STEREO SPECIFIC LC AND LC/MS SEPARATIONS OF SELECTED CHIRAL DRUGS FOR ANALYTICAL AND BIO ANALYTICAL APPLICATIONS

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CERTIFICATE

This is to certify that Mr. K. Nagesh Kumar, a bonafide student of Ph. D. degree of University of Hyderabad, Hyderabad, has carried out research work under my supervision. It is further certified that the investigations, findings and the inferences reported by him in the form of Thesis entitled "**Stereo specific LC and LC/MS separations of selected chiral drugs for analytical and bio analytical applications"**, for the award of Ph.D. degree in Chemistry, are the original contributions to the Chemical Sciences.

It is also certified that he has not submitted the same in part or in full to any other University for the award of a diploma or a degree.

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DECLARATION

I, Nagesh Kumar K, hereby declare that the investigations, findings and inferences reported in the thesis entitled **"Stereo specific LC and LC/MS separations of selected chiral drugs for analytical and bio analytical applications"**, are the original contributions to the basic and applied research in the field of Chemical Sciences and I have not submitted the same in part or in full to this University or to any other University for the consideration of the award of diploma or a degree.

Hyderabad
February, 2014

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Dedicated to

My parents, who believe in me,

My instructors, who educated me and enriched me

List of Abbreviations

2D-LC	Two Dimensional Liquid Chromatography
λ_{max}	Absorption Maximum (nm)
ACN	Acetonitrile
AGP	Acid Glyco Protein
APCI	Atmospheric Pressure Chemical Ionization
API	Active Pharmaceutical Ingredient
AUC	Area Under Curve
CBH	Cellobiohydrolase
CE	Capillary Electrophoresis
CD	Cylcodextrin
CF	Cyclofructan
CS	Calibration standard
CV	Coefficient of Variation
CSP	Chiral Stationary Phase
CZE	Capillary Zone Electrophoresis
DBS	Dried Blood Spot
DCM	Dichloro Methane
DEA	Diethyl Amine
DMF	Dimethylformamide
DRV	Darunavir
EI	Electron Impact Ionization
ESI	Electrospray Ionization
ΔH^o	Enthalpy Change
ΔS^o	Entropy Change
FL	Fluorescence

FDA	Food and Drug Administration
ΔG^o	Free Energy Change
GC-MS	Gas Chromatography-Mass spectrometry
HPLC	High-Performance Liquid Chromatography
HPTLC	High-Performance Thin-Layer Chromatography
HSA	Human Serum Albumin
ICH	International Conference on Harmonization
ICP-MS	Inductively Coupled Plasma-Mass Spectrometry
LC-MS	Liquid Chromatography-Mass Spectrometry
LC-NMR	Liquid Chromatography-Nuclear Magnetic Resonance Spectrometry
LEC	Ligand Exchange Chromatography
LLE	Liquid-Liquid Extraction
LOD	Limit of Detection
LOQ	Limit of Quantitation
LLOQ	Lower Limit of Quantitation
MeOH	Methanol
MIP	Molecularly Imprinted Polymer
MRM	Multiple Reaction Mode
MS^n	Multiple-Stage Mass Spectrometry
MTZ	Mirtazapine
N	Number of Theoretical Plates
NAC	N-acetyl-L-cysteine
OPA	*O*-phtaldialdehyde
PDA	Photo Diode Array
PI	Protease Inhibitor
PRG	Pregabaline

PP	Protein precipitation
PO	Polar Organic
QC	Quality Control
RAM	Restricted Access Media
RRT	Relative Retention Time
Rs	Resolution
RSD	Relative Standard Deviation
SPE	Solid Phase Extraction
t_R	Retention Time (minutes)
k'	Retention Factor or Capacity Factor
RP	Reversed-Phase
SRM	Selected Reaction Mode
SRT	Sertraline
SSRI	Selective Serotonin Reuptake Inhibitor
α	Selectivity or Separation Factor
SNRI	Serotonin-Norepinephrine Reuptake Inhibitor
SPE	Solid Phase Extraction
SFC	Supercritical Fluid Chromatography
TEA	Triethylamine
THF	Tetrahydrofuran
UHPLC	Ultra-High Performance Liquid Chromatography

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CHAPTER 1

Role of chiral selectors in liquid chromatographic separation of enantiomeric drugs

1.1 Introduction

A molecule containing an asymmetric center can exist in two different forms. These are non-superimposable mirror images of each other and called as enantiomers. Enantiomers have identical physical and chemical properties with exceptions that: (a) they rotate plane-polarized light in opposite direction and (b) interact differently with other chiral moieties. An enantiomer which rotates the plane of polarized light to the right (i.e. clockwise) is designated as dextrorotatory ("*d*" or "+"), while one that rotates plane polarized light to the left (i.e. counterclockwise) is called levorotatory ("*l*" or "-"). A molecule with two or more chiral centers can exist as up to 2^n possible stereoisomers, where *n* indicates the number of chiral centers. Stereoisomers that are not mirror images of each other are called diastereomers. Diastereomers have similar chemical properties but different physical properties. A racemic mixture or racemate exhibits no net rotation of plane polarized light since the dextrorotatory and levorotatory components are present in equal amounts. Because of the chiral environment of living systems, two enantiomers may exhibit the same pharmacological activity to different degrees, or may exhibit entirely different kinds of chemical or pharmacological activities. One of the best examples is propranolol which has different pharmacodynamic and pharmacokinetic profiles for the (R)- and (S)-enantiomers. S-propranolol is 100 times more potent than R-propranolol as a β-blocker and has a longer plasma half-life than R-

propranolol [1]. Table: 1.1 gives the list of some of the enantiomeric drugs having different activities.

Table: 1.1 Different enantiomeric drugs showing different activities

Chiral drug	Activity	
	S-enantiomer	R-enantiomer
Carvone	Caraway	Spearmint
Penicillamine	Antiarthritic	Mutagen
Thalidomide	Teratogen	Sadative
Ethambutol	Tuberculostatic	Blindness
Asparagine	Bitter	Sweet

1.1.1 Chiral Separations

Easson and Stedman have presented a significant conceptual advance towards the rationalization of the biological discrimination of a pair of enantiomers in terms of drug action [2]. The interaction between a drug and a receptor is associated with bonding interactions between the functionalities in the drug and complementary sites on the receptor, the three-dimensional spatial arrangement of such functionalities are of considerable significance. It was postulated that the differences in activity arise as a result of differential binding of the pair of enantiomers to a common site. Fig: 1.1 shows the interactions between the stereoisomers having different configurations with receptor a) the more active enantiomer takes part in a minimum of three intermolecular

interactions with the receptor, whereas b) the less active enantiomers could interact at two sites only.

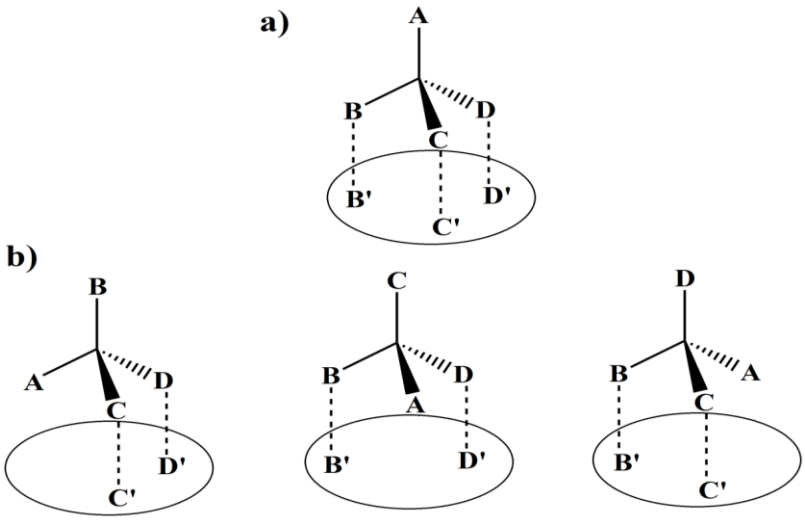


Fig.1.1 Easson–Stedman model of the drug–receptor interaction.

1.1.2 Analytical Techniques

A new trend in drug development is the synthesis of specific enantiomers of chiral drugs. The use of "enantiopure" drugs grew at greater levels and some established drugs are now sold as single enantiomers. Since enantiomers have identical physical properties, they cannot be separated by traditional methods. Nonetheless, they differ in their molecular shape, optical polarity and their interactions with other chiral substances. Thus, they can be separated by chiro-selective chromatographic techniques. In the last four decades, chromatographic methods for enantiomeric separation have became popular due to the relative ease, speed and efficiencies of these separations. Furthermore, a

tremendous number of new chiral techniques have been introduced and are now commercially available. Extensive theoretical and mechanistic studies involving chiral recognition have been published, which can assist in the understanding and optimization of these techniques [3-5]. Several non chromatographic methods have traditionally been used for enantiomeric separations [6, 7]. The most popular method is derivatization of the racemate using another optically-active reagent to form a mixture of diastereomers. The diastereomers are then separated based on differences in their physical properties. Solubility differences may permit separation of diastereomers by fractional crystallization. Crystallization with mechanical separation is useful for few compounds whose enantiomers segregate into morphologically distinct crystals. Seeding a supersaturated racemic solution with small optically-pure crystals also helps in separation. The precipitated crystals thus formed often contain a significant excess of one enantiomer. Enantiomeric excesses have also been obtained via microbiological or enzymatic digestion. In the latter case, the enzyme must preferentially catalyze a reaction involving one enantiomer but not the other. Although, these techniques have been utilized successfully, none can be considered generally useful. All are relatively time consuming and tedious. Moreover, they often fail to afford total enantiomeric separations. Other analytical techniques, i.e. viz; polarimetry, circular dichroism and shifts reagents by nuclear magnetic resonance spectroscopy, have been used for

determination of enantiomeric purity and ratio. Nonetheless, chromatography is the only technique which can be used for the determination of enantiomeric impurities at levels below 1%. Additionally, it has the advantage of permitting the measurement of enantiomeric ratios in low concentration samples, such as those from biological matrices. Most of the published enantiomeric separations involve gas chromatography (GC) [8, 9], high performance liquid chromatography (HPLC) [10, 11], supercritical fluid chromatography (SFC) [12, 13] or capillary zone electrophoresis (CZE) [14]. Considerably a fewer papers describe separations by thin layer chromatography (TLC) [15].

In the last four decades, interest in enantiomeric separation by HPLC has grown tremendously. GC and SFC have certain disadvantages relative to HPLC, the most critical of which is temperature selection. They often require high temperatures which can result in racemization or decomposition of the chiral analyte. HPLC does not suffer from this problem. Many choices of chiral selectors as stationary phases and mobile phase additives are available for TLC, HPLC and CZE. The latter two techniques offer higher column efficiency and greater reproducibility when compared to TLC. Tremendous advances in enantiomeric separation by CZE have occurred in spite of the relatively short development time. However, CZE cannot compete with HPLC in terms of scale-up to larger quantities of analytes. For these reasons, HPLC is one

of the most widely used technique for separation of enantiomers. Many new or improved chiral stationary phases, chiral additives and chiral/achiral derivatizing agents have been used as chiral selectors in HPLC. Many of them are now commercially available. The chiral selectors have a variety of chiral and functional groups inherent to their structure. Thus, they may have very different interactions with a given pair of enantiomers. The fundamental mechanism for chiral recognition is the "three point rule" [2], according to which, at least three interactions are required between the chiral selector and one of the enantiomers in the racemate to be separation. At least one interaction must depend on the stereochemistry at the chiral center of the chiral selector and the enantiomer (Fig: 1.2). Some of the required interactions are hydrogen bonding, π–π interactions and dipole–dipole interactions.

In the early 1970s, the first baseline separation of enantiomers by liquid chromatography was reported by Davankov and in 1981, the chiral stationary phases (CSPs) for HPLC developed by Pirkle were for first time commercialized. In the 1980s, the instrumentation for HPLC had remarkably advanced, and many efficient CSPs for HPLC had also been developed [16–23]. Chromatographic separation of enantiomers on a preparative scale has also been recognized as a powerful alternative of supplying pure enantiomers of bioactive compounds and chiral synthons [24]. Enantiomeric impurities are now measured routinely to the level of 0.01%.

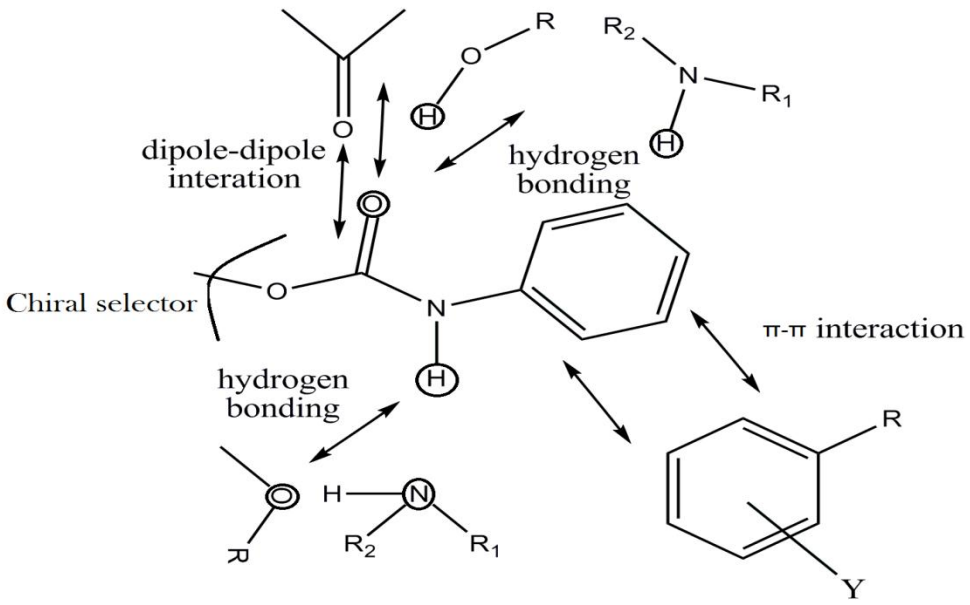


Fig: 1.2 Three point interactions of a chiral selector with enantiomers

1.2 High performance liquid chromatography (HPLC)

To achieve the separation of enantiomers, an enantioselective environment must be created by the addition of a chiral selector to the separation system. There are three general approaches to the separation of enantiomers by HPLC. The first is the separation on chiral stationary phases. The second involves separation using chiral mobile phase additives. The third is the classical separation in which a pair of enantiomers is derivatized with a chiral derivatizing reagent to form diastereomers which can be separated on an achiral stationary phase by HPLC. Thus the chiral separations could be divided into direct and indirect categories depending on the involvement of chiral selector in the chromatographic system. Fig: 1.3 shows the graphical representation of the two types of chiral separations. In this chapter, the mechanistic

aspects of different chiral selectors which are present in chiral stationary phases, chiral mobile phase additives and chiral derivatizing agents are discussed.

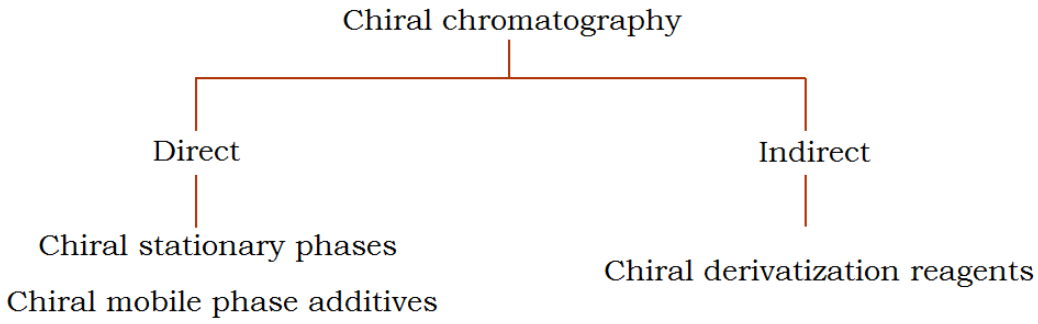


Fig: 1.3 Direct and indirect chiral separations by HPLC

1.2.1 Direct Separation

Direct methods involve separation of racemic mixtures into the corresponding enantiomers using chiral stationary phases (CSPs) or mobile phase additives.

1.2.1.1 Chiral Stationary Phases (CSPs)

On chiral stationary phases, the enantiomeric separations arise due to the formation of transient diastereomeric complexes involving the chiral selector and enantiomers. The retention times of enantiomers depend on the differences in stability of the transient diastereomeric complexes, with the less stable complex eluting earlier than the more stable complex. The disadvantages associated with this approach include the high price of most chiral columns and the fact that some chiral

columns exhibit batch-to-batch variability. Moreover, the prediction of suitable phases is difficult and often empirical. The direct enantioseparation of a chiral compound using a chiral stationary phase (CSP) has had a great impact on pharmaceutical industry. Direct methods based on CSPs are preferred since they are rapid and suitable to resolve racemates on both analytical and preparative scales. There is a wide variety of natural and synthetic CSPs, most of which are commercially available. Polysaccharide based stationary phases, proteins, cyclodextrins and their derivatives, pirkle type phases and macrocyclic antibiotics with different type of chiral selectors have proved to be the most useful phases for resolution of chiral drugs [25].

Chiral selectors are classified in many different ways. One of the more useful classification formats is by structure. Knowing the structure and properties of a chiral selector is the first step in understanding how they function and what they will separate. Today, the semi synthetic chiral selectors dominate the field of enantiomeric separations, although many important contributions are still made by natural and synthetic compounds. Attempts to classify chiral selectors on the basis of perceived function were somewhat arbitrary because a single type of chiral selector can function differently in different environments. For example, the Cyclobond-I-SN (i.e., β-cyclodextrin functionalized with S naphthylethyl carbamate groups) chiral stationary phase (CSP) can form inclusion complexes in the reversed-phase (RP) mode, act as a π-

complex CSP in the normal phase (NP) mode, or show only external surface adsorption in the polar organic (PO) mode [26].

1.2.1.1.1 Polysaccharide based CSPs

The polysaccharide based CSPs were first introduced by Okamoto and his group in 1984. These CSPs for HPLC have been recognized as the most powerful ones for analytical and preparative separations due to their high selectivities, sensitivities and reproducibilities. They are not only effective under normal phase, but also reversed-phase conditions [27]. The most popular polysaccharides are cellulose, amylose, chitosan, xylan, curdlan, dextran and inulin [28] but could not be used as commercial CSPs because of their low resolution capacities and handling problems [29]. Hence the derivatives of these polymers were synthesized. Among them, cellulose and amylose proved to be the best polymers because of their good abundance and capabilities for chiral resolution. Both cellulose and amylose contain glucose units and polymeric chains of D-(+) glucose units joined through β-1,4 and α-1,4 linkages. The degree of polymerization of cellulose ranges from 200-14,000 units of glucose. Similarly, more than 1000 glucose units are found in amylose [30]. Each glucose unit has a chair conformation with 2-OH, 3-OH and 5-CH_2OH groups all in equatorial position as shown in Fig. 1.4. The chains of these units lie side by side in a linear fashion in case of cellulose and helical in amylose and, hence, amylose provides more

chiral grooves for enantiomeric separation. Therefore, amylose is a better chiral selector when compared to cellulose [31].

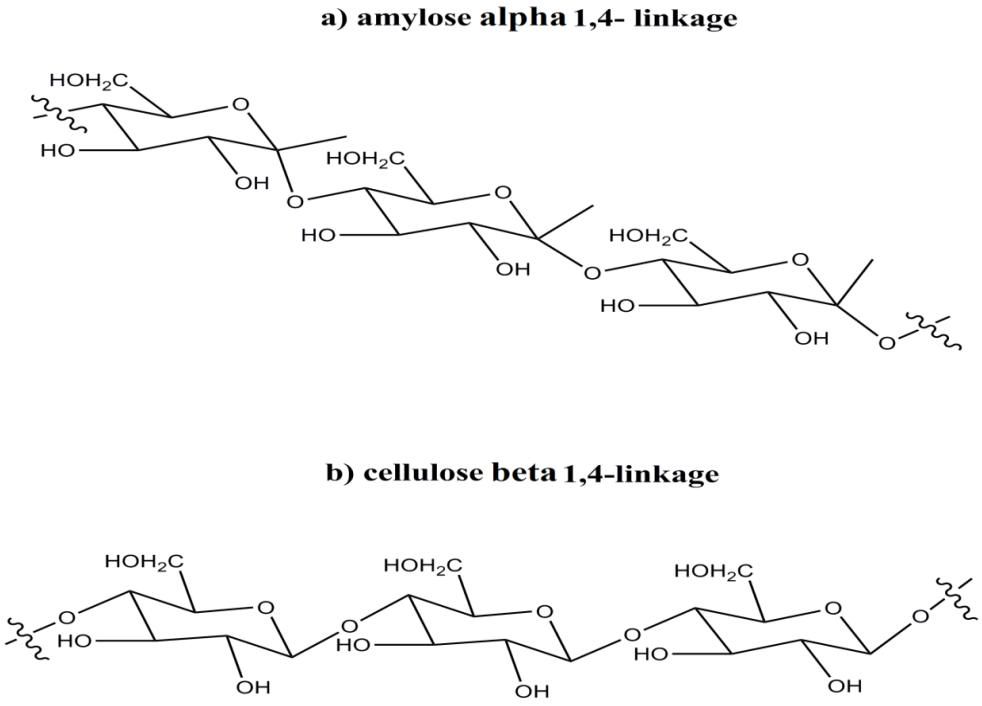


Fig: 1.4 Chemical structures of a) cellulose and b) amylose polymers

Types of Polysaccharide based CSPs

Polysaccharide derivatives, such as phenylcarbamates and benzoates of cellulose, amyloses show high chiral recognition abilities for many racemates when used as CSPs. These can be divided into coated and immobilized polysaccharide CSPs depending upon the nature of connectivity of chiral selector onto silica phase.

Coated Polysaccharide CSPs

These types of CSPs have usually been prepared by coating the polysaccharide derivatives onto a macroporous silica gel without

chemical bonding. These can be used with a limited number of solvents, because, some of the organic solvents, such as THF, chloroform, toluene, ethyl acetate, and acetone, dissolve or swell these derivatives and destroy the packed columns. Due to the solubility of the polysaccharide derivatives, the coated CSPs have usually been used with eluents consisting of alkanes/ alcohol mixtures. However, good solubility of many of samples cannot be achieved by these solvents for analytical and as well as large scale preparative separations with high productivity. These limitations in the mobile phase selection sometimes pose serious problems for efficient analytical and preparative resolution of enantiomers. M/s Daicel, Japan has introduced some popular CSPs of these type and some are shown in Fig: 1.5.

Immobilized polysaccharide derivatives

In order to resolve the limitation of versatility of mobile phases and insolubility of samples encountered in coated polysaccharide CSPs, the derivatives have to be immobilized. In case of immobilized CSPs, even the solutions obtained from various synthetic media can be directly injected. For these reasons, several immobilized polysaccharide-based CSPs have been prepared in the last decade. There are different methods to prepare the immobilized polysaccharide CSPs by immobilization of polysaccharide derivatives using via diisocyanate, vinyl groups by polymerization, via copolymerization with a vinyl monomer and/or immobilization of an amylose derivative at chain end. Among the

different companies M/s Daicel, Japan manufactured the immobilized CSPs and introduced six immobilized polysaccharide CSPs such as CHIRALPAK IA, IB, IC, ID, IE and IF. Fig.1.6 shows the chemical structures of immobilized chiral CSPs.

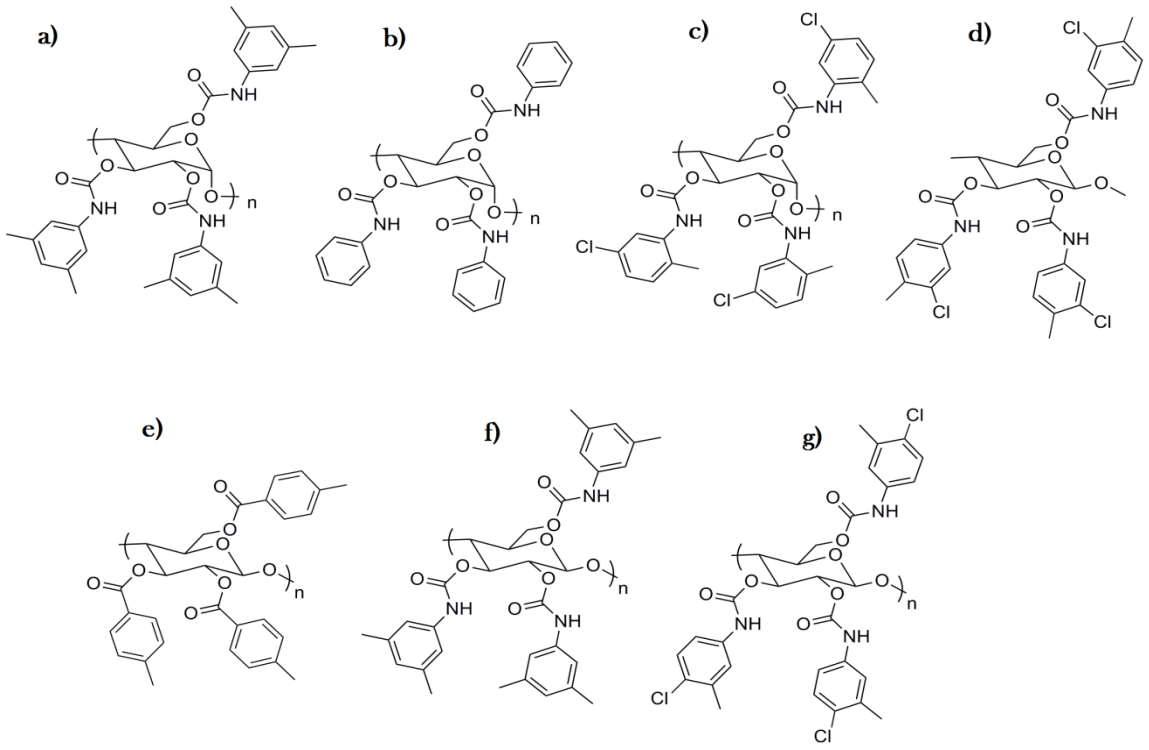


Fig: 1.5 Chemical Structures of coated polysaccharide derived chiral selectors a) CHIRALPAK AD-H: amylose tris (3,5 dimethylphenyl carbamate), b) CHIRALPAK AS-H: amylose tris [(s)-a-methylbenzyl carbamate], c) CHIRALPAK AY-H: amylose tris (5-chloro-2-methylphenyl carbamate), d) CHIRALCEL OZ-H: cellulose tris (3-chloro-4-methyl phenylcarbamate), e) CHIRALCEL OJ: cellulose tris (4-methylbenzoate), f) CHIRALCEL OD-H: cellulose tris (3,5-dimethylphenylcarbamate) and g) CHIRALCEL OX-H: cellulose tris (4-chloro-3-methylphenylcarbamate) coated on silica gel.

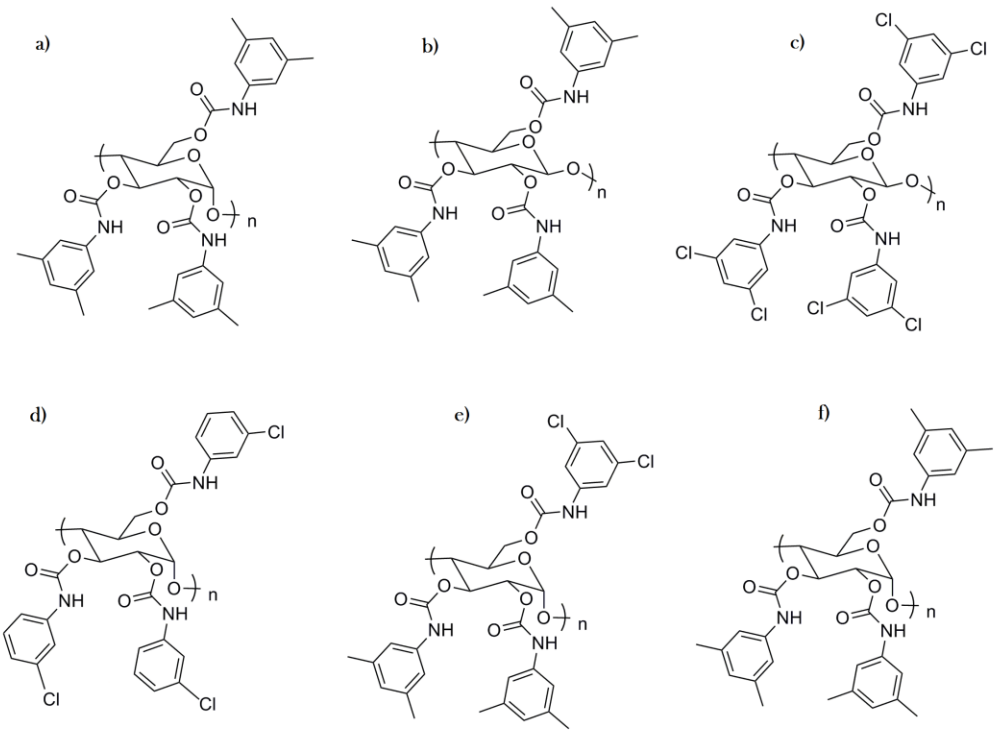


Fig. 1.6 Chemical structures of the immobilized polysaccharide derived chiral selectors on silica gel a) CHIRLAPAK IA: amylose tris (3, 5 dimethylphenylcarbamate), b) CHIRLAPAK IB: cellulose tris (3, 5-dimethylphenylcarbamate), c) CHIRLAPAK IC: cellulose tris (3, 5-dichlorophenylcarbamate), d) CHIRLAPAK ID: amylose tris (3 chlorophenylcarbamate) immobilized on silica gel e) CHIRLAPAK IE: amylose tris (3,5-dichlorophenylcarbamate) and f) CHIRLAPAK IF: amylose tris (3-chloro-4-methyl phenylcarbamate).

1.2.1.1.2 Cyclodextrin based CSPs

Cyclodextrins (CDs) are chiral, toroid-shaped, family of macrocyclic oligosaccharides composed of six to twelve D-glucopyranose units. Cyclodextrin and its derivatives are widely used as selectors of CSPs for HPLC due to their unique molecular structure and resolution capability. CDs afford a direct and simple approach for enantiomer

separation, not only on an analytical scale but also for production at a preparative level. Naturally occurring α-, β- and γ-CDs are cyclic oligosaccharides consisting of six, seven and eight α-1,4-D-glucopyranose units, respectively. Building of glucopyranoside units in the $^4C_1$ conformation with a cavity of a hydrophilic external surface and a hydrophobic internal surface and multiple hydroxyl groups located on the rim of the cavity, has attracted much interests in separation of chiral compounds [32] (Fig: 1.7).

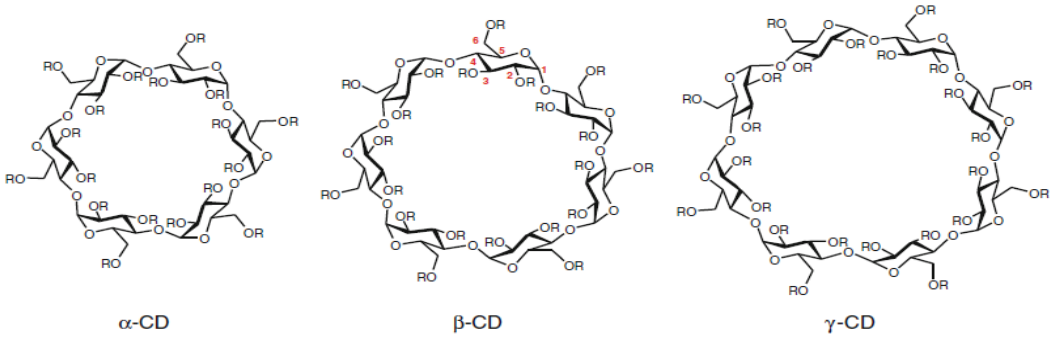


Fig: 1.7 Structures of native cyclodextrins of α-, β- and γ. 6-hydroxyl groups at the primary rim and, the 2- and 3-hydroxyl groups at the secondary rim of CD construct a hydrophilic exterior surface.

Both the architecture and chemistry of cyclodextrins contribute to enantiomer separations. The toroidal cyclodextrin structure has a hydrophilic exterior surface resulting from the 2-, 3-, and 6-position hydroxyl (OH) groups. The interior cyclodextrin cavity is composed of the glucose oxygens and methylene hydrogens, which gives it a non-polar (hydrophobic) character. Chemical interactions that lead to chiral separations occur on both the exterior and interior surfaces of the

cyclodextrin toroid. The most important consideration for retention and chiral recognition is proper fit of the analyte into the cyclodextrin cavity. This fit is a function of both molecular size and shape of the analyte relative to the cyclodextrin cavity. Thus, there are two basic mechanisms at play in chiral separations on cyclodextrins: those that occur on the inside cavity surface (inclusion complexing) (Fig: 1.8a) and those that occur on the outside surface (surface interactions) (Fig: 1.8b) of the cyclodextrin toroid [33].

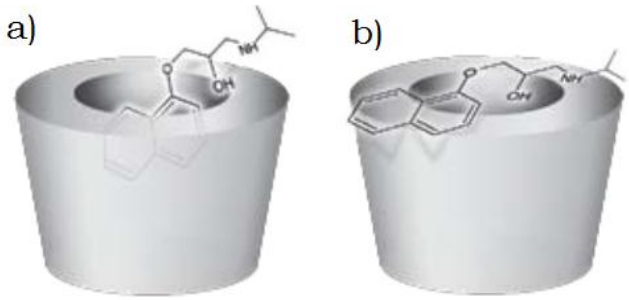


Fig: 1.8 Representation of the inclusion complexing mechanism of an analyte into the cyclodextrin cavity and the surface interaction mechanism of an analyte with the cyclodextrin.

Inclusion mechanism

The basis for many separations on cyclodextrin-based CSPs in the reversed-phase mode is a phenomenon called inclusion complexing. If the analyte can fit into the cyclodextrin cavity and mobile phase conditions are favorable, the inclusion complexing mechanism can occur. It is because of inclusion complexing that reversed-phase is a very successful mode on Cyclobond CSPs. The inclusion complexing

mechanism is attributed to the attraction of the apolar molecule or segment of the molecule to the apolar cyclodextrin cavity, which is very sensitive to structural differences. When the analyte possesses an aromatic group, the orientation in the cavity is selective due to the sharing of electrons between the aromatic methylene groups and the glucoside oxygens on the internal surface of the cyclodextrin toroid. The mechanism is completed by interaction of solute functional groups with the 2- and 3- position secondary hydroxyl groups of the cyclodextrin ring. A schematic of the inclusion mechanism is shown in Fig: 1.8a

Surface interaction mechanism

In surface interactions, the chiral molecule lies across the external surface of the cyclodextrin toroid and interacts with the upper rim of the ring. Surface interactions dominate in polar organic (methanol or acetonitrile containing additives) and normal phase modes because in these modes analytes do not interact with the cyclodextrin cavity for two reasons. First, when acetonitrile is present, it fully inserts into the cavity and blocks analytes from entering it. Second, when the mobile phase is totally non-aqueous, the non-polar interactions between analytes and the interior of the cyclodextrin cavity cannot occur. The surface interaction mechanism is depicted in Fig: 1.8b

Table: 1.2 Enantiomeric separations using different types of CDs by HPLC

Compounds	Selectors
Dansyl amino acids	native CD
Aromatic anionic acids	native CD
Flavonoids	Native CD, MeCD, PhCD
Aryl alcohols	PhCD
Amino alcohols, β-blockers	PhCD, native CD
Alkaloids	PhCD
Antihistamines	MeCD, PhCD
Thiazides, chlorothalidone	Native CD, PhCD

The modified CDs harvest better solubility in certain solvents and improved chiral selectivities towards specific guest molecules in various practical applications. The poplar derivatized groups on β-CD are mainly alkyl, hydroxyl alkyl, acyl, isocyanate, sulfonyl and cation groups are of very important for the separation of enantiomers. Among the CD derivatives developed so far, cationic CDs have attracted considerable interest in separation science and drug/gene delivery [34]. Hydroxypropyl-β-cyclodextrin derivatives are the best example of category of cationic CDs in HPLC and could form inclusion complexes with various organic compounds in aqueous solution [35]. These CDs exhibit satisfactory enantioselectivities for amino acids and acidic racemates. Table: 1.2 lists some examples of enantioseparation by HPLC in presence of native as well as derivative CDs.

1.2.1.1.3 Macrocyclic glycopeptide or antibiotic based CSPs

Macrocyclic glycopeptides, also termed macrocyclic antibiotics were introduced in separation science by Armstrong and coworkers [36]. Macrocyclic antibiotics-based CSPs represent another powerful group of CSPs with wide application possibilities. They can be used in RP, NP and PO separation modes. The best separation efficiency and selectivity is achieved mainly with PO or RP separation systems [37]. The most important selectors of this group are vancomycin, ristocetin, teicoplanin and teicoplanin aglycon. The common structural feature of the glycopeptide selectors is a heptapeptide as a set of inter connected macrocycles each composed of two aromatic rings and a peptide sequence. Vancomycin is composed of three macrocycles, while teicoplanin and ristocetin A contain four macrocycles. As illustrated in Fig: 1.9, the macrocycles possess the structure of a three-dimensional, C-shaped basket in vancomycin. The carbohydrate moieties are positioned at the surface. The macrocycles contain ionizable groups such as a carboxylic acid and amino groups. Thus, a large number of interactions between the glycopeptides and analyte molecules can be envisioned including hydrogen bonds, π-π, dipole–dipole and ionic interactions depending on the experimental conditions. The detailed recognition mechanism on a molecular basis is not yet completely understood [37].

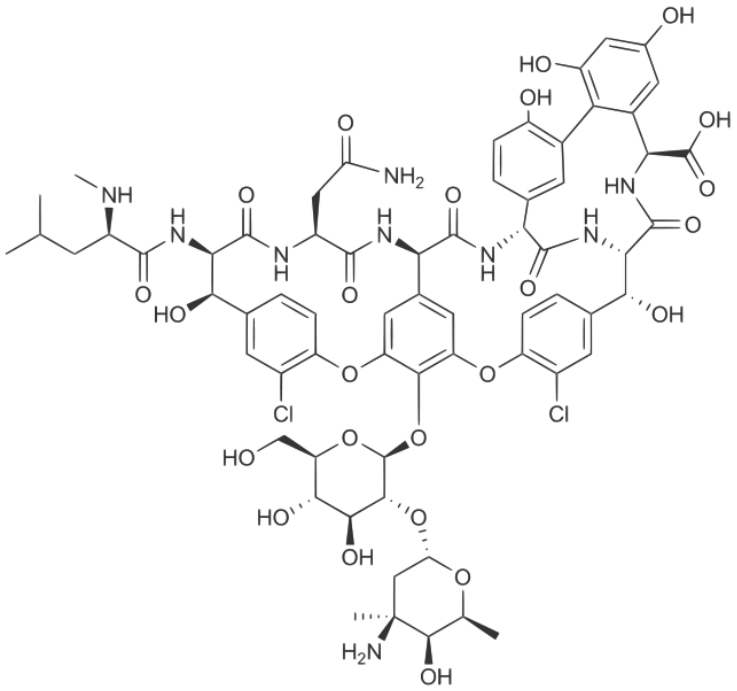


Fig: 1.9 Chemical structural representation of vancomycin

1.2.1.1.4 Cyclofructans

A relatively new class of CSPs is the various derivatized cyclofructans which seem to have great application potential. Cyclofructans (CFs) are cyclic oligosaccharides first described in 1989 as a result of the digestion of inulin by the extracellular enzyme cycloinulo oligosaccharide fructanotransferase of the Bacillus strain circulans OKUMZ 31B [38]. CFs are composed of β (2, 1) linked D-fructofuranose units (Fig: 1.10). In separation science, derivatives containing 6 (CF6) or 7 (CF7) fructose units have been employed so far. These CFs possess a disk-type structure. The inner core has the structure of crown ether and not a hydrophobic cavity as in case of CDs. CF6 possesses inner and outer diameters of 2.3 and 14.6 A°, respectively [39]. The fructose units

are arranged in a spiral array around the 18-crown-6 core with alternating "inward" and "outward" orientations [40, 41]. This way, three of the OH groups in position 3 of the fructofuranose moieties point toward the inner core blocking its access due to hydrogen bonding. As a consequence, only poor or no enantioseparations have been observed using native CF6 by HPLC or CE. Upon derivatization of the fructofuranose OH groups, effective chiral selectors are obtained. Depending on the substitution degree, i.e., partial derivatization or "complete" derivatization, as well as the nature of the substituents, chiral selectors with different recognition abilities are obtained. Partial derivatization with aliphatic or aromatic carbamate moieties yield chiral stationary phases with good enantioselectivity for chiral primary amines. Molecular modeling studies suggested that the hydrogen bonds are disrupted exposing the crown ether core as shown in Fig: 1.10 for the methylcarbamate of CF6. Thus, effective complexation of the amines can be achieved. In contrast, complete derivatization of the OH groups sterically block the core so that the recognition ability toward primary amines is lost.

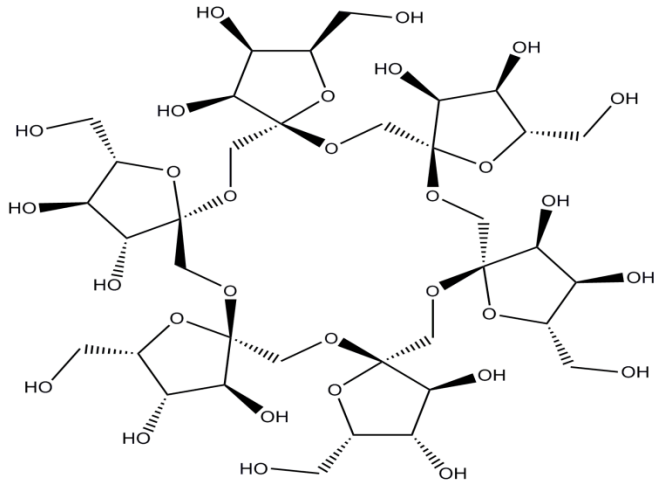


Fig: 1.10 Structure of cyclofructan CF6 containing six D-fructofuranose units.

The derivatives of cyclofructans were successfully applied for separation of chiral molecules. R-naphthylethyl CF6 and dimethylphenyl CF7 CSPs were reported to provide enantioselectivity for a broad range of compounds such as chiral acids, amines, metal complexes, neutral compounds [42]. R-naphthylethyl functionalized cyclofructan based CSP was used to separate racemates of substituted binaphthyl catalysts and was found to be more suitable for enantioseparation of those solutes than R-naphthylethyl β-cyclodextrin CSP. Isopropyl functionalized CF6 CSP demostrate a high efficiency for enantioseparation of compounds possessing primary amino groups [37]. It was the most broadly applicable CSP for primary amine-containing analytes and works more effectively with organic solvents and supercritical fluids than current crown ether CSPs [43]. CF6 and CF7 derivatives containing aliphatic and

aromatic carbamate substituents have been employed as chiral selectors in HPLC [39, 44]. With regard to the nature of the substituents, aliphatic substituted CF6 derivatives proved to be effective selectors for primary amines, while they were much less suitable for enantioseparations of other analytes. In contrast, aromatic substitution of CFs, especially with a high substitution degree, result in effective chiral stationary phases for a large number of aromatic analytes in the normal phase and polar organic mode in HPLC. The nature of the aromatic substituents affects the chiral recognition ability of the individual CF derivative.

1.2.1.1.5 Pirkle-type of CSPs

Donor–acceptor chiral selectors are also called brush-type or Pirkle-type selectors named after William H. Pirkle, one of the pioneers in their development [45]. Such chiral stationary phases are based on small molecule selectors capable of donor–acceptor interactions including hydrogen bonding, π-π interactions (face to face or face to edge) and dipole–dipole stacking. Rigid and bulky moieties as steric barriers may further amplify chiral recognition. The Whelk- O1 phase may be the most widely used Pirkle-type chiral stationary phase (Fig: 1.11a). This selector combines π-donor (tetrahydrophenanthrene) and π-acceptor (3,5-dinitrophenyl) as well as hydrogen bonding sites (amide) and is assumed to possess a cleft-like binding site resulting from the perpendicular orientation of the phenyl and tetrahydrophenanthrene moieties. The preferentially bound enantiomer interacts via face to face π-π

interactions with the dinitrophenyl moiety and hydrogen bonds with the amide function. Further face-to edge π-π interactions enhance the affinity between selector and preferentially bound analyte [36]. The structures of some pirkle type chiral selectors are shown in the Fig: 1.11.

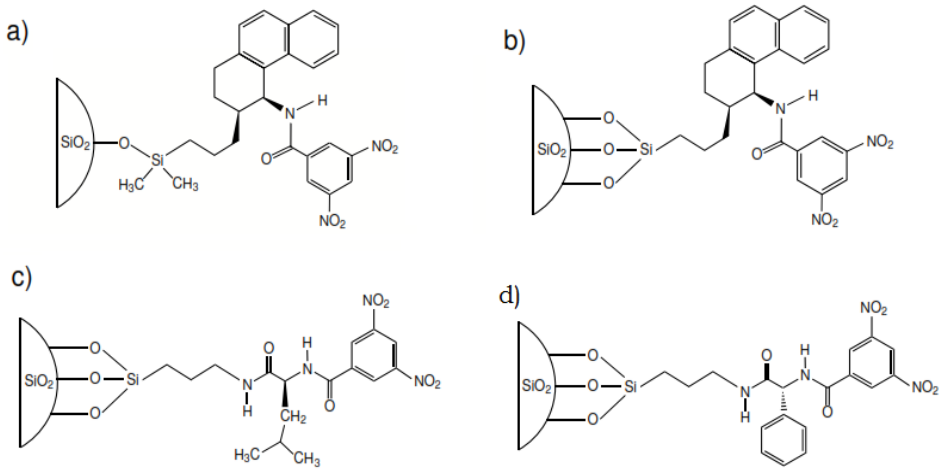


Fig: 1.11 Chemical structures of Pirkle type selectors a) Whelk-O 1, b) Whelk-O 2, c) Leucine and d) Phenylglycine.

1.2.1.1.6 Protein based CSPs

The stereoselective interaction of chiral compounds with proteins is a well-known phenomenon in nature. Proteins have been used as chiral selectors in separation science as pioneered by the groups of Allenmark, Haginaka and others [46, 47]. Hermansson described the use of natural proteins immobilized onto a silica support for chiral separations in 1983 [48]. Proteins contain a large number of chiral centers of one configuration, and many other sites that contribute to the general retention process. These are used less frequently for separation

of enantiomers by HPLC. Their main disadvantage is limited compatibility with mobile phases containing higher amounts of organic modifier. However, they can serve as a model environment for studies of drug interactions in organisms (human body) [27]. Due to the importance of pharmacokinetics of drugs, their stereoselective binding by human serum albumin (HSA) has been investigated. The protein has two major binding sites termed site 1 (warfarin-azapropazone site) and site 2 (indole-benzodiazepine site), as well as several minor sites coordinating a variety of drugs and other compounds [49]. Due to the complexity of the protein selectors, a number of molecular interactions including hydrogen bonds, π-π, dipole and ionic interactions contribute to the complexation of analytes. Molecular modeling was also employed for the understanding of the stereoselectivity of an anti L-amino acid antibody [50]. While L-phenylalanine was bound via hydrogen bonds and hydrophobic interactions, the D-stereoisomer was rejected due to steric hindrance. There are three well known CSPs with proteins as the chiral selectors, CHIRALPAK AGP (α1-acid glycoprotein), CHIRALPAK CBH (cellobio hydrolase) and CHIRALPAK HSA (human serum albumin). They are typically used in reversed-phase mode for a wide variety of chiral separations. CHIRALPAK HSA is also used for drug-binding studies. Solutes are retained by three types of interactions: ionic (for charged solutes), hydrophobic and hydrogen bonding. The relative contribution of the different forces to solute retention depends on the nature of the

analyte. CHIRALPAK AGP has extremely broad applicability and the first choice while developing methods on protein based CSPs.

1.2.1.1.7 Crown ethers

As these CSPs can be used for a limited group of enantiomers they did not gain importance among chiral stationary phases with wide applicability [37]. Chiral crown ethers used in separation science as selectors incorporate chiral moieties such as binaphthyl or tartaric acid units in a polyether macrocycle. Crown ethers form complexes with protonated primary amines, so that their use is essentially limited to this group of analytes [51]. The structure of (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid ((+)-18C6H4) has been given in Fig: 1.12. Analyte complexation is due to the formation of hydrogen bonds between the protonated amine with oxygen atoms of the macrocycle.

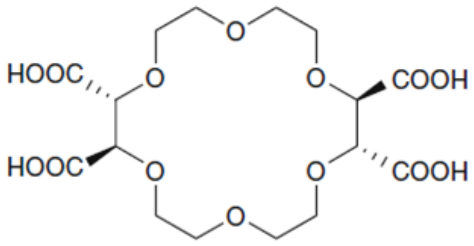


Fig: 1.12 Structures of (+)-(18-crown-6)-2,3,11,12- tetracarboxylic acid

1.2.1.1.8 Imprinted polymers

Molecularly imprinted polymers (MIPs) are synthetic polymers produced by polymerization of functional monomers and cross linkers in

the presence of a template. Two synthetic approaches have been realized, the non-covalent and the covalent approach [52, 53]. MIPs can be regarded as target-specific chiral selectors which possess a pre-determined selectivity for a given analyte or a group of structurally related compounds. Chiral recognition is determined by the steric arrangement of the interacting groups of the template and the polymer. The interaction and the spatial arrangement between the imprinting target molecule and the monomers in solution have been studied [54-56]. Based on the three-point interaction model, the "weakest interaction model" was suggested as chiral recognition mechanism of MIPs [57]. It is assumed that there is no difference between the enantiomers with regard to strong interaction such as ionic interactions, so that differences in the complexation of the solutes will result from the weaker interacting moieties. MIPs typically suffer from poor chromatographic efficiency, peak tailing and poor loading capacity.

1.2.1.1.9 Ligand exchange chromatography (LEC)

LEC represents one of the oldest environments used for the separation of enantiomers. The simplest system can be created using one enantiomer of an amino acid (as chiral ligand) and mostly Cu (II) as a central atom forming the complex with another amino acid as analyte. The principle of chiral ligand exchange in separation science has been introduced in chromatography by Danakov [58]. The technique is based on complexes between ligands and a central ion, typically a metal ion.

Amino acid derivatives are typically employed as chelating agents, although D-quinic acid, D-gluconic acid, D-saccharic acid or L-threonic acid have also been used as complexation agents. The metal ions include divalent metal ions such as Cu^{+2}, Zn^{+2} or Ni^{+2}. Enantioseparation by ligand exchange is restricted to analytes with two or three electron donating groups such as amino acids, hydroxy acids, amino alcohols or diols. In HPLC, chiral ligand exchange is typically performed either by addition of the central ion and the ligand into the mobile phase or using commercial columns based on immobilized ligands such as N,N-dioctyl-L-alanine or L-hydroxyproline [59, 60]. Ligand exchange is based on the reversible chelate coordination of a chiral analyte into the sphere of a metal ion, which is immobilized due to complexation with an enantiopure chelator resulting in a selectand–metal ion-selector complex. The resulting diastereomeric chelates possess different thermodynamic formation ratios or stabilities. The different stabilities of the diastereomeric complexes result in a different distribution of charged species (free analyte and binary complexes) of the enantiomers. It has also been demonstrated that the ionization status of the analyte ligand may affect the stability of the diastereomeric mixed complexes. Thus, different stereo selectivities of complex formation have been observed whether the analyte enantiomers are charged or uncharged. LEC is often performed using achiral columns and mobile phases with chiral additives. Fig: 1.13 shows the chemical structure of ligand exchange CSPs.

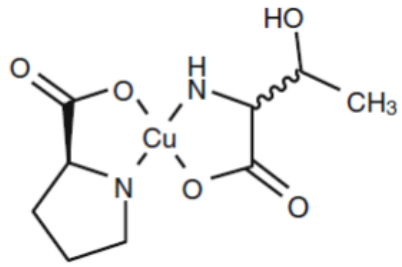


Fig: 1.13 Chemical structure of ligand exchange CSPs

1.2.1.1.10 Synthetic Polymers

Synthetic polymers used as CSPs are obtained by radical polymerization of chiral monomers such as (S)-N-acryloylphenylalanine ethyl ester [61] (Fig: 1.14a), N,N-diallyl-L-tartardiamide derivatives [62] (Fig. 1.14b). Furthermore, helically chiral polymers can be obtained by sparteine-catalyzed anionic polymerization of triphenylmethacrylate [63] (Fig: 1.14 c). Synthetic helical polymers with a controlled helix-sense have been prepared via the noncovalent "helicity induction and memory strategy" [64] (Fig: 1.14 d). In this approach, a poly (4-carboxyphenyl isocyanide) adopted helical conformation upon complexation with a chiral amine and maintained the induced helix when the amine was removed. The polymer could subsequently be immobilized a solid support. Left- and right-handed helices as chiral selectors could also be constructed by living polymerization of enantiomerically pure phenyl isocyanide bearing L-alanine decyl ester pendant [65]. The polymers are typically prepared by so-called "grafting on" approach in which the polymer chains grow in solution and are subsequently attached to the vinyl modified solid support by co-polymerization. According to the

recently introduced "grafting from" approach, the polymer chains grow from the support surface following initial anchoring of an initiator [66]. The latter results in homogenous coverage of the surface of the silica particles and avoids blockage of pores. The chiral discrimination of solutes by the polymer selectors results from interactions such as hydrogen binding and π-π interactions as well as steric factors. In case of chiral stationary phases containing L-alanine-bound polyisocyanides with opposite helicity, it was concluded from structure–separation relationships that the helical structures play a major role for the chiral recognition of the analytes.

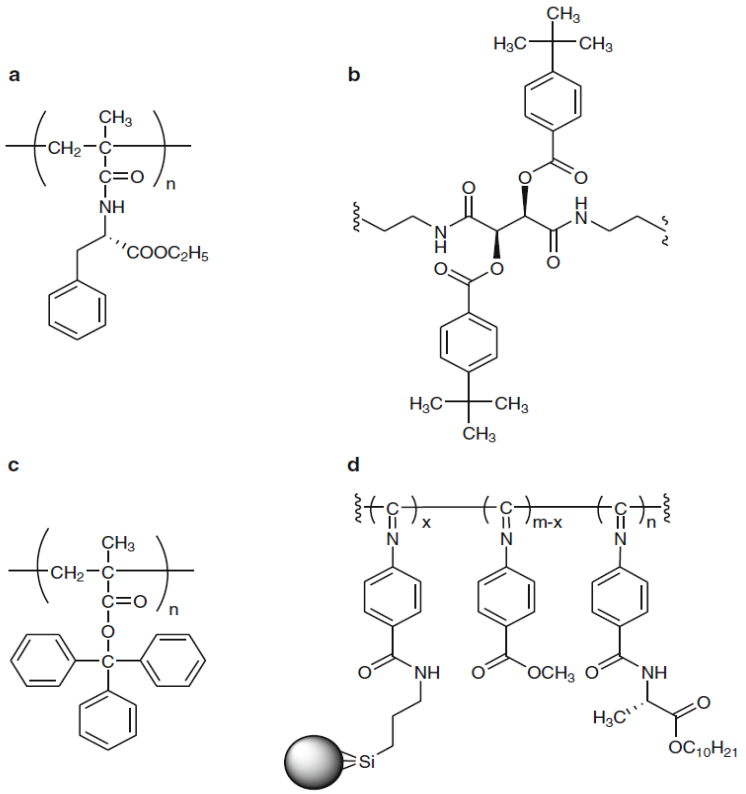


Fig: 1.14 Schematic structures of chiral polymers, poly[(S)-Nacryloylphenylalanine Ethyl ester] (a), poly[O,O0-bis(4-tertbutyl benzoyl)- N,N0-diallyl-Ltartardiamide] (b), poly(triphenyl methacrylate) (c) and a poly(4-carboxyphenyl isocyanide)-based polymer (d)

1.2.1.2 Chiral mobile phase additives (CMPAs)

Among the chiral HPLC techniques available, the CMPA technique is a valuable method for the direct enantioseparation of chiral compounds. In the CMPA method, the chiral selector is dissolved in the mobile phase while the stationary phase is achiral. Interaction with the analyte enantiomers results in the formation of transient diastereomeric complexes. These complexes differ in their formation constants, relative stability as well as differences in their partitioning between the mobile phase and stationary phase and/or distribution between the achiral stationary phase and the mobile phase resulting in an enantioseparation [67]. Advantages of this technique are as follows: (1) less expensive conventional LC columns can be used; (2) a wide variety of possible additives are available; and (3) different selectivities from the chiral phases can be obtained. However, the problems include: (1) many chiral additives are costly, and sometimes, have to be synthesized; (2) the mode of operation is complex and (3) inconvenient for preparative applications because the chiral additive must be removed from the enantiomeric solutes. This section describes the HPLC separation applying CMPA methods by several most useful types of chiral selectors including chiral ligand-exchangers, macrocyclic antibiotics and cyclodextrins.

The enantio recognition mechanism in CMPA systems is widely recognized as quite complex. However, the chiral recognition is generally thought to require unique interactions due to the stereogenic centers of

both, the chiral selector and the chiral analyte simultaneously on at least three positions [68, 69]. Physicochemically, the interactions include inclusion complexation, electrostatic interactions, π–π interactions, hydrogen bonding and dipole–dipole interactions. Because of the multiplicity and complexity of the interactions between the enantiomers and a chiral selector, the surface of the stationary phase, and other components of the chromatographic system, the total separation efficiency can depend strongly on the composition (including the concentration of the chiral selector and other additives), pH, and temperature of the mobile phase [70]. Therefore, it is important to optimize all these parameters when developing a CMPA method. At present, a large number of chiral selectors have been investigated in CMPA methods, and more and more new chiral selectors are being synthesized or evaluated. According to their different separation mechanisms or according to their structure, CMPA can be divided into the following groups: ligand-exchanger, macrocyclic antibiotics, cyclodextrin, etc.

