POPULATION PHARMACOKINETIC ANALYSIS OF CENTRALLY ACTING DRUGS METABOLIZED BY *CYP2D6*

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By

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This is to certify that the Ph.D. thesis entitled **"POPULATION PHARMACOKINETIC ANALYSIS OF CENTRALLY ACTING DRUGS METABOLIZED BY *CYP2D6*"** being submitted to the Tamil Nadu Dr. MGR Medical University, Chennai, for the award of degree of **DOCTOR OF PHILOSOPHY** in **PHARMACY & PHARMACETICAL SCIENCES** was carried out by **Mr. M. SIVA SELVA KUMAR,** at **PSG INSTITUTE OF MEDICAL SCIENCES & RESEARCH,** Peelamedu, Coimbatore, under my direct supervision and guidance to my fullest satisfaction. The contents of this thesis, in full or in parts, have not been submitted to any other Institute or University for the award of any degree or diploma.

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DECLARATION

I hereby certify that I am the sole author of this thesis entitled **"POPULATION PHARMACOKINETIC ANALYSIS OF CENTRALLY ACTING DRUGS METABOLIZED BY *CYP2D6*"** and that neither any part of this thesis nor the whole of the thesis has been submitted for a degree to any other University or Institution. I certify that, to the best of my knowledge, my thesis does not infringe upon anyone's copyright nor violate any proprietary rights and that any ideas, techniques, quotations, or any other material from the work of other people included in my thesis, published or, are fully acknowledged in accordance with the standard referencing practices. I declare that this is a true copy of my thesis, including any final revisions, as approved by my thesis review committee.

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Chapter-1

Introduction

1. INTRODUCTION

The human liver is a major drug metabolizing and xenobiotic organ for which it contains many super-families of drug bio-transforming enzymes. Among them, perhaps the largest group comprises the Cytochrome P450 (*CYP450*) families of oxidoreductases [1]. There exist two successive phases of xenobiotic conversion for detoxification and enhancing elimination from the body. The first phase is the Phase I reactions consisting of oxidation, reduction, hydroxylation and chemical group modifications, whereas the Phase II reactions comprise the conjugation reactions. Phase I reactions help to form a polar water soluble intermediate of the xenobiotic undergoing biotransformation; whereas Phase II reactions add a bulky chemical group to this intermediate and enhance its feco-biliary elimination [2]. Many drug biotransforming enzymes mediate both phases of drug metabolism, which could be alternatively classified as microsomal (in Endoplasmic Reticulum) and non microsomal enzymes. Among the microsomal enzymes which are prone to induction and inhibition by other environmental stimuli like drugs administered concomitantly, the *CYP450* superfamily catalyse oxidation with reduction of their xenobiotic substrates with broad specificity [3]. Related *CYP450* enzymes can also be found in organelles other than the Endoplasmic reticulum, where they serve other functions like cellular respiration through electron transport chain in the mitochondria, and synthesis of thromboxanes in the cytosol through reduction of arachidonic acid in the cell membrane [3]. About 20000 different *CYP450* enzymes have been identified in most organisms across all five kindgoms; perhaps, the most important of their functions is detoxification reactions catalysis through Phase I oxidation and

reduction of xenobiotics. Chemically, they contain heme with ferritin centered in the heme porphyrin ring, which helps in undertaking mono-oxygenation reactions of broadly numerous diverse substrates by a single enzyme [2, 3]. The name *CYP450* comes from the spectrometric peak of the light absorbed by these enzymes, first discovered in plant pigments. Any enzyme belonging to this superfamily is designated in six letters, the first three as '*CYP*' followed by the enzyme superfamily numeral, next alphabet designating the enzyme gene location, and lastly the number designating the isoenzyme in context, eg. *CYP3A4*, *CYP2D6*, etc.

The diversity created by these enzymes are complicated by the presence of certain genetic variant enzymes in certain individuals and populations with a grading of the metabolism potential phenotypes possible due to the variations [4]. As an example, *CYP2D6*10* is an abnormal allele whose frequencies varies in different populations and arises due to a single nucleotide function reduction polymorphism. Accordingly, the phenotypes possible due to the four possible genetic variants viz. wild type, heterozygous mutant, homozygous mutant, and duplication of abnormal allele at a locus are: normal, intermediate, poor and ultra-rapid metabolizers [4, 5]. Many such polymorphisms, INDELs (insertion-deletions), Copy Number Variations (CNVs) and haplotypes are possible with these enzymes [5].

The significance of these genetic variations in drug metabolism and pharmacodynamic targets and transporters is the realm of pharmacogenetics. Such variations are of greater clinical significance in patients receiving the drugs metabolized by the enzymes for chronic disease like depression and schizophrenia

like fluoxetine and risperidone. These polymorphisms determine not only the clinical response but also the adverse effect profiles, drug interaction effects, and patient compliance in terms of either failure or success, by altering the drug's pharmacokinetic and pharmacodynamic actions [4, 5]. These varying effects in individual patients due to genetic variations are not constant among different populations but are subject to both evolutionary pressures and selection in different populations – hence the frequencies of such genetic variants are subject to intense investigation in different races and populations [5, 6].

Schizophrenia is a neuropsychiatric illness characterized in patients by positive, negative and cognitive features of the intellect and perception of the environment by the patient. Many drugs exist now as obsolete treatments for first onset disease, but some of these older generation drugs can be used in resistant psychosis or schizophrenia that is not typical eg. Chlorpromazine, due mainly to the increased incidence of extra-pyramidal effects like tardive dyskinesia, akathisia and dystonia, and parkinsonism. A second generation of the antipsychotic medications devoid of many of such adverse effects to some extent due to their non selectivity to D_2 blocking in the brain, are more commonly used for patients now, along with drugs from other classes (antidepressants like fluoxetine, antimania drugs like lithium) [6].

One such antipsychotic drug, risperidone, is a second generation antipsychotic medication given in many first onset schizophrenia patients as it suppresses the positive symptoms and to a lesser extent, the negative symptoms, but not the cognitive features of this neuropsychiatric disease [6]. But it is not devoid of side effects also – hyperprolactinemias, weight gain and other metabolic

effects and extrapyramidal effects are some of the severe adverse effects of this drug. Its antipsychotic efficacy is due to blocking of central dopamine receptors (D_2) in the mesocortical and mesolimbic pathways in the brain. But the side effects are due to the antagonism of these receptors in the tuberoinfundibular and nigrostriatal pathways, and the blockade of other receptors like serotonin and histamine in the brain [6].

However, these therapeutic and adverse effects are determined in most part also by the genetic variations in certain genes encoding molecules that metabolize the drug or act as targets. One such polymorphism in the *CYP2D6*10* allele has not been evaluated previously in South Indian population in the context of risperidone metabolism to an active antipsychotic metabolite, 9-hydroxyrisperidone in healthy volunteers [7, 8].

"Population pharmacokinetics" deals with the scientific investigation of the variations in therapeutic drug response leading to altered clinical effects in the target patient population and the assessment of the sources of such variations. Such variations in the dose concentration and response relationships are due to certain demographic and anthropometric factors but are mainly due to genetic variations in different ethnic groups [9]. Although the most accurate method of predicting clinical dosing of drugs based on covariates is Bayesian forecasting, a very useful and widely practised method is Population Pharmacokinetic modelling [10].

The increasing utility of omics technologies including pharmacogenomics has helped in such pharmacokinetic modelling of drug use in patient populations. However, in clinical practice, nomograms can also be used for relating covariate

variations between individuals with clinical dosing parameters of drugs [11]. In general, there are two different ways to undertake a population pharmacokinetic analysis – a two stage approach where rich concentration response data sets are used to arrive at relationships between pharmacokinetic alterations with individual covariates; and nonlinear mixed effect models which use sparse data sets and incorporate statistical and mathematical models [12, 13]. In the current study we have used the mixed effect modelling using the software to arrive at some pharmacokinetic covariate relationships.

Methods of quantifying the drugs in biological matrix are highly problematic and are highly dependent on the nature of the biopharmaceutical as well as the analytical protocols. As illustrative examples, new drug development, bioavailability-bioequivalence (BA/BE) studies, clinical pharmacokinetic evaluations, and, basic biomedical research and studies in the pharmaceutical sciences.

In the biopharmaceutical analysis, the blood and urine samples are most commonly obtained from patients and volunteers. Faecal matrices are also employed where the either the parent drug or the active metabolite(s) or both are poorly absorbed from the intestinal mucosa or are extensively eliminated in the bile. A few studies also require saliva and tissue samples. The nature of sampling in biological matrix is mainly influenced by the choice of the study design. In general, plasma is preferred because of the higher magnitude and quality of output from blood and lesser degree of interferences in the biological matrix. Detection of an analyte or its metabolite(s) in samples is typically difficult due to various clean up techniques which are required prior to estimation of the analytes of

interest. For example, solvent extraction and size exclusion chromatography are employed in order to separate the components in the analyte mixture efficiently from the sample matrix. The sensitivity and selectivity obtained from the choice of the assay procedure(s) may become inadequate due to the inefficiency of the clean up methodology.

After clean up techniques are employed successfully, isolation or separation of the required analyte(s) from biological medium is conducted to facilitate partial purification of the sample. Using such ways can help an analyst achieve appropriate sensitivity apart from high selectivity in detecting the particular compound with minimum interference from the biological matrix (ces). A minimum number of steps in the separation procedure can recover loss of drug or metabolite in each of the given samples. Similarly, the biological samples should be frozen immediately to prevent decomposition or other probable physico-chemical changes in the drug before analysis. In a few studies, as the drugs are prone to inactivation by plasma esterase enzymes *in vitro*, the addition of inhibitors of such enzymes like sodium fluoride (NaF) to blood and plasma samples quickly after sample collection prevents drug decomposition. Sample treatment is essential before analysing the drug. Drug analyses are required in samples which vary in nature from plasma to other matrices such as faeces, urine, bile, saliva and seminal specimens. Many factors for each of these samples must be considered before the appropriate pre-treatment method is selected. Factors like the drug-protein binding kinetics, the drug's chemical stability, chemical composition of sample and the presence (or absence) of interferences will affect the final estimation step and hence the yield of the extraction procedures. As an

illustrative example, biological matrices such as blood, plasma, saliva, urine as well as faeces contain significant amounts of protein-rich interferences which possibly will bind the analyte. The drug components may have to be freed from the proteins prior to further treatment. In such instances, protein precipitation step is of key importance because the presence of matrix-interferences like proteins, salts, lipids and other materials in the samples cause rapid deterioration of HPLC / UPLC columns and also interfere with the assay. Such protein elimination procedures require the appropriate use physical methods (heat) and chemical pre-treatments (ammonium sulphate, ethanol, trichloroacetic acid and perchloric acids) [14].

After pre-treatment procedures are successful, the next step is to extract of the required drugs from biological matrices. Two types of extraction methods are usually utilized in analytical chemistry estimations liquid and Solid-Phase Extraction. A liquid-solid inter-phase extraction takes place between a liquid phase and solid phase. This method is mainly suited for polar compounds which being hydrophilic, would otherwise tend to elute in the aqueous medium. Liquid-solid extraction method is also helpful for amphopathic compounds that cannot be extracted easily away from water. This provides an appropriate isolation procedure which can be applied for plasma samples, and is the more commonly utilized technique since removal of a drug or metabolite of interest from the high concentration of the interfering endogenous material(s) that usually interfere with the final bio-analytical yield. Also this technique has the additional advantages of being simple, rapid with a relatively small cost-per-sample factor. Distribution (partitioning) of the required drug between two potential liquid phases is

expressed as the (distribution) partition coefficient. Partition coefficient is constant for the analyte and varies depending on the temperature, particular solute and its vapour pressure for the selected pair of solvents. By knowing the partition coefficient of the extracted drug using dissociation constants of the forward reaction and the absolute volumes of the two phases to be useful in the method chosen, the quantity of the analyte obtained from a single extraction can be determined. Factors which influence this partition coefficient and recovery of drug from matrix in liquid-liquid extraction methods include the selection of the appropriate solvent, pH of the medium and ionic strength of the aqueous phase. It is generally accepted that diethyl ether and chloroform are very useful and appropriate solvents of choice for acidic and basic drugs, respectively, even when the identity of the drugs in the samples cannot be precisely known [15].

The presence of metabolites apart from the parent drug, or the objective of estimating more than one drug in a biological sample frequently demands the employment of more complex separation for their robust measurement, especially when the analytes of interest are comparable in their physical properties and chemical nature. “Affinity chromatography” is a separation technique based upon differing affinities of a mixture of solutes which include different analytes, between at least two diverse physical phases. The interactions or affinities can be classified in terms of a solute (surface active analytes) adhering to the surface of a polar solid phase (adsorption), a solute (solubility of the analytes) dissolving in a liquid medium (partition) and a solute passing through or impeded (physico-chemical activation or deactivation of analyte) by a porous substance based on its molecular size (size-exclusion chromatography). Most of the analyte (or drugs)

contained in a biological sample can be analyzed by using high performance (or High Pressure) liquid chromatography / ultra high performance liquid chromatography (HPLC / UPLC) method because it offers several advantages like specificity, rapidity of method progression, precision, accuracy of results, ease of automated sampling and injection, while eliminating the tedious extraction and isolation procedures.

There are various modes of analytical separation in HPLC / UPLC. They are normal-phase mode, reverse-phase mode, ion-exchange chromatography, reverse-phase ion-pair chromatography, affinity chromatography and size exclusion chromatographic techniques (gel-permeation and gel-filtration chromatography). In the method development, the analyst has to gain knowledge about the physical nature of the sample, namely, its polarity, molecular weight, as well as the chemical properties of the analyte like its ionic character and solubility. Generally, a pharmaceutical analyst usually initiates separation using reverse-phase chromatography, especially when dealing with compounds having high hydrophilicity due to many polar groups attached which determine its water solubility.

The concentration of an organic phase which is required for the mobile phase choice can be computed by gradient elution method. Elution of drug molecules can be altered by changing the polarity of the mobile phase. The magnitude of the elution of a mobile phase, as determined by its strength, depends on properties of polarity since stronger the polarity, higher is the elution. Whenever acidic or basic samples need to be separated in a mixture, it is strongly

advisable to control mobile phase pH by adding a suitable buffer and the pH of the added buffer should be pre-adjusted prior to supplementing the organic phase.

The next step viz. optimization, is initiated only when a reasonable chromatogram is obtained. A "reasonable chromatogram" requires that all the compounds are detected on the chromatogram using more or less symmetrical peaks without much fronting or tailing, also called skewness. By a slight change of the mobile phase composition, the shifting of the peaks occurs. An "optimised chromatogram" is a chromatogram which, after fulfilling the criteria for "reasonable chromatogram" has all the peaks in symmetry are well separated in less run time (good resolution in time).

We have developed for the first time, a method to estimate olanzapine, risperidone and its metabolite in plasma samples using UPLC-DAD analytical protocol, which is then correlated to the pharmacokinetic and pharmacogenetic data along with an adverse effect profile in each volunteer obtained from questionnaire study. We aim to evaluate the pharmacogenetic effects of highly prevalent and polymorphic allele variants of *CYP2D6* on risperidone and its metabolite plasma pharmacokinetics and adverse effect profiles in normal volunteers in South Indian population. As risperidone is a widely prescribed second generation antipsychotic drug in schizophrenia patients, its pharmacogenetics could have profound implications in patients, especially in South Indian population, where the incidence of this neuropsychiatric illness is increasing [16]. Additionally, the results from the pharmacogenetic-pharmacokinetic (genotype-phenotype) correlation along with the data from

adverse drug effect checklist have been supplemented by population pharmacokinetic modelling using NONMEM software.

Chapter-2

Aim & Objectives

2. AIMS & OBJECTIVES

AIM

The study aims to determine the effects of *CYP2D6*10* gene polymorphism on the population pharmacokinetic parameters and pharmacological effects of risperidone enrolling healthy human volunteers.

SPECIFIC OBJECTIVES

- Method development and validation of risperidone, 9-hydroxyrisperidone and olanzapine, by using Ultra-Performance Liquid-Chromatography (UPLC) coupled with diode-array detector in human plasma.
- Assessment of the *CYP2D6* metabolic difference and distribution in South Indian population.
- To assess the effect of *CYP2D6*10* gene polymorphism on the clinical pharmacokinetic parameters and pharmacological actions of risperidone using healthy volunteers.
- Population pharmacokinetic analysis of pharmacokinetic parameters using Non Linear Mixed Effect Modelling (NONMEM).

Chapter-3

Review of literature

3. REVIEW OF LITERATURE

Pharmacokinetics is the study of disposition of drugs in the body and its transport across biological membranes, while pharmacodynamic is the study of dose response relationships and the interactions between the drug (ligand) and the target (receptor). Pharmacogenetics is the science of study of variations in response to drugs, which are determined, by genetic variations between individuals and populations [17].

Pharmacokinetics deals with the study of absorption, distribution, protein and tissue binding, biotransformation (metabolism) and excretion (elimination) of xenobiotics on its passage to target tissues from the site of administration. Clinical pharmacokinetics parameters that are important in this branch include bioavailability, volume of distribution, half-life and clearance. These pharmacokinetic parameters define loading and maintenance doses of drugs in humans and can be calculated using compartmental modeling equations and the nature of kinetics of drug elimination [18].

Bioavailability, defined as the fractional extent of drug administered, which will reach the systemic circulation in the unchanged moiety. Many factors can reduce fractional bioavailability like first pass metabolism; factors can increase the bioavailability like entero-hepatic shunting. Volume of distribution (apparent volume of distribution) defined as the volume that could accommodate the entire drug administered if its concentration were same as that of the compartment wherein it equilibrates. Barriers like the blood brain barrier and reservoirs like melanin in retina exist for some drugs with altered volumes of

distribution, which is governed primarily by the drug solubility in aqueous and lipid media. Half-life is the time taken by a drug to be reduced to half its original plasma concentration in a one-compartment model without redistribution phenomena. Clearance is the extent of drug elimination (metabolism and excretion) per unit time and includes many routes like renal (glomerular filteration, tubular secretion and tubular re-absorption), fecal, biliary, salivary and other minor routes.

Metabolism or Biotransformation of drugs:

CYP enzymes are expressed mostly in the liver and are accountable for the oxidative biotransformation of exogenous compounds including many drugs, alcohol and pro-carcinogens [19]. 57 active *CYP* genes and 58 pseudogenes have been identified in the human genome. The human *CYP* iso-enzymes mainly accountable for drug metabolism comprise *CYP1A2, CYP3A4, CYP2D6, CYP2C9, CYP2C19, CYP2A6* and *CYP3A5* [20]. In order to know the *CYP* family of enzymes, it is better to understand the nomenclature of the system. The *CYP* word, which is used for the mammalian cytochrome P450 family of enzymes [21]. The name origin from a combination of 'cyto' meaning cellular, color from chrome, *P* as of pigment and 450 is the wavelength of light (450 nm) when it used in the analyzing the enzyme in the spectrophotometer [22].

Cytochrome P450 (*CYP450*) are a super family of iron containing xenobiotic and endogenous compound metabolizing enzymes that are almost ubiquitously distributed throughout the human body. They are responsible for undertaking Phase I biotransformation reactions of xenobiotics, including drugs, which include oxidation, reduction, hydrolysis and cyclization [23]. Additionally,

they serve as targets in microorganisms for the actions of certain antifungal given to patients with infections [24]. There are eighteen families of these enzymes in humans, which are encoded by about fifty-seven different enzyme-coding genes in our genomes. Of these enzyme families, which mostly have oxido-reductase activities, the involvement of environmental factors in activating their synthesis, a process called as induction, plays a major role - these factors can include diet, chemicals, therapeutic drugs, steroids, etc. Additionally, they play a key role in mediating endogenous processes like cellular apoptosis, differentiation; and pathophysiology of diseases [25]. They are localized in the plasma membrane, mitochondria apart from endoplasmic reticulum [26]; the latter cellular sub-location is makes they enzymes vulnerable for induction and inhibition by xenobiotic stimuli.

Of the numerous iso-enzymes that constitute these heme containing proteins, *CYP2C, CYP2D, CYP3A* are most abundantly expressed in the human liver, where they catalyze the phase I biotransformation of ~ 50% of clinically relevant drugs. Other iso-enzymes like *CYP1A* and *CYP2E* are activated by pro-carcinogens and environmental toxins like hydrocarbons and do not play a major role in drug metabolism [27]. *CYP2D6, CYP2C9* and *CYP2C19* genes are the valid biomarkers on the FDA list among the others [28]. These biomarkers have the capability to predict phenotypic difference in drug metabolism.

***CYP2D6:* Role in drug biotransformation:**

CYP2D6 is only a relatively minor form in human liver and it constitutes only 1.5% of total cytochrome P-450 iso-forms [29, 30]. It is great importance for the metabolism of clinically used drugs because these enzymes metabolize about

20-25% of the drugs. Other than liver, *CYP2D6* enzyme also expressed in the prostrate [31], heart [32] and brain [33]. *CYP2D6* is located on human chromosome 22 and it contains 4,378 base pair gene with 9 exons and 8 introns. *CYP2D6*1* accepted commonly as wild type and represented in NCBI accession number AY545216.1 and alleles or polymorphisms observed have been explained to differ from this [34].

Among the former iso-enzymes, *CYP2D6, CYP2C9, CYP2C19* are highly polymorphic in their expression profiles in humans. Of the numerous ligands that bind and affect *CYP2D6,* antiarrhythmics, antipsychotics, antidepressants, opioids and tamoxifen, which is a hormone modulator, are its important substrates [35]. *CYP2D6* was identified and characterized in animals and humans in 1980s, a decade after which it was shown in hepatic microsomes to convert dextromethorphan to codeine [36].

CYP2D6 can catalyze a number of reactions of its ligands and it has broad substrate specificity. O-demethylation of opioid analgesics, antitussives like codeine, dextromethorphan; N-demethylation of opioids like hydrocodone, and antidepressants like citalopram and fluoxetine; aromatic hydroxylation of atomoxetine often used in Attention Deficit Hyperactivity Disorder (ADHD) and antiarrhythmics drugs like propafenone, beta adrenergic blockers like alprenolol, antiemetics like Ondanseteron that acts by blocking 5HT3 receptors in the brain, antihypertensive drugs like indoramine, antiestrogen drugs like tamoxifen, antidepressants like desipramine, mianserin, mirtazapine; benzylic hydroxylation of amityptalline and nortryptalline used in the treatment of Major Depressive Disorders (MDD); O-dealkylation of antiarrhythmics like flacainide; N-

dealkylation of dexphenfluramine; and demethylation of the antidepressant paroxetine, are among many other drugs, the key reactions catalyzed by *CYP2D6* [37].

Among the antipsychotics that *CYP2D6* metabolizes, haloperidol, thioradizone, perphenazine, olanzapine, aripiprazole, and risperidone are the important substrates of this enzyme. It also has certain endogenous substrates like progesterone, tyramine and anandamide [38]. Moreover, they have been implicated in the predisposition of amines and neurosteroids like harmine and progesterone. It was also shown that *CYP2D6* is responsible for the regeneration of neurotransmitters like dopamine and 5-hydroxy tryptamine / serotonin in areas like basal ganglia, thalamus, glial cells, and hippocampus – both of which have been implicated in schizophrenia and antipsychotic drug action [36].

Genetics of *CYP2D6*:

The alleles for *CYP2D6* on the short arm of chromosome 22 can be either functional or non-functional alleles [eg. *CYP2D6*6*], the former can be classified as those with normal function [*CYP2D6*35*], decreased function [*CYP2D6*10*] or increased function of *CYP2D6* [*CYP2D6*1XN*], or may even lead to the deletion of the functional protein [*CYP2D6*5*] [37]. For example, a single amino acid substitution at position 34 from proline to serine of the *CYP2D6*10* allele can lead to alteration in response to drugs like metoprolol [23].

Of the numerous polymorphisms that can lead to decreased activity of *CYP2D6* enzyme, *CYP2D6*3*, *CYP2D6*4*, *CYP2D6*5*, *CYP2D6*6*, and *CYP2D6*10* can reduce the clearance of antipsychotics and antidepressants and increase the risk of their adverse effects [39]. The phenotypes that are affected by

such genetic mutations can be categorized as poor metabolizer phenotype [having two defective alleles in a locus], intermediate metabolizer phenotype [one mutated allele and one normal allele at a locus], extensive (or normal) metabolizers [carry two functional alleles, also called wild type alleles], and ultra-rapid drug metabolizers [carry > 2 active alleles] [40]. This is complicated by the presence of hybrid genes of *CYP2D6* with other genes like *CYP2D7* due to the process of genetic recombination in vivo [41]. Additionally, splice variants and haplotypes in linkage equilibrium have also been identified for this highly polymorphic enzyme [42]. The frequencies of *CYP2D6*10* allele hence differ in different populations - ranging from 0-64% across different populations [43]. Hence, it has a dual role of xenobiotic metabolism and possibly a physiologic role in the brain, which is affected by these mutations [44].

Many researchers have stressed that testing for such polymorphisms could partly offset such genetic variant induced differences in clinical response of drugs metabolized by these *CYP450* enzymes – for example the Ampli-Chip-P450 test in a South African population cohort [45]. Clinical dosing guidelines also have been recommended based on such genotype-determined phenotypic variations for the metabolized drugs like Selective Serotonin Reuptake Inhibitors [46], and, codeine [47] due to the unique genetic makeup of the gene encoding this enzyme [48].

Although the frequencies of *CYP2D6*10* are highest in Chinese and Japanese populations [65 % and 41% respectively] [49], this allele is not uncommon in Indian population [50], as reported previously in North India, South India and Tamil populations [3%, 10.2%, 20.3%] [51, 52, 53]. Hence, it is a very

predominant polymorphic allele of *CYP2D6* in South Indian populations, especially the Tamil population. This population is more homogenous and unique than the North Indian population [54]. This depicts a different pattern as compared to the allele frequencies reported in Caucasians (29%) and Native American populations (71%) [55].

Of the numerous variants that have been identified in *CYP2D6,* gene deletions and gene multiplications are most abundantly expressed in populations, whose detection can yield clinically applicable data in patients. Southern blotting RFLP, long template PCR results provide semi-qualitative data on such gene variants, and real time PCR can yield quantitative results about such genetic variants of the enzyme [56].

Schizophrenia and psychosis spectrum disorders:

Schizophrenia is a severe mental disorder characterized by negative, positive features of the patient, along with cognitive impairment, that is also hereditary [57, 58]. Although the genetic variants responsible and the pathophysiological mechanisms have been difficult to delineate and decipher as opposed to other neuropsychiatric disorders like dementias, endo-phenotypes have been proposed to characterize the individual patient in their absence [59].

Antipsychotic drug generations and classes:

Antipsychotic drugs can be broadly classified as older neuroleptics and newer atypical antipsychotics based on their relative affinities to dopamine D_2 receptors and intrinsic efficacy to cause extra-pyramidal side effects in both animal models and human patients. The first generation drugs are the oldest neuroleptics comprising three classes of chemically diverse drugs viz.

Phenothiazines, Butyrophenones, and thioxanthenes. Example of representative drugs in these three classes include chlorpromazine, haloperidol and flupenthixol. The second-generation drugs were discovered later than these drugs and include drugs with less selective actions at D_2 receptors relative to serotonin 5HT receptors in the brain and periphery [59]. Examples of drugs, which belong to first class of this generation, include clozapine, risperidone, olanzapine, quetiapine, ziprasidone, apart from other less commonly prescribed drugs [60]. The second-generation drugs also include a newer second class of drugs with improved pharmacokinetic features – examples include lurasidone, paliperidone, the latter being the active metabolite of risperidone. The third generation drugs include partial agonists at serotonin receptors like aripiprazole and its newer analogs [61].

Antipsychotic medications in Schizophrenia:

Antipsychotic drugs are chemically a heterogeneous group of medication compounds that could be classified based on their chemical nature, pharmacodynamic potencies, or adverse effect profiles. The classification by pharmacodynamic profiles seems to be very useful in predicting their clinical antipsychotic efficacies [62]. All antipsychotics in clinical use currently need to block Dopamine D_2 receptors in the mesocortical and mesolimbic pathways to have a useful antipsychotic effect in patients [63]. Their affinity for these receptors relative to each other and relative to $5HT_2$ receptors in raphe nucleus of the brain seems to determine their class, efficacy and tolerability. To illustrate this rule, for example full D_2 antagonism causes a reduction only in positive symptoms of schizophrenia but preserves the drug's propensity to cause extra-pyramidal features (EPS) like tardive dyskinesia, dystonias and akathesia, as with traditional

I generational antipsychotics like chlorpromazine and flupenthixol. However, a partial antagonism at these receptors along with a degree of selectivity to $5HT_2$ receptors reduces the drug's ability to cause these side effects and increases the benefits in psychotic patients by reducing also the negative and cognitive features of the disease, as with clozapine, an atypical II generation antipsychotic. A third generation of inverse agonists at D_2 receptors also exists including drugs like aripiprazole and brexpiprazole, which are devoid of EPS and are highly potent as antipsychotic drugs. Recently, a novel fourth mechanism has also been proposed for drugs like bitopertin – glycine reuptake inhibition, NMDA potentiation, BDNF stimulation [62]. Both risperidone and olanzapine belong to second generation (atypical or newer) antipsychotic groups used in schizophrenia as first line commonly, or second line drugs in combination with antidepressants and other medications.

A trend towards lower therapeutic efficacy of drugs like haloperidol with increasing copies of abnormal *CYP2D6* alleles, and an increased incidence of adverse effects with poor metabolizer status of the enzyme was recognized previously [64].

Extrapyramidal reactions (EPS) to older antipsychotics –

These are a heterogeneous group of reactions due to side effects of dopamine pathway blockade of neuroleptics and less commonly the newer drugs. There are six different EPS, which can be shown in animal models and in some reactions in patients also. The acute EPS comprise acute muscular dystonia, akathisia, neuroleptic malignant syndrome, and rabbit syndrome of peri-oral tremors. The chronic EPS, which develop only over years of antipsychotic

treatment, include parkinsonism and tardive dyskinesias. Only supportive and symptomatic but unsatisfactory treatment exist for some of these EPS and include beta adrenergic blockers like propranolol; anticholinergic – antihistamine drugs like trihexyphenidyl, and, promethazine; dantrolene which blocks ryanodine receptors; and benzodiazepine drugs like diazepam. Some EPS do not respond to treatment e.g. Tardive dyskinesias [65, 66].

