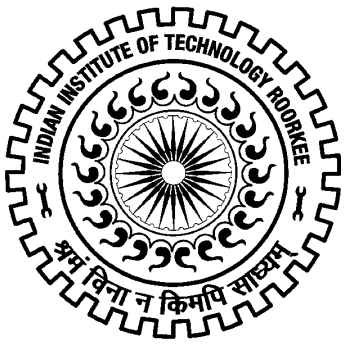
LIQUID CHROMATOGRAPHIC CHIRAL SEPARATION OF CERTAIN IMPORTANT PHARMACEUTICALS

Ph.D. THESIS

by

HARIOM NAGAR



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

APRIL, 2014

LIQUID CHROMATOGRAPHIC CHIRAL SEPARATION OF CERTAIN IMPORTANT PHARMACEUTICALS

A THESIS

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

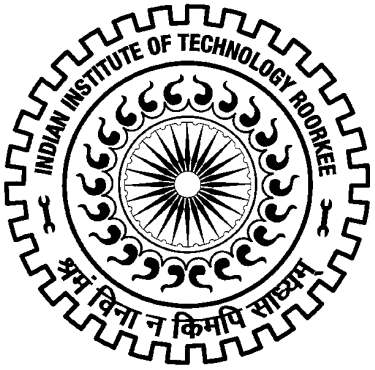
DOCTOR OF PHILOSOPHY

in

CHEMISTRY

by

HARIOM NAGAR



DEPARTMENT OF CHEMISTRY
INDIAN INSTITUTE OF TECHNOLOGY ROORKEE
ROORKEE-247667, UTTARAKHAND (INDIA)
APRIL, 2014

INDIAN INSTITUTE OF TECHNOLOGY ROORKEE
ROORKEE

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled "**LIQUID CHROMATOGRAPHIC CHIRAL SEPARATION OF CERTAIN IMPORTANT PHARMACEUTICALS"** in partial fulfilment of the requirements for the award of the Degree of **Doctor of Philosophy** and submitted in the **Department of Chemistry** of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period from July, 2010 to April, 2014 under the supervision of **Dr. Ravi Bhushan**, Professor, Department of Chemistry, Indian Institute of Technology Roorkee, Roorkee.

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

(HARIOM NAGAR)

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

Date: _____

(Ravi Bhushan)
Supervisor

The Ph. D. Viva-Voce Examination of **Mr. Hariom Nagar**, Research Scholar, has been held on . . . . . .

Signature of Supervisor **Chairman, SRC** **Signature of External Examiner**

Head of the Deptt./Chairman, ODC

CONTENTS

ABSTRACT

Enantiomers are known to show different pharmacodynamic and pharmacokinetic properties after administration. Systematic investigations of biological activities of both enantiomers have become indispensable in areas, such as pharmacology, medicine, life sciences, asymmetric synthesis and studies of structure-function relationship. Various analytical methods have been used for separation of racemic mixtures into enantiomers; TLC and HPLC are widely used for resolution of a variety of chiral pharmaceuticals.

Certain drugs/chiral compounds having wide applications in chemistry, biochemistry, medicine, *etc* have been chosen for present studies for separation of their enantiomers; these include, proteinogenic-α-amino acids, DL-selenomethionine (SeMet), *β*-blockers [(*RS*)-orciprenaline (Orc), (*RS*)-atenolol (Atl), (*RS*)-propranolol (Prl), (*RS*)-betaxolol (Bel) and (±)-isoxsuprine (ISP)] and (*RS*)-fluoxetine (Flx). Both direct and indirect approaches have been applied using different chiral derivatizing reagents (CDRs), chiral stationary phases (CSPs) and chiral selectors.

The **first chapter** covers preamble to present studies and an introduction to chirality and various other related aspects such as enantiomers and their biological significance, chromatographic enantioseparation approaches, chiral selector (as chiral impregnating reagent, chiral mobile phase additive and chiral inducing reagent for TLC), CSPs, CDRs and their applications (for HPLC).

The **Second chapter** presents a detailed description of common experimental procedures used for present studies; it includes instrumentation, materials, preparation of stock solutions, and extraction of active pharmaceutical ingredient from their drug formulations. The methods for synthesis of CDRs along with their characterization data, synthesis of diastereomers have been explained in detail. Separation of diastereomers using reversed phase-HPLC and TLC are described in subsequent chapters.

A total of 17 CDRs were synthesized using DFDNB (1,5-Difluoro-2,4-dinitrobenzene), CC (cyanuric chloride; trichloro-*s*-triazine) and (*S*)-Naproxen ((*S*)-Nap), as starting materials.

Characterization and determination of chiral purity of all the CDRs is also described in this chapter. Theoretical calculation using Gaussian 09 Rev A. 02 program and hybrid

density functional B3LYP with 6-31G basis set was used for developing optimized structures of diastereomers to support the experimental results of elution sequence.

Direct TLC approaches are described for separation of racemic compounds and isolation of their native enantiomers.

The **Third chapter** is devoted to general introduction of *β*-blockers, literature survey on their enantioseparation by liquid chromatographic and to the present study on direct and indirect enantioseparation of *β*-blockers. It has been divided into three sections.

Section A: This section describes use of DFDNB based CDRs (CDR 1-5 and 7-9) for synthesis of diastereomers of *β*-blockers, namely, Orc, Atl, Prl, Bel and ISP, along with their separation by RP-HPLC and RP-TLC.

Following CDRs were used for synthesis of diastereomers of *β*-blockers:

DFDNB as the Structural moiety		
CDR number	Chiral auxiliary	β-blockers
1.	SMLC	Orc, Atl, Prl, Bel
2.	SBLC	Orc, Atl, Prl, Bel, ISP
3.	L-Met	Orc, Atl, Bel
4.	L-Leu	
5.	L-Val	Orc, Atl, Bel, ISP
7.	L-Ala	
8.	(*S*)-(+)-1-cyclohexylethylamine	Orc, Atl, Prl, Bel
9.	(*R*)-(+)-*α*-methyl benzylamine)	

These diastereomers were separated by RP-HPLC on a C_{18} column with detection at 340 nm using a linear gradient of acetonitrile and *aq* trifluoroacetic acid as the mobile phase components. The conditions of derivatization and chromatographic separation were optimized. The method was validated for accuracy, precision, limit of detection, and limit of quantification. The separation behavior (in terms of, retention time and resolution) of the diastereomers was compared.

All the three pairs of diastereomers of ISP were also separated by RP-TLC on DC Kieselgel 60 RP*18 $F_{254}S$ stationary phase using MeCN and TEAP buffer as mobile phase. The influence of pH and temperature was examined on separation. The separated diastereomers were visible as bright yellow spots in ordinary light. LOD was estimated.

Section B: This section deals with the experiment and results for development of RP-HPLC method for enantioseparation of *β*-blockers.

Separation was carried out on certain CC based DCT reagents (CDR 10-15). These reagents were used for synthesis of eighteen pairs of diastereomers of *β*-blockers (Atl, Orc and Bel) under microwave irradiation and stirring method.

Following CC based CDRs were used for synthesis of diastereomers of Atl, Orc, and Bel (the chosen *β*-blockers):

CDR number	Chiral auxiliary in CC moiety
10.	SBLC
11.	SMLC
12.	L-met
13.	L-Leu
14.	L-Val
15.	L-Ala

These diastereomers were separated by RP-HPLC on a C_{18} column with detection at 230 nm using a linear gradient of acetonitrile and *aq* trifluoroacetic acid. Derivatization and chromatographic separation conditions were optimized. The method was validated in terms of accuracy, precision, limit of detection, and limit of quantification.

Section C: This section presents direct TLC separation of enantiomers of *β*-blockers (Atl, Orc and Bel) using L-Glu as chiral selector. Direct TLC separation was

performed using L-Glu as (i) chiral impregnating reagent, (ii) as chiral mobile phase additive (CMPA), and (iii) as chiral inducing reagent (CIR).

Different binary, ternary and quaternary mixtures of a variety of solvents (such as CH_3CN, CH_3OH, H_2O and CH_2Cl_2) were tried systematically to achieve enantiomeric resolution.

Spots were located in iodine chamber. The developed TLC method was validated for precision (RSD) and LOD. The influence of pH, temperature and amount of chiral selector was examined on enantioseparation. Separated enantiomers were isolated and optical purity was determined. Optimized structures of the transient diastereomers of the three β-blockers were drawn by Gaussian 09 Rev A. 02 program, to confirm their migration order.

The **Fourth chapter** deals with literature survey on liquid chromatographic enantioseparation of fluoxetine and its indirect enantioseparation by RP-HPLC and RP-TLC was carried out herein. It comprises of two sections.

Section A: This section describes the synthesis of four DFDNB based CDRs (CDR 1, 2, 4 and 5) and their use for synthesis of diastereomers of Flx. All the four pairs of diastereomers were separated by RP-HPLC on a C_{18} column with detection at 340 nm using a linear gradient of mobile phase containing acetonitrile and *aq* trifluoroacetic acid.

Method validation was carried out for accuracy, precision, limit of detection, and limit of quantification. Simultaneously, RP-TLC of diastereomers of Flx prepared with DFDNB based CDRs (CDR 4 and 5), has been carried out on DC Kieselgel 60 RP*18 $F_{254}S$ stationary phase using MeCN and TEAP buffer as mobile phase. The effect of pH and temperature was examined on separation. The separated diastereomers were detected in ordinary light.

Section B: It deals with the application of four CC based CDRs (CDR 10, 11, 13 and 14), for synthesis of diastereomers of Flx. The four pairs of diastereomers, so synthesized, were separated by RP-HPLC on a C_{18} column with detection at 230 nm using a linear gradient of mobile phase containing acetonitrile and *aq* trifluoroacetic acid.

The **fifth chapter** presents background and literature survey on liquid chromatographic enantioseparation of SeMet. In view of the literature survey direct and indirect enantioseparation of selenomethionine was carried out. It has been divided into three sections.

Section A: It describes the use of CDR 16 (Nap-Phth) for synthesis of diastereomers of selenomethionine prior to their separation. Resulting diastereomers were separated by RP-HPLC using C_{18} column and gradient eluting mixture of MeCN with TEAP buffer as mobile phase. Detection of separated diastereomers was carried out at 231 nm using PDA detector. Validation of the method was carried out in terms of linearity, accuracy, precision, recovery and LOD.

Section B: It deals with separation of diastereomers of SeMet by RP-HPLC and RP-TLC. The diastereomers were synthesized using CDR 2, 3 and 6 under microwave irradiation and conventional heating. The three pairs of diastereomers were separated on C_{18} column using a linear gradient of MeCN and TFA, detection at 340 nm). Migration order of diastereomers of SeMet was supported by theoretical calculation. Simultaneously, these three pairs of diastereomers of SeMet were separated by RP-TLC on DC Kieselgel 60 RP*18 $F_{254}S$ stationary phase using the mobile phase (MeCN and TEAP buffer). Chromatographic conditions were optimized. The developed TLC method was validated in terms of linearity, accuracy, precision, recovery and LOD. The separated diastereomers were detected in ordinary light.

Section C: This section presents direct TLC enantioresolution of SeMet using (–)-Quinine as Chiral Selector. Direct TLC separation was performed using (–) -quinine as (i) chiral impregnating reagent, (ii) as chiral mobile phase additive (CMPA), and (iii) as chiral inducing reagent.

Different binary, ternary and quaternary mixtures of a variety of solvents (such as CH_3CN, CH_3OH, H_2O and CH_2Cl_2) were tried systematically to achieve enantiomeric resolution. The spots were located by ninhydrin solution and also in iodine vapor. Precision (RSD) and LOD were determined for direct TLC method. The influence of pH, temperature and amount of chiral selector was examined on enantioseparation. Spots were scrapped from TLC plates and native enantiomers were isolated.

The **sixth chapter** describes separation of enantiomers of proteinogenic-α-amino acids by HPLC using indirect method. CDR 17 was used as a CDR for the synthesis of diastereomers of eighteen amino acids. The diastereomers were separated by RP-HPLC using C_{18} column and gradient eluting mixture of MeCN with TEAP buffer. Detection was made at 231 nm using PDA detector. Chromatographic conditions were varied to obtain a good resolution. Method validation was carried out in terms of linearity, accuracy, precision, recovery and LOD.

ACKNOWLEDGEMENTS

Foremost, my greatest regards to the almighty **GOD** for bestowing upon me the confidence and patience to fulfill my task.

I would like to record my gratitude to my supervisor, **Professor Ravi Bhushan** for his distinct vision, advice and meticulous guidance throughout this research. He provided me unflinching encouragement and support in various ways. He is a constant oasis of ideas and full of knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my Ph.D. study.

I express my sincere thanks to the Head, Department of Chemistry, IIT Roorkee, Roorkee, for providing basic infrastructural facilities for carrying out the research work. I would like to thank Prof. S.M. Sondhi for his valuable suggestions, moral support and blessings.

It is great pleasure to thanks my colleagues Dr. Shuchi Dixit, Dr. Rituraj Dubey, Dr. Manohar Lal, Sonika Batra, Vinod, Manisha and Shiv for their motivation, suggestions and encouragement in the progress of this research work.

Thanks are due to the AvH-Stiftung Bonn, Germany, for donating Knauer HPLC instrument (to Prof. Ravi Bhushan), and to the Council of Scientific and Industrial Research (CSIR), New Delhi, for providing Junior Research Fellowship (JRF) and Senior Research Fellowship (SRF) during the period of research.

I wish to thank everybody with whom I have shared experiences in life. Specially those who played a significant role in my life, and those who with the gift of their company made my days more enjoyable and worth living. The support and encouragement of my friends Dr. Rajendra Kumar, Dr. Charu Agarwal, Dr. Sandeep, Dr. Satya Prakash, Dr. Arvind Bharti, Dr. Abhishek Singh, Abhishek Baheti, Himanshu Chasta, Himanshu Gupta, Manoj, Sudhir Gupta, Raman, Ambika, Nisha Jarwal, Subodh Bhardwaj, Er. Praveen Kumar, Er. Atul Kumar Singh and Avinash Saini (M.Sc. friend) is gratefully acknowledged. Especially I would like to thanks **Jay Singh Meena** for his selfless cooperation and mental support during the all phase of this work.

My deepest gratitude goes to my family for their unflagging love and support throughout my life, this thesis is simply impossible without them. They have always supported and encouraged me to do my best in all matters of life. I am indebted to my mother, **Mrs. (Late) Sohani Devi** and especially my **father**, **Mr. Prahalad Nagar** for

his care and love. My parents provided the best possible environment for me to grow up and the best available education. They never complained in spite of all the hardships in their life. I have no suitable words that can fully describe their everlasting love to me. To them I dedicate this thesis. My elder brothers, Mr. Mohan Lal and Mr. Hargovind and my sister-in-laws Mrs. Prakashi Devi and Mrs. Yasodha Devi have always been there for me to provide a joyful environment and helped to keep my strains off. A few words for my nieces, **Ms. Mamta Nagar** and **Ms. Laxmi Nagar**, and my Nephews, **Mr. Koshal Nagar, Mr. Kapil Nagar** and **Mr. Mahesh Nagar,** for illuminating my world with their beautiful smile. I feel a deep sense of gratitude for my taoji, **Mr. Rambajan**, maternal uncle, **Mr. Ramswaroop Nagar** and friend **Mr. Banwari Lal Sharma** who taught me good things that formed part of my vision which really matter in life.

My acknowledgement will never be complete without the special mention of the two people who are very important in my life: my beloved better half, **Rakeshi** for her eternal support and understanding of my goals and aspirations, and my daughter, **Bhaavya**, for giving me happiness with her cute smile during the period of my studies. Without her (my wife) support and faith, I would not have been able to complete much of what I have done and become who I am. Her patience and sacrifice will remain my inspiration throughout my life.

Finally I would like to thank everybody who contributed to the successful realization of this thesis, and wish express my apology for not mention them all by name.

Roorkee

Dated: (**HARIOM NAGAR)**

LIST OF ABBREVIATIONS

1.	ε	Molar absorptivity
2.	φ	Quantum yield
3.	(*R*)-MBIC	(*R*)-Methyl benzyl isothiocyanate
4.	(*R*)-MBIT	(*R*)-α-Methyl benzyl isothiocyanate
5.	(*S*)-Nap	(*S*)-Naproxen
6.	(*S*)-NEIC	(*S*)-1-(1-Naphthyl) ethyl isothiocyanate
7.	(*S*)-NEIT	(*S*)-1-(1-Naphthyl) ethyl isothiocyanate
8.	°C	Degree Celsius
9.	AAs	Amino acids
10.	ADME	Absorption, distribution, metabolic conversion and excretion
11.	AITC	2,3,4-Tri–*O*-acetyl-α-D-arabinopyranosil isothiocyanate
12.	Anal. Calcd	Analysis calculated
13.	*aq*	Aqueous
14.	Atl	Atenolol
15.	B3LYP	Becke three- parameters Lee-Yang-Parr
16.	Bel	Betaxolol
17.	BuOH	n-Butanol
18.	CC	Cyanuric chloride
19.	CD	Cyclodextrin
20.	$CDCl_3$	Deuterated chloroform
21.	CDRs	Chiral derivatizing reagents
22.	CE	Capillary electrophoresis
23.	CH_2Cl_2	Dichloromethane
24.	CIR	Chiral inducing reagent
25.	CLC	Centrifugal layer chromatography
26.	cm	Centimetre
27.	CMMNT	2-Chloro-4-methoxy-6-(4-methoxy-1-naphthyl)-[1,3,5]-triazine
28.	CMPA	Chiral mobile phase additive
29.	CS	Chiral selector
30.	CSP	Chiral stationary phases

31.	d	Doublet
32.	DANI	(1*S*,2*S*)-l,3-Diacetoxy-l-(4-nitrophenyl)-2-propyl isothiocyanate
33.	DBD-PyNCS	(*R*/*S*)-4-(3-Isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl-2,1,3-benzoxadiazole
34.	DCC	Dicyclohexylcarbodiimide
35.	DCT	Dichloro-*s*-triazine
36.	dd	Double doublet
37.	DFDNB	1,5-Difluoro-2,4-dinitrobenzene
38.	DFT	Density functional theory
39.	DMAP	4-Dimethylaminopyridine
40.	DMSO	Dimethyl sulfoxide
41.	DNP	Dinitrophenyl
42.	*ee*	Enantiomeric excess
43.	EtOH	Ethanol
44.	FDNP or DNFB	1-Fluoro-2,4-dinitrobenzene
45.	Flx	Fluoxetine
46.	FT-IR	Fourier Transform-infrared spectroscopy
47.	GATC	2,3,4,6-Tetra-*O*-acetyl-*β*-D-galactopyranosyl isothiocyanate
48.	GITC	2,3,4,6-Tetra-*O*-acetyl-*β*-D-glucopyranosyl isothiocyanate
49.	GLC	Gas liquid chromatography
50.	H_3PO_4	Phosphoric acid
51.	HCl	Hydrochloric acid
52.	HETP	Height equivalent to a theoretical plates
53.	HG-ICP-MS	Hydride generation inductively coupled plasma mass spectroscopy
54.	HPLC	High-performance liquid chromatography
55.	HPTLC	High-performance thin layer chromatography
56.	ICH	International Conference on Harmonization
57.	ICP-MS	Inductively coupled plasma mass spectroscopy

58.	IR	Infrared
59.	ISP	(±)-Isoxsuprine
60.	IUPAC	International Union of Pure and Applied Chemistry
61.	*k*	Retention/capacity factor (for HPLC)
62.	KBr	Potassium bromide
63.	LC	Liquid chromatography
64.	L-Glu	L-Glutamic acid
65.	LOD	Limit of detection
66.	LOQ	Limit of quantification
67.	m	Multiplet
68.	M	Molar
69.	mAU	Milli-absorbance units
70.	(*R*)-MBIC	(*R*)-Methyl benzyl isothiocyanate
71.	(*R*)-MBIT	(*R*)-*α*-Methyl benzyl isothiocyanate
72.	MCT	Mono chloro-*s*-triazine
73.	MeCN	Acetonitrile
74.	MeOH	Methanol
75.	MHz	Mega hertz
76.	min	Minute
77.	mL	Micro litre
78.	mM	Millimolar
79.	MS	Mass spectrometry
80.	MWI	Microwave irradiation
81.	$NaHCO_3$	Sodium hydrogen carbonate
82.	Nap-Btz	(*S*)-Naproxen–benzotriazole
83.	NAP-H	(*S*)-2-(6-Methoxynaphthalen-2-yl) propane hydrazide
84.	Nap-Phth	Phthalimidyl-(*S*)-naproxen ester
85.	NBD-COCl	4-(*N*-Chloroformylmethyl-*N*-methyl) amino-7-nitro-2,1,3-benzoxadiazole
86.	(*R*)-NEIC	(*R*)-1-(1-Naphthyl)ethyl isocyanate
87.	(*S*)-NEIC	(*S*)-1-(1-Naphthyl) ethyl isocyanate

88.	(*S*)-NEIT	(*S*)-1-(1-Naphthyl) ethyl isothiocyanate
89.	ng	Nanogram
90.	NIFE	(*S*)-*N*-(4-Nitrophenoxycarbonyl)phenylalanine methoxyethyl ester
91.	nm	Nanometre
92.	nmol	Nanomole
93.	NMR	Nuclear Magnetic resonance
94.	NP	Normal phase
95.	NSAIDs	Non-steroidal anti-inflammatory rugs
96.	Orc	Orciprenaline
97.	p	Pressure
98.	P_2O_5	Phosphorus pentaoxide
99.	PDA	Photodiode array detector
100.	pg	Picogram
101.	pmol	Picomole
102.	Prl	Propranolol
103.	RP	Reversed-phase
104.	rpm	Revolutions per minute
105.	R_s	Resolution
106.	RSD	Relative standard deviation
107.	RT	Room temperature
108.	s	Singlet
109.	SBLC	*S*-Benzyl-L-cysteine
110.	SD	Standard deviation
111.	SeMet	Selenomethionine
112.	SINP	*N*-Succinimidyl-(*S*)-2-(6-methoxynaphth-2-yl) propionate
113.	SMLC	*S*-Methyl-L-cysteine
114.	SSRI	Selective serotonin reuptake inhibitor
115.	*t*	Retention time
116.	t	Triplet
117.	TEA	Triethyl amine
118.	TEAP	Triethyl amine phosphate

119.	TFA	Trifluoro acetic acid
120.	THF	Tetrahydrofuran
121.	TLC	Thin layer chromatography
122.	TLC/MS	Thin layer chromatography/mass spectrometry
123.	UHPLC	Ultrahigh-pressure liquid chromatography
124.	US-FDA	United States Food and Drug Administration
125.	UV/Vis	Ultraviolet/Visible
126.	*v/v*	Volume/Volume
127.	W	Watt
128.	α	Separation factor/enantioselectivity
129.	δ	Chemical Shift
130.	λ_{max}	Wavelength
131.	μg	Microgram
132.	μm	Micrometre

LIST OF PUBLICATIONS

1 Indirect enantioseparation of proteinogenic amino acids using naproxen-based chiral derivatizing reagent and HPLC**.**

Ravi Bhushan and **Hariom Nagar**

***Biomedical Chromatography*, 2013; 27: 750-756.**

(*cf*., Chapter-6)

2 Indirect enantioseparation of Selenomethionine by reversed-phase high-performance liquid chromatography using a newly synthesized chiral derivatizing reagent based on (*S*)-naproxen moiety**.**

Ravi Bhushan and **Hariom Nagar**

***Biomedical Chromatography*, 2014; 28: 106-111.**

(*cf*., Chapter-5)

3 Enantioseparation of Orciprenaline, Betaxolol and Propranolol using HPLC and new chiral reagents based on 1,5-difluoro-2,4-dinitrobenzene.

Ravi Bhushan and **Hariom Nagar**

***Analytical Letter*, 2013: 47; 202–219.**

(*cf*., Chapter-3)

4 Enantioresolution of DL-Selenomethionine by: thin silica gel plates impregnated with (–)-quinine and RP-TLC and HPLC separation of diastereomers prepared with difluorodinitrobenzene based reagents having L-amino acids as chiral auxiliaries. **Hariom Nagar** and Ravi Bhushan

***Analytical Methods*, DOI:10.1039/C3AY41893F.**

(*cf*., Chapter-5)

5 Enantioseparation of (±)-Isoxsuprine by three independent liquid chromatographic methods and isolation of enantiomers.

Ravi Bhushan and **Hariom Nagar**

***Biomedical Chromatography* (communicated), 2014.**

(*cf*., Chapter-3)

6 Separation and isolation of enantiomers from racemic Selenomethionine and three *β*-blockers by achiral phase thin layer chromatography using a chiral inducing reagent.

Ravi Bhushan and **Hariom Nagar**

***Journal of Liquid Chromatography & Related Technologies* (communicated), 2014.**

(*cf*., Chapter-3 and 5)

Chapter-1
Introduction

I. Introduction to Enantiomers and Enantiomeric Resolution

A. Chirality and Biological Relevance of Chiral Pharmaceutical Drugs

Chirality is the property of a molecule which is generated due to asymmetry in its chemical structure arising from different spatial arrangements of groups around the asymmetric center. A molecule that is not identical to its mirror image is termed as 'chiral' (Fig. 1.1 mirror images of alanine). Such non-identical pairs of molecular mirror images are called enantiomers that have identical physicochemical properties, but differ when they are placed in a chiral environment.

Living organisms, for example, are composed of chiral biomolecules such as amino acids, sugars, proteins and nucleic acids. In biological system the body being amazingly chiral selective, will interact with the two enantiomers of a drug differently and metabolize each enantiomer by a separate pathway to produce different pharmacological and pharmacokinetics activity. In this case, one isomer may produce the desired therapeutic activities and other may be inactive or lesser activity (Lee and William, 1990) or, in the worst cases, produce unwanted effects (Rentsch, 2002). The selective nature of receptor sites makes differences in enantiomers activity.

Many biologists and clinical pharmacologists tend to deal with mixtures of isomers as if one compound were involved. Thus a physician, presented with such a drug under a brand name is unaware of isomers and may make mistakes (Ariëns, 1984) because of unavailability of single enantiomer.

Several chiral drugs obtained from the natural sources (*e.g.*, penicillin, morphine and digoxin) are found in single enantiomeric form and are marketed usually as such. However, except for some cases, all the chiral drugs produced synthetically are racemic or non-racemic mixtures of enantiomers and have been used as such. More than 40% of the drugs prescribed as medicine are chiral, only 10% of the total prescribed drugs are enantiomerically pure (Rentsch, 2002).

A few examples of the drug enantiomers, having different biological activities, are shown in Fig 1.2.

For the undesirable effects of racemic mixture of drugs, their prescription in market as racemate has been restricted by drug regulatory agencies such as United

States Food and Drug Administration (USFDA) (FDA, 1992) and European Committee for Proprietary Medicinal Products.

There may be certain advantages of administering enantiomerically pure pharmaceuticals as drugs are as follows:

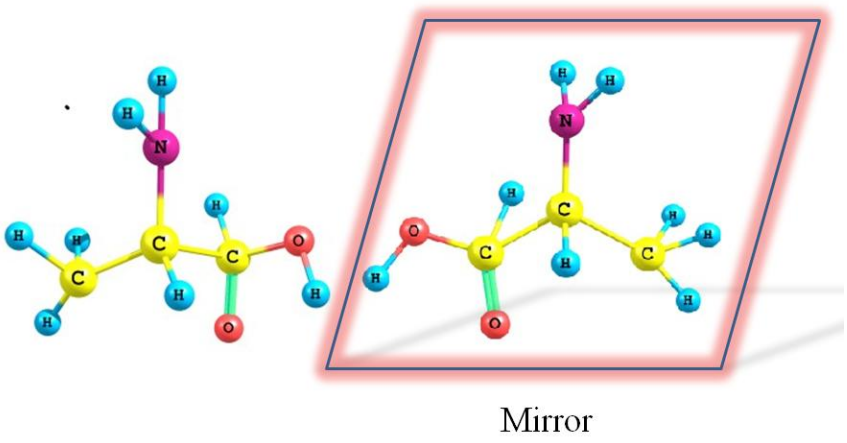


Fig. 1.1: Enantiomers of alanine

- It could reduce the total dose of drug
- Toxicity or unwanted side effects would be minimized or removed
- Elimination of source of interobject variability would be possible
- The relationship of dose–response would increase

Therefore, systematic investigation of biological activities of both enantiomers have become indispensable in areas of prime importance, such as pharmacology, medicine, life sciences, asymmetric synthesis and studies of structure-function relationship of proteins. Various analytical methods have been used for separation of enantiomers; these include fractional crystallization, enzymatic consumption of one of the enantiomers, electrophoresis, and chromatographic techniques particularly, high-performance liquid chromatography (HPLC) and thin layer chromatography (TLC).

B. Chromatographic Method for Enantiomeric Resolution

Chromatography has established its own importance in the fields of separation, identification, quantification and isolation of the components present in multicomponent mixture, and resolution of enantiomers from racemic and non-racemic mixtures. The basis of chromatographic separation is different distribution/affinity of the analyte components for the mobile and stationary phases. The affinities of analyte components depend on the hydrophobic and hydrophilic nature of groups present. If the component is of more hydrophobic in nature then it will have more attraction for the hydrophobic stationary phase and elute later than the less hydrophobic component present in mixture of raw materials i.e., it will have more retention time or have higher R_F value.

LC has importance in pharmaceutical industry and analytical separations due to ease of handling and sample treatment, excellent reproducibility, precision and accuracy. LC provides wide choice of process such as it can be processed in column may be in closed or open column and on plane such as paper or plates.

TLC enjoys a considerable importance as a cost-effective, simple and convenient technique for screening of unknown compounds present in racemic and non-racemic mixture of drugs. It has a high degree of efficiency of separating all the possible components present in multicomponent mixtures. Among all the chromatographic separation techniques, HPLC is the most widely used techniques; it provides wider choice of chromatographic conditions with respect to mobile phases, mode of elution, temperature, detector, and use of various types of chiral and achiral columns.

1. Thin Layer Chromatography (TLC)

Sherma (2002; 2004; 2006; 2008; 2010) presented a comprehensive study on TLC separations and literature survey over the past several years. Certain books have appeared dealing with applications of TLC for analysis of various types of compounds (Sherma, 2003; Kowalska and Sherma, 2007; Sherma and Kowalska, 2007). Martens and Bhushan (1989; 1990; Bhushan and Martens, 2010) reviewed application of TLC in control of enantiomeric purity of amino acids and pharmaceuticals.

TLC has been modernized with the availability of automated sample applicators and commercial plates with a wide variety of adsorbents (e.g., cellulose, alumina, silica, ion-exchangers, polyamides and various other minor organic and inorganic sorbents) on

inert solid surface. Besides, there have been developed centrifugal layer chromatography (CLC) (Scott, 1978), over-pressured thin layer chromatography (Tyihak *et al*., 1979), and high-performance thin layer chromatography (HPTLC) (Bertsch, 1980) which have caused renaissance in the field of TLC. Literature shows various review papers on the importance, opportunities and challenges of the HPTLC technique (Kalasz, 2001; Poole, 2003; Nyiredy, 2002). Since mass spectrometry (MS) and infrared (IR) spectroscopy are highly efficient and potent techniques for molecular structure elucidation these have been coupled with TLC to be known as TLC/MS (Schippert *et al*., 1979) and TLC/FT-IR (Claudia, 2005); these have been used successfully for qualitative and quantitative analysis in various fields such as, food analysis, drug analysis, biological analysis, environment analysis and forensic analysis.

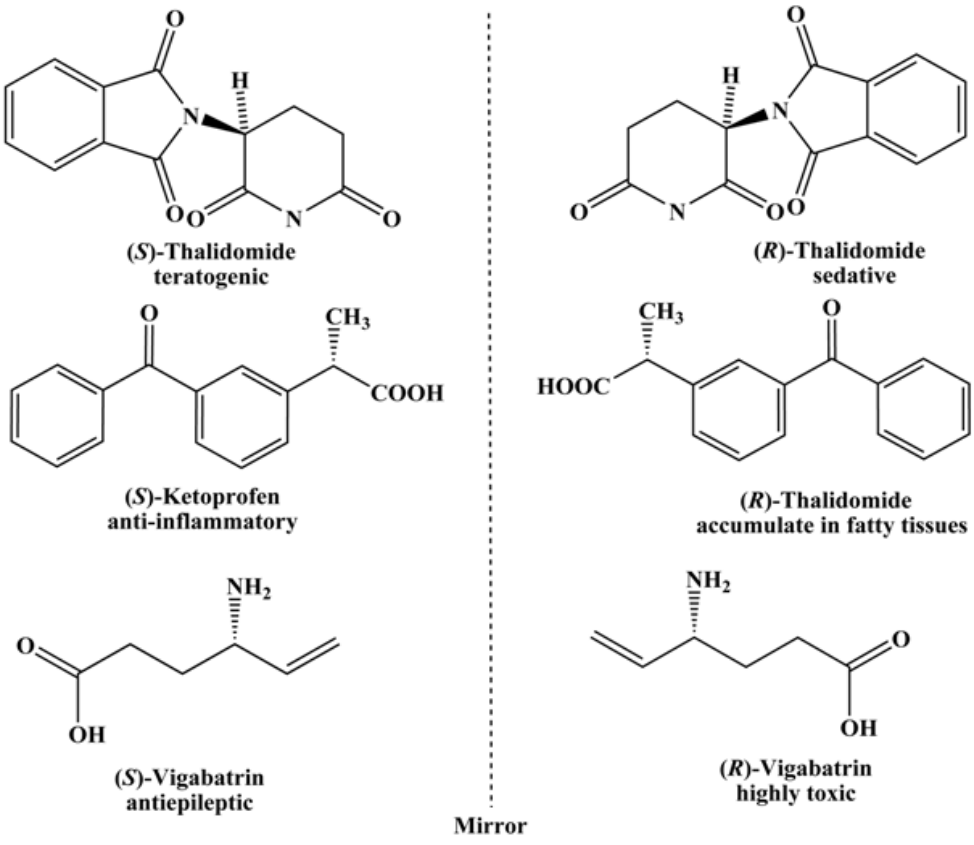


Fig. 1.2: Examples of pharmaceutical enantiomers having different biological essence

2. High-performance Liquid Chromatography (HPLC)

Martin and Synge (1941) put the foundation of partition chromatography with the invention of gas liquid chromatography (GLC) and HPLC. Giddings (1965) developed theoretical framework for GLC which was equally and well applicable for LC. During 1967-1969, different aspects of HPLC were described by Kirkland (1969) and Huber (1969).

Other developments in the area include, core–shell technology, high-temperature LC, monolithic columns, and ultrahigh-pressure liquid chromatography (UHPLC, with backpressure requirement, p > 400 bar) (Novakova and Vlckova, 2009) and (Guillarme *et al*., 2010). The monolith silica column approach was introduced to reduce intrinsic flow resistance which was achieved by increasing flow-through pore size of the packing and the external porosity. In support of this technique a number of commercial column are now available in market (Oberacher *et al*., 2004; Ikegami *et al*., 2004); by enhancing the flow rate of the mobile phase on these columns, analysis time can be reduced.

These characteristic properties of HPLC technique and its recent modifications by assisting with modern instrumentation and columns made it a mostly used chromatographic method as compared to other separation methods. It is most suitable technique for analysis of almost any compound that are thermally fragile, non-volatile and dissolvable in liquid even in trace amount as low as parts per trillion concentration.

Detection in HPLC

HPLC is not only the separation technique but also a detection technique. The detector should have the following characteristics; it should produce quick, compatible, high sensitive, uniform, and reproducible detection responses, and should not be affected by variation in flow rate of mobile phase and temperature. The UV/Vis (of fixed wavelength and variable wavelength) and photodiode array (PDA) detector are the mostly used detectors in HPLC.

a) UV/Vis Detectors

The principle of most of the optical HPLC detectors is based on the measurement of the changes in intensity of electromagnetic radiation passed through the flow-cell and interacts with the analyte present in the HPLC eluent.

b) PDA Detector

PDA detector is the choice of method development and has the importance of monitoring impurities present particularly in complex biological samples. It also provides UV spectra for eluting peaks. In most of the PDA detectors a charge-coupled diode array consisting of 512-1024 diodes have been used, which are capable of resolving the spectrum of~ 1 nm. It allows the access of chromatographic and spectral data conveniently along three axes (absorbance versus wavelength, versus retention time).

Factors Regulating Resolution

Resolution is the calculated value of how two peaks are separated to each other and it can be achieved by, dividing the distance between two peaks by the average 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.

Combination of three major factors (retention factor, selectivity and number of plates), together termed as resolution, control the resolution of bands of two peaks from each other.

1. Retention Factor

It is also called as capacity factor, *k*', it evaluates the degree of retention of analyte molecule. It is defined as the ratio of time of an analyte molecule interacting

with the stationary phase to the time it interacts with the mobile phase. *k*' is calculated by the values obtained from the elution chromatogram by:

$$k = \frac{t - t_0}{t_0}$$

where t_0 is the time taken by the solvent to elute from the column or the first disturbance in baseline (solvent peak).

In TLC, R_F is the ratio of distance travelled by the sample component to the distance travelled by the solvent during the development of chromatogram.

$$R_F = \frac{\text{Distance travelled by the sample component on TLC plate}}{\text{Distance travelled by the solvent on TLC plate}}$$

2. Selectivity

Selectivity (α) may also called as ‘‘separation factor’’ a ratio of net retention time of the two adjacent sample compounds. In other words, α is the measure of relative distribution coefficients. For a baseline separation α must be > 1.0. By reducing the column length α can be increased.

$$\alpha = \frac{t_2 - t_0}{t_1 - t_0} = \frac{k_2}{k_1}$$

3. Theoretical Plates

The term ‘theoretical plates’ is expressed by, *N*; column efficiency is directly proportional to the number of theoretical plates, to which the column is equivalent. Mathematical calculation of this parameter is

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

where t_r representing the retention time of the peak of corresponding eluted solute and *w* is the base width of the peak measured in the same units, *L* express the measure of

column efficiency per unit length, H is the height equivalent of theoretical plates (HETP).

II. Separation of Enantiomers

Enantiomeric separation by chromatographic methods is generally achieved either by 'direct' or 'indirect' approach.

A. Direct Approach

In this approach there is no derivatization process for the formation of diastereomers prior to chromatographic process of separation. Dalgliesh (1952a; 1952b) proposed a model for describing "three-point interaction" involving hydrogen bonding, van der Waal's forces, steric, hydrophobic, dipole–dipole or pi-pi interactions and other forms of electron donation and acceptance that are readily reversible are required to be in action for formation of transient diastereomers and their separation.

Table-1.1 shows a few applications of CSPs, chiral impregnating reagents, CMPAs, and chiral inducing reagents for enantiomeric separation of certain important chiral pharmaceuticals

Direct approach has been performed in LC by using chiral environments in following different ways:

1. Chiral Stationary Phase (CSP)

CSP was first time commercialized in 1981(Beesley, 2011) which was applicable for HPLC separation of enantiomers and since then direct approach using CSPs has become a widely used approach not only for qualitative analysis but also for preparative separation. Today, CSPs of certain categories are available in market which have been prepared by immobilizing the chiral selector on stationary phase or packing in column such as, helical phase (cellulose and amylase esters), Pirkle-type column (π-acceptor), semichiral column (π-donor), affinity-phase (ovomucoid, α_1-acid glycoprotein), crown-ether, ligand-exchange and cavity phase (*α, β and γ*-cyclodextrin etc.) column. Lämmerhofer (2010) presented mechanisms for chiral recognition by enantioselective liquid chromatography using CSPs.

2. Impregnation

This is the most important approach in TLC in which suitable chiral selector is incorporated with the stationary phase without any covalent interaction and without disturbing its inert character, prior to development of chromatogram. The advantages of impregnating reagents have been described in literature for separation of enantiomers by direct method in TLC (Kowalska and Sherma, 2007). TLC plates can be impregnated by following methods, (i) mixing the chiral selector with inert support, (ii) immersing the plain TLC plates into a solution of suitable impregnating reagent, (iii) allowing the plain plate to develop in the solution of impregnating reagent in an ascending or descending manner, (iv) exposing the layer of inert support to the vapours of impregnating reagent or plates have been impregnated by spraying the solution of impregnating reagent.

3. Chiral Mobile Phase Additive (CMPA)

The CMPA approach is adopted in TLC, HPLC and CE for enantiomeric separation; the chiral selector is mixed with the mobile phase system prior to development of chromatogram. The column having good compatibility, ruggedness and high efficiency can be used in HPLC separation by mixing the chiral additive in mobile phase. Stereoseletive separation is considered to be achieved in a system having chiral additive in the mobile phase due to one or a combination of the following 'mechanisms':

(i) Stereoselective complexation in mobile phase,
(ii) Formation of the transient diastereomeric associates between the mobile and stationary phase having different distribution properties,
(iii) Adsorption of the chiral reagent onto the solid stationary phase during the development of chromatogram.

4. Chiral Inducing reagent (CIR)

Literature shows certain nonconventional methods for purification/ separation of enantiomers from the excess enantiomer under totally achiral conditions of physicochemical phase transitions or achiral chromatography. The sporadic reports till 1992 on liquid chromatographic separation of non-racemic mixtures with both achiral

Table-1.1: Certain chiral impregnating reagents/CMPAs/CSPs used for direct enantioseparation of a variety of chiral pharmaceuticals (after 2000)

Chiral stationary phases	**Analytes**	**Reference**
Cyclodextrin (CD) column	Amino alcohols	Bhushan and Kumar, 2009a
	Penicillamine	Merino *et al*., 1992
α_1-Acid glycoprotein column	Propranolol	Xuan and Hage, 2006
	Atenolol	Enquist and Hermansson, 1989
Cellulose column	Betaxolol	Krstulović and Fouchet, 1988
	200 chiral pharmaceuticals	Peng *et al*., 2010
D-Penicillamine based ligand exchange column	Unusual secondary amino acids	Ilisz *et al*., 2006
	Non-protein amino acids	Miyazawa *et al*., 2004
Chiral mobile phase additives in TLC	**Analytes**	**Reference**
L-Tartaric acid	Ketamine and Lisinopril	Bhushan and Agarwal, 2008a
Cu(II)-L-Thr, L-Ser, and L-Tartaric acid complexes	β-blockers	Bhushan *et al*., 2012
Cu(II)-L-Pro complex	Thyroxine	Wang *et al*., 2003
Chiral impregnating reagents in TLC	**Analytes**	**Reference**
L-Asp and L-Glu	β-Blockers	Bhushan and Agarwal, 2008b
D-Tartaric acid	Metoprolol	Lucic *et al*., 2005
Cu(II)-L-Pro, Cu(II)-L-Phe, Cu(II)-L-His, Cu(II)-L-Trp complexes	β-Blockers	Bhushan and Tanwar, 2010

phases were presented as a short review by Martens and Bhushan (1992). In a recent review Martens and Bhushan (2014) have discussed unexpected separation of enantiomers from non-racemic mixtures in a totally achiral environment and the technical terms used in recent literature, along with presentation of scientific terminology in IUPAC background, for explaining the possible mechanism of separation under achiral conditions for both synthetic organic chemists and analytical chemists.

B. Indirect approach

It can be defined as the approach in which transformation of the enantiomers into corresponding diastereomers occurs by reacting them with a chiral derivatizing reagent (CDR) followed by their separation under achiral chromatographic conditions. Enantiomers present in biological samples (blood and/ or urine) in trace level amounts can be analysed by this approach. This is because of high molar absorptivity (ε) of the CDR or high fluorescence quantum yield (φ) of resulting diastereomers. Due to different physicochemical properties diastereomers elute with different rate through the achiral column i.e., they show different retention time. Resolution of chiral drugs by liquid Chromatography based upon diastereomer formation with chiral derivatization reagents has been reviewed by various workers (Ilisz *et al*., 2008; Toyo'oka, 2002; Srinivas, 2004; Görög and Gazdag, 1994; Sun *et al*., 2001).

Advantages of this method are:

- Excellent separation
- Relatively inexpensive achiral columns are required
- A large number of CDRs are commercially available and can be synthesized easily in laboratory
- Greater possibilities to optimize the chromatographic conditions

1. Characteristics of CDRs

For the success of enantioseparation by indirect approach it is very important to choose the appropriate CDR. Certain important criteria should follow during the selection of CDRs:

- CDR should be chemically and optically pure.
- CDR should be soluble freely in water or in other solvents miscible in water (such as alcohol and acetonitrile).

- CDR should be specific for the target functional group and under mild conditions it should quantitatively label the analyte.
- CDR should be stable for long time under the normal refrigeration conditions.
- The reaction conditions should be easily obtainable and should ensure the completion of derivatization reaction and chances of racemization or degradation should be less.
- The reagent should have the capability of absorbing in UV or visible regions

2. CDRs for Amino Compounds

Most of the pharmaceuticals and biologically active compounds, such as drugs, biogenic amines and amino acids contain at least one amino group as functionality in their chemical structure. Literature reveals reactions for synthesis of diastereomers of primary and secondary amines with different CDRs. Reactions with these CDRs result into thioureas, ureas, carbamates and amides as diastereomers. CDRs based on DFDNB and CC has also been employed for diastereomerization of amino group containing compounds.

Fig. 1.3 is shows structures of certain representative CDRs used for derivatization of amino compounds. In Table-1.2 applications of a few CDRs for enantioseparation of amino compounds (after 2002) have been listed.

III. Method Validation

Suitability of newly developed method can be justified by implementing the process of method Validation, i.e., tests for reliability, accuracy and precision of the particular method. A complete validation of the method has significant role for the first time development and implementation of a particular bioanalytical method, for analysis of a new drug entity and a full validation of the revised assay is important if metabolites are added to an existing assay for quantification. Method developer should also consider ruggedness or robustness of the method as the important part of method validation. In analytical chemical laboratories it is the essential activity to validate the method and well implemented in pharmaceutical industry. The US FDA has edited draft guidelines with detailed recommendations for method validation of bioanalytical methods in 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 (ICH-Topic Q2A, 1995; ICH-Topic Q2B, 1996; 1997).

The most common parameters for validation are linearity, accuracy, and precision (repeatability or intra-day precision, and intermediate precision or inter-day precision), limit of detection (LOD) and limit of quantitation (LOQ), recovery, robustness, sample solution stability, and specificity/ selectivity.

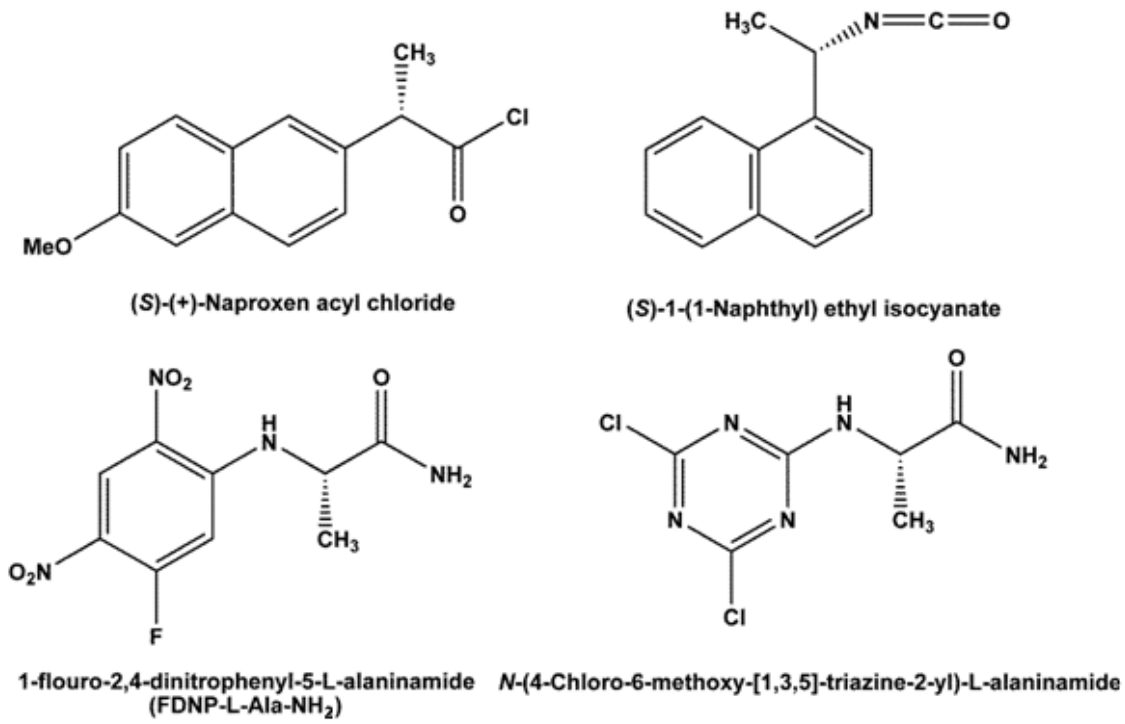


Fig. 1.3: Structures of CDRs applied for derivatization of amino group containing compounds

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

S. No.	Chiral derivatizing reagents (CDRs)	Analytes	Source	Ref. No.
1	*o*-Phenylenediamine and 2-mercaptoethanol	Amino acids	Amino acid oxidase	Oguri *et al*., 2005
2	*o*-Phthaldialdehyde/ *N*-isobutyryl-L-cysteine or *N-tert*-butyloxycarbonyl-L-cysteine	D-Serine and related neuroactive amino acids	Human plasma	Grant *et al*., 2006
3	*o*-Phthalaldehyde/ *N-(R)-mandelyl-L-cysteine*	α-Amino acids	Beer	Chernobrovkin *et al*., 2007
4	*o*-Phthalaldehyde/ *N*-acetyl-L-cysteine	Selenomethionine	Antarctic krill	Bergmann *et al*., 2004
5	2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl isothiocyanate (GATC)	β-Blockers	Standard compounds	Ko *et al*., 2006
6	2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC)	Carvedilol	Human plasma	Yang *et al*., 2004
		Spin-labeled, cyclic and β-amino acids	Standard compounds	Péter *et al*., 2003
7	(*R*)-1-(1-Naphthyl)ethyl isocyanate (NEIC)	Fluoxetine and Norfluoxetine	Rat plasma and brain	Unceta *et al*., 2007
8	(*R*/*S*)-4-(3-Isothiocyanatopyrrolidin-1-yl)-7-(N,N-dimethylaminosulfonyl-2,1,3-benzoxadiazole (DBD-PyNCS)	Thyroxine	Standard componds	Jin *et al*., 2008
			Human serum	Jin *et al*., 2007
9	(1*S*,2*S*)-l,3-diacetoxy-l-(4-nitrophenyl)-2-propyl isothiocyanate (DANI)	Amino acids and β-blockers	Standard compounds	Péter and Fülöp, 2002

Table-1.2 Contd....

S. No.	Chiral derivatizing reagents (CDRs)	Analytes	Source	Ref. No.
10	(*S*)-*N*-(4-Nitrophenoxycarbonyl)phenylalanine methoxyethyl ester (NIFE)	*β*-Alkyl-substituted amino acids	Standard compounds	Vékes *et al*., 2002
		α-Substituted proline analogues		Péter *et al*., 2004a
		β-Amino acids		Péter *et al*., 2004b
11	(*S*)-Naproxen acyl chloride	Vigabatrin	Human Serum	Chun-Yu *et al*., 2008
12	(*S*)-2-(6-methoxynaphthalen-2-yl)propanehydrazide (NAP-H)	Chiral aldehydes and ketones	Standard compounds	Bhushan and Lal, 2012
13	Trichloro-*s*-triazine-based CDRs having optically pure amines as chiral auxiliary	Proteinogenic amino acids	Standard compounds	Bhushan and Lal, 2013a
		Selenomethionine	Standard compounds	Bhushan and Lal, 2013b
14	Trichloro-*s*-triazine-based CDRs having optically pure amino acids as chiral auxiliary	Bupropion	Standard compounds	Bhushan and Batra, 2013a
15	(*S*)-1-(1-naphthyl) ethyl isothiocyanate, (*S*)-NEIT, and (*R*)-α-methyl benzyl isothiocyanate, (*R*)-MBIT	Bupropion	Standard compounds	Bhushan and Batra, 2013b

IV. Preamble to Present Studies

Enantioselective analysis is extremely important in agricultural, environmental, medical, pharmaceutical and food chemistry. Because it plays an important role not only for the pharmacodynamics (involving the interaction of bioactive molecules with enzymes and receptors present in the target organs) but also for pharmacokinetics (involving absorption, distribution, metabolic conversion and excretion; ADME) of the drug compounds. The different activities of the drug enantiomers, such as pharmacodynamics, pharmacokinetics and toxicological activities lead to a variety of effects. However, one enantiomer can be a safe and desired drug, but the other enantiomer can show adverse or deleterious effects. But, many of the drugs developed from organic synthesis are still administered in racemic form.

It's an essential requirement to present the data of investigations of stereospecific fate of drugs in the body and enantiomeric purity determination of chiral drugs, to the regulatory authorities before their introduction into the market and during industrial manufacture. Therefore, essential part of the drug development process is to develop efficient analytical methods for enantioseparation. The chromatographic techniques particularly the LC techniques have become the most preferred methods (or a natural choice) for the resolution and determination of enantiomers.

Considering the importance of chiral analysis and LC techniques, following plan was formulated for present studies:

A. Objectives

1. To separate enantiomers of certain pharmaceutically and biologically important compounds, and
2. To develop simple, inexpensive, sensitive and reproducible methods that could be successfully used for enantiomeric separation and control of enantiomeric purity in different situations
3. To optimize and validate the methods so developed

B. Selection of Chiral Analytes for Enantioseparation

Certain important criteria have been adopted for selection of chiral compounds (as racemic or scalemic mixtures), (i) certain pharmaceuticals that are widely used and marketed as racemic mixtures, (ii) chiral compound that are of low cost, and are easily

available, and (iii) contain a simple functional group having a good reactivity for synthetic modification purposes.

The chiral compounds such as, proteinogenic-*α*-amino acids (or proteinogenic AAs), DL-selenomethionine (SeMet), *β*-blockers (namely, (*RS*)-propranolol (Prl), (*RS*)-atenolol (Atl), (*RS*)-betaxolol (Bel), (*RS*)-orciprenaline (Orc), (±)-isoxsuprine (ISP) and (*RS*)-fluoxetine (Flx) were chosen for the present studies.

C. Approach to Separation

The LC techniques, TLC and HPLC were used for enantioseparation using direct and indirect approaches.

1. Direct Approach

Direct TLC enantioseparation along with isolation of native enantiomers of (i) DL-selenomethionine (SeMet) using (–)-quinine as chiral selector. Four different approaches were adopted; (a) mixing of (–)-quinine with mobile phase as chiral mobile phase additive, (b) Impregnation of plain plates by mixing (–)-quinine (chiral impregnating reagent) with slurry, (c) Impregnation by ascending development of plain plates in a solution of (–)-quinine (chiral impregnating reagent), and (d) pre-mixing of (–)-quinine with SeMet as chiral inducing reagent, (ii) L-SeMet (Glu) was selected as an impregnating reagent for enantioseparation of (±)-ISP by adopting the approach in which TLC was impregnated by mixing of L-Glu with silica gel slurry, (iii) Three *β*-blockers, Atl, Bel and Orc have been separated using L-Glu as chiral impregnating reagents by mixing in the silica gel slurry, as chiral mobile phase additive by mixing with mobile phase and as chiral inducing reagent by pre-mixing with solution of racemic *β*-blockers. The effects of temperature, pH and concentration of chiral selector(s) on enantioseparation have been also studies.

2. Indirect Approach

Indirect approach was adopted for resolution of enantiomers of DL-proteinogenic AAs, SeMet, *β*-blockers (namely, Prl, Atl, Bel, Orc, ISP and Flx).

In view of the literature reports, certain CDRs have been synthesized for derivatization of these selected analytes in the present study. Derivatization was carried

out by microwave irradiation (MWI), vortexing and conventional heating method and the resulting diastereomers have been separated by RP-TLC and RP-HPLC.

D. Selection of Chiral Derivatizing Reagents (CDRs)

It is primary and important task to select CDRs for derivatization prior to formation of diastereomers of selected chiral compounds. The properties (chromophoric or fluorophoric properties) of the diastereomers responsible for their detection and separation are largely dependent on the chromophoric or fluorophoric properties of the CDR.

Certain important criteria were adopted for synthesis of the CDRs. These were the CDRs which could be

- derived from inexpensive and easily available synthons,
- prepared by using straightforward procedures in laboratory and
- used for derivatization of analytes under mild conditions providing stable diastereomers having higher detection sensitivity.

In view of these criteria certain platforms/ synthons/structural moieties such as CC (cyanuric chloride; 2,4,6-trichloro-1,3,5-triazine; trichloro-*s*-triazine; *s*-triazine chloride), DFDNB (1,5-difluoro-2,4-dinitrobenzene) and (*S*)-Naproxen [(*S*)-Nap] were explored to synthesize a wide variety of CDRs. These moieties were selected for their characteristic features described below. These CDRs were having different functional groups to react with analytes for the formation of corresponding diastereomers.

1. CDRs based on DFDNB

DFDNB was chosen as a structural moiety to prepare certain CDRs. For this purpose, two types of chiral auxiliaries were selected for replacement of fluorine of DFDNB by nucleophilic attack. These were, (i) L-amino acids, (*S*-methyl-L-cysteine, *S*-benzyl-L-cysteine, L-Ala, L-Leu, L-Phe, L-Met and L-Val), and (ii) optically pure amines; (*S*)-(+)-1-cyclohexylethylamine, and (*R*)-(+)-α-methyl benzylamine.

2. CDRs Based on CC

Taking into account trifunctionality and high reactivity of CC, it was selected as another moiety for the preparation of CDRs. The chiral auxiliaries chosen for this

purpose were *S*-methyl-L-cysteine, *S*-benzyl-L-cysteine, L-Ala, L-Leu, L-Met and L-Val. Nucleophilic substitution of one chlorine atom in CC provided DCT (dichloro-*s*-triazine) reagents.

3. CDRs based on (*S*)-Nap

(*S*)-Nap was chosen as another structural moiety to form CDRs for preparation of diastereomers of the analytes (chosen for enantioseparation in the present studies) containing 1° and 2° amino groups. Therefore, the CDRs {(*S*)-1-[1H-benzo(d) (1,2,3) triazol-1-yl}-2-{6-methoxynapthalen-2-yl-propan-1-one]; (*S*)-naproxen-benzotriazole; Nap-Btz} and *N*-phthalimidyl-(*S*)-2-(6-methoxynaphth-2-yl) propionate ((Nap-Phth), based on (*S*)-Nap moiety were synthesized by nucleophilic attack of 1*H*-benzotriazole and *N*-hydroxyphthalimide, respectively, on carbonyl carbon of the carboxylic group of (*S*)-Nap in presence of dicyclohexylcarbodiimide (DCC) as coupling agent.

E. Characteristics of DFDNB, CC and (*S*)-Nap

DFDNB: A bifunctional variant of Sanger's reagent (SR) namely ''1,5-difluoro-2,4-dinitrobenzene (DFDNB)'' was used for cross linking of proteins such as wool, silk and insulin for the first time (Zahn and Meienhofer, 1958).

Since SR has good chromophoric groups in its structure which make it identifiable in chromatography, therefore, amino acids have been attached with its dinitrophenyl (DNP) to establish amino acid sequence in peptides (Sanger 1945). Marfey (1984) prepared its chiral variant, namely 1-fluoro-2,4-dinitrophenyl-L-alaninamide (FDNP-L-Ala-NH_2, Marfey's reagent, MR) by substitution of one fluorine atom in DFDNB with L-alanine amide. Besides, the presence of strong electron withdrawing nitro groups in MR facilitates nucleophilic substitution of remaining one fluorine atoms with different chiral auxiliaries with primary or secondary amino group situated on or just near to the stereogenic centre of an enantiomer of racemic mixture and due to presence of the dinitrophenyl chromophore and its conjugation with amino group of chiral moiety in this CDRs make it of high molar absorptivity (at 340 nm). Another feature is that it reacts stoichiometrically, without racemization, under alkaline conditions with a good yielding of diastereomers. Considering these characteristic

feature of MR it has been widely used for structural characterization of peptides, confirmation of racemization in peptide synthesis and detection of small quantities of D-amino acids.

Some review articles have also appeared which shows use and advantages of MR in the enantioseparation of chiral amino acids (B'Hymer *et al*., 2003; Bhushan and Brückner, 2004). Application of MR and comparison with other chiral derivatizing reagents for resolution of multicomponents mixture of DL-amino acids and a large number of other compounds along with mechanism of separation have been discussed (Bhushan and Brückner, 2004) and, assessment and applications to natural products and biological systems (Bhushan and Brückner, 2011). Enantioresolution of SeMet, along with methionine and cysteine has been carried out in this laboratory by reversed-phase (RP) HPLC using 12 CDRs synthesized on DFDNB and CC (Bhushan and Dubey, 2012a) platforms, with UV detection.

CC: Savelova (1990) described that behavior of CC is likely the same as a trifunctional acid chloride (Fig. 1.4). Being a trifunctionality present in CC it shows an interesting feature of successive and controlled nucleophilic substitution of its three chlorine atoms with amines, alcohols, or thiols (as nucleophile) in the presence of a hydrochloride acceptor (usually sodium carbonate, bicarbonate, hydroxide or tertiary amines) under appropriate reaction conditions, *i.e.* temperature, time, solvent etc. (Blotny, 2006). The substitution of three chlorine atoms can be controlled by temperature in a stepwise manner; mono-substitution of chlorine atom occurs below or at 0 °C, di-substitution at room temperature and tri-substitution occurs above 60 °C (Thurston *et al*., 1951; Kempter *et al*., 2000).

By considering the trifunctionality and reactivity features of CC such as, replacement of one chlorine atom by suitable reporter group and another by a residue that alters the polarity or hydrophobicity of the molecule, Brückner and Strecker (1992) synthesized chiral mono chloro-triazine (MCT) reagents for derivatization of α-amino acids (namely, Ala, Arg, Glu, Phe and Val) and corresponding diastereomers were separated by RP-HPLC using mixtures of CH_3CN, water and TFA as mobile phase. In continuation of the above work, Brückner and Wachsmann (2003a) developed certain CDRs (MCT reagents) by nucleophilic substitution of one chlorine atom in CC by

alkoxy (butoxy, methoxy, 1,1,1-trifluoroethoxy) or aryloxy groups (4-methylcoumaryloxy, nitrophenoxy, phenoxy, phenylphenoxy), and the second by different chirally pure amino acid amides (L-Ala-NH_2, L-Phe-NH_2, L-Val-NH_2) or esters (L-Pro *tert*-butyl ester, or Boc-L-Lys *tert*-butyl ester). A fluorescent dichloro-*s*-triazine (DCT) reagent, 3-(4,6-dichloro-1,3,5-triazinylamino)-7-dimethylamino-2-methylphenazine was used for derivatization of DL-amino acids followed by micellar electrokinetic chromatography with *β*-cyclodextrin added to the buffer (Ma, 2002). An MCT reagent, 2-chloro-4-methoxy-6-(4-methoxy-1-naphthyl)-[1,3,5]-triazine (CMMNT) was also synthesized for derivatization of mixtures of *α*-amino acids (Brückner and Wachsmann, 2003b).

Certain chiral DCT and MCT reagents were synthesized by nucleophilic substitution of chlorine atom(s) with different amino acid amides in CC and its 6-methoxy derivative, respectively. These were used for derivatization of amino acids followed by their RP-HPLC separation (Bhushan and Kumar, 2008a). In continuation, two CC based CDRs having piperidinyl as achiral auxiliary and, L-Leu-NH_2 and L-Leu as chiral auxiliaries respectively have also been synthesized for enantioseparation of amino acids (Bhushan and Agarwal, 2011). Enantiomerically pure amino acidss viz., L-Ala, D-Phg, L-Val, L-Leu and L-Met have been used as chiral auxiliaries in CC to prepare certain DCT reagents which were applied for enantioresolution of proteinogenic amino acids (Bhushan and Dixit, 2012a). Two MCT and two DCT reagents were synthesized using chiral amines namely, ((*R*)-(+)-naphthylethyl amine and (*S*)-(+)-1-benzyl-3-aminopyrrolidine) for enantioseparation of proteinogenic amino acids (Bhushan and Lal, 2013a). Bhushan and Martens (2010) has described the structural similarity of diastereomers of amino acids prepared with CC based CDRs to those prepared with Marfey's reagent.

Certain other CC based CDRs having enantiomerically pure amino acids or their amides and amines as chiral auxiliaries have been synthesized and used for RP-HPLC separation of diastereomers of primary or secondary amino group containing pharmaceuticals; these include, (*RS*)-mexilitine (Bhushan and Dixit, 2010a), *β*-blockers (Bhushan and Dixit, 2012b), (*RS*)-baclofen (Bhushan and Dixit, 2012c; 2010b), and DL-SeMet (Bhushan and Dubey, 2012a, Bhushan and Lal, 2013b).