The separation mechanism of ligand-exchange chromatography (LEC) is based on the reversible formation of mixed ternary diastereomeric complexes composed of transition metal ions (Cu^{+2} is the most common ion used), a chiral selector ligand (generally amino acids and their derivatives), and the analyte enantiomers. The chromatographic resolution is due to differences in complex stability

constants of the two ternary complexes with the analyte enantiomers. Typical analytes that can be resolved by this approach contain two or three electron-donating functional groups (e.g., carboxyl groups, amino groups of hydroxyl groups), which can simultaneously enter the coordination sphere of the complexing metal ion and function as tridentate or bidentate chelating ligands. Accordingly, classes of organic compounds that can be resolved by LEC include derivatized and underivatized amino acids, hydroxyl acids, amino alcohols, diamines, dicarboxylic acids, amino amides, or dipeptides. Since the analytes and the chiral selectors used in LEC contain strongly polar functional groups, they are usually better dissolved using water, alcohols, or other strongly polar solvents as mobile phases. Thus, LEC is operated with aqueous or aqueous organic mobile phases, i.e., in the reversed-phase mode.

Another class of chiral selectors, the macrocyclic antibiotics (also known as macrocyclic glycopeptides), was first introduced as chiral selectors by Armstrong et al. in 1994 [71]. The most prominent compound of this group is vancomycin. Glycopeptides have a large number of different functional groups, e.g., aromatic rings, hydroxyl groups, amino groups, carboxylic acid moieties, amide linkages, and hydrophobic pockets, so that a large variety of intermolecular interactions can contribute to the chiral recognition ability of these selectors. The three-dimensional molecular structures of macrocyclic glycopeptides show that they possess a characteristic “basket-shaped”

aglycon, which consists of a peptide core of complex amino acids and linked phenolic moieties. The aglycon basket of all these molecules consists of either three or four fused macrocyclic rings and is responsible for their enantioselective properties. The unique structure of macrocyclic glycopeptides contributes to their wide applicability as chiral selectors. A large variety of anionic, neutral, and cationic compounds such as amino acids, neutral aromatic molecules, and non-steroidal anti-inflamatory drugs can be separated with these chiral selectors. Macrocyclic antibiotics allow a wide variety of chiral separation modes including the normal-phase mode, the reversed-phase mode, and the polar organic mode.

Cyclodextrins (CDs) have been used as chiral selectors in HPLC as well as capillary electrophoresis and represent the most frequently used type of chiral selectors for a broad application range. Most CDs possess sufficient solubility in the mobile phases and low UV absorbance. Moreover, several CD derivatives (native, methylated, and hydroxyl propylated derivatives) are relatively cheap. CDs are cyclic oligosaccharide molecules consisting of D - (+) glucopyranose units connected via a -1,4-glycosidic bonds. The most commonly used CDs, α - CD, β-CD and γ-CD, are composed of six, seven, or eight glucopyranose units, respectively. The molecules possess the form of a truncated cone. The primary C-6 hydroxyl groups are located at the narrower rim while the secondary C-2 and C-3 hydroxyl groups are on the wider rim. CDs

have numerous chiral centers (five in every glucose unit). The formation of inclusion host–guest complexes is thought to be a key interaction in the chiral recognition by CDs. In this case, hydrophobic groups of analyte are included into hydrophobic cavity of CD. Secondary interactions between analyte and the hydroxyl groups on the rims can also contribute to the chiral recognition

1.2.2 Indirect separation

Indirect separations are based on the formation of diastereoisomeric complexes between the enantiomers and suitable chiral derivatizing agents and their subsequent separation by an achiral LC. Furthermore, the indirect methods require extra time for the derivatization reaction. Although the derivatization methods have been widely practiced, they suffer from the possibility of racemization during the derivatization steps and can be used when the enantiomeric molecule possesses an easily derivatized functional group. For these reasons the trend is to use chiral stationary phases in preference to either the derivatization or mobile phase additive approaches. The advantages are good chromatographic properties of derivatives, elution sequence predictable, good chromophoric or fluorophoric properties of the reagent (enhanced sensitivity can be achieved), low cost of achiral columns, method development is simple, selectivity can be increased (better separation is often achieved than with a direct method), possibility of appropriate selection of the elution sequence.

Pre-column derivatization is a useful way to improve both the sensitivity and the selectivity of the method. It is mostly performed off-line because of instrumentation simplicity. HPLC of those derivatives should be preferred that the derivatization reactions and formation of adducts are fast and they can be performed in aqueous solutions and at ambient temperature. The preparation of the selected derivatives should be simple and cost effective resulting in products of high sensitivity and selectivity. Chiral derivatizing agents are used to derivatize the enantiomers of amino acids for separation on achiral stationary phases by converting enantiomers to diastereomers. C_{18} stationary phase is used generally for these purposes. The most popular examples of chiral derivatizing agents (Fig: 1.15) are N_{α}-(2, 4-dinitro-5-fluorophenyl)-L-alaninamide (FDAA, Marfey's reagent), N_{α}-(2,4-dinitro-5-fluorophenyl)-L-valinamide, N_{α}-(2,4-Dinitro-5-fluorophenyl)-D-valinamide, N-Acetyl-L-cysteine, N-Isobutyryl-L-cysteine, N-isobutyryl-D-cysteine and Boc-Cys-OH. Some times phthaldialdehyde used in combination with chiral derivatigng agents to enhance the fluorescence sensitivity. Table: 1.3 gives a comparison of advantages and disadvantages of direct and indirect chiral separation techniques.

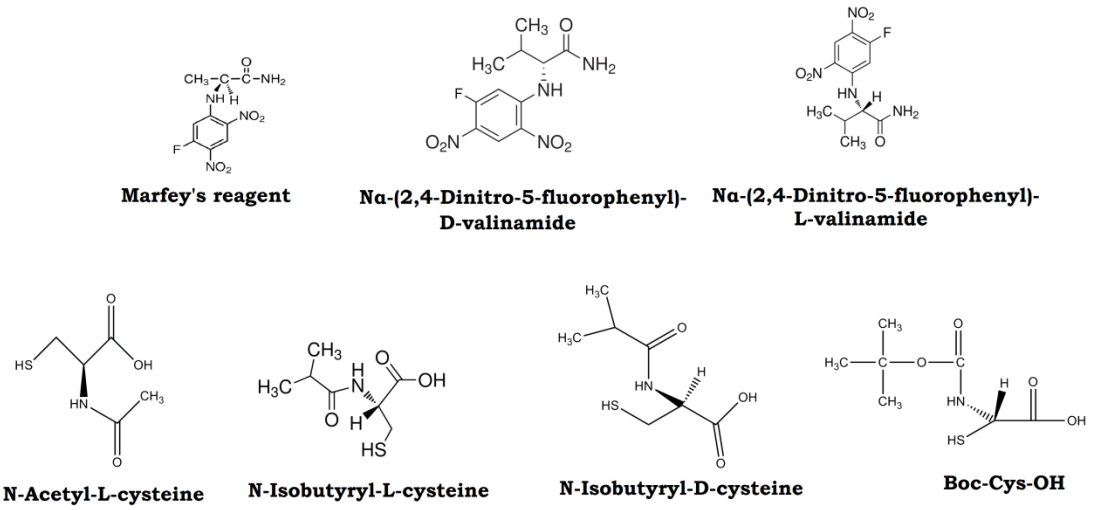


Fig: 1.15 Chemical structures of some of the chiral derivatizing agents

Table: 1.3 Advantages and disadvantages of direct and indirect chiral separation techniques.

Indirect methods	Direct methods
Advantages	
1. Good chromatographic properties of derivatives	1. The purity of the chiral selector is not critica
2. Elution sequence predictable	2. Similar molar absorptivities of enantiomers
3. Good chromophoric or fluoro phoric properties of the reagent (enhanced sensitivity can be achieved)	3. Absence of racemization and kinetic resolution
4. Low cost of achiral columns	4. Racemates without functional groups are separable
5. Method development is simple	5. Preparative applications are available
6. Selectivity can be increased (better separation is often achieved than with a direct method)	6. Change of temperature may be favorable
7. Possibility of appropriate selection of the elution sequence	7. Simple preparation of analytes and simple chromatographic runs

Disadvantages	
1. The purity of the CDA is critical	1. The theoretical plate number of the CSP is small
2. The molar absorptivities of the diastereomers may differ	2. Slow kinetics of desorption
3. The possibility of racemization	3. The elution sequence is not clear
4. The possibility of kinetic resolution	4. No universal column exists
5. The excess of reagent and side products may interfere with the separation	5. CSPs are very sensitive to chromatographic conditions (rather difficult and lengthy method development)
6. Preparative application is restricted	6. High cost of chiral columns
7. Derivatization may be time-consuming	7. Less time consuming

1.3 Statement of the problem

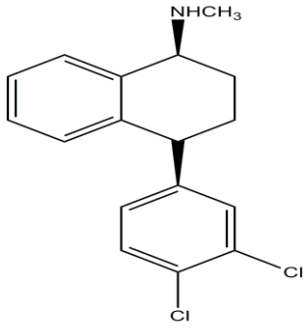
It is evident from the preceding sections that the development of new chiral separations for assurance of quality and safety of chiral drugs and pharmaceuticals is quite important because of these materials are considered to be not only healthcare products but also lifesaving substances. Because of chirality, living organisms show different biological responses to one of a pair of enantiomers in drugs, pesticides, or waste compounds, etc. In development of new chiral drugs, the determination of purity is important in order to establish the acceptability of batches for safety assessment and clinical trials. These activities are considered to be the primary objectives of chiral drug manufacturers. Modern analytical techniques, particularly the chromatographic techniques permit chiral analysis to be carried out

more rapidly in complex matrices than conventional methods. Further, micro quantities of substances are sufficient for carrying out the investigations. Today, the analytical methodology is in a period of transition due to technological advances and modified policies of the governmental regulatory agencies. Presently, a majority of commercially available drugs are both synthetic and chiral. However, a large number of chiral drugs are still marketed as racemic mixtures. Nevertheless, to avoid the possible undesirable effects of a chiral drug, it is imperative that only the pure, therapeutically active form be prepared and marketed. Hence there is a great need to develop the technology for analysis and separation of racemic drugs. Liquid chromatographic chiral separations have proven to be one of the best methods for the direct separation and analysis of stereoisomers. It is more versatile than chiral GC because it can separate a wide variety of nonvolatile compounds. Keeping this in view, the present work has been proposed at LC and LC/MS studies on development of enantiomeric separations for analytical and bio analytical applications of some of the selected chiral drugs, viz; the anti-depressants sertraline, mirtazapine, anticonvulsant pregabalin and anti-retro viral darunavir.

1.4 Aims and objectives

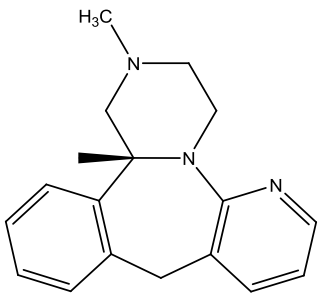
1. To develop and validate a highly sensitive and selective on-line two-dimensional reversed-phase liquid chromatography/ electrospray ionization–tandem mass spectrometric (2D-LC ESI /MS/ MS)

method to determine sertraline (SRT) enantiomers in rat plasma. To develop a column switching method for extraction of steroisomers of SRT from rat plasma, in which the system consisted of RAM column in first dimension for trapping proteineous part of plasma and a chiral Cyclobond column as second dimension for separation of enantiomers and diastereomers of SRT. To apply the developed method to separate and determine the diastereomers as well as enantiomers of SRT simultaneously.

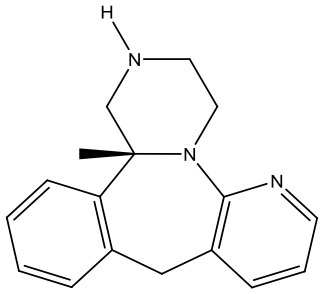


Sertraline

2. To develop and validate a simple and rapid reverse polar ionic LC method for simultaneous enantiomeric separation and determination of mirtazapine, an antidepressant drug, and its main metabolite N-desmethyl mirtazapine. To explore the enantiomeric separation on CHIROBIOTIC V column packed with vancomycin as a stationary phase in an isocratic elution mode. Detection of enantiomers using fluorescence detector and determination of optical rotation (+/−) of the enantiomers by polarimetric detector connected in series. Application of the developed method to invitro rat plasma study.

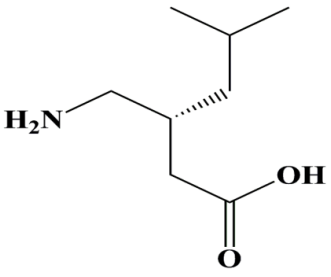


Mirtazapine



Desmethyl mirtazapine

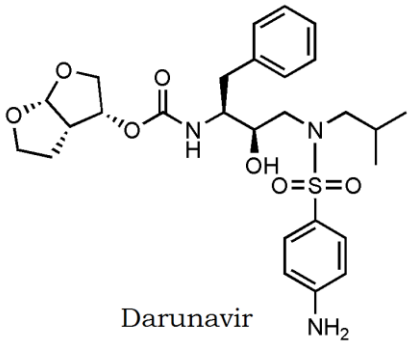
3. To develop and validate an indirect chiral reversed-phase HPLC fluorescence method for the separation of PRG enantiomers on dried blood spots. To explore the derivatization of racemic PRG with chiral derivatizing agent OPA with NAC under alkaline conditions and separation of converted diastereomers on achiral column. To apply the developed HPLC-FL method on dried blood spots to demonstrate its suitability for analysis of plasma samples collected from healthy Wistar rats received single oral dose of 5 mg/Kg of racemic PRG.



Pregabalin

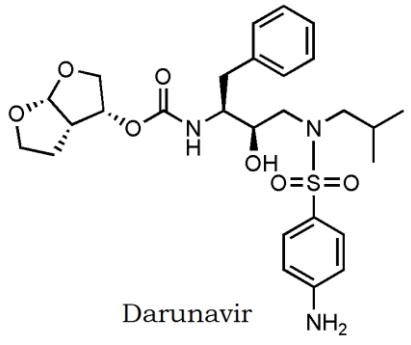
4. To investigate the separation of DRV enantiomers on coated CHIRALPAK AD-H, immobilized CHIRALPAK IA and CHIRALPAK IC CSPs using different chromatographic conditions. Comparison of the separations of DRV enantiomers on coated (CHIRALPAK AD-H) and immobilized (CHIRALPAK IA and IC) CSPs on the basis of the chiral

selector) using a range of mobile phase solvents composed of different organic modifiers. To study the effect of temperature by using chromatographic retention data to derive apparent thermodynamic parameters to determine the strength of interactions between DRV analytes and CHIRALPAK AD-H, CHIRALPAK IA and CHIRALPAK IC CSPs.



Darunavir

5. To develop and validate the stereo selective method for the DRV enantiomers and diastereoisomers on CHIRALPAK AD-H using different chromatographic conditions. To apply the developed method in thermodynamic study to explain the strength of interactions between stereoisomers of DRV and CSP. To apply the developed method in dried blood spots to investigate the chiral selectivity of enantiomers and diastereomers of DRV.



Darunavir

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CHAPTER 2

Two dimensional liquid chromatography-mass spectrometric determination of sertraline stereoisomers in rat plasma

2.1 Introduction

Sertraline hydrochloride (+)-cis-(1S,4S)-N-methyl-4 (3,4-dichlorophenyl)-1,2,3,4-tetrahydro-1-naphthalenamine hydrochloride (SRT) is a selective serotonin reuptake inhibitor (SSRI) used to treat major depression as well as obsessive-compulsive, panic and social anxiety disorders in both adults and children. It is as effective as tricyclic antidepressants with minimal side effects, such as insomnia, nervousness, nausea, diarrhea, dry mouth and dyspepsia [1]. Evidences suggest that SRT may work better when compared to fluoxetine (Prozac) for some subtypes of depression [2]. Treatment of depression with SRT results in a significant decrease of the number of panic attacks and an improved quality of life [3]. Although approved for social phobia and postraumatic stress disorder, SRT only leads to modest improvement in such conditions [4]. The molecule of SRT contains two stereogenic centers and available as four stereoisomers. During its synthesis, the enantiomer (−)-cis-(1R,4R), trans-(1S,4R) and diastereomers trans-(1R,4S)-N-methyl-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-1-naphthal enemine hydrochloride are produced in significant quantities (Fig: 2.1). Therefore, stereo selective separation and determination of SRT is important not only to assure its therapeutic efficacy but also safety.

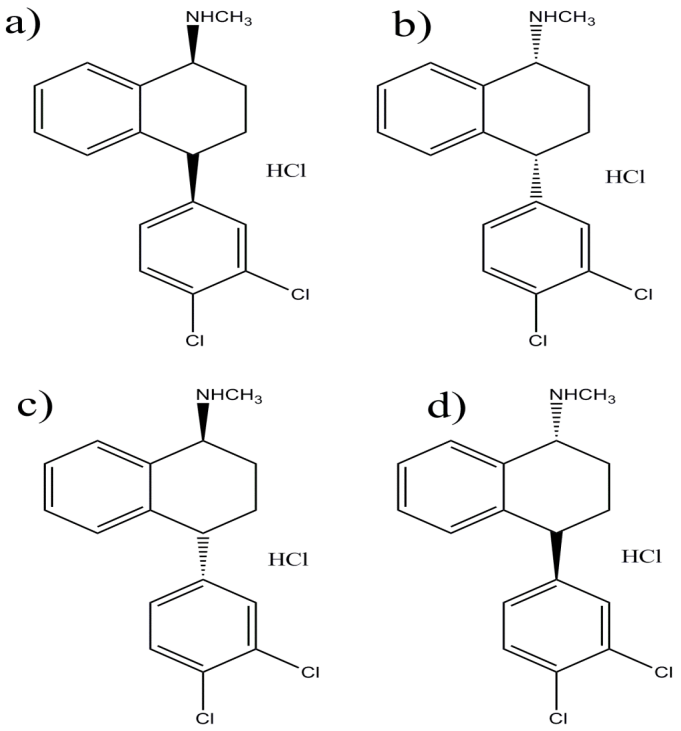

Fig: 2.1 Chemical structures of a) SRT and its stereoisomers b) (-)-cis-1R, 4R SRT, c) trans-1S, 4R SRT and d) trans-1R, 4S SRT

2.2 Literature survey

A wide variety of analytical methods including GC [5], GC–MS [6-8], LC [9-11] and LC–MS [12–16] for determination of SRT in plasma or serum were reported. These methods are suitable to determine SRT either alone or in combination of other drugs. Chung et.al have reported gas chromatography–mass spectrometric method for determination of sertraline in human plasma using selected ion-monitoring (SIM) mode [7]. Margarate et.al have developed a GC-MS method for determination of sertraline and its main metabolite, desmethylsertraline in human serum [8]. Tournel et.al have described HPLC screening method for

determination of seven SSRIs [9]. Several LC methods to determine SRT and its main metabolite N-desmethylsertraline in plasma or serum were reported [10, 11]. Stereoisomers of SRT and its related enantiomeric impurities were separated in bulk drugs and formulations either directly on a dimethyl β-cyclodextrin stationary phase [17] or indirectly using hydroxypropyl *β*-cyclodextrin as a mobile phase additive [18] by HPLC. Foley and Zhou [19] have separated the enantiomers of SRT by CE using sulphated β-cyclodextrin as a chiral selector. However these methods do not address the separation of stereoisomers of SRT in biological fluids. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) in positive-ion electrospray (±ESI) and selected-ion reaction monitoring mode was proved to be a valuable tool in characterization of several antidepressants including SRT and their N-desmethyl metabolites in both raw sewage and primary-treated wastewaters [20].

2.3 Sample preparation

Bio analytical procedures generally involve four major steps: sample preparation, analytical separation, detection and data handling. Sample preparation is often considered the most vital step. Proteins present in the matrix can precipitate or denature and adsorb onto the packing material, leading to back-pressure build-up, changes in retention time and decreased column efficiency. To avoid such problems, there is a need for development of new sample preparation techniques.

Some of the most commonly used sample preparation techniques include liquid–liquid extraction, protein precipitation and solid phase extraction.

2.3.1 Liquid–liquid extraction (LLE)

Liquid–liquid extraction is a method to separate analytes based on their relative solubilities in two different immiscible liquids, usually water and an organic solvent. It is one of the most commonly used sample preparation techniques in bioanalysis. However, LLE has several limitations such as low/variable recovery, the need for a large sample volume (≥1mL) and poor selectivity. Moreover, the other disadvantages could be production of emulsions with organic solvents; large quantities of organic solvents which are often expensive, toxic, carcinogenic and hazardous to the environment; a relatively low extraction efficiency and in some cases; insufficient reproducibility and possibility of contamination.

2.3.2 Protein precipitation (PP)

Protein precipitation (PP) is one of the earliest and least time-consuming sample preparation technique widely used in a bioanalytical laboratory. PP involves denaturation (loss of tertiary and secondary structures) of proteins present in biomatrix by external stress (such as a strong acid/base/heat or, most commonly, the use of an organic solvent such as acetonitrile/methanol [21]. Most of the bioanalytical methods employ addition of a minimum of three parts of organic solvent to one

part biomatrix, followed by cyclomixing and centrifugation. Centrifugation leads to formation of protein pellet and supernatant is separated for bioanalytical quantitation. As denaturation leads to a change in protein structure, the drugs/metabolites/ biomarkers bound to proteins become freely soluble in the denaturation solvent and ready for quantitation. Fig: 2.2 shows the steps involved in protein precipitation. The whole process of PP, however, is time-taking for a large number of samples as it has to handle manually, especially in drug discovery bioanalysis/ clinical bioanalysis.

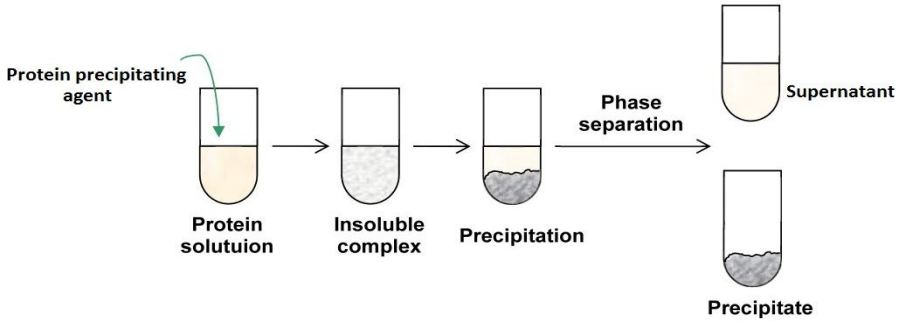

Fig: 2.2 Systematic procedure of protein precipitation (PP).

2.3.3 Solid phase extraction (SPE)

To overcome the limitations of the LLE and PP, one of the first developments in the field of sample preparation was SPE. It is based on the same principle of affinity-based separation as liquid chromatography [22]. Generally, the goals of SPE include retention and elution of analyte from biological fluid [23], removal of contaminants/interfering substances and sample concentration. Traditionally SPE is available in

normal phase, reverse-phase and ion-exchange modes; however, one of the most used formats was reversed-phase [24]. Fig: 2.3 shows the systematic procedure involved in solid phase extraction. Owing to different physicochemical properties (acids/bases/amphoteric, low/high log*P* and single or multiple p*K* values) of analytes of interest, the traditional formats were not always suitable and hence the different stationary phases and different approaches are available.

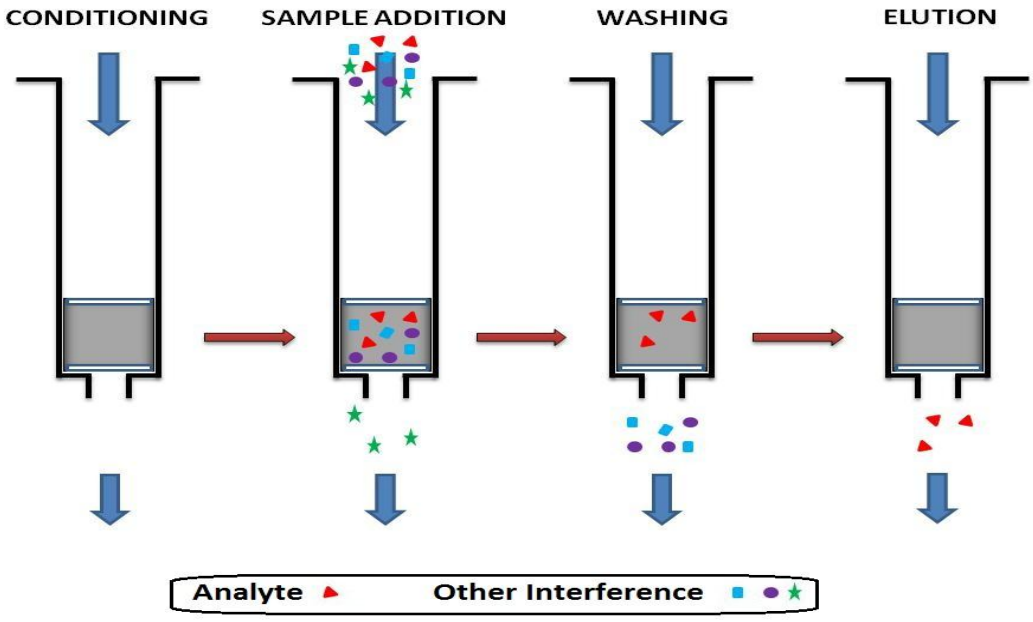

Fig: 2.3 Steps involved in solid phase extraction (SPE).

2.3.4 Two dimensional LC (2D-LC)

Sample preparation is often considered to be the time-limiting step as the extraction techniques such as LLE and SPE are achieved in the off-line mode, increase the total analysis time and reduce the recovery of the analytes of interest. Sample preparation is one of the most important

steps in HPLC analysis of drugs and their metabolites in biological fluids. Proteins in the biological fluids can precipitate or denature and adsorb onto the packing material, leading to an undesirable build-up of back pressure. To eliminate problems such as co precipitation of analytes during extraction and avoid the adsorption of protein onto the analytical column, direct injection of the sample using 2D-LC is becoming the method of choice [25–30]. Two-dimensional liquid chromatography (2D-LC) and 2D-LC coupled with mass spectrometry (2D-LC/MS, 2D-LC/MS/MS) have become popular techniques in bioanalytical chemistry, pharmacology and proteomic research and these systems generate excellent resolution, enabling the comprehensive separation of complex biological matrices. In order to perform a high throughput analysis, efforts have been made in developing faster sample purification procedures. Among different strategies, the introduction of restricted access media (RAM) allowing the direct and repetitive injection of complex biological matrices, represents an attractive approach. This will lead to the facility of direct injection of biological fluid samples into HPLC systems, automation, simplification and speeding up of the sample preparation process.

In the last few decades, 2D-LC has proved to be a useful approach for determination of drugs and metabolites in biological matrices [31-34]. The extraction supports used in sample pre-concentration are coupled to an analytical column allowing the separation of analytes before detection.

For this purpose, an additional pump and a switching-valve are required. A schematic representation of 2D-LC working in back flush mode is shown in Fig.2.4. During the extraction step, the switching-valve is in configuration A. The sample is injected into the extraction pre-column with the loading mobile phase. Simultaneously, the analytical column is conditioned with an elution mobile phase. After the extraction, the valve is switched to configuration B. Analytes are eluted in backflush mode from the extraction support with the mobile phase and transferred to the analytical column and the valve is switched to its initial configuration (configuration A). Analytes are separated onto the analytical column and directed to the detector. Simultaneously, the pre-column is re-equilibrated with the loading mobile phase. Finally, the system is ready for the next sample injection. The specific advantages of 2D-LC over 1D-LC include (i) direct injection of plasma, (ii) on-line sample preparation, (iii) no contact with toxic solvents, (iv) no need of extraction, (v) increased column efficiency, (vi) a huge increase in peak capacity, (vii) no co precipitation of analytes with proteins, (viii) no adsorption of protein onto the analytical column and (ix) avoids extraction losses during evaporation and reconstitution steps. 2D-LC could be performed either on-line or off-line modes. This approach minimizes sample losses, which could be advantageous for improving sensitivity compared to the off-line mode

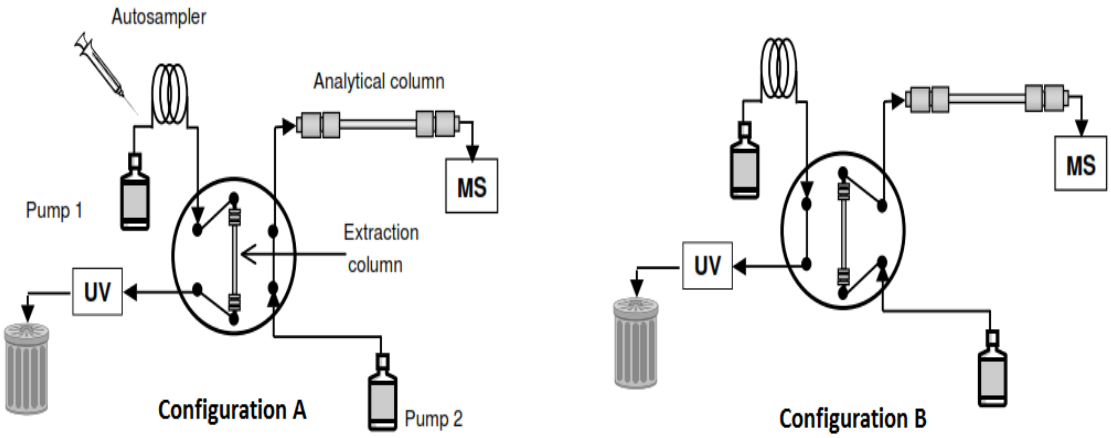

Fig: 2.4 Schematic representation of 2D-LC in configurations A and B.

2.4 Restricted access media (RAM) column

The restricted access media was introduced by Desilets et al. in 1991 [35]. It designates a support family which allows direct injection of biological fluids by limiting the accessibility of interaction sites within the pores to small molecules only (e.g. drugs, endogenous substances and xenobiotics). Macromolecules (most frequently proteins) are excluded and interact only with the outer surface of the particle support coated with hydrophilic groups, which minimises the adsorption of matrix proteins. RAM HPLC columns eliminate the sample clean-up and can be used as a pre-column for the direct injection of biological samples such as serum and plasma. In most cases, they function as a pre-cloumn, in combination with an analytical column in 2D-LC, to remove proteinaceous materials prior to chromatographic separation. RAM columns are characterized by hydrophilic/ hydrophobic, ion-exchange or size exclusion mechanisms. The hydrophilic phase covers the outer

surface of a sorbent and large biomolecules such as proteins are restricted from the adsorptive surfaces inside silica particles. The hydrophobic or ion-exchange phases cover the internal surface of sorbent pores where small analyte molecules are able to penetrate into pores and interact with a stationary phase bonded on the inner surface. As a result, protein molecules pass through the column rapidly and analytes of interest are retained on the adsorptive sites. Depending up on the application, the analyte molecules are directed to MS detection or transferred onto an analytical column for separation prior to MS detection [36-38]. Schematic representation of an embedded network shielded hydrophobic phase is shown in Fig: 2.5.

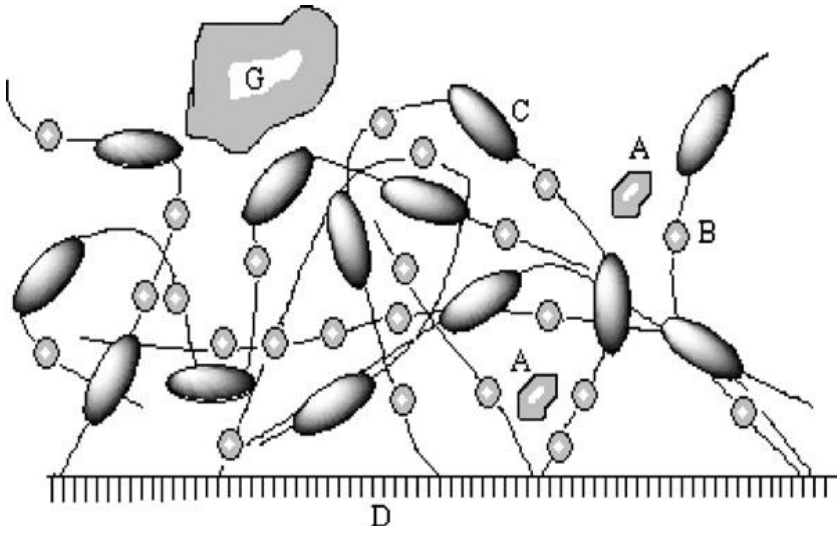

Fig: 2.5 Schematic representation of an embedded-network shielded hydrophobic phase, (A) small analyte molecule; (B) hydrophilic sites; (C) hydrophobic sites; (G) protein; (D) support matrix.

2.5 Objectives of the work

i. To develop and validate a highly sensitive and selective on-line two-dimensional reversed-phase liquid chromatography/ electrospray ionization–tandem mass spectrometric (2D-LC ESI /MS/ MS) method to determine SRT stereoisomers in rat plasma.

ii. To develop 2D-LC methodology for extraction of steroisomers of SRT in rat plasma, in which the system consists of a RAM column in first dimension for trapping proteineous part of plasma and a chiral Cyclobond column as second dimension for separation of stereoisomers of SRT.

iii. To investigate the enantioselective pharmacokinetics of SRT by applying the developed method to a single dose pharmacokinetic study by administering racemic SRT to Wistar rats.

2.6 Experimental

2.6.1 Chemicals and reagents

Racemic mixture of SRT hydrochloride ((±)-cis-N-methyl-4- (3,4-dichlorophenyl)-1,2,3,4-tetrahydro-1-naphthalenemine hydrochloride), (+)-cis-(1S,4S)-N-methyl-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-1 naphthalenemine hydrochloride, (−)-cis- (1R, 4R)-N-methyl-4-(3, 4-dichlorophenyl)-1, 2, 3, 4-tetrahydro-1- naphthalenemine hydrochloride, trans-(1S,4R)-N-methyl-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-1-naphthalenemine hydrochloride, trans-(1R,4S)-N-methyl-4-(3,4-

dichlorophenyl)- 1,2,3,4-tetrahydro-1-naphthalenemine hydrochloride were procured from a local pharmaceutical industry. Purified de-ionized water (Nanopure, Barnstead, USA), HPLC-grade acetonitrile, methanol, trifluoroacetic acid (Qualigens Fine Chemicals, Mumbai, India), ammonium acetate (S.D. Fine Chem., Mumbai, India) were used. The blood samples used for the development and validation of the analytical method were obtained at regular intervals of time for 24 h from male Wistar rats weighing 170±10 g housed one animal per cage under standard conditions. The required environment was controlled with daily feeding of standard chow pellets and water ad libitum.

2.6.2 Instrumentation

The LC system LC-MSD Agilent 1100 series (Agilent Technologies, Waldbronn, Germany) consisting of a binary LC pump, a vacuum degasser, a temperature-controlled microwell plate auto sampler set at 4˚C and a thermostatted column compartment set at 35°C. The compounds were analyzed on a Astec Cyclobond™ I 2000 DM (250×4.6mm, 5µm) (Supelco, PA, USA) column, under isocratic conditions using a mobile phase containing 0.1% aqueous trifluoroacetic acid:acetonitrile (86:14, v/v) at a flow rate of 0.8 mL/min protected by a guard column Hisep-RAM (50×4.0mm, 5µm) (Supelco, USA) under isocratic conditions using a mobile phase containing 0.02M aqueous ammonium acetate (pH 8):acetonitrile (86:14, v/v) at a flow rate of

1mL/min. The analytes were monitored by a mass spectrometer equipped with an electrospray ionization interface, operated in a positive mode (+ESI). Nitrogen was the nebulizer and curtain gas. Collision induced dissociation was achieved using nitrogen as collision gas. The ion source conditions were: temperature 325°C, nebulizer gas pressure 35 psi, dry gas 8.0 L/min, ion spray voltage 3500 V, collision energy 0.8 A, declustering potential 5.0V (lens 1), 60.0V gas (lens 2), entrance potential 40.0V and collision exit potential 113.5 V. The data was captured using a Chemstation software. Transition of m/z 306→274.7 was used for detection of SRT.

2.6.3 Preparation of rat plasma standards

The calibration standards (CS) and quality control samples (QC) were prepared by spiking blank plasma with working solutions of analytes. Calibration standards were at 0.5, 1.0, 2.0, 4.0, 10.0, 20.0, 40.0, 100, 150, 200 ng/mL for all the analytes. A calibration curve was constructed using 200µL plasma of each standard. Quadratic regression equation with peak area against concentration of SRT was used for quantification of unknown concentration of SRT enantiomers in rat plasma. Quality controls were prepared at 0.5, 1.5, 14.0, 150, 200 ng/mL for all analytes and used for determination of accuracy and precision in determination of SRT stereoisomers in rat plasma. The spiked plasma samples at all the levels were stored at −20 ◦C.

The standard stock solutions of 100µg/mL for (±)-cis-SRT, (+)-cis-(1S,4S) SRT, (−)-cis-(1R,4R) SRT, trans-(1S,4R) SRT and trans-(1R,4S) SRT hydrochloride were prepared by dissolving requisite amounts in methanol:water (30:70, v/v). The stock solutions were further diluted with water appropriately to get an intermediate concentration of 4µg/mL. The working solutions of all compounds for spiking calibration and quality control samples were subsequently prepared from standard and intermediate stock solutions. All the standard stock, intermediate stock and working stock solutions were prepared and stored at 4°C until use.

2.6.4 Sample preparation

Frozen plasma samples were thawed at room temperature before processing. The blood samples were centrifuged for 15 min at 4500 rpm. Supernatant was collected, filtered through 0.45µ nylon membrane filter, vortexed for 10 min and directly injected a 20µL aliquot into 2D-LC/MS/MS containing RAM and chiral Cyclobond columns. All plasma samples including calibration curve, QC and samples for pharmacokinetic experiments were prepared as above.

2.6.5 Dosing and sampling

The method was applied to investigate enantioselectivity in the pharmacokinetics of SRT administered in the racemic form in a single dose to rats. The animals received an aqueous solution of (±)-rac-SRT

hydrochloride (25 mg/kg) by oral administration. Blood samples were collected at times 0, 1, 2, 4, 6, 8, 10, 24 h after drug administration. Plasma samples were stored at −70°C until analysis.

2.6.6 Chromatographic conditions

2D-LC–ESI/MS/MS

LC system	: LC-MSD Agilent 1100 series
Ionization and mode	:+ESI
Nebulizer and curtain gas	: Nitrogen
Pressure of nebulizer gas	: 35psi
Temperature of ion source	: 325°C
Flow rate of dry gas	: 8Lmin^{-1}
Ion spray voltage	: 3500 V
Collision energy	: 0.8 A
Declustering potential	: 5.0 V (lens 1); 60.0 V (lens 2)
Entrance potential	: 40.0 V
Collision exit potential	:113.5 V
Temperature of auto sampler	: 4°C
Injection volume	: 20µL

Configuration A

Column	: CYCLOBOND™ I 2000 DM (250x4.6mm, 5µ particle size)
Mobile Phase	: 0.1% aq. TFA: ACN (86:14, v/v)

Flow rate	: 0.8 $mLmin^{-1}$
Column Temperature	: 35°C
Detector	: Mass detector

Configuration B

Column	: RAM column
	(50×4.0mm, 5µ particle size)
Mobile Phase	: 0.02M aq.ammonium acetate
	(pH= 8): ACN (86:14, v/v)
Flow rate	: 1 $mLmin^{-1}$
Column Temperature	: 25°C
Detector	: Mass detector

2.7 Results and discussions

The present chapter describes the importance of extraction procedure by 2D-LC as well as stereo selectivity of SRT stereoisomers on β-Cyclodextrin based cyclobond column using LC/MS/MS. The role of restricted access media (RAM) column in 2D-LC extraction procedure and application of the developed method was applied to a pharmacokinetic study to investigate the enantioselectivity of SRT by oral administration of racemic SRT to Wister rats is also described.

2.7.1 Method development

Initially normal phase screening conditions were tried on CHIRALPAK AD-H (250x4.6mm, 5µm) and CHIRALCEL OD-H (250x4.6mm, 5µm) columns. Separation of SRT stereoisomers was not achieved on both the columns. CHIRAL-AGP (150×4.0mm, 5µm) and CHIROBIOTIC V (150×4.6mm, 5µm) columns in reverse phase using 0.1% aqueous acetic acid with organic modifiers, viz., methanol and acetonitrile of different composition as mobile phase were tried. However, the trails did not result in good separation of the analytes. Fig: 2.6 shows the separation of stereoisomers of SRT on respective columns.

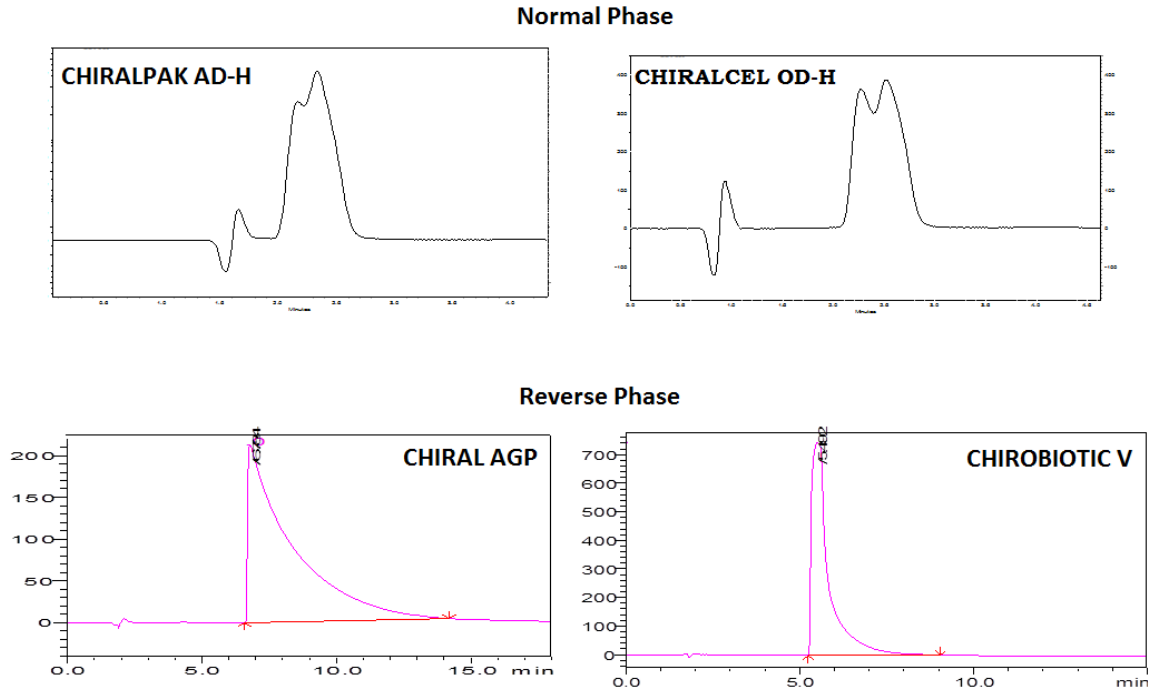

Fig: 2.6 Chromatograms showing the separation of stereoisomers of SRT on CHIRALPAK AD-H, CHIRALCEL OD-H, CHIRAL-AGP and CHIROBIOTIC V columns.

SRT and related substances are polar in nature and fairly soluble in methanol and water. The reversed-phase mode offers greatest possibilities for selectivity by taking advantage of the inclusion mechanism for which cyclodextrins are well known [18]. So reverse phase conditions were tried using a Cyclobond column which is useful for analytes having ring structures includable into the cyclodextrin cavity and form hydrogen bonds. Both the architecture and chemistry of cyclodextrins contribute to enantiomer separations. The toroidal cyclodextrin structure has a hydrophilic exterior surface resulting from the 2-, 3-, and 6-position hydroxyl (OH) groups. The interior cyclodextrin cavity is composed of the glucose oxygens and methylene hydrogens, which gives it a non-polar (hydrophobic) character. Chiral recognition mechanism and chemical interactions that lead to chiral separations occur on both the exterior and interior surfaces of the cyclodextrin toroid was given in chapter 1. The proposed structure of β-cyclodextrin is shown in Fig:2.7.

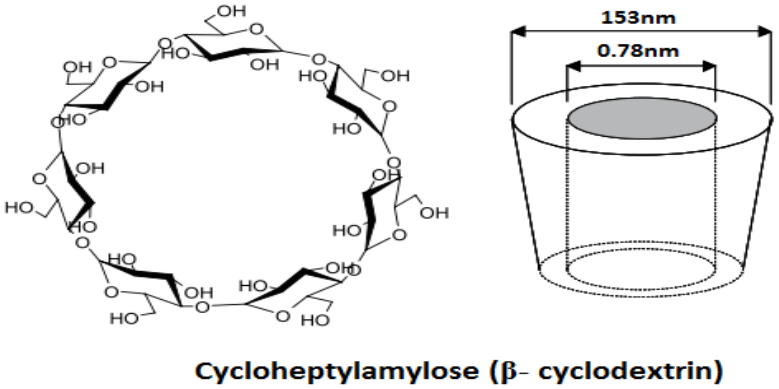

Fig: 2.7 Structural representation of β-cyclodextrin.

A mobile phase containing 0.1% aqueous trifluoroacetic acid:acetonitrile (86:14, v/v) at a flow rate of 0.8 mL/min was used in an isocratic elution mode at room temperature, but all the stereoisomers were not separated. However at elevated column temperatures, i.e. 35°C it was found that all the analytes were well separated. In order to use the 2D-LC technique for the online extraction of SRT in rat plasma, different trails for the separation of SRT stereoisomers on RAM column were carried out. Initially different mobile phase conditions were tried on RAM column to separate SRT stereoisomers. 0.05M aqueous ammonium acetate with different proportions of acetonitrile and 0.05% aqueous acetic acid with acetonitrile of different compositions were tried. These conditions did not result in good separation of the analytes. Isocratic condition with a mobile phase containing aqueous ammonium acetate 0.02M pH 8: acetonitrile (86:14, v/v) at a flow rate of 1mL/min was successful. Depending upon the retention times of the analytes on RAM and Cyclobond columns the method was divided into three steps. The configuration of the system was changed in different steps for excluding proteins through RAM column, separation of analytes through Cyclobond column and conditioning of both columns. The 2D-LC system used for coupling of RAM and Cyclobond columns is shown schematically in Fig: 2.4. The sequential steps used are listed in Table 2.1.

The rat plasma of 20µL sample volume was applied when the system was in configuration A (0–2.5 min) (Fig: 2.4). In this

configuration, the mobile phase (0.02M aqueous NH_4OAc: ACN, 86:14, v/v) was delivered by pump 1 at a flow rate 1.0 mL/min to elute the proteins from RAM column to waste. At the same time the Cyclobond column was conditioned by the mobile phase (0.1% aqueous TFA: ACN, 86:14, v/v) delivered by pump 2. The analytes were retained on the hydrophobic phase of the RAM column. In configuration B, (2.5–10 min) (Fig: 2.4) the mobile phase was delivered by pump 2 at a flow rate 0.8 mL/min and the analytes from RAM to Cyclobond column were eluted. After 10 min the system was changed again to configuration A (Fig: 2.4). In this configuration the mobile phase delivered by pump 2 elutes SRT stereoisomers from Cyclobond column while RAM column was conditioned by the mobile phase delivered by pump 1.

Table 2.1 Sequential steps of on-line 2D-LC.

Step	Time interval (min)	Configuration	Analytical operation
1	0–2.5	A	Exclusion of plasma proteins by RAM column and conditioning of Cyclobond column
2	2.5–10	B	Elution of retained SRT stereoisomers from RAM to Cyclobond
3	10–16	A	Elution of SRT stereoisomers from Cyclobond and conditioning of RAM column

The role of the RAM column was to remove the proteins. It could be characterized by the hydrophilic outer and hydrophobic inner phases which exclude large molecules such as proteins in the void volume while retain selectively the small hydrophobic analytes [39] as discussed in section 2.4. The stationary phase in RAM was a porous chromatographic support specifically designed for the removal of macromolecules, partially based on a size exclusion mechanism [40]. Only small molecules penetrate into the pores and interact with the stationary phase bound to the inner surface. During the analyses, the performance of the RAM column was found to be stable with over 500 plasma injections without significant change in the back pressure. Initially the back pressure of the RAM column was 40 kgf/cm^2 and after analyses of around 500 samples it became 60 kgf/cm^2. The elution order was (+)-cis-1S,4S (−)-cis-1R,4R, trans-1S,4R, trans-1R,4S SRT hydrochloride and was confirmed by injecting individual samples. Typical chromatograms of (a) standard calibration plasma spiked with (+)-cis-1S,4S, (−)-cis-1R,4R, trans-1S,4R, trans- 1R, 4S, (b) SRT enantiomers in rat plasma at 6th hour after oral administration of rac-SRT to Wistar rats and (c) blank plasma are shown in Fig: 2.8a–c. It could be seen from Fig: 2.8 that the trans 1S,4R and 1R,4S isomers were not found in rat plasma (Fig: 2.8b). It was due to the administration of rac SRT containing only the enantiomers of cis-1S,4S and -1R,4R SRT into the Wistar rats. This was studied because of the SRT formulations generally contain enantiomers of cis-1S,4S and -1R,4R

selectively. Further it could be seen that there was no peak corresponding to the metabolite of SRT in the chromatogram of rat plasma (Fig: 2.8b). Probably it might have formed in very traces after 6th hour of injection. However there was no peak detected at 274 corresponding the N-desmethylsertraline. It could be due to two possibilities (i) its concentration could be very much below the detection limit or (ii) it might be not eluted from the column under the conditions used for separation.

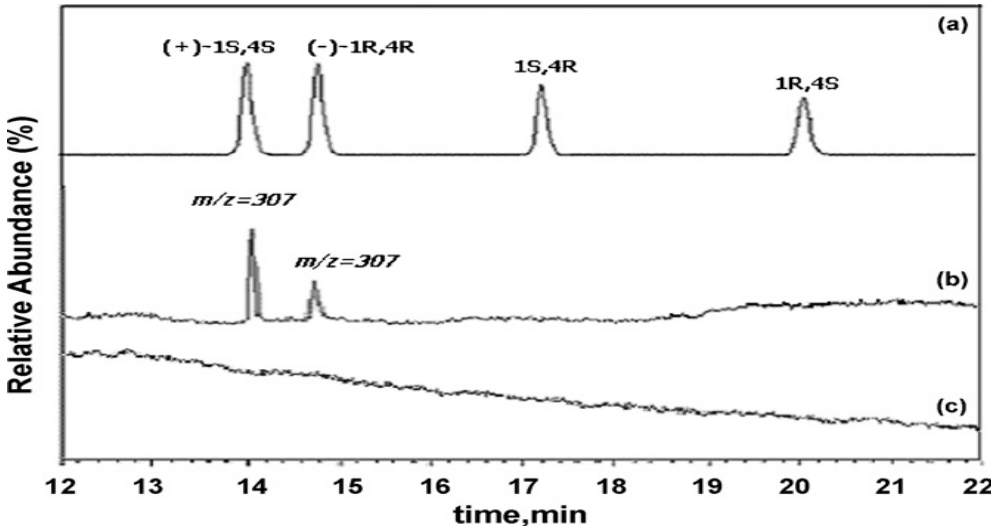

Fig: 2.8 Typical LC chromatograms showing the (a) separation of 50 ng/mL of (+)-cis-1S,4S, (-)-cis-1R,4R, trans-1S,4R, trans-1R,4S SRT hydrochloride. (b) SRT enantiomers (cis-1S,4S, cis-1R,4R) in rat plasma at 6th hour after oral administration of 25 mg/kg (±)-rac-SRT to Wistar rats. (c) Blank plasma

The mass spectrometric detection of SRT was investigated by ESI in (+ve) SRM mode. In the full scan MS/MS chromatogram (Fig: 2.9a), all analytes formed predominately protonated molecules $[M+H]^+$ at m/z 307

and base peak at m/z 274.7. The base peak at m/z 274.7 is due to removal of -NH_2CH_3 group from molecular ion as shown in the Fig: 2.9. On further fragmentation all analytes gave fragments at m/z 158.7 (base peak), m/z 129 and m/z 91 in the full scan of MS/MS^2 chromatogram (Fig: 2.9b). The fragment m/z 158.7 is due to the removal of benzene ring having two chlorine groups from the molecular ion. The fragment m/z 129 is due to removal of groups -NH_2CH_3 and benzene ring having two chlorine groups from the molecular ion. The fragment m/z 91 is due to maclaferty rearrangement from the molecular ion. The proposed fragmentation pathways during MS/MS of the $[M+H]^+$ ions of all the stereoisomers of SRT are shown in Fig: 2.10.

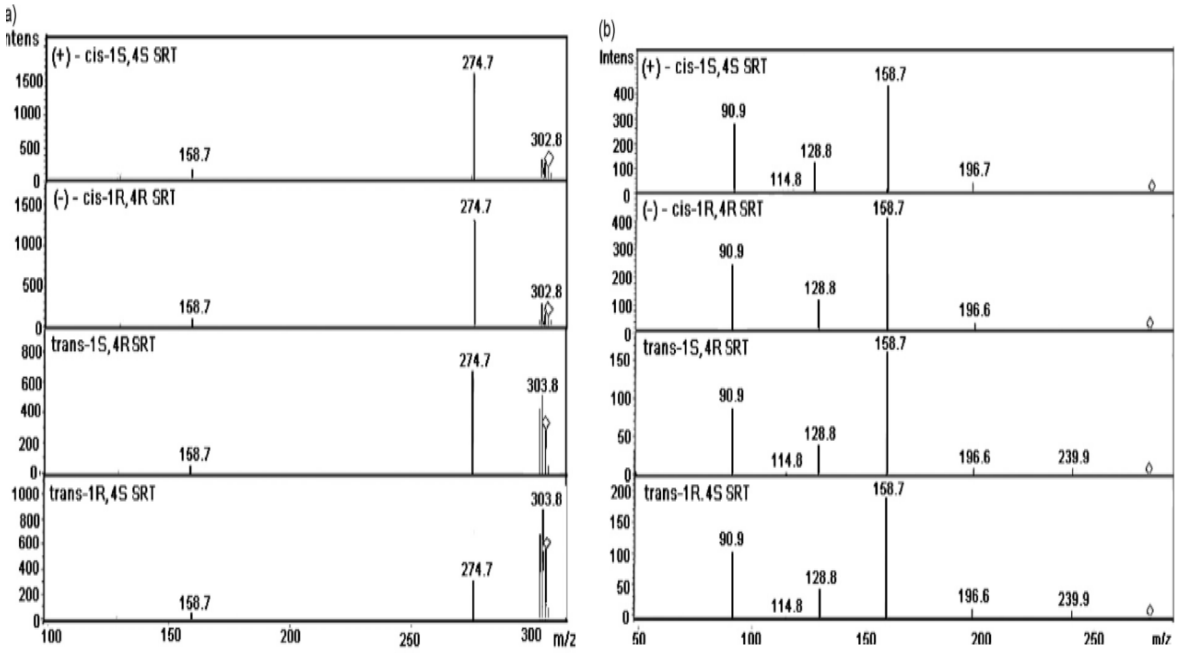

Fig: 2.9 a) MS/MS and b) MS/MS^2 spectra of 50 ng/mL of SRT stereoisomers

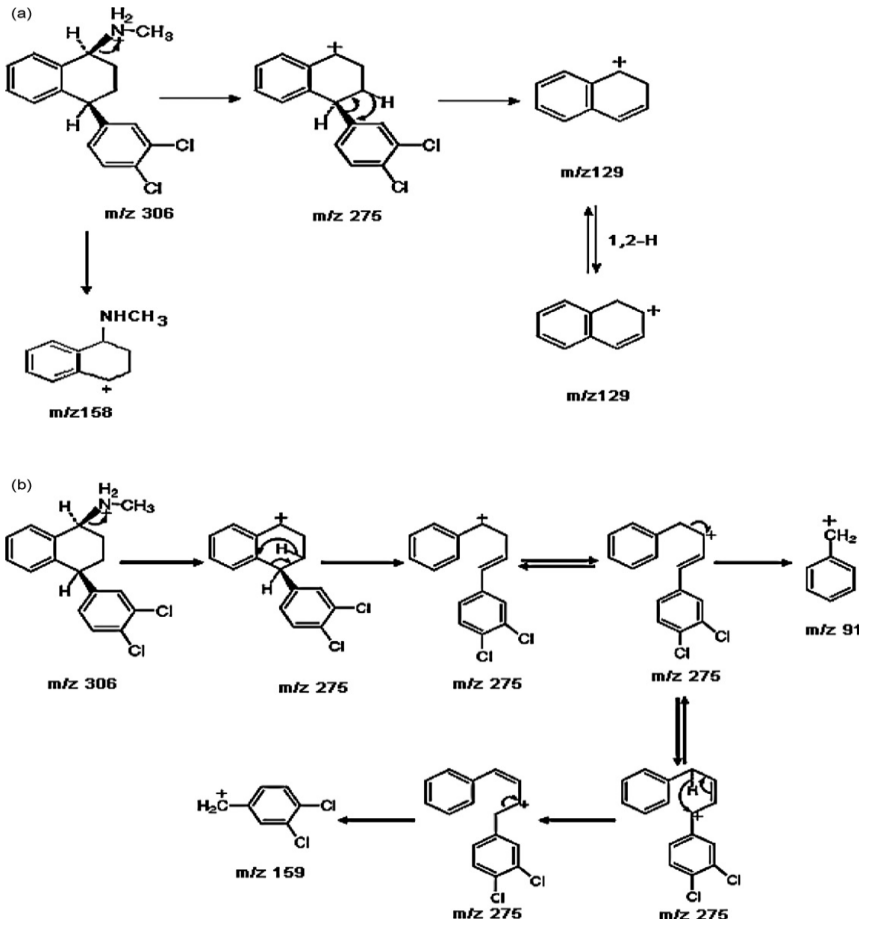

Fig: 2.10 Fragmentation pathways of SRT stereoisomers (a) MS/MS and (b) MS/MS^2.

2.7.2 Validation

2.7.2.1 Selectivity

The selectivity of the method was investigated for potential interferences of endogenous substances by using six independent batches of rat plasma (blank). The chromatograms of the experimental samples obtained after administration of rac-SRT to Wistar rats were compared with standard chromatograms in order to detect interfering

peaks. No interfering peaks were found at the retention times of SRT stereoisomers.

2.7.2.2 Accuracy and precision

The quality control samples (QC) were prepared by spiking blank plasma with working solutions of analytes at 0.5, 1.0, 14.0, 150, 200 ng/mL for all 4 analytes. The accuracy, intra- and inter-day precision of the analytical method were determined by replicate processing. Precision was calculated as coefficient of variation and accuracy as % relative error. The coefficient of variation obtained in the study of intra, inter assay precision and accuracy were less than 5% assuring the reproducibility and repeatability of the results. Table: 2.2 summarizes the data obtained in intra and inter batch accuracy and precision for SRT stereoisomers.

