Pharmacokinetics and Biodistribution Studies of Selected Racemic Drugs

THESIS

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CERTIFICATE

This is to certify that the thesis entitled **"Pharmacokinetics and Biodistribution Studies of Selected Racemic Drugs"** and submitted by **Sunil Kumar Dubey,** ID No 2009PHXF039P for award of Ph.D. Degree of the Institute, embodies original work done by him under my supervision.

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List of Abbreviations and Symbols

ACE	Affinity capillary electrophoresis
ACN	Acetonitrile
AGP	α1acid glycoprotein
ANDA	Abbreviated new drug application
ANOVA	Analysis of Variance
$AUC_{0-\infty}$	Area under the concentration versus time curve for time zero to infinity
CC	Calibration curve
CDA	Chiral derivatising agent
CGE	Capillary gel electrophoresis
CIEF	Capillary isoelectric focusing
CIP	Capillary isotachophoresis
Cl	Clearance
C_{max}	Maximum (or peak) concentration
CMPA	Chiral mobile phase additive
COX	Cyclooxygenase
CPCSEA	Committee for the Purpose of Control and Supervision of Experiments on Animals
CSP	Chiral stationary phase
CSA	Chiral solvating agent
CYP	Cytochrome
CZE	Capillary zone electrophoresis
DSC	Differential scanning calorimetry
EMEA	European Medicines Agency
FDA	Food drug adminstration
Fig	Figure
GC	Gas chromatography
H	Histamine
h	Hour
HLB	Hydrophilic lipophilic balance
HPLC	High performance liquid chromatography
HQC	Highest quality control
HSA	Human serum albumin
HT	Hydroxytryptamine
IAEC	Institutional Animal Ethics Committee
ICH	International Conference on Harmonisation
IM	Intramascular
IPA	Isopropyl alcohol
IV	Intravenous

k	Capacity factor/Retention factor
K_e	Elimination rate constant
LLE	Liquid liquid extraction
LLOQ	Lower limit of quantification
LQC	Lower quality control
LOQ	Limit of quantification
LOD	Limit of detection
mM	Millimole
MAX	Mixed mode anionic exchange
MCX	Mixed mode cationic exchanger
MQC	Middle quality control
MRT	Mean residence time
N	Number of theoretical plates
NMR	Nuclear magnetic resonance
NSAID	Non steroidalantiinflammatory
ODS	Octadecylsilane
ODV	O- desmethylvenlafaxine
pH	Negative log base 10 to the hydrogen ion concentration
p value	Significance level in statistical test
PMDA	Pharmaceuticals and Medical Devices Agency
PP	Protein precipitation
QC	Quality control
QSAR	Quantitative structure activity relationship
R	Rectus
R^2	Regression coefficient
RP	Reverse phase
RSD	Root mean square deviation
S	Sinister
SD	Standard deviation
SNRI	Serotonin–norepinephrine reuptake inhibitors
SPE	Solid phase extraction
$t_{1/2}$	Elimination half life
T_{max}	Time to reach maximum concentration of drug
T_f	Tailing factor
UV	Ultraviolet
USFDA	United States Food and Drug Administration
V_d	Volume of distribution
VEN	Venlafaxine
σ	Standard deviation of Y intercept of regression equation

Abstract

Regulatory considerations and aspect of chirality started in the early 1990's. Regulatory agencies require clinical efficacy and safety data of each enantiomeric form before giving approval. Latest data reveal that 75% of drugs approved after the year 2000 are pure enantiomers. It is well known that asymmetry plays an important role in most of the biological processes. As the physiological environment is chiral, therefore, enantiomeric molecules are expected to differ in their pharmacokinetic profile and pharmacological behavior. However, to study such separation of enantiomers is very important. Chiral separation of enantiomers helps in understanding the pharmacological profiles of racemates and enantiomers. There is an increase in the demand of chiral separation methods in industries as racemates are being replaced by single enantiomeric forms. The method development for enantiomeric separation is challenging and time consuming. Chiral separation and analysis enhances the clarity of the pharmacological and toxicological data obtained and can also help in controlling the quality of synthesized drugs. This thesis, deals with the enantiomeric separation and stereospecific disposition of two important pharmaceutical drugs with respect to their pharmacokinetics and biodistribution. The first drug, ketorolac, is a nonselective COX inhibitor in the family of heterocyclic acetic acid derivatives. Ketorolac is an isostere of ketoprofen. The second drug chosen was venlafaxine which is an antidepressant of SNRI category. Both drugs are available as racemates and are important and widely used. The objective of the present study was to understand the pharmacokinetics and biodistribution behavior of above drugs after developing simple, reliable, rapid and sensitive analytical and bioanalytical methods. As venlafaxine is converted to O-desmethylvenlafaxine in system, therefore, it is also studied along with venlafaxine.

Experimental Work Done on Ketorolac

The studies which have been conducted on ketorolac include analytical and bioanalytical method development on both achiral (Reverse Phase C-18 Oyster BDS columns) as well as chiral columns (Chiral-AGP column-Chrom Tech). Optimized mobile phase system was selected based on peak properties (retention time and asymmetric factor) and sensitivity (height and area). The variables which alter the enantioselectivity like the pH of buffer, organic modifier and organic modifier concentration, the effect of these were studied. The wavelength for estimation of ketorolac and its enantiomers was fixed at 322 nm. All the validation exercises were performed. Pharmacokinetic and biodistribution studies of the drug were conducted on healthy male wistar rats after intra-venous (1.6 mg/kg) and oral (3.2

mg/kg) administration of drug and pharmacokinetic parameters were analyzed by non-compartmental analysis using WinNonlin software.

Discussion and Conclusion of Studies on Ketorolac

The analytical and bioanalytical method was developed for estimation of racemic and enantiomers of ketorolac. Optimized mobile phase consisted of 10 mM sodium dihydrogen phosphate (pH 5.5): acetonitrile (80:20, v/v) for achiral method and 100 mM dihydrogen phosphate (pH 4.5): isopropylalcohol (96:4, v/v) for chiral method. Recovery of ketorolac from plasma samples by solid phase extraction technique using methanol as eluent was found to be efficient. Plasma concentrations of R (+) ketorolac exceeded those of S (-) ketorolac throughout the time course while the total body clearance (CL) of S (-) ketorolac was significantly larger than that of R (+) ketorolac. The elimination half-life ($t_{1/2}$) of R (+) ketorolac was significantly longer than that of S (-) ketorolac. S (-) ketorolac was found to be distributed more to tissues. Thus we can conclude that the S (-) enantiomers which gets widely distributed to the tissues is responsible for its analgesic action. Moreover, bio-distribution studies showed higher concentration of S (-) ketorolac in kidney which probably may be responsible for its nephro-toxicity. Data obtained in oral study followed the same of i.v administration.

Experimental Work Done on Venlafaxine

The studies which have been conducted on venlafaxine (VEN) include simultaneous analytical and bioanalytical method development of VEN and its metabolite O- desmethyl venlafaxine (ODV) on both achiral (Reverse Phase Agilent Eclipse XDB-C18 column) as well as chiral columns (Chiral-AGP column- Chrom Tech). Optimized mobile phase was selected based on peak properties (retention time and asymmetric factor) and sensitivity (height and area). The variables which alter the enantioselectivity like the pH of buffer, organic modifier and organic modifier concentration, the effect of these were studied. The eluents were monitored using fluorescence detector at excitation wavelength 226 nm and emission wavelength of 298 nm. All the validation exercises were performed. Pharmacokinetic and biodistribution studies of the drug were conducted on healthy male wistar rats after intra-venous (40 mg/kg) and oral administration of drug (120 mg/kg) administration of drug and pharmacokinetic parameters were analyzed by non-compartmental analysis using WinNonlin software.

Discussion and Conclusion of Studies on Venlafaxine

The analytical and bioanalytical method was developed for estimation of racemic and enantiomers of VEN and ODV. Optimized mobile phase consisted 10 mM potassium

dihydrogen phosphate buffer (pH 4.5): acetonitrile (75:25, v/v) for estimation of racemic VEN and ODV. For chiral analysis, optimized mobile phase consisted of 10 mM potassium dihydrogen phosphate buffer (pH 6.5): methanol (94:6, v/v). Recovery of VEN and ODV from plasma samples by solid phase extraction using acetonitrile as an eluent was found to be efficient. The pharmacokinetic study revealed that there are distinct differences in pharmacokinetic parameters and distribution character of enantiomers of VEN and ODV. From the pharmacokinetic studies it was found that the plasma concentrations of S (+) VEN was higher than those of R (-) VEN. There was significant difference in most of the pharmacokinetic parameters between S (+) VEN and R (-) VEN. The oral plasma concentration profile was similar to i.v profile. From the biodistribution study it was found that the concentration of R (-) VEN was higher than that of S (+) VEN in all the tissues. Similarly the amount of R (-) ODV was found to be more than that of S (+) ODV in all the tissues. The brain tissue distribution studies revealed that R (-) VEN and R (-) ODV distributed more in comparison to their optical antipode giving inference that antidepressant activity may be more for above enantiomers of VEN and ODV.

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Chapter 1

Introduction

1.1 Stereochemistry: An Overview

The branch of chemistry that deals with spatial arrangements of atoms in molecules and the effects of these arrangements on the chemical and physical properties of substances is called stereochemistry (1). The word “stereos”, in Greek, means solid. The most interesting part of stereochemistry is how beautifully it is associated with chemical structures, and its ability to combine chemistry, geometry and topology in the study of three-dimensional shapes and structures. Chirality is present in several nature substances such as amino acids, nucleotides, and sugars; which are considered as the building blocks of life, and are available in different enantiomeric forms. Most biochemical processes are homochiral in nature, i.e. they show 100 % stereoselectivity (1-5). It is very challenging for pharmaceutical scientists to analyze the activity of their molecule in asymmetrical physiological environment. An example of this is the stereospecific interaction of the carvone with the human olfactory receptors. Carvone exists as two enantiomers R (-) and S (+) carvone, where the R (-) enantiomer is responsible for the distinct taste and smell for its presence in oil of spearmint whereas the S (+) enantiomer is responsible for taste and smell for its presence in caraway (5). The stereochemistry involved in the concept of interaction between drug and receptors has been recognized for a long time; however, the involvement of stereochemistry in the pharmacokinetics and biodistribution of drugs has come into focus more recently.

It has been made mandatory by the Food and Drug Administration (FDA) that drugs be produced in enantiomerically pure forms, and the proper tests are employed to prove the safety of both enantiomers. The concept of stereochemistry is also used to study the properties of synthetic polymers and understanding the reaction mechanisms so as to determine the outcome with respect to the enantiomers possible (1, 2). The thalidomide tragedy of 1961 is a landmark in enantiomeric activity and drug regulation. Thalidomide is a racemate of a glutamic-acid derivative. The following statement appeared in a book on X-ray crystallography "The thalidomide tragedy would probably never have occurred if, instead of using the racemate, the R (+) enantiomer had been brought on to the market. In studies it was shown that after i.p. administration only the S (-) enantiomer exerts an embryotoxic and teratogenic effect. The R (+) enantiomer is devoid of any of those effects under the same experimental conditions" (6, 7). Later it was postulated that, even if there were differences in the toxicity of the enantiomers of thalidomide, their rapid racemization in vivo would blunt them such that they could not be exploited. So, it is very necessary to understand the in vitro and invivo property of racemic drugs and their enantiomers before making any conclusion (7).

1.2 Chemical Evolution of Chirality

Around 1809, the chemical evolution of chirality started with the discovery of Hauy, who postulated from crystal cleavage observations that a crystal and each of its constituent space filling molecules are images of each other in overall shape (8). In 1812, the French physicist Biot discovered optical activity. He concluded that a quartz plate, cut at right angle to its crystal axis, rotates the plane polarized light through an angle proportional to the thickness of the plate (9). This discovery was milestone in the evolutionary process of stereochemistry. Biot also did his experiments on solids and solutions. The difference between the rotation of plane polarized light produced by quartz and the organic substances were postulated by him. First resolution of enantiomers from racemates was done by Louis Pasteur in 1848 (9). He separated manually two forms of ammounium tartrate using a lens and a pair of tweezers. The two kinds of crystals when separately re-dissolved, it was found that it rotated plane polarized light in different direction. Pasteur, postulated that the molecular structures of (+) and (-) tartaric acids were related as a mirror image. However, Pasteur was unable to explain the different degradation rates of the dextro and levorotatory forms of ammonium tartarate by the mould Penicillium glaucum, and why and how the (+) tartarate was used as a carbon source by Penicillium glaucum (10, 11). But in 1874, Le Bel and Vant Hoff independently proposed that the four valences of the carbon atom are directed toward the vertices of an atom-centred tetrahedron and would give two distinctly different non super-imposable forms (12). Vant Hoff also described the carbon atom as asymmetric. This helped in explaining Pasteur's results which previously did not have a scientific rationale (12-14).

Chirality exists in a large number of organic molecules and in few inorganic compounds. In some molecules, the carbon atom is attached to four different atoms or groups, thus making the molecule asymmetric. These type of molecules exist in different three dimensional spatial configuration and exist into two forms, which are mirror images of each other, but non super-imposable. In addition to central chirality, axial chirality can occur in allenes and cumulenes. In central chirality, the substituents are not necessarily different, since the second double bond causes the loss of the C2 rotational symmetry element. In the latter class, only members with an odd number of accumulated carbon atoms are potentially chiral, whereas an even number of carbon atoms results in E/Z isomerism or geometrical isomerism (15). Helicity is a special form of chirality and often occurs in macromolecules such as biopolymers, proteins and polysaccharides. A helix is always chiral owing to its right-handed (clockwise) or left-handed (counter clockwise) arrangement (16). Basic terminology of stereochemistry is presented in table 1.1.

Table 1.1 Basic terminology and definition in stereochemistry

Terminology	Definition
Chiral	Not superimposable with its mirror image, as applied to molecules, conformations, as well as macroscopic objects, such as crystals.
Isomers	Different compounds which have the same molecular formula.
Conformational isomers	Inter-convertible by rotations about single bonds.
Constitutional Isomers	Isomers which differ in "connectivity". Ex: butane and methylpropane, i.e., isobutane, which are different in that butane has a sequence of four carbon atoms in a row, but isobutane has a three carbon chain with a branch.
Stereoisomers	Isomers which have the same connectivity. A more subtle kind of isomerism in which the isomers differ only in their spatial arrangement, not in their connectivity. Cis and Trans-1, 4-dimethylcyclohexane is a good example of a pair of stereoisomers.
Enantiomers	Stereoisomers which are mirror images. Compounds that contain the same atoms linked together in the same way but in a different three-dimensional arrangement. Enantiomers have identical physical properties, but rotate the plane of polarised light in opposite directions. Ex: enantiomers of glucose.
Diastereoisomers	Stereoisomers which are not mirror images. Stereoisomers those are not enantiomeric to each other. They characteristically occur in molecules with two or more chiral centres.
Achiral/ Mirror Image Isomerism	An entity, such as a molecule, is achiral if it is superimposable with its mirror image.
Homochiral	Isomeric molecules are homochiral if they have the same sense of chirality, that is, if they are all R or all S.
Chiral centre / Stereogenic Centre	Four different substitutions, usually attached to carbon that could be swapped to create a new stereoisomer.
Racemate	A mixture of all possible stereoisomers of a compound in equal proportions. It does not have optical activity (50; 50 mixture).
Stereoselective	Relating primarily to one specific stereoisomer. A biological reaction is stereospecific if either the substrate or its binding is chiral.
Atropisomers	Stereoisomers that can be interconverted by rotation about single bonds but for which the barrier to rotation is large enough that the stereoisomers can be separated and do not interconvert readily at room temperature.
Configuration	The relative position or order of the arrangement of atoms in space which characterizes a particular stereoisomer.

Optical Purity	A collection containing only one enantiomeric form of a chiral molecule is called optically pure or enantiopure.
Planar Chirality	It arises from the arrangement of atoms or groups of atoms relative to a stereogenic plane.
Resolution	A process wherein enantiomers are separated.
Conformation	The spatial arrangement of the atoms affording distinction between stereoisomers which can be interconverted by rotations about formally single bonds.

1.3 Stereochemical Aspects And Impact On the Biological System

The research in chirality began in the 19th century, but it was only in the beginning of the 20th century that the role of chirality in the mechanism of action of the drugs was revealed and demonstrated. A lot of problems were faced by research scientist while working with the asymmetric carbon atom. They were unable to synthesize the desired product because of racemisation occurring in compounds having chiral centre. It becomes even more complex when the compounds have more than one chiral centre. In a non-chiral environment, the enantiomers in a racemic mixture possess the same physical and chemical properties (except optical rotation differences).

In 1933, Easson and Stedman proposed a three-point attachment model studying the pressor effect of epinephrine. Their finding led to the basis for the initial understanding of stereochemical differences for enantiospecific interaction of drugs with pharmacological receptors. The R (-) enantiomer of epinephrine was found to be the most potent one (19). The differences in the bio-affinity of enantiomers was attributed to a common chiral site on an enzyme or receptor surface, with the receptor or enzyme requiring to possess three non-equivalent binding sites to discriminate between the enantiomers. The enantiomer that interacts simultaneously with all three sites is called the eutomer (active enantiomer), whereas the other, which binds to fewer than three sites at the same time, is called the distomer (inactive enantiomer) (20).

Between 1940's and 1960's, similar studies were conducted on propranalol, sex hormones and some anti-parkinsonian drugs which showed selective toxicity and activity due to particular enantiomers. But for unknown reasons, drugs were not resolved to their active enantiomers for better activity and reduced toxicity. Ariens, in the late 1980s, proposed that the active enantiomer should be named the eutomer and the less active being called the distomer and that the ratio between the eutomer/distomer (the eudomistic ratio) should be calculated and could be used as to measure the difference in effect (21). In the recent times,

regulatory authorities defined more strict requirements about drug discovery and chiral compounds, with demands to properly characterize the enantiomers of any new molecule. This has stimulated organic chemists to directly synthesize pure enantiomers or to separate the enantiomers on an industrial scale after synthesis of a racemic mixture (22). Study of chirality in drug development is of paramount importance because of differential activity of different enantiomers of the racemic compounds. It is needed to identify the optically active pure forms of the racemic compound in therapeutic drug monitoring, or in clinical and forensic toxicology. A few examples of differential activity of optically pure enantiomers are presented in table 1.2.

Table 1.2: Differential activity of optically pure enantiomers

Drug	(+) Enantiomer	(-) Enantiomer
Ketoprofen	Analgesic and anti-inflammatory	Gastric disorder
Levodopa	Antiparkinsonism	Agraulocytosis
Ketamine	Anaesthetic	Post emergent distress
Morphine	Minimal effect	Analgesic
Thalidomide	Sedation	Teratogenicity
Pencillamine	Antirheumatic (Wilson's disease)	Neurotoxic
Fluoxetine	Migraines	Antidepressant
Cetrizine	Sedation	Antihistaminic

Going for enantiomers may have a lot of benefits which include exclusivity to the company and better price justification, as well as creating a separate market space for commercial benefit.

However, going for enantiomeric form of all drugs are not always beneficial and some examples are mentioned below.

1. More the number of chiral centres more are the complexity of separation.
2. Administering the desired active enantiomer need not always by pass the side effects or organ damage, as there is a high possibility that metabolite formation, chiral inversion or racemisation can take place internally in the biological system.

3. Some molecules might give desired action for a longer duration when there is a racemic form. Ibuprofen when given as a racemic mixture acts in the above manner where the inactive R (-) isomer gets converted metabolically into the active S (+) enantiomer (23), which is an active form and thus produces extended action.
4. In indacrinon which is diuretic, the presence of the distomer was useful, since it promoted the efficacy of the therapeutic eutomer by antagonizing one of its side effects (25).

The drug kinetics after its administration in the body, are expressed by some specific parameters that can be calculated during pharmacokinetic analysis. The analysis is done based on the plasma concentration or other biological samples of the drug or its metabolite at different specific time points after the drug administration. The parameters calculated can be later used for pharmacokinetic characterization of the medicinal substance, for comparing the quality of some pharmaceutical products or for establishing or individualization of a treatment scheme, based on the specific physiological characteristics of the body.

1.4 Enantioselectivity in Pharmacodynamics and Pharmacokinetics

Drug chirality plays an important role in the drug discovery and development as well as in marketing of new drugs (26–29). Chiral drugs are a segment of drug substances that contain one or more chiral centres. More than one-half of marketed drugs are chiral (30). Separation of the enantiomers of chiral drugs has become an important issue in analytical and clinical chemistry in recent years, because of differences in the pharmacological (31), toxicological (32), pharmacodynamic and pharmacokinetics (33, 34) properties of drug enantiomers (35, 36). In 1980's most of the listed drugs in the pharmacopoeia were racemates, but after the emergence of new insights on enantiomers and new technologies involved in the preparation of pure enantiomers led to the increased awareness and interest in the stereochemistry of drug action. The new developments in stereoselective bio-analytical methods led to the increased understanding of stereoselective pharmacodynamics and pharmacokinetics, enabling the differential contributions of enantiomers to overall drug action.

1.4.1 Role of Enantioselectivity in Pharmacodynamics

In light of different spatial configurations, one active isomer may have favourable binding to the receptor sites while an inactive isomer may have an unfavourable binding to the receptor or other unintended targets (37). Pharmacological effects of enantiomeric drugs may be categorized as follows (38).

1. Both enantiomers may act similarly, but they do not have a synergistic effect. S (+) and R (-) enantiomers of Δ-3-tetrahydrocannabinol act on the same receptor, but adding two enantiomers together did not increase the psychoactivity of the drug (39).
2. Both enantiomers have independent therapeutic effects through action on different targets. The best known example is of Quinine and Quinidine. While Quinine has been used for the treatment of malaria for centuries, where as Quinidine, is used as a class 1A antiarrhythmic agent (40).
3. Both enantiomers act on the same biological target(s), but one isomer has higher binding affinity than the other and thus having different level of activity. Carvedilol used in racemic form for the treatment of hypertension and congestive heart failure (41). It is a nonselective β- and α-adrenergic receptor blocking agent. Nonselective β-blocking activity resides mainly in the S (-) carvedilol, and the α-blocking effect is shared by both R (+) and S (-) enantiomers (42).
4. One or both enantiomers have the desired effect; but only one enantiomer can cause unwanted side effects due to binding to different receptors. In case of racemic dropropizine, which has been used an antitussive agent, recent studies have revealed that R (+) dropropizine is more selective on the CNS receptors responsible for side effects whereas S (-) dropropizine possesses the same antitussive activity as the racemic mixture, and hence proved responsible for the therapeutic activity of the drug (43).
5. The inactive enantiomer might antagonize the side effects of the active antipode. Tramadol which is an opioid analgesic is administered in racemic form. The reported side effects are *respiratory* depression, constipation, or sedation (44). It was revealed that the side effects were higher for (+) enantiomer when given alone as compared to racemic tramadol. Later it was found that the (–) enantiomer antagonises the side effects of (+) enantiomer resulting in lesser side effects of racemic forms. Therefore, the racemate of tramadol is superior to the enantiomers for the treatment of severe postoperative pain (45).

It is tedious to predict the pharmacological behavior for two enantiomers. Fokkens and Klebe developed a simple protocol using isothermal titration calorimetry in an attempt to determine the differential binding affinity of two enantiomers to a protein using the racemic form of

drug (46). In some cases, the affinity difference could be explained in terms of differences in the structural fit of the enantiomers into the binding pocket of the protein (47).

1.4.2 Role of Enantioselectivity in Pharmacokinetics and Drug Disposition

In addition to the differences in pharmacodynamics, stereoisomers may differ in their pharmacokinetic properties such as absorption, distribution, metabolism, and excretion (ADME) as a result of stereoselective disposition of drug inside the body (48). Drug absorption, distribution, and excretion are generally processes which do not differentiate between enantiomers, but when it comes to metabolism and the drug's interaction with an enzyme or a transporter system, a chiral discrimination may occur and these enantioselective processes may affect the pharmacokinetics of some drugs.

a) Stereoselectivity in Drug Absorption

One of the prerequisites for stereoselectivity in a biological process is stereospecific recognition of a substrate by a biochemical entity responsible for facilitation of the process. This entity is most often a protein, such as a receptor or enzyme. There is currently great interest in P-glycoprotein-mediated efflux mechanisms, and such transport systems are potentially stereoselective. However, there is limited data in the literature concerning this possibility, and some reports have been the subject of controversy. For example, in vitro data suggested that the S (-) enantiomer of the β-adrenoceptor antagonist, talinolol, is a slightly better substrate than R (+) talinolol for P-glycoprotein (49), which the authors suggested accounted for the lower plasma concentrations of the S (-) than the R (+) enantiomer in in-vivo studies. However, a recent article suggests that modest presystemic metabolism via CYP 3A4, rather than P-glycoprotein efflux, is responsible for the minor but significant differences in R (+) and S (-) talinolol pharmacokinetics after oral administration (50). With respect to the drug absorption, the lymphatic absorption of halofantrine enantiomers has shown to be stereoselective (51–53).

b) Stereoselectivity in Drug Distribution

Stereoselectivity in drug distribution may occur as a result of binding to either plasma or tissue proteins; and transport via specific carrier system, tissue uptake and storage mechanisms. As we know that it is the free fraction of drug and not the bound part which elucidates the action, it is mandatory to understand the extent of binding of each enantiomers to protein or tissue. It is also known that the weakly acidic drugs bind to Human Serum Albumin while weakly basic drugs bind to alpha 1-acid glycoprotein. Plasma binding

capacity for two enantiomers may also be significantly different, thus influencing drug distribution and efficacy of the enantiomers. Methadone has therapeutic benefits that reside in the R (-) enantiomer. Compared to the S (+) enantiomer of methadone, methadone's R (-) enantiomer shows 10-fold higher affinity for μ and κ opioid receptors and up to 50 times the antinociceptive activity in animal model and clinical studies. Methadone's enantiomers show markedly different pharmacokinetics. The R (-) enantiomer shows a significantly greater unbound fraction and total renal clearance than the S (+) enantiomer. These reflect higher plasma protein binding of the S (+) enantiomer (54).

c) Stereoselectivity in Drug Metabolism

Due to the three dimensional nature of substrate recognition by metabolic enzymes, drug metabolism offers many possible avenues for stereoselectivity in plasma concentrations. In drug metabolism, stereo differentiation is the rule rather than the exception, and stereoselectivity in metabolism is probably responsible for the majority of the differences observed in enantioselective drug disposition. Stereoselectivity in metabolism may arise from differences in the binding of enantiomeric substrate to the enzyme active site and/or be associated with catalysis owing to differential reactivity and orientation of the target groups to the catalytic site (55). As a result, enantiomers are frequently metabolized at different rates and/or via different routes to yield alternative products. Plasma protein binding can be an underlying cause of stereoselectivity in plasma concentrations of drug via not only distributive mechanisms, but also by metabolism. For low and moderately extracted drugs, plasma protein binding is a controlling factor for the entry of drug past the cellular membranes of eliminating organs, and can control the effective metabolic rate and clearance (Cl) of total (bound + unbound) drug enantiomer in plasma. The difference in bioavailability, rate of metabolism, metabolite formation, may be influenced by genetic polymorphism in cytochrome P450 (CYP) isoenzymes involved in drug metabolism (56). For example, esomeprazole S (+) isomer of omeprazole, an optical isomer proton pump inhibitor, generally provides better acid control than the current racemic proton pump inhibitors and has a favorable pharmacokinetic profile relative to omeprazole. However, the metabolic profiles of the two enantiomers are different, leading to different systemic exposures and thus different pharmacodynamic effects. Metabolism of the R (-) enantiomer is more dependent on CYP2C19, whereas the S (+) enantiomer can be metabolized by alternative pathways like CYP3A4 and sulfotransferases. This results in the less active R (-) enantiomer achieving higher concentrations in poor metabolizers, which may in the long term cause adverse effects

like gastric carcinoids and hyperplasia (57). In some cases stereoselectivity in first pass metabolism may lead to unexpected differences in the observed concentration vs. effect relationships when a nonstereospecific assay is used to estimate pharmacokinetic data. For example, verapamil, which possesses a high hepatic extraction ratio, displays a steeper concentration–effect relationship after i.v administration compared with oral dosing if a nonstereospecific assay is used (58). This was attributable to a stereoselective first pass metabolism of the more active S (-) enantiomer upon oral dosing. Interspecies differences in the direction of stereoselectivity are also common. As an example, the metabolic oxidation of felodipine in human is greater for the S enantiomer, whereas rat and dog preferentially metabolize the R (+) enantiomer (59).

d) Stereoselectivity in Drug Excretion

Both biliary and urinary excretory processes have some mechanistic components that may lead to stereoselectivity in clearance. Transport proteins such as P-glycoprotein, and organic anion and cation transporters are all implicated in drug transport across canalicular cells and renal tubular cells, and binding site specificity may lead to enantioselectivity in their ability to bind to substrates. Although glomerular filtration across the kidney may be thought of as being nonstereoselective for the unbound drug, plasma protein binding may cause the rates of filtration of enantiomers to differ. As a result of stereoselectivity in the processes of drug disposition, the pharmacokinetic profiles of the individual enantiomer of drugs administered as racemates frequently differ, and pharmacokinetic parameters derived from an analysis of ‘‘total drug’’ concentrations are of limited value and potentially misleading (60). Therapeutic drug monitoring and bioequivalence data derived from investigations employing nonstereospecific analytical methodology provide information of limited value. Stereoselectivity, how it affects the drugs concentration with respect to time at every step can be understood by cetirizine, the potent histamine H1 receptor antagonist, is a racemic mixture of R (-) and S (+) cetirizine. In binding assays, levocetirizine has demonstrated a twofold higher affinity for the human H1 receptor compared to cetirizine, and an approximately 30-fold higher affinity than dextrocetirizin (61). However, levocetirizine is rapidly and extensively absorbed and poorly metabolized and exhibits comparable pharmacokinetic profiles with the racemate. Its apparent volume of distribution is lesser than that of dextrocetirizine. Moreover, the nonrenal clearance of levocetirizine is also significantly lower than that of dextrocetirizine. All evidence available indicates that levocetirizine is intrinsically more active and more efficacious than dextrocetirizine, and for a longer duration

(62). Thus there are commercial products containing levocetrizine are available for antihistamine therapy. An understanding of the stereospecific pharmacokinetics of all chiral drugs may help clinicians to interpret and predict differences among patients in pharmacologic responses to the racemic drugs and to adjust their dosage for each patient.

e) Stereoselectivity in Drug Toxicity

There exists a good correlation between drug toxicity and stereoselectivity because of stereoselective binding of drugs with receptors. As a result of adverse drug reactions, many racemic drugs have been withdrawn from the market. The reason hypothesized for this is the contribution of stereoselectivity in disposition (64). The well known example is of thalidomide, where the use of a specific stereoisomer would have prevented a tragedy. An investigation into this tragedy revealed that hypnotic activity is shown by both the enantiomers when given invidually to mice. But the teratogenic activity was only observed in case S (-) enantiomer (65). Studies with the male antifertility agents 3-chloropropane-1, 2-diol and 3-amino-1-chloropropane-2-ol have indicated that the antifertility activity in rat is due to the S-enantiomers, whereas the R-enantiomers are associated with nephrotoxicity (66, 67).

1.5 Regulatory Aspects of Chirality

Most of the commercial pharmaceutical compounds and new chemical entities in the discovery phase are chiral and their enantiomers show significant differences in their pharmacokinetic and pharmacodynamic properties. Regulatory agencies require clinical efficacy and safety data of each enantiomeric form before giving approval. Latest data reveal that 75% of drugs approved after the year 2000 are pure enantiomers. This has been possible only due to technological advances in enantioselective analytical methods as well as commercial feasibilty of manufacturing enantiomeric forms. Moreover, the regulatory norms have made it difficult for the industries to market racemic products. Statistically speaking, the industrial scenario has changed to such an extent that in 1990's only 3% of the synthetic pharmaceutical or natural derived products were marketed as pure enantiomers; while in the early 2000's the figure grew to 75% (67). A brief touch on to how chiral products got a special place in regulatory guidelines has been given table 1.3 (68). As per USFDA regulatory norms, if the drug is a racemate, then all the toxicological and pharmacological data of the individual enantiomers must be included. For the single enantiomer, a pharmacology and toxicology evaluation of the enantiomer must be conducted, and compared with the existing data of racemate. The evaluation must also include data regarding the

interconversion of the eutomer to a distomer (68). The guidelines are similar in the case of EMEA as well as PMDA. In EMEA, they have specially mentioned that if an enantiomer of a previously marketed racemic product is brought to the market, they must have all the necessary evaluation results of enantiomers. Nowadays, it is mandatory to justify the choice for a racemic product compared to enantiomers.

Table 1.3: Regulatory framework for chiral drugs

1987	USFDA raised the topic of stereochemical regulation in its 1987 guideline on drug substances. Drawback: Little emphasis given on chirality
1989	An internal committee was formed to address the regulatory concerns of stereoisomeric drugs
1990	PMA(Pharmaceutical Manufacturers Association) gave its view on development of stereoisomeric drugs in a paper published by pharmaceutical technology
1992-93	Drug Information Association conference in Paris provided an international platform for examining the regulatory requirements of chiral drugs which was summarised in 1993 paper in Drug Information journal.
1994-95	CPMP (Committe For Properietory Medicinal Products) set its position in a policy in 1994.
1995-Now	Rapid development in chiral drug development with guidelines drafted in different countries and ICH guidelines

1.6 The Importance of Stereospecific Assays

Many pharmacokinetic studies involving chiral drugs utilize nonstereospecific assay techniques, which could potentially limit the value of the information thereby gleaned. The merit of using a nonstereoselective assay is questionable. In a general sense, the complication from using a nonstereospecific assay in assessment of drug bioavailability has been highlighted here by an example of etodolac. In etodolac, marked stereoselectivity is present and the less active enantiomer is predominant in plasma, so use of a nonstereoselective assay can lead to erroneous conclusions with respect to bioequivalence (63). It is certainly true that for many racemic drugs, the plasma AUC ratios of enantiomers yield values of close to 1. Examples include, in humans, ofloxacin, rolipram, tosufloxacin, sotalol, acebutolol, talinolol. For such drugs, it might be argued that use of nonstereospecific methodology can be justified and the information gained is readily interpretable for certain applications, such as determination of bioequivalence or efficacy (64). Even if there is little difference in the concentrations of enantiomers in plasma, in the event that the rate of absorption can affect the enantiomer ratio, then a nonstereospecific assay could give a biased conclusion of bioequivalence (65). After lot of trials, it has been confirmed that the HPLC methods of

separation are a lot preferred over the non chromatographic methods for stereospecific assays.

1.6.1 Chromatographic Chiral Separation

The chromatographic separation of enantiomeric forms from a racemic mixture is preferred due to its ability to overcome most of the limitations of other methods, and it can be achieved by two methods:

1. Indirect Method
2. Direct Method

1.6.2 Indirect Method

The indirect chromatographic separation of racemic mixtures into their enantiomers can be achieved by derivatization of both the enantiomers with a chiral derivatising agent (CDA) resulting in the formation of diastereoisomeric-salt complexes. For a good derivatization the presence of a suitable functional group in the analyte is required. The diastereoisomers can be separated from each other by means of an achiral chromatographic method by virtue of their differences in physical and chemical properties (74). Target functional group should be present in the proximity to the chiral atom to enhance the differences in physicochemical properties. The most important step for this is to convert the enantiomers of a compound into diastereoisomers which in turn lead to separation by normal or reversed phase chromatography on standard columns such as C18 and C8. It is also possible to achieve temporary diastereoisomers by adding an enantiopure counter ion (acid or base) to the mobile phase. It is important to complete the chiral derivatization reaction, since enantiomers may display different kinetics during reaction with another chiral molecule. There is always a risk that the transformation of enantiomers to diastereomers will lead to racemization of the compounds leading in turn to false results (75). Indirect methods have the advantage of predetermining the elution order, but it is a time consuming process due to different reaction times of different enantiomers. It is very difficult to obtain suitable chiral derivatising agents in pure form. For preparative purposes, the indirect chromatographic technique is a lengthy process because it involves an additional synthetic step of cleaving, the derivatising agent from the diastereoisomers after the resolution.

1.6.3 Direct Method

The direct chromatographic method involves the use of the chiral selector either in the mobile phase, called chiral mobile phase additive (CMPA), or in the stationary phase. In the later case, the chiral selector is chemically bonded or covered or adsorbed onto a suitable solid support creating chiral stationary phases (CSPs). The disadvantage in the former case is the high cost as high amount of chiral selector is required for the preparation of the mobile phase. The recovery of this method is very low as compared to the cost involved. Moreover, this approach is not successful in the preparative separation of the enantiomers (74).

In comparison to CMPA, chiral stationary phases (CSPs) are preferred by almost all analytical, biochemical, pharmaceutical, and pharmacological scientists and industries for the chiral separation of enantiomers. Many different types of CSPs are now available for the separation of enantiomers by high performance liquid chromatography, and they have been shown to be very useful in the chromatographic resolution of racemic mixtures. The principle involved in the separation is the formation of temporary diasteromeric complexes on the column. The fundamental mechanism for chiral recognition is the “three point rule”, wherein it is stated that chiral recognition requires a minimum of three simultaneous interactions between the chiral selector and one of the enantiomers in the racemate to achieve separation (75). The most important and useful CSPs are available in the form of open and tubular columns. Gas chromatography is not the method of choice for the chiral resolution of non-volatile racemic compounds because it is essential to convert nonvolatile racemic compound into a volatile species by derivatization. As well as, the separated enantiomers cannot be collected for further pharmacological and other studies. Moreover, it is not possible to use GC at the preparative scale (74). The main advantage of LC is its ability to determine enantiomers in both biological and analytical samples.

Interactions involved in the chiral recognition process include electrostatic, hydrogen bonding, repulsive/attractive Van der Waal, dipolar interactions and inclusion phenomena (76). In the separation of enantiomers by chromatography, the separation factor is determined by the difference between the free energy of adsorption of each enantiomer. Both the methods are described below.

a) Chiral Separation Using Chiral Mobile Phase Additives

In this method, a chiral additive is added to the mobile phase, which is quite costly. The reversed-phase columns which are used here are more rugged, efficient and can sustain

higher sample load. The chiral resolution using CMPAs is carried out on reversed-phase columns and, therefore, a good range of mobile phases including acids, bases and organic solvents can be used without harming the column. The chiral selectors are often identical to those used for covalently bonded CSPs. The finest CMPAs are those having good solubility in the mobile phase and low ultra violet (UV) absorbance. In addition, the chiral selectors can form diastereoisomers with the racemic compounds to be resolved. Some important aspects and factors associated with use of CMPAs are listed below:

1. Many chromatographic and non-chromatographic parameters can affect the base line resolution of racemic compounds using ligand exchange as a mobile phase additive (such as pH, concentration of CMPAs, modifier concentration, and eluent strength).
2. The proteins are not used frequently as the CMPAs, due to their high absorbance in the UV region which causes interference during analysis.
3. Starch is the source of cyclodextrins (CDs) which are cyclic and non-reducing oligosaccharides. CDs are soluble in aqueous mobile phase and, therefore, the majority of resolutions are carried out under the reversed-phase mode. CDs are non-toxic, nonvolatile, having poor UV absorbance, stability over a wide pH range, and are cheaper which makes them the first choice among other CMPA.
4. The chiral resolution with CDs occur by the formation of diastereoisomeric inclusion complex and therefore the composition of the mobile phase, pH, concentration of cyclodextrin, and the temperature are the most critical parameters which need to be controlled.
5. Glycopeptides are less preferred as CMPAs because they absorb UV radiation.

b) Chiral Separation Using Chiral Stationary Phases

Chiral selectors can be obtained from natural sources or can be made by synthetic or semisynthetic route. The natural sources include material such as protein, and cyclodextrin. Chiral selectors can be classified in many ways. The most important method of classification for high-performance liquid chromatography based on the mode of formation of the solute-CSP complex. Different types of CSPs are discussed below.

i) Pirkle type CSPs

In 1979, Mikes et al. developed CSPs by attaching a small chiral molecule to silica gel. In this CSP, the organic groups of the chiral molecule remain directed away from the silica gel,

appearing in the form of a brush; hence, it is called a brush-type phase (78). Afterwards, Pirkle and co-workers worked extensively on these types of CSPs and now these CSPs are commonly called Pirkle-type CSPs (79, 80). These type of chiral stationary phases are very stable and show good chiral selectivities for wide range of componds. Usually, the chiral molecule attached to the silica gel contains an electron donor or an electron acceptor or both types of group. As a result, these CSPs are classified into three groups:

1. π-acidic (with π-electron-acceptor groups)
2. π-basic (with π-electron-donor groups)
3. π-acidic-basic (with π-electron-acceptor and-donor groups)

These type of CSPs are widely used. These CSPs have been used in the resolution of amino acids, amines, α-blockers, β-blockers, amino phosphonates, alkylcarbinols, naphthols, hydantoins, benzodiazepine, barbiturates and many other compounds. The main drawback is the need for pre-derivatization of some racemic compounds which is time consuming and a tiresome job. In addition, these CSPs cannot be used in resolution of natural substances.

ii) Protein based CSPs

Proteins are natural polymers that have a basic skeleton made of L-amino acids, which are chiral molecules. The protein polymer remains in the twisted form because of the difference in spatial arrangements and intramolecular bondings which are responsible for different types of loop/groove present in the protein molecule. This kind of twisted three-dimensional structure of protein makes it enantioselective in nature (81). In 1958, McMenamy and Oncley discovered that the proteins exhibited enantioselectivity (82). They observed that during the isotopic labeling experiment, the L-tryptophan binded more strongly with serum albumin than D-tryptophan. Several types of protein chiral selectors are available such as bovine serum albumin, human serum albumin, α1-acidglycoprotein, ovomucoid, avidin, ovotransferrin, chymotrypsin, and cellobiohydrolase-I. Cellobiohydrolase-I is the only plant based polymer (76, 77). The chiral recognition mechanism of protein CSPs is mainly based on specific interactions between the analyte and CSP itself involving non polar, dipole interactions, hydrogen-bondings, and steric effects (83, 84).

Protein-based CSPs are widely used for the chiral resolution of many drugs. Some applications of protein based CSPs are discussed below:

Bovine serum albumin CSP was used for the enantioseparation of 2-arylpropionic acid derivatives, benzodiazepines, warfarin and benzoin racemates (85). Human serum albumin protein column was utilized for the chiral discrimination of N-(dansyl)-amino acids (86). The influence of solute hydrophobicity on the enantioselectivity of cellobiohydrolase-I CSP using racemic β-blockers was studied by Gotmar et. al (87). The same CSP was used for the enantioseparation of synephrine (88). Two different protein-based CSPs, human serum albumin and α1-acid glycoprotein for the enantioseparation of 10 quinolone antibacterial agents were conducted by Barbato et. al (89). Protein-based CSPs have unique advantages such as the flexibility of the separation systems and the multiple binding interaction sites. The above advantages are responsible for excellent chiral recognition capacities of protein molecules. Analytical and bioanalytical method development is less challenging and chromatographic parameters can be optimized easily to control the chiral resolution on these phases. However, these CSPs are not suitable for the preparative scale. The other drawbacks includes, the less loading capacity, weaker column ruggedness, and limited understanding of the chiral recognition mechanisms (76).

iii) α1-Acid Glycoprotein as CSPs

Orosomucoid (ORM) or α1-acid glycoprotein (AGP or AAG) is an acute phase plasma alpha-globulin glycoprotein and is modulated by two polymorphic genes. It is synthesized primarily in hepatocytes and has a normal plasma concentration between 0.6-1.2 mg/mL (1-3% plasma protein) (90). α1-acid glycoprotein is composed of a single peptide chain containing 181 amino acid units and five heteropolysaccharide units, which include 14 residues of sialic acid, giving the protein a very acidic character with an isoelectric point of 2.7. Since the molecular mass of AGP from human plasma ranges from 38800 to 48000, it is generally assumed to be 40000 and the sugar content of AGP was estimated to be 34%. CSPs based on AGP were developed by Hermansson (91). The factors affecting enantioselectivity and retention of analytes on AGP columns depends on pH of eluent and mobile phase, type and concentration of organic modifier and charged modifier, ionic strength and temperature (92). The difference in hydrogen bonding properties and hydrophobicity of the uncharged organic modifier also influences the enantioselectivity of the analyte significantly. Furthermore, addition of charged modifiers such as N,N-dimethyloctylamine, tetrapropylammonium bromide, tetrabutyl ammonium bromide and sparteine affects the enantioselectivity and retention of uncharged, anionic and cationic analytes, by competing with the solute enantiomers at binding site(s) or bound to allosteric site(s) (93, 94).

iv) Cyclodextrins as CSPs

Schardinger (95) identified three different naturally occurring forms of cyclodextrins, called alpha, beta, and gamma and referred them as Schardinger's sugars. Cyclodextrins (CDs) are cyclic oligosaccharides that form cavities. They are produced by partial degradation of starch and enzymatic coupling of cleaved units into crystalline, homogenous toroidal structures of different molecular weight. The three most characterized CDs, denoted as alpha , beta and gamma, contain six, seven and eight glucose units respectively. The different number of glucose units leads to different internal diameters and size of the cavities. Cyclodextrins (CDs) are cyclic oligosaccharides that form cavities. The presence of the chiral hollow basket, or cavity, makes these molecules suitable for the chiral resolution of a wide range of racemic compounds (96, 97). An α-CD has a size suitable for complexing a single six-membered aromatic ring, a β-CD can easily accommodate a molecule with the size of a bi phenyl or naphthalene and γ-CD can contain molecules as large as substituted pyrenes (98).

The seven α -D-glucose units in β -CD are linked through the 1, 4 position (α -1, 4- linked) having a chair conformation, forming a rigid torus-shaped molecule with a central cavity. The cavity is composed of the skeletal carbons, ether oxygen atoms and methylene hydrogens giving it an apolar character. As a consequence, cyclodextrins can include other apolar molecules of appropriate dimensions and bind them through dipole-dipole interactions, hydrogen bonding or Van der Waals force (99-101).

The enantioselectivity, resolution and retention times depends upon the type and amount of organic modifier, buffer, flow rate, temperature and choice of CD. Chiral discrimination process depends upon the competition between analyte and the organic modifier for the CD cavity. This, in turn controls the retention, as well as the, enantioselectivity (100, 102). The β form of CD is widely used. In HPLC these columns are mainly used in reversed phase modes. Reports are also available for use in normal phase. In normal phase mode the derivatized CDs are mostly used. The enantioselectivity is due to pi-pi interactions with the derivative group. In normal phase chromatography, the inclusion mechanisms do not seem to play an important role, instead difference in hydrogen bonding is resposible for enantioselectivity.

The chiral separation of lorazepam was achieved on a β- cyclodextrin derivative immobilized with silica gel under reversed-phase condition (76).