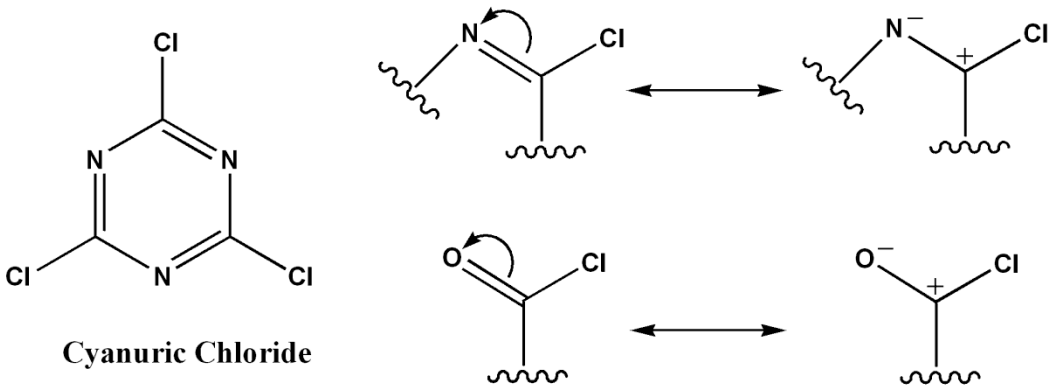


Fig. 1.4: Structure of CC and its analogy with acid chlorides

(*S*)-Nap: There has been a focus in literature on Nap for studies involving different aspects of analytical and bioanalytical studies. It is commercially available as pure (*S*)-enantiomer. (*S*)-Nap provides a chiral platform having a UV absorbing chromophore with a molar absorptivity (ε) higher than 100000 owing to methoxy substituted naphthyl residue. For its characteristic features and presence of a carboxylic acid group, certain CDRs have been synthesized by the reaction of (*S*)-Nap with different activating compounds. These include reaction of (*S*)-Nap with *N*-hydroxysuccinimide (Bhushan and Tanwar, 2008a) for enantioseparation of DL-PenA; with 1*H*-benzotriazole for enantioseparation of DL-PenA, Cys and Homocys (Bhushan and Dubey, 2011), and with hydrazine hydrate for enantioseparation of certain carbonyl compounds (Bhushan and Lal, 2012). Certain other derivatives of (*S*)-Nap that were synthesized and used as CDRs include chloroformate and isothiocyanate derivatives for enantioseparation of β-adrenoceptor antagonists (Büschges *et al.*,1996), and amine derivatives for enantioseparation of 2-arylpropionic acids (Span and Langguth, 1990).

•

Chapter-2
Experimental

I. Materials

All optically pure and racemic proteinogenic α-amino acids, glutamic acid (L-(+)-Glu), *S*-benzyl-L-Cysteine (SBLC), *S*-methyl-L-cysteine (SMLC), (*S*)-(+)-1-cyclohexylethylamine, and (*R*)-(+)-α-methyl benzylamine, (*S*)-naproxen, cyanuric chloride (CC), 1,5-Difluoro-2,4-dinitrobenzene (DFDNB), (*RS*)-propranolol hydrochloride, (*R*)- and (*S*)-propranolol hydrochloride, (*RS*)-atenolol, (*S*)-(−)-atenolol, DL-selenomethionine, L-selenomethionine, were obtained from Sigma-Aldrich (St. Louis, MO, USA). (−)-quinine was obtained from Merck (Mumbai, India). The capsules of (*RS*)-fluoxetine as Funil-60 cap (Intas pharma, Ahmedabad, Gujarat, India). Isoxsuprine hydrochloride as Vasodilan tablets (MDC, Solan, HP, India) was obtained from the local market. Orciprenaline sulfate as Alupent tablets (Zydus Healthcare, East Sikkim, India), betaxolol hydrochloride as Iobet eye drops (FDC Ltd. Bhiwadi, India) and propranolol as Ciplar (Cipla, Bombay, India Ltd.).

Glacial acetic acid, trifluoroacetic acid (TFA), triethyl amine (TEA), phosphoric acid (H_3PO_4), concentrated hydrochloric acid (HCl), sodium hydrogen carbonate ($NaHCO_3$), phosphorus pentaoxide (P_2O_5), ammonia (NH_3), Iodine, HPLC grade n-hexane, acetonitrile (MeCN), methanol (MeOH), ethanol (EtOH), n-butanol (BuOH), dichloromethane (CH_2Cl_2) and ethyl acetate 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). *N*-hydroxyphthalimide, 1*H*-benzotriazole, dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP) was obtained from Sigma-Aldrich (Bangalore, India). Silica gel G, with 13% calcium sulphate as binder having chloride, iron and lead impurities up to 0.02% and with pH 7.0 in a 10% aqueous suspension was from E. Merck (Mumbai, India). The names and abbreviations of proteinogenic *α*-amino acids and selenomethionine are given in Table-2.1 and details of pharmaceutical formulations used in present study are given in Table-2.2.

Table-2.1: Names and abbreviations of *α*-amino acids

S. No.	Amino Acids	Abbreviation	S. No.	Amino Acids	Abbreviation
1	Alanine	Ala	10	Arginine	Arg
2	Phenylalanine	Phe	11	Lysine	Lys
3	Valine	Val	12	Aspartic acid	Asp
4	Leucine	Leu	13	Glutamic acid	Glu
5	Isoleucine	Ile	14	Aspargine	Asn
6	Proline	Pro	15	Threonine	Thr
7	Methionine	Met	16	Serine	Ser
8	Trypthophan	Trp	17	Tyrosine	Tyr
9	Histidine	His	18	Selenomethionine	SeMet

Table-2.2: Details of pharmaceutical formulations

S. No.	**Compound**	**Abbrevi-ation**	**Brand name**	**Marketed By**	**Label amount of API**
1	(*RS*)-Propranolol hydrochloride	Prl	Ciplar	Cipla Ltd. (Goa, India)	40 mg
2	(*RS*)-Atenolol	Atl	Betacard	Torrent, Gujarat, India	100 mg
3	(*RS*)-Betaxolol hydrochloride	Bel	Iobet eye drops	FDC Ltd. Bhiwadi, India	0.5% w/v
4	(*RS*)-Orciprenaline sulfate	Orc	Alupent tablets	Zydus Healthcare, East Sikkim, India	10 mg
5	(±)-Isoxsuprine hydrochloride	ISP	Vasodilan tablets	MDC, Solan, HP, India	10 mg
6	(*RS*)-fluoxetine	Flx	Funil-60 cap	Intas pharma, Ahmedabad, Gujarat, India	60 mg

I. Equipments

a. HPLC System

HPLC consisting of a 10 mL pump head 1000, manager 5000 degasser, photodiode

array detector (PDA) 2600, Knauer manual injection valve and Eurochrom operating software from Knauer (Berlin, Germany).

a. HPLC Column

Reversed-phase C_{18} columns, LiChrospher and Eurospher (250 mm × 4.6 mm, I.D., 5 µm) were from Merck (Darmstadt, Germany) and Knauer (Berlin, Germany), respectively.

b. NMR Instrument

^{1}H spectra were recorded on a Bruker 500 MHz instrument (Billerica, MA, USA) using $CDCl_3$ and DMSO-d_6 as solvents.

c. IR Spectrometer

IR spectra were recorded by Perkin Elmer 1600 Fourier Transform Infrared Spectrophotometer (FT-IR) (Boardman, OH, USA) in KBr pellets.

d. CHN Analyzer

CHN analysis was performed using Elementar Analysensysteme GmbH VarioEL III (Hanau, Germany).

e. Polarimeter

A digital polarimeter from Krüss Optronic instrument (P3002) (Germany) was used to measure the optical rotation.

f. UV-Spectrophotometer

UV spectra were recorded on UV-spectrophotometer model Shimadzu UV-1601 (Japan).

g. pH Meter

Cyberscan 510 pH meter was used to maintain the pH of the solutions/buffers. It was calibrated by using buffer solutions of pH 4.0, pH 7.0 and 10.0 obtained from E. Merck (Mumbai, India).

h. Milli-Q Water Purifier

The Milli-Q system of Millipore (Bedford, USA) to obtain deionised water (18.2MΩ cm^3) from double distilled water.

i. Microwave

The microwave Multiwave 3000 (800 W, Perkin-Elmer, Shelton, CT, USA) was used to conduct the reactions.

II. Isolation of the Racemic Drugs from Pharmaceutical Formulations

A. Extraction of *β*-Blockers from Commercial Tablets

Ten Alupent tablets, each containing 10 mg of (*RS*)-Orc, were ground to a fine powder and dissolved in 20 mL MeOH by sonication at 25 °C. The solution was filtered through Whatman paper (125 mm pore diameter). The same procedure was repeated twice with the residue. Further the combined filtrate was concentrated in vaccuo and kept at 4 °C until crystals appeared. Then the mother liquor was decanted and the crystals were further purified by recrystallization with MeOH. The obtained crystals were washed with diethyl ether and dried in vacuum desiccator. To confirm the purity of product, melting point and UV absorption (λ_{max}) was recorded which was matching with literature reports (United State National library of medicine). Remaining beta blockers were also extracted, isolated and purified by same procedure. The recoveries were of the order of 95–98% of the quantities reported on the commercial labels (*cf*., Table-2.2). The purified compounds were used as racemic standards and their stock solution were prepared in 1 M $NaHCO_3$. These were filtered through a 0.45 μm filter and used for subsequent derivatization.

B. Extraction of Flx

Ten capsules of (*RS*)-Flx, each containing 60 mg, and dissolved in 20 mL MeOH by sonication at 25 °C. The solution was filtered through Whatman paper of 125 mm pore diameter. The same procedure was repeated a few times with the residue. Further the combined extract was filtrate and concentrated in vaccuo and kept at 4 °C until crystals appeared. Then the mother liquor was decanted and the crystals were dried, further purified by recrystallization with methanol-water. Melting point and UV and absorption (λ_{max}) was recorded to confirm the purity of product. The recovered quantity was in the order of 93–95% of the reported on the commercial labels (*cf*., Table-2.2).

III. Preparation of Stock Solutions

The following stock solutions were prepared

a. $NaHCO_3$ (0.1 M and 1M) and HCl (1 M) in purified water.
b. All proteinogenic amino acids in 1M $NaHCO_3$.
c. (*RS*)-Flx (50 mM) was prepared by dissolving appropriate amount in 0.1 M $NaHCO_3$.
d. All racemic *β*-blockers, (*S*)-Atl and (*S*)-Prl (50 mM) in 1 M $NaHCO_3$.
e. DL-SeMet and L-SeMet (1 mM) in 0.1 M $NaHCO_3$

All solutions were filtered through a 0.45 μm filter prior to use.

IV. Synthesis of CDRs

Based on the experimental strategy discussed in preamble to present studies following types of CDRs (17 in total) were synthesized and characterized. The CDRs with their parent moiety and the number assigned to them are mentioned in Table-2.3.

CDRs based on DFDNB (Set A and B): For this purpose, two types of chiral auxiliaries were selected for replacement of fluorine of DFDNB by nucleophilic attack. These were, (i) L-amino acids, (S-methyl-L-cysteine, S-benzyl-L-cysteine, L-Ala, L-Leu, L-Phe, L-Met and L-Val), and (ii) optically pure amines; (*S*)-(+)-1-cyclohexylethylamine, and (*R*)-(+)-α-methyl benzylamine.

Nine CDRs were synthesized

- Seven CDRs having amino acids as chiral auxiliaries (Set A; CDR 1-7; Fig. 2.1)

- Two CDRs having amines as chiral auxiliaries (Set B; CDR 8-9; Fig. 2.2)

CDRs Based on CC (Set C): The chiral auxiliaries chosen for this purpose were *S*-methyl-L-cysteine, *S*-benzyl-L-cysteine, L-Ala, L-Leu, L-Met and L-Val.

- Six CDRs were synthesized using optically pure amino acids auxiliaries on CC backbone (Set C; CDRs 10-15; Fig. 2.3)

CDRs based on (*S*)-Nap (**Set D**): CDRs based on (*S*)-Nap moiety were synthesized by nucleophilic attack of 1*H*-benzotriazole and *N*-hydroxyphthalimide, on carbonyl carbon of the carboxylic group of (*S*)-Nap in presence of dicyclohexylcarbodiimide (DCC) as coupling agent.

The two (*S*)-Nap based CDRs were:

- N-phthalimidyl-(*S*)-2-(6-methoxynaphth-2-yl) propionate, (Nap-Phth) (CDR 16; Fig. 2.4)
- {(*S*)-1-[1H-benzo(d) (1,2,3) triazol-1-yl}-2-{6-methoxynapthalen-2-yl-propan-1-one]; Nap-Btz}(CDR 17; Fig. 2.4)

A. Synthesis of DFDNB based CDRs (*cf.*, Table-2.3)

The structures of all nine CDRs are shown in Fig. 2.1 and 2.2. Representative synthetic procedure for CDR 1 is given below (Fig. 2.5). A few solutions were prepared for the purpose of synthesis of the CDR 1.

(i) 1 M $NaHCO_3$ in distilled water,
(ii) 0.1 M solution of SMLC in (i), and
(iii) 0.2 M solution of DFDNB in acetone-water (6:4, *v/v*); it was maintained at 0–4 ^oC

Synthesis of CDR 1: 10 mL of (ii) was added to 10 mL of (iii)) with constant stirring, in a round bottom flask. The reaction mixture was constantly stirred at 40 °C; after 1 h, the resulting solution was kept at room temperature for 5 min and then 20 mL water was added to it. Further addition of 10 mL HCl (1 M) yielded yellow crystals; these were

Table-2.3: Summary of DFDNB, CC and (*S*)-Nap based CDRs

DFDNB based CDRs				**CC based CDRs**		**(*S*)-Nap based CDRs**	
Set A		**Set B**		**Set C**		**Set D**	
CDR No.	**Chiral auxiliary**	**CDR No.**	**Chiral auxiliary**	**CDR No.**	**Chiral auxiliary**	**CDR No.**	**Chiral auxiliary**
1	SMLC	8	(*S*)-(+)-1-cyclohexylethylamine	10	SBLC	16	(*S*)-Nap coupled with *N*-hydroxyphthalimide
2	SBLC	9	(*R*)-(+)-α-methyl benzylamine	11	SMLC	17	(*S*)-Nap coupled with 1*H*-benzotriazole
3	L-Met			12	L-Met		
4	L-Leu			13	L-Leu		
5	L-Val			14	L-Val		
6	L-Phe			15	L-Ala		
7	L-Ala						

filtered and washed with cold water. The crystals were dried in desiccator over P_2O_5. The characterization data of CDR is given below.

CDR 1: 1-Fluoro-2, 4-dinitrophenyl-5- S-methyl-L-cysteine

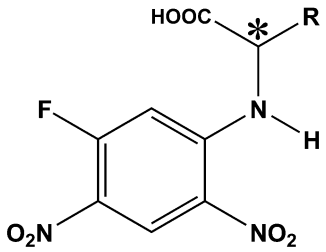
Colour: yellow; Yield: 89%; mp: 236 °C; UV (nm, in MeCN): 331 (λ_{max}); IR (KBr): 3500, 3299, 3126, 3069, 2913, 1734, 1698, 1635, 1613, 1578, 1429, 1293, 1054, 908, 837, 738, 695, 647, cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$-d_6) δ: 2.13 (s, 3H, –S–CH_3), 2.81–2.98 (d, 2H,–CH_2), 3.61(m, 1H,–CH), 3.88-3.91 (d, 1H, –NH), 6.74 (s, 1H, Ar–H), 8.59 (s, 1H, Ar–H), 10.6 (s, 1H, –COOH); Anal. Calcd for $C_{10}H_{10}FN_3O_6S$: C, 37.62%; H, 3.16%; N, 13.16%. Found: C, 37.55%; H, 3.14%; N, 13.12%.

CDR 3: 2-(5-fluoro-2,4-dinitrophenylamino)-4-(methylthio)butanoic acid (FDNP-L-Met)

Color: yellow; Yield: 0.28 g, 86%; mp: 194 °C; UV (nm, in MeCN): 331 (λ_{max}); IR (KBr): 3485, 3089, 2913, 1692, 1614, 1586, 1422, 1293, 1062, 908, 833, 731 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ: 2.09 (3H, s, SCH_3), 2.13-2.19 (2H, m, CH_2), 2.53–2.57 (2H, t, CH_2S), 3.59-3.64 (1H, m, CH), 3.91-3.94 (1H, d, NH), 6.76 (1H, s, Ar–H), 8.65 (1H, s, Ar–H), 10.2 (1H, s, COOH); Elemental analyses, Found: C, 39.49%; H, 3.66%; N, 12.56% Calc. for $C_{10}H_{10}FN_3O_6S$: C, 39.64%; H, 3.63%; N, 12.61%.

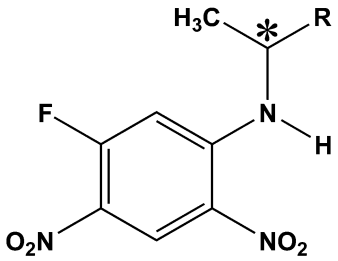
CDR 8: 1-Fluoro-2, 4-dinitrophenyl-5-(S)-(+)-1-cyclohexylethylamine

Colour: yellow; Yield: 90%; mp: 212 °C; UV (nm, in MeCN): 333(λ_{max}); IR (KBr): 3320, 3109, 2925, 2850, 1582, 1525, 1426, 1373, 1327, 1283, 1243, 1122, 1050, 920, 881, 832, 708, 650, 596 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$-d_6) δ: 1.8–1.9 (d, 3H, –CH_3), 1.1-1.4 (m, 11H, –C_6H_{11}), 3.50–3.52 (d,1H, –NH), 6.61(s, 1H, Ar–H), 9.13 (s, 1H, Ar–H); Anal. Calcd for $C_{14}H_{18}FN_3O_4$: C, 54.01%; H, 5.83%; N, 13.50%. Found: C, 54.15%; H, 5.79%; N, 13.76%.



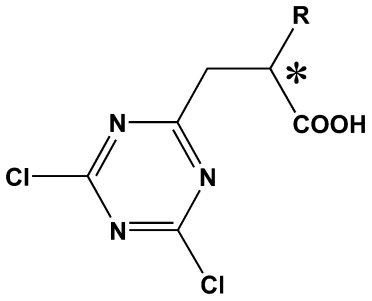
CDR No.	Name	R
1	1-Fluoro-2, 4-dinitrophenyl-5-S-methyl-L-cysteine	$-CH_2SCH_3$
2	1-Fluoro-2, 4-dinitrophenyl-5-S-benzyl-L-cysteine	$-CH_2SCH_2C_6H_5$
3	1-Fluoro-2, 4-dinitrophenyl-5-L-Met	$-CH_2CH_2SCH_3$
4	1-Fluoro-2, 4-dinitrophenyl-5-L-Leu	$-CH_2CH(CH_3)_2$
5	1-Fluoro-2, 4-dinitrophenyl-5-L-Val	$-CH(CH_3)_2$
6	1-Fluoro-2, 4-dinitrophenyl-5-L-Phe	$-CH_2C_6H_5$
7	1-Fluoro-2, 4-dinitrophenyl-5-L-Ala	$-CH_3$

Fig. 2.1: Structures of CDRs of Set A



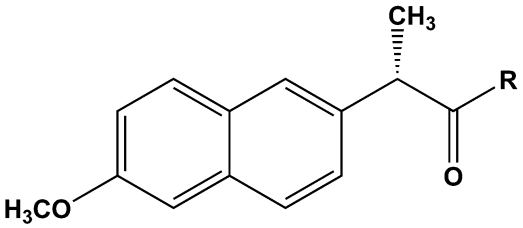
CDR No.	Name	R
8	1-Fluoro-2, 4-dinitrophenyl-5-(*S*)-(+)-1-cyclohexylethylamine	$-C_6H_{11}$
9	1-Fluoro-2, 4-dinitrophenyl-5-(*R*)-(+)-*α*-methyl benzylamine	$-C_6H_5$

Fig. 2.2: Structures of CDRs of Set B



CDR No.	Name	R
10	N-(4,6-Dichloro-[1,3,5] triazine-2-yl)-S-benzyl-L-cysteine	$-CH_2SCH_2C_6H_5$
11	N-(4,6-Dichloro-[1,3,5] triazine-2-yl)-S-methyl-L-cysteine	$-CH_2SCH_3$
12	N-(4,6-Dichloro-[1,3,5] triazine-2-yl)- L-Met	$-CH_2CH_2SCH_3$
13	N-(4,6-Dichloro-[1,3,5] triazine-2-yl)- L-Leu	$-CH_2CH(CH_3)_2$
14	N-(4,6-Dichloro-[1,3,5] triazine-2-yl)- L-Val	$-CH(CH_3)_2$
15	N-(4,6-Dichloro-[1,3,5] triazine-2-yl)-L-Ala	$-CH_3$

Fig. 2.3: Structures of CDRs of Set C



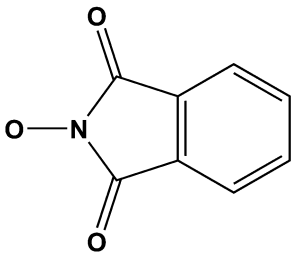
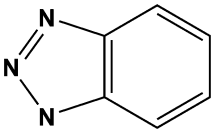
CDR No.	**16**	**17**
Name	*N*-phthalimidyl-(*S*)-2-(6-methoxynaphth-2-yl) propionate, (Nap-Phth)	{(*S*)-1-[1H-benzo(d) (1,2,3) triazol -1-yl}-2-{6-methoxynaptha len-2-yl-propan-1-one]; Nap-Btz}
-R		

Fig. 2.4: Structures of CDRs of Set D

CDR 9: 1-Fluoro-2,4-dinitrophenyl-5-(R)-(+)-α-methyl benzylamine

Colour: yellow; Yield: 85%; mp: 195 °C; UV (nm, in MeCN): 329 (λ_{max}); IR (KBr): 3367, 3098, 2975, 2918, 2879, 1635, 1514, 1423, 1333, 1291, 1051, 920, 873, 827, 741, 703, 566, 527 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$-d_6) δ: 1.70-1.72 (d, 3H, -CH_3), 4.65–4.70 (m, 1H, –CH), 6.41–6.44 (d, 1H, Ar–H), 7.30–7.34 (m, 3H, Ar–H), 7.38–7.41(d, 2H, Ar–H), 8.86–8.87 (d, 1H, Ar–H), 9.13–9.11 (d, 1H, –NH); Anal. Calcd for $C_{14}H_{12}FN_3O_4$: C, 55.08%; H, 3.96%; N, 13.77%. Found: C, 55.21%; H, 3.93%; N, 13.71%. ^1H NMR spectrum of CDR 9 is shown in Fig. 2.6.

The CDRs 1-9 were synthesized having chiral amino acids (CDRs 1-7 of Set A) and chiral amines (CDRs 8-9 of Set B) as chiral auxiliaries (as given in table 2.3 for CDR 1-9). These chiral amino acids (for CDR 1-7) and amines (for CDR 8-9) were chosen because they increase the molar absorptivity of dinitro benzene moiety by direct conjugation of non bonding electron pair of their amino group with the para positioned –NO_2 group. These chiral auxiliaries contain hydrophobic groups (as shown in Fig. 2.1 and 2.2) which enhance the hydrophobicity of the CDRs and are thus expected to influence the separation.

B. Synthesis of CC based CDRs (*cf.*, Table-2.3)

The structures of all 6 CDRs (CDRs 10-15) are shown in Fig. 2.3. Representative synthetic procedure for CDR 10 is given below (Fig. 2.7). A few solutions were prepared for the purpose of synthesis.

(i) 1 M Na_2CO_3 in distilled water
(ii) 0.5 M solution of SMLC in (i), and
(iii) 1.0 M solution of CC in acetone (10 mL); it was maintained at 0–4 ^oC

Synthesis of CDR 10: 10 mL of (ii) was added to 10 mL of (iii)) with constant stirring, in a round bottom flask and 20 mL of acetone was added in it. The reaction mixture was constantly stirred at 20 °C; after 1 h. Then 10 mL of water was added in resultant solution and acetone was removed under reduced pressure. The product was getting crystallization as removing the acetone. Further, the precipitate was filtered and washed with ice cold water. The filtrate was treated with chloroform to extract and then evaporated to dryness in

vaccuo to give another crop of product. The characterization data of CDR is given below. ^{1}H NMR spectrum is shown in Fig. 2.8.

CDR 10: N-(4-Chloro-6-methoxy-[1,3,5]triazine-2-yl)-*S*-benzyl-L-cysteine

Yield: 86%; color: white; UV in MeCN, 231 nm (λ_{max}); mp: 90-93 °C; IR (KBr): 3427, 3031, 2825, 2523, 1625, 1534, 1478, 1401, 1326, 1206, 1128, 1043, 921, 816, 714, 594 cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 3.06-3.08 (d, 2H, -CH_2-S), 3.76 (s, 2H, -S-CH_2), 4.21-4.23 (d, 1H, -CH-N), 6.27-7.26 (m, 5H, -C_6H_5), 8.96–8.98 (d, 1H, -NH), 9.10 (s, 1H, -COOH) ; Anal. Calcd for $C_{13}H_{12}Cl_2N_4O_2S$: C, 43.46%; H, 3.37%; N, 15.60%. Found: C, 43.39%; H, 3.38%; N, 15.64%. ^{1}H NMR spectrum is shown in Fig. 2.8.

CDR 11: N-(4-Chloro-6-methoxy-[1,3,5]triazine-2-yl)-*S*-methyl-L-cysteine

Yield: 84%; color: white; UV in MeCN, 230 nm (λ_{max}); mp: 175-178 °C; IR (KBr): 3425, 2975, 1689, 1631, 1620, 1573, 1472, 1369, 1217, 1137, 1048, 814, 628 cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 3.05 (s, 3H, -S-CH_3), 3.74-3.77 (m, 2H, -CH_2-S), 4.23-4.26 (m, 1H, -CH-N), 8.94–8.95 (d, 1H, -NH), 9.08 (s, 1H, -COOH) ; Anal. Calcd for $C_7H_8Cl_2N_4O_2S$: C, 29.69%; H, 2.85%; N, 19.79%. Found: C, 29.64%; H, 2.86%; N, 23.72%.

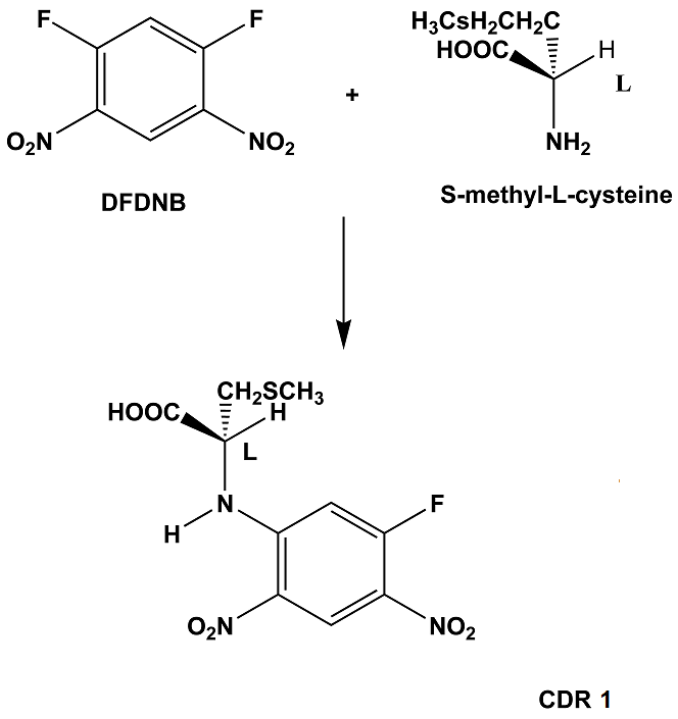


Fig. 2.5: Scheme for synthesis of DFDNB based CDR

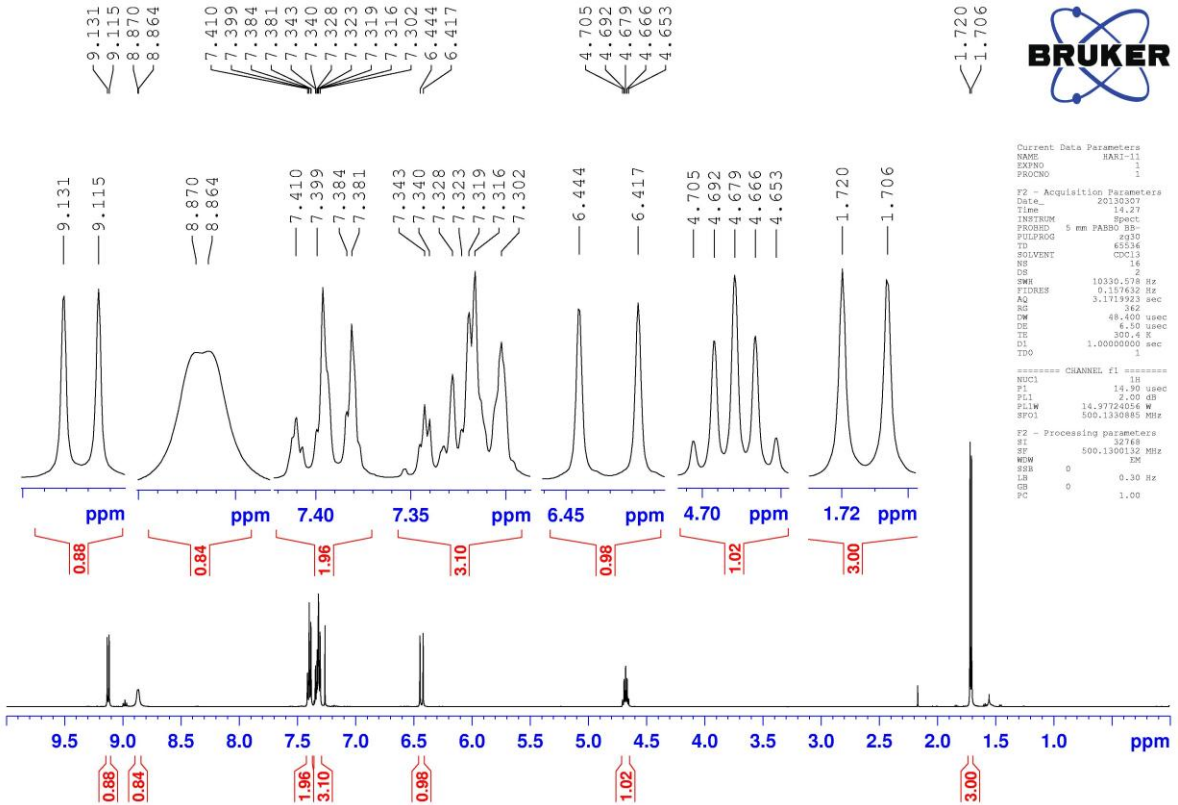


Fig. 2.6: ^{1}H-NMR spectrum of CDR 9

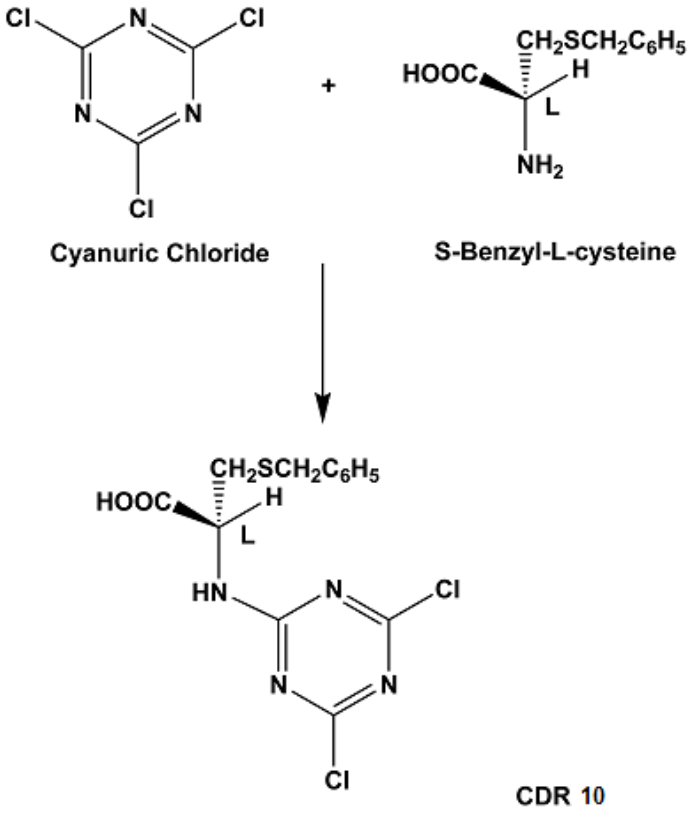


Fig. 2.7: Scheme for synthesis of CC based CDRs

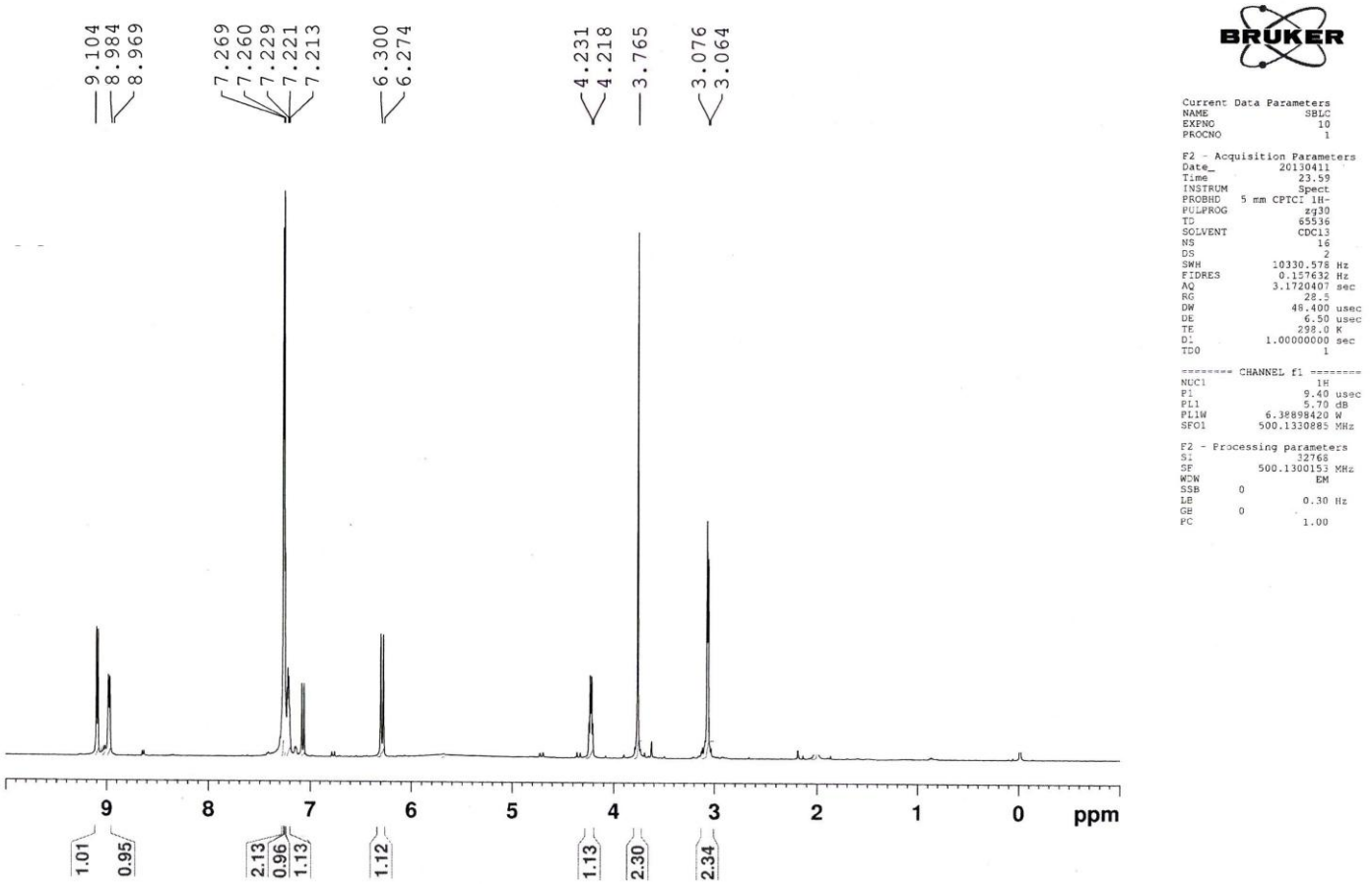


Fig. 2.8: ^{1}H-NMR spectrum of CDR 10

C. Synthesis of (*S*)-Nap based CDRs (*cf*., Table-3.3)

The structures of two CDRs (16 and 17) are shown in Fig. 2.4. Representative synthetic procedure for CDR 16 is given below (Fig. 2.9).

Synthesis of Nap-Phth (CDR 16):

A solution of DCC (262 mg, 1.6 mmol, in 5 mL dry THF) was dropwise added to a stirred solution of 322 mg (1.4 mmol) of (*S*)-Nap and 228 mg (1.4 mmol) of *N*-hydroxyphthalimide (in 3 mL THF) taken in a small round bottomed flask. The methodology for the synthesis of the CDR was the same as reported for the reaction of (*S*)-Nap with N-hydroxysuccinimide in presence of DCC (Bhushan and Tanwar, 2008a). The final product was recrystallized with hot ethanol to give the desired CDR as white solid. The NAP-Phth reagent was characterized by IR, UV, Elemental analysis and ^{1}H-NMR. The general scheme for the synthesis of NAP-Phth reagent is shown in Fig. 2.9. The ^{1}H-NMR spectrum of the CDR is shown in Fig. 2.10. The characterization data is given below.

Yield 515 mg (93.7%); m.p. 150-153°C; $[\alpha]^{25}/_{D}$ = +24.3° (*c* = 0.015, MeOH); UV (nm, in MeOH) 218, 231 (λ_{max}), 263, 273; IR (KBr) 3303, 2933, 2357, 1731, 1652,1673,1604, 1529, 1465, 1387, 1265, 1227,1175, 1052, 1032, 963, 893, 820, 789, and 648 cm^{-1}; ^{1}H NMR (500MHz, DMSO-*d*6) δ 1.35-36 (d, 3H, CH_3CH), 3.71-3.75 (q, 1H, $CHCH_3$), 3.77 (s, 3H, H_3CO), 7.04 (d, 1H, Nap–5–H), 7.06 (d, 1H, Nap–7–H), 7.17 (dd, 1H, Nap–3–H), 7.31 (d, 1H, Nap–1–H),7.56 (d, 1H, Nap–4–H), 7.58 (d, 1H, Nap–8–H), 7.68 (m, 2H, Phth–4,5–H), 8.23 (d, 2H, Phth–3,6–H); Elemental analysis: Calcd for $C_{22}H_{17}NO_5$: C, 70.39%; H, 4.56%; N, 3.73%. Found: C, 70.33%; H, 4.69%; N, 3.67%.

V. Determination of Enantiomeric Purity of CDRs

The enantiomeric purity of the CDRs 1-17 is based on the enantiomeric purity of the chiral auxiliaries (amino acids, amines and (*S*)-Nap) used for their synthesis and was established as per previously reported methods (Bhushan and Kumar, 2008b, Bhushan, 2011). The purity of the sample and its enantiomeric purity are two different parameters. Enantiomeric excess (*ee*) of the chiral auxiliaries (amino acids, amines and (*S*)-Nap) was

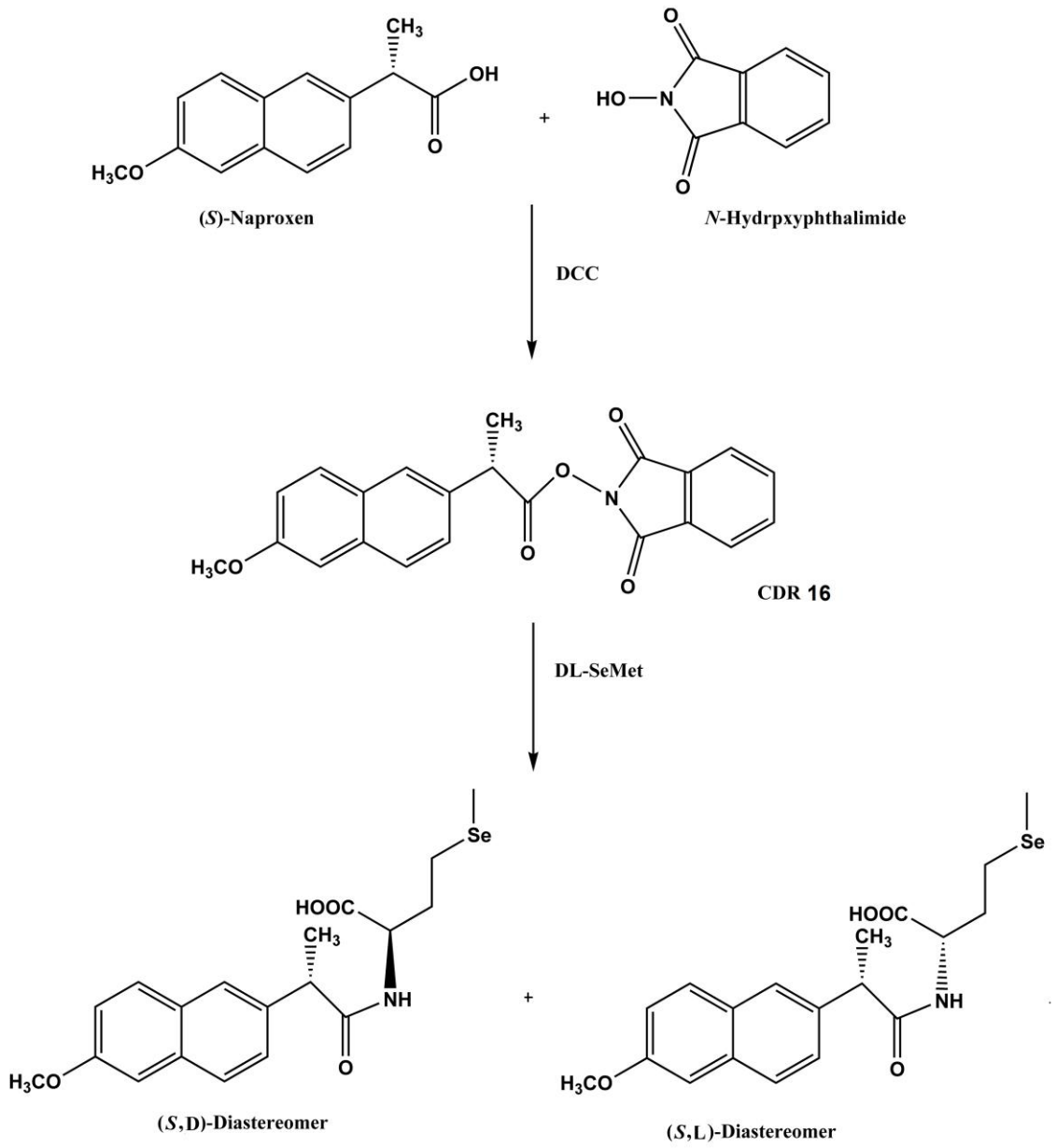


Fig. 2.9: Scheme for synthesis of NAP-Phth reagent and amide diastereomers of SeMet with it

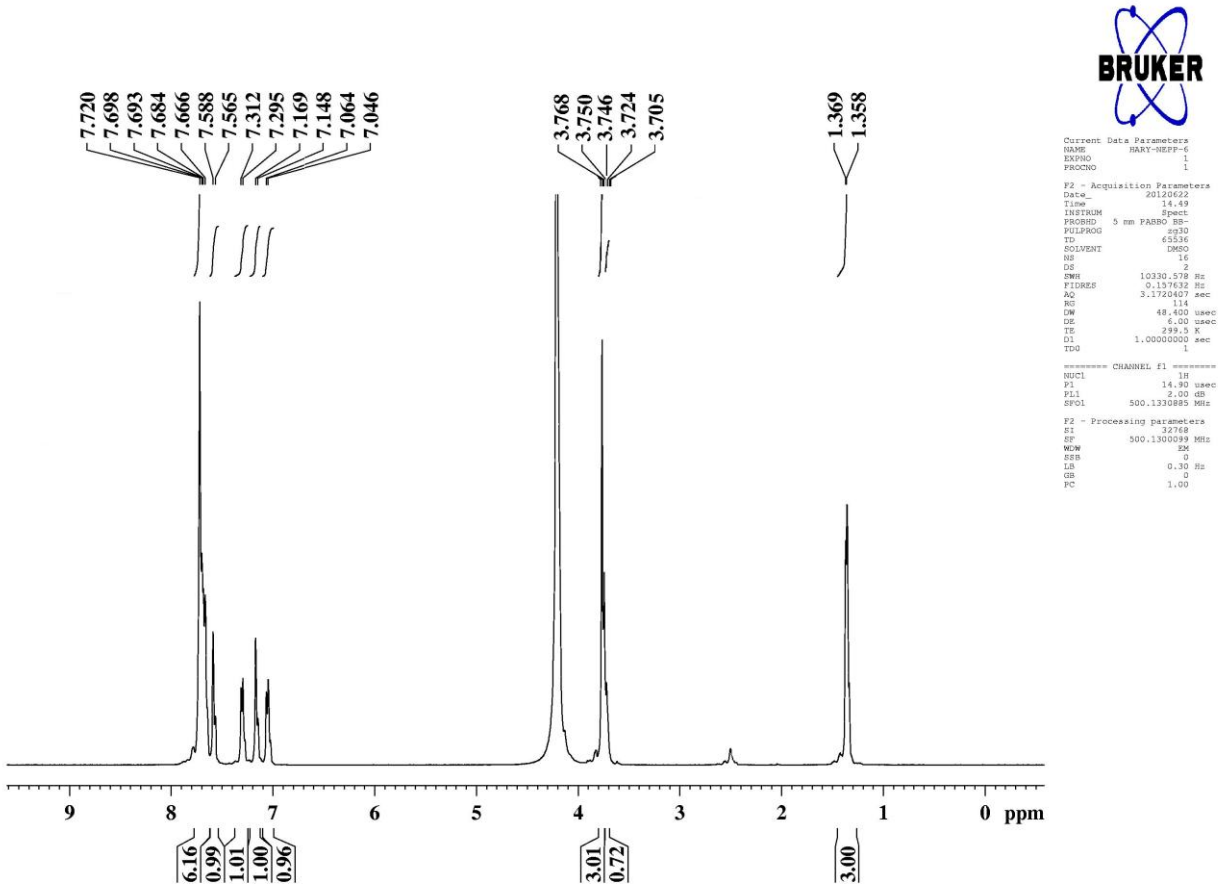


Fig. 2.10: ^{1}H-NMR spectrum of NAP-Phth reagent (CDR 16)

established by comparing their specific rotation as mentioned in the literature against their specific rotation as observed under standard conditions in the laboratory. A representative example of the methodology adopted for establishing enantiomeric purity of CDRs is described below. The chiral auxiliary of CDR 4, *i.e.*, L-Leu was obtained from Sigma–Aldrich and the catalogue (www.sigmaaldrich.com) described its specific rotation, $[\alpha]_D^{25}$ = +15.5° (c=5% in 5 M HCl). The value was further examined under standard conditions in the laboratory and *ee* was found to be 98%. The yield of the CDR was 89%. As a principle of synthesis, the CDR corresponding to the very small % of the other enantiomer (say, 1%, as mentioned above) would be negligible and stands removed during the process of crytallization and recrystallization (remaining in the mother liquor). This, in other words, can be considered as equivalent to fractional crystallization.

When the racemic analyte, say (*RS*)-Flx was reacted with the CDR and the product (i.e. the diastereomeric mixture) so obtained was investigated on RP-column using different mobile phases, appearance of a pair of diastereomic peak detected by PDA detector confirmed the enantiomeric purity of the CDR (and the separation of the two diastereomers of the analyte). Thus the recrystallized CDR was enantiomerically pure. The presence of only two diastereomeric peaks corresponding to (*RS*)-Flx in itself is a confirmation of the enantiomeric purity of the CDR. If the CDR arising from 1% of the D-isomer of Leu was present, the diastereomers of (*RS*)-Flx would have formed resulting in the corresponding diastereomeric peaks in the chromatogram. The same procedure was applied to check the chiral purity of the rest of the CDRs.

The enantiomeric excess (*ee*) of the amines and (*S*)-Nap used as chiral auxiliaries was taken as given in the catalogue (www.sigmaaldrich.com). The procedure for determination of chiral purity of the CDRs prepared from these chiral amines and (*S*)-Nap remains the same as described above.

The diastereomers were synthesized under microwave irradiation (MWI) as described in the following paragraphs. However, diastereomers were also synthesized using conventional heating and vortexing, and results were compared for the separation of diastereomers synthesized using the two approaches.

VI. Synthesis of Diastereomers

The diastereomers were synthesized under MWI and by vortexing as described in the following paragraphs. However, the diastereomers were also synthesized using conventional heating with constant stirring and results were compared for the separation of diastereomers synthesized using the two approaches.

A. Synthesis of Diastereomers of *β*-blockers

Diastereomers of *β*-blockers were synthesized by DFDNB and CC based CDRs.

1. Diastereomers of *β*-blockers Synthesized with DFDNB based CDRs

The diastereomers of five *β*-blockers with all the nine CDRs (1-9) were synthesized according to the literature report (Bhushan and Kumar, 2008b; Bhushan and Kumar, 2009b; Bhushan and Tanwar, 2008b) using MW irradiation as well as conventional heating. Fig. 2.11 is showing the synthesis of diastereomers of Prl with CDR 1.

Following solutions were prepared for the purpose of synthesis of diastereomers.

i. 1M $NaHCO_3$ in distilled water
ii. 50 mM solution of (*RS*)-Prl in (i), and by dissolving 12.97 mg of (*RS*)-Prl in 1 mL of (i)
iii. 25 mM solution of CDR 1 in acetone by dissolving 7.98 mg of CDR 3 in 1 mL acetone; it was maintained at 0–4 ^{o}C.

Solution of (*RS*)-Prl (as at (ii) above) was added to the solution of CDR 1 (as at (iii) above) in a teflon tube. Following reaction conditions were tried to obtain an optimum reaction yield. (*RS*)-Prl: CDR were allowed to react in different ratios such as, 1:1, 1:1.5, 1: 1.7 and 1:2. The reaction mixtures were irradiated in microwave oven for 30, 40, 50, 60 and 70 s at each of the three different power settings, viz, 70, 80 and 90%. Separate sets of reaction mixture were incubated at 30, 40, 45 and 50 °C with constant stirring for 30, 40, 50, 60, 70, 80 and 90 min (at each temperature). The peak areas of each pair of the diastereomers, obtained in HPLC experiments, were taken as a diagnosis for the formation of the diastereomers and their separation.

Same procedure was applied for the synthesis of diastereomers of Prl using the CDRs (2, 8 and 9), Orc, Bel and Atl using the CDRs (1-5 and 7-9) and ISP using the CDRs (2, 5 and 7).

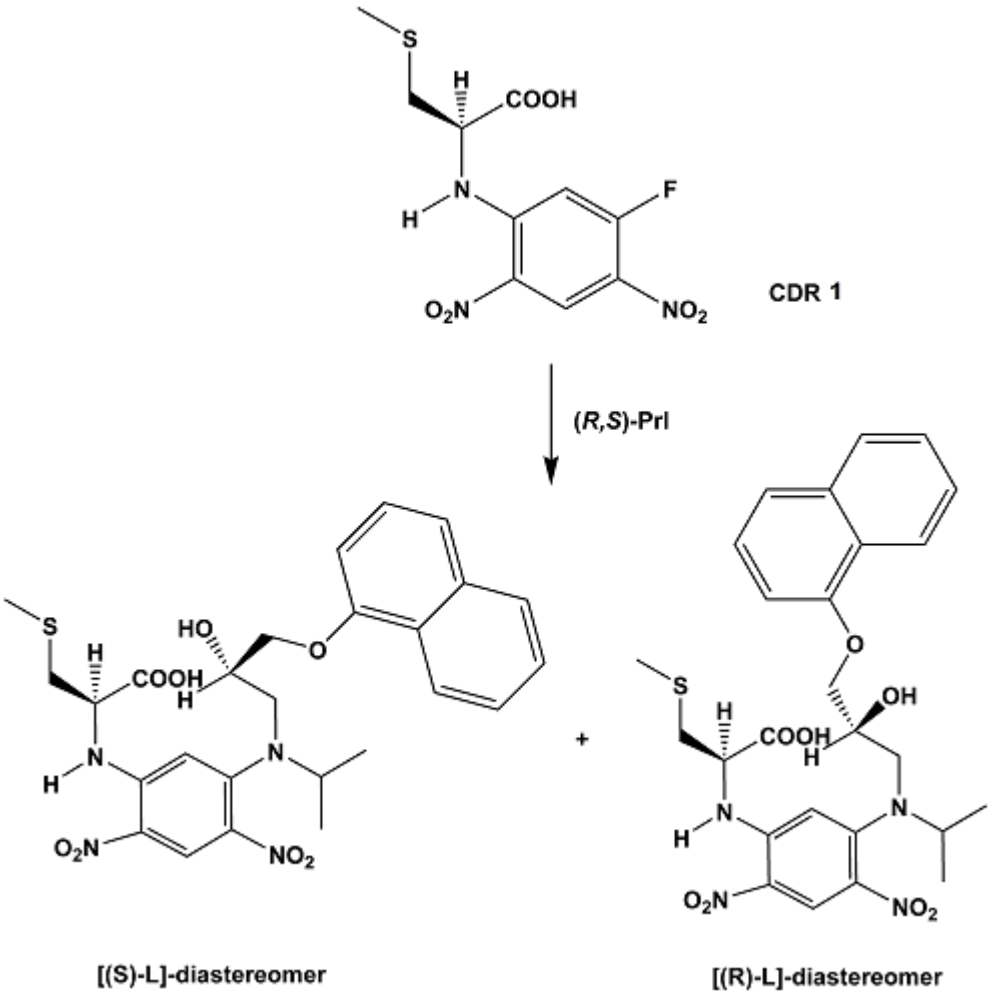


Fig. 2.11: Scheme of derivatization of (*RS*)-Prl with CDR 1

A 10 µL volume of the resulting solution containing diastereomers, was diluted 10 times with MeCN and 20 µL of it was injected onto the column. The resulting solution was degassed and filtered before injection.

2. Diastereomers of *β*-blockers Synthesized with CC based CDRs

The diastereomers of three *β*-blockers with all the six CDRs (10-15) were synthesized. according to the literature report (Bhushan and Kumar, 2008b; Bhushan and dixit, 2012b) using MWI as well as conventional heating. Fig. 2.12 is showing the synthesis of diastereomers of Atl with CDR 10.

Following solutions were prepared for the purpose of synthesis of diastereomers.

(i) 1 M $NaHCO_3$ in distilled water

(ii) 50 mM solution of (*RS*)-Atl in (i), and by dissolving 17.9 mg of (*RS*)-Atl in 1 mL of (i)

(iii) 50 mM solution of CDR 10 in MeCN by dissolving 13.3 mg of CDR 10 in 1 mL acetone; it was maintained at 0–4 ^{o}C.

Solution of (*RS*)-Atl (as at (ii) above) was added to the solution of CDR 10 (as at (iii) above) in a teflon tube. Following reaction conditions were tried to obtain an optimum reaction yield. (*RS*)-Atl: CDR was allowed to react in different ratios such as, 1:1, 1:1.5, 1: 1.7 and 1:2. The reaction mixtures were irradiated in microwave oven for 45, 60, 75 and 90 s at each of the three different power settings, viz, 75, 80, 85 and 90%. Separate sets of reaction mixture were incubated at 35, 40 and 45 °C with constant stirring for 50, 60, 70 and 80 min (at each temperature). 10 µL of the resulting solution of diastereomers was taken and diluted 10 times with MeCN, 20 µL of it was injected onto column. The same method was followed for the synthesis of diastereomers of (*RS*)-Atl using the remaining CC reagents (CDR 11-15) and for synthesis of diastereomers of (*RS*)-Orc and (*RS*)-Bel using all CC reagents (CDR 10-15).

B. Synthesis of Diastereomers of (*RS*)-Flx

Diastereomers of (*RS*)-Flx were synthesized by DFDNB and CC based CDRs.

1. Diastereomers of (*RS*)-Flx with DFDNB based CDRs

The diastereomers of (*RS*)-Flx with all four CDRs (1, 2, 4 and 5) were synthesized according to the literature report (Bhushan and Kumar, 2008b; Bhushan and Kumar, 2009b; Bhushan and Agarwal, 2010) using MW irradiation as well as conventional heating. CDR 1 was taken as representative for synthesis of diastereomers of (*RS*)-Flx. Fig. 2.13 is showing the synthesis of diastereomers of (*RS*)-Flx with CDR 1.

Following solutions were prepared for the purpose of synthesis of diastereomers.

(i) 1M $NaHCO_3$ in distilled water

(ii) 50 mM solution of (*RS*)-Flx in (i), and by dissolving 15.5 mg of (*RS*)-Flx in 1 mL of (i) and,

(iii) 25 mM solution of CDR 1 in acetone by dissolving 7.98 mg of CDR 1 in 1 mL acetone; it was maintained at 0–4 ^{o}C

Solution of (*RS*)-Flx (as at (ii) above) was added to the solution of CDR 1 (as at (iii) above) in a teflon tube. Following reaction conditions were tried to obtain an optimum reaction yield. (*RS*)-Flx and CDR 1 were allowed to react in different ratios such as, 1:1.5, 1:1.7 and 1:2. The reaction was carried out, (i) by MWI (for 50, 60, 70 and 80 s at each of the three different power settings, viz, 70, 80 and 90%) and, (ii) under incubation (at 35, 40, 45 and 50 °C) with constant stirring for 30, 40, 50, 60, 70, 80 and 90 min (at each temperature). A 10 μL volume of the resulting solution containing diastereomers, was diluted 10 times with MeCN and 20 μL of it was injected onto the column. The resulting solution was degassed and filtered before injection. Same reaction condition were applied for the synthesis of diastereomers of; (*RS*)-Flx using the CDRs (2, 4 and 5).

2. Diastereomers of (*RS*)-Flx Synthesized with CC based CDRs

The diastereomers of (*RS*)-Flx with four CDRs (10, 11, 13 and 14) were synthesized according to the literature report (Bhushan and Kumar, 2008b; Bhushan and dixit, 2012b) using MW irradiation as well as conventional heating. CDR 10 was taken

as representative for synthesis of diastereomers of (*RS*)-Flx**.** Fig. 2.14 is showing the synthesis of diastereomers of (*RS*)-Flx with CDR 10.

Following solutions were prepared for the purpose of synthesis of diastereomers.

(i) 1M NaHCO3 in distilled water

(ii) 50 mM solution of (*RS*)-Flx in (i), and by dissolving 15.5 mg of (*R*,*S*)-Flx in 1 mL of

(iii) 50 mM solution of CDR 10 in MeCN by dissolving 13.3 mg of CDR 10 in 1 mL acetone; it was maintained at 0–4 ^{o}C.

Solution of (*RS*)-Flx (as at (ii) above) was added to the solution of CDR 10 (as at (iii) above) in a teflon tube. Following reaction conditions were tried to obtain an optimum reaction yield. (*RS*)-Flx and CDR were allowed to react in different ratios such as, 1:1, 1:1.5, 1: 1.7 and 1:2. The reaction was carried out, (i) by MWI (for 50, 60, 70 and 80 s at each of the three different power settings, viz, 70, 80 and 90%) and, (ii) under incubation (at 35, 40, 45 and 50 °C) with constant stirring for 30, 40, 50, 60, 70, 80 and 90 min (at each temperature). A 10 μL volume of the resulting solution containing diastereomers was diluted 10 times with MeCN and 20 μL of it was injected onto the column. The resulting solution was degassed and filtered before injection. Same reaction condition were applied for the synthesis of diastereomers of; (*RS*)-Flx using the CDRs (11, 13 and 14).

C. Synthesis of Diastereomers of DL-SeMet

Diastereomers of DL-SeMet were synthesized by (*S*)-Nap and DFDNB based CDRs.

i. Diastereomers of DL-SeMet Synthesized with (*S*)-Nap based CDRs

The diastereomers of DL-SeMet were prepared using twofold molar excess of CDR 16 under MWI and vortexing. The structures of diastereomeric pair [(*S*, D)- or (*S*, L)-diastereomers] of DL-SeMet prepared with CDR 16 are shown in Fig. 2.9. The first letter (*S*) represents the configuration of CDR and (L or D) second letter is for the configuration of DL-SeMet.

Solution of DL-SeMet (40 μL, 40 nmol) was placed in a 1.5 mL vial. The solution of the CDR 16 in MeCN (80 μL, 80 nmol) and 5 μL of TEA were added to the vial. Thus, the DL-SeMet and CDR 16 were in the mole ratio of 1:2. The reaction mixture

was then irradiated with microwave (MW) for 40s at 80% (of 800W) and then cooled to room temperature. The reaction was quenched by addition of acetic acid (1M, 40 µL). The diastereomers of all the analytes were also synthesized by vortexing the reaction mixture (in the same mole ratio as used for MW based synthesis) for 10 min (vortexing 500 rpm) at room temperature. Aliquots (10 µL) of resulting solution of diastereomers were diluted 10 times with MeCN and injected (20 µL) on to the column.

The experimental conditions for synthesis of diastereomeric pairs of DL-SeMet with CDR 16 were optimized with respect to the effect of pH, reagent excess, reaction time, MWI and vortexing time. The reaction time was varied from 3 to 12 min under vortexing (500 rpm) and 10 to 60 s at 80% microwave power (800W), within the pH range of 8-11 and SeMet: CDR in a ratio of 1:1 or1:1.5 or 1:2.