2.7.2.3 Linearity

The calibration standards (CS) were prepared by spiking blank plasma with working solutions of analytes at 0.5, 1.0, 2.0, 4.0, 10.0, 20.0, 40.0, 100, 150, 200 ng/mL for all 4 analytes. Plots of plasma concentrations vs peak areas were constructed and the linear regression lines were used for determination of concentrations of enantiomers in plasma samples (Fig: 2.11). Slopes, intercepts and correlation coefficients were calculated. The method showed linearity up to 200.0 ng/mL for all stereoisomers of SRT and the correlation coefficients observed were in

the range of 0.9993–0.9999 for all stereoisomers. Table: 2.3 summarizes the data obtained in recovery and linearity ranges of the SRT stereoisomers. The recovery was calculated by using the formula given in equation 2.1.

Recovery (%) = (area of spiked sample/area of standard) × 100 (2.1)

Table: 2.2 Intra and inter batch accuracy and precision in determination of SRT stereoisomers.

	Precision and accuracy			
Concentration ($ngmL^{-1}$)	(+)-cis-1S,4S SRT (%)	(−)-cis-1R,4R SRT (%)	trans-1S,4R SRT (%)	trans-1R,4S SRT (%)
Intra-assay precision; coefficient of variation (n = 10)				
0.5	3.02	2.45	2.17	3.01
1.5	4.45	3.96	2.17	2.85
14.0	3.53	3.13	4.38	1.05
150.0	1.06	4.62	1.92	3.94
200.0	2.05	2.97	2.84	4.47
Inter-assay precision; coefficient of variation (n=5)				
0.5	2.62	4.45	3.22	3.44
1.5	3.12	2.75	3.83	2.63
14.0	1.44	4.75	3.26	4.87
150.0	4.17	3.48	2.99	1.90
200.0	1.45	1.40	4.94	2.05
Intra-assay accuracy; relative error (n = 10)				
0.5	4.05	−2.04	−3.07	2.03
1.5	2.47	−3.68	1.79	−3.88
14.0	3.83	4.55	4.95	2.85
150.0	2.52	3.88	−4.87	3.85
200.0	5.22	−3.02	−2.46	1.58
Inter-assay accuracy; relative error (n=5)				
0.5	0.0	3.16	−4.64	−3.67
1.5	0.58	−1.99	−3.67	1.99
14.0	3.06	2.45	3.47	1.29
150.0	1.48	3.78	4.98	4.78
200.0	4.96	−3.88	1.58	1.38

Table: 2.3 Recovery and linearity ranges of the SRT stereoisomers

Concentration ($ngmL^{-1}$)	(+)-cis-1S,4S SRT	(−)-cis-1R,4R SRT	trans-1S,4R SRT	trans-1R,4S SRT
Recovery,% (n=3)				
1.5	90.6	98.7	99.1	89.9
14	99.0	96.0	98.7	95.6
150	95.0	94.9	97.6	98.8
Linearity				
Range	0.5–200	0.5–200	0.5–200	0.5–200
Regression equation	y = 3797.9x + 1297.5	y = 3789x + 2747.6	y = 3793.9x + 2344.6	y = 3796.9x + 1235.8
Correlation coefficient (r^2)	0.9999	0.9995	0.9998	0.9993

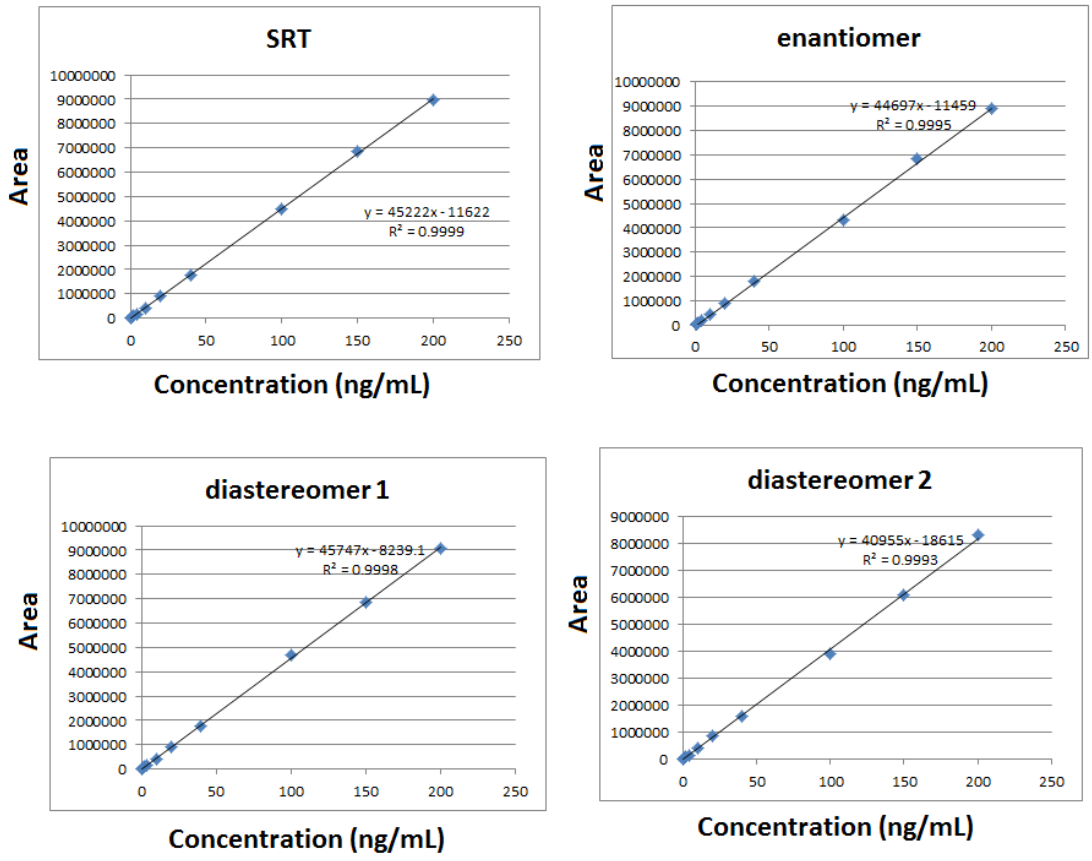

Fig: 2.11 Calibration curves for SRT and its stereoisomers.

2.7.2.4 Stability

The stability of analytes was determined during blood sample collection and handling at room temperature for 2 h and after freezing plasma samples for 1st day, 2nd day, 3rd day, 1 week,15 days and 1month. The results were compared with those obtained by freshly prepared samples. The results are summarized in Table: 2.4.

Table: 2.4 Intra- and inter-stability data of SRT (n = 3)

Storage conditions	Nominal concentration (ng/mL)	RSD (%)
Freeze/thaw stability	0.5	1.81
(three cycles)	14.0	1.69
	150.0	3.22
3 days	0.5	3.32
	14.0	2.75
	150.0	4.32
1 week	0.5	6.68
	14.0	3.22
	150.0	3.35
15 days	0.5	2.78
	14.0	3.57
	150.0	3.27
1 month	0.5	4.89
	14.0	4.47
	150.0	3.78

2.7.2.5 Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ were calculated according to the ICH guidelines. The limit of detection (LOD) (lowest concentration of analyte in a sample matrix that can be detected) and the limit of quantification (LOQ) (lowest that can be quantified with acceptable accuracy and precision) were 0.17 and 0.4 ng/mL. The total ion current (TIC) chromatograms at LLOQ concentration of (+)-cis-1S,4S, (−)-cis-1R,4R, trans-1S,4R, trans-1R,4S SRT hydrochloride are shown in Fig: 2.12, respectively.

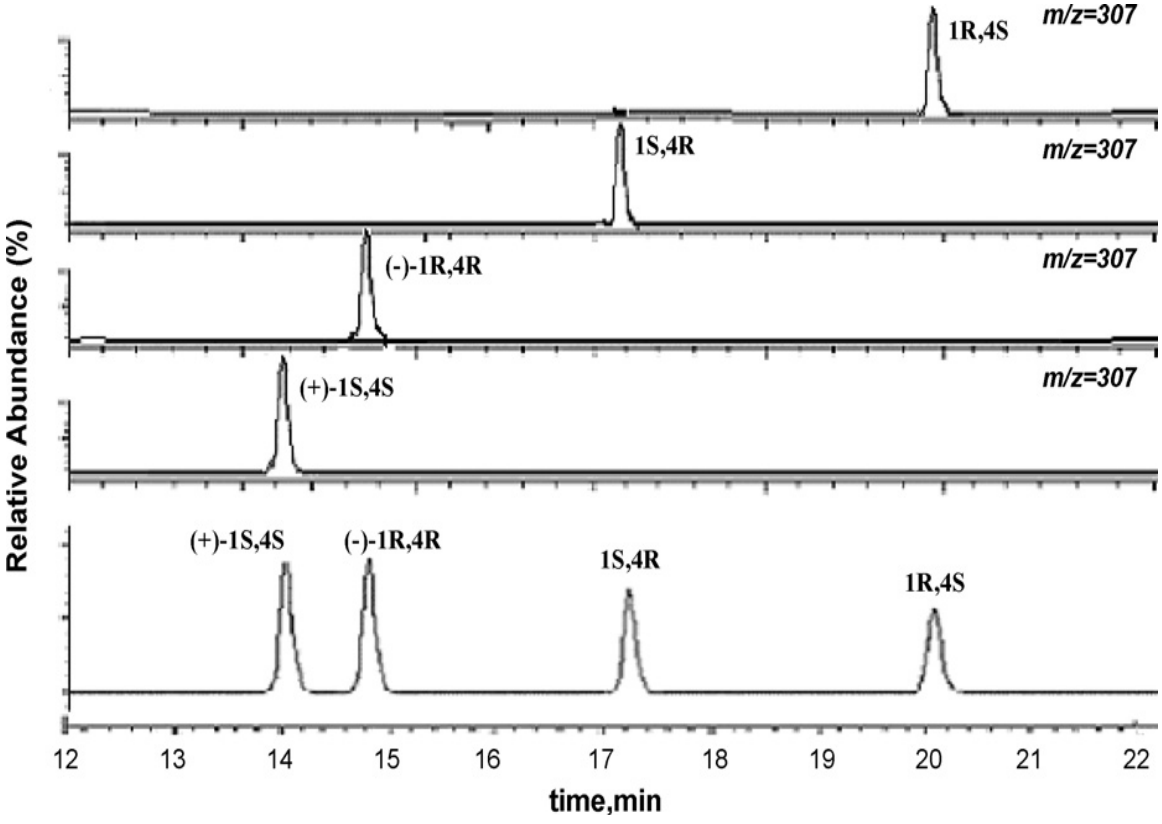

Fig: 2.12. Total ion current (TIC) chromatograms at LLOQ concentration of (+)-cis-1S,4S, (-)-cis-1R,4R, trans-1S,4R, trans-1R,4S SRT hydrochloride.

2.7.3 Application to a pharmacokinetic study in rats

The developed method was applied to study the enantioselectivity in kinetic disposition of SRT administered in the racemic form in a single 25 mg/kg dose to rats. The pharmacokinetics of SRT was enantioselective for the parameters $AUC_{0-\infty}$ (p = 0.68), clearance (p = 0.68) and C_{max} (p = 0.43) calculated by RAMKIN software (Table: 2.5) and p-values by Wilcoxon test. The data obtained for the Wistar male rats showed higher plasma concentrations of (+)-cis-1S,4S SRT enantiomer (Fig: 2.13). The higher $AUC_{0-\infty}$ values obtained for (+)-cis-1S,4S SRT enantiomer compared to (−)-cis-1R,4R SRT enantiomer (113.45 ± 7.45µg h/mL vs 108.69±13.47µg h/mL) could be explained by a lower apparent total clearance (222.21±14.29 mL/h vs 238.16±33.13 mL/h) (Table: 2.5). The kinetic disposition of SRT is enantioselective in male Wistar rats with a (+)/(−) plasma concentration ratio (AUC) close to 1.04. C_{max} was also higher for (+)-cis-1S,4S SRT enantiomer than (−)-cis-1R,4R SRT enantiomer (Fig: 2.13).

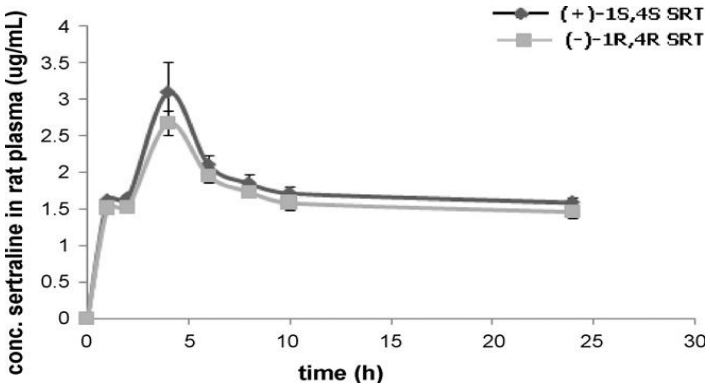

Fig: 2.13 Pharmacokinetic profiles for the cis enantiomers of (±)-SRT of 25 mg/kg by oral administration to rats (n = 6).

Table: 2.5 Pharmacokinetic parameters of (±)-SRT enantiomers after a single oral dose of 25 mg/kg (n = 6) for each time point.

Parameter	(+)-Cis-1S,4S SRT Mean ±S.E.M	(-)-Cis-1S,4S SRT Mean ±S.E.M
C_{max} (µg/mL)	3.09 ± 0.40	2.67 ± 0.16
t_{max} (h)	4.00 ± 0	4.00 ± 0
AUC_{0-24} (µgh/mL)	42.90 ± 2.29	39.38 ± 1.73
$AUC_{0-\infty}$ (µgh/mL)	113.45 ± 7.45	108.69 ± 13.47
$t_{1/2}$	31.02 ± 4.00	32.51 ± 4.37
Clearance (mL/h)	222.21 ± 14.29	238.16 ± 33.13
MRT (h)	45.57 ± 3.90	49.28 ± 6.17

S.E.M: Standard error of the Mean

2.8 Conclusions

A 2D-LC–ESI/MS/MS method for separation and determination of stereoisomers of SRT in rat plasma was established. 2D-LC and MS/MS detection was simple and accurate for determination of SRT stereoisomers in rat plasma. The method was sensitive enough to quantify the low concentration of 0.4 $ngmL^{-1}$ of SRT in rat plasma. The developed 2D-LC technique has only three operating steps, and easy to perform. It helps in maintaining the efficiency and the lifetime of the column. The method showed adequate sensitivity, linearity, precision and accuracy and it has been successfully applied to determine enantioselectivity and the concentration–time profiles in pharmacokinetic studies.

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CHAPTER 3

Evaluation of vancomycin as a chiral stationary phase connected to fluorescence and polarimetric detectors in series for enantiomeric separation of mirtazapine and its metabolite in rat plasma

3.1 Introduction

Mirtazapine [1,2,3,4,10,14b-Hexahydro-2-methylpyrazino-[2,1a] pyrido [2,3-c] [2-benzazepine] (MTZ) (Fig: 3.1) is a novel tetra cyclic anti-depressant used in the treatment of anxiety [1], obsessive–compulsive [2] and post-traumatic stress [3] disorders. MTZ also shows promise in preventing post-chemotherapy nausea and vomiting due to its anti-emetic effects. Chemically, MTZ is a piperazinoazepine which belongs to the class of noradrenergic and specific serotonergic antidepressants and its mechanism of action involve the increased release of serotonin and norepinephrine due to the antagonism on auto-receptors and α-adrenergic hetero-receptors. Its mechanism of action differs from that of second generation antidepressants and shows good efficacy in the treatment of patients who do not respond to the latter [4]. MTZ is available as film-coated tablets; the initial dose is 15–30 mg per day, which can be increased according to the therapeutic efficacy and the needs of the patient [5]. Typical plasma levels corresponding to the daily doses are in the range of 30–80 ng ml^{-1}. Since the plasma half-life of MTZ is about 20 h, it is usually taken once daily, immediately before going to sleep. After oral administration, MTZ is rapidly and completely absorbed, and then extensively bio-transformed in the liver [6]. The biotransformation of MTZ includes 8-hydroxylation, N-demethylation, N-oxidation, as well as direct conjugation of the drug with glucuronic acid and conjugation of its metabolites with glucuronic or sulphuric acid [7].

It is a stereo selective antagonist whose chirality plays an important role due to the potential differences activities and toxicities of drug enantiomers [8]. Its major metabolite, N-desmethyl mirtazapine (DMTZ) (Fig: 3.1) also contributes 3–6% to the total pharmacodynamic profile of the parent drug [9]. Thus it is of great importance to determine the plasma levels of the enantiomers of MTZ and DMTZ for therapeutic drug monitoring.

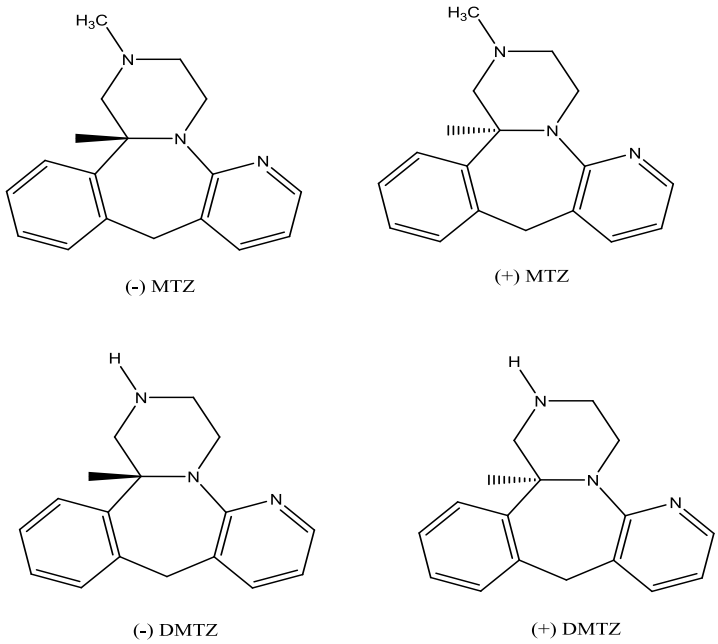

Fig: 3.1 Chemical structures of two enantiomers of MTZ and DMTZ

3.2 Literature survey

Several LC methods have been reported in the literature for analysis of MTZ with other antidepressants in various species [10-22]. Solid-phase microextraction using poly (pyrrole) film and LC with UV detection was reported for analysis of MTZ combined with other

antidepressants in plasma samples [10-12]. Micro extraction packed sorbent followed by liquid chromatography with UV detection was described for the determination antidepressants [13]. A tandem mass spectrometric investigation of the collision-induced dissociation of five commonly prescribed psychoactive pharmaceuticals, risperidone, sertraline, paroxetine, trimipramine, and MTZ, and their metabolites was also carried out [14]. Ultra-high performance liquid chromatography (UHPLC) tandem mass spectrometry (MS/MS) method was developed for rapid quantification of ten psychotropic drugs and metabolites in human plasma [15-17]. Analytical and semi preparative separation of enantiomers of MTZ and its metabolites were carried out on various polysaccharide chiral stationary phases by LC [18]. Off-line solid-phase microextraction [19] and liquid-phase microextraction [20] using porous polypropylene hollow fibre membrane were developed for simultaneous enantioselective determination of MTZ, DMTZ and 8-hydroxymirtazapine in plasma. Santana et al. studied the chromatographic separation of (+)-(S)- and (−)-(R)-enantiomers of MTZ on Chiralpak AD column [21]. Later it was applied to determine the enantiomers of MTZ in human plasma [22]. Mandrioli et al. have used CE for enantioseparation of MTZ and DMTZ in human plasma [23]. The separation was achieved on a fused silica capillary using carboxymethyl-β-cyclodextrine dissolved in phosphate buffer at pH 2.5. However, CE lacks the sensitivity necessary to determine the low levels of MTZ and its metabolite usually found in

patient plasma. For these reasons a careful pretreatment using SPE with hydrophilic–lipophilic balance cartridge was proposed. However, all these procedures involve additional steps of purification and concentration of biological samples, which are tedious and time consuming.

3.3 Objectives of the work

i. To develop and validate a simple and rapid reverse polar ionic LC method for simultaneous separation and determination of MTZ, an antidepressant drug, and its main metabolite DMTZ.

ii. To explore the enantiomeric separation on Chirobiotic V column packed with vancomycin as a stationary phase in reverse polar ionic mode.

iii. To detect the enantiomers using fluorescence detector and determination of optical rotation (+/−) of the enantiomers by using a polarimetric detector connected in series.

iv. To apply the developed method to determine MTZ and DMTZ in rat plasma.

This chapter describes enantiomeric separations of MTZ and its metabolite DMTZ in reverse polar ionic mode on Chirobiotic V column packed with vancomycin as a chiral selector. The reverse polar ionic mode has many advantages not only in terms of speed and sample solubility but also beneficial for high throughput preparative separations. Mobile phases used in this mode are polar organic solvents containing

volatile additives using a mixture of methanol, acetic acid and triethyl amine as a mobile phase ideally suited for LC–MS applications. The column effluents were monitored by both fluorescence and polarimetric detectors connected in series. The plasma proteins were precipitated by acetonitrile as protein precipitation reagent to extract MTZ and DMTZ from rat plasma.

3.4 Experimental

3.4.1 Chemicals and reagents

The rac-MTZ and rac-DMTZ (purity >99.0%) obtained from M/s Neulands laboratory (Hyderabad, India) were used. All the reagents were of analytical grade unless stated otherwise. HPLC-grade methanol, glacial acetic acid and anhydrous triethyl amine (S.D. Fine Chem, Mumbai, India) were used. Blank plasma samples were obtained from Wister rats. Plasma samples were collected by taking permission from ethical committee, pharmacology division, IICT, Hyderabad. All solvents were filtered through a 0.22µm membrane and degassed.

3.4.2 Instrumentation

The HPLC system consisting of two LC-20 AD pumps, a RF- 10AXL fluorescence detector, a SIL-AC auto sampler, a DGU-20 A5 degasser and CBM-20A communications bus module (Shimadzu, Kyoto, Japan) was used. The chromatographic and the integrated data were recorded using HP-Vectra (Hewlett Packard, Waldron, Germany) computer system using

LC-solution data acquiring software (Shimadzu, Kyoto, Japan). The compounds were analyzed on a Chirobiotic V column (250×4.6 mm, 5 µm) (Supelco, PA, USA). The mobile phase was filtered through a 0.22 µm membrane (Millipore) before use. A chiralyser (IBZ Messtechnik GmbH, Hannover, Germany) also known as a polarimetric detector for identification of the optical rotation (+/−) of the peaks corresponding to the enantiomers of MTZ and DMTZ was used. The compounds were analyzed on a Chirobiotic V column using a mobile phase containing methanol: glacial acetic acid:anhydrous triethyl amine (100:0.2:0.1, v/v/v) in an isocratic elution mode at a flow rate of 1 $mLmin^{-1}$. The column temperature was 20°C. The auto sampler temperature was kept at 5°C and the samples of 20µL volume were injected onto the column. The data acquisition run time was 16 min. Compounds were detected by their excitation at 290 nm and emission at 370 nm using fluorescence detector while optical rotation (+/−) of MTZ and DMTZ enantiomers were identified by polarimetric detector connected in series.

3.4.3 Preparation of standards and quality controls

Standard 1 $mgmL^{-1}$ stock solutions of MTZ and DMTZ were prepared separately in methanol. Standard working solutions of MTZ and DMTZ at concentrations of 0.2, 0.5, 1, 5, 10 and 50 μgmL^{-1} were prepared by serial dilution of stock solutions. Methanol was used as a diluent. Drug-free plasma was spiked with standard solutions to prepare

calibration standards with final concentrations of 20, 50, 100, 500, 1000 and 5000 $ngmL^{-1}$ of MTZ and DMTZ. Plasma quality-control (QC) samples containing MTZ and DMTZ at four concentration levels: 20 $ngmL^{-1}$ (lower limit of quantization, LLOQ), 500 $ngmL^{-1}$ (low, LQC), 1000 ngmL-1 (middle, MQC), and (5000 ngmL-1) (high, HQC) were prepared to measure recovery, stability, accuracy and precision of the method. The prepared QC samples cover the therapeutic ranges of both MTZ (20–300 ngmL-1) and DMTZ (50–300 ngmL-1). All solutions were kept at −20°C prior to analysis.

3.4.4 Plasma sample preparation

Plasma samples were stored at −80°C and allowed to thaw gradually to room temperature before processing. After transferring 100µL aliquots of plasma into 1.5 mL centrifuge tubes, 400µL acetonitrile was added as a protein precipitating agent to each tube and the mixture was vortexed for 10 min and centrifuged at 4000 rpm for 20 min. Then, the upper organic layer was transferred to an autosampler vial and injected (20µL) into the HPLC column.

3.4.5 Method validation

The method was validated by evaluating recovery, accuracy, precision, linearity, LOD, LOQ and stability.

3.4.5.1 Recovery

The recoveries were determined by comparing the peak areas of each enantiomer of MTZ and DMTZ in four different QC plasma samples (20, 500, 1000 and 5000 $ngmL^{-1}$) with those of each enantiomer of MTZ and DMTZ in samples prepared by spiking after deproteinization with same amounts of QC plasma samples. The recovery was calculated using equation 3.1:

Recovery (%)= (area of the corresponding peak in the chromatogram of rat plasma spiked with MTZ and DMTZ enantiomers and extracted/ area of the corresponding peak in the chromatogram of deproteinated blank rat plasma spiked with MTZ and DMTZ enantiomers) × 100. (3.1)

3.4.5.2 Accuracy and precision

Accuracy was evaluated by calculating the percentage deviation from the nominal concentration and reported as relative error (RE). Precision was determined by calculating the coefficient of variation (CV) of replicates within one sample run (intra-day) and between samples runs (inter-day). For intra-day accuracy and precision, ten replicates quality control (QC) samples containing MTZ and DMTZ, 20 $ngmL^{-1}$ (LLOQ), 500 $ngmL^{-1}$ (LQC), 1000 $ngmL^{-1}$ (MQC), and (5000 $ngmL^{-1}$) (HQC). Samples were prepared and analyzed on the same day and the cumulation of all five days was used for inter-day accuracy and precision determination.

3.4.5.3 Linearity, limit of detection (LOD) and limit of quantification (LOQ)

Linearity of the analytical method was evaluated by analyzing spiked plasma samples for each concentration (n=3) over the concentration range 20–5000 $ngmL^{-1}$ for all enantiomers of MTZ and DMTZ. The results were used to draw a linear regression curve. The LOD and LOQ were calculated according to the ICH guidelines [24].

3.4.5.4 Stability

The stability of analytes was determined during blood sample collection and after freezing plasma samples for 1st day, 2nd day, 3 rd day, 7 days, 15 days and 30days.

3.4.6 Chromatographic conditions

Column	: Chirobiotic V column (250x4.6mm, 5µ particle size)
Mobile Phase	: Methanol :glacial acetic acid : anhydrous triethyl amine (100:0.2:0.1, v/v/v)
Flow rate	: $1mLmin^{-1}$
Run time	: 16 min.
Auto sampler temperature	: 5°C
Injection volume	: 20 µL
Column Temperature	: 20°C
Detectors	: Fluorescence and polarimetric detectors
λ_{ext} and λ_{emis}	: 290 and 370nm

3.5 Results and discussions

3.5.1 Method development

Various CSPs such as cyclobond and Chiral AGP in reverse phase mode were tried. No elution of analytes on cyclobond and no selectivity on chiral AGP was observed. Later Chirobiotic V column tried in reverse phase mode but not successful. Polar ionic mode was tried as Chirobiotic V works very efficiently in this mode. The vancomycin chiral selector in Chirobiotic CSPs interacts with polar, ionizable and neutral analytes via multiple molecular interactions. This versatility means that the same Chirobiotic column can be successfully used in a variety of mobile phases, a significant benefit over CSPs that operate only in a single mode, normal or reversed-phase and must be dedicated to those mobile phase systems. However, the most interesting feature of Chirobiotic CSPs is the presence of ionic interactions, which allow them to be used in polar ionic and reversed-phase modes for sensitive LC-MS operations. The key application areas of these CSPs are drug discovery, organic synthesis, bioanalytical, drug metabolism, high throughput, MS-compatibility, amino acid and peptide analysis [25-28]. Fig: 3.2 shows the different types of interactions that a Vancomycin chiral selector can form with a variety of analytes. Chirobiotic CSPs offer six different types of molecular interactions: ionic, H-bond, π-π, dipole-dipole, hydrophobic and steric. They also possess multiple inclusion sites that influence selectivity based on the molecular shape of the analyte. The optimization

of enantiomer resolution is achieved by changing the mobile phase to leverage the types and relative strengths of the various interactions [29-32].

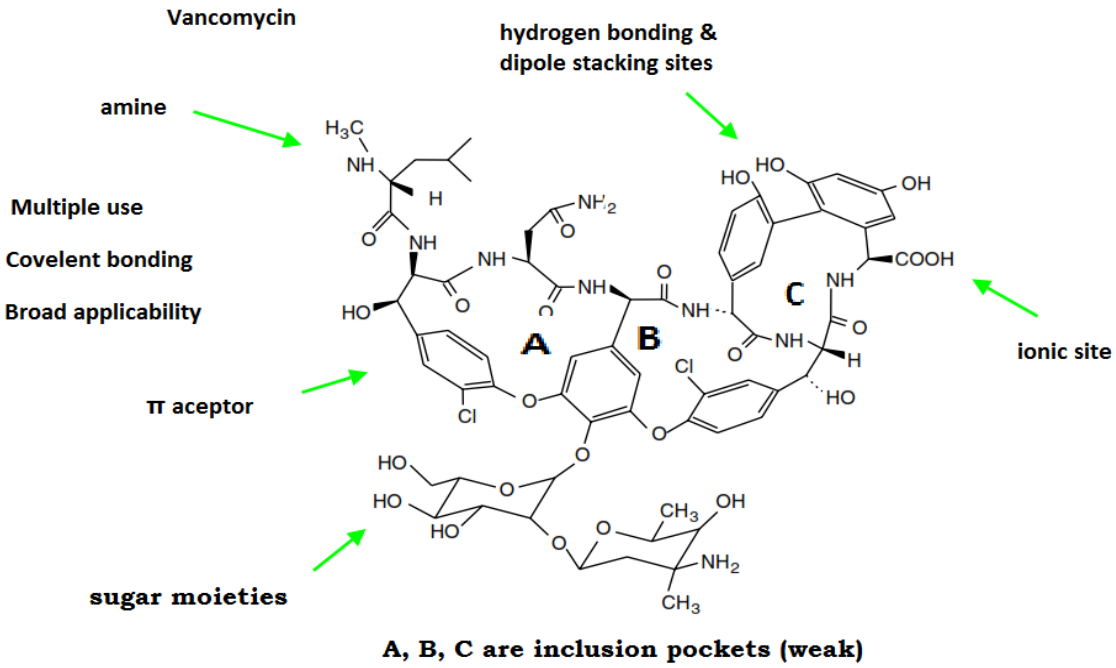

Fig: 3.2 Chemical structure of vancomycin chiral selector.

The bonded macrocyclic glycopeptide itself in terms of its morphology, molecular composition and multiple covalent linkages to the silica surface makes Chirobiotic CSPs unique and gives them significant and valuable benefits over other CSPs. The truly differentiating feature of Chirobiotic CSPs is the presence of ionic interactions. These interactions are unique to Chirobiotic CSPs and are responsible in large part for their desirable retention characteristics toward polar and ionizable analytes in aqueous and non-aqueous solvents. The various Chirobiotic phases share the benefits of robustness, flexibility in mobile phase options, ionic

interactions, compatibility with polar compounds and LC-MS, and preparative scalability. However, Chirobiotic CSPs differ in selectivity, primarily because of their differing number and types of interaction sites, and the number, type and accessibility of ionic sites in the bonded macrocyclic glycopeptid.

Vancomycin, containing 18 chiral centers with various functional groups surrounding its three pockets or cavities is an integral part of the stationary phase of Chirobiotic V column [20]. The strong polar groups present on vancomycin molecule interact with the analytes groups, which are easily ionizable by the reverse polar ionic mobile phases. The enantiomeric separation on chiral stationary phases (CSPs) is generally based on the formation of transient diastereomeric analyte–CSP complexes between the enantiomers and the chiral molecule that is an integral part of the stationary phase [33]. The mobile phase used in reverse polar ionic mode is useful for ionization of groups on or near the chiral centers of the analytes. The reverse polar ionic mode is applicable to all molecules with at least one ionizable group on or near the chiral center. Generally, basic compounds demonstrate more selectivity in reverse polar ionic mode [18]. MTZ and DMTZ are the basic compounds having ionizable group –N near to chiral center. The predicted mechanism of formation of diastereomeric analyte–CSP complexes between enantiomers and vancomycin is shown in Fig: 3.3. Further, the preparation of mobile phase composition is easy and the reverse polar

ionic mode could be described as a novel method to obtain difficult enantioselective separation with macrocyclic antibiotic-based chiral stationary phases by LC [34].

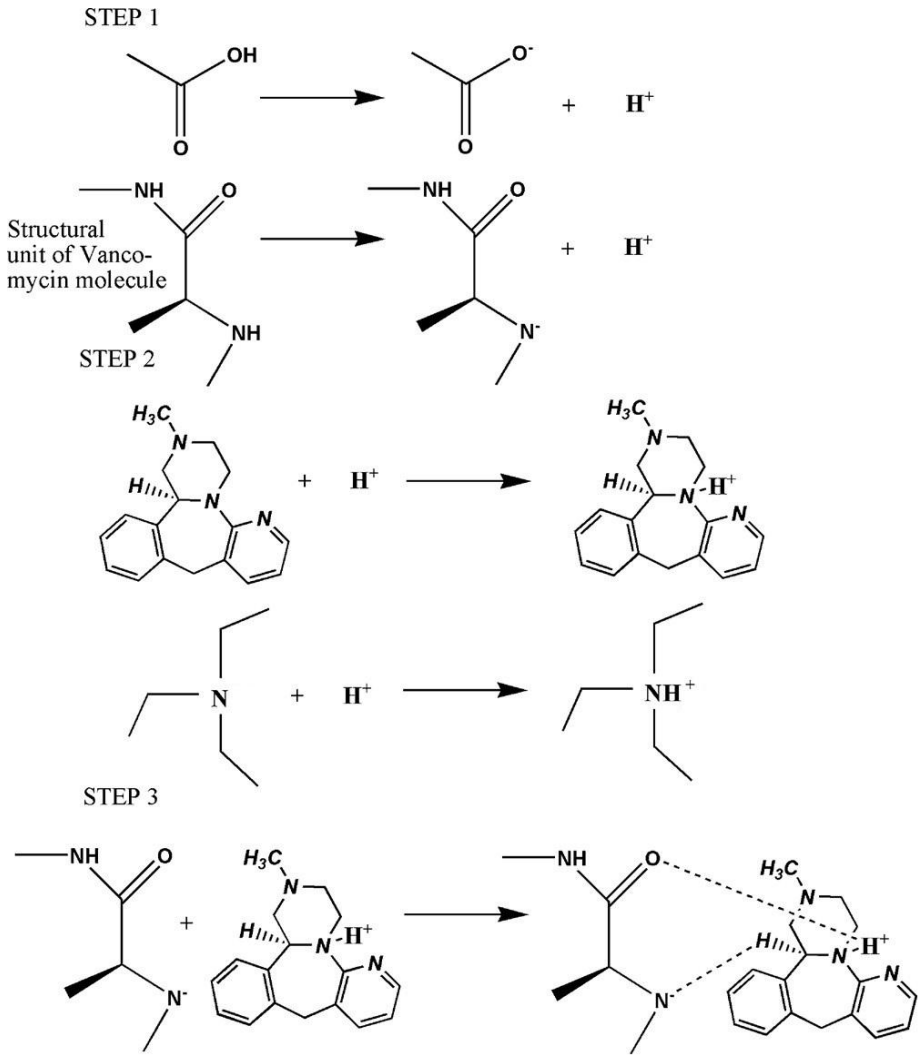

Fig: 3.3 Mechanism of formation of diastereomeric analyte–CSP complexes between enantiomers of MTZ/DMTZ and vancomycin. Step 1: ionization of acetic acid and vancomycin molecule. Step 2: protanation of MTZ enantiomers and triethyl amine. Step 3: interaction of ionized vancomycin molecule and protanated MTZ enantiomers, formation of transient diastereomeric analyte–CSP complexes.

No enantiomeric separation was observed in the absence of triethyl amine when the mobile phase consisted of methanol–acetic acid (100:0.2, v/v). This could be explained on the basis of strong repulsive effects between the protonated amino groups of the analyte molecules and of the CSP. Different mobile phase compositions, flow rate, temperature of the column conditions were tried. The mobile phase composition was optimized by changing the concentration of acetic acid and keeping MeOH, TEA concentration as constant. The chiral separation was found to be very sensitive with Chirobiotic V column when the concentration of AcOH was varied. Initially the mobile phase composition MeOH:AcOH:TEA (100:0.1:0.1, v/v/v) was tried. The enantiomers of MTZ were eluted fast while DMTZ took long time. Later the concentration of AcOH was changed from 0.2 to 0.4%. Except at 0.2% AcOH, the enantiomeric separation of MTZ and DMTZ was not good under all the conditions. The effect of mobile phase composition on retention time (t_R), retention factor (k'), separation (α) and resolution (Rs) parameters at temperature of the column 20°C and flow rate 1 $mLmin^{-1}$ are given in Table: 3.1 and Fig: 3.4. The separation was tried at different temperatures of the column (15°C, 20°C and 25°C), and found to be good at 20°C. Different flow rates of the mobile phase were also tried and 1 $mLmin^{-1}$ flow rate was found to be optimum. Finally, the following chromatographic conditions were optimized using the mobile phase consisting of methanol: glacial acetic acid: anhydrous triethyl amine

(100:0.2:0.1, v/v/v) in an isocratic mode of elution at a flow rate of 1 $mLmin^{-1}$ at 20°C. The polar ionic mode arose from polar organic mode. The polar organic mode was first termed as non-aqueous reversed phase when it was introduced in 1978 by Parris [35, 36]. In this mode initially aprotic solvents of intermediate polarity such as dichloromethane or tetrahydrofuran and aprotic solvent acetonitrile were used. Later alcohol was used to adjust the elution strength and selectivity [37-39]. The polar organic mode uses non-aqueous mobile phases made of 100% organic polar solvents essentially methanol and/or acetonitrile. The polar ionic mode is a variation of the polar organic mode in which the mobile phase acidity is adjusted. The polar ionic mode is defined by the use of 100 % MeOH containing acid and base (typically 0.1%, each with a range of 1.0 to 0.001%), or equivalent volatile salts. Methanol was found to affect H-bond interactions while acetonitrile interacting through π-π interactions [40, 41]. It is an important mode used to separate enantiomers of ionizable molecules such as amino acids, organic acids, and amines [42-46]. Method development in polar ionic mode is very simple and fast by adjustment of acid, base ratio or salt concentration. The mechanism for chiral recognition on the Chirobiotic phases is predominantly ionic, making it essential to add acid and base for ionizable compounds.

Table 3.1: The effect of the mobile phase composition on retention parameters of enantiomers of MTZ and DMTZ.

MeOH:AcOH:TEA (v/v/v)	Retention parameters	R(-)MTZ	S(+)MTZ	R(-)DMTZ	S(+)DMTZ
100:0.1:0.1	t_R (min)	6.413	7.194	15.411	17.281
	k'	1.338	1.623	4.619	5.301
	α	-	1.213	2.846	1.148
	Rs	-	1.019	6.649	1.091
100:0.2:0.1	t_R (min)	6.998	8.294	12.292	13.685
	k'	1.911	2.449	4.054	4.749
	α	-	1.281	1.656	1.171
	Rs	-	2.657	3.796	2.308
100:0.3:0.1	t_R (min)	6.988	8.129	11.503	12.921
	k'	1.552	1.968	3.200	3.718
	α	-	1.268	1.626	1.162
	Rs	-	1.293	2.998	0.999
100:0.4:0.1	t_R (min)	8.632	10.536	11.879	13.542
	k'	2.748	3.574	4.157	4.879
	α	-	1.301	1.163	1.174
	Rs	-	1.713	0.993	1.098

Conditions: column: Chirobiotic V; flow rate: 1 mL/min; detection: fluorescence at excitation at 290nm and emission at 370nm; column temperature: 20°C; t_R: retention time; k': capacity factor; α: selectivity factor; R_S: resolution.

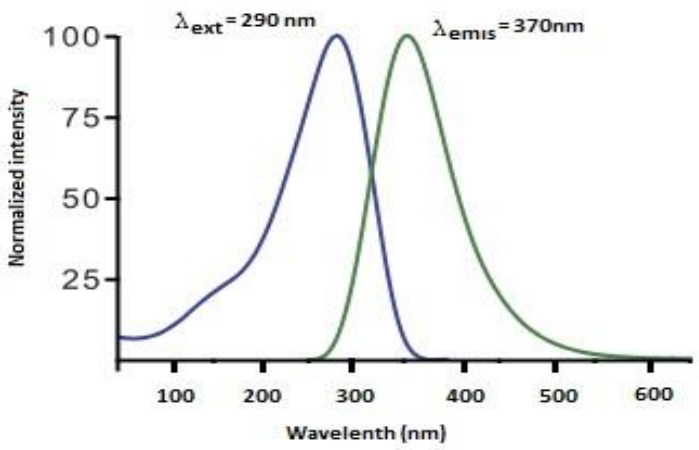

Fig: 3.5 Fluorescence spectra of MTZ

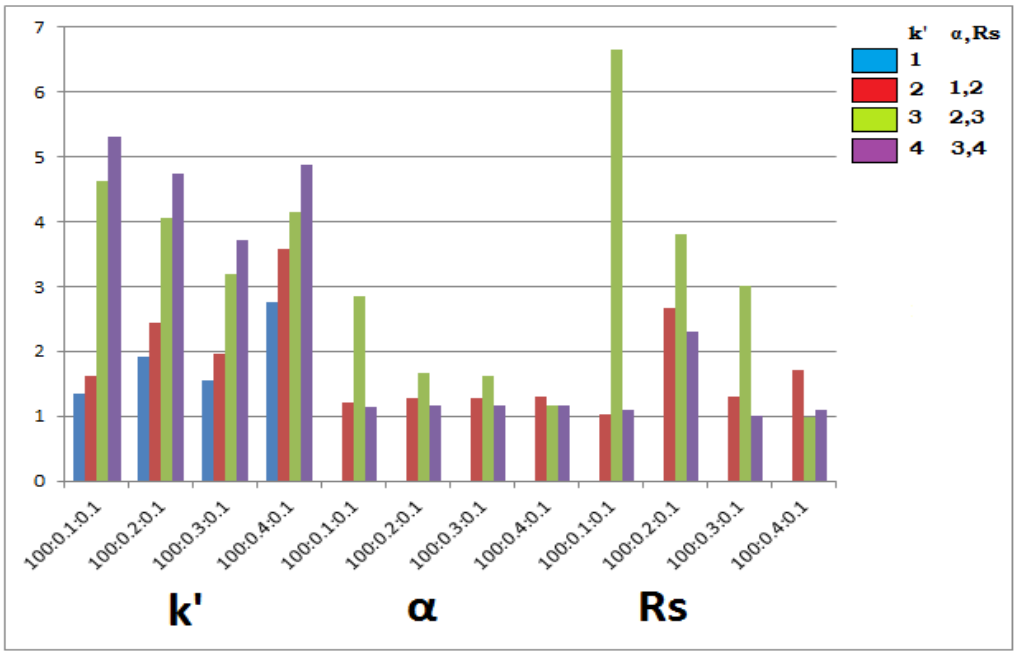

Fig: 3.4 The effect of the mobile phase composition on retention, selectivity and resolution of enantiomers of MTZ and DMTZ

MTZ and DMTZ separated on chirobiotic V were detected by fluorescence detector. The excitation and emission wave lengths were determined by fluorometry spectra. Fig: 3.5 shows the Fluorescence spectra of MTZ showing λ_{ext} and λ_{emis} as 290 and 370nm. When certain molecules or atoms are exposed to an energy source such as high-intensity light, the molecules or atoms absorb the light energy and enter into an excited state. As a molecule or atom moves from this excited state back to its normal state, some of the absorbed energy is released in the form of a photon. This process is called fluorescence. Fluorescence is a type of luminescence. This is a phenomenon in which a substance absorbs light to reach a high-energy level and then emits light to return

to its original level. A UV/UV-VIS detector monitors the absorption of light with a specified wavelength. Some substances have specific wavelengths of light that it absorbs (excitation wavelengths) and emits (emission wavelengths). The atoms and molecules in different elements and compounds emit different levels of fluorescence when exposed to the same energy levels. The fluorescence detector illuminates a sample with a narrow band of high-intensity light from a carefully controlled light source. The detector then measures the low levels of fluorescence emitted from the sample. The emitted light is filtered, amplified and converted to electrical signals that can be recorded and analyzed. As familiar examples, fluorescent paints and highlighters emit fluorescence with a clear color. Fluorescence detection is suitable for trace analysis because of generally having high sensitivity and selectivity. There are not many compounds that originally emit fluorescence (natural fluorescence). However, amino acids, etc. can be detected as fluorescent substances, after reaction with a fluorescence reagent (derivatization). This method makes it possible to measure various components with high sensitivity. Fig: 3.6 shows a fluorescence detector optical system. While a UV/UV-VIS detector detects light that has passed through the flow cell, an FL detector detects fluorescence emitted in the direction orthogonal to the exciting light.

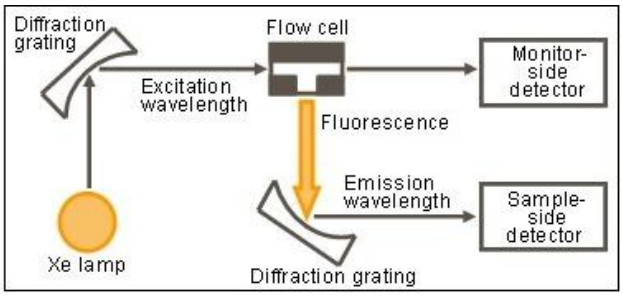

Fig: 3.6 Diagrammatic illustration of fluorescence detector optical system.

The optical rotation of MTZ and DMTZ was determined by using polarimetric detection. Plane polarized light is generated when normal light is passed through an optical polarizing filter. A compound is optically active if linearly polarized light is rotated when passing through it. The degree of rotation is dependent on concentration of a chiral compound and as well as on its molecular structure. Every optically active substance has its own specific rotation (degree of rotation in polarized light) as defined by Biots law:

$$[\alpha]_{\lambda}^{T} = \frac{\alpha_{\lambda}^{T}}{cl}$$

where [α] = specific rotation; l= optical path length in dm; λ =wavelength; T = temperature; α = optical rotation, and c = concentration in g/mL. The specific rotation of the molecule, not the absorption characteristics, is what determines the signal strength using the polarimeter. The Polarimeter uses a diode laser at 670 nm as the light source. Fig: 3.7 shows schematic representation of a polarimetric detector.

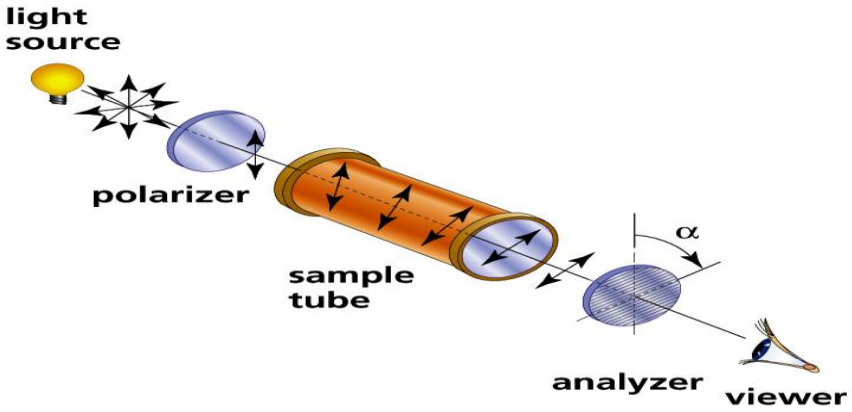

Fig: 3.7 Schematic representation of a polarimetric detector

The compounds were detected by their excitation at 290 nm and emission at 370 nm using fluorescence detector. The chromatographic separation of (a) blank plasma (b) rat plasma spiked MTZ, DMTZ is shown in Fig: 3.8. Polarimetric detector (optical rotation range, 250; average, 10 and offset is 50) connected in series was used for identification of (+/−) enantiomers of MTZ and DMTZ. The chromatographic separation of a mixture of rac MTZ and DMTZ using polarimetric detector is shown in Fig: 3.9. The elution order was R (−) MTZ, S (+) MTZ, R (−) DMTZ and S (+) DMTZ.

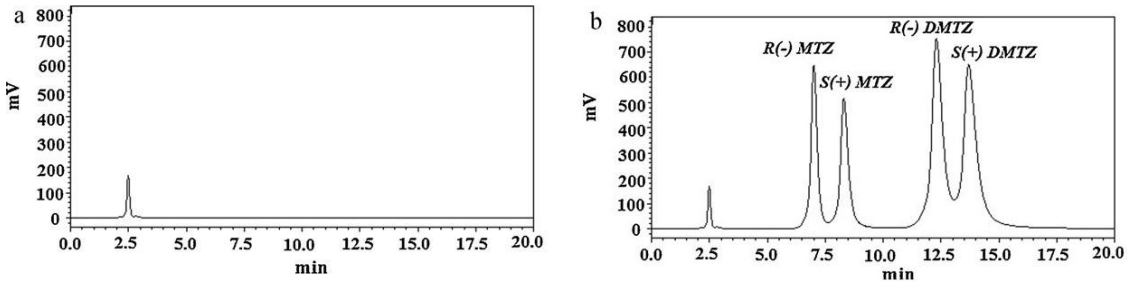

Fig: 3.8 Chromatograms of rat plasma (a) blank and (b) spiked with racemic MTZ and DMTZ. LC conditions: column: Chirobiotic V; eluent:

MeOH:AcOH:TEA (100:0.2:0.1, v/v/v); flow rate: 1 $mLmin^{-1}$; detection: fluorescence at excitation at 290 nm and emission at 370 nm; column temperature: 20°C.

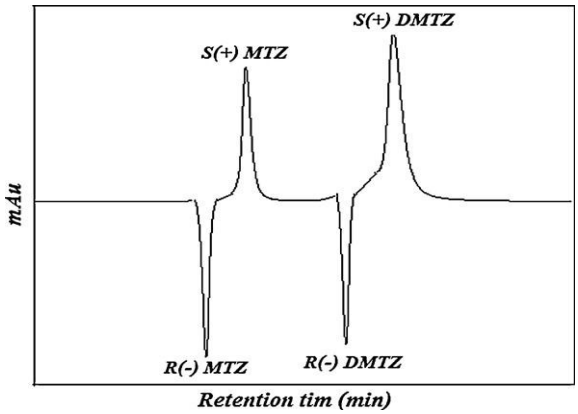

Fig: 3.9 A typical chromatogram showing the identification of optical rotation (+/−) of the enantiomers of standard MTZ and DMTZ by polarimetric detector. For conditions see Fig: 3.8.

3.5.2 Method validation

3.5.2.1 Recovery

The recovery (±RSD %) of each enantiomer of MTZ and DMTZ for QC samples of 20 $ngmL^{-1}$ (lower limit of quantization, LLOQ), 500 $ngmL^{-1}$ (low, LQC), 1000 $ngmL^{-1}$ (middle, MQC), and (5000 $ngmL^{-1}$) (high, HQC) are given in Table: 3.2.

3.5.2.2 Accuracy and precision

Accuracy was evaluated by calculating the percentage deviation from the nominal concentration and is reported as relative error (RE). Precision was determined by calculating the coefficient of variation (CV)

of replicates within one sample run (intra-day) and between samples runs (inter-day). Intra and inter-day accuracy and precision were determined by the performance of four concentrations of QCs and are given in Table: 3.3.

Table 3.2: Recovery data

	Recovery ± RSD (%)[a]			
Enantiomer	20 (ng/mL)	500 (ng/mL)	1000 (ng/mL)	5000 (ng/mL)
R (-) MTZ	99.87±3.27	101.24±0.24	94.99±8.00	100.38±2.69
S (+) MTZ	100.34±7.93	92.23±2.69	88.58±4.66	98.60±2.07
R (-) DMTZ	105.40±10.43	123.02±0.51	103.73±19.9	85.68±1.24
S (+) DMTZ	106.30±17.12	119.82±3.49	104.71±15.71	101.24±0.84

a) n=3 (triplicate determinations)

Intra-day accuracy ranges (RE) observed for the analytes were as follows: R (−) MTZ: 0.002–0.043; S (+) MTZ: 0.005–0.025; R (−) DMTZ: 0.003–0.092 and S (+) DMTZ: 0.002–0.095. Inter-day accuracy ranges (RE) observed for the analytes were as follows: R (−) MTZ: 0.012–0.026; S (+) MTZ: 0.006–0.032; R (−) DMTZ: 0.004–0.059; and S (+) DMTZ: 0.001–0.110. Intra-day precision ranges (CV) observed for the analytes were as follows: R (−) MTZ: 0.006–0.074; S (+) MTZ: 0.013–0.056; R (−) DMTZ: 0.008–0.182 and S (+) DMTZ: 0.005–0.225. Inter-day precision ranges (CV) observed for the analytes were as follows: R (−) MTZ: 0.028–0.080; S (+) MTZ: 0.016–0.055; R (−) DMTZ: 0.045–0.115 and S (+) DMTZ: 0.049–0.200.

Table: 3.3 Intra- and inter- day batch accuracy and precision data

Precision and accuracy	R (-) MTZ	S (+) MTZ	R (-) DMTZ	S (+) DMTZ
Intra-assay precision; Coefficient of variation (n =10)				
20 ng/mL	0.041	0.035	0.102	0.225
500 ng/mL	0.006	0.013	0.012	0.005
1000 ng/mL	0.074	0.056	0.182	0.134
5000 ng/mL	0.025	0.029	0.008	0.012
Inter-assay precision; coefficient of variation (n=5)				
20 ng/mL	0.080	0.055	0.115	0.200
500 ng/mL	0.028	0.016	0.060	0.057
1000 ng/mL	0.043	0.027	0.045	0.057
5000 ng/mL	0.053	0.077	0.058	0.049
Intra-assay accuracy; relative error (n = 10)				
20 ng/mL	0.028	0.025	0.058	0.095
500 ng/mL	0.023	0.019	0.054	0.090
1000 ng/mL	0.002	0.005	0.003	0.002
5000 ng/mL	0.043	0.025	0.092	0.054
Inter-assay accuracy; relative error (n=5)				
20 ng/mL	0.026	0.032	0.059	0.110
500 ng/mL	0.019	0.009	0.004	0.001
1000 ng/mL	0.049	0.006	0.012	0.026
5000 ng/mL	0.012	0.008	0.018	0.023

3.5.2.3 Linearity, limit of detection (LOD) and limit of quantification (LOQ)

Linearity of the method was evaluated by analyzing spiked plasma samples for each concentration (n = 3) over the concentration range 20–5000 $ngmL^{-1}$ for each enantiomer of MTZ and DMTZ. The results obtained were used to draw linear regression curve (Fig: 3.10). The LOD and LOQ were calculated according to the ICH guidelines. The limit of

detection (LOD), the limit of quantification (LOQ), regression equations and regression coefficients (r^2) are given in Table: 3.4. It could be seen from Table: 3.4 that DMTZ has higher slope (2236.9–2295.5) when compared to MTZ (1080.9–1093.6). It could be probably due to emission of more energy by DMTZ at selected excitation and emission wavelengths at 290 nm and 370 nm, respectively. Further the LOQs of both MTZ and DMTZ vary between 12.4 and 17.9 $ngmL^{-1}$ which corresponds to the 2.48–3.58 × 10^{-10} g in an injection volume 20µL. This could be explained in terms of the high UV absorption of MTZ and DMTZ in normal phase solvents viz; n-hexane/iso propanol with DEA buffer where as it was found to be comparatively less in reverse polar ionic buffers viz; AcOH and TEA, which quench the UV absorption of MTZ and DMTZ significantly. Thus the fluorescence detector was used to increase the detection levels of MTZ and DMTZ in the present investigation.

3.5.2.4 Stability

The stability of analytes was determined for QC concentrations except 20 $ngmL^{-1}$ during blood sample collection and after freezing plasma samples for 1st day, 2nd day, 3rd day, 1 week, 15 days and 1 month. The results were compared with those obtained by freshly prepared samples. The results are summarized in Table: 3.5.

Table 3.4 Linearity, (± RSD %)[a] of slope, intercept and correlation coefficient; LOD and LOQ data.

Enantiomer	Range (ng/mL)	Regression equation	r^2	(± RSD %)[a]			LOD (ng/mL)	LOQ (ng/mL)
				m	c	r^2		
R (-) MTZ	20-5000	y = 1093.6x - 26281	0.9997	2.58	61.94	-	4.7	14.1
S (+) MTZ	20-5000	y = 1080.9x - 39598	0.9999	2.95	38.00	0.06	5.9	17.9
R (-) DMTZ	20-5000	y = 2295.5x - 44694	0.9985	0.76	145.89	0.11	4.1	12.4
S (+) DMTZ	20-5000	y = 2236.9x - 36318	0.9997	1.24	152.98	0.04	4.8	14.5

a) n=3
'm' is slope
'c' is intercept
'r^2' is correlation coefficient.