Atypical antipsychotics' pharmacology:

Metabolism of second generation antipsychotics: Risperidone, olanzapine, quetiapine, ziprasidone, aripiprazole are the commonly used second generation antipsychotic drugs, while clozapine, sertindole and amisulpride are less commonly prescribed drugs in this class. Clozapine is metabolized by hepatic *CYP1A2*, with minor contributions for its biotransformation from other *CYP450* enzymes; risperidone is metabolized by *CYP2D6* and to a lesser extent by the *CYP3A4* isoenzyme; olanzapine is directly conjugated by UGTs apart being oxidation by *CYPs*; quetiapine is mainly biotransformed by the *CYP3A4* enzyme; whilst sertindole as well as aripiprazole are oxidized by both *CYP3A4* in addition to *CYP2D6*. Ziprasidone and amisulpride differ greatly in their biotransformation pathways – the former is metabolized by both aldehyde dehydrogenase and *CYP3A4*, the latter drug is eliminated by renal mechanisms with little hepatic metabolism [67].

Risperidone and 9-hydroxyrisperidone:

Pharmacokinetics of risperidone: Although a *CYP3A4* enzyme inducer in rodents [68] and a *CYP2C11* inducer in rats [69] and probably in humans, risperidone is principally metabolized by *CYP2D6* and not *CYP3A4*, to an active

metabolite in the liver. Both risperidone and its more hydrophilic metabolite are antipsychotics with equivalent pharmacologic potencies. It inhibits the metabolism of concomitantly administered clozapine [70].

In order to quantify the drug and its metabolite in patient's blood, appropriate sampling times must be pre-fixed to collect the samples. A recent Monte Carlo simulation research recommends that the absorption phase after dosing of antipsychotic drugs range from 0.2 to 1.1 hours after dosing, distribution-metabolism phase lasts from 1.0 to 8.4 hours, following by a longer elimination phase from 12 to 24 hours [71]. Based on Bayesian analysis of these simulations, they recommend three time points for sampling blood to estimate each risperidone and its metabolite.

Pharmacodynamics of risperidone and 9-hydroxyrisperidone: While both are substrate cum inhibitors of p-glycoprotein in the blood brain barrier, 9-hydroxyrisperidone is less potent in inhibiting this efflux transporter as shown by the higher IC50 values [72] as measured by in vitro assays [73]. Many atypical antipsychotics also inhibit Breast Cancer Resistance Protein (BCRP) *in-vitro* [74]. However, the primary targets of risperidone and its metabolite, for their antipsychotic effects are the dopamine (DA) and serotonin (5-HT) receptors in brain [75].

Clinical / Adverse drug effects of Risperidone

The minimum effective dose of risperidone is 2mg/day, which is greater than the maximum ineffective dose [76] of the drug of 1mg/day on oral administration. Because of unavailability of evidence-based recommendations on the therapeutic levels of many second-generation antipsychotics except probably

clozapine and olanzapine, routine monitoring of their concentration in the plasma of patients is not practiced in many hospitals and clinics [63]. Due to its weak inverse agonism at dopamine receptors and higher affinity for serotonin than central dopamine receptors [62], risperidone and its metabolite (used clinically as a separately approved entity, paliperidone) have few extra-pyramidal adverse effects and higher antipsychotic selectivity as compared to older antipsychotics like chlorpromazine. However, this does not deprive it entirely of toxicities like dizziness, orthostatic hypotension (alpha blocking action), dry mouth and gastrointestinal effects (antiserotonin and anticholinergic effects), and central effects in the brain. Risperidone also has effects on cognitive effects like word recognition, alertness, fine motor movements, and on electroencephalogram and on prolactin levels adversely [77, 78]. Additionally, risperidone can also cause drug induced ECG changes [79]. Apart from an antipsychotic drug, it is emerging as a therapeutic option for bipolar disorder [80, 81].

Risperidone pharmacogenetics

Due to *CYP2D6* mediated pathway predominance for risperidone and the highly polymorphic nature of this enzyme, a higher probability of inter-individual variations and inter population differences in its pharmacokinetics and effects in therapeutic and adverse contexts, can be expected. Not surprisingly, a Spanish study in 111 Schizophrenic patients concluded that such pharmacokinetic-pharmacodynamic target gene variants altered plasma concentrations, therapeutic and toxic responses to risperidone. Such polymorphisms also affect risperidone disposition and effects not only in patients but also in healthy volunteers [82].

Risperidone estimation

A handful of established and novel technologies exist to estimate the levels of risperidone and other atypical antipsychotic medications in biological matrices. For example, risperidone concentrations can be estimated in plasma samples using Multiple injection Method (MIM) coupled to Liquid Chromatography and a tandem Mass Spectrometer for detection of signals (LC-MS/MS) [83]. As this is a high thoroughput method, it is cost effective and time saving while preserving sensitivity [84]. Usually, a high performance column like phenyl methyl column is used and in order to quantify the analytes like risperidone, a multiple ion-monitoring mode using ion transitions at a particular m/z ratio for each analyte is used [85]. Biological matrices other than plasma can also be used for estimating risperidone and related analytes, like saliva of patients, and brain tissue homogenates of rodents [86, 87]. Due to its higher sensitivity and robustness, this method can also used to quantify chemically similar isomers like enantiomers of analytes like risperidone and 9-hydroxyrisperidone present in plasma [88]. Alternatively, Selective reaction monitoring can also be utilized to detect specific analytes of interest in patient's samples [89].

Olanzapine

Olanzapine pharmacokinetics: Olanzapine is conjugated directly by phase II enzymes catalyzing glucuronide conjugation in the liver viz. UGTs, with minor contribution from Cytochrome mediated oxidoreduction. Like risperidone, this antipsychotic is available as both oral and parenteral formulations for use in schizophrenia spectrum disorders. It can be metabolized to N-oxide, which can be reconverted back to olanzapine in blood [90].

Olanzapine pharmacodynamics: A thienobenzodiazepine derivative in its chemical nature, this second-generation antipsychotic drug has high affinities to dopamine, serotonin, histamine, and acetylcholine receptors. Due to its additional actions on the last two groups of receptors, it may have a useful anxiolytic action in some patients with anxiety disorders and bipolar disorder [91, 92].

Olanzapine pharmacogenetics: Therapeutic doses of olanzapine result in a wide range of plasma concentrations as estimated during therapeutic drug monitoring in patients. Smoking cigarettes and ingestion of interacting drugs like carbamazepine which are *CYP1A2* inducers reduce plasma concentration by increasing the extent of oxidation, whereas *CYP450* inhibitors like the selective serotonin reuptake inhibitor, fluoxetine, increase its plasma concentrations which is linearly related to its dose [93]. Although little studied in prior research published, the genetic variants that determine the clinical response to olanzapine are very important and can include variants in metabolism (UGTs and CYPs) as well as the targets [94]. For example, although investigators have evaluated the effects of functional polymorphisms in UGTs on the olanzapine metabolism and pharmacogenetics *in-vitro* using cell culture models, this has yet to confirmed in human volunteers and patients [95].

Olanzapine estimation: Both olanzapine and its metabolite N-oxide olanzapine, can be estimated using Liquid-chromatography / tandem mass-spectrometry (LC-MS/MS) as quantified postmortem using biological samples obtained on dogs [90]. The detectors used to quantify these drugs using HPLC vary from Ultraviolet detection [96, 97, 98], coulometric detection [99], Fluorescence [100, 101], to mass spectrometry [102, 103] with Atmospheric

Pressure Chemical Ionization (APCI) [104], for psychotropic and psychoactive drugs including olanzapine and risperidone.

Olanzapine utility in patients: Although olanzapine causes more extrapyramidal effects and weight gain than most antipsychotics, and more hyperprolactinemia than aripiprazole and ziprasidone, it is very potent among the second-generation antipsychotics [105]. Like risperidone however, olanzapine reduced the probability of reversal to psychosis due to ineffectiveness of the intervention [106].

Combination of risperidone and olanzapine

Although many clinical practice guidelines recommend risperidone and do not advice olanzapine in first episode schizophrenia due to greater incidence of metabolic side effects with olanzapine, many clinicians prefer risperidone followed by olanzapine as sole agents in new onset schizophrenia [107]. This was followed by aripiprazole and paliperidone as the most popular drugs for prescriptions in new onset schizophrenia; however, the combination of antipsychotics has also been favoured although few studies have addressed their superiority to monotherapy and most guidelines do not recommend such irrational combinations [108, 109, and 110].

Modelling of risperidone pharmacokinetics

A number of studies have evaluated the effect of various variables in ab-initio models like NONMEM and WinNonlin with regard to the pharmacokinetic compartmental modelling of risperidone and its main metabolite. Such in modelling techniques have also been applied successfully to neuroactive drugs belonging to groups apart from antipsychotics [111]. Using NONMEM,

population pharmacokinetic modelling was built by first order estimation method to estimate the effects of covariates on risperidone pharmacokinetics [112]. In another study, the effect of covariates like age, *CYP2D6* genotype and sex have been assessed in children and adolescent populations using NONMEM [113]. The researchers in this study have concluded that the active moiety pharmacokinetics along with the covariates' effects on the pharmacokinetic variability of risperidone can be better explained on inclusion of the metabolite effects also. However, since the ratios of p-glycoprotein substrate concentrations like those of risperidone plus 9-hydroxyrisperidone in plasma versus brain interstitium are greater than most neuropharmacologic therapeutic drugs, the cerebrospinal fluid concentrations of the unbound forms are better predictors of their brain concentration as assessed in animal models recently after single dosing [114].

Modelling plasma concentrations of antipsychotics such as risperidone and 9-hydroxyrisperidone can provide a useful surrogate marker of the treatment failure and even compliance and non-adherence effects – as summarized by a recent study in which drug's plasma concentrations were more in patients who either did not adhere to therapy or showed features suggestive of treatment failure [115]. Since risperidone is a class II biopharmaceutical classification drug (BPC II) whose oral absorption depends on dissolution rates significantly, its pharmacokinetic absorption profiles can be predicted using Advance Compartmental Absorption and Transit (ACAT) models as predicted by a recent IVIVC study using GastroPlusTM software [116]. A research performed in patients with schizophrenia has integrated the Physiological Based pharmacokinetic pharmacodynamics models (PKPD) with the usefulness of

antipsychotic medications as assessed by scores of Positive and Negative syndrome scale (PANSS) using a clinical usefulness criterion [117]. They have used NONMEM compartmental modelling of four atypical antipsychotic drugs with the help of Bayesian approaches [118].

Using Monte Carlo simulations and reference values of dextromethorphan metabolic ratios from published literature, investigators have evaluated the effects of *CYP2D6* metabolizer status in Asian and Caucasian populations on the hepatic extraction ratios and intrinsic clearances in different genotypes [119]. They have concluded that *CYP2D6*10* heterozygous mutant and wild type allele carriers are extensive metabolizers of drugs like risperidone, with genetic variability in hepatic metabolism of ~60-70% (CV) in populations, while that of poor metabolizers are ~109% variable across different populations, which is predicated by AUCs of drugs like dextromethorphan (Probe).

Pharmacoproteomic approaches using population pharmacokinetic modelling in mice have assessed the Blood Brain Barrier (BBB) penetrability of psychiatric medications like risperidone [120]. The researchers in this study have used 11 P-glycoprotein substrate concentrations in plasma and have modelled the brain pharmacokinetic in MDR1 knockout mice in vitro utilizing values of dissociation constants and unbound drug fractions.

Since, the plasma concentrations of drugs like risperidone and olanzapine are reflected in the dopamine receptor occupancies of these drugs [121] in imaging studies and ultimately predict the clinical effects, modelling the pharmacokinetics of these drugs which predict and determine their plasma concentrations appears to be rational. Using this approach, investigators have

predicted than among the orally administered antipsychotic medications in patients, paliperidone and aripiprazole extended release preparations caused stable plasma concentration in multiple dosing protocols, as inferred by the fluctuation indices i.e, the differences in the peak-trough plasma levels of these drugs [122]. Finally, although parental preparations produce greater pharmacokinetic coverage than oral formulations of risperidone and paliperidone as investigated recently as producing more patient compliance than extended and intermediate release oral preparations [123], patients prefer oral doses to intramuscular doses.

Clinical response to risperidone and olanzapine

Risperidone has been a commonly used potent antipsychotic with usefulness especially in first onset schizophrenia and treatment resistant psychosis as polypharmacy [124, 125]. It has been found to be useful in resistant depression also with antidepressants like SSRIs [126]. In this condition, the reason for its usefulness may lie in its anti-stress effects on BDNF pathway in the brain [127, 128]. Additionally, it has been useful in patients with mania predominant mood disorders as a second line agent [129]. Finally, its utility in anxiety disorders like Obsessive Compulsive Disorder has been established in research and therapeutic trails in patients [130]. It can also be used in children with autism spectrum disorders.

Apart from first onset schizophrenia, Olanzapine has been useful as augmentation therapy in anxiety disorders like Obsessive Compulsive Disorder (OCD) [131]. Similarly, it has been efficacious in preventing chemotherapy-induced nausea and vomiting caused by highly emetogenic drugs used in cancer patients [132]. In addition, it has been shown to be useful in patients with bipolar

depression along with antidepressants [133]. However, its utility in schizophrenia patients cannot be undermined as it is a commonly efficacious antipsychotic with few side effects unlike earlier generation antipsychotic medications like chlorpromazine [134].

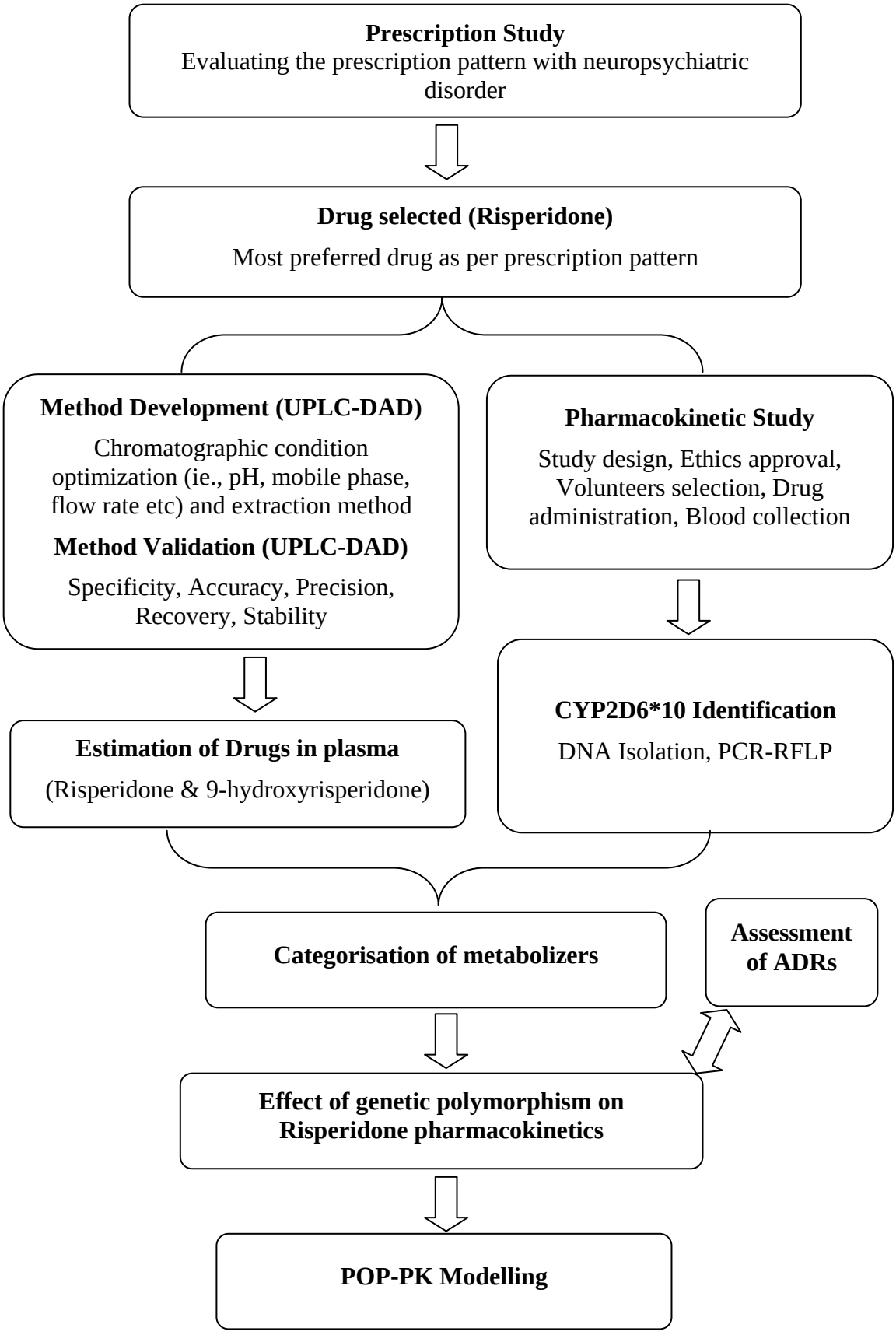
Dosing of risperidone and olanzapine in human volunteers and patients

Risperidone and olanzapine are available for oral administration and intramuscular injection in patients. Many patients however prefer oral formulations including immediate release and extended release preparations to parenteral formulations due to its advantages. Minimum efficacious initiation regimens of risperidone require low doses (2-4 mg/day) in first onset schizophrenia patients [135]. This can be greater for treatment resistant psychosis even if combinations with other such medications are advocated in the same patient. For example, although olanzapine is approved for low dose initiation regimens in first onset schizophrenia (20 mg/day Disintegrating tablets), it has been effective only in higher doses of 50 mg/day in resistant psychosis [136].

Chapter-4

Plan of Work

4. PLAN OF WORK



Chapter-5

Materials

5. MATERIALS

Chemicals cum reagents used

HPLC-grade reagents including the alcohol viz., methanol, the ketone, acetonitrile, and the base, ammonium acetate have been obtained from Sigma-Aldrich, India. HPLC grade of acetic acid and Methyl tert-butyl ether were purchased from Himedia, India. Other reagants like 25% pure Ammonia solution was purchased from Merck, India. Ultrapure water with resistence-tuned purity of 18.2 MΩ cm was procured from MilliQ water purification system (Millipore, USA).

A DNA purification kit was purchased from QIAGen, Germany. Enhanced Avian HS RT-PCR Kit and primers was obtained from Sigma-Aldrich, USA. HphI restriction enzyme was bought from Thermo Fisher scientific, India.

Risperidone, 9-hydroxyrisperidone, olanzapine and clozapine were used in the study from Sigma-Aldrich, India. The human blank-plasma was procured through blood bank services PSG Hospitals, Coimbatore.

Instrument and equipment used

I. Sartorius digital weighting balance (BSA 2245-CW) with printer (YDD 20-OCE)

II. Pico plus pH meter, Lab India

III. Water Acquity H class UPLC system consist of following configurations;

- Quaternary solvent manager with degasser
- Sample manager-FTN
- Column oven
- PDA detector

IV. Ultra sonic cleaner, Sonica, Italy

V. Deep freezer

VI. Centrifuge 5810R, Eppendrof, Germany

VII. Turbo vap LV, Biotage, USA

VIII. Spinix vortex shaker, Tarsons, India

IX. Test tube rotator, Tarsons, India

X. Thermal cycle, Arkthik, Thermo Scientific, Finland

XI. Gel dock Image Analyser, Syngene, G:Box, India

XII. Analytical column such as,

- Acquity UPLC-BEH C_{18} (50 × 2.1 mm, 1.7µ)
- Acquity UPLC-BEH C_{18} (100 ×2.1 mm, 1.7µ)
- Phenomenex Onyx Monolithic C (50 ×2.0 mm)
- Inertsil-ODS 3 (2.1 ×100 mm, 5µ)
- Zorbox SBCN (2.1 ×100 mm, 3.5µ)

Chapter-6

Methodology

6. METHODOLOGY

6.1 Prescription screening

We had undertaken a preliminary study evaluating the prescription pattern for outpatients attending the PSG Hospital psychiatry clinics with a neuropsychiatric disorder. We then scrutinized their prescription under the treatment section for the drugs that they were receiving for schizophrenia. Additionally we noted other concomitant drugs that they were prescribed. Our focus was mainly on deciphering a pattern of antipsychotic drugs that the patients were being prescribed for schizophrenia either for the first time or as a follow up.

6.2 Estimation of drugs in human plasma

6.2.1 Chromatographic conditions optimization

Chromatographic method selection depends on the sample nature (neutral or ionic molecule), solubility and molecular weight. In the present study, the selected drugs have high polarity and so ion pair or ion exchange or reverse phase chromatography will be appropriate. In the current study reverse phase ultra-performance liquid chromatography coupled with diode array detection (UPLC-DAD) methods are considered to be more suitable since they are particularly specific, sensitive, linear, accurate, precise and rapid methods.

6.2.2 Wavelength selection for the detection of risperidone, 9-hydroxy risperidone and olanzapine

10 µg/mL stock solutions of risperidone, 9-hydroxyrisperidone and olanzapine were prepared separately in solvent mixtures of acetonitrile and water (1:1). These solutions were scanned in the Ultraviolet (UV) region of 200 – 400

nm and the UV spectrums were recorded for risperidone, 9-hydroxyrisperidone and olanzapine (Figure 1, 2 and 3). Using the UV absorption spectra, detection at wavelength 277 nm was fixed for each of risperidone, 9-hydroxyrisperidone and olanzapine respectively.

Figure 1. Risperidone UV Spectrum

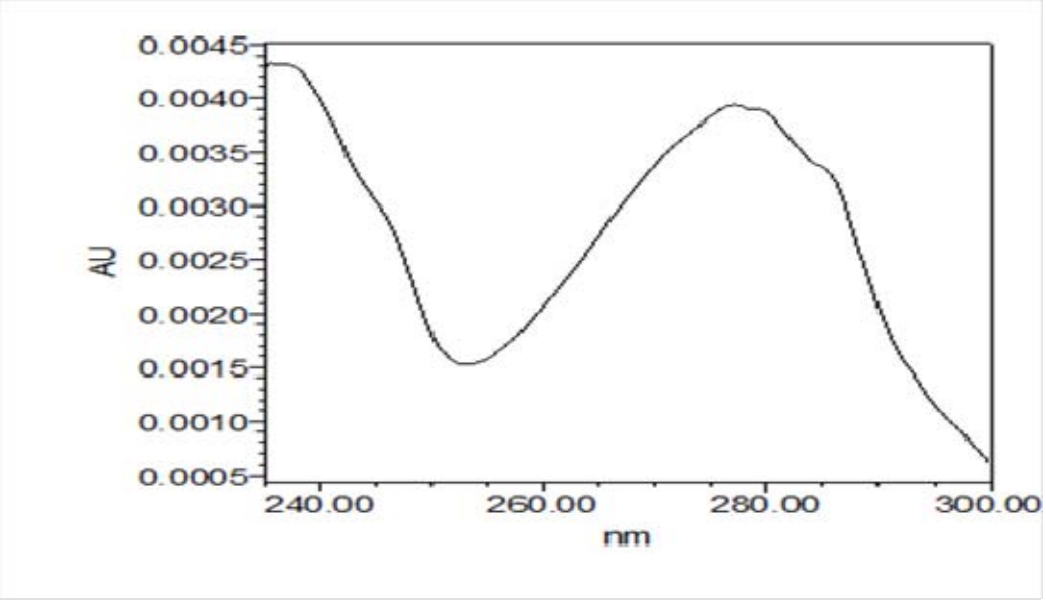


Figure 2. 9-Hydroxyrisperidone UV Spectrum

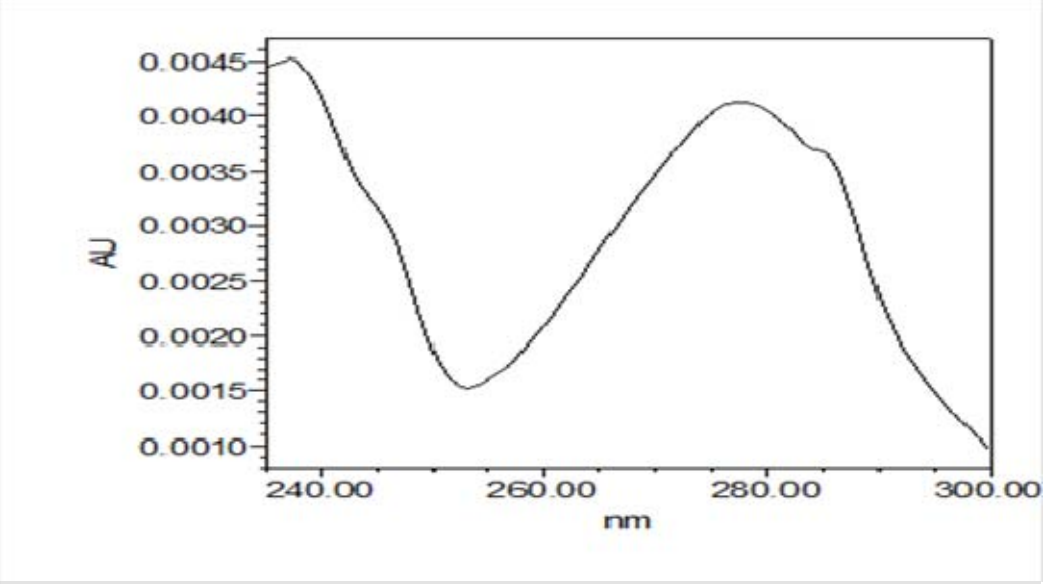
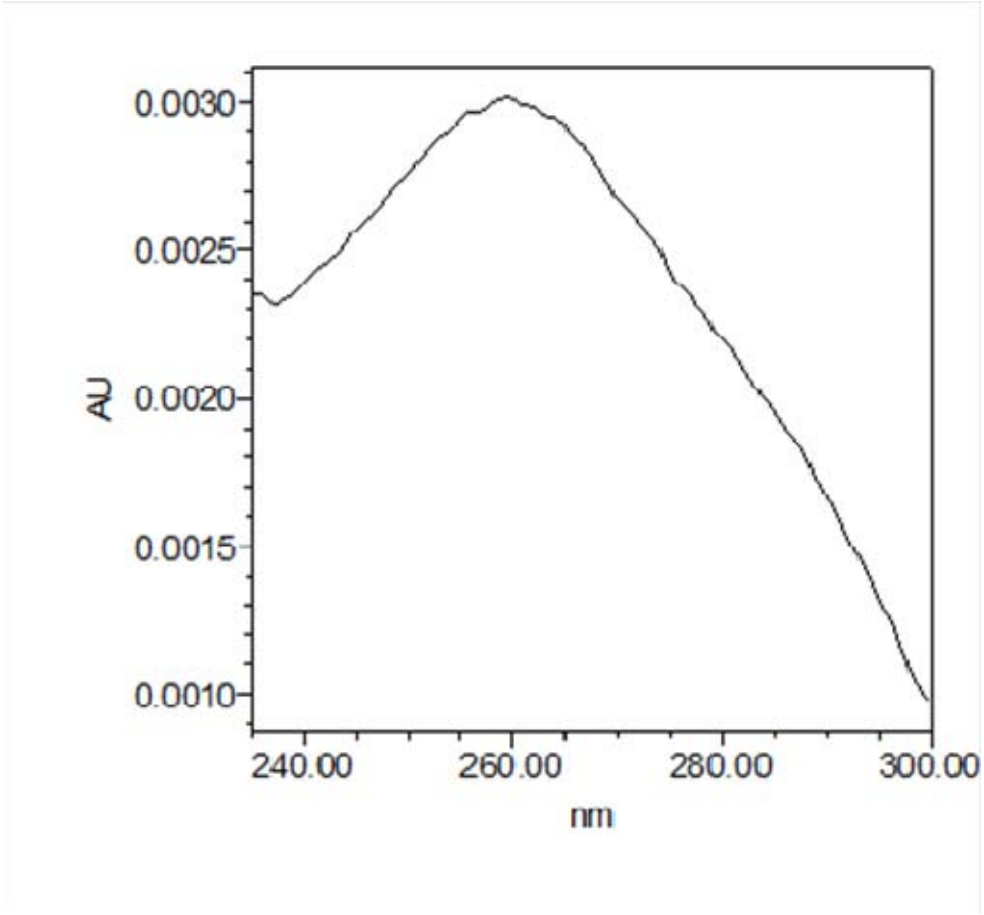


Figure 3.Olanzapine UV Spectrum



6.2.3 Initial separation conditions

A gradient run was performed for the initial separation. In this preliminary separation, the approximate ratio of buffer solution in the organic phase to elute the drugs from the column was determined. An aliquot of the standard solution was prepared and chromatogrammed using the following chromatographic conditions;

Stationary phase : Acquity UPLC BEH C_{18} column, (1.7 µm, 2.1 X 50 mm i.d., and 1.7 µm, 2.1 X 100 mm i.d.,)

Mobile phase : Solvent A: 20mM ammonium acetate / 0.2% acetic acid

Solvent B: Methanol / Acetonitrile

Solvent ratio : Gradient run, 5 to 100% solvent B for 15 min

Flow rate : 0.5 mL/min

Injection volume : 10 μL

Column temperature : 30ºC

Based on the above gradient run, the approximate percentage of methanol or acetonitrile in the acetate buffer or acetic acid buffer required to elute the drugs from the column was determined. The mobile phase mixture of 15mM ammonium acetate (70% v/v) and acetonitrile (30% v/v) was used for the following isocratic separation and the chromatograms were recorded.

6.2.4 Effects of chromatograph technique related variables

The chromatographic condition optimization, the effect of variables such as pH, nature of the mobile phase, solvent's strength, flow rate, addition of chromatographic peak modifiers, solvent-ratio and the nature of stationary phase on the peak separation were assessed. The resulting chromatograms were recorded and the chromatographic parameters related to asymmetric factor, capacity factor, column efficiency and resolution were calculated. From the above parameters, the optimum symmetry factor and capacity factor were selected for the further estimation.

The standard solution of risperidone, 9-hydroxyrisperidone and olanzapine were chromatogrammed for 10min using buffer solution in acetonitrile at different pH ranging from 2.0 to 7.0 as the mobile phase at a flow rate of 0.5 mL. It was observed that an increase in pH decrease the retention time of risperidone, 9-hydroxyrisperiodne and increase retention time of olanzapine.

At pH 3.5, the risperidone, 9-hydroxyrisperidone and olanzapine were well retained and symmetrical peaks were achieved.

6.2.5 Nature of stationary phase

A variety of reverse phase stationary phase (C_{18}, SB-CN and Monolithic) were used and the chromatograms were recorded. Based on the peak shape and retention time, Acquity UPLC BEH C_{18} column was selected for risperidone, 9-hydroxyrisperidone and olanzapine.

Different mobile phase, namely, acetonitrile, methanol and acetic acid in aqueous phase were used at a flow rate of 0.3, 0.4 or 0.5 mL/min. Initial separation condition methanol was used in the mobile phase and it exhibited broad peaks without much symmetry in addition to a long retention time. When acetonitrile was used as the organic solvent of choice in the mobile phase, the analysis time was diminished and the peak shape became more symmetrical in shape.