1.7 Sample Preparation

Sample preparation is an important preanalytical step in drug analysis, and includes isolation, cleanup and concentration (or occasionally dilution) of samples. The purpose of sample preparation is to enhance assay selectivity and sensitivity, and to reduce amounts of interfering matrix components. The extent of sample preparation depends on the complexity of the sample, and has great importance when drugs in biological matrices such as plasma, urine and tissue homogenates are analysed (103). There are different strategies for sample preparation such as protein precipitation, liquid liquid extraction and solid phase extraction. In bioanalysis, different matrices often are of interest for analysis of drugs. Urine, plasma and whole blood samples are commonly used; however, alternative matrices such as brain, liver, bile, lungs, kidney, heart tissues can also be of interest.

1.7.1 Liquid-Liquid Extraction/ Solvent Extraction

The principle of using two immiscible liquids for extraction of analyte, commonly termed as liquid liquid extraction (LLE) or solvent extraction is one of the most commonly used sample preparation techniques in bioanalysis. One phase in LLE often is aqueous and second phase is an organic solvent. More hydrophilic compounds prefer the polar aqueous phase; whereas more hydrophobic compounds will be found mainly in the organic solvents. Analyte extracted into the organic phase are easily recovered by evaporation of the solvent, while analytes extracted into the aqueous phase can often be injected directly on to a reversed-phase column. The technique is simple, rapid and has relatively small cost factor per sample when compared to others (104). However, LLE has several limitations such as low and variable recovery, the need for a large sample volume, poor selectivity and matrix effects in liquid chromatography–mass spectroscopic (LCMS) methods (105, 106). LLE also has a limitation for the extraction of a wide variety of compounds with varying lipophilicities (107). Recently, efforts have been made to improve the limitations associated with LLE.

1.7.2 Protein Precipitation

Protein precipitation was one of the earliest and is the least time-consuming sample preparation technique and has been widely used in bioanalytical methods. Protein precipitation 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 (108). 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 active change in protein structure, the drug/metabolite/ biomarker bound to these proteins becomes freely soluble in the denaturation solvent, ready for quantitation. The whole process of protein precipitation, however, is time-consuming when handled manually for a large number of samples, especially in drug discovery bioanalysis or clinical bioanalysis.

1.7.3 Solid Phase Extraction

To overcome the limitations of the LLE and protein precipitation, one of the first developments in the field of sample preparation was solid phase extraction (SPE) technique. SPE is based on the same principle of affinity-based separation as liquid chromatography (109). Analytes to be extracted are portioned between a solid and liquid phase (110). For extraction, the analytes must have a greater affinity for the solid phase than for the sample matrix. Interfering compounds are rinsed of the stationary phase by one or several washing steps and then the analytes are desorbed with the solvents. The principles for separation involve intermolecular forces (i.e. hydrophobic interaction and ion ion forces) between the analyte, active sites on the adsorbent and in the liquid phase or sample matrix. Extraction can be performed in reversed- phase or normal- phase mode. Reversed phase partitions solutes from a polar phase to non polar phase, which may be in the form of a hydrocarbon chain or polymeric sorbent. In normal phase SPE, polar compound, dissolved in a nonpolar solvent are extracted by adsorption to a polar sorbent. The most common sorbents used are chemically bonded silica phases. In bioanalysis, normally the reversed phase mode is used as the substances of interest, most often, is dissolved in an aqueous phase such as plasma or serum. Generally, the goals of SPE are retention and elution of analyte from biological fluid (111), 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 (112). Owing to varying physicochemical properties of analytes of interests, these traditional formats were not always suitable and the availability of different stationary phases and different approaches were needed.

1.7.4 Sorbents used for SPE

Oasis HLB is a hydrophilic – lipophilic water wettable reversed phase sorbent. It is made from a balanced ratio of two monomers, the hydrophilic N-vinylpyrrolidone and lipophilic divinylbenzene. This unique chemistry makes it resistance to extreme pH, extraordinary

retention of polar compounds and three times retention capacity as compared to silica based SPE sorbents like C18. It also gives high batch to batch reproducibility. MCX (Mixed Mode Cationic Exchanger) is another sorbent designed to overcome the limitations of the traditional silica based mixed mode SPE sorbents. MCX is a strong cation exchanger, mixed mode water wettable, polymeric sorbent. It is sulfonic acid based cationic exchanger. The ability to fully manipulate pH (0-14) during the method development optimization and use of SPE method on a mixed mode sorbents, enables not only straight forward method development but also helps to ensure very rugged and robust procedure. It allows extraction of basic compounds. Another sorbent designed to overcome the limitation of traditional silica based mixed mode SPE sorbents is MAX (Mixed Mode Anionic Exchange). It consists of Quaternary Amine Anion exchanger. MAX allows complex samples to be separated into two fractions, acidic compound and basic/ neutral compounds (113).

In this research work Oasis HLB sorbents has been used for extraction of ketorolac and venlafaxine (VEN) as well as its metabolite O-desmethylvenlafaxine (ODV). After several trials extraction procedure was optimized for all the analytes.

1.8 Method Development and Validation

Reliable information is a requirement for right understanding of analytical and bioanalytical methods and analytical and bioanalytical methods must be fully validated in order to demonstrate their applicability for the proposed use. Precise and accurate analytical and bioanalytical methods for the quantitative evaluation of drugs and their metabolites in biological matrices are needed for pharmacokinetics and biodistribution studies. The selection of analytical and bioanalytical methods is dependent on the specific needs and purposes. The best suited chromatographic method and best suited type of detection have to be taken into consideration. In pharmacokinetics, biodistribution and toxicological studies it is required to develop bioanalytical methods that cover both therapeutic and toxic concentrations of drugs, hence a broad calibration concentration range is required. When it is necessary to analyse parent drug its metabolites or their enantiomers, a selective and simultaneous method need to be developed for their analysis. Before analyzing the sample with HPLC or LCMS/MS, it is needed to have cleaner sample devoid of endogenous products. For this, the sample need to be prepared by sample pre-treatment. The extent of pre-treatment more often depends on the nature of the sample. Different types of matrices have their own uniqueness and they differ in their protein, sugar and lipid contents. So a effective sample preparation technique is essential to remove all above substances.

Method validation procedures helps in knowing that a method used for quantitative analysis of analytes, metabolites or enantiomers in a given biological sample is reliable and reproducible for the proposed use. Various factors, for example calibration model, selectivity, short and long term stability, dilution integrity are investigated. If the method is intended to quantify more than one analyte, method need to be validated for each analyte. The fundamental parameters for method validation include accuracy, precision, selectivity, sensitivity, reproducibility, and stability. Also, the limit of detection (LOD) and the limit of quantification (LOQ) have to be determined. The validation of a chiral bioanalytical methods is similar to any bioanalytical method. The number of analytes are more which need to be separated when analysing chiral compounds and their metabolites, the run time can be quite long in order to achieve an acceptable resolution. As a consequence of long analysis time, a limited number of samples can be processed each day. Hence, the extent of validation of the method can be hampered. To verify correct quantitations, freshly prepared quality control (QC) samples should be interspersed consecutively in each run (114).

Normally, in analytical data, the variance increases with concentration. For the fitting and analysis of regression lines, a weighted analysis has been used by several researchers, with each value weighted by empirical weights, such as, $1/x$, $1/x^2$, $1/y$, $1/y^2$ and where x is the concentration and y is the area of the peak (115). For analysis of variance, either a weighted analysis or a log transformation of the data can be used to get rid of the variance heterogeneity (heteroscedasticity). In this work, the weighted analysis has been used to characterize the reproducibility and linearity of the data when the heteroscedasticity of the data was found.

1.9 Problem Definition and Research Objectives

As discussed in this chapter most of the commercial pharmaceutical compounds and new chemical entities in the discovery phase are chiral and their enantiomers show significant differences in their pharmacokinetic and pharmacodynamic properties. The significance of chirality of drugs have been acknowledged since long and the arguments of using them as racemates or as enantiomers has been a matter of discussion in the scientific community. The biological environment is extremely stereoselective and it interacts with each enantiomeric form of the drugs differently and metabolize each enantiomer by a unique pathway to produce different or same pharmacological activity. Thus, one enantiomer may produce the desired therapeutic activity, while the other may be inactive or can produce side effects or toxic effects and, in some instances, new indications resulting in therapeutic benefits. There are many such drugs available as racemates in the market, of which very little is known about

the stereoselective pharmacokinetics and biological properties. In future drug discovery and development, single stereoisomers will play an important role and racemic drugs will require logical and scientific explanation before commercial approval. Racemates in the market will continue to be reevaluated for their toxicological profiles and will be reintroduced in the market with cleaner pharmacological profiles.

Thus analysis of drug enantiomers requires the development of very selective methods to separate the analytes from the dissolution and biosamples in addition to chiral separation. With increasing evidence of problems related to stereoselectivity in drug action, enantioselective analysis, by chromatographic methods, has become the focus of intensive research of analytical and bioanalytical scientists.

All these developments have been attributed to the emergence of new technologies that has allowed the preparation of pure enantiomers in significant quantities and the awareness of the importance of stereoselective pharmacokinetics and pharmacodynamics, responsible for the relative contributions of enantiomers to overall drug action. Chiral separation and analysis enhances the clarity of the pharmacological and toxicological data and can also be used as a quality control parameter of the drug. Pharmacokinetic study of drugs is very important for design of new drugs, design of delivery systems and clinical therapy. Thus it is necessary to study pharmacokinetic characters of drugs for proper use and understanding its therapeutic action, side effects and toxicity. Characters of enantiomeric drugs have become important in last few decades and it has become important to study individual enantiomers and their pharmacokinetic profiles.

Ketorolac tromethamine is a nonselective COX inhibitor in the family of heterocyclic acetic acid derivatives. Venlafaxine (VEN) acts by blocking the transporter "reuptake" proteins for key neurotransmitters affecting mood, thereby leaving more active neurotransmitters in the synapse. Both the drugs are available as a racemic mixture of an equimolar ratio of R and S stereoisomers. It is well known that the enantiomers of chiral drugs can present differences in pharmacological activity or efficacy. The objective of the present study was to develop a simple, reliable, rapid and sensitive analytical, bioanalytical as well as biodistribution study methods with better detection range, with less retention time for the estimation of ketorolac tromethamine, VEN, its metabolite O- desmethyl venlafaxine (ODV) and their enantiomers. Thus this project is taken up to understand the stereospecific disposition of enantiomers and their relationship with therapeutic efficacy and unwanted effects of drugs.

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Chapter 2

Drug Profile

2.1 Ketorolac

2.1.1 Introduction

Ketorolac is a pyrrolizine carboxylic acid derivative, chemically, (±)-5-benzoyl-2, 3-dihydro-1H-pyrrolizine-1-carboxylic acid (Fig 2.1). It is a non steroidal anti inflammatory drug (NSAID) and is used principally for its analgesic activity (1). It is a non selective cylooxygenase (COX) inhibitor (2, 3). It acts by nonselective inhibition of COX, leading to decreased formation of precursors of prostaglandins and thromboxanes from arachidonic acid (4, 5).

2.1.2 Drug information

Drug name	: Ketorolac Tromethamine
Category	: NSAID (COX inhibitor)
Therapeutic activity	: Analgesic, antipyretic, anti-inflammatory
Chemical name	: (±)-5-benzoyl-2, 3-dihydro-1H-pyrrolizine-1-carboxylic acid
Chemical formula	: $C_{15}H_{13}NO_3$
Molecular weight	: 255.27
log P	: 2.26
pK_a	: 3.5
CAS Registry Number	: 74103-07-4
Melting point	: 165-167 ^oC (tromethamine salt)
Water solubility	: 25 mg/mL (tromethamine salt)
BCS Class	: I

Chemical structure

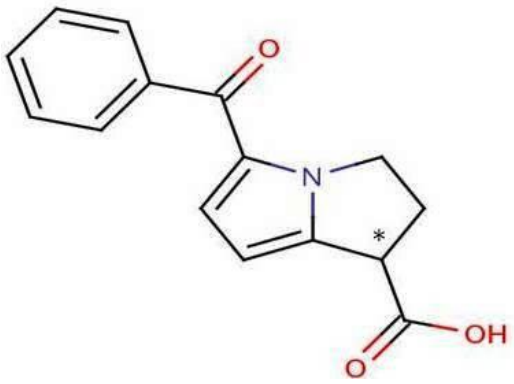

Fig 2.1: Chemical structure of ketorolac.

2.1.3 Pharmacodynamics and Mechanism of Action

Ketorolac, an anti-inflammatory agent with analgesic and antipyretic properties, is used to treat post operative pain, osteoarthritis and control of acute pain. It is a peripherally acting analgesic. Ketorolac tromethamine possesses no sedative or anxiolytic properties. Most NSAIDs, including ketorolac, are non-selective prostaglandin G/H synthase (better known as cyclooxygenase or COX) inhibitors that act on both prostaglandin G/H synthase 1 and 2 (COX-1 and 2). Prostaglandin G/H synthase catalyzes the conversion of arachidonic acid to prostaglandin G2 and prostaglandin G2 to prostaglandin H2. Prostaglandin H2 is the precursor of a number of prostaglandins which in turn lead to fever, pain, swelling and inflammation. Ketorolac antagonizes COX by binding to the upper portion of the active site, preventing its substrate, arachidonic acid, from entering the active site. The analgesic, antipyretic and anti-inflammatory effects of ketorolac are due to inhibition of prostaglandin synthesis. Analgesia is probably takes place via a peripheral action in which blockade of pain impulse generation results from decreased prostaglandin activity. Due to inhibition of the synthesis of other substances that sensitize pain receptors to mechanical or chemical stimulation may also contribute to the analgesic effect. Ocular administration of ketorolac reduces prostaglandin E2 levels in aqueous humor, in addition to inhibition of prostaglandin biosynthesis (6, 7, 8).

2.1.4 Pharmacokinetics

a) Absorption

The absorption of ketorolac is rapid, C_{max} being attained between 20 to 60 min, and almost completely absorbed by oral administration. Oral bioavailability is reported to be 80–100%. Food decreases rate of absorption but not extent of absorption. Onset of action followed by oral administration is 30–60 min, and duration of action is about 6–8 hours (9).

b) Distribution

It has high plasma protein binding (>99%) and not distributed widely. It crosses the blood-brain barrier poorly but crosses the placenta and distributed into milk. It has a volume of distribution between 0.1 to 0.3 L/kg, comparable with those of other NSAIDs (10).

c) Metabolism

It is primarily metabolized by hepatic metabolism, by hydroxylation and also undergoes conjugation with glucuronic acid and excreted mainly by urine (11). Neither metabolite has significant analgesic activity.

d) Elimination

Ketorolac is excreted mainly by urine (92%), 60% of it as parent drug and the remaining 40% as metabolites. Rest about 6% is excreted by feaces. Half-life of the drug is about 4–6 hours in adults and 3.8–6.1 hours in pediatric patients.

2.1.5 Side Effects

Side effects of ketorolac include rashes, headaches, dizziness, drowsiness, abdominal pain, dizziness, nausea, diarrhea, constipation, heartburn, and fluid retention. Ketorolac may cause ulcers and bleeding in the stomach and intestines, particularly with sucessive use for more than five days. Sometimes, stomach ulceration and intestinal bleeding can occur without any abdominal pain. Like other NSAIDs ketorolac also reduces the flow of blood to the kidneys and impair function of the kidneys. The impairment is most likely to occur in patients with preexisting impairment of kidney function or congestive heart failure, and use of NSAIDs in these patients should be done cautiously. Liver failure has also been associated with ketorolac. NSAIDs reduce the ability of blood to clot and therefore increase bleeding time after an injury (12).

2.1.6 Need for Enantioselectivity Study

The drug is available as a racemic mixture of an equimolar ratio of R (+) and S (−) stereoisomers. It is well known that the enantiomers of chiral drugs can present differences in pharmacological activity or efficacy. Animal studies have shown that the pharmacological activity of ketorolac resides in the S (-) enantiomer and that the R (+) enantiomer is pharmacologically inactive (13). Hence enantioselective bio-analytical technique has to be developed for the simultaneous estimation of the two enantiomers of ketorolac for biodistribution and pharmacokinetic studies to understand pharmacological and toxicological responses of enantiomers.

2.2 Venlafaxine

2.2. 1 Introduction

Venlafaxine (VEN) is a bicyclic phenylethylamine derivative (Fig 2.2), which is a unique anti-depressant, structurally differs from other currently available anti-depressants (14). VEN and its active metabolite, O-desmethylvenlafaxine (ODV), inhibit the neuronal uptake of

norepinephrine, serotonin, and, to a lesser extent, dopamine, but have no monoamine oxidase inhibitory activity and affinity for brain muscarinic, cholinergic, histaminergic, or alpha-adrenergic receptors (15-16). Hence, it is devoid of the adverse anti-cholinergic, sedative, and cardiovascular effects of tricyclic anti-depressants (17). VEN has an established tolerability and efficacy profile for the treatment of depressive disorders (15). VEN is the first drug to be marketed that inhibits both noradrenalin and 5-HT re-uptake without actions in other receptors (18). VEN is unique among the antidepressant as it down regulates β-receptors after a single dose, a property that leads to speculation that VEN may have a more rapid onset of antidepressant activity than other antidepressant (19).

2.2.2 Drug information

Drug name	: Venlafaxine Hydrochloride
Category	: Antidepressant
Therapeutic activity	: 1. Noradrenaline (NA) synaptic reuptake inhibitor
	: 2. Serotonin ($5HT_3$) synaptic reuptake inhibitor
Chemical name	: 1-[2-(dimethylamino)-1-(4-methoxyphenyl)ethyl]cyclohexan-1- ol hydrochloride salt (IUPAC)
Chemical formula	: $C_{17}H_{77}NO_7$. HCl
Molecular weight	: 313.87
log P	: 2.48
Pk_a	: 9.27
CAS registry number	: 93413-69-5
Melting point	: 215 ^{0}C – 217 ^{0} C
Water solubility	: 572 mg/ml in water
BCS Class	: I
Chemical structure :	

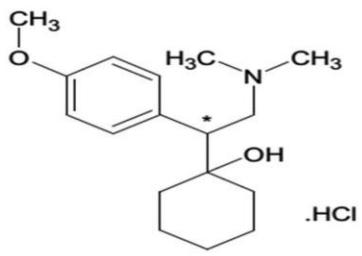

Fig 2.2: Chemical structure of Venlafaxine Hydrochloride.

2.2.3 Pharmacodynamics and Mechanism of Action

At lower doses, VEN is a potent serotonin reuptake inhibitor, while at higher doses, it inhibits noradrenaline (NA) reuptake. R (-) enantiomer inhibit both the NA and 5HT synaptic reuptake, whereas the S (+) enantiomer inhibits only the 5HT (20, 21). VEN has no affinity for adrenergic, muscarinic or histaminergic receptors, but is to lesser extent an inhibitor of presynaptic reuptake of dopamine (15,16).

2.2.4 Pharmacokinetics

a) Absorption

VEN is well absorbed. From a single dose, VEN is absorbed at least 92% and after absorption it undergoes first pass metabolism in the liver. The mean ratio of S (+)/ R (-) of enantiomers of VEN in human over 2-6 h interval ranged from 1.33 to 1.35 with an overall ratio of 1.34 ± 0.26 (20, 21). Post first pass metabolism about 40-45% of drug reaches systemic circulation. VEN ond ODV exhibit linear kinetics over the dose range of 75-450 mg/day (22,23).

b) Distribution

Protein binding of VEN is about 27 % (24).

c) Metabolism

The phase I metabolism of VEN occurs in the liver, mainly by the cytochrome system. The well-known major pathway for the metabolism of VEN is illustrated in fig 2.3. In humans, VEN is metabolized by CYP2D6 to its major metabolite ODV and by CYP3A4 to N-desmethylvenlafaxine (NDV).NDV is then further metabolized to N, O-didesmethylvenlafaxine (DDV), probably by CYP2D6 (16). The amount of ODV formed is 56% while 16 % of DDV and 1% of NDV are produced (15). Some studies support the possible involvement of CYP2C9 and CYP2C19 in the metabolism of VEN (25, 26). ODV contributes to the overall pharmacological effects of VEN since it exhibits a pharmacological profile similar to that of VEN. NDV and DDV display less potent effects on 5-HT and NA reuptake compared with VEN and ODV (27, 28).

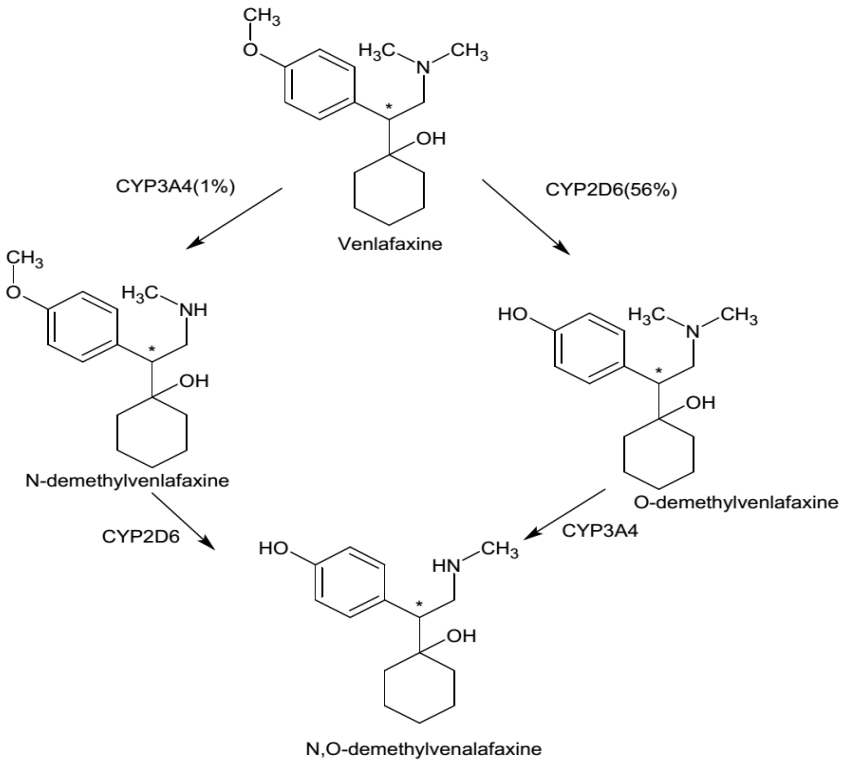

Fig 2.3: Metabolism of Venlafaxine.

d) Excretion

Most of the VEN, about 87%, is eliminated by kidneys within 48 h, including the unchanged form (5%), free ODV (29%), coupled ODV (26%), or inactive metabolites (27%). In patients suffering from renal dysfunction, the half-life in the elimination phase increases, which results in the need to reduce the dose taken by the patient (29, 30).

2.2.5 Side Effects

In comparison to older tricyclic antidepressants, the newer generation antidepressants, including VEN have low toxicity profile (29, 31). However, higher dose can lead to serious side effects. Further investigation suggests that VEN has higher toxicity compared to selective serotonin reuptake inhibitors ($SSRI_S$) such as paroxetine and fluoxetine (23). Overdoses with VEN can lead to a number of adverse effects such as tachycardia, sedation, hypertension, seizures and serotonin syndrome (32). In addition, it has been suggested that VEN may be more toxic in CYP2D6 poor metabolisers (29, 30). Therefore, CYP2D6 poor metabolisers or who are taking interacting drugs may achieve higher drug concentration similar to those found in overdose.

2.2.6 Need for Enantioselectivity Study

The drug is available as a racemic mixture of an equimolar ratio of R (-) and S (+) stereoisomers (20). It is well known that the enantiomers of chiral drugs can present differences in pharmacological activity or efficacy. Animal studies have shown that the pharmacological activity of VEN resides in both the enantiomeric forms. The metabolite ODV also shows the same pharmacological response as that of VEN. Hence, for pharmacokinetic and biodistribution studies of enantiomers of VEN and ODV, simultaneous and enantioselective bio-analytical technique has to be developed.

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Chapter 3

Analytical Method Development and Validation

3.1 Introduction

Analysis is an essential and integrated part of any pharmaceutical product design. Analytical methods significantly influence the product design and development process and it is essential to ensure that the employed analytical methods provide accurate and reliable information. Thus, it is important to develop a simple, sensitive and accurate analytical method for assessing critical quality and performance attributes (1-3).

The science of analyzing chemical charateristics such as identity and purity is well established and still advancing with use of hyphenated analytical techniques coupled with mathematical principles such as multivariate design, design of experimentation etc (4, 5). Although estimation of pure drug(s) in pharmaceutical product is complex with one or more product components such as excipients and degradation substances, the liquid chromatography remains the most common technique for potency and impurity analysis.

With the FDA's process analytical technology (PAT) initiative, the current view of 'quality by design' is further strengthened by stating that quality should be built in the product and should not be inspected within. Drug control and regulatory agencies of several countries have recognized the importance of analytical sciences in quality product design and development and have released extensive guidelines on validation requirements in recent years (6, 7, 8, 9).

Although analytical validation requirements depend upon the type of analyte and analytical instrument, it broadly includes specificity and selectivity, linearity and range, accuracy and precision, sensitivity, reproducibility, stability etc (10).

Literature survey showed that very few analytical methods have been reported for the estimation of ketorolac alone or in combination. They are spectrophotometric (11), flow injection analysis (12), HPLC (13-20), which are either costly or complex process. For routine analysis, a simple, rapid and highly sensitive analytical method is preferred.

Various spectrophotometric methods have been reported for estimation of VEN and ODV. These methods include spectro ultraviolet and spectrofluorimetric methods. Main advantages of these methods are convenience and they are more economical than HPLC, but are less sensitive (21-33). Although chromatographic methods have been reported for determination of VEN and ODV in bulk and pharmaceuticals (34- 41), no method has been reported for simultaneous estimation of VEN and ODV. Also no enantioselective method for determination of VEN and ODV in pharmaceuticals is reported.

The objective of the present study was to develop a simple, accurate, precise and sensitive achiral and chiral (enantioselective) RP-HPLC method for estimation of ketorolac and simultaneous estimation of VEN, ODV and their enantiomers in bulk and formulations. Moreover, in house developed methods were validated as per current regulatory guidelines for analytical methods using suitable statistical tests.

3.2 Analytical Method Development and Validation of Ketorolac

3.2.1 Estimation of Racemic Ketorolac

3.2.1.1 Experimental

a) Materials

Ketorolac tromethamine reference standard was provided by TRC, Canada. Ketorol DT with a 10 mg label claim, manufactured by Dr. Reddys Laboratories Ltd., and eye drop, Acular LS with a labelled claim of 4 mg/mL, manufactured by Allergan were procured from local market. HPLC grade acetonitrile, methanol and analytical grade hydrochloric acid, sodium hydroxide pellets and sodium dihydrogen phosphate were obtained from Merck India Limited, Mumbai, India. High purity deionized water was prepared by TKA smart2pure, Niederelbert, Germany purification system. In addition, an electronic balance (AG-135, Mettler-Toledo, Germany), pH meter (pH tutor, Eutech Instruments, Singapore), a sonicator (Toshiba, New Delhi) were used.

b) Chromatographic System and Conditions

The chromatographic system used to perform development and validation of this method consisted of an LC-20AD binary pump, an SPD-M20A photodiode array detector, SIL 20 AC auto sampler, connected to a communication & Bus module CBM 20A (Shimadzu, Kyoto, Japan). Chromatographic analysis was performed on OYSTER BDS (150×4.6mm id., 5µm particle size) column. Separation was achieved using a mobile phase of sodium di hydrogen phosphate (10mM, pH 5.5): acetonitrile (80:20, v/v) at a flow rate of 1.0 mL/min. The eluent was monitored using PDA detector at wavelengths 322 for ketorolac. The column was maintained at ambient temperature and injection volume of 50 µl was used.

c) Preparation of Stock and Standards

Stock solution was prepared by weighing 5 mg of ketorolac and was transferred to 5 mL volumetric flask and the volume was made up to the mark with methanol to obtain a solution containing 1000 µg/mL of ketorolac. Appropriate aliquots of ketorolac stock

solution were taken in different 10 mL volumetric flasks, and diluted up to the mark with mobile phase to obtain final concentrations of 0.02, 0.1, 0.5, 1.0, 5.0, 10.0, 15.0 µg /mL of ketorolac. The solutions were analysed and chromatograms were recorded. Calibration curve was constructed by plotting average peak area versus ketorolac concentration and regression equation was computed. Similarly quality control (QC) standards were prepared at low (LQC = 0.05 µg/mL), medium (MQC = 4 µg/mL) and high (HQC =12 µg/mL) concentration levels of calibration curve and analysed.

d) Sample Preparation

i) Tablet (Ketorol DT, 10mg)

For commercial tablet of ketorolac, the average weight of 20 tablets was recorded and tablets were powdered and mixed. A quantity equivalent to 10 mg of ketorolac was weighed accurately and was transferred into a 100 mL volumetric flask; 10mL of water was added and sonicated for 10 min. After sonication 40 mL of methanol was added and again sonicated for 10 min and finally volume was made up to the mark by adding methanol. This solution was centrifuged for 10 min. 5 mL of supernatant was taken and volume was made up to 50 mL with mobile phase. From this solution 0.5 mL was taken and volume was made up to 1 mL with mobile phase and this solution was analyzed.

ii) Eye Drop (Acular LS, 4mg/mL, 5mL)

Five mL of ophthalmic solution was transferred into 100 mL volumetric flask and the volume was made by using methanol. Then 5 mL of above solution was taken, the volume was made up to 50 mL with mobile phase. From this solution 0.5 mL was taken and made up to 1 mL with mobile phase and it was analyzed.

e) Method Development

For the development of analytical method mobile phase composition and flow rate were optimized by trying different aqueous phase and non-aqueous phase combinations at different flow rates. Various buffers such as phosphate (pH 3-7 and 10 mM, 100 mM), citrate buffer (pH 3-5 and 10 mM, 100 mM), ammonium acetate buffer (pH 3-7 and 10 mM, 100 mM) and acetic acid buffer (pH 3-5 and 10 mM, 100 mM) were studied in combination with methanol (20, 25 and 30 %), acetonitrile (20, 25 and 30 %) and isopropanol (20, 25 and 30 %). Mobile phase composition and flow rate were finally selected based on the criteria of peak properties (retention time and asymmetric factor) and sensitivity (height and area).

f) Method Validation

The method was validated for the parameters like selectivity, linearity, accuracy, precision, detection limit, quantification limit and robustness. The method was applied for drug content analysis from commercial product.

i) Selectivity

Selectivity of the method was accessed by placebo and spiked placebo analysis technique. Placebo and formulation standards were prepared in triplicate and processed as described in sample preparation. Obtained chromatograms were compared with the fresh calibration standards.

ii) Linearity

It was obtained by preparing three sets of the drug solutions in mobile phase containing ketorolac at a concentration of 0.02 - 15 µg/mL; 50 µL of these drug solutions were injected in to column and the peak area and retention time was recorded. Average peak area was plotted against concentration and curves were subjected to linear regression analysis by least square method. Regression equation was used to calculate the corresponding predicted concentration. One way analysis of variance (ANOVA) was performed on each replicate response.

iii) Accuracy and Precision

Accuracy was assessed by placebo spiking and standard addition method. In placebo spiking method, a known amount of pure drug standard, at three concentration levels 75, 100 and 125% of labeled claim of tablet, was added to placebo blank. Similarly, a known amount of pure drug standard was added to pre-analysed sample at two concentration level, 50 and 100 % of the labeled claim. At each concentration, three sets were prepared and analyzed. Results are expressed as % Bias and % RSD. Precision was determined through repeatability (intra-batch) and intermediate (inter-batch) precision. Study was conducted by using quality control (QC) standards prepared at lower (0.05 µg/mL), medium (4 µg/mL) and higher (12 µg/mL) concentration levels. Precision of the method was expressed as percent relative standard deviation (% RSD).

For repeatability (intra batch) and intermediate precision (inter-batch) three replicates of the QC standards were prepared fresh and analysed at three different occasions. The intra batch and inter-batch assay results are expressed as % RSD.

iii) Sensitivity

Sensitivity was determined based on standard deviation of intercept **(**σ) and slope (s) of the calibration curve. The limit of detection (LOD) and limit of quantification (LOQ) were calculated using following formulae: LOD = 3.3σ/s and LOQ = 10 σ/s, where σ = standard deviation of intercept of calibration curve and s = average of the slope of the calibration curves.

iv) Robustness

Robustness study was conducted by making small but deliberate changes to the optimized method parameters. Flow rate, pH of mobile phase and % organic phase in mobile phase were changed at two levels individually, one below and one above the optimized value. Flow rate was changed by 0.1 mL/min, pH by 1 unit and % organic phase by 5%. ANOVA was applied to show that changes in these critical parameters does not change the obtained responses significantly.

v) System Suitability and Drug Stability

As an integral part of the analytical procedure, various chromatographic performance parameters such as tailing factor and number of theoretical plates (T_f, N) were recorded in system suitability study. Bench-top stability of the drug solution was performed for 8.5 h. The stock solution stability of drug was evaluated at refrigerated temperature for 16 days by comparing the response of stability stock with fresh stock.

vi) Analysis of Formulation

Samples prepared from the commercial tablet and eyedrop were analysed in triplicate for drug content.

3.2.1.2 Results and Discussion

a) Analytical Method Development

In the preliminary study, peak properties and response function were optimized by changing type of organic modifier, organic to aqueous ratio, buffer type, buffer strength and pH. Among the buffers, phosphate buffer gave peak with good symmetry and resolution. Optimum concentration of phosphate buffer was found to be 10 mM because increasing the concentration led to peak tailing and decreased the resolution, and use at lower concentration decreased its sensitivity. Reduction in pH led to peak asymmetry, where pH below 4 showed poor peak properties and loss in selectivity with incomplete resolution. Use of acetonitrile

gave good resolution together with good peak symmetry. Increase in pH above 6.0 showed peak fronting. Optimized concentration of acetonitrile was found to be 20 % of the mobile phase. OYSTER BDS (150×4.6mm id., 5µm particle size) column was used for better peak symmetry and enhanced stability. Moreover, wavelength was optimized at 322 nm for better sensitivity and selectivity. Thus optimized mobile phase consisted of 10 mM sodium di hydrogen phosphate (pH 5.5): acetonitrile (80:20, v/v) with better peak properties, selectivity and reproducibility.

b) Analytical Method Validation

i) Selectivity

Placebo samples showed no interference within the vicinity of the ketorolac peak, which indicated selectivity of the method for ketorolac in presence of formulation excipients shown in fig 3.1. Representative chromatogram is shown in fig 3.2.

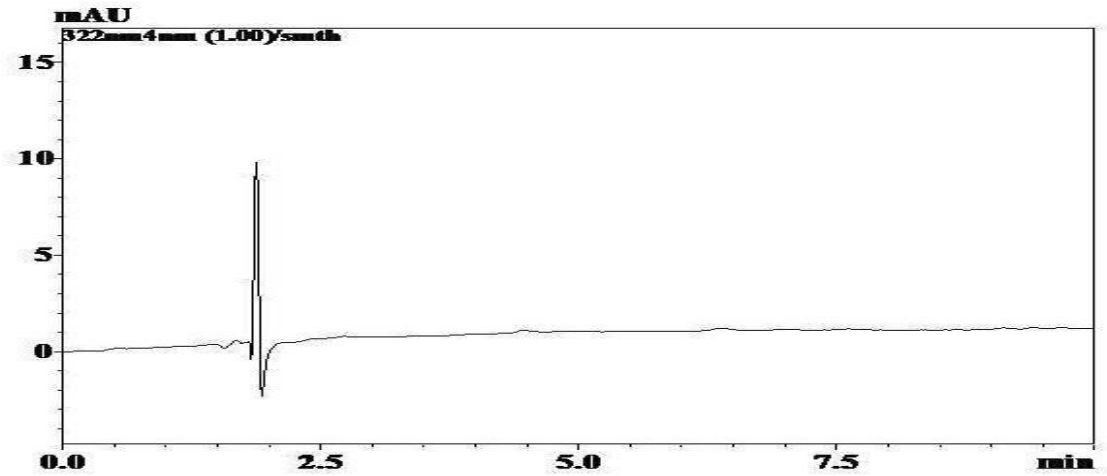

Fig 3.1: Representative chromatogram of placebo sample.

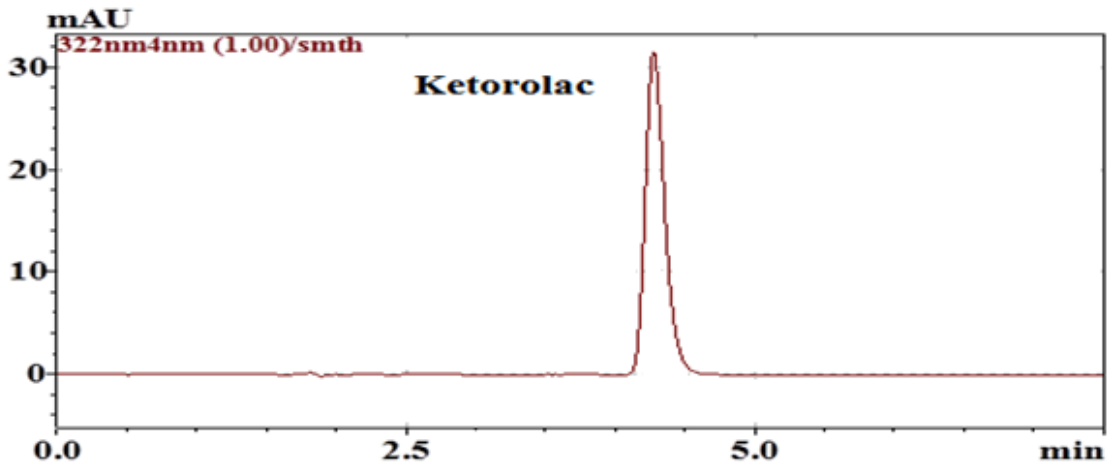

Fig 3.2: Representative chromatogram of ketorolac of 1µg/mL.

ii) Linearity and Range

The linearity regression analysis indicated linear relationship between average peak area and concentration over the range 0.02 – 15 µg/mL with weighted regression equation (Table 3.1).

peak area =165010 x concentration (µg/mL) + 130 (weighted $1/x^2$) ; $R^2 = 0.9997$

Table 3.1: Calibration curve of ketorolac

Conc (µg/mL)	Mean peak area[a]	S.D	% RSD
0.02	3410.000	35.511	1.041
0.1	17190.667	273.908	1.593
0.5	81607.667	910.085	1.115
1	163840.000	1538.247	0.939
5	824294.333	9111.618	1.105
10	1652143.333	28754.632	1.740
15	2456583.667	40524.498	1.650

[a] Each value represents the average of three independent determinations.

One way ANOVA was performed for peak area obtained at individual concentration and F (1.453×10^{-4}) value was found to be much lower than theoretical F (3.555) value at 5 % level of significance. Therefore there was no significant difference between the measured calibration curve standards.

iii) Accuracy and Precision

The method showed consistent and high absolute recoveries at all concentrations with mean absolute recovery ranging from 98.350 % - 99.435 %. There was no significant interference of excipients and the method was found to be accurate with low % bias of 0.565 – 1.650 (Table 3.2). Recovery study indicated that the method was suitable for determination of ketorolac from tablets and eye drops. Method was found to be precise with % RSD not exceeding 1.113 and 1.162 for intra and inter batch, respectively (Table 3.3).

Table 3.2: Recovery studies by placebo spiking technique and standard addition method

Analyte	Technique	Amount of drug added (% Label claim)	Mean[a] absolute recovery (%)	% RSD	% Bias
Ketorolac	Placebo spiking	75	98.988	1.134	1.012
		100	98.350	1.490	1.650
		125	99.214	1.667	0.786
	Standard addition	50	99.435	1.769	0.565
		100	98.893	1.320	1.107

[a] Each value represents the average of three independent determinations.

Table 3.3: Results of repeatability and intermediate precision study

Nominal Conc (µg/mL)	Repeatability (n=3)		Intermediate (n=9)	
	Mean	% RSD	Mean	% RSD
0.05	0.049	0.564	0.049	1.162
4.00	3.984	1.113	3.989	0.910
12.00	11.985	0.822	11.974	0.557

iv) Sensitivity

The LOD and LOQ were found to be 0.0004 µg/mL and 0.001 µg/mL, respectively. Upon repeated injection at quantitation limit, the peak properties (retention time, peak area, and tailing factor) were not affected and mean absolute recovery was consistently high and acceptable with low % bias and % RSD. Thus, the method was found to be highly sensitive.

v) Robustness

Robustness study indicated that obtained responses remains unaffected by the small changes in critical method parameters such as flow rate, pH of mobile phase and % organic phase in mobile phase (Table 3.4).

Table 3.4: Results of robustness study

Factor	Level	Area[a]	Asymmetry[b]
Flow rate (mL/min)		F value calculated	F value calculated
0.9	-1	0.288	0.03614
1.0	0		
1.1	+1		
pH of mobile phase			
5.4	-1	0.33	1.182
5.5	0		
5.6	+1		
% Organic phase			
15%	-1	0.046	0.4444
20%	0		
25%	+1		

[a, b] Each value represents the average of three independent determinations.

F tabulated (5.143) is greater than F calculated in all the cases, therefore there is no significant difference between the area, asymmetry and resolution values obtained by deliberately changing the critical method parameters such as flow rate, pH of mobile phase and % organic phase.

vi) System Suitability and Drug Stability

The method was found to be suitable in terms of system performance as obtained values for suitability parameters such as tailing factor and number of theoretical plates (T_f, N) was within acceptable limits. The method showed better peak symmetry. System suitability study confirmed that the method was specific, precise, and stable for determination of ketorolac. Further, the drug peak exhibited no chromatographic or response change for 16 days at refrigerated temperature when compared against freshly prepared standards (Table 3.5).

Table 3.5: Results of stability studies

Storage period and Storage condition	Nominal Conc (µg/mL)	Mean[a]	% RSD	% Accuracy
Stock solution ~ 16 days, refrigerated temperature	0.05 12.00	0.049 11.984	0.607 0.437	98.818 99.866
Bench top ~ 8.5 h, room temperature	0.05 12.00	0.050 11.990	0.741 0.305	99.610 99.918

[a] Each value represents the average of three independent determinations.

vii) Analysis of Formulation

The mean recoveries for each formulation were found to be in good agreement with the labeled claim of individual products. The method was found to be accurate with mean absolute recovery of 99.93 % for tablet and 99.65 % for tablets and eye drops respectively (Table 3.6). Summary of the validation parameter is given in table 3.7.

Table 3.6: Assay data of ketorolac tromethamine dosage forms

Dosage form	Labeled claim	Found amount (mg)	Mean[a] amount (mg)	% Recovery
Tablet (Ketorol DT, 10 mg)	10 mg	10.04 9.94 9.78	9.93	99.93
Eye drop (Acular LS, 4 mg/mL, 5mL)	4 mg/mL (20 mg)	19.92 19.89 19.94	19.93	99.65

[a] Each value represents the average of three independent determinations.

Table 3.7: Summary of the validation parameters of ketorolac

Parameter	Value
Calibration range	0.02-15 µg/mL
Regression coefficient	$R^2 = 0.9996$
Regression equation	y (peak area) =165010 x concentration (µg/mL) + 130 (weighted $1/x^2$)
Limit of detection	0.0004 µg/mL
Limit of quantification	0.001 µg/mL
Absolute recovery	98.350 % - 99.435 %
Accuracy (% Bias)	0.565 – 1.650 %
Precision (% RSD)	0.564 - 1.113 % (Intraday) 0.557 - 1.162 % (Interday)
System sutability	$T_f = 1.15 \pm 0.025$ $R_t = 4.20 \pm 0.10$ $N = 4578.79 \pm 123.89$
Selectivity	Selective
Robustness	No significant change in the parameters on making small changes in critical parameters

3.2.2 Estimation of Enantiomers of Ketorolac

3.2.2.1 Experimental

a) Material

Ketorolac tromethamine reference standard and pure enantiomers were provided by TRC, Canada. HPLC grade acetonitrile, methanol and analytical grade hydrochloric acid, sodium hydroxide pellets and sodium dihydrogen phosphate were obtained from Merck India Limited, Mumbai, India. High purity deionized water was prepared by TKA smart2pure, Niederelbert, Germany purification system. In addition, an electronic balance (AG-135, Mettler-Toledo, Germany), pH meter (pH tutor, Eutech Instruments, Singapore), a sonicator (Toshiba, New Delhi) were used.

b) Chromatographic System and Conditions

The same HPLC system as mentioned under the method I was used. The column used was Chiral-AGP column (100 x 4.0 mm I.D., particle size 5 µ, Chrom tech Ltd, Sweden). Optimized mobile phase, phosphate buffer pH 4.5; 0.1M: isopropanol (96:4, v/v) was filtered before use through a 0.22 µm membrane filter, degassed in a bath sonicator for 20 min and was pumped from the solvent reservoir to the column at a flow rate of 1mL/min to equilibrate the system. Injection volume used was 50 µl. The back pressure yielded was 75-95 Kgf. The run time was set for 10 min and eluents were monitored at 322 nm.

c) Preparation of Stock and Standards

Primary stock solution of 1000 µg/mL ketorolac racemic mixture was prepared by dissolving 5 mg of racemic ketorolac in a 5 mL volumetric flask containing methanol and the volume was made by the same. Appropriate aliquots of ketorolac stock solution were taken in different 10 mL volumetric flasks, then diluted up to the mark with mobile phase to obtain final concentrations of 0.01, 0.05, 0.25, 0.5, 2.5, 5, and 7.5 µg/mL of R(+)ketorolac and S (-) ketorolac. Similarly three quality control (QC) standards were prepared at low (LQC = 0.025 µg/mL), medium (MQC = 2.00 µg/mL) and high (HQC = 6.00 µg/mL) concentration levels of calibration curve.

d) Analytical Method Development

In the process of analytical method development for R (+) and S (-) ketorolac, mobile phase composition and flow rate were optimized by trying different aqueous phase and non-aqueous phase combinations at different flow rates. The variables which altered the enantioselectivity

when using the chiral AGP column where chosen and effect of each variable was studied. The variables selected were type of buffer, buffer strength, pH of buffer, nature and concentration of organic modifiers. Here the effect of individual variable was studied by keeping all other chromatographic conditions constant. Various buffers such as phosphate (pH 3-7 and10 mM, 100mM), citrate buffer (pH 3-5 and 10 mM, 100 mM), ammonium acetate buffer (pH 3-5 and 10 mM, 100 mM) and acetic acid buffer (pH 3-5 and 10 mM, 100mM) were studied in combination with methanol (2, 4, 8 %), acetonitrile (2, 4, 8 %) and isopropanol (2, 4, 8 %). Mobile phase composition and flow rate were finally selected based on the criteria of peak properties (retention time and asymmetric factor), sensitivity (height and area) and ease of preparation.

e) Analaytical Method Validation

The developed chromatographic method was validated for selectivity, linearity, range, precision, accuracy, sensitivity and robustness.

i) Selectivity

Selectivity of the method was accessed by placebo and spiked placebo analysis technique. Placebo and formulation standards were prepared in triplicate and processed as described in sample preparation section.

ii) Linearity and Range

It was obtained by preparing three sets of the drug solutions in mobile phase containing ketorolac enantiomers at a concentration of 0.01- 7.5 µg/mL; 50 µl of these drug solutions were injected in to column and the peak area and retention time was recorded. Average peak area at each level was plotted against concentration. Regression equation was used to calculate the corresponding predicted concentration. One way analysis of variance (ANOVA) was performed on each replicate response.

iii) Precision and Accuracy

Accuracy was assessed by placebo spiking and standard addition method. In placebo spiking method, a known amount of pure drug standard, at three concentration levels 75, 100 and 125% of labeled claim of tablet, was added to placebo blank. Similarly, a known amount of pure drug standard was added to pre -analysed sample at two concentration level, 50 and 100 % of the labeled claim. At each concentration, three sets were prepared and analyzed. Results are expressed as % Bias and % RSD. Precision was determined through repeatability

(intra-batch) and intermediate (inter-batch) precision. Study was conducted by quality control (QC) standards prepared at lower (0.025 µg/mL), medium (2 µg/mL) and higher (6 µg/mL) concentration levels. Precision of the method is expressed as percent relative standard deviation (% RSD).