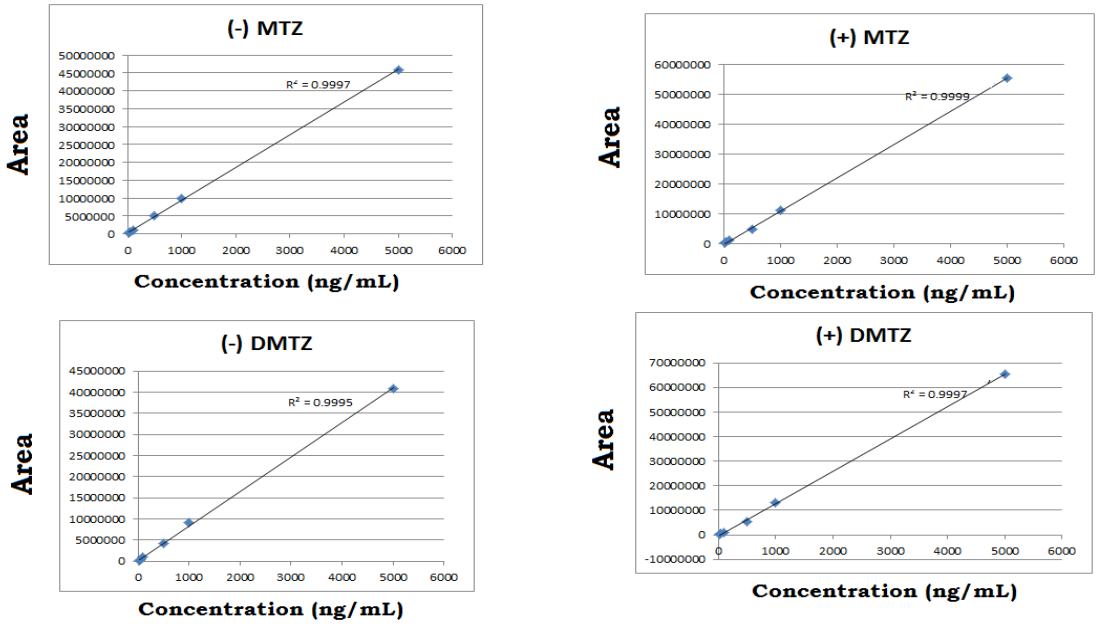

Fig: 3.10 Linearity curves for (-) MTZ, (+) MTZ, (-) DMTZ and (+) DMTZ

Table: 3.5 Intra-and inter-stability data (± RSD %)[a] of spiked (-)MTZ.

	(± RSD %)[a]		
	Nominal concentration (ng/mL)		
	500	1000	5000
Storage conditions			
Freeze/thaw stability (three cycles)	0.629	7.361	2.495
3 days	1.320	5.576	2.875
1 week	1.184	18.240	0.859
15 days	0.498	13.409	1.223
1 month	0.859	0.498	1.223

a) n=3 (triplicate determinations)

3.6 Conclusions

Liquid chromatographic separation of the enantiomers of MTZ and its metabolite DMTZ was achieved on a new Chirobiotic V column packed with vancomycin. Evaluation of vancomycin was done in polar ionic mode with different concentrations of mobile phase additives. Method was optimized using simple mobile phase mixture. The method was validated for determination of MTZ and DMTZ enantiomers in rat plasma using HPLC-fluorescence-polarimetric detectors connected in series. The method showed adequate sensitivity, linearity, precision and accuracy.

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CHAPTER 4

Pre column O-phthalaldehyde-N-acetyl-L-cysteine derivatization followed by separation of pregabalin enantiomers on dried blood spots

4.1 Introduction

Pregabalin (PRG), (S)-3-(amino methyl)-5-methylhexanoic acid), is an antiepileptic and analgesic drug used in regulating the brain neuronal activity (Fig: 4.1). It exhibits anti-seizure activity and is useful in treatment of pain, physiological conditions associated with psychomotor stimulants, inflammation, gastrointestinal damage, alcoholism, insomnia and various psychiatric disorders, including mania and bipolar disorders [1]. It has been approved by Food and Drug Administration (FDA) for treatment of spinal cord injury and central nervous system (CNS) disorders including epilepsy, fibromyalgia and also neuropathic pain. It has been recently approved by the European Union (EU) as an adjunctive therapy for treatment of partial seizures with and without generalized tonic–clonic seizures and peripheral neuropathic pain in adults. PRG is minimally metabolized and primarily excreted through urine in an unchanged form.

PRG is a structural analogue of the endogenous inhibitory neurotransmitter γ aminobutric acid (GABA) containing one chiral carbon. The S enantiomer is active while the R (−) enantiomer has been reported to be 10 times less active in biological assays. Thus R (−) enantiomer is considered to be an optical impurity [2] and controlled in production processes.

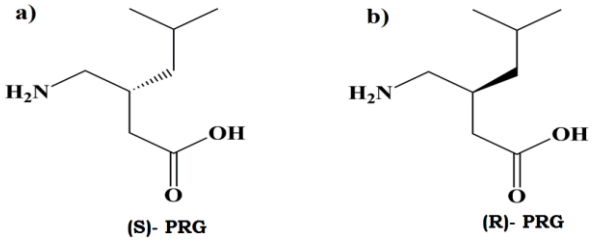

Fig: 4.1 Chemical structures of a) (S)-PRG and its enantiomeric impurity b) (R)-PRG.

4.2 Literature survey

Several achiral separation methods were reported for separation of PRG and PRG with related drugs of various compositions [3-9]. Berry et al., have reported a method for the separation of PRG by using Picryl sulfonic acid (PSA) as a derivatizing agent with HPLC on C_8 column [3]. *O*-phthalaldehyde (OPA) and 3-mercaptopropionic acid were used as derivatizing agent for the simultaneous determination of PRG, gabapentin and vigabatrin in human serum [4]. Dousa et al., have developed an HPLC-fluorescence detection method for the separation of PRG and its possible impurities by derivatization of PRG with OPA and 2-mercaptoethanol [5]. Several mass spectrometric methods such as GC-MS [6] and LC/MS/MS methods were also reported in human plasma [7-9].

Few direct and indirect enantiomeric separations were reported for the determination of (S)-PRG from its enantiomer (R)-PRG using various analytical separation techniques [10-13]. Xiaohui et. al., and Shingare et

al., have reported chiral derivatization method by using Marfey's reagent (FDAA) as chiral derivatizing agent. Marfey's reagent was able to form the diastereomers from the corresponding enantiomers. The obtained diastereomers were separated by using C_{18} column for the quality control of PRG [10, 11]. The direct chiral separation method was reported by using serial coupling of vancomycin chiral columns by using HPLC/MS/MS [12]. Capillary zone electrophoresis (CZE) method was developed by using uncoated fused-silica capillary [13].

4.3 Dried blood spots

Blood spot testing was originally developed in 1960s out of a need to screen newborns for phenylketonuria (PKU), since a simple heel stick is more practical than a conventional blood draw in young infants. Later it was extended to include tests for congenital hypothyroidism [14-17]. Today neonatal screening for PKU and thyroid deficiencies using blood spot tests is a routine procedure, and assays for a wide range of other analytes on blood spots have been successfully developed [18-28]. The simplicity of sample collection, stability of samples in storage and transport, and excellent correlation of blood spot assays with serum tests, have made it an ideal method for epidemiological and field research studies for a variety of health conditions in both children and adults [29]. The main advantages of DBS are the low blood volume required which is compliant with the overall objectives of the 3Rs (reduce, refine and

replace), simplification of sample collection, storage and reduction of transportation costs (Table: 4.1). Because of the several advantages as described in Table: 4.1, the application of DBS has been extended to many other fields which include therapeutic drug monitoring, toxicokinetics and pharmacokinetics. These studies require high throughput and high sensitive method for analysis.

Table: 4.1: Comparison of DBS and conventional plasma sampling.

S.No.	**Dried blood spots**	**Conventional plasma**
1	Small blood volumes required for sampling.	Large blood volumes required for sampling.
2	Suitable for pediatric studies	Unsuitable for pediatric studies.
3	Less invasive sampling (finger or heel prick).	More invasive sampling (venous cannula).
4	Patients need not to leave their homes and no skilled personnel are required for sample collection.	Patients need to go clinic and skilled personnel are required for sample collection.
5	Easy storage and shipment.	Refrigerated storage and shipment required for sample stability.
6	Antimicrobial properties of the DBS sample, removing the requirements for special biohazard arrangements [30].	Special biohazard arrangements required
7	Significant reduction in blood volume required for DBS, allows reduction in animal slaughter [31].	Animal slaughter is more.
8	DBS requires extensive method development and more sample processing (necessitates automation)	Method development and sample processing are simple.
9	The limited sampling volume can sometimes hinder sensitive assays from being developed.	Higher sampling volume leads to more sensitivity.

4.4 Objectives of the work

i. To develop and validate an indirect HPLC method for separation of PRG enantiomers on dried blood spots.

ii. To optimize the derivatization procedure of racemic PRG with chiral derivatizing agent OPA with NAC under alkaline conditions and separation of diastereomers on a reversed phase C_{18} column.

iii. To demonstrate the developed HPLC method is suitable for DBS analysis of samples collected from healthy wistar rats received single oral dose of 5 mg/Kg of racemic PRG on dried blood spots.

This chapter describes an indirect separation of PRG enantiomers using pre column derivatization by o-phthalaldehyde-NAC as a chiral derivatizing agent. Separation of enantiomers on DBS spots was carried out to investigate the enantioselectivity in a pharmacokinetic study of PRG in rat plasma.

4.5 Experimental

4.5.1 Chemicals and materials

OPA and NAC (Sigma–Aldrich, USA), boric acid, sodium hydroxide and disodium hydrogen phosphate (S.D. fine-chem., Mumbai, India), methanol and acetonitrile (Merck, Mumbai, India), glass-distilled and de-ionized water (Nanopure, Barnsted, USA) were used. S-PRG, R-PRG and R,S-PRG were gift samples from a local pharmaceutical company, Hyderabad, India.

4.5.2 Instrumentation

The HPLC system consisting of two LC-20AD pumps, an RF-10AXL fluorescence detector, a SIL-20AC auto sampler, a DGU-20A5 degasser and CBM-20A controller (all from Shimadzu, Kyoto, Japan), was used. An Elico, model LI 120, pH meter equipped with a combined glass calomel electrode was used for pH measurements. It was calibrated using standard buffers of pH 4.0, 7.0 and 9.2. The chromatographic and the integrated data were recorded using an HP-Vectra (Hewlett Packard, Waldron, Germany) computer system using LC-Solution data acquiring software (Shimadzu, Kyoto, Japan). Before delivering the mobile phase into the system, it was filtered through 0.45µm PTFE filters and degassed. Chromatographic separation was achieved on an Inertsil ODS (250 × 4.6 mm, 5 µm; Merck, Darmstadt, Germany) column using mobile phase consisting of 0.02 M ammonium acetate (pH=7.5): methanol (40:60 v/v) in an isocratic elution mode at a flow rate of 1.0 $mLmin^{-1}$ at 25°C. Fluorescence detection was performed at 330 and 450 nm as excitation and emission wavelengths respectively. The injection volume was 20 µL.

4.5.3 Preparation of reagents

0.1M Borate buffer was prepared by dissolving 0.62g of boric acid in approximately 80mL of water, adjusted pH to 10.0 using 2.0 N NaOH and made up to the 100.0mL with water. Stock solutions of 50 mM OPA was prepared by dissolving 10.07g of OPA in 10 mL methanol and 50

mM NAC was prepared by dissolving 0.041g of NAC in 10 mL of methanol. The derivatization reagent was prepared by mixing 1.0 mL each of 50 mM OPA and 50 mM NAC in a 10 mL volumetric flask and made up to 10 mL with 0.1M borate buffer (pH 10.0). The storage life of the stock solution was at least 4 weeks at 4°C. The working solution was prepared freshly at the time of derivatization. Stock solutions of the racemic-PRG, and S-PRG were prepared in water to give a final concentration 1mgmL^{-1}. Working standards of PRG were prepared by appropriate dilutions of the stock solution with water. All the solutions were stored in dark at 4°C and brought to room temperature before use.

4.5.4 Dried blood spot (DBS) sample preparation

The DBSs were prepared by spotting 30 µL of the respective whole blood standard onto FTA blood spot cards. The samples were allowed to dry in the dark at 4°C for at least 3 h prior to analysis. QC samples thus prepared were used in validation of the developed method. When required, QC samples were stored at room temperature in a sealed plastic bag containing desiccant until analysis.

4.5.5 Dried blood spot sample extraction

At the middle of the DBS sample, 10 mm diameter disc was punched and transferred to a centrifuge tube. A 500 µL volume of methanol (extraction solvent) was added and the tube was vortex mixed for 10 min. The extract was centrifuged at 2500rpm for 10 min and the

supernatant was transferred to an auto sampler vial for analysis. The extraction procedure is shown schematically in Fig: 4.2.

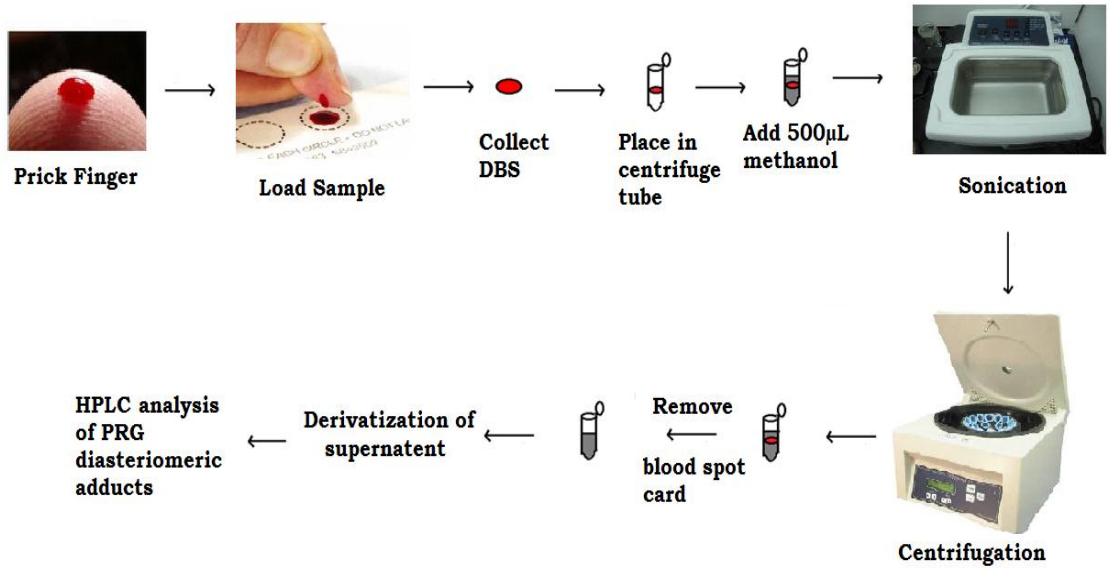

Fig: 4.2 Spotting, extraction, and derivatization of PRG on DBS

4.5.6 Method validation

4.5.6.1 Enantioseparation

Enantioseparation of (S) and (R) PRG and sequence of elution was verified by injection of pure enantiomers prepared in both deionized water.

4.5.6.2 Lower limits of quantification and detection

The lower limit of quantification (LLOQ) was determined as the lowest concentration where the signal-to-noise ratio was 10. The LLOQ was determined by eight replicates within one analytical run with concentrations determined by the calibration curve. The limit of detection

(LOD) was determined as the concentration where the signal-to-noise ratio was greater than 3.

4.5.6.3 Linearity of the calibration curve

The calibration curve was constructed by plotting peak area against the corresponding nominal concentration for the eight calibration standards and by evaluation of different regression models. Detector response (peak height or area) were linearly regressed, including intercept, against nominal analyte concentrations.

4.5.6.4 Accuracy, precision and recovery

Accuracy and precision was evaluated by analysis of five replicates of spiked plasma at each QC level (3, 400 and 1000 µg/mL of each enantiomer) over 5 days. The extraction recovery was determined over 3 days at the three enantiomer QC levels (3, 400 and 1000 µg/mL for each enantiomer). Duplicates of (S) and (R) PRG were prepared in deionized water (corresponding to nominal concentrations of 3, 400 and 1000 µg/mL of each enantiomer) were placed for pre column derivatization and analysis. The peak areas of (S) and (R) PRG diastereomers in water were compared with peak areas for the QC samples on rat dried blood spots (n = 5 for each level) in the same analytical run. The peak area ratios between QC samples and the water samples of identical concentrations were used to determine recovery.

4.5.6.5 Stability

The stability of the derivatization reagent at 20°C (bench top stability) was evaluated up to 48 h. Every sixth hour three samples, prepared in deionized water, were analyzed (one at each QC level). Stability was evaluated by comparing deviation in peak area between the first three injections, at time 0 to 48 h.

4.5.7 Chromatographic conditions

Column	: C_{18} column (250x4.6mm, 5µm particle size)
Mobile Phase	: 0.02 M ammonium acetate (pH=7.5): methanol (40:60 v/v)
Flow rate	: 1.0 $mLmin^{-1}$
Run time	: 15min
Auto sampler temperature	: 10 °C
Injection volume	: 20µL
Column temperature	: 25°C
Detector	: fluorescence detector
λ_{ext} and λ_{emis}	: 330 and 450nm

4.6 Results and discussions

4.6.1 Method development

Preliminary experiments for direct separation of PRG enantiomers were performed on polysaccharide stationery phases viz., CHIRALCEL OD-H, OJ-H, CHIRALPAK AD-H and IE in normal phase but no resolution and clear separation between S-PRG and its enantiomer was obtained. CHIRALPAK IE showed the separation among all the columns but the selectivity, resolution and peak shapes were not good. Fig: 4.3 shows the separation of PRG enantiomers on CHIRALCEL OD-H, OJ-H and CHIRALPAK AD-H under normal phase screening conditions and Fig: 4.4 shows the separation on CHIRALPAK IE using mobile phase consisting of n-Hexane: EtOH: TFA (80/20/0.1, v/v/v) at a flow rate of 1 mL/min.

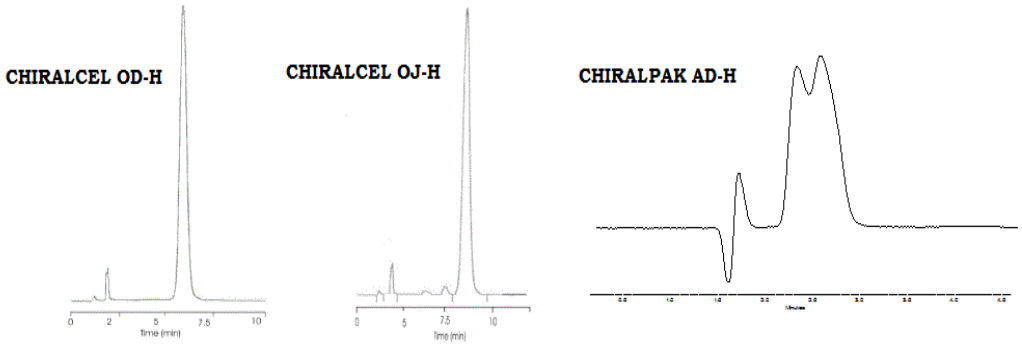

Fig: 4.3 Separation of PRG enantiomers on CHIRALCEL OD-H, OJ-H and CHIRALPAK AD-H using n-Hexane: EtOH: TFA (80/20/0.1, v/v/v); Flow Rate: 1 mL/min.

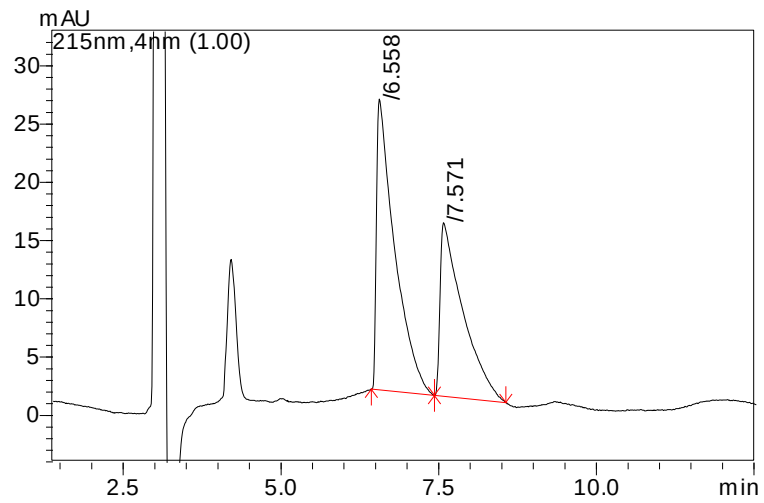

Fig: 4.4 Separation of DRV enantiomers on CHIRALPAK IE by using n-Hexane: EtOH: TFA (80/20/0.1, v/v/v); Flow Rate: 1 mL/min.

Thus an indirect chiral separation through derivatization was tried. Considering the structural factors of PRG being amine, derivatization with chiral Marfey's, Sanger's and OPA–NAC reagents followed by reverse phase HPLC separation of the derivatized diastereomers were studied. However, derivatization with OPA–NAC yielded a very good separation and the reaction was found to be complete at room temperature (25 ±2)°C itself. Therefore, OPA–NAC was selected as a suitable derivatization reagent as it was very economical, easy to operate, rugged and forms stable diastereomer derivatives. Derivatization time was studied from 5 to 60 min and no significant change in the peak area was found, hence, 10 min was considered as adequate and optimum. The reactivity of all common derivatization reagents is directed towards the amino group(s) in the amino acid structure. Several

fluorogenic reagents are available, among which OPA based reagents play the most significant role in modern amino acid analysis [32]. OPA in presence of thiol reacts with primary amine forms fluorescence product. Fig: 4.5 shows the reaction scheme of OPA/thiol reagent. In this reaction, 2-mercapto ethanol reacts with a primary amine to fluorescence the amine. Several chiral derivatizing agents available along with OPA; however NAC is the best forms stable derivatives with fluorescent yields comparable to other derivatizing agents. The popular reaction for the chiral derivatization of amino acids is their reaction with OPA and chiral thiols to form fluorometrically highly active isoindole derivatives. OPA with chiral thiol system is widely applied for determination of the amino acid enantiomer contents in living systems, food analyses and geological systems, with fluorescence detection.

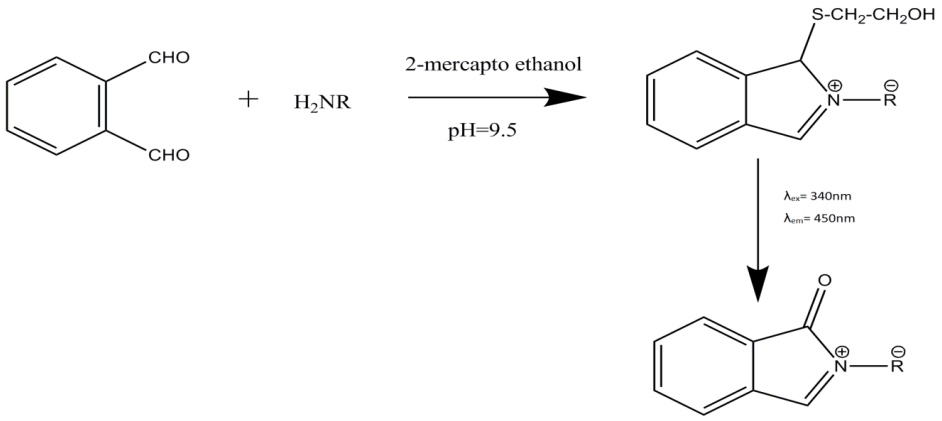

Fig: 4.5 Reaction scheme of OPA/thiol reagent with a primary amine (amino acid) in alkaline medium.

Initially, separation of diastereomers was carried out on a number of columns viz., Phenominex Luna C_{18} column, Zorbax Eclipse XDB-C_{18}

and Merck LichrospherR 100 RP18e columns packed with octadecyl silane as stationary phase. The column that gave the best results in terms of separation and endogenous compounds was inertsil ODS. Clear baseline resolution was obtained with shorter retention time. The effect of variation in pH of the mobile phase on retention and resolution was studied. When the pH of the buffer solution was reduced to 6.5, a 3–4 min increase in retention was found with no significant change in the resolution (Rs) but with poor peak height (broad peak), however, increase in Rs with reduced retention was obtained when the pH was increased to 7.5. Table: 4.2 gives the retention parameters (t_R, α and Rs) obtained at pH (6.5, 7.0 and 7.5). After optimizing the pH, different buffer concentrations and temperatures tried to get the better chromatographic separation. Table: 4.3 gives effects of buffer concentration and temperature on retention parameters of the separation of PRG enantiomers. The optimal chromatographic conditions finalized for separation of PRG enantiomers were 0.02 M ammonium acetate (pH=7.5): methanol (40:60 v/v) at column temperature 25°C. Fig: 4.6 represents the derivatization reaction and structures of the diastereomers and Fig: 4.7 represents the typical chromatograms of a) S-PRG, b) S-PRG with its enantiomer and c) blank. It could be seen from Fig: 4.7, R-PRG enantiomer was well resolved from S-PRG with Rs > 1.5. Typical retention times of S-PRG and R-PRG were 8.5 and 10.5 min, respectively.

Table 4.2: Effect of pH on separation of PRG enantiomers

pH	$t_{R\ (S\text{-}PRG)}$	$t_{R\ (R\text{-}PRG)}$	α	Rs
6.5	14.06	16.4	1.3	1.3
7	11	13.5	1.5	1.4
7.5	8.5	10.5	1.8	2.2

Table: 4.3: Effect of buffer concentration, temperature on chromatographic retention parameters for the separation of PRG enantiomers

		Retention times (min)		Resolution	S/N ratio		Theoretical plates	
		t_1	t_2	$Rs_{1,2}$	$(S/N)_1$	$(S/N)_2$	N_1	N_2
	(mM)							
effect of buffer concentration	25	8.3	10.3	2.1	9.8	8.6	7038	7617
	20	8.5	10.5	2.2	7.5	7.5	7491	8818
	15	8.1	10.5	1.8	17.8	18.0	7344	7765
	10	5.24	6.32	1.6	15.0	13.7	7166	7792
	5	5.42	6.68	1.4	20.2	19.8	7143	7590
	(^{0}C)							
effect of temperature	15	9.5	8.04	2.2	20.5	18.7	6555	6650
	20	9	7.86	2.1	18.2	19.1	7765	7018
	25	8.5	10.5	2.2	24.3	22.5	7377	7813
	30	6.18	7.53	1.5	20.4	17.1	7711	7844
	35	6.05	7.39	1.4	18.3	15.7	7847	8130
	40	5.92	7.22	1.2	11.0	10.5	7653	9120

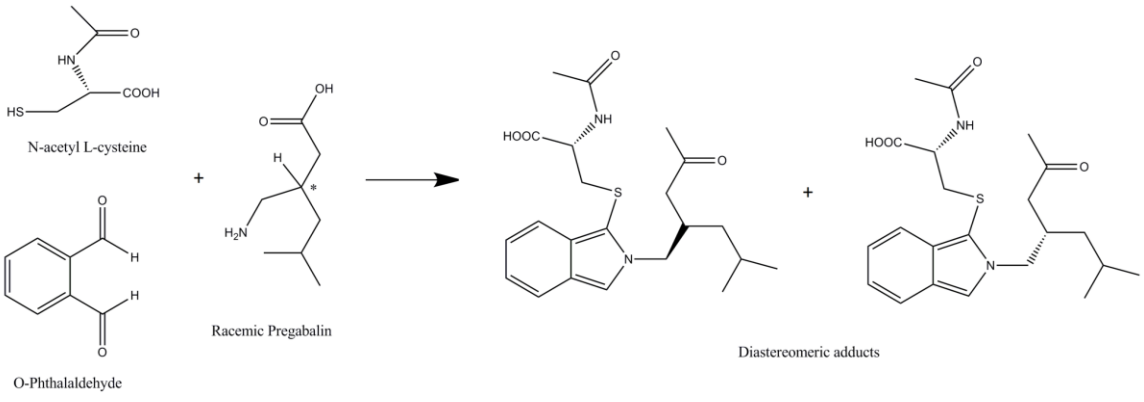

Fig: 4.6 Derivatization of PRG enantiomers with OPA + NAC.

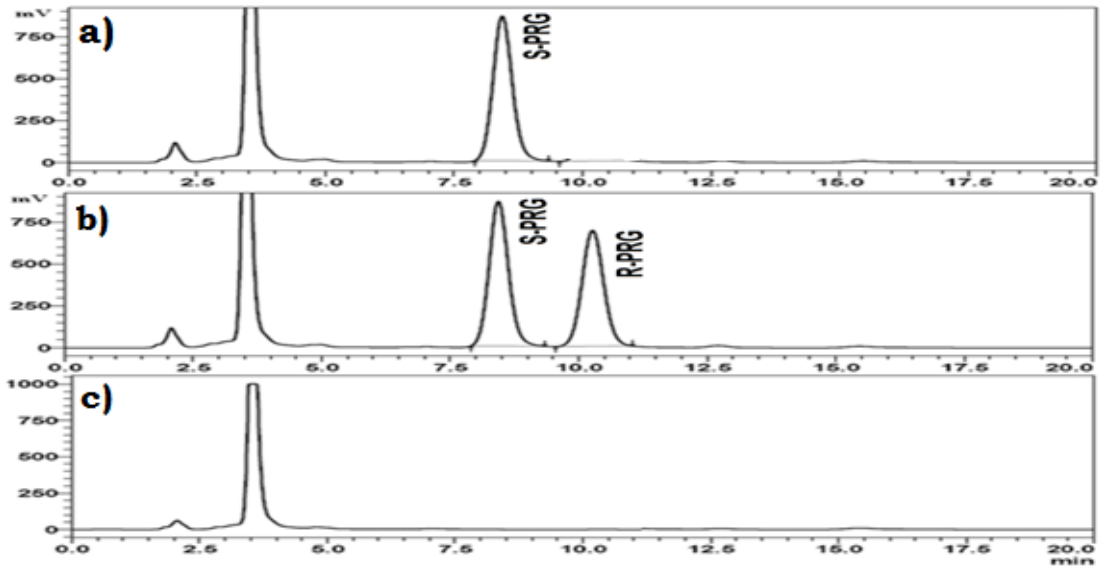

Fig: 4.7 Typical chromatograms showing the separation of a) S (-) PRG, b) S (-) R (+) enantiomers of PRG and c) blank.

4.6.2 Method validation

4.6.2.1 Lower limit of quantification and lower limit of detection

The LLOQ in 30µL rat plasma was 1.5 µg/mL at which the precision was 14.9 and 9.9% and accuracy -11.4 and -0.4% (n = 8) for both enantiomers. LOD was 0.75 µg/mL for each enantiomer at which the concentration the signal-to-noise ratio was greater than 3:1.

4.6.2.2 Linearity, accuracy, precision and recovery and selectivity

Calibration curves using peak area against the corresponding concentration with linear regression was selected as the most appropriate model. The coefficient of determination (r^2) was >0.995 ± 0.005 (mean ± SD, n = 5) for both enantiomers. Fig: 4.8 shows the linearity curves for S-PRG and R-PRG. Accuracy in terms of % relative

error and precision in terms of % RSD for the QC samples are presented in Table: 4.4. Recovery was reproducible over the days evaluated (3 days) (Table: 4.4). Blank plasma from six healthy blood samples from different rats did not show interfering peaks at the retention times of (S) and (R) PRG.

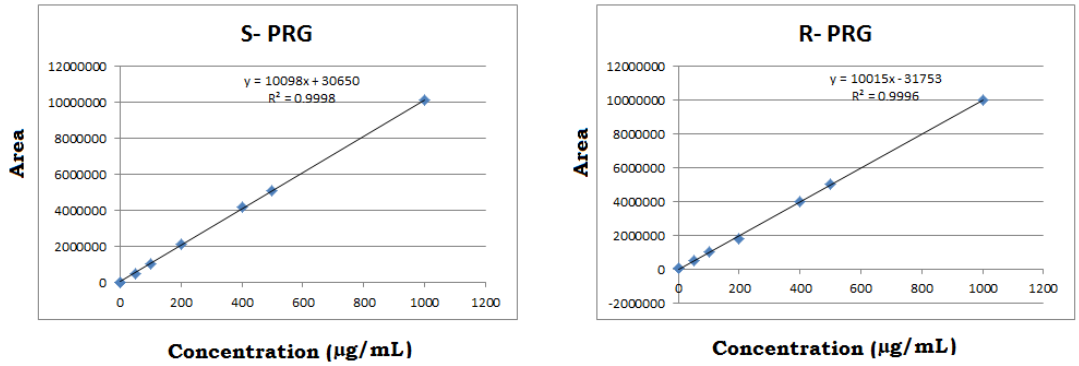

Fig: 4.8 Linearity curves for S-PRG and R-PRG.

Table: 4.4: Accuracy, intra- and inter-assay precision, and extraction recovery for the determination of PRG enantiomers in 30 µL rat plasma

	QC 1 (3 µg/mL)		QC 2 (400 µg/mL)		QC 3 (1000 µg/mL)	
	(S) - PRG	(R) - PRG	(S) - PRG	(R) - PRG	(S) - PRG	(R) - PRG
Experimental concentration (mean)	2.8	2.9	373	383	1047	1078
Within-day precision (%) n=25	9.7	7.6	4.9	4.8	6.9	6.8
Between-day precision (%) n=5	8.4	2.3	4.0	5.1	2.0	3.7
Total assay precision (%)	13	7.9	6.3	7.0	7.2	7.7
Accuracy (%)	-7.9	-4.2	-6.7	-4.3	5.5	9.0
Recovery (mean %)	72	71	66	67	72	73

4.6.3 Application to a pharmacokinetic study

Six Wistar rats (200–220 g) housed under standard conditions and had ad libitum access to water and standard laboratory diet throughout the experiments were used in the present study. After a single dose by oral administration of 5 mg/kg of racemic PRG to healthy wistar rats (n = 6), blood samples (30 µL) were collected from prick from the tail. Serial blood samples were spotted onto the FTA blood spot cards at 0, 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12 and 24 h post-dose. The spotted DBS sample was transferred to a centrifuge tube and added 500 µL extraction solvent and the tube was vortex mixed for 10 min. The extract was centrifuged at 2500rpm for 10 min and the supernatant was transferred to an autosampler vial. To this vial 200 µL of derivatization reagent and 700 µL of borate buffer were added. After sonicating for 1 min, 20µL of the reaction mixture was injected on to the HPLC system. The concentrations of PRG were determined from the calibration curve on the same day. Concentration–time and logarithmic concentration (Y-axis)–time profiles of PRG are shown in Fig: 4.9. The maximum DBS concentration (C) and time at which the concentration reached the maximum (T_{max}), the DBS concentration–time curve from 0 h to the last measurable concentration (AUC_{max}) and area under DBS concentration–time curve from 0 h to infinity (AUC_{0-t}) and the terminal half-life ($t_{1/2}$) for PRG enantiomers were calculated and given in Table: 4.5 and the enantioselectivity of both PRG enantiomers was shown in Fig: 4.9. Statistical analysis was performed

using Microsoft Excel 2007 while pharmacokinetic software, 'Ramkin', based on non-compartment model was used to calculate the [AUC] from the DBS drug concentration vs. time profiles. It could be seen from the results that as small as 30 µL blood is sufficient enough to determine all the pharmacokinetic parameters instead of 500 µL plasma samples which is required by other extraction procedures.

Table: 4.5 Pharmacokinetic parameters

Parameter	S-PRG	R-PRG
C_{max} (ng/mL)	545	380
t_{max} (h)	3.0	3.0
AUC_{0-24} (h ng /mL)	3966	6160
$t_{1/2}$ (h)	8.0	8.5

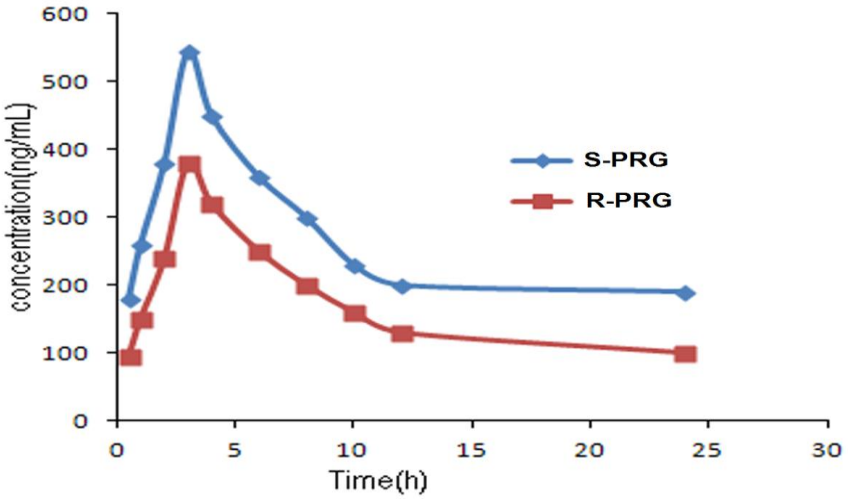

Fig: 4.9 Concentration-time profiles of S- and R- PRG enantiomers.

4.7 Conclusions

Pre column derivatization using OPA-NAC was successfully adopted in separation and determination of PRG enantiomers on dried blood spots. Further, the proposed method is simple and rapid as time required for derivatization was less than 2 min at room temperature. OPA-NAC based reagent provided successful conversion of PRG enantiomers completely into preferred diastereomers and their chromatographic resolution with good limits of detection. The method is simple, rapid, economical and high throughput as it utilizes an inexpensive achiral column with short run time. The high sensitivity combined with high robustness of pre column derivatization, good reproducibility and high sample throughput, makes the method ideal for clinical as well as therapeutic drug monitoring of the optical impurity R-PRG in a bioanalytical laboratory.

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CHAPTER 5

Separation of darunavir enantiomers on polysaccharide chiral stationary phases: A thermodynamic investigation

5.1 Introduction

Darunavir [(-)-[(3R, 3aS, 6aR)-hexahydrofuro [2, 3-b] furan-3-yl(2S,3R)-4-(4-amino-N-isobutylphenylsulfonamido)-3-hydroxy-1 phenyl butan-2-ylcarbamate] (DRV) (I) (Fig: 5.1) is a new HIV-1 protease inhibitor (PI) effective against viral strains that are not sensitive to other antiretrovirals. It is a prescription medicine approved by the U.S. Food and Drug Administration (FDA) for treatment of HIV infection in adults and children 3 years of age and older [1]. It works by blocking protease, and prevents HIV from replicating and lowers the amount of HIV in the blood. It is one of the key components of many salvage therapies in multi experienced patients [2]. The metabolism of DRV is cytochrome P450 (CYP) 3A4-dependent and is generally co-administered with low-dose of ritonavir [3]. Like other PIs, DRV is highly protein bound (~95%), while a small unbound fraction (5%) is available to penetrate the central nervous system [4]. Chemically, it has five stereogenic centers resulting in the possibility of 32 stereoisomers. Its enantiomer is (3S,3aR,6aS)-hexahydrofuro [2,3-b]furan-3-yl(2R,3S)-4-(4-amino-*N* isobutylphenyl sulfonamido)-3-hydroxy-1-phenylbutan-2-ylcarbamate and having low biological activity than DRV (I). Therefore, enantiomeric separation of DRV and its enantiomer is very important to assure the therapeutic efficacy and safety of its pharmaceutical preparations.

5.2 Literature survey

Various liquid chromatographic separation techniques were used for quantification of DRV along with other antiretroviral drugs in various species [5-18]. Dailly et al., have reported mass spectrometric assay for simultaneous determination of the plasma concentration of 11 antiretroviral agents by using column switching method [5]. Heine et al., have determined clinical evaluation method for plasma concentrations of DRV with etravirine, raltegravir and ritonavir in dried blood spots [6]. Some of the bio analytical methods were reported for the determination of most commonly prescribed protease inhibitors (PIs) and non-nucleoside reverse transcriptase inhibitors along with DRV in HPLC [7-11]. LC/MS/MS methods were also reported for the determination of DRV with metabolites [12-15]. Liquid chromatography–triple quadrupole mass spectrometric method [16] and UPLC MS/MS [17] for the determination of most commonly prescribed protease inhibitors and non-nucleoside reverse transcriptase inhibitors was developed and validated. Steady-state plasma and intracellular pharmacokinetics of darunavir with other anti-retro virals viz; raltegravir, etravirine and ritonavir in heavily pre-treated patients was studied [18]. A few methods were reported for determination of plasma concentrations of DRV without any combination of other anti-retroviral using HPLC with UV detection [19, 20]. However no single chiral method was reported till date.

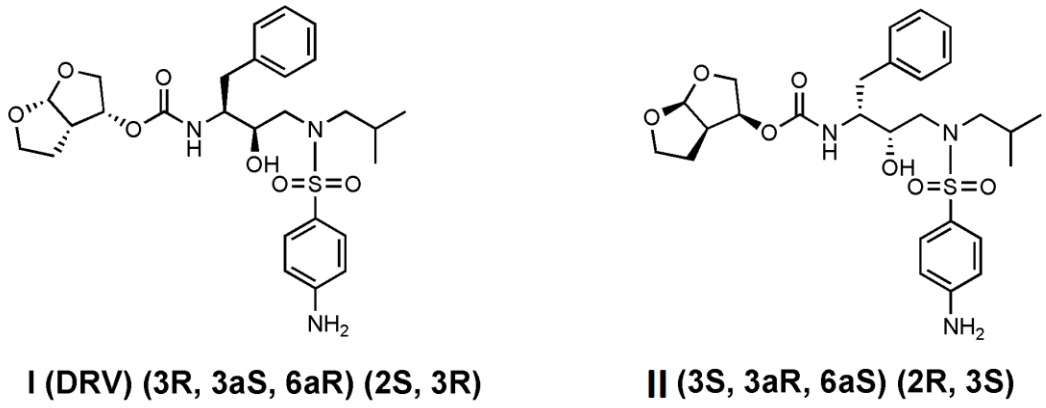

Fig: 5.1 Chemical structures of DRV (I) and its enantiomer (II).

5.3 Poly saccharide chiral stationary phases

Liquid chromatographic separation of enantiomers has been considerably advanced during the last few decades [21-23]. The coated polysaccharides, viz. cellulose and amylose on silica gel supports introduced by Okamoto et al., [24] have become routine chiral stationary phases (CSPs) for analytical and preparative enantiomeric separations [25-27]. The popular CSPs include CHIRALPAK AD-H, CHIRALPAK AS-H, CHIRALPAK AY-H, CHIRALCEL OZ-H, CHIRALCEL OJ, CHIRALCEL OD-H and CHIRALCEL OX-H. However, their utility for monitoring of synthetic organic reactions was found to be limited because most of the synthetic organic reactions are generally performed in nonstandard solvents such as dichloromethane (DCM), toluene, chloroform ($CHCl_3$), ethyl acetate (EtOAc), tetrahydrofuran (THF), acetone, methyl tert-butyl ether, and 1,4-dioxane. These solvents may partially or totally dissolve the coated stationary phases leading to decrease of column's life. Thus,

monitoring of enantiomeric excess of synthetic organic reactions was found to be difficult on these columns. It requires a tedious workup to remove nonstandard solvents prior to injection on coated chiral columns. To overcome such problems, various research groups have investigated the CSPs by immobilizing polysaccharides on silica gel supports [28-30]. These CSPs were generally prepared by chemically bonding polysaccharide phenylcarbamates to silica gel through hydroxyl groups either randomly or regioselectively. Enomoto et al. prepared amylose tris(3,5-dimethylphenylcarbamate) CSPs using enzyme catalyzed polymerization, in which amylose was chemically bonded non regio selectively to silica gel at the reducing terminal [31]. These CSPs have shown higher resolving power and used with nonstandard organic solvents such as THF and chloroform etc. Recently, three types of immobilized polysaccharide-based columns: CHIRALPAK IA, CHIRALPAK IB and CHIRALPAK IC have become commercially available [32-35]. Fig: 5.2 shows the chemical structures of chiral selectors of CHIRALPAK AD-H, IA and IC studied in the present work. Although the chiral selectors were the same, the immobilized CSPs show different enantioselectivity when compared with the corresponding coated columns under similar conditions.

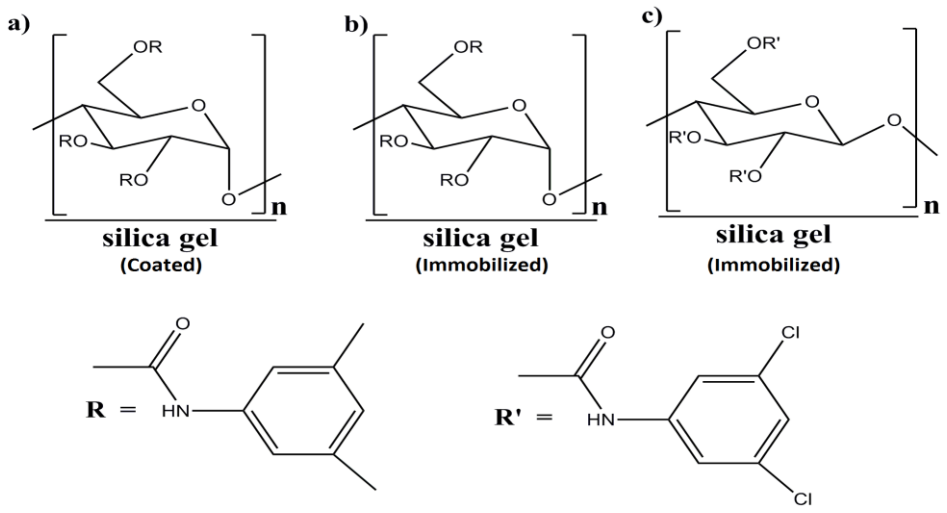

Fig: 5.2 Chemical structures of a) coated CHIRALPAK AD-H and immobilized b) CHIRALPAK IA and c) CHIRALPAK IC columns.

5.4 Objectives of the work

i. To investigate the separation of enantiomers on coated CHIRALPAK AD-H, immobilized CHIRALPAK IA and CHIRALPAK IC columns using a variety of mobile phases at different temperatures.

ii. To compare the DRV enantiomeric separations on coated (CHIRALPAK AD-H) and immobilized (CHIRALPAK IA) columns containing the same chiral selector amylose tris (3, 5-dimethylphenylcarbamate) using a range of mobile phase solvents composed of different organic modifiers.

iii. To study of effect of temperature and to derive apparent thermodynamic parameters to determine the strength of interactions between DRV enantiomers and CHIRALPAK AD-H, CHIRALPAK IA and CHIRALPAK IC CSPs.

5.5 Experimental

5.5.1 Chemicals and materials

All solvents used were of HPLC grade (E. Merck, Mumbai, India). Analytical reagent grade diethyl amine (DEA) (Spectrochem, Mumbai, India) was used. DRV and its enantiomers produced by a local pharmaceutical unit were obtained as gift samples.

5.5.2 Instrumentation

The HPLC system consisting of two LC-20AD pumps, photodiode array detector, an SIL-20AC auto sampler, a DGU-20A5 degasser, and CBM-20A controller (Shimadzu, Kyoto, Japan) was used. The chromatographic and the integrated data were recorded using HP-Vectra (Agilent, Waldbronn, Germany) computer system using LC-solution data acquiring software (Shimadzu, Kyoto, Japan).

5.5.3 Chromatography

The DRV (I) and its enantiomer were separated on CHIRALPAK AD-H, CHIRALPAK IA and CHIRALPAK IC columns (250 x 4.6mm, 5 µm particle size) (Daicel, Tokyo, Japan). The prepared mobile phases were filtered through a 0.45 mm membrane filter paper and degassed with an ultrasonic bath before use. The analytes were dissolved in 2-propanol at approximately 0.1mgmL^{-1} and filtered through a 0.45 mm membrane filter paper prior to injection. The flow rate was set at 1mLmin^{-1}. The ultraviolet detection wavelength was set at 266nm. The injection volume

was 5 mL. The void volume (v_0) of the column was determined by injecting 1, 3, 5-tri-tert-butylbenzene and determining the retention time (t_0) of the unretained peak. Capacity factors, k', were calculated using the formula $(t_R-t_0)/t_0$, where t_R is the retention time of particular enantiomer of DRV and t_0 is the retention time of the unretained peak. The separation factors (α) were calculated using the formula k_2' / k_1', where k_1' and k_2' are the capacity factors of first and second eluted enantiomers respectively. Their elution order was determined by injecting the individual compounds under similar conditions. Origin software was used to plot the linear Van't Hoff plots.

5.5.4 Chromatographic conditions

Columns	: CHIRALPAK AD-H, CHIRAL PAK IA and CHIRALPAK IC (250x4.6mm, 5µm particle size)
Mobile Phase	: Different combinations of n-hexane with organic modifiers: 2-propanol, 1-propanol, ethanol and diethylamine additive
Flow rate	: 1mLmin^{-1}
Auto sampler temperature	: 5°C
Injection volume	: 5µL

Column temperatures	: 20 to 50°C
Detector	:Photo diode array (PDA)
λ_{max}	: 266nm

5.6 Results and discussions

The separation of DRV enantiomers on both coated and immobilized versions of columns: CHIRALPAK AD-H, CHIRALPAK IA and CHIRALPAK IC using different mobile phase conditions at different temperatures was studied. The chromatographic retention data was used to derive the thermodynamic parameters and to interpret the strength of interactions between respective enantiomers of DRV and CSPs. DRV (I) and its enantiomer (V) were separated on three different columns, viz. CHIRALPAK AD-H, CHIRALPAK IA and CHIRALPAK IC columns under normal phase conditions. The mobile phases were under composed of n-hexane and different organic modifiers. Different volumes of standard solvents ethanol, 1-propanol and 2-propanol were tried on all the three columns. The separation was studied at different temperatures on all the three columns.

5.6.1 CHIRALPAK AD-H

Three types of alcohols, viz. ethanol, 2-propanol and 1-propanol in different combinations of n-hexane and 0.1% DEA were tried on CHIRALPAK AD-H. Chromatographic retention parameters such as retention factor (k'), selectivity (α) and resolutions (Rs) were determined

by changing % volume (25-45%) of alcohol at different temperatures (20-40°C). Fig: 5.3 illustrates the effect of volume and type of alcohol at column temperature 25°C.

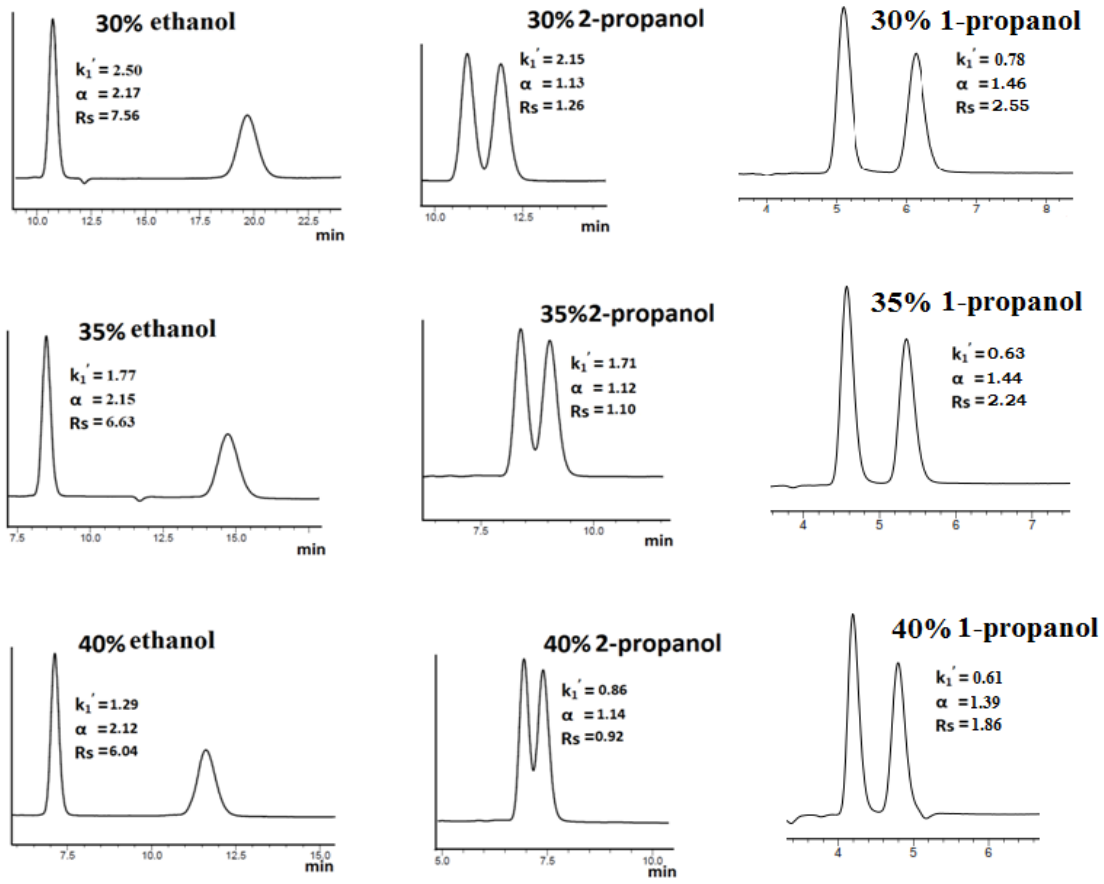

Fig: 5.3 Effect of volume (vertical comparison) and type of alcohol (horizontal comparison) on retention, selectivity and resolution of DRV (I) and its enantiomer DRV (II) on CHIRALPAK AD-H at 25°C.

Table: 5.1: Effect of volume of 2-propanol, ethanol and 1-propanol at different temperatures of CHIRALPAK AD-H on the retention (k'), selectivity (α) and resolution (Rs) of DRV enantiomers (I & II).

Organic modifier	T(°C)	25%				30%				35%				40%				45%			
		k_1'	k_2'	α_{12}	Rs_{12}	k_1'	k_2'	α_{12}	Rs_{12}	k_1'	k_2'	α_{12}	Rs_{12}	k_1'	k_2'	α_{12}	Rs_{12}	k_1'	k_2'	α_{12}	Rs_{12}
2-propanol	20	4.38	5.00	1.14	1.47	2.28	2.58	1.13	1.3	2.65	2.67	1.00	1.12	1.26	1.45	1.15	1.05	-	-	-	-
	25	4.13	4.64	1.12	1.44	2.15	2.43	1.13	1.26	1.71	1.91	1.12	1.10	0.86	0.98	1.14	0.92	-	-	-	-
	30	3.97	4.42	1.11	1.43	2.03	2.29	1.13	1.21	1.08	1.24	1.15	1.08	0.62	0.70	1.12	0.86	-	-	-	-
	35	3.79	4.18	1.10	1.39	1.90	2.15	1.13	1.15	0.77	0.96	1.25	1.07	0.44	0.46	1.04	0.75	-	-	-	-
	40	3.63	3.97	1.09	1.30	1.80	2.02	1.12	1.11	0.54	0.70	1.30	1.06	0.31	0.35	1.12	0.69	-	-	-	-
ethanol	20	-	-	-	-	2.68	6.08	2.27	8.08	1.87	4.29	2.30	6.80	1.58	3.30	2.09	6.11	-	-	-	-
	25	-	-	-	-	2.50	5.43	2.17	7.56	1.77	3.79	2.15	6.63	1.30	2.74	2.12	6.05	1.01	2.15	2.13	5.22
	30	-	-	-	-	2.33	4.73	2.03	8.01	1.65	3.38	2.05	6.45	1.03	2.16	2.10	6.02	-	-	-	-
	35	-	-	-	-	2.16	4.09	1.90	7.96	1.51	2.97	1.96	6.40	0.87	1.60	1.85	6.00	-	-	-	-
	40	-	-	-	-	2.00	3.55	1.77	7.63	1.42	2.53	1.78	6.82	0.65	1.32	2.01	5.98	1.46	2.30	1.57	5.30
1-propanol	20	-	-	-	-	0.81	1.19	1.48	2.42	0.66	0.96	1.46	2.10	0.83	1.11	1.33	1.80	-	-	-	-
	25	-	-	-	-	0.78	1.14	1.46	2.55	0.63	0.91	1.44	2.24	0.61	0.85	1.39	1.86	-	-	-	-
	30	-	-	-	-	0.75	1.09	1.45	2.67	0.61	0.87	1.42	2.25	0.79	1.03	1.31	1.89	-	-	-	-
	35	-	-	-	-	0.72	1.03	1.43	2.59	0.59	0.83	1.41	2.17	0.68	0.89	1.31	1.86	-	-	-	-
	40	-	-	-	-	0.66	0.91	1.37	2.12	0.54	0.73	1.35	1.81	0.59	0.76	1.28	1.52	-	-	-	-

Conditions: flow rate: 1 $mLmin^{-1}$; UV: 266nm; injection volume: 5µL

"-"experiment not performed.

It could be seen from Fig: 5.3, better separation was obtained using ethanol as an organic modifier. The retention factors were almost same for both ethanol and 2-propanol but were less in case of 1-propanol. However, there was a large difference in selectivity and resolution between ethanol and other alcohols (horizontal comparison). Similar trends were observed in other experiments. It could be seen from Fig: 5.3 that better selectivity (α = 2.17) and resolution (Rs = 7.56) were obtained using 30% of ethanol at 25°C. Table: 5.1 gives the retention data showing the effect of different volumes of 2-propanol, ethanol and 1-propanol on selectivity and resolution of DRV enantiomers at various column temperatures.

5.6.2 CHIRALPAK IA

Initially, the separation of enantiomers of DRV with non standard range of solvents such as THF, DCM, EtOAc and $CHCl_3$ was attempted on CHIRALPAK IA, but not successful. Later, three different standard solvents viz; ethanol, 2-propanol and 1-propanol were tried on CHIRALPAK AD-H in different combinations of n-hexane and 0.1% DEA and good separations were achieved. The retention factor, selectivity and resolutions were determined by changing the volume (15-45%) alcohol at various temperatures (20-40°C). Fig: 5.4 illustrates the effect of volume and type of alcohol on the retention, selectivity and resolutions of the enantiomers of DRV at 25°C. As shown in Fig: 5.4, ethanol clearly shows

better separation among the three alcohols in all the cases. Retention factors were increased (horizontal comparison) from1-propanol to ethanol to 2-propanol. It could be due to the incorporation of different size or shape of alcohol modifiers into the chiral cavities of the CSP. Thus, the modified stereo environment influences the solute retention as reported elsewhere [36]. However, the selectivity and resolutions were increased from 2-propanol to 1-propanol to ethanol. Similar trends were observed in other experiments.

In case of different volumes of alcohol, as the volume of ethanol increased from 30% to 40% (vertical comparison) retention factors and resolutions were decreased. This was in accordance with the general understanding that the H-bonding interactions between the enantiomers and CSPs decrease with increased alcohol content in the mobile phase leading to decreased retentions. However, selectivities did not change much. Similar trend was noticed in the case of 2-propanol and 1-prpanol. It could be seen from Fig: 5.4, better selectivity (α = 1.33) and resolution (Rs = 2.71) were obtained using 35% of ethanol at 25°C. Table: 5.2 gives the retention data showing the effect of volume of 2-propanol, ethanol and 1-propanol on selectivity and resolution of enantiomers of DRV at different column temperatures. However, better separation was obtained on CHIRALPAK AD-H compared to CHIRALPAK IA.