6.2.6 Selection of internal standard

To minimize the error in the assay due to loss of drugs that takes place during the extraction procedure, internal standard may be used along with the standard drugs. The selection of internal standard based on the polarity, purity, solubility and absorption characteristics. In present study, clozapine used as internal standard (IS). This internal standard provides well resolved and symmetrical peaks.

6.2.7 Optimization of chromatographic variables

Based on the above set of evaluations, the subsequent chromatography related conditions were used for the quantification of risperidone and 9-hydroxyrisperidone along with olanzapine in human blank plasma.

Optimized chromatographic conditions

Stationary phase	: Acquity UPLC BEH C_{18} column, (1.7 µm, 2.1 X (2.1 X 100 mm, i.d., 1.7µm)
Mobile phase	: Acetonitrile: 10mM ammonium acetate with pH 3.5 adjusted using acetic acid
Mobile phase ratio	: 30:70 % v/v
Flow rate	: 0.3 mL/min
Column temperature	: 40ºC
Sample tray temperature	: 10ºC
Injection volume	: 5 µL
Detection	: 277 nm
Data acquisition	: Empower 3 software version 1.0 (waters)
Internal standard	: Clozapine

6.3 Preparation of stock solutions

6.3.1 Preparation of Risperidone stock solution (1 mg/mL)

Weighed accurately 10 mg of risperidone reference substance and transferred into a 10 mL volumetric flask, dissolved and diluted to the mark with methanol. The stock solution was stored in refrigerator between 2º to 8ºC until analysis.

6.3.2 Preparation of 9-Hydroxyrisperidone stock solution (1 mg/mL)

10 mg of 9-hydroxyrisperidone reference standard was accurately weighed and transferred into a 10 mL volumetric flask, dissolved and diluted to the mark with methanol. The stock solution was stored in refrigerator between 2º to 8ºC until analysis.

6.3.3 Preparation of Olanzapine stock solution (1 mg/mL)

10 mg of olanzapine reference standard was accurately weighed and transferred into a 10 mL volumetric flask, dissolved and diluted to the mark with methanol. The stock solution was stored in refrigerator between 2º to 8ºC until analysis.

6.3.4 Preparation of mixture of stock solution of Risperidone, 9-Hydroxyrisperidone and olanzapine (100 µg/mL)

Transferred accurately 5.0 mL of each stock solution (Risperidone, 9-Hydroxyrisperidone and Olanzapine) into a 50 mL volumetric flask and dilute to the mark with methanol: water (50:50, v/v).

The stock solution of the drugs was diluted till suitable concentrations for spiking plasma was obtained for the purpose of calibration curve (CC) standards and quality control (QC) samples.

6.3.5 Calibration standards and Quality control (QC) samples

Calibration curve and quality control working solutions were prepared from the stock solution by an adequate dilution using methanol: water (50:50, v/v). Calibration standard for plasma were prepared by spiking the stock solution to obtain the concentration levels of 1.0 to 100 ng/mL in human plasma for all analytes. Quality control samples were prepared at a concentration of 1.0 ng/mL (LLOQ QC), 5.0 ng/mL (LQC), 45.0 ng/mL (MQC) and 85.0 ng/mL (HQC). All the samples were stored at -80º ± 10ºC until analysis.

6.3.6 Stock solution of Clozapine (Internal standard, 1mg/mL)

10mg of clozapine was accurately weighed and transferred into a 10 mL volumetric flask, dissolved and diluted to the mark with methanol. The stock solution was stored in refrigerator between 2º to 8ºC until analysis. The stock solution was diluted with methanol: water (50:50, v/v) to obtain a working solution of 20 µg/mL.

6.3.7 Preparation of calibration curve standards and quality control samples

Transferred 240 µL of each sample to pre-labelled 5 mL storage vials. Added 10 µL of working stock solutions, 25 µL internal standard and 250 µL of 10mM ammonium acetate (base with alkaline pH of 9.0) were added to the

aliquot. Mixing the sample for 20 seconds at each step was performed using a vortex shaker.

6.3.8 Preparation of subject samples

Transferred 250 µL of subject sample to pre-labelled 5 mL storage vials. Added 25 µL internal standard and 250 µL of 10 mM ammonium acetate (pH - 9.0) were added. Mixing of this sample for 20 seconds at each step using vortex shaker was carried out.

6.3.9 Extraction procedure for sample processing

Adding 3.0 mL of tert-butylmethylether and vortex mixing it for 5 minutes, and then centrifuging this at 10000 rpm for 5 min at 4ºC resulted in the formation of a twin layer fluid. The clear top organic layer (~2.5 mL) was transferred onto 5 mL poly-propylene tubes. The samples underwent evaporation to dry state at 40ºC using nitrogen evaporator. After evaporation, the resultant residue in the dry state was reconstituted using 200 µL of the mobile phase, which was vortex mixed for 1.0 minutes. The samples were centrifuged at 10000 rpm for 5 minutes. The clear supernatant of 190 µL each samples of was transferred into clean and dry micro-vials made of glass, and 5 µL was injected through an autosampler into the UPLC system for further analysis.

6.4 Method-validation

Validation is a process which involves establishment or confirmation by laboratory studies that a method / system / procedure / analyst can give the required sensitivity, precision, accuracy, ruggedness, etc. In the most fundamental form, validation of an analytical procedure demonstrates that the procedure developed is suitable for its intended purpose. Validation process was carried out after the development of the UPLC method.

Validation parameters were,

1. Specificity / Selectivity
2. Carry over and Sensitivity
3. Linearity
4. Accuracy and precision

a. Within-batch / Intra-day precision and accuracy

b. Between batch / inter-day precision and accuracy

5. Stability studies

a. Stock solution stability

b. Bench top stability

c. Autosampler stability

d. Freeze-thaw stability

e. Long-term stability

6. Recovery
7. Ruggedness

6.4.1 Specificity and Selectivity

A method is said to be specific when it produces a maximum response only for an analyte of interest. Method selectivity is defined as the capability of the method to produce the given response for the analyte even in the presence of other interferences. To facilitate and confirm that the chosen method has qualified to be highly specific and selective, the subsequent two sets of samples were processed and injected into the UPLC column via the appropriate extraction procedure.

1. Six different lot of blank human plasma obtained from different sources (Plasma containing K_2EDTA as anticoagulant) were chromatographically screened for interference without analytes and internal standard.
2. Above blank plasma samples were chromatographically screened for interfering substances by spiked analyte at the lower limit of quantification (LLOQ) level with the internal standard for each bag.

To calculate the percent interference, the response obtained for each blank plasma peak area response compared with the blank plasma spiked with LLOQ peak area response.

% Interference =Peak area response of blank / peak area response of LLOQ X 100

At least five out of six lots should have responses less than five times the LLOQ level response in the same matrix

6.4.2 Carry-over and Sensitivity

Carryover of the experiment was evaluated using injections of the standard with the highest calibration curve immediately after the blank human plasma injection. The percent carry-over should not be more than 0.5% for analytes and

internal standard. Sensitivity assessed by quantitative determination of lowest concentration (signal-to-noise ratio ≥ 10:1) of analyte in biological matrix that can be calculated with an acceptable precision and accuracy. Sensitivity was calculated by using signal to noise ratio (H/h), where as H-mean signal of analyte at lowest calibration level in terms of height and h-mean noise for the blank plasma samples in the terms of height.

$$\textit{\% Carry over} = \frac{\textit{Mean area of analyte in blank plasma}}{\textit{Mean area of analyte highest calibration standard}} X \ 100$$

6.4.3 Linearity assessment

Linearity of the calibration curves was assessed by the use of linear regression statistical analysis along with the solution of working standard which were spiked in plasma having the analytes of interest at different concentrations within the range of 1.00-100.00 ng/mL for all analytes. The calibration curve was constructed by plotting the peak area ratio of analytes: internal standard against the nominal concentration of the calibration standards in blank human plasma. The intercept and slopes were calculated with least square linear regression analysis with the use of a $1/X^2$ (X = concentration) weighting factor. The calibration standard had to have a correlation coefficient (r) of 0.99 or greater. The acceptance criteria for each back-calculated standard concentration were ± 15% deviation from the nominal value except at LLOQ, which was set as ± 20% [137]. Calibration of the curves consisted of:

- Injection of middle concentration level of the working standard solution with IS to confirm retention times.

- Injection of processed blank sample to verify interferences and retention time (RT) values of the analytes and IS
- Zero sample processed appropriately to verify the interference at the RT of analyte
- A set of eight non-zero standard solutions covering the expected range. The lowest and the highest standards were prepared in duplicated.

6.4.4 Accuracy and Precision

The method used for validation consisted of the assessments of analytical accuracy plus the precision after the experiments were carried out using four independent and distinct series in blank plasma. From these assessments carried out with an adequate ruggedness, the intra-day and inter-day analytical accuracy with precision was determined using six replicates containing analytes at four different QC levels. The lower limit of quantification (LLOQ) was 1.0 ng/mL; the low QC (LQC) was 5.0 ng/mL; the calculated medium QC (MQC) was 45.0 ng/mL and the value of high QC (HQC) 80.0 ng/mL.

Accuracy: definition and estimation

Accuracy represents the degree of closeness of the mean values which are computed from a series of measurement using the method and the true value in agreement. It is reported as percentage nominal value of the analysed concentration and is calculated as;

% Nominal value = (Calculated Concentration / True Concentration) x 100

Intraday Accuracy

Intraday (within a day) accuracy is determined by calculating the numerical value of the percentage nominal of the calculated concentration from the actual values for quality control samples at each concentration level analysed in a single run / single day and the mean of percentage nominal at each levels was reported.

Inter day Accuracy

Inter day accuracy is computed as the percentage nominal of the calculated concentration from the actual value for quality control samples at each concentration level analyzed over at least two days and the mean of percentage nominal at each level was reported.

Precision: definition and determination

This term represents the closeness of the agreement of values among a series of measurements that are obtained from sampling the analyte over many times. This can be estimated with variances (square root of standard deviation) of repeatability (Intraday variance values) and convergent precision (Sum of inter day and intraday variances). Precision is expressed as the percentage coefficient of variation (%CV) calculated as per the following expression:

% CV = (Standard Deviation / Mean) x 100

Intraday Precision

Intraday precision analysis is done by calculating percentage coefficient of variation (% CV) of the results obtained during a single run / same day.

Inter day Precision

Inter day precision can be determined after calculating the percentage coefficient of variation (%CV) of the results obtained over a period of at least two days. Such precision values obtained must not exceed at least ± 15 % of the relative standard deviation (RSD), except for LLOQ, for which the value should be within ± 20 % of RSD [137].

6.4.5 Recovery

Recovery of a bio-analytical method is the absolute values of the measured response obtained when a certain amount of analyte is added to and extracted from the biological matrices, and it is expressed as the percentage of the actual response yielded for the true concentration of the pure authentic standard which has not yet been subjected to the extraction.

The recovery of each method was determined by using six similar replicates of quality control (pure QC) samples un-extracted from the mobile phase, with each concentration close to spiked Quality Control samples at Low level Concentration (LQC), middle level concentration (MQC) and high level concentration (HQC) were extracted and prepared. These un-extracted sample volumes are injected simultaneously with samples belonging to precision and accuracy batches.

Percent recovery of analyte at each level was calculated using the following expression:

$$\% \text{ Recovery} = \frac{\text{Area of analyte in extracted plasma sample}}{\text{Mean area of analyte in un-extracted solution level}} \times 100$$

The percentage of recovery from the internal standard was calculated additionally. The peak area of response using internal standard obtained for the extracted MQC sample volume (as analyzed in the precision and accuracy batch) is compared for differences with the mean area of response for the internal standard obtained for the un-extracted MQC aliquots.

6.4.6 Stock Solution Stability

The stability of stock solution of analytes and internal standard was evaluated at room temperature for 24 hours and refrigerator for 7 days. Aqueous stock solution of analytes and the internal standard were prepared. One portion of the stock solution was placed in the refrigerator between 2º to 8ºC for 7 days, whereas the second part of the solution was stored at room temperature for 24 hours. The stability samples were compared with freshly prepared stock solution. The stability of individual analytes after 24 hours at room temperature and 7 days at refrigerated should be between 90 to 110% or the change should be 10%.

$$\% \text{ Stability} = \frac{\text{Mean response of stability samples}}{\text{Mean response of freshly prepared samples}} \times 100$$

6.4.7 Benchtop-Stability

Six aliquots of the Low quality control (LQC) and high quality control (HQC) levels each were placed on the bench at ambient conditions for 8 ± 1 h. The above samples were analyzed along with freshly prepared quality control samples (Six each of LQC and HQC) and calibration curve standards. The stability of the analytes was assessed by comparing the means of concentration of stability samples with those of freshly prepared quality control samples with their respective nominal concentrations.

6.4.8 Autosampler Stability

To assess the sample stability in the autosampler unit after processing the samples for duration equal to the anticipated run time, six samples of QC's each at the lowest and highest quality control were placed in this unit for 26 hours at 8ºC. The quality control samples were retained in the autosampler to prove auto sampler stability. After the lapse of the test time, the samples placed in the auto sampler were injected into the system along with freshly prepared calibration curve standards and quality control samples. The stability of the analytes was evaluated by comparing the means of concentration of stability samples with those of freshly prepared quality control samples with their respective nominal concentrations.

6.4.9 Freeze-thaw Stability

Freeze thaw stability measurements were evaluated in order to ensure that the analytes were stable when present in the biological medium after even multiple freeze-thaw cycles (FTC). Withdraw six aliquots each of LQC and HQC samples from the deep freezer after 24 hours of freezing followed by thawing without much disturbance at room temperature. These samples were refreezing under the same conditions for 12-24 hours. After 12-24 hours, the samples were removed and thawed them unassisted at room temperature (1^{st} FTC).

Again refreeze, the samples were kept under the same conditions for 12-24 hours. After 12 -24 hours, the samples were removed and thawed them unassisted at room temperature (2^{nd} FTC).

Again refreeze, the samples were kept under the same conditions for 12-24 hours. After 12-24 hours, the samples were removed and thawed them unassisted

at room temperature (3rd FTC). Analyze each of the lower and higher quality control samples after 3rd FTC along with a set of freshly thawed quality control samples (Six each of LQC and HQC).

The sample stability was determined by comparing the means of concentrations of the stability samples with those of freshly thawed samples.

6.4.10 Long Term stability (LTS)

To quantitatively emphasize the analytes' stability in the given matrix under the same physico-chemical conditions of storage similar to those followed with the study samples for duration between the date of first sample collection and the date of last sample analysis. Long-term stability was determined by storing the six aliquots of each of low and high quality control samples under the same condition as the study samples. The concentration of all the stability samples were compared with the mean of back-calculated values of stability obtained for quality control sample with the mean concentration of corresponding quality control samples analyzed with the initially accepted precision and accuracy batches.

The percent accuracy of fresh QC samples was calculated by using following formula;

$$\frac{\textit{Back calculated concentration from CC}}{\textit{Actual concentration}} \textit{ x 100}$$

The percent stability was calculated by using following formula;

$$\frac{\textit{Mean back calculated concentration of stability samples}}{\textit{Mean back calculated concentration of freshly thawed samples}} \textit{ x 100}$$

The stability samples were considered stable if assay values were within the acceptable limits of accuracy (85-115% from the fresh samples) and precision (± 15% RSD) [137]

6.4.11 Robustness

Ruggedness of the method (also termed robustness) was evaluated on a single precision and accuracy batch by altering the experimental conditions such as another analyst and different lot of column.

6.5 Pharmacokinetic study design and data handling

6.5.1 Study design

Single dose, single period, open label, oral bioavailability study of risperidone under fasting condition in healthy adult human subjects.

6.5.2 Number of subjects

Twenty healthy adult human subjects were recruited for this study.

6.5.3 Screening procedures

Subjects were screened for physical examination, assessment of well-being, vital signs examination and haematological testing (which will include RBC and WBC counts, platelet count, haemoglobin, blood sugar levels).

Inclusion criteria

- During the pre-study screening male healthy willing adult human volunteers above 18 years of age.
- No history of chronic illness (asthma, epilepsy and cardiac disease).
- Subjects not on any medications, which are metabolized by the CYP450 metabolic pathway.
- The physical examination and laboratory evaluations should be clinically acceptable limits.
- Volunteers are required to refrain for from taking any medications one week before study initiation.
- Subjects willing to give informed written consent and comply with the protocol requirements will be taken in the study.

Exclusion criteria

- Those who fail the screening procedure, and are not declared healthy by the physician.
- A positive history of allergic responses to drugs or a known food allergy provided by the subject.
- Subjects who informed of any clinically significant illness within a week of study initiation or who told of prior hospitalized within three months.
- Subjects who have been prescribed and have consumed a non-OTC medication within the past 14 days or any topical product within 7 days prior to administration of study medication.
- Subjects who have been administered a parenteral depot formulation or an implant releasing any drug 3 months before instructing the ingestion of study medication.
- Subjects who gave a history confirming chronic alcoholism.
- Subjects who indicated the habit of heavy smoking (at least 6 units / day of cigarettes or any other form of nicotine) or habit of chewing or inhaling nicotine containing products in an uncontrollable fashion.
- Subjects with an intake of any other investigational drug apart from that related to the current study within the last ninety days prior to dosing.
- Subjects who have donated blood for transfusion (1 unit or 350 mL) in a period including the 90 days prior to receiving the first dose of study medication or during the study period.
- Subjects who have drugs abuse.

6.5.4 Housing

Subjects were checked-in the clinical facility at least 11 hours before administration of the drug and they were continued to remain in the facility for 24 hours.

6.5.5 Dietary status

Subjects were fast for at least 11 hours prior to being served the standard breakfast. The drug was administered three hours before the subjects begin the standard breakfast. Subjects were instructed to eat their whole breakfast in 30 minutes. Only the fluids given with breakfast and water (except within one hour prior to and until one hour after drug administration) were allowed. Standard meals, consisting of caffeine-free, xanthine-free and grapefruit-free foods and beverages were served at scheduled time.

6.5.6 Subject withdrawal

- Serious adverse effects
- Major violation of the protocol
- Withdrawal of consent

The subjects were monitored for abnormal symptoms / signs during the study period and for one week after the study period and if noticed, the details were entered in the case report sheets.

6.5.7 Ethics approval

The study protocol was submitted to the Institutional Human Ethics Committee (IHEC) at PSG prior to commencing study and has been performed in accordance with the latest principles laid down in the updated World Medical Association's

Declaration of Helsinki. This has been obtained through prior consent of the volunteers participated in the study was taken.

6.5.8 Informed consent

All the study volunteers participating in the current study received a full verbal as well as written explanation regarding the purpose and objectives of the study, the procedures to be followed, and the risk-benefit ratios involved. They were provided with Informed Consent form which was typed in appropriate universal and local linguistic tools, Tamil or English, each providing the subject with general information and frequently asked questions (FAQs) about the study. Before they take part, the volunteers were asked to voluntarily sign (or not sign) the Informed Consent form to confirm (or reject) their voluntary informed agreement to undergo and proceed with the study.

6.5.9 Drug administration

All the volunteers were made to assemble in the facility 11 hours prior to the initiation of the study. After overnight fasting, the volunteers were given code numbers. Their pulse rates and blood pressures were recorded and sterile intravenous cannula introduced with strict aseptic precautions for blood collection. Volunteers were instructed to consume the study drug after each of them were given a single 2 mg risperidone oral tablet (RESPIDON-2, Torrent Pharmaceuticals Ltd, India) formulation, along with 200 mL of potable water.

6.5.10 Blood sample collection

Blood samples (2 mL) were collected using disposable syringe in pre-labelled K_2EDTA tubes at 0.00 (before drug administration), at 0.50, 1.00, 2.00,

4.00, 8.00, 12.00, and 24.00 hours after dosing. Immediately after blood is drawn in each tube, it will be inverted gently several times to ensure the mixing of tube contents. Vacuum collection tubes was be placed upright in a rack kept in iced water bath at approximate temperature of 2ºC to 8ºC before centrifugation and during separation. Before centrifugation the blood samples were divided into two aliquots. One aliquot was used for drug estimation and another one used for the DNA isolation.

The samples were centrifuged at 4º ± 2°C and at 2500-3000 rpm for 10 minutes to separate plasma. The blood samples were kept in iced water bath before centrifugation and during separation. The separated plasma samples were transferred to pre-labeled polypropylene tube (aliquots) and stored upright at a temperature below –80°C until analysis.

6.5.11 Assessment of drug adverse effects

The effects of the parent drug were observed cross-sectionally using a set of questions elucidating the clinical features that could probably be related to drug effects. These effects can be classified as neuropsychiatric, central nervous system (CNS), dizziness, tiredness, hypotension, cardiovascular (CVS), and gastrointestinal (GIT) effects. These questions were instituted four hours after single oral dosing of the drug in healthy volunteers.

6.6 Genotype analysis and *CYP2D6*10* allele Identification

6.6.1 Isolation of DNA from blood

Buffy-coat obtained after centrifugation of blood was added with 20 µL of protease (Proteinase K) was transferred into 2.0 mL centrifuge tube. Then 200 µL of buffer AL was added to the above sample and vortex mixed for 15 seconds to ensure efficient lysis. The sample was incubated at 56ºC for 10 minutes to get the maximum yield. Then 200 µL of ethanol was added to the sample and vortex mixed for 15 seconds. The DNA sample was carefully transferred into QIAamp spin column without wetting the rim, and seals the cap then placed in the QIAamp spin column in a 2 mL collection tube. The DNA sample was centrifuged at 8000 rpm for 1.0 minute and discarded the filtrate. Then 500 µL of buffer AW1 to QIAamp spin was added to column without wetting the rim, and then placed the QIAamp spin column in a 2 mL collection tube. The above step was repeated once to get the clear filtrate. The DNA sample was centrifuged at 14000 rpm for 3 minutes and discarded the filtrate. QIAamp spin column was placed in 1.5 mL clean micro centrifuge tube and added 200 µL of buffer AE. The DNA was incubated at room temperature (15º to 25ºC) for 5 minutes. Purified DNA was eluted by using buffer AE and stored at -20ºC.

6.6.2 Quantification of DNA using Nano-Spectrophotometer

The NanoDrop® ND-1000 was a full-spectrum (220 - 750nm) spectrophotometer that measures 1.0 µL samples with high accuracy and reproducibility. A patented sample retention technology was employed in surface tension alone to hold the sample in place. It measured the quality and quantity of nucleic acid samples up to 3700 ng/mL without dilution. DNA was absorbed at

260 nm. The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of DNA. A ratio of ~1.8 was generally accepted purity of DNA.

6.6.3 Primer dilution

The primer was obtained as lyophilized power and reconstituted in appropriate volume of sterile distilled water to get 100 µM. A 2.0 µM of working stock solution was prepared and stored at -20ºC.

Primers

*CYP2D6*10*

F: 5′- GTG CTG AGA GTG TCC TGC C - 3′

R: 5′- CAC CCA CCA TCC ATG TTT GC - 3′

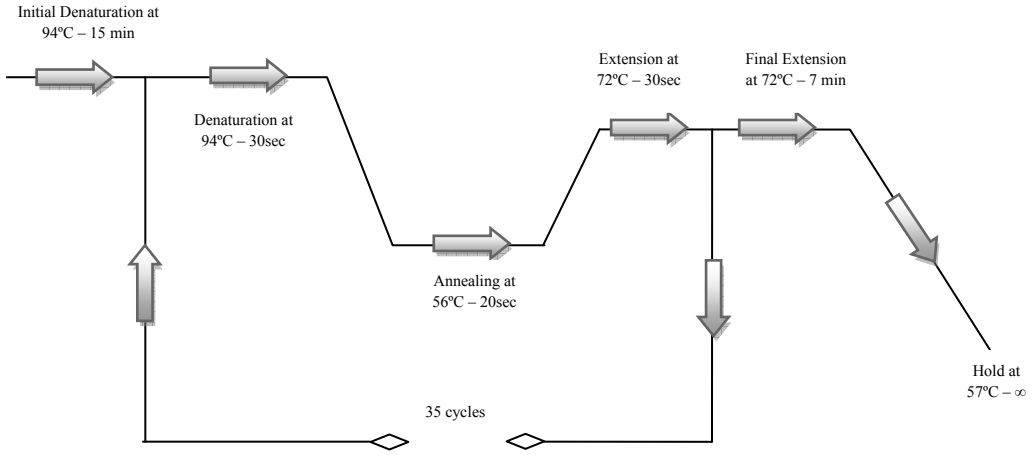
6.6.4 Polymerase Chain Reaction (PCR) Reaction mixer

The reactions were carried out in a volume of 20µL containing (final concentration) using Enhanced Avian HS RT-PCR Kit.

1x PCR

- Water - 14.2 µL
- 10x Buffer - 2.0 µL
- DNTP (10mM) - 1.0 µL
- Taq DNA polymerase - 1.0 µL
- Forward Primer (10 µM) - 0.2 µL
- Reverse Primer (10 µM) - 0.2 µL
- DNA - Equivalent to 100ng

PCR Cycle



This cycle was programmed for *CYP2D6*10*. Sample was amplified with the above thermal cycle to produce an amplified product of 325 bp and resolved in 2% Agarose gel for amplification [138].

6.6.5 Restriction Fragment Length Polymorphism (RFLP)

RFLP Mix (20 µL)

PCR Amplified Product	:	10 µL
Required Buffer	:	2.0 µL
Required Enzyme	:	0.25 µL
water	:	7.75 µL

Restriction Digestion of genotype

After the completion of the restriction digestion, the samples were resolved in 2% Agarose gel using TBE buffer at 135V for 35 min. UV-Gel doc image analyzer was used to identify the genotype of each sample based on their restriction pattern.

The digestion pattern and their corresponding amplicon size are tabulated below,

SNP Annotation	PCR Product	RFLP Enzyme	Digestion pattern and Genotype	
*CYP2D6*10*	325	Hphl	263, 62 – bp	CC *(*1/*1)*
			263, 183, 80 – bp	CT *(*1/*2)*
			183, 80 – bp	TT *(*2/*2)*

6.7 Pharmacokinetic parameter

After estimating the selected drugs in human plasma, the following pharmacokinetic parameters were calculated using WinNonlin software 5.1 (Pharsight Corporation, Mountain View, CA, USA).

- Cmax Maximum plasma concentration
- Tmax Time of maximum plasma cocentratin
- AUC_{0-t} Area under the plasma concentration-time curve from time 0 to 24 hour
- $AUC_{0-\infty}$ Area under the plasma concentration-time from zero to infinity
- K_{el} Elimination rate constant
- $T_{1/2}$ Elimination half-life
- V_d volumes of distribution
- CL Clearance

6.8 Statistical analysis

All the statistical analyses were carried out using SPSS 19.0 for windows (SPSS IBM, USA) at 5% statistical significance level. *CYP2D6* genotype frequencies of participants were analysed and the *Hardy-Weinberg* equilibrium was evaluated using *chi- square* test followed by *Kruskal Wallis H* test. This was followed by the quantitative analysis of our data for testing our hypothesis of the effect of polymorphism on disposition of the drugs using un-paired *t*-test and *Mann-Whitney U* test with a two tailed analysis.

6.9 Population pharmacokinetic analysis

Population pharmacokinetic analyses were performed via Non-Linear Mixed Effect Modelling (NONMEM) modelling program the help of NONMEM 7.1 (ICON Development Solutions) and Wings for NONMEM (WFN) used as the output file end. The analysis was carried out on a Windows® 7 personal computer with an Intel® i3 processor and g95 FORTRAN compiler. Graphical analyses of the NONMEM output raw data were performed using Microsoft® Excel®. All the NONMEM model runs were carried out with a protocol for First Order Conditional Estimation with Interaction (FOCEI) method. Selection of the best model was based on various criteria, like objective function values (OFV), visual examination of the basic goodness of fit plots, plausibility of the parameter estimated. In this analysis the following pharmacokinetic (PK) parameters were estimated: the absorption rate constant (KA), first pass metabolism (FPM), central compartment volume (V2), peripheral compartment volume (V3), inter compartment clearance (Q), non reversible clearance of RIS by formation of 9-OHRIS (CLPM), clearance of RIS by other routes (CL) and clearance of 9-OHRIS (CLM).

Chapter-7

Results & Analysis

7. RESULTS & ANALYSIS

This chapter elaborates on the obtained results with reference to the experiment in the form of tables and figures along with a detailed account on results of optimization and validation of various bio-analytical methods used to the quantitation of risperidone, 9-hydroxyrisperidone and olanzapine in human plasma, amount of the selected drugs in plasma samples, pharmacokinetic study design, data handling, determination of pharmacokinetic parameters, genotype analysis, statistical evaluation, *in-vivo* data analysis and population pharmacokinetic analysis using NONMEM.

7.1 Drug selection based on prescription pattern

After a thorough evaluation of the patients' prescription pattern, we have noted that of all the antipsychotic drugs that could have been possibly prescribed in schizophrenia, risperidone, olanzapine, quetiapine and haloperidol were the drugs that were prescribed at PSG hospitals psychiatry clinics. Risperidone scored the most preferred drug in terms of the number of prescriptions (Figure 4). The following is the order of the prescribing pattern of these four antipsychotics in percentages of total antipsychotics prescribed: risperidone (48%) > haloperidol (26%) > olanzapine (20%) > quetiapine (6%). Also, it is one of the few antipsychotics that are metabolized by *CYP2D6*, and is also influenced by its polymorphisms. Hence, we have selected this drug for our pharmacogenetic evaluation due to the above reasons, and also due to the fact that it is most prescribed drug in antipsychotic for schizophrenia without co-morbidities for which other drugs like quetiapine and haloperidol are prescribed.

7.2 Estimation of selected drugs in human plasma

7.2.1 Chromatographic conditions optimization

A reversed-phase UPLC technique was developed to quantify risperidone, 9-hydroxyrisperidone and olanzapine in human plasma sample medium using diode array detection. The development of UPLC method was carried out with a focus on accomplishing sufficiently high resolution of target drugs of interest and endogenous interferences present in biological medium in a short analytical run time, with simple buffer usage. For the above reason various different mobile phases each containing numerous stoichiometric combination of buffers (e.g. ammonium acetate buffer and phosphate buffer) along with organic solvents (methanol and acetonitrile) combined with altered flow rates (range of 0.200 to 0.500 mL/min) were evaluated to optimize an efficient chromatographic resolution between chromatographic peaks of risperidone, 9-hydroxyrisperidone and olanzapine as well as the IS. Using methanol as the preferred solvent for the mobile phase resulted in broader peaks without much symmetry in longer analytical time periods. When acetonitrile was used as the main organic solvent in the mobile phase, analysis time decreased whilst the peaks turned out more symmetrical and sharp in shapes. Hence, the optimum resolution of peaks was attained with an isocratic mobile phase that consists of a mixture of acetonitrile-10mM ammonium acetate (30:70; v/v) containing formic acid with the pH of the medium appropriately adjusted to an optimum value of 3.5 at a flow rate of 0.3 mL/min on Acquity UPLC BEH C_{18} column. The consumption of volatile buffers like ammonium acetate in the selected mobile phase gives enhanced column life

and can be suitably used with Mass spectrometry, not like phosphate containing buffers.