For repeatability (intra batch) and intermediate (inter batch), three batches of QC standards were prepared fresh and analysed at three different time intervals to get intra batch and inter batch precision. The intra batch and inter batch assay results are expressed as % RSD.

iv) Sensitivity

Sensitivity was determined based on standard deviation of intercept **(**σ) and slope (s) of the calibration curve. The limit of detection (LOD) and limit of quantification (LOQ) were calculated using following formulae: LOD = 3.3σ/s and LOQ = 10 σ/s, where σ = standard deviation of intercept of calibration curve and s = average of the slope of the calibration curves.

v) Robustness

Robustness study was conducted by making small but deliberate changes to the optimized method parameters. Flow rate, pH of mobile phase and % organic phase in mobile phase was changed at two levels one below and one above the optimized value. Flow rate was changed by 0.1 mL/min, pH by 0.1 unit and percent organic phase by 1%. ANOVA was applied to show that change in these critical parameters does not change the obtained responses significantly.

vi) System Suitability and Drug Stability

As an integral part of the analytical procedure, various chromatographic performance parameters tailing factor and number of theoretical plates (T_f, N) were recorded in system suitability study. Bench-top stability of the drug solution was performed for 8.5 h. The stock solution stability of drug was evaluated at refrigerated temperature for 16 days by comparing the response of stability stock with fresh stock.

3.2.2.2 Results and Discussion

a) Method Development

Among the buffers, phosphate buffer gave the peak with good symmetry and resolution, and thus phosphate buffer was selected for method development. Optimum concentration of phosphate buffer was found to be 100 mM because increasing concentration leads to peak

tailing and decreases the resolution and use at reduced concentration decreases its sensitivity. Increase in the pH increased the negative charge on column and thus retention of ketorolac decreased, and it eluted much faster. Decreasing the pH led to opposite effect. Optimized pH was found to be 4.5 by several trials. The use of organic modifier also played a major role. IPA gave good resolution together with good peak symmetry. The maximum amount of organic modifier to be used was 20 % of mobile phase, as the concentration increased the resolution increased and the retention time decreased. The optimized mobile phase was found to be 96:4 (0.1M NaH_2PO_4, pH 4.5: IPA). The order of elution was found to be the R (+) enantiomer followed by S (-) enantiomer and their retention time were 3.45 min and 4.62 min respectively. During the development of the method on the chiral AGP column, it was shown that method was selective because the enantiomers were separated to baseline (R_S=2.65).

b) Method Validation

i) Selectivity

Placebo samples showed no interference within the vicinity of the ketorolac enantiomer peak, which indicated selectivity of the method for ketorolac enatiomers in presence of formulation excipients (Fig 3.3). Representative chromatograms of R (+) ketorolac, S (-) ketorolac, and racemic mixture is given in fig 3.4-3.6 respectively.

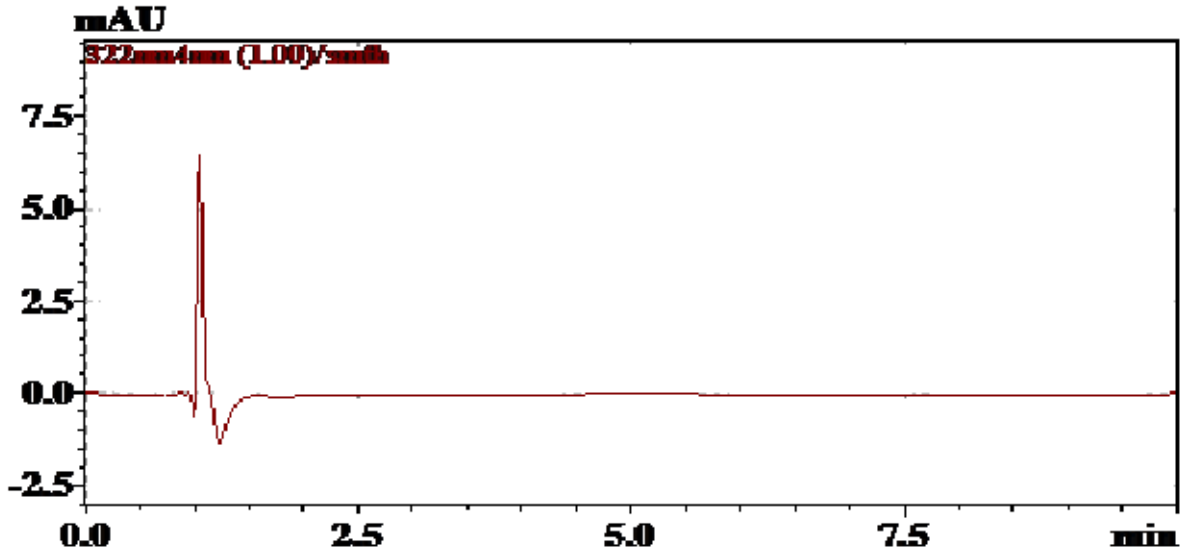

Fig 3.3: Representative chromatogram of placebo sample.

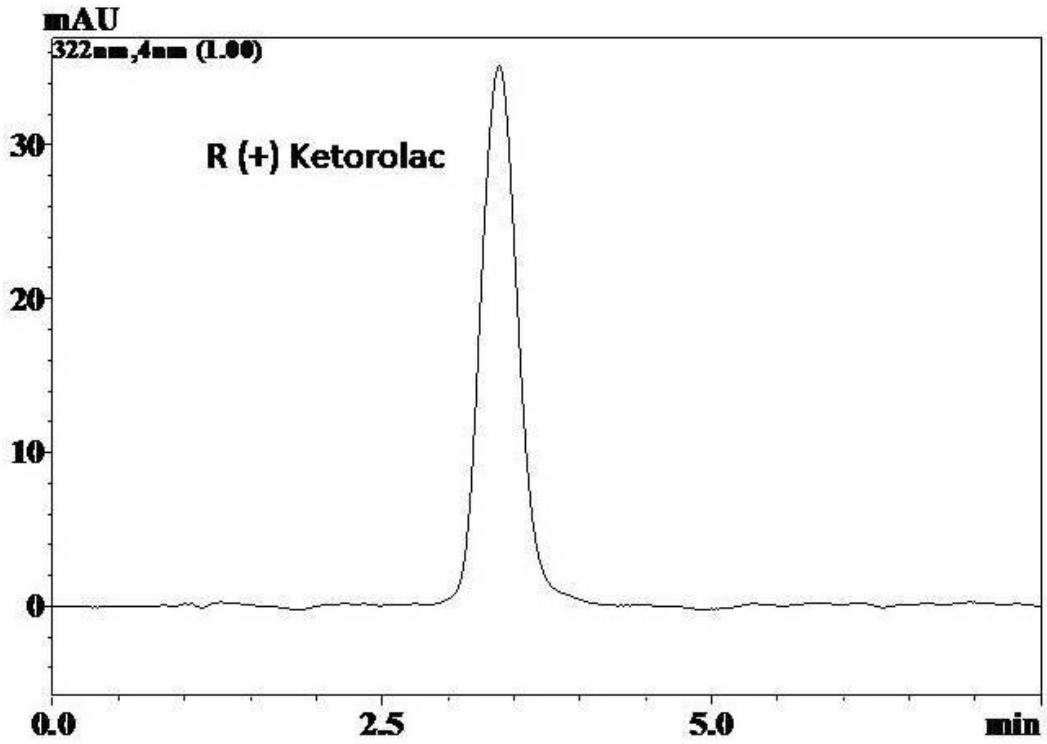

Fig 3.4: Representative chromatogram of pure R (+) enantiomer of ketorolac (2 µg/mL).

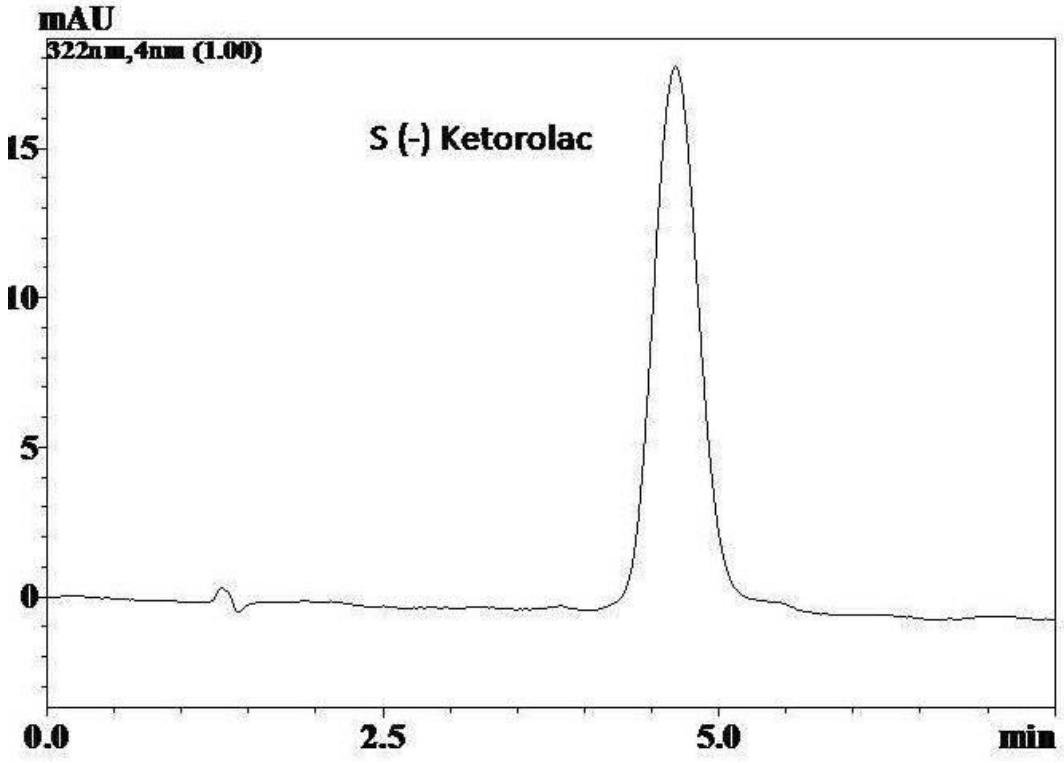

Fig 3.5: Representative chromatogram of pure S (-) enantiomer of ketorolac (1µg/mL).

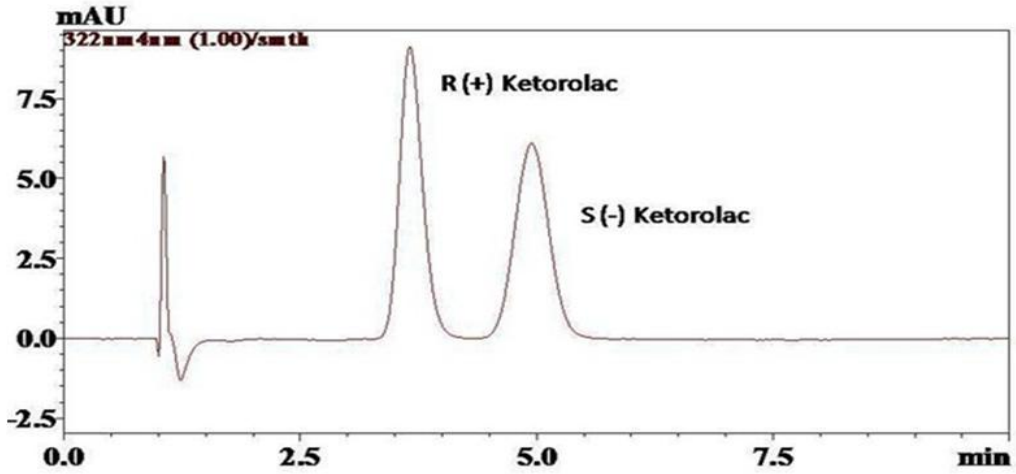

Fig 3.6: Chromatogram showing the two completely resoluted peaks of R (+) and S (-) ketorolac (0.5µg/mL each).

ii) Linearity and range

The linearity regression analysis indicated linear relationship between average peak area and concentration over the range 0.01 – 7.5µg/mL with weighted regression equation (Table 3.8 and 3.9).

peak area =163806 x concentration (µg/mL) + 64.02 (weighted $1/x^2$); R^2 = 0.9995 for R (+) ketorolac

peak area =164428 x concentration (µg/mL) + 54.92 (weighted $1/x^2$); R^2 = 0.9995 for S (-) ketorolac

Table 3.8: Calibration curve of R (+) enantiomer of ketorolac

Conc (µg/mL)	Mean peak area[a]	S.D	% RSD
0.01	1692.333	20.984	1.240
0.05	8540.667	97.167	1.138
0.25	40232.333	635.841	1.580
0.5	80869.000	1535.834	1.899
2.5	407107.000	3758.607	0.923
5	822965.000	10505.967	1.277
7.5	1234556.333	15101.323	1.223

[a] Each value represents the average of three independent determinations.

Table 3.9: Calibration curve of S (-) enantiomer of ketorolac

Conc (µg/mL)	Mean peak area[a]	S.D	% RSD
0.01	1688.667	22.189	1.314
0.05	8583.333	79.027	0.921
0.25	40242.000	642.704	1.597
0.5	81637.667	577.497	0.707
2.5	408232.333	2481.081	0.608
5	825845.000	12407.950	1.502
7.5	1235360.667	13025.899	1.054

[a] Each value represents the average of three independent determinations.

One way ANOVA was performed for peak area obtained at individual concentration and F= 1.415×10^{-4} value for R (+) ketorolac and 3.830×10^{-4} for S (-) ketorolac were found to be much lower than F tabulated (3.555) at 5 % level of significance. Therefore there was no significant difference between the measured calibration curve standards for both the enantiomers.

iii) Accuracy and Precision

The method showed consistent and high absolute recoveries at all the five concentration levels with mean absolute recovery ranging from 98.245 % - 99.735 % for R (+) ketorolac and 98.417 % - 99.606 % for S (-) ketorolac. There was no significant interference of excipients and the method was found to be accurate with low % bias of 0.265 -1.755 and 0.394 -1.583 for R (+) ketorolac and S (-) ketorolac respectively (Table 3.10). Method was found to be precise with % RSD not exceeding 1.791 and 1.363 (intra batch) and 1.651 and 1.353 (inter batch), for R (+) ketorolac and S (-) ketorolac respectively (Table 3.11).

Table 3.10: Recovery study by placebo spiking technique and standard addition method for R (+) and S (-) ketorolac

Analyte	Technique	Amount of drug added (% Label claim)	Mean[a] absolute recovery (%)	% RSD	% Bias
R (+) Ketorolac	Placebo spiking	75	98.533	1.549	1.467
		100	98.245	1.101	1.755
		125	99.032	1.664	0.968
	Standard addition	50	98.668	0.854	1.332
		100	99.735	1.528	0.265

Analyte	Technique	Amount of drug added (% Label claim)	Mean[a] absolute recovery (%)	% RSD	% Bias
S (-) Ketorolac	Placebo spiking	75	98.445	1.206	1.555
		100	98.574	1.125	1.426
		125	99.606	1.331	0.394
	Standard addition	50	98.417	0.681	1.583
		100	99.597	1.243	0.403

[a] Each value represents the average of three independent determinations.

Table 3.11: Results of repeatability and intermediate precision study

Analyte	Nominal Conc (µg/mL)	Repeatability (n=3)		Intermediate Precision (n=9)	
		Mean	% RSD	Mean	% RSD
R (+) Ketorolac	0.025	0.025	1.791	0.025	1.505
	2.000	1.978	1.333	1.963	1.615
	6.000	5.959	0.929	5.961	0.852
S (-) Ketorolac	0.025	0.025	1.363	0.025	1.353
	2.000	1.963	1.121	1.967	1.015
	6.000	5.962	0.569	5.968	0.809

iv) Sensitivity

The LOD and LOQ were 0.001 µg/mL and 0.002 µg/mL respectively for both the enantiomers. Upon repeated injection at quantitation limit, the peak properties (retention time, peak area, and tailing factor) were not affected and mean absolute recovery was consistently high and acceptable with low % bias and % RSD. Thus, the method was found to be highly sensitive.

v) Robustness

Robustness study indicated that obtained responses remain unaffected by the small changes in critical method parameters such as flow rate, pH of mobile phase and % organic phase in mobile phase composition (Table 3.12).

vi) System suitability and drug stability

The method was found to be suitable in terms of system performance as obtained values for primary suitability parameters such as tailing factor, resolution, number of theoretical plates were above acceptable limits. The method showed better peak symmetry. System suitability

study confirmed that the method was specific, precise, and stable for determination of ketorolac (Table 3.14). Further, the drug peak exhibited no chromatographic or response change for 16 days at refrigerated temperature when compared against freshly prepared standards (Table 3.13). Summary of the validation parameter of R (+) and S (-) ketorolac is given in Table 3.14.

Table 3.12: Results of robustness studies

Factor	Level	Area[a]		Asymmetry[b]		Resolution[c]
Flow rate (mL/min)		R (+)	S (-)	R (+)	S (-)	
		F value calculated		F value calculated		F value calculated
0.9 1.0 1.1	-1 0 +1	0.0756	0.690	0.5714	0.2727	0.1489
pH of mobile phase						
4.4 4.5 4.6	-1 0 +1	0.153	0.170	0.1429	0.120	0.1489
% Organic phase						
3% 4% 5%	-1 0 +1	0.070	0.577	1.400	0.654	0.6129

[a, b, c] Each value represents the average of three independent determinations.

F tabulated (5.143) is greater than F calculated in all the cases, indicating there are no significant difference between the area, asymmetry and resolution values obtained by deliberately changing the critical method parameters such as flow rate, pH of mobile phase and % organic phase of mobile phase.

Table 3.13: Results of stability studies

Storage period and Storage condition	Analyte	Nominal Conc (µg/mL)	Mean[a]	% RSD	% Accuracy
Stock solution ~ 16 days, refrigerated temperature	R (+) Ketorolac	0.025	0.025	1.463	98.734
		6.000	5.951	0.870	99.176
	S (-) Ketorolac	0.025	0.025	1.313	98.509
		6.000	5.954	0.904	99.226

Storage period and Storage condition	Analyte	Nominal Conc (µg/mL)	Mean[a]	% RSD	% Accuracy
Bench top ~ 8.5 h, room temperature	R (+) Ketorolac	0.025	0.025	1.574	98.856
		6.000	5.949	1.004	99.155
	S (-) Ketorolac	0.025	0.025	0.605	98.339
		6.000	5.940	0.491	99.002

[a] Each value represents the average of three independent determinations.

Table 3.14: Summary of the validation parameters of ketorolac enantiomers

Parameter	Values	
	R (+) Ketorolac	S (-) Ketorolac
Calibration range	0.01-7.5 µg/mL	0.01-7.5 µg/mL
Regression Coefficient	$R^2 = 0.9995$	$R^2 = 0.999$
Regression equation	y (peak area) =163806 x concentration (µg/mL) + 64.02 (weighted $1/x^2$)	y (peak area) =164428 x concentration (µg/mL) + 54.92 (weighted $1/x^2$)
Limit of detection	0.001 µg/mL	0.001 µg/mL
Limit of quantification	0.002 µg/mL	0.002 µg/mL
Accuracy (% Bias)	0.265 - 1.755 %	0.394 - 1.583 %
Precision (% RSD)	0.929 – 1.791 % (Intraday) 0.852 – 1.615 % (Interday)	0.569 – 1.363 % (Intraday) 0.809 - 1.353 % (Interday)
System sutability	$T_f = 1.11 \pm 0.008$ $R_t = 3.47 \pm 0.004$ $N = 1258.17 \pm 11.64$	$T_f = 1.08 \pm 0.010$ $R_t = 4.64 \pm 0.004$ $N = 1347.51 \pm 4.11$ $R_s = 2.65 \pm 0.002$
Selectivity	Selective	Selective
Robustness	No significant change in the parameters on making small change in critical parameters	No significant change in the parameters on making small change in critical parameters

3.3 Analytical Method Development and Validation of Venlafaxine & O-Desmethyl Venlafaxine

3.3.1 Estimation of Racemic Venlafaxine and O-Desmethyl Venlafaxine

3.3.1.1 Experimental

a) Materials

Pure VEN and ODV were provided by Toronto Research Chemicals Inc. (Canada). Analytical grade sodium hydroxide pellets (NaOH) was obtained from Merck India Limited, Mumbai, India. Both ortho phosphoric acid (H_3PO_4), potassium dihydrogen orthophosphate (KH_2PO_4) were of analytical grade delivered by S.D. Fine Chemicals, India. Venlor XR capsules (75mg) manufactured by Cipla Ltd. India and D-Venz tablets (50mg) manufactured by Sun Pharmaceutical Industries Ltd. India were procured from local market of New Delhi, India. Milli-Q water purification system (Millipore, USA) was used for obtaining high quality HPLC grade water. In addition, an electronic balance (AG-135, Mettler-Toledo, Germany), pH meter (pH tutor, Eutech Instruments, Singapore), a sonicator (Toshiba, New Delhi) were used.

b) Chromatographic System and Conditions

The chromatographic system used to perform development and validation of this method consisted of a LC-2010 CHT (Schimadzu, Japan) high performance liquid chromatographic instrument equipped with RF-20A fluorescence detector. Data collection and integration was accomplished using LC Solutions software. Chromatographic analysis was performed on Eclipse XDB-C18 column (4.6 × 150 mm I.D., particle size 5 µ, Agilent Technologies). Separation was achieved using a mobile phase of 10 mM potassium phosphate pH 4.5: acetonitrile (75:25, v/v) at a flow rate of 1.0 mL/min. The eluent was monitored using fluorescence detector at excitation wavelength of 226 nm and emission wavelength of 298 nm. The column was maintained at ambient temperature and injection volume of 50µl was used.

c) Preparation of Stock and Standards

Primary stock solution of 1mg/mL racemic VEN and ODV were seperately prepared by dissolving 5 mg VEN and ODV in 5 mL volumetric flask containing methanol and the volume was made by methanol. A common standard working stock solutions containing 10, 20, 30, 40, 50, 100, 200 and 400 µg/mL comprising of VEN and ODV were prepared by serial dilution of primary stock solutions with phosphate buffer pH 6.5. Standards containing 0.1, 0.2, 0.6, 1, 1.5, 2.0, 4 and 8 µg/mL of VEN and ODV were prepared by diluting the

working standards. The standards were vortexed for 5 min. Simultaneously Quality Control (QC) samples were prepared in the same manner as that of CC standards for VEN and ODV (0.15, 1.6 and 6.0 µg/mL), representing QC samples at low (LQC), medium (MQC) and high (HQC) levels, respectively.

d) Sample Preparation

Weighed contents of 20 capsules of VEN are taken and triturated in pestle mortar. Powder equivalent to 100 mg of VEN was weighed. This was added to 8 mL of ACN and shaken for 40 min. To this mobile phase was added and shaken for another 20 min. Finally volume was made to 100 mL with mobile phase. Above solution was passed through 0.22 µm filter. 1 mL of filtrated was diluted to 10 mL with mobile phase.

20 tablets of ODV were weighed and triturated in pestle mortar. Powder equivalent to 100 mg of ODV was weighed. This was added to 8 mL of ACN and shaken for 40 min. To this mobile phase was added and shaken for another 20 min. Finally volume was made to 100 mL with mobile phase. Above solution was passed through 0.22 µm filter. 1 mL of filtrate was diluted to 10 mL with mobile phase.

300 µl of the above solutions were diluted to 1 mL with buffer pH 6.5. From this 20 µl was taken and diluted to 1 mL with buffer pH 6.5. 50 µl of this solution was injected.

e) Method Development

In the process of analytical method development for simultaneous determination of VEN and ODV, mobile phase composition and flow rate were optimized by trying different aqueous phase and non-aqueous phase combinations at different flow rates. Various buffers such as phosphate (pH 3-7 and 10 mM, 100 mM), citrate buffer (pH 3-5 and 10 mM, 100 mM), ammonium acetate buffer (pH 3-5 and 10 mM, 100 mM) and acetic acid buffer (pH 3-5 and 10 mM, 100 mM) were studied in combination with methanol (20, 25 and 30 %), acetonitrile (20, 25 and 30 %) and isopropanol alcohol (20, 25 and 30 %). Mobile phase composition and flow rate were finally selected based on the criteria of peak properties (retention time and asymmetric factor), sensitivity (height and area) and ease of preparation and applicability of the method.

e) Method Validation

The method of analysis was validated for the parameters like selectivity, linearity, accuracy, precision, detection limit, quantification limit and robustness. The method was applied for drug content analysis from commercial products.

i) Selectivity

Selectivity of the method was accessed by placebo and spiked placebo analysis technique. Placebo and formulation standards were prepared in triplicate and processed as described in sample preparation. Obtained chromatograms were compared with the fresh calibration standards.

ii) Linearity

It was obtained by preparing three sets of the drug solutions in phosphate buffer pH 6.5 containing VEN and ODV at a concentration of 0.1- 8.0 µg/mL; 50 µl of these drug solutions were injected in to column and the peak area and retention time was recorded. Average peak area at each level was plotted against concentration and curves were subjected to linear regression analysis by least square method. Regression equation was used to calculate the corresponding predicted concentration. One way analysis of variance (ANOVA) was performed on each replicate response obtained at eight concentration levels.

iii) Accuracy and Precision

Accuracy was assessed by placebo spiking and standard addition method. In placebo spiking method, a known amount of pure drug standard, at three concentration levels 75, 100 and 125 % of labeled claim of capsule and tablet, was added to placebo blank. Similarly, a known amount of pure drug standard was added to preanalysed sample at two concentration level, 50 and 100 % of the labeled claim. At each concentration, three sets were prepared and analyzed. Results were expressed as % Bias and % RSD. Precision was determined through repeatability (intra-batch) and intermediate (inter-batch) precision. Study was conducted by quality control (QC) standards prepared at lower (0.150 µg/mL), medium (1.60 µg/mL) and higher (6.00 µg/mL) concentration levels. Precision of the method was expressed as percent relative standard deviation (% RSD). Three batches were run for precision study.

iv) Sensitivity

Sensitivity was determined based on standard deviation of intercept **(**σ) and slope (s) of the calibration curve. The limit of detection (LOD) and limit of quantification (LOQ) were calculated using following formulae: LOD = 3.3σ/s and LOQ = 10 σ/s, where σ = standard deviation of intercept of calibration curves and s = average of the slope of the calibration curves.

v) Robustness

Robustness study was conducted by making small but deliberate changes to the optimized method parameters. Flow rate, pH of mobile phase and % organic phase in mobile phase was changed at two levels one below and one above the optimized value. Flow rate was changed by 0.1mL/min, pH by 0.1 unit and % organic phase by 5 %. ANOVA was applied to show that changes in these critical parameters do not change the obtained responses significantly.

vi) System Suitability and Drug Stability

As an integral part of the analytical procedure, various chromatographic performance parameters (T_f, N, R_s) were recorded in system suitability study. Bench-top stability of the drug solution was performed for 8.5 h. The stock solution stability of drug was evaluated at refrigerated temperature for 16 days by comparing the response of stability stock with fresh stock.

vii) Analysis of Formulation

As a part of validation procedure, the method was tested for drug content analysis of commercial formulations of VEN and ODV.

3.3.1.2 Results and Discussion

a) Analytical Method Development

In the preliminary study, peak properties and response function were optimized by changing type of organic modifier, organic to aqueous ratio, buffer type, buffer strength and pH. Among the buffers phosphate buffer gave peak with good symmetry and resolution. Optimum concentration of phosphate buffer was found to be 10 mM because increasing concentration led to peak tailing and decreased the resolution where as using reduced concentration decreased its sensitivity. Reduction in pH led to peak asymmetry, where pH below 4 showed poor peak properties and loss in selectivity with incomplete resolution. Acetonitrile gave good resolution together with good peak symmetry for both VEN and ODV. Optimized concentration of acetonitrile was found to be 25 % of the mobile phase. Eclipse XDB-C18 column (4.6 × 150 mm I.D., particle size 5 µ, Agilent Technologies) column was used for better peak symmetry and enhanced stability at higher pH. Moreover, excitation and emission wavelength were optimized to 226 and 298 nm, respectively for better sensitivity and selectivity. Thus optimized mobile phase consisted of 10 mM potassium

di hydrogen phosphate pH 4.5: acetonitrile (75:25, v/v) with better peak properties, selectivity and reproducibility.

b) Analytical Method Validation

i) Selectivity

Placebo samples showed no interference within the vicinity of the VEN and ODV peak (Fig 3.7), which indicated selectivity of the method for VEN and ODV in presence of formulation excipients. Representative chromatogram of VEN & ODV is given in fig 3.8.

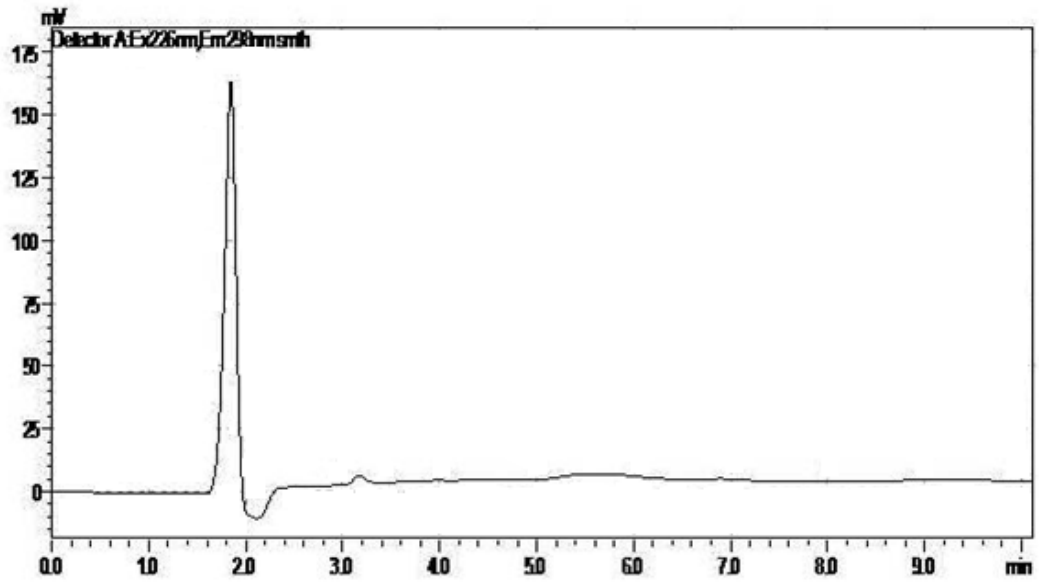

Fig 3.7: Representative chromatogram of placebo sample.

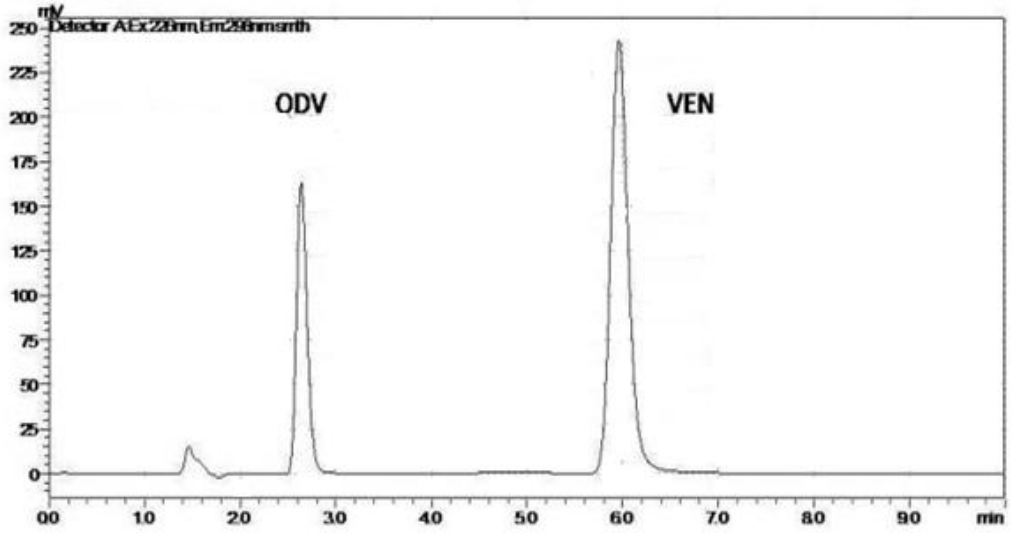

Fig 3.8: Representative chromatogram of VEN and ODV (1.5 µg/mL each).

ii) Linearity and Range

The linearity regression analysis indicated linear relationship between average peak area and concentration over the range 0.1 – 8.0 µg/mL with weighted regression equations (Table 3.15 & 3.16).

peak area = 5954000 x concentration (µg/mL) – 24834 (weighted $1/y^2$) ; R^2 = 0.9999 (VEN)

peak area = 1644000 x concentration (µg/mL) - 2304 (weighted $1/y^2$) ; R^2 =0.9999 (ODV)

Table 3.15: Calibration curve of VEN

Conc (µg/mL)	Mean peak area[a]	S.D	% RSD
0.1	574827.667	7508.358	1.306
0.2	1151146.333	8182.735	0.711
0.6	3495650.667	46527.749	1.331
1	5943190.667	65529.256	1.103
1.5	9035384.000	148972.004	1.649
2	11885366.333	105558.843	0.888
4	23816844.667	295473.446	1.241
8	47712330.667	376886.792	0.790

[a] Each value represents the average of three independent determinations.

Table 3.16: Calibration curve of ODV

Conc (µg/mL)	Mean peak area[a]	S.D	% RSD
0.1	162469.333	2561.417	1.577
0.2	326072.667	2860.420	0.877
0.6	980380.333	8808.404	0.898
1	1634049.667	22046.868	1.349
1.5	2460235.333	29770.076	1.210
2	3273286.000	33230.001	1.015
4	6609890.333	101906.788	1.542
8	13273295.000	97986.047	0.738

[a] Each value represents the average of three independent determinations.

One way ANOVA was performed for peak area obtained at individual concentration and F 3.073×10^{-4} for VEN and 3.758×10^{-4} for ODV values were found to be much lower than theoretical F (3.467) value at 5 % level of significance. Therefore there was no significant difference between the measured calibration curve standards.

iii) Accuracy and Precision

The method showed consistent and high absolute recoveries at all five concentration levels with mean absolute recovery ranging from 98.723 % - 99.781 % for VEN and 98.572 % - 99.798 % for ODV. There was no significant interference of excipients and the method was found to be accurate with low % bias of 0.219- 1.334 % for VEN and 0.202-1.428 % for ODV. Recovery study indicates that the method was suitable for determination of VEN and ODV from capsules and tablets respectively (Table 3.17). Method was found to be precise with % RSD not exceeding 1.761 and 1.672 % (intra batch) and 1.458 % and 1.784 % (Inter batch) for VEN and ODV respectively.

Table 3.17: Recovery studies by placebo spiking technique and standard addition method

Analyte	Technique	Amount of drug added (% Label claim)	Mean[a] absolute recovery (%)	% RSD	% Bias
VEN	Placebo spiking	75	98.666	1.516	1.334
		100	98.723	1.207	1.277
		125	99.583	1.132	0.417
	Standard addition	50	99.061	1.457	0.939
		100	99.781	1.329	0.219
ODV	Placebo spiking	75	98.572	1.199	1.428
		100	99.286	1.011	0.714
		125	98.752	1.385	1.248
	Standard addition	50	99.798	1.481	0.202
		100	99.478	1.093	0.522

[a] Each value represents the average of three independent determinations.

Table 3.18: Results of repeatability and intermediate precision study

Analyte	Nominal Conc (µg/mL)	Repeatability (n = 3)		Intermediate Precision (n = 9)	
		Mean	% RSD	Mean	% RSD
VEN	0.150	0.148	1.761	0.149	1.130
	1.600	1.586	1.450	1.590	1.458
	6.000	5.985	1.157	5.956	0.858
ODV	0.150	0.149	1.672	0.148	1.188
	1.600	1.590	0.897	1.588	1.784
	6.000	5.990	1.195	5.990	0.765

iv) Sensitivity

The LOD and LOQ were 0.002 µg/mL and 0.007 µg/mL for VEN and 0.005 µg/mL and 0.0150 µg/mL for ODV. Upon repeated injection at quantitation limit, the peak properties (retention time, peak area, and tailing factor) were not affected and mean absolute recovery was consistently high and acceptable with low % bias and % RSD. Thus, the method was found to be highly sensitive.

v) Robustness

Robustness study indicated that obtained responses remains unaffected by the small changes in critical method parameters such as flow rate, pH of mobile phase and % organic content in mobile phase (Table 3.19).

vi) System Suitability and Drug Stability

The method was found to be suitable in terms of system performance as obtained values for primary suitability parameters such as tailing factor, resolution, number of theoretical plates were above acceptable limits. The method showed better peak symmetry. System suitability study confirmed that the method was specific, precise, and stable for determination of VEN and ODV (Table 3.22). Further, the drug peak exhibited no chromatographic or response change for 16 days at refrigerated temperature when compared against freshly prepared standards (Table 3.20).

vii) Analysis of Formulation

The mean recoveries for each formulation were found to be in good agreement with the labeled claim of individual products. The method was found to be accurate with mean absolute recovery of VEN and ODV as 99.93 and 99.88%, respectively (Table 3.21). Summary of validation parameters is given in table 3.22.

Table 3.19: Results of Robustness Study

Factor	Level	Area[a]		Asymmetry[b]		Resolution[c]
Flow rate (mL/min)		VEN	ODV	VEN	ODV	F value calculated
		F value calculated		F value calculated		
0.9 1.0 1.1	-1 0 +1	0.319	0.023	0.7742	0.5808	0.1110

Factor	Level	Area[a]		Asymmetry[b]		Resolution[c]
		VEN	ODV	VEN	ODV	F value calculated
		F value calculated		F value calculated		
pH of mobile phase						
4.4 4.5 4.6	-1 0 +1	0.078	0.002	0.4414	0.3022	0.8749
% Organic phase						
20 % 25 % 30 %	-1 0 +1	0.193	0.042	0.3925	0.7697	0.5437

[a, b, c] Each value represents the average of three independent determinations.

F tabulated (5.143) is greater than F calculated in all the cases, therefore there is no significant difference between the area, asymmetry and resolution values obtained by deliberately changing the critical method parameters such as flow rate, pH of mobile phase and % organic phase.

Table 3.20: Results of stability studies

Storage period and Storage condition	Analyte	Nominal Conc (µg/mL)	Mean[a]	% RSD	% Accuracy
Stock solution ~ 16 days, refrigerated temperature	VEN	0.15	0.148	1.292	98.889
		6.00	5.968	0.605	99.469
	ODV	0.15	0.148	1.537	98.776
		6.00	5.995	1.184	99.922
Bench top ~ 8.5 h, room temperature	VEN	0.15	0.149	1.400	99.385
		6.00	5.973	0.749	99.874
	ODV	0.15	0.149	1.405	99.051
		6.00	5.992	1.183	99.874

[a] Each value represents the average of three independent determinations.

Table 3.21: Assay data of dosage forms

Dosage form	Label claim	Found amount (mg)	Mean[a] amount (mg)	% Recovery
VENLOR XR capsules (VEN)	75 mg	74.98 74.92 74.95	74.95	99.93
D- VENIZ tablets (ODV)	50 mg	49.94 49.92 49.96	49.94	99.88

[a] Each value represents the average of three independent determinations.

Table: 3.22 Summary of the validation parameters of VEN and ODV

Parameter	Values	
	VEN	ODV
Calibration range	0.1 – 8.0 µg/mL	0.1 - 8.0 µg/mL
Regression coefficient	$R^2 = 0.9999$	$R^2 = 0.9999$
Regression equation	y (peak area) = 5954000 x concentration (µg/mL) – 24834 (weighted $1/y^2$)	y (peak area) = 1644000 x concentration (µg/mL) - 2304 (weighted $1/y^2$)
Limit of detection	0.002 µg/mL	0.005 µg/mL
Limit of quantification	0.007 µg/mL	0.015 µg/mL
Absolute recovery/ Recovery efficiency	98.723 - 99.781 %	98.572 - 99.798%
Accuracy (% Bias)	0.219-1.334	0.202- 1.428
Precision (% RSD)	1.157 – 1.761 (Intraday) 0.858 - 1.458 (Interday)	1.672 – 1.879 (Intraday) 0.765 – 1.784 (Interday)
System sutability	$T_f = 1.16 \pm 0.01$ $R_t = 5.95 \pm 0.005$ $N = 4824 \pm 6.98$ $R_s = 2.52 \pm 0.03$	$T_f = 1.09 \pm 0.01$ $R_t = 2.64 \pm 0.004$ $N = 2366 \pm 8.18$
Selectivity	Selective	Selective
Robustness	No significant change in the parameters on making small change in critical parameters	No significant change in the parameters on making small change in critical parameters

3.3.2 Estimation of Enantiomers of VEN & ODV

3.3.2.1 Experimental

a) Material

Pure VEN, ODV and their enantiomers were provided by Toronto Research Chemicals Inc. (Canada). Analytical grade sodium hydroxide pellets (NaOH) was obtained from Merck India Limited, Mumbai, India. Both orthophosphoric acid (H_3PO_4), potassium dihydrogen orthophosphate (KH_2PO_4) were of analytical grade delivered by S.D. Fine Chemicals, India. Milli-Q water purification system (Millipore, USA) was used for obtaining high quality HPLC grade water. In addition, an electronic balance (AG-135, Mettler-Toledo, Germany), pH meter (pH tutor, Eutech Instruments, Singapore), a sonicator (Toshiba, New Delhi) were used.

b) Chromatographic system and conditions

The chromatographic system used to perform development and validation of this method consisted of a LC-2010 CHT (Schimadzu, Japan) high performance liquid chromatographic instrument equipped with RF-20A fluorescence detector. Data collection and integration was accomplished using LC Solutions software. The column used was Chiral-AGP column (100 x 4.0 mm I.D., particle size 5 µ, Chrom tech Ltd, Sweden). Mobile phase, 10 mM phosphate buffer pH 6.5: methanol (94:6, v/v) was filtered through a 0.22 µm membrane filter before use, degassed in a bath sonicator for 20 min and was pumped from the solvent reservoir to the column at a flow rate of 1mL/min to equilibrate the system. Then a sample, injection volume of 50 µl was given. The run time was set for 20 min and eluents were monitored at excitation wavelength of 226 nm and emission wavelength of 298 nm.

c) Preparation of Stock and Standards

Primary stock solution of 1mg/mL VEN and ODV racemic was prepared by dissolving 5 mg of racemic VEN and ODV in a 5 mL volumetric flask containing methanol and the volume was made by the same. A common standard working stock solution containing 10, 20, 30, 40, 50, 100, 200 and 400 µg/mL of comprising VEN and ODV were prepared by serial dilution of primary stock solutions with phosphate buffer pH 6.5. Calibration curve standards containing 0.05, 0.1, 0.3, 0.5, 0.75, 1, 2 and 4 µg/mL of each enantiomer of VEN and ODV were prepared by diluting working stock solution. Simultaneously Quality Control (QC) samples were prepared in the same manner as that of CC standards for each R (-) VEN, S (+)

VEN, R (-) ODV and S (+) ODV (0.075, 0.80 and 3.0 µg/mL), representing QC samples at low (LQC), medium (MQC) and high (HQC) levels, respectively.

d) Analytical Method Development

In the process of analytical method development for enantiomers of VEN and ODV, mobile phase composition and flow rate were optimized by trying different aqueous phase and non-aqueous phase combinations at different flow rates. Various buffers such as phosphate (pH 3-7 and 10 mM, 100 mM), citrate buffer (pH 3-5 and 10 mM,100 mM), ammonium acetate buffer (pH 3-5 and 10 mM, 100 mM) and acetic acid buffer (pH 3-5 and 10 mM, 100 mM) were studied in combination with methanol (2, 4, 6 and 8 %), acetonitrile (2, 4, 6 and 8 %) and isopropanol (2, 4, 6 and 8 %). Mobile phase composition and flow rate were finally selected based on the criteria of peak properties (retention time and asymmetric factor), sensitivity (height and area) and ease of preparation.

e) Analytical Method Validation

The developed chromatographic method was validated for selectivity, linearity, range, precision, accuracy, sensitivity and robustness.

a) Selectivity

Selectivity of the method was accessed by placebo and spiked placebo analysis technique. Placebo and formulation standards were prepared in triplicate and processed as described in sample preparation. Obtained chromatograms were compared with the fresh calibration standards.

b) Linearity and Range

It was obtained by preparing three sets of the drug solutions in mobile phase containing VEN and ODV enantiomers at a concentration of 0.050 – 4 µg/mL; 50 µl of these drug solutions were injected into column and the peak area and retention time was recorded. Average peak area at each level was plotted against concentration and curves were obtained.

Regression equation was used to calculate the corresponding predicted concentration. One way analysis of variance (ANOVA) was performed on each replicate response obtained at eight concentration levels.

c) Precision and Accuracy

Accuracy was assessed by placebo spiking and standard addition method. In placebo spiking method, a known amount of pure drug standard, at three concentration levels 75, 100 and 125% of labeled claim of capsule and tablet, was added to placebo blank. Similarly, a known amount of pure drug standard was added to pre -analysed sample at two concentration level, 50 and 100 % of the labeled claim. At each concentration, three sets were prepared and analyzed. Results were expressed as % Bias and % RSD.

