeCN from 35 to 65% within 45 min at a flow rate of 1.0 mL/min and UV detection at 231 nm

Optimization of HPLC Conditions

✸ **Effect of pH and Concentration of Buffer Used in Mobile Phase**

Keeping the concentration of buffer used in the mobile phase at 10 mM, the effect of pH of the mobile phase on the resolution of diastereomers of Leuol prepared with (*S*)-Nap-BImz was studied within the pH range of 2.5-6.0. TEAP buffer was used to investigate within the range of pH 2.5-4.5 and the best resolution was observed at pH 4.0. To study the effect of pH within the range 4.5-6.0, triethylammonium acetete (TEAA) buffer was used and it was found that the diastereomers were not resolved in this pH range. Thus keeping the pH of TEAP buffer constant at 4.0, effect of buffer concentration was studied by varying it within the range of 05-40 mM. Peak broadening was found with decrease in concentration of TEAP buffer below 10 mM and little difference in resolution was found when buffer concentration was above 10 mM (upto 40 mM; with a change of 5 mM at a time). Since high buffer concentration may harm the column, hence 10 mM buffer concentration was taken as optimized concentration.

✸ **Effect of Organic Solvent**

Both the gradient and isocratic elution modes were investigated using binary mobile phases consisting of an organic modifier (MeCN or methanol) and a buffer (TEAP or TEAA). Sharp peaks were obtained under gradient elution. MeCN was found to be a better organic modifier in comparison to methanol as broader peaks (and even no resolution in a few cases) and larger retention times were observed with methanol. Thus mobile phase composed of MeCN and TEAP buffer was found to be the best.

✸ **Effect of Flow Rate**

The effect of change of flow rate on the resolution of diastereomers was examined by varying the flow rate in portions of 0.5 mL/min within the range of 0.5-1.5 mL/min. With the decrease in the flow rate from 1.0 to 0.5 mL/min, increase in retention time along with broadening

of the diastereomeric peaks was observed. However, retention time and resolution value decreased when flow rate was increased from 1.0 to 1.5 mL/min. Hence, flow rate of 1.0 mL/min was used throughout the experiment.

4. Resolution Mechanism

Since the pH of the mobile phase is around 4.0 (pH of TEAP buffer, used in the mobile phase) and is considerably acidic the hydroxy group of the analyte will be ionized; therefore, only this ionic interaction play its role in resolution of the diastereomers. Then the factors contributing to the hydrophobicity of diastereomers are taken into consideration.

The following factors may contribute to the hydrophobicity of diastereomers, (i) presence of naphthyl group in the reagent platform of the diastereomers, (ii) partial double bond character of the amide bond formed due to feasible delocalization of lone pair of electrons between the nitrogen and the carbonyl group of the amide group, and (iii) the side chain present in the amino alcohols (-R; Fig. 7.2).

It can be interpreted that the hydrophobic interactions of the two diastereomers with the reversed phase material of the column are responsible for their different partition coefficients resulting into different retention times and thus resolution. The characteristic feature of diastereomers is that they have different physical properties.

The amino alcohols (Alaol, Leuol, Pheol, Valol and Metol) can be arranged in their decreasing hydrophobicity order as Leuol > Valol > Pheol > Metol > Alaol; based on partial specific volumes (values given in parenthesis) of corresponding amino acids, Leu (0.842) > Val (0.777) > Phe (0.756) > Met (0.709) > Ala (0.691), calculated by Bull and Breese [250]. This is reflected in the observed increase in the retention times and resolution values of the synthesized diastereomers of amino alcohols (Table 7.1; entries 1, 2, 4, 6 and 8) with increase in the hydrophobicity of corresponding amino alcohols.

5. Method Validation

Linearity

A good linear relationship was obtained over the range mentioned in "Present Work" section. The regression equations were y=1.173x+1.056 (R^2=0.998) and y=1.245x-0.991 (R^2 = 0.999) for the ((*S*), L) - diastereomer and ((*S*), D) - diastereomer, respectively.