7.2.2 Method Validation

Measurements of risperidone, 9-hydroxyrisperidone and olanzapine in plasma samples obtained from the volunteers were undertaken with the help of the optimized chromatographic conditions. The parameters qualifying method validation such as specificity, sensitivity, accuracy, linearity, were evaluated, along with stability and precision.

7.2.3 Specificity / Selectivity

The blank plasma were evaluated as shown in Figure 5 no interfering peaks at the retention time of risperidone, 9-hydroxyrisperidone, olanzapine, and IS. Specificity of the method was assessed by evaluating the chromatograms of human blank plasma, IS spiked in blank plasma and among analyte with IS in the blank human plasma. No interfering peak observed in the chromatogram from endogenous compound at the retention time of risperidone, 9-hydroxyrisperidone, olanzapine and IS. The representative chromatograms are shown in Figure 6 and Figure 7.

7.2.4 Carry-over and Sensitivity

No peak area response was detected at the retention time of analytes and IS in blank plasma after analysing the highest calibration standard. This showed that the mobile phase flow configuration through the needle scheme in the injector unit and the subsequent cleaning steps in the column at the last part of the chromatography separation were enough to eliminate the possible residues

associated with the analytes. Limit of quantitation was 1.0 ng/mL obtained for all the analytes used for the calibration curve. Signal to noise ratio was ≥ 10 for plasma.

7.2.5 Linearity

A linear equation was used to produce the best fit for the concentration/response relationship. The weighting of regression line was $1/(\text{concentration})^2$. An eight-point calibration curve between peak area ratio and concentration was found to be linear from 1.0 ng/mL to 100.0 ng/mL for risperidone, 9-hydroxyrisperidone and olanzapine. The goodness of fit (correlation coefficient 'r') was consistently greater than 0.99 during the course of validation (Figure 8, 9 and 10). The parameters of validation experiment were evaluated using total four calibration curves.

The accuracy (% nominal) of the mean back-calculated concentration (BCC) of calibration curve levels was ranged from 99.09% to 100.39% for risperidone, 99.08% to 100.38% for 9-hydroxyrisperidone and 99.17% to 101.12% for olanzapine.

The precision (%CV) of the back-calculated concentration of calibration curve levels ranged from 0.53% to 2.14% for risperidone, 0.28% to 2.03% for 9-hydroxyrisperidone and 0.62% to 2.425 for olanzapine. The results were given in Table 1, 2 and 3.

7.2.6 Accuracy

The accuracy of the assay was defined as the absolute value of the ratio of the back calculated mean values of the quality control standards (LLOQ QC,

LQC, MQC and HQC) to their respective nominal values, expressed as percentage.

Intra-day accuracy was ranged from 99.79% to 102.31% for risperidone, 98.17% to 103.34% for 9-hydroxyrisperidone and 97.55% to 103.41% for olanzapine.

Inter-day accuracy was ranged from 99.18% to 105.41% for risperidone, 101.04% to 104.59% for 9-hydroxyrisperidone and 99.20% to 104.13% for olanzapine. The results were given in Table 4.

7.2.7 Precision

The precision was measured as the corresponding percent coefficient of variation values over the concentration range used for LLOQ, low concentration, medium concentration and high concentration quality control standards over the course of validation.

Intra-day precision was ranged from 1.54% to 5.31% for risperidone, 0.92% to 3.74% for 9-hydroxyrisperidone and 1.67% to 3.11% for olanzapine.

Inter-day precision was ranged from 4.11% to 6.96% for risperidone, 4.09% to 6.14% for 9-hydroxyrisperidone and 3.59% to 5.56% for olanzapine. The results were given in Table 4.

7.2.8 Recovery

The area of analyte at low, medium and high quality control standards were compared against the mean area of respective un-extracted quality control standards. The mean recovery for risperidone, 9-hydroxyrisperidone and olanzapine at low, medium and high QCs was 86.26%, 90.25, 91.98 and 88.69%, 91.92%, 89.77% and 86.43%, 90.94%, 90.88 respectively. The global mean

recovery for risperidone, 9-hydroxyrisperidone and olanzapine was 90.16%, 90.13% and 89.42%.

The internal standard peak area of low, medium, and high quality control standards were compared to the internal standard mean peak area of all un-extracted QC standards. The mean recovery for the Internal Standard was 84.25%.

The detailed results were presented in Table 5, 6, 7 and 8. The results show that the recovery of all analytes was consistent at all levels.

7.2.9 Stock Solution Stability

A stock solution prepared each for risperidone, 9-hydroxyrisperidone and olanzapine were made (100 µg/mL) in methanol and kept on bench at room temperature as well as refrigerator at 20º to 80ºC. The stability after 24 hours (room temperature and refrigerated) and 7 days (refrigerated) was evaluated by performing assay against freshly prepared stock solution.

The percentage stability was found to be 98.83%, 95.85% and 98.14 for stock solutions prepared for risperidone, 9-hydroxyrisperidone and olanzapine at room temperature for 24 hours. The percentage stability in refrigerated at 24 hours was found to be 101.21%, 98.56% and 101.12% for risperidone, 9-hydroxyrisperidone and olanzapine respectively. The percent stability *in-vitro* of risperidone, 9-hydroxyrisperidone and olanzapine in refrigerated at 7 days was found to be 94.15%, 95.45% and 99.28%.

Internal standard stock solution stability was found to be 98.88% and 99.60% for room temperature as well as refrigerated at 24 hours and 101.28% for refrigerated at 7 days. The results were within acceptance limit of 90.0% to 110.0% and comprehensive results were presented in Table 9, 10, 11 and 12.

7.2.10 Short term Stability

The bench top stability of low and high quality control standards was determined by comparing the back-calculated concentrations of risperidone, 9-hydroxyrisperidone and olanzapine from the freshly prepared samples with those kept on bench at ambient conditions for 8 hours.

The percentage stability of low and high quality control standards were 97.07%, 98.82% for risperidone and 102.94%, 100.79% for 9-hydroxyrisperidone and 99.00%, 98.22% for olanzapine respectively. The results were within the acceptance limit of 85.00% to 115.00% and detailed results were presented in the Table 13, 14 and 15.

7.2.11 Autosampler Stability

The stability of low and high quality control standards was evaluated by storing the samples at 8ºC for 26 hours. After 26 hours, the samples were compared with freshly prepared quality control samples.

The percentage stability of low and high quality control standards was 101.37%, 101.47% for risperidone and 98.32%, 97.85% for 9-hydroxyrisperidone and 101.81%, 97.20% for olanzapine respectively. The results were within the acceptance limit of 85.00% to 115.00% and comprehensive results were presented in the Table 16, 17 and 18.

7.2.12 Freeze-thaw Stability

The stability of low and high quality control standards was determined after third freeze-thaw cycles. The stability was determined by comparing the mean of back-calculated concentration of risperidone, 9-hydroxyrisperidone and

olanzapine from the third freeze thaw samples with that of freshly thawed quality control standards.

The percentage stability of low and high quality standards were 95.90% and 98.62% for risperidone, 102.55% and 98.23% for 9-hydroxyrisperidone, 101.00% and 98.45% for olanzapine. The results obtained were within the acceptance limit of 85.00% to 115.00%. The detailed results were shown in Table 19, 20 and 21.

7.2.13 Long-Term stability

Long-term stabilities of risperidone, 9-hydroxyrisperidone and olanzapine compounds in the plasma, quality control samples was stored in deep freezer at -80ºC ± 10ºC and the analysis was carried out after 30 days of storage. The percent stability after 30 days was found to be 102.54% for LQC and 102.22% for HQC samples of risperidone, 97.84% for LQC and 101.14% for HQC samples of 9-hydroxyrisperidone, 100.80% for LQC and 97.04% for HQC samples of olanzapine. The obtained results were within the acceptance limit of 85.00% to 115.00%. The results were presented in Table 22, 23 and 24.

In brief, the validated method for the simultaneous estimation of risperidone, 9-hydroxyrisperiodne and olanzapine in human plasma was accurate, precise, linear, selective and stability. Hence, the above validated method was used for further pharmacokinetic studies for quantifying the risperidone and 9-hydroxyrisperidone analytes in plasma.

7.3 Pharmacokinetic analysis of risperidone and 9-hydroxyrisperidone

A novel method of quantification of analytes viz., risperidone, 9-hydroxyrisperidone after dosing of 2mg risperidone (RISPIDONE–2, Torrent Pharmaceuticals Ltd., India) tablets administered as a single dose orally in twenty healthy volunteers was successfully completed. The represented chromatogram of risperidone and 9-hydroxyrisperidone in human plasma collected at 2 hours after single oral dose of 2mg risperidone as shown in Figure 11. We have obtained maximum plasma concentrations of risperidone in our study for each volunteer whose average value was almost twice greater that of the average of its major metabolite measured using the newly validated assay method, viz. 9-hydroxyrisperidone. Both the compounds of interest were quantified 24 hours after dosing of risperidone in the samples [16.48 ± 5.07 vs. 10.33 ± 1.90, (mean ± SD, ng/mL)]. Similarly, the time taken to reach maximum concentration in plasma for the parent drug was nearly one fourth of that for 9-hydroxyrisperidone [0.80 ± 0.25 vs. 4.00 ± 1.12, (h)]. The Area Under plasma concentration-time Curves (AUC) of risperidone from 0-12 hours and 0-infinity hours was 92.64 ± 27.79 and 103.93 ± 32.38 (ng.h/mL). These average values of AUC were greater for the metabolite compared to their values for its parent drug, (141.80 ± 32.41 and 191.80 ± 49.81 (ng.h/mL), respectively).

The average plasma half-lives of risperidone and 9-hydroxyrisperidone were 6.17 ± 1.76 (h) and 11.54 ± 3.11 (h) respectively. Finally, the first order elimination pharmacokinetic rate constants corresponding with the terminal part of the first order elimination rate constants associate with the terminal part of plasma concentration time curves were 0.12 ± 0.04 and 0.06 ± 0.02 (h^{-1})

respectively. The volume of distribution and total clearance of the risperidone were 2.49 ± 0.53 (L/kg) and 4.95 ± 1.36 (mL/min.kg). The 9-hydroxyrisperidone volume of distribution in the participants was 2.56 ± 0.60 (L/kg) and its total clearance was 2.67 ± 0.79 (mL/min.kg). The mean plasma concentration vs. time profiles for both analytes is shown in Figure 12. The pharmacokinetic parameters were calculated by using non-compartmental pharmacokinetic model and summarized in Table 25.

7.4 Baseline and genotype analysis

The study sample comprised twenty healthy volunteers and the allele frequencies of *CYP2D6*10* were analysed. The *CYP2D6*10* frequencies distribution were found to be 70%, 25% and 5% of normal, intermediate and poor metabolizers (Figure 13). *CYP2D6*10* genotyping revealed that fourteen volunteers were homozygous for the normal metabolizers (having two functional copies of gene), five volunteers were heterozygous mutant for intermediate metabolizers (having only one functional copy) and one volunteers was homozygous mutant for poor metabolizers (carrying two abnormal alleles). Based on the genotype, the fourteen volunteers were defined as normal metabolizers; five volunteers were defined as intermediate metabolizers. In addition, one volunteer was defined as poor metabolizers. The demographical data were compared between the normal and intermediate genotype groups. No significant differences were observed in body weight, age, body surface area and body mass index among the groups as shown in Table 26.

The frequencies of all three genotypes in the *CYP2D6* gene in our study population were compared for statistical difference with those reported elsewhere.

This comparison did not reveal a significant difference in the reported values of frequencies in reference to our allelic frequencies. The allelic frequencies of all SNPs were in agreement with *Hardy Weinberg equilibrium* ($P < 0.05$).

7.5 Effect of genetic polymorphism on risperidone pharmacokinetics

We observed the association between the *CYP2D6* genotypes and the pharmacokinetic parameters of risperidone and 9-hydroxyrisperidone. The difference between the mean C_{max} in normal (14.44 ± 1.78 ng/mL) and intermediate (18.35 ± 0.76 ng/mL) metabolizers was significant ($P < 0.001$) in risperidone (Figure 14). The poor metabolizer (35.70 ng/mL) C_{max} was higher as compared to normal metabolizers in risperidone. The average AUC_{0-t} and $AUC_{0-\infty}$ were higher in intermediate (108.38 ± 6.87 and 120.63 ± 7.68 ng.h/mL) and poor metabolizer (179.41 and 210.35 ng.h/mL) as compared to normal metabolizer (80.81 ± 16.99 and 90.37 ± 18.38 ng.h/mL) in risperidone. A significant difference ($P < 0.05$) was observed in the pharmacokinetic parameters of half-life and clearance of risperidone.

The pharmacokinetic parameters (C_{max}, $AUC_{0-\infty}$, $T_{1/2}$ and CL) of 9-hydroxyrisperidone were significant ($P < 0.05$) between the genotype groups (Table 27). The mean C_{max} in active moiety was significant ($P < 0.01$) between the normal (19.85 ± 2.47 ng/mL) and intermediate (24.47 ± 1.99 ng/mL) metabolizers (Figure 15). The active moiety was higher in poor metabolizer (41.18 ng/mL). In active moiety, the pharmacokinetic parameters (AUC_{0-t}, $AUC_{0-\infty}$, $T_{1/2}$ and CL) were significant ($P < 0.01$) between the genotype groups. The metabolic ratio was higher in poor (4.22) and intermediate (2.04 ± 0.20) metabolizers as compared to the normal metabolizers (1.40 ± 0.22) (Table 28).

The T_{max} was remain unaffected with respect to *CYP2D6*10* genotype among the pharmacokinetics of risperidone and its metabolite. The area under the plasma concentration time curve for the different genotype groups has shown in Figure 16.

The maintenance dosing rate for risperidone in normal metabolizers is 0.38 mg/hr, whereas that for risperidone in poor metabolizers is 0.48 mg/hr. Since the time taken to reach steady state after initiation of maintenance dosing of risperidone is 4-5 half lives, the total maintenance dose of risperidone is 10.41mg in normal metabolizers and 22.18 mg in poor metabolizers for the duration of five half lives. Since the net dose of risperidone is twice in poor metabolizers compared to normal metabolizers, and that the incidence of adverse effects due to risperidone is greater in poor metabolizers, this stresses the importance of halving the maintenance dose in poor metabolizers, in order to reduce cumulative toxicity.

7.6 Pharmacological effects

The adverse effects after oral dosing of risperidone observed were extra-pyramidal effects (56.67%), tiredness (80%) followed by GIT effects like hypotension (40%), nausea (50%), and effects in CVS (35%). The neuropsychiatric effect was observed with 30% of the volunteers. Excepting neuropsychiatric effects and tiredness, the prevalence of adverse effects was found to be greater in intermediate metabolizers than normal metabolizers. Hence slow metabolizers exhibited a higher prevalence of CNS ($P = 0.001$), CVS ($P < 0.001$), dizziness ($P = 0.005$), hypotension ($P = 0.01$), and GIT ($P = 0.01$) as compared to normal metabolizers (Figure 17).

7.7 Population pharmacokinetic analysis

Pharmacokinetic of risperidone plasma concentration vs time data of risperidone and its metabolite 9-hydroxyrisperidone was well fitted simultaneously using a one compartment model followed by a two compartment model during the model development. Based on OFV, and the goodness of fit plots, a PK model with 2-compartment disposition for risperidone and 1-compartment disposition for 9-hydroxyrisperidone appeared to be adequately describing the observed data (Figure 18).

Based on the model, the following parameters were estimated: Absorption rate constant (KA), Fraction of parent metabolized while absorbing from the depot compartment, central compartment volume of risperidone (V2), peripheral compartment volume for risperidone (V3), inter compartment clearance (Q), non reversible clearance of risperidone by formation of 9-hydroxyrisperidone (CLPM), clearance or risperidone by other routes (CL), volume of 9-hydroxyrisperidone was assumed to be the same as that of parent (V4=V2), and clearance of 9-hydroxyrisperidone (CLM).

The basic pharmacokinetic model was implemented by using subroutine ADVAN13 and TOL=9 in NONMEM. All the models were ran using the first order conditional estimation method with eta-epsilon interaction (FOCEI).

A specific parameter and the between subject variability of the parameter was estimated using the below equation:

$$Pi=Ptv*exp\#(\eta i)$$

Where 'Pi' is the individual subject parameter, 'Ptv' is the typical value of the parameter in the studied population and 'η' is the log normally distributed

between subject variability, with a mean of '0' and variance of 'ω2'. The residual variability was evaluated using additive, proportional and combined error models. The combined error model appeared to be describing the data adequately. The combined error model is described as below:

$$Obs_{ij} = Pred_{ij} * (i + \varepsilon_{prop\ ij}) + \varepsilon_{add\ ij}$$

Where Obs_{ij} is the jth observed value in ith subject, $Pred_{ij}$ is the model predicted jth value in the ith subject, and $\varepsilon_{prop\ ij}$ and $\varepsilon_{add\ ij}$ are the proportional and additive error respectively. These represent the residual intra subject variability with mean of '0' and variance of σ2.

Covariate analysis

Since the objective was to evaluate the influence of genetic polymorphism in *CYP2D6* enzyme there by on the pharmacokinetic profiles of risperidone and 9-hydroxyrisperidone, the most important covariate in this analysis was the genotype of the subjects. Out of the 20 subjects included in this study, 14 subjects were normal metabolizers and 6 subjects were poor metabolizers.

The effect of two different pharmacogenetic groups on the PK parameters like CLPM, FPM, KA and CLM was evaluated as below.

TVCLPM=THETA (6)*GENE + (THETA (9)*(1-GENE))

GENE is 1 for poor metabolizers and 0 for normal metabolizers. Where THETA (6) is the typical CLPM value of poor metabolizer population and THETA (9) is the typical CLPM value of the normal metabolizer population.

The effect of genotype on the PK parameter was considered significant if the OFV reduced by at least 3.84 from the base model.

The final model was evaluated by plots of observed and predicted individual subject concentrations, and by plots of population predicted concentrations and weighted residuals.

Based on the OFV and the goodness of fit plots, a 2-compartment model was considered best for risperidone and 1-compartment model for 9-hydroxyrisperidone. The best residual error model that described the entire data was combined additive and proportional error model. A combined model of risperidone and 9-hydroxyrisperidone was considered as the base model. The model predicted and the observed plasma concentration profiled of risperidone and 9-hydroxyrisperidone are presented below (Figure 19 and 20).

Incorporation of genotype as a covariate on the fraction metabolized appeared to be significant (~ 6 point reduction in OFV from baseline). The FPM estimates indicated approximately 10% lower fraction is metabolized in mutant alleles in comparison to the wild.

Similarly, addition of genotype as a covariate also appeared to be significant on rate of absorption from the depot (KA), with approximately 5 point reduction in OFV from base line. The KA appeared to be faster in mutant alleles relative to that in wild type.

Addition of genotype as the covariate on CLPM (metabolic conversion of risperidone to 9-hydroxyrisperidone) showed the highest reduction in OFV (~ 17 points), indicating the metabolic conversion of risperidone to 9-hydroxyrisperidone is highly influenced by the genotype of the subject. The CLPM in mutant subjects were estimated to be approximately 30% lower than that of wild type.

Further addition of covariates, over the model incorporating CLPM appeared to be not reducing the OFV substantially, this could probably because of the lower number subjects in the study. The results of the covariate analysis are presented below Table 29.The PK parameter estimates and the between subject variability estimates are tabulated below Table 30. The goodness of fit plots for the final model for risperidone and 9-hydroxyrisperidone are presented in Figure 21 and 22

Table 1: Back-Calculated Calibration Curve Concentration for Risperidone in Human Plasma

Calibration Curve Level	Nominal Concentration (ng/mL)	P & A I		P & A II		P & A III		P & A IV		Mean BCC (ng/mL)	%CV	% Nominal for mean BCC
		BCC (ng/mL)	% Nominal	BCC (ng/mL)	% Nominal	BCC (ng/mL)	% Nominal	BCC (ng/mL)	% Nominal			
Level 1	1.10	1.08	98.18	1.12	101.82	1.09	99.09	1.07	97.27	1.09	1.98	99.09
Level 2	5.10	5.02	98.43	5.04	98.82	5.21	102.16	5.23	102.49	5.12	2.14	100.39
Level 3	10.54	10.45	99.15	10.68	101.33	10.48	99.43	10.45	99.15	10.52	1.05	99.81
Level 4	22.91	23.18	101.18	22.79	99.48	22.68	99.00	23.26	101.53	22.98	1.24	100.31
Level 5	45.82	45.79	99.93	45.92	100.22	45.98	100.35	44.76	97.69	45.61	1.26	99.54
Level 6	68.67	68.76	100.13	69.41	101.08	68.87	100.29	68.24	99.37	68.82	0.70	100.22
Level 7	80.78	80.88	100.12	79.21	98.06	81.60	101.02	80.88	100.12	80.64	1.26	99.83
Level 8	104.94	104.54	99.62	105.34	100.38	103.98	99.09	104.67	99.74	104.63	0.53	99.70

BCC: Back Calculate Concentration, CV: Coefficient of variation

Table 2: Back-Calculated Calibration Curve Concentration for 9-Hydroxyrisperidone in Human Plasma

Calibration Curve Level	Nominal Concentration (ng/mL)	P & A I		P & A II		P & A III		P & A IV		Mean BCC (ng/mL)	%CV	% Nominal for mean BCC
		BCC (ng/mL)	% Nominal	BCC (ng/mL)	% Nominal	BCC (ng/mL)	% Nominal	BCC (ng/mL)	% Nominal			
Level 1	1.09	1.10	100.92	1.06	97.25	1.09	100.00	1.07	98.17	1.08	1.69	99.08
Level 2	5.10	5.00	98.04	5.11	100.20	5.17	101.37	4.99	97.84	5.07	1.72	99.41
Level 3	10.53	10.43	99.05	10.65	101.14	10.48	99.53	10.72	101.80	10.57	1.30	100.38
Level 4	22.61	21.78	96.33	22.18	98.10	22.21	98.23	22.87	101.15	22.26	2.03	98.45
Level 5	45.21	44.86	99.23	44.74	98.96	45.02	99.58	46.10	101.97	45.18	1.38	99.93
Level 6	68.61	67.80	98.82	68.23	99.45	68.02	99.14	67.86	98.91	67.98	0.28	99.08
Level 7	81.48	83.52	102.50	82.69	101.49	80.80	99.17	80.12	98.33	81.78	1.94	100.37
Level 8	101.57	101.04	99.48	102.21	100.63	100.23	98.68	103.04	101.45	101.63	1.22	100.06

BCC: Back Calculate Concentration, CV: Coefficient of variation

Table 3: Back-Calculated Calibration Curve Concentration for Olanzapine in Human Plasma

Calibration Curve Level	Nominal Concentration (ng/mL)	P & A I		P & A II		P & A III		P & A IV		Mean BCC (ng/mL)	%CV	% Nominal for mean BCC
		BCC (ng/mL)	% Nominal	BCC (ng/mL)	% Nominal	BCC (ng/mL)	% Nominal	BCC (ng/mL)	% Nominal			
Level 1	1.21	1.18	97.52	1.20	99.17	1.19	98.35	1.23	101.65	1.20	1.80	99.17
Level 2	4.98	5.10	102.41	4.89	98.19	4.92	98.80	4.96	99.60	4.97	1.87	99.80
Level 3	10.63	10.81	101.69	10.54	99.15	10.68	100.47	10.22	96.10	10.56	2.42	99.34
Level 4	22.82	23.10	101.23	23.03	100.92	22.67	99.34	22.77	99.78	22.89	0.90	100.31
Level 5	44.74	44.89	100.34	45.04	100.67	45.41	101.50	45.61	101.94	45.24	0.73	101.12
Level 6	68.63	67.95	99.01	68.33	99.56	69.54	101.33	68.53	99.85	68.59	0.99	99.94
Level 7	81.32	81.88	100.69	82.28	101.18	81.08	99.70	81.94	100.76	81.80	0.62	100.59
Level 8	104.94	104.72	99.79	105.89	100.91	104.59	99.67	104.42	99.50	104.91	0.64	99.97

BCC: Back Calculate Concentration, CV: Coefficient of variation

Table 4: Precision and Accuracy of Risperidone, 9-Hydroxyrisperidone and Olanzapine in Human Plasma

Actual concentration (ng/mL)	Intra-day (n = 6)			Inter-day (n = 18)		
	Measured concentration (mean ± SD, ng/mL)	CV (%)	Accuracy (%)	Measured concentration (mean ± SD, ng/mL)	CV (%)	Accuracy (%)
Risperidone						
1.12	1.13 ± 0.06	5.31	100.89	1.15 ± 0.08	6.96	102.68
5.12	5.18 ± 0.13	2.51	101.17	5.28 ± 0.36	6.82	103.13
45.82	46.88 ± 0.72	1.54	102.31	48.30 ± 2.20	4.55	105.41
80.78	80.61 ± 1.62	2.01	99.79	80.12 ± 3.29	4.11	99.18
9-Hydroxy risperidone						
1.09	1.07 ± 0.04	3.74	98.17	1.14 ± 0.07	6.14	104.59
5.10	5.06 ± 0.13	2.57	99.22	5.18 ± 0.31	5.98	101.57
45.21	46.72 ± 0.80	1.71	103.34	45.68 ± 2.34	5.12	101.04
81.48	80.49 ± 0.74	0.92	98.78	84.15 ± 3.44	4.09	103.28
Olanzapine						
1.21	1.20 ± 0.02	1.67	99.17	1.26 ± 0.07	5.56	104.13
4.98	5.15 ± 0.16	3.11	103.41	4.94 ± 0.22	4.45	99.20
44.74	46.02 ± 1.04	2.26	102.86	45.35 ± 1.94	4.28	101.36
81.32	79.33 ± 1.65	2.08	97.55	81.27 ± 2.92	3.59	99.94

SD: Standard deviation, CV: Coefficient of variation

Table 5: Observed Recovery of Risperidone from Human Plasma

Sr. no	LQC			MQC			HQC		
	Area of Un-extracted	**Area of extracted**	**% Recovery**	**Area of Un-extracted**	**Area of extracted**	**% Recovery**	**Area of Un-extracted**	**Area of extracted**	**% Recovery**
1	1716	1564	91.14	12582	11388	90.51	25906	23398	90.32
2	1744	1438	82.45	12688	11383	89.71	25351	23187	91.46
3	1622	1428	88.04	12992	12106	93.18	26237	23666	90.20
4	1669	1430	85.68	12953	11530	89.01	26014	23728	91.21
5	1721	1543	89.66	12632	11531	91.28	25700	24126	93.88
6	1639	1530	93.35	12661	11528	91.05	25343	24121	95.18
7	1759	1539	87.49	12911	11235	87.02	26207	24011	91.62
Mean	**1696**	**1496**	**88.26**	**12774**	**11529**	**90.25**	**25823**	**23748**	**91.98**
% CV	**--**	**--**	**4.06**	**--**	**--**	**2.15**	**--**	**--**	**2.02**
Global Mean Recovery			**90.16**						
			LQC		**MQC**			**HQC**	
Difference from global mean			**1.90**		**0.09**			**1.82**	

CV: Coefficient of variation

Table 6: Observed Recovery of 9-Hydroxyrisperidone from Human Plasma

Sr. no	LQC			MQC			HQC		
	Area of Un-extracted	**Area of extracted**	**% Recovery**	**Area of Un-extracted**	**Area of extracted**	**% Recovery**	**Area of Un-extracted**	**Area of extracted**	**% Recovery**
1	1584	1381	87.18	14463	12820	88.64	28440	24776	87.12
2	1574	1410	89.58	14758	13533	91.70	27950	24730	88.48
3	1641	1387	84.52	14374	12894	89.70	27348	25217	92.21
4	1421	1320	92.89	14170	13269	93.64	27727	24578	88.64
5	1556	1369	87.98	14719	13293	90.31	27243	25207	92.53
6	1564	1311	83.82	14933	13704	91.77	26967	24740	91.74
7	1528	1449	94.83	14220	13894	97.71	27802	24365	87.64
Mean	**1553**	**1375**	**88.69**	**14520**	**13344**	**91.92**	**27640**	**24802**	**89.77**
% CV	--	--	**4.60**	--	--	**3.29**	--	--	**2.57**
Global Mean Recovery			**90.13**						
			LQC		**MQC**			**HQC**	
Difference from global mean			**1.44**		**1.79**			**0.36**	

CV: Coefficient of variation

Table 7: Observed Recovery of Olanzapine from Human Plasma

Sr. no	LQC			MQC			HQC		
	Area of Un-extracted	**Area of extracted**	**% Recovery**	**Area of Un-extracted**	**Area of extracted**	**% Recovery**	**Area of Un-extracted**	**Area of extracted**	**% Recovery**
1	1822	1613	88.53	16754	14152	84.47	38303	35912	93.76
2	1798	1539	85.60	15972	14149	88.59	38725	35243	91.01
3	1816	1529	84.20	16878	15686	92.94	37230	34341	92.24
4	1705	1554	91.14	16910	15241	90.13	38874	34302	88.24
5	1750	1469	83.94	16256	15270	93.93	37076	34152	92.11
6	1721	1564	90.88	16184	15789	97.56	36289	31807	87.65
7	1741	1405	80.70	16941	15074	88.98	37445	34133	91.16
Mean	**1765**	**1525**	**86.43**	**16556**	**15052**	**90.94**	**37706**	**34270**	**90.88**
% CV	--	--	**4.51**	--	--	**4.68**	--	--	**2.43**
Global Mean Recovery			**89.42**						
			LQC		**MQC**			**HQC**	
Difference from global mean			**2.99**		**1.52**			**1.46**	

CV: Coefficient of variation

Table 8: Observed Recovery of IS (Clozapine) from Human Plasma

Sr. no	Area of Un-extracted IS	Area of extracted IS	% Recovery
1	21517	18180	84.49
2	21078	18216	86.42
3	22139	18675	84.35
4	23262	19854	85.35
5	21663	18470	85.26
6	21279	18946	89.04
7	22147	18911	85.39
8	22664	18980	83.75
9	21609	18185	84.15
10	22558	19117	84.75
11	22927	19477	84.95
12	22119	18989	85.85
13	22413	18634	83.14
14	23833	19591	82.20
15	23037	18879	81.95
16	22021	18609	84.51
17	22408	18738	83.62
18	21089	18870	89.48
19	23104	18799	81.37
20	22393	18629	83.19
21	22535	19290	85.60
Mean	**22370**	**18831**	**84.25**
% CV	**--**	**--**	**3.23**