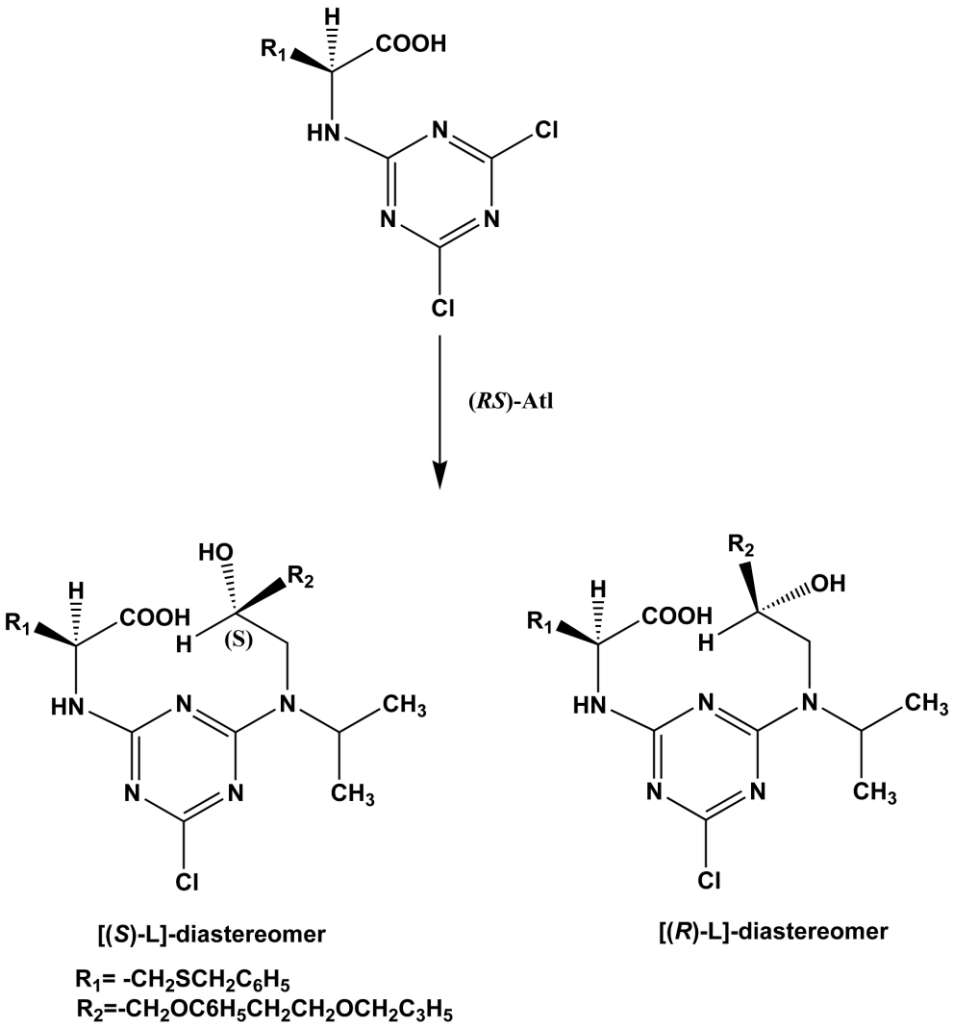


Fig. 2.12: Scheme of derivatization of (*RS*)-Atl with CDR 10

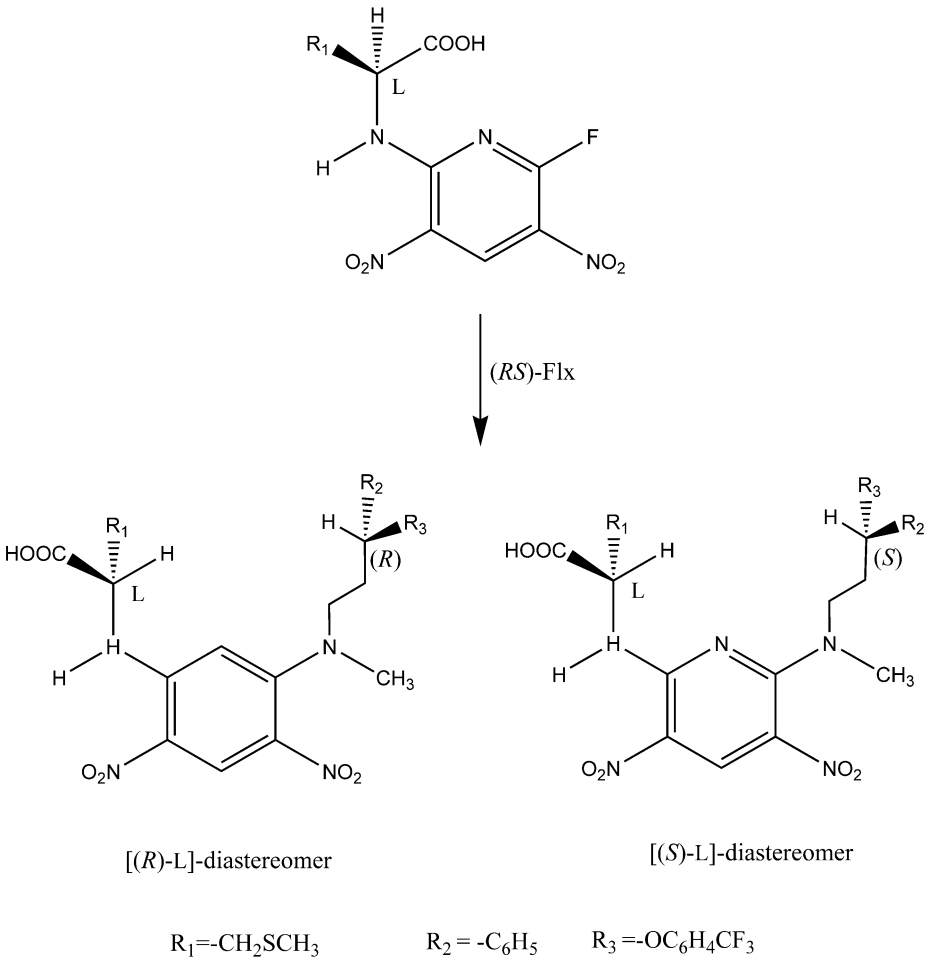


Fig. 2.13: Scheme of derivatization of (*RS*)-Flx with CDR 1

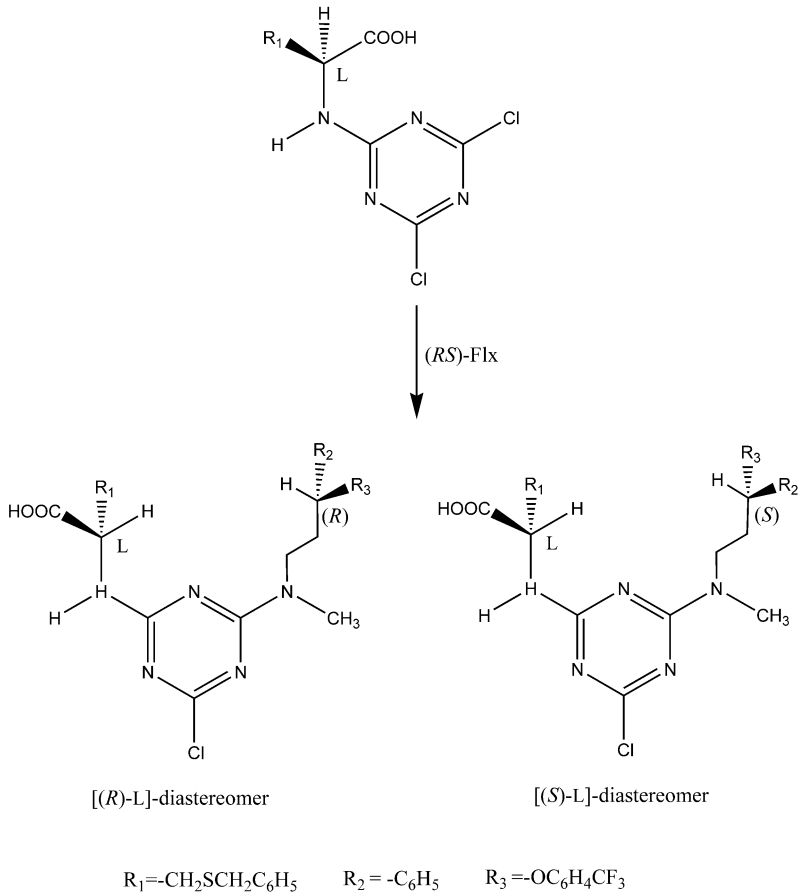


Fig. 2.14: Scheme of derivatization of (*RS*)-Flx with CDR 10

ii. Diastereomers of DL-SeMet Synthesized with DFDNB based CDRs

Diastereomers of DL-SeMet were synthesized using the three CDRs (CDR 2, 3 and 6) according to the literature report (Bhushan and Kumar, 2009b) using MWI as well as conventional heating. Synthesis of representative diastereomers with CDR 3 is shown in Fig. 2.15. The reaction conditions for derivatization were optimized by performing following variations: MWI time (40, 45, 50, 55, 60, 65 and 70 s) and MWI power 70 to 85%; conventional heating time of 20, 30, 40, 50 and 60 min; temperature between 35 to 50 °C; and ratio of DL-SeMet:CDR (1:1 to 1:2.5).

MWI for 55 s at 75% of 800 W, or conventional heating for 50 min at temperature of 45 °C, and a ratio of DL-SeMet:CDR (1:1.7) were found as optimized conditions for completion of reaction for the synthesis of diastereomers. Diastereomers of L-SeMet were prepared under the optimized conditions with all the three CDRs 2, 3 and 6.

D. Synthesis of Diastereomers of DL-Proteinogenic AAs

The diastereomers of 18 proteinogenic AAs were prepared using twofold molar excess of CDRs under MWI and vortexing. A total of 18 diastereomeric pairs were synthesized. The structures of diastereomeric pair [D-(*S*) or L-(*S*) diastereomers] of DL-Leu prepared with CDR 17 are shown in Fig. 2.16. The first letter (L or D) represents the configuration of AAs and second letter (*S*) is for the configuration of CDR.

Solution of DL-Leu (50 µL, 50 nmol, as the representative) was placed in a 1.5 mL vial. The solution of chiral derivatizing reagent in MeCN (100 µL, 100 nmol,) and 5 µL of TEA were added to the vial. Thus, the amino acid and CDR17 were in the mole ratio of 1:2. A ratio of 1:5 of amino acid(s) to CDR was applied in the case of amino acids forming bis derivatives (Cys, Lys and Tyr). The reaction mixture was then irradiated with microwave (MWI) for 45s at 75% (of 800W) and then cooled to room temperature. The reaction was quenched by addition of acetic acid (1M, 50 µL).

The diastereomers of all the analytes were also synthesized by vortexing the reaction mixture (in the same mole ratio as used for MW based synthesis) at 700 rpm for 10 min at room temperature. Aliquots (10 µL) of resulting solution of diastereomers were diluted 10 times with MeCN and injected (20 µL) on to the column.

The experimental conditions for the synthesis of diastereomeric pairs of proteinogenic AAs with CDR 17 using microwave-irradiation were optimized with respect to the effect of pH, reagent excess, reaction time and microwave power.

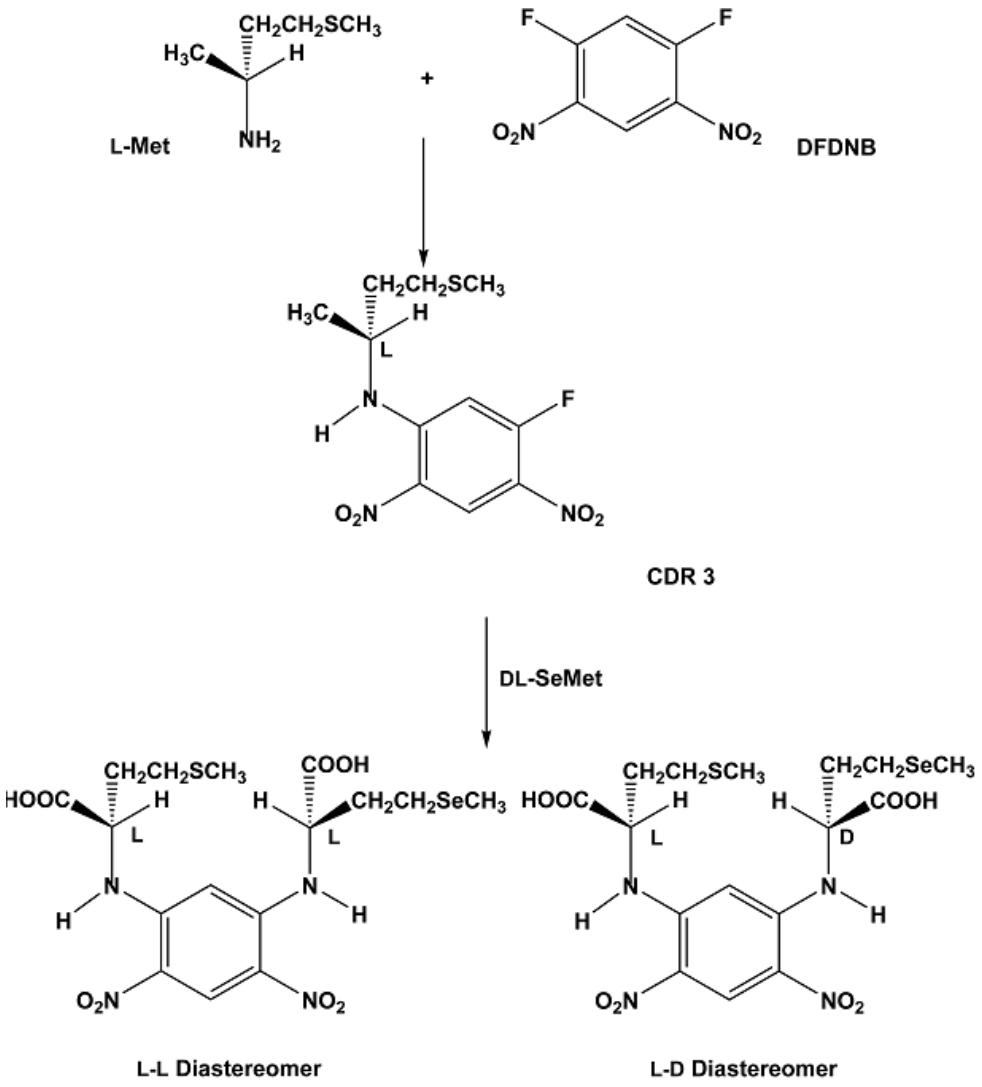


Fig. 2.15: Scheme of derivatization of DL-SeMet with CDR 3

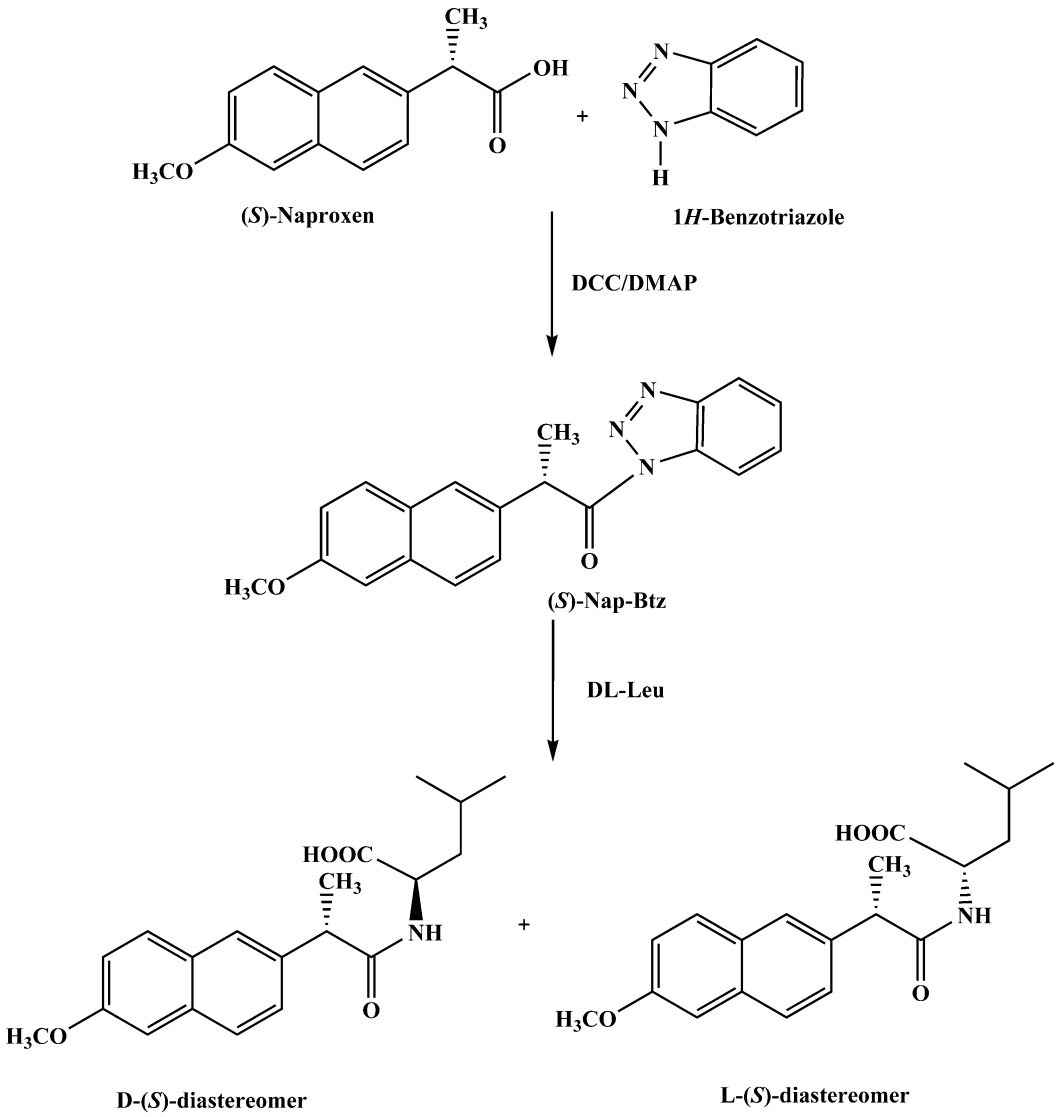


Fig. 2.16: Scheme of derivatization of DL-Leu with CDR 17

VII. Thin Layer Chromatography (TLC)

TLC separation was performed by direct method on home-made plates and by indirect method on commercial TLC. Following types of TLC plates were prepared for TLC separation by direct method

A. Direct TLC

1. Preparation of Plain Thin Layer Silica Gel Plates

To prepare the slurry of silica gel 25 g of it was dissolved in 50 mL of water and the slurry was spread on glass plates by using Stahl-type applicator to prepare TLC plates (of 10 cm height x 5 cm width, thickness of 0.5 mm). The plates were activated by keeping overnight in oven at 60±2 °C and were considered as plain plates.

2. Preparation of Impregnated Thin layer Plates

Following two different approaches were used:

(a) *Impregnation of plain plates by mixing the chiral selector with slurry:* While preparing the plain thin layer silica gel plates, slurry of silica gel G (25 g) was prepared in the solution of chiral selector (50 mL, 0.1% (–)-quinine). A few drops of diluted HCl were added to adjust the required pH 8. Plates were also prepared by adjusting the pH of the slurry at 6 and 9. These impregnated TLC plates were used for enantioseparation of DL-SeMet.

TLC plates were also impregnated by the same approach using L-Glu (0.5% in 50 mL double-distilled water) and were used for enantioseparation of *β*-blockers (Atl, Orc, Bel and ISP).

(b) *Impregnation by ascending development of plain plates in a solution of chiral selector:*

The plain plates, were impregnated with (–)-quinine by ascending development of the plates in the solutions of the chiral selector (0.1% in MeOH-H_2O, 2:8, *v/v*, with pH adjusted to 6, or 8 or 9) for 15 to 20 min. The plates were then dried in air. Different concentrations of chiral selector were used in the range of 0.05 to 0.2% with each

change of 0.05% to prepare impregnated plates under (a) and (b). These TLC plates were used for enantioseparation of DL-SeMet.

3. Use of Chiral Selector as Mobile Phase Additive (CPMA)

DL- and L-SeMet sample were applied on plain plates while the chiral selector was used as mobile phase additive. (–)-quinine, as chiral selector, was dissolved in every combination of solvent system (e.g., CH_3CN-CH_3OH-CH_2Cl_2-H_2O) to bring its concentration in the range 0.05 to 0.2% (with each change of 0.02%) and finally the pH was adjusted every time (to 6, or 8 or 9) before performing TLC.

To investigate the effect of concentration of the impregnating reagent different concentrations of (–)-quinine (0.05% to 0.2% with each change of 0.05% were tried. pH of the solvent systems was adjusted by the addition of a few drops of NH_3 or HCl, as the case may be.

This approach was also used for enantioseparation of *β*-blockers (Atl, Orc and Bel) using the L-Glu as CMPA. Racemic and pure (*S*)-isomer of Atl were spotted on plain plates. Chiral selector was added to the mobile phase (e.g., CH_3CN-CH_3OH-CH_2Cl_2-H_2O) in the concentration range of 0.1 to 1.0% with each change of 0.2%. The pH of all the solutions was adjusted at 5 and by addition of NH_3 and/or HCl; investigations were carried out by varying pH in the range of 3 to 7.

4. Pre-mixing of Chiral Selector with Racemic Mixture

Solutions of racemic SeMet and (−)-quinine were mixed in a mole ratio of (1:1). Solutions of all the three *β*-blockers (10^{-3} M) were mixed with L-Glu in the same mole ratio. Solution of L-SeMet was mixed with that of (−)-quinine (in a mole ratio of 1:1).

These were spotted (10 μL) on plain plates (having no chiral impregnating reagent in the slurry) using Hamilton syringe, 2 cm above the margin.

This approach was also used for enantioseparation of *β*-blockers (Atl, Orc and Bel) using the L-Glu as CIR. Separate sets were prepared for *β*-blocker by mixing the each with L-Glu (in a mole ratio of 1:1).

5. Development of Chromatograms (for Direct TLC Resolution)

Solutions of DL- and L-isomers of SeMet were spotted (10 μL, using Hamilton syringe, on the start of the impregnated chiral phase thin-layer plates as described under impregnation approaches (a) and (b), and CMPA approach while under the approach ‘‘Pre-mixing of chiral selector with racemic mixture’’ the mixture of racemic SeMet and (−)-quinine was applied on achiral phase plain plates.

Different solvent systems were tried for development of chromatograms. Cleaned, dried and paper-lined rectangular glass chambers, pre-equilibrated with the mobile phase for 15 min, were used for each experiment. Effect of temperature on enantioresolution of DL-SeMet was investigated by developing chromatograms at 15, 25 and 35°C. Temperature was controlled using an incubator. After development, the chromatograms were dried in air at room temperature for 10 to 15 min.

The spots of separated enantiomers of D- and L-SeMet were located by (i) spraying freshly prepared ninhydrin solution (0.2 % in acetone containing a few drops of glacial CH_3COOH followed by heating at 60 °C for 10 min, and (ii) spraying ninhydrin solution (0.3 g in 100 mL *n*-BuOH containing 3 mL CH_3COOH) followed by heating at 70 °C for 10 min. There appeared characteristic pink-purple spots corresponding to enantiomers of SeMet. Detection was also done using iodine vapors and dark brown spots were located.

Chromatograms of enantiomers of *β*-blockers (Atl, Orc, Bel and ISP) were also developed under same experimental conditions and the resulting chromatograms were located by iodine vapors.

6. Separation and Isolation of Enantiomers

The enantiomers of DL-SeMet were separated by performing TLC experiments as given above. The spots, representing the two enantiomers, were marked on the plates that were exposed to iodine vapors. Iodine was allowed to evaporate off. The silica gel corresponding to different spots was scrapped from nearly 30 chromatograms. The silica gel so collected was extracted with water. The combined extracts pertaining to each of the enantiomers of SeMet were filtered and concentrated in vaccuo.

It was expected that only enantiomers of SeMet, from the two cut spots, would go into solution since (–)-quinine is insoluble in cold water. To compare and verify the isomers getting separated from DL-mixture, L-enantiomer was applied in parallel on the impregnated plates. The optical purity of separated enantiomers was examined by polarimeter which was matching with the literature data. The optical rotation of L-SeMet was found to be $[\alpha]^{25}/_{D}$ +17.89^{o}, which is in agreement with $[\alpha]^{25}/_{D}$ +18.0^{o} (*c* 2, in 2M HCl) (Sigma-Aldrich Catalogue, 2003), thus it was considered to be single enantiomerically pure L-isomer obtained by separation and isolation.

The two spots representing enantiomers of Atl were marked on the plate and iodine was allowed to evaporate off. The spots were cut (from several plates, nearly 40) and extracted with methanol separately. The solutions were filtered and concentrated in *vacuum*. Each of these solutions was expected to contain both L-Glu and one of the enantiomers. Since the two compounds were soluble in many of the common solvents removal of chiral selector was not easily possible by difference of solubility. Each of these solutions was subjected to column chromatography using silica gel packed in acetonitrile. Fractions of 2 mL each were collected and polarity was varied by using MeCN–MeOH (5:1). The fractions were examined for L-Glu. After a few initial blank fractions, first 30 fractions contained the enantiomer of the analyte. Identical fractions were mixed and concentrated when pure enantiomer of Atl was obtained. The purity was further ascertained by polarimetry. The chiral selector was finally removed from the diastereomers by column chromatography to get enantiomer. The separated enantiomers of other three *β*-blockers (Orc, Bel and ISP) were also extracted with methanol.

B. Indirect TLC

Indirect TLC separation of diastereomers of DL-SeMet and (±)-ISP was carried out on commercial RP C_{18} silica gel plates. Following CDRs were used for synthesis of diastereomers; CDRs 2, 3 and 6 for synthesis of diastereomers of DL-SeMet, CDRs 2, 5 and 7 for (±)-ISP and CDRs 4 and 5 for Flx. These diastereomers were applied on Silica gel 60 RP-18 $F_{254}S$ or DC Kieselgel 60 RP-18 $F_{254}S$ plates and the mobile phase (MeCN-TEAP buffer) was used for separation. Different ratio of MeCN and TEAP

were tried for separation. Other chromatographic conditions, temperature (15 to 35°C), pH (3 to 6) were also varied to obtain a good resolution. Different temperature conditions were maintained in incubator. All the separated diastereomers were detected in ordinary light.

XI. Method Validation

The method validation for enantioseparation of *α*-AAs, DL-SeMet, *β*-blockers (Atl, Bel, Orc, Prl and ISP) and (*RS*)-Flx was carried out according to ICH guidelines (1996). The details description has been furnished in respective chapters.

•

Chapter-3
Enantioseparation of *β*-blockers using HPLC and TLC

Contents **Page No.**

Chapter-3
Enantioseparation of β-blockers using HPLC and TLC

Chapter-3
Enantioseparation of *β*-blockers using HPLC and TLC

I. *β*-Blockers

β-Blockers are amino alcohols, also referred as *β*-adrenergic blocking agents, *β*-adrenergic antagonists and *β*-antagonists. Sir James W. Black invented (*RS*)-propranolol as the first clinically used *β*-blocker which revolutionized the medical management of angina pectoris in the 20th century (Stapleton, 1997). *β*-blockers are known to have several side effects such as gastrointestinal irritation, tiredness, dizziness, depression, paresthesias, muscle aching, and asthmatic wheezing. Nevertheless, they are used widely in the treatment of cardiovascular diseases such as arterial hypertension, coronary heart disease arrhythmia and angina pectoris. They are also beneficial in hyperkinetic heart syndrome, hypotensive circulatory disorders, portal hypertension, glaucoma and migraine (Borchard, 1998; Thomas, 1996; Ruffolo, 1983; Frishman, 1984). There are three main types of *β*-blockers, i.e., β_1-selective agents, β_2-selective agents and mixed α_1/β-adrenergic antagonists. β_1-adrenergic receptors are mainly located in heart and kidneys; β_2-adrenergic receptors are located mainly in lungs, gastrointestinal tract, liver, uterus, vascular smooth muscle, and skeletal muscle while certain other *β*-blockers exhibit mixed antagonism of both *β*- and α_1-adrenergic receptors and provide additional arteriolar vasodilating action.

Though the enantiomers of *β*-blockers show different pharmacological effects and activities (Mehvar and Brocks, 2001; Mehvar and Brocks, 2004) most of them are marketed and applied in therapy as racemic mixtures. The cardiac *β*-blocking activity of the *β*-blockers usually resides with the (*S*)-enantiomers [(*S*) :(*R*), activity ratio ranging from 33 to 530] which is mainly due to their higher receptor affinities, better stereospecific fitting, and a high stereospecific hepatic oxidation (Carr *et al*., 1995; Walle, *et al*., 1983; Albani *et al*., 1983; Belpaire *et al*., 1995).

II. Literature Survey on Liquid Chromatographic Enantioseparation of Orc, Bel, Prl, Atl and ISP

Orc is a moderately selective β_2-adrenergic receptor agonist and a bronchodilator used in the treatment of asthma. Bel is a β_1 receptor blocker used in the treatment of hypertension and glaucoma. It shows greater affinity for β_1-receptor than metoprolol. Prl is a non-selective beta blocker used to treat hypertension, anxiety and panic. Atl in the treatment of hypertension, and for randomised controlled trials of hypertension it has

also been used as a reference drug (Dahlöf *et al*., 2002). **ISP** is a beta-adrenergic agonist that stimulates beta-2 receptors for relaxation of uterine and vascular smooth muscles (Falkay and Kovács, 1986). It may also produce positive inotropic and chronotropic effects.

Generally, the (*S*)-(–)-enantiomer of these drugs is pharmacologically effective, showing about 50–500 fold higher activities (Lee and William, 1990). In most cases *β*-blockers are administered as racemic mixtures.

Application of L-amino acids and complex of L-amino acids with a metal ion, particularly Cu(II), as impregnating reagent in TLC or as chiral ligand exchange reagent or as CMPA both in TLC and HPLC has been reviewed for direct enantiomeric resolution of *β*-blockers, NSAIDs, amino acids (and their derivatives) and certain other compounds (Bhushan and Dixit, 2012d).

Both direct and indirect approaches have been developed to achieve enantiomeric separation of *β*-blockers. TLC separation using ligand exchange mechanism has been reported for resolving racemic Prl and Atl, on plates impregnated with Cu(II)-L-Arg complex (Bhushan and Gupta, 2006), for resolving Atl using Cu(II)-complexes of L-amino acids, L-Pro, L-Phe, L-His, and *N*,*N*-Me_2-L-Phe (Bhushan and Tanwar, 2009a) and Cu(II) complexes of L-threonine, L-serine, and L-tartaric acid for separation of Prl (Bhushan *et al*., 2012). Direct TLC separation of Atl and Prl has been carried out by impregnation approach using L-Asp (Bhushan and Arora, 2003), L-Lys or L-Arg (Bhushan and Thiongo, 1998), (*R*)-mandelic acid and erythromycin (Bhushan and Tanwar, 2008c) and vancomycin (Bhushan and Agarwal, 2010b) as chiral impregnating reagents while L-tartaric acid, L-Asp and L-Glu were used as chiral impregnating reagents (Bhushan and Agarwal, 2008b) for TLC resolution of Atl.

Direct resolution of enantiomers of Prl has been achieved using CSPs consisting of cellulose tris(3,5-dimethylphenylcarbamate)-coated zirconia monolithic column (Kumar and Park, 2011a; 2011b) and immobilized acid glycoprotein, and ovomucoid (Haginaka *et al*., 1990). CSPs based on amylose tris(3,5-dimethylphenylcarbamate) (Ates *et al*., 2013), cellulose tris(3-chloro-4-methylphenylcarbamate) (Peng *et al*., 2010)

and *β*-cyclodextrins (Gagyi *et al*., 2008) were used for direct HPLC enantioseparation of Prl and Bel.

CDRs based on DFDNB and certain other CDRs were synthesized and used for HPLC enantioseparation of Prl and Atl by Bhushan and Tanwar (2009b). Bhushan and Dixit (2012b) prepared CC based CDRs having amino acids or their amides as chiral auxiliaries and used the same for HPLC enantioseparation of some *β*-blockers (other than Prl and Bel).

Chiral derivatives of isothiocyanate (Thompson *et al*., 1982; Sedman and Gal, 1983; Büschges *et al*., 1996a; Péter *et al*., 2001; Ko *et al*., 2006) have been used for HPLC enantioseparation of Prl and Bel via indirect approach. Besides, anhydrides of tert-butoxycarbonyl-L-leucine (Guttendorf *et al*., 1989), and (*R,R*)-*O,O*-diacetyl tartaric acid (Lindner and Rath, 1989), different chloroformates (Mehvar, 1989), and alkyl/aryl substituted acetyl chloride (Kim *et al*., 2001) have also been used for the same. Carboxymethyl-*β*-cyclodextrin (Zhang *et al*., 2008) has been used for enantioseparation of Bel as CSP.

Resolution of Orc has been achieved using sulfobutyl ether *β*-cyclodextrin (Ngim *et al*., 2012), methyl-*β*-cyclodextrin (Amini *et al*., 2000) and sulfated-*β*-cyclodextrin (Yang *et al*., 2005) as chiral selector on C_{18} column. It was also separated on cellulose tris(3,5-dimethylphenylcarbamate) based column (Ullrich *et al*., 2001) and brush type CSP (Hoffmann *et al*., 2007).

Literature reveals that enantioresolution of (±)-ISP was carried out by HPLC using chiral columns of cellulose and amylose (Peng *et al*., 2010), and brush type chiral stationary phase (CSP) (Hoffmann et al., 2007). Two independent methods using HPLC on polysaccharide type CSP and capillary electrophoresis, (CE) with native and derivatized cyclodextrins (CD) have been proposed for enantioseparation of ISP (Chankvetadze *et al*., 2002). CE has also been used for its enantioresolution using (*S*)-*N*-(4-allyloxy-3,5-dichlorobenzoyl)-2-amino-3,3-dimethylbutane phosphonic acid immobilized onto the reactive sulfhydryl surface of silica based capillary column (Preinerstorfer *et al*., 2006); potassium polypectate as chiral selector (Phinney *et al*., 1999); and sulfated cyclodextrins (Stalcup and Gahm,1996). In these HPLC or in

capillary methods chiral columns have been used for separation and detection of ISP (Peng *et al*., 2010; Hoffmann *et al*., 2007; Chankvetadze *et al*., 2002; Preinerstorfer *et al*., 2006; Phinney *et al*., 1999; Stalcup and Gahm, 1996). A relatively elevated cost and poor selectivity are the major drawbacks of some of these chiral columns.

In this chapter enantioseparation of Prl, Atl, Orc, Bel, and ISP by HPLC and TLC has been described.

III. Present Work

A. HPLC Enantioseparation of Orc, Bel, Atl and Prl using CDRs based on DFDNB Containing SMLC, SBLC, L-Met, L-Leu, L-Val, L-Ala, (*S*)-(+)-1-Cyclohexylethylamine and (*R*)-(+)-*α*-Methyl benzylamine as Chiral Auxiliaries

In view of the characteristics of DFDNB, the literature cited above and the references cited therein on the synthesis of DFDNB based CDRs, and in search of new CDRs, following studies were carried out,

(a) to introduce SMLC, SBLC, L-Met, L-Leu, L-Val, L-Ala and (*S*)-(+)-1-cyclohexylethylamine and (*R*)-(+)-*α*-methyl benzylamine as chiral auxiliaries in DFDNB for synthesis of eight CDRs.

These chiral auxiliaries were chosen because the non bonding electron pair of their amino group would show direct conjugation, by resonance, with the para positioned $–NO_2$ group and would be expected to further increase molar absorptivity of dinitro benzene moiety. These chiral auxiliaries contain $–CH_2SCH_3$, $–CH_2CH_2SC_6H_5$, $–CH_2CH_2SCH_3$, $–CH_2CH\ (CH_3)_2$, $–CH\ (CH_3)_2$, $–CH_3$, $–C_6H_{11}$ and $–C_6H_5$ as the hydrophobic groups (in the eight chiral selectors, respectively) which enhance the hydrophobicity of the CDRs and are thus expected to influence the separation.

(b) to use these CDRs (i.e., CDR 1-5 and 7, Set A; and CDR 8-9, Set B; Chapter-2) for HPLC enantioseparation of the four title compounds since there are no reports on their enantioseparation using these DFDNB based CDRs. The method was validated for linearity, accuracy, LOD and LOQ. This is the first report on synthesis of the four CDRs (*cf*., Table 2.3; CDR 1 and 2, set B, Fig. 2.1; and 8 and 9, Fig. 2.2) and their application for enantioseparation of the four title compounds (Fig. 3.1).

1. Results and Discussion

(a) DFDNB based CDRs

The presence of nitro groups in DFDNB enhances the molar absorptivity as chromophores and their strong electron withdrawing nature facilitates nucleophilic substitution of one of the fluorine atoms with different chiral auxiliaries to yield the CDR. The eight CDRs were synthesized and characterized as described in Chapter-2. These were:

- Six CDRs (CDR 1-5 and 7, set A) containing SMLC, SBLC, L-Met, L-Leu, L-Val and L-Ala as chiral auxiliaries in DFDNB (*cf.*, Fig. 2.1; Table-2.3).
- Two CDRs (CDR 8-9, set B) containing (*S*)-(+)-1-cyclohexylethylamine and (*R*)-(+)-*α*-methyl benzylamine (*cf.*, Fig. 2.2; Table-2.3).

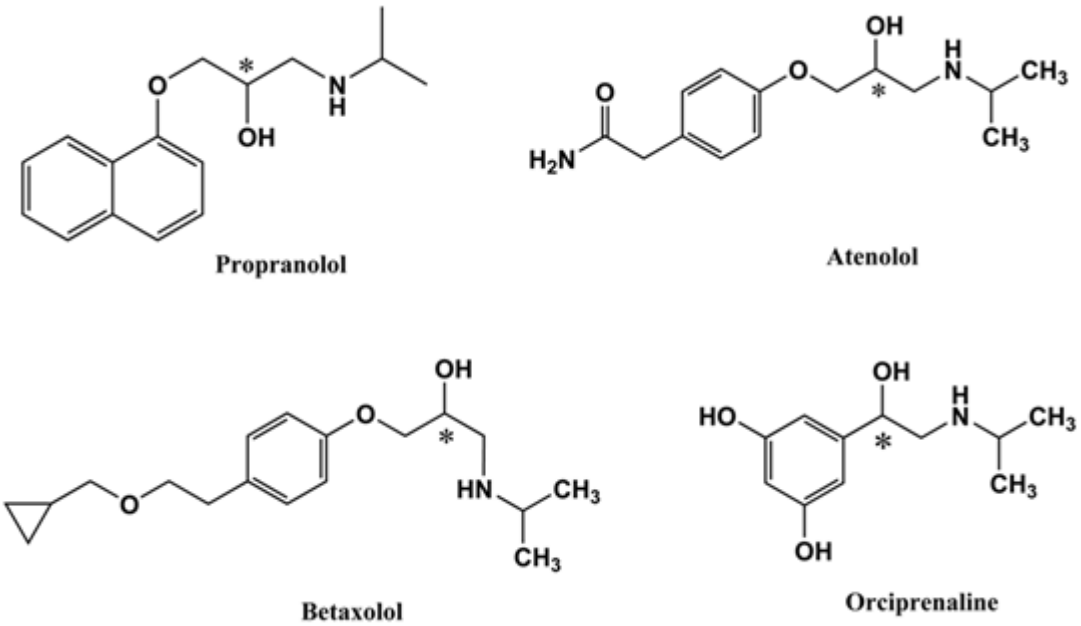


Fig. 3.1: Structures of *β*-blockers

(b) Synthesis of Diastereomers

Experimental details for synthesis of diastereomers of *β*-blockers under MWI and conventional heating with constant stirring are described in Chapter-2. The effects of variation in molar ratio of CDR: analyte, pH and MWI were investigated to establish completion of reaction in quantitative yield. Derivatization conditions were optimized for the reactions of (*RS*)-Prl with CDR 1.

(i) Role of Variation in Molar Ratios of CDR: Analyte

Various molars ratios of Prl:CDR (i.e., 1:1, 1:1.5, 1:1.7 and 1:2) were used to find the optimum reagent concentration for derivatization. The 1.7 fold molar excess of CDR was successful for quantitative derivatization and to prevent kinetic resolution, because at lower ratios slight kinetic resolution was observed. At higher ratios no significant change in reaction time and yield of derivatization was observed.

(ii) Role of pH

A detailed systematic study with respect to role of pH on derivatization was carried out by addition of $NaHCO_3$. pH around 8 provided the best yield for derivatization. Diastereomeric peaks appeared only when the reaction was carried out in the presence of $NaHCO_3$ as it makes amino group free to increase the nucleophilicity of the reactant. The formation of diastereomers was not observed without addition of $NaHCO_3$. Thus for further derivatization reactions, pH around 8 was maintained during the course of reaction, to obtained the quantitative yields.

(iii) Role of MWI and Conventional Heating

To investigate the effect of MWI time and power, reaction mixtures of Prl with CDR 1 were irradiated in microwave oven for 30, 40, 50, 60 and 70 s at each of the three different power settings, viz, 70, 80 and 90%. Separate sets of reaction mixture were incubated at 30, 40, 45 and 50 °C with constant stirring for 30, 40, 50, 60, 70, 80 and 90 min (at each temperature) to optimize the time and temperature conditions. Heating of reaction mixture for 50 s (at 80% of 800 W) under microwave while in a separate set of reaction mixture heating at 45 °C in an incubator for 50 min with constant stirring for conventional heating were found successful to give best yield of derivatization. Fig. 3.2

is showing the effect of time for the derivatization reaction of Prl with CDR 1, in conventional heating condition; as increasing the reaction time from 30 to 50 min, there was found an increment of derivatization yield while on increasing it from 50 to 90 min, no significant increase in derivatization yield.

The peak areas of each pair of the diastereomers, obtained in HPLC experiments, were taken as a diagnosis for the formation of the diastereomers and their separation.

The optimized conditions for the synthesis of diastereomers are, (*RS*)-Prl:CDR 1 in the mole ratio of 1:1.7; heating of reaction mixture at 45 °C in an incubator for 50 min with constant stirring for conventional heating while MWI for 50 s (at 80% of 800 W). After cooling to room temperature, HCl (2 M, 20 μL) was added to terminate the reaction. Synthesis of diastereomers of (*RS*)-Orc, (*RS*)-Bel and (*RS*)-Atl with CDR 1 and diastereomers of the four *β*-blockers with remaining CDRs was performed under the said optimized conditions. The 1.7 fold molar excess of CDR was successful for quantitative derivatization and to prevent kinetic resolution. The diastereomers of the type [(*R*)-L]-, and [(*S*)-L]-, are formed where the first letter refers to the configuration of the analyte and the second to that of the chiral auxiliary of the CDR.

(c) HPLC Separation

A total of twenty-eight pairs of diastereomers, as synthesized, were separated using RP- HPLC.

Following mobile phases were tried;

Mobile phase 1, MeCN with TEAP buffer (10 mM).

Mobile phase 2, MeCN with TFA buffer (0.1%)

Mobile phase 3, MeOH with TFA buffer (0.1%).

Mobile phase were tested in linear gradient of MeCN from 35 to 80, 35 to 65, 35 to 60, 30 to 65, 25 to 65, 25 to 70, 20 to 65, 20 to 70 and 10 to 90% and in isocratic mode (with the ratio of organic modifier: buffer as, 80:20, 60:40, 40:60, 20:80 and 10:90) in 45 min run.

Each mobile phase was filtered through a 0.45μm filter and degassed by sonication and passing nitrogen before use.

The data for resolution (R_S), separation factor (α) and retention factor (k) for separation of diastereomers are given in Table-3.1. Sharper peaks were observed with the mobile phase under gradient conditions in comparison to those under isocratic conditions. MeCN used in mobile phase 1 and 2 was found to be a better organic modifier in comparison to MeOH as larger retention times and broader peaks were obtained with the later. The reason for broader peak shapes and higher retention times with methanol is because of its lower dielectric constant (33 D) and higher viscosity (0.59 cP at 25 °C) in comparison to acetonitrile which has a higher dielectric constant (37.5 D) and lower viscosity (0.343 cP at 25 °C). Thus, the mobile phase 2, with gradient elution containing MeCN as organic modifier and 0.10% TFA, in 45 min at a flow rate of 1.0 mL/min and detection at 340 nm was successful for separation of all the diastereomers.

Diastereomers of Prl, Atl and Bel were better resolved under linear gradient (35 to 65%) of MeCN while diastereomers of Orc under linear gradient (30 to 75%) of MeCN (Table-3.1). The chromatogram showing separation of diastereomers of the three *β*-blockers (Atl, Bel and Orc) prepared with CDR 1, 5 and 8 are given in Fig. 3.3 (a), (b) and (c), respectively and the chromatogram showing separation of diastereomers of the Prl prepared with CDR 1 and 8, are shown in Fig. 3.3 (a) and (c), respectively.

The diastereomers synthesized by the two approaches (using MWI and conventional heating) were found to be identical in terms of their characterization and chromatographic data. The peak areas corresponding to all the diastereomers, obtained for each change of the above mentioned derivatization conditions, were calculated by system software; these values served as a measure of completion of reaction and yield of derivatization.

The elution sequence of diastereomers was confirmed by examining elution of the diastereomers prepared from enantiomerically pure (*R*)-Prl and (*S*)-Prl with CDR 1. The first eluted peak was for the diastereomer corresponding to (*R*)-Prl (i.e. [(*R*)-L]-diastereomer), and second one was for its (*S*)-counterpart (i.e. [(*S*)-L]-diastereomer).

A comparative account of separation efficiencies of two sets of CDRs (A and B), among themselves and among the two sets, on the basis of R_S values observed for the diastereomers has been presented in Table-3.2.

(i) Comparison of Resolution (R_S) for the Diastereomers Prepared with DFDNB based CDRs Having Amino Acids as Chiral Auxiliaries

As shown in Table-3.1, baseline resolutions of diastereomers of Atl, Bel and Orc prepared with all the six CDRs (CDR 1-5 and 7) and the diastereomers of Prl prepared with CDRs 1 and 2 were achieved. The highest resolution among the twenty pairs of diastereomers was observed for the diastereomeric pair of (*RS*)-Prl prepared with CDR 2 (DFDNB having SBLC as chiral auxiliary) and the lowest resolution was observed for the diastereomeric pair of (*RS*)-Atl prepared with CDR 7 (DFDNB having L-Ala as chiral auxiliary) (Table-3.2).

(ii) Comparison of Resolution (R_S) for the Diastereomers Prepared with DFDNB based CDRs Having Amines as Chiral Auxiliaries

Among all eight diastereomeric pairs of four *β*-blockers (Atl, Bel, Orc and Prl) synthesized with the CDRs 8 and 9, the lowest resolution was found for the diastereomers of (*RS*)-Atl prepared with CDR 9 and the highest for diastereomers of (*RS*)-Prl prepared with CDR 8 (Table-3.2).

In conclusion, CDRs having chiral auxiliaries either L-Leu (i.e., CDR 4 among the CDRs of Set A) or (*S*)-(+)-1-cyclohexylethylamine (i.e., CDR 8 among the CDRs of Set B) provided the best resolution for the diastereomers of Atl, Bel and Orc. In case of diastereomers of Prl the CDR 2 and CDR 8 provided better resolution than the CDRs 1 and 9, respectively.

(iii) Effect of Side Chain of L-Amino Acids (used as Chiral Auxiliaries) on Resolution (R_S)

The hydrophobicity of the alkyl side chain of chiral auxiliaries (amino acids) also affects the interaction of the diastereomers with the ODS material of the column and the separation. Observation of chromatographic data reveals that the CDRs having more hydrophobic side chain are more strongly held by the column showing longer retention times and providing better resolution (R_S) of the diastereomers. According to

hydrophobicity scale given by Bull and Breese (1974) for amino acids, its decreasing order is; Leu (0.842) > Val (0.777) > Met (0.709) > Ala (0.691); values given in parenthesis are representing the apparent partial specific volume. The chromatographic data of diastereomers of *β*-blockers are shown in Table-3.1; it is in very good correlation, with respect to retention time and the hydrophobicity, for the diastereomers of Atl, Bel and Orc prepared with CDRs 3, 4, 5 and 7. There is also a very good correlation with respect to R_S and hydrophobicity of the diastereomers of these three *β*-blockers.

(d) Recovery and %Yield of diastereomers

To determine %yield of diastereomers, small-scale preparative separation of diastereomers was achieved; the sample of diastereomeric mixture (without dilution) was injected onto C_{18} column, using 200 µL injection loop. After 5 repeated injections, approximately 15 mL fraction of the mobile phase, containing first eluting diastereomer, was collected; it was the [(*R*)-L]-diastereomer. To this fraction, 10 mL water and 5 mL of 1 M HCl were added. It was cooled in an ice bath and the corresponding precipitate *i.e.* [(*R*)-L]-diastereomer was obtained. The precipitate was centrifuged and washed with cold water; it was dried in desiccator over P_2O_5. The same procedure was applied for every eluting diastereomer. The %yield of the recovered diastereomers was calculated and is given in Table-3.3.

The recovery studies of the two eluted diastereomers (as described in section ‘‘Method validation’’), also served as a measure of their yields of the order of 99% since recovery of all eluted diastereomers was found to be more than 98%. In ‘method validation’, recovery was considered according to peak area of injected diastereomers while, in small-scale preparative separation % yield was calculated by obtaining the diastereomers after workup described under ‘Recovery and %Yield of diastereomers’. Since, in the experimental process some quantity of diastereomers remains unrecovered the yield of diastereomers is, therefore, little less in comparison to the one shown under ‘method validation’.

2. Comparison of Derivatization Conditions and Separation of Diastereomers with Literature Reports

In the present study, derivatization of *β*-blockers using DFDNB based CDRs (in which *S*-methyl-L-Cysteine, *S*-(+)-1-cyclohexylethylamine and (*R*)-(+)-*α*-methyl benzylamine were introduced as chiral auxiliaries) was carried out under MWI which required only 50 s (at 80% power of 800 W); it is much less in comparison to certain reports in literature (Table-3.4) wherein derivatization is reported under conventional heating. Diastereomers of Prl and Bel have been separated with better resolution R_S (given in Table-3.1) as compared to the resolution reported in literature (Table-3.4) for the diastereomers of Prl and Bel prepared with different kinds of CDRs.

With respect to Orc it is the first report on its enantioseparation by indirect approach. The diastereomers were separated with better resolution (given in Table-3.1) and R_S is higher than the R_S obtained with chiral column (direct approach); for example, R_S was 4.68 using cellulose tris(3,5-dimethylphenylcarbamate) (Ullrich *et al.,* 2001), 0.8 using sulfobutyl ether *β*-cyclodextrin (Ngim *et al.,* 2012), 3.95 using methyl-*β*-cyclodextrin (Amini *et al.,* 2000) and 2.031 using sulfated-*β*-cyclodextrin (Yang *et al.,* 2005).

3. Separation Mechanism

The mechanism for HPLC separation of diastereomers of certain amino acids, prepared with MR, along with explanation for elution sequence and difference in retention time for diastereomers has been explained in the literature (Marfey, 1984; Bhushan *et al*., 2009; Fujii *et al*., 1997). In the present case, the diastereomers of all the four *β*-blockers, prepared with

Table-3.1: Chromatographic separation data of diastereomers of four *β*-blockers prepared with different CDRs (Set A and Set B)

CDR	Atenolol			Betaxolol			Orciprenaline			Propranolol		
	k_1	α	R_S	k_1	α	R_s	k_1	α	R_S	k_1	α	R_S
Set A												
1	6.87	1.15	6.39	9.64	1.13	11.72	7.84	1.13	9.79	9.90	1.15	14.12
2	9.01	1.26	7.51	12.20	1.13	11.50	11.66	1.14	9.91	14.96	1.11	14.50
3	7.63	1.14	6.84	10.15	1.14	10.56	8.28	1.15	9.96	NA		
4	8.89	1.15	8.65	11.80	1.15	12.50	10.84	1.19	12.78			
5	7.72	1.21	7.63	10.24	1.16	11.95	8.31	1.23	10.31			
7	6.05	1.21	4.36	7.59	1.13	6.65	5.15	1.14	4.68			
Set B												
8	11.16	1.11	7.31	16.08	1.06	9.65	14.45	1.08	10.22	18.00	1.09	13.48
9	10.15	1.10	6.27	15.56	1.06	8.70	13.67	1.03	8.26	17.13	1.06	9.92

Chromatographic conditions: LiChrospher C_{18} (250 × 4.6 mm i.d., 5 µm particle size); mobile phase 2, linear gradient (35 to 65%) of MeCN with 0.10% TFA for diastereomers of Prl, Atl and Bel, and 30 to 75% for diastereomers of Orc in 45 min; flow rate, 1.0 mL/min; detection, 340 nm. t_{R1} is retention time, k_1 is retention factor of [(*R*)-L]-diastereomers; α, separation factor; R_S, resolution. NA, resolution not attempted

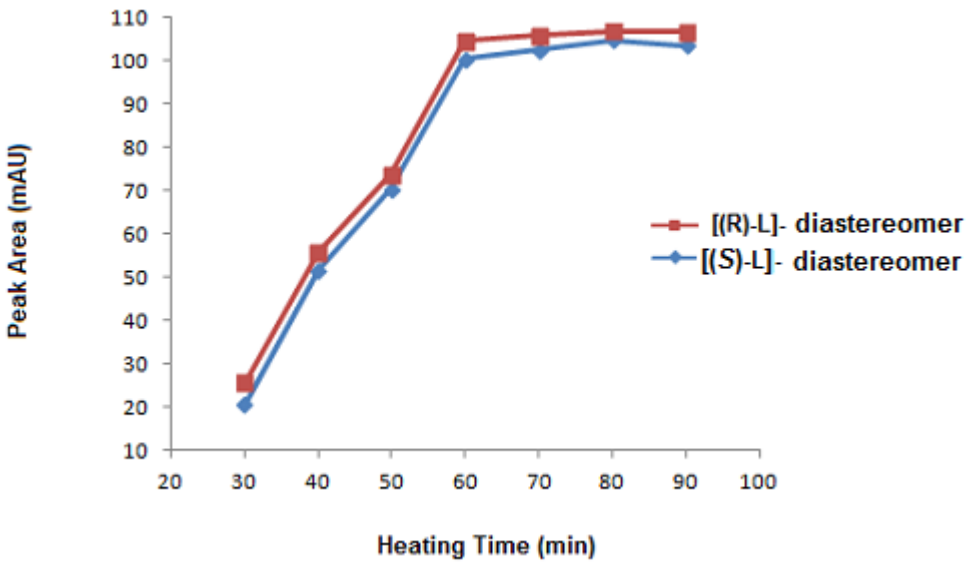
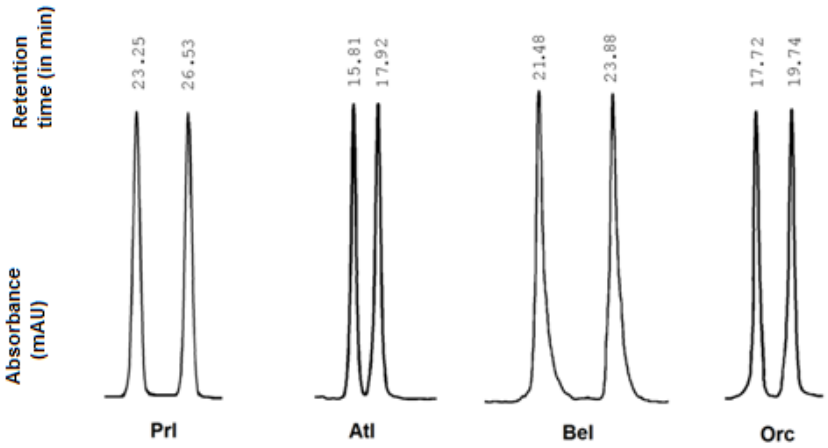
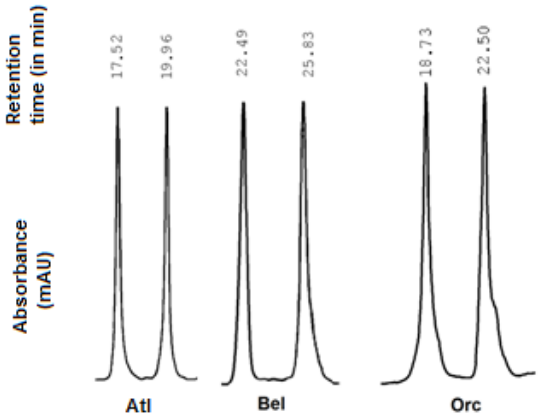


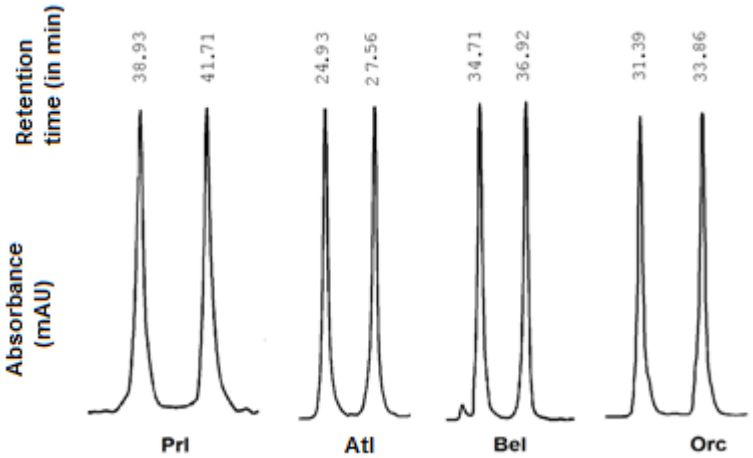
Fig. 3.2: Effect of heating time on derivatization of Prl with CDR 1



(a)



(b)



(c)

Fig. 3.3: Sections of chromatograms in, (a), (b) and (c), are representing the chromatograms of diastereomers of *β*-blockers prepared with CDR 1, 5 and 8, respectively

Table-3.2: Comparison of separation efficiencies of two sets of CDRs on the basis of R_S values observed for the diastereomers of four *β*-blockers

***β*-blockers**	**CDRs arranged on the basis of decreasing R_S values for the diastereomers**
(*RS*)-Prl	2>1>8>9
(*RS*)-Atl	4>5>2>3>1>7
(*RS*)-Bel	4>5>1>2>3>8>9>7
(*RS*)-Orc	4>5>8>3>2>1>9>7

Table-3.3: Showing % yield of diastereomers of *β*-blockers recovered after separation

Analyte	Diastereomers with CDR 1			Diastereomers with CDR 8			Diastereomers with CDR 9		
	Theoretical yield (mg)	1	2	Theoretical yield (mg)	1	2	Theoretical yield (mg)	1	2
Prl	6.31	2.97 (47.06)	2.94 (46.59)	6.22	2.85 (45.82)	2.81 (45.18)	6.15	2.75 (44.72)	2.81 (45.69)
Orc	5.76	2.72 (47.22)	2.70 (46.88)	5.68	2.65 (46.65)	2.60 (45.77)	5.61	2.46 (43.85)	2.41 (43.00)
Bel	6.86	3.09 (45.04)	3.07 (44.75)	6.77	3.06 (45.20)	3.02 (44.61)	6.70	3.22 (48.06)	3.19 (47.61)

1 and 2 are representing experimental yield of [(*R*)-L]- and [(*S*)-L]-diastereomer, respectively in mg and % yield of diastereomer is shown below in parentheses.

Mol wt of Prl, Orc and Bel are 259.34, 211.26 and 307.43 gm mol^{-1}, respectively. The molecular weights of the diastereomers of three title compounds prepared with CDR 1, 8 and 9 respectively are, 558.60, 550.65 and 544.60 gm mol^{-1} for diastereomers of Prl; 510.52, 502.56 and 496.51 for diastereomers of Orc; and 606.69, 598.73 and 592.68 gm mol^{-1} for diastereomers of Bel.

Table-3.4: Summary of literature reports on derivatization conditions of Prl and Bel using different CDRs and resolution of corresponding diastereomers

S. No.	CDR	Derivatization time	Temperature	R_S	Reference
1	2,3,4,6-tetra-*O*-acetyl-*β*-D-galact-opyranosyl isothiocyanate	30 min	RT	6.23^B	Ko *et al*., 2006
2	2,3,4,6-tetra-*O*-acetyl-*β*-D-gluco-pyranosyl isothiocyanate	30 min	35 ˚C	4.70^B	Ko *et al*., 2006
3	l-acetoxy-1-phenyl-2-propyl isothiocyanate	3 h	RT	4.22^A	Péter and Fülöp, 2002
4	1,3-diacetoxy-1-(4-nitro-phenyl)-2-propyl isothiocyanate	3 h	RT	2.32^A	Péter and Fülöp, 2002
5	*N*-3,5-dinitrobenzoyl-*trans*-l, 2-diaminocyclohexane isothiocyanate	2 h	60 ˚C	6.67^A	Kleidernigg *et al*., 1996
6	4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole	1.5 h	65 ˚C	3.5^A	Toyo'oka *et al*., 1997
7	(–)-(*α*-methoxy-(*α*-(trifluorometh-yl)phenylacetyl chloride	5 min	60 ˚C	1.18^B	Kim *et al*., 2001
8	(*R*,*R*)-*O*,*O*-diacetyl tartaric acid anhydride	4 h	40 ˚C	5.00^A	Lindner and Rath, 1989
9	1,3-diacetoxy-1-(4-nitro-phenyl)-2-propyl isothiocyanate	3 h	RT	2.33^A 2.00^B	Péter *et al*., 2001
10	1-phenylethyl isocyanate	5 min	RT	4.30^A	Olsen *et al*., 1993
11	1-(6-methoxy-2-naphthyl)ethyl isothiocyanate)	30 min	RT	1.8^A	Büschges *et al*., 1996

^{A} and ^{B} represent the R_S values for the diastereomers of (*RS*)-propranolol and (*RS*)-betaxolol

different CDRs (Set A and Set B), corresponding to (*R*)-enantiomer were eluted prior to those corresponding to (*S*)-isomer. The same explanation holds good to explain the elution order of the diastereomers.

Marfey (1984), Brückner and Keller-Hoehl (1990) and Brückner and Gah (1991) discussed independently that the resolution of diastereomers of L- and D-amino acids prepared with Marfey's reagent (containing L-Ala-NH_2 as chiral auxiliary) is essentially due to intramolecular H-bonding.

Marfey (1984) attributed the reason for the [(L), L]-diastereomer eluting before the [(D), L]-isomer to a stronger intramolecular H-bonding in D- than in L-isomer. He suggested that the carboxyl group of analysed amino acid can H-bond either to an *ortho* situated nitro group producing a nine member ring or, more likely, to the carbonyl oxygen of the *meta*-situated L-Ala–NH_2 forming a 12-membered ring. Stronger H bonding in a [(D), L]-diastereomer would produce a more hydrophobic molecule which would be expected to interact more strongly with the RP column and thus have a stronger retention time than an [(L), L]-diastereomer. According to Fujii *et al*., (1997), the resolution of the L- and D- amino acid derivatives was due to difference in their hydrophobicity, which is derived from the *cis*- or *trans*-type arrangement of two more hydrophobic substituents at both *α*-carbons of an amino acid and L-alanine amide, i.e., orientation of hydrophobic groups with respect to the plane of dinitrobenzene. The diastereomer having *cis*-type arrangement of the hydrophobic groups and more extent of hydrogen bonding is more hydrophobic and interacts more strongly with ODS material of C_{18} column.

The same mechanism is further supported in the present case by developing optimized (lowest energy) structures of the two diastereomers using the Gaussian 09 Rev A. 02 program and hybrid density functional B3LYP with 6-31G (d, p) basis set (Fig. 3.4). In [(*S*)-L]-diastereomer, the hydrophobic groups –CH_2SCH_3 on the stereogenic centre (C-1) of CDR 1 and –$C_6H_3(OH)_2$ on stereogenic centre (C-2) of Orc are oriented on the same side with respect to the plane of dinitrobenzene moiety and are considered to be *cis* to each other (Fig. 3.4a). On the other hand, in [(*R*)-L]-diastereomer, the –CH_2SCH_3 and –$C_6H_3(OH)_2$ groups are oriented in space opposite to the plane of dinitrobenzene moiety and thus have *trans*-type arrangement (Fig. 3.4b). The program

Gaussian 09 Rev A. 02 and hybrid density functional B3LYP with 6-31G basis set was used to obtain optimized structures. A graphical representation for the same is also shown in Fig. 5.

The mechanism in the present work gets confirmed with the experimental observation that the [(*R*)-L]-diastereomer is eluted first from the column which means it is retained for lesser time in comparison to that of the [(*S*)-L]-diastereomer. In other words, the [(*S*)-L]-diastereomer (having *cis*-type arrangement) interacts more strongly with ODS material of C_{18} column than the *trans*-type arrangement, hence has a longer retention time. These retention times of the diastereomers are thus reflecting the overall hydrophobicity of the two molecules. It can therefore be concluded that the hydrophobicity of *cis*-type arrangement is more than that of *trans*-type arrangement. The diastereomers of all the three *β*-blockers, prepared with CDRs 1, 8 and 9, corresponding to (*R*)-enantiomer were eluted prior to those corresponding to (*S*)-isomer.

The *cis*- and *trans*-type arrangements (as explained above), hydrogen bonding between meta-situated nitro with hydroxyl group of analyte, large size of S atom in CDR and the presence of benzene group in CDR (contributing to the overall hydrophobicity) along with rheological properties of the mobile phase, are responsible for different partition coefficients and different retention times of [(*R*)-L]-and [(*S*)-L]-diastereomers. Therefore, the diastereomers elute one after another for these different physical properties.

4. Method Validation

Method validation studies were performed using diastereomers of (*RS*)-Prl prepared with CDR 1.

Linearity: The peak area (Y-axis) response of the diastereomers of (*R*)-(first eluting diastereomer) and (*S*)-(second eluting diastereomer) enantiomer prepared with CDR 1 were plotted against the corresponding concentration (20 to 100 ng mL^{-1}; X-axis). The linear regression was computed by using the least square method. The relative standard deviation (RSD) values of slope, intercept and correlation coefficient were obtained (less than 1.4%) as a evidence of good linear relationship over this range. The regression

equations were y = 0.942x –11.42 (R^2 = 0.999) and y = 0.943x–10.37 (R^2 = 0.999) for the diastereomers of (*R*)- and (*S*)-Prl, respectively Table-3.5.

Accuracy, precision and limit of detection: Inter-day (5 days) and intra-day assay studies for accuracy and precision were carried out by replicate analysis (n = 3) of four standard solutions of diastereomeric mixtures (20, 40, 60, 100 ng mL^{-1}). The recovery and mean SD (standard deviation) for each of the diastereomers were calculated on the basis of peak areas of first and second eluting diastereomer from the slope and intercept of the calibration plots. These are shown in Table-**3.5.** The values of relative standard deviation for [(*R*)-L]- and [(*S*)-L]-diastereomers, respectively, were 0.49% to 1.28% and 0.59% to 1.30% for intra-day precision, and 0.58% to 1.39% and 0.65% to 1.26% for inter-day precision. The recovery values for the [(*R*)-L]- and [(*S*)-L]-diastereomers were 98.87% to 101.13% and 99.25% to 101.06% for intra-day assay and 99.37% to 101.98% and 99.24% to 101.25% for inter-day assay, respectively. LOD, corresponding to signal-to-noise ratio of 3, was found to be 12 pg mL^{-1} and 13 pg mL^{-1} for [(*R*)-L]- and [(*S*)-L]-diastereomers, respectively and LOQ corresponding to signal-to-noise ratio of 10, was found to be 36 pg mL^{-1} and 39 pg mL^{-1} for [(*R*)-L]- and [(*S*)-L]-diastereomers Table-3.5.