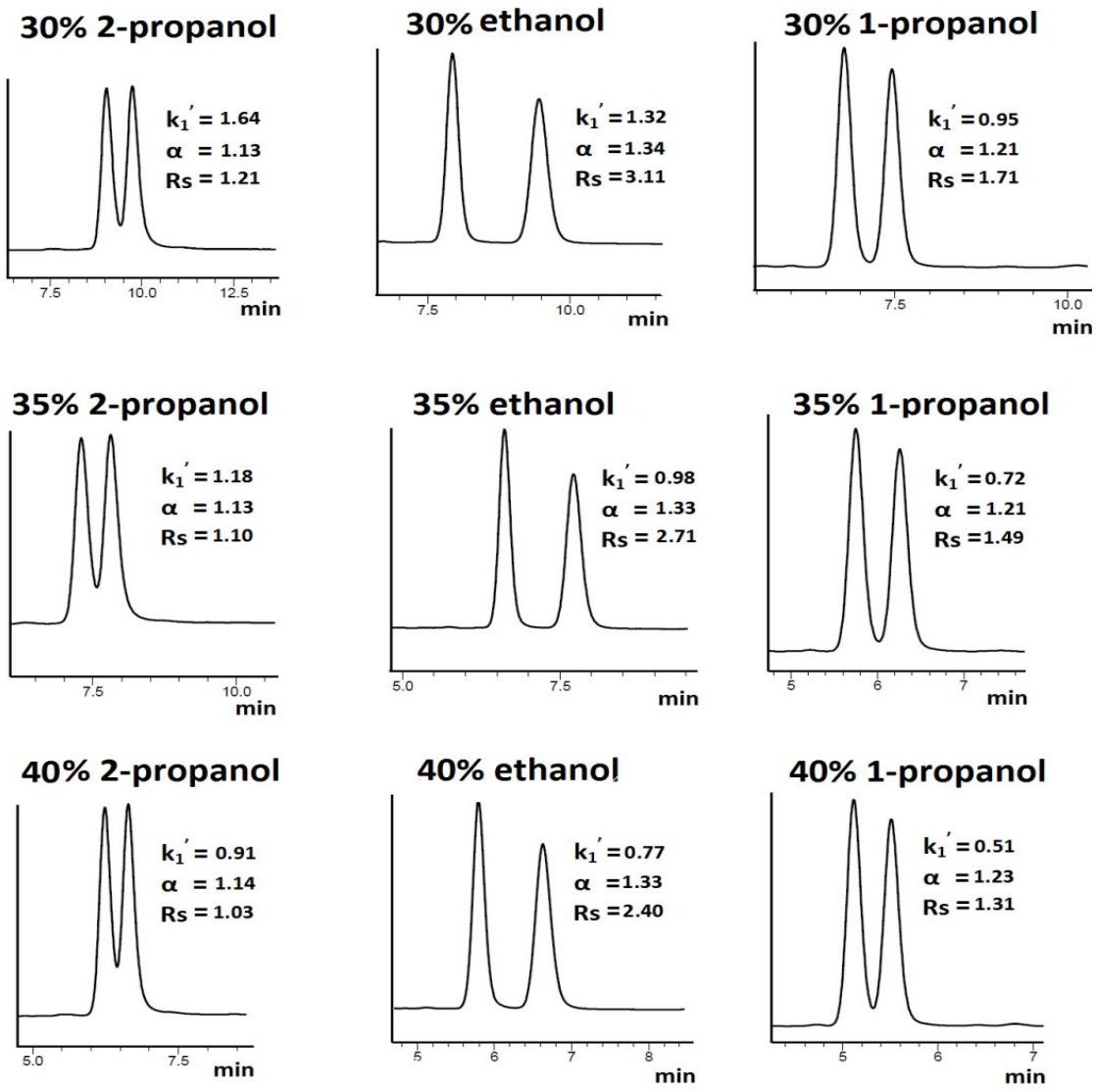

Fig: 5.4 Effect of volume (vertical comparison) and type of alcohol (horizontal comparison) on retention, selectivity and resolution of DRV (I) and its enantiomer DRV (II) on CHIRALPAK IA column at 25°C.

Table: 5.2 Effect of volume of 2-propanol, ethanol and 1-propanol at different column temperatures CHIRALPAK IA on the retention, selectivity and resolution of DRV enantiomers (I) & (II).

Organic modifier		15%				20%				25%				30%				35%				40%				45%			
		k_1'	k_2'	α_{12}	Rs_{12}	k_1'	k_2'	α_{12}	Rs_{12}	k_1'	k_2'	α_{12}	Rs_{12}	k_1'	k_2'	α_{12}	Rs_{12}	k_1'	k_2'	α_{12}	Rs_{12}	k_1'	k_2'	α_{12}	Rs_{12}	k_1'	k_2'	α_{12}	Rs_{12}
	T(ºC)																												
	20	-	-	-	-	4.32	4.91	1.14	1.50	2.60	2.99	1.15	1.38	1.75	2.00	1.14	1.27	1.28	1.46	1.15	1.17	0.98	1.13	1.15	1.09	0.82	0.94	1.15	1.02
	25	-	-	-	-	4.06	4.54	1.12	1.44	2.48	2.83	1.14	1.33	1.64	1.85	1.13	1.21	1.18	1.34	1.13	1.10	0.91	1.03	1.14	1.03	0.75	0.86	1.14	0.95
2-propanol	30	10.09	11.10	1.10	1.73	3.76	4.29	1.14	1.62	2.32	2.59	1.12	1.44	1.54	1.69	1.10	1.27	1.12	1.22	1.09	1.13	0.84	0.94	1.12	0.96	0.68	0.76	1.12	0.85
	35	9.78	10.61	1.08	1.55	3.58	3.95	1.10	1.44	2.22	2.44	1.10	1.26	1.44	1.59	1.10	1.19	1.02	1.14	1.11	1.06	0.77	0.86	1.11	0.86	0.61	0.68	1.11	0.75
	40	9.25	9.97	1.08	1.50	3.38	3.67	1.08	1.35	2.11	2.29	1.09	1.22	1.38	1.48	1.07	1.07	0.97	1.06	1.09	0.89	0.72	0.80	1.10	0.77	-	-	-	-
	20	-	-	-	-	2.75	3.78	1.95	3.89	1.99	2.76	1.39	3.16	1.38	1.91	1.38	2.73	1.03	1.41	1.38	2.40	0.80	1.09	1.37	2.14	0.64	0.87	1.36	1.92
	25	-	-	-	-	2.66	3.62	1.36	3.73	1.78	2.41	1.35	3.16	1.32	1.76	1.34	3.11	0.98	1.31	1.33	2.71	0.77	1.02	1.33	2.40	0.62	0.83	1.33	2.13
ethanol	30	-	-	-	-	2.53	3.05	1.32	3.79	1.68	2.22	1.32	3.35	1.17	1.54	1.32	2.84	0.87	1.14	1.31	2.43	0.68	0.88	1.31	2.11	0.55	0.71	1.30	1.84
	35	-	-	-	-	2.27	2.93	1.29	3.77	1.52	1.95	1.28	3.14	1.10	1.41	1.28	2.70	0.80	1.01	1.27	2.22	0.64	0.81	1.27	1.97	0.51	0.64	1.26	1.65
	40	-	-	-	-	2.09	2.63	1.26	3.53	-	-	-	-	1.02	1.22	1.19	2.23	0.75	0.90	1.20	1.79	0.58	0.70	1.20	1.52	0.47	0.57	1.20	1.32
	20	-	-	-	-	2.24	2.70	1.21	2.27	1.49	1.80	1.21	1.96	1.04	1.26	1.21	1.72	0.77	0.94	1.22	1.51	0.54	0.67	1.23	1.30	-	-	-	-
	25	3.59	4.33	1.21	2.80	2.10	2.54	1.21	2.38	1.36	1.65	1.22	1.99	0.95	1.15	1.21	1.71	0.72	0.87	1.21	1.49	0.51	0.63	1.23	1.31	-	-	-	-
1-propanol	30	-	-	-	-	1.95	2.35	1.21	2.45	1.27	1.57	1.22	2.11	0.89	1.08	1.21	1.79	0.65	0.80	1.22	1.53	0.48	0.58	1.23	1.31	-	-	-	-
	35	3.18	3.84	1.21	3.09	1.82	2.20	1.21	2.53	1.20	1.44	1.21	2.13	0.83	1.00	1.21	1.79	0.60	0.73	1.22	1.52	0.44	0.54	1.23	1.30	-	-	-	-
	40	3.02	3.64	1.21	3.18	1.70	2.04	1.20	2.57	1.12	1.35	1.20	2.13	0.77	0.93	1.21	1.76	0.56	0.68	1.21	1.48	0.41	0.50	1.23	1.27	-	-	-	-

Conditions: flow rate: 1 $mLmin^{-1}$; UV: 266nm; injection volume: 5µL

"-"experiment not performed.

5.6.3 CHIRALPAK IC

As in the case of CHIRALPAK IA, three different standard solvents ethanol, 2-propanol and 1-propanol were tried in different combinations of n-hexane and 0.1% DEA on CHIRALPAK IC. The retention factor, selectivity and resolutions were measured by changing the volume (30-55%) of alcohol at different temperatures (20-40°C). Fig: 5.5 illustrates the effect of volume and type of alcohol on the retention, selectivity and resolutions of the enantiomers of DRV at 25°C. As shown in Fig: 5.5, 2-propanol gave better separation among the three alcohols. The retention factors, selectivities and resolutions were increased (horizontal comparison) from ethanol to 1-propanol to 2-propanol as in the order of their polarity indices. Similar trends were followed in all combinations of volumes of alcohols. It could be observed from the Fig: 5.5, better selectivity (α = 2.20) and resolution (Rs = 6.96) were obtained using 35% of 2-propanol at 25°C. Table: 5.3 gives the retention data showing the effect of concentration of 2-propanol, ethanol and 1-propanol on selectivity and resolution of enantiomers of DRV at different column temperatures. However, better separations were obtained with all alcohols on CHIRALPAK IC when compared to CHIRALPAK AD-H and CHIRALPAK IA.

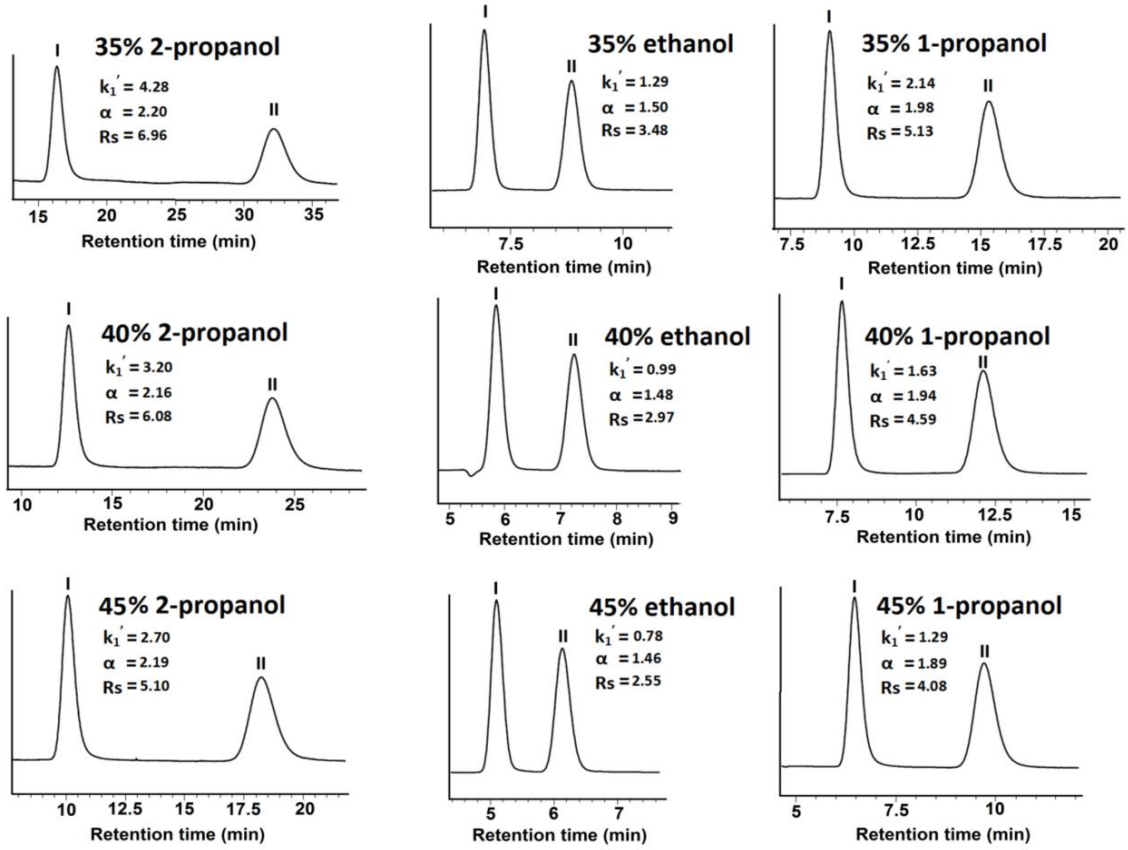

Fig: 5.5 Effect of volume (vertical comparison) and type of alcohol (horizontal comparison) on retention, selectivity and resolution of DRV (I) and its enantiomer DRV (II) on CHIRALPAK IC at 25°C.

Table: 5.3 Effect of volume of ethanol, 1-propanol and 2-propanol at different column temperatures of CHIRALPAK IC on the retention, selectivity and resolution of DRV enantiomers (I) & (II).

Organic modifier		30%				35%				40%				45%				50%				55%			
		k_1'	k_2'	α	Rs	k_1'	k_2'	α	Rs	k_1'	k_2'	α	Rs	k_1'	k_2'	α	Rs	k_1'	k_2'	α	Rs	k_1'	k_2'	α	Rs
	T(ºC)																								
Ethanol	20	1.85	2.82	1.53	3.90	1.35	2.03	1.51	3.29	1.03	1.54	1.50	2.80	0.81	1.19	1.48	2.42	0.66	0.96	1.46	2.10	0.83	1.11	1.33	1.80
	25	1.78	2.70	1.52	4.10	1.29	1.94	1.50	3.48	0.99	1.47	1.48	2.97	0.78	1.14	1.46	2.55	0.63	0.91	1.44	2.24	0.81	1.06	1.32	1.86
	30	1.72	2.56	1.49	4.33	1.25	1.84	1.47	3.56	0.97	1.41	1.46	3.10	0.75	1.09	1.45	2.67	0.61	0.87	1.42	2.25	0.79	1.03	1.31	1.89
	35	1.67	2.45	1.47	4.49	1.21	1.76	1.45	3.68	0.92	1.33	1.44	3.08	0.72	1.03	1.43	2.59	0.59	0.83	1.41	2.17	0.68	0.89	1.31	1.86
	40	1.12	1.55	1.38	3.05	1.12	1.55	1.38	3.05	0.85	1.17	1.37	2.49	0.66	0.91	1.37	2.12	0.54	0.73	1.35	1.81	0.59	0.76	1.28	1.52
1-propanol	20	3.11	6.42	2.06	5.25	2.25	4.55	2.02	4.66	1.72	3.40	1.98	4.15	1.37	2.64	1.93	3.76	ND	ND	ND	ND	ND	ND	ND	ND
	25	2.96	5.99	2.02	5.83	2.14	4.24	1.98	5.13	1.63	3.16	1.94	4.59	1.29	2.44	1.89	4.08	1.31	2.31	1.76	3.68	ND	ND	ND	ND
	30	2.83	5.57	1.97	6.32	2.04	3.93	1.93	5.52	1.55	2.93	1.89	4.93	1.22	2.27	1.85	4.40	1.26	2.18	1.72	3.93	ND	ND	ND	ND
	35	2.72	5.21	1.91	6.78	1.98	3.67	1.86	5.92	1.48	2.74	1.84	5.22	1.17	2.11	1.80	4.64	1.21	2.04	1.68	4.12	1.12	1.80	1.60	3.65
	40	2.61	4.88	1.87	7.06	1.90	3.44	1.81	6.15	1.43	2.55	1.79	5.41	1.12	1.97	1.75	4.76	1.16	1.89	1.64	4.19	1.07	1.67	1.56	3.69
2-propanol	20	ND	ND	ND	ND	4.71	10.67	2.27	6.18	3.52	7.85	2.23	5.72	2.70	5.93	2.19	5.10	2.15	4.64	2.16	4.67	1.77	3.75	2.12	4.35
	25	ND	ND	ND	ND	4.28	9.41	2.20	6.96	3.20	6.92	2.16	6.08	2.46	5.24	2.13	5.50	1.94	4.07	2.10	5.01	1.60	3.29	2.05	4.67
	30	ND	ND	ND	ND	3.98	8.46	2.12	7.30	2.97	6.15	2.07	6.55	2.24	4.60	2.05	5.87	1.76	3.56	2.02	5.40	1.45	2.88	1.98	4.96
	35	ND	ND	ND	ND	2.74	5.52	2.01	6.91	2.74	5.52	2.01	6.91	2.07	4.11	1.99	6.28	1.61	3.14	1.95	5.66	1.33	2.54	1.91	5.19
	40	4.42	7.81	1.77	7.85	3.54	6.96	1.96	7.96	2.59	5.02	1.94	7.20	1.93	3.70	1.92	6.50	1.51	2.86	1.90	5.93	1.23	2.29	1.86	5.38

Conditions: flow rate: 1 $mLmin^{-1}$; UV: 266nm; injection volume: 5µL

"-"experiment not performed.

5.6.4 Vis-à-vis comparison.

The three columns with respect to type of alcohol and temperature for separation of DRV enantiomers were compared. Chiral selectivity of all the three columns was studied.

5.6.4.1 Effect of alcohol

The effect of alcohol on chromatographic retention parameters (k', α and R_s) was evaluated by fixing one alcohol on three different columns. Fig: 5.6 shows the comparison of retention parameters on CHIRALPAK AD-H, CHIRALPAK IA and CHIRALPAK IC with different alcohols using n-hexane: ethanol: DEA (60:40:0.1, v/v/v), n-hexane: 2-propanol: DEA (60:40:0.1, v/v/v) and n-hexane: 1-propanol: DEA (60:40:0.1, v/v/v) as mobile phases at 25°C; Flow rate was 1mLmin^{-1}. In case of ethanol, both CHIRALPAK IA and CHIRALPAK IC showed similar retention, selectivity and resolutions; however large selectivity and resolutions obtained on CHIRALPAK AD-H. Where as in case of 2-propanol, both CHIRALPAK AD-H and CHIRALPAK IA showed similar retention, selectivity and resolutions; however large selectivity and resolutions obtained on CHIRALPAK IC. In case of 1-propanol, improved selectivity and resolutions with more retention times observed on CHIRALPAK IC than CHIRALPAK AD-H and CHIRALPAK IA. Overall In other words ethanol on CHIRALPAK AD-H, 2-propanol and 1-propanol on CHIRALPAK IC showed good separations. The enantiomeric separations were in the order of

CHIRALPAK AD-H > CHIRALPAK IC > CHIRALPAK IA in case of ethanol, CHIRALPAK IC > CHIRALPAK IA > CHIRALPAK AD-H in case of 2-propanol and CHIRALPAK IC > CHIRALPAK AD-H > CHIRALPAK IA in case of 1-propanol. This trend was followed not only in the case of 40% alcohol but also in other compositions. In all these cases, CHIRALPAK IC showed better separation when compared to CHIRALPAK IA.

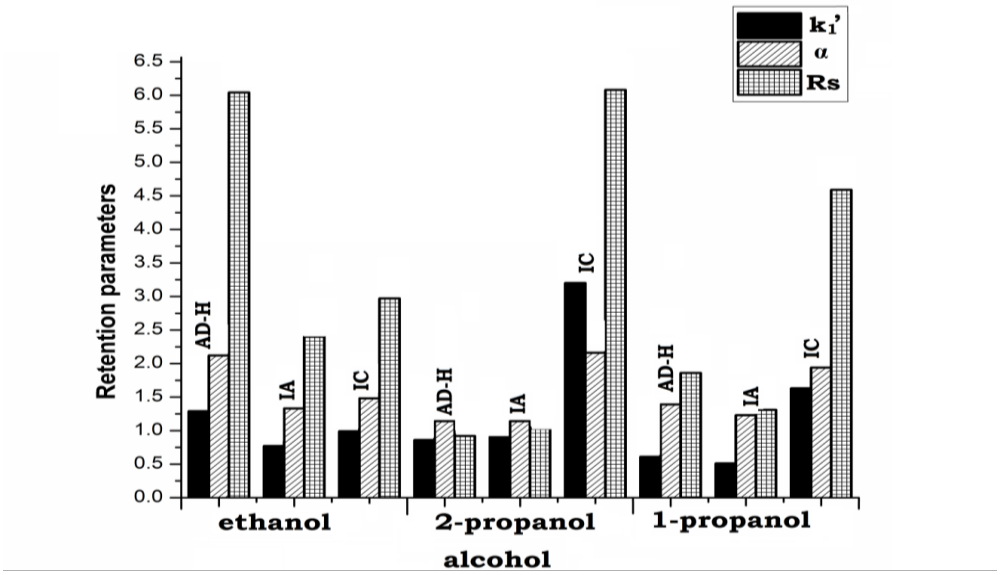

Fig: 5.6 Comparison of retention parameters (k', α and Rs) of DRV and its enantiomer on CHIRALPAK AD-H, CHIRALPAK IA and CHIRALPAK IC using alcohols ethanol, 2-propanol and 1-propanol. For mobile phase conditions, refer section 5.6.4.1.

5.6.4.2 Effect of temperature

The effect of temperature was studied by changing the temperature between 20 and 40°C with an interval of 5^0C. Fig: 5.7 shows the comparison of retention parameters (k', α and Rs) of DRV and its enantiomer on CHIRALPAK AD-H, CHIRALPAK IA and CHIRALPAK IC with ethanol as an organic modifier in the mobile phase (n-hexane:

ethanol: DEA (60:40:0.1, v/v/v)). As shown in Fig: 5.7, as the temperature of CHIRALPAK AD-H increases from 20 to 40°C, retention factors were decreased, selectivities were not changed much, however high resolutions were obtained at all temperatures except 40°C. In case of CHIRALPAK IA, as temperature increases from 20 to 40°C, all the retention parameters were decreased. In case of CHIRALPAK IC, as the temperature increases from 20 to 40°C, retention factors were decreased, selectivities were not changed much, however resolutions were increased from 20 to 35°C and decreased at 40°C. Overall, good separations were observed at 35°C on all the columns. Thus the enantiomeric separations were in the order of CHIRALPAK AD-H > CHIRALPAK IC > CHIRALPAK IA at all the temperatures.

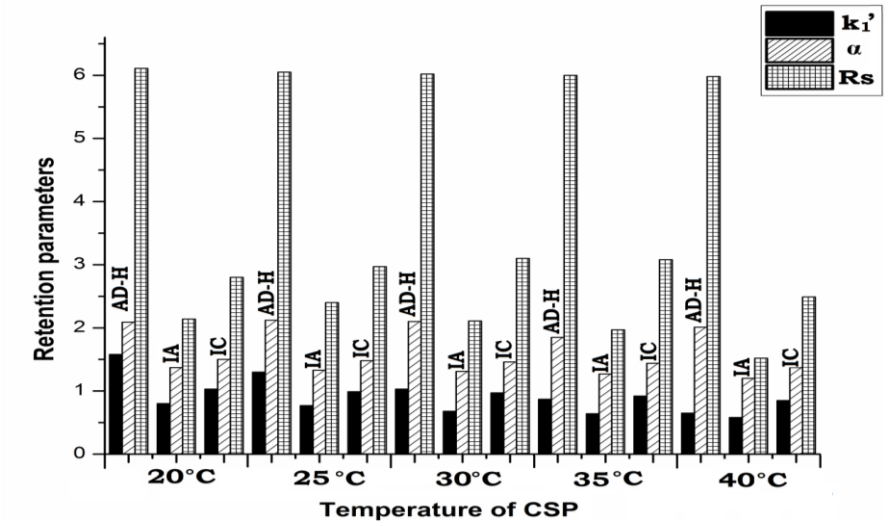

Fig: 5.7 Comparison of retention parameters (k', α and Rs) of DRV and its enantiomer (+) DRV on CHIRALPAK AD-H, CHIRALPAK IA and CHIRALPAK IC using ethanol as an organic modifier. For mobile phase conditions, refer section 5.6.4.2.

Fig: 5.8 shows the same comparison but with 2-propanol as organic modifier (n-hexane: 2-propanol: DEA (60:40:0.1, v/v/v)). As shown in Fig: 5.8, as the temperature CHIRALPAK AD-H increases from 20 to 40°C, retention factors were decreased; however, interestingly low selectivities and resolutions were obtained without changing much. This was totally reverse to the case of ethanol where high selectivities and resolutions were obtained. In case of CHIRALPAK IA, as temperature increases from 20 to 40°C, retention factors were decreased, selectivities and resolutions were not changed much. In case of CHIRALPAK IC, retention factors and selectivities were decreased; however high resolutions with increasing trend were obtained at all temperatures except at 20°C which was reverse to the case of ethanol where low resolutions were obtained. Overall, good separations were observed at 40°C on all the columns. Thus the enantiomeric separations were in the order of CHIRALPAK IC > CHIRALPAK IA > CHIRALPAK AD-H at all the temperatures. However, good separations were obtained on CHIRALPAK AD-H at all temperatures.

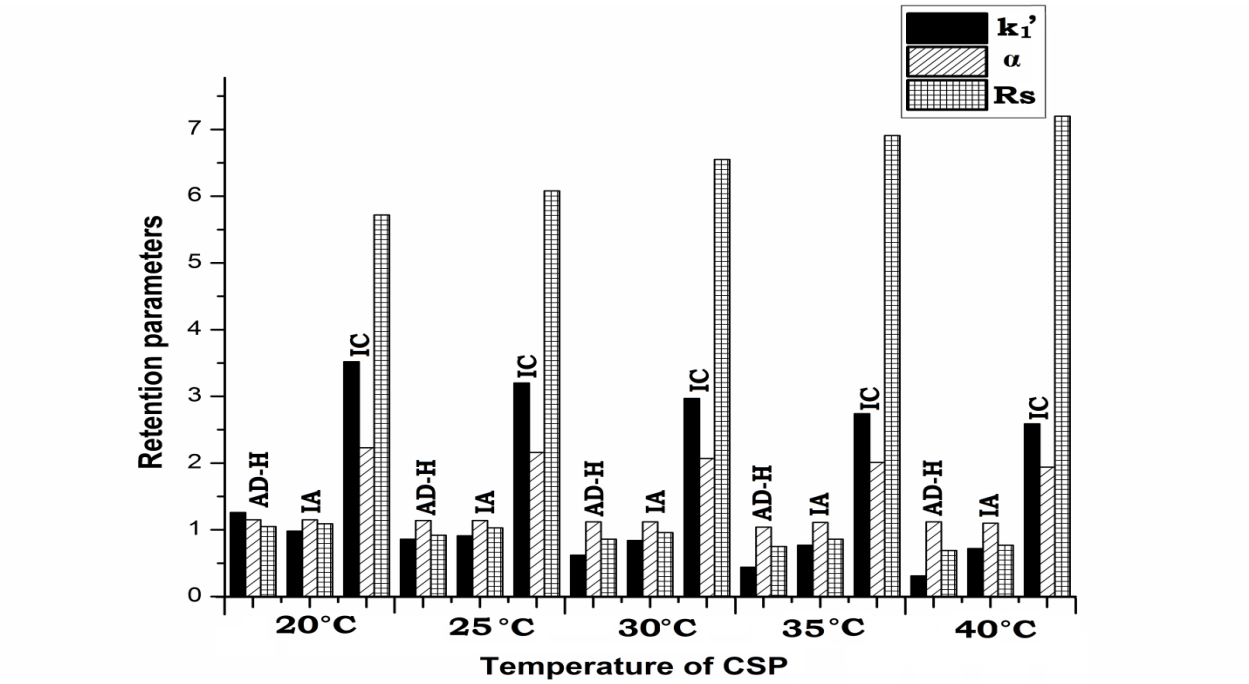

Fig: 5.8 Comparison of retention parameters (k', α and Rs) of DRV and its enantiomer on CHIRALPAK AD-H, CHIRALPAK IA and CHIRALPAK IC using 2-prpanol. For mobile phase conditions, refer section 5.6.4.2.

Fig: 5.9 shows the comparison of retention parameters (k', α and Rs) as 1-propanol as organic modifier (n-hexane: 1-propanol: DEA (60:40:0.1, v/v/v)). As shown in Fig: 5.9, as the temperature increases from 20 to 40°C, retention factors did not follow any trend, however, selectivities and resolutions were obtained without changing much. In case of CHIRALPAK IA and CHIRALPAK IC, as temperature increased from 20 to 40°C, the similar trend was followed as in case of 2-propanol. Overall, well defined separations were observed at 35°C on all the three columns. Thus the enantiomeric separations were in the order of CHIRALPAK IC > CHIRALPAK AD-H > CHIRALPAK IA at all temperatures.

Table: 5.4 shows the chiral selectivity for separation of DRV enantiomers using various alcohols in different volumes on CHIRALPAK AD-H, CHIRALPAK IA and CHIRALPAK IC columns at different temperatures. As shown in Table: 5.4, CHIRALPAK IC yielded good separation with all percentages of all polar organic modifiers among three columns. Thus the order of alcohols in terms of enantiomeric separation was ethanol >1propanol> 2-propanol on all the three columns.

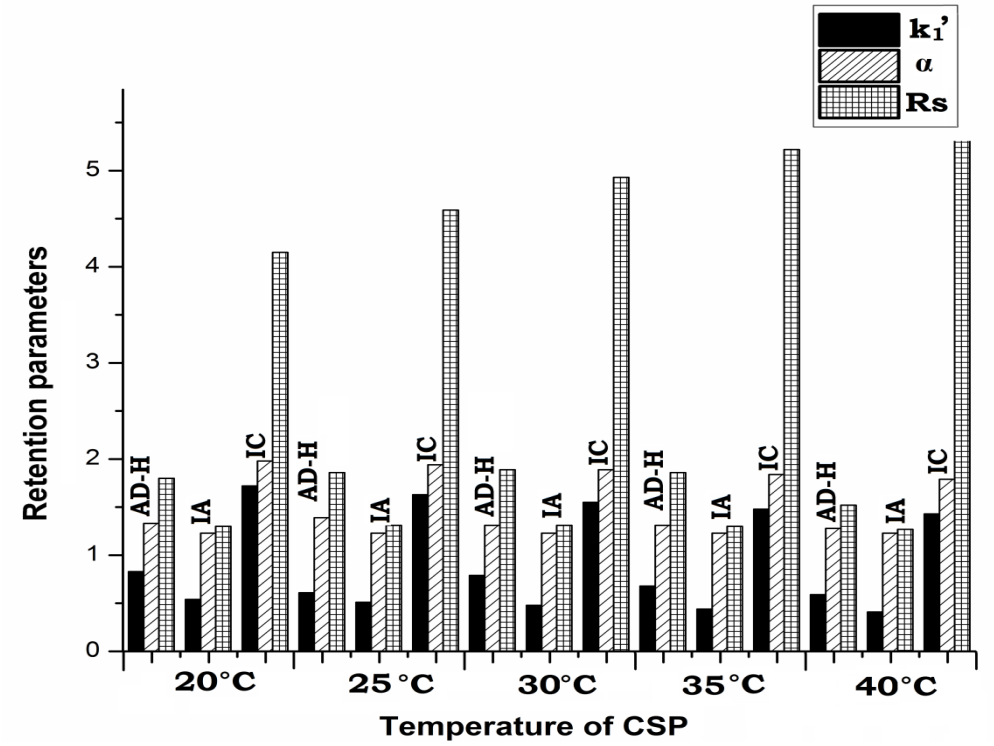

Fig: 5.9 Comparison of retention parameters (k', α and Rs) of DRV and its enantiomer (+) DRV on CHIRALPAK AD-H, CHIRALPAK IA and CHIRALPAK IC using 1-propanol. For mobile phase conditions, refer section 5.6.4.2.

Table: 5.4 Chiral selectivity for separation of DRV enantiomers using various alcohols in different volumes on CHIRALPAK AD-H, CHIRALPAK IA and CHIRALPAK IC at different temperatures

		CSP																
		CHIRALPAK AD-H						CHIRALPAK IA						CHIRALPAK IC				
Organic modifier	(°C) / (%)	20	25	30	35	40		20	25	30	35	40		20	25	30	35	40
2-propanol	30																	
	35																	
	40																	
	45																	
ethanol	30																	
	35																	
	40																	
	45																	
1-propanol	30																	
	35																	
	40																	
	45																	

: Well separation

: Base line separation

: Partial separation

: not determined

5.6.5 Thermodynamic investigation

In a chromatographic enantioseparation, the relation between chromatographic retention data and temperature of the chiral column can be described by the Van't Hoff equation [37-40].

$$ln\ k' = -\Delta H^0/RT + \Delta S^0/R + ln\ \Phi \equiv ln\ k' = -\Delta H^0/RT + \Delta S^* \quad (1)$$

$$ln\ \alpha = -\Delta\Delta H^0/RT + \Delta\Delta S^0/R \quad (2)$$

Where, k' is retention factor of the particular enantiomer, α is separation factor for a given enantiomeric pair. ΔH^0 and ΔS^0 represent the differences in the enthalpy and entropy respectively when one

enantiomer transfers from mobile phase to the stationary phase. *R* is universal gas constant. *Φ* is phase ratio. *ΔS** is used to substitute for the expression $\Delta S^0/R+ln\ \Phi$. The *ΔS** values were used mainly for comparison, but not for determination of the Δ*S* values as the phase ratio was unknown. The $\Delta\Delta H^0$ and $\Delta\Delta S^0$ represent the differences of ΔH^0 and ΔS^0 for a given pair of enantiomers respectively. If the plots of *ln k'* or *ln α* against *1/T* are linear in a temperature range, the corresponding thermodynamic parameters ΔH^0 and ΔS^*, which are temperature-independent, can be deduced from the slope (ΔH^0= *-slope x R*) and the intercept (*ΔS**= *intercept x R*) of the straight lines. The linear character also suggests that the conformation of the CSP does not change substantially within the range of experimental temperatures. The thermodynamic parameters derived by this method are apparent, not intrinsic [41] and comparison of these thermodynamic parameters ΔH^0, ΔS^*, $\Delta\Delta H^0$ and $\Delta\Delta S^*$ may give some understanding of chiral recognition mechanism and information regarding strength of interactions between the analytes and CSP. Attempts were made to calculate thermodynamic parameters for the separation of DRV enantiomers on CHIRALPAK AD-H using ethanol and 2-propanol, CHIRALPAK IA and CHIRALPAK IC using alcohols 2-propanol and 1-propanol. Because of fluctuation of the α values under these conditions, the $\Delta\Delta H^0$ and $\Delta\Delta S^*$ were determined directly from the differences of ΔH_2^0 and ΔH_1^0, ΔS_2^* and ΔS_1^*.

5.6.5.1 CHIRALPAK AD-H

As shown in Table: 5.1, in case of 2-propanol, the k' values decreased as the column temperature was increased for all volumes of 2-propanol. Fig: 5.10 shows the Van't Hoff plots of a) ln k_1' vs 1/T and ln k_2' vs 1/T were highly linear in the temperature range of 20 to 40°C indicating that the conformation of the CSP did not change in the studied temperature range.

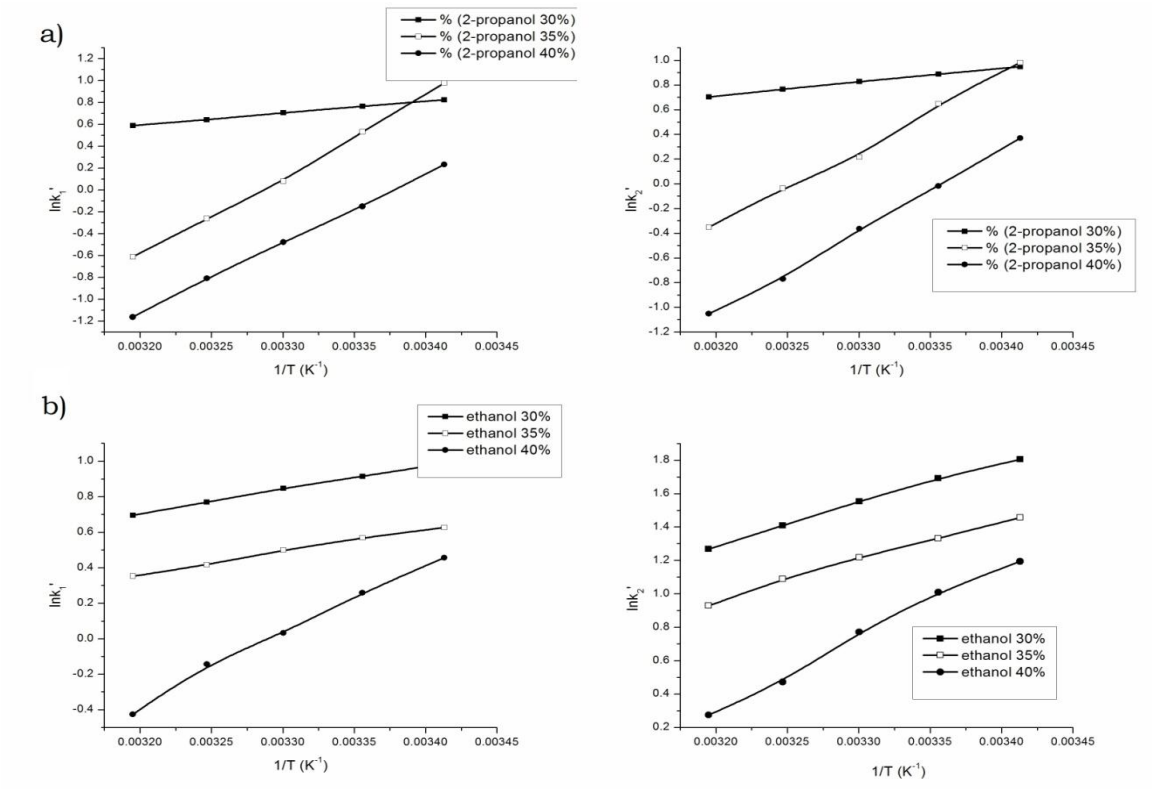

Fig: 5.10 Typical Van't Hoff plots for the separation of DRV enantiomers on CHIRALPAK AD-H using alcohols a) 2-propanol and b) ethanol in different volumes.

The corresponding thermodynamic parameters ΔH^0 and ΔS^* were calculated from these plots of ln k_1' vs 1/T and ln k_2' vs 1/T respectively

(Table: 5.5). As shown in Table: 5.5, as the volume 2-propanol increases, the absolute ΔH_1^0 and ΔH_2^0 values of DRV increased (-9.056 to -52.608 $kJmol^{-1}$ and -9.370 to -54.847 $kJmol^{-1}$) increasing from 30 to 40% indicating increase of heat of absorption reflecting that the interactions between enantiomers of DRV and the CSP increase from 30 to 40%.

Table: 5.5 Effect of volume of 2-propanol and 1-propanol on thermodynamic parameters using CHIRALPAK AD-H column

Alcohol	volume (%)	ΔH_1^0 ($kJmol^{-1}$)	ΔS_1^*	R^2_1	ΔH_2^0 ($kJmol^{-1}$)	ΔS_2^*	R^2_2	$\Delta\Delta H^0$ ($kJmol^{-1}$)	$\Delta\Delta S^0$ ($kJ\ K^1mol^{-1}$)	$R^2_{1,2}$
2-propanol	30	-9.056	-0.024	0.999	-9.370	-0.199	0.9993	-0.313	0.241	<0.995
	35	-51.046	-0.199	0.9975	-51.127	-0.166	0.9953	-0.081	0.035	<0.995
	40	-52.608	-0.178	0.9994	-54.847	-0.184	0.9983	-2.239	0.006	<0.995
ethanol	30	-11.091	-0.030	0.9982	-20.685	-0.055	0.9963	-9.594	0.026	<0.995
	35	-10.659	-0.031	0.9951	-19.796	-0.055	0.9932	-9.137	0.024	<0.995
	40	-32.990	-0.109	0.9927	-36.207	-0.113	0.9924	-3.218	0.005	<0.995

As shown in Table: 5.1, in case of ethanol, the k' values were decreased as the column temperature increased for all volumes of 1-propanol. Fig: 5.10 shows that the Van't Hoff plots of b) ln k_1' vs 1/T and ln k_2' vs 1/T were highly linear in the temperature range of 20 to 40°C indicating that the conformation of the CSP did not change in the studied temperature range. The corresponding thermodynamic parameters ΔH^0 and ΔS^* were calculated from these plots of ln k_1' vs 1/T and ln k_2' vs 1/T respectively (Table: 5.5). As shown in Table: 5.5, as volume of ethanol increases, the absolute ΔH_1^0 values of DRV decreased

(-11.091 to -10.659 $kJmol^{-1}$) indicating decrease of heat of absorption increasing from 30 to 35% and increased (-10.659 to -32.990) from 35 to 40% indicating increase of heat of absorption reflecting that interactions between the first eluted enantiomer (-) DRV and the CSP decrease increasing from 30 to 35% and increased from 35 to 40%, however the absolute ΔH_2^0 values of DRV decreased (-20.685 to -19.796 $kJmol^{-1}$) indicating decrease of heat of adsorption increasing from 30 to 35% and increased (-19.796 to-36.207 $kJmol^{-1}$) from 35 to 40% indicating increase of heat of absorption reflecting that interactions between the second eluted enantiomer (+) DRV and the CSP decrease increasing from 30 to 35% and increased from 35 to 40%, The absolute $\Delta\Delta H^0$ and $\Delta\Delta S^0$ values in all volumes of 1- and 2-propanol were negative and suggest that the enantioseparation was enthalpy driven.

5.6.5.2 CHIRALPAK IA

As shown in the Table: 5.2, in case of 2-propanol, the k' values were decreased as the column temperature was increased for all volumes. Fig: 5.11 shows the Van't Hoff plots of a) ln k_1' vs 1/T and ln k_2' vs 1/T were highly linear in the temperature range of 20 to 40°C indicating that the conformation of the CSP did not change in the studied temperature range.

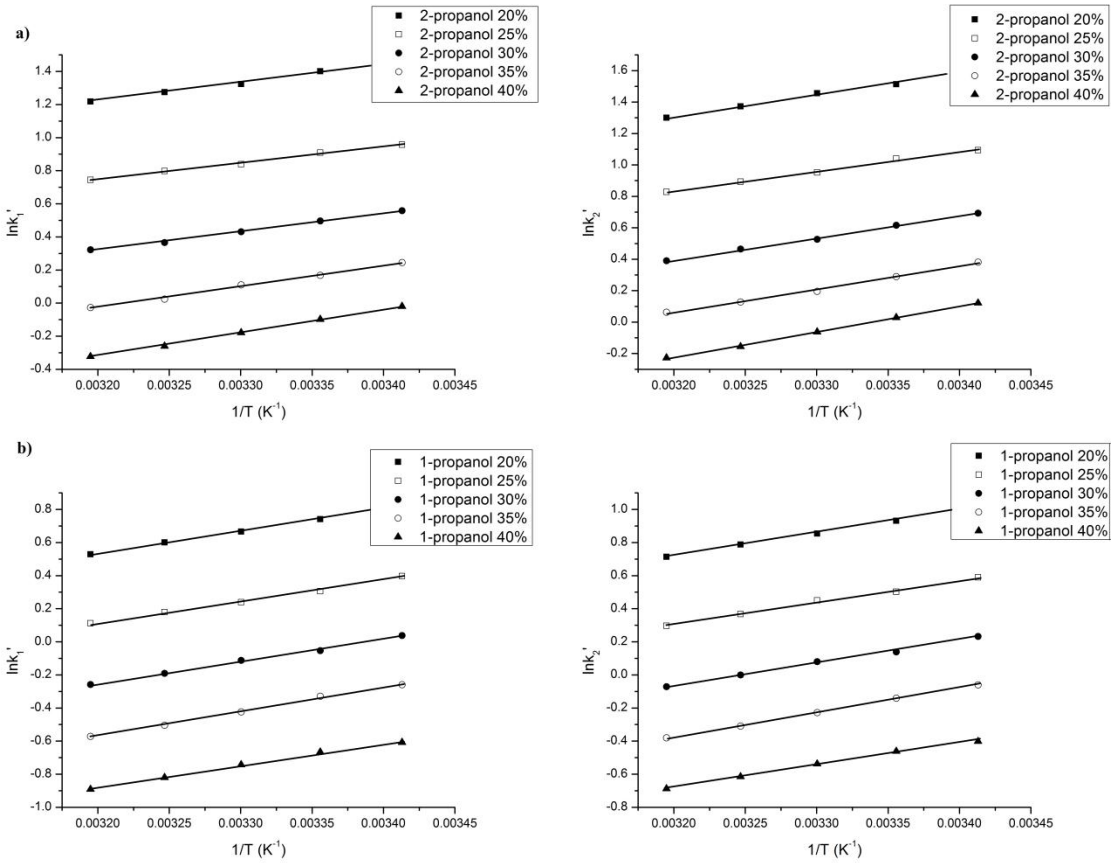

Fig: 5.11 Typical Van't Hoff plots for the separation of DRV enantiomers on CHIRALPAK IA using a) 2-propanol and b) 1-propanol in different volumes.

The corresponding thermodynamic parameters ΔH^0 and ΔS^* were calculated from these plots of ln k_1' vs 1/T and ln k_2' vs 1/T respectively (Table: 5.6). As shown in Table: 5.6, as the volume 2-propanol increases, the absolute ΔH_1^0 and ΔH_2^0 values of DRV decreased (-9.372 to -8.150 kJmol^{-1} and -11.029 to -10.328 kJmol^{-1}) increasing from 20 to 25% indicating decrease of heat of absorption from 20 to 25% and increased from 25 to 40% (-8.150 to -11.723 kJmol^{-1} and -10.328 to -13.465 kJmol^{-1}) indicating increase of heat of absorption reflecting that the

interactions between enantiomers of DRV and the CSP decrease from 20 to 25% and increases from 25 to 40%.

Table: 5.6 Effect of volume of 2-propanol and 1-propanol on thermodynamic parameters using CHIRALPAK IA for separation of DRV enantiomers

Organic modifier	volume (%)	ΔH_1^0 (kJmol^{-1})	ΔS_1*	R^2_1	ΔH_2^0 (kJmol^{-1})	ΔS_2*	R^2_2	$\Delta\Delta H^0$ (kJmol^{-1})	$\Delta\Delta S^0$ (kJ K^{1}mol^{-1})	$R^2_{1,2}$
2-propanol	20	-9.372	-0.020	0.9966	-11.029	-0.024	0.9966	-1.658	-0.005	<0.995
	25	-8.150	-0.020	0.9958	-10.328	-0.026	0.9953	-2.178	-0.006	<0.995
	30	-9.181	-0.027	0.9969	-11.521	-0.034	0.9983	-2.340	-0.007	<0.995
	35	-10.502	-0.034	0.9960	-12.214	-0.039	0.9953	-1.712	-0.005	<0.995
	40	-11.723	-0.040	0.9989	-13.465	-0.045	0.9989	-1.742	-0.005	0.997
1-propanol	20	-10.524	-0.029	0.9986	-10.750	-0.028	0.9983	-0.226	0.0008	<0.995
	25	-10.643	-0.033	0.9954	-10.980	-0.033	0.9958	-0.338	0.0005	<0.995
	30	-11.140	-0.038	0.9974	-11.396	-0.037	0.9972	-0.256	0.0007	<0.995
	35	-12.203	-0.044	0.9973	-12.346	-0.043	0.9995	-0.143	0.0011	<0.995
	40	-10.981	-0.042	0.9963	-11.065	-0.041	0.9965	-0.084	0.0014	<0.995

In case of 1-propanol as shown in Table: 5.2, the k' values decreased as the column temperature increased for all volumes. Fig: 5.11 shows the Van't Hoff plots of b) ln k_1' vs 1/T and ln k_2' vs 1/T were highly linear in the temperature range of 20 to 40°C indicating that the conformation of the CSP did not change in the studied temperature range. The corresponding thermodynamic parameters ΔH^0 and ΔS^* were calculated from these plots of ln k_1' vs 1/T and ln k_2' vs 1/T respectively (Table: 5.6). As shown in Table: 5.6, as volume of 1-propanol increases, the absolute ΔH_1^0 and ΔH_2^0 values of DRV increased (-10.524 to -12.203 kJmol^{-1} and -10.750 to -12.346 kJmol^{-1}) indicating increase of heat of absorption from 20 to 35% and decreased from 35 to 40% (-12.203 to -10.981 kJmol^{-1} and -12.346 to -11.065 kJmol^{-1}) indicating decrease of

heat of absorption reflecting that the interactions between enantiomers of DRV and the CSP increase from 20 to 35% and decrease from 35 to 40%. The absolute $\Delta\Delta H^0$ and $\Delta\Delta S^0$ values in all volumes for both alcohols were negative and suggest that the enantioseparation was enthalpy driven.

5.6.5.3 CHIRALPAK IC

As shown in Table: 5.3, in case of 1-propanol, the k' values were decreased as the column temperature was increased for all volumes. Fig: 5.12 shows the Van't Hoff plots of a) ln k_1' vs 1/T and ln k_2' vs 1/T were highly linear in the temperature range of 20 to 40°C indicating that the conformation of the CSP did not change in the studied temperature range.

The corresponding thermodynamic parameters ΔH^0 and ΔS^* were calculated from these plots of $ln\ k_1'$ vs $1/T$ and lnk_2' vs $1/T$ respectively (Table: 5.7). As shown in Table: 5.7, as the volume of 1-propanol increases, the absolute ΔH_1^0 values of DRV decreased (-6.640 to -6.409 kJmol^{-1}) indicating decrease of heat of adsorption from 30 to 35% and increased (-6.409 to -7.510 kJmol^{-1}) indicating increase of heat of adsorption from 35 to 45% reflecting that interactions between the first eluted enantiomer (-) DRV and the CSP decrease increasing from 30 to 35% and increase from 35 to 45%, however the absolute ΔH_2^0 values of DRV increased (-10.517 to -11.211 kJmol^{-1}) indicating increase of heat of adsorption increasing from 30 to 45% reflecting that interactions between the second eluted enantiomer (+) DRV and the CSP increase

increasing from 30 to 45%. The $\Delta\Delta H^0$ and $\Delta\Delta S^*$ values in all volumes were negative, suggesting that the enantioseparation was enthalpy driven.

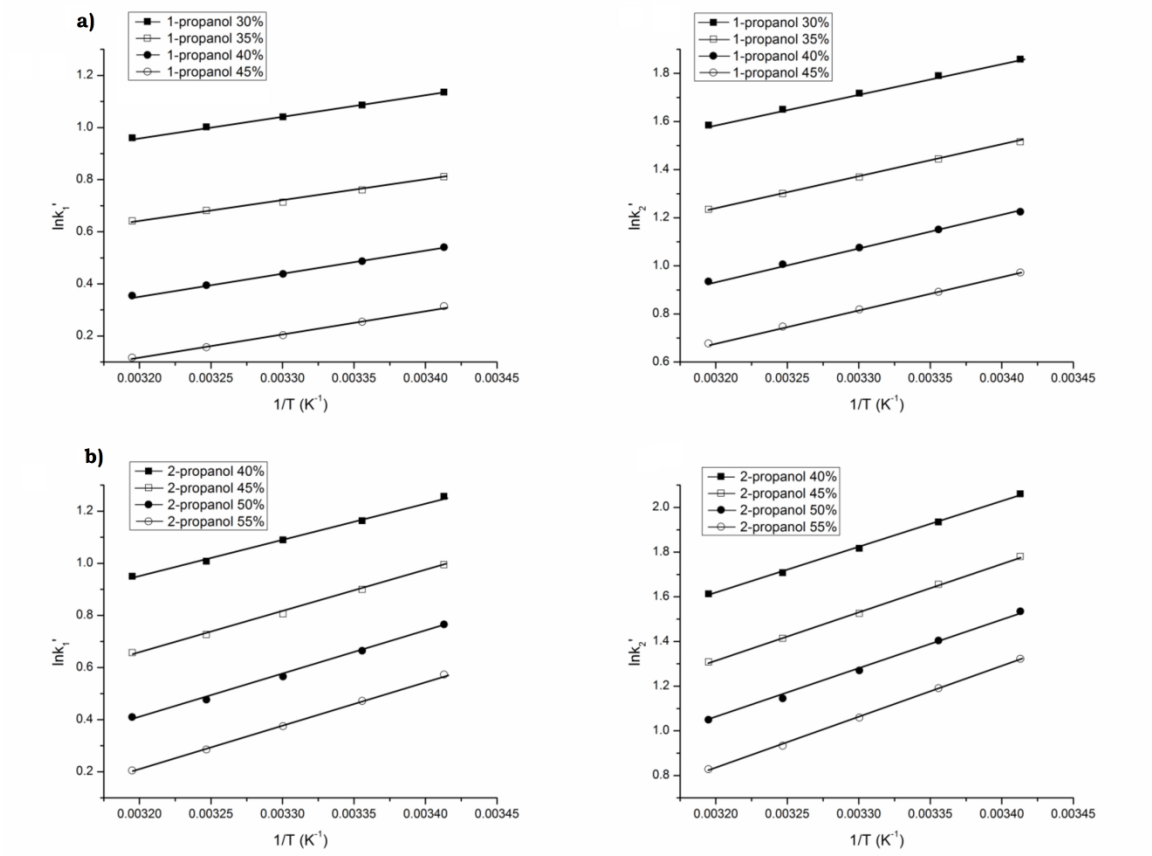

Fig: 5.12 Typical Van't Hoff plots for the separation of DRV enantiomers on CHIRALPAK IC using a) 2-propanol and b) 1-propanol in different volumes.

Table: 5.7 Effect of volume of 1-propanol and 2-propanol on thermodynamic parameters using CHIRALPAK IC for separation of DRV enantiomers

Organic modifier	Volume (%)	ΔH_1^0 (kJmol^{-1})	ΔS_1*	R^2_1	ΔH_2^0 (kJmol^{-1})	ΔS_2*	R^2_2	$\Delta\Delta H^0$ (kJmol^{-1})	$\Delta\Delta S$*
1-propanol	30	-6.640	-0.013	0.999	-10.517	-0.020	0.999	-3.877	-0.007
	35	-6.409	-0.015	0.995	-10.762	-0.024	0.999	-4.354	-0.009
	40	-7.080	-0.020	0.998	-11.046	-0.028	0.999	-3.966	-0.008
	45	-7.510	-0.023	0.997	-11.211	-0.030	0.999	-3.701	-0.007
2-propanol	40	-11.746	-0.030	0.996	-17.123	-0.041	0.999	-5.376	-0.012
	45	-12.951	-0.036	0.998	-18.120	-0.047	0.999	-5.169	-0.011
	50	-13.726	-0.041	0.997	-18.761	-0.051	0.998	-5.034	-0.011
	55	-14.080	-0.043	0.999	-19.019	-0.054	0.999	-4.940	-0.011

As shown in Table: 5.3, in case of 2-propanol, the *k'* values were decreased as the column temperature was increased for all volumes of 2-propanol. Fig: 5.12 shows that the Van't Hoff plots of b) *ln k_1' vs 1/T* and *ln k_2' vs 1/T* were highly linear in the temperature range of 20 to 40°C indicating that the conformation of the CSP did not change in the studied temperature range. The corresponding thermodynamic parameters ΔH^0 and ΔS^* were calculated from these plots of ln k_1' vs 1/T and lnk_2' vs 1/T (Table: 5.7). As shown in Table: 5.7, as the volume of 2-propanol increases, the absolute ΔH^0 values of enantiomers of DRV increased (-11.746 to -14.080 kJmol^{-1} and -17.123 to -19.019 kJmol^{-1}) indicating increase of heat of adsorption increasing from 40 to 55% reflecting that the interactions between the enantiomers of DRV and the CSP were increasing. The absolute $\Delta\Delta H^0$ and $\Delta\Delta S^*$ values in all volumes were also negative, suggesting that the enantioseparation was enthalpy-driven.

5.7 Conclusions

Separation of DRV enantiomers was investigated on both coated and immobilized polysaccharide based columns. Effect of the type and strength of organic modifiers on retention and enantioseparation of DRV enantiomers on coated and immobilized amylose tris(3,5-dimethylphenylcarbamate) CSPs was studied at different column temperatures. The resolutions on all the columns were greater than 1.5 in most of the experiments. The differences between the coated and immobilized type of the chiral columns were discussed in terms of their efficiency and applicability. The effect of temperature on all CSPs was studied, and the apparent thermodynamic values derived from the Van't Hoff plots (ln k0 vs. 1/T, ln α vs. 1/T) were used to explain some aspects about chiral recognition mechanism. The plots of ln k' against 1/T were highly linear, and the negative values of all the deduced thermodynamic parameters in the temperature range of 20 to 40°C in all organic modifiers for different percentages on all columns suggest that the conformation of the CSP did not change in the studied temperature range. The optimized conditions in all polar modifier systems may be of use in the development of LC/APCI/MS/MS and LC/APPI/MS/MS methods to determine DRV and its enantiomeric impurity not only in pharmaceutical formulations but also in biological matrices.

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CHAPTER 6

Separation of darunavir stereoisomers on dried blood spots and its pharmacokinetic study: Method development and validation.