Precision was determined through repeatability (intra-batch) and intermediate (inter-batch) precision. Study was conducted by quality control (QC) standards prepared at lower (0.075 µg/mL), medium (0.80µg/mL) and higher (3.0 µg/mL) concentration levels. Precision of the method was expressed as percent relative standard deviation (% RSD). Three batches were run for precision study.

d) Sensitivity

Sensitivity was determined based on standard deviation of intercept **(**σ) and slope (s) of the calibration curve. The limit of detection (LOD) and limit of quantification (LOQ) were calculated using following formulae: LOD = 3.3σ/s and LOQ = 10 σ/s, where σ = standard deviation of intercept of calibration curves and s = average of the slope of the calibration curves.

e) Robustness

Robustness study was conducted by making small but deliberate changes to the optimized method parameters. Flow rate, pH of mobile phase and % organic phase in mobile phase was changed at two levels one below and one above the optimized value. Flow rate was changed by 0.1mL/min, pH by 0.1 unit and % organic phase by 1 %. ANOVA was applied to show that change in these critical parameters does not change the obtained responses significantly.

f) System Suitability and Drug Stability

As an integral part of the analytical procedure, various chromatographic performance parameters such as tailing factor (T_f), resolution (R_s) and number of theoretical plates (N) were recorded in system suitability study. Bench-top stability of the drug solution was performed for 8.5 h. The stock solution stability of drug was evaluated at refrigerated temperature for 16 days by comparing the response of stability stock with fresh stock.

3.3.2.2 Results and Discussion

a) Method Development

In the preliminary study, peak properties and response function were optimized by changing type of organic modifier, organic to aqueous ratio, buffer type, buffer strength and pH. Among the buffers, phosphate buffer gave peak with good symmetry and resolution. Optimum concentration of phosphate buffer was found to be 10 mM because increasing concentration led to peak tailing and decreased the resolution where as use at reduced concentration decreased its sensitivity. The selection of pH was the key factor in method development, as the column is of protein having its isoelectric point normally between 2.7-3.8. As pH increased the negative charge on column increased and it retained the VEN (having positive charge at this pH) for longer duration and it thus took more time for elution and produced improper resolution of VEN enantiomers. Also by decreasing the pH, the retention time was decreased but there was no proper resolution of ODV enantiomers. Optimized pH was found to be 6.5 by several trials. The use of organic modifier played a major role. Their effect on enantiomeric separation was studied. By using ACN, good response (peak area) and shorter retention time was achieved, but proper resolution was not attained. As ACN concentration was increased, the relatively non polar VEN enantiomers were resolved but of ODV were not resolved and by decreasing the ACN concentration vice-versa happened. So, use of methanol which is less nonpolar compared to ACN was used for optimization. The maximum amount of organic modifier to be used is 20% of mobile phase, which prolongs column life, as the concentration increased the resolution increased and the retention time was decreased, the optimized ratio was found to be 94:6 (buffer: methanol) as it gave better resolution, sensitivity and reduced time of analysis.

Moreover, excitation and emission wavelength were optimized to 226 and 298 nm, respectively for better sensitivity and selectivity from endogenous product. Thus optimized mobile phase consisted of 10 mM potassium di hydrogen phosphate pH 6.5: methanol (94:6, v/v) with better peak properties, selectivity and reproducibility.

b) Method Validation

i) Selectivity

Placebo samples showed no interference within the vicinity of the R (-) VEN, S (+) VEN, R (-) ODV and S (+) ODV peak, which indicated selectivity of the method for in presence of formulation excipients. Representative chromatograms are given in fig 3.9 - 3.14.

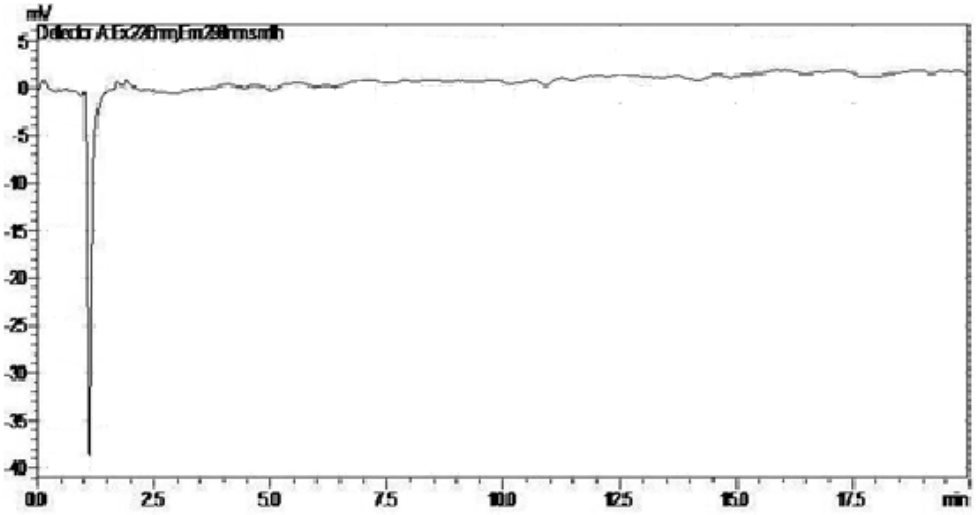

Fig 3.9: Representative chromatogram for placebo blank.

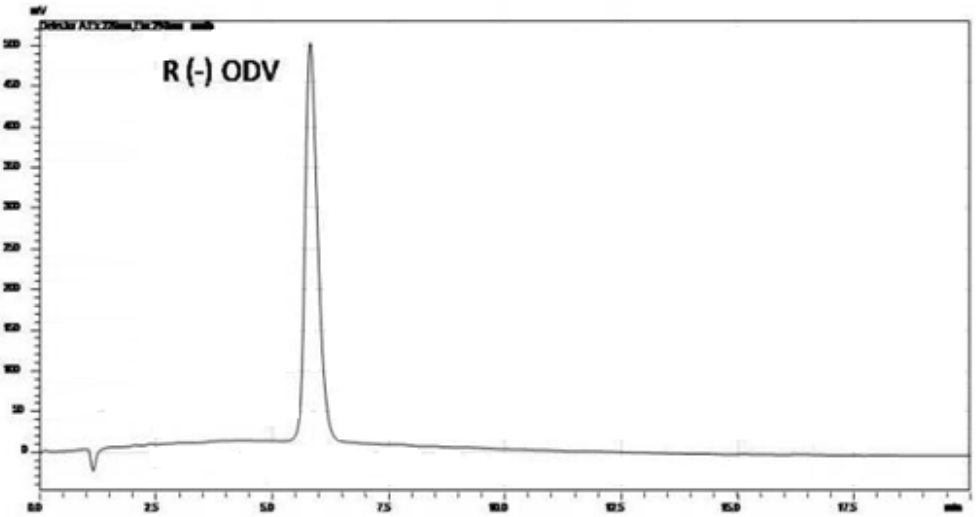

Fig 3.10: Representative chromatogram of pure R (-) ODV (2 µg/mL).

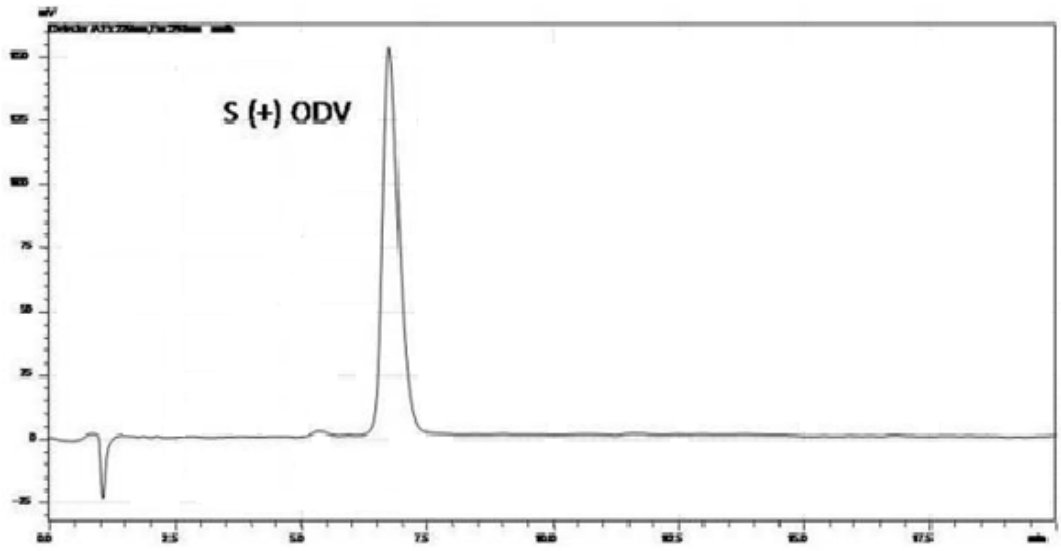

Fig 3.11: Representative chromatogram of pure S (+) ODV (1 µg/mL).

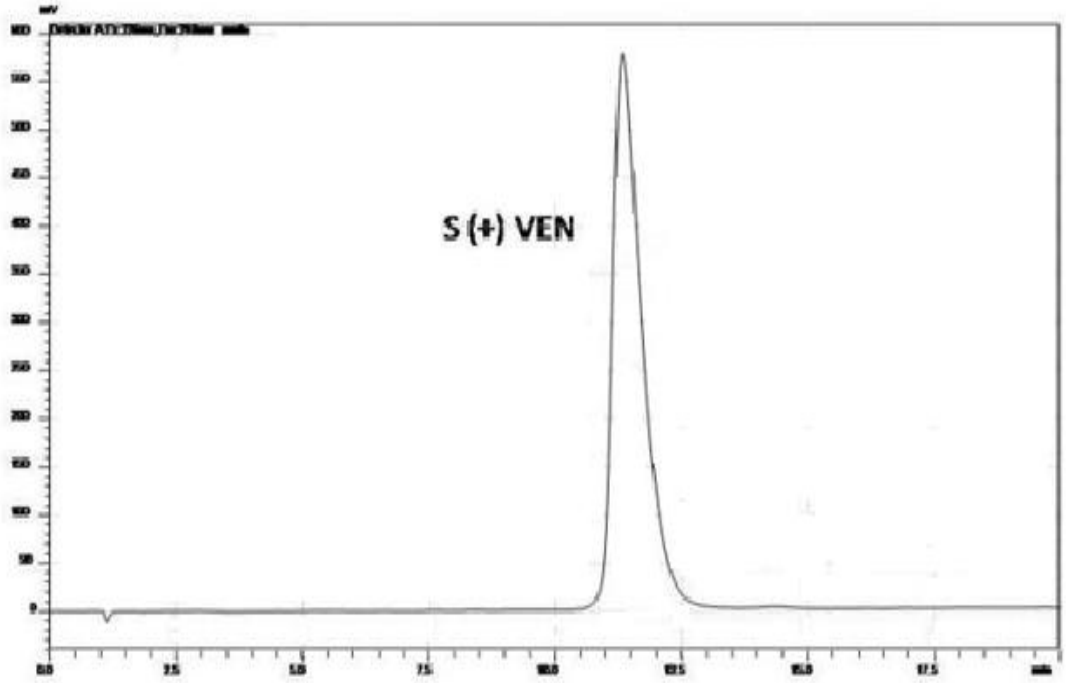

Fig 3.12: Representative chromatogram of pure S (+) VEN (4 µg/mL).

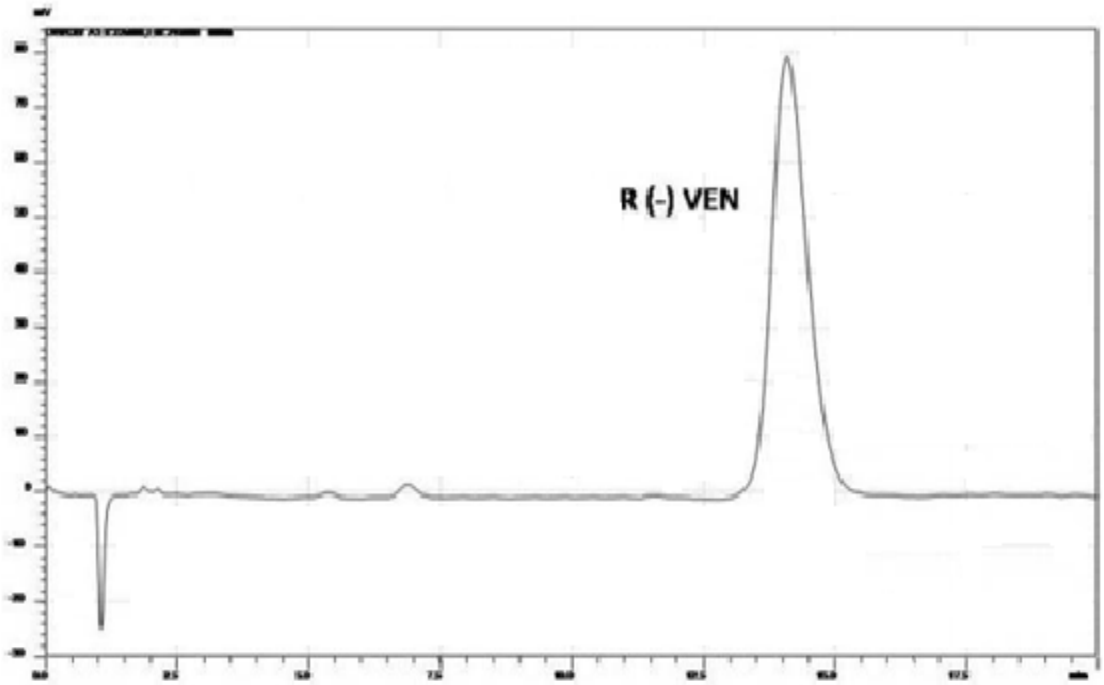

Fig 3.13: Representative chromatogram of pure R (-) VEN (0.75 µg/mL).

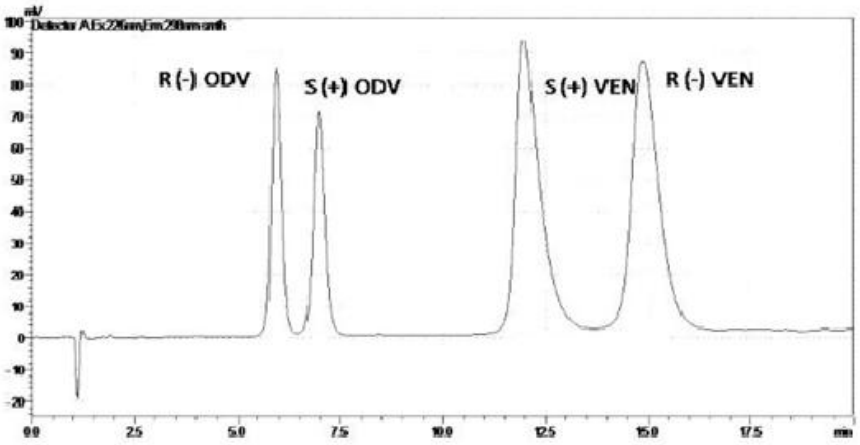

Fig 3.14: Representative chromatogram of completely resoluted peak of VEN and ODV enantiomers (0.5 µg/mL each).

ii) Linearity and Range

The linearity regression analysis indicated linear relationship between average peak area and concentration in plasma over the range 0.05- 4µg/mL with weighted regression equation for each eanantiomer (Table 3.23- 3.26).

peak area = 5930000 x concentration (µg/mL) – 12722; (weighted $1/y^2$) R^2 = 0.9999 for R (-) VEN

peak area = 5961000 x concentration (µg/mL) - 11948; (weighted $1/y^2$) R^2 = 0.9999 for S (+) VEN

peak area = 1633000 x concentration (µg/mL) - 1076; (weighted $1/y^2$) R^2 = 0.9999 for R (-) ODV

peak area = 1650000 x concentration (µg/mL) - 1373; (weighted $1/y^2$) R^2 = 0.9999 for S (+) ODV

Table 3.23: Calibration curve of R (-) VEN

Conc (µg/mL)	Mean peak area[a]	S.D	% RSD
0.05	285762.000	2966.142	1.038
0.1	573463.333	2451.699	0.428
0.3	1739413.000	17540.097	1.008
0.5	2956113.333	39731.725	1.344
0.75	4491665.667	64845.328	1.444
1	5929675.667	59909.141	1.010
2	11856458.333	195949.880	1.653
4	23767643.000	421714.093	1.774

[a] Each value represents the average of three independent determinations.

Table 3.24: Calibration curve of S (+) VEN

Conc (µg/mL)	Mean peak area[a]	S.D	% RSD
0.05	288249.000	4187.828	1.453
0.1	576189.333	3092.330	0.537
0.3	1753076.667	28465.414	1.624
0.5	2983726.333	29631.649	0.993
0.75	4511405.667	65702.882	1.456
1	5938999.000	55908.044	0.941
2	11918254.333	151430.469	1.271
4	23905104.333	295366.194	1.236

[a] Each value represents the average of three independent determinations.

Table 3.25: Calibration curve of R (-) ODV

Conc (µg/mL)	Mean peak area[a]	S.D	% RSD
0.05	80759.667	1370.674	1.697
0.1	161876.000	2473.408	1.528
0.3	487146.667	5343.497	1.097
0.5	811772.667	6431.754	0.792
0.75	1219968.667	17874.522	1.465
1	1627108.333	16785.867	1.032
2	3285661.333	58375.592	1.777
4	6582999.000	63479.360	0.964

[a] Each value represents the average of three independent determinations.

Table 3.26: Calibration curve of S (+) ODV

Conc (µg/mL)	Mean peak area[a]	S.D	% RSD
0.05	81349.333	851.654	1.047
0.1	163186.333	2662.505	1.632
0.3	489333.000	6247.087	1.277
0.5	818429.000	11240.163	1.373
0.75	1236884.000	16847.081	1.362
1	1643074.333	20401.423	1.242
2	3321576.667	59139.209	1.780
4	6656074.333	49027.819	0.737

[a] Each value represents the average of three independent determinations.

One way ANOVA was performed for peak area obtained at individual concentration and F 8.776×10^{-4} for R (-) VEN and 3.152×10^{-4} for S (+) VEN while 3.092×10^{-4} for R(-) ODV and 3.130×10^{-4} for S(+) ODV values were found to be much lower than theoretical F (3.467) value at 5 % level of significance. Therefore there was no significant difference between the measured calibration curve standards.

iii) Accuracy and Precision

The method showed consistent and high absolute recoveries at all three concentration levels with mean absolute recovery ranging from 98.890 - 99.840 % for R (-) VEN and 98.240 - 99.421 % for S (+) VEN whereas 98.524 -99.460 % for R (-) ODV and 98.379 - 99.850 % for S (+) ODV. There was no significant interference of excipients and the method was found to be accurate with low % bias of 0.224-1.110 for R (-) VEN and 0.579-1.760 for S (+) VEN (Table-3.27), whereas 0.540-1.476 for R (-) ODV and 0.150-1.621 for S (+) ODV (Table-3.28). Recovery study shows that the method is suitable for determination of VEN and ODV enantiomers.

Method was found to be precise with % RSD not exceeding 1.697 and 1.755 for R (-) VEN and S (+) VEN (Intra-batch) whereas 1.326 for R (-) ODV and 1.610 for S (+) ODV (intra batch) while % RSD not exceeding 1.592 and 1.197 for R (-) VEN and S (+) VEN (Inter-batch) whereas 1.112 for R (-) ODV and 1.172 for S (+) ODV (inter batch). The detail is shown in table 3.29.

Table 3.27: Recovery study by placebo spiking technique for VEN enantiomers

Analyte	Technique	Amount of drug added (% Label claim)	Mean[a] absolute recovery (%)	% RSD	% Bias
R (-) VEN	Placebo spiking	75	98.731	1.389	0.269
		100	98.890	1.272	1.110
		125	99.840	1.300	0.160
	Standard addition	50	99.625	1.013	0.375
		100	99.776	1.292	0.224
S (+) VEN	Placebo spiking	75	98.611	1.554	1.389
		100	99.058	1.288	0.942
		125	98.240	1.945	1.760
	Standard addition	50	99.552	1.506	1.448
		100	99.421	1.340	0.579

[a] Each value represents the average of three independent determinations.

Table 3.28: Recovery study by placebo spiking technique for ODV enantiomers

Analyte	Technique	Amount of drug added (% Label claim)	Mean[a] absolute recovery (%)	% RSD	% Bias
R (-) ODV	Placebo spiking	75 100 125	98.586 99.460 98.914	1.656 1.633 1.191	1.414 0.540 1.086
	Standard addition	50 100	98.524 99.260	1.755 1.041	1.476 0.740
S (+) ODV	Placebo spiking	75 100 125	98.379 99.850 98.937	1.589 1.005 1.098	1.621 0.150 1.063
	Standard addition	50 100	98.765 99.686	1.512 1.462	1.234 0.314

[a] Each value represents the average of three independent determinations.

Table 3.29: Results of repeatability and intermediate precision study

Analyte	Nominal Conc (µg/mL)	Repeatability (n=3)		Intermediate Precision (n=9)	
		Mean	% RSD	Mean	% RSD
R (-) VEN	0.075	0.074	1.436	0.075	1.592
	0.80	0.796	1.697	0.794	1.052
	3.0	2.985	1.329	2.980	1.083
S (+) VEN	0.075	0.074	1.336	0.074	1.145
	0.80	0.796	1.755	0.795	1.197
	3.0	2.975	1.238	2.983	0.984
R (-) ODV	0.075	0.074	1.219	0.074	0.869
	0.80	0.793	1.326	0.794	1.019
	3.0	2.994	0.858	2.990	1.112
S (+) ODV	0.075	0.074	1.610	0.074	1.015
	0.80	0.788	1.157	0.790	0.870
	3.0	2.979	1.199	2.992	1.172

iv) Sensitivity

The LOD and LOQ were 0.001 and 0.002 µg/mL for R (-) VEN and 0.002 and 0.007 µg/mL for S (+) VEN respectively. For R (-) ODV, LOD and LOQ were 0.001 µg/mL and 0.003 µg/mL while for S (+) ODV, LOD and LOQ were 0.003 µg/mL and 0.009 µg/mL. At quantitation limit, the peak properties (retention time, peak area, and tailing factor) were not affected and mean absolute recovery was consistently high and acceptable with low % bias and % RSD. Thus, the method was found to be highly sensitive.

v) Robustness

Robustness study indicated that obtained responses remained unaffected by the small changes in critical method parameters such as flow rate, pH of mobile phase and % organic phase in mobile phase composition. Results of robustness study of VEN and ODV enantiomers are given in table 3.30 and 3.31.

Table 3.30: Results of robustness study of VEN enantiomers

Factor	Level	Area[a]		Asymmetry[b]		Resolution[c]
Flow rate (mL/min)		R (-) VEN	S (+) VEN	R (-) VEN	S (+) VEN	F value calculated
		F value calculated		F value calculated		
0.9	-1	0.033	0.004	0.682	0.196	0.595
1.0	0					
1.1	+1					
pH of mobile phase						
6.4	-1	0.054	0.075	0.123	0.189	0.711
6.5	0					
6.6	+1					
% Organic phase						
5 %	-1	0.019	0.025	0.301	0.235	0.089
6 %	0					
7 %	+1					

[a, b, c] Each value represents the average of three independent determinations.

Table 3.31: Results of robustness study of ODV enantiomers

Factor	Level	Area[a]		Asymmetry[b]		Resolution[c]
Flow rate (mL/min)		R (-) ODV	S (+) ODV	R (-) ODV	S (+) ODV	F value calculated
		F value calculated		F value calculated		
0.9	-1	0.009	0.240	0.052	0.528	0.062
1.0	0					
1.1	+1					
pH of mobile phase						
6.4	-1	0.630	0.614	0.061	0.194	1.180
6.5	0					
6.6	+1					

Factor	Level	Area[a]		Asymmetry[b]		Resolution[c]
% Organic phase						
5%	-1	0.274	0.138	0.417	0.017	0.544
6%	0					
7%	+1					

[a, b, c] Each value represents the average of three independent determinations.

F tabulated (5.143) is greater than F calculated in all the cases, therefore there is no significant difference between the area, asymmetry and resolution values obtained by deliberately changing the critical method parameters.

vi) System Suitability and Drug Stability

The method was found to be suitable in terms of system performance as obtained values for primary suitability parameters such as retention factor, resolution, number of theoretical plates were above acceptable limits. The method showed better peak symmetry. System suitability study confirmed that the method was specific, precise, and stable for determination of VEN and ODV enantiomers (Table 3.32, 3.33). Summary of validation parameters of VEN and ODV enantiomers is given in table 3.34 and 3.35.

Further, the drug peak exhibited no chromatographic or response change for 16 days at refrigerated temperature when compaired against freshly prepared standards.

Table 3.32: Stability studies of VEN enantiomers

Storage period and Storage condition	Analyte	Nominal Conc (µg/mL)	Mean[a]	% RSD	% Accuracy
Stock solution ~ 16 days, refrigerated temperature	R (-) VEN	0.075	0.075	1.251	99.349
		3.0	2.974	1.120	99.349
	S (+) VEN	0.075	0.074	0.973	98.900
		3.0	2.979	1.181	99.289
Bench top ~ 8.5 h, room temperature	R (-) VEN	0.075	0.075	1.808	99.594
		3.0	2.989	1.401	99.634
	S (+) VEN	0.075	0.074	1.120	99.053
		3.0	2.976	1.144	99.215

[a] Each value represents the average of three independent determinations.

Table 3.33: Stability studies of ODV enantiomers

Storage period and Storage condition	Analyte	Nominal Conc (µg/mL)	Mean[a]	% RSD	% Accuracy
Stock solution ~ 16 days, refrigerated temperature	R (-) ODV	0.075	0.074	1.385	99.023
		3.000	2.985	0.761	99.488
	S (+) ODV	0.075	0.075	1.675	99.614
		3.000	2.989	1.357	99.645
Bench top ~ 8.5 h, room temperature	R (-) ODV	0.075	0.074	1.260	98.795
		3.000	2.993	0.728	99.750
	S (+) ODV	0.075	0.074	1.771	98.852
		3.000	2.984	1.106	99.481

[a] Each value represents the average of three independent determinations.

Table 3.34: Summary of validation parameters of VEN enantiomers

Parameter	Values	
	R (-) VEN	S (+) VEN
Calibration range	0.05 - 4 µg/mL	0.05 - 4 µg/mL
Regression coefficient	$R^2 = 0.9999$	$R^2 = 0.9999$
Regression equation	y (peak area) = 5930000 x concentration (µg/mL) - 12722 (weighted $1/y^2$)	y (peak area) = 5961000 x concentration (µg/mL) - 11948 (weighted $1/y^2$)
Limit of detection	0.001µg/mL	0.002 µg/mL
Limit of quantification	0.002 µg/mL	0.007 µg/mL
Accuracy (% Recovery)	98.731 % - 99.840	98.240 % - 99.421 %
Accuracy (% Bias)	0.224 % - 1.110	0.579 % - 1.760 %
Precision (% RSD)	1.329 % - 1.697 % (Intraday) 1.052 % - 1.592 % (Interday)	1.238 % - 1.755 % (Intraday) 0.984 % - 1.197 % (Interday)
System sutability	$T_f = 1.16 \pm 0.001$ $R_t = 14.67 \pm 0.012$ N = 2369.33 ± 117	$T_f = 1.14 \pm 0.008$ $R_t = 11.78 \pm 0.015$ N = 2360 ± 284.02 $R_s = 2.58 \pm 0.001$
Selectivity	Selective	Selective
Robustness	No significant change in the parameters on making small change in critical parameters	No significant change in the parameters on making small change in critical parameters

Table 3.35: Summary of validation parameters of ODV enantiomers

Parameter	Values	
	R (-) ODV	S (+) ODV
Calibration range	0.05 - 4 µg/mL	0.05 - 4 µg/mL
Regression coefficient	$R^2 = 0.9999$	$R^2 = 0.9999$
Regression equation	y (peak area) = 1633000 x concentration (µg/mL) - 1076 (weighted $1/y^2$)	y (peak area) = 1650000 x concentration (µg/mL) - 1373 (weighted $1/y^2$)
Limit of detection	0.001 µg/mL	0.003 µg/mL
Limit of quantification	0.003 µg/mL	0.009 µg/mL
Accuracy (% Recovery)	98.524 % - 99.460 %	98.379 % - 99.850 %
Accuracy (% Bias)	0.540 % - 1.476 %	0.150 % - 1.621%
Precision (% RSD)	0.858 % - 1.326 % (Intraday) 0.869 % - 1.112 % (Interday)	1.157 % - 1.610 % (Intraday) 0.870 % - 1.172 % (Interday)
System suitability	$T_f = 1.08 \pm 0.011$ $R_t = 5.92 \pm 0.023$ N = 2779 ± 83.58	$T_f = 1.11 \pm 0.004$ $R_t = 6.94 \pm 0.017$ N = 2731.67 ± 94.26 $R_s = 2.68 \pm 0.02$
Selectivity	Selective	Selective
Robustness	No significant change in the parameters on making small change in critical parameters	No significant change in the parameters on making small change in critical parameters

3.4 Conclusion

Simple, accurate and sensitive RP-HPLC analytical methods have been developed to analyze ketorolac, VEN, ODV and their enantiomers and validated according to guidelines. These methods have sufficiently low limit of quantification along with the very short analysis time which can be useful in analyzing many samples in a single day which is the most advantageous aspect in comparison to other analysis techniques available in published literature. The method is also very robust and enables quality-control analysis of enantiomer composition and purity with large sample throughput.

The method had advantage over problems of poor chromatography, questionable uncharacterized peak, high injection load in comparison to other reported methods. Moreover, environment friendly solvents have been used. Use of chiral AGP and the approach to optimize the chromatographic conditions in order to obtain better resolution,

selectivity and reduced time of analysis column has been described by studying effect of each variable. The developed methods were found to be simple, sensitive and selective for analysis of above analytes in bulk and formulations. Statistical analysis proved that method is accurate, precise and reproducible.

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Chapter 4

Bioanalytical Method Development and Validation

4.1 Introduction

Development of a suitable and effective bioanalytical method for the quantitative determination of drugs and their metabolites in biological fluids not only plays a significant role in the evaluation and interpretation of bioavailability, bioequivalence, pharmacokinetic, and toxicokinetic study of a drug but also helps in designing suitable delivery systems for proper clinical process or therapy. These studies generally support regulatory requirements. The quality of these studies is directly related to the quality of the underlying bioanalytical data.

Ketorolac is available as a racemic mixture of an equimolar ratio of R (+) and S (−) stereo isomers. Studies have shown that the pharmacological activity of ketorolac resides in the S (-) ketorolac and that the R (+) ketorolac is pharmacologically inactive (1). The pharmacokinetics of ketorolac has been documented in humans (2-5), and animals like dogs and rats (6, 7). Enantiomeric separations and estimation of ketorolac with indirect methods include formation of diastereomer esters with (-)-α-phenethyl alcohol followed by separation with normal-phase high performance liquid chromatography (7); derivatization of ketorolac with ethylchloroformate/L-leucinamide and subsequent reverse phase chromatography of the diastereomers (8); formation of diastereomers with thionyl chloride-S-l-phenylethylamine (9); using achiral Partisil 5 octadecyl silane (10). Direct methods include Human serum albumin (HSA) as chiral stationary phase, using α1-acid glycoprotein (AGP) chiral column (11, 12) amylose tris (3,5-dimethylphenylcarbamate) chiral column (13).

Chromatographic methods have been reported for estimation of VEN and its metabolites in different matrices (14-34). All LC-MS/MS determinations are having good sensitivity (14-30). Gas chromatography (32) and some methods with fluorescence detection (15, 22, 28) also have appreciable sensitivity. For most of the HPLC methods (17, 24, 26, 31, 34) UV detector was used for estimation. Detection wavelength of all methods was found to be at or near to λ_{max} of VEN (225 nm).

Chiral discrimination is frequently encountered in biological systems. The pharmacological and pharmacokinetic differences between the enantiomers are often significant (33). The biological activity of chiral substances often depends upon their stereochemistry, since the living body has chiral environment (34). Therefore, health and regulatory authorities, such as the US Food and Drug administration (FDA) defined more strict requirements to patent racemic drugs, demanding a full documentation of separate pharmacological and pharmacokinetic profiles of the individual enantiomers and their combination (35). Few

enantioselective methods for determination of VEN and ODV have been reported (36-41). Most of the methods used either complex gradient system or ion pairing agents leading to higher analytical variations and increased cost of analysis. In addition, HPLC-UV methods demonstrated inadequate sensitivity, poor peak symmetry and use of complex extraction protocols. In most of the reported methods sophisticated analytical instruments have been used. There is no simple, rapid, cost effective analytical method based on spectrofluorimetry in literature, which could be used for routine analysis of VEN and its metabolite.

As part of objective of this study it was planned to develop a suitable bioanalytical procedure that may be applicable in the quantification of the ketorolac, VEN, ODV and their enantiomers and to use this for the stereoselective pharmacokinetic study. Final objective is to investigate, the difference of pharmacokinetic behavior and tissue distribution of ketorolac, VEN, ODV and their enantiomers in rats.

4.2 Bioanalytical Method Development and Validation of Ketorolac

4.2.1 Estimation of Racemic Ketorolac

4.2.1.1 Experimental

a) Materials

Ketorolac tromethamine reference standard was provided by TRC, Canada. HPLC grade acetonitrile, methanol and analytical grade hydrochloric acid, sodium hydroxide pellets and sodium dihydrogen phosphate were obtained from Merck India Limited, Mumbai, India. High purity deionized water was prepared by TKA smart2pure, Niederelbert, Germany purification system. In addition, an electronic balance (AG-135, Mettler-Toledo, Germany), pH meter (pH Tutor, Eutech Instruments, Singapore), a sonicator (Toshiba, New Delhi) were used.

b) Chromatographic System and Conditions

The chromatographic system used to perform development and validation of this method consisted of an LC-20AD binary pump, an SPD-M20A photodiode array detector, SIL 20 AC auto sampler, connected to a communication & Bus module CBM 20A (Shimadzu, Kyoto, Japan). Chromatographic analysis was performed on OYSTER BDS (150×4.6mm id., 5µm particle size) column. Separation was achieved using a mobile phase of sodium di hydrogen phosphate (10mM, pH 5.5): acetonitrile (80:20, v/v) at a flow rate of 1.0 mL/min. The eluent was monitored using PDA detector at wavelengths 322 nm for ketorolac. The column was maintained at ambient temperature and injection volume of 50µl was used.

c) Preparation of Stock and Standards

Primary stock solution of 1mg/mL ketorolac racemic mixture was prepared by dissolving 5 mg of racemic ketorolac in a 5 mL volumetric flask containing methanol and the volume was made by the same. Working standard solutions containing 1, 5, 25, 50, 250, 500 and 750 µg/mL of ketorolac were prepared by serial dilution of primary stock solutions with mobile phase. Plasma standards containing 0.02, 0.1, 0.5, 1, 5, 10 and 15 µg/mL of ketorolac were prepared. Similarly four quality control (QC) standards were prepared at lower limit of quantification (LOQQC= 0.020 µg/mL), low (LQC= 0.05µg/mL), medium (MQC= 4 µg/mL) and high (HQC= 12 µg/mL) concentration levels of calibration curve were prepared. Similarly, brain, lungs, liver, kidney and heart standards were prepared from 0.05 µg/mL to 5 µg/mL concentration of ketorolac. The prepared biosamples were processed as described in the sample preparation section and analyzed by the proposed method. All experimental protocols were approved by the Institutional Animal Ethics Committee (approval number IAEC/RES/13/07/REV-2/17/14).

d) Sample Preparation

The OASIS HLB (Hydrophilic Lipophilic Balance) cartridges were used for analyte extraction from plasma samples. The cartridges were conditioned with methanol (1 mL) and equilibrated with de-ionised water (1 mL). To 500 µL of prepared samples, 200 µL of mobile phase and 100 µL of 5% v/v formic acid were added and vortexed for 5 min and then loaded onto cartridges and the cartridges were washed with 5% methanol (1 mL) finally eluted with methanol (1 mL). The eluates were evaporated to dryness at 40^0C under N_2 gas. Residues were then reconstituted in 500 µL of mobile phase.

e) Bioanalytical Method Development

Successful analysis of an analyte in biological fluids relies on the optimization of sample preparation, chromatographic separation and interference free detection.

In the process of bio analytical method development for ketorolac, mobile phase composition and flow rate were optimized by trying different aqueous phase and non-aqueous phase combinations at different flow rates. Various buffers such as phosphate (pH 3-7 and 10 mM, 100 mM), citrate buffer (pH 3-5 and 10 mM, 100 mM), ammonium acetate buffer (pH 3-7 and 10 mM, 100 mM) and acetic acid buffer (pH 3-5 and 10 mM, 100 mM) were studied in combination with methanol (20, 25 and 30 %), acetonitrile (20, 25 and 30 %) and isopropanol (20, 25 and 30 %). Mobile phase composition and flow rate were finally selected based on the criteria of peak properties (retention time and asymmetric factor) and sensitivity (height and

area) and ease of preparation and applicability of the method for in vivo studies in rats. During sample preparation, addition of ortho-phosphoric acid and formic acid at various concentrations were tried to increase recovery.

f) Bioanalaytical Method Validation

The developed chromatographic method was validated for selectivity, linearity, range, precision, accuracy, sensitivity, stability and dilution integrity in plasma samples.

i) Selectivity

The test for selectivity was carried out using six different lots of rat blank plasma batches processed by the same extraction method and analyzed to determine the extent to which endogenous substances may contribute to the interferences for analytes. These samples were compared with those containing ketorolac, at the lower limit of quantitation (LLOQ). The aim of performing the selectivity check with these different batches of plasma samples is to ensure the quality of the results of study sample analysis. The peak area of interfering peak at the retention time of analyte should be <20% of analyte peak area response of the analyte in LLOQ sample.

ii) Linearity and Range

It was obtained by preparing three sets of the drug solutions in mobile phase containing ketorolac racemates at a concentration of 0.02- 15 µg/mL; 50 µl of these drug solutions were injected in to column and the peak area and retention time were recorded. Average peak area at each level was plotted against concentration and curves were subjected to linear regression analysis by least square method. Regression equation was used to calculate the corresponding predicted concentration. One way analysis of variance (ANOVA) was performed on each replicate response.

iv) Precision and Accuracy

Accuracy and precision of the method in individual matrix was determined by analyzing QC standards prepared at LOQQC (0.02 µg/mL), LQC (0.05 µg/mL), MQC (4 µg/mL) and HQC (12 µg/mL) levels. Each QC standard was processed and analysed in six replicates and analysis was repeated on three different occasions to study intra and inter batch precision and accuracy. Concentration of ketorolac in QC standard was calculated from regression equation. Accuracy was expressed as % Bias and precision was determined as intra and inter batch variation, expressed as percent relative standard deviation (% RSD).

v) Recovery

Recovery of ketorolac enantiomers from plasma samples were assessed (n=6) at LQC (0.05 µg/mL), MQC (4µg/mL) and HQC (12 µg/mL) respectively. The extraction efficiency was determined by comparing the areas obtained from processed samples to those solutions of corresponding concentration of analytical samples injected directly in the HPLC system.

vi) Sensitivity

Sensitivity of the method was obtained by determining the lowest concentration of ketorolac that can be estimated with acceptable accuracy and precision (% RSD < 20 %) and it was the lower limit of quantification (LLOQ), in the individual matrix. The QC standards were prepared at LLOQ (0.02 µg/mL) concentration in six replicates and analyzed by the proposed method on three different occasions. Concentration of ketorolac in the QC standard was calculated from the regression equation and parameters such as mean calculated concentration, % Bias and % RSD were determined.

vii) Dilution Integrity

If it is expected that some sample concentrations may exceed the upper limit of quantitation, a test for sample dilution with blank matrix during validation should be performed. One or more QC samples at concentrations several times higher than the upper limit of the calibration curve should be prepared, covering the maximum expected dilution. These QC samples are diluted with blank matrix to bring the concentration to within the calibration range and then analyzed. The acceptance criteria for the diluted QC are the same as that of accuracy and precision. If during sample analysis a dilution higher than the one covered during validation is needed, further dilution can be validated during sample analysis by analyzing the required diluted QC samples. To study the over curve dilution integrity, standards were prepared in rat plasma at 20, 24 and 28 µg/mL concentrations of ketorolac and they were diluted 5 and 10 times. The dilution integrity standards were vortexed for 5 min and processed as mentioned previously.

viii) Stability Studies

Various factors determine the stability of a drug, viz. the chemical properties, matrix, container system and storage conditions. Stability experiments in stock solution and plasma were performed very extensively to evaluate the stability of ketorolac. All the experimental conditions which the drugs actually encountered during sample analysis were simulated during method validation to evaluate the various stabilities like long-term stability at -20 ^{0}C

for 90 days, freeze–thaw stability, room temperature stability of drug in plasma (bench-top stability), stock solution stability and post-preparative stability at auto-sampler temperature of 4 ^{0}C (auto-sampler stability).

To evaluate long-term stability, a long period was defined as the time elapsed between the start of sampling and the end of sample analysis. The aliquots of QCs were first frozen at -20 ^{0}C for 90 days and then thawed to be extracted and tested against fresh CC and QC samples. The difference between the starting concentration and the concentration after 90 days will show whether the drug in plasma can degrade under these conditions. Evaluation of freeze–thaw stability involves estimation of analytes stability after three freeze–thaw cycles. Similarly, bench-top stability of the spiked samples was performed for 8.5 h. The stock stability of drug was evaluated at refrigerated temperature for 16 days by comparing the response of stability stock with fresh stock. Stability of samples in auto sampler at a temperature (4 ^{0}C) was evaluated for 54 h. All stability studies were carried out using six replicates of QC samples at two different concentration levels, that is, LQC and HQC against freshly spiked CC standards and the results were compared with the fresh QCs.

4.2.1.2 Results and Discussion

a) Bioanalytical Method Development

In the preliminary study, peak properties and response function were optimized by changing type of organic modifier, organic to aqueous ratio, buffer type, buffer strength and pH. Among the buffers, phosphate buffer gave peak with good symmetry and resolution. Optimum concentration of phosphate buffer was found to be 10 mM because increasing concentration led to peak tailing and decrease of resolution. Use of reduced concentration decreased its sensitivity. Reduction in pH led to peak asymmetry, where pH below 4 showed poor peak properties and loss in selectivity with incomplete resolution from the endogenous products. Increase in pH above 6.5 led to loss in selectivity. Acetonitrile gave good resolution together with good peak symmetry. Optimized concentration of acetonitrile was found to be 20 % of the mobile phase. OYSTER BDS (150×4.6mm id., 5µm particle size) column was used for better peak symmetry and enchanced stability at higher pH. Moreover, wavelenghth was optimized at 322 nm for better sensitivity and selectivity from endogenous product. Thus optimized mobile phase consisted of 10 mM sodium di hydrogen phosphate pH 5.5: acetonitrile (80: 20, v/v) with better peak properties, selectivity and reproducibility. Addition of 5% v/v formic acid during the sample preparation led to increased recovery of the analyte by the solid phase extraction. Thus optimized extraction protocol showed consistent and high

recovery at all concentration levels without any interference from endogenous components and impurities.

b) Bioanalytical Method Validation

a) Selectivity

Chromatogram of six blank plasma samples revealed that there was no peak present at the retention time of ketorolac (Fig 4.1). A lack of response in blank biological matrix confirmed the selectivity of the method from endogenous substances. Further test samples obtained from oral pharmacokinetic studies proved that there was no interference from the metabolite or degradation product in the near vicinity of the drug. Representative chromatogram of standard of ketorolac is given in fig 4.2. Thus the proposed method was found to be selective in determination of ketorolac from the spiked as well as test sample.

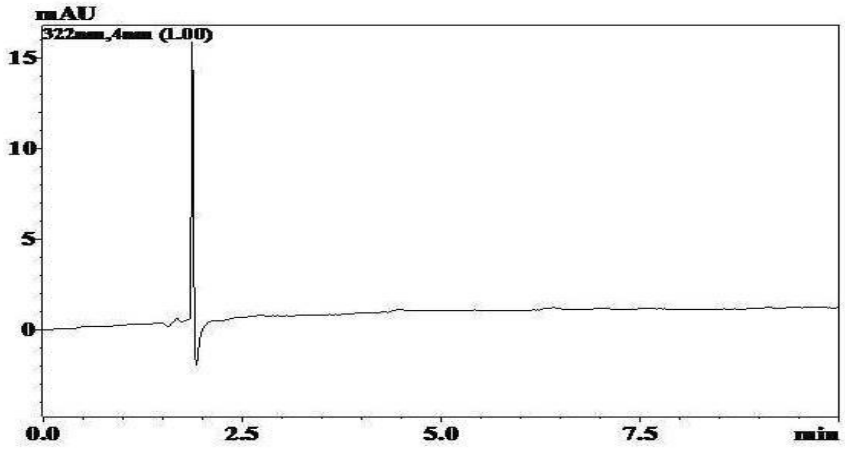

Fig 4.1: Representative chromatogram of blank rat plasma sample.

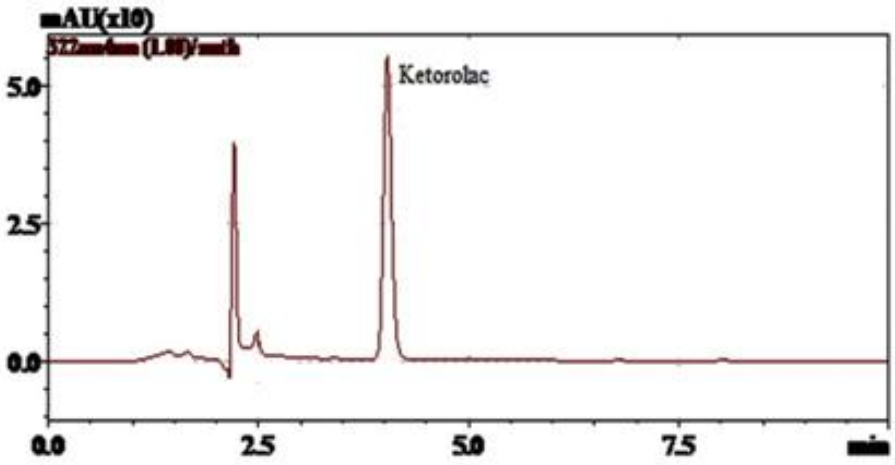

Fig 4.2: Representative chromatogram of standard ketorolac in rat plasma (0.1 µg/ml).

b) Linearity and Range

The linearity regression analysis indicated linear relationship between average peak area and concentration in plasma over the range 0.02 – 15 µg/mL with weighted regression equation.

peak area =150218 x concentration (µg/mL) + 36.11 (weighted $1/x^2$); $R^2 = 0.9983$

Table 4.1: Calibration curve of ketorolac in rat plasma

Conc (µg/mL)	Mean peak area [a]	S.D	% RSD
0.02	3038.000	154.350	5.081
0.1	15096.333	599.950	3.974
0.5	75719.000	3171.868	4.189
1	150732.667	6509.994	4.319
5	736488.000	19088.125	2.592
10	1528880.000	65463.242	4.282
15	2228949.000	114209.533	5.124

[a] Each value represents the average of three independent determinations.