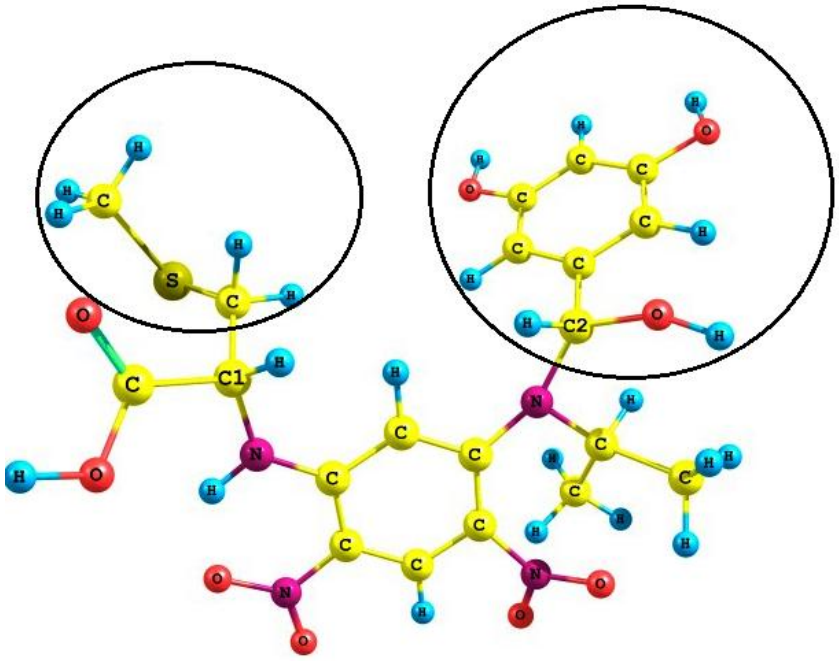


Fig. 3.4a: [(*S*)-L]-diastereomers

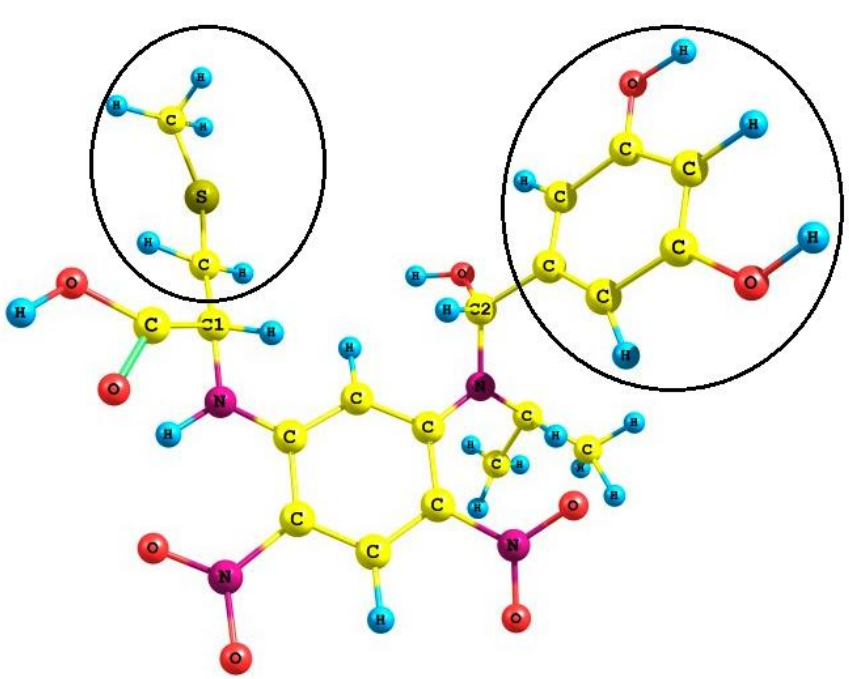


Fig. 3.4b: [(*R*)-L]-diastereomers

Fig. 3.4: Optimized structures of [(*S*)-L]-and [(*R*)-L]-diastereomers of (*RS*)-Orc prepared with CDR 1

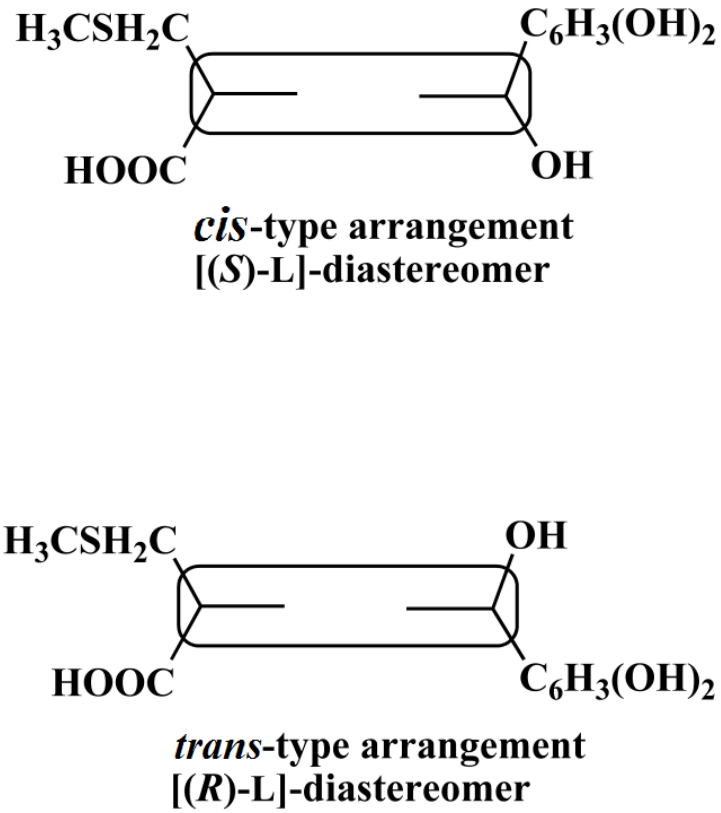


Fig. 3.5: ***Cis*** **and** ***trans*** **type arrangement of hydrophobic groups in diastereomers of (*RS*)-Orc prepared with CDR 1**

Table-3.5: Summary of HPLC method validation containing linearity, intra- and inter-day precision, and recovery studies

	First eluting diastereomer			Second eluting diastereomer		
Linearity						
Range	20 to100 ng mL^{-1}			20 to 100 ng mL^{-1}		
Slope	0.942			0.943		
Intercept	–11.42			–10.37		
Correlation Coefficient (R^2)	0.999			0.999		
Actual concentration ng mL^{-1}	**Mean±SD (measured) ng mL^{-1}**	**Recovery (%)**	**RSD (%)**	**Mean±SD (measured) ng mL^{-1}**	**Recovery (%)**	**RSD (%)**
Intra-day precision (n = 3)						
20	10.113 ± 0.129	101.13	1.28	10.106 ± 0.131	101.06	1.30
40	19.774 ± 0.148	98.87	0.75	19.850 ± 0.141	99.25	0.71
60	30.306 ± 0.167	101.02	0.55	30.255 ± 0.185	100.85	0.61
100	49.960 ± 0.245	99.92	0.49	50.015 ± 0.295	100.03	0.59
Inter-day precision (n = 3)						
20	9.849 ± 0.137	99.37	1.39	9.937 ± 0.125	99.37	1.26
40	19.922 ± 0.173	99.61	0.95	19.848 ± 0.173	99.24	0.87
60	30.231 ± 0.203	100.77	0.67	29.988 ± 0.204	99.96	0.68
100	50.990 ± 0.167	101.98	0.58	50.625 ± 0.329	101.25	0.65
Sensitivity						
LOD(pg mL^{-1})	12			13		
LOQ (pg mL^{-1})	36			39		

B. HPLC Enantioseparation of Orc, Bel and Atl using CDRs based on CC Containing SMLC, SBLC, L-Met, L-Leu, L-Val and L-Ala as Chiral Auxiliaries

Keeping in view the advantages of trifunctionality of CC, six DCT reagents (CDR 10-15 *cf*., Table-2.3; Fig. 2.3) were synthesized. These were used for the synthesis of diastereomers of three *β*-blockers (Fig. 3.1), namely, (*RS*)-Atl, (*RS*)-Orc and (*RS*)-Bel.

Total 18 pairs of diastereomers were synthesized under MWI. These diastereomeric pairs were separated by RP-HPLC on a C_{18} column with detection at 230 nm using gradient elution with mobile phase containing aq TFA and acetonitrile in different compositions. Enantiomerically pure, (*S*)-Atl was converted into its diastereomer for its use as standard for the determination of elution order of diastereomers of racemic *β*-blockers. Optimized structures of diastereomers of Orc (prepared with CDR 11) were developed with the help of density functional theory (using the Gaussian 09 Rev A. 02 program and hybrid density functional B3LYP with 6-31G (d, p) basis set); these supported the explanation for elution order observed experimentally.

Method validation was carried out for linearity, accuracy, LOD and LOQ.

1. Results and Discussion

(a) CC based CDRs (*cf*., Table-2.3)

CDRs 10-15 were used for the synthesis of diastereomers of three *β*-blockers. The synthesis and characterization data of newly synthesized CDRs (CDR 10 and 11), have been described in Chapter-2.

(b) Synthesis of Diastereomers

Experimental details for synthesis of diastereomers of *β*-blockers under MWI and conventional heating with constant stirring are described in Chapter-2. The structures of diastereomeric pair [(*R*)-L]- or [(*S*)-L]-diastereomers] of (*RS*)-Atl prepared with CDR 10 are shown in Chapter-2 (Fig. 2.12). Stability of the CDRs was checked by performing HPLC experiments at an interval of 10 days up to 100 days from the day of synthesis of

diastereomers using the diastereomers of Atl (prepared with CDR 10) as a representative, by comparing each time with a freshly prepared sample.

The effects of variation in molar ratio of CDR: analyte, pH and MWI were investigated to establish completion of reaction in quantitative yield. Derivatization conditions were optimized for the reactions of (*RS*)-Atl with CDR 10 (as representatives for the reactions of (*RS*)-Atl with CDRs 11-15 and, (*RS*)-Orc and (*RS*)-Bel with CDRs 10-15).

(i) Role of Variation in Molar Ratios of CDR: Analyte

(*RS*)-Atl and CDR 10 were allowed to react in different ratios such as, 1:1, 1:1.5, 1: 1.7 and 1:2. The 1.7 fold molar excess of CDR was successful for quantitative derivatization. At a ratio lower than 1:1.7 slight kinetic resolution was observed, while at higher ratios there was no significant change in reaction time and yield of derivatization. Therefore, to prevent kinetic resolution 1.7 fold molar excess of CDR was used throughout all synthesis process of diastereomers.

(ii) Role of pH

pH was varied to investigate its effect on derivatization by addition of $NaHCO_3$. At the addition of 50 µL (50 µmol) of 1 M $NaHCO_3$ a pH around 8 achieved which was found successful to obtain the best yield. No derivatization was observed in the absence of $NaHCO_3$. Thus pH around 8, maintained by 1 M $NaHCO_3$, was used for all derivatization reactions which provided quantitative yields.

(iii) Role of MWI and Conventional Heating

To investigate the effect of MWI time and power reaction mixtures of Atl with CDR 10 were irradiated in microwave oven for 30, 45, 60, 75 and 90 s at each of the three different power settings, viz, 75, 80, 85 and 90%. Separate sets of reaction mixture were incubated at 35, 40 and 45 °C with constant stirring for 50, 60, 70 and 80 min (at each temperature) to optimize the time and temperature conditions. Heating of reaction mixture for 75 s (at 80% of 800 W) under microwave was found as optimized condition to complete the derivatization reaction (as shown in Fig. 3.6) while in a separate set of

reaction mixture conventional heating at 45 °C in an incubator for 70 min with constant stirring was found successful to give the best yield of derivatization.

The peak areas of each pair of the diastereomers, obtained in HPLC experiments, were taken as a diagnosis for the formation of the diastereomers and their separation.

(c) HPLC Separation

A total of eighteen pairs of diastereomers, as synthesized, were separated using RP-HPLC. Aliquots (10 µL) of diastereomers were diluted 10 times with MeCN, filtered and 20 µL were injected onto the column.

Following mobile phases were prepared and used,

Mobile phase 1, MeCN with TFA (0.1%)

Mobile phase 2, MeOH with TFA (0.1%)

Both the mobile phase were tested in linear gradient of MeCN from 40 to 70, 35 to 75, 30 to 80, 25 to 85, 20 to 90, 15 to 95 and 10 to 90%) and in isocratic mode with the ratio of MeCN/MeOH : TFA as, 90:10, 80:20, 60:40, 40:60, 20:80 and 10:90) in 45 min run.

The mobile phase was filtered through a 0.45 µm filter and degassed by passing nitrogen and sonication, before use, to protect the column from any types of impurity and air bubbles.

(i) Optimization of Chromatographic conditions

Chromatographic conditions were optimized with respect to the concentrations of organic modifier and TFA in the mobile phase and its flow rate. Detection of diastereomers was made at 230 nm. An increase in content of organic modifier from the linear gradient of 35 to 75% resulted into decreased retention time and resolution.

Concentration of buffer varied in the range of 0.02% to 0.20%, on increase in concentration from 0.02% to 0.10% there was found an increment in resolution while at the concentration greater than 0.10% there was no significant changes in resolution.

The flow rate of mobile phase was also varied from 0.8 to 1.5 mL/min in portion of 0.2 mL min^{-1} to optimize separation conditions. On decreasing the flow rate from 1 to

0.8 mL min^{-1} an increase of retention time and slight broadening of peaks were observed while on increasing in the flow rate from 1 to 1.5 mL min^{-1} resulted in lowering of the retention time and decreasing the resolution.

It was found that both the mobile phases were capable of separating all the eighteen pairs of diastereomers of *β*-blockers. With the mobile phase 1 (MeCN-TFA), under both the isocratic and gradient elution modes, there appeared sharper peaks in comparison to mobile phase 2 (MeOH-TFA). But with mobile 1, better sharpness was observed in the gradient elution of MeCN from 35 to 75% and 0.1% TFA, in 45 min at a flow rate of 1.0 mL/min. It means the best results were observed with mobile phase 1 in the gradient elution of MeCN from 35 to 75%, in 45 min at a flow rate of 1.0 mL/min.

(ii) Separation of Diastereomers

All the synthesized 18 pairs of diastereomers were synthesized and separated on C_{18} column using RP-HPLC. HPLC conditions were optimized as described above. The results, in terms of retention factor (*k*), separation factor (α), and resolution (R_S) are mentioned in Table-3.6. The sections of chromatograms showing separation of diastereomers of all three *β*-blockers using the CDR 10 are shown in Fig. 3.7. The peak areas corresponding to the two diastereomers (resolved under successful HPLC conditions) obtained for each change in the above mentioned derivatization conditions, were calculated by system software; these served as a measure of completion of reaction and yield of derivatization. The diastereomers synthesized by the two approaches (MWI and vortex) were found to be identical in terms of their chromatographic data.

The elution sequence of diastereomers of (*RS*)-Atl was confirmed by examining elution of the diastereomers prepared from enantiomerically pure (*S*)-Atl with CDR 10, as representative. The first eluted peak was for the diastereomer corresponding to (*R*)-Atl (i.e. [(*R*)-L]-diastereomer), and second one was for its (*S*)-counterpart (i.e. [(*R*)-L]-diastereomer).

Analysis of the Table-3.6, based on the comparison of the resolution reveals that, the highest resolution was found for the diastereomers of the Bel prepared with the CDR 13 and lowest for the diastereomers of the Atl prepared with the CDR 15.

As discussed above, retention time of the diastereomers was highly effected by hydrophobicity of the side chain of L-amino acids used as chiral auxiliaries in CDRs. The chromatographic data of diastereomers of *β*-blockers, shown in Table-3.6; are in very good correlation, with respect to retention time and the hydrophobicity.

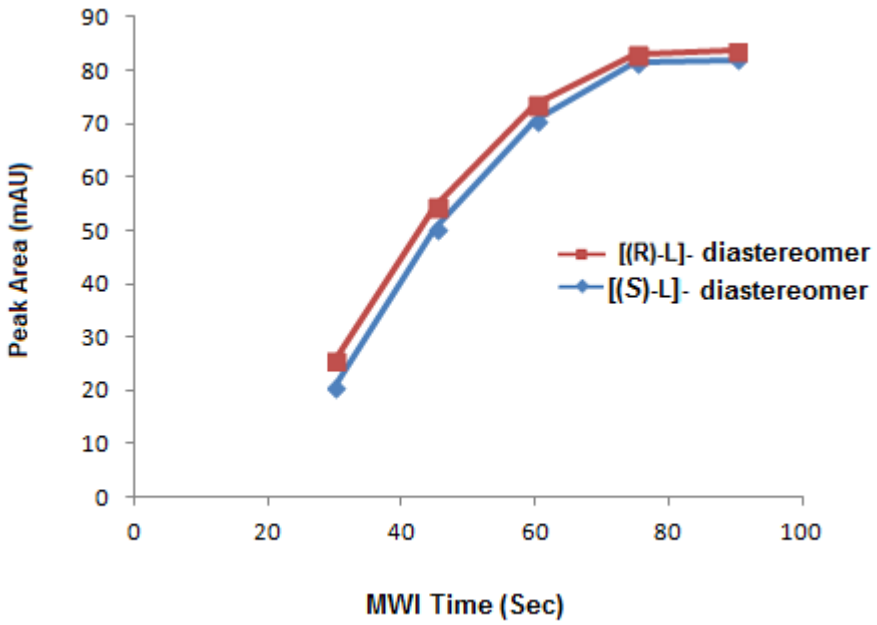


Fig. 3.6: Effect of MWI (at 80% power) on completion of derivatization reaction of Atl with CDR 10

2. Separation Mechanism

Experiments and the results clearly showed that the diastereomers of (*R*)-Atl eluted earlier than the diastereomer of the corresponding (*S*)-isomer. The elution sequence of the diastereomers and mechanism of separation can be explained by taking analogy from the model proposed (Brückner and Keller-Hoehl, 1990; Brückner and Gah, 1991; Fujii *et al*., 1997) for HPLC separation of diastereomers of amino acids prepared with Marfey's reagent.

The Gaussian 09 Rev A. 02 program and hybrid density functional B3LYP with 6-31G basis set were used to draw optimized (lowest energy) structures of the two diastereomers of Orc, prepared with CDR 11 (Fig. 3.8a and 3.8b). In [(*S*)-L]-diastereomer the hydrophobic groups are oriented in *cis*-type arrangement (Fig. 3.8a). In [(*R*)-L]-diastereomer, the groups are oriented, in *trans*-type arrangement (Fig. 3.8b). Orientations of these hydrophobic groups in optimized structures give further support to the experimental study. Explanation of *cis* and *trans* arrangement of hydrophobic groups in [(*S*)-L]-diastereomer and [(*R*)-L]-diastereomer, and elution sequence of these diastereomers, is the same as given in ''Section A'' under subheading ''Separation mechanism''.

Table-3.6: Chromatographic separation data of diastereomers of *β*-blockers prepared with different CDRs (10–15)

CDR Set C	Atl			Bel			Orc		
	k_1	α	R_s	k_1	α	R_s	k_1	α	R_s
10	15.00	1.11	8.64	15.75	1.14	10.62	13.90	1.12	9.37
11	11.00	1.10	6.14	12.05	1.12	9.30	9.21	1.11	6.68
12	12.08	1.11	8.03	13.00	1.13	10.43	10.94	1.13	8.94
13	14.14	1.13	10.49	15.42	1.14	12.71	13.50	1.16	12.17
14	12.49	1.13	9.63	14.40	1.14	11.81	12.26	1.13	11.05
15	9.42	1.09	4.74	7.59	1.13	6.65	8.87	1.10	5.86

Chromatographic conditions: RP-HPLC using LiChrospher C_{18} (250 × 4.6mm i.d., 5 µm Particle size) column; mobile phase 1, linear gradient (35 to 75%) of MeCN with 0.10% TFA for diastereomers of SeMet in 45 min; flow rate, 1.0 mL/min; detection, 340 nm. t_{R1} is retention time, k_1 is retention factor of [(*R*)-L]-diastereomers; α, separation factor; R_S, resolution; hR_F, 100 x retardation factor.

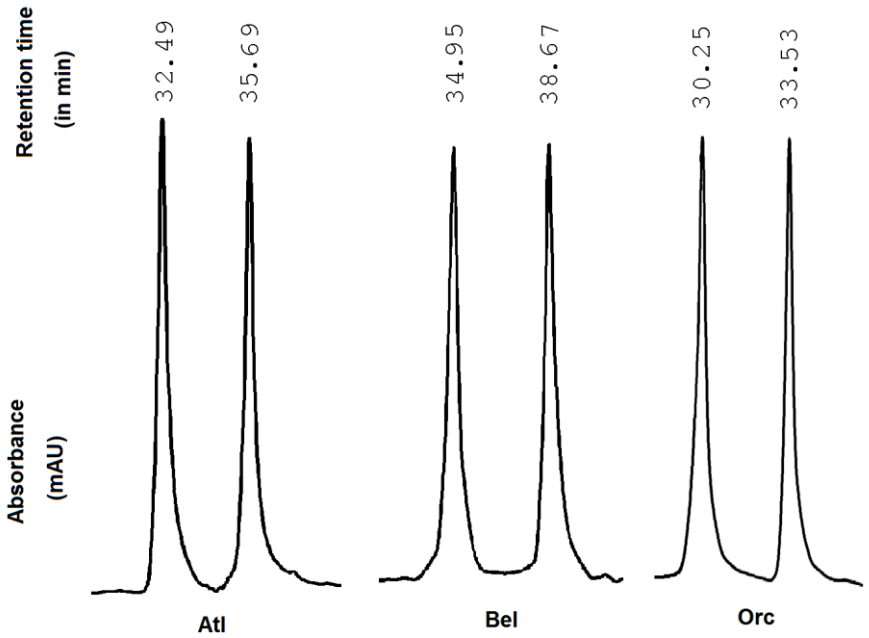


Fig. 3.7: Sections of chromatograms showing baseline resolution of diastereomers of *β*- blockers prepared with CDR 10. Chromatographic conditions: as mentioned in the legend of Table-3.6.

3. Method Validation

Method validation studies have been carried out using diastereomers of (*RS*)-Atl prepared with CDR 10.

Linearity: The calibration graphs were plotted between the peak area responses (Y-axis) of the diastereomers of (*R*)-Atl (first eluting diastereomer) and (*S*)-Atl (second eluting diastereomer) prepared with CDR 10 and the corresponding concentration (80 to 200 ng mL^{-1}; X-axis). The linear regression equation was developed by the least square method using Microsoft Excel program and was used to determine the slopes and correlation coefficients as shown in Table-3.7. A good linear relationship was obtained over this range. The regression equations were $y = 0.94x - 38.62$ ($R^2 = 0.99$) and $y = 0.93x - 39.24$ ($R^2 = 0.99$) for the first and second-eluted diastereomer, respectively.

Accuracy, Precision and Limit of Detection: The intra-day assay and inter-day assay studies for accuracy and precision were carried out by replicate HPLC analysis (n=3) of diastereomers of (*RS*)-Atl prepared with CDR10 at five concentrations levels (80, 120, 160, 200 ng mL^{-1}). The recovery and mean standard deviation (SD) for each of the diastereomers were calculated on the basis of peak areas of first and second eluting diastereomer. These are shown in Table-3.7. The relative standard deviation (RSD) for first eluted diastereomer varied from 0.55% to 1.16% for intra-day precision and from 0.44% to 1.23% for inter-day precision; these values were from 0.65% to 1.04% and from 0.49% to 1.21% for second-eluted diastereomer. Intra-day recovery for first and second-eluted diastereomer varied from 99.48% to 100.61% and from 99. 76% to 101.19%; the respective values for inter-day recoveries were from 98.67% to 99.94% and from 98.62% to 100. 21%. The LOD was 2.20ng $mL^{-1}$and 2.12ng mL^{-1} for (*R*)- and (*S*)-Atl, respectively. The LOQ was 6.60ng mL^{-1} and 6.36ng mL^{-1} for (*R*)- and (*S*)-Atl, respectively.

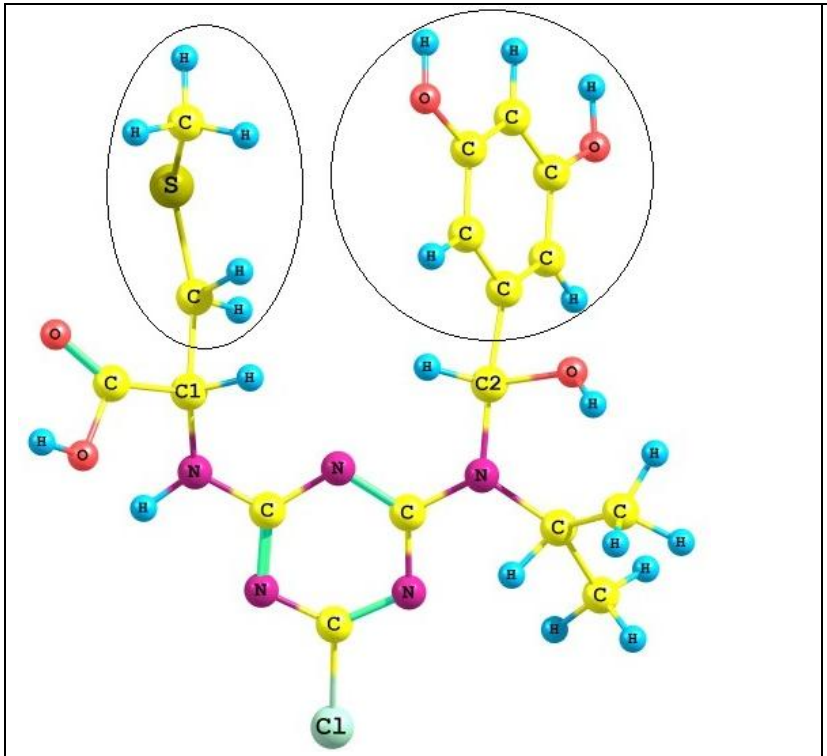


Fig. 3.8a

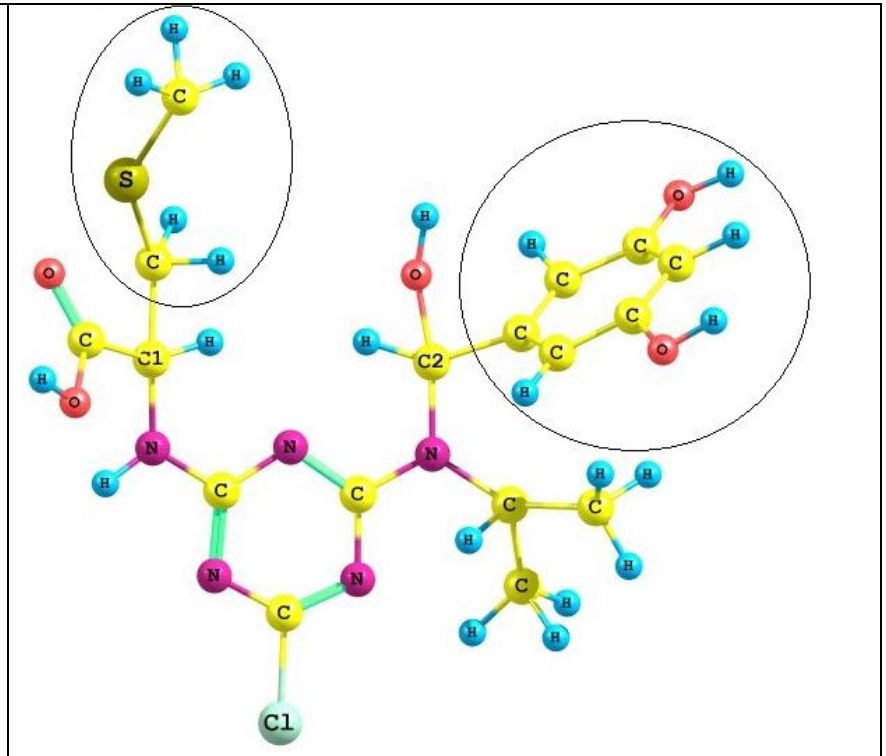


Fig. 3.8b

Fig. 3.8: Optimized structures of [(*S*)-L]-**and** [(*R*)-L]-**diastereomers of (*RS*)-Orc prepared with CDR 11.**

The program Gaussian 09 Rev A. 02 and hybrid density functional B3LYP with 6-31G basis set was used to obtain optimized structures.

Table-3.7: Results of method validation for HPLC separation of diastereomers of (*RS*)-Atl prepared with CDR 10

	First eluting diastereomer			Second eluting diastereomer		
Linearity						
Range	80 to 200 ng mL^{-1}			80 to 200 ng mL^{-1}		
Slope	0.94			0.93		
Intercept	38.62			39.24		
Correlation Coefficient (R^2)	0.99			0.99		
Actual concentration ng mL^{-1}	Mean±SD (measured) ng mL^{-1}	Recovery (%)	RSD (%)	Mean±SD (measured) ng mL^{-1}	Recovery (%)	RSD (%)
Intra-day precision (n = 3)						
80	80.49±0.94	100.61	1.16	80.95±0.84	101.19	1.04
120	119.38±1.01	99.48	0.85	119.71±0.99	99.76	0.83
160	160.19±1.09	100.12	0.68	159.79±1.15	99.87	0.72
200	199.78±1.10	99.89	0.55	200.06±1.30	100.03	0.65
Inter-day precision (n = 3)						
80	79.95±0.98	99.94	1.23	79.31±0.96	99.14	1.21
120	118.54±0.91	98.79	0.77	118.34±0.88	98.62	0.74
160	157.87±0.92	98.67	0.58	160.33±0.85	100. 21	0.53
200	199.62±0.88	99.81	0.44	199.86±0.98	99.93	0.49

C. Direct Enantioseparation of Orc, Bel and Atl by TLC using L-Glu as Impregnating Reagent, Mobile Phase Additive and as Chiral Inducing Reagent

Keeping in view the success of L-Glu for enantioseparation of *β*-blockers by TLC (Bhushan and Agarwal, 2008b), direct TLC separation of enantiomers of three *β*-blockers (namely, (*RS*)-Atl, (*RS*)-Bel, and (*RS*)-Orc) along with isolation into their native enantiomers was achieved by TLC using L-Glu as chiral selector. The chiral selector was used in the following ways,

Approach (A): Chiral Impregnating Reagent

Approach (B): CMPA

Aapproach C: chiral inducing reagent (CIR)

The detailed description of preparation of impregnated thin layer plates, use of CMPA and, pre-mixing of chiral selector with racemic mixture (achiral phase chromatography) and developments of chromatograms have been described in Chapter-2. The results of TLC resolution along with the studies related to the effects of temperature, pH and concentration of chiral selectors on enantiomeric resolution are discussed herein.

Different binary, ternary and quaternary mixtures of a variety of solvents (such as CH_3CN, CH_3OH, H_2O and CH_2Cl_2) were tried systematically to achieve enantiomeric resolution. RSD and LOD were determined in each case.

The spots were located in iodine vapor. The influence of pH, temperature and amount of chiral selector was examined on enantioseparation. Spots were scrapped from TLC plates and native enantiomers were isolated (method described in Chapter-2, under Experimental).

1. Results and Discussion

L-Glu (Fig. 3.9) is a proteinogenic amino acid. It is soluble in purified water. The present study showed all the three approaches were found successful for enantioseparation of these three *β*-blockers.

(a) Enantioseparation

Different combinations and proportions of solvents were found successful in the three different approaches. The successful mobile phase was, CH_3CN-CH_3OH-CH_2Cl_2-H_2O in the ratio of (5:1.5:1.5:1, *v*/*v*) for approach (A); (7:1:1:1.5, *v*/*v*) containing 0.15% of L-Glu for approach (B); and (6:3:3:1.5, *v*/*v*) for approach (C), respectively. Chromatograms were developed in 10 min.. hR_F (R_F x 100) values for all the three *β*-blockers are listed in Table-3.8. Some of the solvent systems that were tried but were not successful are given in Table-3.9. The resolution (R_S) values were calculated by dividing the distance between two spots by the sum of the two spot radii (Kowalska, 1990); in the present study, the highest resolution value was calculated to be 3.7 for enantiomers of Orc using the approach (A) and the lowest was 1.7 for enantiomers of Atl using the approach (C). To verify the migration order of the isomers getting separated from racemic mixture, pure enantiomer was applied in parallel on the TLC plates and results were matched. The migration distance of (*R*)-isomer was higher than that of (*S*)-isomer.

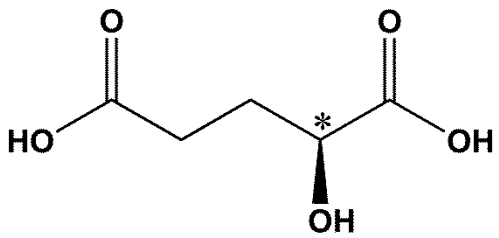


Fig 3.9: Structures of L-Glu (* represents chiral centre)

Photograph of actual chromatogram showing resolution of (*RS*)-Atl is given in Fig. 3.10, while Fig. 3.11 represents the photograph of an actual chromatogram for resolution of (*RS*)-Orc and (*RS*)-Bel on the plates impregnated with L-Glu (approach A). Fig. 3.12 and 3.13 show the photograph of actual chromatogram for resolution of all the three *β*-blockers obtained by using approach (B) and (C), respectively.

Enantiomers of each of the analytes were isolated as described in Chapter-2, under the subheading 'Separation and Isolation of Enantiomers'. Specific rotation $[\alpha]^{25}/_D$ for lower spot of Atl was found to be −10.76° (c = 0.5, MeOH) which was in agreement with the literature reports (Sigma-aldrich.com, 2013).

Enantiomers of all the three *β*-blockers were successfully detected using the three approaches in a range lower than the limits prescribed (1%) for pharmaceuticals in industry. Approach (A) was applied to separate the mixture of (*R*)- and (*S*)-enantiomers of *β*-blockers in the ratio of 1:99. Fig. 3.14 shows a representative chromatogram for separation of (*R*)-, and (*S*)-Atl (in a ratio of 1:99).

It was observed that approach (A) was better than the approach (B) in terms of R_S. This may be due to better availability of chiral selector on silica surface and also throughout the chromatographic planar bed (due to immobilization) which provided enhanced interactions between chiral selector and analyte. This argument is in agreement with the difference in experimental conditions that in approach (A) a little more quantity of the chiral selector was required to impregnate the TLC plates while in approach (B) the quantity of chiral selector required was relatively less, because chiral selector was added as a component to the mobile phase. The approach (B), was better than the approach (C), as it may be due to more quantity of the chiral selector was used in approach (B) than the approach (C). Because in approach (C), L-Glu was only mixed with the solution of *β*-blockers in 1:1 ratio and not incorporated in stationary phase.

(b) Effect of Concentration of Chiral Selector, pH and Temperature

To investigate the effect of concentration of the impregnating reagent different concentrations of L-Glu (0.05 % to 0.25% with each change of 0.05% for all three approaches) were tried. Effect of temperature on enantioresolution of *β*-blockers was investigated at 15±2, 25±2 and 35±2 °C**.** The pH of the solution (of silica gel slurry, pre-mixed solution of *β*-blockers and CIR and mobile phases) in all approaches was adjusted at 3, 5 and 7 to investigate its effect on the enantiomeric resolution. At the chromatographic conditions like, temperature (15±2 and 35±2 °C), concentrations of chiral selector (0.05, 0.1, 2.0 and 2.5%) and pH (3 and 7) there was observed eight shaped spots or tailing in spots or no resolution in spot**.**

The concentration 0.1% was not sufficient to form transient diastereomers with analyte so there was observed tailing in spots while with the concentration of chiral selector above 0.15% there was no increment in resolution than that was obtained at

0.15% concentration. The temperature of 25±2 °C was found successful for resolution via all the three approaches. Successful resolution was observed at pH 5. The pH of mobile phase in all the three approaches was adjusted around 5.

Thus, successful and optimized chromatographic conditions for direct enantioresolution, via all three (A), (B) and (C) approaches were, a temperature of 25 °C, pH 5 and concentration of chiral selector (0.15%) i.e., a ratio of 1:1 [of (*RS*)-*β*-blocker: L-Glu].

2. Separation Mechanism

Dalgliesh, (1952b) suggested that at least three *non-covalent* interactions must be involved between the analyte and the chiral selector (along with stationary phase), for enantioresolution to occur; these may be van der Waals and electrostatic interactions, hydrogen bonding, pi–pi, steric, hydrophobic or dipole–dipole, and other forms of electron donation and acceptance that are readily reversible. Electrostatic interaction between $-COO^-$ of *β*-blockers and $=NH^+$ of the chiral selector were thus responsible for maintaining the diastereomer. For these interactions to operate chiral selector and analyte should be present in oppositely charged form. In all the approaches, pH of all the solutions and the plates was maintained at 5; because at this pH, *β*-blockers existed in protonated cationic form (Burger, 1970) while L-Glu (pI 3.1) existed in the anionic form which provided electrostatic interaction between $-COO^-$ of L-Glu and $=NH_2^+$ of the chiral selector. Therefore, due to these interactions the transient diastereomers were formed and their successful separation was achieved using all the three approaches. Besides, the temperature of 25±2 °C was the optimum one to allow such non-covalent molecular interactions required for formation of the transient diastereomers and their suitable mobility for resolution to occur.

In case of approach (C), pre-mixing of racemic mixture with L-Glu, the chiral selector, resulted into formation of two diastereomeric salts, [(*R*)-L]- and [(*S*)-L], as mentioned above. The diastereomers were formed without resorting to any covalent linkage between the racemate and the chiral selector, *i.e*. these were simply due to

ionic linkages. It was the movement of diastereomers on TLC plate that resulted into separation. As mentioned above, the electrostatic interaction between COO^{-} of L-Glu and $\equiv NH^{+}$ of the analyte, hydrogen bonding and van der Waal's forces were responsible for maintaining the diastereomer.

3. Theoretical Approach for Separation Mechanism and Migration Order

The explanation, as given above, for formation and separation of transient diastereomers, has been supported by DFT using Gaussian 09 Rev. A.02 program and B3LYP hybrid density functional with 6-31G* basis set to draw optimized structures of transient diastereomers of *β*-blockers formed with L-Glu (Fig. 3.15).

Optimized structures of transient diastereomers of Orc formed with L-Glu: In [(*S*)-L]-diastereomer, $-COO^{-}$ group present on stereogenic centre C^{1} of L-Glu and $=NH_2^{+}$ group (in $-CH_2NHCH(CH_3)_2$ moiety) present on stereogenic centre C^{2} of (*S*)-Orc, are oriented closely in space and provide a strong electrostatic interaction (Fig 3.15a). The structure (in Fig 3.15a) shows that (i) amino group ($-NH_3^{+}$) on stereogenic centre C^{1} of L-Glu and –OH on stereogenic centre C^{2} of (*S*)-Orc, and (ii) carboxyl group ($-COO^{-}$) of L-Glu and –OH group present on benzene ring of Orc are in linear positions (and in the same plane) and provide strong H-bonding. Besides, there may be van der Waal's forces, steric, and hydrophobic interactions and other forms of electron donation and acceptance that are readily reversible and are responsible for formation and separation of transient diastereomers (to support three point interaction rule) which cannot be depicted in this figure (Fig 3.15a).

Table-3.8: TLC Resolution data of the three *β*-blockers by three TLC approaches using L-Glu as chiral selector

Approach	Mobile Phase	Atenolol			R_S	Orciprenaline			R_S	Betaxolol			R_S
		hR_F Pure	hR_F From racemic mixture			hR_F Pure	hR_F From racemic mixture			hR_F Pure	hR_F From racemic mixture		
		(*S*)	(*R*)	(*S*)		(*S*)	(*R*)	(*S*)		(*S*)	(*R*)	(*S*)	
(A)	1	32	53	32	2.7	41	67	41	3.7	35	54	35	3.1
(B)	2	30	47	30	2.2	37	58	37	2.8	34	56	34	2.9
(C)	3	28	44	28	1.7	33	53	33	2.1	31	50	31	2.0

(A), chiral selector mixed in the slurry of silica gel used for making the plates; (B), chiral selector added to mobile phase; and (C), Pre-mixing of CIR with analyte**.**
Mobile phase **1**, CH_3CN-CH_3OH-CH_2Cl_2-H_2O (5:1.5:1.5:1, *v*/*v*);
Mobile phase **2**, CH_3CN-CH_3OH-H_2O (7:1:1:1.5 *v*/*v*) containing 0.15% of L-Glu; and
Mobile phase 3, CH_3CN-CH_3OH-CH_2Cl_2-H_2O (6:3:3:1.5, *v*/*v*).
R_S, resolution; $hR_F = R_F \times 100$.

Table-3.9: Solvent systems unsuccessful for resolution of four *β*-blockers by L-Glu as impregnating agent, mobile phase additive and CIR

Solvent system	Composition (*v/v*)	Observation
(A), L-Glu as chiral impregnating reagent		
MeCN-MeOH-H_2O	4:4:3	NR
	6:3:2	ES
	8:2:1	RT
MeCN-H_2O-CH_2Cl_2	3:6:3	NR
	5:4:1	NR
	7:3:1	ES
CH_3CN-CH_3OH-CH_2Cl_2-H_2O	12:0.5:0.5:1	NR
	9:1:1:1.5	NR
	8:1:2:1.5	NR
(B), L-Glu as chiral mobile phase additive		
MeCN-MeOH-H_2O containing 0.15% of L-Glu	4:4:3	NR
	6:3:2	ES
	8:2:1	RT
MeCN-H_2O-CH_2Cl_2 containing 0.15% of L-Glu	3:6:3	NR
	5:4:1	ES
	7:3:1	RT
CH_3CN-CH_3OH-CH_2Cl_2-H_2O containing 0.15% of L-Glu	12:0.5:0.5:1	NR
	9:1:1:1.5	ES
	7:1.5:1:2	RT
(C), L-Glu as chiral inducing reagent		
MeCN-MeOH-H_2O	4:4:3	NR
	6:3:2	ES
	8:2:1	RT
MeCN-H_2O-CH_2Cl_2	3:7:2	NR
	4:4:1	ES
	7:3:1	RT
CH_3CN-CH_3OH-CH_2Cl_2-H_2O	12:0.5:0.5:1	NR
	9:1:1:1.5	ES
	7:1.5:1:2	RT

NR: not resolved; RT: resolved with tailing; CS: chiral selector; ES: eight shape spot.

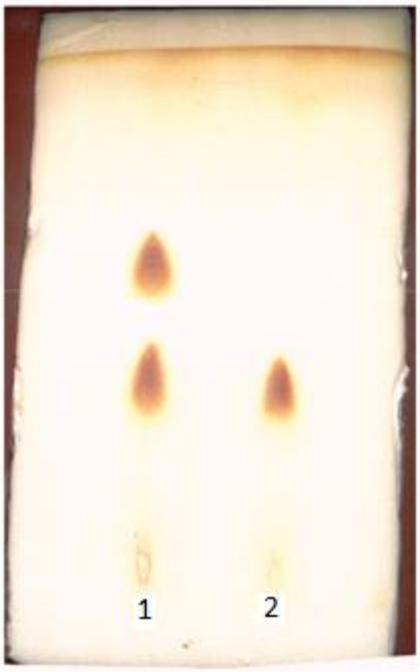


Fig. 3.10: Photograph of an actual chromatogram showing resolution of (*RS*)-Atl on the plates impregnated with L-Glu (approach (A)); from left to right: Line 1, lower spot is of (*S*)-isomer and the upper spot is of (*R*)-isomer resolved from the racemic mixture; Line 2, pure (*S*)-isomer. Mobile phase: CH_3CN–CH_3OH–CH_2Cl_2–H_2O (5:1.5:1.5:1, *v/v*). Solvent front: 8 cm; temperature: 25 ± 2 °C; detection: iodine vapor; development time: 10 min.

Fig. 3.11: Photograph of an actual chromatogram showing resolution of (*RS*)-Orc and (*RS*)-Bel on the plates impregnated with L-Glu (approach (A)); from left to right: Line 1 and 2, respectively, for (*RS*)-Orc and (*RS*)-Bel, lower spot is of (*S*)-isomer, and the upper spot is of (*R*)-isomer resolved from the racemic mixture. Chromatographic conditions are the same as in Fig. 3.10.

Fig. 3.12: Photograph of an actual chromatogram showing resolution of (*RS*)-Atl, (*RS*)-Orc and (*RS*)-Bel using L-Glu as CMPA (approach (B)). From left to right: Line 1, 2 and 3, respectively, for (*RS*)-Orc, (*RS*)-Bel and (*RS*)-Atl, lower spot is of (*S*)-isomer, and the upper spot is of (*R*)-isomer resolved from the racemic mixture. Mobile phase: CH_3CN-CH_3OH-H_2O (7:1:1:1.5, *v*/*v*) containing 0.15% of L-Glu. Other chromatographic conditions are the same as in Fig. 3.10.

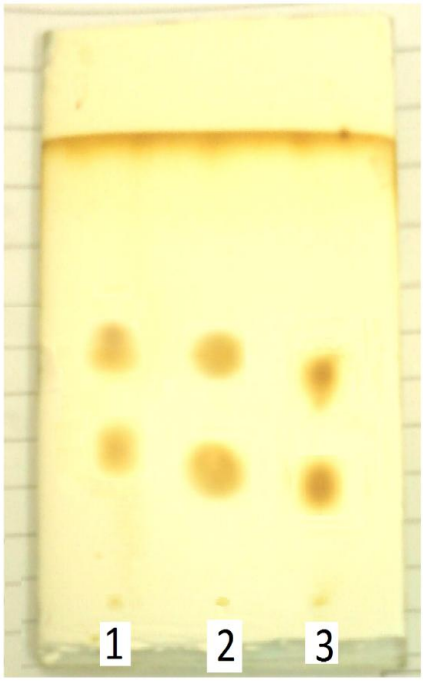


Fig. 3.13: Photograph of an actual chromatogram showing resolution of (*RS*)-Atl, (*RS*)-Orc and (*RS*)-Bel using approach (C). From left to right: Line 1, 2 and 3, respectively, for (*RS*)-Orc, (*RS*)-Bel and (*RS*)-Atl, lower spot is of (*S*)-isomer, and the upper spot is of (*R*)-isomer resolved from the racemic mixture. Mobile phase: CH_3CN–CH_3OH–CH_2Cl_2–H_2O (6:3:3:1.5, *v/v*). Solvent front: 8 cm; temperature: 25 ± 2 °C; detection: iodine vapor; development time: 10 min.

Fig. 3.14: Photograph of an actual chromatogram showing resolution of (*RS*)-Atl using approach (A). From left to right: Line 1, (*R*:*S*), 1:99, lower spot for (*S*)-isomer and upper spot for (*R*)-isomer; line 2, is of (*S*)-isomer. Chromatographic conditions are the same as in Fig. 3.10.

In [(*R*)-L]-diastereomer (Fig. 3.15b), the $–COO^–$ group on C^1 of L-Glu and $=NH_2^+$ group (in, $–CH_2NHCH(CH_3)_2$ moiety) on C^2 of (*R*)-Orc appear to be away from each other. Similarly, $–NH_3^+$ group on C^1 of L-Glu and –OH present on C^2 of (*R*)-Orc are also not seen in the same plane. Thus, there are weaker interactions as compared to those seen in [(*S*)-L]-diastereomer. However, carboxyl group ($–COO^–$) of L-Glu and –OH of benzene ring in Orc appear to be in the same plane and show H-bonding.

Optimized structures of transient diastereomers of Atl formed with L-Glu: Explanation for optimized structures, [(*R*)-L]- and [(*S*)-L]-diastereomer of Atl with L-Glu was the same as of the diastereomers of Orc with L-Glu (Fig 3.16a and Fig. 3.16b).

Fig 3.16a represents [(*S*)-(+)]-diastereomer; it shows that $–COO^–$ group present on stereogenic centre C^1 of L-Glu (being anionic at pH 5 under experimental conditions), and $=NH_2^+$ group (in $–CH_2NHCH(CH_3)_2$ moiety) present on stereogenic centre C^2 of (*S*)-Atl (being cationic at pH 5), are oriented closely in space and provide a strong electrostatic interaction. Besides, amino group ($–NH_3^+$) on stereogenic centre C^1 of L-Glu and –OH on stereogenic centre C^2 of (*S*)-Atl are oriented in linear positions and provide strong H-bonding. Besides, there may be steric, van der Waal's forces, and hydrophobic interactions and other forms of electron donation and acceptance that are readily reversible and are responsible for formation and separation of transient diastereomers (to support three point interaction rule) which cannot be depicted in this figure.

Fig. 3.16b represents [(*R*)-L]-diastereomer; it shows that the $–COO^–$ group on C^1 of L-Glu and $=NH_2^+$ group (in, $–CH_2NHCH(CH_3)_2$ moiety) on C^2 of L-Atl appear away from each other. At the same time, $–NH_3^+$ group on C^1 of L-Glu and –OH on C^2 of L-Atl are not seen in the same plane. Thus, there are weaker interactions as compared to those seen in [(*S*)-L]-diastereomer.

4. RSD and Limit of Detection

The mean values (n = 5) for RSD (precision) for the *β*-blockers were in the ranges 0.40–1.09% for separation on plates impregnated with chiral selector. To determine the detection limit, solutions of different concentrations of (*R*)-Atl (in the range of 0.1 to 1.0%) were spiked with fixed amount of (*S*)-Atl. These were subjected to TLC separation. The results indicated that this method was successful for detection of (*R*)-Atl up to 0.2% in (*S*)-Atl. However, the detection limits for enantiomers of the selected compounds were found within the range 1.2–1.8 μg (i.e., 1.2, 1.3 and 1.8 μg for Atl, Bel and Orc, respectively). Fig. 3.14 shows a representative chromatogram of resolution of (*R*)- and (*S*)-Atl (in a ratio of 1:99).

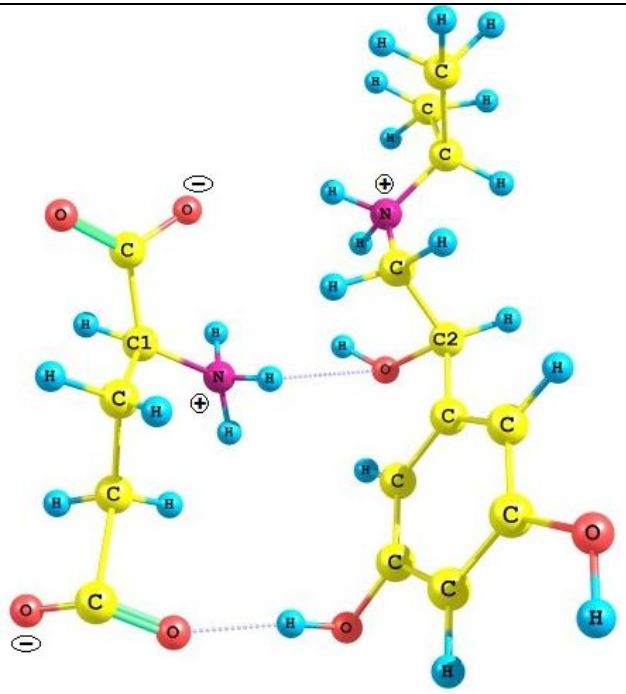


Fig. 3.15a

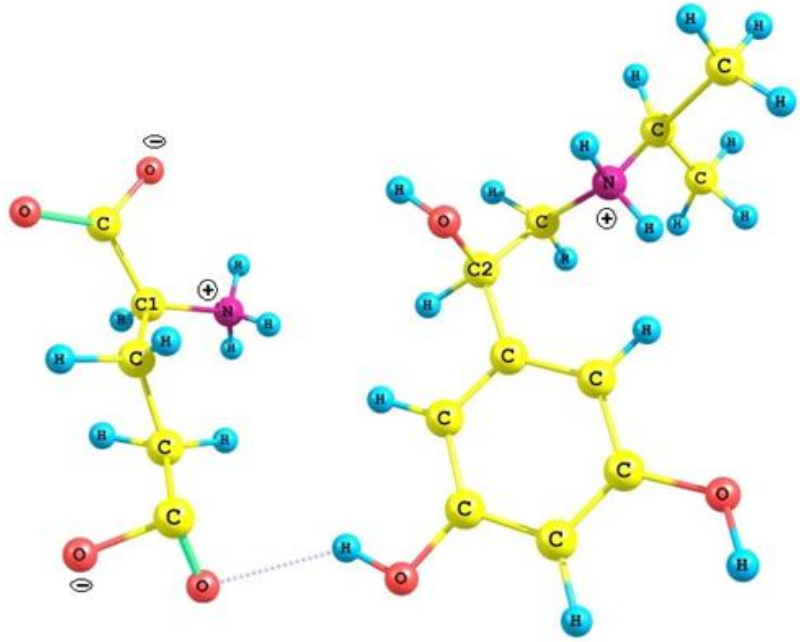


Fig. 3.15b

Fig. 3.15: Structures of transient diastereomers of Orc with L-Glu, optimized and drawn using the Gaussian 09 Rev. A.02 program and B3LYP hybrid density functional with 6-31G* basis set. Fig. 3.15a, [(*S*)-L]-diastereomer, and **Fig.** 3.15b, [(*R*)-L]-diastereomer; explanation described under "Theoretical Approach for Separation Mechanism and Elution Order".

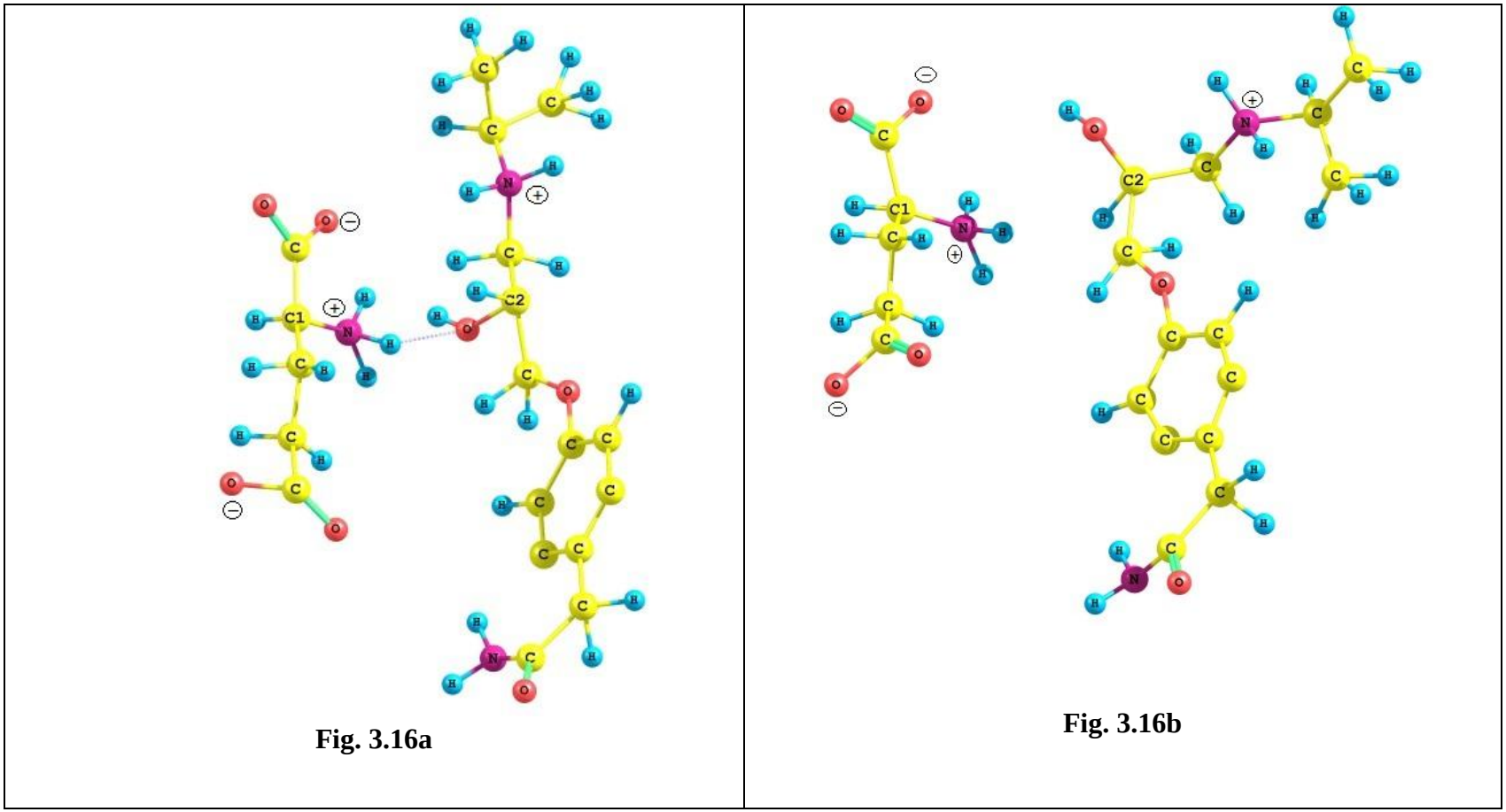


Fig. 3.16a

Fig. 3.16b

Fig. 3.16: Optimized structures of transient diastereomers of Atl with L-Glu. Fig. 3.16a, [(*S*)-L]-diastereomer, and Fig. 3.16b, [(*R*)-L]-diastereomer; explanation described under "Theoretical Approach for Separation Mechanism and Elution Order". The program Gaussian 09 Rev A. 02 and hybrid density functional B3LYP with 6-31*G basis set was used to obtain optimized structures.

D. Separation of Enantiomers of ISP by direct TLC using L-Glu as Chiral Impregnating Reagent and Separation of Diastereomers (Prepared with DFDNB based CDRs) by HPLC and TLC

Considering the literature reports on resolution of (±)-ISP (Fig. 3.17) using expensive chiral columns and other aspects pointed out herein the issues involved with respect to separation of (±)-ISP were to develop a few new, cost effective and simple methods of enantioseparation of (±)-ISP. Thus, following approaches were adopted

(i) Resolution of enantiomers of (±)-ISP using TLC plates impregnated with L-Glu as chiral selector and verification of native enantiomers by isolation (because of advantages of TLC compared to HPLC as summarized by Sherma (2001) for pharmaceutical and drug analysis, and earlier reports in this area (Bhushan *et al*., 2012; Bhushan and Thiongo, 1998; Bhushan and Arora, 2003; Bhushan and Agarwal, 2008b),

(ii) Three chiral derivatizing reagents, based on DFDNB moiety, were synthesized having L-Alanine, L-Valine and S-Benzyl-L-cysteine as chiral auxiliaries. These chiral amino acids were chosen because they increase the molar absorptivity (already discussed in Chapter-2). These were used for synthesis of diastereomers of (±)-ISP (because of the ease of synthesis of such CDRs and our experience on their application (Bhushan and Tanwar, 2008b; Bhushan and Tanwar, 2009b),

(iii) RP-HPLC and RP-TLC have been performed for separation of diastereomers. Enantiomerically pure, (+)-ISP was also converted into its diastereomer which was used as standard for the determination of elution order of diastereomers of (±)-ISP. Their elution order in experimental study of RP-TLC and RP-HPLC separation was supported by developing optimized structures of diastereomers based on density functional theory. The method was validated for accuracy, precision, LOD and LOQ.

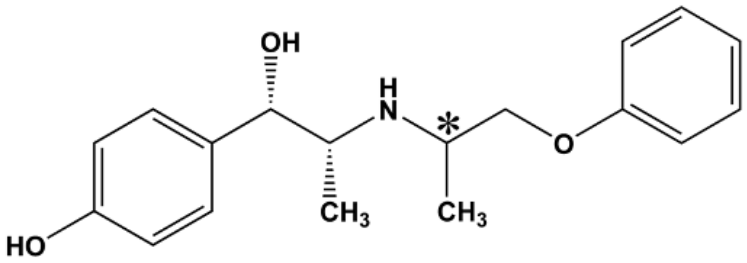


Fig. 17(a)

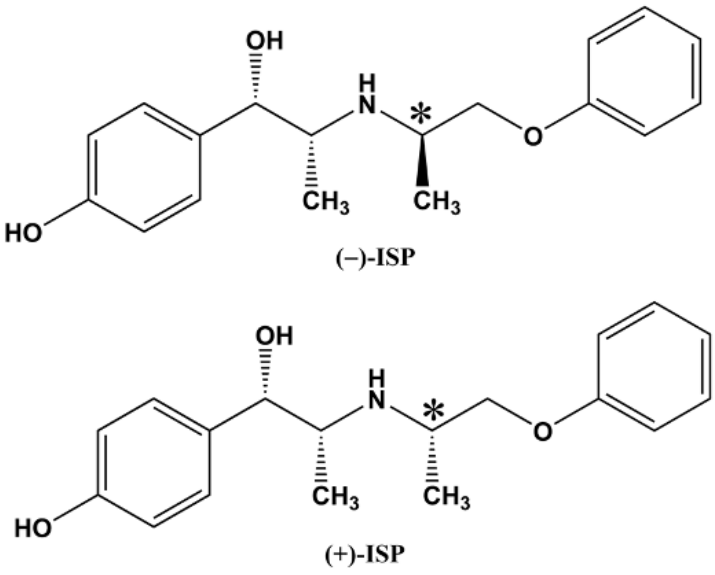


Fig. 17(b)

Fig. 3.17: (a), Structures of (±)-ISP (* represents chiral centre); (b), Structures of (−)-ISP and (+)-ISP (* represents chiral centre)

1. Results and Discussion

(a) Resolution Using Impregnated Plates

L-Glu was used as chiral selector for impregnation of thin layer plate. Enantiomers of (±)-ISP were successfully resolved using the solvent system, CH_3CN–CH_3OH–CH_2Cl_2–H_2O (5:1.5:0.5:1 *v*/*v*) in 10 min. Spots were located by exposure to iodine vapors. R_F values are averages from at least five experiments performed under identical conditions on the same day and on different days. R_F values of 0.83 and 0.52 were obtained for (−)-ISP and (+)-ISP, respectively, and resolution was found as 3.06. The chromatogram showing resolution of (±)-ISP on plates impregnated with L-Glu are given in Fig. 3.18.

The pI value of L-Glu is 3.1 and therefore it existed in anionic form at pH 5 under the experimental conditions. The beta blocking agents could exist as protonated cations at this pH value (Burger, 1970). Therefore, it can be considered that ionic interactions between -COO^- of the chiral selector and the cation of ISP molecule, hydrogen bonding and steric interactions are playing a role, resulting *in situ* formation of diastereomers and hence enantioresolution.

Formation of such diastereomers was further evidenced by detecting the presence of amino acid in the two resolved spots by treating the chromatogram with ninhydrin solution; the plate acquired a light pink background but the resolved spots were visible with greater intensity of characteristic color and sharpness (Bhushan and Thiongo, 1998).

Chiral selector in concentration less than 0.5 %, temperature less than 25 °C and pH 3, 4, 6, 7 were not found to be the appropriate conditions for separation of enantiomers. Elongation or tailing of spots was observed at concentration > 0.5 % and temperature > 25 °C. Chiral selector at 0.5 % concentration, pH 5 and a temperature of 25 °C were established as the optimized conditions for successful resolution.

(b) CDRs and Diastereomers

The three CDRs **(2**, 5 and 7; *cf*. Fig. 2.1; Chapter-2) were to form diastereomers of (±)-ISP which were then separated by RP-HPLC and RP-TLC (indirect approach).

Substitution of the remaining fluorine atom in DNFB based CDRs resulted in the formation of three pairs of diastereomers of (±)-ISP. Reaction at pH around 11 using 30 μL of 10% TEA was found to be optimum for derivatization of (±)-ISP using each of the three CDRs. The derivatization was free from side products and was quantitative. Diastereomers of the type [(+)-L]-, and [(−)-L]-, were formed where the first letter refers to the configuration of the analyte and the second to that of the chiral auxiliary of the CDR. Diastereomer of (+)-ISP was also synthesized which had [(+)-L]- configuration.

In the absence of TEA no derivatization occurred both under conventional heating method and by using MW irradiation for 1 to 5 min. In both the methods (conventional heating for 60 min at 45 °C) or under MW irradiation (80 s at 75 % of 800 W), 1.7 molar excess of CDR was successful for complete derivatization. At a lower ratio a slight kinetic resolution was observed. Diastereomers prepared by using conventional heating and MW irradiation were found to be identical in terms of their spectroscopic and chromatographic characteristics. The peak areas corresponding to all the diastereomers obtained for each change of derivatization conditions (mentioned under Experimental) were calculated by system software; these served as a measure of completion of reaction and yield of derivatization.

(c) Yields and Stability of Diastereomers

The recovery studies of the two eluted diastereomers (as described in "Accuracy, precision and limit of detection"), served as a measure of their yields of the order of 99%, since recovery of all eluted diastereomers was found to be more than 98.56%.

Stability of the diastereomers was investigated after long-term (refrigerated at a temperature of 3 to 5 °C) and short-term (room temperature) storage as a function of storage conditions including the container system and their chemical properties. The evaluation also included stability of the analyte in stock solution and situations encountered during actual sample handling and analysis. Based on HPLC experiments carried out at an interval of 10 days up to 50 days from the day of synthesis the diastereomers were found to be stable for 30 days under refrigeration conditions (4 °C).