CV: Coefficient of variation

Table 9: Stock Solution Stability for Risperidone

Stability of the Drug		
Time point	**% Stability**	
	Room temperature	**Refrigerator**
24 hrs	98.83	101.21
7 Days	---	94.15

Table 10: Stock Solution Stability for 9-Hydroxyrisperidone

Stability of the Drug		
Time point	**% Stability**	
	Room temperature	**Refrigerator**
24 hrs	95.85	98.56
7 Days	---	95.45

Table 11: Stock Solution Stability for Olanzapine

Stability of the Drug		
Time point	**% Stability**	
	Room temperature	**Refrigerator**
24 hrs	98.14	101.12
7 Days	---	99.28

Table 12: Stock Solution Stability for IS

Stability of the Drug		
Time point	**% Stability**	
	Room temperature	**Refrigerator**
24 hrs	98.88	99.60
7 Days	---	101.28

Table 13: Short Term Stability for Risperidone after 8 hours on Bench-Top

Run No	LQC		HQC	
	Concentration (ng/mL)			
	Fresh QC's	After 8 hrs	Fresh QC's	After 8 hrs
1	4.98	4.98	80.56	79.56
2	5.26	4.88	80.55	78.21
3	4.98	4.82	79.24	79.47
4	5.21	5.03	82.32	81.29
5	5.14	5.08	82.45	80.32
6	5.12	5.02	79.54	80.12
Mean	**5.12**	**4.97**	**80.78**	**79.83**
% Stability	**---**	**97.07**	**---**	**98.82**

Table 14: Short Term Stability for 9-Hydroxyrisperiodne after 8 hours on Bench-Top

Run No	LQC		HQC	
	Concentration (ng/mL)			
	Fresh QC's	After 8 hrs	Fresh QC's	After 8 hrs
1	5.04	5.24	80.17	84.98
2	5.21	5.28	81.36	80.12
3	5.10	5.38	82.14	82.34
4	5.19	5.18	81.10	83.84
5	4.94	5.38	82.24	83.21
6	5.14	5.02	81.87	78.23
Mean	**5.10**	**5.25**	**81.48**	**82.12**
% Stability	**---**	**102.94**	**---**	**100.79**

Table 15: Short Term Stability for Olanzapine after 8 hours on Bench-Top

Run No	LQC		HQC	
	Concentration (ng/mL)			
	Fresh QC's	After 8 hrs	Fresh QC's	After 8 hrs
1	4.95	4.98	80.27	80.98
2	5.12	4.88	81.32	78.54
3	4.86	4.78	82.42	82.34
4	4.88	5.18	81.10	77.68
5	5.08	4.72	80.71	81.45
6	4.98	5.02	82.12	78.23
Mean	**4.98**	**4.93**	**81.32**	**79.87**
% Stability	**---**	**99.00**	**---**	**98.22**

Table 16: Post Preparative Stability for Risperidone after 26 hours at 8°C

Run No	LQC		HQC	
	Concentration (ng/mL)			
	Fresh QC's	After 26 hrs	Fresh QC's	After 26 hrs
1	4.98	5.10	80.56	80.64
2	5.26	5.21	80.55	82.35
3	4.98	5.16	79.24	84.21
4	5.21	5.21	82.32	81.21
5	5.14	5.24	82.45	82.45
6	5.12	5.24	79.54	80.98
Mean	**5.12**	**5.19**	**80.78**	**81.97**
% Stability	**---**	**101.37**	**---**	**101.47**

Table 17: Post Preparative Stability for 9-Hydroxyrisperidone after 26 hours at 8°C

Run No	LQC		HQC	
	Concentration (ng/mL)			
	Fresh QC's	After 26 hrs	Fresh QC's	After 26 hrs
1	5.04	4.98	80.17	79.34
2	5.21	5.21	81.36	82.35
3	5.10	4.88	82.14	77.28
4	5.19	5.21	81.10	81.21
5	4.94	4.72	82.24	77.21
6	5.14	5.24	81.87	80.98
Mean	**5.10**	**5.04**	**81.48**	**79.73**
% Stability	**---**	**98.82**	**---**	**97.85**

Table 18: Post Preparative Stability for Olanzapine after 26 hours at 8°C

Run No	LQC		HQC	
	Concentration (ng/mL)			
	Fresh QC's	After 26 hrs	Fresh QC's	After 26 hrs
1	4.95	4.91	80.27	79.38
2	5.12	5.12	81.32	81.15
3	4.86	5.14	82.42	76.28
4	4.88	5.24	81.10	80.21
5	5.08	4.78	80.71	76.21
6	4.98	5.24	82.12	80.98
Mean	**4.98**	**5.07**	**81.32**	**79.04**
% Stability	**---**	**101.81**	**---**	**97.20**

Table 19: Freeze Thaw Stability for Risperidone after 3 Cycles

Run No	LQC		HQC	
	Concentration (ng/mL)			
	Fresh QC's	After 3 cycles	Fresh QC's	After 3 cycles
1	5.03	4.88	79.32	79.45
2	5.12	4.98	80.75	78.38
3	4.94	4.86	79.85	80.52
4	5.21	4.92	82.54	80.64
5	5.20	4.82	82.55	80.88
6	5.24	5.02	79.69	79.56
Mean	**5.12**	**4.91**	**80.78**	**79.91**
% Stability	**---**	**95.90**	**---**	**98.92**

Table 20: Freeze Thaw Stability for 9-Hydroxyrisperiodne after 3 Cycles

Run No	LQC		HQC	
	Concentration (ng/mL)			
	Fresh QC's	After 3 cycles	Fresh QC's	After 3 cycles
1	5.01	4.88	80.12	79.45
2	5.18	5.34	81.52	79.21
3	5.32	4.86	82.36	80.52
4	5.02	5.43	81.26	80.64
5	4.97	5.58	82.14	80.88
6	5.08	5.29	81.46	79.56
Mean	**5.10**	**5.23**	**81.48**	**80.04**
% Stability	**---**	**102.55**	**---**	**98.23**

Table 21: Freeze Thaw Stability of Olanzapine after 3 Cycles

Run No	LQC		HQC	
	Concentration (ng/mL)			
	Fresh QC's	After 3 cycles	Fresh QC's	After 3 cycles
1	5.08	4.88	82.51	79.45
2	4.88	5.04	81.62	79.21
3	5.12	4.86	81.42	80.52
4	4.98	5.03	80.24	80.64
5	5.04	5.08	80.02	80.88
6	4.78	5.29	82.12	79.56
Mean	**4.98**	**5.03**	**81.32**	**80.04**
% Stability	**---**	**101.00**	**---**	**98.43**

Table 22: Long-Term Stability of Risperidone at 30 Days

Run No	LQC		HQC	
	Concentration (ng/mL)			
	Initial	After 30 days	Initial	After 30 days
1	4.98	5.21	80.56	81.68
2	5.26	5.36	80.55	83.28
3	4.98	5.34	79.24	84.21
4	5.21	5.25	82.32	81.24
5	5.14	5.16	82.45	82.45
6	5.12	5.18	79.54	82.58
Mean	**5.12**	**5.25**	**80.78**	**82.57**
% Stability	**---**	**102.54**	**---**	**102.22**

Table 23: Long-Term Stability of 9-Hydroxyrisperidone at 30 Days

Run No	LQC		HQC	
	Concentration (ng/mL)			
	Initial	After 30 days	Initial	After 30 days
1	5.04	4.72	80.17	78.34
2	5.21	5.30	81.36	84.38
3	5.10	4.82	82.14	84.21
4	5.19	5.08	81.10	82.48
5	4.94	4.84	82.24	82.45
6	5.14	5.18	81.87	82.58
Mean	**5.10**	**4.99**	**81.48**	**82.41**
% Stability	**---**	**97.84**	**---**	**101.14**

Table 24: Long-Term Stability of Olanzapine at 30 Days

Run No	LQC		HQC	
	Concentration (ng/mL)			
	Initial	After 30 days	Initial	After 30 days
1	4.95	4.72	80.27	78.34
2	5.12	5.30	81.32	77.38
3	4.86	4.82	82.42	78.21
4	4.88	5.18	81.10	77.48
5	5.08	4.84	80.71	79.45
6	4.98	5.28	82.12	82.58
Mean	**4.98**	**5.02**	**81.32**	**78.91**
% Stability	**---**	**100.80**	**---**	**97.04**

Table 25: The Mean Pharmacokinetic Parameters of Risperidone and 9-Hydroxyrisperidone in healthy Human Volunteers

Parameters	Risperidone (mean ± SD)	9-Hydroxyrisperidone (mean ± SD)
C_{max} (ng/mL)	16.48 ± 5.07	10.33 ± 1.90
T_{max} (h)	0.80 ± 0.25	4.00 ± 1.12
AUC_{0-t} (ng h/mL)	92.64 ± 27.79	141.80 ± 32.41
$AUC_{0-\infty}$ (ng h/mL)	103.93 ± 32.38	191.80 ± 49.81
$T_{1/2}$ (h)	6.17 ± 1.76	11.54 ± 3.11
Kel (h^{-1})	0.12 ± 0.04	0.06 ± 0.02
Vz (L/kg)	2.49 ± 0.53	2.56 ± 0.60
CL (mL/min/Kg)	4.95 ± 1.36	2.67 ± 0.79

SD: Standard deviation

Table 26: Demographical data between the groups in *CYP2D6* metabolizers

Parameter	Groups			*P*-value*
	Normal Metabolizers (mean ± SD, N=14)	**Intermediate Metabolizers (mean ± SD, N=5)**	**Poor Metabolizers (N=1)**	
Age (years)	30.36 ± 5.27	25.80 ± 3.49	24.00	n.s.
Body weight (kg)	70.44 ± 7.51	74.88 ± 11.57	81.50	
Height (cm)	172.86 ± 9.16	174.0 ± 5.05	189.00	
BMI (kg/m^2)	23.65 ± 2.52	24.72 ± 3.49	25.90	
BSA (m^2)	1.84 ± 0.13	1.89 ± 0.14	2.20	

n.s., not significant; **P*-value shown in the table depicts the difference in the mean values between normal and intermediate metabolizers; SD: Standard deviation

Table 27: Risperidone and 9-Hydroxyrisperidone Pharmacokinetic Parameters in *CYP2D6* Metabolizers

Pharmacokinetic parameters	*CYP2D6* genotype			*P*-value*
	Normal Metabolizers (mean ± SD, N=14)	Intermediate Metabolizers (mean ± SD, N=5)	Poor Metabolizers (N=1)	
Risperidone				
C_{max} (ng/mL)	14.44 ± 1.78	18.35 ± 0.76***	35.70	<0.000
T_{max} (h)	0.79 ± 0.26	0.90 ± 0.22	0.50	0.390
AUC_{0-t} (ng.h/mL)	80.81 ± 16.99	108.38 ± 6.87**	179.41	0.003
$AUC_{0-\infty}$ (ng.h/mL)	90.37 ± 18.38	120.63 ± 7.68**	210.35	0.003
$T_{1/2}$ (h)	5.48 ± 1.51	7.49 ± 1.03*	9.24	0.141
CL/F (mL/min/kg)	5.49 ± 3.68	3.96 ± 0.25*	2.26	0.013
9-hydroxyrisperidone				
C_{max} (ng/mL)	10.44 ± 1.36	9.05 ± 0.57*	8.45	0.043
T_{max} (h)	4.14 ± 1.23	3.60 ± 0.89	4.00	0.382
AUC_{0-t} (ng.h/mL)	127.62 ± 25.4	151.01 ± 10.46	149.73	0.065
$AUC_{0-\infty}$ (ng.h/mL)	158.84 ± 35.45	228.78 ± 9.62***	259.82	0.001
$T_{1/2}$ (h)	9.58 ± 2.37	14.59 ± 1.21***	18.61	0.000
CL/F (mL/min/kg)	3.13 ± 0.67	2.08 ± 0.09**	1.83	0.003

*P<0.05; **P<0.01; ***P<0.001; *P-value shown in the table depicts the difference in the mean values between normal and intermediate metabolizers; SD: Standard deviation

Table 28: Pharmacokinetic Parameters of Active Moiety and Metabolic ratio of *CYP2D6* Metabolizers

Pharmacokinetic parameters	*CYP2D6* genotype			*P*-value*
	Normal Metabolizers (mean ± SD, N=14)	Intermediate Metabolizers (mean ± SD, N=5)	Poor Metabolizers (N=1)	
Active Moiety				
C_{max} (ng/mL)	19.85 ± 2.47	24.47 ± 1.99**	41.18	0.002
T_{max} (h)	1.29 ± 1.22	0.90 ± 0.22	0.50	0.500
AUC_{0-t} (ng.h/mL)	211.65 ± 29.67	259.39 ± 7.22**	329.14	0.003
$AUC_{0-\infty}$ (ng.h/mL)	246.82 ± 40.46	338.30 ± 11.29***	447.68	0.000
$T_{1/2}$ (h)	8.22 ± 1.48	11.32 ± 0.92***	12.80	0.000
CL/F (mL/min/kg)	1.98 ± 0.32	1.41 ± 0.05**	1.06	0.001
Metabolic Ratio				
(Risperidone / 9-hydroxyrisperidone)	1.40 ± 0.22	2.04 ± 0.20***	4.22	<0.000

*P<0.05; **P<0.01; ***P<0.001; *P-value shown in the table depicts the difference in the mean values between normal and intermediate metabolizers; SD: Standard deviation

Table 29: Summary of Covariate Analysis

Serial Number	Model	Change in OFV	Compared with Model
1	Base Model	---	---
2	Base Model with genotype as covariate on CLPM	-17.5	1
3	Base Model with genotype as covariate on FPM	-6.165	1
4	Base Model with genotype as covariate on KA	-5.071	1
5	Model 2 + genotype as covariate on FPM	-0.029	2
6	Model 2 + genotype as covariate on KA	1.363	2

Table 30: The PK Parameter Estimates and the Between Subject Variability Estimates

Parameters	Population PK Estimates	Eta SD
CL (L/hr)	0.0014	1.40
V2 (L/hr)	78.90	0.09
KA (hr^{-1})	2.13	0.56
Q (L/hr)	12.10	---
V3 (L/hr)	115.00	---
CLPM (L/hr)	11.10	0.11
CLPM (L/hr)	15.60	---
CLM (L/hr)	10.80	0.26
FPM	0.20	0.35
Risperidone ε_{add} (ng/mL)	0.001	---
Risperidone ε_{prop} (ng/mL)	0.308	---
9-hydroxyrisperidone ε_{add} (ng/mL)	0.001	---
9-hydroxyrisperidone ε_{prop} (ng/mL)	0.158	---

Figure 4: Antipsychotic Drugs Prescription Pattern and Rationale for Selecting Risperidone for Pharmacogenetic Assessment

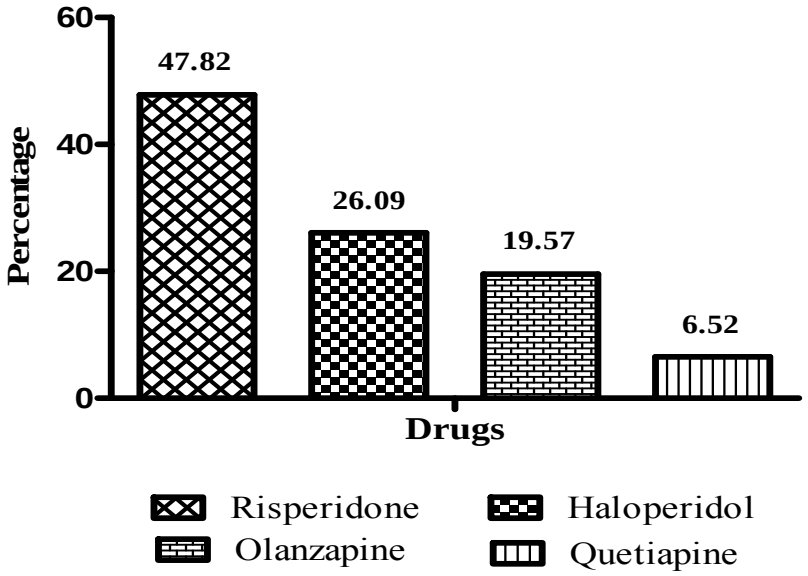


Figure 5: Representative Chromatogram of Blank Plasma

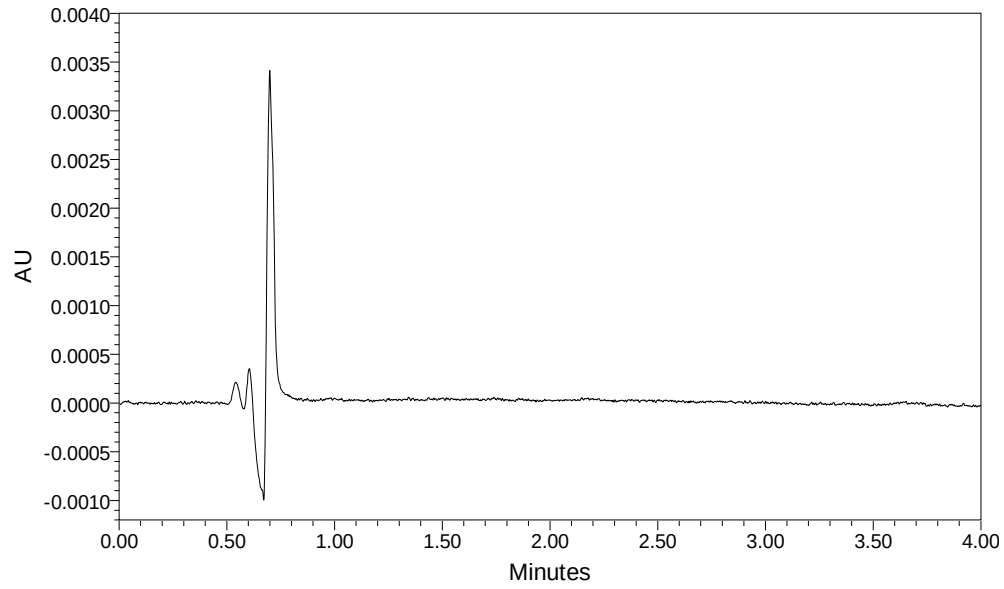


Figure 6: Representative Chromatogram of Zero Plasma

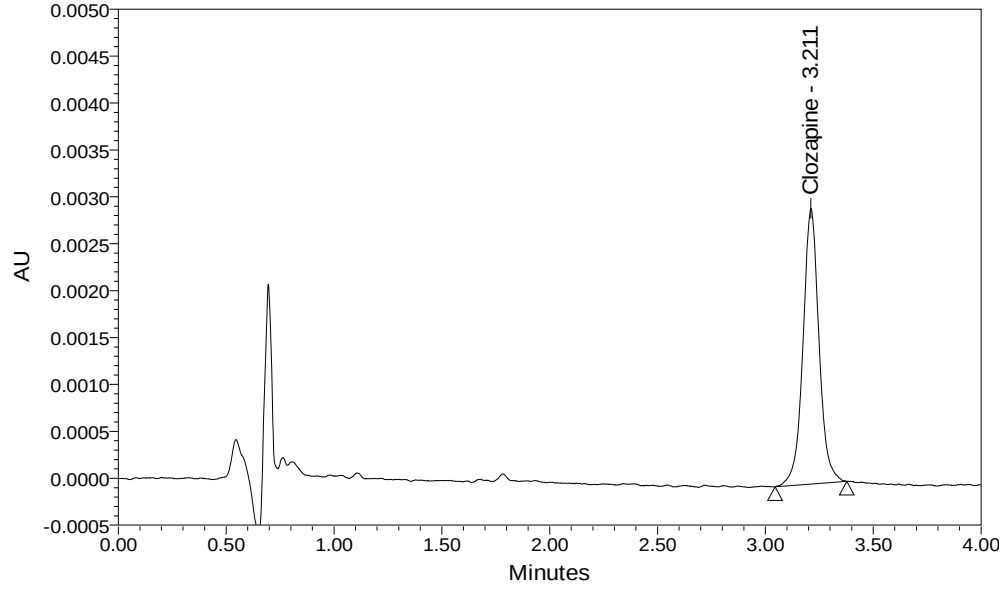


Figure 7: Representative Chromatogram of LLOQ QC

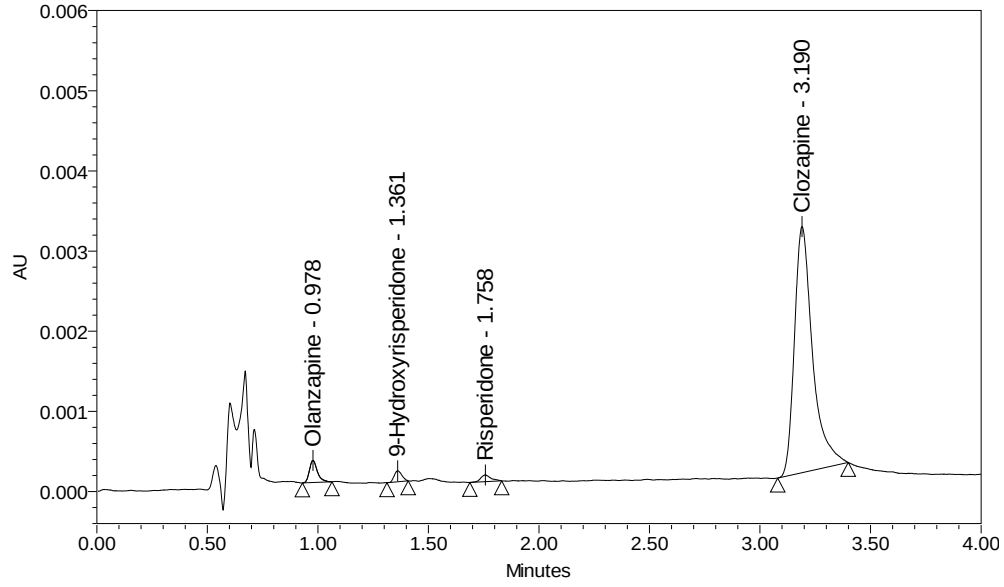


Figure 8: Representative Calibration Curve Plot for Risperidone

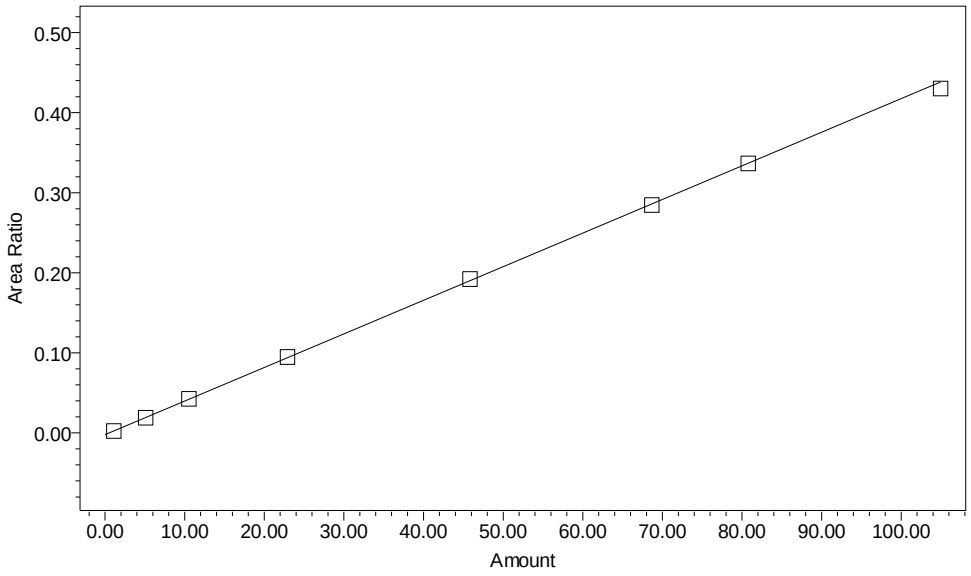


Figure 9: Representative Calibration Curve Plot for 9-Hydroxyrisperidone

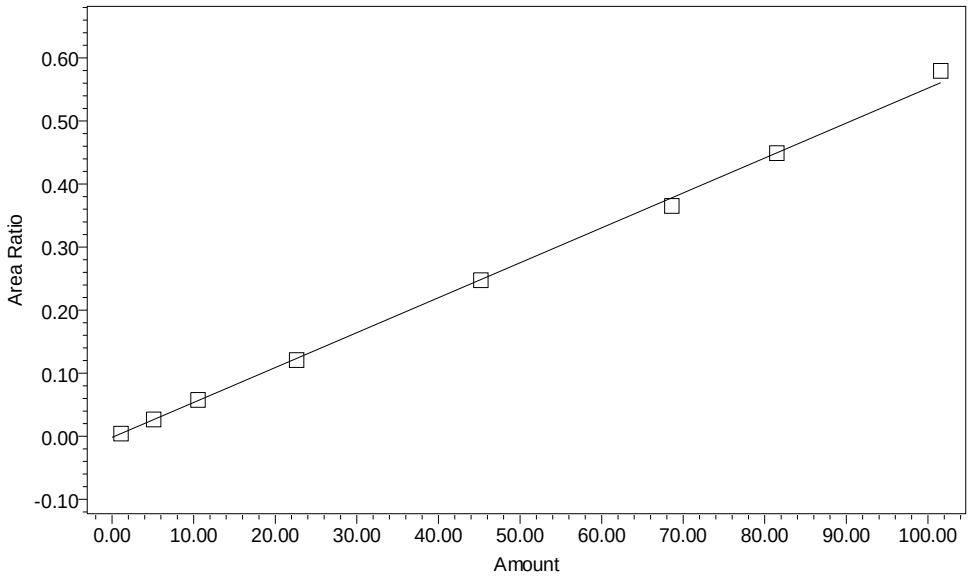


Figure 10: Representative Calibration Curve Plot for Olanzapine

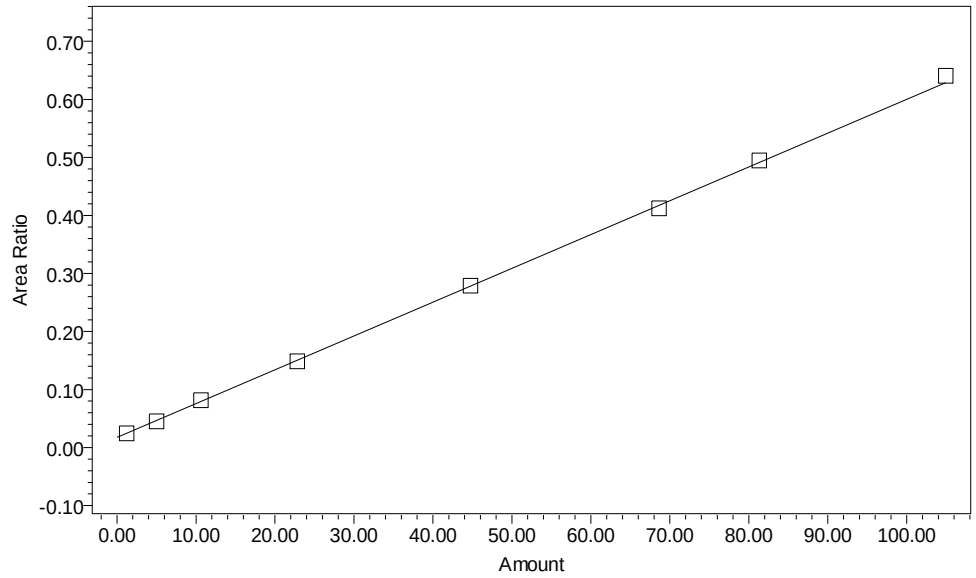


Figure 11: Representative Chromatogram of Plasma Risperidone and 9-Hydroxyrisperidone 2 hours Post Oral Dosing

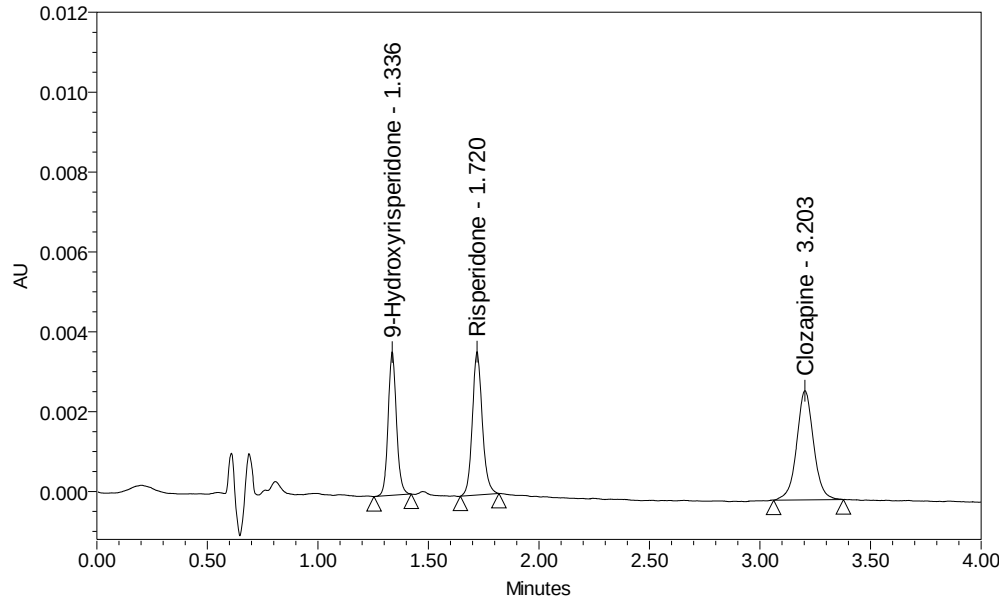


Figure 12: Concentration-Time Plot of Risperidone and its Active Metabolite, 9-Hydroxyrisperidone in Human Plasma