Accuracy and Precision

The relative standard deviation (%) for L- and D-Leuol varied from 0.48 to 1.04 and 0.92 to 0.97 for intra-day assay precision and 0.83 to 1.26 and 0.96 to 1.24 for inter-day assay precision. The percentage recovery for L- and D-Leuol varied from 98.2 to 100.2 and 99.0 to 100.1 for intra-day assay and 96.4 to 100.1 and 96.7 to 100.3 for inter-day assay (Table-7.2).

Validation studies concludes that the chiral derivatizing reagent, (*S*)-Nap-BImz was quite stable for one month while the solution of diastereomers of Leuol was quite stable up to one week under refrigerated condition (4 °C). The results indicate the ability of this method for detection up to 0.08% of L-Leuol in D-Leuol by HPLC.

Table 7.2: Intra-day assay and inter-day assay precision of diastereomers of DL-Leuol prepared with (*S*)-Nap-BImz

Concentration (pmol)	First eluting diastereomer			Second eluting diastereomer		
	Mean±SD	Recovery*	RSD*	Mean±SD	Recovery*	RSD*
Intra-day						
50	24.80±0.12	99.2	0.48	24.88±0.23	99.5	0.92
100	49.12±0.52	98.2	1.04	49.51±0.48	99.0	0.96
150	75.16±0.61	100.2	0.81	75.11±0.73	100.1	0.97
Inter-day						
50	24.11±0.23	96.4	0.92	24.18±0.31	96.7	1.24
100	48.98±0.63	97.8	1.26	48.99±0.47	97.9	0.96
150	75.10±0.62	100.1	0.83	75.20±0.82	100.3	1.09

*** represents percentage values;**
CV coefficient of variation;
SD standard deviation

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Conclusion

CONCLUSION

In spite of different pharmacological and toxicological behavior of the enantiomers of chiral drugs, many racemic drugs continue to be marketed, and clinically administered as such. Thus, enantioseparation remains essential in studies on racemization of drugs in living organisms, in the analysis of enantiomeric purity of drugs during their different steps of synthesis, in the isolation of drugs from natural sources, in the establishment of specifications for drug marketing and in monitoring stereospecific fate of drugs. Hence, it has become indispensable from industry and academic points of view.

Enantioseparation can be achieved by either converting them into diastereomeric derivatives prior to chromatography (indirect method) or by using a chiral environment during the chromatographic process (direct method). Sensitive, flexible, versatile, less expensive and reproducible high-performance liquid chromatographic enantioseparation methods have been developed for several compounds, including (*R*, *S*)-cinacalcet, DL-penicillamine, DL-cysteine, DL-homocysteine, (*R*, *S*)-mexiletine, (*R*, *S*)-omeprazole, (*R*, *S*)-rabeprazole, (*R*, *S*)-lansoprazole, (*R*, *S*)-pantoprazole, DL-alaninol, DL-leucinol, DL-prolinol, DL-phenylalaninol, DL-phenylglycinol, DL-valinol, DL-homophenylalaninol and DL-methioninol. A successful impregnated thin layer chromatographic enantioseparation of (*R*, *S*)-cinacalcet has also been achieved.

DFDNB is a strong UV absorbing moiety due to presence of highly reactive fluorine atoms and due to presence of chromophoric dinitrobenzene group. On account of this fact six CDRs based on DFDNB, having L-Ala-NH_2, L-Phe-NH_2, L-Val-NH_2, L-Leu-NH_2, L-Met-NH_2, and D-Phg-NH_2 as chiral auxiliaries were synthesized and further used to synthesize the diastereomers of (*R*, *S*)-Cin easily under microwave irradiation (for 3 min at 75% power) without racemization. The synthesized diastereomers were well separated ($\alpha > 1.0$ and $R_S > 1.5$) along with high sensitivity (LOD; 60 pmol) by RP-HPLC. The only drawback of these CDRs is their light sensitivity, so that the whole synthesis procedure (synthesis of CDR and synthesis of diastereomers of (*R*, *S*)-Cin using these CDRs) is protected from light. This method can be utilised not only for

enantioseparation but also to check the impurity of (*S*)-Cin in the pharmacologically active (*R*)-Cin.