(d) RP-TLC Separation of Diastereomers

The hR_F and R_S are given in Table-3.10 and are also compared with separation data

obtained with RP-HPLC. Among all the three diastereomeric pairs, the best resolution was found for the diastereomers prepared with CDR 2 (Table-3.10). Resolution was calculated as described above for enantioresolution on impregnated plates. Chromatograms are shown in Fig. 3.19. Mobile phase A containing TEAP buffer (50 mM, pH 4.6) – MeCN (4: 5, *v*/*v*) at 25 °C was found to be successful for separation of all the three diastereomeric pairs; an increase of MeCN concentration resulted in increase of hR_F and decrease of R_S. With mobile phase D, resolution was poorer than that obtained with mobile phase A.

Table-3.10 shows that R_S was higher for diastereomers prepared with CDR 2 AND 5 separated by RP-TLC in comparison to R_S (3.06) obtained for enantiomers by direct TLC.

(e) RP-HPLC Separation of Diastereomers

The data for resolution (R_S), separation factor (α) and retention factor (k) for separation of three pairs of diastereomers are given in Table-3.10. The chromatogram showing separation of diastereomers of (±)-ISP prepared with CDR 2 and, 5 and 7 are given in Fig. 3.20 and 3.21, respectively. The chromatogram of diastereomer of (+)-ISP prepared with CDR 2 was compared with chromatogram of diastereomers of (±)-ISP prepared with CDR 2 (Fig. 3.20).

Chromatographic conditions were optimized, for separation of diastereomers of (±)-ISP prepared with CDR 2, with respect to the concentrations of TFA, TEAP and of organic modifier in the mobile phase and its flow rate and run time using C_{18} column. These conditions affect retention time, separation factor and resolution (R_S). Sharper peaks were observed with the mobile phase used under gradient conditions in comparison to those obtained under isocratic conditions. Presence of TEAP in the mobile phase resulted into broad peaks. Therefore, the effect of TFA was investigated in the concentration range of 0.05% to 0.20% and 0.10% TFA was established as the optimized concentration when sharp peaks showing base line resolution were obtained.

Diastereomers of (±)-ISP prepared with CDR 2, 5 and 7 were better resolved under linear gradient (35 to 75%) of MeCN as organic modifier and 0.10% TFA, in 60 min run at a flow rate of 1.0 mL/min and detection at 340 nm.

Fig. 3.18: Actual photograph of chromatogram showing resolution of (±)-ISP using L-Glu impregnated plates. From left to right: Line 1, lower spot is of (−)-isomer, and the upper spot is of (+)-isomer resolved from the racemic mixture; Line 2, pure (−)-isomer. Development time, 10 min; temperature, 25±2 °C; Solvent system, CH_3CN–CH_3OH–CH_2Cl_2–H_2O (5:1.5:0.5:1, v/v). Solvent front: 8.4 cm. Detection, iodine vapors.

Table-3.10: Chromatographic separation data of diastereomers of (±)-ISP prepared with CDRs 2, 5 and 7

	RP- HPLC				RP- TLC		
	t_{R1}	k_1	α	R_s	hR_F		R_s
CDR					[(+)-L]-,	[(−)-L]-,	
2	36.45	16.65	1.12	7.79	20.0	53.3	4.4
5	26.75	11.95	1.11	6.56	25.0	56.7	4.2
7	23.33	10.29	1.09	4.56	36.7	63.3	2.9

Chromatographic conditions: RP-HPLC using LiChrospher C_{18} (250 × 4.6mm i.d., 5 µm particle size); mobile phase 2, linear gradient (35 to 75%) of MeCN with 0.10% TFA in 60 min; flow rate, 1.0mL/min; detection, 340 nm.

Silica gel 60 RP-18 $F_{254}S$ plates were used for RP-TLC, mobile phase: MeCN with TEAP (4: 5 *v*/*v*); detection, in ordinary light. Configuration of diastereomers is represented as, [(+)-L], and [(−)-L]-, where the first letter refers to the configuration of the analyte and the second to that of the chiral auxiliary of the CDR.

t_{R1} is retention time, k_1 is retention factor of [(+)-L]-diastereomer; α, separation factor; R_S, resolution; hR_F, 100 x R_F.

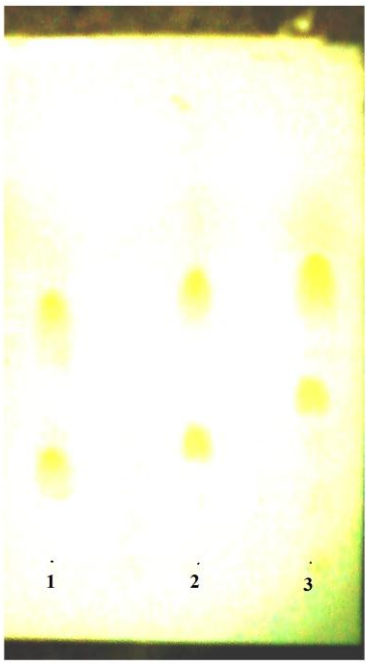


Fig. 3.19: Chromatogram obtained by RP-TLC showing separation of diastereomers of (±)-ISP. Line 1, 2 and 3 show spots of diastereomers of (±)-ISP prepared with CDR 2, 5 and 7, respectively. The upper spot is of [(+)-L]-diastereomer and the lower spot is of [(−)-L]-diastereomer. The mobile phase was triethylamine phosphate buffer (50 mM, pH 4.6)-acetonitrile (5:4, *v*/*v*), the solvent front was 6.5 cm and development time, 10 min.

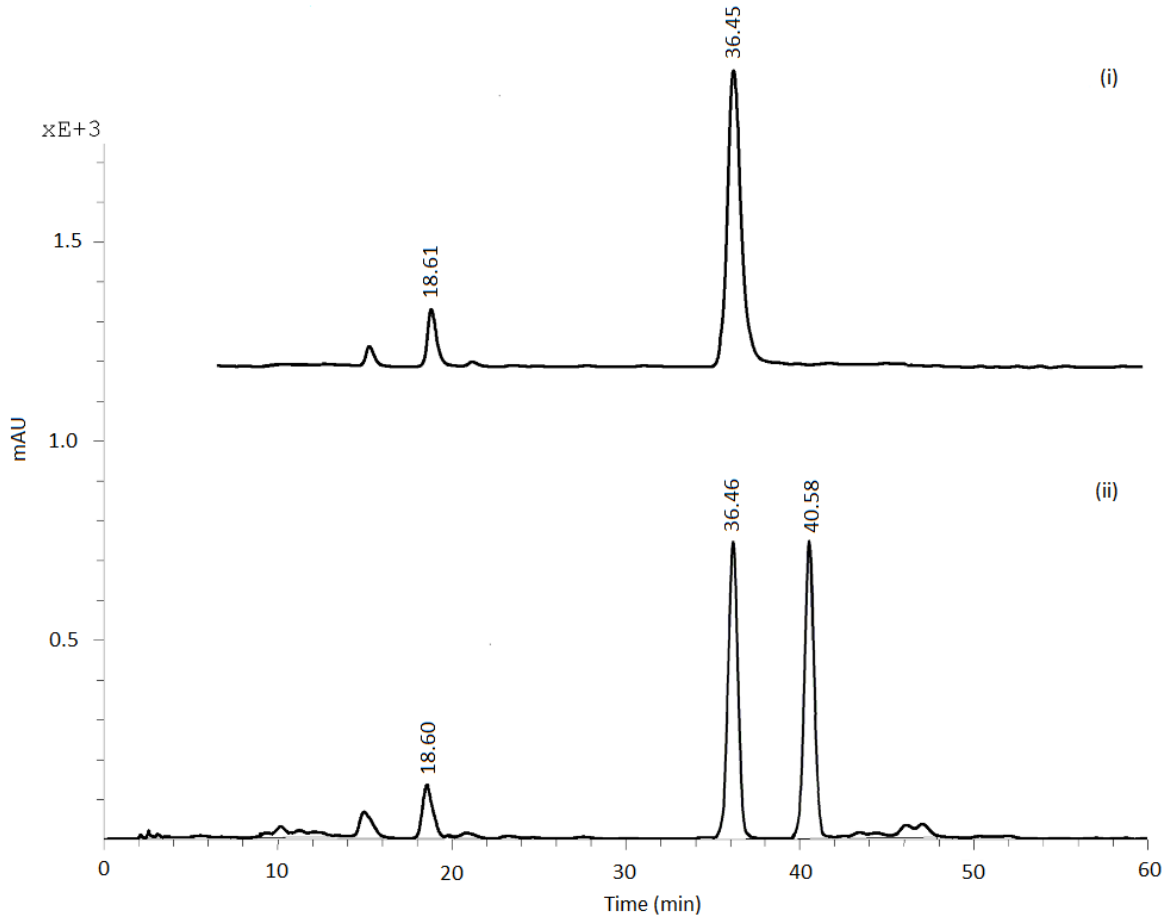


Fig. 3.20: Chromatogram of diastereomers of (±)-ISP prepared with CDR 2. Chromatogram (i) is of the derivative of (+)-ISP and the peaks appeared at 18.61, 36.45 min belong to retention time of CDR 2 and the diastereomer of (+)-ISP, respectively, (ii) is showing the enantioseparation of (±)-ISP using CDR 2. Peaks appeared at 18.60, and 36.46 and 40.58 min belong to retention time of CDR 2, and diastereomers of (+)-ISP and (−)-ISP, respectively. Chromatographic conditions: as mentioned in the legend of Table-3.10, for RP-HPLC.

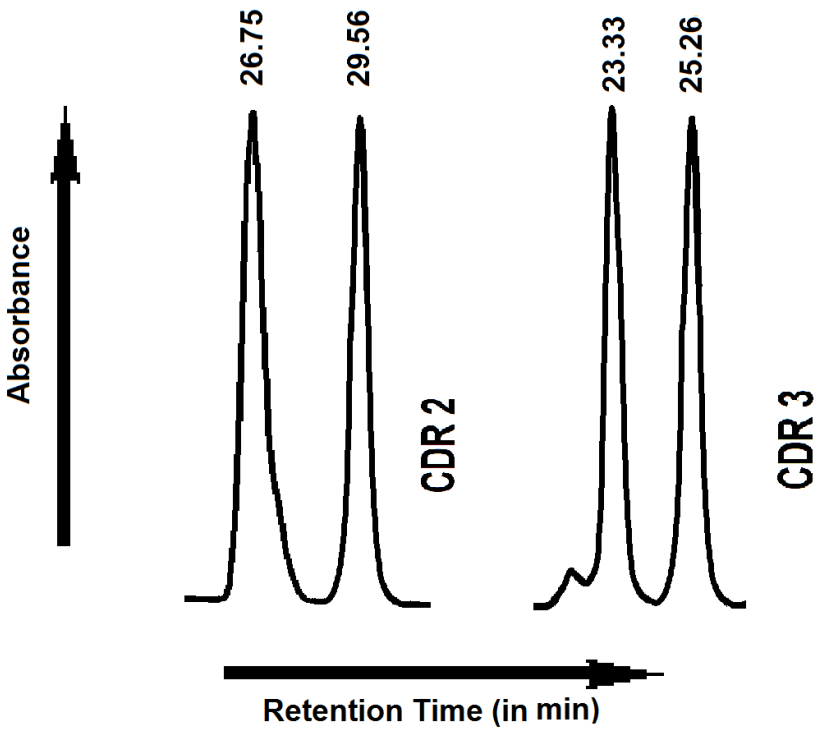


Fig. 3.21: Sections of chromatograms are showing resolution of diastereomers of (±)-ISP using CDR 5 and 7. Chromatographic conditions: as mentioned in the legend of Table-3.10, for RP-HPLC.

2. Separation Mechanism

Experiments and the results clearly showed that the diastereomers of (+)-ISP eluted earlier than the diastereomer of the corresponding (−)-isomer. The elution sequence of the diastereomers and mechanism of separation can be explained by taking analogy from the model proposed (Brückner and Keller-Hoehl, 1990; Brückner and Gah, 1991; Fujii *et al*., 1997) for HPLC separation of diastereomers of amino acids prepared with Marfey's reagent.

The Gaussian 09 Rev A. 02 program and hybrid density functional B3LYP with 6-31G basis set (**Fig. 3.22**) were used to draw optimized (lowest energy) structures of the two diastereomers. Orientations of these optimized structures give further support to the experimental study. In [(–)-L]-diastereomer, the hydrophobic groups, $–CH_2SC_6H_5$ and $–CH_2OC_6H_5$ present on the stereogenic centre of CDR (C-1 in Fig. 3.22a) and ISP (C-2 in Fig. 3.22a), respectively, are oriented on the same side with respect to the plane of dinitrobenzene moiety and are considered to be *cis* to each other (Fig. 3.22a). On the other hand, in [(+)-L]-diastereomer, these hydrophobic groups are oriented in space opposite to the plane of dinitrobenzene moiety and thus have *trans*-type arrangement (Fig. 3.22b). A graphical representation for the same is also shown in Fig. 3**.23**. The *cis*- or *trans*-type arrangements of the two diastereomers are responsible for difference in their hydrophobicities.

The *cis*- and *trans*-type arrangements (as explained above), large size of S atom in CDR and the presence of benzene group in CDR (contributing to the overall hydrophobicity) along with rheological properties of the mobile phase, are responsible for different partition coefficients and different retention times of [(+)-L]-and [(−)-L]-diastereomers. Therefore, the diastereomers eluted one after another.

3. Method Validation

Method validation studies have been carried out using diastereomers of (±)-ISP prepared with CDR 2.

Linearity: The calibration graphs were plotted between the peak area responses (Y-axis) of the diastereomers of (+)-ISP (first eluting diastereomer) and (−)-ISP (second eluting diastereomer) prepared with CDR 2 and the corresponding concentration (90 to 180 ng mL^{-1}; X-axis). The linear regression equation was developed by the least square method using Microsoft Excel program and was used to determine the slopes and correlation coefficients as shown in Table-3.11. A good linear relationship was obtained over this range. The regression equations were $y = 1.24x – 2.51$ ($R^2 = 0.99$) and $y = 1.26x – 0.84$ ($R^2 = 0.99$) for the first and second-eluted diastereomer, respectively.

Accuracy, Precision and Limit of Detection: The intra-day assay and inter-day assay studies for accuracy and precision were carried out by replicate HPLC analysis (n=3) of diastereomers of (±)-ISP prepared with CDR 2 at five concentrations levels (90, 120, 150, 180 µg mL^{-1}). The recovery and mean standard deviation (SD) for each of the diastereomers were calculated on the basis of peak areas of first and second eluting diastereomer. These are shown in Table-3.11. The relative standard deviation (RSD) for first eluted diastereomer varied from 0.58% to 1.16% for intra-day precision and from 0.43% to 1.24% for inter-day precision; these values were from 0.61% to 1.02% and from 0.46% to 1.20% for second-eluted diastereomer. Intra-day recovery for first and second-eluted diastereomer varied from 99.51% to 100.45% and from 99.64% to 101.08%; the respective values for inter-day recoveries were from 98.66% to 99.92% and from 98.56% to 100.12%. LOD was found to be 23 and 22 pg mL^{-1}, respectively, for diastereomers of (+)-ISP and (−)-ISP. LOQ was found to be 69 pg mL^{-1} and 66 pg mL^{-1} for the diastereomers of (+)-ISP and (−)-ISP, respectively, using HPLC and 13 ng mL^{-1} and 11 ng mL^{-1} for the same, using RP-TLC. In direct TLC the LOD for (+)-ISP and (−)-ISP was found to be in the range of 0.1-0.09 µg mL^{-1}.

4. Comparison of Resolution with Literature Reports

The diastereomers of (±)-ISP prepared with the CDR 2, 5 and 7 in the present study have been separated using indirect HPLC with R_S of 7.79, 6.56 and 4.56, respectively, which is better than the resolution reported in literature, for example, R_S was 1.5 using chiral cellulose and amylose column (Peng *et al*., 2010); 4.18 using brush type CSP (Hoffmann *et al*., 2007); 0.6 using potassium polypectate as chiral selector in capillary electrophoresis (Phinney *et al*., 1999); 2.1 using chiral selector (*S*)-*N*-(4-allyloxy-3,5-dichlorobenzoyl)-2-amino-3,3-dimethylbutane phosphonic acid immobilized onto the reactive sulfhydryl surface of silica based capillary column (Preinerstorfer *et al*., 2006), 1.0 using sulfated cyclodextrins by capillary zone electrophoresis (Stalcup and Gahm, 1996], and 1.38 (Wolrab *et al*., 2013a) and 1.12 (Wolrab *et al*., 2013b) using strong cation exchange-type chiral stationary phase based on syringic acid amide derivative of trans-(*R,R*)-2-aminocyclohexanesulfonic acid.

5. Comparison of R_S, LOD and Elution Time in the Present Study

A comparative study of these three methods concluded that R_S obtained by HPLC was 7.79, 6.56 and 4.56 respectively, for the diastereomers prepared with CDRs 2, 5 and 7, while R_S obtained by TLC were 4.4, 4.2 and 2.9 respectively, for the diastereomers prepared with same CDRs. R_S was 3.06 for the enantiomers separated via direct approach. Thus R_S of diastereomers of (±)-ISP was found higher than that obtained for the enantiomers of (±)-ISP. The limit of detection under indirect approach using HPLC was in the range of pg mL^{-1} because a DNB chromophore was present in the diastereomers that helped in on-line detection by UV and the detector was also very sensitive. TLC was found to be a rapid approach as it took 10 min in developing the chromatogram while it took 23 to 40 min in HPLC.

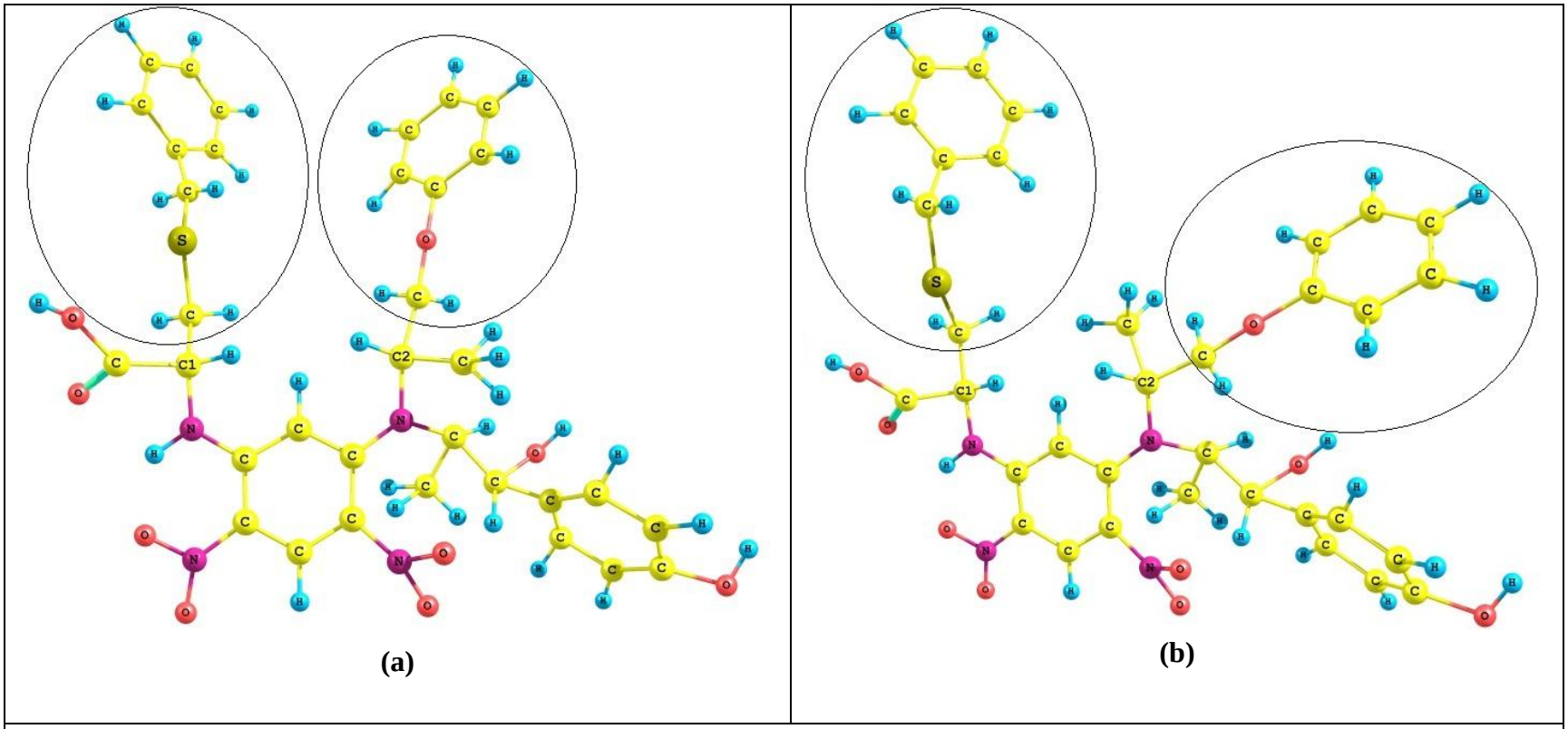
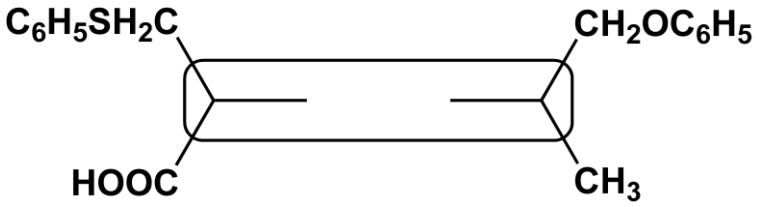
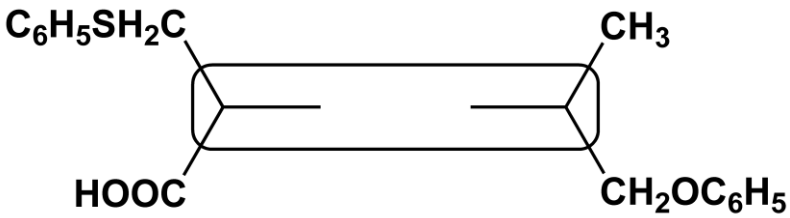


Fig. 3.22: Optimized structures of [(−)-L]- and [(+)-L]-diastereomer of ISP.
The program Gaussian 09 Rev A. 02 and hybrid density functional B3LYP with 6-31G basis set was used to obtain optimized structures.



***cis*- type arrangement**



***trans*- type arrangement**

Fig. 3.23: Schematic representation of *cis* and *trans* type arrangement of hydrophobic groups in [(−)-L]- and [(+)-L]-diastereomers of (±)-ISP

Table-3.11: Results of method validation for HPLC separation of diastereomers of (±)-ISP prepared with CDR 2

	First eluting diastereomer			Second eluting diastereomer		
Linearity						
Range	90 to 180 ng mL^{-1}			90 to 180 ng mL^{-1}		
Slope	1.24			1.26		
Intercept	2.51			0.84		
Correlation Coefficient (R^2)	0.99			0.99		
Actual concentration ng mL^{-1}	Mean±SD (measured) ng mL^{-1}	Recovery (%)	RSD (%)	Mean±SD (measured) ng mL^{-1}	Recovery (%)	RSD (%)
Intra-day precision (n = 3)						
90	90.41±1.01	100.45	1.16	90.97±0.93	101.08	1.02
120	119.41±0.99	99.51	0.83	119.57±0.97	99.64	0.81
150	150.23±1.01	100.15	0.67	149.84±1.05	99.89	0.70
180	179.75±1.04	99.86	0.58	180.11±1.10	100.06	0.61
Inter-day precision (n = 3)						
90	89.93±1.12	99.92	1.24	89.22±1.07	99.13	1.20
120	118.79±0.88	98.99	0.74	118.27±0.92	98.56	0.78
150	147.99±0.81	98.66	0.55	150.18±0.89	100.12	0.59
180	178.67±0.77	99.26	0.43	179.95±0.83	99.97	0.46
Sensitivity						
LOD (pg mL^{-1})	23			22		
LOQ (pg mL^{-1})	69			66		

E. Inference

Diastereomers were synthesized under MWI in shorter reaction time and derivatization conditions were easily optimized.

The increased molar absorptivity of DNB moiety due to *para* positioned $–NO_2$ groups lead to detection at concentration lower than the limit of 1% prescribed for pharmaceutical industry and proved to be better in terms of resolution in comparison to certain other CDRs reported in literature (Table-3.3).

The explanation for separation mechanism and elution order is supported by developing optimized structures of the two diastereomers using Gaussian 09 Rev A. 02 program and hybrid density functional B3LYP with 6-31G (d, p) basis set.

The three approaches for direct TLC enantioresolution of Orc, Bel, and Atl using L-glu as chiral selector can be successfully used in routine analysis in pharmaceutical industry and R & D activities, as well, since they are capable to detect enantiomers of various *β*-blockers (Orc, Bel, Atl, and ISP) at concentration lower than the limit of 1% prescribed for pharmaceutical industry while RP-HPLC provided better resolution than TLC in the range of pg mL^{-1}, for (±)-ISP in particular. The direct approach of enantioresolution by TLC is simple and is less expensive in terms of commercial availability of the materials and not requiring expensive instrumentation and is also capable of providing native enantiomers. The present approach opens another area for general application and utility of TLC as a complimentary technique for HPLC separations and there is no competition between HPLC and TLC.

•

Chapter-4

Enantioseparation of (*RS*)-Flx using HPLC and TLC

Contents **Page No.**

Chapter-4

Enantioseparation of (*RS*)-Flx using HPLC and TLC

I. Flx

Fluoxetine (Flx, Fig. 4.1) {*N*-methyl-γ-[4-(trifluoromethyl)-phenoxy] benzenepro-panamine} is a widely used antidepressant due to its efficacy and reduced side effects (Friegner and Boyer, 1991). It belongs to the selective serotonin reuptake inhibitor (SSRI) family of drugs. In 1987, FDA approved it initially for the treatment of depression, and has since been prescribed to over 40 million patients; fluoxetine's prominence was manifested when it was featured on the cover of Newsweek's March 1990 issue as "A breakthrough drug for depression", and listed as one of the "Pharmaceutical Products of the Century" in the November 1999 issue of Fortune (Wong *et al*., 2005). It is also one of a few antidepressants that is approved for children and adolescents (Ludwig *et al*., 2009), and is increasingly used during pregnancy (Cooper *et al*., 2007). Currently, it is approved by the FDA for the treatment of major depression, bulimia nervosa, obsessive-compulsive disorder, panic disorder, and premenstrual dysphoric disorder (US-FDA, 2010).

The (*S*)-(–)-enantiomer of Flx has small but distinguishable stereospecificity and is slightly more potent in inhibiting serotonin reuptake than (*R*)-(+)-Flx *in vitro* (Wong *et al*., 1990). The duration of serotonin inhibition was 24 h for a single dose of pure (*S*)-Flx while with a single dose of (*R*)-Flx inhibition persisted up to 8 h only. In other words, (*S*)-Flx is approximately 1.5 times more potent than (*R*)-Flx and displays a three-fold longer duration of action. (*S*)-enantiomer is effective in the treatment of migraine headaches while (*R*)-enantiomer is effective for treatment of depression. Due to these features enantioseparation of racemic Flx becomes significant in the area of pharmaceutical science.

II. Literature Survey on Liquid Chromatographic Enantioseparation of Flx

Direct HPLC enantioseparation of Flx has been achieved using CSPs based on ovomucoid and tris(3,5-dimethylphenyl carbamate) cellulose (Olsen, 1998), tris-3,5-dimethylphenyl carbamate derivative of cellulose and amylose (Bonato *et al*., 1999), perphenyl carbamate of *β*-cyclodextrin (Yu *et al*., 2002), dinitrophenylated cyclodextrin derivative (Zhong *et al*., 2006). Piperaki *et al*., (1993) used *β*-Cyclodextrin as CSPs for enantioseparation of fluoxetine by CE and LC (Piperaki *et al*., 1995).

ChiralCel OD column was used for HPLC separation of pre-column derivatized Flx. The derivatizing reagents used were 4-(*N*-chloroformylmethyl-*N*-methyl) amino-7-nitro-2,1,3-benzoxadiazole (NBD-COCl) and 4-(*N*-chloroformylmethyl-N-methyl)amino-7-dimethylaminosulfonyl-2,1,3-benzoxadiazole (DBD-COCl) reagents (Yu *et al*., 2006).

For HPLC enantioseparation by indirect approach, Flx was derivatized with (*R*)-1-(1-napthylethyl) isocyanate and resulting diastereomers were separated in NP elution mode (Potts and Parli, 1992) and RP elution mode (Unceta *et al*., 2007). Derivatization of Flx has also been done using *R*-(−)-mandelic acid as CDR (Bopp, 1998) which was followed by NP-HPLC separation of corresponding diastereomers. Marfey's reagent and (*S*)-*N*-(4-Nitrophenoxycarbonyl) phenylalanine methoxyethyl ester were used for RP-HPLC enantioseparation of Flx (Bhushan and Agarwal, 2010a) while direct TLC separation and isolation of the enantiomers of Flx was achieved using L-tartaric acid as mobile phase additive.

In this chapter enantioseparation of (*RS*)-Flx by HPLC and TLC has been described.

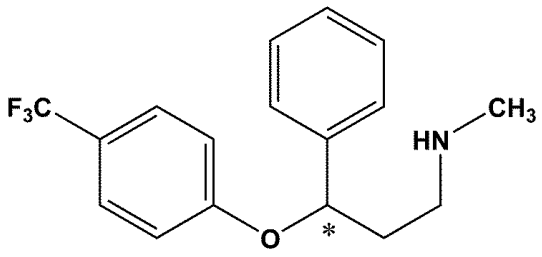


Fig. 4.1: Structure of (*RS*)-Flx

III. Present work

A. Enantioseparation of (*RS*)-Flx by HPLC and TLC using DFDNB based CDRs Containing SMLC, SBLC, L-Leu and L-Val as Chiral Auxiliaries

1. DFDNB based CDRS

The characteristic features of DFDNB and the success of CDRs based on it for separation of pharmaceutically important compounds have already been described in Chapter-2 and 3.

In view of the literature cited above, following studies were carried out in the present case,

1. The CDRs 1, 2, 4 and 5 (as described in Chapter-2) were used for synthesis of diastereomers of (*RS*)-Flx. The explanation for choosing SMLC, SBLC, L-Leu and L-Val as chiral auxiliaries for the synthesis of DFDNB based CDRs and the characteristic features imparted by these chiral auxiliaries to the CDRs have already been described in Chapter-3, Section II A.

2. Four pairs of diastereomers (prepared with CDRs 1, 2, 4 and 5) were separated by RP-HPLC and two diastereomeric pairs of (*RS*)-Flx (prepared with CDRs 4 and 5) were separated by RP-TLC. Experimental studies carried out to determine the elution order of diastereomers in RP-TLC and RP-HPLC were confirmed by developing optimized structures of diastereomers based on density functional theory. The method was validated for accuracy, precision, Limit of detection (LOD) and limit of quantitation (LOQ).

2. Results and Discussion

(a) Synthesis of Diastereomers

Four diastereomeric pairs of (*RS*)-Flx were synthesized by nucleophilic substitution of the remaining fluorine atom in the DNFB based CDRs. The general scheme for synthesis of diastereomers of (*RS*)-Flx with CDR 1 (as a representative) is shown in Chapter-2 (Fig. 2.13). Diastereomers of the type [(*R*)-L]-, and [(*S*)-L]-, were formed where the first letter refers to the configuration of the analyte and the second to that of the chiral auxiliary of the CDR. These diastereomers were kept under refrigerated

condition (at 0-5 °C). Stability of these diastereomers was checked by measuring the peak area ratio of the stored samples at an interval of 10 days up to 80 days from the day of synthesis of diastereomers and comparing the same with freshly prepared samples under identical conditions of separation. It was found that these were stable for 50 days.

Derivatization conditions were optimized in terms of molar ratio of CDR: analyte, MWI time and conventional heating time to establish completion of reaction of (*RS*)-Flx with CDR **1** (as representative for the reactions of (*RS*)-Flx with the remaining three CDRs).

Following reaction conditions were tried to obtain an optimum reaction yield.

(i) Role of Variation in Molar Ratios of Analyte: CDR

(*RS*)-Flx and CDR were allowed to react in different ratios such as, 1:1.5, 1:1.7 and 1:2. Quantitative derivatization was achieved at 1.7 fold molar excess of CDR. At higher ratios there was no significant change in reaction time and yield of derivatization while at lower ratio there was observed a slight kinetic resolution. Therefore, 1.7 fold molar excess of CDR was chosen for the synthesis of all diastereomers.

(ii) Role of pH

Solutions of analytes were prepared in 1M $NaHCO_3$ and it was also added to the reaction mixture during derivatization to establish pH 8.

(iii) Role of MWI and Conventional Heating

The effect of the MWI time and power on derivatization was investigated by varying the MWI for 50, 60, 70 and 80 s at each of the three different power settings, viz, 70, 80 and 90%. Heating of reaction mixture for 70 s (at 80% of 800 W) under microwave was found as optimized condition to complete the derivatization reaction (as shown in Fig. 4.2). To optimize the heating time and temperature in conventional method separate sets of reaction mixture were incubated at 35, 40, 45 and 50 °C with constant stirring for 30, 40, 50, 60, 70, 80 and 90 min (at each temperature). Conventional heating at 45 °C in an incubator for 70 min with constant stirring was found successful to give best yield of derivatization.

In both the methods (conventional heating for 70 min at 45 °C) or under MW irradiation (70 s at 80 % of 800 W), 1.7 molar excess of CDR and pH 8 were found successful for complete derivatization.

The peak areas of each of the diastereomers (from every pair), obtained in HPLC experiments, confirmed the formation of the diastereomers and their separation.

(b) RP-HPLC Separation of Diastereomers

All four synthesized diastereomeric pairs of (*RS*)-Flx were synthesized and separated by RP-HPLC on C_{18} column. Different mobile phases containing MeCN or MeOH as organic modifier and *aq* TFA were tried for separation. The mobile phase was filtered through a 0.45 µm filter and degassed by passing nitrogen and sonication, before use, to protect the column from any types of impurity and air bubbles.

Following mobile phases were prepared and used,

Mobile phase 1: MeCN and aqueous TFA (0.1%), applied in isocratic mode (10:90, 20:80, 40:60, 60:40 and 80:20 *v/v*) and gradient mode (35 to 80, 35 to 60, 30 to 80, 30 to 60, 30 to 50, 25 to 80, 25 to 60, 25 to 50, 20 to 70 and 10 to 90% of MeCN) for 45 min.

Mobile phase 2: MeOH and aqueous TFA (0.1%); it was used in a manner similar to described for mobile phase 1 (both isocratic and linear gradient modes).

(i) Optimization of Separation Conditions

Chromatographic conditions were optimized with respect to the concentrations of organic modifier and TFA in the mobile phase and its flow rate. Detection of diastereomers was made at 340 nm. Concentration of aqueous TFA was varied in the range of 0.01% to 0.20%, on increase in concentration from 0.01% to 0.10% there was found an increment in resolution while at the concentration greater than 0.10% there was no significant change in resolution. Since concentration of aqueous TFA higher than the 0.10% may cause the damage to the column, therefore, 0.10% was considered as optimized concentration.

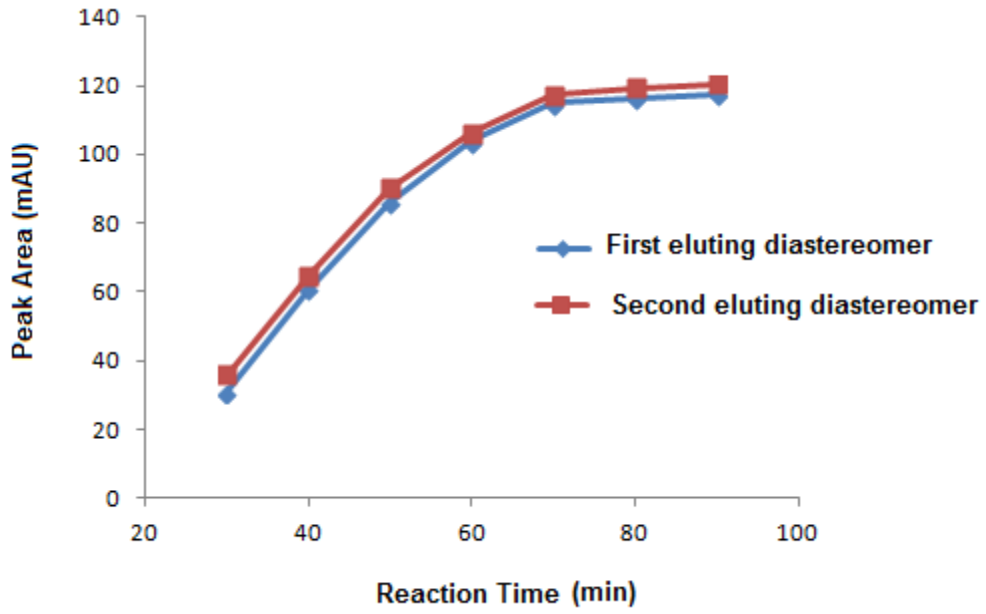


Fig. 4.2: Effect of conventional heating time for derivatization of (*RS*)-Flx with CDR 1

The flow rate (0.5 to 1.5 mL min^{-1}) of mobile phase was also varied in portion of 0.5 mL min^{-1} to optimize separation conditions. On decreasing the flow rate from 1 to 0.5 mL min^{-1} an increase of retention time and slight broadening of peaks was observed while on increasing the flow rate from 1 to 1.5 mL min^{-1} resulted in lowering the retention time and decreasing the resolution.

It was found that both the mobile phases were capable of separating all the four pairs of diastereomers of DL-SeMet. With the mobile phase 1 (aqueous TFA-MeCN), under both the isocratic and gradient elution modes, there appeared sharper peaks in comparison to mobile phase 2 (TFA-MeOH). But mobile phase 1, in the gradient elution of MeCN from 30 to 60% and 0.1% TFA, in 45 min at a flow rate of 1.0 mL/min provided sharper peaks than in other elution modes.

In mobile phase 2, MeOH was used as organic modifier with aqueous TFA. MeCN could be considered as better organic modifier because MeOH has lower dielectric constant (33 D) and higher viscosity (0.59 cP at 25 °C) than MeCN (having a

dielectric constant of 37.5 D and viscosity of 0.343 cP at 25 °C). Due to these features of MeCN, smaller retention times and higher resolution was observed with it.

(ii) ***Separation of Diastereomers***

All the 4 pairs of diastereomers were separated on C_{18} column using RP-HPLC. The mobile phase 1 in linear gradient elution of MeCN from 30 to 60% and 0.1% TFA, in 45 min at a flow rate of 1.0 mL/min was found as successful chromatographic condition for separation of all these four pairs of diastereomers. The chromatographic data obtained for resolution of diastereomers of (*RS*)-Flx with mobile phase 1, in terms of retention factor (k), separation factor (α), and resolution (R_S) are listed in Table-4.1. Among all the four pairs of diastereomers, the best R_S was found for the diastereomeric pairs of (*RS*)-Flx prepared with CDR 4 while the least resolution was observed for the diastereomeric pairs of (*RS*)-Flx prepared with CDR 1. The sections of chromatograms showing separation of diastereomers of (*RS*)-Flx prepared with CDRs 1, 2, 4 and 5 are shown in Fig. 4.3. The peak areas calculated by the system software, corresponding to diastereomers obtained under varying conditions of derivatization, served as a measure of completion of reaction and yield of derivatization.

(c) RP-TLC Separation of Diastereomers

The two diastereomers of (*RS*)-Flx prepared with CDRs 4 and 5 were separated by RP-TLC on DC Kieselgel 60 RP*18 $F_{254}S$ stationary phase. Stock solutions of diastereomers of (*RS*)-Flx were diluted 10 times with MeCN; these were applied on RP-TLC plates using 10 µL Hamilton syringe.

Following mobile phases were tried,
Mobile phase A: TEAP buffer–MeCN (as organic modifier)
Mobile phase B: TEAP buffer–MeOH (as organic modifier)

The hR_F and R_S values are given in Table-4.2. The resolution (R_S) values were calculated by dividing the distance between two spots by the sum of the two spot radii (Kowalska, 1990). Chromatograms are shown in Fig. 4.4. Mobile phase A containing TEAP buffer (50 mM, pH 4.5) – MeCN (5:5, *v*/*v*) at 25 °C was found to be successful for separation of diastereomeric pairs; an increase of MeCN concentration resulted in

increase of hR_F and decrease of R_S. With mobile phase B, resolution was poorer than that obtained with mobile phase A. With mobile phase A, R_S of diastereomeric pairs of (*RS*)-Flx prepared with CDR 4 was found better than the R_S obtained for diastereomeric pairs of (*RS*)-Flx prepared with CDR 5.

Effect of the temperature on R_S was investigated by varying the temperature as 15 ± 2 ℃, 25 ± 2 ℃ and 35 ± 2 ℃. Table-4.3 is showing that, at 15 ± 2 ℃ temperature no resolution was observed and at 25 ± 2°C spots were resolved with good resolution value. While at 35 ± 2 ℃ spots were resolved but there was tailing in spots. Thus, the temperature 25 ± 2 ℃ was considered as optimized condition.

Table-4.1: RP-HPLC data of diastereomers of (*RS*)-Flx prepared with different CDRs (1, 2, 4 and 5)

CDRs	t_{R1}	t_{R2}	k_1	k_2	α	R_S
1	16.68	18.18	5.96	6.82	1.14	5.52
2	28.87	31.29	9.69	10.59	1.09	10.68
4	27.54	30.88	9.20	10.44	1.13	10.77
5	21.97	24.14	7.50	8.70	1.16	7.62

Chromatographic conditions: RP-HPLC using LiChrospher C_{18} (250 × 4.6mm i.d., 5 µm particle size) column; mobile phase 2, linear gradient (30 to 60%) of MeCN with 0.10% TFA for diastereomers of Flx in 45 min; flow rate, 1.0mL/min; detection, 340 nm. t_{R1} and t_{R2} are retention time, k_1 and k_2 are retention factors of [(*S*)-L]- and [(*R*)-L]-diastereomer, respectively; α, separation factor; R_S, resolution.

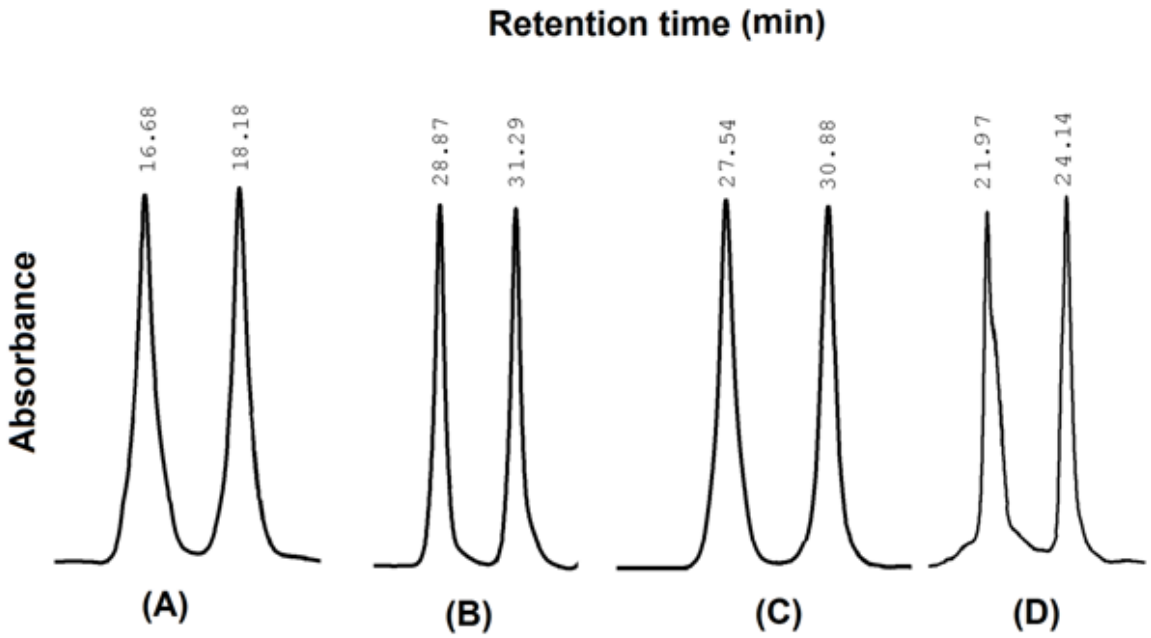


Fig. 4.3: Sections of chromatograms (A), (B), (C) and (D) showing resolution of diastereomers of (*RS*)-Flx prepared with CDRs 1, 2, 4 and 5, respectively. Chromatographic conditions: as mentioned in the legend of Table 4.1.

Table-4.2: RP-TLC separation data of diastereomers of (*RS*)-Flx prepared with different CDRs (4 and 5)

CDR	hR_F		R_S
	[(*R*)-L]-	[(*S*)-L]-	
4	23	48	3.75
5	32	55	3.50

Silica gel 60 RP-18 $F_{254}S$ plates were used for RPTLC, mobile phase: TEAP buffer (50 mM, pH 4.5) – MeCN (5:5, *v*/*v*); detection, in ordinary light. Configuration of diastereomers is represented as, [(*R*)-L], and [(*S*)-L]-, where the first letter refers to the configuration of the analyte and the second to that of the chiral auxiliary of the CDR. hR_F, 100 x R_F; R_S, resolution.

Fig. 4.4: Chromatograms in line (a) and (b) are showing resolution of diastereomers of (*RS*)-Flx prepared with CDR 4 and 5, respectively.
Chromatographic conditions: as mentioned in the legend of Table 4.2.

Table-4.3: Effect of temperature on resolution of (*RS*)-Flx using successful mobile phase

CDR	Temperature ^{o}C		
	15 ±2 ^{o}C	25 ±2 ^{o}C	35 ±2 ^{o}C
4	NR	R	TS
5	NR	R	TS

The successful mobile phase MeCN with TEAP (5: 5 *v*/*v*); were used during all these studies; **R**: Resolved into enantiomers; **NR**: not resolved; **TS**: spot with tailing; temperature 15±2°C, and spots were located with iodine vapour.

(d) Recovery and %Yield of diastereomers

Yield (%) of diastereomers was determined by performing small-scale preparative separation of diastereomers. The detail of the method is given in Chapter-3 under the subheading ‘‘Recovery and %Yield of diastereomers’’. The %yield of the recovered diastereomers was calculated and is given in Table-4.4. The recovery studies of the two eluted diastereomers (as described in section ‘‘Method validation’’), also served as a measure of their yields of the order of 98% since recovery of all eluted diastereomers was found to be more than 97.36%.

3. **Separation Mechanism**

The elution sequence of diastereomers of (*RS*)-Flx was confirmed by optimizing structures of diastereomers of (*RS*)-Flx (prepared with CDR 1, as a representative) with the help of density functional theory using the Gaussian 09 Rev A. 02 program and hybrid density functional B3LYP with 6-31G (d, p) basis set. In both the methods (RP-HPLC and RP-TLC) the elution sequence of the diastereomers was the same.

The elution sequence of the diastereomers of (*RS*)-Flx and mechanism of separation can be explained by taking analogy from the model proposed (Brückner and Keller-Hoehl, 1990; Brückner and Gah, 1991; Fujii *et al*., 1997) for HPLC separation of

diastereomers of amino acids prepared with Marfey's reagent. The detail is described in Chapter-3 under the section A (subheading ''Separation mechanism'').

The optimized structures of the [(*R*)-L]- and [(*S*)-L]-diastereomers of (*RS*)-Flx (prepared with CDR 1) are given in Fig 4.5. In [(*R*)-L]-diastereomer, the hydrophobic groups, $–CH_2SCH_3$ and $–C_6H_5$ present on the stereogenic centre of CDR (C-1 in Fig. 4.5a) and Flx (C-2 in Fig. 4.5a), respectively, are oriented on the same side with respect to the plane of dinitrobenzene moiety and are considered to be *cis* to each other (Fig. 4.5a). On the other hand, in [(*S*)-L]-diastereomer, these hydrophobic groups are oriented in space opposite to the plane of dinitrobenzene moiety and thus have *trans*-type arrangement (Fig. 4.5b). A graphical representation for the same is also shown in Fig. 4.6. The *cis*- or *trans*-type arrangements of the two diastereomers are responsible for difference in their hydrophobicities.

The proposed mechanism gets confirmed with the experimental observation that the [(*S*)-L]-diastereomer is eluted first from the column which means it is retained for lesser time in comparison to that of the [(*R*)-L]-diastereomer. In other words, the [(*R*)-L]-diastereomer (having *cis*-type arrangement) interacts more strongly with ODS material of C_{18} column than the *trans*-type arrangement, hence has a longer retention time. These retention times of the diastereomers are thus reflecting the overall hydrophobicity of the two molecules. It can therefore be concluded that the hydrophobicity of *cis*-type arrangement is more than that of *trans*-type arrangement. The diastereomers of (*RS*)-Flx, prepared with CDRs 2, 4 and 5, corresponding to (*S*)-enantiomer were eluted prior to those corresponding to (*R*)-isomer.

The *cis*- and *trans*-type arrangements (as explained above), large size of $–CH_2SCH_3$ present in CDR (contributing to the overall hydrophobicity) along with rheological properties of the mobile phase, are responsible for different partition coefficients and different retention times of [(*R*)-L]-and [(*S*)-L]-diastereomers. Therefore, the diastereomers eluted one after another.

Table-4.4: Showing % yield of diastereomers of (*RS*)-Flx recovered after separation

CDR	Diastereomers of (*RS*)-Flx		
	Theoretical yield (mg)	1	2
CDR 1	7.30	3.37 (46.26)	3.41 (46.75)
CDR 2	8.21	3.94 (47.98)	3.89 (47.42)
CDR 4	7.25	3.27 (45.07)	3.32 (45.81)
CDR 5	7.08	3.41 (48.12)	3.35 (47.28)

1 and 2 are representing experimental yield of [(*R*)-L]- and [(*S*)-L]-diastereomer, respectively in mg and % yield of diastereomer is shown below in parentheses.
Mol wt of Flx is 309.33 gm mol^{-1}. The molecular weights of the diastereomers of Flx prepared with CDR 1, 2, 4 and 5, respectively are, 608.58, 684.19, 604.57 and 590.20 gm mol^{-1}.

4. Method Validation

Method validation studies have been carried out using diastereomers of (*RS*)-Flx prepared with CDR 1.

***(i) Linearity*:** The calibration graphs were plotted between the peak area responses (Y-axis) of the diastereomers of (*R*)-Flx (first eluting diastereomer) and (*S*)-Flx (second eluting diastereomer) prepared with CDR 1 and the corresponding concentration (30 to 120 ng mL^{-1}; X-axis). The linear regression equation was developed by the least square method using Microsoft Excel program and was used to determine the slopes and correlation coefficients as shown in Table-4.5. A good linear relationship was obtained over this range. The regression equations were $y = 1.05x - 13.84$ ($R^2 = 0.998$) and $y = 1.12x - 14.04$ ($R^2 = 0.999$) for the first and second-eluted diastereomer, respectively.

***(ii) Accuracy, Precision and Limit of Detection*:** The intra-day assay and inter-day assay studies for accuracy and precision were carried out by replicate HPLC analysis (n=3) of diastereomers of (*RS*)-Flx prepared with CDR 1 at five concentrations levels (30, 60, 90, 120 µg mL^{-1}). The recovery and mean standard deviation (SD) for each of the diastereomers were calculated on the basis of peak areas of first and second eluting diastereomer. These are shown in Table-4.5. The relative standard deviation (RSD) for first eluted diastereomer varied from 0.53% to 1.26% for intra-day precision and from 0.55% to 1.35% for inter-day precision; these values were from 0.65% to 1.33% and from 0.62% to 1.24% for second- eluted diastereomer. Intra-day recovery for first and second-eluted diastereomer varied from 98.78% to 102.12% and from 99.47% to 101.82%; the respective values for inter-day recoveries were from 98.81% to 100.02% and from 98.59% to 100.46%. LOD was found to be 14 pg mL^{-1}, respectively, for the diastereomers of each enantiomer of (*RS*)-Flx prepared with CDR 1. LOQ was found to be 42 pg mL^{-1}, respectively, for the diastereomers of each enantiomer of (*RS*)-Flx prepared with CDR 1, using HPLC and 17 ng mL^{-1} for diastereomers of each enantiomer of (*RS*)-Flx prepared with CDR 4, using RP-TLC.

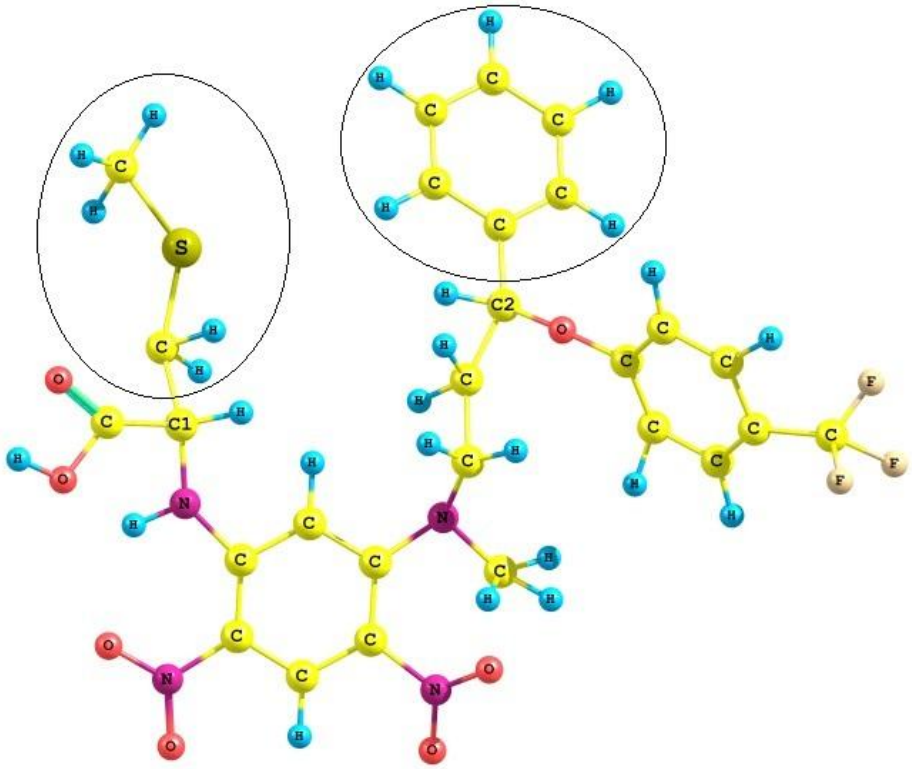


Fig. 4.5a: [(*R*)-L]-diastereomer

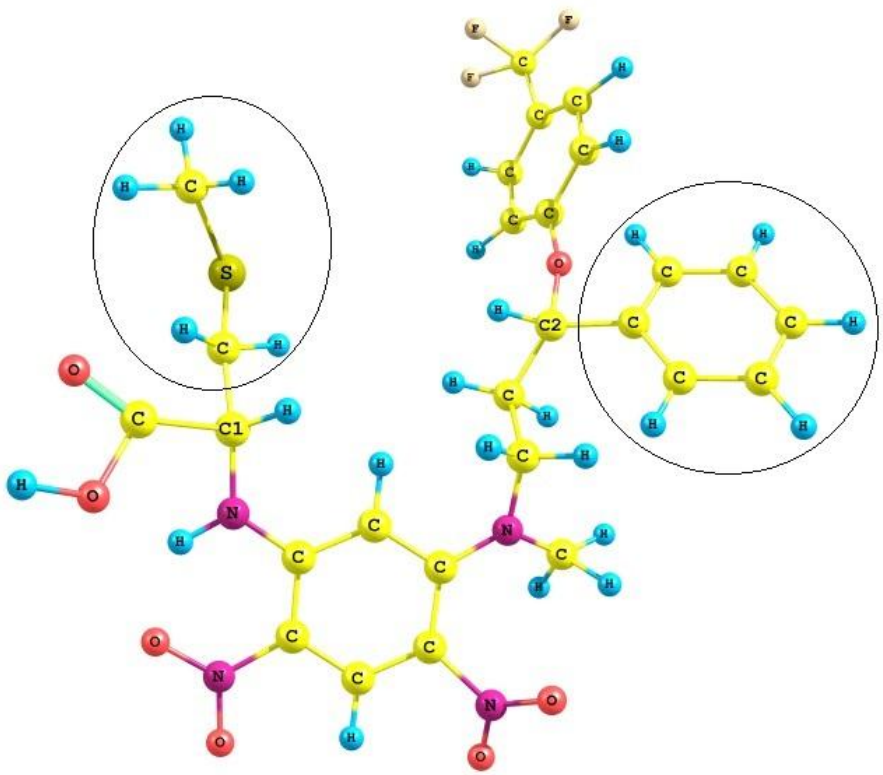
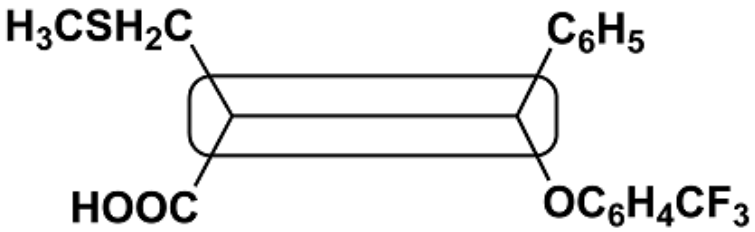
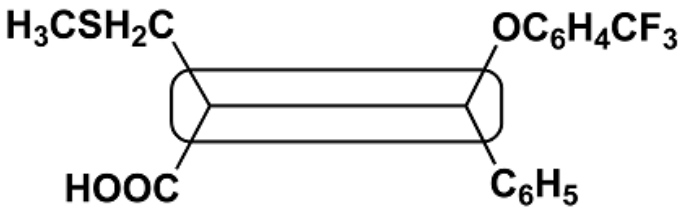


Fig. 4.5b: [(*S*)-L]-diastereomer

Fig. 4.5: Optimized structures of [(*R*)-L]- and [(*S*)-L]-diastereomer of (*RS*)-Flx prepared with CDR 1



[(*R*)-L]-Diastereomer



[(*S*)-L]-Diastereomer

Fig. 4.6: Schematic representation of *cis* and *trans* type arrangement of hydrophobic groups in diastereomers of (*RS*)-Flx

Table-4.5: Summary of HPLC method validation containing linearity, intra- and inter-day precision, and recovery studies

	First eluting diastereomer			Second eluting diastereomer		
Linearity						
Range	30 to120 ng mL^{-1}			30 to 120 ng mL^{-1}		
Slope	1.05			1.12		
Intercept	–13.84			–14.04		
Correlation Coefficient (R^2)	0.998			0.999		
Actual concentration ng mL^{-1}	Mean±SD (measured) ng mL^{-1}	Recovery (%)	RSD (%)	Mean±SD (measured) ng mL^{-1}	Recovery (%)	RSD (%)
Intra-day precision (n = 3)						
30	15.32 ± 0.19	102.12	1.26	15.27 ± 0.20	101.82	1.33
60	29.63± 0.25	98. 78	0.85	29.84 ± 0.23	99.47	0.78
90	45.62± 0.30	101.39	0.65	45.38 ± 0.28	100.85	0.61
120	59.95± 0.32	99.92	0.53	60.02 ± 0.39	100.03	0.65
Inter-day precision (n = 3)						
30	14.96 ± 0.20	99.73	1.35	15.07 ± 0.19	100.46	1.24
60	29.68 ± 0.26	98.93	0.89	29.91 ± 0.25	99.69	0.82
90	45.01± 0.32	100.02	0.71	44.88 ± 0.34	99.74	0.75
120	59.29 ± 0.33	98. 81	0.55	59.15 ± 0.37	98.59	0.62

B. Enantioseparation of (*RS*)-Flx by HPLC using CDRs based on CC Containing SMLC, SBLC, and L-Leu, L-Val as Chiral Auxiliaries

1. CC based CDRs

In view of the advantages of trifunctionality of CC and literature cited in Chapter-1 under the subheading ‘‘Characteristics of Cyanuric Chloride’’ on applications of its derivatives as CDRs, four CDRs (CDR 10, 11, 13 and 14 cf., Table-2.3; Fig. 2.3), based on CC moiety, were synthesized containing SBLC, SMLC, L-Leu and L-Val as chiral auxiliaries. These synthesized CDRs (i.e., 10, 11, 13 and 14; Chapter-2) were used for synthesis of diastereomers of (*RS*)-Flx (because of the ease of synthesis of such CDRs and our experience on their application for certain important pharmaceuticals (Bhushan and Dixit, 2012b; Bhushan and Dubey, 2012a; Bhushan and Lal, 2013b). These total four pairs of diastereomers have been separated by RP-HPLC on a C_{18} column with detection at 230 nm using gradient elution with mobile phase containing aqueous TFA and MeCN in different compositions. Experimental study in terms of elution order of diastereomers in RP-HPLC was confirmed by developing optimized structures of diastereomers based on density functional theory. Method validation was carried out for linearity, accuracy, LOD and LOQ.

2. Results and Discussion

(a) Synthesis of Diastereomers

The total four pairs of diastereomers of (*RS*)-Flx with all the four above said CDRs were synthesized using MWI as well as conventional heating. Method of synthesis was the same as used in the literature (Bhushan and Kumar, 2008a; Bhushan and Dixit, 2012b). The details for synthesis of diastereomers of (*RS*)-Flx using both the methods (MWI and conventional heating with constant stirring) are described in Chapter-2. The structures of diastereomeric pair [(*R*)-L]- or [(*S*)-L]-diastereomers] of (*RS*)-Flx prepared with CDR 10 are shown in chapter-2 (Fig. 2.14). Stability of the CDRs was checked by performing HPLC experiments at an interval of 10 days up to 100 days from the day of synthesis of diastereomers using the diastereomers of (*RS*)-Flx (prepared with CDR 10) as a representative, by comparing each time with a freshly prepared sample. The diastereomers were found stable for 50 days from the day of synthesis of diastereomers.

Following reaction conditions were tried to obtain an optimum reaction yield. (*RS*)-Flx and CDR were allowed to react in different ratios such as, 1:1, 1:1.5, 1: 1.7 and 1:2. The reaction was carried out by varying the following factors, (i) microwave irradiation (for 50, 60, 70 and 80 s at each of the three different power settings, viz, 70, 80 and 90%) and, (ii) incubation (at 35, 40, 45 and 50 °C) with constant stirring for 30, 40, 50, 60, 70, 80 and 90 min (at each temperature). pH of the solution was maintained around 8 for achieving the quantitative yield of derivatization. The following reaction conditions were found successful to complete the derivatization reaction; molar ratio of (*RS*)-Flx and CDR 1:1.7, microwave irradiation for 60 s at 80 % power (of 800 W) and pH 8. In conventional heating method incubation of reaction mixture (at 40 °C) with constant stirring for 60 min was found as optimized condition and the ratio of (*RS*)-Flx and CDR, and pH of the reaction medium was the same as found successful for MWI method.

Derivatization conditions were optimized for the reactions of (*RS*)-Flx with CDR 10 (as representatives for the reactions of (*RS*)-Flx with CDRs 11, 13 and 14). The peak areas of each pair of the diastereomers, obtained in HPLC experiments, were taken as a diagnosis for the formation of the diastereomers and their separation.

(b) HPLC Separation

RP-HPLC separation of total four diastereomeric pairs of (*RS*)-Flx synthesized with CDRs (CDR 10, 11, 13 and 14) was carried out on C_{18} column. Mobile phases were the same as used for separation of diastereomeric pairs of (*RS*)-Flx synthesized with CDRs (CDR 1, 2, 4 and 5), in section A, under the subheading ‘‘RP-HPLC Separation of Diastereomers’’.

(i) Optimization of Separation Conditions

The method for optimization of separation conditions is the same as used for separation of diastereomeric pairs of (*RS*)-Flx synthesized with CDRs (CDR 1, 2, 4 and 5), in section A, under the subheading ‘‘Optimization of Separation conditions’’.

(iii) Separation of Diastereomers

The successful chromatographic condition for separation of all these four pairs of diastereomers was found as, mobile phase 1 in linear gradient elution of MeCN from 25 to 80% and 0.1% TFA, in 45 min at a flow rate of 1.0 mL/min. The chromatographic data obtained for resolution of diastereomers of (*RS*)-Flx with mobile phase 1, in terms of retention factor (k), separation factor (α), and resolution (R_S) are listed in Table-4.6. Among the all four pairs of diastereomers, the best R_S was found for the diastereomeric pairs of (*RS*)-Flx prepared with CDR 13 while it was found poorer for the diastereomeric pairs of (*RS*)-Flx prepared with CDR 11. The sections of chromatograms showing separation of diastereomers are shown in Fig. 4.7. The peak areas corresponding to the two diastereomers (resolved under successful HPLC conditions) obtained for each change in the above mentioned derivatization conditions, were calculated by system software; these served as a measure of completion of reaction and yield of derivatization. The diastereomers synthesized by the two approaches (MWI and vortex) were found to be identical in terms of their chromatographic data.