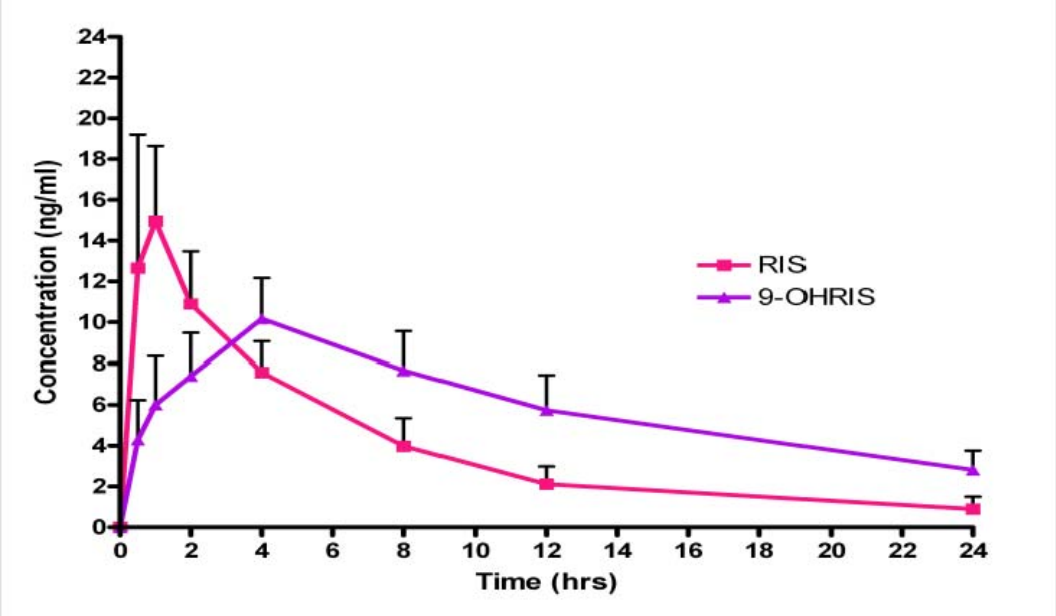


Figure 13: Ethidium bromide-stained 10% polyacrylamide gel showing digested amplicons used for *CYP2D6*10* genotyping. Samples in lanes 'a' and 'b' are homozygous (263-bp and 62-bp) for the wild type 1/1 genotype. Sample in lane 'c' is heterozygous (263-bp, 183-bp, 80bp, 62-bp) for the 1/10 genotype and sample in lane'd' is homozygous (182-bp, 80-bp, 62-bp) for the 10/10 genotype

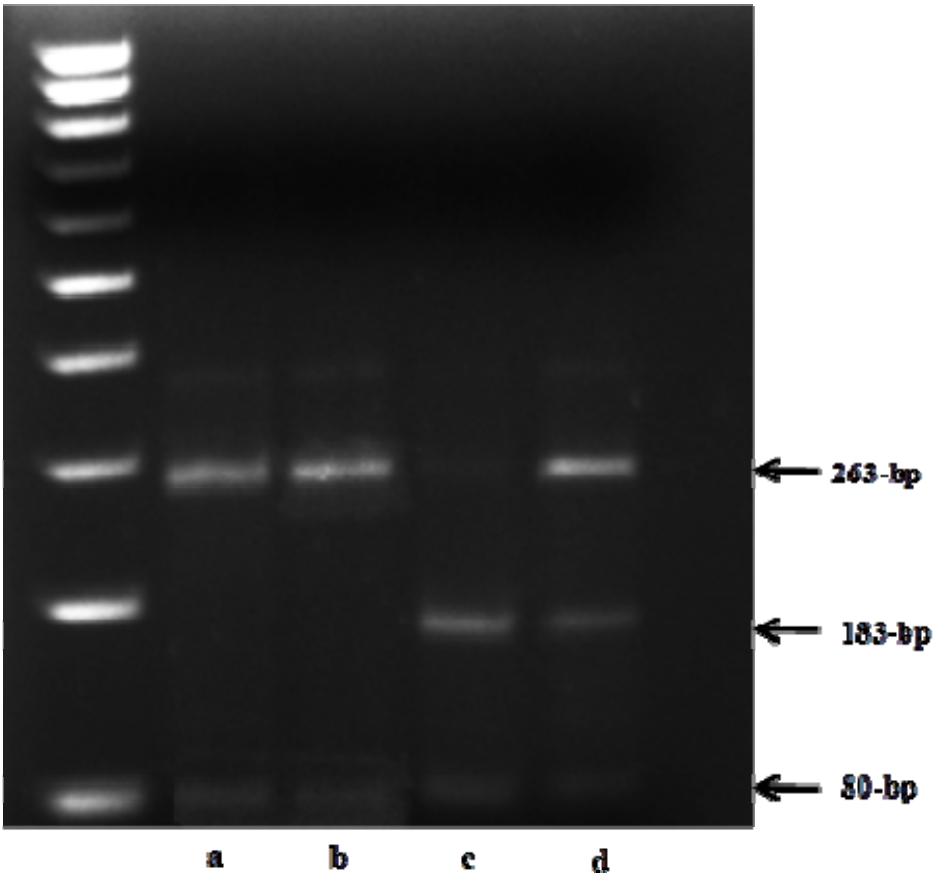


Figure 14: The Peak Plasma Concentration (C_{max}) of Risperidone and *CYP2D6* Metabolizers

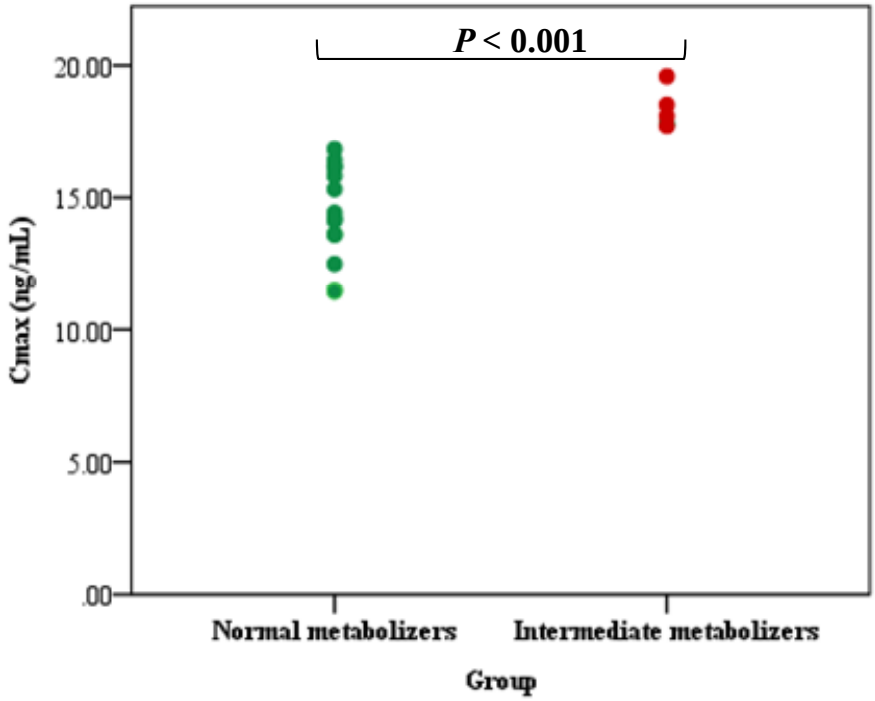


Figure 15: The Peak Plasma Concentration (C_{max}) of Active Moiety and *CYP2D6* Metabolizers

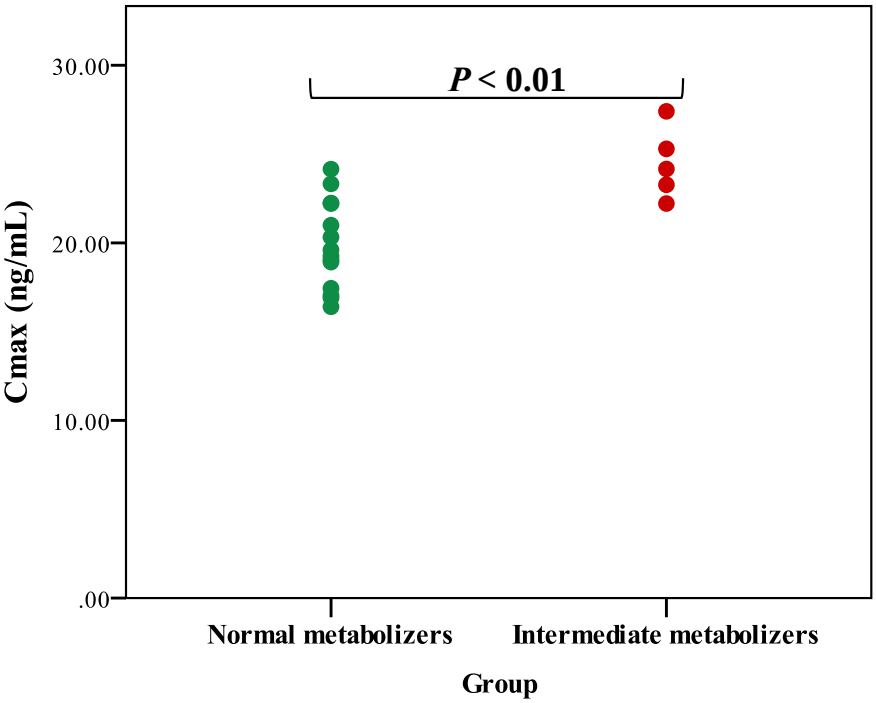


Figure 16: The Mean Risperidone Concentration-Time Profile in three Genotype with *CYP2D6*10* after Single Oral Administration of Risperidone (2mg)

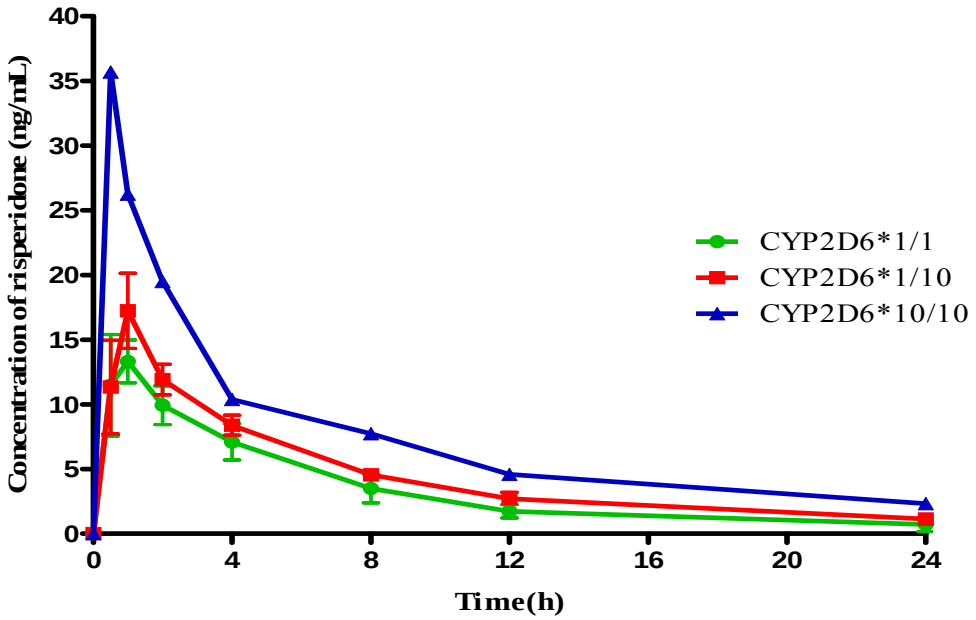
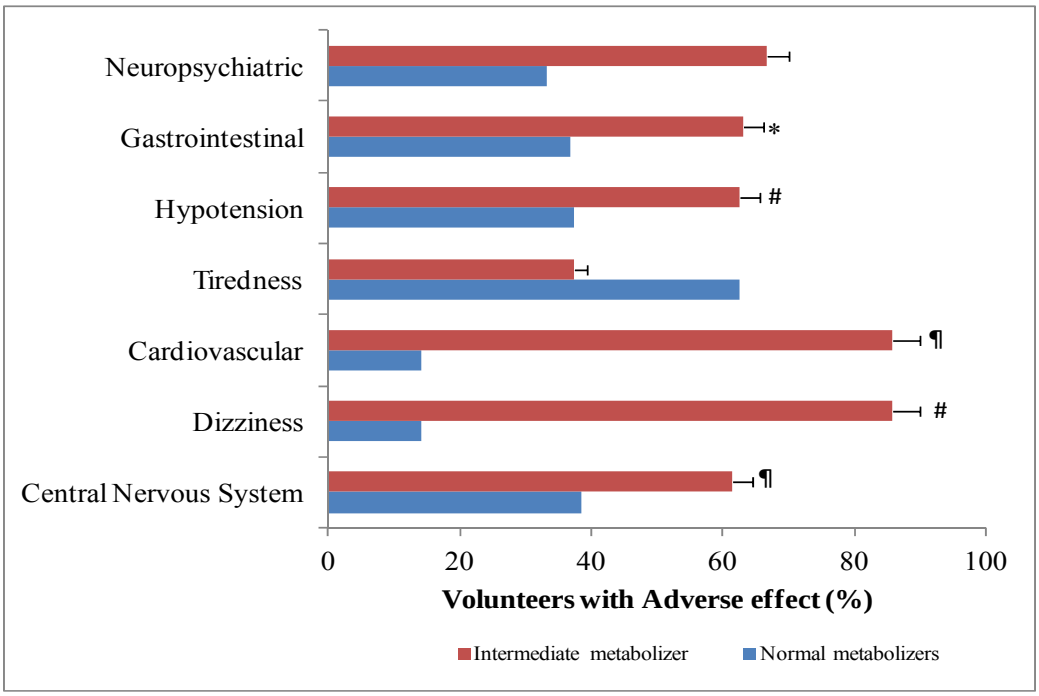


Figure 17: Incidence of Adverse Reactions in Healthy Volunteers: Association with Drug



*$P<0.05$, #$P<0.01$, ¶$P<0.001$

Figure 18: Scheme of the Compartment Model Describing Risperidone and 9-Hydroxyrisperidone Disposition

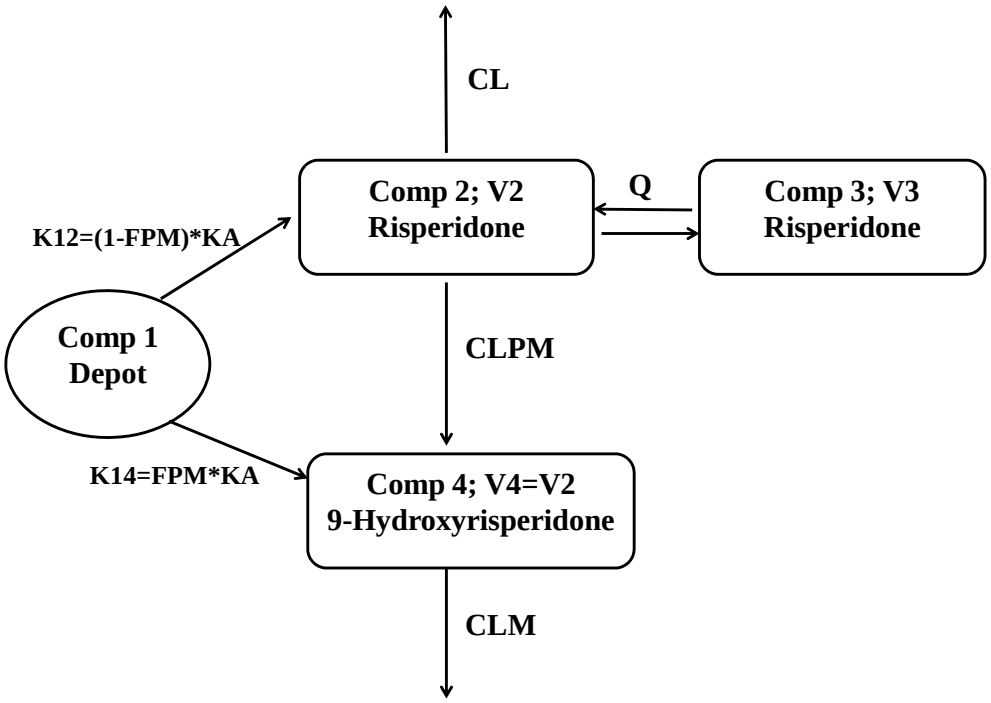


Figure 19: The Model Predicted and the Observed Plasma Concentration Profiled of Risperidone

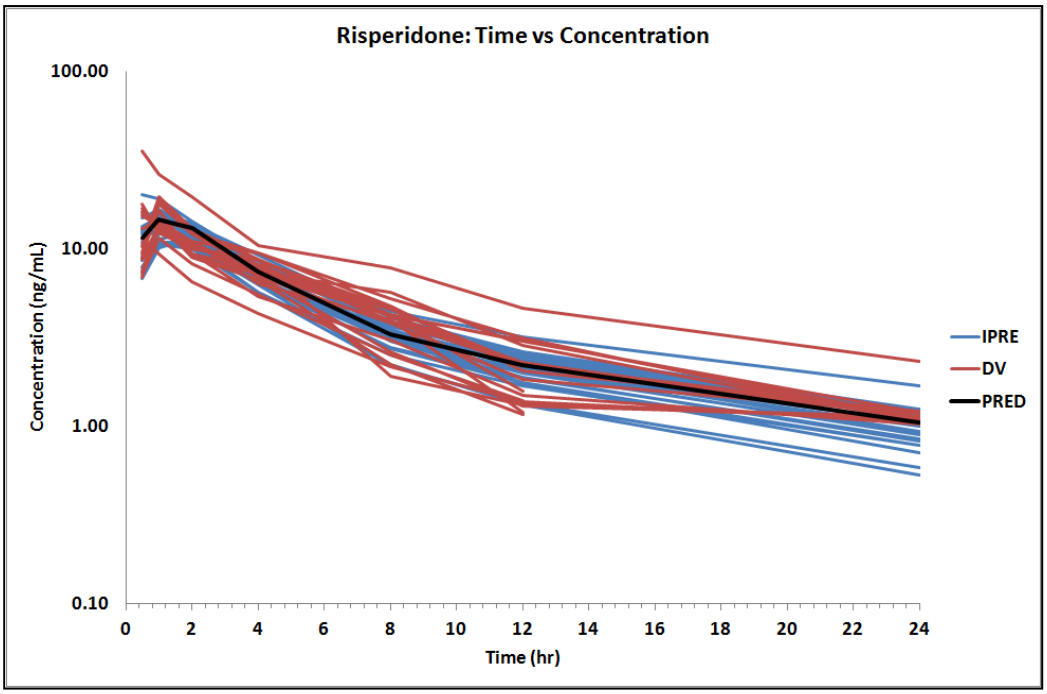


Figure 20: The Model Predicted and the Observed Plasma Concentration Profiled of 9-Hydroxyrisperidone

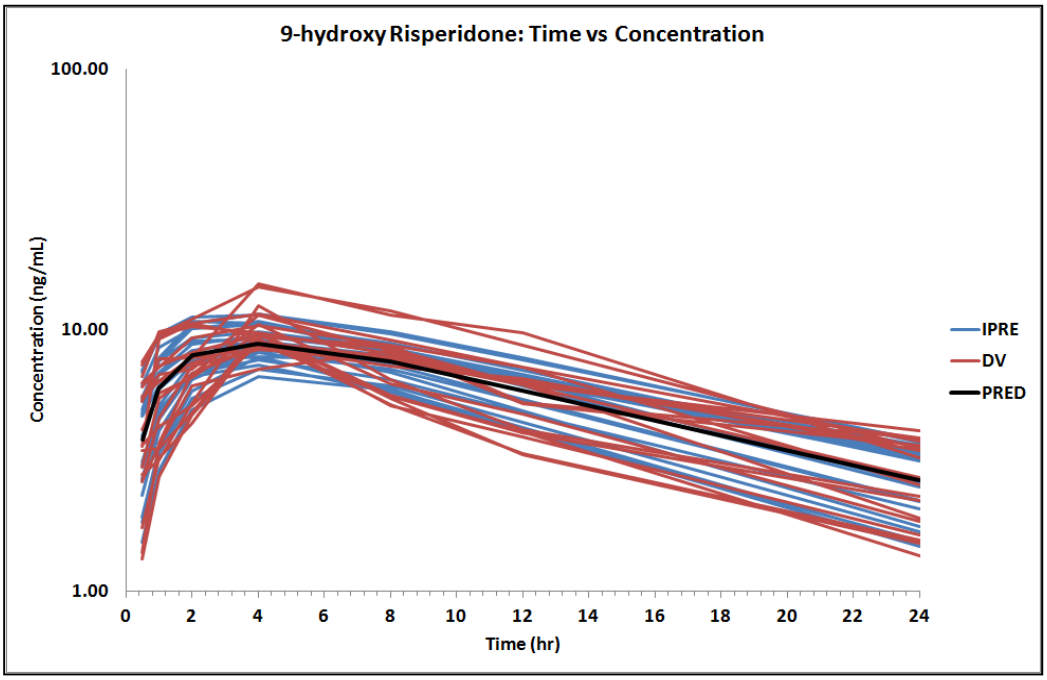
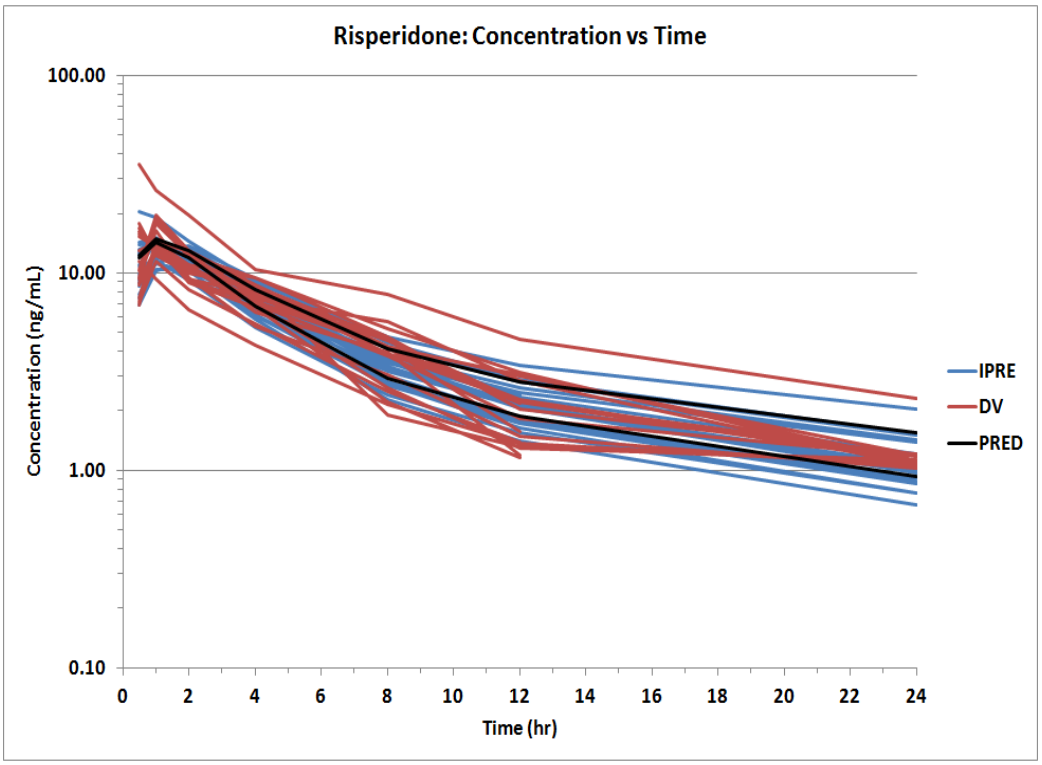


Figure 21: The Goodness of Fit Plots for the Final Model of Risperidone (Continued)



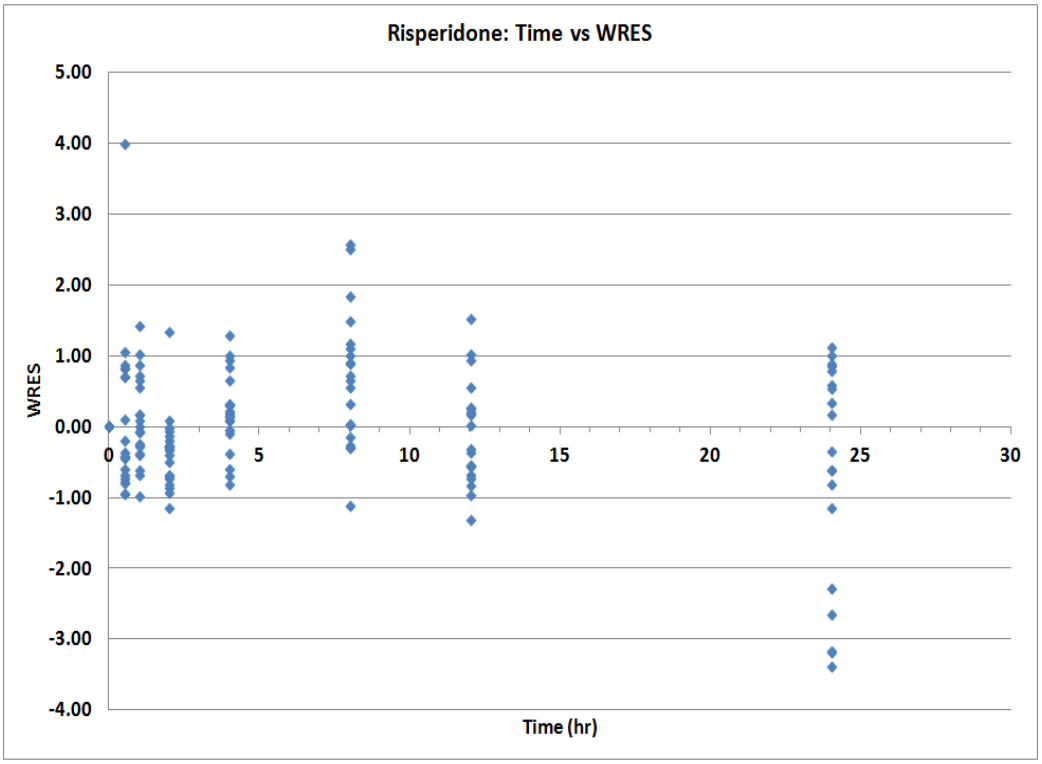
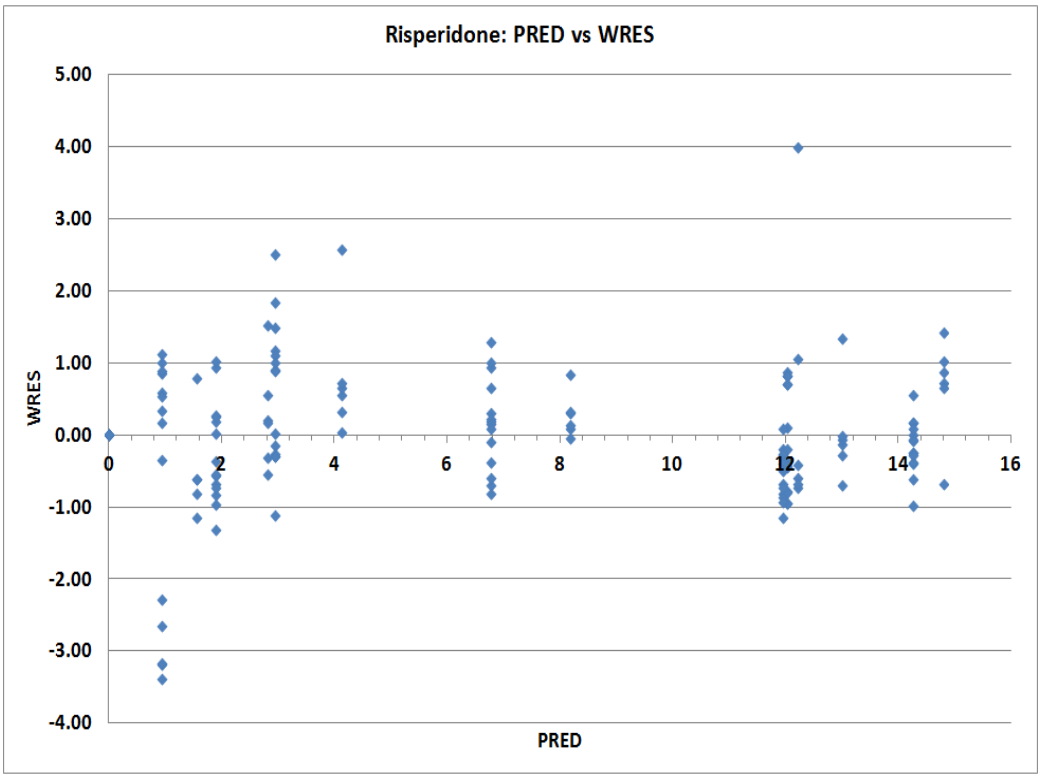


Figure 21: The Goodness of Fit Plots for the Final Model of Risperidone (Continued)