One way ANOVA was performed for peak area obtained at individual concentration and F ($1.097x10^{-3}$) value was found to be much lower than theoretical F (3.467) value at 5 % level of significance. Therefore there was no significant difference between the measured calibration curve standards.

Good linear relationship existed between average peak area and concentration with weighted regression equation in various tissues for ketorolac (Table 4.2)

Table 4.2: Calibration curve of ketorolac in various tissues of rat

Biological sample	Standard curve	R^2	Range (µg/mL)
Brain	peak area = 369100 x concentration (µg/mL) + 29650 (weighted $1/x^2$)	0.992	0.05 - 5.00
Heart	peak area = 302000 x concentration (µg/mL) + 22260 (weighted $1/x^2$)	0.993	0.05 - 5.00
Kidney	peak area = 408200 x concentration (µg/mL) + 30220 (weighted $1/x^2$)	0.992	0.05 - 5.00
Liver	peak area = 294200 x concentration (µg/mL) + 22300 (weighted $1/x^2$)	0.997	0.05 - 5.00
Lungs	peak area =304900 x concentration (µg/mL) + 32470 (weighted $1/x^2$)	0.993	0.05 - 5.00

c) Recovery Studies

The proposed method showed high and consistent recovery of ketorolac from rat plasma. Mean absolute recovery in plasma ranged from 88.503 - 91.114 % over the calibration range (Table 4.3).

Table 4.3: Mean absolute recovery of ketorolac from rat plasma

Analyte	Quality control	Recovery [a] (n=6)	
		Mean ± SD	% RSD
Ketorolac	LQC	88.503 ± 3.633	4.105
	MQC	90.358 ± 3.087	3.416
	HQC	91.114 ± 3.248	3.565

[a] Recovery= [(Peak area of plasma standard/Peak area of analytical standard)*100].

d) Accuracy and Precision

The obtained results confirmed the accuracy of the proposed method as the % Bias ranged from 4.956 to 6.717. The method was found to be precise with % RSD of 4.541 to 6.637 (intrabatch) and 5.096 to 6.315 (interbatch). Results of precision and accuracy study were in acceptable limits and indicated that method was accurate and precise (Table 4.4).

Table 4.4: Intra and inter batch accuracy and precision in rat plasma

Nominal Conc (µg/mL)	Repeatability (n=6)			Intermediate (n=18)		
	Mean	% RSD	% Bias	Mean	% RSD	% Bias
0.02	0.019	6.137	6.717	0.019	6.315	5.060
0.05	0.047	5.918	5.122	0.047	5.112	5.417
4	3.767	6.637	5.819	3.794	6.252	5.145
12	11.382	4.541	5.150	11.405	5.096	4.956

e) Sensitivity

Plasma standards prepared at LLOQ showed quantifiable amount of ketorolac, when analyzed in replicate of six on three different days. Further it confirmed that the method was precise and accurate at LLOQ with % RSD not more than 6.315 % and % bias not exceeding 6.717. The method was found to be sensitive with high signal to noise ratio at 322 nm detection wavelength. It can be suggested that the developed method is suitable for various pharmacokinetic investigations in the rat plasma, which demands high sensitivity.

f) Dilution Integrity

The dilution integrity of the method was found to be acceptable with accuracy (% Bias) of 5.748, 5.013 and 5.533 for 5 times dilution and 5.444, 5.545 and 5.427 for 10 times dilution for 20, 24 and 28 µg/mL concentration levels respectively. Precision for dilutions were within the acceptable limits with % RSD of 4.318, 5.528 and 5.899 for 5 times dilution and 5.585, 4.785 and 4.296 for 10 times dilution for 20, 24 and 28 µg/mL concentration levels respectively.

g) Stability studies

Results obtained for bench top stability studies at two QC levels (LQC and HQC) demonstrated that ketorolac was stable in rat plasma under bench top conditions. Ketorolac does not show significant change (% RSD) in response up to 8.5 h, when compared with the response obtained from fresh standards. Similarly, in long term stability study, ketorolac was found to be stable in rat plasma at -20 ^{0}C at all QC level, as there was no significant difference between response of the standard at zero time and at the end of 90 days. The maximum deviation observed was within acceptable limits. There was no significant degradation detected in QC standards up to 3 freeze thaw cycles. Results are expressed as % recovery, which was 94.215 % at LQC and 95.886 % at HQC level following three freeze-thaw cycles and the results were within acceptable limits. Thus, the drug was found to be stable for three freeze thaw cycles making it suitable for subzero storage conditions. Percent deviation calculated for all stability studies were within the acceptable limit of ± 15 % at LQC and HQC level, demonstrating the stability of ketorolac under various processing and storage conditions stated in the method (Table 4.5). Summary of validation parameters is given in table 4.6.

Table 4.5: Stability studies of ketorolac in rat plasma

Storage period and Storage condition	Nominal Conc (µg/mL)	Mean[a]	% RSD	% Accuracy
Stock solution ~ 16 days, refrigerated temperature	0.05 12.0	0.048 11.406	5.506 3.837	95.564 95.048
Bench Top ~ 8.5 h, room temperature	0.05 12.0	0.047 11.370	5.581 4.056	93.816 94.751
Three freeze thaw cycle (-20 ^{0}C)	0.05 12.0	0.047 11.506	4.828 4.594	94.215 95.886
In - Injector stability (4 ^{0}C, ~54 h)	0.05 12.0	0.047 11.390	5.434 4.451	94.914 94.916
Long term stability (-20^0 C, ~ 90 days	0.05 12.0	0.047 11.227	6.321 3.398	93.851 93.555

[a] Each value represents the average of six independent determinations.

Table 4.6: Summary of validation parameters of ketorolac in rat plasma

Parameter	Value
Calibration range	0.02-15 µg/mL
Linearity (coefficient)	$R^2 = 0.9983$
Regression equation	peak area =150218 x concentration (µg/mL) + 36.11(weighted $1/x^2$)
Limit of detection	0.003 µg/mL
Lower limit of quantification	0.020 µg/mL
Absolute recovery/ Recovery efficiency	88.503 % – 91.114 %
Accuracy (% Bias)	5.122 – 6.717 % (Intraday) 4.956 – 5.417 % (Interday)
Precision (% RSD)	4.541 % - 6.637 % (Intraday) 5.096 - 6.315 % (Interday)
System sutability	$T_f = 1.15 \pm 0.05$ $R_t = 4.21 \pm 0.05$ $N = 3938.04 \pm 12.41$
Selectivity	Selective

4.2.2 Estimation of Enantiomers of Ketorolac

4.2.2.1 Experimental

a) Materials

Ketorolac tromethamine reference standard and pure enantiomers were provided by TRC, Canada. HPLC grades Acetonitrile (ACN), Methanol (MeOH), Isopropyl alcohol (IPA), analytical grade phosphoric acid, sodium hydroxide pellets and sodium di-hydrogen phosphate (NaH_2PO_4) were procured from Merck India Limited, Mumbai. High purity deionized water was prepared by TKA smart2pure, Niederelbert, Germany purification system. In addition, an electronic balance (AG-135, Mettler-Toledo, Germany), pH meter (pH tutor, Eutech Instruments, Singapore), a sonicator (Toshiba, New Delhi) were used.

b) Chromatographic System and Conditions

The chromatographic system used to perform development and validation of this method consisted of an LC-20AD binary pump, an SPD-M20A photodiode array detector, SIL 20 AC auto sampler, connected to a communication & Bus module CBM 20A (Shimadzu, Kyoto, Japan). The column used was Chiral-AGP column (100 x 4.0 mm I.D., particle size 5 µm,

Chromtech Ltd, Sweden). Mobile phase, phosphate buffer pH 4.5; 0.1M: isopropanol (96:4, v/v) was filtered before use through a 0.22 µm membrane filter, degassed in a bath sonicator for 20 min and was pumped from the solvent reservoir to the column at a flow rate of 1mL/min to equilibrate the system. Then a sample, injection volume of 50 µl was given. The run time was set for 10 min and eluents were monitored at 322 nm.

c) Preparation of Stock and Standards

A stock solution was prepared by dissolving 5mg of racemic ketorolac tromethamine in 10 mL of methanol. The stock solution was further diluted with mobile phase to prepare working standard solutions containing 1, 5, 25, 50, 250, 500 and 750 µg/mL of ketorolac. Rat plasma standards containing 0.01, 0.05, 0.25, 0.5, 2.5, 5.0 and 7.5 µg/mL each enantiomer were prepared. Similarly four quality control (QC) standards were prepared at lower (LOQQC = 0.010 µg/mL) low (LQC = 0.025 µg/mL), medium (MQC = 2 µg/mL) and high (HQC = 6 µg/mL) concentration levels of calibration curve in triplicates for three days. Similarly, brain, lungs, liver, kidney and heart standards were prepared from 0.025 µg/mL to 2.5 µg/mL concentration of each enantiomer. The standards were prepared in triplicates. The prepared biosamples were processed as described in the sample preparation section and analyzed by the proposed method. All experimental protocols were approved by the Institutional Animal Ethics Committee (approval number IAEC/RES/13/07/REV-2/17/14).

d) Sample preparation

The OASIS HLB (Hydrophilic Lipophilic Balance) cartridges were used for analyte extraction from plasma samples. The cartridges were conditioned with methanol (1 mL) and equilibrated with de-ionised water (1 mL). To 500 µL of prepared samples, 200 µL of mobile phase and 100 µL of 5% v/v formic acid were added and vortexed for 5 min and then loaded into cartridges and the cartridges were washed with 5% methanol (1 mL) finally eluted with methanol (1 mL). The eluates were evaporated to dryness at 40^0C under N_2 gas. Residues were then reconstituted in 500 µL of mobile phase.

e) Bioanalytical Method Development

In the process of bioanalytical method development for R (+) and S (-) ketorolac, mobile phase composition and flow rate were optimized by trying different aqueous phase and non-aqueous phase combinations at different flow rates. The variables which altered the enantioselectivity when using the chiral AGP column were chosen and effect of each variable was studied. The variables selected were type of buffer, buffer strength, pH of buffer, organic

modifier and organic modifier concentration. Here the effect of individual variable was studied by keeping all other chromatographic conditions constant. Various buffers such as phosphate (pH 3-7 and 10 mM, 100 mM), citrate buffer (pH 3-5 and 10 mM, 100 mM), ammonium acetate buffer (pH 3-7 and 10 mM, 100 mM) and acetic acid buffer (pH 3-5 and 10 mM, 100 mM) were studied in combination with methanol (2, 4, 8 %), acetonitrile (2, 4, 8 %) and isopropanol (2, 4, 8 %). Mobile phase composition and flow rate were finally selected based on the criteria of peak properties (retention time and asymmetric factor), sensitivity (height and area) and ease of preparation.

f) Bioanalaytical Method Validation

The developed chromatographic method was validated for selectivity, linearity, range, precision, accuracy, sensitivity, stability and dilution integrity in plasma samples.

a) Selectivity

The test for selectivity was carried out using six different lots of blank plasma batches processed by the same extraction method and analyzed to determine the extent to which endogenous substances may contribute to the interferences for analytes. These samples were compared with those containing ketorolac, at the lower limit of quantitation (LLOQ). The aim of performing the selectivity check with these different batches of plasma samples is to ensure the quality of the results of study sample analysis. The peak area of interfering peak at the retention time of analyte should be <20% of analyte peak area response of the analyte in LLOQ sample.

b) Linearity and Range

It was obtained by preparing three sets of the drug solutions in mobile phase containing ketorolac enantiomer at a concentration of 0.01 µg/mL– 7.5 µg/mL; 50 µl of these drug solutions were injected in to column and the peak area and retention time were recorded. Average peak area at each level was plotted against concentration and curves were subjected to weighted linear regression analysis by least square method. Regression equation was used to calculate the corresponding predicted concentration. One way analysis of variance (ANOVA) was performed on each replicate responses.

c) Precision and Accuracy

Accuracy and precision of the method in individual matrix was determined by analyzing QC standard prepared at LOQQC (0.01 µg/mL), LQC (0.025 µg/mL), MQC (2 µg/mL) and HQC

(6 µg/mL) levels. Each QC standard was processed and analysed in six replicates and analysis was repeated on three different occasions to study intra and inter batch precision and accuracy. Concentration of ketorolac in QC standard was calculated from regression equation. Accuracy was expressed as % Bias and precision was determined as intra and inter batch variation, expressed as percent relative standard deviation (% RSD).

d) Recovery

Recovery of ketorolac enantiomers from plasma samples were assessed (n=6) at LQC (0.025 µg/mL), MQC (2 µg/mL) and HQC (6 µg/mL) respectively and the above concentrations were attained by spiking equivalent amount of racemic ketorolac into 0.5mL rat plasma. The extraction efficiency was determined by comparing the areas obtained from processed samples to those solutions of corresponding concentration of analytical samples injected directly in the HPLC system.

e) Sensitivity

Sensitivity of the method was obtained by determining the lowest concentration of ketorolac that can be estimated with acceptable accuracy and precision (%RSD < 20 %) and it was a lower limit of quantification (LLOQ), in the individual matrix. The QC standards were prepared at LLOQ (0.01 µg/mL), concentration for both enantiomers of ketorolac, replicate of six and analyzed by the proposed method, on three different occasions. Concentration of ketorolac in the QC standards was calculated from the regression equation and parameters such as mean calculated concentration, % Bias and % RSD were determined.

f) Dilution Integrity

To study the over curve dilution integrity (DI), standards were prepared in rat plasma at 10, 12 and 14 µg/mL concentrations of enantiomers of ketorolac and they were diluted 5 and 10 times. The dilution integrity standards were vortexed for 5 min and processed as mentioned previously.

g) Stability Studies

Various factors determine the stability of a drug, viz. the chemical properties, matrix, container system and storage conditions. Stability experiments in stock solution and plasma were performed very extensively to evaluate the stability of ketorolac. All the experimental conditions which the drugs actually encountered during sample analysis were simulated during method validation to evaluate the various stabilities like long-term stability at -20 ^{0}C

for 90 days, freeze–thaw stability, room temperature stability of drug in plasma (bench-top stability), stock solution stability and post-preparative stability at auto-sampler temperature of 4 ^{0}C (auto-sampler stability).

To evaluate long-term stability, a long period was defined as the time elapsed between the start of sampling and the end of sample analysis. The aliquots of QCs were first frozen at -20^{0}C for 90 days and then thawed to be extracted and tested against fresh CC and QC samples. The difference between the starting concentration and the concentration after 90 days will show whether the drug in plasma can degrade under these conditions. Evaluation of freeze–thaw stability involves estimation of analytes stability after three freeze–thaw cycles. Similarly, bench-top stability of the spiked samples was performed for 8.5 h. The stock stability of drug was evaluated at refrigerated temperature for 16 days by comparing the response of stability stock with fresh stock. Stability of samples in auto sampler at a temperature (4 ^{0}C) was evaluated for 54 h. All stability studies were carried out using six replicates of QC samples at two different concentration levels, that is, LQC and HQC against freshly spiked CC standards and the results were compared with the fresh QCs.

4.2.2.2 Results and Discussion

a) Bioanalytical Method Development

Among the buffers, phosphate buffer gave the peak with good symmetry and resolution, and thus phosphate buffer was selected for method development. Optimum concentration of phosphate buffer was found to be 100 mM because increasing concentration led to peak tailing and decrease in the resolution, using reduced concentration of buffer decreased sensitivity. Increase in the pH increased the negative charge on column and thus retention of ketorolac decreased, and it eluted much faster. Decreasing the pH led to opposite effect. Optimized pH was found to be 4.5 by several trials. The use of organic modifier also played a major role. IPA gave good resolution together with good peak symmetry. The maximum amount of organic modifier to be used was 20 % v/v of mobile phase, as the concentration increased the resolution and the retention time decreased. The optimized mobile phase was found to be 96:4 (0.1M NaH_2PO_4, pH 4.5: IPA). The order of elution was found to be the R (+) enantiomer followed by S (-) enantiomer and their retention time were 3.45 min and 4.62 min respectively. During the development of the method on the chiral AGP column, it was shown that method was selective because the enantiomers were separated to baseline (R_S=2.65). Addition of 5% v/v formic acid during the sample preparation led to increased

recovery of the analyte by the solid phase extraction. Thus optimized extraction protocol showed consistent and high recovery at all concentration levels without any interference from endogenous components and impurities. The performance of the HPLC assay was assessed by peak shape, selectivity, linearity, precision, accuracy, recovery, limit of quantification (LOQ), freeze-thaw stability and stability of reconstituted extracts.

b) Bioanalytical Method Validation

i) Selectivity

Chromatogram of six blank plasma samples revealed that there was no peak present at the retention time of ketorolac enantiomers (Fig 4.3). A lack of response in blank biological matrix confirmed the selectivity of the method from endogenous substances. Further test samples obtained from oral pharmacokinetic studies proved that there was no interference from the metabolite or degradation product in the near vicinity of the drug. Comparison of the chromatogram of the blank and spiked sample indicated selectivity of the method (Fig 4.4). Thus the proposed method was found to be selective in determination of ketorolac from the spiked as well as test sample.

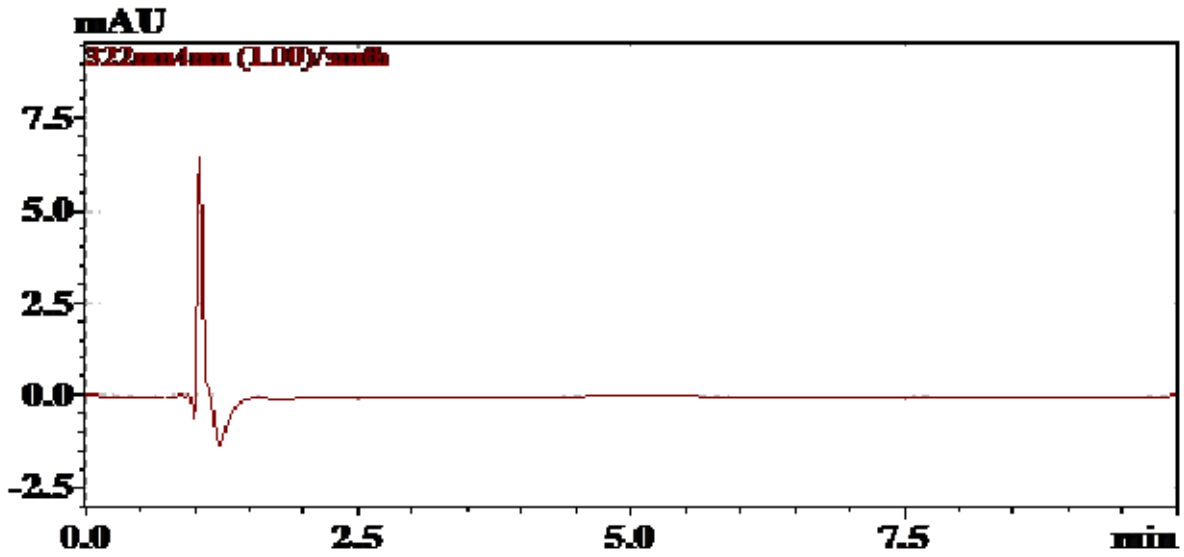

Fig 4.3: Representative chromatogram of blank rat plasma sample.

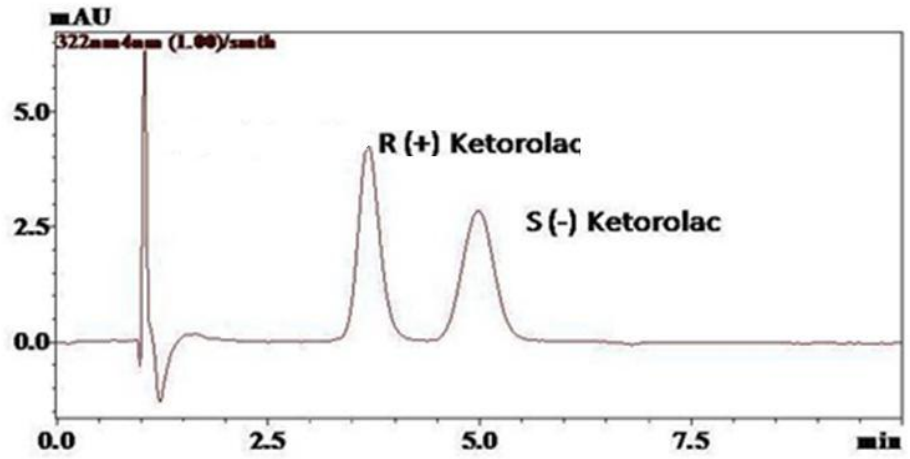

Fig 4.4: Representative chromatogram of standard R (+) ketorolac and S (-) ketorolac (0.25 µg/mL each) in rat plasma.

ii) Linearity and Range

Calibration standards from 0.01 to 7.5 µg/mL of each enantiomer were used in the weighted linear regression to construct a calibration curve (Table 4.7, 4.8).

peak area = 150499 x concentration (µg/mL) + 14.01(weighted $1/x^2$) ; R^2 = 0.9981 for R (+) ketorolac

peak area = 150354 x concentration (µg/mL) + 21.99; (weighted $1/x^2$); R^2 = 0.9984 for S (-) ketorolac

Table 4.7: Calibration curve of R (+) ketorolac in rat plasma

Conc (µg/mL)	Mean peak area [a]	S.D	%RSD
0.01	1519.333	94.553	6.223
0.05	7525.333	243.988	3.242
0.25	37812.667	1978.857	5.233
0.5	75300.667	2924.369	3.884
2.5	368347.333	21474.463	5.830
5	764522.333	28991.566	3.792
7.5	1130509.333	25552.933	2.260

[a] Each value represents the average of three independent determinations.

Table 4.8: Calibration curve of S (-) ketorolac in rat plasma

Conc (µg/mL)	Mean peak area[a]	S.D	% RSD
0.01	1526.333	86.327	5.656
0.05	7515.333	244.723	3.256
0.25	37747.667	1687.600	4.471
0.5	75258.333	3012.403	4.003

Conc (µg/mL)	Mean peak area[a]	S.D	% RSD
2.5	367805.000	17625.492	4.792
5	764326.333	28266.491	3.698
7.5	1131235.667	26977.403	2.385

[a] Each value represents the average of three independent determinations.

One way ANOVA was performed for peak area obtained at individual concentration and F value of 7.116×10^{-4} for R (+) ketorolac and 8.900×10^{-4} for S (-) ketorolac were found to much lower than F tabulated (3.555) at 5 % level of significance. Therefore, there was no significant difference between the measured calibration curve standards for both the enantiomers.

Good linear relationship existed between average peak area and concentration with weighted regression equation in various tissues for ketorolac enantiomers which is depicted in table 4.9.

Table 4.9: Calibration curve of ketorolac enantiomers in different tissues of rat

Biological sample	Form of Ketorolac	Standard curve	R^2	Range (µg/ml)
Brain	R (+) Ketorolac	peak area = 361000 x concentration (µg/mL) +16290 (weighted $1/x^2$)	0.993	0.025 – 2.50
	S (-) Ketorolac	peak area = 378700 x concentration (µg/ mL) +11590 (weighted $1/x^2$)	0.993	0.025 – 2.50
Heart	R (+) Ketorolac	peak area = 308700 x concentration (µg/ mL) +12240 (weighted $1/x^2$)	0.994	0.025 – 2.50
	S (-) Ketorolac	peak area = 335400 x concentration (µg/mL) + 9381 (weighted $1/x^2$)	0.996	0.025 – 2.50
Kidney	R (+) Ketorolac	peak area = 414500 x concentration (µg/ mL) +11880 (weighted $1/x^2$)	0.992	0.025 – 2.50
	S (-) Ketorolac	peak area = 435100 x concentration (µg/mL) +10230 (weighted $1/x^2$)	0.994	0.025 – 2.50
Liver	R (+) Ketorolac	peak area = 301900 x concentration (µg/mL) +15880 (weighted $1/x^2$)	0.994	0.025 – 2.50
	S (-) Ketorolac	peak area = 341100 x concentration (µg/mL) +14270 (weighted $1/x^2$)	0.992	0.025 – 2.50

Biological sample	Form of Ketorolac	Standard curve	R^2	Range (µg/ml)
Lungs	R (+) Ketorolac	peak area= 335500 x concentration (µg/mL) +12990 (weighted $1/x^2$)	0.996	0.025 – 2.50
	S (-) Ketorolac	peak area = 348700 x concentration (µg/mL) +11200 (weighted $1/x^2$)	0.994	0.025 – 2.50

iii) Recovery Studies

The proposed method showed high and consistent recovery of ketorolac from rat plasma. Mean absolute recovery in plasma was ranged from 87.948 % - 90.033 % for R (+) ketorolac and 88.657 % - 91.030 % for S (-) ketorolac over the calibration range (Table 4.10)

Table 4.10: Mean absolute recovery of ketorolac enantiomers from rat plasma

Analyte	Quality control	[a] Recovery (n=6)	
		Mean[a] ± SD	% RSD
R (+) Ketorolac	LQC	87.948 ± 3.702	4.209
	MQC	89.846 ± 4.378	4.873
	HQC	90.033 ± 4.040	4.488
S (-) Ketorolac	LQC	88.657 ± 3.839	4.331
	MQC	90.459 ± 4.127	4.562
	HQC	91.030 ± 3.139	3.448

[a] Recovery= [(Peak area of plasma standard/Peak area of analytical Standard)*100].

iv) Precision, Accuracy and Recovery

The obtained results confirmed the accuracy of the proposed method as the % Bias ranged from –5.039 to 6.303 and -4.561 to 6.849 for R (+) ketorolac and S (-) ketorolac respectively. The method was found to be precise with % RSD not exceeding 7.864 % and 6.165 % (Intra-batch) and 6.600% and 6.918 % (Inter-batch) for R (+) ketorolac and S (-) ketorolac respectively (Table 4.11).

v) Sensitivity

Plasma standards prepared at LLOQ showed quantifiable amount of ketorolac, when analyzed in replicate of six on three different days. Further it confirmed that the method was precise and accurate at LLOQ with % RSD 7.864 and 6.165 (intrabatch) and 6.600 and 6.918 (inter batch) for R (+) ketorolac and S (-) ketorolac respectively. % Bias was found to be not more than -5.356 and - 5.807 for R (+) ketorolac and S (-) ketorolac respectively at LLOQ level. The method was found to be sensitive with high signal to noise ratio at 322 nm detection wavelength. It can be suggested that the present method is suitable for various pharmacokinetic investigations in the rat plasma, which demands high sensitivity.

vi) Dilution Integrity

The dilution integrity of the method was found to be acceptable with accuracy (% Bias) of 5.950, 5.779 and 5.530 for 5 times dilution and 5.867 , 4.958 and 5.198 for 10 times dilution of 10, 12 and 14 µg/mL concentration levels for R (+) ketorolac respectively. Precision for dilutions were within the acceptable limits with % RSD of 5.722 , 5.129 and 5.627 for 5 times dilution and 4.210 , 4.740 and 5.377 for 10 times dilution for 10, 12 and 14µg/mL respective concentration levels of R (+) ketorolac respectively. For S (-) ketorolac, % bias was found to be 5.288, 5.064 and 5.688 for 5 times dilution and 5.384 , 5.144 and 4.759 for 10 times dilution for 10, 12 and 14 µg/mL concentration levels respectively. Precision for dilutions were within the acceptable limits with % RSD of 5.137, 5.672 and 5.768 for 5 times dilution and 4.976 , 4.683 and 4.714 % for 10 times dilution for 10, 12 and 14 µg/mL concentration levels of S (-) ketorolac respectively.

Table 4.11: Accuracy, intra and inter precision in rat plasma

Analyte	Nominal Conc (µg/mL)	Repeatability (n=6)			Intermediate (n=18)		
		Mean	% RSD	% Bias	Mean	% RSD	% Bias
R (+) Ketorolac	0.01	0.011	7.864	-5.039	0.011	6.600	-5.356
	0.025	0.023	6.049	6.303	0.024	4.116	5.958
	2.0	1.897	5.964	5.151	1.877	5.782	5.649
	6.0	5.675	5.896	5.421	5.696	4.980	5.074
S (-) Ketorolac	0.01	0.011	6.165	-5.807	0.010	6.918	-4.561
	0.025	0.024	3.925	5.547	0.023	5.891	6.849
	2.0	1.894	6.913	5.280	1.887	5.834	5.668
	6.0	5.670	4.557	5.493	5.701	4.857	4.985

vi) Stability Studies

Results obtained for bench top stability studies at two QC level (LQC and HQC) demonstrate that ketorolac enantiomers were stable in rat plasma under bench top conditions. Enantiomers of ketorolac does not show significant change (% RSD) in response up to 8.5 h, when compared with the response obtained from fresh standards. Similarly, in long term stability study, R (+) and S (-) ketorolac were found to be stable in rat plasma at -20 ^{0}C at all QC levels, as there was no significant difference between response of the standard at zero time

and at the end of 90 days. The maximum deviation observed was within acceptable limits. There was no significant degradation observed in QC standards up to 3 freeze thaw cycles. The recoveries of R (+) ketorolac were, from 94.098 % and 95.706 % and 93.949 % and 94.35 % for S (-) ketorolac at LQC and HQC level respectively, following three freeze-thaw cycles. Thus, the drug was found to be stable for three freeze thaw cycle making it suitable for subzero storage conditions. Percent deviation calculated for all stability studies were within the acceptable limit of ± 15 % at LQC and HQC level (Table 4.12) demonstrating the stability of ketorolac under various processing and storage condition stated in the method. Summary of the validation parameter is given in table 4.13.

Table 4.12: Stability studies of ketorolac enantiomers in rat plasma

Storage period and Storage condition	Analyte	Nominal Conc (µg/mL)	Mean[a]	% RSD	% Accuracy
Stock solution ~ 16 days, refrigerated temperature	R (+) Ketorolac	0.025	0.023	4.391	93.300
		6.000	5.682	4.123	94.698
	S (-) Ketorolac	0.025	0.024	3.311	94.415
		6.000	5.729	3.890	95.490
Bench top ~ 8.5 h, room temperature	R (+) Ketorolac	0.025	0.023	4.483	93.179
		6.000	5.612	5.400	95.571
	S (-) Ketorolac	0.025	0.023	4.367	93.949
		6.000	5.729	4.211	94.635
Three freeze thaw Cycle (-20^{o}C)	R (+) Ketorolac	0.025	0.024	4.662	94.098
		6.000	5.742	4.779	95.706
	S (-) Ketorolac	0.025	0.023	5.008	93.949
		6.000	5.678	4.558	94.635
In - Injector stability ~ 54 h	R (+) Ketorolac	0.025	0.024	5.030	94.373
		6.000	5.688	3.853	94.808
	S (-) Ketorolac	0.025	0.024	3.047	94.733
		6.000	5.695	4.088	94.923
Long term stability (-20^{o}C, ~ 90 days)	R (+) Ketorolac	0.025	0.024	4.373	94.018
		6.000	5.82	4.343	94.701
	S (-) Ketorolac	0.025	0.023	3.425	93.436
		6.000	5.733	4.841	95.551

[a] Each value represents the average of six independent determinations.

Table 4.13: Summary of validation parameters of ketorolac enantiomers in rat plasma

Parameter	Values	
	R (+) Ketorolac	S (-) Ketorolac
Calibration range	0.01-7.5 µg/mL	0.01-7.5 µg/mL
Regression coefficient	R^2 = 0.9981	R^2 = 0.9984
Regression equation	y (peak area) =150499 x concentration (µg/mL) +14.01	y (peak area)=150354 x concentration (µg/mL) + 21.99
Limit of detection	0.0021 µg/mL	0.0020 µg/mL
Lower limit of quantification	0.01 µg/mL	0.01 µg/mL
Absolute recovery / Recovery efficiency	87.948 % - 90.033 %	88.657 % - 91.030 %
Accuracy (% Bias)	-5.039 - 6.303 % (Intrady) -5.356 – 5.958 % (Interday)	-5.807% – 5.547% (Intrady) -4.561– 6.849 % (Interday)
Precision (% RSD)	5.896 % - 7.864 % (Intraday) 4.116 – 6.600 % (Interday)	3.925 % - 6.165% (Intraday) 4.857 % - 6.918 % (Interday)
System sutability	T_f = 1.20 ± 0.02 R_t = 3.46 ±0.002 N = 1306 ± 15.52	T_f = 1.11 ±0.02 R_t = 4.62±0.004 N =1399.23 ± 21.16 R_s = 2.65 ± 0.01
Selectivity	Selective	Selective

4.3 Bioanalytical Method Development and Validation of Venlafaxine and O-Desmethyl Venlafaxine

4.3.1 Estimation of Racemic Venlafaxine and O-Desmethyl Venlafaxine

4.3.1.1 Experimental

a) Material

Pure VEN and ODV were provided by Toronto Research Chemicals Inc. (Canada). Analytical grade sodium hydroxide pellets (NaOH) was obtained from Merck India Limited, Mumbai, India. Both orthophosphoricacid (H_3PO_4), potassium dihydrogen orthophosphate (KH_2PO_4) were of analytical grade delivered by S.D. Fine Chemicals, India. Milli-Q water purification system (Millipore, USA) was used for obtaining high quality HPLC grade water. In addition, an electronic balance (AG‑135, Mettler-Toledo, Germany), pH meter (pH tutor, Eutech Instruments, Singapore), a sonicator (Toshiba, New Delhi) were used.

b) Chromatographic System and Conditions

The chromatographic system used to perform development and validation of this method consisted of a LC-2010 CHT (Schimadzu, Japan) high performance liquid chromatographic instrument equipped with RF-20A fluorescence detector. Data collection and integration was accomplished using LC Solutions software. Chromatographic analysis was performed on Eclipse XDB-C18 column (4.6 × 150 mm I.D., particle size 5 µm, Agilent Technologies). Separation was achieved using a mobile phase of 10 mM potassium phosphate pH 4.5: acetonitrile (75:25, v/v) at a flow rate of 1.0 mL/min. The eluent was monitored using fluorescence detector at excitation wavelength of 226 nm and emission wavelength of 298 nm. The column was maintained at ambient temperature and injection volume of 50µl was used.

c) Preparation of Stock and Standards

Primary stock solution of 1 mg/mL racemic VEN and ODV were prepared by seperately dissolving 5 mg VEN and ODV in 5 mL volumetric flask containing methanol and the volume was made by the methanol. A common standard working stock solution containing 5, 10, 30, 50, 75, 100, 200 and 400 µg/mL of VEN and ODV were prepared by serial dilution of primary stock solutions with phosphate buffer pH 6.5. Eight rat plasma standards containing 0.1, 0.2, 0.6, 1, 1.5, 2, 4 and 8 µg/mL of VEN and ODV were prepared in rat plasma from working standards. The standards were vortexed for 5 min. Simultaneously Quality Control (QC) samples in rat plasma were prepared in the same manner as that of CC standards for VEN and ODV (0.1, 0.15, 1.6 and 6.0 µg/mL), representing QC samples at lower limit of quantification (LOQQC), low (LQC), medium (MQC) and high (HQC) levels, respectively. All experimental protocols were approved by the Institutional Animal Ethics Committee (approval number IAEC/RES/13/07/REV-2/17/14).

d) Sample Preparation

The OASIS HLB (Hydrophilic Lipophilic Balance) cartridges were used for analyte extraction from plasma samples. The cartridges were conditioned with methanol (1 mL) and equilibrated with de-ionised water (1 mL). To 500 µl of prepared samples, 200 µL buffer (pH 4.5) was added and vortexed for 5min and then loaded into cartridges. After that cartridges were washed with 1 mL 5% methanol finally eluted with 1 mL acetonitrile two times. The eluates were evaporated to dryness at 40^0C under N_2 gas. Residues were then reconstituted in 500 µL of phosphate buffer (pH 6.5).

e) Bioanalytical Method Development

Successful analysis of an analyte in biological fluids relies on the optimization of sample preparation, chromatographic separation and interference free detection.

In the process of bio analytical method development for simultaneous determination of VEN and ODV, mobile phase composition and flow rate were optimized by trying different aqueous phase and non-aqueous phase combinations at different flow rates. Various buffers such as phosphate buffer (pH 3-7 and 10 mM, 100 mM), citrate buffer (pH 3-5 and 10 mM, 100 mM), ammonium acetate buffer (pH 3-5 and 10 mM, 100 mM) and acetic acid buffer (pH 3-5 and 10 mM, 100 mM) were studied in combination with methanol (20, 25 and 30 %), acetonitrile (20, 25 and 30 %) and isopropanol alcohol (20, 25 and 30 %). Mobile phase composition and flow rate were finally selected based on the criteria of peak properties (retention time and asymmetric factor), sensitivity (height and area) and ease of preparation and applicability of the method for in vivo studies in rats.

f) Method Validation

The developed chromatographic method was validated for selectivity, linearity, range, precision, accuracy, sensitivity, stability and dilution integrity in plasma samples.

i) Selectivity

The test for selectivity was carried out using six different lots of blank plasma batches processed by the same extraction method and analyzed to determine the extent to which endogenous substances may contribute to the estimation of analytes. These samples were compared with those containing VEN and ODV, at the lower limit of quantitation (LLOQ). The aim of performing the selectivity check with these different batches of plasma samples is to ensure the quality of the results of study sample analysis. The peak area of interfering peak at the retention time of analyte should be <20% of peak area response of the analyte in LLOQ sample.

ii) Linearity and Range

It was obtained by preparing three sets of the drug solutions in mobile phase containing VEN and ODV at a concentration of 0.1- 8 µg/mL; 50 µl of these drug solutions were injected in to column and the peak area and retention time was recorded. Average peak area at each level was plotted against concentration and curves were subjected to weighted linear regression analysis by least square method. Regression equation was used to calculate the corresponding

predicted concentration. One way analysis of variance (ANOVA) was performed on each replicate response obtained at eight concentration levels.

iii) Precision and Accuracy

Accuracy and precision of the method in individual matrix was determined by analyzing QC standard prepared at LOQQC (0.1 µg/mL), LQC (0.15µg/mL), MQC (1.6 µg/mL) and HQC (6.0 µg/mL) levels. Each QC standard was processed and analysed in six replicates and analysis was repeated for three batches to study intra and inter batch precision and accuracy. Concentrations of VEN and ODV in QC standard were calculated from weighted regression equation. Accuracy was expressed as % Bias and precision was determined as intra and inter batch variation, expressed as percent relative standard deviation (% RSD).

iv) Recovery

Recovery of VEN and ODV from plasma samples were assessed (n=6) at LQC (0.15 µg/mL), MQC (1.6 µg/mL) and HQC (6.0 µg/mL) respectively and the above concentrations were attained by spiking equivalent amount of racemic VEN and ODV into rat plasma. The extraction efficiency was determined by comparing the concentration obtained from processed samples to corresponding concentration of analytical samples injected directly in the HPLC system.

v) Sensitivity

Sensitivity of the method was obtained by determining the lowest concentration of VEN and ODV that can be estimated with acceptable accuracy and precision (% RSD < 20 %) and it was a lower limit of quantification (LLOQ), in the individual matrix. The QC standards were prepared at LLOQ (0.02 µg/mL) concentration in six replicate and analyzed by the proposed method on three different occasions. Concentrations of VEN and ODV in the QC standard were calculated from the regression equation and parameters such as mean calculated concentration, % Bias and % RSD were determined.

vi) Dilution Integrity

To study the over curve dilution integrity, standards were prepared in rat plasma at 12, 18 and 24 µg/mL concentrations levels of VEN and ODV and they were diluted 5 and 10 times. The dilution integrity standards were vortexed for 5 min and processed as mentioned previously.

vii) Stability Studies

Various factors determine the stability of a drug, viz. the chemical properties, matrix, container system and storage conditions. Stability experiments in stock solution and plasma were performed very extensively to evaluate the stability of VEN and ODV. All the experimental conditions which the drugs actually encountered during sample analysis were simulated during method validation to evaluate the various stabilities like long-term stability at-20 ^{0}C for 90 days, freeze–thaw stability, room temperature stability of drug in plasma (bench-top stability), stock solution stability of VEN and ODVand post-preparative stability at auto-sampler temperature of 4 ^{0}C (auto-sampler stability).

To evaluate long-term stability, a long period was defined as the time elapsed between the start of sampling and the end of sample analysis. The aliquots of QCs were first frozen at -20 ^{0}C for 90 days and then thawed to be extracted and tested against fresh CC and QC samples. The difference between the starting concentration and the concentration after 90 days will show whether the drug in plasma can degrade under these conditions. Evaluation of freeze–thaw stability involves estimation of analytes stability after three freeze–thaw cycles. Similarly, bench-top stability of the spiked samples was performed for 8.5 h. The stock stability of drug was evaluated at refrigerated temperature for 16 days by comparing the response of stability stock with fresh stock. Stability of samples in auto sampler at a temperature (4 ^{0}C) was evaluated for 54 h. All stability exercises were carried out using six replicates of QC samples at two different concentration levels, that is, LQC and HQC against freshly spiked CC standards and the results were compared with the fresh QCs.

4.3.1.2 Results and Discussion

a) Bioanalytical Method Development

In the preliminary study, peak properties and response function were optimized by changing type of organic modifier, organic to aqueous ratio, buffer type, buffer strength and pH. Among the buffers, phosphate buffer gave peak with good symmetry and resolution. Optimum concentration of phosphate buffer was found to be 10 mM because increased concentration led to peak tailing and decreased the resolution where as using reduced concentration decreased its sensitivity. Reduction in pH led to peak asymmetry, where pH below 4 showed poor peak properties and loss in selectivity with incomplete resolution. Acetonitrile gave good resolution together with good peak symmetry for both VEN and ODV. Optimized concentration of acetonitrile was found to be 25 % of the mobile phase.

Eclipse XDB-C18 column (4.6 × 150 mm I.D., particle size 5 µ, Agilent Technologies) column was used for better peak symmetry and enhanced stability at higher pH. Moreover, excitation and emission wavelength were optimized to 226 and 298 nm, respectively for better sensitivity and selectivity. Thus optimized mobile phase consisted of 10 mM potassium dihydrogen phosphate pH 4.5: acetonitrile (75:25, v/v) with better peak properties, selectivity and reproducibility.

b) Bioanalytical Method Validation

i) Selectivity

Chromatogram of six blank rat plasma samples revealed that there was no peak present at the retention time of VEN and ODV (Fig 4.5). A lack of response in blank biological matrix confirmed the selectivity of the method from endogenous substances. Further test samples obtained from oral pharmacokinetic studies proved that there was no interference from the metabolite or degradation product in the near vicinity of the drug. Comparison of the chromatogram of the blank and spiked sample indicated selectivity of the method (Fig 4.6). Thus the proposed method was found to be selective in determination of VEN and ODV in the spiked as well as test sample.

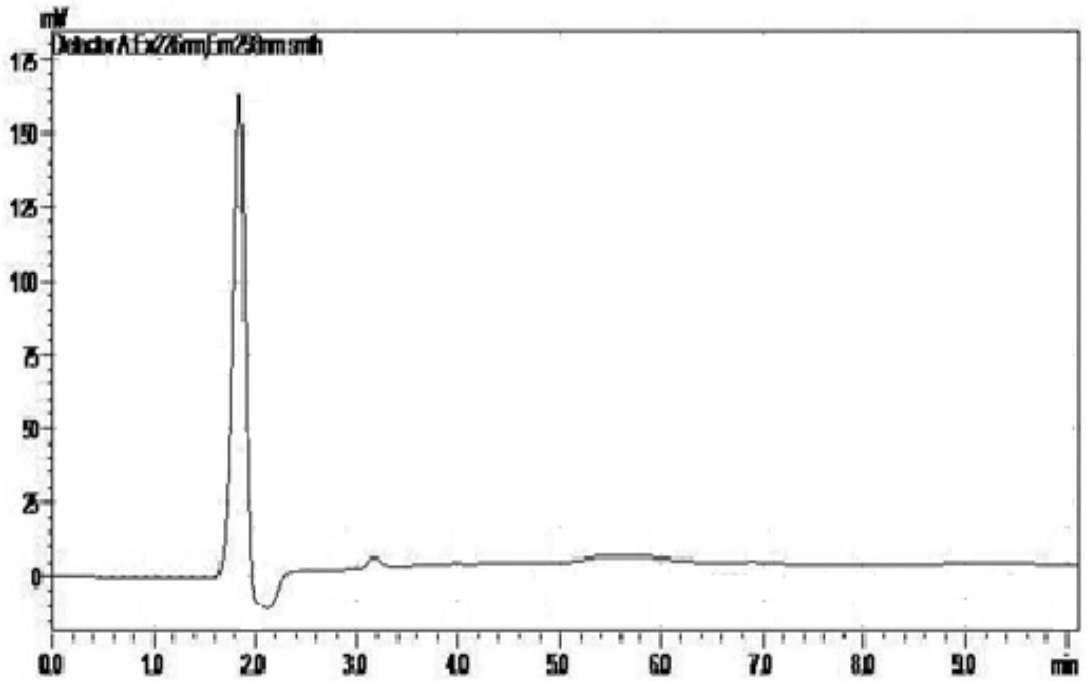

Fig 4.5: Representative chromatogram of blank rat plasma.

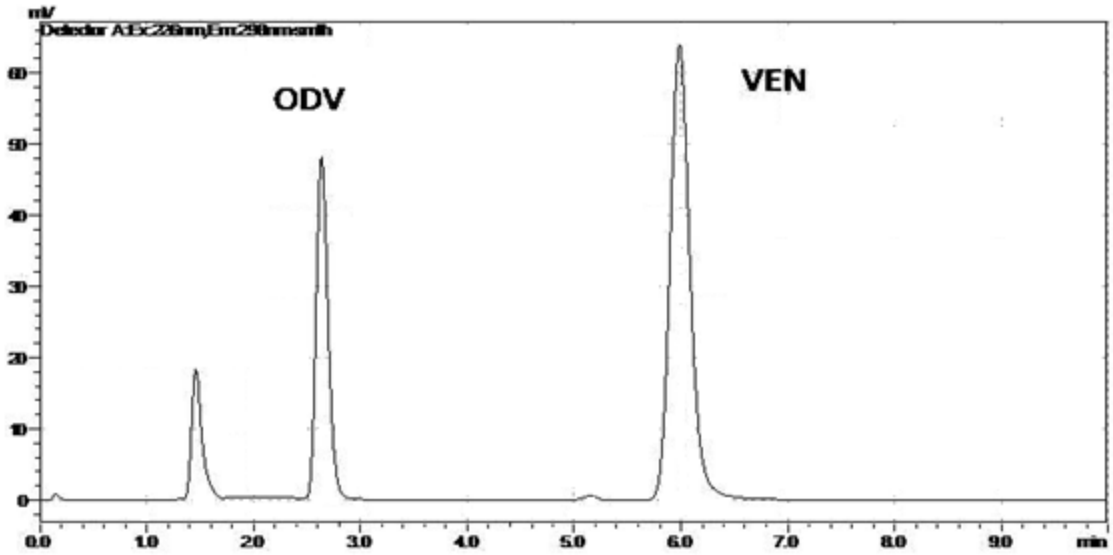

Fig 4.6: Representative chromatogram of VEN and ODV (0.6 µg/mL each) in rat plasma.

ii) Linearity and Range

The linearity regression analysis indicated linear relationship between average peak area and concentration in plasma over the range 0.10 – 8 µg/mL for VEN and ODV respectively (Table 4.14 and 4.15) with weighted regression equation.

peak area = 5460000 x concentration (µg/mL) – 382.7 (weighted $1/y^2$) ; R^2 = 0.9991 (VEN)

peak area = 1507000 x concentration (µg/mL) – 2102 (weighted $1/y^2$) ; R^2 = 0.9988 (ODV)

Table 4.14: Calibration curve of VEN in rat plasma

Conc (µg/mL)	Mean peak area[a]	S.D	% RSD
0.1	544808.667	31704.903	5.819
0.2	1091511.667	37020.536	3.392
0.6	3287430.000	97587.657	2.969
1	5463751.667	194297.039	3.556
1.5	8275423.333	355478.579	4.296
2	11004276.333	544116.853	4.945
4	21804653.667	905809.282	4.154
8	42821797.333	1142551.206	2.668

[a] Each value represents the average of three independent determinations.