3. Separation Mechanism

The elution sequence of diastereomers of (*RS*)-Flx was confirmed by optimizing structures of diastereomers of (*RS*)-Flx (prepared with CDR 11, as a representative) with the help of density functional theory using the Gaussian 09 Rev A. 02 program and hybrid density functional B3LYP with 6-31G (d, p) basis set (Fig. 4.8a and b).

In [(*R*)-L]-diastereomer the hydrophobic groups $–CH_2SCH_3$ and $–C_6H_5$ present on the stereogenic centre of CDR (C-1 in Fig. 4.8a) and Flx (C-2 in Fig. 4.8a) are oriented in *cis*-type arrangement (Fig. 4.8a). While in [(*S*)-L]-diastereomer, the groups are oriented, in *trans*-type arrangement (Fig. 4.8b). Explanation of *Cis* and *Trans* arrangement of hydrophobic groups in [(*R*)-L]-diastereomer and [(*S*)-L]-diastereomer, and elution sequence of these diastereomers, is the same as given in ‘‘Section A’’ under subheading ‘‘Separation mechanism’’. A graphical representation for the diastereomers of (*RS*)-Flx

prepared with CDR 11 is also shown in Fig. 4.9. The all four diastereomers of (*RS*)-Flx, prepared with CDRs 10, 11, 13 and 14, corresponding to (*S*)-enantiomer were eluted prior to those corresponding to (*R*)-isomer.

Table-4.6: RP-HPLC data of diastereomers of (*RS*)-Flx prepared with different CDRs (10, 11, 13 and 14)

CDRs	t_{R1}	t_{R2}	k_1	k_2	α	R_S
10	24.73	27.12	7.92	8.79	1.11	6.82
11	17.10	18.82	5.17	5.79	1.12	4.05
13	23.65	26.79	7.54	8.67	1.15	8.49
14	20.85	22.95	6.53	7.29	1.12	6.56

Chromatographic conditions: RP-HPLC using LiChrospher C_{18} (250 × 4.6 mm i.d., 5 µm particle size) column; mobile phase 2, linear gradient (25 to 80%) of MeCN with 0.10% TFA for diastereomers of Flx in 45 min; flow rate, 1.0mL/min; detection, 230 nm. t_{R1} and t_{R2} are retention time, k_1 and k_2 are retention factors of [(*S*)-L]- and [(*R*)-L]-diastereomer, respectively; α, separation factor; R_S, resolution.

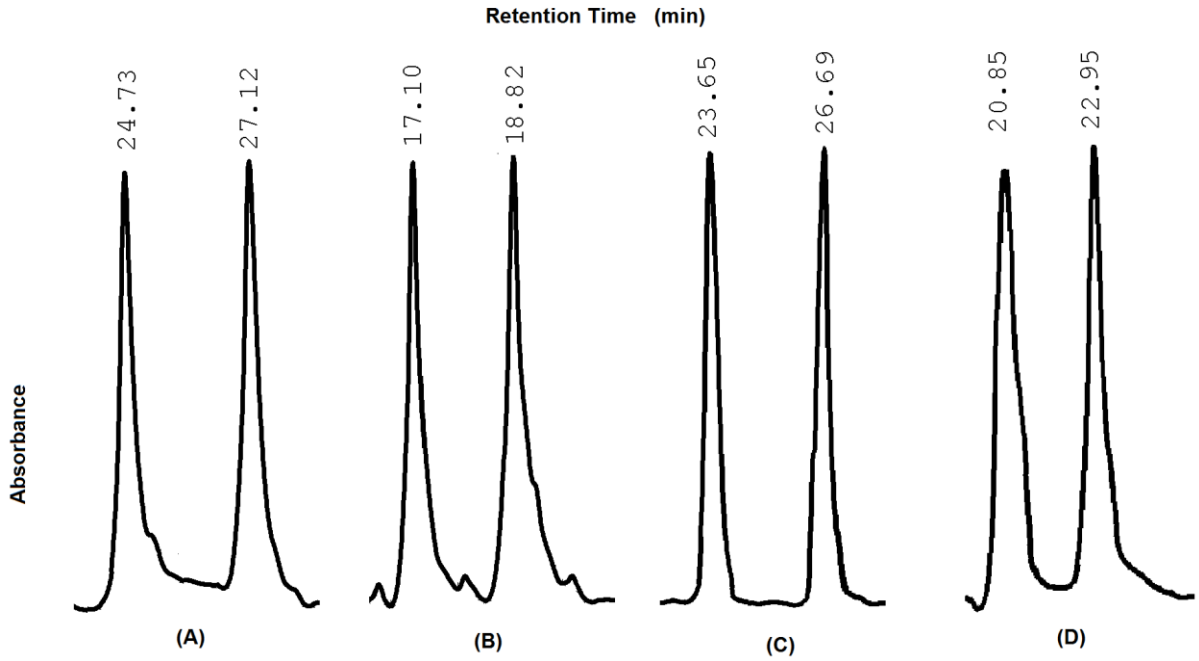


Fig. 4.7: Sections of chromatograms (A), (B), (C) and (D) are showing resolution of diastereomers of (*RS*)-Flx prepared with CDRs 10, 11, 13 and 14, respectively. Chromatographic conditions: as mentioned in the legend of Table 4.6.

4. Method Validation

Method validation studies have been carried out using diastereomers of (*RS*)-Flx prepared with CDR 10.

***(i) Linearity*:** The calibration graphs were plotted between the peak area responses (Y-axis) of the diastereomers of (*S*)-Flx (first eluting diastereomer) and (*R*)-Flx (second eluting diastereomer) prepared with CDR 10 and the corresponding concentration (70 to 160 ng mL^{-1}; X-axis). The linear regression equation was developed by the least square method using Microsoft Excel program and was used to determine the slopes and correlation coefficients as shown in Table-4.7. A good linear relationship was obtained over this range. The regression equations were $y = 1.23x+18.12$ ($R^2 = 0.997$) and $y = 1.25x+21.15$ ($R^2 = 0.999$) for the first and second-eluted diastereomer, respectively.

***(ii) Accuracy, Precision and Limit of Detection*:** The intra-day assay and inter-day assay studies for accuracy and precision were carried out by replicate HPLC analysis (n=3) of diastereomers of (*RS*)-Flx prepared with CDR 10 at five concentrations levels (70, 100, 130, 160 ng mL^{-1}). The recovery and mean standard deviation (SD) for each of the diastereomers were calculated on the basis of peak areas of first and second eluting diastereomer. These are shown in Table-4.7. The relative standard deviation (RSD) for first eluted diastereomer varied from 0.57% to 1.18% for intra-day precision and from 0.41% to 1.28% for inter-day precision; these values were from 0.62% to 1.07% and from 0.49% to 1.25% for second-eluted diastereomer. Intra-day recovery for first and second-eluted diastereomer varied from 98.60% to 101.63% and from 100.06% to 102.13%; the respective values for inter-day recoveries were from 97.41% to 100.84% and from 98.70% to 100. 09%. The LOD was 0.20 ng mL^{-1} for the diastereomer of each enantiomer of (*RS*)-Flx prepared with CDR 10 and LOQ of these diastereomers was 2.60 ng mL^{-1}.

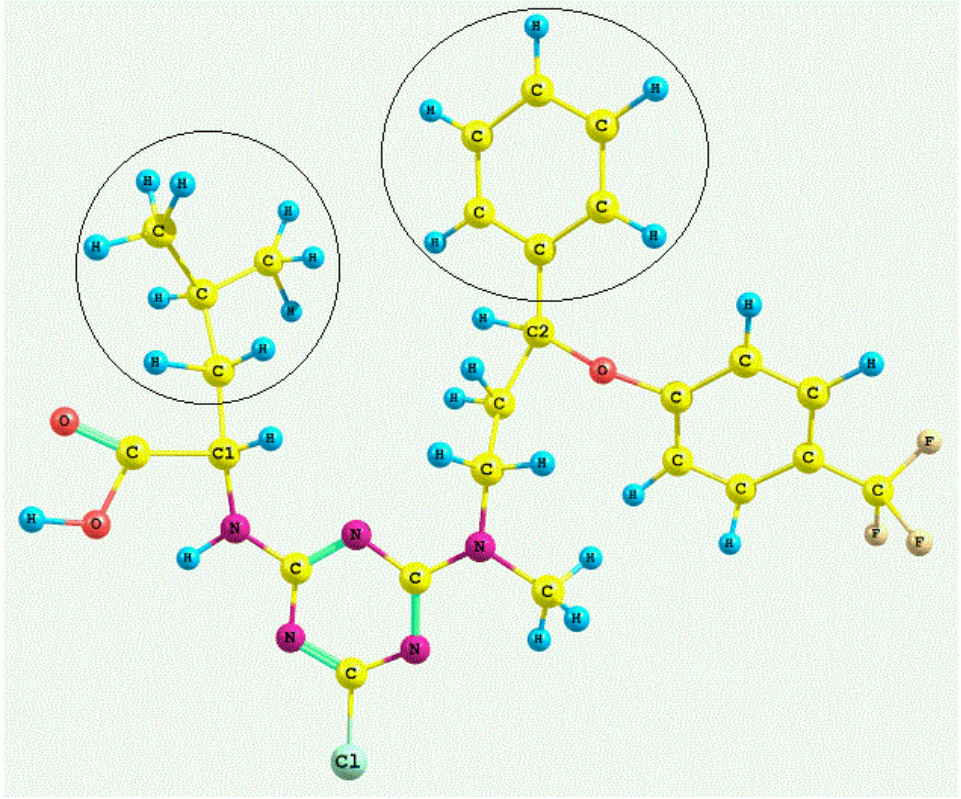


Fig. 4.8a: [(*R*)-L]-diastereomer

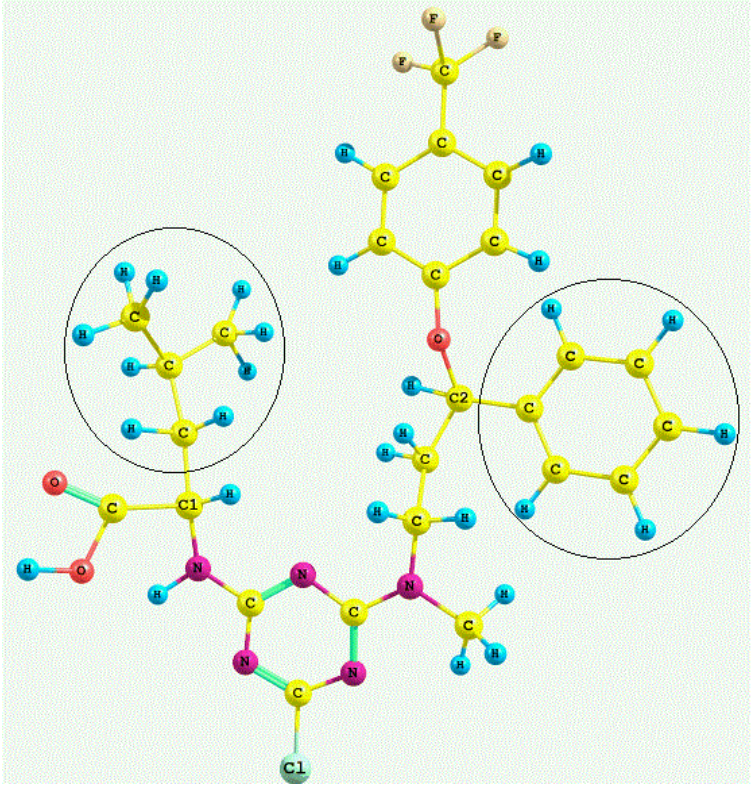
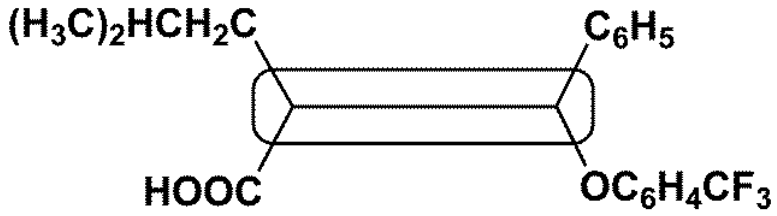
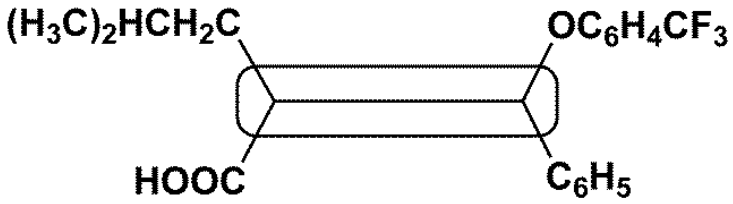


Fig. 4.8b: [(*S*)-L]-diastereomer

Fig. 4.8: Optimized structures of [(*R*)-L]- and [(*S*)-L]-diastereomer of (*RS*)-Flx prepared with CDR 11



[(*R*)-L]-Diastereomer



[(*S*)-L]-Diastereomer

Fig. 4.9: Graphical representation of *cis* and *trans* type arrangement of hydrophobic groups in diastereomers of (*RS*)-Flx prepared with CDR 11

Table-4.7: Summary of HPLC method validation containing linearity, intra- and inter-day precision, and recovery studies

	First eluting diastereomer			Second eluting diastereomer		
Linearity						
Range	70 to 160 ng mL^{-1}			70 to 160 ng mL^{-1}		
Slope	1.23			1.25		
Intercept	18.12			21.05		
Correlation Coefficient (R^2)	0.997			0.999		
Actual concentration ng mL^{-1}	Mean±SD (measured) ng mL^{-1}	Recovery (%)	RSD (%)	Mean±SD (measured) ng mL^{-1}	Recovery (%)	RSD (%)
Intra-day precision (n = 3)						
70	35.57±0.42	101.63	1.18	35.75±0.84	102.13	1.07
100	49.69±0.43	99.38	0.86	49.86±0.45	99.72	0.91
130	65.01±0.44	100.01	0.68	64.88±0.66	99.81	0.71
160	79.68±0.45	98.60	0.57	80.05±0.50	100.06	0.62
Inter-day precision (n = 3)						
70	35.29±0.45	100.84	1.28	34.63±0.43	98.95	1.25
100	49.54±0.37	99.07	0.74	48.81±0.39	99.61	0.80
130	64.19±0.42	98.76	0.65	65.06±0.36	100.09	0.56
160	77.92±0.32	97.41	0.41	78.96±0.39	98.70	0.49

IV. Inference

The present study was successful in achieving HPLC and TLC separation by indirect approach in RP conditions. CDRs based on DFDNB (CDR 1, 2, 4 and 5) and CC (CDR 10, 11, 13 and 14) were synthesized. Of these, four CDRs (CDR 1, 2, 10 and 11) were newly synthesized. The DFDNB and CC based CDRs have high molar absorptivity due to direct conjugation of amino group of chiral auxiliary with dinitrophenyl chromophore of DNB moiety and CC moiety. These CDRs were used for the synthesis of eight pairs of diastereomers of (*RS*)-Flx and the corresponding diastereomers were successfully resolved by indirect HPLC. Besides, two diastereomeric pairs of (*RS*)-Flx prepared with CDRs 4 and 5 were separated successfully by RP-TLC. Elution sequence of the diastereomers was confirmed by optimizing their structures using DFT by Gaussian 09 Rev A. 02 program. LOD was found to be in pg mL^{-1} range for the diastereomers of (*RS*)-Flx prepared with CDRs 1, 2, 4 and 5, using HPLC and in ng mL^{-1} range for the diastereomers of (*RS*)-Flx prepared with CDRs 4 and 5, using RP-TLC. For the diastereomers of (*RS*)-Flx prepared with CDRs 10, 11, 13 and 14, LOD was found to be in ng mL^{-1} range. It means these method can be successfully applied in pharmaceutical industry, as well, since they are capable to detect the isomer of (*RS*)-Flx at concentration lower than the limit of 1% prescribed for pharmaceutical industry.

•

Chapter-5

Enantioseparation of SeMet using HPLC and TLC

Contents **Page No.**

Chapter-5

Enantioseparation of SeMet using HPLC and TLC

I. SeMet

Selenomethionine (2-amino-4-methylselanylbutanoic acid, SeMet; Fig. 5.1) is a naturally occurring chiral amino acid. It contains Se which is well known for its biological and dietary importance. L-SeMet is a major natural food form of Se for humans while its D-isomer is toxic at high levels (Bubba *et al*., 2013). Se deficiency may create a negative impact on the immune system (Spallholz *et al*., 1990) and leads to higher risk in case of infection with HIV (Baum *et al*., 1997). Some physiological functions, related to cardiovascular system (Néve, 1996), central nervous system (Castano *et al*., 1997), inflammatory processes (Behne *et al*., 1996), and reproduction ability (Knekt *et al*., 2000) are influenced by selenium. The development of pharmaceutical preparations involving Se compounds has become a growing area because of use of selenium supplementation for cancer chemoprevention (Clark *et al*., 1996).

Importance of SeMet as a source of Se in humans, other mammalians and plants has been covered in certain books and reviews (Hatfield *et al*., 2006; Papp *et al*., 2007; Schrauzer, 2000). L-SeMet is better absorbed and better incorporated into body than any other known form of selenium (Thompson *et al*., 1984. D-SeMet is degraded to inorganic selenium and returned to inorganic selenium body pool, and is only one-fifth bioavailable as L-selenomethionine (Thomson *et al*., 1982).

In this chapter enantioseparation of SeMet by HPLC and TLC has been discussed.

I. Literature Survey on liquid chromatographic enantioseparation of SeMet

HPLC based separation: Liquid chromatography and plasma mass spectrometric detection (ICP-MS) has been applied for identification and quantification of the species present in nutritional supplements. Most of these make use of commercially available chiral selective columns for determination of enantiomeric forms of Se-amino acids (Méndez *et al*., 1998; Méndez *et al*., 1999; Méndez *et al*., 2000a; Sutton *et al*., 2000). A relatively poor selectivity and the elevated cost are the major drawbacks of some of these columns (Montes-Bayón *et al*., 2001). Application of ICP-MS detector is associated with special method for sample preparation and high equipment cost in comparison to those required for UV-Vis detectors.

Direct enantioresolution of SeMet on CSPs along with their limitations and advantages has been reviewed by Sanz-Medel and Blanco-González (1998). The major drawbacks of these columns are high cost and relatively poor selectivity (Montes-Bayón *et al*., 2001).

Lindner and co-workers reported cinchona alkaloid-based zwiterionic ion exchange type chiral selectors which were immobilized on to silica gel yielding brush-type CSPs; these columns enabled three modes of ion exchange in HPLC, anion exchange mode for separation of chiral acids, cation exchange mode for chiral amines, and zwitterion exchange for enantioresolution of amphoteric compounds like native amino acids and small peptides (Pell *et al*., 2012; Hoffmann *et al*., 2008; Wernisch *et al*., 2012). Quinine and quinidine based zwitterionic CSPs have also been commercialized (CHIRALPAK ZWIX (+) and ZWIX (–)™, Chiral Technologies Inc., West Chester, PA 19380).

Enantioresolution of DL-SeMet by HPLC using certain CDRs such as, *o*-phthaldialdehyde and *N*-isobutyryl-L-cysteine (Bergmann *et al*., 2004) and Marfey's reagent supplemented with elemental specific induced coupled plasma mass spectrometric detector (Montes-Bayón *et al*., 2001) has been reported.

In recent years, enantioseparation of DL-SeMet has been successful using (*R*)-methyl benzyl isothiocyanate, and (*S*)-1-(1-naphthyl) ethyl isothiocyanate, (Bhushan and Dubey, 2012b) as CDRs. Besides, DFDNB and monochloro-, and dichloro triazine (MCT, and DCT) based CDRs containing amino acid amides as chiral auxiliaries were synthesized and used successfully for enantioseparation of DL-SeMet (Bhushan and Dubey, 2012a).

Enantioresolution by TLC: Literature search showed that there was no report on TLC enantioresolution of DL-SeMet by direct approach.

III. Present Work

A. Indirect enantioseparation of SeMet by RP-HPLC using a new chiral reagent based on (*S*)-Nap moiety

In view of the literature cited as above and cross references, taking into account the importance of enantioseparation of SeMet, and Nap based CDR (CDR 16) has been

used to synthesize diastereomers of SeMet via MWI and vortexing. The reaction conditions were optimized. The diastereomers were separated by RP-HPLC. Detection of separated diastereomers was carried out at 231 nm using PDA detector. Optimised structures of (*S*, D)- and (*S*, L)-diastereomers of SeMet were developed using Gaussian 09 Rev A. 02 program and hybrid density functional B3LYP with 6-31G basis set, to support the experimental results (in terms of elution sequence of diastereomers).

There is no earlier report on the synthesis and application of phthalimidyl-(*S*)-Nap ester, as the CDR, for enantioseparation of DL-SeMet.

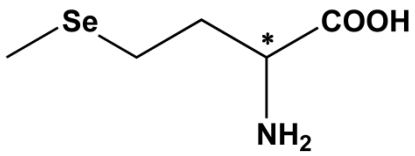


Fig. 5.1: Structure of DL-SeMet

1. Results and Discussion

(a) Synthesis of CDR 16 (Nap-Phth)

The synthesis and characterization data of naproxen based reagent (CDR 16) are described in Chapter-2 (Fig. 2.4). Activation of naproxen, in the present case, by *N*-hydroxyphthalimide has been advantageous for the following reasons, (i) the synthesis of *N*-hydroxyphthalimide activated naproxen was achieved by vigorously stirring the reaction mixture at room temperature for 2.5 hr using DCC while synthesis of benzotriazole activated naproxen required 4 hr stirring at room temperature using hazardous thionyl chloride (Katritzky *et al*., 2009), and (ii) *N*-hydroxyphthalimide, as the activating agent, is neither explosive in nature like benzotriazole nor hygroscopic like *N*-hydroxysuccinimide. *N*-hydroxyphthalimide can also be stored at room temperature easily. The new CDR so synthesized was endowed with a high molar absorptivity due to methoxy substituted naphthyl residue attached to the chiral centre and gives detection at very low concentration and the hydrophobic property of the naphthyl ring facilitates enantioresolution. The chiral purity of the CDR was established as per literature reports (Bhushan and Kumar, 2010; Bhushan, 2011).

(b) Synthesis of diastereomers

The synthesis of diastereomers of DL-SeMet with (*S*)-Nap-Phth (CDR 16) has been described in Chapter-2. The diastereomers so prepared are represented as (*S*, L)-, and (*S*, D)-diastereomers where L, and D represent the configuration of SeMet while (*S*) belongs to configuration of (*S*)-Nap.

The experimental conditions for synthesis of diastereomeric pairs of DL-SeMet with Nap-Phth were optimized with respect to the effect of pH, reagent excess, reaction time MWI and vortexing time.

Effect of reagent excess, pH, MWI time and Vortexing time: Effect of reagent excess was investigated on derivatization by varying the ratio of SeMet and CDR such as 1:1, 1:1.5 or 1:2. Slight kinetic resolution was observed when SeMet and CDR were reacted in a ratio of 1:1 or 1:1.5 while there was no kinetic resolution on two fold molar excess of reagent.

Effect of pH on derivatization was investigated by varying the pH from 8 to 11.5 using different volume of TEA in separate sets of reaction mixtures and it was observed that yield of the diastereomers increased with increase in pH from 8.0 to 11.0 (Fig. 5.2). Therefore, a pH 11.0 was chosen for further experiments on derivatization.

Separate sets of reaction mixture were irradiated with MW for 10 to 60 s at 40 to 80% power (of 800 W) to check completion of synthesis. On increasing the MWI time from 10 to 40 s (and on varying MW power from 40 to 80% at each reaction time) an increase in derivatization yield was observed while on increasing irradiation time from 40 to 60 s at 80% MW power there was no significant increment in derivatization yield. Thus, MWI time of 40 s at 80% power (of 800 W) was considered successful as the peak areas corresponding to the two diastereomers (obtained from the system software) became constant (under the same HPLC mobile phase conditions that were optimized for enantioseparation, see "Separation of Diastereomers") and were found in the ratio of around 1:1. These conditions served as a measure/indicator of completion of derivatization reactions (Fig. 5.3).

The reaction time was varied from 3 to 12 min under vortexing (500 rpm). It was found that on increasing the vortexing time from 3 to 10 min there was observed an

increase in peak area and on increasing the vortexing time from 10 to 12 min there were no significant change in peak areas.

The optimized conditions for derivatization can be summarised as: pH 11 provided by TEA (5 μL) since SeMet required a sufficiently basic medium to act as a good nucleophile, a twofold molar excess of CDR and vortexing for 10 min or MWI for 40 s at 80% power.

N-hydroxyphthalimide group, in the CDR (Nap-Phth), underwent nucleophilic substitution by SeMet via its amino group (*cf.*, Fig. 2.9; Chapter 2). Thus the diastereomers of SeMet, containing (*S*)-Nap moiety, were produced easily in 40 s using MWI or in 10 min by vortexing the reaction mixture at room temperature, in comparison to the literature reporting coupling of (*S*)-naproxen activated by *1H*-benzotriazole in 3-12 h at 20˚C in aqueous acetonitrile to give diastereomeric mixtures of two different racemic amino acids (Katritzky *et al*., 2009).

The diastereomers were kept under refrigerated condition (0-5 °C) and found to be stable for 30 days. The stability was checked by measuring the ratio of peak area of the stored samples and comparing the same with freshly prepared samples under identical conditions of separation.

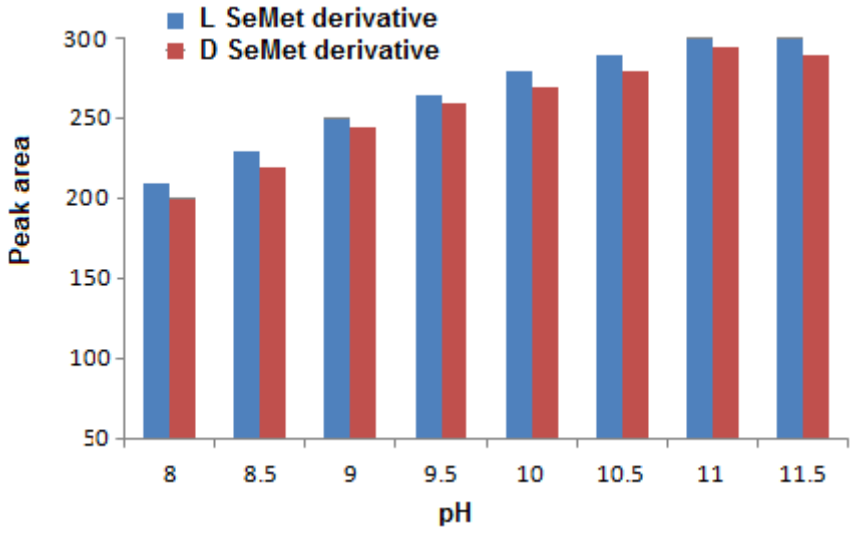


Fig. 5.2: Effect of pH on derivatization of SeMet with CDR 16

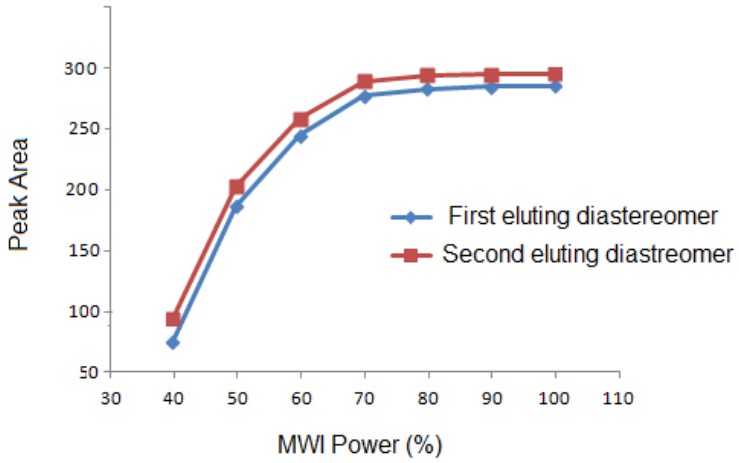


Fig. 5.3: Effect of MWI power on completion of derivatization reaction of DL-SeMet with CDR 16

(c) HPLC Analysis

Aliquots of the solution of diastereomers were diluted 10 times with MeCN and injected (20 µL) on to the column. Following binary combinations of mobile phase were tried;

Mobile phase 1, MeOH with TEAP buffer (10mM)
Mobile phase 2, MeCN with TEAP buffer (10mM)

Both the mobile phases were tried in linear gradient (35 to 70%, 35 to 65%, 30 to 70%, 30 to 65%, 25 to 65 % and 10 to 90 %) and isocratic mode (10:90, 20:80, 40:60, 60:40 and 80:20).

Mobile phase was filtered through a 0.45µm filter and degassed by sonication and passing nitrogen before use.

(i) Optimization of Chromatographic Conditions

Chromatographic conditions were optimized by changing the concentration of TEAP buffer (5 to 15 mM) and organic modifier (as shown above), pH (in the range 2.5 to 5.5), and flow rate (0.5 to 2.0 mL/min) of mobile phase.

The diastereomers were successfully resolved using mobile phase consisting of aqueous TEAP (10 mM, pH 3.4)-MeCN in a linear gradient of MeCN from 25 to 65% in 45 min at a flow rate of 1.0 mL/min and detection at 231nm. The mobile phase under

isocratic conditions provided broader peaks. Larger retention times and broader peaks were obtained with the mobile phase containing MeOH because of the same reasons as discussed previously.

(ii) Separation of Diastereomers

The diastereomers were separated under reversed phase conditions by HPLC. Mobile phase 2, consisting of aqueous TEAP (10mM, pH 3.4)-MeCN in a linear gradient of MeCN from 25 to 65% in 45 min was found better than the isocratic mode (MeCN: TEAP buffer in 60:40 ratio in 45 min run); the flow rate was 1.0 mL min^{-1} in both cases. The chromatographic data obtained for separation of diastereomers in terms of retention factor (k), separation factor (α), and resolution (R_S) are listed in Table-5.1. It shows that on linear gradient elution the k values obtained were higher than those observed under isocratic conditions and R_S was also better under gradient condition in comparison to that obtained under isocratic condition. The chromatogram showing resolution is shown in Fig. 5.4.

The peak areas corresponding to the two diastereomers (resolved under successful HPLC conditions) obtained for each change of the above mentioned derivatization conditions, were calculated by system software; these served as a measure of completion of reaction and yield of derivatization. The diastereomers synthesized by the two approaches (using MWI and vortex) were found to be identical in terms of their chromatographic data.

The elution sequence of diastereomers was confirmed by examining elution of the diastereomers prepared from enantiomerically pure D-SeMet and L-SeMet with CDR 16. The first eluted peak was for the diastereomer corresponding to D-SeMet (i.e. (*S*, D)-diastereomer), and second one was for its L-counterpart (i.e. (*S*, L)-diastereomer).

2. Separation Mechanism

Diastereomer of D-SeMet eluted earlier than the diastereomer of the corresponding L-isomer. The elution sequence of the diastereomers and mechanism of separation can be explained by taking analogy from the model proposed (Fujii *et al*., 1997; Brückner and Keller-Hoehl, 1990; Brückner and Gah, 1991) for HPLC separation of diastereomers of amino acids prepared with Marfey's reagent (Marfey, 1984).

Optimized structures of the two diastereomers were drawn using the Gaussian 09 Rev A. 02 program and hybrid density functional B3LYP with 6-31G basis set (Fig. 5.5a and 5.5b). In (*S*, L)-diastereomer, the hydrophobic groups, $-CH_3$ on the stereogenic centre of CDR (C1 in Fig. 5.5a) and $-CH_2CH_2SeCH_3$ on stereogenic centre of SeMet (C2 in Fig. 5.5a), are oriented on the same side with respect to the amide bond and are considered to be *cis* to each other. The *cis*-type arrangement stays since there is a partial double bond character in the amide bond with restricted rotation around C-N. On the other hand, in (*S*, D)-diastereomer, the $-CH_3$ and $-CH_2CH_2SeCH_3$ groups are oriented in space above and below the amide bond and thus have *trans*-type arrangement (Fig. 5.5b). A graphical representation for the same is also shown in Fig. 5.6. The *cis*- or *trans*-type arrangements of the two diastereomers are responsible for difference in their hydrophobicities.

The mechanism gets confirmed with the experimental observation that the (*S*, D)-diastereomer is usually eluted first from the column which means it is retained for lesser time in comparison to that of the (*S*, L)-diastereomer. In other words, the (*S*, L)-diastereomer (having *cis*-type arrangement) interacts more strongly with ODS material of C_{18} column than the *trans*-type arrangement, hence has a longer retention time. These retention times of the diastereomers are thus reflecting the overall hydrophobicity of the two molecules. It can therefore be concluded that the hydrophobicity of *cis*-type arrangement is more than that of *trans*-type arrangement.

The *cis*- and *trans*-type arrangements (as explained above), the partial double bond character of amide bond between amino nitrogen of SeMet and carbonyl carbon of naproxen, less electro negativity and large size of Se atom in SeMet and the presence of naphthyl group in CDR (contributing to the overall hydrophobicity) along with rheological properties of the mobile phase, are responsible for different partition coefficients and different retention times of (*S*, L)- and (*S*, D)-diastereomers. Therefore, the diastereomers elute one after another for these different physical properties.

3. Method validation

Validation studies in terms of linearity, accuracy and precision were carried out for diastereomers of DL-SeMet prepared with CDR 16.

Linearity: The calibration graphs were plotted between the peak area responses of (*S*, D)-diastereomer and (*S*, L)-diastereomer and the corresponding concentration (7-14 pmol mL^{-1}). The linear regression equation was developed by the least square method using Microsoft Excel program and was used to determine the slopes and correlation coefficients as shown in Table-5.2. A good linear relationship was obtained over this range. The regression equations were $y = 144.3x - 9.952$ ($R^2 = 0.999$) and $y = 141.0x - 11.10$ ($R^2 = 0.999$) for the (*S*, D)-diastereomer and (*S*, L)-diastereomer, respectively.

Accuracy, precision and limit of detection: The intra-day assay and inter-day assay studies for accuracy and precision were carried out by replicate HPLC analysis (n=6) of standard solutions of diastereomeric mixtures of DL-SeMet at three concentrations (7, 10 and 14 pmol mL^{-1}). The recovery and mean standard deviation (SD) for each of the diastereomers were calculated on the basis of peak areas of first and second eluting diastereomer. These are shown in Table-5.2. The relative standard deviation for (*S*, D)- and (*S*, L)-diastereomers, respectively, varied from 0.84 to 1.29% and 0.97 to 1.08% for intra-day assay precision and 0.78 to 1.47% and 0.69 to 1.35% for inter-day assay precision. The recovery for diastereomers of D- and L-SeMet varied from 98.4 to 100.5% and 101.1 to 103.6% for intra-day assay and 96.9 to 98.7% and 99.3 to 100.7% for inter-day assay.

LOD corresponding to signal-to-noise ratio of 3, was found to be 0.11pmol $mL^{-1}$and 0.10pmol mL^{-1} for diastereomers of D- and L-SeMet, respectively and LOQ corresponding to the signal-to-noise ratio of 10, was found to be 0.33pmol mL^{-1} and 0.30 pmol mL^{-1} for diastereomers of D- and L-SeMet, respectively.

Table-5.1: Chromatographic data for separation of diastereomers of DL-SeMet prepared with CDRs 16

Chromatographic parameters	Gradient condition	Isocratic condition
k_1	10.68	9.23
k_2	12.52	10.73
α	1.17	1.16
R_S	22.57	17.525

Chromatographic conditions: Lichrospher C_{18} column (250 x 4.6 mm I.D., 5 μm particle size); mobile phase 2, aq TEAP (10 mM, pH 3.4)-MeCN in a linear gradient of MeCN from 25 to 65% and under isocratic conditions (MeCN: TEAP buffer in 60:40 ratio) in 45 min at a flow rate of 1.0 mL/min: detection, 231nm. k_1 and k_2 are retention factors for diastereomer corresponding to L- and D-SeMet, respectively; α, separation factor; R_S, resolution.

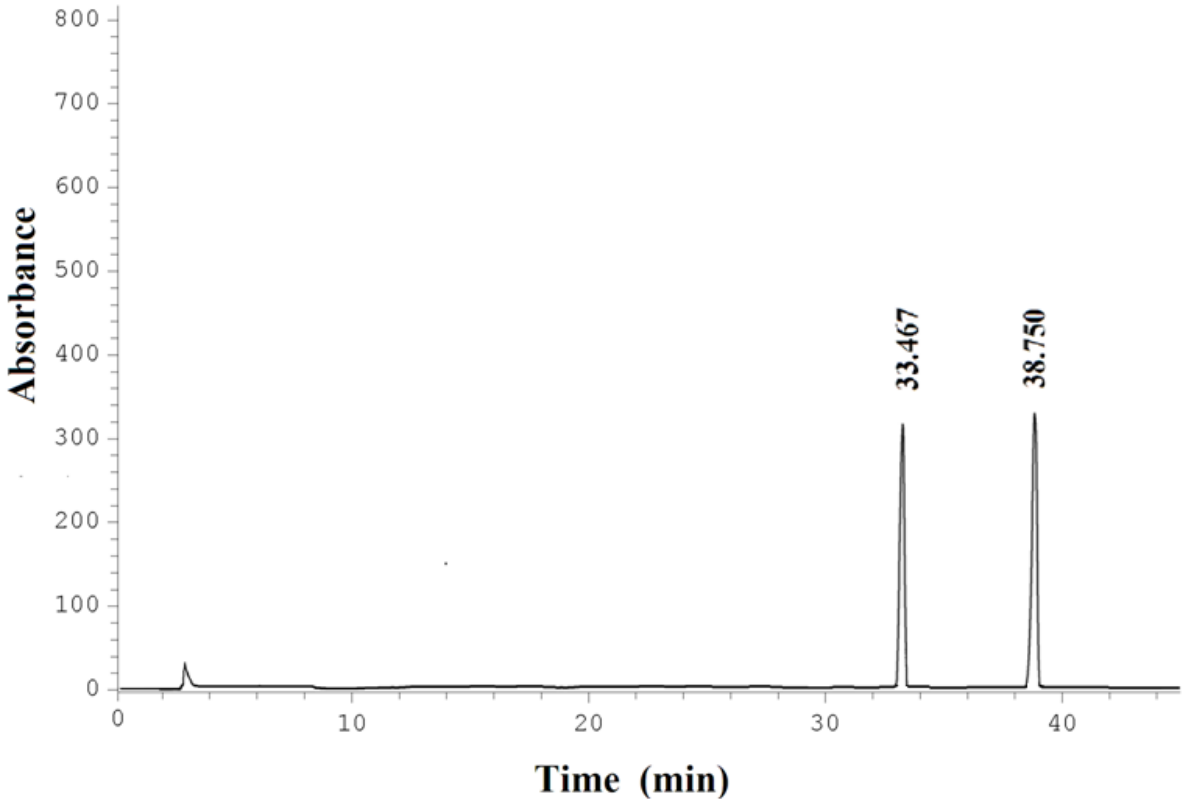
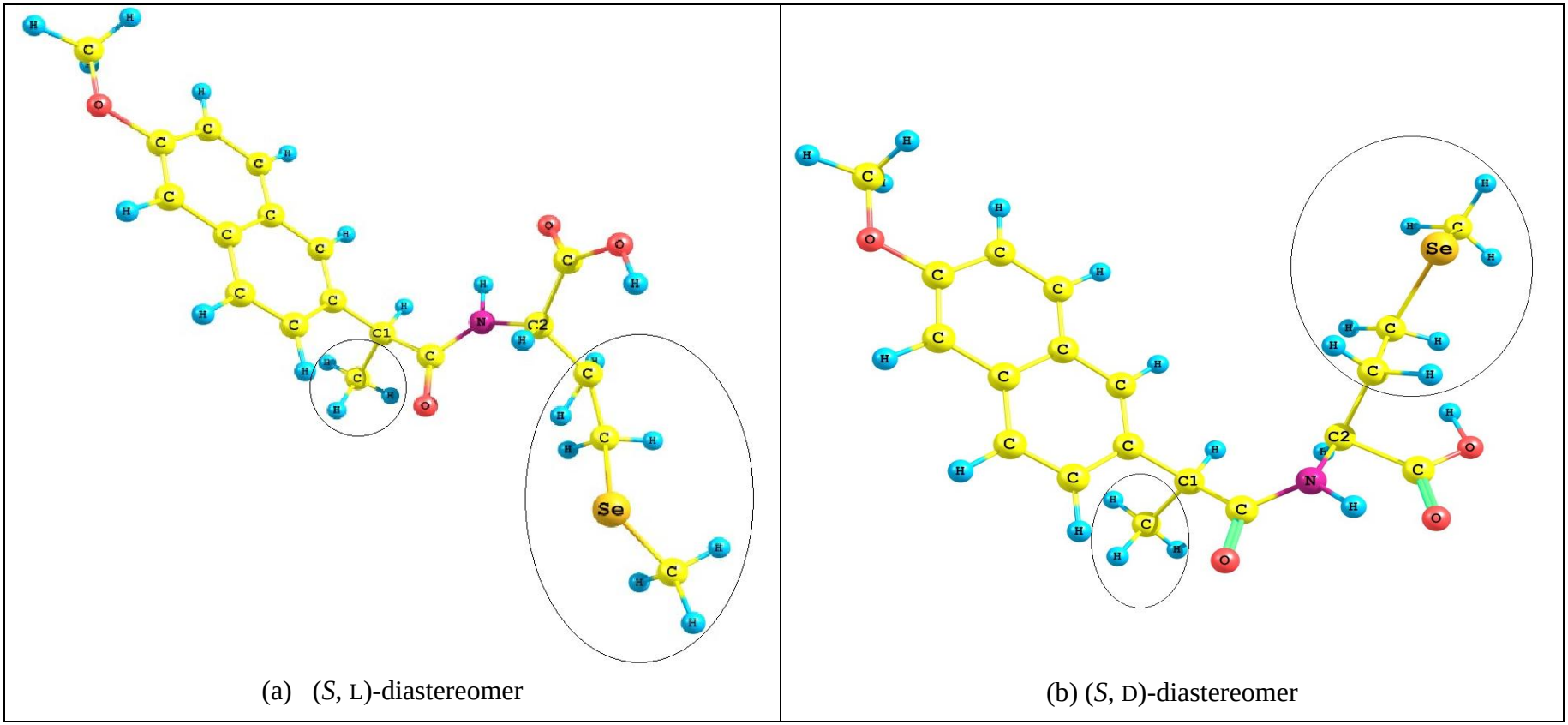


Fig. 5.4: Chromatogram showing resolution of diastereomers of DL-SeMet with CDR 16. *Chromatographic conditions*: as mentioned in legend of Table-5.1. The peaks of retention time 33.467 and 38.750 represents (*S*, D)- and (*S*, L)- diastereomer peak, respectively.

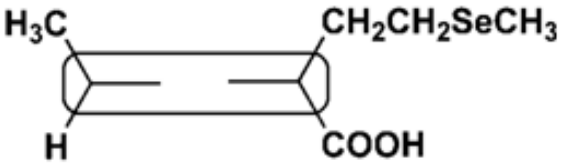
4. Comparison of R_S and LOD with literature reports

In the present study diastereomers of SeMet have been separated with better resolution (R_S 22.57) in comparison to the resolution reported in literature, for example, R_S was 2.34 and 3.23 using (*R*)-MBIC, and (*S*)-NEIC as CDRs, respectively (Bhushan and Dubey, 2012b); 15.66, 4.24, and 7.46, respectively, using Marfey's reagent, and MCT and DCT type CDRs containing L-alaninamide as chiral auxiliary (Bhushan and Dubey, 2012a). The resolution in the present case was also better/higher than that obtained using direct approach, e.g., R_S was 1.2 using *β*-cyclodextrin capillary column, (Méndez *et al*., 2000b); 1.00 on Cyclobond I *β*-cyclodextrin column (Méndez *et al*., 1998); ≈2.00 using ligand exchange Cu(II)-(L-*N*-(2-hydroxy-propyl)-phenylalanine complex (Duan *et al*., 2012); 0.96 and 1.8 using Chirasil-L-Val CSP (Méndez *et al*., 1999; Devos *et al*., 2002).

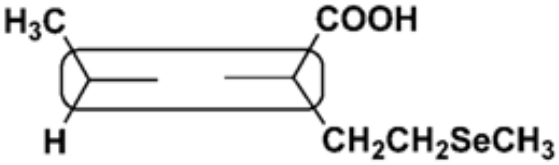


(a) (*S*, L)-diastereomer (b) (*S*, D)-diastereomer

Fig. 5.5: Optimized structures of the (*S*, L)- and (*S*, D)-diastereomers of SeMet with CDR 16.
The program Gaussian 09 Rev A. 02 and hybrid density functional B3LYP with 6-31G basis set was used to obtain optimized structures.



***cis*-type arrangement**
(*S*, L)-diastereomer



***trans*-type arrangement**
(*S*, D)-diastereomer

Fig. 5.6: *Cis* and *trans* type arrangement of hydrophobic groups in diastereomers of DL-SeMet prepared with CDR 16

Table-5.2: Summary of HPLC method validation data obtained for diastereomers of DL-SeMet

	First eluting diastereomer			Second eluting diastereomer		
Linearity						
Range	7-14 pmol mL^{-1}			7-14 pmol mL^{-1}		
Slope	141.0			144.3		
Intercept	–11.10			–9.952		
Correlation Coefficient (R^2)	0.999			0.999		
Precision						
Actual concentration (pmol mL^{-1})	Mean±SD (measured) (pmol mL^{-1})	Recovery (%)	RSD (%)	Mean±SD (measured) (pmol mL^{-1})	Recovery (%)	RSD (%)
Intra-day precision (n = 6)						
7	3.518 ± 0.045	100.5	1.29	3.623 ± 0.039	103.7	1.08
10	4.920 ± 0.041	98.4	0.84	5.055 ± 0.049	101.1	0.97
14	6.986 ± 0.075	99.8	1.07	7.175 ± 0.074	102.5	1.03
Inter-day precision (n = 6)						
7	3.427 ± 0.050	97.6	1.47	3.497 ± 0.047	99.9	1.35
10	4.845 ± 0.038	96.9	0.78	4.965 ± 0.034	99.3	0.69
14	6.909 ± 0.066	98.7	0.95	7.049 ± 0.059	100.7	0.84
Sensitivity						
LOD (pmol mL^{-1})	0.11			0.10		
LOQ (pmol mL^{-1})	0.33			0.30		

Values are mean ± SD, SD: standard deviation; RSD: relative standard deviation.

The values for limit of detection for each diastereomer of SeMet prepared with certain other CDRs, are reported to be 0.32 μg mL^{-1} (UV detector), and about 4 μg L^{-1}(ICP-MS detector), respectively, for the diastereomers prepared with (*S*)-NEIC (Bhushan and Dubey, 2012b), and *o*-phthalaldehyde and *N*-isobutyryl-L-cysteine (Bergmann *et al*., 2004). In the present case, LODs were found to be 0.11 pmol mL^{-1} (44 ng L^{-1}) and 0.10 pmol mL^{-1} (41 ng L^{-1}) for diastereomers of D- and L-SeMet, respectively; these are much lower than any previously reported value. These LOD values are also much lower than those reported (to be 70 μg L^{-1} or 20 μg L^{-1} using HG-ICP-MS detector) for direct enantioseparation of the derivatives prepared with *o*-phthalaldehyde or 2,3-naphthalenedicarboxaldehyde (Méndez *et al*., 1998).

B. Separation of Diastereomers of SeMet Prepared with DFDNB based CDRs Containing L-Met, SBLC and L-Phe as Chiral Auxiliaries, using RP-HPLC and TLC

Literature search showed that there was no report on enantioseparation of DL-SeMet by indirect HPLC and TLC. The characteristics and application of DFDNB, as cited above, literature reports on indirect enantioresolution of DL-SeMet by HPLC (Bhushan and Dubey, 2012a) and quest for searching/establishing new methods for enantioresolution following studies were attempted,

(i) Application of new DFDNB based CDRs (CDR 2, 3 and 6, having SBLC, L-Met, and L-Phe as chiral auxiliaries, *cf.*, Table-2.3; Fig. 2.1), to synthesize diastereomers of DL-SeMet via nucleophilic substitution of the remaining fluorine atom in these reagents under microwave irradiation and conventional heating.

(ii) Separation of diastereomers by RP-TLC, and RP-HPLC.

(iii) Correlation of experimental results of migration order of L-D and L-L diastereomers of SeMet with their structural features; the optimized structures (of lowest energy) of the two diastereomers of SeMet (prepared with CDR 3) were developed using Gaussian 09 Rev A. 02 program and hybrid density functional B3LYP with 6-31G basis set.

As per literature search, this is the first report on these aspects, mentioned above. The method was validated for linearity, accuracy, LOD.

1. Results and Discussion

(a) Synthesis of CDRs

Of the three, two new DFDNB based CDRs were synthesized (*cf.*, Table-2.3; Fig. 2.1). The scheme of synthesis and characterization data of CDRs 2, 3 and 6, used for synthesis of diastereomers of DL-SeMet have been described in Chapter-2. Other characteristics have already been described in Chapter-2.

(b) Synthesis of Diastereomers

The CDRs were used for synthesis of diastereomeric pairs of DL-SeMet. The structures of diastereomeric pair (L-D or L-L diastereomers) of DL-SeMet prepared with CDR 3 are shown in chapter-2 (Fig. 2.15). Microwave irradiation for 55 s at 75% of 800 W, or conventional heating for 50 min at temperature of 45 °C, and a ratio of DL-SeMet and CDR (1:1.7) were found as optimized conditions for completion of reaction for the synthesis of diastereomers. The reaction conditions for derivatization were optimized by performing following variations: microwave irradiation (MWI) time (40, 45, 50, 55, 60, 65 and 70 s) and MWI power 70 to 85%; conventional heating time of 20, 30, 40, 50 and 60 min; temperature between 35 to 50 °C; and ratio of DL-SeMet:CDR (1:1 to 1:2.5). Fig. 5.7 showing the effect of MWI time on derivatization on SeMet with CDR 3. On increasing the time from 40 to 55s there was found increment in peak area of diastereomers and after this from 55 to 70s, no significant increment was there.

Diastereomers of L-SeMet were prepared under the optimized conditions with all the three CDRs. The first letter L represents the configuration of chiral auxiliary of the CDR and second letter (D or L) is for the configuration of SeMet. These diastereomers were used for separation studies by RP-TLC and RP-HPLC. Analogy for structural representation showing H-bonding in Fig. 2.15 is based on the model proposed (Brückner and Keller-Hoehl, 1990; Fujii *et al.* 1997; Brückner and Gah, 1991) for HPLC separation of diastereomers of amino acids prepared with Marfey's reagent (Marfey, 1984).

Stability of the diastereomers of DL-SeMet prepared with CDR 3 (as a representative) was checked by HPLC experimental at an interval of 10 days up to 50 days from the day of synthesis of diastereomers, by comparing each time with a freshly prepared sample. It was found that diastereomers were stable up to 40 days.

(c) RP-TLC Separation of Diastereomers

All the three pairs of diastereomers of DL-SeMet were separated by RP-TLC on DC Kieselgel 60 RP*18 $F_{254}S$ stationary phase**.** Stock solutions of diastereomers of DL-SeMet and L-SeMet were diluted 10 times with MeCN; these were applied on RP-TLC plates using 10 µL Hamilton syringe.

Following mobile phases were tried,

Mobile phase 1; consisting of TEAP buffer–MeCN (as organic modifier)
Mobile phase 2; containing TEAP buffer–MeOH (as organic modifier)

(i) Optimization of Chromatographic Conditions

Several trials of mobile phase 1 with different ratio of MeCN as organic modifier and TEAP (triethylammonium phosphate) as buffer (of 50 mM, pH 4.8) were tried by keeping the total volume of mobile phase same for each ratio; 1:5.7, 2.7: 4.0, 3.2:3.5 and 5.0:1.7 (*v/v*) to achieve successful resolution. TEAP buffer solution of different concentrations (10 to 60 mM with a change of 10 mM) was used to optimize separation results. pH of mobile phase was also varied in the range of 2 to 5 by adjusting the pH of TEAP buffer (by adding phosphoric acid in TEA solution). Mobile phase 2, containing MeOH as organic modifier and TEAP as buffer was also tried in similar conditions to achieve resolution.

Chromatograms were developed (for 10 min) in rectangular glass chambers, pre-equilibrated with mobile phase for 10 min, at different temperatures (in the range, 15 to 35 °C with a variation of 10 °C); the chromatograms were dried with hair-dryer. The separated diastereomers were visible as bright yellow spots in ordinary light.

(ii) Separation of Diastereomers

Mobile phase 1, consisting of TEAP buffer (50 mM, pH 4.8)–MeCN (3.2:3.5, *v/v*) at 25 °C was found to be successful for the separation of all diastereomeric pairs. An increase in MeCN concentration from 3.5 to 5 mL resulted in an increase of hR_F values and decrease of R_S. A decrease of MeCN from 3.2 to 1:5.7 decreases the migration distance of diastereomers and spots were not clearly separated. Mobile phase 2, containing TEAP buffer and MeOH (as organic modifier) was also used in similar conditions and the R_S values were 3.24, 2.99 and 2.73, for the diastereomers prepared

with CDR 2, 3 and 6, respectively; resolution was poorer than that obtained with mobile phase 1. With mobile phase 2, consisting of TEAP buffer (50 mM, pH 4.8)–MeOH (3.2:3.5, *v/v*) at 25 °C resolution was obtained as, 2.75, 2.14 and 1.53, for the diastereomers of SeMet prepared with CDR 2, 3 and 6, respectively. The unsuccessful solvent combinations in mobile phase 1 and 2 are shown in Table-5.3. Among all the three diastereomeric pairs, the best resolution was found for diastereomers prepared with CDR 2 (Table-5.4). Resolution (R_S) was calculated by dividing the distance between two spots by the sum of their spot radii, a value of 1.50 was taken as indicative of complete resolution whereas a value of 1.20 or below indicated incomplete resolution (Kowalska and Sherma, 2007). Photographs of actual chromatograms are shown in Fig. 5.8.

The diastereomers were visible in ordinary light as bright yellow spots and no locating reagent was required in contrast to the requirement of locating reagents on impregnated plates used for direct enantioresolution. In this case elution sequence for the diastereomers was same as obtained in RP-HPLC mentioned below.

(d) RP-HPLC Separation of Diastereomers

Aliquots (10 µL) of diastereomers were diluted 10 times with MeCN, filtered and 20 µL were injected onto the column.

Following mobile phases were prepared and used,

Mobile phase 1; MeCN and TEAP buffer (10 mM, pH 4), applied in isocratic mode (10:90, 20:80, 40:60, 60:40 and 80:20) and gradient mode (35 to 80, 35 to 75, 35 to 65, 35 to 60, 30 to 65, 25 to 65, 25 to 70, 20 to 65, 20 to 70 and 10 to 90% of MeCN) for 45 min run. Mobile phase 2 and 3, contained MeCN and MeOH (as organic modifier, respectively) and TFA (0.1%); these were used in a manner similar to described for mobile phase 1 (both isocratic and linear gradient modes).

The mobile phase was filtered through a 0.45 µm filter and degassed by passing nitrogen and sonication, before use, to protect the column from any types of impurity and air bubbles.

It was found that all the three mobile phases were capable of separating all the three pairs of diastereomers of DL-SeMet. With the mobile phase 1 (TEAP buffer-

MeCN), under both the isocratic and gradient elution modes, there appeared broadening of peaks. There were obtained sharper peaks with mobile phase 2 (TFA-MeCN) under the isocratic mode, but sharpness was better with gradient elution of MeCN from 25 to 65% and 0.1% TFA, in 45 min at a flow rate of 1.0 mL/min. Different concentrations of TFA (in the range of 0.05% to 0.20%) were applied for optimizing its concentration. In mobile phase 3, MeOH was used as organic modifier with aq TFA. MeCN could be considered to have been successful as organic modifier since decreased retention time and increased R_S was observed with it in comparison to that with MeOH

The chromatographic data obtained for resolution of diastereomers of DL-SeMet with mobile phase 2, in terms of retention factor (k), separation factor (α), and resolution (R_S) are listed in Table-5.4. The sections of chromatograms showing separation of diastereomers are shown in Fig. 5.9**.** The peak areas calculated by the system software, corresponding to diastereomers obtained under varying conditions of derivatization, served as a measure of completion of reaction and yield of derivatization.

The elution sequence of diastereomers of DL-SeMet was confirmed by examining elution of the diastereomers prepared from enantiomerically pure D- and L-SeMet with CDR 3, as representative. The first eluted peak was for the diastereomer corresponding to L-SeMet (i.e. L-L diastereomer), and second one was for its D-counterpart (i.e. L-D diastereomer).

2. Separation Mechanism: Experimental Observations *Vs* Theoretical Considerations

The experiments showed that the L-L diastereomer was retained for lesser time in comparison to that of the L-D diastereomer (i.e., L-L diastereomer eluted before L-D from the column). Optimized structures (of lowest energy) of the two diastereomers were drawn using Gaussian 09 Rev A. 02 program and hybrid density functional B3LYP with 6-31G basis set (Fig. 5.10a and 5.10b). These show spatial orientations of hydrophobic groups. In L-D diastereomer, the hydrophobic groups, $-CH_2CH_2SCH_3$ on the stereogenic centre of CDR (C-1 in Fig. 5.10a) and $-CH_2CH_2SeCH_3$ on stereogenic centre of SeMet (C-2 in Fig. 5.10a), are oriented on the same side with respect to the plane of dinitrobenzene moiety and are considered to be *cis* to each other. On the other hand, in L-L diastereomer, the same groups are oriented (in space) opposite to the plane

of dinitrobenzene moiety and thus have *trans*-type arrangement (Fig. 5.10b). A graphical representation for the same is also shown in Fig. 5.11.

The said *cis*- or *trans*-type arrangements are considered to be responsible for hydrophobicity difference in these two diastereomers. The L-D diastereomer having *cis*-type arrangement can be considered to be more hydrophobic than the *trans*-type arrangement of L-L diastereomer. Therefore, L-D diastereomer interacts more strongly with ODS material of C_{18} column than the L-L diastereomer, hence has a longer retention time. Thus the experimentally observed elution sequence gets confirmed by the theoretical approach as well.

Different partition coefficients and different retention times (of L-D and L-L diastereomers are the results of their different physical properties, such as, (i) overall hydrophobicity due to *cis*- and *trans*-type arrangements (as explained above) and intra-molecular hydrogen bonding, and (ii) large size of S atom in CDR, Se in SeMet and the presence of benzene group in CDR, and thus good separation results on C_{18} column with different rheological properties of the mobile phase.

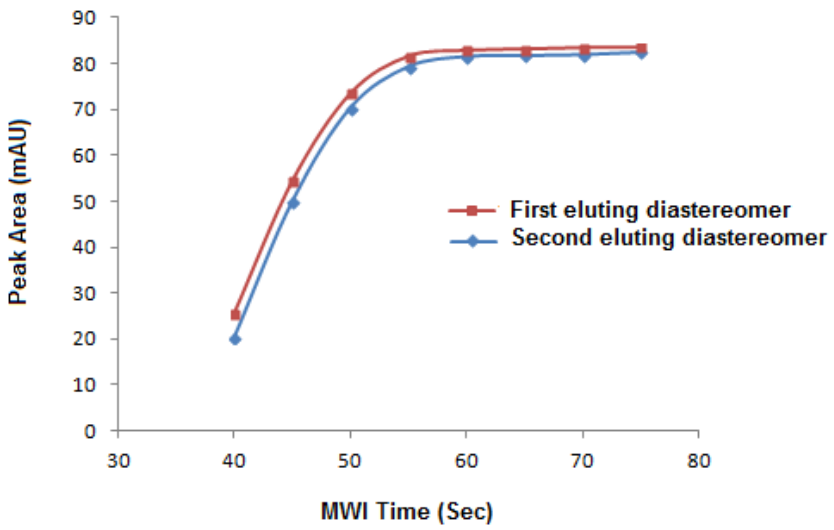


Fig. 5.7: Effect of MWI time (at 75% power) on completion of derivatization reaction of DL-SeMet with CDR 3

Table-5.3: Different solvent systems unsuccessful to separate the diastereomers of SeMet on RP-TLC plates

Sr. No.	Solvent System	Remarks
1	**MeCN-H_2O**	
(a)	1:5.7	NR, spot was very close to the base point
(b)	2.7: 4.0	Spots not clearly separated (Not baseline resolution)
(c)	4.5:2.2	Elongated spots, Not baseline resolution
(d)	5.0:1.7	NR, spots moved with solvent front
2	**MeCN-H_2O**	
(a)	1:5.7	NR, spot was very close to the base point
(b)	2.7: 4.0	Spots not clearly separated (Not baseline resolution)
(c)	4.5:2.2	NR, elongated spots
(d)	5.0:1.7	NR, spots moved with solvent front

NR: not resolved

Table-5.4: Chromatographic separation data of diastereomers of DL-SeMet prepared with different CDRs (2, 3, and 6)

	Reversed-phase HPLC				Reversed-phase TLC		
	t_{R1}	k_1	α	R_S	hR_F		R_S
CDR					L-D	L-L	
2	19.43	8.57	1.37	21.69	21.7	45.0	3.24
3	22.41	10.04	1.30	19.81	18.3	41.7	2.99
6	23.93	10.78	1.26	18.22	16.7	40.0	2.73

Chromatographic conditions: RP-HPLC using LiChrospher C_{18} (250 × 4.6mm i.d., 5 µm particle size) column; mobile phase 3, linear gradient (25 to 65%) of MeCN with 0.10% TFA for diastereomers of DL-SeMet in 45 min; flow rate, 1.0 mL/min; detection, 340 nm. t_{R1} is retention time; k_1 is retention factor of L-L diastereomers; α, separation factor; RP-TLC using TLC silica gel 60 RP-18 $F_{254}S$; solvent system, TEAP buffer (50 mM, pH 4.8)–MeCN, (3.2:3.5, *v/v*); solvent front, 6 cm; development time, 10 min; detection, in ordinary light; R_S, resolution; $hR_F = R_F$ x100.

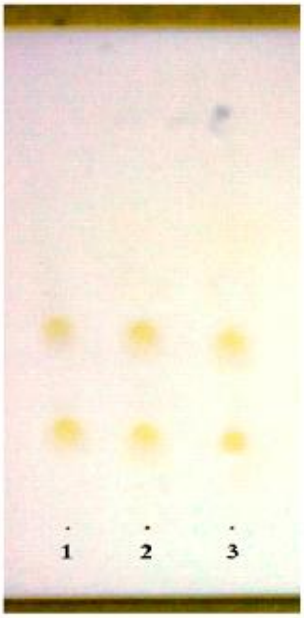


Fig. 5.8: Photograph of chromatogram showing resolution of diastereomers of DL-SeMet, prepared with CDR 2, 3, and 6.
Chromatographic conditions: as mentioned in the legend of Table-5.4 (for RP-TLC): Chromatograms in line 1, 2 and 3 are representing the diastereomers of DL-SeMet prepared with CDR 2, 3 and 6. Lower spot is of L-D diastereomer in each case.

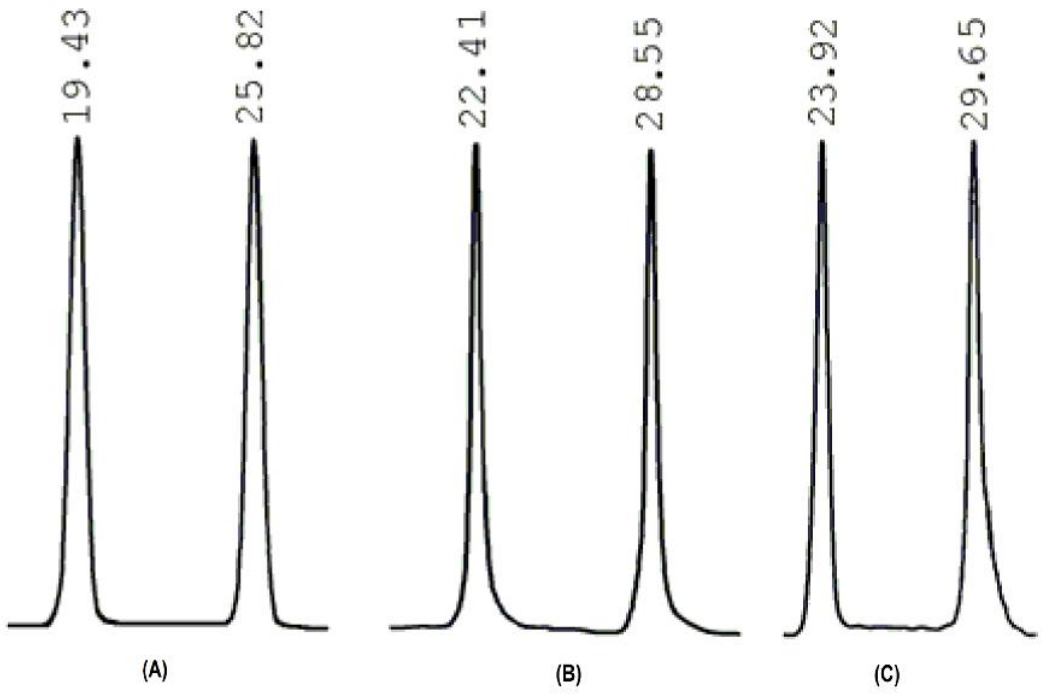


Fig. 5.9: Sections of chromatograms showing baseline resolution of the diastereomers of DL-SeMet prepared with CDR 2, 3, and 6.
Chromatographic conditions: as mentioned in legend of Table-5.4; the first peak corresponds to L-L diastereomer in each case; Chromatograms (A), (B) and (C) are, respectively, of the diastereomers of DL-SeMet prepared with CDR 2, 3 and 6.