6.1 Introduction

As described in chapter 5, Darunavir [(-)-[(3R, 3aS, 6aR)-hexahydrofuro [2, 3-b] furan-3-yl(2S,3R)-4-(4-amino-N-isobutylphenyl sulfonamido)-3-hydroxy-1 phenylbutan -2-ylcarbamate] (DRV) (I) (Fig: 6.1) is a new HIV-1 protease inhibitor (PI) effective against viral strains that are not sensitive to other antiretrovirals [1, 2]. It is a prescription medicine approved by the U.S. Food and Drug Administration (FDA) for treatment of HIV infection in adults and children 3 years of age and older [3, 4]. Chemically, it has five stereogenic centers resulting in the possibility of 32 stereoisomers. During the synthesis of DRV, four of the stereoisomers are produced in small quantities as impurities affecting the quality of the drug. The four stereoisomers obtained as impurities of DRV (I) are three diastereoisomers viz; (3S,3aR,6aS)-hexahydrofuro[2,3-b]furan-3-yl(2S,3R)-4-(4-amino-*N* isobutylphenylsulfonamido)- 3-hydroxy-1-phenylbutan-2-ylcarbamate (II), (3R,3aS,6aR)- hexahydrofuro [2,3-b]furan-3-yl(2S,3S)-4-(4-amino-*N*-isobutylphenylsulfonamido)-3-hydroxy-1-phenylbutan-2- ylcarbamate (III), (3S,3aS,6aR)-hexahydro furo[2,3-b]furan-3-yl(2S,3R)-4-(4-amino-*N*-isobutylphenylsulfonamido)-3-hydroxy-1-phenylbutan-2-ylcarbamate (IV) and one enantiomer (3S,3aR, 6aS)- hexahydrofuro[2,3-b]furan-3-yl(2R,3S)-4-(4-amino-*N* isobutyl phenylsulfonamido)-3-hydroxy-1-phenylbutan-2-ylcarbamate (V) (Fig: 6.1). The biological activity of DRV (I) is more compared to all other stereoisomers. Therefore, stereoselective separation of DRV and its

stereoisomers is very important to assure the therapeutic efficacy and safety of its pharmaceutical preparations.

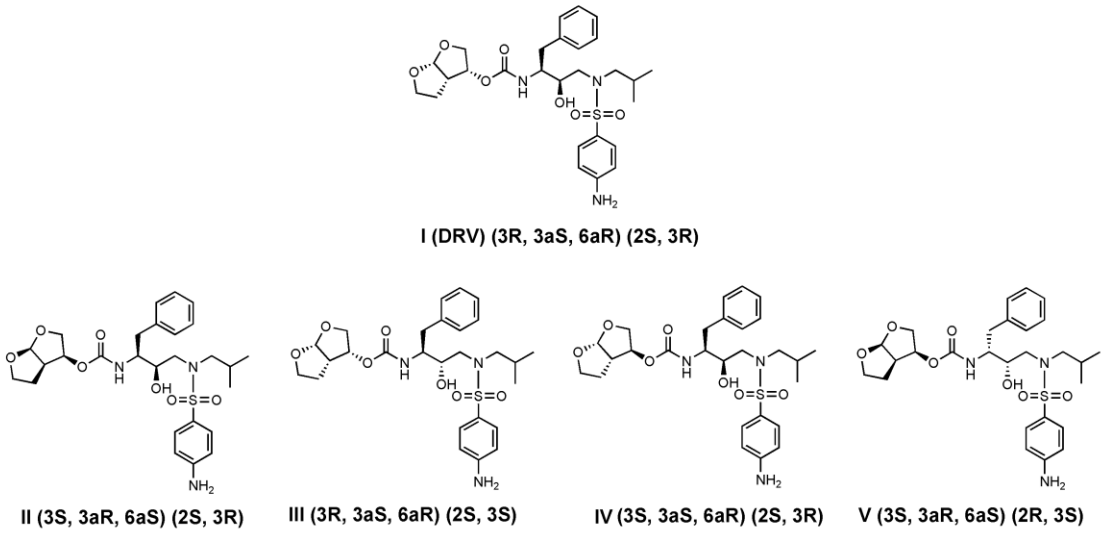

Fig: 6.1 Chemical structures of DRV and its stereoisomers.

6.2 Objectives of the work

i. To investigate the separation of i) enantiomers and ii) diastereomers on coated CHIRALPAK AD-H column (Fig: 6.2) using a variety of mobile phases at different temperatures.

ii. To study of effect of temperature and to derive apparent thermodynamic parameters to determine the strength of interactions between DRV stereoisomers and the CSP.

iii. To optimize LC condition for separation of DRV (I) from its stereoisomers (II-V).

iv. To apply the developed HPLC method on dried blood spots to demonstrate its suitability for analysis of blood samples collected

from healthy wistar rats received single oral dose of 10mg/Kg of DRV and stereoisomers.

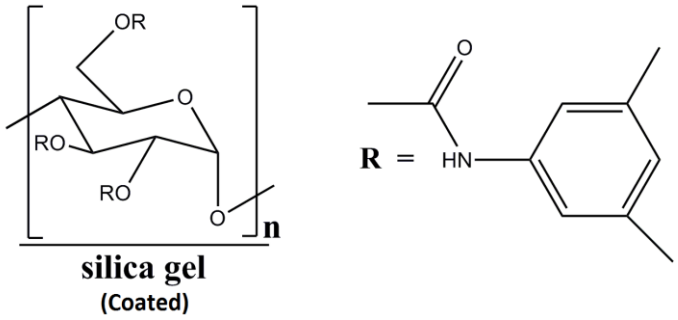

Fig: 6.2 Chemical structures of chiral selector for CHIRALPAK AD-H column.

6.3 Experimental

6.3.1 Chemicals and materials

All solvents used were of HPLC grade (E. Merck, Mumbai, India). Analytical reagent grade diethyl amine (DEA) (Spectrochem, Mumbai, India) was used. DRV and its stereoisomers produced by a local pharmaceutical unit were obtained as gift samples.

6.3.2 Dried blood spot (DBS) sample preparation

The DBSs were prepared by spotting 30 µL of the respective whole blood standard onto FTA blood spot cards [5-15]. The samples were allowed to dry in the dark at 4°C for at least 3 h prior to analysis. QC samples thus prepared were used in validation of the developed method. When required, QC samples were stored at room temperature in a sealed plastic bag containing desiccant until analysis.

6.3.3 Dried blood spot sample extraction

At the middle of the DBS sample, 10 mm diameter disc was punched and transferred to a centrifuge tube. A 500 μL volume of methanol (extraction solvent) was added and the tube was vortex mixed for 10 min. The extract was centrifuged at 2500rpm for 10 min and the supernatant was transferred to an auto sampler vial for analysis.

6.3.4 Instrumentation

The HPLC system consisting of two LC-20AD pumps, photodiode array detector, an SIL-20AC auto sampler, a DGU-20A5 degasser, and CBM-20A controller (Shimadzu, Kyoto, Japan) was used. The chromatographic and the integrated data were recorded using HP-Vectra (Agilent, Waldbronn, Germany) computer system using LC-solution data acquiring software (Shimadzu, Kyoto, Japan).

6.3.5 Chromatography

The DRV (I) and its stereoisomers were separated on CHIRALPAK AD-H column (250 x 4.6mm, 5 mm particle size) (Daicel, Tokyo, Japan). The prepared mobile phases were filtered through a 0.45 mm membrane filter paper and degassed with an ultrasonic bath before use. The analytes were dissolved in 2-propanol at approximately 0.1mgmL^{-1} and filtered through a 0.45 mm membrane filter paper prior to injection. The flow rate was set at 1mLmin^{-1}. The ultraviolet detection wavelength was set at 266nm. The injection volume was 5 mL. The void volume (v_0) of the

column was determined by injecting 1, 3, 5-tri-tert-butylbenzene and determining the retention time (t_0) of the unretained peak. Capacity factors, k_0, were calculated using the formula $(t_R-t_0)/t_0$, where t_R is the retention time of particular stereoisomer of DRV and t_0 is the retention time of the unretained peak. The separation factors (α) were calculated using the formula k_2' / k_1', where k_1' and k_2' are the capacity factors first and second eluted stereoisomers respectively. Their elution order was determined by injecting the individual compounds under similar conditions. Origin software was used to plot the linear Van't Hoff plots.

6.3.6 Chromatographic conditions

Columns	: CHIRALPAK AD-H (250x4.6mm, 5µm particle size)
Mobile Phase	: n-hexane/ethanol/DEA (80:20:0.1 v/v/v).
Flow rate	: 1mLmin^{-1}
Auto sampler temperature	: 5°C
Injection volume	: 5µL
Column temperatures	: 40°C
Detector	: Photo diode array (PDA)
λ_{max}	: 266nm

6.4 Results and discussions

The separation of DRV from its stereoisomers on CHIRALPAK AD-H using different mobile phase conditions at different temperatures was studied. The chromatographic retention data was used to derive the thermodynamic parameters and to interpret the strength of interactions between respective stereoisomers of DRV and CSPs [16-18].

6.4.1 Method development

It could be seen from Fig: 6.1 that II, III, IV, and V are stereoisomers of DRV (I). Among the four stereoisomers, V is an enantiomer of DRV (I). In order to extend the study to stereoisomers II, III, and IV, the separation was initially tried on CHIRALPAK IA, CHIRALPAK IC and CHIRALPAK AD-H columns under normal-phase conditions. Preliminary experiments gave better results on coated CHIRALPAK AD-H column while immobilized CHIRALPAK IA and CHIRALPAK IC columns were partially successful in resolving the stereoisomers. Thus, CHIRALPAK AD-H column was selected for further investigation. Mobile phases containing different proportions of alcohols i.e. 2-propanol, 1-propanol, methanol, and ethanol were tried and it was found that the peaks were not resolved completely with all the alcohols except ethanol. Thus, a thorough investigation on the effects of ethanol (%) in mobile phase and column temperature on separation of stereoisomers was carried out.

6.4.1.1 Effect of ethanol (%) in mobile phase

Mobile phases comprising of n-hexane and 0.1% DEA in different proportions ethanol were tried to study its effect on separation of DRV and its stereoisomers on CHIRALPAK AD-H column. On varying the ethanol (%) in mobile phase a significant effect on retention (t_R), separation (α) and resolution (*R*s) of the stereoisomers of DRV was observed. Typical chromatograms obtained with 20, 25, and 30% ethanol are shown in Fig: 6.3

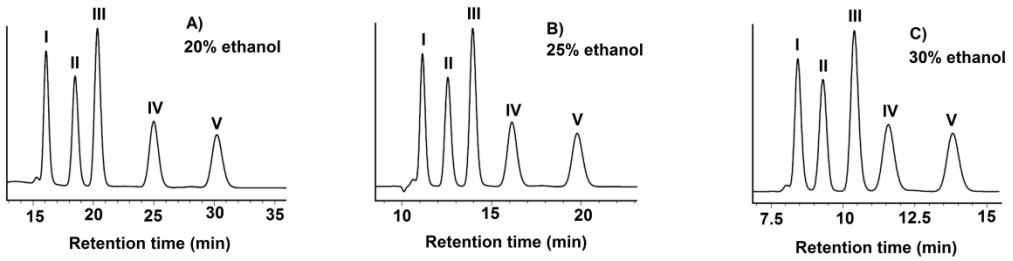

Fig: 6.3 Effect of ethanol (%) on retention (t_R), separation (α), and resolution (Rs) of DRV and its stereoisomers on CHIRALPAK AD-H at 40°C. (A) n-hexane/ethanol/diethyl amine (80:20:0.1 v/v/v). (B) n-hexane/ethanol/diethyl amine (75:25:0.1 v/v/v). (C) n-hexane/ethanol/diethyl amine (70:30:0.1 v/v/v).

As shown in Fig: 6.3, the retention times (t_R) for all the four stereoisomers of DRV were decreased as the percentage of ethanol increased from 20 to 30%. It may be due to the decrease of H-bonding interactions between the stereoisomers and CSP with increased alcohol content in the mobile phase leading to decreased retentions. Separation factors (α) did not change much and resolutions (*R*s) were decreased as

the percentage of ethanol increased from 20 to 30%. Table: 6.1 gives the retention (t_R), separation (α), and resolution (Rs) factors obtained with different proportions of ethanol in mobile phase at temperatures between 20 and 50°C.

Table: 6.1 Effect of ethanol (%) and column temperature on capacity factor (k'), separation factor (α) and resolution (R_s) of DRV and its stereoisomers

Ethanol (%) (v/v)	Column Temperature (°C)	Retention parameters											
		Capacity factor (k')					Separation factor (α)				Resolution (R_s)			
		k_I'	k_{II}'	k_{III}'	k_{IV}'	k_V'	$\alpha_{I,II}$	$\alpha_{II,III}$	$\alpha_{III,IV}$	$\alpha_{IV,V}$	$Rs_{I,II}$	$Rs_{II,III}$	$Rs_{III,IV}$	$Rs_{IV,V}$
20	20	4.89	6.08	7.63	9.35	11.82	1.24	1.25	1.23	1.26	2.47	2.92	2.32	2.46
	25	4.40	5.36	6.58	8.31	10.55	1.22	1.23	1.26	1.27	2.56	2.85	2.65	2.61
	30	4.10	4.92	5.89	7.35	9.20	1.20	1.20	1.25	1.25	2.65	2.77	2.94	2.88
	35	3.81	4.51	5.29	6.51	8.00	1.18	1.17	1.23	1.23	2.60	2.59	3.10	3.01
	40	3.25	3.88	4.37	5.42	6.98	1.19	1.12	1.24	1.29	2.83	1.96	3.38	3.91
	45	3.01	3.38	3.89	5.13	6.42	1.12	1.15	1.32	1.25	3.01	1.46	3.57	4.26
	50	2.76	3.10	3.40	4.63	5.68	1.12	1.09	1.36	1.23	3.24	1.24	3.84	4.46
25	25	2.54	3.19	3.88	4.90	ND	1.26	1.22	1.26	ND	2.28	2.18	2.02	ND
	30	2.62	3.12	3.83	4.69	5.86	1.19	1.23	1.22	1.25	2.11	2.60	2.21	2.41
	35	2.33	2.79	3.29	4.10	5.30	1.20	1.18	1.24	1.29	2.44	2.38	2.72	3.08
	40	2.07	2.46	2.84	3.44	4.45	1.19	1.16	1.21	1.29	2.36	2.10	2.60	3.39
30	25	1.69	2.12	2.64	3.25	4.55	1.25	1.25	1.23	1.40	1.88	2.08	1.55	2.18
	30	1.69	2.12	2.64	3.25	4.55	1.25	1.25	1.23	1.40	1.88	2.08	1.55	2.18
	35	1.48	1.78	2.14	2.56	3.37	1.20	1.20	1.19	1.32	1.96	2.18	1.84	2.80
	40	1.40	1.64	1.95	2.29	2.93	1.18	1.19	1.17	1.28	1.91	2.17	1.86	2.87

ND- Not determined

6.4.1.2 Effect of column temperature

Fig: 6.4 shows the effect of the column temperature on the retention, separation, and resolution of the stereoisomers of DRV using

an optimum composition of a mobile phase containing 20% ethanol. The study was conducted in the temperature range of 20–50°C. Within this range, seven experiments at an interval of 5°C were conducted. The lower limit was set as –10°C to the ambient temperature of the investigated laboratory. As expected, the retention times for all the compounds were decreased as the temperature of the column increased from 20 to 50°C. On further decreasing the temperature below 20°C, it was observed that the retention times were too long with decreased resolution. As shown in Table: 6.1, the retention factors for all stereoisomers of DRV were decreased, separation factors did not change much, and resolutions were increased (an opposite trend to percentage of ethanol) as the temperature of the column was increased from 20 to 50°C at all percentages of ethanol. Overall, reasonable retention, better separation, and resolutions were obtained at 40°C using a mobile phase consisting of *n*-hexane/ethanol/DEA (80:20:0.1 v/v/v). Fig: 6.5 shows the Van't Hoff plots of ln k' vs 1/T for DRV (I) and its four stereoisomers (II, III, IV and V). It could be seen from Fig: 6.5 that the plots are highly linear in the temperature range of 20 to 50°C indicating that the conformation of the CSP did not change in the investigated range of temperatures

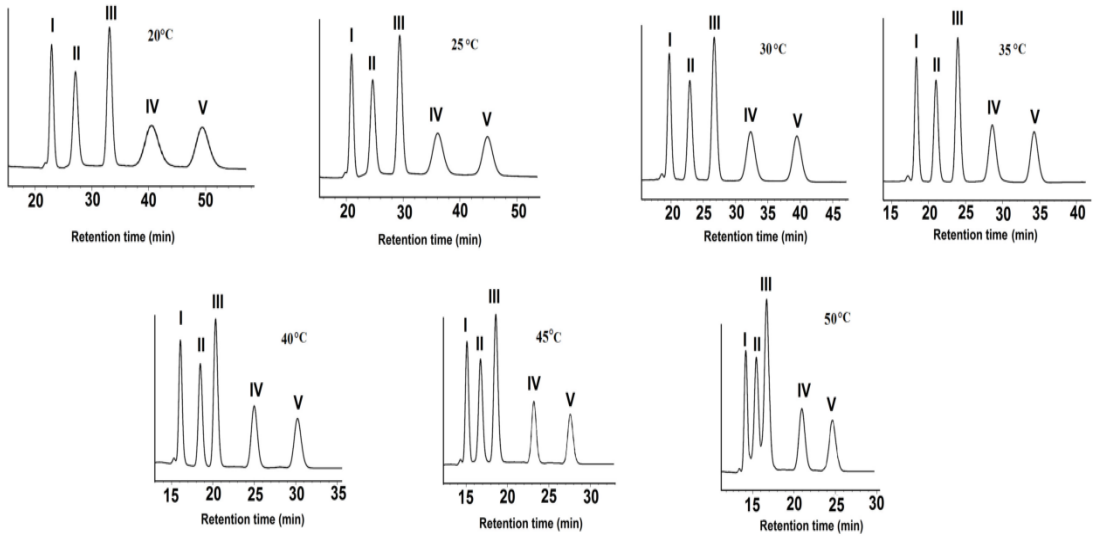

Fig: 6.4 Effect of column temperature on retention (t_R), separation (α), and resolution (Rs) of DRV and its stereoisomers on Chiralpak AD-H using n-hexane/ethanol/diethyl amine (80:20:0.1 v/v/v) as optimum mobile-phase composition.

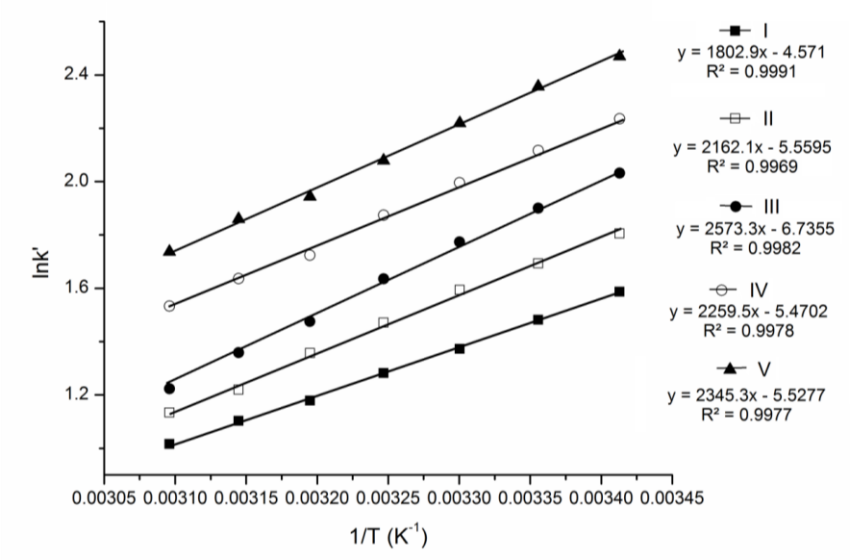

Fig: 6.5. Van't Hoff plots of DRV and its four stereoisomers (II, III, IV, and V).

Table: 6.2 gives the estimated coefficients ΔH^0 and ΔS^* along with their standard errors and regression coefficients (r^2). The corresponding

thermodynamic parameters calculated from these plots were used to explain the strengths of interactions between the stereoisomers and CSP. Because of fluctuation of separation factors (α) under these conditions, the $\Delta\Delta H^0$ and $\Delta\Delta S^*$ were determined directly from the differences of ΔH_2^0 and ΔH_1^0, ΔS_2^* and ΔS_1^*. As shown in Table: 6.2, a very low value of absolute ΔH^0 (-14.989) was obtained for stereoisomer I. It indicates lesser heat of adsorption reflecting lesser interactions with CSP. In case of stereoisomer III a high value of absolute ΔH^0 (-21.394) indicates larger heats of adsorption reflecting stronger interactions with CSP. The absolute ΔH^0 values obtained for DRV and its stereoisomers were increased in the order of I (-14.989) < II (-17.976) < IV (-18.785) < V (-19.499) < III (-21.394) indicating that the strength of interactions between the stereoisomer and CSP increase in the order of I < II < IV < V < III. The $\Delta\Delta H^0$ and $\Delta\Delta S^*$ values for all the stereoisomers were also negative (Table: 6.2) suggesting that the separation was driven by enthalpy.

Table: 6.2 Thermodynamic parameters

Stereoisomer	ΔH^0 ($kJmol^{-1}$)	Standard error	ΔS^*	Standard error	r^2		$\Delta\Delta H^0$ ($kJmol^{-1}$)	$\Delta\Delta S^*$
I	-14.989	0.072	-0.038	0.0008	0.999			
II	-17.976	0.088	-0.046	0.0006	0.997	I, II	-2.986	-0.008
III	-21.394	0.137	-0.056	0.0006	0.998	II, III	-3.419	-0.010
IV	-18.785	0.062	-0.045	0.0006	0.998	III, IV	-2.609	-0.011
V	-19.499	0.151	-0.046	0.0002	0.998	IV, V	-0.713	-0.001

6.4.2 Method validation

The linearity of detector response was checked by preparing calibration standards of DRV (I) and its four stereoisomers (II, III, IV and V) at concentration ranges from their LOQ to 50µgmL^{-1}. The obtained regression coefficients were greater than 0.995 showing a good linearity between the concentration and detector response. LOD and LOQ were determined at a signal-to-noise ratio of 3 and 10 respectively. The linearity and LOD, LOQ data are given in Table: 6.3.

Table: 6.3 Linearity, LOD and LOQ data.

Stereoisomer	Range (µg/mL)	Regression equation	r^2	LOD (µg/mL)	LOQ (µg/mL)
I	3.24–50	y = 42890x - 1135.3	0.999	1.08	3.24
II	3.30–50	y = 44466x - 2543.6	0.999	1.10	3.30
III	2.94–50	y = 80112x - 3361.8	0.999	0.98	2.94
IV	3.51–50	y = 42735x - 1895.8	0.999	1.17	3.51
V	3.63–50	y = 35762x - 1251.8	0.996	1.21	3.63

The accuracy of the method was determined by spiking the four stereoisomers to DRV at 5, 10 and 50µgmL^{-1}. Each solution was analyzed in 6 replicates and evaluated by calculating the percentage deviation from the nominal concentration and reported as relative error (%RE). The precision was determined using three standard solutions of stereoisomers of DRV individually at 5, 10 and 50 µgmL^{-1}, analyzing each solution in 6 replicates and calculating the coefficient of variation (% CV). The accuracy in terms of (%RE) was obtained ranging from 0.02 to 28.58 and the precision values in terms of (%CV) were obtained ranging from 0.01 to 27.07. The accuracy and precision data are given in Table: 6.4.

Table: 6.4 Accuracy and precision data.

	I	II	III	IV	V
Precision					
Concentration (µg/mL)	Coefficient of Variation (% CV)				
5	17.82	18.45	2.72	27.07	19.94
10	0.27	0.92	0.38	3.75	1.62
50	0.01	0.06	0.47	0.21	0.29
Accuracy					
Concentration (µg/mL)	Relative Error (% RE)				
5	18.09	18.23	2.10	28.58	21.21
10	0.31	1.05	0.34	3.04	1.51
50	0.02	0.04	0.29	0.14	0.32

6.4.3 Application to a pharmacokinetic study

Six wistar rats (200–220 g) housed under standard conditions and had ad libitum access to water and standard laboratory diet throughout the experiments were used in the present study. After a single dose by oral administration of 10mg/kg of DRV stereoisomers having each stereoisomer of 2mg to healthy wistar rats (n = 6), blood samples (30 µL) were collected from prick from the tail. Serial blood samples were spotted onto the FTA blood spot cards at 0, 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12 and 24 h post-dose. The spotted DBS sample was transferred into a centrifuge tube and added 500 µL extraction solvent and the tube was vortex mixed for 10 min. The extract was centrifuged at 2500rpm for 10 min and the supernatant was transferred to an auto sampler vial and was injected on to the HPLC system. The concentrations of DRV were determined from the calibration curve on the same day. Concentration–time and logarithmic concentration (Y-axis)–time profiles of DRV are shown in Fig: 6.6. The maximum DBS concentration (C_{max}) and time at which the

concentration reached the maximum (T_{max}), the DBS concentration–time curve from 0 h to the last measurable concentration (AUC_{max}) and area under DBS concentration–time curve from 0 h to infinity (AUC_{0-t}) and the terminal half-life ($t_{1/2}$) for DRV stereoisomers were calculated and given in Table: 6.5. It could be observed from the Table: 6.5. C_{max}, AUC_{0-24} and $AUC_{0-\infty}$ were obtained in the order of I> II> IV> III> V, $t_{1/2}$ in the reverse order and T_{max} remains constant. Statistical analysis was performed using Microsoft Excel 2007 while pharmacokinetic software, 'Ramkin', based on non-compartment model was used to calculate the [AUC] from the DBS drug concentration vs. time profiles.

Table: 6.5 Pharmacokinetic parameters

	Stereoisomers				
	I	II	III	IV	V
Parameter	Mean ±S.E.M	Mean ±S.E.M	Mean ±S.E.M	Mean ±S.E.M	Mean ±S.E.M
C_{max} (µg/mL)	4.50 ± 0.40	4.00 ± 0.16	2.50±0.18	3.00±0.15	2.00±0.38
T_{max} (h)	6.00 ± 0	6.00 ± 0	6.00 ± 0	6.00 ± 0	6.00 ± 0
AUC_{0-24} (µgh/mL)	42.90 ± 2.29	39.38 ± 1.73	30.05 ±1.56	26.12 ±2.78	21.56 ±4.78
$AUC_{0-\infty}$ (µgh/mL)	113.45 ± 7.45	108.69 ± 13.47	95.45 ±25.12	100.58 ±12.45	85.45 ±09.78
$t_{1/2}$	31.02 ± 4.00	32.51 ± 4.37	35.29 ±5.78	33.36 ±12.89	39.69 ±6.78

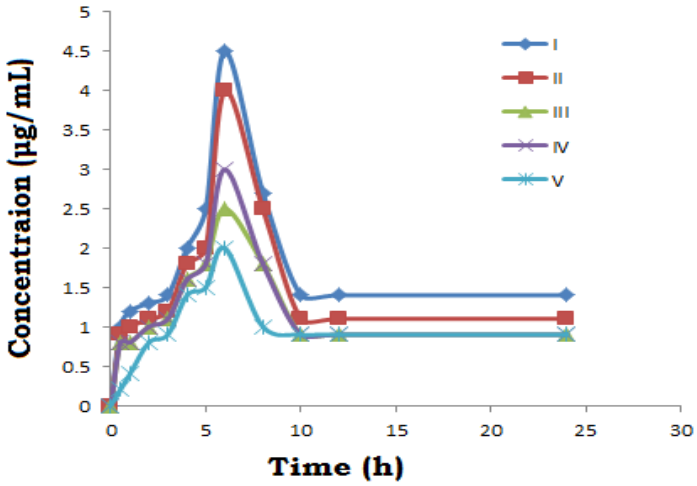

Fig: 6.6 Concentration-time profiles of DRV and its stereoisomers.

6.5 Conclusions

Stereomeric separation of DRV was investigated on coated and immobilized columns. Effect of the type and strength of organic modifiers on retention and enantioseparation of DRV enantiomers on coated amylose tris(3,5-dimethylphenylcarbamate) CSP was studied at different column temperatures. The differences between the coated and immobilized type of the chiral columns were discussed in terms of efficiency and applicability. The effect of temperature on CSP was studied, and the apparent thermodynamic values derived from the Van't Hoff plots (ln k_0 vs 1/T, ln α vs 1/T) were used to explain some aspects about chiral recognition mechanism. The plots of ln k' against 1/T were highly linear, and the negative values of all the deduced thermodynamic parameters in the temperature range of 20 to 50°C suggest that the conformation of the CSP did not change in the studied temperature range. The developed method was applied on DBS to investigate the stereo selectivity of all stereoisomers.

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List of Publications

1. Determination of rat plasma levels of sertraline enantiomers using direct injection with achiral–chiral column switching by LC–ESI/MS/MS.
   R. Nageswara Rao, **K. Nagesh Kumar**, Dhananjay D. Shinde, *Journal of Pharmaceutical and Biomedical Analysis* 52 (2010) 398–405.

2. Enantiomeric separation of mirtazapine and its metabolite in rat plasma by reverse polar ionic liquid chromatography using fluorescence and polarimetric detectors connected in series.
   R. Nageswara Rao, **K. Nagesh Kumar**, S. Ramakrishna, *Journal of Chromatography B* 879 (2011) 1911– 1916

3. Liquid chromatographic separation of darunavir enantiomers on coated and immobilized amylose tris (3, 5-dimethylphenylcarbamate) chiral stationary phases.
   R. Nageswara Rao, **K. Nagesh Kumar**, Ch. Gangu Naidu, *Chirality* 24 (2012) 652–660.

4. Liquid chromatographic separation and thermodynamic study of the stereoisomers of darunavir on Chiralpak AD-H column.
   R. Nageswara Rao, **K. Nagesh Kumar**, B. Sravan Kumar, Journal of separation Science 35 (2012) 2671-2677.

5. Thermodynamic evaluation of immobilized cellulose tris-(3,5dichlorophenyl carbamate) as a stationary phase for liquid chromatographic separation of darunavir enantiomers.
   R. Nageswara Rao, **K. Nagesh Kumar**, Journal of Chromatographic Science (Communicated).

6. Pre column O-phthalaldehyde-N-acetyl-L-cysteine derivatization followed by separation of pregabalin enantiomers on dried blood spots.
   R. Nageswara Rao, **K. Nagesh Kumar**, (Communicated).

Work Shops/Seminars Attended/Papers Presented

1. "20th International Symposium on Pharmaceutical & Biomedical Analysis" at Agra, India, 1-4, March 2009, conducted by Journal of Pharmaceutical Biomedical Analysis. Determination of rat plasma levels of sertraline enantiomers using direct injection with achiral–chiral column switching by LC–ESI/MS/MS.

2. "National Seminar on Recent advances in Analytical Sciences-Indian perspective (RAASI)" at IICT, Hyderabad, India, 20-21, January, 2011, conducted by IICT. Enantiomeric separation of mirtazapine and its metabolite in rat plasma by reverse polar ionic liquid chromatography using fluorescence and polarimetric detectors connected in series.

3. "2nd International Symposium on: Mass Spectrometry in Life Sciences" at NCBS, Bangalore, India 23-26, August 2011, conducted by NCBS. Evaluation of thermodynamic parameters in liquid chromatographic separation of darunavir enantiomers on Chiralpak IA, IC columns and comparative study with AD-H column.

4. "International Conference on Emerging Trends in Chemical Sciences" at School of Chemical Sciences, Central University of Gujarat, Gandhinagar, 14-15th March 2013, conducted by Central University of Gujarat. Indirect chiral separation of pregabalin enantiomers by o-phthalaldehyde-N-acetyl-L-cysteine as chiral derivatizing agent followed by fluorescence detection.

5. Workshop on Drug Discovery: Drug Design, Development, Delivery and Preclinical Studies (D_4PS), NIPER-IICT, Hyderabad, 2010.

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Determination of rat plasma levels of sertraline enantiomers using direct injection with achiral–chiral column switching by LC–ESI/MS/MS

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Column switching
RAM column
Cyclobond column
LC–ESI/MS/MS
Pharmacokinetics

ABSTRACT

A highly sensitive and selective on-line two-dimensional reversed-phase liquid chromatography/electrospray ionization–tandem mass spectrometric (2D-LC–ESI/MS/MS) method to determine sertraline (SRT) enantiomers in rat plasma was developed and validated. The method was applied to separate and determine the diastereomers and enantiomers of SRT simultaneously. The 2D-LC–ESI/MS/MS system consisted of RAM column in first dimension for trapping proteineous part of plasma and a chiral Cyclobond column as second dimension for separation of enantiomers and diastereomers of SRT using 0.1% aqueous trifluoroacetic acid:acetonitrile (86:14, v/v) as mobile phase in an isocratic elution mode. The linear dynamic range was 0.5–200 ng/mL ($r^2 > 0.999$). Acceptable precision and accuracy were obtained over the calibration range. The assay was successfully used in the analysis of SRT enantiomers in rat plasma to support pharmacokinetic studies.

1. Introduction

Sertraline hydrochloride (+)-cis-(1S,4S)-*N*-methyl-4 (3,4-dichlorophenyl)-1,2,3,4-tetrahydro-1-naphthalenamine hydrochloride (SRT) is a selective serotonin reuptake inhibitor (SSRI) used to treat major depression as well as obsessive-compulsive, panic and social anxiety disorders in both adults and children. It is as effective as tricyclic antidepressants (TCA) [1] with minimal side effects, such as insomnia, nervousness, nausea, diarrhea, dry mouth and dyspepsia. During its synthesis, the (−)-cis-(1R,4R)-*N*-methyl-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-1-naphthalenemine hydrochloride, trans-(1S,4R) and (1R,4S)-*N*-methyl-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-1-naphthalenemine hydrochloride are introduced in significant quantities (Fig. 1). Therefore, stereo selective separation and determination of SRT is important to assure its therapeutic efficacy and safety.

A wide variety of analytical methods including GC-ECD [2], GC–MS [3], HPLC-PDA, HPLC–UV and HPLC–MS/MS [4–13] for determination of SRT in plasma or serum were reported. These methods are suitable to determine SRT either alone or in combination of other drugs. Stereoisomers of SRT and its related enantiomeric impurities were separated (i) directly on a dimethyl β-cyclodextrin stationary phase [14] and (ii) indirectly using hydroxypropyl β-cyclodextrin as a mobile phase additive [15] by HPLC in bulk drugs and formulations. Foley and Zhou [16] separated the enantiomers of SRT by CE using highly sulphated β-cyclodextrin as a chiral selector. However these methods do not address the separation of enantiomers of SRT in biological fluids. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) in positive-ion electrospray (±ESI) and selected-ion reaction monitoring modes [17] was proved to be a valuable tool in characterization of several antidepressants including SRT and their *N*-desmethyl metabolites in raw sewage and both primary-treated wastewaters. Several LC methods to determine SRT and its main metabolite *N*-desmethylsertraline in plasma or serum suitable pharmacokinetic studies were reported [7,8,12]. All these methods follow the precipitation of proteins with organic solvents [13] and use either liquid–liquid or solid phase extraction for sample clean-up. Sample preparation is one of the most important steps in HPLC analysis of drugs and their metabolites in biological fluids. Proteins in the biological fluids can precipitate or denature and adsorb onto the packing material, leading to the build-up of back pressure. To eliminate problems such as co precipitation of analytes during extraction and avoid the adsorption of protein onto the analytical column, direct injection of the sample using column switching is becoming the method of choice [18–23]. Recently Cass and Galatti [24] reported a bidimensional achiral–chiral chromatography for determination of the plasma levels of modafinil enantiomers and its major metabolites by direct injection of human

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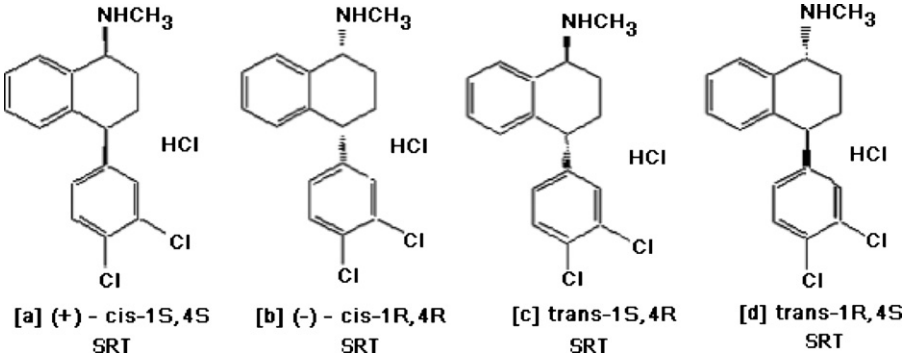

Fig. 1. Chemical structures of: (a) (+)-cis-1S,4S; (b) (−)-cis-1R,4R; (c) trans-1S,4R; (d) trans-1R,4S SRT hydrochloride.

plasma with on-line sample preparation. Two-dimensional liquid chromatography (2D-LC) and 2D-LC coupled with mass spectrometry (2D-LC/MS, 2D-LC/MS/MS) have become popular techniques in bioanalytical chemistry, pharmacology and proteomic research and these systems generate excellent resolution, enabling the comprehensive separation of complex biological matrices [19]. The specific advantages of 2D-LC over 1D-LC include (i) direct injection of plasma, (ii) on-line sample preparation, (iii) no contact with toxic solvents, (iv) no need of extraction, (v) increased column efficiency, (vi) a huge increase in peak capacity, (vii) no co precipitation of analytes with proteins, (viii) no adsorption of protein onto the analytical column and (ix) avoids extraction losses during evaporation and reconstitution steps. 2D-LC could be performed either on-line or off-line modes. The on-line approach narrows the choice of LC mode due to the mobile phase compatibility for direct transfer to the second dimension. This approach minimizes sample losses, which could be an advantage for sensitivity compared to the off-line mode.

The present paper describes the development and validation of a LC method for simultaneous determination of SRT enantiomers and diastereomers using direct injection with RAM–Cyclobond column switching by LC–ESI/MS/MS. The method was applied for the investigation of enantioselectivity in the pharmacokinetic studies of SRT administered in racemic form in a single dose to rats. The method was sensitive enough to quantify the low concentration of 0.4 ng/mL SRT in rat plasma.

2. Experimental

2.1. Chemicals and materials

Racemic mixture of SRT hydrochloride ((±)-cis-*N*-methyl-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-1-naphthalenemine hydrochloride), (+)-cis-(1S,4S)-*N*-methyl-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-1-naphthalenemine hydrochloride, (−)-cis-(1R, 4R)-*N*-methyl-4-(3, 4-dichlorophenyl)-1, 2, 3, 4-tetrahydro-1-naphthalenemine hydrochloride, trans-(1S,4R)-*N*-methyl-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-1-naphthalenemine hydrochloride, trans-(1R,4S)-*N*-methyl-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-1-naphthalenemine hydrochloride were procured from a local pharmaceutical industry. Purified de-ionized water (Nanopure, Barnstead, USA), HPLC-grade acetonitrile, methanol, trifluoroacetic acid (Qualigens Fine Chemicals, Mumbai, India), ammonium acetate (S.D. Fine Chem., Mumbai, India) were used. The blood samples used for the development and validation of the analytical method were obtained at regular intervals of time for 24 h from male Wistar rats weighing 170 ± 10 g housed one animal per cage under standard conditions. The required environment was controlled with daily feeding of standard chow pellets and water ad libitum.

2.2. Liquid chromatography–mass spectrometry

The LC system LC-MSD Agilent 1100 series (Agilent Technologies, Waldbronn, Germany) consisting of a binary LC pump, a vacuum degasser, a temperature-controlled microwell plate auto sampler set at 4 °C and a thermostatted column compartment set at 35 °C. The compounds were analyzed on a Astec CYCLOBOND™ I 2000 DM (25 cm × 4.6 mm, 5 μm) (Supelco, PA, USA) column, under isocratic conditions using a mobile phase containing 0.1% aqueous trifluoroacetic acid:acetonitrile (86:14, v/v) at a flow rate of 0.8 mL/min protected by a guard column Hisep-RAM (50 mm × 4.0 mm; particle size 5 μm) (Supelco, USA) under isocratic conditions using a mobile phase containing 0.02 M aqueous ammonium acetate (pH 8):acetonitrile (86:14, v/v) at a flow rate of 1 mL/min.

The analytes were monitored by mass spectrometer equipped with an electrospray ionization interface, operated in a positive mode (+ESI). Nitrogen was the nebulizer and curtain gas. Collision induced dissociation was achieved using nitrogen as collision gas. The ion source conditions were: temperature 325 °C, nebulizer gas pressure 35 psi, dry gas 8.0 L/min, ion spray voltage: 3500 V, collision energy 0.8 A, declustering potential 5.0 V (lens 1), 60.0 V (lens 2), entrance potential 40.0 V and collision exit potential 113.5 V. The data was captured using a Chemstation software. Transition of m/z 306 → 274.7 was used for detection of SRT.

2.3. Preparation of plasma standards

The calibration standards (CS) and quality control samples (QC) were prepared by spiking blank plasma with working solutions of analytes. Calibration standards were at 0.5, 1.0, 2.0, 4.0, 10.0, 20.0, 40.0, 100, 150, 200 ng/mL for all the analytes. A calibration curve was constructed using 200 μL plasma of each standard. Quadratic regression equation with peak area against concentration of SRT was used for quantification of unknown concentration of SRT enantiomers in rat's plasma. Quality controls were prepared at 0.5, 1.5, 14.0, 150, 200 ng/mL for all analytes and used for determination of accuracy and precision in determination of SRT enantiomers and diastereomers in rat plasma. The spiked plasma samples at all the levels were stored at −20 °C.

The standard stock solutions of 100 μg/mL for (±)-cis-SRT, (+)-cis-(1S,4S) SRT, (−)-cis-(1R,4R) SRT, trans-(1S,4R) SRT and trans-(1R,4S) SRT hydrochloride were prepared by dissolving requisite amounts in methanol:water (30:70, v/v). The stock solutions were further diluted with water appropriately to get an intermediate concentration of 4 μg/mL. The working solutions of all compounds for spiking calibration and quality control samples were subsequently prepared from standard and intermediate stock solutions. All the standard stock, intermediate stock and working stock solutions were prepared and stored at 4 °C until use.

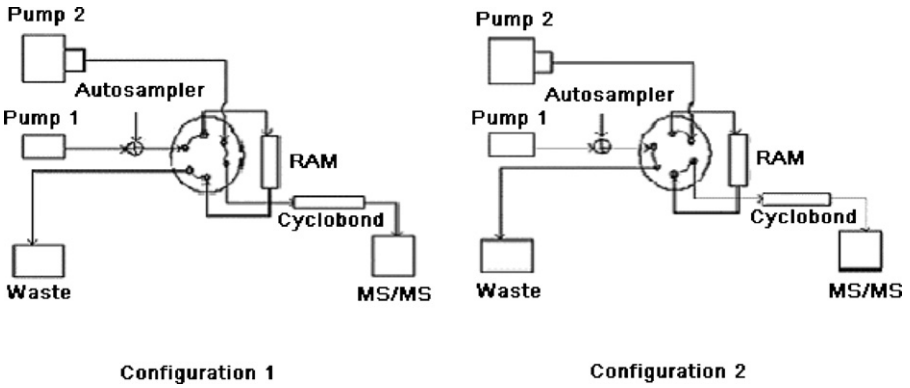

Fig. 2. Schematic diagram of the column switching system in configurations 1 and 2.

2.4. Sample preparation

Frozen plasma samples were thawed at room temperature before processing. The blood samples were centrifuged for 15 min at 4500 rpm. Supernatant was collected, filtered through 0.45μ nylon membrane filter, vortexed for 10 min and directly injected a 20 μL aliquot into 2D-LC/MS/MS containing RAM and chiral Cyclobond columns. All plasma samples including calibration curve, QC and samples for pharmacokinetic experiments were prepared as above.

2.5. Dosing and sampling

The method was applied to the investigation of enantioselectivity in the pharmacokinetics of SRT administered in the racemic form in a single dose to rats. The animals received an aqueous solution of (±)-rac-SRT hydrochloride (25 mg/kg) by oral administration. Blood samples were collected at times 0, 1, 2, 4, 6, 8, 10, 24 h after drug administration. Plasma samples were stored at −70 °C until analysis.

3. Results and discussion

3.1. Method development

Initially CHIRAL-AGP (150 mm × 4.0 mm) and CHIROBIOTEC V (150 mm × 4.6 mm) column using 0.1% aqueous acetic acid with organic modifiers, viz., methanol and acetonitrile of different composition as mobile phase were tried to separate the enantiomers and diastereomers of SRT. However, the trails did not result in good separation of the analytes. SRT and related substances are polar in nature and fairly soluble in methanol and water. The reversed-phase mode offers greatest possibilities for selectivity by taking advantage of the inclusion mechanism for which cyclodextrins are well known [15]. So reverse phase conditions were tried using a Cyclobond column which is useful for analytes having ring structures includable into the cyclodextrin cavity and form hydrogen bonds. The inclusion mechanism of cyclodextrins resolves the enantiomeric drugs which are difficult to separate on other chiral stationary phases [25]. A mobile phase containing 0.1% aqueous trifluoroacetic acid:acetonitrile (86:14, v/v) at a flow rate of 0.8 mL/min was used in an isocratic elution mode at room temperature, but all the enantiomers were not separated. However at elevated column temperatures, i.e. 35 °C it was found that all the analytes were well separated.

Initially different mobile phase conditions were tried on RAM column to separate SRT analytes. 0.05 M aqueous ammonium acetate with different proportions of acetonitrile and 0.05% aqueous acetic acid with acetonitrile of different compositions were

Table 1
Sequential steps of on-line column switching.

Step	Time interval (min)	Configuration	Analytical operation
1	0–2.5	1	Exclusion of plasma proteins by RAM column and conditioning of Cyclobond column
2	2.5–10	2	Elution of retained SRT from RAM to Cyclobond
3	10–16	1	Elution of SRT enantiomers, diastereomers from Cyclobond and conditioning of RAM column

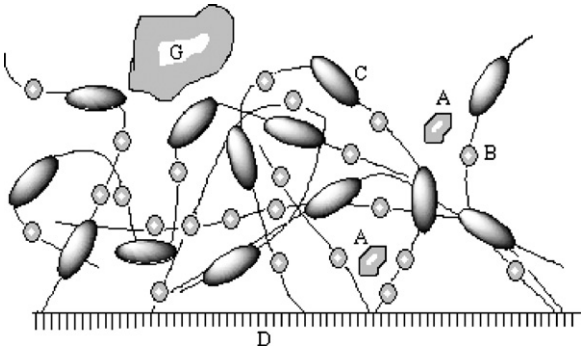

Fig. 3. Schematic representation of an embedded-network shielded hydrophobic phase, (A) small analyte molecule; (B) hydrophilic sites; (C) hydrophobic sites; (G) protein; (D) support matrix. (Reproduced from [26] with kind permission.)

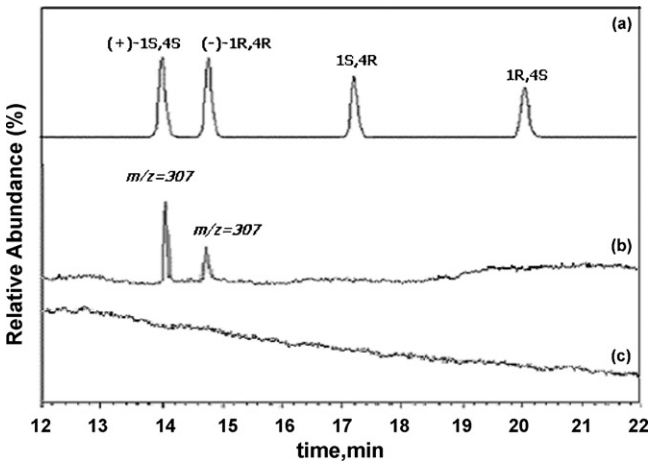

Fig. 4. Typical liquid chromatograms showing the (a) separation of 50 ng/mL of (+)-cis-1S,4S, (−)-cis-1R,4R, trans-1S,4R, trans-1R,4S SRT hydrochloride. (b) SRT enantiomers (cis-1S,4S, cis-1R,4R) in rat plasma at 6th hour after oral administration of 25 mg/kg (±)-rac-SRT to Wistar rats. (c) Blank plasma.

tried. These conditions did not result in good separation of the analytes. Isocratic conditions with a mobile phase containing aqueous ammonium acetate 0.02 M pH 8:acetonitrile (86:14, v/v) at a flow rate of 1 mL/min were successful. Depending upon the retention times of the analytes on RAM and Cyclobond columns the method was divided into three steps. The configuration of the system was changed in different steps for excluding proteins through RAM column and separation of analytes through Cyclobond column. The column switching system used for coupling of RAM and Cyclobond columns is shown schematically in Fig. 2. The sequential steps used are listed in Table 1. The rat plasma of 20 μL sample volume was applied when the system was in configuration (1) (0–2.5 min) (Fig. 2). In this configuration, the mobile phase (0.02 M aqueous NH_4OAc:ACN, 86:14, v/v) was delivered by pump 1 at a flow rate 1.0 mL/min to elute the proteins from RAM column to waste. At the same time the Cyclobond column was conditioned by the mobile phase (0.1% aqueous TFA:ACN, 86:14, v/v) delivered by pump 2. The analytes were retained on the hydrophobic phase of the RAM column. In configuration (2), (2.5–10 min) (Fig. 2) the mobile phase was delivered by pump 2 at a flow rate 0.8 mL/min and the analytes from RAM to Cyclobond column were eluted. After 10 min the system was changed to configuration (1) (Fig. 2). In this configuration the mobile phase delivered by pump 2 elutes SRT enantiomers and diastereomers from Cyclobond column while RAM column was conditioned by the mobile phase delivered by pump 1. The role of the RAM column was to remove the proteins. It could be characterized by the hydrophilic outer and hydrophobic inner phases which exclude large molecules such as proteins in the void volume while retain selectively the small hydrophobic analytes [26]. The stationary phase in RAM was a porous chromatographic support specifically designed for the removal of macromolecules, partially based on a size exclusion mechanism [27]. Only small molecules penetrate into the pores and interact with the stationary phase bound to the inner surface. During the analyses the performance of the RAM column was found to be stable with over 500 plasma injections without significant change in the back pressure. Initially the back pressure of the RAM column was 40 kgf/cm^2 and after analyses of around 500 samples

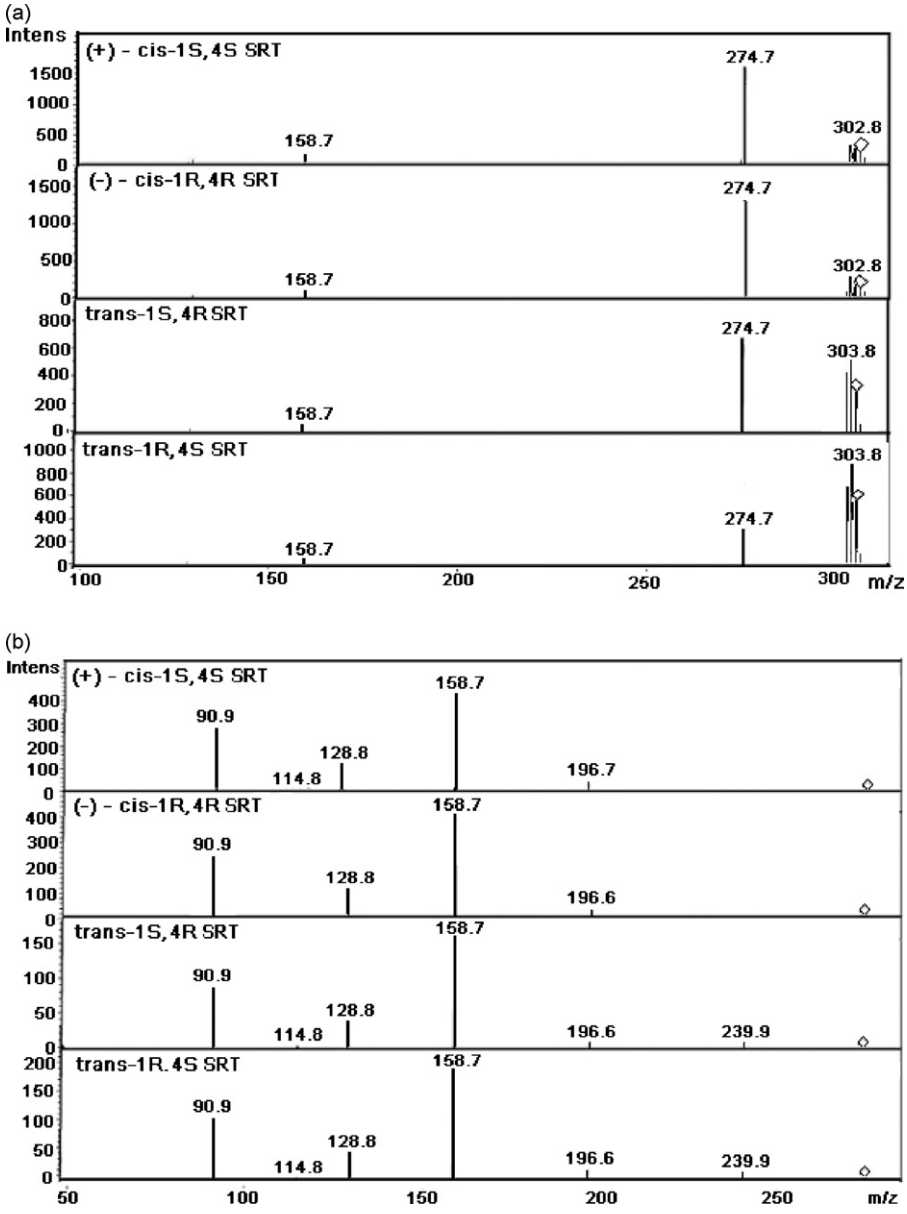

Fig. 5. (a) MS/MS1 and (b) MS/MS2 spectra of 50 ng/mL of SRT enantiomers and diastereomers.

it became 60 kgf/cm^2. Schematic representation of an embedded-network shielded hydrophobic phase is shown in Fig. 3. The elution order was (+)-cis-1S,4S (−)-cis-1R,4R, trans-1S,4R, trans-1R,4S SRT hydrochloride. Typical chromatograms of (a) standard calibration plasma spiked with (+)-cis-1S,4S (−)-cis-1R,4R, trans-1S,4R, trans-1R, 4S, (b) SRT enantiomers in rat plasma at 6th hour after oral administration of rac-SRT to Wistar rats and (c) blank plasma are shown in Fig. 4a–c. It could be seen from Fig. 4 that the trans 1S,4R and 1R,4S isomers were not found in rat plasma (Fig. 4b). It was due to the administration of rac SRT containing only the enantiomers of cis-1S,4S and -1R,4R SRT into the Wistar rats. This was studied because of the SRT formulations generally contain enantiomers of cis-1S,4S and -1R,4R selectively. Further it could be seen that there was no peak corresponding to the metabolite of SRT in the chromatogram of rat plasma (Fig. 4b). Probably it might have formed in very traces after 6th hour of injection. To detect the metabolite LC–MS/MS was studied. However there was no peak detected at 274 corresponding the *N*-desmethylsertraline. It could be due to two possibilities (i) its concentration could be very much below the detection limit or (ii) it might be not eluted from the column under the conditions used for separation. The mass spectrometric detection of SRT was investigated by ESI (+ve) SRM mode. In the full scan MS/MS1 chromatogram (Fig. 5a), all analytes formed predominately protonated molecules $[M+H]^+$ at *m*/*z* 307 and base peak at *m*/*z* 274.7. On further fragmentation all analytes gave fragments at *m*/*z* 158.7 (base peak), *m*/*z* 129 and *m*/*z* 91 in the full scan

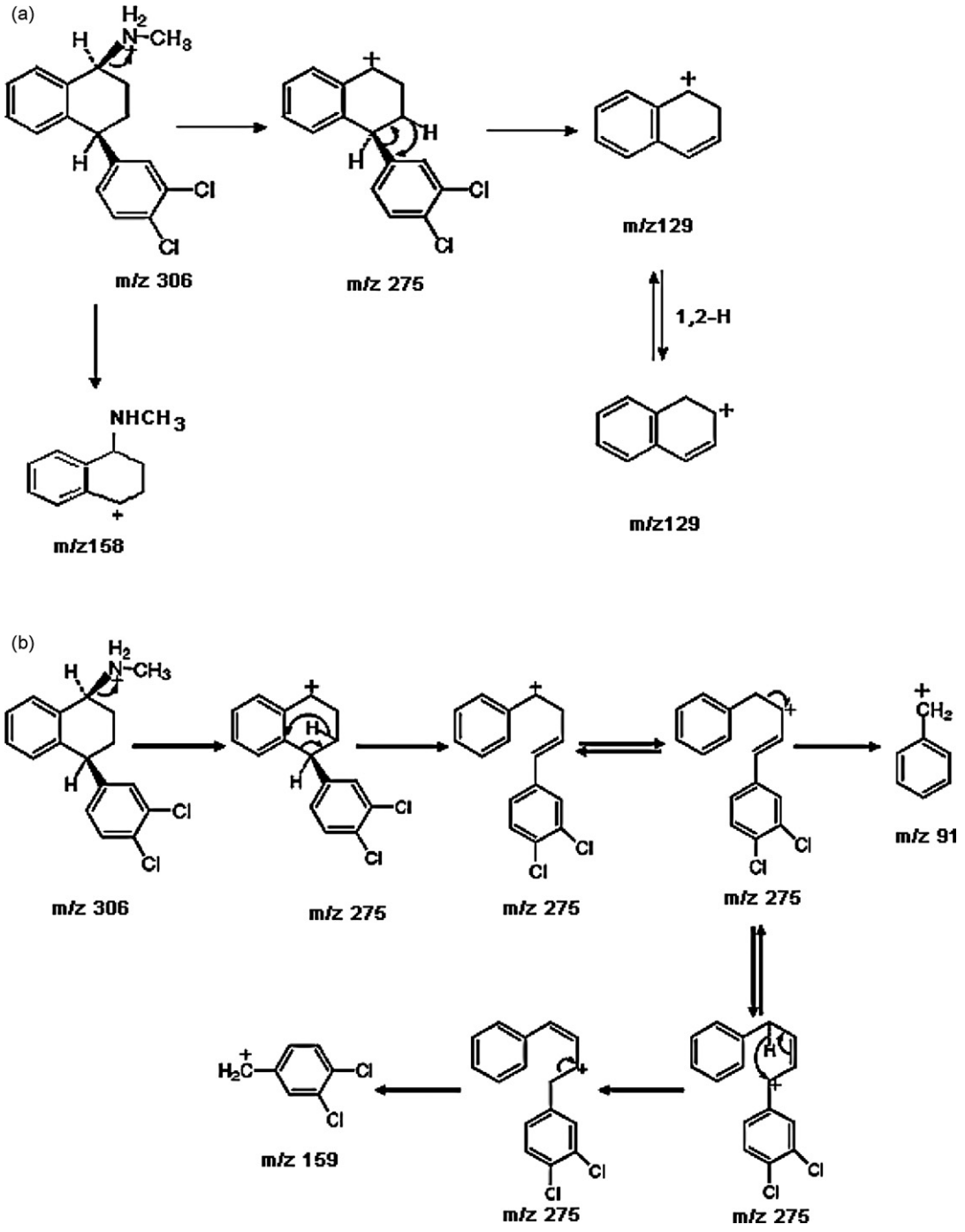

Fig. 6. (a) MS/MS1 and (b) MS/MS2. Fragmentation pathways of SRT enantiomers and diastereomers.