Table 4.15: Calibration curve of ODV in rat plasma

Conc (µg/mL)	Mean peak area[a]	S.D	% RSD
0.1	148450.333	7576.584	5.104
0.2	299311.000	13976.504	4.670
0.6	904609.667	37345.932	4.128
1	1510737.333	57450.895	3.803
1.5	2248237.000	130519.375	5.805
2	3009745.000	171369.024	5.694
4	6002522.667	279696.974	4.660
8	12067423.000	550051.597	4.558

[a] Each value represents the average of three independent determinations.

One way ANOVA was performed for peak area obtained at individual concentration and F 5.834×10^{-4} for VEN and 2.551×10^{-4} for ODV values were found to be much lower than theoretical F (3.467) value at 5 % level of significance. Therefore there was no significant difference between the measured calibration curve standards.

Good linear relationship existed between average peak area and concentration with weighted regression equation in various tissues for VEN and ODV which is depicted in table 4.16 and 4.17.

iii) Recovery Studies

The proposed method showed high and consistent recovery of VEN and ODV from rat plasma. Mean absolute recovery in plasma was ranged from 89.449 to 92.045 % for VEN and ODV 88.606 to 91.983 % for ODV over the calibration range (Table 4.18).

Table 4.16: Calibration curve of VEN in various tissues of rat

Biological sample	Standard curve	R^2	Range (µg/mL)
Brain	peak area = 3284000 x concentration (µg/mL) + 9029 (weighted $1/y^2$)	0.9983	0.10 - 8.00
Heart	peak area = 3220000 x concentration (µg/mL) + 92453 (weighted $1/y^2$)	0.9984	0.10 - 8.00
Kidney	peak area = 3223000 x concentration (µg/mL) + 90175 (weighted $1/y^2$)	0.9985	0.10- 8.00

Biological sample	Standard curve	R^2	Range (µg/mL)
Liver	peak area = 3124000 x concentration (µg/mL) + 97342 (weighted $1/y^2$)	0.9980	0.10 - 8.00
Lungs	peak area = 3160000 x concentration (µg/mL) + 3457 (weighted $1/y^2$)	0.9983	0.10 - 8.00

Table 4.17: Calibration curve of ODV in various tissues of rat

Biological sample	Standard curve	R^2	Range (µg/mL)
Brain	peak area = 1146000 x concentration (µg/mL) + 9024 (weighted $1/y^2$)	0.9986	0.10 - 8.00
Heart	peak area = 1143000 x concentration (µg/mL) + 13100 (weighted $1/y^2$)	0.9986	0.10 - 8.00
Kidney	peak area = 1141000 x concentration (µg/mL) + 14570 (weighted $1/y^2$)	0.9986	0.10 - 8.00
Liver	peak area = 1146000 x concentration (µg/mL) + 10735 (weighted $1/y^2$)	0.9980	0.10 - 8.00
Lungs	peak area = 1150000 x concentration (µg/mL) + 8671 (weighted $1/y^2$)	0.9984	0.10 - 8.00

Table 4.18: Mean absolute recovery of VEN and ODV from rat plasma

Analyte	Quality Control	[a] Recovery (n=6)	
		Mean ± SD	% RSD
VEN	LQC	89.449 ± 3.167	3.540
	MQC	91.485 ± 3.082	3.369
	HQC	92.045 ± 3.021	3.283
ODV	LQC	88.606 ± 3.806	4.291
	MQC	90.047 ± 3.238	3.596
	HQC	91.983 ± 3.022	3.286

[a] Recovery= [(Peak Area of plasma standard/Peak area of analytical Standard)*100].

iv) Accuracy and Precision

The obtained results confirmed the accuracy of the proposed method as the % bias ranged from 5.090 - 6.980 for VEN and 5.213 – 7.361 for ODV. Method was found to be precise with % RSD not exceeding 5.556 and 7.373 for VEN and ODV (Intra-batch) and 5.070 for

VEN and 5.447 for ODV (inter batch). Results of precision and accuracy study were in acceptable limits and indicated that method was accurate and precise (Table 4.19)

Table 4.19: Intra and inter batch accuracy and precision

Analyte	Nominal Conc. (µg/mL)	Repeatability (n=6)			Intermediate (n=18)		
		Mean	% RSD	% Bias	Mean	% RSD	% Bias
VEN	0.1	0.094	5.218	5.505	0.093	4.662	6.980
	0.15	0.141	4.201	6.185	0.141	3.157	5.968
	1.6	1.517	4.793	5.191	1.518	3.571	5.145
	6	5.695	5.556	5.090	5.673	5.070	5.454
ODV	0.1	0.093	7.373	7.361	0.094	5.447	6.460
	0.15	0.141	5.099	6.079	0.141	3.884	6.029
	1.6	1.506	6.184	5.855	1.517	4.913	5.213
	6	5.657	3.706	5.713	5.655	3.217	5.751

v) Sensitivity

Plasma standards prepared at LLOQ showed quantifiable amount of VEN and ODV, when analyzed in replicate of six. Further it confirmed that the method was precise and accurate at LLOQ with % RSD not more than 5.218 and 7.373, with % bias not exceeding 6.980 and 7.361 for VEN and ODV respectively. The method was found to be sensitive with high signal to noise ratio at excitation and emission wavelength of 226 and 298 nm. It can be suggested that the developed method is suitable for various pharmacokinetic investigations in the rat plasma, which demands high sensitivity.

vi) Dilution Integrity

The dilution integrity of the method was found to be acceptable with accuracy (% Bias) of 5.546, 5.736 and 5.148 for 5 times dilution and 4.691, 5.520 and 5.422 for 10 times dilution of 12, 18 and 24 µg/mL concentration levels for VEN respectively. Precision for dilutions were within the acceptable limits with % RSD of 4.754 , 4.656 and 4.494 for 5 times dilution and 5.231, 5.122 and 4.739 for 10 times dilution of 12, 18 and 24 µg/mL concentration levels of VEN respectively. For ODV, % bias was found to be 5.457, 5.232 and 5.804 for 5 times dilution and 4.659, 5.135 and 4.837 for 10 times dilution of 12, 18 and 24 µg/mL concentration levels respectively. Precision for dilutions were within the acceptable

limits with % RSD of 5.939, 5.686 and 5.199 for 5 times dilution and 4.867, 5.680 and 5.976 for 10 times dilution of 12, 18 and 24 µg/mL concentration levels of ODV respectively.

vii) Stability Studies

Results obtained for bench top stability studies at two QC levels (LQC and HQC) demonstrated that ODV and VEN were stable in rat plasma under bench top conditions. VEN and ODV did not show significant change (% RSD) in response up to 8.5 h, when compared with the response obtained from fresh standards. Similarly, in long term stability study, VEN and ODV were found to be stable in rat plasma at -20 ^{0}C at all QC levels, as there was no significant difference between response of the standard at zero time and at the end of 90 days. The maximum deviation observed was within acceptable limit.

There was no significant degradation detected in QC standards up to three freeze thaw cycles. Results are expressed as % recovery, which was 94.492 % and 93.756 % at LQC while being 94.319 and 93.778 % at HQC level for VEN and ODV following three freeze-thaw cycles and the results were within acceptable limits. Thus, the drug was found to be stable for three freeze thaw cycle making it suitable for subzero storage conditions. Percent deviation calculated for all stability studies were within the acceptable limit of ± 15 % at LQC and HQC level, demonstrating the stability of VEN and ODV under various processing and storage conditions stated in the method (Table 4.20). Summary of validation parameters is given in table 4.21.

Table 4.20: Stability studies of VEN and ODV in rat plasma

Storage period and Storage condition	Analyte	Nominal Conc (µg/mL)	Mean[a]	% RSD	% Accuracy
Stock solution ~ 16 days, refrigerated temperature	VEN	0.150	0.141	4.531	93.811
		6.000	5.625	4.143	93.756
	ODV	0.150	0.140	5.093	93.486
		6.000	5.652	4.734	94.202
Bench top ~ 8.5 h, room temperature	VEN	0.150	0.142	4.035	94.780
		6.000	5.624	4.627	93.734
	ODV	0.150	0.141	4.063	94.020
		6.000	5.658	3.963	94.300

Storage period and Storage condition	Analyte	Nominal Conc (µg/mL)	Mean[a]	% RSD	% Accuracy
Three freeze thaw Cycle (-20°C)	VEN	0.150	0.142	3.224	94.492
		6.000	5.659	4.059	94.319
	ODV	0.150	0.141	4.788	93.756
		6.000	5.627	4.126	93.778
In - Injector stability ~ 54 h	VEN	0.150	0.141	3.490	93.913
		6.000	5.640	6.520	94.005
	ODV	0.150	0.142	4.254	94.755
		6.000	5.711	4.493	95.186
Long term stability (-20°C, ~ 90 days)	VEN	0.150	0.143	3.457	95.431
		6.000	5.660	4.935	94.334
	ODV	0.150	0.141	5.137	93.860
		6.000	5.609	3.335	93.484

[a] Each value represents the average of six independent determinations.

Table 4.21: Summary of validation parameters of VEN and ODV in rat plasma

Parameter	Values	
	VEN	ODV
Calibration range	0.10 – 8.0 µg/mL	0.10 - 8.0 µg/mL
Regression coefficient	R^2 = 0.9991	R^2 = 0.9988
Regression equation	peak area = 5460000 x concentration (µg/mL) – 382.7	peak area = 1507000 x concentration (µg/mL) - 2120
Limit of detection	0.0178 µg/mL	0.0183 µg/mL
Lower limit of quantification	0.10 µg/mL	0.10 µg/mL
Absolute recovery / Recovery efficiency	89.449 – 92.045 %	88.606 - 91.983 %
Accuracy (% Bias)	5.090 – 6.185 (Intraday) 5.145 – 6.980 (Interday)	5.713 – 7.361 (Intraday) 5.213 – 6.460 (Interday)
Precision (% RSD)	4.201 – 5.556 (Intraday) 3.157 – 5.07 (Interday)	3.706 – 7.373 (Intraday) 3.217 – 5.447 (Interday)
System sutability	T_f = 1.16 ±0.016 R_t = 5.96 ± 0.018 N = 4864 ± 48.86 R_s = 2.55 ± 0.06	T_f = 1.09 ± 0.010 R_t = 2.64 ±0.008 N = 3536.33 ± 65.96
Selectivity	Selective	Selective

4.3.2 Estimation of Enantiomers of Venlafaxine and O-Desmethyl Venlafaxine

4.3.2.1 Experimental

a) Material

Pure VEN, ODV and their enantiomers were provided by Toronto Research Chemicals Inc. (Canada). Analytical grade sodium hydroxide pellets (NaOH) was obtained from Merck India Limited, Mumbai, India. Both orthophosphoric acid (H_3PO_4), potassium dihydrogen orthophosphate (KH_2PO_4) were of analytical grade delivered by S.D. Fine Chemicals, India. Milli-Q water purification system (Millipore, USA) was used for obtaining high quality HPLC grade water. In addition, an electronic balance (AG-135, Mettler-Toledo, Germany), pH meter (pH tutor, Eutech Instruments, Singapore), a sonicator (Toshiba, New Delhi) were used.

b) Chromatographic System and Conditions

The chromatographic system used to perform development and validation of this method consisted of a LC-2010 CHT (Schimadzu, Japan) high performance liquid chromatographic instrument equipped with RF-20A fluorescence detector. Data collection and integration was accomplished using LC Solutions software. The column used was Chiral-AGP column (100 x 4.0 mm I.D., particle size 5 µm, Chromtech Ltd, Sweden). Mobile phase, 10 mM phosphate buffer pH 6.5: methanol (94:6, v/v) was filtered through a 0.22 µm membrane filter before use, degassed in a bath sonicator for 20 min and was pumped from the solvent reservoir to the column at a flow rate of 1mL/min to equilibrate the system. Then a sample, injection volume of 50 µl was given. The run time was set for 20 min and eluents were monitored at excitation wavelength of 226 nm and emission wavelength of 298 nm

c) Preparation of Stock and Standards

Primary stock solution of 1mg/mL VEN and ODV racemic was prepared by seperately dissolving 5 mg of both racemic VEN and ODV in 5 mL volumetric flask containing methanol and the volume was made by the same. A common standard working stock solution containing 5, 10, 30, 50, 75, 100, 200 and 400 µg/mL of comprising VEN and ODV were prepared by serial dilution of primary stock solutions with phosphate buffer pH 6.5. Eight rat plasma standards containing 0.05, 0.1, 0.3, 0.5, 0.75, 1, 2 and 4 µg/mL of each enantiomers of VEN and ODV were prepared by spiking equivalent amount of each working standard into blank rat plasma. The standards were vortexed for 5 min. Simultaneously Quality Control

(QC) samples in rat plasma were prepared in the same manner as that of CC standards for each R (-) VEN, S (+) VEN, R (-) ODV and S (+) ODV (0.05, 0.075, 0.80 and 3.0 µg/mL), representing QC samples at lower limit of quantification (LOQQC), low (LQC), medium (MQC) and high (HQC) levels, respectively. All CC and QC standards were prepared in three sets containing six replicates of each concentration. The prepared bio samples were processed as described in the sample preparation section and analyzed by the proposed method. All experimental protocols were approved by the Institutional Animal Ethics Committee (approval number IAEC/RES/13/07/REV-2/17/14).

d) Sample Preparation

The OASIS HLB (Hydrophilic Lipophilic Balance) cartridges were used for analyte extraction from rat plasma samples. The cartridges were conditioned with methanol (1 mL) and equilibrated with de-ionised water (1 mL). To 500 µl of prepared samples, 200 µL buffer (pH 4.5) was added and vortexed for 5 min and then loaded into cartridges. After that cartridges were washed with 1 mL 5% methanol finally eluted with 1 mL acetonitrile two times. The eluates were evaporated to dryness at 40^0C under N_2 gas. Residues were then reconstituted in 500 µL of phosphate buffer (pH 6.5).

e) Bioanalytical Method Development

Successful analysis of an analyte in biological fluids relies on the optimization of sample preparation, chromatographic separation and interference free detection.

In the process of bio analytical method development for enantiomers of VEN and ODV, mobile phase composition and flow rate were optimized by trying different aqueous phase and non-aqueous phase combinations at different flow rates. Various buffers such as phosphate buffer (pH 3-7 and 10 mM, 100 mM), citrate buffer (pH 3-5 and 10 mM, 100 mM), ammonium acetate buffer (pH 3-7 and 10 mM, 100 mM) and acetic acid buffer (pH 3-5 and 10 mM, 100 mM) were studied in combination with methanol (2, 4, 6, 8 %), acetonitrile (2, 4, 6, 8 %) and isopropanol (2, 4, 6, 8 %). Mobile phase composition and flow rate were finally selected based on the criteria of peak properties (retention time and asymmetric factor), sensitivity (height and area) and ease of preparation.

f) Bioanalaytical Method Validation

The developed chromatographic method was validated for selectivity, linearity, range, precision, accuracy, sensitivity, stability and dilution integrity in plasma samples.

i) Selectivity

The test for selectivity was carried out using six different lots of blank plasma batches processed by the same extraction method and analyzed to determine the extent to which endogenous substances may contribute to the interferences for analytes. These samples were compared with those containing R (-) VEN, S (+) VEN, R (-) ODV and S (+) ODV at the lower limit of quantitation (LLOQ). The aim of performing the selectivity check with these different batches of plasma samples is to ensure the quality of the results of study sample analysis. The peak area of interfering peak at the retention time of analyte should be <20% of analyte peak area response of the analyte in LLOQ sample.

ii) Linearity and Range

It was obtained by preparing three sets of the drug solutions in mobile phase containing VEN and ODV enantiomers at a concentration of 0.05- 4 µg/mL; 50 µL of these drug solutions were injected in to column and the peak area and retention time was recorded. Average peak area at each level was plotted against concentration and curves were subjected to linear regression analysis by least square method. Regression equation was used to calculate the corresponding predicted concentration. One way analysis of variance (ANOVA) was performed on each replicate response obtained at eight concentration levels.

iii) Precision and Accuracy

Accuracy and precision of the method in individual matrix was determined by analyzing QC standard prepared at LOQQC (0.05 µg/mL), LQC (0.075 µg/mL), MQC (0.8 µg/mL) and HQC (3.0 µg/mL) levels. Each QC standard was processed and analysed in six replicates and analysis was repeated for three bathes to study intra and inter batch precision and accuracy. Concentrations of R (-) VEN, S (+) VEN, R (-) ODV and S (+) ODV in QC standard were calculated from weighted regression equation. Accuracy was expressed as % Bias and precision was determined as intra and inter batch variation, expressed as % RSD.

iv) Recovery

Recovery of VEN and ODV enantiomers from plasma samples were assessed (n=6) at LQC (0.075 µg/mL), MQC (0.80 µg/mL) and HQC (3.00 µg/mL) respectively and the above concentrations were attained by spiking equivalent amount of racemic VEN and ODV into 0.5 mL rat plasma. The extraction efficiency was determined by comparing the areas obtained from processed samples to those solutions of corresponding concentration of analytical samples injected directly in the HPLC system.

v) Sensitivity

Sensitivity of the method was obtained by determining the lowest concentration of R (-) VEN, S (+) VEN, R (-) ODV and S (+) ODV that can be estimated with acceptable accuracy and precision (% RSD < 20 %) and it was the lower limit of quantification (LLOQ), in the individual matrix. The QC standards were prepared at LLOQ (0.05 µg/mL) concentration in six replicates and analyzed by the proposed method for three different batches. Concentrations of R (-) VEN, S (+) VEN, R (-) ODV and S (+) ODV in the QC standard were calculated from the weighted regression equation and parameters such as mean calculated concentration, % Bias and % RSD were determined.

vi) Dilution Integrity

To study the over curve dilution integrity, standards were prepared in rat plasma at 6, 9 and 12 µg/mL concentrations levels of enantiomers of VEN and ODV and they were diluted 5 and 10 times. The dilution integrity standards were vortexed for 5 min and processed as mentioned previously.

vii) Stability Studies

Various factors determine the stability of a drug, viz. the chemical properties, matrix, container system and storage conditions. Stability experiments in stock solution and plasma were performed very extensively to evaluate the stability of R (-) VEN, S (+) VEN, R (-) ODV and S (+) ODV. All the experimental conditions which the drugs actually encountered during sample analysis were simulated during method validation to evaluate the various stabilities like long-term stability at-20 ^{0}C for 90 days, freeze–thaw stability, room temperature stability of drug in plasma (bench-top stability), stock solution stability and post-preparative stability at auto-sampler temperature of 4 ^{0}C (auto-sampler stability).

To evaluate long-term stability, a long period was defined as the time elapsed between the start of sampling and the end of sample analysis. The aliquots of QCs were first frozen at -20 ^{0}C, for 90 days and then thawed to be extracted and tested against fresh CC and QC samples. The difference between the starting concentration and the concentration after 90 days will show whether the drug in plasma can degrade under these conditions. Evaluation of freeze–thaw stability involves estimation of analytes stability after three freeze–thaw cycles. Similarly, bench-top stability of the spiked samples was performed for 8.5 h. The stock stability of drug was evaluated at refrigerated temperature for 16 days by comparing the response of stability stock with fresh stock. Stability of samples in auto sampler at a

temperature (4 ^{0}C) was evaluated for 54 h. All stability exercises were carried out using six replicates of QC samples at two different concentration levels, that is, LQC and HQC against freshly spiked CC standards and the results were compared with the fresh QCs.

4.3.2.2 Results and Discussion

a) Bioanalytical Method Development

In the preliminary study, peak properties and response function were optimized by changing type of organic modifier, organic to aqueous ratio, buffer type, buffer strength and pH. Among the buffers phosphate buffer gave peak with good symmetry and resolution. Optimum concentration of phosphate buffer was found to be 10 mM because increasing the concentration led to peak tailing and decreased the resolution where as using at reduced concentration decreased its sensitivity.

The selection of pH was the key factor in method development, as the column is of protein having its isoelectric point between 2.7-3.8. As pH increased the negative charge on column increased and it retained the VEN for longer duration and took more time for elution and there was also no proper resolution of VEN enantiomers. Also by decreasing the pH, the retention time was decreased but there is no proper resolution of ODV enantiomers. Optimized pH was found to be 6.5 by several trials.

The use of organic modifier played a major role. Their effect on enantiomeric separation was studied. By using ACN, good response (peak area) and shorter retention time was achieved, but proper resolution was not attained. As ACN concentration was increased, the relatively non polar VEN enantiomers were resolved but ODV were not resolved and by decreasing the ACN concentration vice-versa happened. So, use of methanol which is less nonpolar compared to ACN was used for optimization. The maximum amount of organic modifier to be used was 20% of mobile phase, which prolongs column life, as the concentration increased the resolution increased and the retention time was decreased. The optimized ratio was found to be 94:6 (buffer: methanol) as it gave better resolution, sensitivity and reduced time of analysis. Moreover, emission and excitation wavelength were optimized to 226 and 298 nm, respectively for better sensitivity and selectivity from endogenous product. Thus, optimized mobile phase consisted of 10 mM potassium di hydrogen phosphate pH 6.5: methanol (94:6, v/v) with better peak properties, selectivity and reproducibility. The optimized extraction protocol showed consistent and high recovery at all concentration levels without any interference from endogenous components and impurities.

b) Bioanalytical Method Validation

i) Selectivity

Chromatogram of six blank rat plasma samples revealed that there was no peak present at the retention time of VEN and ODV enantiomers (Fig 4.7). A lack of response in blank biological matrix confirmed the selectivity of the method from endogenous substances. Further test samples obtained from oral pharmacokinetic studies proved that there was no interference from the metabolite or degradation product in the near vicinity of the drug. Comparison of the chromatogram of the blank and spiked sample indicated selectivity of the method (Fig 4.8). Thus the proposed method was found to be selective in determination of VEN and ODV enantiomers from the spiked as well as test sample.

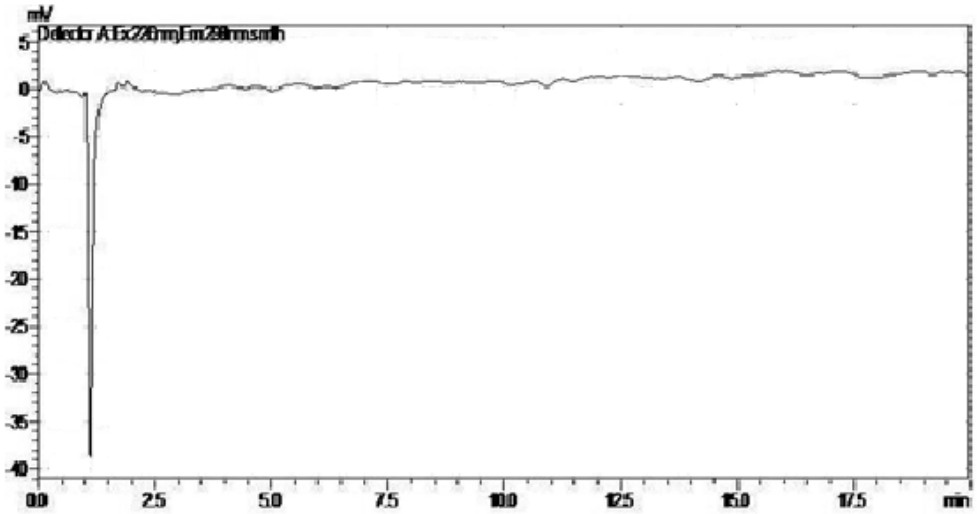

Fig 4.7: Representative chromatogram of blank rat plasma sample.

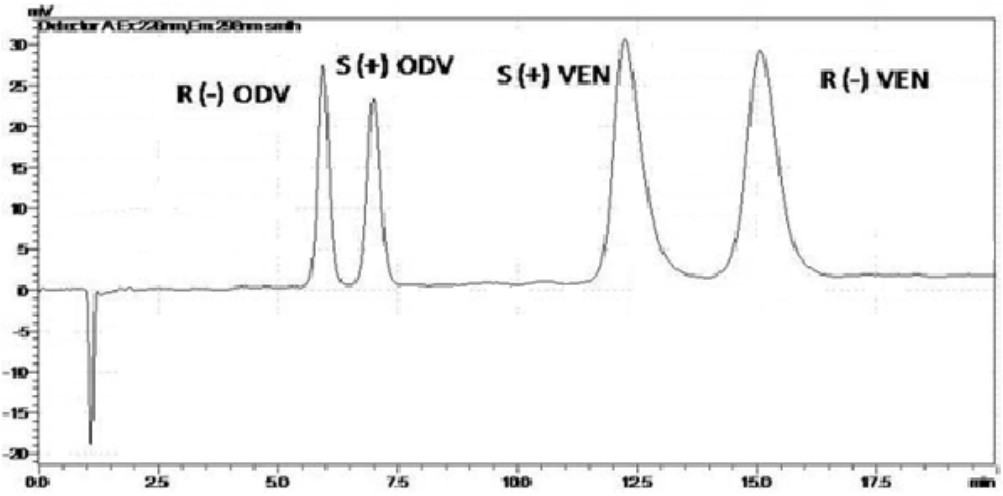

Fig 4.8: Representative chromatogram of R (-) ODV, S (+) ODV, S (+) VEN and R (-) VEN (0.3 µg/mL each) in rat plasma

ii) Linearity and Range

The linearity regression analysis indicated linear relationship between average peak area and concentration in plasma over the range 0.05 – 4 µg/mL with weighted regression equation for R (-) VEN, S (+) VEN, R (-) ODV & S (+) ODV (Table 4.22- 4.25).

peak area = 5424000 x concentration (µg/mL) + 1237(weighted $1/y^2$); R^2 = 0.9987 for R (-) VEN
peak area = 5424000 x concentration (µg/mL) + 1981 (weighted $1/y^2$); R^2 = 0.9987 for S (+) VEN
peak area = 1505000 x concentration (µg/mL) – 1147 (weighted $1/y^2$); R^2 = 0.9989 for R (-) ODV
peak area = 1491000 x concentration (µg/mL) – 574.8 (weighted $1/y^2$); R^2 = 0.9990 for S (+) ODV

Table 4.22: Calibration curve of R (-) VEN in rat plasma

Conc (µg/mL)	Mean peak area [a]	S.D	% RSD
0.05	271390.000	17189.567	6.334
0.1	544992.667	22002.161	4.037
0.3	1638419.667	48993.054	2.990
0.5	2737362.333	103643.245	3.786
0.75	4131878.000	193657.479	4.687
1	5488155.000	299176.260	5.451
2	10616036.333	629825.198	5.933
4	21296280.000	969294.367	4.551

[a] Each value represents the average of three independent determinations.

Table 4.23: Calibration curve of S (+) VEN in rat plasma

Conc (µg/mL)	Mean peak area[a]	S.D	% RSD
0.05	272334.667	14605.193	5.363
0.1	545392.667	21278.310	3.901
0.3	1630647.667	54883.536	3.366
0.5	2745808.667	108560.612	3.954
0.75	4134603.667	207687.510	5.023
1	5494685.000	258698.826	4.708
2	10652220.333	658442.681	6.181
4	21240223.333	1073345.524	5.053

[a] Each value represents the average of three independent determinations.

Table 4.24: Calibration curve of R (-) ODV in rat plasma

Conc (µg/mL)	Mean peak area [a]	S.D	% RSD
0.05	74159.000	3955.134	5.333
0.1	149386.667	8638.397	5.783
0.3	449207.333	21068.799	4.690
0.5	748292.333	32685.383	4.368
0.75	1133416.333	30875.464	2.724
1	1506785.667	54901.381	3.644
2	2972069.667	139379.516	4.690
4	6091891.667	334599.358	5.493

[a] Each value represents the average of three independent determinations.

Table 4.25: Calibration curve of S (+) ODV in rat plasma

Conc (µg/mL)	Mean peak area [a]	S.D	% RSD
0.05	73907.667	3949.527	5.344
0.1	148074.000	6683.690	4.514
0.3	453738.333	20338.536	4.482
0.5	742254.000	29511.760	3.976
0.75	1112165.667	54722.605	4.920
1.00	1490972.667	50208.707	3.368
2.00	2971505.667	125587.827	4.226
4.00	5946710.667	249460.875	4.195

[a] Each value represents the average of three independent determinations.

One way ANOVA was performed for peak area obtained at individual concentration and F 1.803×10^{-4} for R (-) VEN and 4.974×10^{-4} for S (+) VEN while 7.511×10^{-3} for R (-) ODV and 1.386×10^{-3} for S(+) ODV values were found to be much lower than theoretical F (3.467) value at 5 % level of significance. Therefore there was no significant difference between the measured calibration curve standards.

Good linear relationship existed between average peak area and concentration with weighted regression equation in various tissues for VEN and ODV enantiomers which is depicted in table 4.26 and 4.27.

Table 4.26: Calibration curve of VEN enantiomers in various tissues of rat

Biological sample	Form of VEN	Standard curve	R^2	Range (µg/mL)
Brain	R (-) VEN	peak area = 3387000 x concentration (µg/mL) + 2202 (weighted $1/y^2$)	0.9977	0.05 – 4.00
	S (+) VEN	peak area = 3402000 x concentration (µg/mL) + 6905 (weighted $1/y^2$)	0.9979	0.05 – 4.00
Heart	R (-) VEN	peak area = 3196000 x concentration (µg/mL) + 61022 (weighted $1/y^2$)	0.9971	0.05 – 4.00
	S (+) VEN	peak area = 3259000 x concentration (µg/mL) + 61454 (weighted $1/y^2$)	0.9978	0.05 – 4.00
Kidney	R (-) VEN	peak area = 3221000 x concentration (µg/mL) +35532 (weighted $1/y^2$)	0.9980	0.05 – 4.00
	S (+) VEN	peak area = 3254000 x concentration (µg/mL) + 35858 (weighted $1/y^2$)	0.9984	0.05 – 4.00
Liver	R (-) VEN	peak area = 3000000 x concentration (µg/mL) + 49366 (weighted $1/y^2$)	0.9958	0.05 – 4.00
	S (+) VEN	peak area = 3004000 x concentration (µg/mL) + 49543 (weighted $1/y^2$)	0.9952	0.05 – 4.00
Lungs	R (-) VEN	peak area = 3193000 x concentration (µg/mL) + 21477 (weighted $1/y^2$)	0.9971	0.05 – 4.00
	S (+) VEN	peak area = 3210000 x concentration (µg/mL) + 23024 (weighted $1/y^2$)	0.9974	0.05 – 4.00

[a] Each value represents the average of three independent determinations.

Table 4.27: Calibration curve of ODV enantiomers in various tissues of rats

Biological sample	Form of ODV	Standard curve	R^2	Range (µg/mL)
Brain	R (-) ODV	peak area = 1151000 x concentration (µg/mL) + 1252 (weighted $1/y^2$)	0.9989	0.05 – 4.00
	S (+) ODV	peak area = 1150000 x concentration (µg/mL) + 2911 (weighted $1/y^2$)	0.9987	0.05 – 4.00
Heart	R (-) ODV	peak area = 1128000 x concentration (µg/mL) + 3213 (weighted $1/y^2$)	0.9983	0.05 – 4.00
	S (+) ODV	peak area = 1133000 x concentration (µg/mL) + 3205 (weighted $1/y^2$)	0.9980	0.05 – 4.00
Kidney	R (-) ODV	peak area = 1150000 x concentration (µg/mL) +1569 (weighted $1/y^2$)	0.9985	0.05 – 4.00
	S (+) ODV	peak area = 1148000 x concentration (µg/mL) +1539 (weighted $1/y^2$)	0.9988	0.05 – 4.00
Liver	R (-) ODV	peak area = 1138000 x concentration (µg/mL) + 4582 (weighted $1/y^2$)	0.9984	0.05 – 4.00
	S (+) ODV	peak area = 1137000 x concentration (µg/mL) + 4802 (weighted $1/y^2$)	0.9983	0.05 – 4.00

Biological sample	Form of ODV	Standard curve	R^2	Range (µg/mL)
Lungs	R (-) ODV	peak area = 1143000 x concentration (µg/mL) + 2392 (weighted $1/y^2$)	0.9978	0.05 – 4.00
	S (+) ODV	peak area = 1148000 x concentration (µg/mL) + 2100 (weighted $1/y^2$)	0.9980	0.05 – 4.00

iii) Recovery studies

The proposed method showed high and consistent recovery of VEN and ODV enantiomers from rat plasma and tissue samples. Mean absolute recovery in plasma was 89.338 % - 91.199 % and 89.555 % - 92.176 % for R (-) VEN and S (+) VEN respectively while 89.063 - 91.249 % and 89.606 - 92.064 % for R (-) ODV and S (+) ODV respectively depicted in Table 4.28.

Table 4.28: Recovery studies of VEN and ODV enantiomers

Analyte	Quality control	[a] Recovery (n=6)	
		Mean ± SD	% RSD
R (-) VEN	LQC	89.338 ± 4.438	4.967
	MQC	90.956 ± 3.364	3.698
	HQC	91.199 ± 3.316	3.316
S (+) VEN	LQC	89.555 ± 4.208	4.699
	MQC	91.993 ± 3.689	4.010
	HQC	92.176 ± 3.067	3.327
R (-) ODV	LQC	89.063 ± 5.314	5.967
	MQC	90.112 ± 3.403	3.776
	HQC	91.249 ± 3.477	3.477
S (+) ODV	LQC	89.606 ± 4.922	5.493
	MQC	90.363 ± 3.368	3.727
	HQC	92.064 ± 3.054	3.318

[a] Recovery = [(Peak area of plasma standard/Peak area of analytical standard)*100].

iv) Accuracy and Precision

The obtained results confirmed the accuracy of the proposed method as the % Bias ranged from 5.002 - 6.989 for R (-) VEN and 5.148 - 6.572 for S (+) VEN while 5.032 - 7.162 for R (-) ODV and 5.059 - 6.658 for S (+) VEN. Method was found to be precise with % RSD not exceeding 5.464 and 5.195 for R (-) VEN and S (+) VEN (Intra batch) and 7.235 for R (-) ODV and 6.310 for S (+) ODV (intra batch) while % RSD not exceeding 5.388 and 5.084

for R (-) VEN and S (+) VEN (inter batch) whereas 6.751 for R (-) ODV and 6.044 % for S (+) ODV (inter batch). Results of precision and accuracy study were in acceptable limits and indicated that method was accurate and precise (Table 4.29).

Table 4.29: Intra and inter batch accuracy and precision

Analyte	Nominal Conc (µg/mL)	Repeatability (n=6)			Intermediate (n=18)		
		Mean	% RSD	% Bias	Mean	% RSD	% Bias
R (-) VEN	0.05	0.047	5.436	5.410	0.047	5.388	5.002
	0.075	0.070	5.464	6.125	0.070	4.098	6.989
	0.8	0.759	4.934	5.179	0.759	4.069	5.072
	3	2.842	4.684	5.259	2.835	4.597	5.509
S (+) VEN	0.05	0.047	5.195	6.484	0.047	4.769	5.868
	0.075	0.071	4.487	5.148	0.070	5.084	6.572
	0.8	0.756	5.153	5.534	0.756	3.318	5.558
	3	2.825	3.977	5.819	2.830	4.613	5.662
R (-) ODV	0.05	0.047	7.235	6.802	0.046	6.751	7.162
	0.075	0.070	5.711	6.123	0.071	5.826	5.812
	0.8	0.756	4.691	5.541	0.750	6.559	6.217
	3	2.849	4.178	5.032	2.828	3.340	5.729
S (+) ODV	0.05	0.047	6.310	6.528	0.047	6.044	6.658
	0.075	0.071	6.038	5.672	0.070	5.367	6.032
	0.8	0.753	4.227	5.883	0.752	5.340	5.984
	3	2.848	3.786	5.059	2.824	3.437	5.879

v) Sensitivity

Plasma standards prepared at LLOQ showed quantifiable amount of R (-) ODV, S (+) ODV, R (-) VEN and S (+) VEN, when analyzed in replicate of six on three different days. Further it confirmed that the method was precise and accurate at LLOQ with % RSD less than 5.436 and 5.195 and % Bias not exceeding 5.002 and 6.484 for R (-) VEN and S (+) VEN respectively whereas % RSD less than 7.235 and 6.310 and % Bias not exceeding 7.162 and 6.658 for R (-) ODV and S (+) ODV respectively. The method was found to be sensitive with high signal to noise ratio at emission and excitation wavelength of 226 and 298 nm. It can be suggested that the present method is suitable for various pharmacokinetic investigations in the rat plasma, which demands high sensitivity.

vi) Dilution Integrity

The dilution integrity of the method was found to be acceptable with accuracy (% Bias) of 5.405, 5.213 and 5.655 for 5 times dilution and 5.173, 5.120 and 5.744 for 10 times dilution of 6, 9 and 12 µg/mL concentration levels for R (-) VEN . Precision for dilutions were within the acceptable limits with % RSD of 5.573, 4.348 and 5.368 for 5 times dilution and 4.784, 5.775 and 4.494 for 10 times dilution of 6, 9 and 12 µg/mL respective concentration levels for R (-) VEN respectively. Accuracy (% Bias) of 5.410, 5.145 and 5.472 for 5 times dilution and 5.265, 5.905 and 5.138 for 10 times dilution of 6, 9 and 12 µg/mL concentration levels for S (+) VEN. Precision for dilutions were within the acceptable limits with % RSD of 4.874, 4.454 and 5.439 for 5 times dilution and 5.003, 5.825 and 4.068 for 10 times dilution of 6, 9 and 12 µg/mL respective concentration levels for S (+) VEN respectively. For R (-) ODV % Bias was found to be 5.485 , 5.451 and 5.762 for 5 times dilution and 5.964, 4.659 and 5.145 for 10 times dilution of 6, 9 and 12 µg/mL concentration levels respectively. Precision for dilutions were within the acceptable limits with % RSD of 5.051, 4.790 and 5.142 for 5 times dilution and 5.898, 4.420 and 4.348 for 10 times dilution of 6, 9 and 12 µg/mL concentration levels of R (-) ODV respectively while for S (+) ODV % Bias was found to be 5.577, 5.285 and 5.238 for 5 times dilution and 5.736, 5.538 and 4.869 for 10 times dilution of 6, 9 and 12 µg/mL concentration levels respectively. Precision for dilutions were within the acceptable limits with % RSD of 4.504, 4.625 and 5.608 for 5 times dilution and 5.911, 4.509 and 4.375 for 10 times dilution of 6, 9 and 12 µg/mL concentration levels for S (+) ODV respectively.

vii) Stability Studies

Results obtained for bench top stability studies at two QC level (LQC and HQC) demonstrated that R (-) ODV, S (+) ODV, R (-) VEN and S (+) VEN stable in rat plasma under bench top conditions, with no significant change (% RSD) in response up to 8.5 h , when compared with the response obtained from fresh standards. Similarly, in long term stability study, R (-) ODV, S (+) ODV, R (-) VEN and S (+) VEN was found to be stable in rat plasma at –20 ^{0}C at all QC levels, as there was no significant difference between response of the standard at zero time and at the end of 90 days. The maximum deviation observed was within acceptable limits. There was no significant degradation detected in QC standards up to three freeze thaw cycles. Results are expressed as % recovery, which was 93.015 % and 94.571 % at LQC and 93.858 % and 94.234 % at HQC level for R (-) VEN and S (+) VEN, respectively following three freeze-thaw cycles, results were within acceptable limits. %

Recovery was found to be 94.180 % and 94.017 % at LQC and 93.575 % and 93.889 % at HQC level for R (-) ODV and S (+) ODV, respectively following three freeze-thaw cycles and the results were within acceptable limits. Thus, the enantiomers were found to be stable for three freeze thaw cycles making it suitable for subzero storage conditions. Percent deviation calculated for all stability studies were within the acceptable limit of ± 15 % at LQC and HQC level, demonstrating the stability R (-) VEN, S (+) VEN, R (-) ODV and S (+) ODV under various processing and storage conditions8 stated in the method (Table 4.30, 4.31). Summary of validation parameters of VEN and ODV enantiomers are given in table 4.32 and 4.33 respectively.

Table 4.30: Stability studies of VEN enantiomers in rat plasma

Storage period and Storage condition	Analyte	Nominal Conc (µg/mL)	Mean[a]	% RSD	% Accuracy
Stock solution ~ 16 days, room temperature	R (-) VEN	0.075	0.071	6.561	94.664
		3.000	2.835	4.420	94.508
	S (+) VEN	0.075	0.070	5.125	93.401
		3.000	2.833	4.254	94.419
Bench top ~ 8.5 h, room temperature	R (-) VEN	0.075	0.070	2.069	93.091
		3.000	2.803	5.010	93.438
	S (+) VEN	0.075	0.070	4.332	93.502
		3.000	2.819	6.560	93.959
Three freeze thaw Cycle (-20 ^{o}C)	R (-) VEN	0.075	0.070	3.245	93.015
		3.000	2.816	5.498	93.858
	S (+) VEN	0.075	0.071	4.735	94.571
		3.000	2.827	4.391	94.234
In - Injector stability (~ 54 h)	R (-) VEN	0.075	0.071	2.400	94.080
		3.000	2.855	5.386	95.171
	S (+) VEN	0.075	0.071	5.204	94.941
		3.000	2.845	4.853	94.826
Long term stability (-20^{o}C, ~ 90 days)	R (-) VEN	0.075	0.071	5.975	94.706
		3.000	2.839	4.543	94.635
	S (+) VEN	0.075	0.071	5.537	94.270
		3.000	2.812	4.801	93.736

[a] Each value represents the average of six independent determinations.

Table 4.31: Stability studies of ODV enantiomers in rat plasma

Storage period and Storage condition	Analyte	Nominal Conc (µg/mL)	Mean[a]	%RSD	% Accuracy
Stock solution ~ 16 days, room temperature	R (-) ODV	0.075	0.071	6.007	94.134
		3.000	2.813	4.144	93.782
	S (+) ODV	0.075	0.070	5.776	93.429
		3.000	2.805	2.971	93.499
Bench top ~ 8.5 h, room temperature	R (-) ODV	0.075	0.071	6.009	94.002
		3.000	2.828	3.906	94.281
	S (+) ODV	0.075	0.072	6.293	95.835
		3.000	2.93	3.126	93.732
Three freeze thaw Cycle (-20^{o}C)	R (-) ODV	0.075	0.071	5.948	94.180
		3.000	2.807	4.165	93.575
	S (+) ODV	0.075	0.071	5.356	94.017
		3.000	2.92	3.128	93.889
In - Injector stability ~ 54 h	R (-) ODV	0.075	0.072	5.944	95.424
		3.000	2.822	3.891	94.081
	S (+) ODV	0.075	0.071	5.319	94.320
		3.000	2.829	3.127	94.762
Long term stability (-20^{o}C, ~ 90 days)	R (-) ODV	0.075	0.070	5.804	93.975
		3.000	2.827	3.823	94.218
	S (+) ODV	0.075	0.071	5.709	94.200
		3.000	2.829	3.367	94.306

[a] Each value represents the average of six independent determinations.

Table 4.32: Summary of validation parameters of VEN enantiomers in rat plasma

Parameter	Values	
	R (-) VEN	S (+) VEN
Calibration range	0.05 - 4.0 µg/mL	0.05 – 4.0 µg/mL
Regression coefficient	R^2 = 0.9987	R^2 = 0.9987
Regression equation	y (peak area) = 5424000 x concentration (µg/mL) + 1237 (weighted 1/y^2)	y (peak area) = 5424000 x concentration (µg/mL) + 1981 (weighted 1/y^2)
Limit of detection	0.006 µg/mL	0.010 µg/mL
Lower limit of quantification	0.05 µg/mL	0.05 µg/mL
Absolute recovery / Recovery efficiency	88.338 - 91.199%	89.555 - 92.176 %
Accuracy (% Bias)	5.179 – 6.125 (Intraday) 5.002 – 6.989 (Interday)	5.148 – 6.484 (Intraday) 5.558 – 6.572 (Interday)

Parameter	Values	
	R (-) VEN	S (+) VEN
Precision (% RSD)	4.684 - 5.464 (Intraday) 4.069 - 5.388 (Interday)	3.977 - 5.195 (Intraday) 3.318 - 5.084 (Interday)
System sutability	T_f = 1.16 ± 0.002 R_t = 14.67 ±0.012 N = 2443.67 ± 74.85	T_f = 1.14 ±0.005 R_t = 11.76 ±0.019 N = 2479.33 ± 115.14 R_s = 2.45 ± 0.042
Selectivity	Selective	Selective

Table 4.33: Summary of validation parameters of ODV enantiomers in rat plasma

Parameter	Values	
	R (-) ODV	S (+) ODV
Calibration range	0.05 - 4.0 µg/mL	0.05 - 4.0 µg/mL
Regression coefficient	R^2 = 0.9989	R^2 = 0.9990
Regression equation	y (peak area) =1505000 x concentration (µg/mL) – 1147 (weighted $1/y^2$)	y (peak area)= 1491000 x concentration (µg/mL) – 574.8 (weighted $1/y^2$)
Limit of detection	0.011 µg/mL	0.005 µg/mL
Lower limit of quantification	0.05 µg/mL	0.05µg/mL
Absolute recovery /Recovery efficiency	89.063 - 91.249	89.606 – 92.064
Accuracy (% Bias)	5.032 – 6.802 (Intraday) 5.729 – 7.162 (Interday)	5.059 – 6.528 (Intraday) 5.879 – 6.658 (Interday)
Precision (% RSD)	4.178 – 7.235 (Intraday) 3.340 – 6.751 (Interday)	3.786 – 6.310 (Intraday) 3.437 – 6.044 (Interday)
System sutability	T_f = 1.09 ± 0.010 R_t = 5.93 ±0.022 N = 2876.33 ± 35.02	T_f = 1.11 ± 0.010 R_t= 6.94 ± 0.016 N = 2765.33 ± 107.05 R_s = 2.56 ± 0.048
Selectivity	Selective	Selective

4.4 Conclusion

Reproducible, accurate and sensitive RP-HPLC methods using solid phase extraction technique were developed to analyze ketorolac, VEN, ODV and their enantiomers in the rat plasma and different tissue samples. The developed methods have a suitable low limit of quantification along with the very short analysis time which can be useful in analyzing many samples in a single day. Thus, these methods are advantageous in comparison to other analysis techniques available in published literature. These methods employed a very simple cost effective sample preparation technique. The method had advantages over problems of

poor chromatography, questionable uncharacterized peak, tedious extraction steps, high injection load. The methods have been found to be simple, robust and can be used for analysis. The developed methods can be used not only for pharmacokinetics and biodistribution but also can be used in evaluating novel delivery systems.