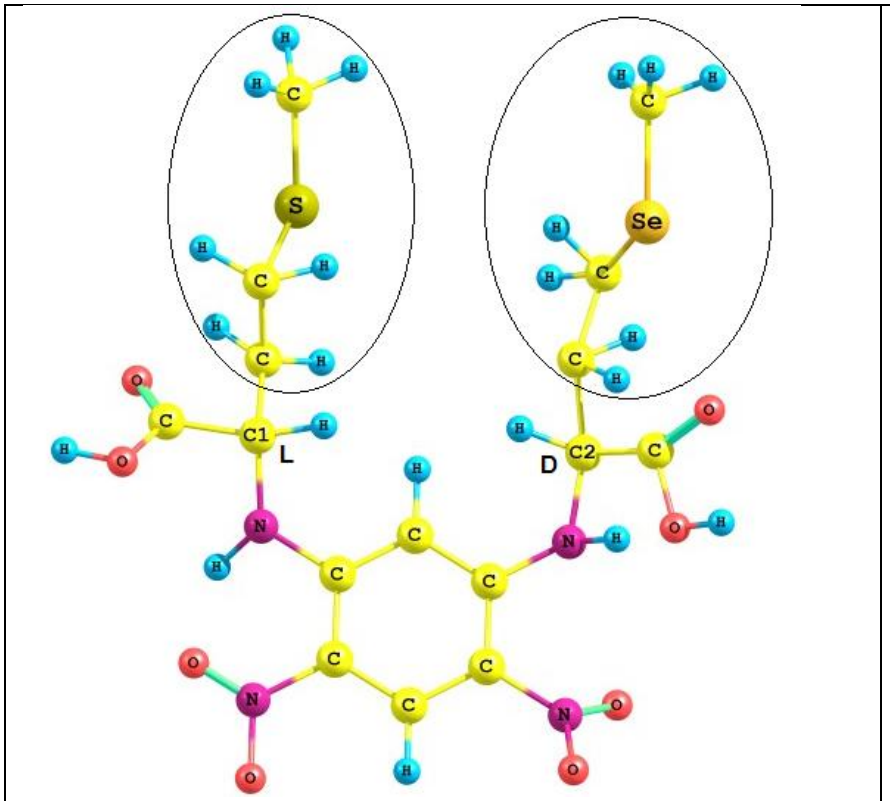


Fig. 5.10a

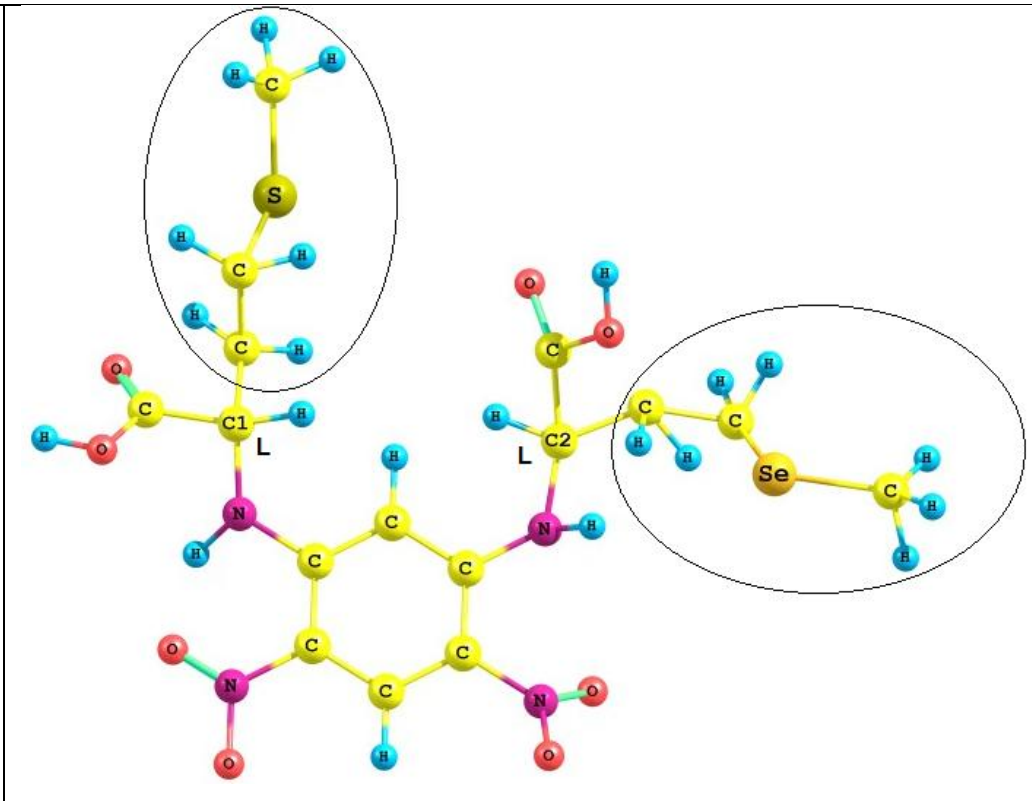


Fig. 5.10b.

Fig. 5.10: Optimized structures of the two diastereomers of DL-SeMet prepared with CDR 3.
The program Gaussian 09 Rev A. 02 and hybrid density functional B3LYP with 6-31G basis set was used to obtain optimized structures.

3. Method Validation

Linearity: For RP-HPLC, the concentrations, 100 to 1000 ng mL^{-1} of L-L diastereomer and L-D diastereomer were injected in C_{18} column and the observed corresponding peak area responses have been used to plot the calibration graphs (Table-5.5). The regression parameters, slopes, intercept and correlation coefficients were determined by developing the regression equation which was found strictly linear over this range and produced by the least square method using Microsoft Excel program. The regression equations were $y = 0.595 + 3.667$ ($R^2 = 0.999$) and $y = 0.589x - 5.707$ ($R^2 = 0.999$) for the L-L diastereomer and L-D diastereomer, respectively.

Accuracy, precision and limit of detection: The standard solutions of diastereomeric mixtures of DL-SeMet in the concentrations 100, 200, 500 and 1000 ng mL^{-1} have been applied in HPLC for intra-day assay and inter-day assay studies of accuracy and precision by replicate HPLC analysis (n=3). The recovery and mean standard deviation (SD) for each of the diastereomers were calculated on the basis of peak areas of first and second eluting diastereomer. These are shown in Table-5.5. The relative standard deviation for L-L diastereomer was varied from 0.51 to 1.23% for intra-day assay precision and 0.71 to 1.34% for inter-day assay precision and for L-D diastereomer it was varied from 0.63 to 1.32 and 0.66 to 1.27, respectively, for intra-day and inter-day assay precision.

To determine the accuracy of the RP-TLC and RP-HPLC methods, different amounts of D-SeMet were spiked with fixed amount of L-SeMet. Recovery of the L-D diastereomer from the solution containing excess of L-L diastereomer was investigated. The results indicate that this method can be applied for detection of D-SeMet in L-SeMet up to 0.06% by TLC and 0.008% by HPLC.

RP-TLC and RP-HPLC were found capable to detect 36 ng mL^{-1} (18 ng mL^{-1} of each enantiomer) and 48 pg mL^{-1} (24 pg mL^{-1} of each enantiomer), respectively, of DL-SeMet using CDR 3. Thus, samples containing such small amounts of any of these enantiomers could be separated and detected.

4. Comparison of R_S and LOD with Literature Reports

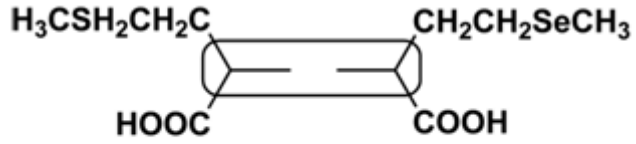
Literature reported the LOD values, 70 μg L^{-1} or 20 μg L^{-1} (using HG-ICP-MS detector) for direct chiral separation of the derivatives prepared with *o*-phthalaldehyde or 2,3-naphthalenedicarboxaldehyde (Méndez *et al*., 1998). LOD values reported for the each diastereomer of SeMet prepared with certain other CDRs, *o*-phthalaldehyde and *N*-isobutyryl-L-cysteine (Bergmann *et al*., 2004); and (*S*)-NEIC (Bhushan and Dubey, 2012b) were about 4 μg L^{-1} (ICP-MS detector), and 0.32 μg mL^{-1} (UV detector), respectively. In the present case, LOD was found to be 24 pg mL^{-1} for each diastereomers of D- and L-SeMet using RP-HPLC method; this is much lower than the LOD values reported in literature.

Resolution in the present case (as shown in Table-5.4, by RP-HPLC) was found better than that reported in literature, e.g., R_S 15.66, 4.24, and 7.46, respectively, using Marfey's reagent, MCT and DCT type CDRs containing L-alaninamide as chiral auxiliary (Bhushan and Dubey, 2012a); 2.34 and 3.23, respectively, using (*R*)-MBIC, and (*S*)-NEIC as CDRs, (Bhushan and Dubey, 2012b). The resolution was also better than those reported in literature using direct approach, R_S was 0.96 and 1.8 using Chirasil-L-Val CSP (Méndez *et al*., 1999; Devos *et al*., 2002); 1.00 on Cyclobond I *β*-cyclodextrin column (Méndez *et al*., 1998); 1.2 using *β*-cyclodextrin capillary column (Méndez *et al*., 2000b); ≈2.00 using ligand exchange Cu(II)-(L-*N*-(2-hydroxy-propyl)-phenylalanine) complex (Duan *et al*., 2012). Resolution obtained in RP-TLC (as shown in **Table-5.4**), was found also better in comparison to most of the literature reports.

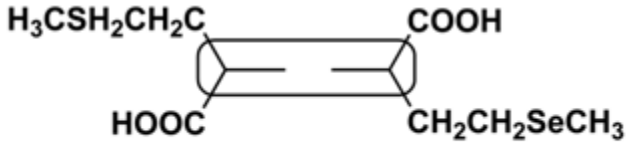
5. Comparison of R_S and LOD of Present Study

The resolution of diastereomers by RP-HPLC was better than that obtained by RP-TLC (as given in Table-5.4).

The LOD was in the range of pg mL^{-1} and ng mL^{-1}, respectively, for RP-HPLC and RP-TLC (as given in ''method validation''). It is simply because HPLC is more sensitive, and the diastereomers having a DNB chromophore could also be detected/visible in very small amounts.



L-D Diastereomer



L-L Diastereomer

Fig. 5.11: The plausible conformations of diastereomers of the D- and L-SeMet prepared with CDR 3.

In L-D diastereomer the hydrophobic groups of L-Met and D-SeMet were oriented in *cis* arrangement and in L-L diastereomers they are in *trans* type arrangement with respect to the plane of dinitrobenzene moiety.

Table-5.5: Results from validation of RP-HPLC method for diastereomers of DL-SeMet

	First eluting diastereomer			Second eluting diastereomer		
Linearity						
Range	100 to 1000 ng mL^{-1}			100 to 1000 ng mL^{-1}		
Slope	0.595			0.589		
Intercept	3.667			5.707		
Correlation Coefficient (R^2)	0.999			0.999		
Actual concentration ng mL^{-1}	Mean±SD (measured) ng mL^{-1}	Recovery (%)	RSD (%)	Mean±SD (measured) ng mL^{-1}	Recovery (%)	RSD (%)
Intra-day precision (n = 3)						
100	50.60±0.622	101.20	1.23	50.54±0.667	101.09	1.32
200	98.95±0.752	98.95	0.76	99.31±0.775	99.31	0.78
500	251.19±1.683	100.46	0.67	247.15±1.557	98.86	0.63
1000	499.75±2.549	99.95	0.51	500.30±4.152	100.06	0.83
Inter-day precision (n = 3)						
100	49.31±0.607	98.61	1.34	39.804±0.506	99.52	1.27
200	99.97±0.947	99.72	0.95	58.962±0.542	98.27	0.92
500	243.83±1.731	97.53	0.71	77.688±0.536	97.11	0.69
1000	499.20±4.243	99.84	0.85	493.40±3.256	98.68	0.66

C. Direct TLC Enantioseparation of DL-SeMet by using (–)-Quinine as Chiral Selector

(–)-quinine has been used as an impregnating reagent for enantioresolution of DL-amino acids on silica gel plates (Bhushan and Arora 2001). Enantioresolution of DL-SeMet compounds on chiral phase TLC using (–)-quinine as the chiral selector has not been reported yet.

Quinine naturally occurs in the bark of the cinchona tree and is a natural white crystalline alkaloid contains two major fused-ring systems: the aromatic quinoline and the bicyclic quinuclidine (Fig. 5.12). It shows antipyretic (fever reducing), antimalarial, analgesic (painkilling), and anti-inflammatory properties with a bitter taste. It is a stereoisomer of quinidine, which, unlike quinine, is an antiarrhythmic agent. It is freely soluble in lower alcohols (as MeOH, EtOH etc.).

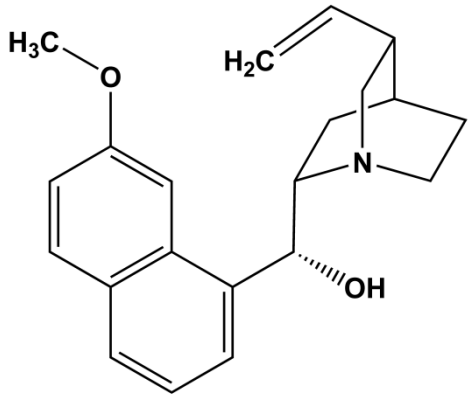


Fig. 5.12: Structure of (–)-quinine

1. Results and Discussion

Direct TLC separation of enantiomers of SeMet along with isolation of native enantiomers was achieved by TLC using (−)-quinine (an alkaloid) as chiral selector. Following different approaches were used for preparing the TLC plates and to develop the chromatograms:

(A) Impregnation of plain plates by mixing the chiral selector with slurry,

(B) Impregnation by ascending development of plain plates in a solution of chiral selector,

(C) Use of chiral selector as mobile phase additive and

(D) Mixing of (−)-quinine, as chiral inducing reagent (CIR) with racemic SeMet in various mole ratio and the resulting mixtures were applied on plain thin layer silica gel (achiral) plates.

The detailed description of preparation of impregnated thin layer plates, CMPA and, pre-mixing of chiral selector with racemic mixture and developments of chromatograms have been described in Chapter-2. The results of TLC resolution along with the studies related to the effects of temperature, pH and concentration of chiral selectors on enantiomeric resolution are discussed herein. The influence of pH, temperature and amount of chiral selector was examined on enantioseparation. The spots were located by spraying freshly prepared ninhydrin solution and also in iodine vapor. The developed TLC method was validated and RSD and LOD were determined.

(a) Enantiseparation of DL-SeMet on TLC using (–)-Quinine as an Impregnating Reagent (Approach A and B) and Mobile Phase Additive (C)

(i) Enantioseparation

Different binary, ternary and quaternary mixtures of a variety of solvents (such as CH_3CN, CH_3OH, H_2O and CH_2Cl_2) were tried systematically, to achieve enantiomeric resolution. In approach (C) quinine was added to each of the solvent combinations tried.

The successful solvent systems and hR_F (R_F x 100) values are listed in Table-5.6. Solvent system consisting of CH_3CN-CH_3OH-CH_2Cl_2-H_2O, in slightly varying proportions, was commonly successful for the approaches (A), (B), and (C). It was interesting to observe that all the three approaches were capable of separating enantiomers of DL-SeMet. The unsuccessful solvent combinations tried in all three approaches are given in Table-5.7. Representative photographs of chromatograms are shown in Fig. 5.13 (A, B and C). R_F values are averages from at least five experiments performed under identical conditions on the same day and on different days.

The method was found to be successful to separate the mixture of D-, and L-enantiomers in the ratio of 1:100 of DL-SeMet. A representative chromatogram, showing resolution of D- and L-SeMet (in a ratio of 1:100) using approach (A) is given as Fig. 3.13 (D).

It was observed that the highest R_S was obtained with approach (A) while approach (B) gave better separation than (C). Of the various approaches attempted herein, mixing of chiral selector with silica gel slurry (approach A) was found to be the best for direct enantiomeric separation of DL-SeMet in terms of R_S. It may be due to better availability (immobilization) of chiral selector not only on silica surface but also throughout the chromatographic planar bed providing enhanced interactions between analyte and chiral selector.

Literature reports spraying of a 0.3 % solution of ninhydrin prepared in *n*-BuOH (100 mL) containing CH_3COOH (3 mL) followed by heating for 30 min at 60 °C or for 10 min at 110 °C (Bhushan and Martens, 2003) for detection of amino acids. In the present case, heating at 70 °C for 10 min was successful using the ninhydrin solution of the same composition.

The formation of diastereomers in the present case was confirmed by isolating the two enantiomers from TLC plates. The solutions of the isolated enantiomers were examined by polarimeter. The specific rotation value was in agreement with literature (Sigma-Aldrich catalogue, 2003; 2013). The polarimetric experiments suggested that the two isomers were in 1:1 ratio and the L-isomer had a higher R_F than the D-isomer under all the three conditions. Further, the presence of chiral selector, in both the spots, on TLC plate was evidenced when the silica gel residue (supposed to retain chiral selector) tested positive for the presence of (–)-quinine. Detecting the presence of amino acid in the two resolved spots with characteristic color, by treating the chromatogram with ninhydrin solution, was also an evidence for formation of such diastereomers; the plate acquired a light pink background but the resolved spots were visible with greater intensity of characteristic color and sharpness (Bhushan and Thiongo, 1998).

(ii) Effect of concentration of chiral selector, pH and temperature

In approach (A) and (B) a ratio of 1:3 (DL-SeMet:(–)-quinine) was required for successful resolution while in approach (C) a ratio of 1:2 (DL-SeMet:(–)-quinine) was found to be successful (as sufficient concentration) for resolution. In approach (C) chiral selector is present in mobile phase so it formed diastereomers with the analyte quickly. Though it is difficult to interpret the requirement of a little higher amount of the chiral selector in approach (A) and (B) but it may be argued that there is a

possibility of some amount of chiral selector is irreversibly adsorbed on the silica gel surface when the silica gel was impregnated with the chiral selector and thus all of it is not available for interaction with the enantiomers.

The best resolution was obtained at 25±2 °C in all approaches. Increase of temperature from 25 to 35 °C resulted in tailing of spots and a decrease in temperature to 15 °C showed no resolution. The successful resolution was observed at pH 8.

The solvent systems and the silica plates were maintained at pH 8 where DL-SeMet existed in the anionic form (pI 5.75) while (−)-quinine (pka_1=5.07 at 18 °C and pka_2=9.7) (Kar, 2003) existed in the protonated cationic form. Literature suggests that at least three *non-covalent* interactions must be involved between the analyte and the chiral selector (along with stationary phase), (Dalgliesh, 1952a) for enantioresolution to occur; these may be van der Waals and electrostatic interactions, hydrogen bonding, pi–pi, steric, hydrophobic or dipole–dipole, and other forms of electron donation and acceptance that are readily reversible. Electrostatic interaction between COO^- of SeMet and $\equiv NH^+$ of the chiral selector were thus responsible for maintaining the diastereomer.

Table-5.6: TLC separation of DL-SeMet using (–)-quinine as chiral selector

Approach	Mobile phase (Solvents combinations) (*v/v*)	hR_F			Time (min)	R_S
		Pure	From racemic mixture			
		(L)	(L)	(D)		
(A), CS mixed in the slurry of silica gel before making the plates	CH_3CN-CH_3OH-CH_2Cl_2-H_2O, (11:1:1:1.5)	30	30	13	10	4.31
(B), ascending development of the plate in the solution of CS	CH_3CN-CH_3OH-CH_2Cl_2-H_2O, (8:1:2:1.5)	40	40	19	9	2.84
(C), CS added to mobile phase	CH_3CN-CH_3OH-CH_2Cl_2-H_2O (9:1:1:1.5) having 0.07% of (–)-quinine	35	35	18	10	1.86

R_S, resolution; hR_F, retardation factor x 100 (R_F x 100); CS, Chiral selector.

Unsuccessful solvent system in different approaches:

Approach (A); CH_3CN-CH_3OH-CH_2Cl_2-H_2O (12:0.5:0.5:1, 9:1:1:1.5 and 8:1:2:1.5)

Approach (B); CH_3CN-CH_3OH-CH_2Cl_2-H_2O (12:0.5:0.5:1, 11:1:1:1.5, 9:1:1:1.5 and7:1.5:1:2)

Approach (C); CH_3CN-CH_3OH-CH_2Cl_2-H_2O (11:1:1:1.5, 8:1.5:1:2, and 7:1.5:1:2), varying the concentration of (–)-quinine from 0.05% to 0.2% for every solvent combination.

Table-5.7: Unsuccessful solvents combinations used for TLC separation of DL-SeMet using (–)-quinine as chiral selector

Solvent system	Composition (v/v)	Observation
(A), CS mixed in the slurry of silica gel before making the plates		
MeCN-MeOH-H2O	5:4:2	NR
	6:2:1	ES
	8:2:1	ES
MeCN-H_2O-CH_2Cl_2	3:7:2	NR
	4:4:1	NR
	7:3:1	NR
CH_3CN-CH_3OH-CH_2Cl_2-H_2O	12:0.5:0.5:1	ES
	9:1:1:1.5	RT
	8:1:2:1.5	NR
(B), ascending development of the plate in the solution of CS		
MeCN-MeOH-H_2O	5:4:2	NR
	6:2:1	ES
	8:2:1	RT
MeCN-H2O-CH_2Cl_2	3:7:2	ES
	4:4:1	NR
	7:3:1	RT
CH_3CN-CH_3OH-CH_2Cl_2-H_2O	12:0.5:0.5:1	NR
	9:1:1:1.5	ES
	7:1.5:1:2	RT
(C), CS added to mobile phase		
MeCN-MeOH-H2O having 0.07% of (–)-quinine	5:4:2	RT
	6:2:1	NR
	8:2:1	ES
MeCN-H2O-CH_2Cl_2 having 0.07% of (–)-quinine	3:7:2	NR
	4:4:1	ES
	7:3:1	RT
CH_3CN-CH_3OH-CH_2Cl_2-H_2O having 0.07% of (–)-quinine	12:0.5:0.5:1	ES
	9:1:1:1.5	NR
	7:1.5:1:2	RT

NR: not resolved; RT: resolved with tailing; CS: chiral selector; ES: eight shape spot; development distance 8 cm, temperature 25±2°C, and spots were located with iodine vapor

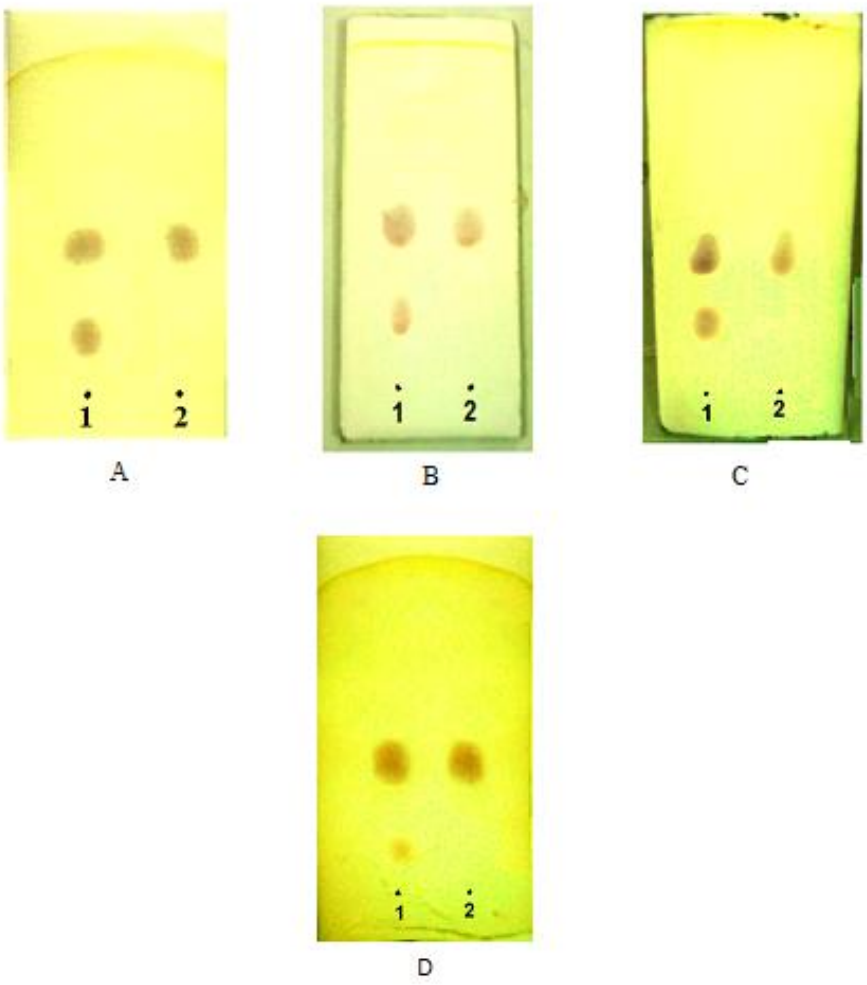


Fig. 5.13: Photograph of an actual chromatogram showing resolution of DL-SeMet using (–)-quinine.

(A): Mobile phase: $CH_3CN–CH_3OH–CH_2Cl_2–H_2O$ (11:1:1:1.5, *v/v*). Development time, 10 min. (**B**): Mobile phase: $CH_3CN–CH_3OH–CH_2Cl_2–H_2O$ (8:1:2:1.5, *v/v*). Development time, 9 min. (**C**): Mobile phase: $CH_3CN–CH_3OH–CH_2Cl_2–H_2O$ (9:1:1:1.5, *v/v*) having 0.07% of (–)-quinine. Development time, 10 min. (**D**): From left to right: Line 1, (D:L), 1:100, 10 µL of 1 mM solution, lower spot for D-isomer and upper spot for L-isomer; line 2, 10 µL of 1 mM solution of L-isomer. Mobile phase: $CH_3CN–CH_3OH–CH_2Cl_2–H_2O$ (11:1:1:1.5, *v/v*).

In all approaches, solvents front is 8 cm; temperature, 25±2 °C; detection, in ninhydrin solution (0.3% in *n*-BuOH) and from left to right: Line 1, lower spot is of D-isomer and the upper spot is of L-isomer resolved from the mixture of DL-SeMet; Line 2, pure L-isomer.

(b) Enantioseparation of DL-SeMet on TLC using (–)-Quinine as Chiral Inducing Reagent (Approach D)

(i) Enantioseparation

Solvent compositions used in this approach were same used in approach A. The successful mobile phase was, acetonitrile-methanol-dichloromethane-water in the ratio of (10:1.5:0.5:1.5, *v*/*v*) for enantioseparation of SeMet; chromatograms were developed in 10 min. hR_F (R_F x 100) values are shown in Table-5.8. The mobile phases given in Table-5.7 (for approach A) have been used for this study in same manner; either not resolution or elongation of spots or eight shaped spots were found. Fig. 5.14 shows photograph of the chromatogram showing TLC resolution of L- and D-SeMet. The (R_S) values were calculated by the same method described above for approaches (A-C).

To compare and verify the migration order of the isomers getting separated from racemic mixture, pure enantiomer was applied in parallel on the TLC plates. It was found that the migration distance of L-SeMet was more than that of L-isomer.

Since (−)-quinine is insoluble in cold water so it was assumed that only enantiomers of SeMet, from the two cut spots, would go into solution. The optical purity of separated enantiomers was examined by polarimeter and it was found in agreement with the literature report. The specific rotation of the isolated L-SeMet was found to be (+) 17.90^o which was in agreement with literature (specific rotation for L-SeMet is (+) 18.0^o, c=2, 2M HCl) (Sigma-aldrich.com, 2003; Sigma Aldrich Catalogue, 2013), thus it was considered to be enantiomerically pure.

In the present studies (approach D), racemic mixture of SeMet was pre-mixed with (−)-quinine (a CIR) before applying on plates. There occurred resolution of the racemic mixture and the two enantiomers were recovered in 1:1 ratio (established by polarimetric studies). There occurred formation of diastereomeric associates, of the type (L-SeMet-(−)-quinine) and (D-SeMet-(−)-quinine), leading to transformation of the initial racemate into mixture of diastereomers with different chromatographic mobilities in achiral phases and hence resolution.

Isolation of two enantiomers from TLC plates confirmed the formation of diastereomers via non-covalent reversible interactions. Presence of the CIR in both the spots, resolved under chromatographic conditions (where chiral selector was not used either as impregnating reagent or mobile phase additives), was evidenced when the residual silica gel (remaining after extracting enantiomers of SeMet) tested positive for the presence of (–)-quinine. Further evidence for formation of such diastereomers was obtained when the chromatogram was sprayed with ninhydrin and each spot was visible in characteristic pink-purple colour for the presence of SeMet. However, there appeared a light pink background on the plate but the resolved spots were visible with more intensity and sharpness.

(ii) Effect of Concentration of Chiral Selector, pH and Temperature

Among several possible combinations, using different ratio of CIR a ratio of 1:1 (DL-SeMet:(–)-quinine) gave the best result because this proved to be the required ratio for the formation of diastereomers**.** On increasing the ratio of SeMet and CIR from 1:1 to 1:3 there was no changes in results and at ratio lower than the 1:1 was not sufficient stiocheometrically to form diastereomers. The effect of temperature and pH was investigated on the formation of diastereomers (formed through noncovalent interactions); by varying the temperature at 15±2 °C, 25±2 °C and 35±2 °C and pH at 6, 8 and 9. The best results for enantioresolution of DL-SeMet were obtained at 25 °C and pH 8.

Table-5.8: Resolution data of enantiomers of SeMet

Analyte	CIR	Mobile Phase (*v*/*v*)	hR_F Pure	hR_F From racemic mixture		R_S
			L	L	D	
SeMet	(−)-Quinine	CH_3CN-CH_3OH-CH_2Cl_2-H_2O (10:1.5:0.5:1.5)	26	26	13	1.69

R_S, resolution; $hR_F = R_F$ x 100.
Approach (Pre-mixing of CIR with analyte)

(iii) Separation Mechanism

DL-SeMet was pre-mixed with (−)-quinine (acting as CIR) and the pH of the resulting mixture was maintained around 8, at which DL-SeMet existed in the anionic form (pI 5.75) while (−)-quinine (pka_1=5.07 at 18 °C and pka_2=9.7) (Kar, 2003) existed in the protonated cationic form. Electrostatic interaction between $-COO^-$ of SeMet and $\equiv NH^+$ of the chiral selector, along with three point interaction (Dalgliesh, 1952a) involving hydrogen bonding, van der Waal's forces, steric, hydrophobic, dipole–dipole or pi-pi interactions and other forms of electron donation and acceptance that are readily reversible were in action for formation of transient diastereomers and their separation. This approach was found to be successful for direct and sensitive resolution of DL-SeMet from racemic mixture under achiral phases of chromatography and for detection of their enantiomers in a range lower than the limits prescribed (1%) for pharmaceuticals in industry. It may be considered better and a novel approach in comparison to the methods/approaches (Stephani and Cesare, 1998; Nicoud *et al*., 1996; Baciocchi *et al*., 2002; Ogawa *et al*., 2010; Mayani *et al*., 2009; 2011; Katagiri *et al*., 2011; Soloshonok, 2006; Soloshonok and Berbasov, 2006a; 2006b; Martens and Bhushan, 2014; Nakamura *et al*., 2012; Tateishi *et al*., 2013) where enantiomeric enrichment from a non racemic mixture was obtained, and in particular the method reported by Tateishi *et al*., (2013) on separation of racemate via chiral initiator-induced approach using achiral chromatography which indeed is another approach to enantiomeric enrichment rather than resolution of racemic mixture. Nevertheless, the present approach is successful in resolving racemic mixture under achiral phases of chromatography but has the difficulty in getting quantitative yields.

2. RSD and Limit of Detection

The mean values (n = 5) for precision (RSD) for the *β*-blockers were in the ranges 0.40–0.45 to 1.38% for separation on plates impregnated with chiral selector.

To determine the limit of detection, solutions of different concentrations of DL-SeMet (in the range of 0.1 to 1.0%) were spiked with fixed amount of L-SeMet. These were subjected to TLC separation under approach (A), (B), (C) and (D). The results indicated that this method was successful for detection of D-SeMet up to 0.2% in L-

SeMet. However, a representative chromatogram, showing resolution of D- and L-SeMet (in a ratio of 1:100) using approach (A) is given as Fig. 5.13 (D).

Fig. 5.14: Photograph of an actual chromatogram showing resolution of DL-SeMet.
From left to right: Line 1, lower spot is of D-isomer and the upper spot is of L-isomer resolved from the racemic mixture; Line 2, pure L-isomer. Mobile phase: $CH_3CN–CH_3OH–CH_2Cl_2–H_2O$ (10:1.5:0.5:1.5, *v/v*). Solvent front: 8 cm; temperature: 25 ± 2 °C; detection: ninhydrin solution (0.3% in *n*-BuOH); development time: 10 min.

IV. Inference

Section A: A new CDR (Nap-Phth) has been developed, containing *N*-hydroxyphthalimide group which acted as a good leaving group, leading to easy formation of diastereomers which in turn have high UV absorbing chromophore in the form of (*S*)-Nap moiety. Diastereomers were synthesized under mild derivatization conditions with shorter reaction time in comparison to that required by other CDRs reported in literature. *N*-hydroxyphthalimide, used as the activating agent, is neither explosive in nature nor hygroscopic and it can also be stored at room temperature easily. There occurred a better resolution with low LOD of diastereomers of SeMet in comparison to those prepared with several other CDRs reported in literature. The method can be applied for detection of trace amount of DL-SeMet and other Se or sulphur containing amino acids.

Section B: Formation of diastereomers under MW irradiation in a few seconds is another attractive feature of the work. The newly developed CDRs have high molar absorption properties with the capability of detecting diastereomers at low limits. The advantages in terms of LOD and in terms of higher R_S (for the separation of diastereomers) have been described under 'Comparison of R_S and LOD with literature reports'. Besides, a theoretical justification (based on development of optimized structures of the diastereomers of SeMet) has been provided in support of separation mechanism and for determination of elution order of diastereomers.

Comparison of the results (in terms of chromatographic analysis time, resolution and detection limit) of D- and L-enantiomers with literature establishes advantages of DFDNB based CDRs over other reagents reported in the literature reports.

Section C: The present approaches has the following features, (i) resolution of a racemic mixture is possible with the help of a chiral selector in TLC, (ii) isolation of native enantiomer is possible, (iii) the approach is expected to be of general application for obtaining enantiomerically pure samples from racemic mixtures, and (iv) it is overall simple, less expensive and does not require analysis/determination of enantiomeric composition by chiral HPLC.

Chromatographic analysis took only a few minutes so the method could be useful to determine enantiomeric purity of the samples obtained from different sources even without much sample preparation requirements, and detection is possible at a concentration lower than the limit of 1% prescribed for pharmaceutical industry.

The present approach (achiral phase TLC) opens another area for general application and utility of TLC.

•

Chapter-6

Enantioseparation of Proteinogenic AAs using (*S*)-Nap based CDR and HPLC

Contents **Page No.**

I. Amino Acids and Literature Survey on Their Enantioseparation

Chirally pure amino acids (AAs) constitute a ready, commercial, cost effective chiral pool with prospects of simple derivatization of their functional groups. They find application in asymmetric synthesis, enantiomeric separation and determination of enantiomeric purity of certain pharmaceutically important compounds. They have been used as chiral selectors in the form of impregnating reagents/mobile phase additive (Bhushan and Dixit, 2012d) and chiral auxiliaries to synthesize derivatizing reagents (Marfey, 1984; Bhushan and Brückner, 2004; 2011). In addition, they are used as suitable starting materials to explore/establish new methods/protocols for enantioseparation (Bhushan and Martens, 2010). Nevertheless, separation of enantiomers of AAs is of great importance in various fields of science both in industry and academics. Some review articles have appeared in literature which deals with separation of AAs (Bhushan and Brückner, 2004; 2011; Bojarski, 1997; Görög, 2000; Srinivas, 2004) by both direct and indirect approaches.

Literature (as summarized in Table-6.1) reveals that different CDRs used for enantioseparation of AAs require a derivatization time ranging from 30 to 180 min and a temperature range of 30 ˚C to 80 ˚C; the different diastereomers so obtained showed a resolution value in the range of 2.20 to 13.

II. Present Work

In view of the above and in search of certain new CDRs enantioseparation of 18 proteinogenic AAs (Fig. 6.1a and 6.1b) was achieved by HPLC using indirect method. By considering the advantages of (*S*)-Nap, Nap-Btz (CDR 17; Table-2.3; Chapter-2; Fig. 2.4) was synthesized. This was used as a CDR for the synthesis of diastereomers of eighteen AAs. Diastereomers of 18 proteinogenic AAs were synthesized under MWI and by vortexing. The diastereomers synthesized by two approaches were found to be identical in terms of their characterization and chromatographic data. Separation of these diastereomers was performed by RP-HPLC using C_{18} column and mixture of MeCN with TEAP buffer as mobile phase was used in gradient elution mode. The separation method was validated for accuracy, precision and LOD.

1. Results and Discussion

(a) Synthesis of CDR, (*S*)-Nap-Btz (CDR 17)

CDR 17 (yield >94%) was obtained by nucleophilic attack of 1*H*-benzotriazole on carbonyl carbon of the carboxylic acid of Nap in the presence of coupling reagent DCC/DMAP under mild conditions with the removal of dicyclohexylurea. The CDR, was characterized by IR, UV, CHN and ^{1}H NMR. The chiral purity of CDR 17 was established as described elsewhere (Bhushan and Kumar, 2010; Bhushan, 2011.

Synthesis of benzotriazole activated naproxen required 4 hr using hazardous thionyl chloride (Katritzky *et al*., 2009) while for the present work synthesis of Nap-Btz has been achieved by vigorously stirring the reaction mixture at room temperature for 2.5 hr using DCC/DMAP coupling reagent (Bhushan and Dubey, 2011). In the absence of DMAP the reaction took 4 hr for completion. The CDR (Nap-Btz) is an amide and is more stable than *N*-succinimidyl-(*S*)-2-(6-methoxynaphth-2-yl) propionate (SINP, an ester) (Bhushan and Tanwar, 2008a), due to higher thermodynamic stability of amides over esters.

(b) Synthesis of diastereomers

The scheme for the synthesis of diastereomers is shown in Fig. 2.16 with DL-Leu as the representative. The diastereomers can be designated as (L-(*S*))- and (D-(*S*))-diastereomers (the first letter refers to the configuration of amino acid to be analyzed and the second to that of the reagent). The conditions for derivatization were optimazed using DL-Leu; these were pH around 11.0, two fold molar excess of the CDR and MW irradiation of 45 s at 75% power (800 W). A twofold molar excess of the CDR to the analyte was successful for the AAs producing a mono derivative (i.e., the AAs containing only one primary amino group as the reacting functional group) while a fivefold molar excess of the CDR was required in other cases (e.g., Lys, Cys and Tyr). The diastereomers of all the analytes were also synthesized by vortexing the reaction mixture (in the same mole ratio as used for MW based synthesis) at 700 rpm for 10 min in room temperature condition. Aliquots (10 µL) of resulting solution of diastereomers were diluted 10 times with MeCN and injected (20µL) on to the column.

Table-6.1: Summary of literature reports on derivatization conditions of AAs using certain CDRs and chromatographic characteristics for separation of corresponding diastereomers

S. no.	CDR	Derivatization time	Temp / MWI power	R_s	Column	Reference
1	*N*-(4,6-Dichloro-(1,3,5)-triazine-2-yl)-L-leucinamide	90 min	80˚C	NA	Lichrospher C_{18} (250 × 4.6 mm I.D.,5-µm particle size	Bhushan and Kumar, 2008a
2	*N*-(4-Chloro-6-methoxy-(1,3,5)-triazine-2-yl)-L-leucinamide	180 min	50˚C	NA	Lichrospher C_{18} (250 × 4.6 mm I.D.,5-µm particle size)	Bhushan and Kumar, 2008a
3	*N*-(4,6-Dichloro-(1,3,5)-triazine-2-yl)-L-leucine	60 s	75% (800 W) MWI	13.34	Lichrospher C_{18} (250 × 4.6 mm I.D.,5-µm particle size)	Bhushan and Dixit, 2012a
4	1-Fluoro-2,4-dinitrophenyl-D-phenylglycinamide	60 min	45˚C	NA	Agilent BDS C_{18} (250 × 4.6 mm, 4-µm particle size)	Bhushan and Kumar, 2008b
5	DBD-PyNCS	20 min	55˚C	4.53	Wakosil-II $3C_{18}$ RS (150 x 4.6 mm I.D., 3-µm particle size)	Jin D et al., 1998
6	DANI	120 min	60˚C	2.83	Nova-Pak C_{18} column, (150 x 3.9 mm I.D., 4-µm particle size)	Péter et al., 2000a
7	NIFE	20 min	RT	12.33	Vydac 218TP54 C_{18} (250 x 4.6 mm I.D., 5-µm particle size)	Péter et al., 2000b
8	*N*-(4-Chloro-6-pipredinyl-(1,3,5)-triazine-2-yl)-L-leucine	60 min	80˚C	2.20	Licrospher C $_{18}$ (250 x 4.6 mm I.D., 5-µm particle size)	Bhushan and Agarwal, 2011
9	N^2-(5-Fluoro-2,4-dinitrophe-nyl)-L-phenylalanine amide	60 min	30-40˚C	NA	Spherisorb ODS2 (125 x 4.6 mm I.D. , 3-µm particle size)	Brückner and Keller-Hoehl, 1990
10	GITC	30 min	RT	5.43	Develosil ODS 5 (250 x 4.6 mm I.D., 5-µm particle size)	Kinoshita et al., 1981
11	2,3,4-Tri–*O*-acetyl-α-D-arabin-opyranosil isothiocyanate (AITC)	30 min	RT	7.75	Develosil ODS 5 (250 x 4.6 mm I.D., 5-µm particle size)	Kinoshita et al., 1981

NA not available, *RT* room temperature, *MWI* microwave irradiation

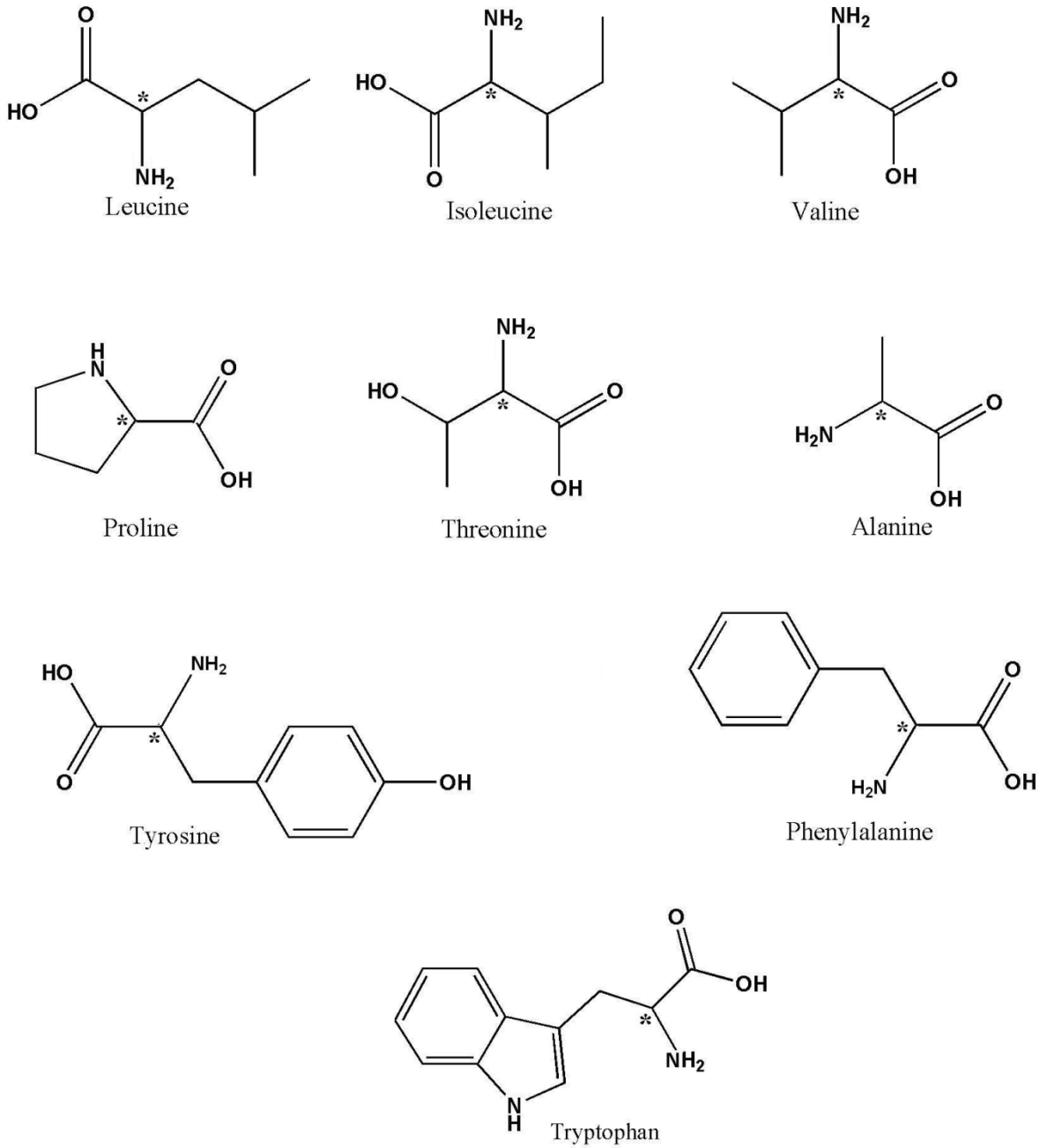


Fig. 6.1a: Structures of proteinogenic *α*-amino acids

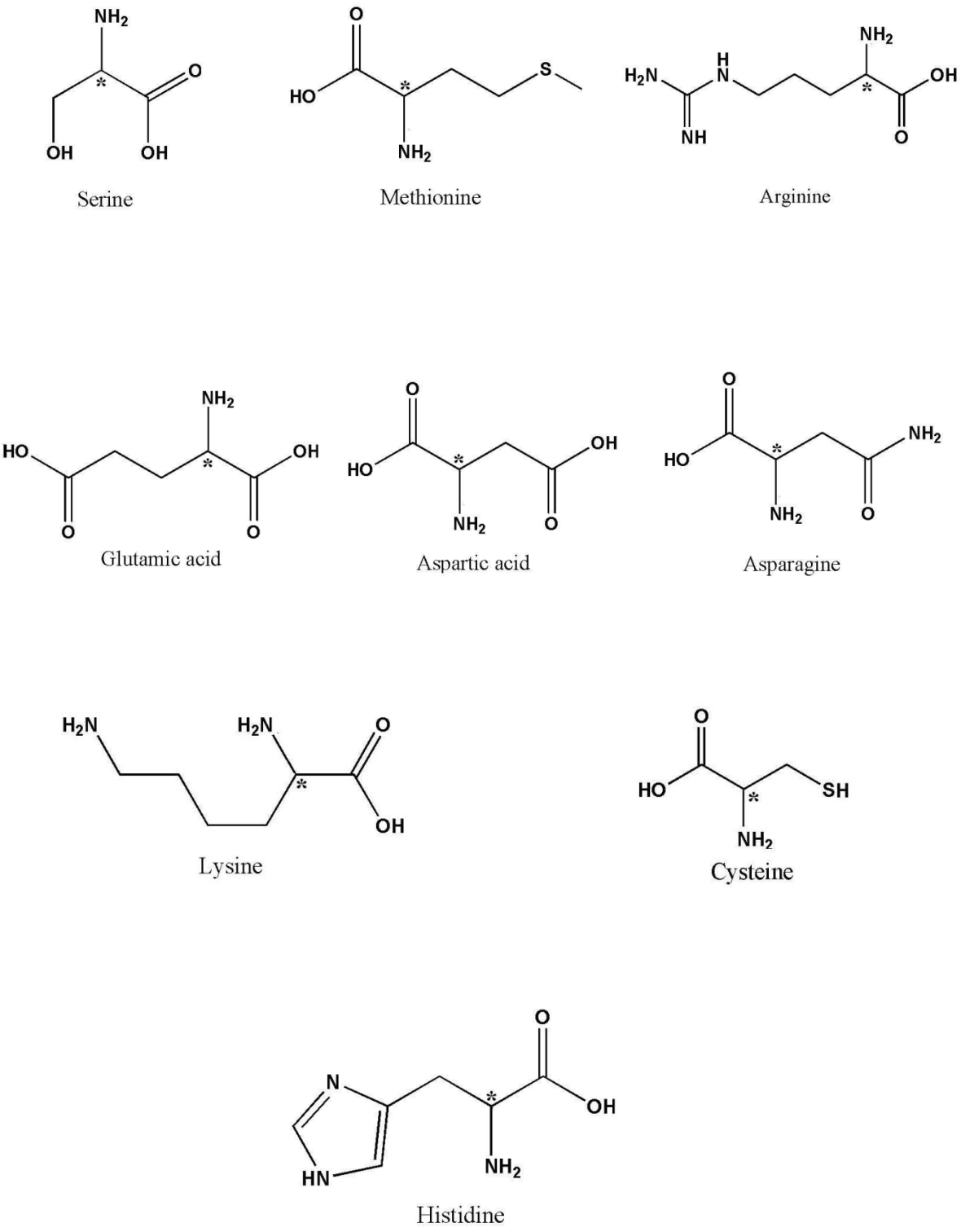


Fig. 6.1b: Structures of proteinogenic *α*-amino acids

Katritzky et al., (2009) reported coupling of L-Nap-Btz with two different racemic AAs at 20˚C in aqueous acetonitrile in the presence of triethylamine requiring 3-12 h to give the diastereomeric mixtures. In the present case the diastereomers of 18 proteinogenic AAs could be easily obtained in 45 s using MWI or in 10 min by vortexing the reaction mixture at room temperature. It was mainly because AAs acted as good nucleophiles in the basic medium, provided by Et_3N, for the substitution of benzotriazole moiety from the CDR 17. No racemization of the CDR was observed under the reaction conditions applied. The CDR 17, was stable for 30 days and the solutions of diastereomers of AAs were quite stable up to 10 days under refrigerated condition (4 °C). The diastereomers synthesized by two approaches (using MWI and vortexing at room temperature) were found to be identical in terms of their characterization and chromatographic data.

The experimental conditions for the synthesis of diastereomers of DL-Leu (as a representative for synthesis of diastereomers of remaining racemic AAs) with CDR 17 were optimized with respect to the role of pH of buffer, reagent excess, MWI time and power) for synthesis of diastereomers under MWI were optimized.

(i) Effect of Reagent Excess

Various molar ratios of CDR: analyte (i.e., 1:1, 2:1, 3:1, 4:1 and 5:1) were used to find the optimum reagent concentration for derivatization. Due to the slight difference in reaction rates of enantiomers, there observed a slight kinetic resolution when lower ratios of CDR were applied. Use of CDR in excess as 2:1 and 5:1 was found best for derivatization of AAs which form mono derivatives and bis derivatives (Cys, Lys and Tyr), respectively, and to overcome the kinetic resolution during derivatization process.

(ii) Role of pH

The reaction of DL-Leu with CDR 17 requires basic medium as it is a nucleophilic substitution reaction. TEA was added in different fractions for maintaining the pH to facilitate the derivatization. The pH was varied in the range of 7.5 to 11.5 to investigate its effects on derivatization. At pH around 11.0 considerable yield was obtained for derivatization of DL-Leu with CDR 17. The yield of derivatization product

(i.e., peak area) increased on increasing the pH from 7.5 to 11.0 and after that its increment up to 11.5 showed no significant changes in yield. Therefore, a pH 11.0 was maintained for further derivatization process of other AAs. In absence of TEA, no derivatization was observed.

(i) Role of MWI

Separate sets were prepared of the reaction mixture and irradiated with MWI in the microwave oven at an interval of 10 s from 30 to 60 at 70-85% power (of 800 W) to check the derivatization yield and to ensure the completion of synthesis process. The MWI times of 45 s at 75% power (800 W) was considered successful as the peak areas of two diastereomers (obtained from the system software) became constant (under the same HPLC mobile phase conditions that were optimized for enantioseparation, see "Separation of Diastereomers") and were found in the ratio of 1:1. These conditions served as a measure/indicator of completion of derivatization reactions and were used for further derivatization of rest of the AAs (Fig. 6.2).

The diastereomer of enantiomerically pure L-Leu was synthesized with CDR 17 showed a sharp and single peak, detected by PDA detector under the same HPLC mobile phase conditions that were optimized for enantioseparation. It thus confirmed that no racemization of AAs was there during the course of derivatization.

(ii) Role of Vortexing time

Effect of the vortexing time on derivatization was investigated by varying the time form 5 to 12 min. It was found that on increasing the vortexing time from 5 to 10 there was observed an increase in peak area and from 10 to 12 min there were no significant changes in peak areas (Fig. 6.3).

(c) HPLC analysis

Aliquots (10 μL) of diastereomers were diluted 10 times with MeCN, filtered and 20 μL were injected onto the column. Different binary combinations of mobile phase were used;

Mobile phase 1: MeOH (linear gradient from 35 to 70%, 30 to 65%, 25 to 70%, 25 to 80% and 10 to 90 %) with TEAP buffer (10 mM).

Mobile phase 2: MeCN (linear gradient from 35 to 70%, 30 to 65%, 25 to 70%, 25 to 80% and 10 to 90 %) with TEAP buffer (10 mM).

Mobile phase 3: MeCN in isocratic mode with TEAP (10mM) buffer (40:60, 50:50, 60:40 and 70:30).

Mobile phase was filtered through a 0.45μm filter and degassed by sonication and passing nitrogen before use. The flow rate was 1.0 mL min^{-1} with UV detection at 231 nm.

(i) Optimization of Chromatographic Conditions

The chromatographic conditions were optimized for separation of diastereomers of DL-Leu prepared with CDR 17, by varying the nature and concentration of organic modifier, TEAP concentration and flow rate. Various linear gradients of organic modifier and TEAP buffer (while using mobile phase 1 and 2) were applied. Different ratios of organic modifier and TEAP in isocratic mode were also applied (while using the mobile phase 3). An increase in amount of organic modifier there was observed an overlapping of peaks showing a decrease in resolution. The eluting mode, linear gradient used in mobile phases 1 and 2 was found better than the isocratic mode used in mobile phase 3. MeOH (mobile phase 1) and MeCN (mobile phase 2) were tried as organic modifiers. The diastereomers eluted faster with mobile phase containing MeCN in comparison to mobile phase containing MeOH since MeOH is more viscous than MeCN. As a consequence, broader peaks with larger retention times were obtained for MeOH. Therefore, MeCN was used as organic modifier in mobile phase. Flow rate was varied within the range of 0.5 to 2.0 mL/min (in portions of 0.5 mL/min) to establish the successful flow rate of 1.0 mL/min. On decreasing the flow rate from 1 to 0.5 mL/min there observed an increase of retention time and slight broadening of peaks, and on increasing the flow rate from 1 to 2, there observed a decrease in resolution (R_S). Thus, 1 mL/min was taken as the optimized value of flow rate.

The effect of TEAP was investigated in the concentration range of 5 to 30 mM of buffer using a linear gradient of MeCN from 5 to 30 mM of buffer from 30 to 65 % in 35 min at a flow rate of 1.0 mL min^{-1} and 10 mM of TEAP was established as the optimized concentration. Mobile phase without TEAP resulted in poor reproducibility and asymmetric peaks having a heading. On increasing the concentration from 5 to 10 mM, there observed an increase in separation factor while on further increment there experienced only slight difference. High concentration could be harmful for column; therefore, 10 mM concentration of TEAP was taken as optimized concentration. Thus, the mobile phase; linear gradient of MeCN with TEAP buffer (10 mM, pH 3.5) from 30 to 65 % in 35min at a flow rate of 1.0mL min^{-1} was found successful.

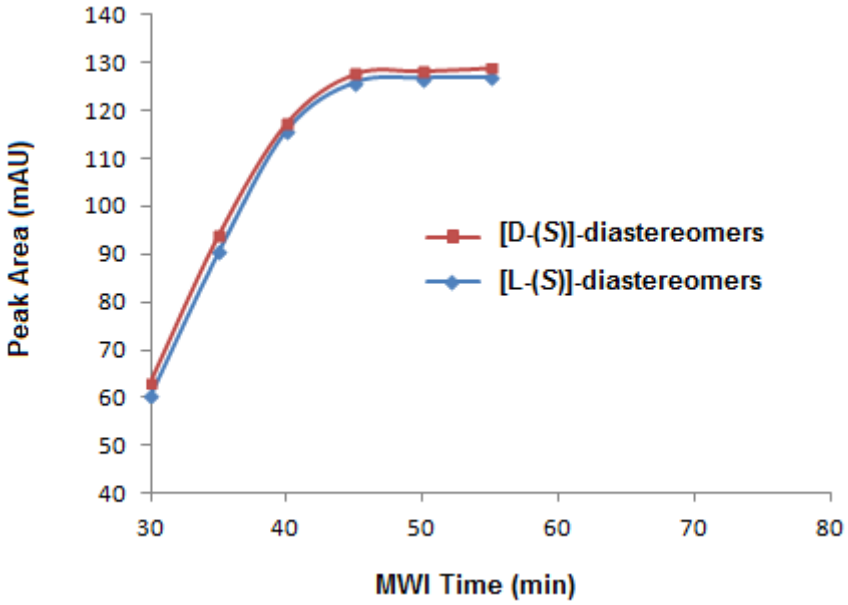


Fig. 6.2: Effect of MWI time on completion of derivatization reaction of DL-Leu with CDR 17

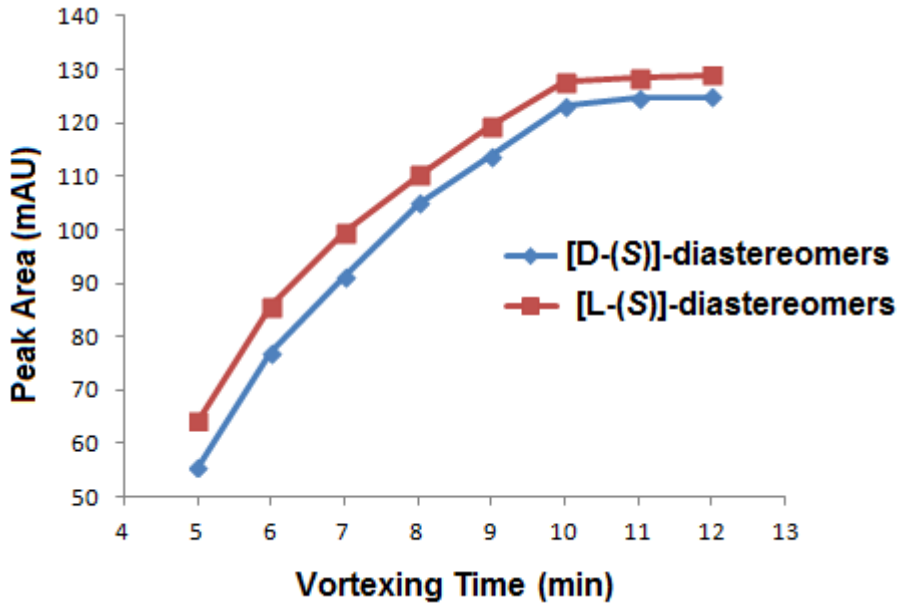


Fig. 6.3: Effect of vortexing time on completion of derivatization reaction of DL-Leu with CDR 17

(ii) Separation of Diastereomers

The 18 pairs of diastereomers were well separated by RP- HPLC. The chromatographic data obtained for separation of diastereomers of 18 AAs as retention time (t_R), retention factor (k), separation factor (α), and resolution (R_S) are listed in Table-6.2 and 6.3. Sections of representative chromatograms showing resolution of five diastereomeric pairs are shown in Fig. 6.4. The successful mobile phase was MeCN and TEAP buffer (10 mM, pH 3.5) in 35 min (linear gradient from 30 to 65 %).

Separation and elution sequence of diastereomers in terms of these above mentioned parameters is describing in section ‘‘Separation Mechanism’’ on the basis of hydrophobic and polar nature of the side chain of AAs.

2. Separation Mechanism

The elution sequence of the diastereomers was determined by taking separate HPLC run for the diastereomers prepared with standard AAs (L- and D-Leu as representatives). This can be explained as follows.

(i) The presence of naphthyl group in the reagent and the partial double bond character in the diastereomers (of amide bond between amino nitrogen of amino acid and carbonyl carbon of naproxen) may contribute to their hydrophobicity. It can be interpreted that the hydrophobic interaction of the two diastereomers with

the reversed phase material of the column along with the influence of rheological properties of the mobile phase are responsible for their different partition coefficients and different retention times. Thus, the diastereomers elute one after another for these different physical properties and it can be stated that the (L-(*S*))-diastereomers interact more strongly and are retained for longer time in comparison to the (D-(*S*))-diastereomers.

(ii) The hydrophobicity of the alkyl side chain of AAs was also a factor to affect the interaction of the diastereomers with the ODS material of the column and the separation.

The chromatographic data for this set of AAs is shown in Table-6.2; it is in good correlation, with respect to retention time and the hydrophobicity, for the diastereomers of AAs at serial number 1 to 10 with the exception of Val. There is a very good correlation with respect to R_S and hydrophobicity of these ten AAs (Fig. 6.5).

The chromatographic data for the remaining AAs is shown in Table-6.3; the AAs are arranged in decreasing order of their resolution. The diastereomers of AAs forming bis derivatives (1, 2 and 4) become more hydrophobic because of the presence of two units of (*S*)-Nap in comparison to other AAs (at serial number 3, 5, 6, 7 and 8). The AAs at serial number 5, 6, 7 and 8 contain polar side chain. Therefore, the diastereomers of these AAs (5, 6, 7 and 8) have shorter retention times and poor resolution values in comparison to the diastereomers of AAs (1, 2 and 4) and Pro.

3. Method Development and Validation

In the form of linearity, accuracy and precision, validation studies were carried out for diastereomers of DL-Leu prepared with CDR 17.

Linearity: The linear regression was developed 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 (D-(*S*))-diastereomer and (L-(*S*))-diastereomer and the corresponding concentration (1.67-167 pmol). A good linear relationship was obtained over this range. The regression equations were $y = 23.98x + 26.97$ ($R^2 = 0.999$) and $y = 23.91x + 27.11$($R^2 = 0.999$) for the (D-(*S*))-diastereomer and (L-(*S*))-diastereomer, respectively (Table-6.4).

Accuracy, precision and limit of detection: Inter-day (5 days) and intra-day recoveries were calculated by replicate analysis (n = 3) of five standard solutions of diastereomeric mixtures (1.67, 8.35, 16.7, 83.5, 167 pmol mL^{-1}). The recovery and mean SD (standard deviation) for each of the diastereomers were calculated on the basis of peak areas of first and second eluting diastereomer from the slope and intercept of the calibration plots. These are shown in Table-6.4. The calculated relative standard deviation for (D-(*S*))- and (L-(*S*))-diastereomers, respectively, varied from 0.46 to 1.18% and 0.58 to 1.30% for intra-day assay precision, and 0.62 to 1.32% and 0.68 to 1.23% for inter-day assay precision. The recovery for the diastereomers corresponding to D- and L-Leu varied from 98.85 to 101.35% and 99.73 to 101.84% for intra-day assay and 99.07 to 100.94% and 98.88 to 100.97% for inter-day assay, respectively. To determine the limit of detection (LOD), corresponding to the signal-to-noise ratio of 3, different amounts of

L-Leu (within the range of 0.15 - 0.01%) were spiked with fixed amount of D-Leu. The recovery of (L-(*S*))-diastereomer from the solution containing excess of (D-(*S*))-isomer was investigated. The results indicate that this method can be applied for detection of L-Leu in D-Leu up to 0.06% by HPLC.