Table 2
Intra- and inter-batch accuracy and precision in determination of SRT enantiomers and diastereomers.

Precision and accuracy	(+)-cis-1S,4S SRT (%)	(−)-cis-1R,4R SRT (%)	trans-1S,4R SRT (%)	trans-1R,4S SRT (%)
Intra-assay precision; coefficient of variation ($n = 10$)				
0.5 ng/mL	3.02	2.45	2.17	3.01
1.5 ng/mL	4.45	3.96	2.17	2.85
14.0 ng/mL	3.53	3.13	4.38	1.05
150 ng/mL	1.06	4.62	1.92	3.94
200 ng/mL	2.05	2.97	2.84	4.47
Inter-assay precision; coefficient of variation ($n = 5$)				
0.5 ng/mL	2.62	4.45	3.22	3.44
1.5 ng/mL	3.12	2.75	3.83	2.63
14.0 ng/mL	1.44	4.75	3.26	4.87
150 ng/mL	4.17	3.48	2.99	1.90
200 ng/mL	1.45	1.4	4.94	2.05
Intra-assay accuracy; relative error ($n = 10$)				
0.5 ng/mL	4.05	−2.04	−3.07	2.03
1.5 ng/mL	2.47	−3.68	1.79	−3.88
14.0 ng/mL	3.83	4.55	4.95	2.85
150 ng/mL	2.52	3.88	−4.87	3.85
200 ng/mL	5.22	−3.02	−2.46	1.58
Inter-assay accuracy; relative error ($n = 5$)				
0.5 ng/mL	0.0	3.16	−4.64	−3.67
1.5 ng/mL	0.58	−1.99	−3.67	1.99
14.0 ng/mL	3.06	2.45	3.47	1.29
150 ng/mL	1.48	3.78	4.98	4.78
200 ng/mL	4.96	−3.88	1.58	1.38

of MS/MS2 chromatogram (Fig. 5b). The proposed fragmentation pathways during MS/MS for the $[M+H]^+$ ions of all the enantiomers and diastereomers of SRT are shown in Fig. 6.

3.2. Validation

3.2.1. Selectivity

The selectivity of the method was investigated for potential interferences of endogenous substances by using six independent batches of rat plasma (blank). Moreover, the chromatograms of the experimental samples obtained after administration of rac-SRT to Wistar rats were compared with standard chromatograms in order to detect interfering peaks. No interfering peaks were found at the retention times of SRT enantiomers and diastereomers.

3.2.2. Accuracy and precision

The quality control samples (QC) were prepared by spiking blank plasma with working solutions of analytes at 0.5, 1.0, 14.0, 150, 200 ng/mL for all 4 analytes. The accuracy, intra- and inter-day precision of the analytical method were determined by replicate processing. Precision was calculated as coefficient of variation and accuracy as % relative error. The coefficient of variation obtained in the study of intra-, inter-assay precision and accuracy were less than 5% assuring the reproducibility and repeatability of the results. Table 2 summarizes the data obtained in intra- and inter-batch accuracy and precision for SRT enantiomers and diastereomers.

3.2.3. Linearity

The calibration standards (CS) were prepared by spiking blank plasma with working solutions of analytes at 0.5, 1.0, 2.0, 4.0, 10.0, 20.0, 40.0, 100, 150, 200 ng/mL for all 4 analytes. Plots of plasma concentrations vs peak areas were constructed and the linear regression lines were used for determination of concentrations of enantiomers in plasma samples. Slopes, intercepts and correlation coefficients were calculated. The method showed linearity up to 200.0 ng/mL for all enantiomers and diastereomers of SRT and the correlation coefficients observed were in the range of 0.9995–0.9999 for all enantiomers and diastereomers. Table 3 summarizes the data obtained in recovery and linearity ranges of the SRT enantiomers and diastereomers. The recovery was calculated by using the formula given below.

$$\text{Recovery (\%)} = \left(\frac{\text{area of spiked sample}}{\text{area of standard}}\right) \times 100.$$

3.2.4. Stability

The stability of analytes was determined during blood sample collection and handling at room temperature for 2 h and after freezing plasma samples for 1st day, 2nd day, 3rd day, 1 week,15 days and 1 month. The results were compared with those obtained by freshly prepared samples. The results are summarized in Table 4.

Table 3
Recovery and linearity ranges of the SRT enantiomers and diastereomers.

	(+)-cis-1S,4S SRT	(−)-cis-1R,4R SRT	trans-1S,4R SRT	trans-1R,4S SRT
Recovery,% ($n = 3$)				
1.5 ng/mL	90.6	98.7	99.1	89.9
14 ng/mL	99.0	96.0	98.7	95.6
150 ng/mL	95.0	94.9	97.6	98.8
Linearity				
Range (ng/mL)	0.5–200	0.5–200	0.5–200	0.5–200
Regression equation	$y = 3797.9x + 1297.5$	$y = 3789x + 2747.6$	$y = 3793.9x + 2344.6$	$y = 3796.9x + 1235.8$
Correlation coefficient (r^2)	0.9999	0.9997	0.9996	0.9999

Table 4
Intra- and inter-stability data of SRT ($n = 3$).

Storage conditions	Nominal concentration (ng/mL)	RSD (%)
Freeze/thaw stability (three cycles)	0.5	1.81
	14.0	1.69
	150.0	3.22
3 days	0.5	3.32
	14.0	2.75
	150.0	4.32
1 week	0.5	6.68
	14.0	3.22
	150.0	3.35
15 days	0.5	2.78
	14.0	3.57
	150.0	3.27
1 month	0.5	4.89
	14.0	4.47
	150.0	3.78

3.2.5. Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ were calculated according to the ICH guidelines. The limit of detection (LOD) (lowest concentration of analyte in a sample matrix that can be detected) and the limit of quantification (LOQ) (lowest that can be quantified with acceptable accuracy and precision) were 0.17 and 0.4 ng/mL. The total ion current (TIC) chromatograms at LLOQ concentration of (+)-cis-1S,4S, (−)-cis-1R,4R, trans-1S,4R, trans-1R,4S SRT hydrochloride is shown in Fig. 7, respectively.

3.2.6. Application of the analytical method to pharmacokinetic study in rats

The developed method was applied to the study of enantioselectivity in the kinetic disposition of SRT administered in the racemic form in a single 25 mg/kg dose to rats. Fouda et al. [3] reported pharmacokinetic study on (±)-cis-1S,4S SRT. However this method does not address the enantioselectivity of SRT. The pharmacokinetics of SRT was enantioselective for the parameters $AUC_{0-\alpha}$ ($p = 0.68$), clearance ($p = 0.68$) and C_{max} ($p = 0.43$) calculated by RAMKIN software and p-values by Wilcoxon test. (Table 5). The data obtained for the Wistar male rats showed higher plasma concentrations of (+)-cis-1S,4S SRT enantiomer (Fig. 8). The higher $AUC_{0-\alpha}$ values obtained for (+)-cis-1S,4S SRT enantiomer compared to (−)-cis-1R,4R SRT enantiomer (113.45 ± 7.45 μg h/mL vs 108.69 ± 13.47 μg h/mL) could be explained by a lower apparent total clearance (222.21 ± 14.29 mL/h vs 238.16 ± 33.13 mL/h) (Table 5). The kinetic disposition of SRT is enantioselective in male Wistar rats with a (+)/(−) plasma concentration ratio (AUC) close to 1.04. C_{max} was also higher for (+)-cis-1S,4S SRT enantiomer than (−)-cis-1R,4R SRT enantiomer (Fig. 7).

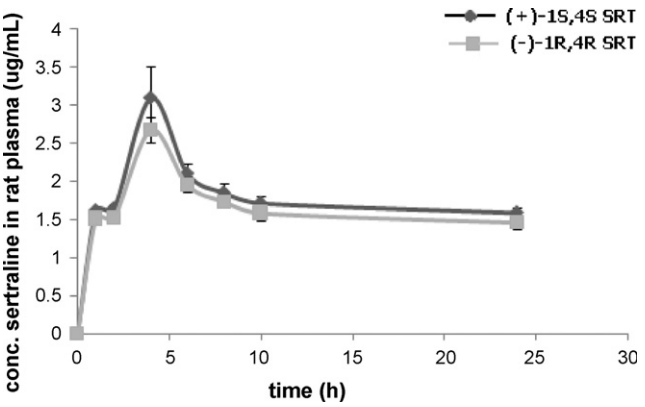

Fig. 7. Total ion current (TIC) chromatograms at LLOQ concentration of (+)-cis-1S,4S, (−)-cis-1R,4R, trans-1S,4R, trans-1R,4S SRT hydrochloride.

Table 5
Pharmacokinetic parameters of (±)-SRT enantiomers after a single oral dose of 25 mg/kg ($n = 6$) for each time point.

Parameters	(+)-cis-1S,4S SRT Mean ± S.E.M.	(−)-cis-1R,4R SRT Mean ± S.E.M.
C_{max} (μg/mL)	3.09 ± 0.40	2.67 ± 0.16
t_{max} (h)	4.00 ± 0	4.00 ± 0
AUC_{0-24} (μg h/mL)	42.90 ± 2.29	39.38 ± 1.73
$AUC_{0-\alpha}$ (μg h/mL)	113.45 ± 7.45	108.69 ± 13.47
$t_{1/2}$	31.02 ± 4.00	32.51 ± 4.37
Clearance (mL/h)	222.21 ± 14.29	238.16 ± 33.13
MRT (h)	45.57 ± 3.90	49.28 ± 6.17

C_{max}: maximum concentration of drug in rat plasma was observed at a particular time point. t_{max}: time at which maximum concentration of drug in rat plasma was observed. AUC: area under plasma concentration–time curve. $t_{1/2}$: time required for decomposition of the drug into half of the concentration. Clearance: the rate at which a substance is removed or cleared from the body. MRT (Mean Residence Time): the average time those molecules of a drug reside in the body. S.E.M.: standard deviation of standard error of the mean.

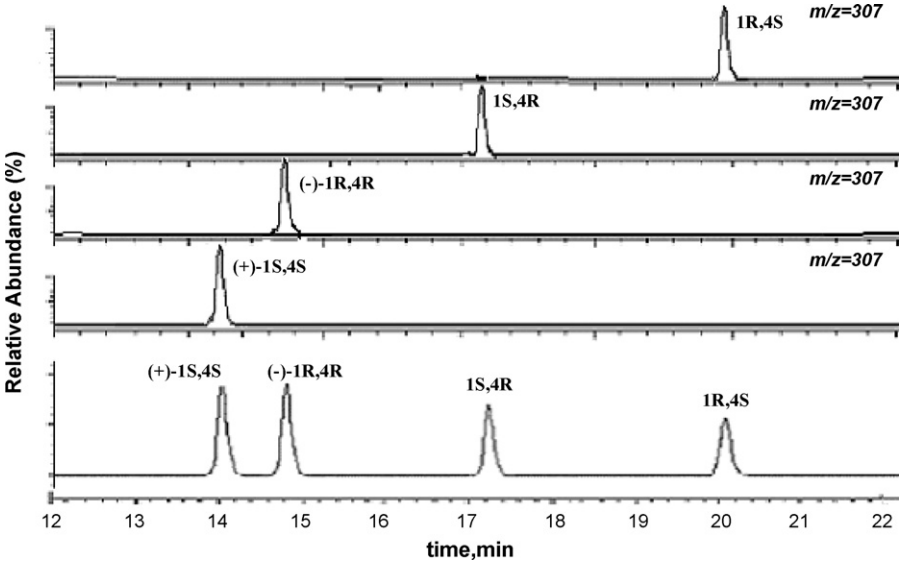

Fig. 8. Pharmacokinetic profiles for the cis enantiomers of (±)-SRT of 25 mg/kg by oral administration to rats ($n = 6$).

4. Conclusions

A LC–ESI/MS/MS column switching method for separation and determination of both enantiomers and diastereomers of SRT in rat plasma was established. LC with column switching and MS/MS detection was suitable for simple and accurate determination of SRT enantiomers and diastereomers in rat plasma. The developed method is simple and rapid, without sample pretreatment. The developed column switching technique has only three operating steps, and easy to perform. It helps in maintaining the efficiency and the lifetime of the column. The method showed adequate sensitivity, linearity, precision and accuracy and it has been successfully applied to determine enantioselectivity and the concentration–time profiles in pharmacokinetic studies.

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Enantiomeric separation of mirtazapine and its metabolite in rat plasma by reverse polar ionic liquid chromatography using fluorescence and polarimetric detectors connected in series

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ABSTRACT

A simple and rapid reverse polar ionic LC method was developed and validated for simultaneous separation and determination of mirtazapine, an antidepressant drug, and its main metabolite N-desmethyl mirtazapine using fluorescence and polarimetric detectors connected in series. The chromatographic separation was achieved on Chirobiotic V column packed with vancomycin as a stationary phase in an isocratic mode of elution of methanol:glacial acetic acid:anhydrous triethyl amine (100:0.2:0.1, v/v/v) as a mobile phase. The compounds were detected by their excitation at 290 nm and emission at 370 nm using fluorescence detector while the optical rotation (+/−) of the enantiomers was identified by polarimetric detector. The analytes were extracted from rat plasma by precipitation of proteins and the average yield was 88–111% for mirtazapine and 85–123% for N-desmethyl mirtazapine. The method was linear over the concentration range of 20–5000 ng/mL. The method was successfully applied on rat plasma spiked with the enantiomers of mirtazapine and N-desmethyl mirtazapine.

1. Introduction

Mirtazapine (1,2,3,4,10,14b-hexahydro-2-methyl-pyrazino [2,1-a]-pyrido [2,3-c] [2-benzazepine] (MTZ) (Fig. 1a) is a novel tetra cyclic anti depressant used not only in psychotherapy treatment but also alcoholic detoxification and post-traumatic disorders [1]. It is a stereo selective antagonist whose chirality plays an important role due to the potential of different activities and toxicities of drug enantiomers [2]. Its major metabolite, N-desmethyl mirtazapine (DMTZ) (Fig. 1b) contributes 3–6% to the total pharmacodynamic profile of the parent drug [3]. Thus it is of great importance to determine the plasma levels of the enantiomers of MTZ and DMTZ for therapeutic drug monitoring.

Several HPLC methods have been reported in the literature for analysis of MTZ and its metabolites in biological fluids. RP-HPLC [4,5] and LC–MS/MS [6] with electrospray ionization were extensively used for analysis of antidepressants including MTZ and DMTZ in plasma. Analytical and semi preparative separation of enantiomers of MTZ and its metabolites were carried out on various polysaccharide chiral stationary phases by LC [7]. Santana et al. studied the chromatographic separation of (+)-(S)- and (−)-(R)-enantiomers of MTZ on Chiralpak AD column [8]. Later it was applied to determine the enantiomers of MTZ in human plasma [9]. Mandrioli et al. used CE for enantioseparation of MTZ and DMTZ in human plasma [1]. The separation was achieved on a fused silica capillary using carboxymethyl-β-cyclodextrine dissolved in phosphate buffer at pH 2.5. However, CE lacks the sensitivity necessary to reliably determine the low levels of MTZ and its metabolite usually found in patient plasma. For this reasons a careful pretreatment using SPE with hydrophilic–lipophilic balance cartridge was proposed. Recently off-line solid-phase microextraction [10] and liquid-phase microextraction (LPME) [11] using porous polypropylene hollow fibre membrane were developed for simultaneous enantioselective determination of MTZ, DMTZ and 8-hydroxymirtazapine in plasma. However, all these procedures are not only tedious and time consuming but also involve additional steps of purification and concentration of biological samples.

Macrocyclic antibiotic CSPs have become popular for separation of a wide range of structurally different chiral compounds [12]. For example, vancomycin (Fig. 1c) produced by *Streptomyces orientalis*, has many of the separation characteristics of protein based stationary phases with exceptional stability and higher sample capacity [13]. However, so far no method has been reported in the literature using the reverse polar ionic mode of chiral separation of MTZ and DMTZ on columns packed with vancomycin as a stationary phase. The reverse polar ionic mode has many advantages not only in terms of speed and sample solubility but also beneficial for high throughput preparative separations. Mobile phases used

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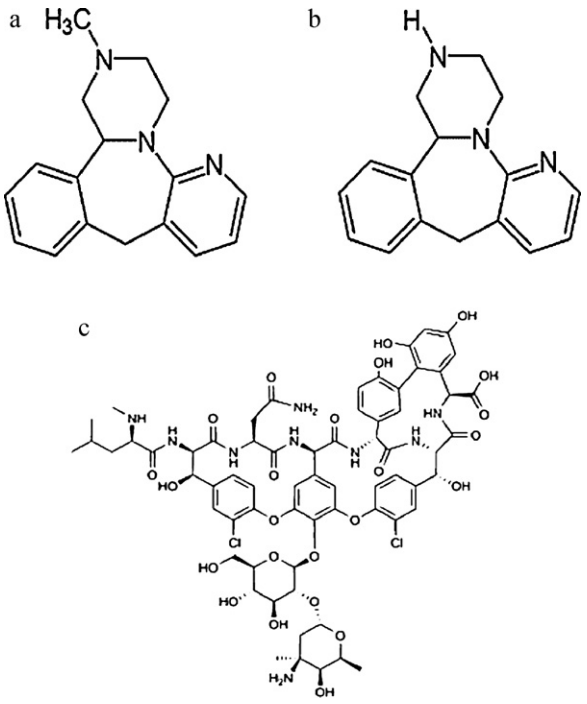

Fig. 1. Chemical structures of (a) MTZ, (b) DMTZ and (c) vancomycin.

in this mode are polar organic solvents containing volatile additives ideally suited for LC–MS applications. Thus it is of important to investigate the separation of enantiomers of MTZ and DMTZ on macrocyclic antibiotic stationary phases in reverse polar ionic mode by LC.

In the present study, the liquid chromatographic separation of enantiomers of MTZ and DMTZ was studied on a chirobiotic V column containing vancomycin as a stationary phase in reverse polar ionic mode using a mixture of methanol, acetic acid and triethyl amine as a mobile phase. The column effluents were monitored by both fluorescence and polarimetric detectors connected in series. The plasma proteins were precipitated by acetonitrile to extract MTZ and DMTZ quantitatively.

2. Experimental

2.1. Chemicals and reagents

The rac-MTZ and rac-DMTZ (purity >99.0%) obtained from Neulands laboratory (Hyderabad, India) were used. All the reagents were of analytical grade unless stated otherwise. HPLC-grade methanol, glacial acetic acid and anhydrous triethyl amine (S.D. Fine Chem; Mumbai, India) were used. Blank plasma samples were obtained from wister rats. All solvents were filtered through a 0.22 μm membrane and degassed.

2.2. Instrumentation

The HPLC system consisting of two LC-20 AD pumps, a RF-10AXL fluorescence detector, a SIL-20 AC auto sampler, a DGU-20 A_5 degasser and CBM-20A communications bus module (Shimadzu, Kyoto, Japan) was used. The chromatographic and the integrated data were recorded using HP-Vectra (Hewlett Packard, Waldron, Germany) computer system using LC-solution data acquiring software (Shimadzu, Kyoto, Japan). The compounds were analyzed on a Chirobiotic V column (25 cm × 4.6 mm, 5 μm) (Supelco, PA, USA). The mobile phase was filtered through a 0.22 μm membrane (Millipore) before use. A chiralyser (IBZ Messtechnik GmbH, Hannover, Germany) also known as a polarimetric detector for identification of the optical rotation (+/−) of the peaks corresponding to the enantiomers of MTZ and DMTZ was used.

2.3. Chromatographic conditions

The compounds were analyzed on a Chirobiotic V column (25 cm × 4.6 mm, 5 μm) (Supelco, PA, USA), using a mobile phase containing methanol:glacial acetic acid:anhydrous triethyl amine (100:0.2:0.1, v/v/v) in an isocratic elution mode at a flow rate of 1 mL/min. The column temperature was 20 °C. The auto sampler temperature was kept at 5 °C and the samples of 20 μL volume were injected onto the column. The data acquisition run time was 16 min. Compounds were detected by their excitation at 290 nm and emission at 370 nm using fluorescence detector while optical rotation (+/−) of MTZ and DMTZ enantiomers were identified by polarimetric detector connected in series.

2.4. Preparation of standards and quality controls

Standard 1 mg/mL stock solutions of MTZ and DMTZ were prepared separately in methanol. Standard working solutions of MTZ and DMTZ at concentrations of 0.2, 0.5, 1, 5, 10 and 50 μg/mL were prepared by serial dilution of stock solutions. Methanol was used as a diluent. Drug-free plasma was spiked with standard solutions to prepare calibration standards with final concentrations of 20, 50, 100, 500, 1000 and 5000 ng/mL of MTZ and DMTZ. Plasma quality-control (QC) samples containing MTZ and DMTZ at four concentration levels: 20 ng/mL (lower limit of quantization, LLOQ), 500 ng/mL (low, LQC), 1000 ng/mL (middle, MQC), and (5000 ng/mL) (high, HQC) were prepared to measure recovery, stability, accuracy and precision of the method. The prepared QC samples cover the therapeutic ranges of both MTZ (20–300 ng/mL) and DMTZ (50–300 ng/mL). All solutions were kept at −20 °C prior to analysis.

2.5. Plasma sample preparation

Plasma samples were stored at −80 °C and allowed to thaw gradually to room temperature before processing. After transferring 100 μL aliquots of plasma into 1.5 mL centrifuge tubes, 400 μL acetonitrile was added as a protein precipitating agent to each tube and the mixture was vortexed for 10 min and centrifuged at 4000 rpm for 20 min. Then, the upper organic layer was transferred to an autosampler vial and injected (20 μL) into the column.

2.6. Method validation

The method was validated by evaluating recovery, accuracy, precision, linearity, LOD, LOQ and stability.

2.6.1. Recovery

The recoveries were determined by comparing the peak areas of each enantiomer of MTZ and DMTZ in four different QC plasma samples (20, 500, 1000 and 5000 ng/mL) with those of each enantiomer of MTZ and DMTZ in samples prepared by spiking after deproteinization with same amounts of QC plasma samples. The recovery was calculated using the formula:

$$\text{Recovery (\%)} = \frac{\text{area of the corresponding peak in the chromatogram of rat plasma spiked with MTZ and DMTZ enantiomers and extracted}}{\text{area of the corresponding peak in the chromatogram of deproteinated blank rat plasma spiked with MTZ and DMTZ enantiomers}} \times 100$$

2.6.2. Accuracy and precision

Accuracy was evaluated by calculating the percentage deviation from the nominal concentration and reported as relative error (RE). Precision was determined by calculating the coefficient of variation (CV) of replicates within one sample run (intra-day) and between samples runs (inter-day).

For intra-day accuracy and precision, ten replicates quality-control (QC) samples containing MTZ and DMTZ, 20 ng/mL (LLOQ), 500 ng/mL (LQC), 1000 ng/mL (MQC), and (5000 ng/mL) (HQC). Samples were prepared and analyzed on the same day; and the cumulation of all five days was used for inter-day accuracy and precision determination.

2.6.3. Linearity, limit of detection (LOD) and limit of quantification (LOQ)

Linearity of the analytical method was evaluated by analyzing spiked plasma samples for each concentration ($n = 3$) over the concentration range 20–5000 ng/mL for all enantiomers of MTZ and DMTZ. The results were used to draw a linear regression curve. The LOD and LOQ were calculated according to the ICH guidelines [14].

2.6.4. Stability

The stability of analytes was determined during blood sample collection and after freezing plasma samples for 1st day, 2nd day, 3rd day, 1 week, 15 days and 1 month.

3. Results and discussion

3.1. Method development

3.1.1. Selection of chirobiotic V column in reverse polar ionic mode

The enantiomeric separation on chiral stationary phases (CSPs) is generally based on the formation of transient diastereomeric analyte–CSP complexes between the enantiomers and the chiral molecule that is an integral part of the stationary phase [15]. Vancomycin, containing 18 chiral centers with various functional groups surrounding its three pockets or cavities is an integral part of the stationary phase of chirobiotic V column [11]. The strong polar groups present on vancomycin molecule interact with the analytes groups, which are easily ionizable by the reverse polar ionic mobile

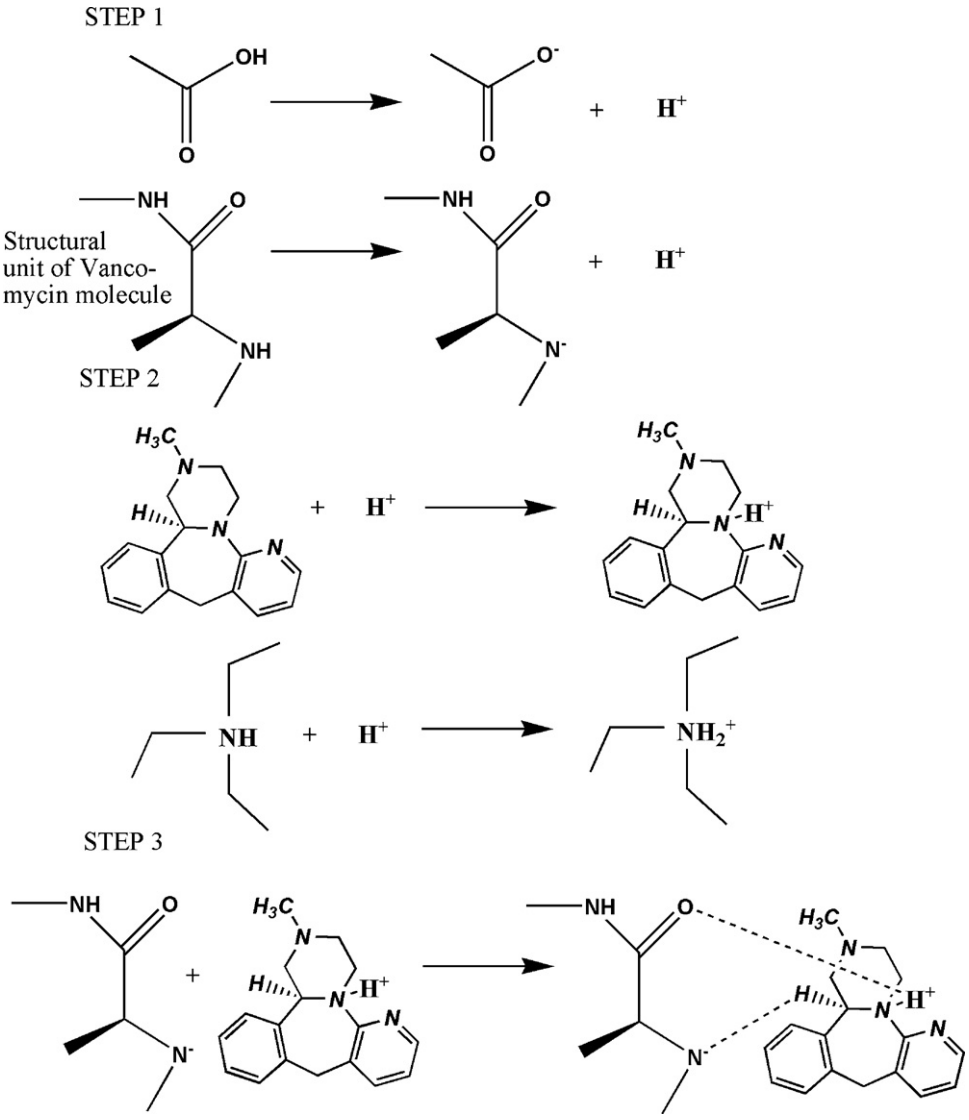

Fig. 2. Mechanism of formation of diastereomeric analyte–CSP complexes between enantiomers of MTZ/DMTZ and vancomycin. Step 1: ionization of acetic acid and vancomycin molecule. Step 2: protanation of MTZ enantiomers and triethyl amine. Step 3: interaction of ionized vancomycin molecule and protanated MTZ enantiomers, formation of transient diastereomeric analyte–CSP complexes.

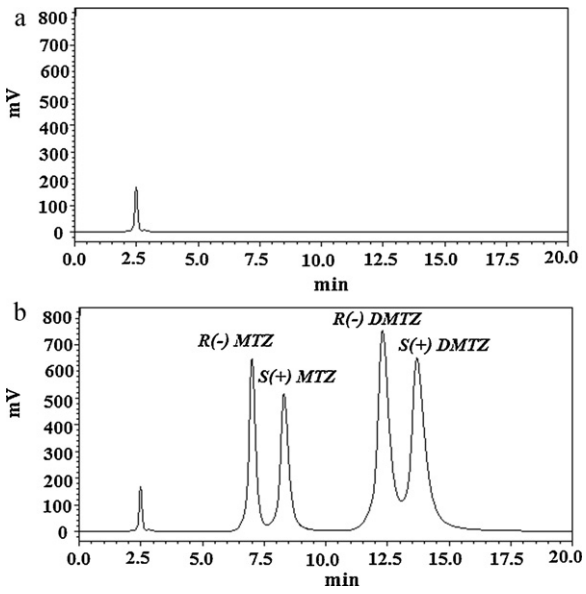

Fig. 3. Chromatograms of rat plasma (a) blank and (b) spiked with racemic MTZ and DMTZ. LC conditions: column: chirobiotic V; eluent: MeOH:AcOH:TEA (100:0.2:0.1, v/v/v); flow rate: 1 mL/min; detection: fluorescence at excitation at 290 nm and emission at 370 nm; column temperature: 20 °C.

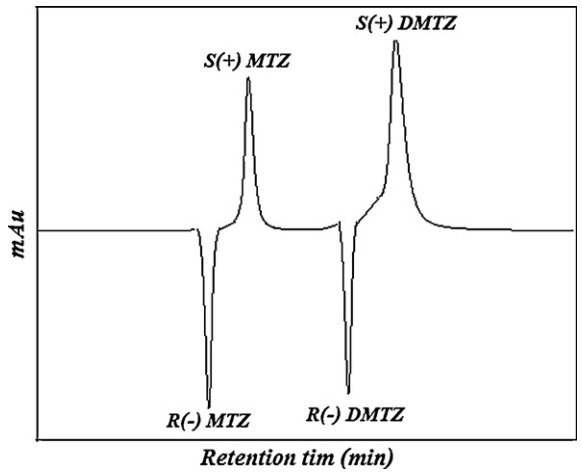

Fig. 4. A typical chromatogram showing the identification of optical rotation (+/−) of the enantiomers of standard MTZ and DMTZ by polarimetric detector. For conditions see Fig. 3.

phases. The mobile phase used in reverse polar ionic mode is useful for ionization of groups on or near the chiral centers of the analytes. The reverse polar ionic mode is applicable to all molecules with at least one ionizable group on or near the chiral center. Generally, basic compounds demonstrate more selectivity in reverse polar ionic mode [13]. MTZ and DMTZ are the basic compounds having ionizable group –N< near to chiral center. The mechanism of formation of diastereomeric analyte–CSP complexes between enantiomers and vancomycin is shown in Fig. 2. Further, the preparation of mobile phase composition is easy and the reverse polar ionic mode could be described as a novel method to obtain difficult enantioselective separation with macrocyclic antibiotic-based chiral stationary phases by LC [16].

3.1.2. Fluorescence and polarimetric detection

The compounds were detected by their excitation at 290 nm and emission at 370 nm using fluorescence detector. In the present study, baseline separation of the MTZ and DMTZ enantiomers was achieved on vancomycin CSP with fluorescence detection in the reverse polar ionic mode. The chromatographic separation of (a) blank plasma (b) rat plasma spiked MTZ, DMTZ is shown in Fig. 3. Polarimetric detector (optical rotation range, 250; average, 10 and offset is 50) connected in series was used for identification of (+/−) rotations of enantiomers of MTZ and DMTZ. The chromatographic separation of a mixture of rac MTZ and DMTZ using polarimetric detector is shown in Fig. 4. The elution order was R (−) MTZ, S(+) MTZ, R (−) DMTZ and S(+) DMTZ.

3.1.3. Mobile phase optimization

No enantiomeric separation was observed in the absence of triethyl amine when the mobile phase consisted of methanol–acetic acid (100:0.2, v/v). This could be explained on the basis of strong repulsive effects between the protonated amino groups of the analyte molecules and of the CSP. Different mobile phase compositions, flow rate, temperature of the column conditions were tried. The mobile phase composition was optimized by changing the concentration of acetic acid and keeping MeOH, TEA concentration as constant. The chiral separation was found to be very sensitive with chirobiotic V column when the concentration of AcOH was varied [13]. Initially the mobile phase composition MeOH:AcOH:TEA (100:0.1:0.1, v/v/v) was tried. The enantiomers of MTZ were eluted fast while DMTZ took long time. Later the concentration of AcOH was changed from 0.2 to 0.4%. Except at 0.2% AcOH, the enantiomeric separation of MTZ and DMTZ was not good under all the conditions. The effect of mobile phase composition on retention time (t_R), retention factor (k'), separation (α) and resolution (R_s) parameters at temperature of the column 20 °C and flow rate 1 mL/min are given in Table 1. The separation was tried at different temperatures of the column (15 °C, 20 °C, and 25 °C), and found to be good at 20 °C. Different flow rates of the mobile phase were also tried and 1 mL/min flow rate was found to be optimum. Finally, the following chromatographic conditions were optimized using the mobile phase of methanol:glacial acetic acid:anhydrous triethyl amine (100:0.2:0.1, v/v/v) in an isocratic mode of elution at a flow rate of 1 mL/min at 20 °C.

3.2. Method validation

3.2.1. Recovery

The recovery (±RSD%) of each enantiomer of MTZ and DMTZ for QC samples of 20 ng/mL (lower limit of quantization, LLOQ), 500 ng/mL (low, LQC), 1000 ng/mL (middle, MQC), and (5000 ng/mL) (high, HQC) are given in Table 2.

3.2.2. Accuracy and precision

Accuracy was evaluated by calculating the percentage deviation from the nominal concentration and is reported as relative error (RE). Precision was determined by calculating the coefficient of variation (CV) of replicates within one sample run (intra-day) and between samples runs (inter-day). Intra and inter-day accuracy and precision were determined by the performance of four concentrations of QCs and are given in Table 3.

Intra-day accuracy ranges (RE) observed for the analytes were as follows: R (−) MTZ: 0.002–0.043; S (+) MTZ: 0.005–0.025; R (−) DMTZ: 0.003–0.092; and S (+) DMTZ: 0.002–0.095. Inter-day accuracy ranges (RE) observed for the analytes were as follows: R (−) MTZ: 0.012–0.026; S (+) MTZ: 0.006–0.032; R (−) DMTZ: 0.004–0.059; and S (+) DMTZ: 0.001–0.110. Intra-day precision ranges (CV) observed for the analytes were as follows: R (−) MTZ: 0.006–0.074; S (+) MTZ: 0.013–0.056; R (−) DMTZ: 0.008–0.182; and S (+) DMTZ: 0.005–0.225. Inter-day precision ranges (CV)

Table 1
The effect of the mobile phase composition on retention and separation of enantiomers of MTZ and DMTZ.

MeOH:AcOH:TEA (v/v/v)	R (−)MTZ	S (+)MTZ	R (−)DMTZ	S (+)DMTZ
100:0.1:0.1				
t_R (min)	6.413	7.194	15.411	17.281
k'	1.338	1.623	4.619	5.301
α	–	1.213	2.846	1.148
R_s	6.297	1.019	6.649	1.091
100:0.2:0.1				
t_R (min)	6.998	8.294	12.292	13.685
k'	1.911	2.449	4.054	4.749
α	–	1.281	1.656	1.171
R_s	8.083	2.657	3.796	2.308
100:0.3:0.1				
t_R (min)	6.988	8.129	11.503	12.921
k'	1.552	1.968	3.200	3.718
α	–	1.268	1.626	1.162
R_s	6.569	1.293	2.998	0.999
100:0.4:0.1				
t_R (min)	8.632	10.536	11.879	13.542
k'	2.748	3.574	4.157	4.879
α	–	1.301	1.163	1.174
R_s	7.223	1.713	0.993	1.098

Conditions: column: Chirobiotic V; flow rate: 1 mL/min; detection: fluorescence at excitation at 290 nm and emission at 370 nm; column temperature: 20 °C.
t_R, retention time; k', capacity factor; α, selectivity factor; R_S, resolution.

Table 2
Recovery data.

Enantiomer	Recovery ± RSD (%)[a]			
	20 (ng/mL)	500 (ng/mL)	1000 (ng/mL)	5000 (ng/mL)
R (−) MTZ	99.868 ± 3.27	101.240 ± 0.24	94.995 ± 8.00	100.380 ± 2.69
S (+) MTZ	100.340 ± 7.93	92.226 ± 2.69	88.576 ± 4.66	98.601 ± 2.07
R (−) DMTZ	105.397 ± 10.43	123.022 ± 0.51	103.734 ± 19.9	85.679 ± 1.24
S (+) DMTZ	106.298 ± 17.12	119.824 ± 3.49	104.710 ± 15.71	101.245 ± 0.84

[a] $n = 3$ (triplicate determinations).

observed for the analytes were as follows: R (−) MTZ: 0.028–0.080; S (+) MTZ: 0.016–0.055; R (−) DMTZ: 0.045–0.115; and S (+) DMTZ: 0.049–0.200.

3.2.3. Linearity, limit of detection (LOD) and limit of quantification (LOQ)

Linearity of the analytical method was evaluated by analyzing spiked plasma samples for each concentration ($n = 3$) over the concentration range 20–5000 ng/mL for each enantiomer of MTZ and DMTZ. The results obtained were used to draw linear regression curve. The LOD and LOQ were calculated according to the ICH guidelines. The limit of detection (LOD), the limit of quantification (LOQ), regression equations and regression coefficients (r^2) are given in Table 4. It could be seen from Table 4 that DMTZ has higher slope (2236.9–2295.5) when compared to MTZ (1080.9–1093.6). It could be probably due to emission of more energy by DMTZ at selected excitation and emission wavelengths at 290 nm and 370 nm, respectively. Further the LOQs of both MTZ and DMTZ vary between 12.4 and 17.9 ng/mL which corresponds to the $2.48–3.58 \times 10^{-10}$ g in an injection volume 20 μL which was almost close to the value of 3.12×10^{-10} g (LOQ is 6.25 ng/mL) with an injection volume 50 μL as reported by de Sanatana et al. [8,9]. This could be explained in terms of the high UV absorption of MTZ and DMTZ in normal phase solvents viz; n-hexane/iso propanol with DEA buffer where as it was found to be comparatively less in reverse polar ionic buffers viz; AcOH and TEA, which quench the UV absorption of MTZ and DMTZ significantly. Thus the fluorescence detector was used to increase the detection levels of MTZ and DMTZ in the present investigation.

Table 3
Intra- and inter-day batch accuracy and precision data.

Precision and accuracy	R (−) MTZ	S (+) MTZ	R (−) DMTZ	S (+) DMTZ
Intra-assay precision; coefficient of variation ($n = 10$)				
20 ng/mL	0.041	0.035	0.102	0.225
500 ng/mL	0.006	0.013	0.012	0.005
1000 ng/mL	0.074	0.056	0.182	0.134
5000 ng/mL	0.025	0.029	0.008	0.012
Inter-assay precision; coefficient of variation ($n = 5$)				
20 ng/mL	0.080	0.055	0.115	0.200
500 ng/mL	0.028	0.016	0.060	0.057
1000 ng/mL	0.043	0.027	0.045	0.057
5000 ng/mL	0.053	0.077	0.058	0.049
Intra-assay accuracy; relative error ($n = 10$)				
20 ng/mL	0.028	0.025	0.058	0.095
500 ng/mL	0.023	0.019	0.054	0.090
1000 ng/mL	0.002	0.005	0.003	0.002
5000 ng/mL	0.043	0.025	0.092	0.054
Inter-assay accuracy; relative error ($n = 5$)				
20 ng/mL	0.026	0.032	0.059	0.110
500 ng/mL	0.019	0.009	0.004	0.001
1000 ng/mL	0.049	0.006	0.012	0.026
5000 ng/mL	0.012	0.008	0.018	0.023

3.2.4. Stability

The stability of analytes was determined for QC concentrations except 20 ng/mL during blood sample collection and after freezing plasma samples for 1st day, 2nd day, 3rd day, 1 week, 15 days and 1 month. The results were compared with those obtained by freshly prepared samples. The results are summarized in Table 5.

Table 4
Linearity (±RSD%)[a] of slope, intercept and correlation coefficient; LOD and LOQ data.

Enantiomer	Range (ng/mL)	Regression equation	r^2	(±RSD%)[a]			LOD (ng/mL)	LOQ (ng/mL)
				m	c	r^2		
R (−) MTZ	20–5000	$y = 1093.6x - 26281$	0.9998	2.58	61.94	–	4.7	14.1
S (+) MTZ	20–5000	$y = 1080.9x - 39598$	0.9995	2.95	38.00	0.06	5.9	17.9
R (−) DMTZ	20–5000	$y = 2295.5x - 44694$	0.9988	0.76	145.89	0.11	4.1	12.4
S (+) MTZ	20–5000	$y = 2236.9x - 36318$	0.9999	1.24	152.98	0.04	4.8	14.5

"m" is slope; "c" is intercept; and"r^2" is correlation coefficient.
[a] $n = 3$ (triplicate determinations).

Table 5
Intra-and inter-stability data (±RSD%)[a] of spiked (−) MTZ.

Storage conditions	(±RSD%)[a] Nominal concentration (ng/mL)		
	500	1000	5000
Freeze/thaw stability (three cycles)	0.629	7.361	2.495
3 days	1.320	5.576	2.875
1 week	1.184	18.240	0.859
15 days	0.498	13.409	1.223
1 month	0.859	0.498	1.223

[a] $n = 3$ (triplicate determinations).

4. Conclusions

Enantiomeric separation of MTZ and its metabolite DMTZ was achieved on a new chirobiotic V column packed with vancomycin in a reverse polar ionic phase by LC. The method was developed and validated for determination of MTZ and DMTZ enantiomers in rat plasma. The repeatability of the HPLC-fluorescence-polarimetric detector was acceptable. The method showed adequate sensitivity, linearity, precision and accuracy.

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Liquid Chromatographic Separation of Darunavir Enantiomers on Coated and Immobilized Amylose Tris (3, 5-Dimethylphenylcarbamate) Chiral Stationary Phases

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ABSTRACT Liquid chromatographic separation of darunavir enantiomers on covalently bonded and physically adsorbed polysaccharide chiral stationary phases was studied at different temperatures. The separations were accomplished under normal-phase conditions by using different combinations of hexane, organic modifiers (2-propanol, 1-propanol and ethanol), and diethylamine as mobile phase solvents. The effect of organic modifiers and the column temperature on retention, separation, and resolution was investigated. The observed differences were explained in terms of the coated and immobilized nature of the two columns. Van't Hoff plots (ln k' vs. 1/T, ln α vs. 1/T) and apparent thermodynamic parameters were derived to understand the effect of temperature on separation. *Chirality 24:652–660, 2012.*

KEY WORDS: chiral separations; darunavir enantiomers; covalently bonded; physically adsorbed; polysaccharide stationary phases

INTRODUCTION

Chiral separations by liquid chromatography (LC) have been considerably advanced during the last few decades.[1–3] The coated polysaccharides, viz. cellulose and amylose on silica gel supports introduced by Okamoto *et al.*,[4] have become routine chiral stationary phases (CSPs) for analytical and preparative enantiomeric separations.[5–7] However, their utility for monitoring of synthetic organic reactions was found to be limited because most of the synthetic organic reactions are generally performed in nonstandard solvents such as dichloromethane (DCM), toluene, chloroform ($CHCl_3$), ethyl acetate (EtOAc), tetrahydrofuran (THF), acetone, methyl tert-butyl ether, and 1,4-dioxane. These solvents may partially or totally dissolve the coated stationary phases leading to decrease of column's life. Thus, monitoring of enantiomeric excess of synthetic organic reactions was found to be difficult on these columns. It requires a tedious workup to remove nonstandard solvents prior to injection on coated chiral columns. To overcome such problems, various research groups have investigated the CSPs by immobilizing (covalent linkage) polysaccharides on silica gel supports.[8–10] These CSPs were generally prepared by chemically bonding polysaccharide phenylcarbamates to silica gel through hydroxyl groups randomly and regioselectively. However, such CSPs have shown lower chiral recognition ability than the corresponding coated-type CSPs as the regular higher order structures of the polysaccharides were disturbed. Enomoto *et al*. prepared amylose tris(3,5-dimethylphenylcarbamate) CSPs by using enzyme-catalyzed polymerization, in which amylose was chemically bonded nonregioselectively to silica gel only at the reducing terminal residue.[11] These CSPs have shown higher resolving power and also used with nonstandard organic solvents such as THF and chloroform. Recently, three types of immobilized polysaccharide-based CSPs (Chiralpak IA, Chiralpak IB and Chiralpak IC) have become commercially available. These columns were prepared by covalently bonding amylose tris(3,5-dimethylphenylcarbamate), cellulose tris (3,5-dimethylphenylcarbamate), and cellulose tris(3,5-dichlorophenylcarbamate) to silica gel, respectively.[12–15] Although the chiral selectors were the same, the immobilized CSPs have shown different enantioselectivity compared with the corresponding coated columns under similar conditions. According to the reported literature,[16,17] Chiralpak AD-H has higher resolving power than Chiralpak IA. This indicates that there may be conformational changes of the polysaccharide chains upon immobilization. An understanding of the extent of immobilization and its effect on separation and resolution ability is of great interest not only in designing more effective CSPs but also in selecting better chromatographic conditions. Thermodynamic investigations[18–20] may be of some use to evaluate the effect of column temperature on chiral discrimination. Apparent thermodynamic parameters corresponding to chromatographic retention data may give some meaningful understanding of the mechanism of chiral separations.

Darunavir [(−) - [(3*R*, 3a*S*, 6a*R*)-hexahydrofuro [2, 3-b] furan-3-yl (2*S*, 3*R*)-4-(4-amino-*N*-isobutylphenylsulfonamido) -3-hydroxy-1-phenylbutan-2-ylcarbamate] (DRV) (I) (Fig. 1) is a new HIV-1 protease inhibitor and effective against viral strains that are not sensitive to other antiretrovirals.[21] It is one of the key components of many salvage therapies in multiexperienced patients.[22] During its synthesis, the [(+) - (3*S*, 3a*R*, 6a*S*)-hexahydrofuro [2, 3-b] furan-3-yl (2*R*, 3*S*)-4-(4-amino-*N*-isobutylphenylsulfonamido)-3-hydroxy-1-phenylbutan-

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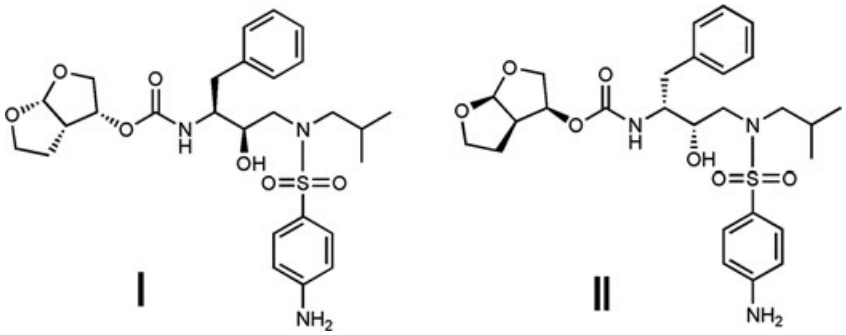

Fig. 1. Chemical structures of (–) DRV (I) and (+) DRV (II).

2-ylcarbamate] enantiomer (II) (Fig. 1) may be formed as an impurity in significant quantity. Therefore, stereoselective separation of DRV (I) and (II) enantiomers is important to assure the therapeutic efficacy and safety of its pharmaceutical preparations. Several LC, LC/MS, LC/MS/MS methods have been reported for achiral separation of DRV from other antiretroviral drugs in human plasma. LC with UV detection[21] has been routinely used for this purpose. A few LC–MS methods for simultaneous determination of DRV along with other antiretroviral agents,[22] on dried blood spots,[23] and in plasma of HIV-infected patients[24] were reported. LC/MS/MS[25–29] was used for quantification of DRV in human plasma. It was also used for quantification of DRV in the presence of protease inhibitors (atazanavir, lopinavir, and ritonavir) and non-nucleoside reverse transcriptase inhibitors (efavirenz and nevirapine) on dried blood spots.[30] Dailly *et al*. proposed a column switching LC/MS/MS method for determination of plasma concentration of DRV along with other 10 antiretroviral agents.[31] However, to the best of the authors' knowledge, there are no reports on chiral separation of DRV enantiomers till today.

The present article compares the separation of DRV enantiomers on (i) immobilized and (ii) coated CSPs on the basis of the chiral selector amylose tris(3, 5-dimethylphenylcarbamate) (Fig. 2) CSPs by using a range of mobile phase solvents composed of different organic modifiers. The differences between coated and immobilized type of the chiral columns in terms of efficiency and applicability were explained. The chromatographic retention, separation, and resolution were investigated by varying the percentage and type of polar modifier at different column temperatures. The data, in turn, were used to derive apparent thermodynamic parameters to understand the effect of temperature on separation.

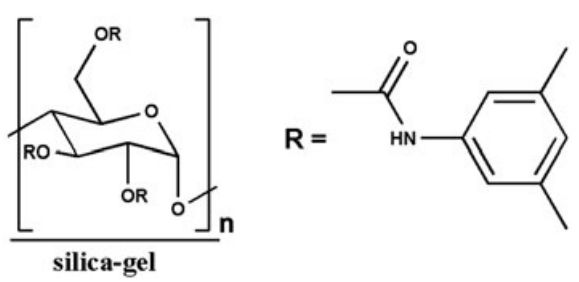

Fig. 2. Chemical structure for the chiral selector of Chiralpak IA and AD-H chiral stationary phases based on tris(3,5-dimethylphenylcarbamate) of amylose on silica gel.

MATERIALS AND METHODS

Materials

All the solvents used were of high-performance liquid chromatography grade (E.Merck, India). Analytical reagent grade diethylamine (DEA) (Spectrochem, Mumbai, India) was used. The enantiomers of DRV were obtained from a local manufacturing unit as gift samples.

Chromatography

The high-performance liquid chromatography system consisting of two LC-20 AD pumps, photodiode array detector, an SIL-20 AC auto sampler, a DGU-20 A_5 degasser, and CBM-20A controller (Shimadzu, Kyoto, Japan) was used. The chromatographic and the integrated data were recorded using HP-Vectra (Agilent, Waldbronn, Germany) computer system using LC-solution data acquiring software (Shimadzu, Kyoto, Japan). The DRV (I) and (II) enantiomers were separated on Chiralpak IA (25 cm × 4.6 mm I.D., 5 μm particle size) and Chiralpak AD-H columns (25 cm × 4.6 mm I. D., 5 μm particle size) (Daicel, Tokyo, Japan). The prepared mobile phases were filtered through a 0.45 μm membrane filter paper and degassed with an ultrasonic bath before use. The analytes were dissolved in 2-propanol at approximately 0.1 mg ml^{-1} and filtered through a 0.45 μm membrane filter paper prior to injection. The flow rate was set at1.0 ml min^{-1}. The ultraviolet detection wavelength was set at 266 nm. The injection volume was 5 μl. The void volume (v_0) of the column was determined from the first perturbation of the baseline following the procedure as reported elsewhere.[18] Later, it was also confirmed by injecting 1, 3, 5-tri-tert-butylbenzene and determining the retention time (t_0) of the unretained peak. Capacity factors, k', were calculated from the formula $(t_R - t_0)/t_0$, where t_R is the retention time of particular enantiomer of DRV and t_0 is the retention time of the unretained peak. The separation factors (α) were calculated from k_2'/k_1', where k_1' and k_2' are the capacity factors DRV (I) and (II) enantiomers, respectively. Their elution order was determined by injecting the individual enantiomers under similar conditions. It was observed that the first and second peaks correspond to DRV (I) and (II) enantiomers, respectively. Origin software was used to plot the linear Van't Hoff plots.

RESULTS AND DISCUSSION

DRV (I) and (II) enantiomers were separated on two different columns, viz. Chiralpak IA and AD-H under normal-phase conditions. The mobile phases were composed of hexane and different organic modifiers. Different volumes of ethanol, 1-propanol, and 2-propanol were tried with Chiralpak IA column, whereas only ethanol and 2-propanol were used with Chiralpak AD-H column. The separation was studied at different temperatures between 20 and 40 °C on both the columns.

Effect of Organic Modifiers

Chiralpak IA column. Initially, we have tried the separation of enantiomers of DRV with extended range of solvents such as THF, DCM, EtOAc, and $CHCl_3$ but not successful in

separation. Later, three different standard solvents ethanol, 2-propanol, and 1-propanol in different combinations of hexane and 0.1% DEA were tried. Figure 3 shows the chromatograms demonstrating the effect of percentage and type of polar modifier on the retention, separation, and resolution of DRV (I) and (II) enantiomers at 25 °C. As shown in Figure 3, as the percentage of 2-propanol increased (vertical comparison), retention factors were decreased, enantioseparations did not change much, and resolutions were decreased. Similar trends were observed in the case of 1-propanol and ethanol. This was in accordance with the general understanding that the H-bonding interactions between the enantiomers and CSPs decrease with increased alcohol content in the mobile phase leading to decreased retentions. Table 1 gives the retention data showing the effect of concentration of 2-propanol, ethanol, and 1-propanol on separation and resolution of enantiomers of DRV at different column temperatures. It could be seen from Table 1 that the better enantioseparation ($\alpha = 1.39$) and resolution ($Rs = 3.77$) were obtained using 25% and 20% of ethanol at 20 and 35 °C, respectively. Figure 3 also shows the increase in retention of DRV enantiomers (horizontal comparison) from1-propanol to ethanol to 2-propanol. It could be due to the incorporation of different size/shape alcohol modifiers into the chiral cavities of the CSP. Thus, the modified stereo-environment influences the solute retention (k') as reported elsewhere.[32] However, the enantioseparation increased from 2-propanol to 1-propanol to ethanol, whereas resolution increased from 2-propanol to 1-propanol to ethanol. Similar trends were followed in all combinations of polar modifiers. Overall, better enantioseparation and resolution were obtained using ethanol and 2-propanol as organic modifiers, respectively.

Chiralpak AD-H column. Two types of polar modifiers, viz. ethanol and 2-propanol, in different combinations of hexane and 0.1% DEA were tried. Figure 4 shows the effect of percentage and type of polar modifier on the retention, separation, and resolution of the enantiomers of DRV at 25 °C. As percentage of ethanol increased from 30% to 40% (vertical comparison), retention factors were decreased, enantioseparations did not change much, and resolutions were decreased. Similar trend was noticed in the case of 2-propanol. However, better enantioseparation and resolution were obtained on Chiralpak AD-H compared with Chiralpak IA column by using ethanol and 2-propanol as polar modifiers. Overall, better enantioseparation ($\alpha = 2.50$) and resolution ($Rs = 7.56$) were obtained using 30% of ethanol at

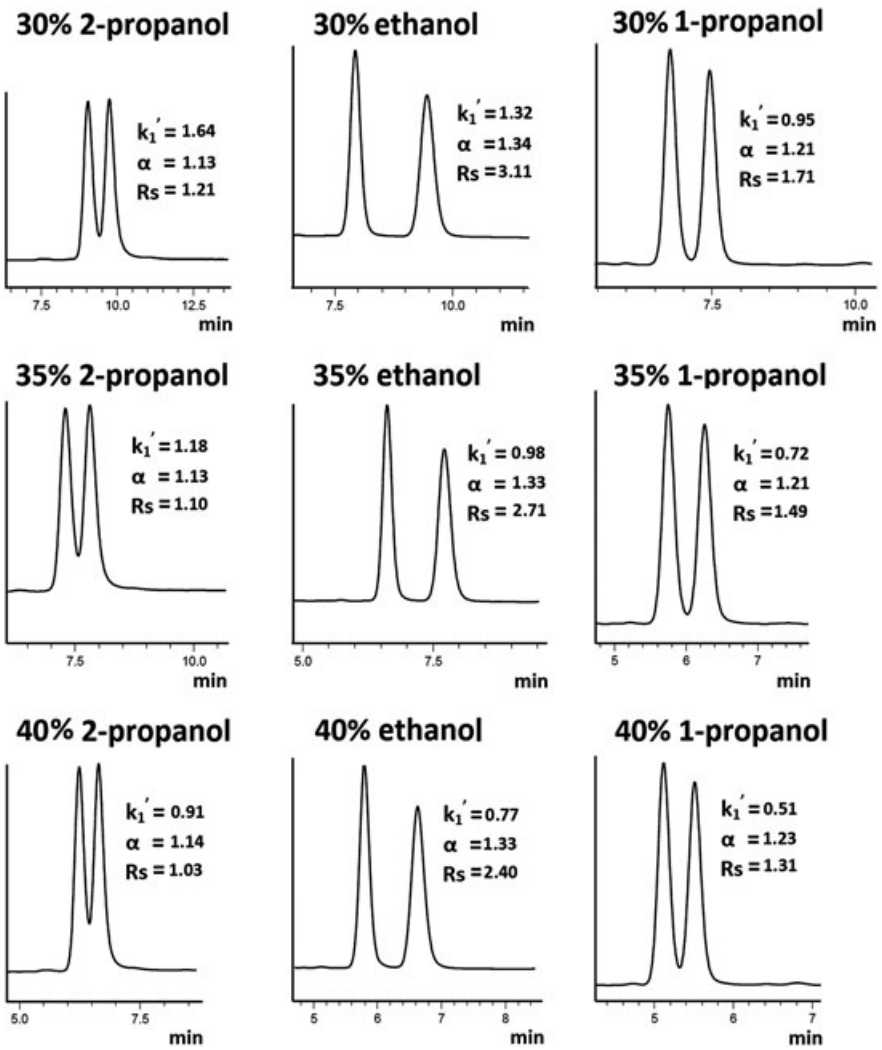

Fig. 3. Effect of strength (vertical comparison) and type of polar modifier (horizontal comparison) on retention, separation, and resolution of DRV (I) and (II) enantiomers on Chiralpak IA column at 25 °C.