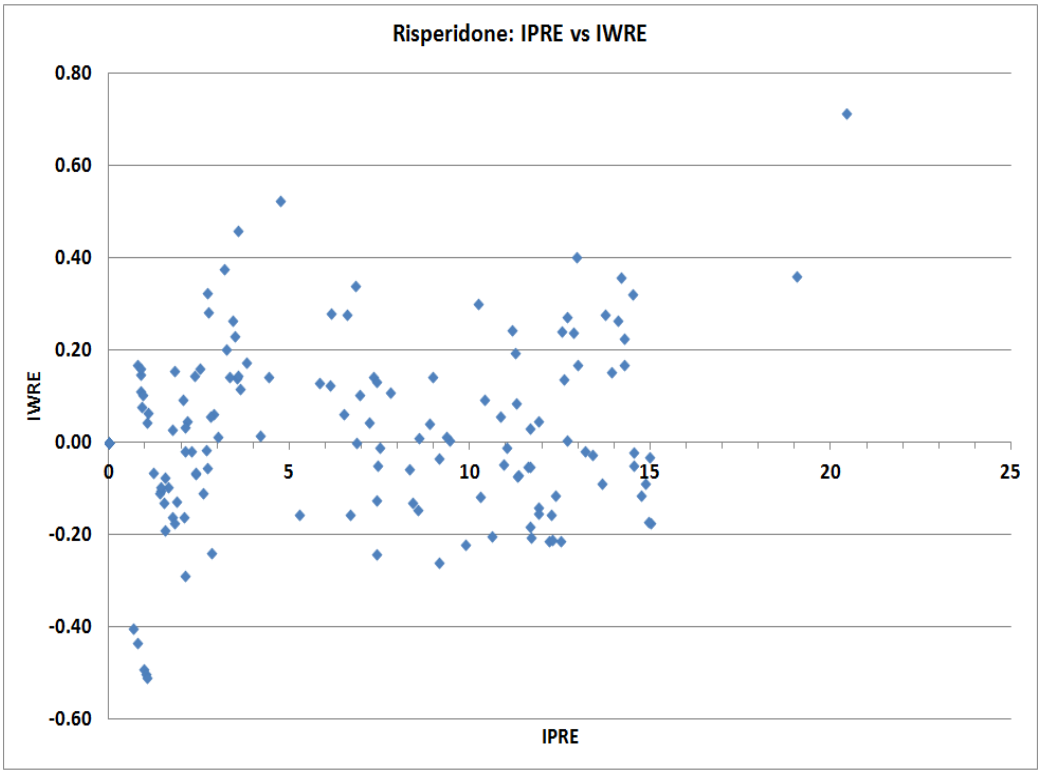
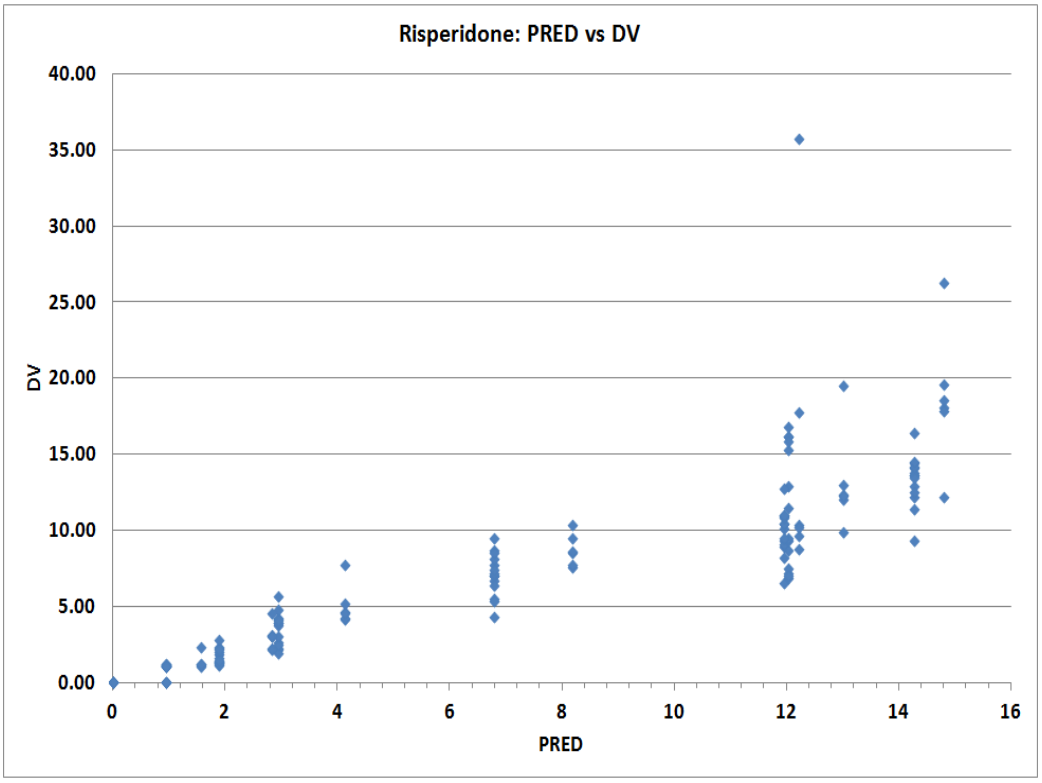


Figure 21: The Goodness of Fit Plots for the Final Model of Risperidone



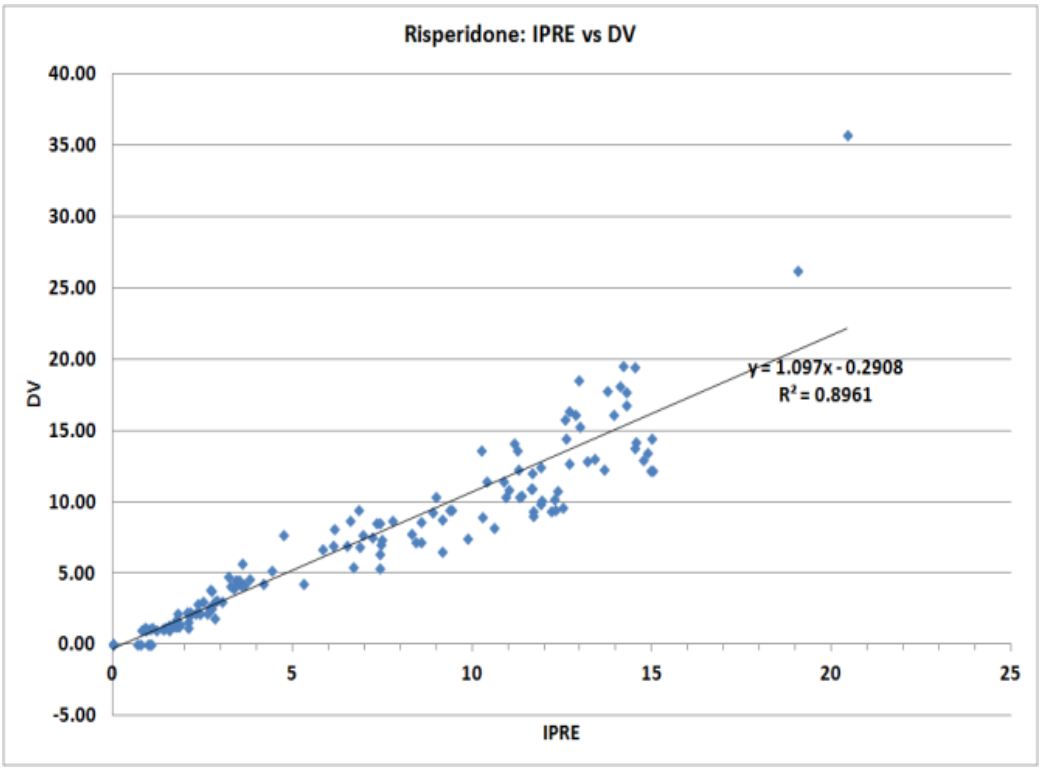
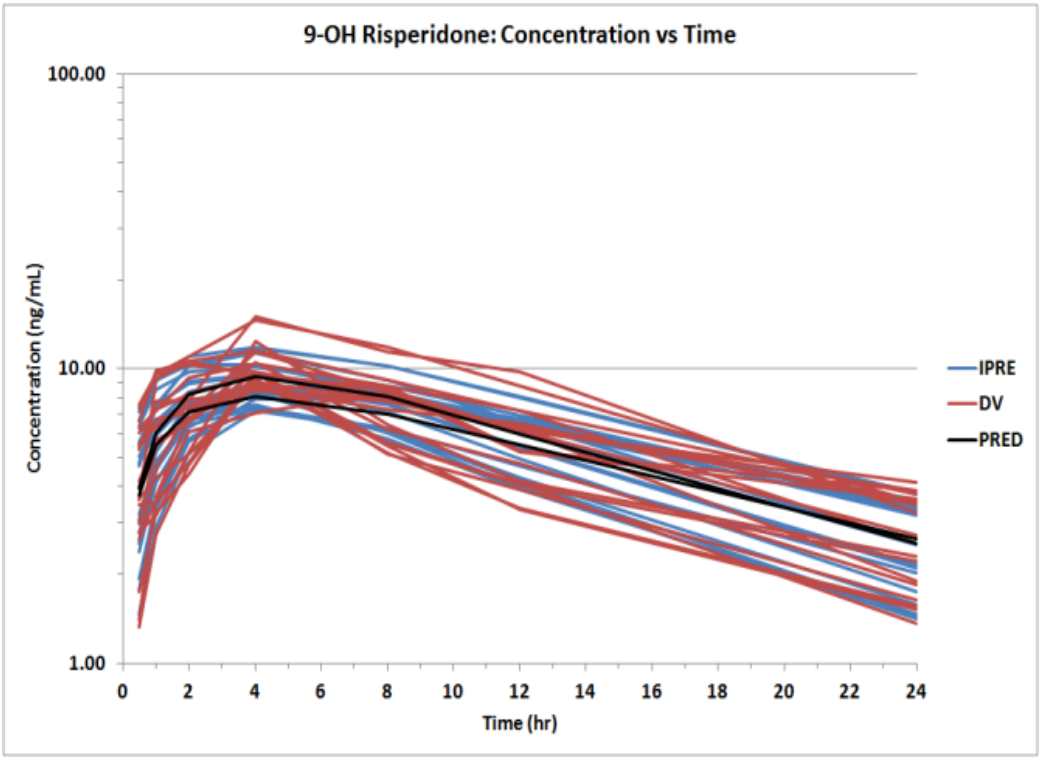


Figure 22: The Goodness of Fit Plots for the Final Model of 9-Hydroxyrisperidone (Continued)



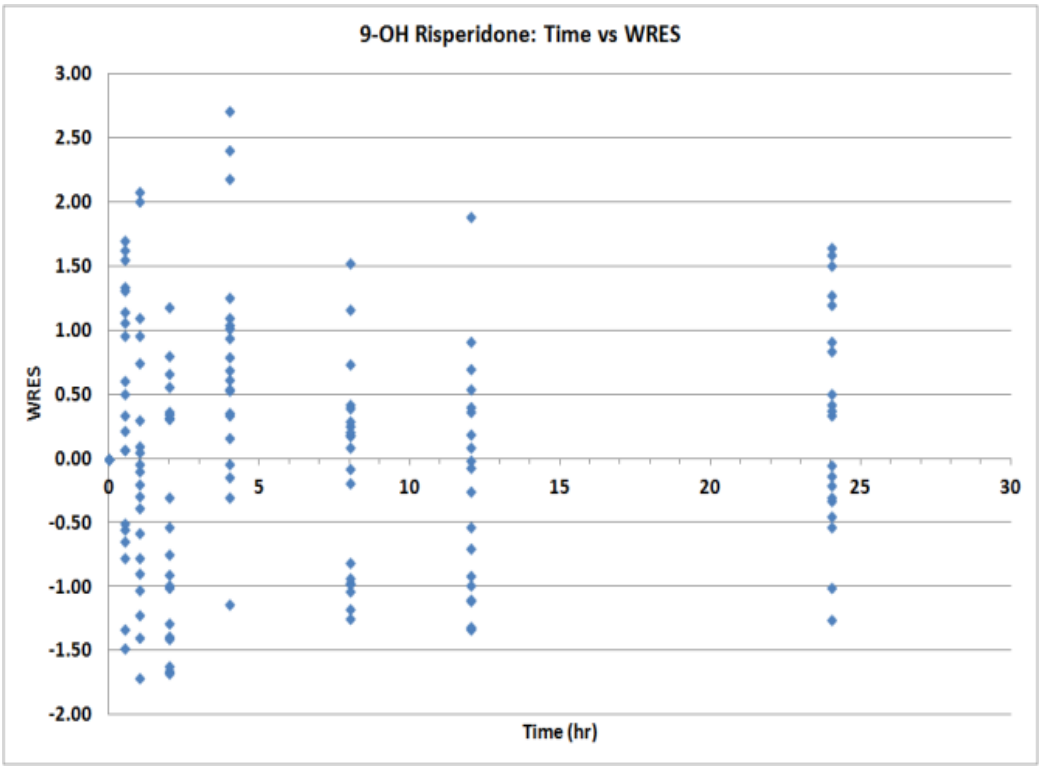
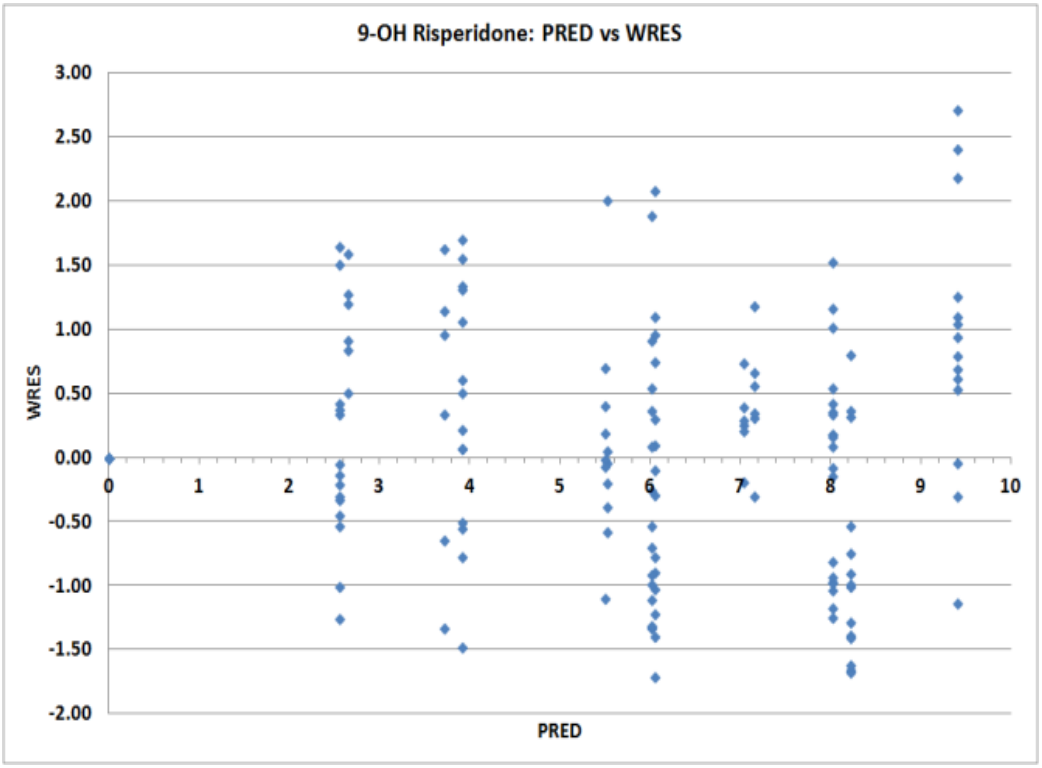


Figure 22: The Goodness of Fit Plots for the Final Model of 9-Hydroxyrisperidone (Continued)



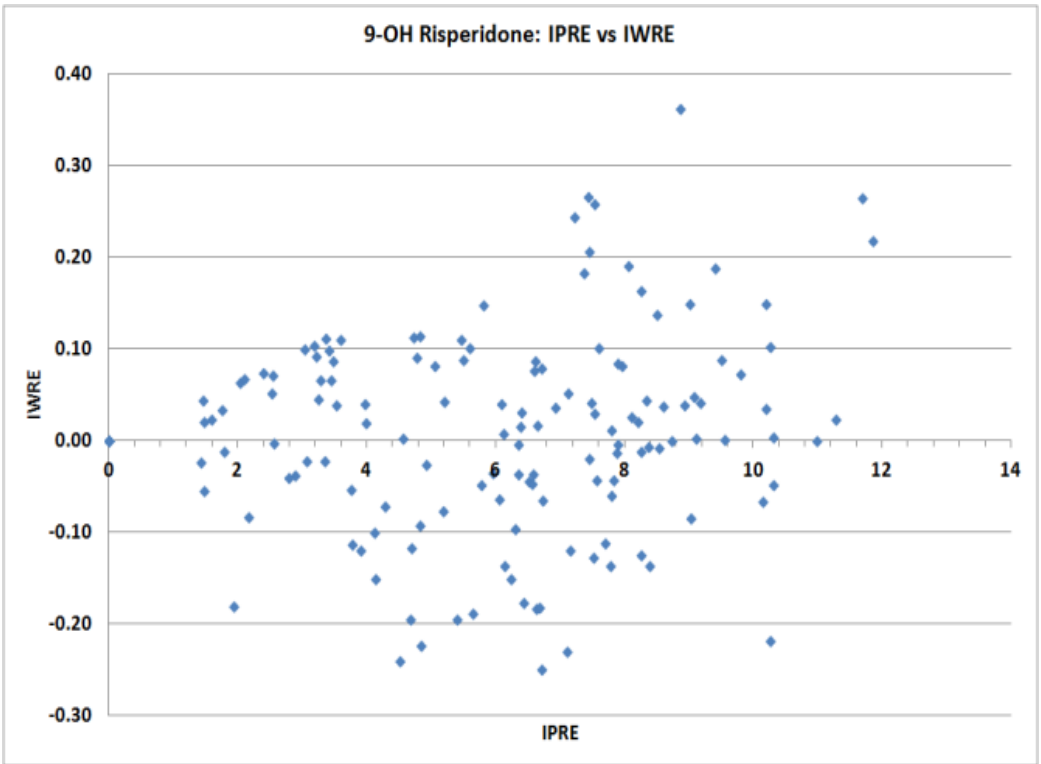
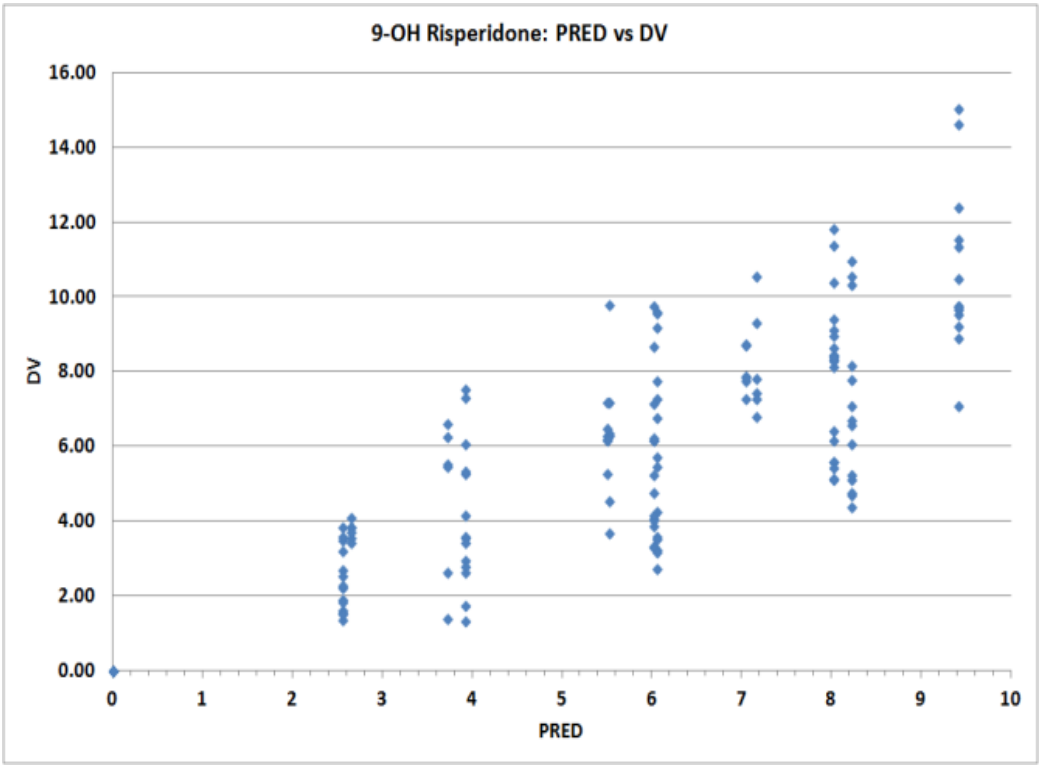
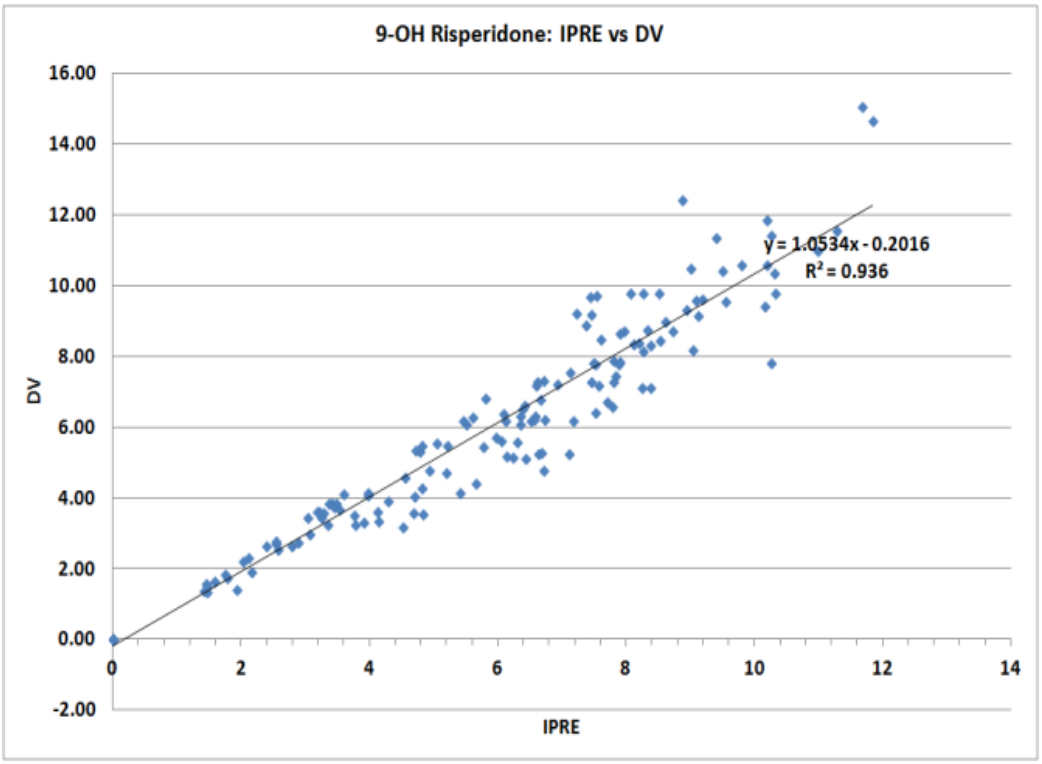


Figure 22: The Goodness of Fit Plots for the Final Model of 9-Hydroxyrisperidone





Chapter-8

Discussion

8. DISCUSSION

CYP enzyme and its functions:

Biotransformation is a pharmacokinetic process carried out by various metabolizing enzymes in order to enhance the extent of elimination of chemical substances after detoxification. Enzymes undertake these reactions can be classified as microsomal or non-microsomal, or in terms of the catalyzed reactions, as Phase-I and Phase-II. The most abundant and diverse of the phase-I microsomal and non-microsomal enzymes include the *CYP450* enzymes. Based on their substrates, these *CYP450* enzymes can be grouped into those families that catalyze metabolism of drugs and other xenobiotics, and those which catalyze the biotransformation of endogenous compounds like eicosanoids, prostanoids, steroids, etc. [139]. The first group includes clinically significant enzyme families like *CYP 1*, *CYP 2* and *CYP 3*.

CYP 3A4, CYP2D6, CYP2C9, and, *CYP2C19* are the major isoenzymes catalyzing the metabolic transformation of most of the prescription and Over-The-Counter (OTC) drugs. *CYP2D6* is the primary enzyme that catalyzes many drugs used in neuropsychiatric illnesses. Moreover, certain *CYP450* enzymes also play a role in maintaining the levels of such drugs across the Blood Brain Barrier [140]. In our study, we have hypothesized that this enzyme is clinically relevant in determining the disposition of a commonly used antipsychotic medication. We have observed that a genetic polymorphism of this enzyme commonly prevalent in individuals of South Indian origin is associated with altered pharmacokinetics and adverse effects of the drug, which in turn affects the clinical outcome.

In the present study, we have evaluated the pharmacokinetic parameters of risperidone in normal and *CYP2D6* polymorphism participants from the region of South India. Risperidone is a second generation antipsychotic drug commonly prescribed to patients suffering from schizophrenia and related disorders. It is metabolized *in-vivo* to an active metabolite, 9-hydroxyrisperidone with the potency comparable to the parent drug. Moreover it is the only major metabolite of the antipsychotic drug. This conversion is catalyzed by the enzyme *CYP2D6* in the hepatic microsomes. Further, this active metabolite is eliminated by both renal and hepatic routes after exerting its CNS activity [141]. We have considered both this metabolite and the parent drug in our study for the comparison of the effects of the genetic polymorphism on their disposition [142]. Previous studies have evaluated the effects of such polymorphisms after a single oral dosing in healthy volunteers but in different populations [143, 144]. Of the numerous polymorphisms exhibited by the *CYP2D6* gene, *CYP2D6*10* is one of the commonly assessed alleles, on its effects on the pharmacokinetics of risperidone, but in populations elsewhere in Asia as well as other continents [145].

Analytical method

Antipsychotics medications can be estimated and quantified in a variety of biological matrices such as plasma using High Performance liquid chromatographic techniques coupled with various detectors [146, 147]. Each technique can yield differing values of optimization outputs such as sensitivity, specificity, precision, and accuracy, due to various interferences, and can be applied to both patients and healthy volunteers [148].

In our study, the estimation of the three such compounds including olanzapine, risperidone and its metabolite, 9-hydroxyrisperidone in plasma samples from healthy volunteers. For the first time, we have successfully developed a UPLC-DAD method and applied this for estimating the above study compounds. This method has surpassed other currently used methods in terms of the run time and efficiency, and the cost factors involved.

CYP mutations reported and our observations

Various mutations can affect the *CYP2D6* enzyme metabolism functions in many different ways. Generally, genetic mutations can be divided into insertion-deletions (INDELs), Copy Number Variations (CNVs) and Single Nucleotide Polymorphisms (SNPs), and can be denoted as per star notation [149, 150]. Likewise, the mutations affecting the *CYP2D6* functions can be designated as *CYP2D6*3*, *CYP2D6*4* and its splice variants, *CYP2D6*7*, *CYP2D6*9*, *CYP2D6*10*, and *CYP2D6*41*, all of which reduce its metabolising activity [151, 152]. Further, variants such as *CYP2D6*5* deletes the gene coding this enzyme, while *CYP2D6*6* frame shift mutation makes the enzyme non functional [153].
The frequencies of one of the common allelic variants of the enzyme, *CYP2D6*10* varies among different populations across the world as reported previously [154, 155]. In the European population the frequency of this allele is 0.9-8.0%, whereas in Canadian populations, this ranges from 2.3-3.0%. In the other parts of the Americas, the frequencies range from 1.8-7.1% (South American origin), to 2.7-7.5% (Central American descent), to 2.7-7.5% among the African Americans. In Asian continent, the allele is prevalent at 22.4-64.1% in Chinese population and 8.6-45.9% in the Japanese.

We have related the changes in pharmacokinetic of these two study compounds due to the genotype variants on the pharmacological effects of the parent drug in healthy volunteers. The distribution of frequencies in each of the *CYP2D6*10* genotypes studied in our South Indian population is 70%, 25%, and 5%. These frequencies are slightly different from those reported in other Asian populations [144, 156 and 157]. We have noticed exhibition of Hardy Weinberg equilibrium, with *CYP2D6*10* frequencies i.e., this genotype seems to be evolutionarily conserved among the various ethnic sub groups of Asia. A few studies observed high *CYP2D6*10* frequencies in Asians, unlike in other populations where a maximum frequency of 9% mutants reported in Saudi Arabians [158] and 10% mutants reported in Sri Lankan population [159]. In Sri Lankan population, the investigators have used dextromethorphan as a *CYP2D6* marker without the inclusion of risperidone in either healthy volunteers or in schizophrenia patients.

Due to the high incidence of this neuropsychiatric illness and the lack of adherence generally observed with antipsychotic drugs, knowledge of the genetic basis of the response to risperidone might play a key role in the management of patients with schizophrenia [160].

Pharmacogenetic-Pharmacokinetic correlations with antipsychotics

In addition to regulation of function by microRNAs [161], the importance of the genetic variants on the clinical responses to a variety of drugs metabolised by key *CYP* enzymes, ranging from antidepressants to anticancer drugs cannot be undermined [162, 163]. The relationship between the steady state plasma concentrations and the therapeutic response is strong for many classical

antipsychotic and atypical antipsychotics as reviewed elsewhere [164]. However, for the first time, we have evaluated the influence of such an important polymorphism in a South Indian population of healthy volunteers on the actions and adverse effects of risperidone. Our study revealed a strong evidence for our hypothesis that the pharmacokinetics and adverse effect profiles of the parent antipsychotic and its metabolite are strongly influenced by the commonly encountered *CYP2D*10* allelic variants in our population.

In this study, the genotype status of *CYP2D6*10* has a significant influence on the plasma levels of risperidone and 9-hydroxyrisperidone. Nearly twofold increase in the plasma concentration of risperidone was observed in the poor metabolizers than normal metabolizers. Yoo *et al.,* [165] reported that the *CYP2D6* genotype status influences the plasma level of risperidone and its metabolite 9-hydroxyrisperidone. The current study showed that the poor and intermediate metabolizers had higher C_{max} than normal metabolizers. The 9-hydroxyrisperidone levels were found to be reduced in poor and intermediate metabolizers as compared to the normal metabolizers. However, recent studies have reported that the inhibitors of *CYP2D6* increased plasma risperidone levels, without any significant change in the 9-hydroxyrisperidone levels [166, 167].

Risperidone and its metabolites persist in plasma for shorter duration in normal metabolizer. The half-life of risperidone in poor metabolizer is twice as compared to normal metabolizer. The poor metabolizer more accumulates the metabolite, where as the normal metabolizer show a greater tendency to metabolize the parent to its 9-hydroxyrisperidone. Huang *et.al.,* reported that poor metabolizer had higher half-life of risperidone than the normal metabolizer [168].

The present study showed that, among the other pharmacokinetic parameter only the T_{max} was unaltered by the *CYP2D6*10* genotypes of risperidone and its metabolite. However, AUC_{0-t} and $AUC_{0-\infty}$ comparisons across genotypes have yielded statistically significant differences; their mean values were greater in intermediate and poor metabolizers as compared to normal metabolizers [165, 182].

Risperidone is eliminated much more quickly in poor and intermediate metabolizers than the normal metabolizers, as the 9-hydroxyrisperidone. The elimination capacity of risperidone across the three genotypes is greater than that of the metabolite, as shown by the clearance values. Normal metabolizers convert the risperidone to its 9-hydroxyrisperidone much more rapidly than the intermediate metabolizers followed by the poor metabolizers, which is in agreement with earlier published results evaluating the pharmacogenetic of risperidone in healthy volunteers [168].

Mannheimer *et al.,* [169] have recently reported that the risperidone metabolic ratio is a useful therapeutic biomarker to predict the metabolizer status, implying that normal metabolizers have lower metabolic ratio. These results agree with our conclusions, since we found that intermediate and poor metabolizers had high metabolic ratio than normal metabolizers. In our study, *CYP2D6* genotype clearly modified the plasma levels of active moiety (sum of risperidone and 9-hydroxyrisperidone levels). Higher plasma concentration of active moiety was associated with higher prevalence of adverse effects [170].

Risperidone and 9-hydroxyrisperidone have similar pharmacological properties [171]. Whereas the two molecules have similar affinities for the

dopamine D2 receptor, higher risperidone plasma levels are more toxic than comparable levels of 9-hydroxyrisperidone [172]. More often, risperidone is discontinued owing to noneffectiveness (27%) and intolerable side effects (10%) [159]. The latter may be due to the greater penetrability of risperidone across the blood-brain barrier.