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Chapter 5

Pharmacokinetic and Biodistribution Study

5.1 Introduction

The understanding of pharmacokinetic behavior of drugs in biological system has always been the subject of primary importance in treatment of diseases. Preclinical pharmacokinetic, tissue distribution and metabolism studies provide valuable information on efficacy and safety profile of a drug, since normally a good correlation exists between pharmacokinetic and therapeutic as well as toxicological profiles (1-3). A comprehensive knowledge of the absorption, distribution, metabolism and elimination processes of a compound are important for the interpretation of pharmacological and toxicological studies and also for designing delivery systems. Tissue distribution studies are essential in providing information on distribution and accumulation of the drug and/or metabolites, especially in relation to potential sites of action or toxicity. These information may be useful for designing pharmacological and toxicological studies and for interpreting the results of these experiments.

In case of enantiomeric drugs, knowledge of pharmacokinetic parameters and distribution profile of each enantiomer can provide insight of role played by each enantiomer in therapeutic efficacy, toxicity or side effects, as pharmacokinetic parameters are likely to differ.

Ketorolac is available as a racemic mixture of equimolar ratio of R (+) and S (−) stereo isomers. Animal studies have shown that the pharmacological activity of ketorolac resides in the S (-) ketorolac and that the R (+) ketorolac is pharmacologically inactive (4). The pharmacokinetic studies of racemic ketorolac as well as enantiomers have been documented in humans (5-8), and animals such as goats, rats (9, 10). However, no study has been reported on tissue distribution.

Although pharmacokinetic studies have been reported for VEN and its metabolite, most of them have been done in humans (11-31). There are only few reports of pharmacokinetics of VEN and its metabolite in rats (32). There is only one paper describing the pharmacokinetics of enantiomers of VEN (33). Biodistribution studies have been reported in pig using radiolabeling techniques (34).

Knowledge of pharmacokinetic and biodistribution profiles of enantiomers of ketorolac, venlafaxine and its metabolite will help to understand role of enantiomers in therapeutic efficacy, side effects or toxic effects. Further they may help in proper planning of therapy and delivery.

5.2 Pharmacokinetic and Biodistribution of Ketorolac and its Enantiomers

5.2.1 Experimental

a) Materials & Instruments

Drug (ketorolac tromethamine) and all chemicals were obtained from sources as mentioned in chapter 4. A tissue tearer Sorvall (Dupont Instruments, USA) and ultrasonic cell disruptor-Microson^{TM} (Misonix, USA) were used for tissue homogenization. All surgical instruments such as scissors, forceps, glass syringes, etc were used after sterlization.

b) Animals

Healthy male wistar rats were selected for pharmacokinetic and biodistribution studies of racemic ketorolac in plasma and tissues. Rats weighing 200 ± 20 g were used in the study. Animals were fed with the standard laboratory pellet food with water *ad libitum*. Rats were maintained at controlled temperature and humidity and artificial light and dark cycles (12h). Rats under study were fasted overnight before the study but water was provided. All experimental protocols were approved by the Institutional Animal Ethics Committee (approval number IAEC/RES/13/07/REV-2/17/14) and were accordance with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

c) Pharmacokinetic and Biodistribution Studies

Single dose oral and i.v administration of pure racemic drug solutions were made for the studies. The drug concentration levels in plasma and various organs were determined at different time intervals. The objective of this study was to find the enantioselective disposition of the selected drug after administration.

i) IV Dosing and Plasma Sample Collection

Rats were administered 1.6 mg/kg of ketorolac tromethamine solution through the caudal vein using 1 mL syringe (25 G x ½ " needle), after dilating the vein with hot water**.** From each animal, one blood sample was collected by retro orbital puncture and three animals were used for each time point, at predetermined intervals of 4 min, 8 min, 16 min, 32 min, 46 min, 1 h, 2 h, 4 h, and 8 h after administration of drug. At each time point, 1mL of blood sample was withdrawn under anaesthetized condition. Blood samples were collected in tubes containing 100 µL of 10% w/v sodium EDTA (Ethylene diamine tetraacetate), as anticoagulant and centrifuged at 12000 rpm for 30 min at -4^{o}C. The resulting plasma samples were stored at -20^{o}C until analysis.

ii) Oral Dosing and Plasma Sample Collection

Rats were administered 3.2 mg/kg of ketorolac tromethamine solution using oral feeding catheter. From each animal, one blood sample was collected by retro orbital puncture and three animals were used for each time point, at predetermined intervals of 5 min, 10 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 9 h and 12 h after administration of drug. At each time point, 1mL of blood sample was withdrawn under anaesthetized condition. Blood samples were collected in tubes containing 100 µL of 10% w/v sodium EDTA (Ethylene diamine tetraacetate), as anticoagulant and centrifuged at 12000 rpm for 30 min at -4^{0}C. The resulting plasma samples were stored at -20^{o}C until analysis.

iii) Tissue Sample Collection

In oral pharmacokinetic study, at 15 min, 30 min, 2 h and 6 h, time points, post blood sample collection, the whole blood was drained off from inferior venacava using 10 mL syringe (21 G x 1" needle) and heart, kidney, liver, lungs and brain were collected by surgical process. For this purpose, under ether anesthesia the abdominal incision was made to expose all organs. The collected tissues were perfused with saline to remove residual blood. Separated organs were transferred to an ice cold petridish containing saline. All tissues were cleaned with 2-5 mL of saline. Tissue was blotted on a filter paper, weighed and homogenized in the saline solution. This homogenates were centrifuged at the same conditions as that of plasma samples and the supernatant were collected. Biosamples were frozen and maintained at -20 ^{0} C until analysis.

c) Analysis of Biological Samples

To 500 µL of plasma and tissue samples, 200 µL mobile phase, 100 µL of 5% v/v formic acid was added and the drug was eluted by methanol by using solid phase extraction. The eluates were evaporated to dryness at 40^{0}C under N_2 gas. Residues were then reconstituted in 500 µL of mobile phase. Concentration of drug in biological samples was determined by the developed and validated bioanalytical methods reported in chapter 4.

d) Pharmacokinetic Data analysis

The drug amount in various plasma and tissue at different time intervals were analyzed by the non-compartmental analysis method using WinNonlin ver 2.1 software. Pharmacokinetic parameters like C_{max}, $AUC_{0-\infty}$, MRT, V_d, K_e , $t_{1/2}$ and Cl were determined for ketorolac and its enantiomers. Finally the results of the invivo pharmacokinetics of enantiomers at different doses were assessed using suitable statistical tests with $P< 0.05$ level of significance.

5.2.2 Results and Discussion

a) I.V Administration

The log plasma concentration vs time profile of ketorolac and its enantiomers after i.v. administration is shown in fig 5.1. Plasma concentrations of R (+) ketorolac exceeded those of S (-) ketorolac throughout the time course. Thus $AUC_{0-\infty}$ of R (+) ketorolac was significantly higher than that of S (-) ketorolac. The total body Cl of S (-) ketorolac was significantly larger than that of R (+) ketorolac as calculated from the result. There was no significant difference between MRT, K_e and $t_{1/2}$ of R (+) ketorolac and S (-) ketorolac. The V_d of S (-) ketorolac was almost twice to that of R (+) ketorolac. The pharmacokinetic parameters of each enantiomer and their paired t-test probability values of the difference between each enantiomer using the non-compartmental technique is shown in table 5.1.

Table 5.1: Pharmacokinetic parameters of ketorolac and its enantiomers in plasma after I.V administration in rat

Parameters	Ketorolac	R (+) Ketorolac	S (-) Ketorolac	Probability*
$AUC_{0-\infty}$ (µg.h/mL)	7.57 ± 0.77	5.06 ± 0.62	2.37 ± 0.12	0.019
MRT(h)	2.47 ± 0.10	2.47 ± 0.14	2.35 ± 0.03	0.347
V_d (L/kg)	0.53 ± 0.04	0.38 ± 0.01	0.79 ± 0.03	0.021
K_e (h^{-1})	0.40 ± 0.02	0.42 ± 0.04	0.43 ± 0.002	0.375
$t_{1/2}$ (h)	1.74 ± 0.08	1.66 ± 0.15	1.60 ± 0.01	0.403
Cl (L/h/kg)	0.21 ± 0.02	0.16 ± 0.02	0.34 ± 0.02	0.006

*If probability value less than 0.05 ($p<0.05$), there exists a significant difference between the values at 95% confidence interval between enantiomers

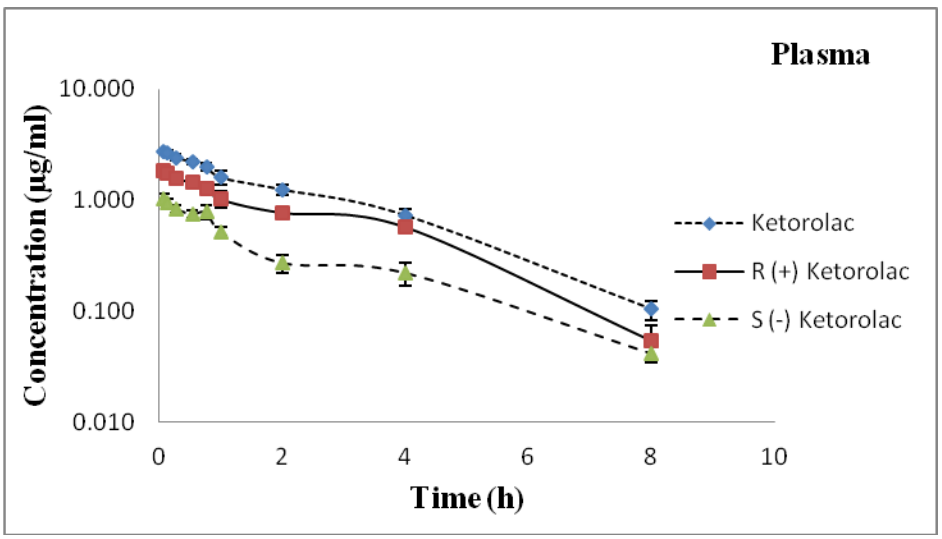

Fig 5.1: Log plasma concentration - time profiles of ketorolac and its enantiomers after I.V administration in rat.
(Each value represents the mean ± standard deviation of three independent determinations)

b) Oral Route of Administration

The log plasma concentration vs time profile of ketorolac and its enantiomers after oral administration is shown in fig 5.2. Plasma concentrations of R (+) ketorolac was found to be higher than those of S (-) ketorolac. At 95 % confidence interval, parameters like $AUC_{0-\infty}$, C_{max}, V_d of R (+) ketorolac were significantly different from S (-) ketorolac. MRT and $t_{1/2}$ of R (+) ketorolac was not significantly different from S (-) ketorolac. Cl of R (+) ketorolac was not statistically different from S (-) ketorolac but it was almost, half the value of its antipode. Oral plasma concentration profile of ketorolac and their enantiomers were similar to i.v profile. As it is evident from both the profiles that the concentration of R (+) ketorolac is higher than S (-) ketorolac in the plasma through out the time course suggesting higher tissue distribution of S (-) ketorolac. The bioavailibity of S (-) ketorolac found to be slightly higher than R (+) ketorolac. The pharmacokinetic parameters of each enantiomer and their paired t-test probability values of the difference between each enantiomers is shown in table 5.2.

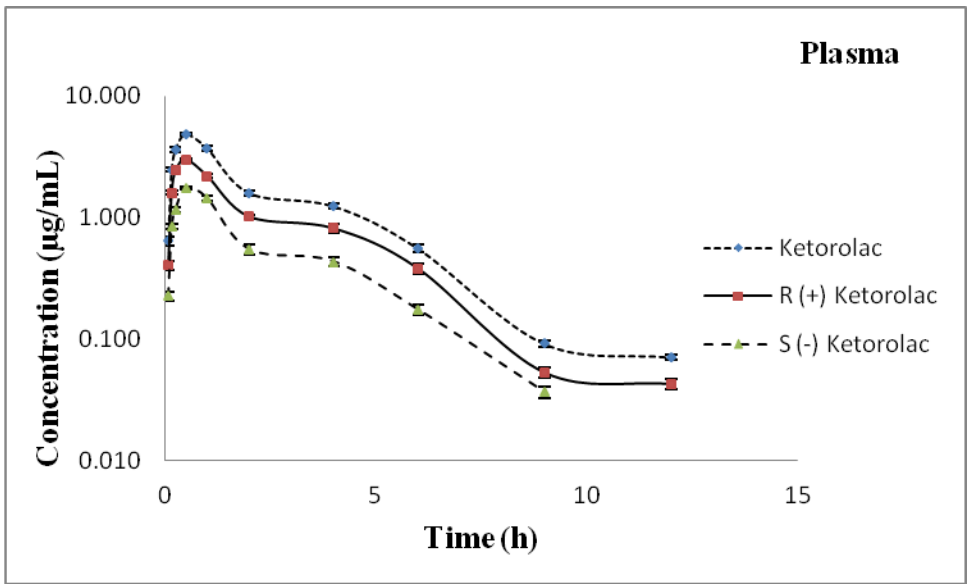

Fig 5.2: Log plasma concentration - time profiles of ketorolac and its enantiomers after oral administration in rat.

(Each value represents the mean ± standard deviation of three independent determinations)

Table 5.2: Pharmacokinetic parameters of ketorolac and its enantiomers after oral administration in rat

Parameters	Ketorolac	R (+) Ketorolac	S (-) Ketorolac	Probability*
T_{max} (h)	0.5	0.5	0.5	-
C_{max} (µg/mL)	4.82 ± 0.15	2.99 ± 0.10	1.76 ± 0.05	0.027
$AUC_{0-\infty}$ (µg.h/mL)	12.25 ± 0.74	7.79 ± 0.59	4.34 ± 0.43	0.021
MRT (h)	2.71 ± 0.03	2.76 ± 0.08	2.63 ± 0.11	0.091
K_e (h^{-1})	0.38 ± 0.01	0.38 ± 0.01	0.40 ± 0.02	0.251
V_d (L/kg)	0.69 ± 0.04	0.54 ± 0.03	0.93 ± 0.05	0.023
$t_{1/2}$ (h)	1.81 ± 0.01	1.81 ± 0.03	1.75 ± 0.07	0.232
Cl (L/h/kg)	0.26 ± 0.02	0.21 ± 0.01	0.37 ± 0.04	0.057
F	0.81 ± 0.019	0.77 ± 0.01	0.92 ± 0.02	0.056

*If probability value less than 0.05 ($p<0.05$), there exists a significant difference between the values at 95% confidence interval between the enantiomers.

b) Biodistribution Studies: Oral Route of Administration

The drug amount in different tissues following single oral dose of 3.2 mg/kg racemic drug were determined and plotted as a function of time. The various pharmacokinetic parameters calculated are listed in table 5.3. The drug amount (per unit weight of tissue) vs time profile of heart, kidney, liver and lungs are depicted in fig 5.3 to 5.6 respectively. The drug amount in the brain was below limit of quantification except at 30 min indicating very poor permeability to brain and thus not presented.

Maximum tissue amounts were observed within 30 min of administration in all the tissues heart, lungs, liver and kidney. The pharmacokinetic parameters C_{max}, AUC_{tissue}, MRT, K_e, $t_{1/2,}$ and Cl were calculated for heart, kidney, liver and lungs from drug amount vs time profiles. Pharmacokinetic parameters of both the enantiomers in heart was found to be more or less similar indicating no difference between the isomers though C_{max} for S (-) ketorolac was found to be marginally higher. In kidney, there was a significant difference in pharmacokinetic parameters. The C_{max} was nearly four times and AUC was found to be more than double for S (-) ketorolac than that of R (+) ketorolac. MRT, K_e and $t_{1/2}$ differ significantly in kidney. In liver, C_{max} was found to be much higher for S (-) ketorolac but AUC did not differ significantly. In liver, S (-) ketorolac eliminated very fast in comparison to R (+) ketorolac having $t_{1/2}$ (one third) in comparison to R (+) ketorolac. In lungs, there was

no difference observed for C_{max} and other parameters but AUC was found to be marginally higher for S (-) ketorolac.

From the results, it seems that the order of distribution of S (-) ketorolac with respect to C_{max} was kidney > liver > lungs > heart. Thus it was found that the ketorolac enantiomers exhibited difference in their disposition. From the tissue distribution studies it is evident that ketorolac and its enantiomers have poor permeation to brain. The difference in distribution of two enantiomers may be due to enantioselective binding with proteins.

Table 5.3: Pharmacokinetic parameters of ketorolac and their enantiomers in tissues of rat after oral administration

Biological sample	Parameters	Ketorolac	R (+) Ketorolac	S (-) Ketorolac	Probability*
Heart	T_{max} (h)	0.5	0.5	0.5	-
	C_{max} (µg/g)	0.86 ± 0.06	0.39 ± 0.03	0.44 ± 0.02	0.0072
	AUC $_{0-\infty}$ (µg.h/g)	1.71 ± 0.12	0.73 ± 0.05	0.81 ± 0.05	0.0560
	MRT (h)	2.50 ± 0.01	1.56 ± 0.02	1.41 ± 0.04	0.1624
	Ke (h^{-1})	0.36 ± 0.001	0.53 ± 0.01	0.58 ± 0.02	0.2160
	$t_{1/2}$ (h)	1.95 ± 0.01	1.31 ± 0.01	1.21 ± 0.04	0.2098
	Cl (L/h/kg)	1.88 ± 0.13	2.21 ± 0.16	1.98 ± 0.12	0.1087
Kidney	T_{max} (h)	0.5	0.5	0.5	-
	C_{max} (µg/g)	6.95 ± 0.48	1.41 ± 0.12	5.35 ± 0.57	0.0509
	AUC $_{0-\infty}$ (µg.h/g)	14.17 ± 1.64	4.28 ± 0.39	9.68 ± 0.97	0.0478
	MRT (h)	1.93 ± 0.03	2.89 ± 0.10	1.62 ± 0.02	0.0294
	Ke (h^{-1})	0.46 ± 0.001	0.33 ± 0.01	0.52 ± 0.02	0.004
	$t_{1/2}$ (h)	1.52 ± 0.03	2.10 ± 0.07	1.33 ± 0.02	0.0215
	Cl (L/h/kg)	0.23 ± 0.03	0.38 ± 0.03	0.17 ± 0.02	0.0385
Liver	T_{max} (h)	0.5	0.5	0.5	-
	C_{max} (µg/g)	2.08 ± 0.24	0.79 ± 0.12	1.34 ± 0.19	0.0572
	AUC $_{0-\infty}$ (µg.h/g)	3.06 ± 0.39	1.88 ± 0.25	1.83 ± 0.23	0.1124
	MRT (h)	4.51 ± 0.44	4.46 ± 0.01	0.97 ± 0.003	0.0018
	K_e (h^{-1})	0.90 ± 0.01	0.24 ± 0.004	0.70 ± 0.01	0.0001
	$t_{1/2}$ (h)	0.77 ± 0.01	2.93 ± 0.01	0.99 ± 0.01	0.0027
	Cl (L/h/kg)	1.06 ± 0.13	0.86 ± 0.11	0.87 ± 0.11	0.0413
Lungs	T_{max} (h)	0.5	0.5	0.5	-
	C_{max} (µg/g)	1.73 ± 0.02	0.86 ± 0.03	0.89 ± 0.02	0.5353
	$AUC_{0-\infty}$ (µg.h/g)	5.48 ± 0.48	3.70 ± 0.37	4.09 ± 0.49	0.0467
	MRT(h)	0.92 ± 0.02	4.37 ± 0.25	4.66 ± 0.48	0.1920
	K_e (h^{-1})	0.22 ± 0.02	0.23 ± 0.01	0.22 ± 0.01	0.1704
	$t_{1/2}$ (h)	3.13 ± 0.30	3.04 ± 0.17	3.21 ± 0.33	0.2339
	Cl (L/h/kg)	0.42 ± 0.6	0.44 ± 0.04	0.40 ± 0.05	0.1581

*If probability value less than 0.05 ($p<0.05$), there exists a significant difference between the values at 95% confidence interval between the enantiomers.

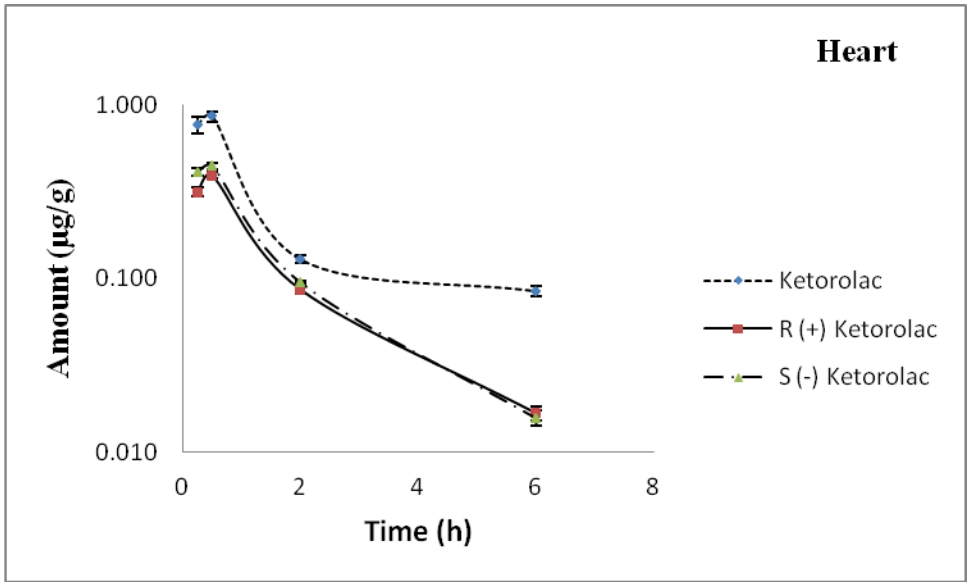

Fig 5.3: Log amount - time profiles of ketorolac and its enantiomers in rat heart.

(Each value represents the mean ± standard deviation of three independent determinations)

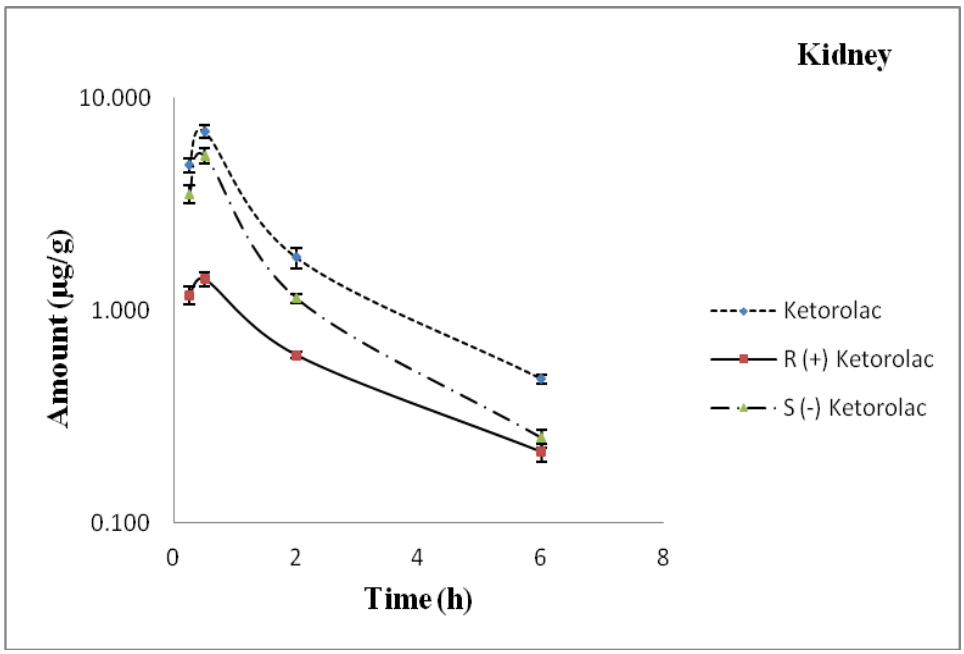

Fig 5.4: Log amount - time profiles of ketorolac and its enantiomers in rat kidney.

(Each value represents the mean ± standard deviation of three independent determinations)

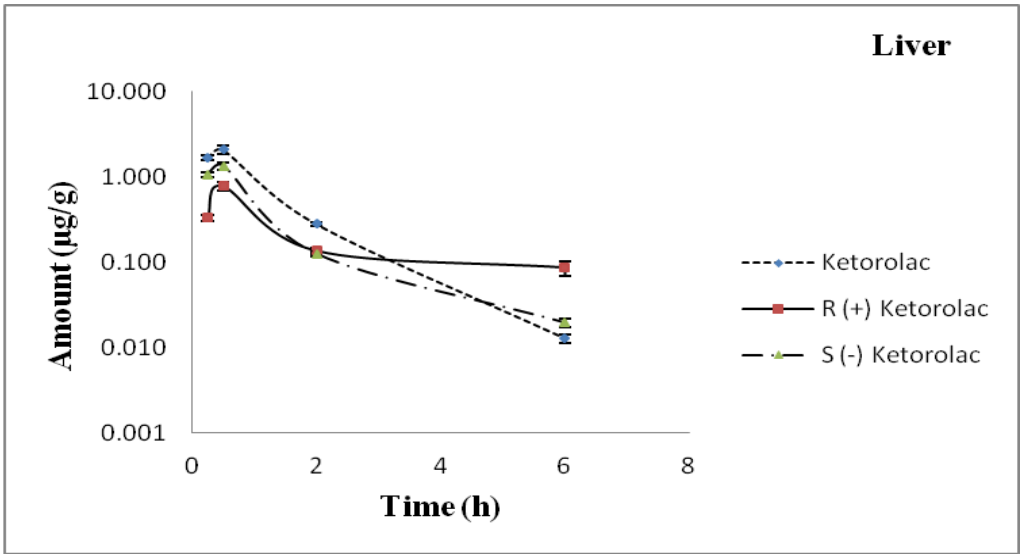

Fig 5.5: Log amount - time profiles of ketorolac and its enantiomers in rat liver.

(Each value represents the mean ± standard deviation of three independent determinations)

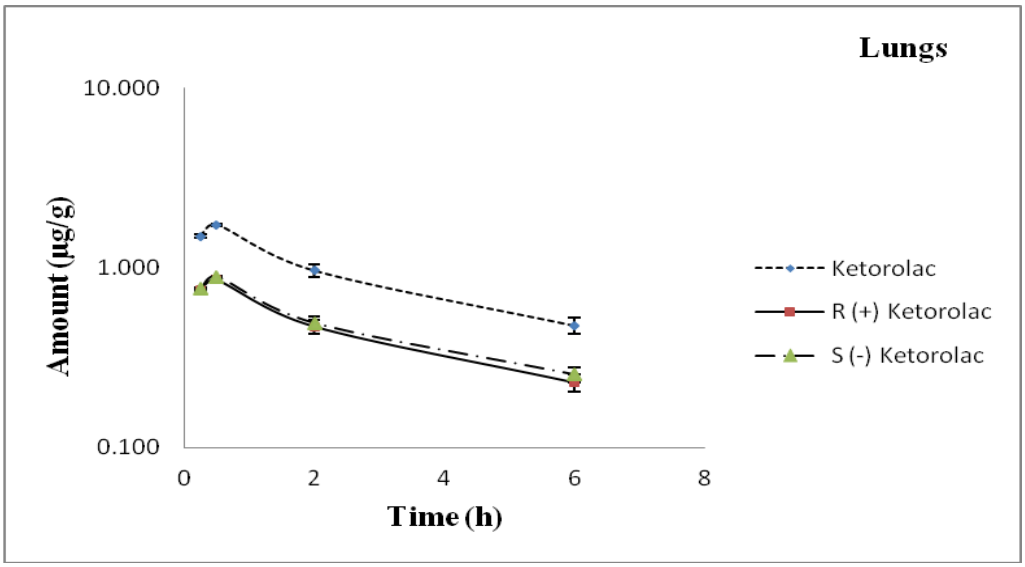

Fig 5.6: Log amount - time profiles of ketorolac and its enantiomers in rat lungs.

(Each value represents the mean ± standard deviation of three independent determinations)

5.3 Pharmacokinetic and Biodistribution of VEN and ODV

5.3.1 Experimental

a) Materials & Instruments

Drugs (Venlafaxine hydrochloride) and all chemicals were obtained from sources as mentioned in chapter 4. A tissue tearer Sorvall (Dupont Instruments, USA) and ultrasonic cell disruptor Microson^{TM} (Misonix, USA) were used for tissue homogenization. All surgical instruments such as scissors, forceps, glass syringes, silk suture etc were used after sterlization.

b) Animals

Healthy male wistar rats were selected for pharmacokinetic and biodistribution studies of racemic venlafaxine in plasma and tissues. Rats weighing 200 ± 20 g were used in the study. Animals were fed with the standard laboratory pellet food with water *ad libitum*. Rats were maintained at controlled temperature and humidity and artificial light and dark cycles (12h). Rats under study were fasted overnight before the study but water was provided. All experimental protocols were approved by the Institutional Animal Ethics Committee (approval number IAEC/RES/13/07/REV-2/17/14) and were accordance with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

c) Pharmacokinetic and Biodistribution Studies

Single dose oral and i.v administration of pure racemic drug solutions were made for the studies. The drug concentration levels in plasma and various organs were determined at different time intervals. The objective of this study was to find the enantioselective disposition of the selected drug after administration.

i) IV Dosing and Plasma Sample Collection

Rats were administered 40 mg/kg of venlafaxine hydrochloride solution through the caudal vein using 1 mL syringe (25 G x ½" needle), after dilating the vein with hot water**.** From each animal, one blood sample was collected by retro orbital puncture and three animals were used for each time point, at predetermined intervals of 2 min, 4 min, 8 min, 16 min, 32 min, 1, 2, 4, and 8 h after administration of drug. At each time point, 1mL of blood sample was withdrawn under anaesthetized condition. Blood samples were collected in tubes containing 100 µL of 10% w/v sodium EDTA (Ethylene diamine tetraacetate), as anticoagulant and centrifuged at 12000 rpm for 30 min at -4^{0}C. The resulting plasma samples were stored at -20^{o}C until analysis.

ii) Oral Dosing and Plasma Sample Collection

Rats were administered 120 mg/kg of venlafaxine hydrochloride solution using oral feeding catheter. From each animal, one blood sample was collected by retro orbital puncture and three animals were used for each time point, at predetermined intervals of 5 min, 10 min, 15 min, 30 min, 1, 2, 4, 6, 9 and 12 hrs after administration of drug. Blood samples were collected in tubes containing 100 µL of 10% w/v sodium EDTA (Ethylene diamine tetraacetate), as anticoagulant and centrifuged at 12000 rpm for 30 min at -4^{0}C. The resulting plasma samples were stored at -20^{o}C until analysis.

iii) Tissue Sample Collection

In oral pharmacokinetic study, at 10 min, 15 min, 30 min, 1h, 3 h and 6 h, time points, post blood sample collection, the whole blood was drained off from inferior venacava using 10 mL syringe (21 G x 1" needle) and heart, kidney, liver, lungs and brain were collected by surgical process. For this purpose, under ether anesthesia the abdominal incision was made to expose all organs. The collected tissues were perfused with saline to remove residual blood. Separated organs were transferred to an ice cold petridish containing saline. All tissues were cleaned with 2-5 mL of saline. Tissue was blotted on a filter paper, weighed and homogenized in the saline solution. This homogenates were centrifuged at the same conditions as that of plasma samples and the supernatant were collected. Biosamples were frozen and maintained at -20 ^{0} C until analysis.

d) Analysis of Biological Samples

To 500 µL biosamples, 200 µL buffer of pH 4.5 was added and the drug was eluted by methanol by using solid phase extraction. The eluates were evaporated to dryness at 40^{0}C under N_2 gas. Residues were then reconstituted in 500 µL of mobile phase. Concentration of drug in biological samples was determined by the developed bioanalytical methods, reported in chapter 4.

e) Pharmacokinetic Data Analysis

The drug amount in various tissues and plasma at different time intervals were analyzed by the non-compartmental analysis method using WinNonlin ver 2.1 software. Pharmacokinetic parameters like C_{max}, $AUC_{0-\infty}$, MRT, K_e, V_d, $t_{1/2}$ and Cl were determined for VEN, ODV and their enantiomers using different techniques. Finally the results of the invivo pharmacokinetics of enantiomers at different doses were assessed using suitable statistical tests with $P < 0.05$ level of significance.

5.3.2 Results and Discussions

a) I.V administration

The log plasma concentration vs time profile of VEN and its enantiomers after i.v. administration of 40 mg/kg is shown in fig 5.7. Plasma concentration of S (+) VEN was higher than those of R (-) VEN. There was significant difference in most of the pharmacokinetic parameters between the two enantiomers. $AUC_{0-\infty}$ of S (+) VEN was significantly higher than that of R (-) VEN. $AUC_{0-\infty}$ was nearly three times for S (+) VEN than that of R (-) VEN. K_e was nearly more than twice for R (-) VEN than its enantiomer. $t_{1/2}$ of S (+) VEN was more than double compared to that of R (-) VEN. The total body Cl of R (-) VEN was more than three times than its enantiomer. The pharmacokinetic parameters of each enantiomer and their paired t-test probability values of the difference between each enantiomer using the non-compartmental technique is shown in table 5.5. The log concentration vs time profile of ODV and its enantiomers after i.v. dose of 40 mg/kg of VEN is depicted in fig 5.8. Plasma concentrations of S (+) ODV was higher than those of R (-) ODV. There was significant difference in the pharmacokinetic parameters between the enantiomers. $AUC_{0-\infty}$ was more than five times for S (+) ODV than that of R (-) ODV. K_e was nearly 37% more for R (-) ODV than that of S (+) ODV. $t_{1/2}$ of S (+) ODV was 36 % more than its enantiomer. The pharmacokinetic parameters of each enantiomer and their paired t-test probability values of the difference between each enantiomer using the non-compartmental technique is shown in table 5.4 and 5.5.

Table 5.4: Pharmacokinetic parameters of VEN and its enantiomers in plasma after I.V adminstration of VEN in rat

Parameters	VEN	R (-) VEN	S (+) VEN	Probability*
$AUC_{0-\infty}$ (µg.h/mL)	3.49 ± 0.51	0.50 ± 0.05	1.40 ± 0.13	0.027
MRT (h)	1.99 ± 0.56	0.68 ± 0.002	1.45 ± 0.05	0.002
K_e (h^{-1})	0.50 ± 0.15	1.38 ± 0.01	0.63 ± 0.02	0.002
V_d (L/kg)	22.58 ± 3.20	27.35 ± 2.65	19.08 ± 1.86	0.078
$t_{1/2}$ (h)	1.46 ± 0.44	0.50 ± 0.002	1.11 ± 0.04	0.001
Cl (L/h/mL)	11.63 ± 1.60	40.12 ± 3.76	13.14 ± 1.03	0.012

*If probability value less than 0.05 ($p<0.05$), there exists a significant difference between the values at 95% confidence interval between the enantiomers.

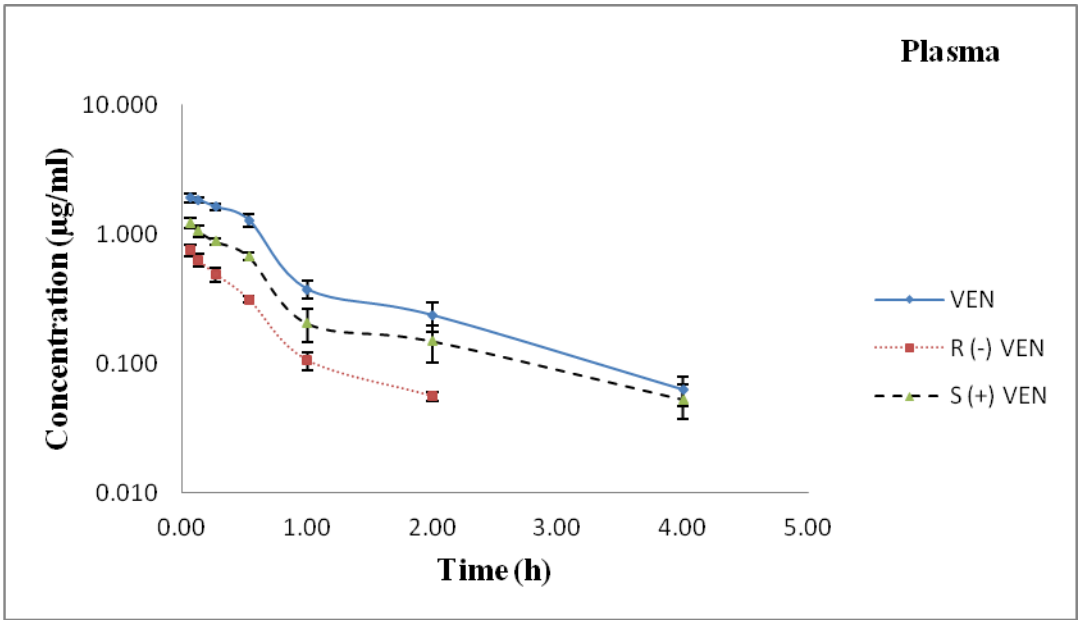

Fig 5.7: Log plasma concentration - time profiles of VEN and its enantiomers after I.V administration of VEN in rat.

(Each value represents the mean ± standard deviation of three independent determinations)

Table 5.5: Pharmacokinetic parameters of ODV and its enantiomers in plasma after I.V administration of VEN in rat

Parameters	ODV	R (-) ODV	S (+) ODV	Probability*
T_{max} (h)	0.27	0.27	0.27	-
C_{max} (µg/mL)	0.70 ± 0.14	0.12 ± 0.01	0.56 ± 0.08	0.010
AUC $_{0-\infty}$ (µg.h/mL)	1.07 ± 0.08	0.17 ± 0.01	0.90 ± 0.07	0.002
MRT (h)	1.55 ± 0.001	1.25 ± 0.02	1.62 ± 0.04	0.006
K_e (h^{-1})	0.65 ± 0.01	0.86 ± 0.02	0.63 ± 0.01	0.001
$t_{1/2}$ (h)	1.06 ± 0.02	0.81 ± 0.02	1.10 ± 0.02	0.0007

*If probability value less than 0.05 ($p<0.05$), there exists a significant difference between the values at 95% confidence interval between the enantiomers.

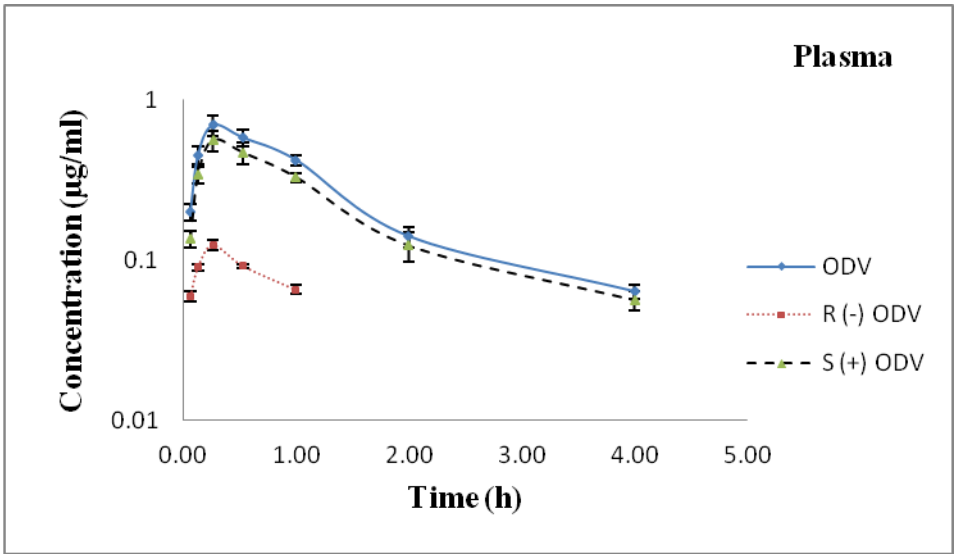

Fig 5.8: Log plasma concentration - time profiles of ODV and its enantiomers after I.V administration of VEN in rat.

(Each value represents the mean ± standard deviation of three independent determinations)

b) Oral route of administration

The log plasma concentration vs time profile of VEN, ODV and their enantiomers is given fig 5.9 and 5.10 respectively. The pharmacokinetic parameters of VEN, ODV and their enatiomers and their paired t-test probability value of the difference between each enantiomers are given in table 5.6 and 5.7 respectively.

Table 5.6: Pharmacokinetic parameters of VEN and its enantiomers in plasma after oral administration of VEN in rat

Parameters	VEN	R (-) VEN	S (+) VEN	Probability*
T_{max} (h)	0.25	0.25	0.25	-
C_{max} (µg/mL)	3.820 ± 0.517	1.065 ± 0.061	2.738 ± 0.106	0.005
$AUC_{0-\infty}$ (µg.h/mL)	5.768 ± 0.426	1.012 ± 0.050	4.660 ± 0.275	0.0013
MRT (h)	1.316 ± 0.070	0.666 ± 0.057	1.446 ± 0.059	0.0073
K_e (h^{-1})	0.725 ± 0.036	1.683 ± 0.235	0.682 ± 0.027	0.0221
V_d (L/kg)	28.762 ± 1.584	35.972 ± 7.151	18.922 ± 0.621	0.0501
$t_{1/2}$ (h)	0.982 ± 0.006	0.418 ± 0.062	1.018 ± 0.040	0.0095
Cl (L/h/kg)	20.883 ± 1.584	59.402 ± 2.932	12.906 ± 0.106	0.0007
F	0.590 ± 0.055	0.675 ± 0.085	0.895 ± 0.235	0.1733

*If probability value less than 0.05 ($p<0.05$), there exists a significant difference between the values at 95% confidence interval between the enantiomers.

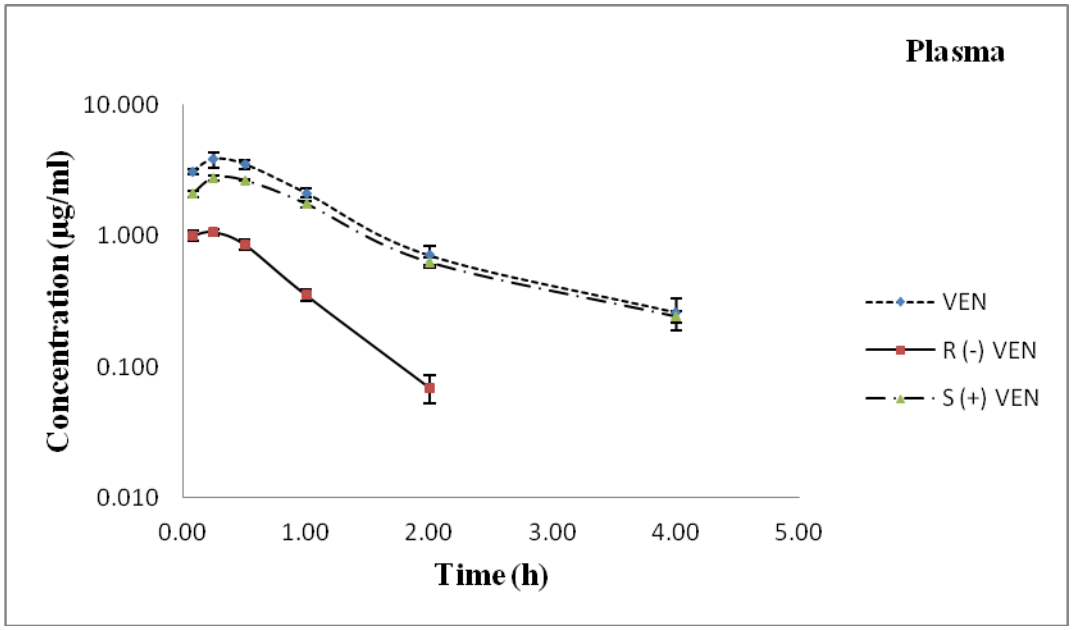

Fig 5.9: Log plasma concentration - time profiles of VEN and their enantiomers after oral administration of VEN in rat.

(Each value represents the mean ± standard deviation of three independent determinations)

Table 5.7: Pharmacokinetic parameters of ODV and its enantiomers in plasma after oral administration of VEN in rat

Parameters	ODV	R (-) ODV	S (+) ODV	Probability*
T_{max} (h)	0.5	0.5	0.5	-
C_{max} (µg/mL)	1.799 ± 0.448	0.330 ± 0.026	1.440 ± 0.105	0.0017
AUC $_{0-\infty}$ (µg.h/mL)	3.923 ± 0.776	0.348 ± 0.025	3.509 ± 0.097	0.002
MRT(h)	1.861 ± 0.111	1.016 ± 0.027	1.858 ± 0.071	0.004
K_e (h^{-1})	0.567 ± 0.044	1.095 ± 0.032	0.591 ± 0.10	0.0021
$t_{1/2}$ (h)	1.228 ± 0.092	0.634 ± 0.018	1.173 ± 0.019	0.0015

*If probability value less than 0.05 ($p<0.05$), there exists a significant difference between the values at 95% confidence interval between the enantiomers.

A significant difference in most of the pharmacokinetic parameters was observed in rat plasma. Plasma concentrations of S (+) VEN was found to be higher than those of R (-) VEN. There was significant difference in most of the pharmacokinetic parameters between S (+) VEN and R (-) VEN. C_{max} was nearly two point five times and $AUC_{0-\infty}$ was more than four times for S (+) VEN than that of R (-) VEN. There was significant difference between $t_{1/2}$ and K_e of R (-) VEN and S (+) VEN. $t_{1/2}$ of S (+) VEN was 144 % more than that of R (-) VEN. The total body Cl of R (-) VEN was nearly five times than that of S (+) VEN.

Plasma concentrations of S (+) ODV was found to be higher than those of R (-) ODV. There was significant difference in all the pharmacokinetic parameters between the enantiomers. C_{max} was nearly five times and $AUC_{0-\infty}$ was ten times for S (+) ODV compared to that of R (-) ODV. There was significant difference between $t_{1/2}$ and K_e of R (-) VEN and S (+) VEN. $t_{1/2}$ of S (+) ODV was nearly 85 % more than its enantiomer.