TABLE 1. Effect of strength of 2-propanol, ethanol, 1-propanol, and column temperature on the retention, enantioseparation, and resolution of DRV by using Chiralpak IA column

		15%				20%				25%				30%			
Solvent		k_1'	k_2'	α_{12}	Rs_{12}	k_1'	k_2'	α_{12}	Rs_{12}	k_1'	k_2'	α_{12}	Rs_{12}	k_1'	k_2'	α_{12}	Rs_{12}
	T (°C)																
2-Propanol	20	–	–	–	–	4.32	4.91	1.14	1.50	2.60	2.99	1.15	1.38	1.75	2.00	1.14	1.27
	25	–	–	–	–	4.06	4.54	1.12	1.44	2.48	2.83	1.14	1.33	1.64	1.85	1.13	1.21
	30	10.09	11.10	1.10	1.73	3.76	4.29	1.14	1.62	2.32	2.59	1.12	1.44	1.54	1.69	1.10	1.27
	35	9.78	10.61	1.08	1.55	3.58	3.95	1.10	1.44	2.22	2.44	1.10	1.26	1.44	1.59	1.10	1.19
	40	9.25	9.97	1.08	1.50	3.38	3.67	1.08	1.35	2.11	2.29	1.09	1.22	1.38	1.48	1.07	1.07
Ethanol	20	–	–	–	–	2.75	3.78	1.95	3.89	1.99	2.76	1.39	3.16	1.38	1.91	1.38	2.73
	25	–	–	–	–	2.66	3.62	1.36	3.73	1.78	2.41	1.35	3.16	1.32	1.76	1.34	3.11
	30	–	–	–	–	2.53	3.05	1.32	3.79	1.68	2.22	1.32	3.35	1.17	1.54	1.32	2.84
	35	–	–	–	–	2.27	2.93	1.29	3.77	1.52	1.95	1.28	3.14	1.10	1.41	1.28	2.70
	40	–	–	–	–	2.09	2.63	1.26	3.53	–	–	–	–	1.02	1.22	1.19	2.23
1-Propanol	20	–	–	–	–	2.24	2.70	1.21	2.27	1.49	1.80	1.21	1.96	1.04	1.26	1.21	1.72
	25	3.59	4.33	1.21	2.80	2.10	2.54	1.21	2.38	1.36	1.65	1.22	1.99	0.95	1.15	1.21	1.71
	30	–	–	–	–	1.95	2.35	1.21	2.45	1.27	1.57	1.22	2.11	0.89	1.08	1.21	1.79
	35	3.18	3.84	1.21	3.09	1.82	2.20	1.21	2.53	1.20	1.44	1.21	2.13	0.83	1.00	1.21	1.79
	40	3.02	3.64	1.21	3.18	1.70	2.04	1.20	2.57	1.12	1.35	1.20	2.13	0.77	0.93	1.21	1.76

Condition: flow rate: 1 ml min^{-1}.
"–", experiment not performed.

25 °C on Chiralpak AD-H column. By using 30% of polar modifier as shown in Figure 4 (horizontal comparison), the retention factors were almost the same for both ethanol and 2-propanol. However, there was a large difference of enantioseparation and resolutions between ethanol and 2-propanol. In the case of 35% and 40% polar modifiers, similar trend was followed as in the case of 30% polar modifier. Overall, better enantioseparation and resolution were obtained using ethanol as a polar modifier.

Comparison of Chiralpak IA and AD-H columns. The effect of immobilization (Chiralpak IA column) on chiral recognition ability was noted when compared with the coated phase (Chiralpak AD-H column). The retention, separation, and resolution of the enantiomers of DRV on both the columns under the same conditions were compared. Even though both columns were composed of same molecules because of their type of composition, these columns showed differences in enantioseparation and resolutions in separation of DRV (I) and (II) enantiomers. Figure 5 shows the comparison of the separations on Chiralpak IA and AD-H columns by using hexane : ethanol : DEA (70:30:0.1, v/v/v) at different temperatures. There was a prominent difference between Chiralpak IA and AD-H columns in terms of retention, separation, and resolutions at all temperatures (25–40 °C) by using ethanol as a polar modifier. The separation factor (α) and resolution (Rs) were decreased while moving from Chiralpak AD-H to Chiralpak IA column. Figure 6 also shows the comparison of Chiralpak IA and AD-H columns for the separation of enantiomers of DRV by using hexane : 2-propanol : DEA (75:25:0.1, v/v/v) at different temperatures. However, there was a negligible difference in terms of retention, separation, and resolutions within the temperature range by using 2-propanol as a polar modifier.

Effect of Temperature

In chromatographic separation of enantiomers, the relation between chromatographic retention and temperature can be described by the Van't Hoff equation.[33–36]

$$\ln k' = -\Delta H^0/RT + \Delta S^0/R + \ln \Phi = -\Delta H^0/RT + \Delta S^* \quad (1)$$

$$\ln \alpha = -\Delta\Delta H^0/RT + \Delta\Delta S^0/R \quad (2)$$

where k' is the retention factor of the particular enantiomer, α is the separation factor for a given enantiomeric pair, ΔH^0 and ΔS^0 represent the differences in the enthalpy and entropy, respectively, when one enantiomer transfers from mobile to the stationary phase, R is universal gas constant, Φ is phase ratio, and ΔS^* is used to substitute for the expression $\Delta S^0/R + \ln \Phi$. The ΔS^* values were used mainly for comparison but not for determination of the ΔS values as the phase ratio was unknown. The $\Delta\Delta H^0$ and $\Delta\Delta S^0$ represent the differences of ΔH^0 and ΔS^0 for a given pair of enantiomers, respectively. If the plots of ln k' or ln α against 1/T are linear in a temperature range, the corresponding thermodynamic parameters ΔH^0 and ΔS^0, which are temperature-independent, can be deduced from the slope ($\Delta H^0 = -\text{slope} \times R$) and the intercept ($\Delta S^* = \text{intercept}$ R) of the straight lines. The linear character also suggests that the conformation of the CSP does not change substantially within the range of experimental temperatures. The thermodynamic parameters derived by this method are apparent, not intrinsic,[18] and comparison of these thermodynamic parameters ΔH^0, ΔS^0, $\Delta\Delta H^0$, and $\Delta\Delta S^0$ may give some understanding of chiral recognition mechanism.

In this study, 2-propanol and 1-propanol systems gave linear Van't Hoff plots indicating that the conformation of the CSP did not change in the studied temperature range, whereas ethanol system unusually did not give linear Van't Hoff plots[35] on Chiralpak IA column indicating that the change in conformation of the CSP in the studied temperature range. Table 1 shows the effect of column temperature on the retention and separation of DRV (I) and (II) enantiomers by using 2-propanol and 1-propanol as polar modifiers on Chiralpak IA column. In the case of 2-propanol, the k' values were decreased as the column temperature was increased for all percentages of 2-propanol. Figure 7(a) shows the Van't Hoff plots of ln k_1' vs. 1/T and ln k_2' vs. 1/T were highly linear in the temperature

TABLE 1. Effect of strength of 2-propanol, ethanol, 1-propanol, and column temperature on the retention, enantioseparation, and resolution of DRV by using Chiralpak IA column

35%				40%				45%				50%			
k_1'	k_2'	α_{12}	Rs_{12}	k_1'	k_2'	α_{12}	Rs_{12}	k_1'	k_2'	α_{12}	Rs_{12}	k_1'	k_2'	α_{12}	Rs_{12}
1.28	1.46	1.15	1.17	0.98	1.13	1.15	1.09	0.82	0.94	1.15	1.02	–	–	–	–
1.18	1.34	1.13	1.10	0.91	1.03	1.14	1.03	0.75	0.86	1.14	0.95	–	–	–	–
1.12	1.22	1.09	1.13	0.84	0.94	1.12	0.96	0.68	0.76	1.12	0.85	–	–	–	–
1.02	1.14	1.11	1.06	0.77	0.86	1.11	0.86	0.61	0.68	1.11	0.75	–	–	–	–
0.97	1.06	1.09	0.89	0.72	0.80	1.10	0.77	–	–	–	–	–	–	–	–
1.03	1.41	1.38	2.40	0.80	1.09	1.37	2.14	0.64	0.87	1.36	1.92	0.68	0.88	1.30	1.73
0.98	1.31	1.33	2.71	0.77	1.02	1.33	2.40	0.62	0.83	1.33	2.13	0.70	0.88	1.27	1.92
0.87	1.14	1.31	2.43	0.68	0.88	1.31	2.11	0.55	0.71	1.30	1.84	0.59	0.74	1.25	1.63
0.80	1.01	1.27	2.22	0.64	0.81	1.27	1.97	0.51	0.64	1.26	1.65	0.58	0.70	1.21	1.49
0.75	0.90	1.20	1.79	0.58	0.70	1.20	1.52	0.47	0.57	1.20	1.32	0.52	0.61	1.17	1.18
0.77	0.94	1.22	1.51	0.54	0.67	1.23	1.30	–	–	–	–	–	–	–	–
0.72	0.87	1.21	1.49	0.51	0.63	1.23	1.31	–	–	–	–	–	–	–	–
0.65	0.80	1.22	1.53	0.48	0.58	1.23	1.31	–	–	–	–	–	–	–	–
0.60	0.73	1.22	1.52	0.44	0.54	1.23	1.30	–	–	–	–	–	–	–	–
0.56	0.68	1.21	1.48	0.41	0.50	1.23	1.27	–	–	–	–	–	–	–	–

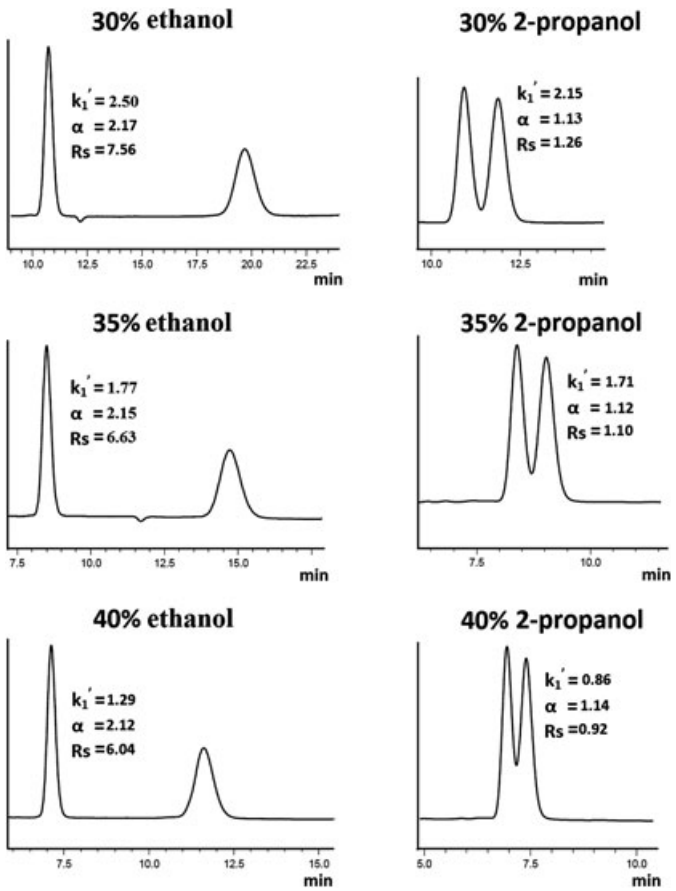

Fig. 4. Effect of strength (vertical comparison) and type of polar modifier (horizontal comparison) on retention, separation and resolution DRV (I) and (II) enantiomers on Chiralpak AD-H column 25 °C.

range of 20–40 °C, indicating that the conformation of the CSP did not change in the studied temperature range. The corresponding thermodynamic parameters ΔH^0 and ΔS^0 were calculated from these plots of ln k_1' vs. 1/T and ln k_2' vs. 1/T, respectively (Table 2). As shown in Table 2, as the percentage of 2-propanol increases, the ΔH_1^0 and ΔH_2^0 values of DRV decreased (−9.372 to −8.150 kJmol^{-1} and −11.029 to −10.328 kJmol^{-1}) moving from 20% to 25% and increased from 25% to 40% (−8.150 to −11.723 kJmol^{-1} and −10.328 to −13.465 kJmol^{-1}), reflecting that the interactions between

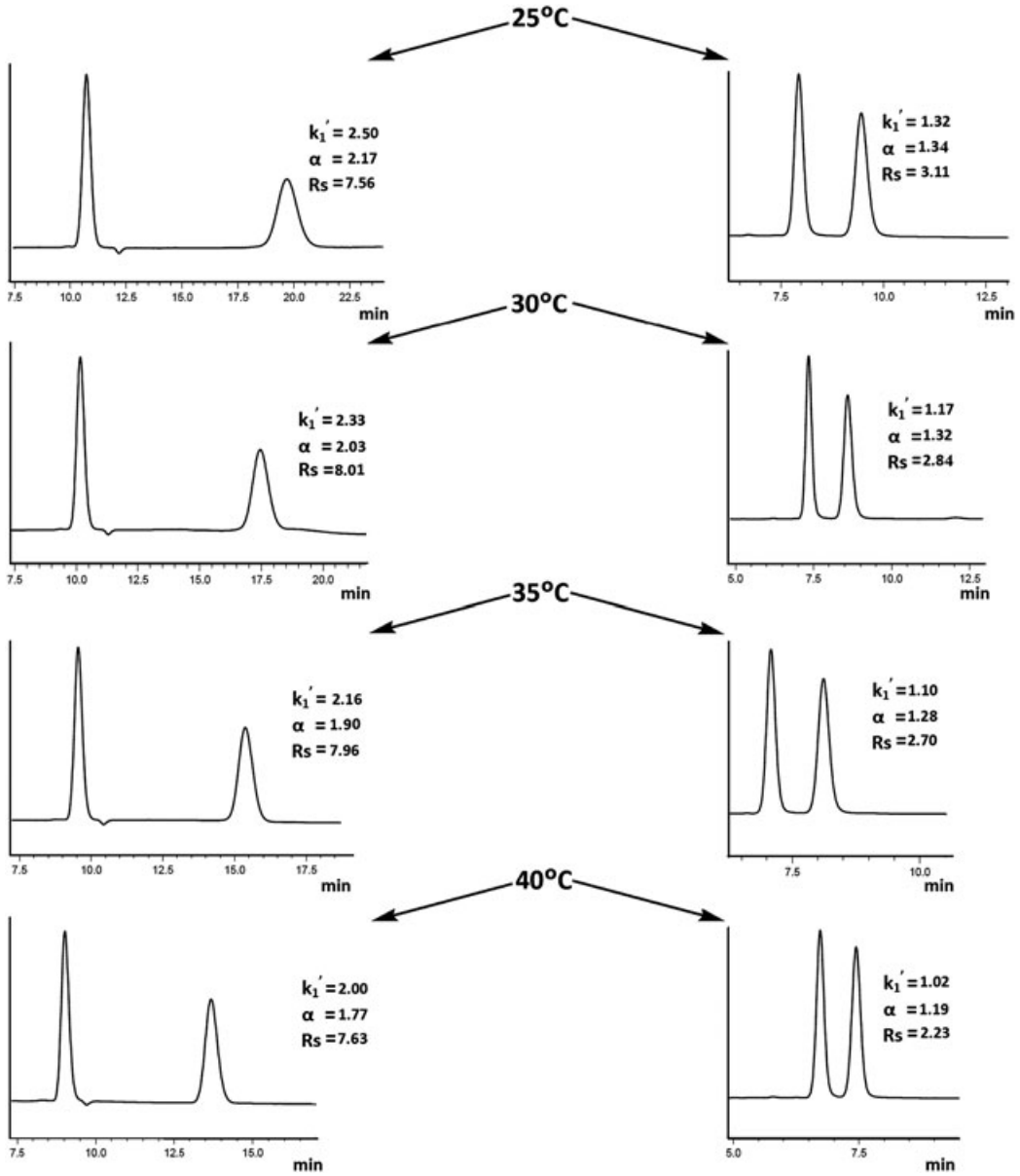

Fig. 5. Comparison of Chiralpak AD-H (left) and Chiralpak IA (right) columns for the separation of DRV (I) and (II) enantiomers at different temperatures. Mobile phase composition: hexane : ethanol : DEA (70:30:0.1, v/v/v).

enantiomers of DRV and the CSP decrease from 20% to 25% and increase from 25% to 40%. The $\Delta\Delta H^0$ and $\Delta\Delta S^0$ values were negative and suggest that the enantioseparation was enthalpy driven.

In the case of 1-propanol as shown in Table 1, the k' values were decreased as the column temperature increased for all strengths of 1-propanol. Figure 7(b) shows the Van't Hoff plots of ln k_1' vs. 1/T and ln k_2' vs. 1/T were highly linear in the temperature range of 20–40 °C, indicating that the conformation of the CSP did not change in the studied temperature range. The corresponding thermodynamic parameters ΔH^0 and ΔS^0 were calculated from these plots of ln k_1' vs. 1/T and ln k_2' vs. 1/T, respectively (Table 2). As shown in Table 2, as percentage of 1-propanol increases, the ΔH_1^0 and ΔH_2^0 values of DRV increased (-10.524 to $-12.203\,\mathrm{kJmol^{-1}}$ and -10.750 to $-12.346\,\mathrm{kJmol^{-1}}$), moving from 20% to 35%, and decreased from 35% to 40% (-12.203 to $-10.981\,\mathrm{kJmol^{-1}}$ and -12.346 to $-11.065\,\mathrm{kJmol^{-1}}$), reflecting that the interactions between enantiomers of DRV and the CSP increase from 20% to 35% and decrease from 35% to 40%.

CONCLUSIONS

Effect of the type and strength of organic modifiers on retention and enantioseparation of DRV enantiomers on coated and immobilized amylose tris(3,5-dimethylphenylcarbamate) CSP was studied at different column temperatures. The resolutions achieved on both the columns were greater than 1.5 in most of the experiments. The differences between the coated and immobilized type of the chiral columns were discussed in terms of efficiency and applicability. The effect of temperature was studied, and the apparent thermodynamic values derived from the Van't Hoff plots (ln k' vs. 1/T, ln α vs. 1/T) were used to explain some aspects about chiral recognition mechanism. The plots of ln k' against 1/T were highly linear, and the negative values of all the deduced

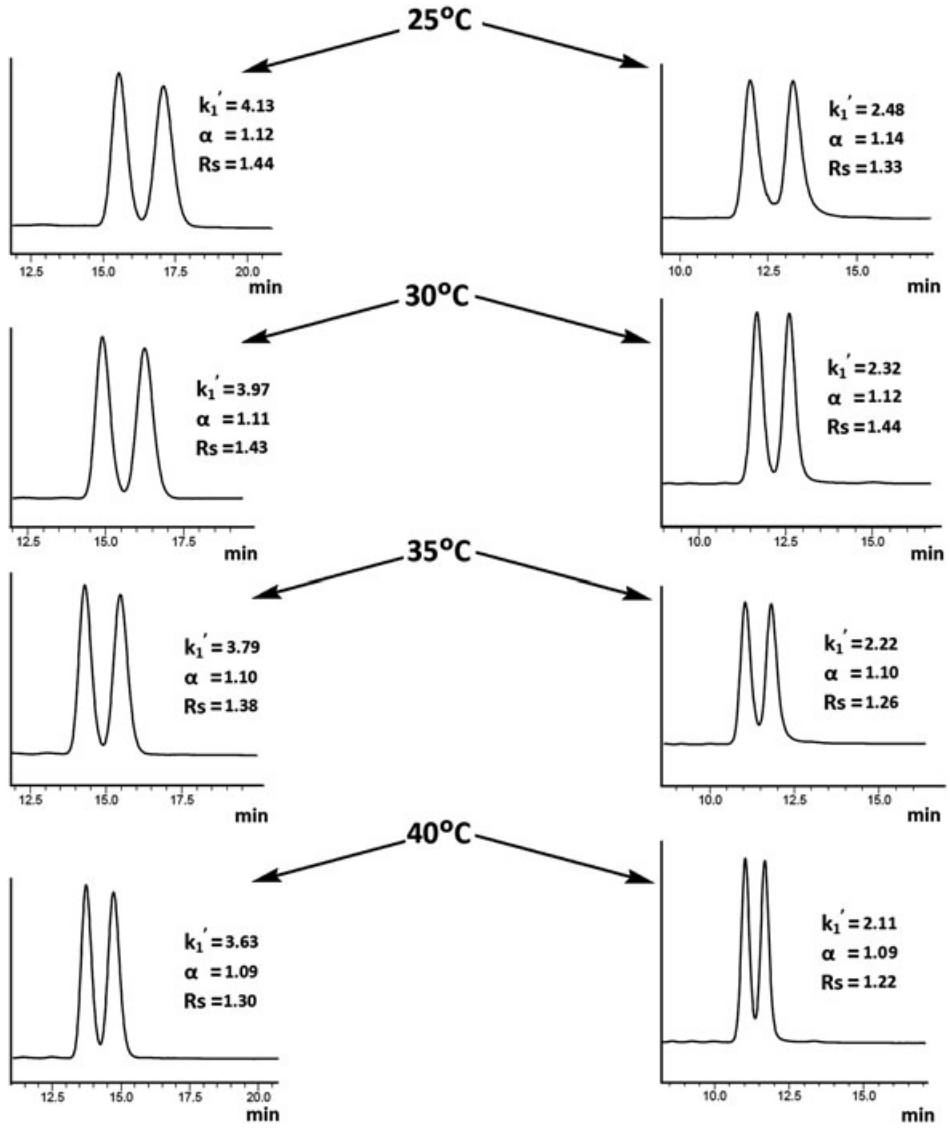

Fig. 6. Comparison of Chiralpak AD-H (left) and Chiralpak IA (right) columns for the separation of DRV (I) and (II) enantiomers at different temperatures. Mobile phase composition: hexane : 2-propanol : DEA (75:25:0.1, v/v/v).

TABLE 2. Effects of content of 2-propanol and 1-propanol on thermodynamic parameters by using Chiralpak IA column

Solvent	Volume (%)	ΔH_1^0 (kJmol^{-1})	ΔS_1*	R_1^2	ΔH_2^0 (kJmol^{-1})	ΔS_2*	R_2^2	$\Delta\Delta H^0$ (kJmol^{-1})	$\Delta\Delta S^0$ (kJ K^1 mol^{-1})	$R_{1,2}^2$
2-Propanol	20	−9.372	−0.020	0.9966	−11.029	−0.024	0.9966	−1.658	−0.005	<0.995
	25	−8.150	−0.020	0.9958	−10.328	−0.026	0.9953	−2.178	−0.006	<0.995
	30	−9.181	−0.027	0.9969	−11.521	−0.034	0.9983	−2.340	−0.007	<0.995
	35	−10.502	−0.034	0.9960	−12.214	−0.039	0.9953	−1.712	−0.005	<0.995
	40	−11.723	−0.040	0.9989	−13.465	−0.045	0.9989	−1.742	−0.005	0.9969
1-Propanol	20	−10.524	−0.029	0.9986	−10.750	−0.028	0.9983	−0.226	0.0008	<0.995
	25	−10.643	−0.033	0.9954	−10.980	−0.033	0.9958	−0.338	0.0005	<0.995
	30	−11.140	−0.038	0.9974	−11.396	−0.037	0.9972	−0.256	0.0007	<0.995
	35	−12.203	−0.044	0.9973	−12.346	−0.043	0.9995	−0.143	0.0011	<0.995
	40	−10.981	−0.042	0.9963	−11.065	−0.041	0.9965	−0.084	0.0014	<0.995

thermodynamic parameters in the temperature range of 20–40 °C in 1-propanol, 2-propanol systems on Chiralpak IA column suggest that the conformation of the CSP did not change in the studied temperature range. The optimized conditions in all polar modifier systems may be of use in the development of LC/APCI/MS/MS and LC/APPI/MS/MS methods to determine DRV (I) and (II) enantiomers not only in pharmaceutical formulations but also in biological matrices.

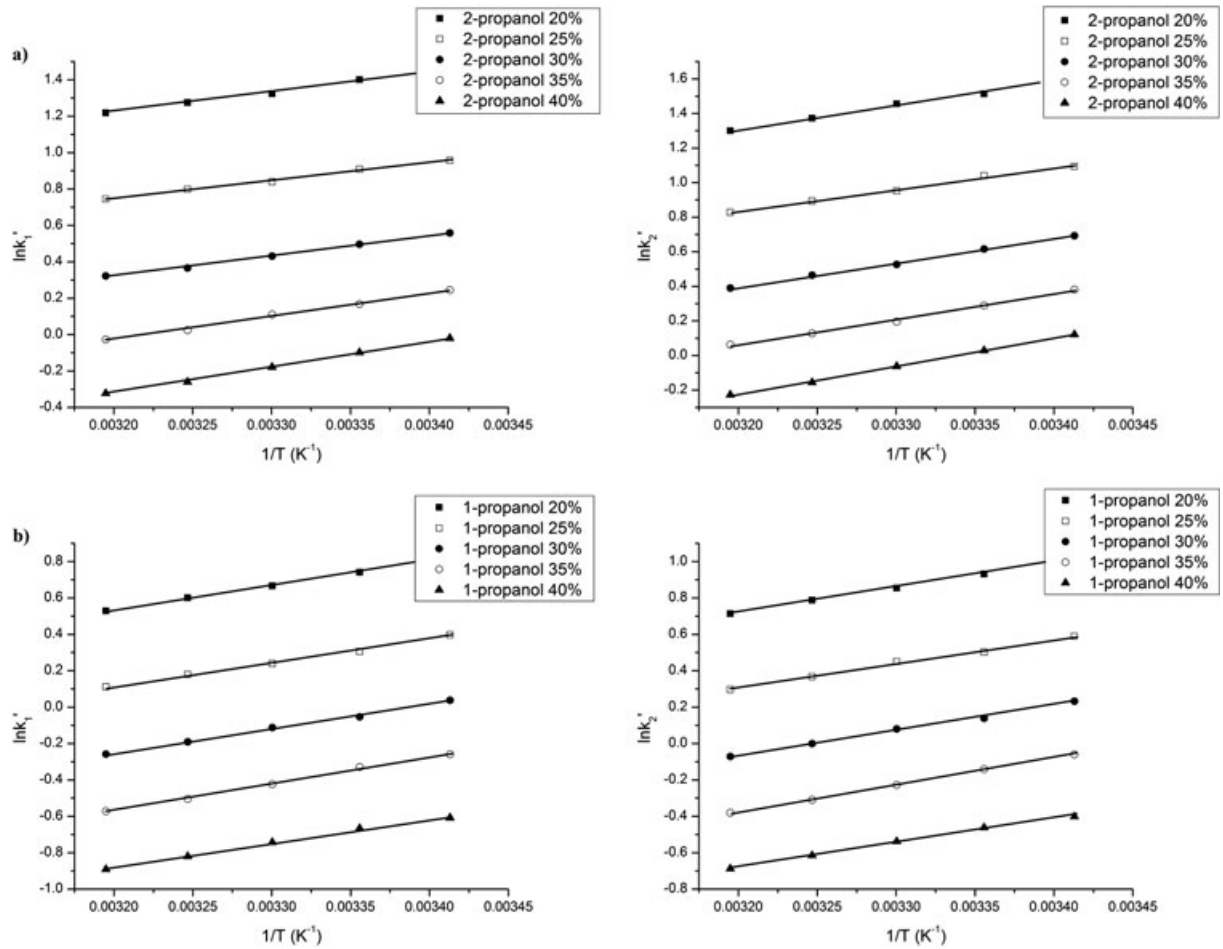

Fig. 7. Typical Van't Hoff plots for the separation of DRV (I) and (II) enantiomers on Chiralpak IA column (**a**) 2-propanol system and (**b**) 1-propanol system.

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Research Article

Liquid chromatographic separation and thermodynamic investigation of stereoisomers of darunavir on Chiralpak AD-H column

Liquid chromatographic separation of stereoisomers of darunavir on Chiralpak AD-H, a column containing the stationary phase coated with amylose tris(3,5-dimethylphenylcarbamate) as a chiral selector, was studied under normal-phase conditions at different temperatures between 20 and 50°C. The effect of quality and quantity of different polar organic modifiers viz: methanol, ethanol, 1-propanol, and 2-propanol in the mobile phase as well as column temperature on retention, separation, and resolution was investigated and optimized. The optimum separation was accomplished using a mobile phase composed of *n*-hexane/ethanol/diethyl amine (80:20:0.1 v/v/v) at 40°C. Apparent thermodynamic parameters ΔH^0 and ΔS^* were derived from the Van't Hoff plots ($\ln k'$ versus $1/T$) and used to explain the strength of interactions between the stereoisomers and amylose tris(3,5-dimethylphenylcarbamate) coated chiral stationary phase.

Keywords: Antiretrovirals / Chiralpak AD-H / Darunavir / Stereoisomers / Van't Hoff plots
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1 Introduction

Nowadays, most of the drugs available in market are chiral and 25% of them are stereoisomers with enhanced bioactivity [1]. Generally, these are produced by synthetic procedures as mixtures containing two or more stereoisomers depending upon the asymmetric centers within the molecule. Their biological activities also differ dramatically since the physiological environment within living organisms is chiral [2]. Evaluation of their purity is of great importance not only for assurance of quality but also safety by manufacturers and regulatory authorities. For these reasons, there is a strong demand for rapid, selective, and sensitive analytical methods that facilitate separation of chiral drugs. Chromatography with chiral stationary phases (CSPs) has been the most frequently used technique as it is rapid, effective, reproducible, and preparative in nature. Chiral liquid chromatography has become a major method for stereomeric separation as well as purification of enantiomeric drugs. Several methods including LC [3, 4] and LC-MS [5, 6] were reported for separation of stereoisomers. Cellulose and amylose derivatives are the most abundant optically active biopolymers with perfectly defined structures, which can resolve enantiomers including amino acid derivatives [7]. Chiralpak AD-H is one of the most popular and useful polysaccharide-based column containing a stationary phase coated with tris(3,5-dimethylphenylcarbamate) derivative of amylase for stereoisomeric separation of chiral drugs [8–10]. An understanding of the chiral recognition mechanism on these columns help us in designing of more effective CSPs. Thermodynamic investigations [11–13] may be of some use to evaluate the effect of column temperature on chiral discrimination and deduce some apparent thermodynamic parameters corresponding to chromatographic retention data to give meaningful understanding of the mechanistic aspects of stereo isomeric separations.

Darunavir (–)– [(3R,3aS,6aR)-hexahydrofuro[2,3-b]furan-3-yl(2S, 3R)-4-(4-amino-*N*-isobutylphenylsulfonamido)-3-hydroxy-1-phenylbutan-2-ylcarbamate] (DRV) (I) is a new HIV-1 protease inhibitor effective against viral strains that are not sensitive to other antiretrovirals [14]. It is one of the key components of many salvage therapies in multidrug-experienced patients [15]. Chemically, it has five stereogenic centers resulting in the possibility of 32 stereoisomers. During the synthesis of DRV, four of the stereoisomers were obtained as impurities affecting the quality of the drug. The remaining stereoisomers were not formed due to the selectivity of the synthetic scheme adopted. The present work was focused on separating these four stereoisomers from DRV (I) rather than synthesizing all the 32 stereoisomers, which is tedious requiring different synthetic protocols. However, in a continuous process of producing other stereoisomers, their separation may be reported in a

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Abbreviations: **CSP**, chiral stationary phase; **DEA**, diethyl amine; **DRV**, darunavir

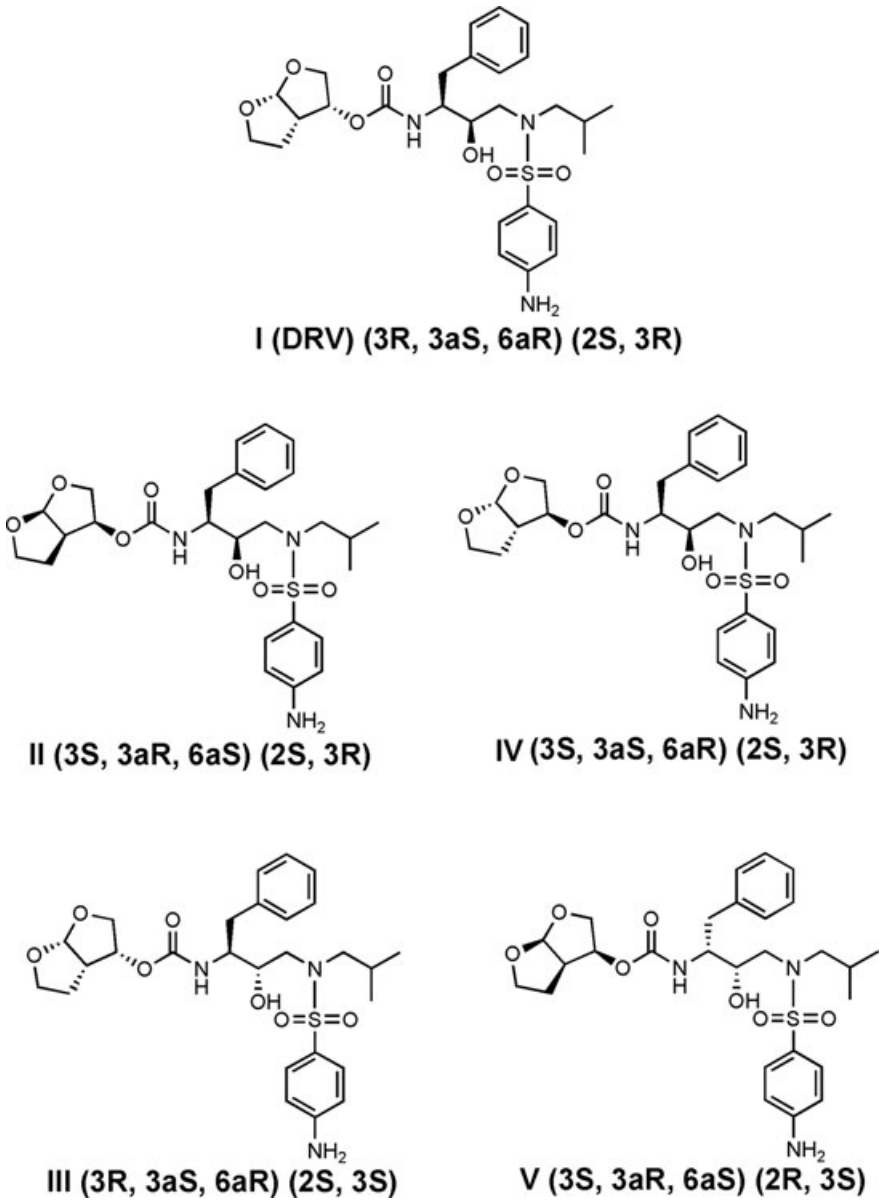

Figure 1. Chemical structures of I (DRV) and its stereoisomers II, III, IV, and V.

future work. At present, the four stereoisomers obtained as impurities of DRV (I) are (3S,3aR,6aS)-hexahydrofuro[2,3-b] furan-3-yl(2S,3R)-4-(4-amino-*N*-isobutylphenylsulfonamido)-3-hydroxy-1-phenylbutan-2-ylcarbamate (II), (3R,3aS,6aR)-hexahydrofuro[2,3-b]furan-3-yl(2S,3S)-4-(4-amino-*N*-isobutylphenylsulfonamido)-3-hydroxy-1-phenylbutan-2-ylcarbamate (III), (3S,3aS,6aR)-hexahydrofuro[2,3-b]furan-3-yl(2S,3R)-4-(4-amino-*N*-isobutylphenylsulfonamido)-3-hydroxy-1-phenylbutan-2-ylcarbamate (IV), and (3S,3aR,6aS)-hexahydrofuro[2,3-b]furan-3-yl(2R,3S)-4-(4-amino-*N*-isobutylphenylsulfonamido)-3-hydroxy-1-phenylbutan-2-ylcarbamate (V) (Fig. 1). The biological activity of DRV (I) is more compared to all other stereoisomers. Therefore, stereoselective separation of DRV and its stereoisomers is very important to assure the therapeutic efficacy and safety of its pharmaceutical preparations. Several LC, LC-MS, LC/MS/MS methods were reported for separation of DRV from other antiretroviral drugs in human plasma. LC with UV [14] and MS [15] detection for simultaneous determination of DRV and other antiretroviral agents on dried blood spots [16] and in plasma of HIV-infected patients [17] was reported. LC/MS/MS [18–22] was used not only for quantification of DRV in human plasma but also on dried blood spots [23] in presence of protease inhibitors and non-nucleoside reverse transcriptase inhibitors. Dailly and colleagues reported a column-switching LC/MS/MS method for determination of the plasma concentration of DRV along with other ten antiretroviral agents [24]. Previously, the authors' compared separation of darunavir and its enantiomer (V) on two different viz., Chiralpak AD-H and Chiralpak IA columns containing stationary phases (i) coated and (ii) immobilized with amylose tris(3,5-dimethylphenylcarbamate),

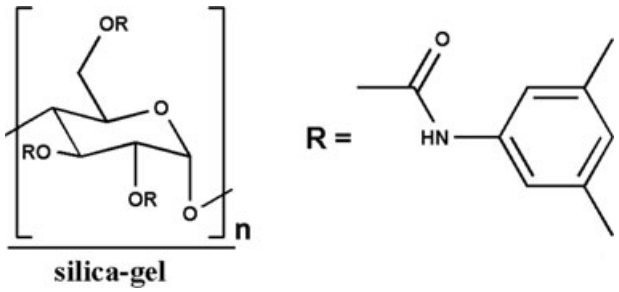

Figure 2. Chemical structure of tris(3,5-dimethylphenylcarbamate) of amylose coated on silica gel.

respectively [25]. However, to the best of authors' knowledge, there are no reports on separation of DRV from its stereoisomers till today.

The present manuscript describes the separation of DRV and four of its stereoisomers on a CSP coated with amylose tris(3,5-dimethylphenylcarbamate) as a chiral selector (Fig. 2). The chromatographic retention, separation, and resolution were investigated by varying the percentage of ethanol at different column temperatures. The data were used to derive apparent thermodynamic parameters, which in turn explained some of the mechanistic aspects of interactions of stereisomers with CSP.

2 Materials and methods

2.1 Chemicals

All solvents used were of HPLC grade (E. Merck, Mumbai, India). Analytical reagent grade diethyl amine (DEA) (Spectrochem, Mumbai, India) was used. DRV and its four stereoisomers II, III, IV, and V produced by a local pharmaceutical unit were obtained as gift samples.

2.2 Chromatography

The HPLC system consisting of two LC-20 AD pumps, photodiode array detector, an SIL-20 AC auto sampler, a DGU-20 A_5 degasser, and CBM-20A controller (Shimadzu, Kyoto, Japan) was used. The chromatographic and the integrated data were recorded using HP-Vectra (Hewlett Packard, Waldron, Germany) computer system using LC-solution data-acquiring software (Shimadzu). The stereoisomers of DRV were separated on Chiralpak AD-H column (25 cm × 4.6 mm I.D., 5-μm particle size) (Daicel, Tokyo, Japan). The prepared mobile phases were filtered through a 0.45-μm membrane filter paper and degassed with an ultrasonic bath before use. The analytes were dissolved in 2-propanol at approximately 0.1 mg mL^{-1} and filtered through a 0.45-μm membrane filter paper prior to injection. The flow rate was set at 1.0 mL min^{-1}. The UV detection wavelength was set at 266 nm. The injection volume was 20 μL. Retention factors (k') were calculated from the formula $(t_R - t_0)/t_0$, where t_R is the retention time of a particular stereoisomer and t_0 is the retention time of an unretained peak. Separation factors (α) for pair of stereoisomers of DRV were calculated from k_2'/k_1', where k_1' and k_2' are the retention factors for the first and second eluted stereoisomers,respectively.

2.3 Validation

Linearity of detector response was checked by preparing calibration standards of DRV and its stereoisomers at a concentration range of their LOQ to 50 μg/mL using 2-propanol as a diluent. Linear regression curves were obtained by plotting peak area versus concentration. Accuracy of the method was evaluated by spiking the four stereoisomers to DRV at 5, 10, and 50 μg/mL and analyzing each solution in six replicates. The precision was calculated using three standard solutions of stereoisomers of DRV individually at 5, 10, and 50 μg/mL and analyzing each solution in six replicates. LOD and LOQ were determined at a signal-to-noise ratio of 3 and 10, respectively.

3 Results and discussion

3.1 Method development

It could be seen from Fig. 1 that II, III, IV, and V are stereoisomers of DRV (I). Among the four stereoisomers, V is an enantiomer of DRV (I). In an earlier study, the separation of DRV (I) and its enantiomer (V) was investigated on Chiralpak IA and AD-H columns [25]. In order to extend the study to stereoisomers II, III, and IV, the separation was initially tried on Chiralpak IA, IC, and AD-H columns under normal-phase conditions. Preliminary experiments gave better results on coated Chiralpak AD-H column while immobilized Chiralpak IA and IC columns were partially successful in resolving the stereoisomers. Thus, Chiralpak AD-H column was selected for further investigation. Mobile phases containing different proportions of alcohols i.e. 2-propanol, 1-propanol, methanol, and ethanol were tried and it was found that the peaks were not resolved completely with all the alcohols except ethanol. Thus, a thorough investigation on the effects of ethanol (%) in mobile phase and column temperature on separation of stereoisomers was carried out.

3.1.1 Effect of ethanol (%) in mobile phase

Mobile phases comprising of *n*-hexane and 0.1% DEA with different proportions ethanol were tried to study its effect on separation of DRV and its stereoisomers on Chiralpak AD-H column. On varying the ethanol (%) in mobile phase a significant effect on retention (t_R), separation (α) and resolution (R_s) of the stereoisomers of DRV was observed. Typical chromatograms obtained with 20, 25, and 30% ethanol are shown in Fig. 3. As shown in Fig. 3, the retention times (t_R)

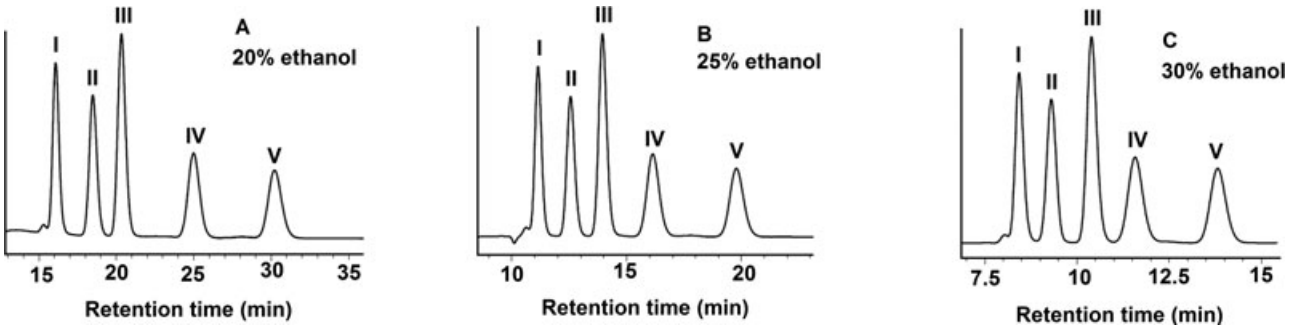

Figure 3. Effect of ethanol (%) on retention (t_R), separation (α), and resolution (R_s) of DRV and its stereoisomers on Chiralpak AD-H at 40°C. (A) *n*-hexane/ethanol/diethyl amine (80:20:0.1 v/v/v). (B) *n*-hexane/ethanol/diethyl amine (75:25:0.1 v/v/v). (C) *n*-hexane/ethanol/diethyl amine (70:30:0.1 v/v/v).

for all the four stereoisomers of DRV were decreased as the percentage of ethanol increased from 20 to 30%. It may be due to the decrease of H-bonding interactions between the stereoisomers and CSP with increased alcohol content in the mobile phase leading to decreased retentions. Separation factors (α) did not change much and resolutions (R_s) were decreased as the percentage of ethanol increased from 20 to 30%. Table 1 gives the retention (t_R), separation (α), and resolution (R_s) factors obtained with different proportions of ethanol in mobile phase at temperatures between 20 and 50°C.

3.1.2 Effect of column temperature

Figure 4 shows the effect of the column temperature on the retention, separation, and resolution of the stereoisomers of DRV using an optimum composition of a mobile phase containing 20% ethanol. The study was conducted in the temperature range of 20–50°C. Within this range, seven experiments at an interval of 5°C were conducted. The lower limit was set as –10°C to the ambient temperature of the investigated laboratory. As expected, the retention times for all the compounds were decreased as the temperature of the column increased from 20 to 50°C. On further decreasing the temperature below 20°C, it was observed that the retention times were too long with decreased resolution. As shown in Table 1, The retention factors for all stereoisomers of DRV were decreased, separation factors did not change much, and resolutions were increased (an opposite trend to percentage of ethanol) as the temperature of the column was increased from 20 to 50°C at all percentages of ethanol. Overall, reasonable retention, better separation, and resolutions were obtained at 40°C using a mobile phase consisting of *n*-hexane/ethanol/DEA (80:20:0.1 v/v/v).

Table 1. Effect of ethanol (%) and column temperature on capacity factor (k'), separation factor (α), and resolution (R_s) of DRV and its stereoisomers

Ethanol (%) (v/v)	Column temperature (°C)	Retention parameters												
		Capacity factor (k')					Separation factor (α)				Resolution (R_s)			
		k_I'	k_{II}'	k_{III}'	k_{IV}'	k_V'	$\alpha_{I,II}$	$\alpha_{II,III}$	$\alpha_{III,IV}$	$\alpha_{IV,V}$	$Rs_{I,II}$	$Rs_{II,III}$	$Rs_{III,IV}$	$Rs_{IV,V}$
20	20	4.89	6.08	7.63	9.35	11.82	1.24	1.25	1.23	1.26	2.47	2.92	2.32	2.46
	25	4.40	5.36	6.58	8.31	10.55	1.22	1.23	1.26	1.27	2.56	2.85	2.65	2.61
	30	4.10	4.92	5.89	7.35	9.20	1.20	1.20	1.25	1.25	2.65	2.77	2.94	2.88
	35	3.81	4.51	5.29	6.51	8.00	1.18	1.17	1.23	1.23	2.60	2.59	3.10	3.01
	40	3.25	3.88	4.37	5.42	6.98	1.19	1.12	1.24	1.29	2.83	1.96	3.38	3.91
	45	3.01	3.38	3.89	5.13	6.42	1.12	1.15	1.32	1.25	3.01	1.46	3.57	4.26
	50	2.76	3.10	3.40	4.63	5.68	1.12	1.09	1.36	1.23	3.24	1.24	3.84	4.46
25	25	2.54	3.19	3.88	4.90	ND	1.26	1.22	1.26	ND	2.28	2.18	2.02	ND
	30	2.62	3.12	3.83	4.69	5.86	1.19	1.23	1.22	1.25	2.11	2.60	2.21	2.41
	35	2.33	2.79	3.29	4.10	5.30	1.20	1.18	1.24	1.29	2.44	2.38	2.72	3.08
	40	2.07	2.46	2.84	3.44	4.45	1.19	1.16	1.21	1.29	2.36	2.10	2.60	3.39
30	25	1.69	2.12	2.64	3.25	4.55	1.25	1.25	1.23	1.40	1.88	2.08	1.55	2.18
	30	1.69	2.12	2.64	3.25	4.55	1.25	1.25	1.23	1.40	1.88	2.08	1.55	2.18
	35	1.48	1.78	2.14	2.56	3.37	1.20	1.20	1.19	1.32	1.96	2.18	1.84	2.80
	40	1.40	1.64	1.95	2.29	2.93	1.18	1.19	1.17	1.28	1.91	2.17	1.86	2.87

ND, not determined.

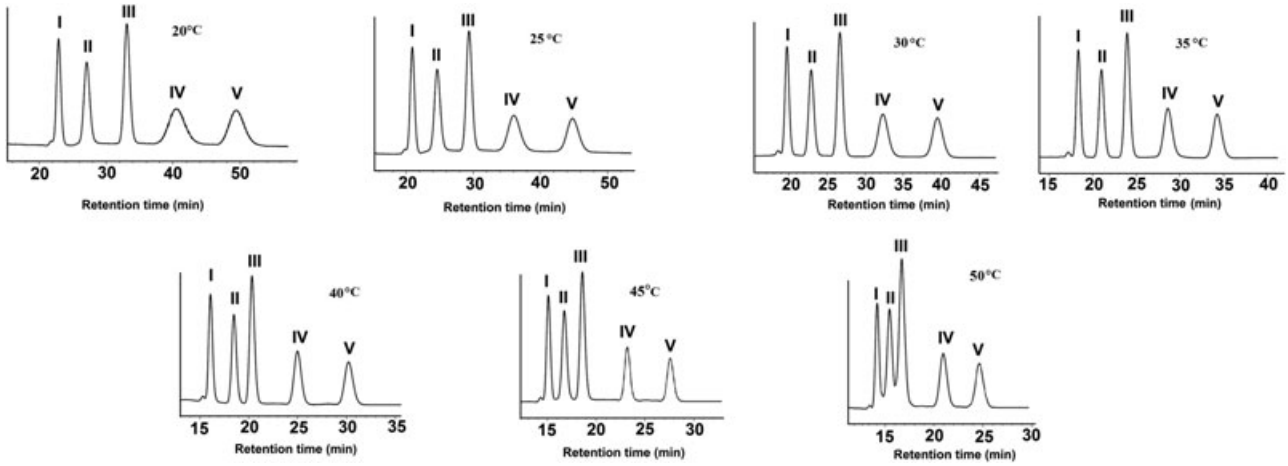

Figure 4. Effect of column temperature on retention (t_R), separation (α), and resolution (R_s) of DRV and its stereoisomers on Chiralpak AD-H using *n*-hexane/ethanol/diethyl amine (80:20:0.1 v/v/v) as optimum mobile-phase composition.

3.1.3 Evaluation of thermodynamic parameter

In a stereoisomeric separation, the relation between chromatographic retention and column temperature can be described by the van't Hoff equation [26–28].

$$\ln\ k' = -\Delta H^0/RT + \Delta S^0/R + \ln\Phi$$
$$\ln\ k' = -\Delta H^0/RT + \Delta S^* \quad (1)$$

$$\ln\ \alpha = -\Delta\Delta H^0/RT + \Delta\Delta S^0/R \quad (2)$$

where, k' is retention factor of a particular stereoisomer, α is separation factor for a given stereomeric pair. ΔH^0 and ΔS^0 represent the differences in the enthalpy and entropy, respectively, when one stereoisomer transfers from mobile phase to the stationary phase. R is universal gas constant. Φ is phase ratio. ΔS^* is used to substitute for the expression $\Delta S^0/R + \ln\Phi$. The ΔS^* values were used mainly for comparison, but not for determination of ΔS values as the phase ratio is unknown. The $\Delta\Delta H^0$ and $\Delta\Delta S^0$ represent the differences of ΔH^0 and ΔS^0 for a given pair of stereoisomers, respectively. If the plots of lnk' or ln α against $1/T$ are linear in a temperature range, the corresponding thermodynamic parameters ΔH^0 and ΔS^0, which are temperature-independent, can be deduced from the slope ($\Delta H^0 = -$ slope $\times$ R) and the intercept ($\Delta S^* =$ intercept $\times$ R) of the straight lines. The linear character also suggests that the conformation of the CSP does not change substantially within the range of experimental temperatures. The thermodynamic parameters derived by this method are not intrinsic in nature but apparent and reflect the combined effects of nonselective and enantioselective retention mechanisms [29–31]. The comparison of these thermodynamic parameters ΔH^0, ΔS^0, $\Delta\Delta H^0$, and $\Delta\Delta S^0$ may give some understanding of chiral recognition mechanism.

Figure 5 shows the van't Hoff plots of lnk' versus $1/T$ for DRV (I) and its four stereoisomers (II, III, IV, and V). It could be seen from Fig. 5 that the plots are highly linear in the temperature range of 20–50°C indicating that the conformation of the CSP did not change in the investigated range of temperatures. Table 2 gives the estimated coefficients ΔH^0 and ΔS^* along with their standard errors and regression coefficients (r^2). The corresponding thermodynamic parameters calculated from these plots were used to explain the strengths of interactions between the stereoisomers and CSP. Because of fluctuation of separation factors (α) under these conditions, the $\Delta\Delta H^0$ and $\Delta\Delta S^*$ were determined directly from the differences of $\Delta H_2{}^0$ and $\Delta H_1{}^0$, $\Delta S_2{}^*$ and $\Delta S_1{}^*$. As shown in Table 2, a very low value of absolute ΔH^0 (–14.989) was obtained for stereoisomer I. It indicates lesser heat of adsorption reflecting lesser interactions with CSP. In case of stereoisomer III, a high value of absolute ΔH^0 (–21.394) indicates larger heats of adsorption reflecting stronger interactions with CSP. The absolute ΔH^0 values obtained for DRV and its stereoisomers were increased in the order of I (–14.989) < II (–17.976) < IV (–18.785) < V (–19.499) < III (–21.394) indicating that the strength of interactions between the stereoisomer and CSP increase in the order of I < II < IV < V < III. The $\Delta\Delta H^0$ and $\Delta\Delta S^*$ values for all the stereoisomers were also negative (Table 2) suggesting that the separation was driven by enthalpy.

3.2 Method validation

The linearity of detector response was checked by preparing calibration standards of DRV (I) and its four stereoisomers (II, III, IV, and V) at concentration ranges from their LOQ to 50 μg/mL. The obtained regression coefficients were greater than 0.995 showing a good linearity between the concentration and detector response. LOD and LOQ were determined at

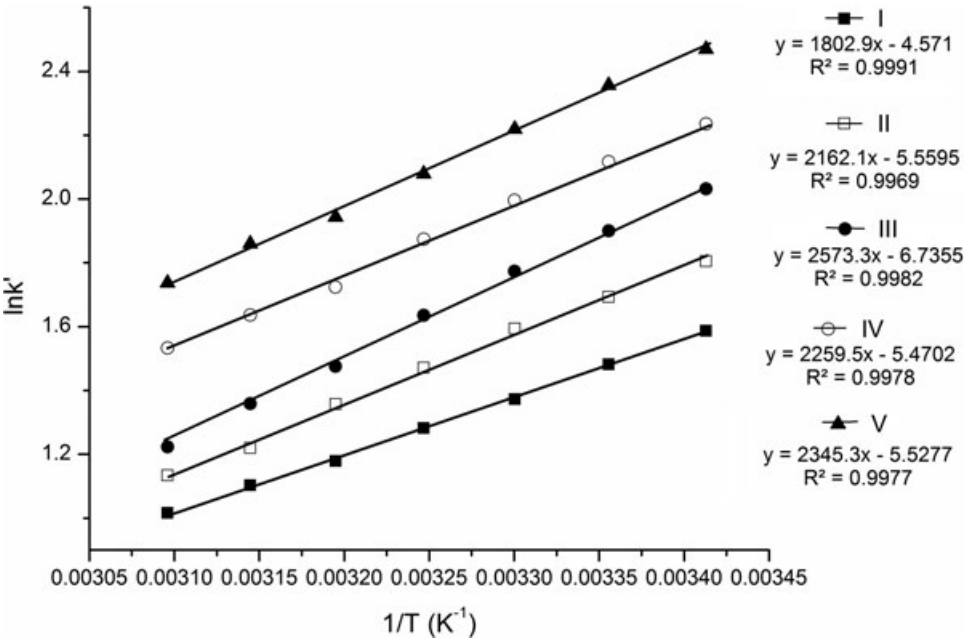

Figure 5. Van't Hoff plots of DRV and its four stereoisomers (II, III, IV, and V).

a signal-to-noise ratio of 3 and 10, respectively. The linearity, LOD, and LOQ data are given in Table 3. The accuracy of the method was determined by spiking the four stereoisomers to DRV at 5, 10, and 50 μg/mL. Each solution was analyzed in six replicates and evaluated by calculating the percentage deviation from the nominal concentration and reported as relative error (%RE). The precision was determined using three standard solutions of stereoisomers of DRV individually at 5, 10, and 50 μg/mL, analyzing each solution in six replicates and calculating the coefficient of variation (%CV). The accuracy in terms of (%RE) was obtained ranging from 0.02 to 28.58 and the precision values in terms of (%CV) were obtained ranging from 0.01 to 27.07. The accuracy and precision data are given in Table 4.

4 Conclusions

The effect of the type and percentage of organic modifier by varying column temperature on retention and separation was studied on Chiralpak AD-H column for separation of DRV and four of its stereoisomers. The effect of column temperature was studied and the apparent thermodynamic parameters derived from the van't Hoff plots (lnk' versus $1/T$, lnα versus $1/T$) were used to explain some aspects about chiral recognition mechanism of Chiralpak AD-H column. The plots of lnk' against $1/T$ were highly linear and the negative values of all apparent thermodynamic parameters suggested that the conformation of the CSP did not change in the studied temperature range of 20–50°C. The developed LC

Table 2. Thermodynamic parameters

Stereoisomer	ΔH^0 (kJ/mol)	Standard error	ΔS^*	Standard error	r^2		$\Delta\Delta H^0$ (kJ/mol)	$\Delta\Delta S^*$
I	−14.989	0.072	−0.038	0.0008	0.999			
II	−17.976	0.088	−0.046	0.0006	0.997	I, II	−2.986	−0.008
III	−21.394	0.137	−0.056	0.0006	0.998	II, III	−3.419	−0.010
IV	−18.785	0.062	−0.045	0.0006	0.998	III, IV	−2.609	−0.011
V	−19.499	0.151	−0.046	0.0002	0.998	IV, V	−0.713	−0.001

Table 3. Linearity, LOD, and LOQdata

Stereoisomer	Range (μg/mL)	Regression equation	r^2	LOD (μg/mL)	LOQ (μg/mL)
I	3.24–50	$y = 42890x - 1135.3$	0.999	1.08	3.24
II	3.30–50	$y = 44466x - 2543.6$	0.999	1.10	3.30
III	2.94–50	$y = 80112x - 3361.8$	0.999	0.98	2.94
IV	3.51–50	$y = 42735x - 1895.8$	0.999	1.17	3.51
V	3.63–50	$y = 35762x - 1251.8$	0.996	1.21	3.63

Table 4. Accuracy and precision data

	I	II	III	IV	V
Precision					
Concentration (μg/mL)	Coefficient of variation (%CV)				
5	17.82	18.45	2.72	27.07	19.94
10	0.27	0.92	0.38	3.75	1.62
50	0.01	0.06	0.47	0.21	0.29
Accuracy					
Concentration (μg/mL)	Relative error (%RE)				
5	18.09	18.23	2.10	28.58	21.21
10	0.31	1.05	0.34	3.04	1.51
50	0.02	0.04	0.29	0.14	0.32

$n = 6$.

conditions may be of use in determination of DRV stereoisomers not only in pharmaceutical formulations but also in biological matrices.

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