Table-6.2: Experimental data (HPLC data) for diastereomeric resolution of 10 AAs (proteinogenic) prepared with CDR 17

S. No.	Diastereomer of	t_{R1}	t_{R2}	k_1	k_2	α	R_S
1	Leu	24.433	28.217	12.207	14.252	1.167	15.136
2	Ile	23.733	27.434	12.040	14.073	1.168	14.774
3	Val	18.550	22.167	11.045	13.394	1.212	12.493
4	Phe	23.583	26.218	11.957	13.404	1.121	11.742
5	Trp	21.683	24.150	11.390	12.800	1.123	11.667
6	Met	18.967	21.683	10.157	11.754	1.157	10.320
7	Ala	14.867	17.667	8.911	10.778	1.209	9.090
8	Thr	12.152	14.119	6.595	7.824	1.188	5.132
9	Glu	9.250	10.574	5.423	6.346	1.170	5.092
10	Asp	8.650	9.633	4.967	5.785	1.164	3.354

Chromatographic conditions: Eurospher C_{18} column (250 x 4.6 mm I.D., particle size, 5 μm); the mobile phase was aqueous TEAP (pH 3.5, 10 mM,)-MeCN (in a linear gradient) from 30 to 65% in 35 min, flow rate 1.0 mL/min: detection at, 231 nm; t_{R1} and t_{R2} are the retention times of (D-(*S*))- and (L-(*S*))-diastereomers respectively, k_1 and k_2 are the retention factors of (D-(*S*))- and (L-(*S*))-diastereomers, respectively, α is stereoseletive factor and R_S is the resolution for the diastereomers of AAs. AAs (1-10) arranged with respect to their hydrophobicity order (decreasing order) as per scale given by Bull and Breese (1974).

Table-6.3: Experimental data (HPLC data) for resolution of diastereomers of 8 AAs (proteinogenic) prepared with CDR 17

S. No.	Diastereomer of	t_{R1}	t_{R2}	k_1	k_2	α	R_S
1	Cys	21.883	25.117	10.682	12.630	1.182	12.089
2	Tyr	25.067	29.433	12.333	14.655	1.188	8.959
3	Pro	16.533	18.183	6.595	7.824	1.186	7.710
4	Lys	22.533	25.050	12.739	14.270	1.120	5.782
5	Arg	9.866	11.300	5.491	6.434	1.171	5.597
6	His	8.268	9.147	4.844	5.508	1.137	4.854
7	Asn	6.957	7.750	3.804	4.344	1.141	2.910
8	Ser	9.526	9.834	5.393	5.600	1.038	2.467

Chromatographic conditions: Same as in Table-6.2

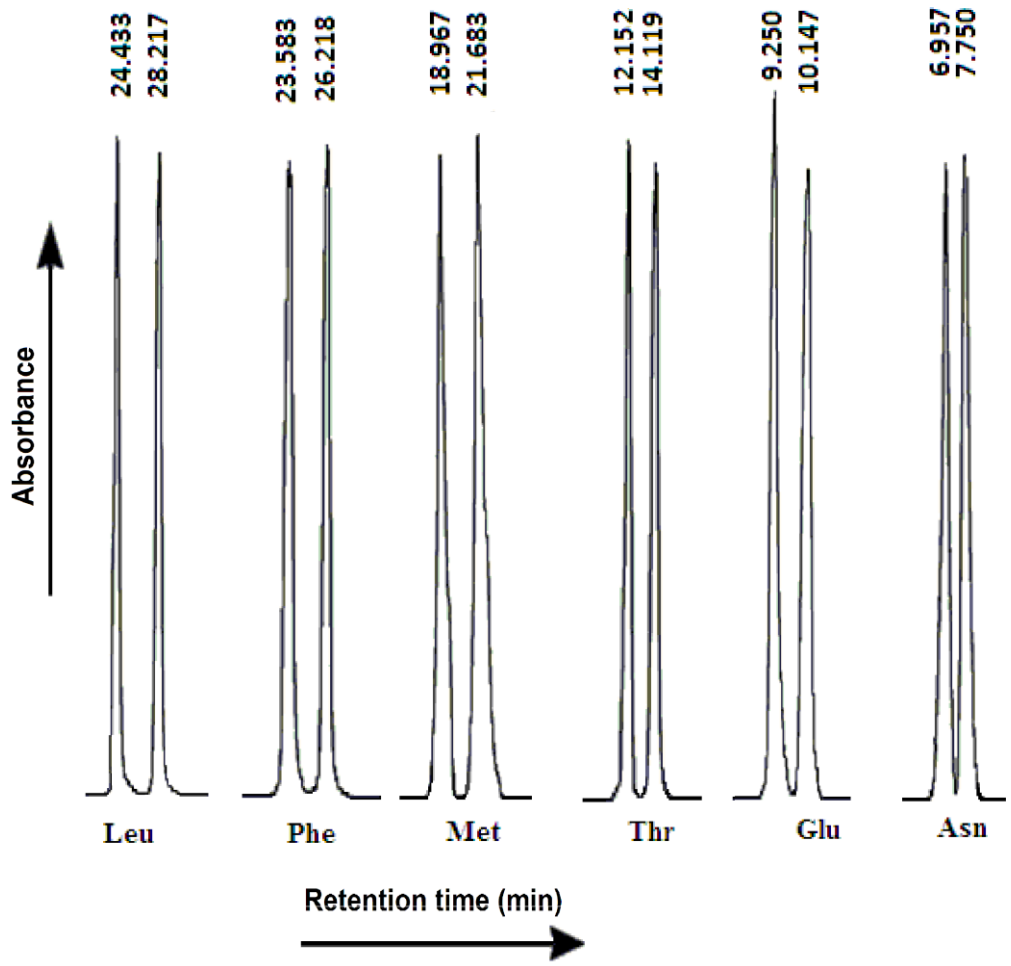


Fig. 6.4: Sections of Chromatograms (showing resolution) of diastereomers of representative AAs prepared with CDR 17.
Chromatographic conditions: Same as in Table-6.2

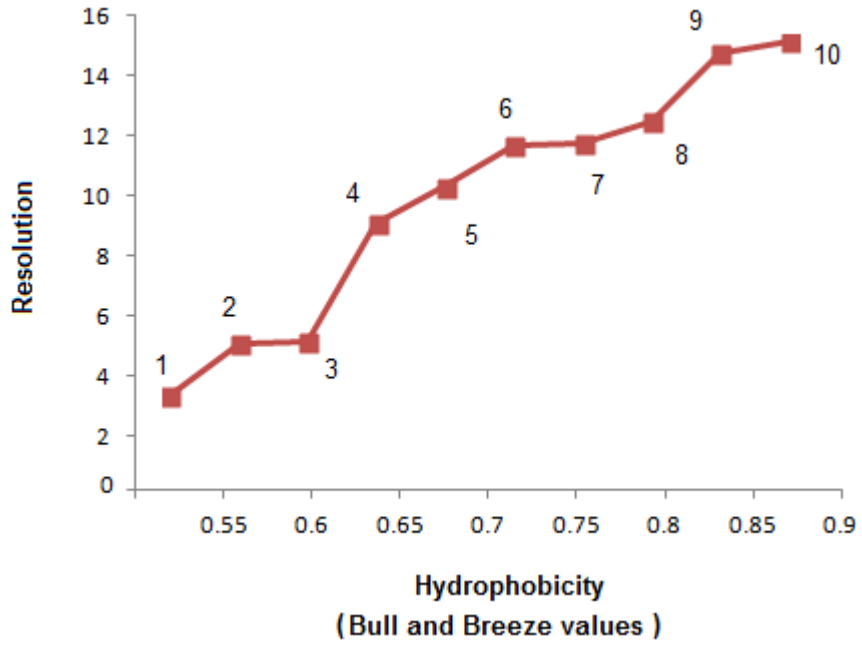


Fig. 6.5: Hydrophobicity vs resolution of diastereomers of AAs (proteinogenic) 1-10 (as given in Table-2)

Table-6.4: Method validated results (Intra- and inter-day precision and recovery studies)

	First eluting diastereomers			Second eluting diastereomers		
Actual concentration (pmol mL^{-1})	Observed concentration[a] (pmol mL^{-1})	Recovery	RSD (%)	Observed concentration[a] (pmol mL^{-1}	Recovery	RSD (%)
Intra-day precision (n = 3)						
1.67	0.851 ± 0.010	101.35	1.18	0.849 ± 0.011	101.19	1.30
8.35	4.174 ± 0.024	99.85	0.67	4.183 ± 0.026	100.07	0.62
16.7	8.446 ± 0.039	101.15	0.46	8.424 ± 0.049	100.89	0.58
83.5	41.299 ± 0.301	98.92	0.73	41.637 ± 0.358	99.73	0.86
167	84.068 ± 0.471	100.68	0.56	85.036 ± 0.663	101.84	0.78
Inter-day precision (n = 3)						
1.67	0.844 ± 0.011	100.52	1.32	0.848 ± 0.010	100.97	1.23
8.35	4.141 ± 0.036	99.07	0.87	4.175 ± 0.035	98.88	0.84
16.7	8.428 ± 0.052	100.94	0.62	8.410 ± 0.057	100.72	0.68
83.5	41.533 ± 0.386	99.48	0.93	41.378 ± 0.410	99.11	0.99
167	83.834 ± 0.637	100.40	0.76	83.224 ± 0.774	99.67	0.93

III. Inference

With the characteristics of (*S*)-Nap, the present study has established that the CDR based on it is very successful for indirect enantioresolution of proteinogenic AAs and is better than many other CDRs reported in literature (Table-6.1) in terms of derivatization conditions and resolution. The derivatization conditions using MWI (or vortexing) were less time consuming and easily optimized. The large, highly conjugated naphthyl ring attached to the chiral centre offers high UV absorbance and gives detection at very low concentration (LOD 0.1–1.2 pmol range) and the hydrophobic property of naphthyl ring facilitates enantioresolution.

•

References

Albani F, Riva R, Contin M and Baruzzi A. *Stereoselective Binding of Propranolol Enantiomers to Human Alpha 1-Acid Glycoprotein and Human Plasma*. Br. J. Clin. Pharmacol. 1984; **18**: 244.

Amini A, Paulsen-Sörman U and Westerlund D. *Dependence of Chiral Separations on the Amount of Cyclodextrins as Selectors, Employing the Partial Filling Technique in Capillary Zone Electrophoresis*. Chromatographia 2000; **51**: 226.

Ariëns EJ. *Stereochemistry, a Basis for Sophisticated Nonsense in Pharmacokinetics and Clinical Pharmacology*. Eur. J. Clin. Pharmacol. 1984; **26**: 663.

Ates H, Younes AA, Mangelings D and Heyden YV. *Enantioselectivity of Polysaccharide-Based Chiral Selectors in Polar Organic Solvents Chromatography: Implementation of Chlorinated Selectors in a Separation Strategy*. J. Chromatogr. Biomed. 2013; **74**: 1.

Baciocchi R, Zenoni G, Mazzotti M and Morbidelli M. *Separation of Binaphthol Enantiomers through Achiral Chromatography*. J. Chromatogr. A 2002; **944**: 225.

Baum MK, Shor- Posner G, Lai SH, Zhang GY, Fletcher MA, Sauberlich H and Page JB. *High Risk of HIV Related Mortality is Associated with Selenium Deficiency*. JAIDS-J. Acq. Imm. Def. 1997; **15**: 370.

Behne D, Weiler H, Kyriakopoulos A, *Effects of Selenium Deficiency on Testicular Morphology and Function in Rats*. J. Reprod. Fertil. 1996; **106**: 291.

Belpaire FM, Wynant P, Van Trappen P, Dhont M, Verstraete A and Bogaert MG. *Protein Binding of Propranolol and Verapamil Enantiomers in Maternal and Foetal Serum*. Br. J. Clin. Pharmacol. 1995; **39**: 190.

Bergmann J, Lassen S and Prange A. *Determination of the Absolute Configuration of Selenomethionine from Antarctic Krill by RP-HPLC/ICP-MS Using Chiral Derivatization Agents*. Anal. Bioanal. Chem. 2004; **378**: 1624.

B´Hymer C, Montes-Bayon M and Caruso JA. *Marfey´s Reagent: Past, Present, and Future Uses of 1-Fluoro-2,4-Dinitrophenyl-5-L-Alanine Amide*. J. Sep. Sci. 2003; **26**: 7.

Beesley TE. *Review of Chiral Stationary Phase Development and Chiral Applications*. LC-GC Eur. 2011; **24**: 270.

Bertsch W, Hara S, Kaiser RE and Zlatkis A. *Instrumental HPTLC*, Dr. Alfred Huethig Verlag, New York (1980).

Bhushan R and Agarwal C. *Direct TLC Resolution of (±)-Ketamine and (±)-Lisinopril by Use of (+)-Tartaric Acid or (-)-Mandelic Acid as Impregnating Reagents or Mobile Phase Additives. Isolation of the Enantiomers*. Chromatographia 2008a; **68**: 1045.

Bhushan R and Agarwal C. *Direct Resolution of Six Beta Blockers into their Enantiomers Using Thin Silica Plates Impregnated with L-Asp and L-Glu*. J. Planar Chromatogr.- Mod. TLC 2008b; **21**: 129.

Bhushan R and Agarwal C. *High-Performance Liquid Chromatography Enantioseparation of (RS)-Fluoxetine Using Marfey's Reagent and (S)-N-(4-Nitrophenoxycarbonyl) Phenylalanine Methoxyethyl Ester as Chiral Derivatizing Reagents along with Direct Thin-Layer Chromatographic Resolution and Isolation of Enantiomers Using L-Tartaric Acid as Mobile Phase Additive*. Biomed. Chromatogr. 2010a; **24**: 1152.

Bhushan R and Agarwal C. *Resolution of Beta Blocker Enantiomers by TLC with Vancomycin as Impregnating Agent or as Chiral Mobile Phase Additive*. J. Planar Chromatogr. 2010b; **23**: 7.

Bhushan R and Agarwal C. *Reversed-Phase Liquid Chromatographic Resolution of Diastereomers of Protein and Non-Protein Amino Acids Prepared with Newly Synthesized Chiral Derivatizing Reagents Based on Cyanuric Chloride*. Amino Acids 2011; **40**: 403.

Bhushan R and Arora M. *Resolution of Enantiomers of DL-Amino Acids on Silica Gel Plates Impregnated with Optically Pure (−)-Quinine*. Biomed. Chromatogr. 2001; **15**: 433.

Bhushan R and Arora M. *Direct Enantiomeric Resolution of (±)-Atenolol, (±)-Metoprolol, and (±)-Propranolol by Impregnated TLC Using L-Aspartic Acid As Chiral Selector*. Biomed. Chromatogr. 2003; **17**: 226.

Bhushan R and Batra S. *Enantioresolution of (RS)-Bupropion by Reversed Phase High-Performance Liquid Chromatography Using Cyanuric Chloride Based Chiral Derivatizing Reagents Having Amino Acids as Chiral Auxiliaries*. J. Liq. Chromatogr. Rel. Tech. 2013b, **DOI:** 10.1080/10826076.2013.850722.

Bhushan R and Batra S. *High-Performance Liquid Chromatographic Enantioseparation of (RS)-Bupropion Using Isothiocyanate-Based Chiral Derivatizing Reagents*. Biomed. Chromatogr. 2013a; **27**: 956.

Bhushan R and Brückner H. *Marfey's Reagent for Chiral Amino Acids Analysis: A Review*. Amino Acids 2004; **27**: 231.

Bhushan R and Brückner H. *Use of Marfey's Reagent and Analogs for Chiral Amino Acid Analysis: Assessment and Applications to Natural Products and Biological Systems*. J. Chromatogr. B 2011; **879**: 3148.

Bhushan R and Dixit S. *Application of Cyanuric Chloride-Based Six New Chiral Derivatizing Reagents having Amino Acids and Amino Acid Amides as Chiral Auxiliaries for Enantioresolution of Proteinogenic Amino Acids by Reversed-Phase High-Performance Liquid Chromatography*. Amino Acids 2012a; **42**: 1371.

Bhushan R and Dixit S. *Enantioresolution of Five β-Blockers by Reversed-Phase High-Performance Liquid Chromatography Using Fifteen Chiral Derivatizing Reagents having Amino Acids or their Amides as Chiral Auxiliaries on a Cyanuric Chloride Platform*. Biomed. Chromatogr. 2012b; **26**: 239.

Bhushan R and Dixit S. *HPLC Enantioresolution of (RS)-Baclofen using Three Newly Synthesized Dichloro-s-Triazine Reagents having Amines and Five others having Amino Acids as Chiral Auxiliarie*s. Biomed. Chromatogr. 2012c; **26**: 743.

Bhushan R and Dixit S. *Amino Acids as Chiral Selectors in Enantioresolution* by *Liquid Chromatography*. Biomed. Chromatogr. 2012d; **26**: 962.

Bhushan R and Dixit S. *Microwave-Assisted Synthesis and Reversed-Phase High-Performance Liquid Chromatographic Separation of Diastereomers of (RS)-Baclofen Using Ten Chiral Derivatizing Reagents Designed from Trichloro-s-Triazine*. J. Chromatogr. A 2010b; **1217**: 6382.

Bhushan R and Dixit S. *Reversed-Phase High-Performance Liquid Chromatographic Separation of Diastereomers of (RS)-Mexiletine Prepared by Microwave Irradiation with four New Chiral Derivatizing Reagents Based on Trichloro-s-Triazine having Amino Acids as Chiral Auxiliaries and 10 others having Amino Acid Amides*. J. Chromatogr. A 2010a; **1217**: 7669.

Bhushan R and Dubey R. *Synthesis of (S)-Naproxen-Benzotriazole and its Application as Chiral Derivatizing Reagent for Microwave-Assisted Synthesis and Indirect High-Performance Liquid Chromatographic Separation of Diastereomers of Penicillamine, Cysteine and Homocysteine*. J. Chromatogr. A 2011; **1218**: 3648.

Bhushan R and Dubey R. *Application of Amino Acid Amides as Chiral Auxiliaries in Difluoro Dinitro Benzene and Cyanuric Chloride Moieties for High performance Liquid-Chromatographic Enantioseparation of Selenomethionine and its Mixture with Methionine and Cysteine*. Amino Acids 2012a; **42**: 1417.

Bhushan R and Dubey R. *Validated High-Performance Liquid Chromatographic Enantioseparation of Selenomethionine Using Isothiocyanate Based Chiral Derivatizing Reagents.* Biomed. Chromatogr. 2012b; **26**: 471.

Bhushan R and Gupta D. *Ligand-Exchange TLC Resolution of Some Racemic β-Adrenergic Blocking Agents*. J. Planar Chromatogr.-Mod. TLC 2006; **19**: 241.

Bhushan R and Kumar R. *Comparative Application of Microwave, Ultrasonication, Ultracentrifugation and Conventional Heating for Preparation of Sample as Dinitrophenyl Derivative for Direct Enantioseparation of Certain Amino Alcohols and 1-Amino-2-Propanol from Vitamin B_{12} Hydrolysate on α_1-Acid Glycoprotein and β-Cyclodextrin Columns*. J. Chromatogr. A 2009a; **1216**: 7941.

Bhushan R and Kumar R. *Reversed-Phase High Performance Liquid Chromatographic Separation of Diastereomers of α-Amino Alcohols and Microwave Assisted Synthesis of Marfey's Reagent, Its Chiral Variants and Diastereomers*. J. Chromatogr. A 2009b; **1216**: 2592.

Bhushan R and Kumar R. *Enantioresolution of DL-penicillamine*. Biomed. Chromatogr. 2010; **24**: 66.

Bhushan R and Kumar V. *Indirect Enantioseparation of α-Amino Acids by Reversed-Phase Liquid Chromatography Using New Chiral Derivatizing Reagents Synthesized from s-Triazine Chloride.* J. Chromatogr. A 2008a; **1201**: 35.

Bhushan R and Kumar V. *Synthesis and Application of New Chiral Variants of Marfey's Reagent for Liquid Chromatographic Separation of the Enantiomers of α-Amino Acids*. Acta Chromatogr. 2008b; **20**: 329.

Bhushan R, Kumar V and Tanwar S. *Chromatographic Separation of Enantiomers of Non-Protein α-Amino Acids after Derivatization with Marfey's Reagent and its Four Variants*. Amino Acids. 2009; **36**: 571.

Bhushan R and Lal M. *LC Enantioseparation of 30-Component Diastereomeric Mixture of Amino Acids and Detection of D-Isomers Using New Reagents with Amines as Chiral Auxiliaries in Cyanuric Chloride*. Chromatographia 2013a; **76**: 1087.

Bhushan R and Lal M. *Application of Optically Pure Amines as Chiral Auxiliaries to Develop Trichloro-S-Triazine-Based New Chiral Derivatizing Reagents for Reversed-Phase High-Performance Liquid Chromatographic Enantioseparation of* DL-*Selenomethionine*. Biomed. Chromatogr. 2013b; **27**: 968.

Bhushan R and Lal M. *(S)-Naproxen as a Platform to Develop Chiral Derivatizing Reagent for Reversed-Phase High-Performance Liquid Chromatographic Enantioseparation of Analytes having a Carbonyl Functional Group*. Biomed. Chromatogr. 2012; **26**: 1582.

Bhushan R and Martens J. *Handbook of Thin Layer Chromatography*. Sherma J and Fried B (3rd Ed). Marcel Dekker: New York, 2003, p. 373.

Bhushan R and Martens J. *Amino acids: Chromatographic Separation and Enantioresolution*. HNB: New York, 2010.

Bhushan R, Martens J, Agarwal C and Dixit S. *Enantioresolution of Some β-Blockers and a β_2-Agonist Using Ligand Exchange TLC*. J. Planar Chromatogr.- Mod. TLC 2012; **25**: 463.

Bhushan R and Tanwar S. *Synthesis of Succinimidyl-(S)-Naproxen Ester and its Application for Indirect Enantioresolution of Penicillamine* by *Reversed-Phase High-Performance Liquid Chromatography*. J. Chromatogr. A 2008a; **1209**: 174.

Bhushan R and Tanwar S. *RP-LC Resolution of (RS)-Atenolol Via Diastereomerization with Marfey's Reagent and its Structural Variants Under Conventional and Microwave Heating*. Chromatographia 2008b; **68**: 849.

Bhushan R and Tanwar S. *Direct TLC Resolution of the Enantiomers of Three β-Blockers by Ligand Exchange with Cu(II)-L-Amino Acid Complex Using Four Different Approaches*. Chromatographia 2009a; **70**: 1001.

Bhushan R and Tanwar S. *Reversed-Phase High-Performance Liquid Chromatographic Enantioresolution of Six B-Blockers Using Dinitrophenyl-L-Pro-N-Hydroxysuccinimide Ester, N-Succinimidyl-(S)-2-(6-Methoxynaphth-2-Yl) Propionate and Twelve Variants of Sanger's Reagent as Chiral Derivatizing Reagents*. Biomed. Chromatogr. 2009b; **23:** 1291.

Bhushan R and Tanwar S. *Different Approaches of Impregnation for Resolution of Enantiomers of Atenolol, Propranolol and Salbutamol Using Cu(II)-L-Amino Acid Complexes for Ligand Exchange on Commercial Thin Layer Chromatographic Plates*. J. Chromatogr. A 2010; **1217:** 1395.

Bhushan R and Tanwar S. *Direct TLC Resolution of Atenolol and Propranolol into their Enantiomers Using Three Different Chiral Selectors as Impregnating Reagents*. Biomed. Chromatogr. 2008c; **22**: 1028.

Bhushan R. *Enantiomeric Purity of Chiral Derivatizing Reagents for Enantioresolution. Bioanalysis* 2011; **3**: 2057.

Bhushan R and Thiongo GT. *Direct Enantioseparation of Some β-Adrenergic Blocking Agents Using Impregnated Thin Layer Chromatography*. J. Chromatogr. B 1998; **708**: 330.

Blotny, G. *Recent Applications of 2,4,6-Trichloro-1,3,5-Triazine and its Derivatives in Organic Synthesis*. Tetrahedron 2006; **62**: 9507.

Bojarski J. *Recent Progress in Chromatographic Enantioseparations*. Chem. Anal-Warsaw 1997; **42**: 139.

Borchard U. *Pharmacological Properties of β-Adrenoceptor Blocking Drugs*. J. Clin. Cardiol. 1998; **1**: 5.

Bonato PS, Lanchote VL, Bortocan R, Jabor, VAP, Paias FO, Ricci-Júnior E and Carvalho R. *Chiral Stationary Phases Based on Cellulose and Amylose Tris-3,5-Dimethylphenylcarbamate Derivatives for the Resolution of Selected Chiral Drugs and Metabolites*. J. Liq. Chromatogr. Rel. Tech. 1999; **22**: 1813.

Bopp RJ and Kennedy JH. *Practical Considerations for Chiral Separations of Pharmaceutical Compounds*. LC·GC 1988; **6:** 514.

Brückner H and Gah C. *High-Performance Liquid Chromatographic Separation of DL-Amino Acids Derivatized with Chiral Variants of Sanger's Reagent*. J. Chromatogr. A 1991; **555**: 81.

Brückner H and Keller- Hoehl C. *HPLC Separation of DL-Amino Acids Derivatized with N^2-(5-Fluoro-2,4-Dinitrophenyl)-L-Amino Acid Amides*. Chromatographia 1990; **30**: 621.

Brückner H and Strecker B. *Use of Chiral Monochloro-s-Triazines Reagents for the Liquid Chromatographic Resolution of DL-Amino Acids*. J. Chromatogr. 1992; **627:** 97.

Brückner H and Wachsmann M. *Design of Chiral Monochloro-s-Triazine Reagents for the Liquid Chromatographic Separation of Amino Acid Enantiomers*. J. Chromatogr. A 2003a; **998**: 73.

Brückner H and Wachsmann M. *Evaluation of Fluorescent Monochloro-s-Triazine Reagent for Amino Acid Analysis*. Chromatographia Suppl. 2003b**; 57:** S143.

Bull HB and Breese K. *Surface Tension of Amino Acid Solutions. A Hydrophobicity Scale of the Amino Acid Residues*. Arch. Biochem. Biophys. 1974; **161**: 665.

Burger. *Medicinal Chemistry*, Part II, 3rd edn, Wiley Inter-science, New York, 1970.

Büschges R, Linde H, Mutschler E and Spahn-Langguth H. *Chloroformate and Isothiocyanate Derived from 2-Arylpropionic Acids as Chiral Reagents: Synthetic Routes and Chromatographic Behaviour of the Derivatives*. J. Chromatogr. A 1996; **725**: 323.

Castano A, Ayala A, Rodriguez-Gomez JA, Herrera AJ, Cano J and Machado A. *Low Selenium Diet Increases the Dopamine Turnover in Prefrontal Cortex of the Rat*. Neurochem. Int. 1997; **30**: 549.

Carr RA and Pasutto FM, Lewanczuk RZ, and Foster RT. *Protein Binding of Sotalol Enantiomers in Young and Elderly Human and Rat Serum Using Ultrafiltration*. Biopharm. Drug Dispos. 1995; **16:** 705.

Chankvetadze B, Burjanadze N and Blaschke G. *Enantioseparation of Chiral Vasodilator Drug Isoxsuprine in High-Performance Liquid Chromatography and Capillary Electrophoresis*. J. Pharm. Biomed. Anal. 2002; **27**: 153.

Chernobrovkin MG, Shapovalova EN, Guranda DT, Kudryavtsev PA, Švedas VK and Shpigun OA. *Chiral High-Performance Liquid Chromatography Analysis of α-Amino Acid Mixtures Using a Novel SH Reagent N-(R)-Mandelyl-L-Cysteine and Traditional Enantiomeric Thiols for Precolumn Derivatization*. J. Chromatogr. A 2007; **1175**: 89.

Chun-Yu H, Shing-Yaw W, Aij-Lie K and Hsin-Lung W. *Fluorescent High-Performance Liquid Chromatographic Analysis of Vigabatrin Enantiomers after Derivatizing with Naproxen Acyl Chloride*. J. Chromatogr. A 2008; **1178:** 166.

Clark LC, Combs G, Turnball BW, Slate EH, Chalker DK, Chow J, Davis LS, Glover RA, Graham DK, Gross EG, Krongrad A, Lesher JL, Park HK, Sanders BB, Smith CL, Taylor JR, Alberts DS, Allison RJ, Bradshaw JC, Curtus D, Deal DR, Dellasega M, Hendrix JD, Herlong JH, Hixon LJ, Knight F, Moore J, Rice JS, Rogers AI, Schuman B, Smith EH and Woodard JC. *Effects of Selenium Supplementation for Cancer Prevention in Patients with Carcinoma of the Skin a Randomized Controlled Trial*. J. Am. Med. Assoc. 1996; **276**: 1957.

Claudia C. *Qualitative and Quantitative Analysis by Hyphenated (HP) TLC-FTIR Technique,* J. Liq. Chromatogr. Rel. Technol. 2005; **28**: 1203.

Cooper WO, Willy ME, Pont SJ, Ray WA. *Increasing Use of Antidepressants in Pregnancy*. Am. J. Obstet. Gynecol. 2007; **196:** 544.

Dahlöf B, Devereux RB, Kjeldsen SE, Julius S, Beevers G, Faire U, Fyhrquist F, Ibsen H, Kristiansson K, Lederballe-Pedersen O, Lindholm LH, Nieminen MS, Omvik P, Oparil S and Wedel H. *Cardiovascular morbidity and mortality in the Losartan*

Intervention for Endpoint reduction in hypertension study (LIFE): a randomised trial against atenolol. Lancet 2002; **359**: 995.

Dalgliesh CE. *Relation Between Pyridoxine and Tryptophan Metabolism, Studied in the Rat*. Biochem. J. 1952a; **52:** 3.

Dalgliesh CE. *The Optical Resolution of Aromatic Amino Acids on Paper Chromatograms*. J. Chem. Soc. 1952b; **37**: 3940.

Enquist M and Hermansson J. *Separation and Quantitation of (R)- and (S)-Atenolol in Human Plasma and Urine Using an α_1-AGP Column*. Chirality 1989; **1**: 209.

Del Bubba M, Checchini L and Lepri L. *Thin-layer chromatography enantioseparations on chiral stationary phases: a review.* Anal. Bioanal. Chem. 2013; **405**: 533.

Devos C, Sandra K and Sandra P. *Capillary Gas Chromatography Inductively Coupled Plasma Mass Spectrometry (CGC-ICPMS) for the Enantiomeric Analysis of DL-Selenomethionine in Food Supplements and Urine*. J. Pharm. Biomed. Anal. 2002; **27**: 507.

Duan J, He M and Hu B. *Chiral Speciation and Determination of Selenomethionine Isomers in Selenized Yeast* by *Ligand-Exchange Micellar Electrokinetic Capillary Chromatography after Solid Phase Extraction*. J. Chromatography. A 2012; **1268**: 173.

Falkay G and Kovács L. *Affinity of tocolytic agents on human placental and myometrial beta-adrenergic receptor*. J. Perinat. Med. 1986; **14**: 109.

FDA. *Policy Statement for the Development of New Stereoisomeric Drugs*. Chirality 1992; **4:** 338.

Frishman WH. *Clinical Pharmacology of the β-Adrenoreceptor Blocking Drugs*. Appleton-Century-Crofts, New York, 1984.

Friegner JP and Boyer WF. *Selective Serotonin Re-uptake Inhibitors*. JohnWiley & Sons, New York, 1991.

Fujii K, Ikai Y, Mayumi T, Oka H, Suzuki M and Harada K. *A Nonempirical Method Using LC/MS for Determination of the Absolute Configuration of Constituent Amino Acids in a Peptide: Combination of Marfey's Method with Mass Spectrometry and Its Practical Application*. Anal. Chem. 1997; **69**: 3346.

Gagyi L, Gyéresi Á and Kilár F. *Role of Chemical Structure in Stereoselective Recognition of b-Blockers by Cyclodextrins In Capillary Zone Electrophoresis*. J. Chromatogr. Biomed. 2008; **70**: 1268.

Giddings JC. *Dynamics of Chromatography*. *Part I. Principles and Theory*, Marcel Dekker, New York, 1965.

Görög S and Gazdag M. *Enantiomeric Derivatization for Biomedical Chromatography*. J. Chromatogr. B 1994; **659**: 51.

Görög S. *Chiral derivatization*, In: Wilson ID, Adlard ER, Cooke M and Poole CF (Eds). Encyclopedia of separation science. Academic Press: London, 2000, p. 2310.

Grant SL, Shulman Y, Tibbo P, Hampson DR and Baker GB. *Determination of D-Serine and Related Neuroactive Amino Acids in Human Plasma by High-Performance Liquid Chromatography with Fluorimetric Detection*. J. Chromatogr. B 2006; **844**: 278.

Guillarme D, Ruta J, Rudaz S and Veuthey JL, *New trends in fast and high-resolution liquid chromatography: a critical comparison of existing approaches.* Anal. Bioanal. Chem. 2010; **397**: 1069.

Guttendorf RJ, Kostenbauer HB and Wedlund EJ. *Quantification of Propranolol Enantiomers in Small Blood Samples from Rats by Reversed-Phase High-Performance Liquid Chromatography after Chiral Derivatization*. J. Chromatogr. Biomed. 1989; **489**: 333.

Haginaka J, Wakai J, Takahashi K, Yasuda H and Katagi T. *Chiral Separation of Propranolol and its Ester Derivatives on an Ovomucoid-Bonded Silica: Influence of pH, Ionic Strength and Organic Modifier on Retention. Enantioselectivity and Enantiomeric Elution Order*. Chromatographia 1990; **29**: 587.

Hatfield DL, Berry MJ and Gladyshev VN. *Selenium: its Molecular Biology and Role in Human Health*. 2nd ed., Springer-Verlag, New York Inc., New York, 2006.

Hoffmann CV, Pell R, Lämmerhofer M and Lindner W. *Synergistic Effects on Enantioselectivity of Zwitterionic Chiral Stationary Phases for Separations of Chiral Acids, Bases, and Amino Acids by HPLC.* Anal. Chem. 2008; **80**: 8780.

Hoffmann CV, Laemmerhofer M and Lindner W. *Novel Strong Cation-Exchange Type Chiral Stationary Phase for the Enantiomer Separation of Chiral Amines by High-Performance Liquid Chromatography*. J. Chromatogr. A 2007; **1161:** 242.

Huber JFK. *Analytical Techniques for Enviromental Monitoring and Control*. J. Chromatogr. Sci. 1969; **7:** 85.

ICH-Topic Q2A: *Validation of Analytical Procedures. International Conference on Harmonization of Technical Requirement for Registration of Pharmaceuticals for Human Use. Geneva*, 1996.

ICH-Topic Q2A: *Validation of Analytical Procedures, International Conference on Harmonization of Technical Requirement for Registration of Pharmaceuticals for Human Use*. Geneva, 1995.

ICH-Topic Q2B: *Validation of Analytical Procedures, International Conference on Harmonization (ICH)*. Geneva, 1996.

Ikegami T, Dicks E, Kobayashi H, Morisaka H, Tokuda D, Cabrera K and Tanaka N. *How to Utilize the True Performance of Monolithic Silica Columns*. J. Sep. Sci. 2004; **27:** 1292.

Ilisz I, Tourwé D, Armstrong DW and Péter A. *High-Performance Liquid Chromatographic Enantioseparation of Unusual Secondary Amino Acids on a D-Penicillamine-Based Chiral Ligand Exchange Column*. Chirality 2006; **18:** 539.

Ilisz I, Berkecz R and Péter A. *Application of Chiral Derivatizing Agents in the High-Performance Liquid Chromatographic Separation of Amino Acid Enantiomers: A Review*. J. Pharm. Biomed. Anal. 2008; **47**: 1.

Jin D, Kumar AP, Song GC and Yong-Ill L. *Determination of Thyroxine Enantiomers in Pharmaceutical Formulation by High-Performance Liquid Chromatography–Mass Spectrometry with Pre-Column Derivatization*. Microchem. J. 2008; **88:** 62.

Jin D, Nagakura K, Murofushi S, Miyahara T and Toyo'oka T. *Total Resolution of 17 DL-Amino Acids Labelled with a Fluorescent Chiral Reagent R(-)-4-(3-Isothiocyanatopyrrolidin-1-Yl)-7-(N,N-Dimethylaminosulfonyl-2,1,3-Benzoxadiazole, by High-Performance Liquid Chromatography*. J. Chromatogr. 1998; **822**: 215.

Jin D, Zhang M, Jin S, Lee M, Song G, Back G and Lee Y. *Enantioselective Resolution of Thyroxine Hormone by High-Performance Liquid Chromatography Utilizing a Highly Fluorescent Chiral Tagging Reagent*. Chirality 2007; **19:** 625.

Kalasz H and Bathori M. *Pharmaceutical Applications of TLC*, LC–GC. Eur. 2001; **10:** 311.

Kar A. *Pharmacognosy and Pharmacobiotechnology*. New Delhi: New Age International (P) Limited. Publishers, 2003.

Katagiri T, Yoda C, Furuhashi K, Ueki K and Kubota T. *Separation of an Enantiomorph and Its Racemate by Distillation: Strong Chiral Recognizing Ability of Trifluorolactates*. Chem. Lett. 1996; **2**: 115.

Katritzky AR, Jishkariani D and Narindoshvili T. *Convenient Synthesis of Ibuprofen and Naproxen Aminoacyl, Dipeptidoyl and Ester Derivatives*. *Chem. Bio. & Drug Design* 2009; **73**: 618.

Kempter C, Pötter W, Binding N, Kläning H, Witting U and Karst U. *Tailor Made Derivatizing Agents for Carbonyl Compounds Using Liquid Chromatography*. Anal. Chim. Acta 2000; **410**: 47.

Kirkland JJ. *Introduction to Modern Liquid Chromatography*. J. Chromatogr. Sci. 1969; **7**: 7.

Kim KH, Lee JH, Ko YM, Hong SP and Youm JR. *Chiral Separation of b-Blockers After Derivatization with (–)-A-Methoxy-A-(Trifluoromethyl)Phenylacetyl Chloride by Gas Chromatography*. Arch. Pharm. Res. 2001; **24**: 402.

Kinoshita T, Kasahara Y and Nimura N. *Reversed-Phase High-Performance Liquid Chromatographic Resolution of Nonesterified Enantiomeric Amino Acids by Derivatization with 2, 3, 4, 6-Tetra-O-Acetyl-β-D-Glucopyranosyl Isothiocyanate and 2, 3, 4-Tri-O-Acetyl-β-D-Arabinofpyranosyl Isothiocyanate*. J. Chromatogr. 1981; **210**: 77.

Kleidernigg OP, Posch K and Lindner W. *Synthesis and Application of a New Isothiocyanate as a Chiral Derivatizing Agent for the Indirect Resolution of Chiral Amino Alcohols and Amines*. J. Chromatogr. A 1996; **729:** 33.

Knekt P, Heliövaara M, Aho K, Alfthan G, Marniemi and Aromaa AJ. *Serum Selenium, Serum Alpha-Tocopherol and the Risk of Rheumatoid Arthritis*. Epidemiology 2000; **11**: 402.

Ko MY, Shin DH, Oh JW, Asegahegn WS and Kim KH. *Chiral Separation of β-Blockers After Derivatization with a New Chiral Derivatization Agen*t. GATC. Arch. Pharm. Res. 2006; **29**: 1061.

Kowalska T and Sherma J (Eds.). *Thin Layer Chromatography in Chiral Separations and Analysis*, CRC Press. Boca Raton, 2007.

Kowalska T. *Handbook of Thin Layer Chromatography*. In: Sherma J and Fried B (Eds). Marcel Dekker: New York, 1990, p. 43.

Krstulović AM and Fouchet MH. *Direct Enantiomeric Separation of Betaxolol with Applications to Analysis of Bulk Drug and Biological Samples*. J. Chromatogr. A 1988; **452**: 477.

Kumar AP and Park JH. *Chiral Separation of Basic Compounds on a Cellulose Tris (3,5-Dimethylphenylcarbamate)-Coated Zirconia Monolithin Basic Eluents by Capillary Electrochromatography*. J. Chromatogr. A 2011a; **1218**: 6548.

Kumar AP and Park JH. *Fast Separations of Chiral β-Blockers on a Cellulose 3,5-Dimethylphenylcarbamate-Coated Zirconia Monolithic Column by Capillary Electrochromatography*. J. Chromatogr. A 2011b; **1218**: 5369.

Lämmerhofer M. *Chiral Recognition by Enantioselective Liquid Chromatography: Mechanisms and Modern Chiral Stationary Phases.* J. Chromatogr. A 2010; **1217:** 814.

Lee EJ and William KM. *Chirality: Clinical Pharmacokinetic and Pharmacodynamic Considerations.* Clin. Pharmacokinet. 1990; **18**: 339.

Lindner W and Rath M. *Enantioselective Drug Monitoring of (R)- and (S)-Propranolol in Human Plasma Via Derivatization with Optically Active (R,R)-O,O-Diacetyl Tartaric Acid Anhydride*. J. Chromatogr. 1989; **487**: 375.

Lucic B, Radulovic D, Vujic Z and Agbaba D. *Direct Separation of the Enantiomers of (±)-Metoprolol Tartrate on Impregnated TLC Plates with D-(-)-Tartaric Acid as a Chiral Selector*. J. Planar Chromatogr.-Mod. TLC 2005; **18**: 294.

Ludwig J, Marcotte DE and Norberg K. *Anti-depressants and Suicide*. J. Health Econ. 2009; **28**: 659.

Ma HM, Wang ZH and Su MH. *New Triazine Spectroscopic Reagent for the Separation of DL-Amino Acids by Micellar Electrokinetic Chromatography*. J. Chromatogr. A 2002; **955**: 125.

Marfey P. *Determination of D-Amino Acids. II. Use of A Bifunctional Reagent, 1,5-Difluoro-2,4-Dinitrobenzene*. Carlsberg. Res. Commun. 1984; **49**: 591.

Martens J and Bhushan R. *Purification of Enantiomeric Mixtures in Enantioselective Synthesis: Overlooked Errors and Scientific Basis of Separation in Achiral Environment.* Helv. Chim. Acta 2014; **97**: 161.

Martens J and Bhushan R. *Importance of Enantiomeric Purity and its Control by Thin-Layer Chromatography*. J. Pharm. Biomed. Anal. 1990; **8**: 259.

Martens J and Bhushan R. *TLC Enantiomeric Separation of Amino Acids*. Int. J. Pept. Protein Res. 1989; **34**: 433.

Martens J and Bhushan R. *Resolution of Enantiomers with Achiral Phase Chromatography*. J. Liq. Chromatogr. 1992; **15**: 1.

Martin AJP and Synge RLM. *New Form of Chromatogram Employing Two Liquid Phases, 1. Theory of Chromatography, 2. Application to the Micro Determination of the Higher Mono Amino Acids in Proteins*. J. Biochem. 1941; **35:** 1358.

Mayani VJ, Abdi SHR, Mayani SV, Kim HC and Park SK. *Enantiomer Self-Disproportionation and Chiral Stationary Phase Based Selective Chiral Separation of Organic Compounds*. Chirality 2011; **23**: 300.

Mayani VJ, Abdi SHR, Kureshy RI. Khan NH, Agrawal S and Jasra RV. *Enantiomer Self-Disproportionation of Chiral Compounds on Achiral Ordered Mesoporous Silica M41S and Regular Silica Gel as a Stationary Phase*. *Chirality* 2009; **21**: 255.

Mehvar R and Brocks DR. *Stereospecific Pharmacokinetics and Pharmacodynamics of Beta-Adrenergic Blockers in Humans*. J. Pharm. Pharm. Sci. 2001; **4:**185.

Mehvar R and Brocks DR. *Stereospecific Pharmacokinetics and Pharmacodynamics: Cardiovascular Drugs*. In: Reddy IK and Mehvar R (Eds). Chirality in Drug Design and Development. Marcel Dekker: New York, 2004, p. 281.

Mehvar R. *Stereospecific Liquid Chromatographic Analysis of Racemic Adrenergic Drugs Utilizing Precolumn Derivatization with (–)-Menthyl Chloroformate*. J. Chromatogr. Biomed. 1989; **493:** 402.

Méndez SP, Gonzálec EB and Sanz-Medel A. *Chiral Speciation and Determination of Selenomethionine Enantiomers in Selenized Yeast by HPLC-ICP-MS Using a Teicoplanin-Based Chiral Stationary Phase*. J. Anal. At. Spectrom. 2000a; **15**: 1109.

Méndez SP, Gonzálec EB, Sánchez MLF and Sanz-Medel A. *Speciation of DL-Selenomethionine Enantiomers on a β-Cyclodextrin Column with Fluorimetric and on-Line Hydride Generation Inductively Coupled Plasma Mass Spectrometric Detection*. J. Anal. At. Spectrom. 1998; **13:** 893.

Méndez SP, González EB and Sanz-Medel A. *Enantiomeric Separation of Seleno-Amino Acid Derivatives by Cyclodextrin-Modified Micellar Electrokinetic Chromatography Using A Mixed Micellar System of Sodium Dodecylsulphate and Taurodeoxycholic Acid*. Anal. Chim. Acta 2000b; **416**: 1.

Méndez SP, Montes-Bayón M, Gonzálec EB and Sanz-Medel A. *Selenomethionine Chiral Speciation in Yeast and Parenteral Solutions by Chiral Phase Capillary Gas Chromatography-ICP-MS*. J. Anal. At. Spectrom. 1999; **14:** 1333.

Merino MI, Blanco GE and Sanz-Medel A. *Liquid Chromatographic Separation Penicillamine Enantiomers Derivatized with OPA/2-ME on a β-Cyclodextrin Bonded Phase.* Mikrochim. Acta 1992; **107:** 73.

Miyazawa T, Minowa H, Imagawa K and Yamada T. *Separation of Enantiomers of Non-Protein Amino Acids by High-Performance Liquid Chromatography on a Chiral Ligand-Exchange Column*. Chromatographia 2004; **60:** 45.

Montes-Bayón M, B'Hymer C, Ponce De León CA and Karuso JA. *Resolution of Seleno-Amino Acid Optical Isomers Using Chiral Derivatization and Inductively Coupled Plasma Mass Spectrometric (ICP-MS) Detection*. J. Anal. At. Spectrom. 2001; **16**: 945.

Nakamura T, Tateishi K, Tsukagoshi S, Hashimoto S, Watanabe S, Soloshonok VA, Aceña JA and Kitagawa O. *Self-Disproportionation of Enantiomers of Non-Racemic Chiral Amine Derivatives Through Achiral Chromatography*. Tetrahedron 2012; **68**: 4013.

Néve J. *Selenium as a Risk Factor for Cardiovascular Diseases*. J. Cardiovasc. Risk. 1996; **3:** 42.

Ngim KK, Zhong Q, Mistry K and Chetwyn N. *Effect of Sulfobutyl Ether β-Cyclodextrin Modifier on Selectivity of Reversed Phase HPLC Separations*. J. Liq. Chromatogr. Rel. Technol. 2012; **35**: 2845.

Nicoud RM, Jaubert JN, Rupprecht I and Kinkel J. *Enantiomeric Enrichment of Non-Racemic Mixtures of Binaphthol with Non-Chiral Packings*. Chirality 1996; **8**: 234.

Novakova L and Vlckova H. *A Review of Current Trends and Advances in Modern Bio-Analytical Methods: Chromatography and Sample Preparation.* Anal. Chim. Acta 2009; **656**: 8.

Nyiredy S. *Planar Chromatographic Method Development using the PRISMA Optimization System and flow Charts*. J. Chromatogr. Sci. 2002; **40:** 553.

Oberacher H, Premstaller A and Huber CG. *Characterization of Some Physical and Chromatographic Properties of Monolithic Poly(Styrene-Co-Divinylbenzene) Columns*. J. Chromatogr. A 2004; **1030:** 201.

Oguri S, Nomura M and Fujita Y. *A New Strategy for the Selective Determination of D-Amino Acids: Enzymatic and Chemical Modifications for Pre-Column Derivatization*. J. Chromatogr. A 2005; **1078**: 51.

Ogawa S, Nishimine T, Tokunaga E, Nakamura S and Shibata N. *Self-Disproportionation of Enantiomers of Heterocyclic Compounds Having a Tertiary Trifluoromethyl Alcohol Center on Chromatography with a Non-Chiral System.* J. Fluorine Chem. 2010; **131**: 521.

Olsen L, Bronnum-Hansen K, Helboe P, Jørgensen GH and Kryger S. *Chiral Separations of β-Blocking Drug Substances Using Derivatization with Chiral Reagents and Normal-Phase High-Performance Liquid Chromatography*. J. Chromatogr. 1993; **636**: 231.

Olsen, BA, Wirth DD and Larew JS. *Determination of Fluoxetine Hydrochloride Enantiomeric Excess Using High-Performance Liquid Chromatography with Chiral Stationary Phases*. J. Pharmaceut. Biomed. 1998; **17:** 623.

Pell R, Sić S and Lindner W. *Mechanistic Investigations of Cinchona Alkaloid-Based Zwitterionic Chiral Stationary Phases*. J. Chromatogr. A 2012; **1269**: 287.

Peng L, Jayapalan S, Chankvetadze B and Farkas T. *Reversed-Phase Chiral HPLC and LC/MS Analysis with Tris(Chloromethylphenylcarbamate) Derivatives of Cellulose and Amylose as Chiral Stationary Phases*. J. Chromatogr. A 2010; **1217:** 6942.

Péter A, Árki A, Vékes E, Tourwé D, Lázár L, Fülöp F and Armstrong DW. *Direct and Indirect High-Performance Liquid Chromatographic Enantioseparation of β-Amino Acids*. J. Chromatogr. A 2004b; **1031:** 171.

Péter M and Fülöp F. *Comparison of Isothiocyanate Chiral Derivatizing Reagents for High-Performance Liquid Chromatography*. Chromatographia 2002; **56:** 631.

Péter M, Gyéresi Á and Fülöp F. *Liquid Chromatographic Enantioseparation of β-Blocking Agents with (1R,2R)-1,3-Diacetoxy-1-(4-Nitrophenyl)-2-Propyl Isothiocyaate as Chiral Derivatizing Agent*. J. Chromatogr. A 2001; **910**: 247.

Péter M, Péter A and Fülöp F. *Application of (1S, 2S)- and (1R, 2R)-1, 3-Diacetoxy-1-(4-Nitrophenyl)-2-Propylisothiocyanate to the Indirect Enantioseparation of Racemic Proteinogenic Amino Acids*. J. Chromatogr. A 2000a; **871**: 115.

Péter A, Török G, Vékes E, Van Betsbrugge J and Tourwé D. *HPLC Separation of Enantiomers of α-Substituted Proline Analogues by the Application of (S)-N-(4-Nitrophenoxy-Carbonyl) Phenylalanine Methoxyethyl Ester as Chiral Derivatizing Agent*. J. Liq. Chromatogr. Rel. Technol. 2004a; **27:** 17.

Péter A, Török R, Wright K, Wakselman M and Mazaleyrat JP. *Liquid Chromatographic Enantioseparation of Spin-Labelled β-Amino Acids*. J. Chromatogr. A 2003; **1021**: 1.

Péter A, Vékes E and Török G. *Application of (S)-N-(4-Nitrophenoxycarbonyl) Phenylalanine Methoxyethyl Ester as a New Chiral Derivatizing Agent for Proteinogenic Amino Acid Analysis by High-Performance Liquid Chromatography*. Chromatographia 2000b; **52**: 821.

Piperaki S and Parissi-Poulou M. *Use of Cyclodextrins as Chiral Selectors for Direct Resolution of the Enantiomers of Fluoxetine and its Metabolite Norfluoxetine by HPLC*. Chirality 1993; **5:** 258.

Piperaki S, Tsantili-Kakaulidou A and Parissi-Poulou M. *Solvent Selectivity in Chiral Chromatography using a β-Cyclodextrin-Bonded Phase*. Chirality 1995; **7**: 257.

Phinney KW, Jinadu LA and Sander LC. *Chiral Selectors from Fruit: Application of Citrus Pectins to Enantiomer Separations in Capillary Electrophoresis*. J. Chromatogr. A 1999; **857**: 285.

Poole CF. *Layer Chromatography: Challenges and Opportunities*. J. Chromatogr. A 2003; **1000**: 963.

Potts BD and Parli CJ. *Analysis of the Enantiomers of Fluoxetine and Norfluoxetine in Plasma and Tissue Using Chiral Derivatization and Normal-Phase Liquid Chromatography*. J. Liq. Chromatogr. 1992; **15**: 665.

Preinerstorfer B, Lubda D, Lindner W and Lämmerhofer M. *Monolithic Silica-Based Capillary Column with Strong Chiral Cation-Exchange Type Surface Modification for Enantioselective Non-Aqueous Capillary Electrochromatography*. J. Chromatogr. A 2006; **1106**: 94.

Rentsch KM. *The Importance of Stereoselective Determination of Drugs in the Clinical Laboratory*. J. Biochem. Biophys. Methods 2002; **54:** 1.

Ruffolo RR. *Stereoselectivity in Adrenergic Agonists and Adrenergic Blocking Agents*. In: Ariëns EJ, Soudijn W and Timmermans PBMWM (Eds). Stereochemistry and Biological Activity of Drugs. Blackwell, Oxford (1983) p. 103.

Sanz-Medel A and Blanco GE. *Chiral Trace-Element Speciation in Biological Samples: Present Importance and Application to Speciation for Seleno-Amino Acids*. Trends Anal. Chem. 2002; **21**: 709.

Sanger F. *Free Amino Group of Insulin*. Biochem. J. 1945; **39:** 507.

Savelova VA, Piskunova ZP, Drizhd LP and Taran NA. *Nucleophilic Substitution Mechanism in 1,3,5-Triazine Derivatives*. Org. Reactivity+ 1990; **27**: 184.

Schrauzer GN. *Selenomethionine: A Review of its Nutritional Significance, Metabolism and Toxicity*. J. Nutr. 2000; **130**: 1653.

Sedman, AJ and Gal J. *Resolution of the Enantiomers of Propranolol and other Beta-Adrenergic Antagonists by High-Performance Liquid Chromatography*. J. Chromatogr. Biomed. 1983; **278**: 199.

Schippert B, Kovar KA and Sewing KFr. *Determination of Histamine and its Metabolic Products in the Pig Gastric Mucosa*. Pharmacology 1979; **19:** 86.

Scott RPW. *Highlights from Contemporary Analytical Liquid Chromatography*. Analyst 1978; **103:** 37.

Sherma J and Fried B (Eds.). *Handbook of Thin-Layer Chromatography*, 3rd Edn., Marcel Dekker, New York, 2003.

Sherma J. *Modern Thin Layer Chromatography in Pharmaceutical and Drug Analysis. Pharmacopeial Forum* 2001; **27**: 3420.

Sherma J and Kowalska T. (Eds.), *Thin Layer Chromatography in Phytochemistry*, CRC Press, Boca Raton, 2007.

Sherma J. *Planar Chromatography*. Anal. Chem. 2002; **74:** 2653.

Sherma J. *Planar Chromatography*. Anal. Chem. 2004; **76:** 3251.

Sherma J. *Planar Chromatography*. Anal. Chem. 2006; **78:** 3841.

Sherma J. *Planar Chromatography*. Anal. Chem. 2008; **80**: 4253.

Sherma J. *Planar Chromatography*. Anal. Chem. 2010; **82**4: 895.

Sigma-Aldrich Catalogue, 2003.

Sigma-aldrich. com, 2013.

Sigma-aldrich.com, 2003.

Sigma Aldrich Catalogue, 2013.

Soloshonok VA and Berbasov DO. *Self-Disproportionation of Enantiomers of (R)-Ethyl-3-(3,5-Dinitrobenzamido)-4,4,4-Trifluorobutanoate on Achiral Silica Gel Stationary Phase*. J. Fluorine Chem. 2006; **127:** 597.

Soloshonok VA. *Remarkable Amplification of the Self-Disproportionation of Enantiomers on Achiral-Phase Chromatography Column*s. Angew. Chem. Int. Edit. 2006; **45**: 766.

Soloshonok, VA and Berbasov DO. *Self-Disproportionation of Enantiomers on Achiral Phase Chromatography. One More Example of Fluorine's Magic Powers*. *Chim. Oggi* 2006; **24**: 44.

Spallholz JE, Boylan LM and Larsen HS. *Advances in Understanding Selenium's Role in the Immune System*. Ann. Ny. Acad. Sci. 1990; **587**: 123**.**

Span H and Langguth P. *Chiral Amines Derived from 2-Arylpropionic Acids: Novel Reagents for the Liquid Chromatographic (LC) Fluorescence Assay of Optically Active Carboxylic Acid Xenobiotics*. Pharmaceut. Res. 1990; **7**: 1262.

Srinivas NR. *Evaluation of Experimental Strategies for the Development of Chiral Chromatographic Methods Based on Diastereomer Formation*. *Biomed.* Chromatogr. 2004; **18**: 207.

Stalcup AM and Gahm KH. *Application of Sulfated Cyclodextrins to Chiral Separations by Capillary Zone Electrophoresis.* Anal. Chem. 1996; **68**: 1360.

Stapleton MP. *Sir James Black and Propranolol, the Role of the Basic Sciences in the History of Cardiovascular Pharmacology*. J. Tex. Heart I. 1997; **24:** 336.

Stephani R and Cesare V. *Enantiomeric Enrichment of Non-Racemic Antihistamines by Achiral High-Performance Liquid Chromatography*. J. Chromatogr. A 1998; **813**: 79.

Sun XX, Sun LZ and Aboul-Enein HY. *Chiral Derivatization Reagents for Drug Enantioseparation by High-Performance Liquid Chromatography Based Upon Precolumn Derivatization and Formation of Diastereomers: Enantioselectivity and Related Structure,* Biomed. Chromatogr. 2001; **15:** 116.

Sutton KL, Ponce CA, De Leon, Ackley KL, Sutton RMC and Stalcup AM, Caruso JA. *Development of Chiral HPLC for Seleno-Amino Acids with ICP-MS Detection: Application to Selenium Nutritional Supplements*. Analyst 2000; **125:** 281.

Tateishi K, Tsukagoshi S, Nakamura T, Watanabe S, Soloshonok VA and Kitagawa O. *Chiral Initiator-Induces Self-Disproportionation of Enantiomers Via Achiral Chromatography: Application To Enantiomer Separation of Racemate*. Tetrahedron Lett. 2013; **54**: 5220.

Thomas RE. *Cardiac Drugs*. In: Wolff ME (Eds). Burger's Medicinal Chemistry and Drug Discovery. Vol. 2. Wiley: New York, 1996) p. 153.

Thompson JA, Holtzman JL, Tsuru M and Lerman CL. *Procedure for the Chiral Derivatization and Chromatographic Resolution of R-(+)- and S-(–)-Propranolol*. J. Chromatogr. 1982; **238**: 470.

Thompson HJ, Meeker LD and Kokoska S. *Effect of an Inorganic and Organic form of Dietary Selenium on the Promotional Stage of Mammary Carcinogenesis in the Rat*. Cancer Res. 1984; **44**: 2803.

Thomson CD, Robinson MF, Campbell DR and Rea HM. *Effect of Prolonged Supplementation with Daily Supplements of Selenomethionine and Sodium Selenite on Glutathione Peroxidase Activity in Blood of New Zealand Residents.* Am. J. Clin. Nutr. 1982; **36**: 24.

Thurston J, Dudley J, Kaiser D, Hechenbleikner I, Schaefer F and Holm-Hansen D. *Cyanuric Acid Derivatives. I. Aminochloro-s-Triazines*. J. Am. Chem. Soc. 1951: **73;** 2981.

Toyo'oka T. *Resolution of Chiral Drugs by Liquid Chromatography Based Upon Diastereomer Formation with Chiral Derivatization Reagents*. J. Biochem. Biophys. Methods 2002; **54:** 25.

Toyo'oka T, Toriumi M and Ishii Y. *Enantioseparation of β-Blockers Labelled with a Chiral fluorescent Reagent, (R)-(–)-DBD-Pyncs, by Reversed-Phase Liquid Chromatography*. J. Pharmaceut. Biomed. 1997; **15**: 1467.

Tyihak E, Mincsovics E and Kalasz HJ. *New Planar Liquid Chromatographic Techniques: Over-Pressured Thin-Layer Chromatography*. J. Chromatogr. 1979; **174:** 75.

Ullrich T, Menge S, Schmid M, Gübitz G and Krauss GJ. *Enantioselective High-Performance Liquid Chromatography of Therapeutically Relevant Amino Alcohols as Their Fluorescent 1-Naphthyl Isocyanate Derivatives.* Biomed. Chromatogr. 2001; **15**: 212.

Unceta N, Barrondo S, Ruiz De Azúa I, Gómez-Caballero A, Goicolea MA, Sallés J and Barrio RJ. *Determination of Fluoxetine, Norfluoxetine and their Enantiomers in Rat Plasma and Brain Samples by Liquid Chromatography with Fluorescence Detection*. J. Chromatogr. B 2007; **852:** 519.

U. S. Department of Health, Human Services, Food and Drug Administration, Fluoxetine information, 2010. http://www.fda.gov/Drugs/DrugSafety/PostmarketDrug Safety Information for Patients and Providers/ucm109352.htm, last assessed July 24, 2010.

Vékes E, Török G, Péter A, Sápi J and Tourwé D. *Indirect High-Performance Liquid Chromatographic Separation of Stereoisomers of β-Alkyl-Substituted Amino Acids by the Application of (S)-N-(4-Nitrophenoxycarbonyl)-Phenylalanine Methoxyethyl Ester as Chiral Derivatizing Agent*. J. Chromatogr. A 2002; **949:** 125.

Walle UK, Walle T, Bai SA and Olanoff LS. *Stereoselective Binding of Propranolol to Human Plasma, Alpha 1-Acid Glycoprotein, and Albumin*. Clin. Pharmacol. Ther. 1983; **34** : 718.

Wang R, Jia ZP, Hu XL, Xu LT, Li YM and Chen LR. *Determination of Serum Thyroxine Enantiomers in Patients by Liquid Chromatography with a Chiral Mobile Phase*. J. Chromatogr. B 2003; **785:** 353.

Wernisch S, Pell R and Lindner W. *Increments to Chiral Recognition Facilitating Enantiomer Separations of Chiral Acids, Bases and Ampholytes Using* Cinchona-*Based Zwitterion Exchanger Chiral Stationary Phases*. J. Sep. Sci., 2012; **35**: 1560.

Wolrab D, Kohout M, Boras M and Lindner W. *Strong Cation Exchange-Type Chiral Stationary Phase for Enantioseparation of Chiral Amines in Subcritical fluid Chromatography*. J. Chromatogr. A 2013a; **1289**: 94.

Wolrab D, Kohout M, Macíková P, Boras M and Lindner W. *Strong Cation Exchange Chiral Stationary Phase-A Comparative Study in High-Performance Liquid Chromatography and Subcritical fluid Chromatography*. J. Chromatogr. A 2013b; **1317**: 59.

Wong DT, Perry KW and bymaster FP. *The Discovery of Fluoxetine Hydrochloride (Prozac)*. Nat. Rev. Drug Discov. 2005; **4:** 764.

Wong DT, Fuller RW and Robertson DW. *Fluoxetine and its Two Enantiomers as Selective Serotonin Uptake Inhibitors*. Acta Pharm. Nord. 1990; **2:** 171.

Xuan H and Hage DS. *Evaluation of a Hydrazide-Linked α_1-Acid Glycoprotein Chiral Stationary Phase: Separation of (R)- and (S)-Propranolol*. J. Sep. Sci. 2006; **29:** 1412.

Yang E, Wang S, Kratz J and Cyronak MJ. *Stereoselective Analysis of Carvedilol in Human Plasma Using HPLC/MS/MS after Chiral Derivatization*. J. Pharm. Biomed. Anal. 2004; **36:** 609.

Yang GS, Chen DM, Yang Y, Tang B, Gao JJ, Aboul-Enein HY and Koppenhoefer B. *Enantioseparation of Some Clinically Used Drugs by Capillary Electrophoresis Using Sulfated β-Cyclodextrin as a Chiral Selector*. Chromatographia 2005; **62**: 441.

Yu H, Ching CB, Fu P and Ng CS. *Enantioseparation of fluoxetine on a new β-Cyclodextrin bonded phase column by HPLC*. Separ. Sci. Technol. 2002; **37:** 1401.

Yu L, Mei-Li F and Jie-Guo X. *Enantiomeric Separation of Fluoxetine Derivatives on Polysaccharide-Based Chiral Columns*. Arch. Pharm. (Weinheim) 2006; **339:** 461.

Zhang H, Shao H, Youmei A and Zhang Z. *Optimized Conditions of Enantioseparation of β-Blockers by Cze Using Carboxymethyl-β-Cyclodextrin as Chiral Selector*. Chromatogrphia 2008; **68**: 653.

Zhong Q, He L, Beesley TE, Trahanovsky WS, Sun P, Wang C and Armstrong DW. *Development of Dinitrophenylated Cyclodextrin Derivatives for Enhanced Enantiomeric Separations by High-Performance Liquid Chromatography*. J. Chromatogr. A 2006; **1115**: 19.

APPENDIX-I: LIST OF FIGURES

APPENDIX-II: LIST OF TABLES