L-His and L-Arg have been used as chiral selectors for impregnated thin layer chromatographic enantioresolution of (*R*, *S*)-Cin. The possibility to obtain (*R*)-Cin in the native form within 10 min using L-His and L-Arg as readily available and cost effective chiral selectors. In addition to this, good resolution values ($R_S > 1.5$) and good detection limits (> 0.26 μg) prove that the direct approach is an excellent choice for enantioresolution.

An azole derivative of (*S*)-naproxen, (*S*)-naproxen-benzotriazole has been synthesized under mild conditions within 2.5 h and was found very well with respect to its stability (for one month) and separation characteristics. It has been used as CDR for microwave assisted synthesis of diastereomers of PenA, Cys and Homocys in 30 s (at 80% power). The HPLC separation of diastereomers showed an LOD of 0.0001- 0.0015 nmol. Thus, this CDR can successfully be applied to check the enantiomeric purity of commercial D-penicillamine and other amino group containing pharmaceuticals under less expensive and simple RP-HPLC conditions with a very good LOD.

(*S*)-(-)-(*N*)-trifluoroacetyl-prolyl chloride and camphanic chloride have been used as CDRs for simple, rapid and successful analytical enantioresolution of (*R*, *S*)-MEX. The analytical resolution has been scaled upto small-scale preparative resolution of diastereoselective-enriched diastereomers. Prior elution of (*R*)- to (*S*)-MEX enhances the success of the method as (*R*)-MEX has the desired pharmacological activity. Thus, the method can be used for isolation of more potent enantiomer from a racemic mixture of pharmaceutically important compounds having primary amino group. Use of (*R*)-MEX and the CDR in the ratio of 2:1 or performing the reaction in the presence of pyridine has provided a method to neutralizing the one mol of HCl formed during the reaction of the CDR (the acyl chloride) and with (*R*)-MEX (the amine).

(*R*, *S*)-omeprazole, (*R*, *S*)-rabeprazole, (*R*, *S*)-lansoprazole, and (*R*, *S*)-pantoprazole have been enantioseparated by high-performance liquid chromatography using monochlorinated polysaccharide based Lux cellulose-2 CSP under normal and polar-organic phase conditions and ultraviolet detection at 285 nm. The switching between normal to polar organic phases and vice

versa is feasible to the enantioseparation of chiral sulfoxides on Lux cellulose-2 column and could be further applied to a wide range of compounds. The optimized method could be successfully applied to check the enantiomeric purity of dexRAB to the extent of 18 $ngml^{-1}$. The polysaccharide based CSP containing both the -Cl and $-CH_3$ substituents on the phenyl group of phenyl carbamate moiety of the CSP provided better resolution than several other CSPs containing dimethyl- or dichloro- phenyl carbamate cellulose and amylose.

(*S*)-Naproxen-benzimidazole, an amide derivative of (*S*)-naproxen, has been synthesized under mild conditions within 4 h and used as CDR for MWI synthesis of diastereomers of β-amino alcohols in just 35 s (at 75 % of 800 W power). The HPLC resolution of diastereomers showed an LOD of 0.5- 2.5 pmol. Thus, this CDR can successfully be applied to check the trace amount of an enantiomer of any of aforementioned amino alcohols available in its antipode. Successful enantioseparation of these amino acid analogues β-amino alcohols contradicted the fact that presence of α-carboxyl group is an obligation for the enantioseparation of amino acids.

Application of (*S*)-Nap-Btz and (*S*)-Nap-BImz do not require special protection from light at any stage of experiment i.e. neither during their synthesis nor during synthesis of diastereomers of thiol-group containing amino acids and β-amino alcohols, respectively though this type of light protection was essential with MR based CDRs, which were used previously for the enantioseparation of these compounds.

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