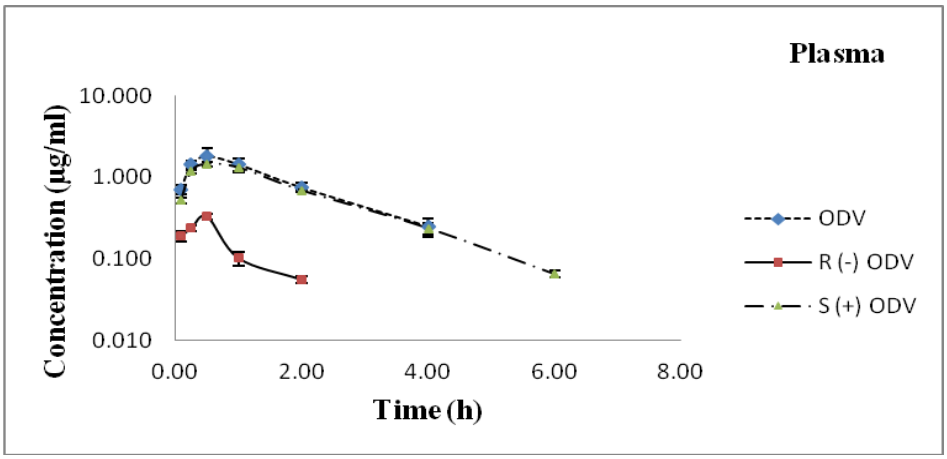

Fig 5.10: Log plasma concentration - time profiles of ODV and their enantiomers after oral administration of VEN in rat.

(Each value represents the mean ± standard deviation of three independent determinations)

c) Biodistribution studies: Oral route of administration

The drug amount in the rat tissues following single oral dose of 120 mg/kg racemic drug was determined and plotted as a function of time. The various pharmacokinetic parameters calculated are listed in table 5.8 and 5.9 for VEN, ODV and their enatiomers respectively. The tissue amount time profile of VEN, ODV and their enantiomers in brain, heart, kidney, liver, lungs are given in fig 5.11 to 5.20 respectively.

Table 5.8: Pharmacokinetic parameters of VEN and its enantiomer in various tissues of rat after oral administration of VEN

Biological sample	Parameters	VEN	R (-) VEN	S (+) VEN	Probability*
Brain	T_{max} (h)	0.25	0.25	0.25	-
	C_{max} (µg/g)	3.469 ± 0.257	2.043 ± 0.203	1.372 ± 0.025	0.0054
	$AUC_{0-\infty}$ (µg.h/g)	4.754 ± 0.111	3.445 ± 0.223	1.191 ± 0.018	0.0030
	MRT (h)	2.003 ± 0.130	2.035 ± 0.0.034	1.753± 0.060	0.0377
	K_e (h^{-1})	0.455 ± 0.061	0.475 ± 0.009	0.404 ± 0.023	0.0575
	$t_{1/2}$ (h)	1.543 ± 0.223	1.459 ± 0.023	1.718 ± 0.099	0.0685
	Cl (L/h/kg)	25.152 ± 0.589	17.393 ± 1.103	50.398 ± 0.763	0.0003

Biological sample	Parameters	VEN	R (-) VEN	S (+) VEN	Probability*
Heart	T_{max} (h)	0.25	0.25	0.25	
	C_{max} (µg/g)	3.498 ± 0.042	1.966 ± 0.103	1.508 ± 0.056	0.0043
	AUC $_{0-\infty}$ (µg.h/g)	3.957 ± 0.142	2.732 ± 0.143	1.118 ± 0.026	0.0021
	MRT (h)	1.886 ± 0.053	2.022 ± 0.106	1.583± 0.235	0.1155
	K_e (h^{-1})	0.476 ± 0.014	0.450 ± 0.058	0.517 ± 0.084	0.4139
	$t_{1/2}$ (h)	1.456 ± 0.043	1.561 ± 0.219	1.366 ± 0.237	0.4602
	Cl (L/h/kg)	30.349 ± 1.076	22.0 ± 0.1.732	53.674 ± 1.259	0.0003
Kidney	T_{max} (h)	0.25	0.25	0.25	
	C_{max} (µg/g)	13.794 ± 0.726	8.214 ± 0.363	5.560 ± 0.083	0.0089
	$AUC_{0-\infty}$ (µg.h/g)	13.017 ± 0.547	9.171 ± 0.278	3.760 ± 0.061	0.0008
	MRT (h)	1.281 ± 0.026	1.299 ± 0.022	1.210 ± 0.219	0.5409
	K_e (h^{-1})	0.553 ± 0.018	0.589 ± 0.023	0.522 ± 0.112	0.4439
	$t_{1/2}$ (h)	1.241 ± 0.040	1.179 ± 0.048	1.337 ± 0.336	0.4388
	Cl (L/h/kg)	9.229 ± 0.0.395	6.547 ± 0.196	15.961± 0.258	0.0003
Liver	T_{max} (h)	0.25	0.25	0.25	
	C_{max} (µg/g)	17.129 ± 0.330	10.125 ± 0.478	7.072 ± 0.342	0.0215
	$AUC_{0-\infty}$ (µg.h/g)	14.107 ± 0.242	9.061 ± 0.365	5.611 ± 0.067	0.0043
	MRT (h)	1.073 ± 0.041	1.129 ± 0.097	0.844 ± 0.034	0.0162
	K_e (h^{-1})	0.663 ± 0.045	0.626 ± 0.060	0.893 ± 0.057	0.0004
	$t_{1/2}$ (h)	1.048 ± 0.073	1.114 ± 0.103	0.778 ± 0.049	0.0088
	Cl (L/h/kg)	8.524 ± 0.153	6.629 ± 0.264	10.694 ± 0.0.127	0.0022
Lungs	T_{max} (h)	0.25	0.25	0.25	
	C_{max} (µg/g)	6.414 ± 0.498	4.229 ± 0.473	2.166 ± 0.629	0.0815
	AUC $_{0-\infty}$ (µg.h/g)	8.171 ± 0.305	5.097 ± 0.261	3.0 ± 0.337	0.0235
	MRT(h)	1.904 ± 0.055	1.868 ± 0.033	1.924 ± 0.088	0.2389
	K_e (h^{-1})	0.456 ± 0.022	0.446 ± 0.076	0.483 ± 0.036	0.1790
	$t_{1/2}$ (h)	1.22 ± 0.075	1.556 ± 0.056	1.441 ± 0.105	0.1623
	Cl (L/h/kg)	14.7 ± 0.540	11.732 ± 0.613	20.164 ± 2.185	0.0327

*If probability value less than 0.05 ($p<0.05$), there exists a significant difference between the values at 95% confidence interval between the enantiomers.

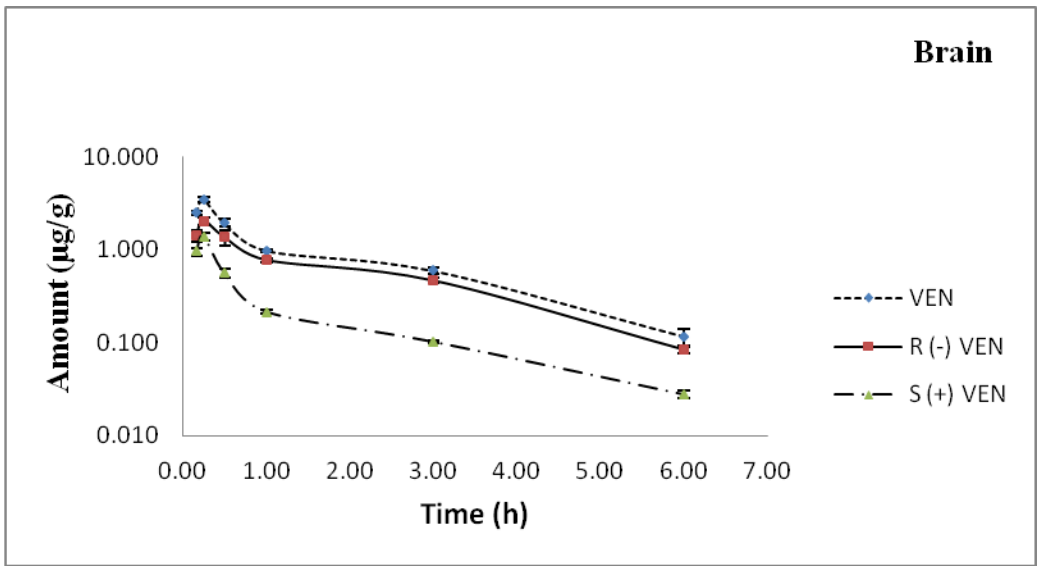

Fig 5.11: Log amount - time profiles of VEN and its enantiomers in rat brain.

(Each value represents the mean ± standard deviation of three independent determinations)

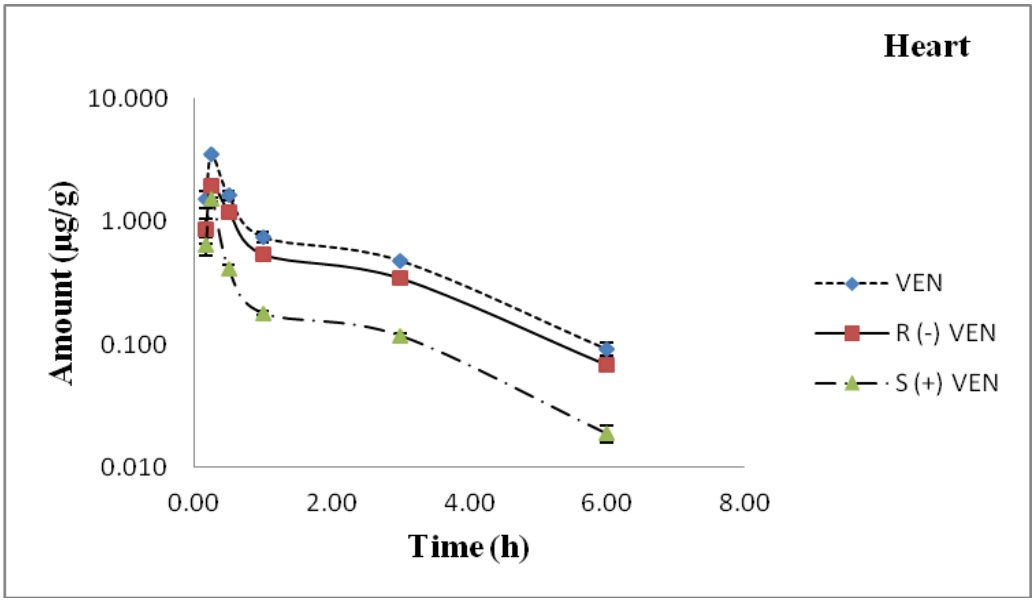

Fig 5.12: Log amount - time profiles of VEN and its enantiomers in rat heart.

(Each value represents the mean ± standard deviation of three independent determinations)

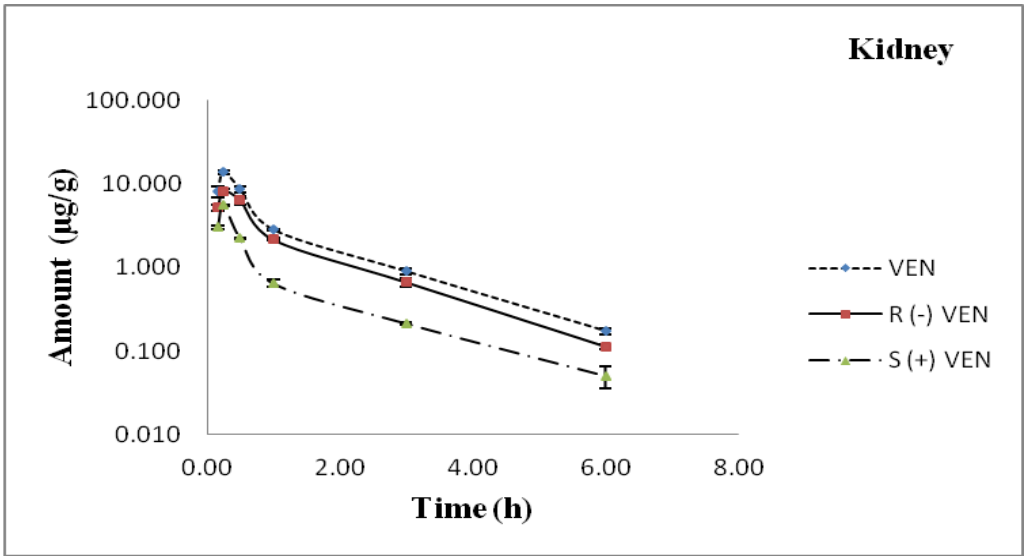

Fig 5.13: Log amount - time profiles of VEN and its enantiomers in rat kidney.
(Each value represents the mean ± standard deviation of three independent determinations)

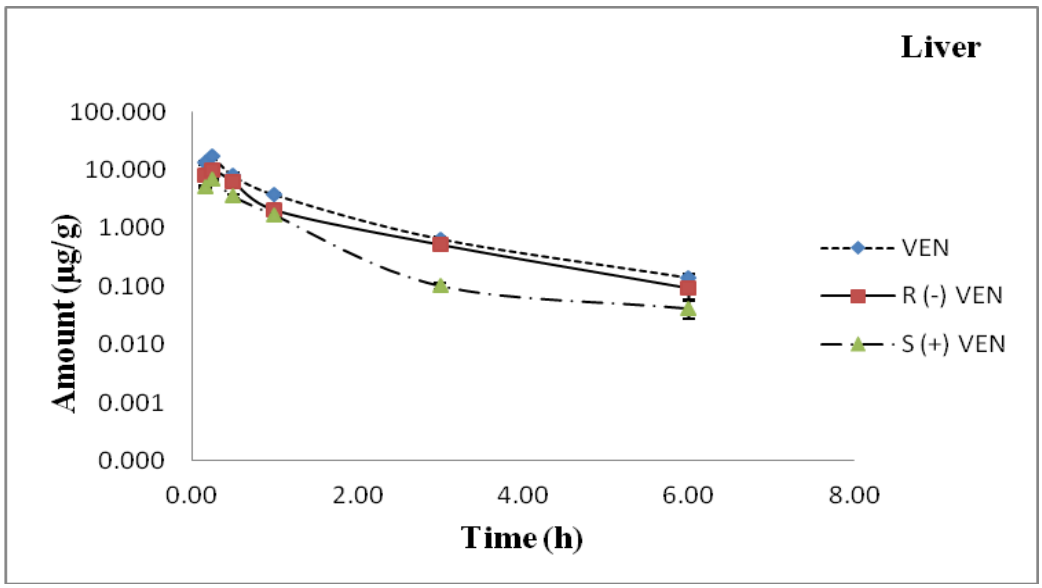

Fig 5.14: Log amount - time profiles of VEN and its enantiomers in rat liver.
(Each value represents the mean ± standard deviation of three independent determinations)

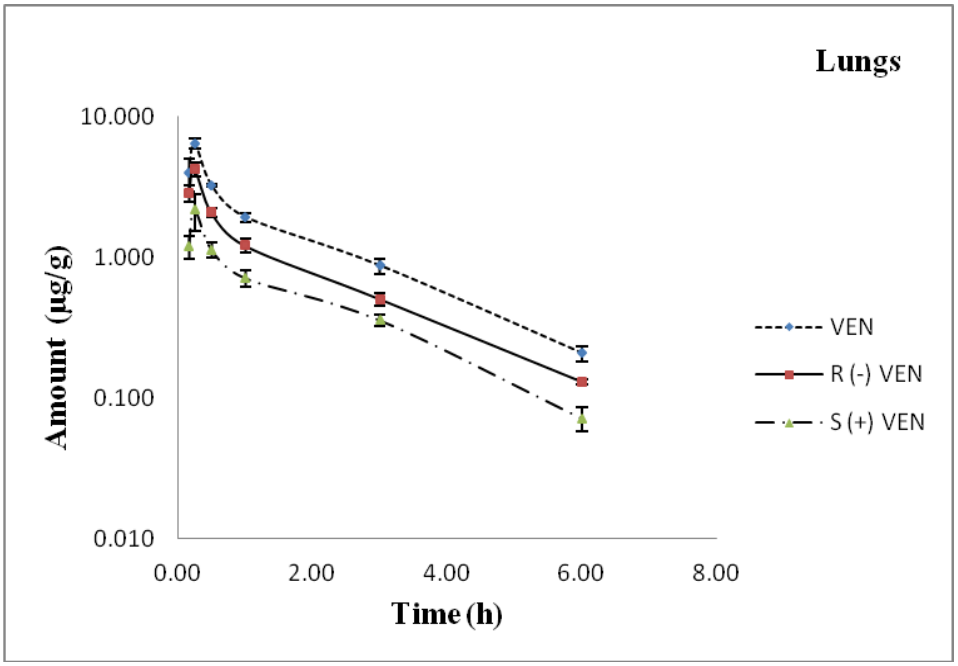

Fig 5.15: Log amount - time profiles of VEN and its enantiomers in rat lungs.

(Each value represents the mean ± standard deviation of three independent determinations)

Table 5.9: Pharmacokinetic parameters of ODV and its enantiomer in various tissues of rat after oral administration of VEN

Biological sample	Parameters	ODV	R (-) ODV	S (+) ODV	Probability*
Brain	T_{max} (h)	0.5	0.5	0.5	-
	C_{max} (µg/g)	1.730 ± 0.137	0.955 ± 0.045	0.799 ± 0.025	0.0601
	$AUC_{0-\infty}$ (µg.h/g)	3.876 ± 0.252	2.501 ± 0.181	1.315 ± 0.181	0.0086
	MRT (h)	3.661 ± 0.662	3.933 ± 0.755	3.437 ± 0.204	0.4556
	K_e (h^{-1})	0.261 ± 0.487	0.282 ± 0.016	0.223 ± 0.040	0.0263
	$t_{1/2}$ (h)	2.716 ± 0.051	2.461 ± 0.529	3.167 ± 0.529	0.2073
Heart	T_{max} (h)	0.5	0.5	0.5	-
	C_{max} (µg/g)	0.894 ± 0.061	0.558 ± 0.062	0.311 ± 0.019	0.0257
	$AUC_{0-\infty}$ (µg.h/g)	2.821 ± 0.115	1.851± 0.085	0.873 ± 0.073	0.0079
	MRT (h)	3.817 ± 0.254	4.472 ± 1.034	2.880 ± 0.308	0.1752
	K_e (h^{-1})	0.259 ± 0.017	0.240± 0.063	0.355 ± 0.033	0.1718
	$t_{1/2}$ (h)	2.689 ± 0.187	3.048 ± 0.933	1.961 ± 0.173	0.2307
Kidney	T_{max} (h)	0.5	0.5	0.5	-
	C_{max} (µg/g)	3.330 ± 0.213	1.884 ± 0.103	1.4237± 0.043	0.0131
	$AUC_{0-\infty}$ (µg.h/g)	7.853 ± 0.380	5.647 ± 0.229	2.121 ± 0.140	0.0006
	MRT (h)	1.299 ± 0.022	2.242 ± 0.024	1.698 ± 0.069	0.0087
	K_e (h^{-1})	0.530 ± 0.026	0.501 ± 0.006	0.556 ± 0.012	0.0195
	$t_{1/2}$ (h)	1.310 ± 0.065	1.384 ± 0.016	1.248 ± 0.0271	0.0182
Liver	T_{max} (h)	0.5	0.5	0.5	-
	C_{max} (µg/g)	5.43 ± 0.0.255	3.657 ± 0.051	1.721 ± 0.051	0.0001

Biological sample	Parameters	ODV	R (-) ODV	S (+) ODV	Probability*
	$AUC_{0-\infty}$ (µg.h/g)	8.22 ± 0.509	5.260 ± 0.251	2.760 ± 0.061	0.0026
	MRT (h)	1.830 ± 0.109	1.860 ± 0.032	1.716 ± 0.041	0.0733
	K_e (h^{-1})	0.463 ± 0.033	0.450 ± 0.026	0.510 ± 0.005	0.0773
	$t_{1/2}$ (h)	1.502 ± 0.105	1.545 ± 0.087	1.361 ± 0.013	0.0852
Lungs	T_{max} (h)	0.5	0.5	0.5	-
	C_{max} (µg/g)	2.521 ± 0.279	1.471± 0.214	1.032 ± 0.073	0.0918
	$AUC_{0-\infty}$ (µg.h/g)	5.510 ± 0.297	3.614 ± 0.471	1.828 ± 0.058	0.0201
	MRT (h)	2.899 ± 0.344	3.205 ± 0.572	2.358 ± 0.093	0.0951
	K_e (h^{-1})	0.347± 0.042	0.319 ± 0.062	0.435 ± 0.032	0.0297
	$t_{1/2}$ (h)	2.019 ± 0.264	2.230 ± 0.456	1.599 ± 0.122	0.0860

*If probability value less than 0.05 ($p<0.05$), there exists a significant difference between the values at 95% confidence interval between the enantiomers.

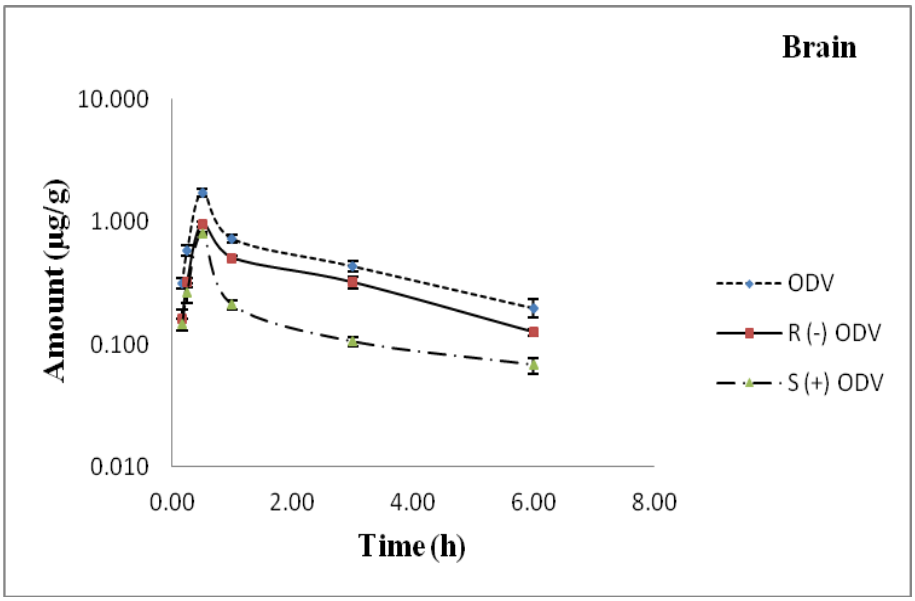

Fig 5.16: Log amount - time profiles of ODV and its enantiomers in rat brain.

(Each value represents the mean ± standard deviation of three independent determinations)

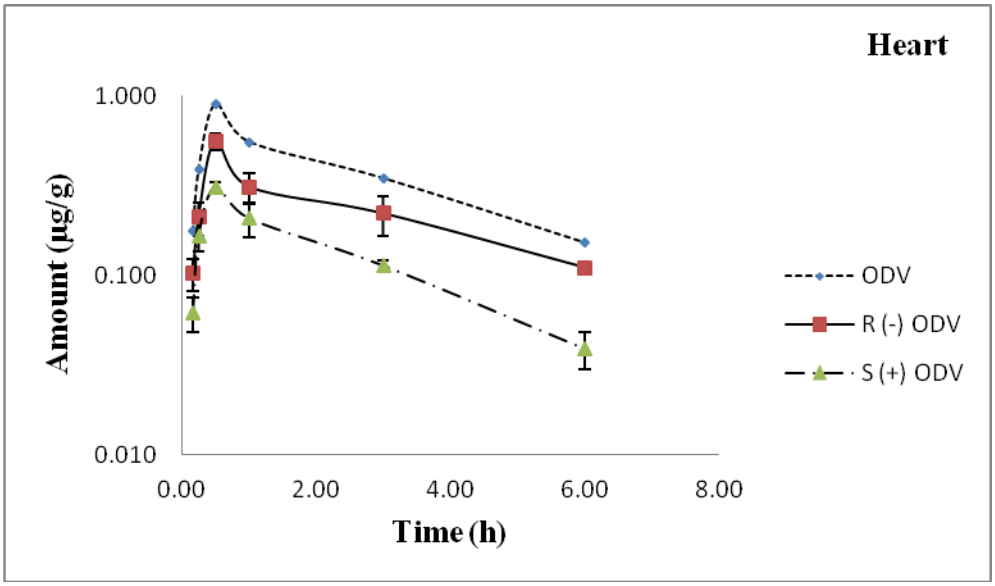

Fig 5.17: Log amount - time profiles of ODV and its enantiomers in rat heart.

(Each value represents the mean ± standard deviation of three independent determinations)

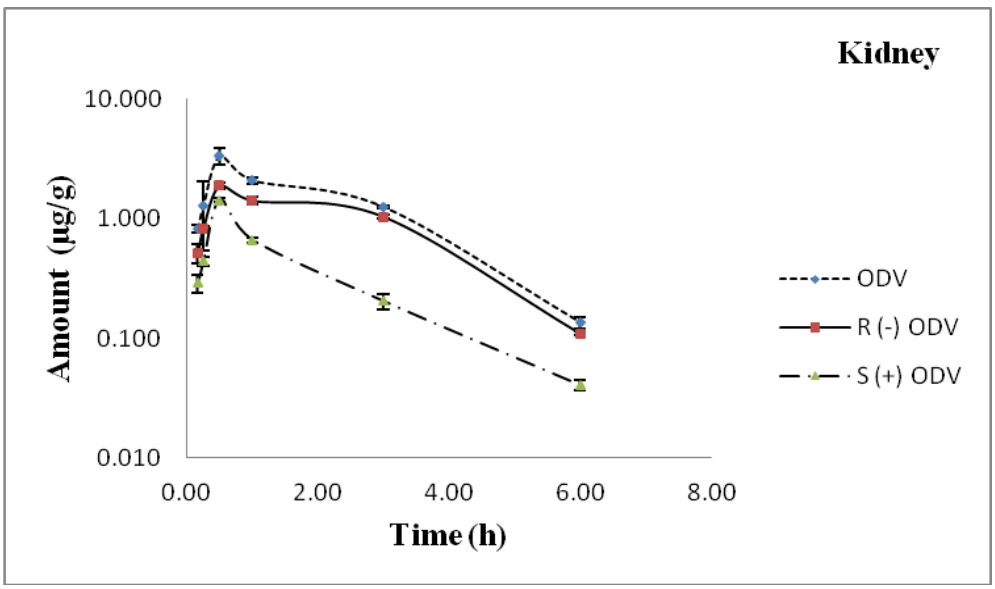

Fig 5.18: Log amount - time profiles of ODV and its enantiomers in rat kidney.

(Each value represents the mean ± standard deviation of three independent determinations)

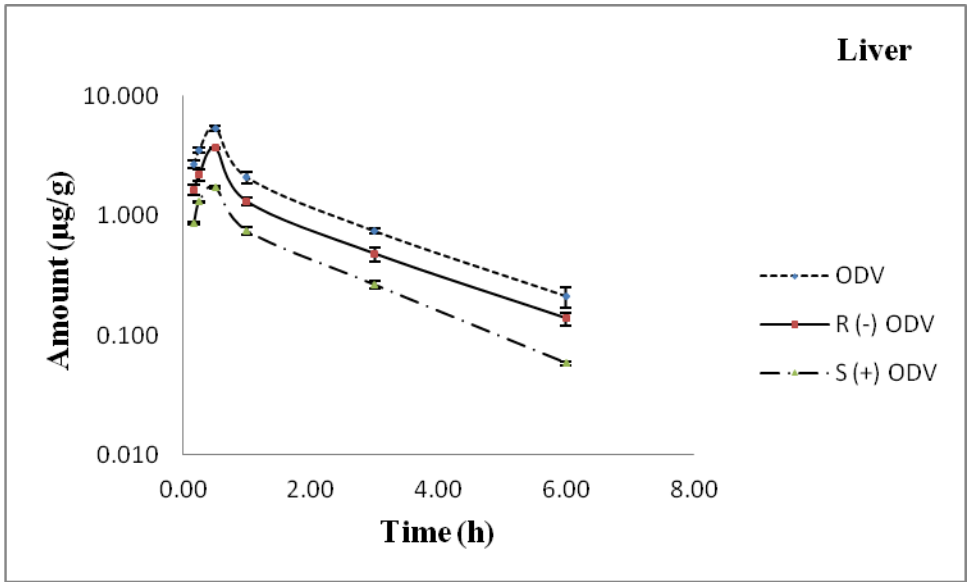

Fig 5.19: Log amount - time profiles of ODV and its enantiomers in rat liver.

(Each value represents the mean ± standard deviation of three independent determinations)

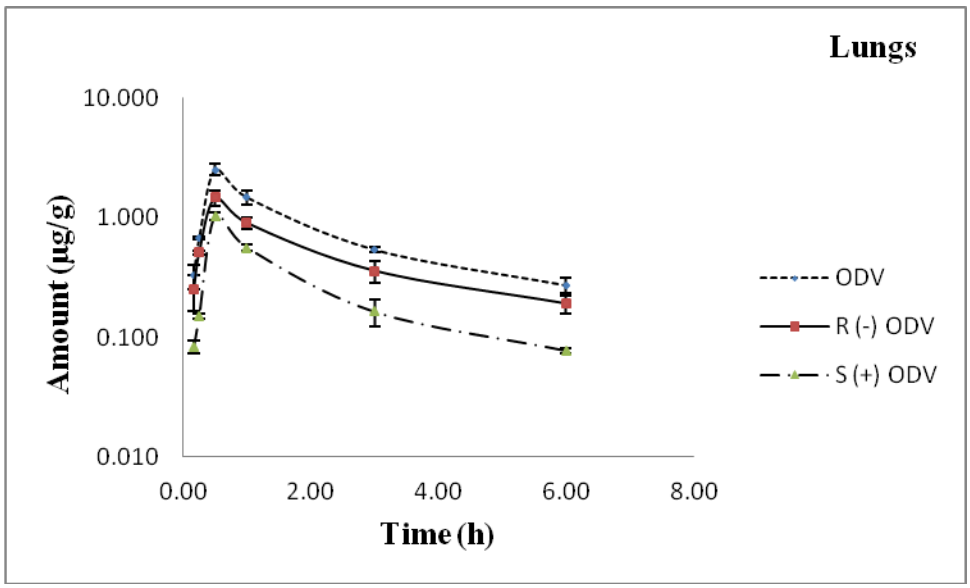

Fig 5.20: Log amount - time profiles of ODV and its enantiomers in rat lungs.

(Each value represents the mean ± standard deviation of three independent determinations)

Maximum tissue concentration was attained within 15 min for VEN and 30 min for ODV in the brain, heart, kidney, liver and lungs. Maximum tissue amount was attained within 15 min for VEN and its enantiomers. There was significant difference in most of the pharmacokinetic parameters between S (+) VEN and R (-) VEN. Maximum amount was found to be more for R (-) VEN compared to that of S (+) VEN in all the tissues. In brain, the difference between

the C_{max} and $AUC_{0-\infty}$ has been found to be significant. C_{max} was one point five times and $AUC_{0-\infty}$ was nearly thrice for R (-) VEN compared to those of S (+) VEN in brain. Cl of S (+) VEN was nearly three times to that of R (-) VEN in brain. In heart there was a significant difference in C_{max}, $AUC_{0-\infty}$ and Cl between the enantiomers. In kidney, there was a significant difference in pharmacokinetic parameters. The C_{max} and $AUC_{0-\infty}$ for R (-) VEN were 48 % and nearly 144 % more than that of S (+) VEN in kidney respectively. MRT, K_e and $t_{1/2}$ were not significantly different in kidney. In liver, there was a significant difference in all pharmacokinetic parameters between the enantiomers. The C_{max} and $AUC_{0-\infty}$ for R (-) VEN were 43 % and nearly 62 % more than that of S (+) VEN in liver respectively. Cl was nearly twice for S (+) VEN than that of R (-) VEN in liver. In lungs, the C_{max} and $AUC_{0-\infty}$ for R (-) VEN were 95 % and nearly 70 % more than that of S (+) VEN respectively. There was no significant difference observed for MRT, K_e and $t_{1/2}$ between the enantiomers in lungs.

There was significant difference in most of the pharmacokinetic parameters between S (+) ODV and R (-) ODV. Maximum amount was found to be more for R (-) ODV than that of S (+) ODV in all the tissues. In brain, the $AUC_{0-\infty}$ of R (-) ODV was nearly twice than that of S (+) ODV. There was no significant difference observed for MRT and $t_{1/2}$ between the enantiomers in brain. In heart there was a significant difference in C_{max} and $AUC_{0-\infty}$ between the enantiomers. The C_{max} and $AUC_{0-\infty}$ for R (-) ODV were 79 % and nearly 112 % more than that of S (+) ODV in heart respectively. In kidney, there was a significant difference in pharmacokinetic parameters. The C_{max} and $AUC_{0-\infty}$ for R (-) ODV were 32 % and nearly 166 % more than that of S (+) ODV in kidney respectively. MRT, K_e and $t_{1/2}$ differ significantly in kidney. In liver, there was no significant difference in the pharmacokinetic parameters between the enantiomers except for C_{max} and $AUC_{0-\infty}$. The C_{max} and $AUC_{0-\infty}$ for R (-) ODV were 112 % and nearly 90 % more than its enantiomer in liver. In lungs, the C_{max} and $AUC_{0-\infty}$ for R (-) ODV were 42 % and nearly 98 % more than that of S (+) ODV respectively. There was significant difference observed for MRT and $t_{1/2}$ between the enantiomers in lungs.

5.4 Conclusions

Pharmacokinetic study of ketorolac revealed that there is a chiral discrimination in the pharmacokinetics profile of ketorolac entantiomers, reflected by the result in difference in pharmacokinetic behavior. The pharmacokinetic analysis of ketorolac plasma concentrations obtained after i.v and oral administration revealed enantioselective pharmacokinetics of each enantiomer. After i.v and oral dose, it was found that the plasma concentrations of R (+) ketorolac exceeded those of S (-) ketorolac throughout the time course, indicating less distribution of R (+) ketorolac to other tissues or organs. The $AUC_{0-\infty}$ in plasma of R (+)

ketorolac was significantly higher than that of S (-) ketorolac. The total body Cl of S (-) ketorolac was significantly larger than that of R (+) ketorolac. Based on plasma AUC of two enantiomers of i.v and oral absorption of racemic drug, the bioavailability (F value) of S (-) ketorolac was found to be slightly higher than R (+) ketorolac, though it is required to be studied by administering individual enantiomers to have further confirmation.

Biodistribution studies indicated that the T_{max} values of each organ match with the plasma except brain where concentration obtained were below limit of quantification. It is evident from the tissue distribution studies that $AUC_{0-\infty}$ of S (-) ketorolac, was more than that of its antipode in most of the studied organs. The difference in distribution of two enantiomers may be due to enantioselective binding with proteins. However, $AUC_{0-\infty}$ of S (-) ketorolac was significantly higher in kidneys as compared to heart, lung and liver tissues, probably accountable for the NSAIDs associated acute renal failure upon long term usage. From the study, it is observed that the levo form of ketorolac is distributed more to the tissues.

The pharmacokinetic analysis of VEN plasma concentrations obtained after i.v and oral dosing revealed enantioselective pharmacokinetics of each enantiomer of VEN and its metabolite. It was revealed after study that there was chiral discrimination in pharmacokinetic study of VEN and ODV enantiomers. After i.v and oral dose, it was found that the plasma concentrations of S (+) VEN and S (+) ODV exceeded those of R (-) VEN and R (-) ODV throughout the time course. There were significant differences in most of the pharmacokinetic parameters between VEN and ODV enantiomers. Pharmacokinetic study indicated that R (-) VEN and R (-) ODV have high tissue distribution and rapid elimination from plasma as compared to S (+) VEN and S (+) ODV which was in accordance with i.v study. Based on plasma AUC of two enantiomers of VEN, after i.v and oral absorption of racemic drug, the bioavailability (F value) of S (+) VEN was found to be higher than the R (-) VEN, though it is required to be studied by administering individual enantiomers to have further confirmation.

Maximum tissue amount was attained within 15 min for VEN and 30 min for ODV for all the tissues studied from the time of administration. There were significant difference in most of the pharmacokinetic parameters between S (+) VEN and R (-) VEN. Maximum amount was found to be more for R (-) VEN than that of S (+) VEN in all the tissues. The difference in distribution of two enantiomers may be due to enantioselective binding with proteins. There was significant difference in most of the pharmacokinetic parameters between S (+) ODV and R (-) ODV. Maximum amount was found to be more for R (-) ODV than that of S (+)

ODV in all the tissues. The brain tissue distribution studies revealed that R (-) VEN and R (-) ODV distributed more in comparison to their optical antipode giving inference that antidepressant activity may be more for above enantiomers of VEN and ODV. Further, it can be concluded from the study that the levo form of the enantiomers of VEN and ODV are distributed more to the tissues.

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Chapter 6

Conclusions and Future Scope

Conclusions

Pharmacokinetic and Biodistribution studies of enantiomeric drugs and their individual enantiomers have become important and essential in last few decades and necessitated by regulatory bodies. Thus this thesis was taken up to understand the stereospecific disposition of selected two drugs ketorolac and venlafaxine (VEN) and its metabolite O- desmethyl venlafaxine (ODV). As analytical and bioanalytical methods are integral part of pharmacokinetic and biodistribution studies and suitable stereospecific methods were not found in literature for these drugs, new methods were planned to develop.

Chromatographic enantioseparations are under constant development in methodology and applications in various fields. The most important of these applications are within pharmaceutical, environmental and clinical analysis, where the optical purity of drugs, toxins, and pollutants, as well as their enantioselective fate in the living organism may have very important implications in human health and/or effective therapy.

A reproducible, accurate, sensitive and stereospecific analytical and bioanalytical RP-HPLC method has been developed for racemic ketorolac, VEN, ODV and their enantiomers. These methods have a suitable limit of quantification along with the very short analysis time. The method has advantage over problems of poor chromatography, questionable uncharacterized peak, and tedious extraction steps. The developed and validated methods were applied for the pharmacokinetics and biodistribution behavior of above drugs.

The pharmacokinetic analysis of ketorolac plasma concentrations obtained after i.v and oral dose revealed enantioselective pharmacokinetics of each enantiomer. After i.v and oral dose, it was found that the plasma concentrations of R (+) ketorolac exceeded those of S (-) ketorolac throughout the time course, indicating less distribution of R (+) ketorolac to other tissues or organs. The $AUC_{0-\infty}$ in plasma of R (+) ketorolac was significantly higher than that of S (-) ketorolac. The total body Cl of S (-) ketorolac was significantly larger than that of R (+) ketorolac. The bioavailability (F value) of S (-) ketorolac was higher than R (+) ketorolac. It is evident from the tissue distribution studies that $AUC_{0-\infty}$ of S (-) ketorolac, was more than that of its antipode in most of the studied organs. However, $AUC_{0-\infty}$ of S (-) ketorolac was significantly higher in kidneys as compared to heart, lung, brain and liver tissues, probably accountable for the NSAIDs associated acute renal failure upon long term usage.

The pharmacokinetic analysis of VEN plasma concentrations obtained after i.v and oral dosing revealed enantioselective pharmacokinetics of each enantiomer of VEN and its

metabolite. It was revealed after study that there was chiral discrimination in pharmacokinetics of VEN and ODV enantiomers. Pharmacokinetic study of VEN upon i.v dose of 40 mg/kg showed that plasma concentrations of S (+) VEN exceeded those of R (-) VEN throughout the time course. $AUC_{0-\infty}$ of S (+) VEN was significantly higher than that of R (-) VEN. The total body Cl of R (-) VEN was significantly larger than that of S (+) VEN. These findings indicated that the pharmacokinetics of enantiomers in rats were stereo-selective.

In case of oral pharmacokinetic studies in rat plasma bioavailability (F value) of S (+) VEN was found to be higher than the R (-) VEN. Cmax, $AUC_{0-\infty}$, Cl, V_d, $t_{1/2}$ and Ke of S (+) VEN were significantly different from that of R (-) VEN. Similarly Cmax, $AUC_{0-\infty}$, $t_{1/2}$ and K_e of S (+) ODV were significantly different than R (-) ODV. Cmax, $AUC_{0-\infty}$ and $t_{1/2}$ of S (+) VEN and S (+) ODV is higher than R (-) VEN and R (-) ODV respectively. Pharmacokinetic study indicated that R (-) VEN has high tissue distribution and rapid elimination from plasma as compared to S (+) VEN which is in accordance with i.v study. Maximum tissue amount was attained within 15 min for VEN and 30 min for ODV from the time of administration in the brain, heart, kidney, liver and lungs. The brain tissue distribution studies revealed that R (-) VEN and R (-) ODV distributed more in comparison to their optical antipode giving inference that antidepressant activity may be more for above enantiomers of VEN and ODV.

The study has provided useful information on pharmacokinetic and biodistribution of the selected drugs in rat and provided distribution fate of different enantiomers.

Future Scope

Though the present studies are carried out in rat, it is necessary to carry out further studies in human to understand the real role played by individual enantiomer in therapeutic efficacy and toxic effects. It, further, can help in establishing relationship between animal and human studies.

It is possible to further improve the chiral analysis method for ketorolac, VEN, ODV and their enantiomers by changing from ultraviolet and fluorescence detection to mass spectrometric detection. Different type of column may be used instead of AGP column with a mobile phase that is more compatible with a mass spectrometric detector.

Chiral separation was a technical and scientific challenge, because the enantiomers are chemically and physically very similar, and show relatively small separation ratios on various types of column, including chiral AGP column which is chiral selective. Chiral separation was achieved, by choosing a distribution system which gave the greatest possible resolution between the enantiomers (*i.e.* a stationary phase of AGP column and mobile phase combination that exhibited maximum enantioselectivity). There is further scope of increasing efficiencies by possibly using smaller particles and using different techniques to pack them for better separation. If such a system could be developed, a single chiral stationary phase might be all that is necessary to separate the majority of enantiomeric compounds. Such a system is, certainly, the part of future scope. It must, however, be complemented by pumps and sample valves that can provide and tolerate the necessary high pressures.

An improvised method can be developed for the above drugs in future by automatizing the solid phase extraction, their separation on a chiral column and detection simultaneously. Another combination would be on-line extraction and detection by mass spectrometry. The developed enantioselective methods can be used for evaluation of novel drug delivery system. The possibility of interactions between enantiomers of these drugs and their enantioselective interactions in biological environment can be evaluated in future. Enantioselective pharmacokinetic studies of these drugs can be carried out following single and multiple doses in healthy human volunteers and patients.

Today most of the new drugs reaching the market are single enantiomers, rather than the racemic mixtures which have dominated up to last decade. As patents for the racemic drugs are expiring “chiral switches” provides a strategy to extend the profitable life of a pharmaceutical blockbuster drug, and may result in extended patent protection and provide an advantage against generic competition.

There will be a need for enantioselective analysis for therapeutic drug monitoring and pharmacokinetic studies. It should not be forgotten that metabolic processes can give unwanted effects, e.g. chiral inversion as illustrated by the famous example of thalidomide. Thus it may be useful to study enantioselective metabolites produced from enantiomeric drugs and their role in toxicities.

Knowledge of pharmacokinetic and biodistribution profiles of enantiomers and metabolites will help to understand role of enantiomers in therapeutic efficacy, side effects or toxic effects. Further they may help in proper planning of therapy and delivery.

Appendix I

Publications and Conferences

Publications

1. **Sunil K. Dubey**, Jangala Hemanth, K.CH.Venkatesh, R.N.Saha and S.Pasha New chiral reverse phase HPLC method for enantioselective analysis of ketorolac using chiral AGP column, Journal of Pharmaceutical Analysis, 2012, 2(6):462–465.
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3. **Sunil Kumar Dubey**, R.N. Saha and S. Pasha. Rapid, Sensitive, Validated Ultra-Performance Liquid Chromatography/Mass Spectrometric Method for determination of venlafaxine and its metabolite: Application to pharmacokinetic study. Journal of Pharmaceutical Analysis, 2013, 3(6): 466-471.
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5. **Sunil K. Dubey**, Amit Anand, Sugandha Saboo, Ranendra N. Saha. New, Sensitive and Validated Spectrofluorimetric Method for Simultaneous Estimation of Venlafaxine and O-desmethylvenlafaxine in Bulk and Pharmaceutical Dosage Form. Journal of Analytical Chemistry (Accepted).
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Conferences

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2. **Sunil K Dubey**, Jangala Hemanth, K.CH.Venkatesh, R.N.Saha and S.Pasha. Ultra-Performance Liquid Chromatography/Mass Spectrometric Method for the determination of venlafaxine and its metabolite in plasma: Application to pharmacokinetic study. Presented in 19th International Mass Spectrometry Conference (IMSC2012), Kyoto, 15- 21 September, 2012.
3. **Sunil K Dubey**, R.N.Saha, Amit Anand. A new enantioselective HPLC method for analysis of Ketorolac: Application to pharmacokinetic study Presented in 39th International Symposium on High-Performance-Liquid-Phase Separations and Related Techniques HPLC2013, Amsterdam, The Netherlands, 16th to 20th June, 2013.

Appendix II

Biography of Sunil Kumar Dubey

Mr. Sunil Kumar Dubey is currently working as a Lecturer in Department of Pharmacy in Birla Institute of Technology and Science, Pilani, India. He has completed his B.Pharm from central university Bilaspur and M. Pharm from Birla Institute of Technology, Mesra, Ranchi. He has worked in Ranbaxy Research Laboratories, in Clinical Pharmacology and Pharmacokinetics Division, Gurgaon for 3 years before joining BITS Pilani. Until now, he has published 17 research articles, 20 conference papers and 1 book. Apart from this, he has attended several international and national conferences, workshops, and given guest lectures. He has received young scientist and best paper award by pharmahelpline society and IPA respectively. He has successfully completed minor project approved by University Grant Commission, New Delhi. He has achieved "excellent" annual performance appraisal for consecutively three years of working in Ranbaxy. He was the coordinator for many group activities, and has given trainings on GLP, method development and validation.

Biography of Prof. Ranendra N. Saha

Dr. Ranendra N. Saha is Professor of Pharmacy, Deputy Director, Research & Educational Development and Shri B K Birla & Smt Sarala Birla Chair Professor BITS, Pilani, (Rajasthan), India. He completed his Bachelor and Master of Pharmacy degrees from Jadavpur University, Kolkata and obtained his Doctor of Philosophy from BITS Pilani. He has been awarded Pharmacy Teacher of India in 2005 by APTI for his contribution in research, teaching and profession of Pharmacy. He has more than 33 years of teaching and research experience and guided several doctoral, postgraduates and undergraduate students. He has published research articles in renowned international and national journals and presented papers in conferences in India and abroad. Dr. Saha has successfully completed several government and industry sponsored projects. He has also developed commercial products, granted patent, and transferred technologies to production and analytical departments. He is a member of selection committees of CSIR, several universities, colleges etc. He is also a member of Board of studies of several universities and colleges and acting as a visting Professor to few universities.