Linking pharmacology with expected ADR profiles

Subjects with *CYP2D*10* polymorphism that give their phenotypes a status of poor and intermediate metabolizers, have significantly elevated plasma concentrations and AUC extrapolated to infinity as reported previously [173]. These patients hence have theoretically higher exposures to drugs such as risperidone and 9-hydroxyrisperidone in their plasma and tissues. Though practice guidelines to alter the doses or change the drugs in patients exist based on the pharmacokinetic parameters in various genotypes [174], there is an increasing need to integrate the therapeutic drug monitoring (TDM) data with the genotyping results to recommend rational therapeutic guidelines to maximise efficacy and compliance and minimise toxicity in different genotypes or populations of *CYP2D6* allele variants. For example, though the incidence of extra-pyramidal side effects seen with newer antipsychotics is lower than that with neuroleptics, it does occur with drugs like risperidone in patients which necessitates the importance of genotyping in patients along with TDM analysis in schizophrenia [175]. But in order to start initially in our population due to lack of such evidence, we have successfully undertaken a triple approach to identify any relationships between the pharmacogenetics variants and pharmacokinetics on the adverse

effect profile in healthy volunteers in our population using a questionnaire approach, after single oral dosing of risperidone.

In our study, genotype and pharmacokinetic parameters were evaluated with adverse effects' profiles, as accumulation of risperidone leading to such adverse effects has been observed in previous studies [176]. The most common adverse reactions associated with risperidone dosing were somnolence, nausea, abdominal pain, dizziness, vomiting, agitation, and akathisia [177]. In fact, extensive research has been carried out to elucidate the genetic basis of the response to risperidone in order to decrease its adverse effects [178]. However, a paradoxical lower incidence of tiredness in poor metabolizers may be due low level of 9-hydroxyrisperidone. Novalbos *et al.* [179] concluded that poor metabolizers in general report less frequent tiredness and related adverse effects.

Higher incidences of hypotensive episodes including orthostatic hypotension have been observed in intermediate and poor metabolizers as compare to normal metabolizers due to higher sensitivity of noradrenergic alpha-1 receptors in the brain to risperidone induced antagonism. In our study, incidence of neuropsychiatric adverse effects does not show significant differences in both poor and normal metabolizers. This might highlight actions at higher cortical centres that are either inaccessible or not susceptible to the central effects of risperidone as reflected in our clinical score. Our results agree with similar conclusions from Lopez-Rodriguez *et al.* [180], who have evaluated the importance of pharmacodynamic markers in determining adverse effects to multiple antipsychotics including risperidone. Mas *et al.* [181] have discussed the clinical importance of using low risperidone doses in patients. Our data supports

these above conclusions in our sample population, i.e., poor metabolizers require half the risperidone dose to reduce the incidence of adverse effects.

Population Pharmacokinetics

Further, the results of compartmental analysis and population pharmacokinetic modelling showed that pharmacokinetics of the parent drug including absorption and distribution is well described in one compartment model and the metabolism of risperidone to its active metabolite, 9-hydroxyrisperidone and their elimination is well described by two compartment model which hold good power for predicting the effects of covariates on the pharmacokinetics of the drugs in plasma.

The two parts of the model are connected by the unidirectional clearance of the parent drug to its metabolite in the liver which describes the elimination of the parent drug by metabolism and the elimination of the active metabolite through excretion. Very commonly, researchers have modelled the pharmacokinetics of risperidone and 9-hydroxyrisperidone using one compartment models, using first order absorption kinetics of both risperidone and 9-hydroxyrisperidone plasma concentrations [182] and parenteral 9-hydroxyrisperidone using first order elimination kinetics [183]. Less commonly, two compartment models of first and zero order absorption rate constants were used to model the pharmacokinetics of risperidone and the two stereoisomers of the metabolite [184].

Certain assumptions were made before the modelling was initiated – for example, the clearance of risperidone to 9-hydroxyrisperidone was non reversible, and the volume of distribution of risperidone and 9-hydroxyrisperidone were

equivalent. The metabolism of risperidone to its metabolite was assumed to follow a first order rate kinetics. All modelling parameters were computed based on the absorption of a depot preparation of risperidone into the central compartment using NONMEM.

The effects of covariates with a probable influence on the pharmacokinetics of risperidone and its active metabolite were evaluated using the model developed. Such covariates included genotype versus non reversible risperidone clearance, first pass metabolism at pre systemic sites, absorption rate constant, with each of the two parts of the model viz. model 1 and model 2.

Of all the covariates that could theoretically influence the plasma and tissue pharmacokinetics of risperidone and its metabolite, the genotype has the most significant and single major influence as shown by OFV value changes. The model was also used to estimate the pharmacokinetic parameters of the drugs in the population and the effect of genotype of these parameters.

Overall, the covariate analysis indicated, when evaluated individually, genotype appeared to have significant impact on CLPM, FPM and KA. However when evaluated the effect of genotype on FPM and KA along with CLPM there were no additional benefit. Thus the PK model with genotype as a covariate on CLPM was considered as the final model adequately explaining the plasma concentration profiles of this study.

In previous studies, the clearance of risperidone was significantly altered by *CYP2D6* genotype [168, 185]. The deposition of the risperidone is influenced by *CYP2D6* expression in the enterocytes of the intestine which is altered by the

genetic polymorphism of *CYP2D6*10* allele, further effecting the plasma pharmacokinetics, and the clinical response to risperidone [186, 187 and 188].

Our data supports these above conclusions in our sample population, i.e., poor metabolizers require half the risperidone dose than that for normal metabolizers to reduce the incidence of adverse effects. The results can be further examined and used to extrapolate to a larger sample of patients on multiple drugs including risperidone, and can help in rational dosing of this commonly prescribed antipsychotic medication. This can further enable clinicians in their rationale psychiatric practice by increasing patients compliance and reducing therapeutic failure due to non-adherence, under efficacy and toxicities by tailoring the dose regimen.

Chapter-9

Summary & Conclusion

9. SUMMARY AND CONCLUSION

Summary

- A simple, sensitive, and reliable UPLC method has been developed and validated for simultaneous determination of risperidone, 9-hydroxyrisperidone and olanzapine in human plasma *in vitro*.
- The developed method has been successfully adopted to evaluate the pharmacokinetic parameters of second generation antipsychotic drugs, risperidone and its major metabolite, 9-hydroxyrisperidone, in human plasma.
- The distributions of normal, intermediate and poor metabolizers were found to be 70%, 25% and 5% respectively. The allelic frequencies of all SNP were in Hardy-Weinberg equilibrium ($P \geq 0.05$).
- The metabolic ratio was higher in intermediate metabolizers (2.04 ± 0.20) as compared to normal metabolizers (1.40 ± 0.22).
- The pharmacokinetic parameters (C_{max}, $AUC_{0\text{-}t}$, $T_{1/2}$ and CL) of active moiety were found to be significant in normal and intermediate metabolizers.
- Poor metabolizers exhibited a higher prevalence of CNS ($P = 0.001$), CVS ($P < 0.001$), dizziness ($P = 0.005$), hypotension ($P = 0.01$) and GIT ($P = 0.01$) as compared to normal metabolizers.
- Our data supports these above conclusions in our sample population, i.e., poor metabolizers require half the risperidone dose than that for normal metabolizers to reduce the incidence of adverse effects.

Conclusion

In the current study, we have demonstrated significant association between the genotype status and pharmacokinetic parameter of normal subjects from our locale in South India with respect to the *CYP2D6*10* genotype. Our data suggest that *CYP2D6*10* polymorphism have significant association between risperidone and 9-hydroxyrisperidone pharmacokinetics. Active moiety is highly predictive of the clinical response to risperidone in healthy volunteers, which is dependent on the *CYP2D6*10* genotype status. Additionally, we demonstrated that using NONMEM and multi-compartment mixed effect modelling of the population pharmacokinetics of risperidone and its metabolite, genotype has a major influence on determining the plasma concentrations of both risperidone and 9-hydroxyrisperidone. The pharmacogenetic variations in clearance of the risperidone and 9-hydroxyrisperidone may be due to differential expressions of *CYP2D6* in intestinal epithelium in different genotypes of *CYP2D6*10* allele. Further, this could be applied to clinical decision making such as determination of dosing intervals. We recommend that the dose of risperidone in slow metabolizers must be less that used in normal metabolizers, though it has to be confirmed in further studies in our population.

Chapter-10

Impact of Study

10. IMPACT OF STUDY:

1. Prescription pattern has been successfully integrated in our pharmacogenetic study in order to rationally choose the commonly prescribed medications in schizophrenia patients for further analysis of pharmacokinetics. This will contribute to the study design in further such studies and will improve the success rates and benefits of pharmacogenetic studies in patients
2. This study is probably the first of its kind in novelty in South Indian population in healthy volunteers using pharmacogenetic influence on the pharmacokinetics of risperidone and its metabolite with a focus on *CYP2D6*10* allele
3. For the first time, we have developed a cost effective and yield efficient UPLC-DAD method of concurrent estimation of olanzapine, risperidone and 9-hydroxyrisperidone in human plasma, whose sensitivity and robustness is comparable to LC-MS methodologies. Additionally this UPLC-DAD method can be successfully adapted to LC-MS analytical methodology whilst reducing time and cost factors of methods and preserving yield success of LC-MS methods
4. It can yield results from pharmacogenetic-pharmacokinetic correlations which can be used to extrapolate and apply to patients from healthy volunteers
5. Further, the study could enable proper clinical use and rationale dosing of antipsychotics based on pharmacogenetic testing in patients administered risperidone and other related antipsychotic drugs.

6. As a novel tool, we have for the first time used a questionnaire to cross sectionally enquire about the presence or absence of adverse effect related to risperidone or its metabolite, and relate it to pharmacokinetics, pharmacodynamic alterations due to pharmacogenetics as a surrogate marker.
7. Unlike in previously published research, we have shown that the active moiety is of paramount importance in determining pharmacogenetics of risperidone and its active metabolite in healthy volunteers which could be further evaluated and extrapolated in patients.
8. We prove that a two compartment model with a one compartment extension can predict to a high probability the influence of genetics polymorphism on the pharmacokinetics of risperidone, which could be used in rational dose modification and safer use in patients.

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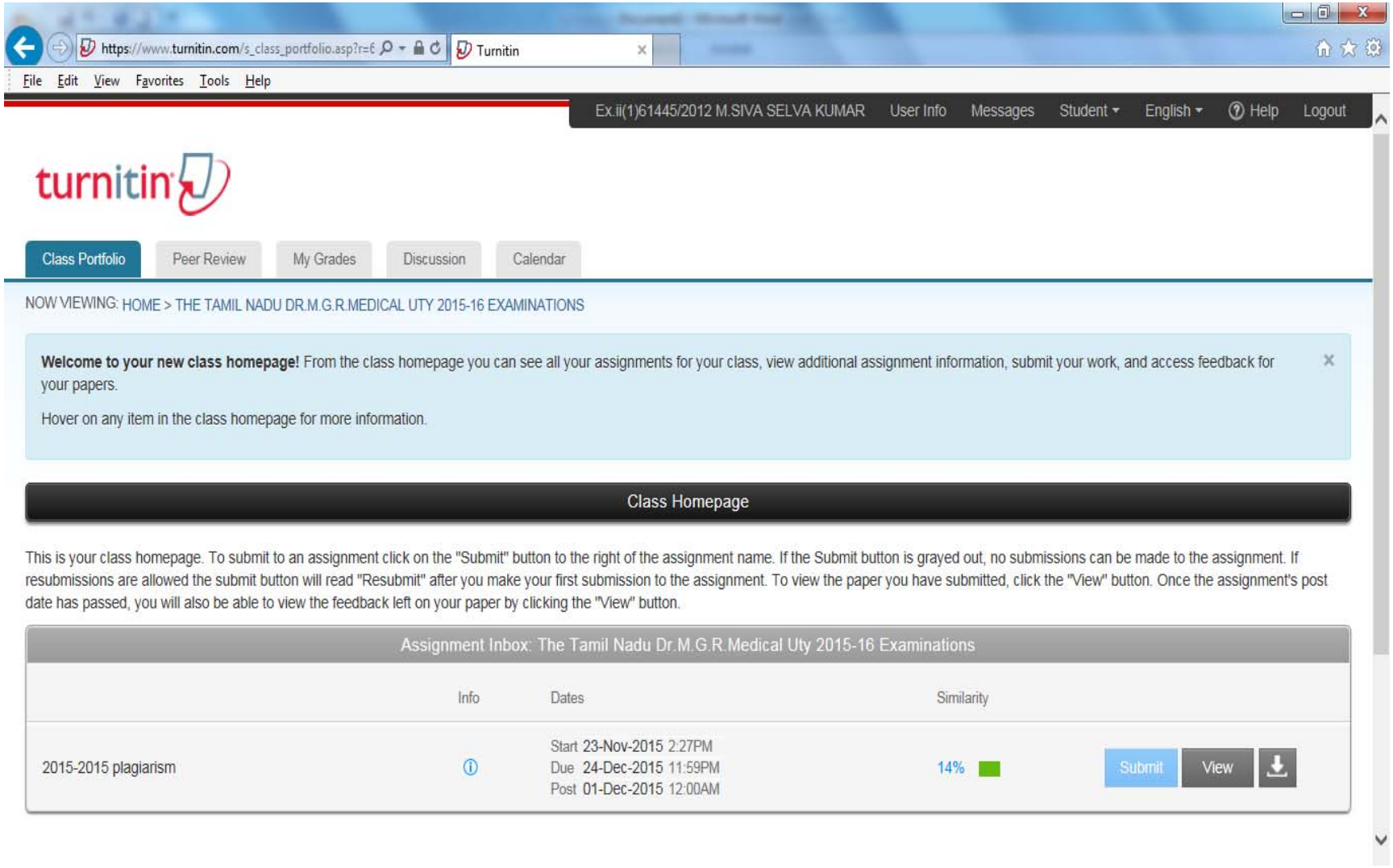
Annexure

Plagiarism Report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List of Publications

Publications

1. Siva Selva Kumar M, Ramanathan M. Concurrent determination of olanzapine, risperidone and 9-hydroxyrisperidone in human plasma by ultra performance liquid chromatography with diode array detection method: application to pharmacokinetic study. Biomed Chromatogr. 2015 Jun 30.

2. Siva Selva Kumar M, Ranjithkumar R, Ramanathan M. Fast and sensitive validated method for quantification of haloperidol in human plasma using ultra performance liquid chromatography (UPLC) with diode array detection. World Journal of Pharmaceutical Research. 2015 Nov 30.

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Concurrent determination of olanzapine, risperidone and 9-hydroxyrisperidone in human plasma by ultra performance liquid chromatography with diode array detection method: application to pharmacokinetic study

M. Siva Selva Kumar[a] and M. Ramanathan[b]*

ABSTRACT: A simple and sensitive ultra-performance liquid chromatography (UPLC) method has been developed and validated for simultaneous estimation of olanzapine (OLZ), risperidone (RIS) and 9-hydroxyrisperidone (9-OHRIS) in human plasma *in vitro*. The sample preparation was performed by simple liquid–liquid extraction technique. The analytes were chromatographed on a Waters Acquity H class UPLC system using isocratic mobile phase conditions at a flow rate of 0.3 mL/min and Acquity UPLC BEH shield RP_{18} column maintained at 40°C. Quantification was performed on a photodiode array detector set at 277 nm and clozapine was used as internal standard (IS). OLZ, RIS, 9-OHRIS and IS retention times were found to be 0.9, 1.4, .1.8 and 3.1 min, respectively, and the total run time was 4 min. The method was validated for selectivity, specificity, recovery, linearity, accuracy, precision and sample stability. The calibration curve was linear over the concentration range 1–100 ng/mL for OLZ, RIS and 9-OHRIS. Intra- and inter-day precisions for OLZ, RIS and 9-OHRIS were found to be good with the coefficient of variation $<$6.96%, and the accuracy ranging from 97.55 to 105.41%, in human plasma. The validated UPLC method was successfully applied to the pharmacokinetic study of RIS and 9-OHRIS in human plasma. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: UPLC; risperidone; 9-hydroxyrisperidone; olanzapine; pharmacokinetics

Introduction

Schizophrenia is a common chronic neuropsychiatric illness affecting patients worldwide. This condition requires the initiation of treatment with antipsychotic medications, of which second-generation drugs [risperidone (RIS) and olanzapine (OLZ)] are the most commonly prescribed in patients. Second-generation antipsychotic drugs exhibit beneficial effects on anxiety, depression and mania, and exert reduced extra-pyramidal side effects (Leucht *et al.*, 2009; Ansari and Mulla, 2014; Lieberman *et al.*, 2005). Both risperidone and olanzapine are widely prescribed second generation antipsychotic drugs, and olanzapine is often co-administered with risperidone (Jayaram *et al.*, 2006). 9-Hydroxyrisperidone (9-OHRIS) is the major metabolite of RIS, formed *in-vivo* through hepatic oxidation, and itself is biologically active. Correlation of its plasma levels, parent drug levels and clinical effects is complicated (Sheehan *et al.*, 2010). Of the numerous metabolic enzymes, the cytochrome P450 family account for the metabolism of the majority of psychotropic medications. Cytochrome P450 has a variable expression pattern and these microsomal enzymes are subjected to genetic, pharmacogenetic and epigenetic variations, which leads to pharmacokinetic variations and results in unexpected alterations and clinical response (Basile *et al.*, 2002; Crisafulli *et al.*, 2011). Quantification of antipsychotic medications in human biological matrices requires high precision and greater sensitivity since most of these compounds are present in low concentrations in plasma. Moreover, as most of the antipsychotic medications are prescribed along with other drugs in schizophrenia patients, drug interactions can be expected.

In the past, various high-performance liquid chromatography (HPLC) methods with UV detection (Zhang *et al.*, 2007; Shen *et al.*, 2002; Dusci *et al.*, 2002), coulometric (Schatz and Saria, 2000) and fluorescence detection (Suckow *et al.*, 1992; Kristoffersen *et al.*, 1999; Mandrioli *et al.*, 2007) have been reported to detect and quantify antipsychotic medications. Recently, several studies have utilized methods based on liquid chromatography coupled with mass spectrometry (LC-MS/MS) to increase sensitivity in detection with shorter run times (Urinovska *et al.*, 2012, Ansermot *et al.*, 2013). Although LC-MS/MS methods are advantageous in many ways, the expensive instrumentation and decreased accessibility to routine hospital practice and laboratories are their major limitations.

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Abbreviations used: DAD, diode array detection; OLZ, olanzapine; RIS, risperidone; 9-OHRIS, 9-hydroxyrisperidone.

Considering these facts, an ultra high performance liquid chromatography (UPLC) with diode array detection (DAD) for OLZ, RIS and 9-OHRIS was developed and validated. Further, the method was designed using a simple mobile phase in isocratic conditions, devoid of strong buffers and ion pairing agents to achieve better reproducibility with less interference. The method has a shorter run time, and can even be transferred to LC-MS/MS if needed as compatible buffer and chromatographic conditions were used. The proposed method was successfully applied to a pharmacokinetic study of risperidone in human volunteers. The developed method can also be utilized for therapeutic drug monitoring of OLZ, RIS and 9-OHRIS.

Materials and methods

Chemicals and reagents

Acetonitrile, methanol and ammonium acetate of HPLC grade were purchased from Sigma-Aldrich, India. The reference standards of RIS, 9-OHRIS, OLZ and clozapine (IS) were purchased from Sigma-Aldrich, India. Methyl *tert*-butyl ether and acetic acid of HPLC grade were obtained from Himedia, India. Ammonia solution (25% pure) was purchased from Merck, India. Blank human plasma was obtained from blood bank services PSG Hospitals, Coimbatore. Ultrapure water (18.2 MΩ cm) was obtained from a MilliQ water purification system from Millipore (Milford, USA).

Instrumentation and chromatography conditions

The UPLC instrument consisted of a Waters Acquity H class UPLC system equipped with a quaternary pump and 96-vial autosampler coupled with a diode array UV detector (Waters, Milford, MA, USA). The chromatographic separation was performed on an Acquity UPLC BEH C_{18} column from Waters (2.1 × 100 mm; 1.7 μm). The column temperature was set at 40°C and the autosampler was kept at 10°C. The mobile phase composed of a mixture of 10 mM ammonium acetate buffer at a pH of 3.5, which was adjusted with acetic acid (70%, v/v) and acetonitrile (30%, v/v) at a flow rate of 0.3 mL/min. Before analysis, the mobile phase was filtered through a 0.22 μm membrane filter and degassed by ultra-sonication. A 5 μL injection of each sample was loaded on to the system and total analysis time was 4 min. DAD was set at 277 nm. The sampling needle was washed with 400 μL of strong wash (ACN–water, 65:35) to reduce carry-over and 400 μL of weak wash (ACN–water, 30/70). Data acquisition was done using Empower 3 software version 1.0 (Waters).

Preparation of stock and working standard solution

OLZ, RIS and 9-OHRIS stock solutions of 1 mg/mL were prepared by dissolving suitable amounts of the single drugs in methanol. Mixtures of stock solutions OLZ, RIS and 9-OHRIS (100 μg/mL) were prepared in methanol. Clozapine was selected as IS based on a previous study (Idris and Elgorashe, 2013). Further, clozapine has been used widely only in treatment-resistant schizophrenia (Meyer, 2011). Therefore we selected clozapine as IS. The IS stock solution of 200 μg/mL was prepared in methanol. The stock solution of OLZ, RIS, 9-OHRIS and IS were stable at 4°C for 1 month. Further dilution was made in methanol–water (50:50, v/v) to produce working stock solutions for the calibration standards and quality control (QC) standards. The IS working solution (5 μg/mL) was prepared in methanol–water (50:50 v/v). Calibration curve samples were prepared by spiking 240 μL of human blank plasma with the appropriate mixture of working solutions of OLZ, RIS and 9-OHRIS (10 μL) on the day of analysis. All the samples were stored together at −80 ± 10°C until analysis.

Sample preparation

Sample preparation was carried out by the liquid–liquid extraction procedure. To a 250 μL of aliquot of plasma, 25 μL of IS working solution and 250 μL of 10 mM ammonium acetate (pH 9.0) were added and mixed for 20 s on a spinix vortex shaker (Tarsons, India). The mixture was vortexed for 30 s. A 3.0 mL aliquot of tert-butylmethylether was added and vortex mixed for 5 min, and then centrifuged at 10,000 rpm for 5 min at 4°C on an Eppendorf 5810R centrifuge (Eppendorf AG, Hamburg, Germany). The clear supernatant organic layer (2.5 mL) was transferred into 5 mL polypropylene tubes and evaporated to dryness at 40°C using a nitrogen evaporator (Turebovap®, Biotage, USA). The residue was reconstituted in 200 μL of the mobile phase, vortex mixed for 1.0 min and centrifuged at 10,000rpm for 5 min. Finally, 190 μL of the clear supernatant was transferred into a glass micro-vial and 5 μL was injected onto the UPLC system for analysis.

Method validation

The method was validated for selectivity, specificity, recovery, linearity, accuracy, precision and stability using US Food and Drug Administration guidelines (US DHHS *et al*., 2001) for assay in human plasma.

Carry-over and limit of quantification

Carry-over effect was assessed by injecting the highest calibration standard just before the blank plasma. The lower limit of quantitation (LLOQ) was defined as the first point of the calibration standard with lowest concentration (signal-to-noise ratio ≥10:1) that can be calculated with an acceptable precision and accuracy.

Selectivity and specificity

Selectivity of the method was assessed by analyzing six different donors human blank plasma injected at the beginning of the validation and investigating the potential interferences at the LC peak region for analytes and IS. At least five out of six lots should have responses less than five times the LLOQ level response in the same matrix.

Recovery

The recoveries of OLZ, RIS and 9-OHRIS were assessed by the analyses of the three concentrations (5, 45 and 80 ng/mL) whereas the recovery of IS was determined at a single concentration of 5 μg/mL. Recovery was calculated as the extracted spiked plasma peak area response compared with the response obtained for neat standard solution at equivalent concentrations by the liquid–liquid extraction process. The analyte responses from the extracted samples at known concentrations were compared with responses of un-extracted standards.

Linearity

Linearity was evaluated by linear regression analysis with the use of working standard solutions and spiked plasma samples containing the drugs of interest at different concentrations within the range 1–100 ng/mL for all analytes. The calibration curves were constructed by plotting the ratio of the peak area of each analyte to the peak area of internal standard vs the nominal drug concentration. The slopes and intercept were calculated with least square linear regression analysis of the data with the use of a $1/x^2$ (where x is the concentration) weighting factor. The acceptance criterion for each back-calculated standard concentration was ±15% deviation from the nominal value except at LLOQ, which was set as ±20% (US DHHS *et al*., 2001).

Accuracy and precision

The validation method evaluation of the accuracy and precision in the experiments were performed for four independent series (including ruggedness) in plasma. The intra- and inter-day assay accuracy and precision were estimated by analyzing six replicates containing analytes at three different QC levels, that is, 1, 5, 45 and 80 ng/mL. Accuracy represents the closeness of agreement between the mean values obtained from the series of measurements by the method and the actual value. The accuracy should be within 85–115% of the nominal value, except at LLOQ, where it should not deviate by no more than 20% (US DHHS *et al*., 2001). Precision, which represents the closeness of agreement among a series of measurements obtained from multiple sampling, was estimated with variances of repeatability (intra-day variances) and intermediate precision (sum of intra-day and inter-day variances). The precision should not exceed ±15% relative standard deviation (RSD), except for the LLOQ, where it should be within ±20% of RSD (US DHHS *et al*., 2001).

Stability

Stability tests were performed to evaluate the stability of analytes in plasma samples under different conditions. The bench-top stability study assessed the stability of analytes in plasma at room temperature for 8 h, whereas a post-preparative stability study assessed the stability of analytes in the treated samples (autosampler stability for 26 h at 10°C). These were determined at low (5 ng/mL) and high (80 ng/mL) QC concentrations. The freeze–thaw stability was determined after three freeze and thaw cycles and long-term stability was determined by accessing QC samples stored at −80°C for 30 days.

Pharmacokinetic study

Twenty healthy male volunteers aged between 24 and 31 years (26.8 ± 2.4 years) and weighing from 62 to 82 kg (70.7 ± 6.8 kg) were enrolled in the study and informed consent was obtained. The study was approved by the Institutional Human Ethics Committee prior to commencing and was performed in accordance with the principles of the World Medical Association's Declaration of Helsinki. The volunteers underwent screening examinations that included a medical history and physical examination. Volunteers received a single 2 mg risperidone tablet (Respidon-2, Torrent Pharmaceuticals Ltd, India) along with 200 mL water. Serial venous blood samples (2 mL) for the determination of plasma RIS and 9-OHRIS concentrations were obtained from forearm vein before dosing and at 0.5, 1.0, 2.0, 4.0, 8.0, 12.0 and 24 h after dosing. Samples were collected in K_2·EDTA tube and plasma was separated by centrifugation at 4000 rpm and stored at −80°C until analysis. WinNonlin software 5.1 was used to calculate the pharmacokinetics parameters using a noncompartmental model (Pharsight Corporation, Mountain View, CA, USA). Pharmacokinetic parameters including C_{max} (maximum plasma concentration), T_{max} (the time to reach C_{max}), the area under the plasma concentration–time curve from time zero to last measurable time point (AUC_{0-t}), the area under the plasma concentration–time curve from zero to infinity ($AUC_{0-\infty}$), K_{el} (first-order rate constant associated with the terminal (log–linear) portion of the curve) and $T_{1/2}$ (the terminal half-life) were calculated.

Results and discussion

Optimization of the chromatographic conditions

A reverse-phase liquid chromatographic method was developed for the determination of OLZ, RIS and 9-OHRIS in human plasma samples using diode array detection. LC method development was carried out focusing on achieving sufficient resolution of target drugs and endogenous interferences in matrix within a short run time, with simple buffers. For this purpose different mobile phases comprising several combinations of buffers (e.g. phosphate buffer and ammonium acetate buffer) and organic solvents (acetonitrile and methanol) along with altered flow rates (range of 0.200–0.500 mL/min) were tested to optimize for an effective chromatographic resolution of OLZ, RIS, 9-OHRIS and IS. Using methanol in mobile phase produced broad peaks without symmetry and a long analysis time. When acetonitrile used as organic solvent in the mobile phase, the analysis time was reduced and peaks were more symmetrical and sharp. The best resolution of peaks achieved with isocratic mobile phase consisted of a mixture of acetonitrile–10 mM ammonium acetate (30:70; v/v) containing acetic acid with the pH adjusted to 3.5 at a flow rate of 0.3 mL/min on an Acquity UPLC BEH C_{18} column. The utilization of a volatile buffer like ammonium acetate in mobile phase gives better column life and can be compatible with mass spectrometry, unlike phosphate buffers.

Carry-over and limit of quantification

No carry-over was observed when the highest calibration standard was analyzed just before the blank plasma. This indicated that a flow-through needle configuration of the injector and a washing step of the column at the end of the chromatography separation were adequate to remove potential residues of the analytes. The LLOQ on the calibration curve was 1.0 ng/mL for all of the compounds and the signal-to-noise ratio in plasma was ≥10.

Selectivity and specificity

Selectivity was evaluated by comparing the chromatograms of blank plasma, blank plasma spiked with IS and with analytes (1 ng/mL) and IS, and a kinetic study sample obtained 2 h after oral administration (2 mg risperidone tablet). As shown in Fig. 1, no interfering peaks from endogenous compounds were observed at the retention times of analytes and IS. The total chromatographic run time was 4 min.

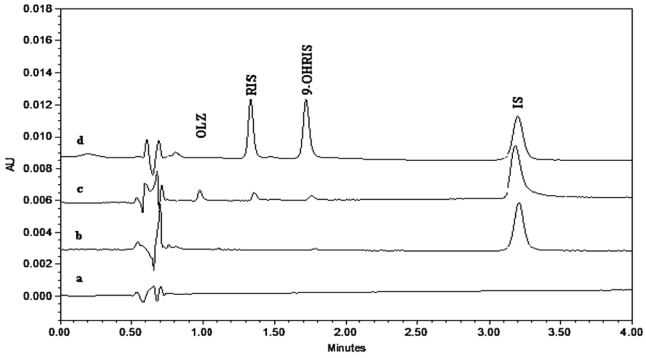

Figure 1. Representative UPLC chromatograms of olanzapine (OLZ), risperidone (RIS), 9-hydroxyrisperidone (9-OHRIS) and IS in human plasma. (a) Blank plasma sample; (b) blank plasma sample spiked with IS; (c) blank plasma spiked with analytes (1ng/mL) and IS; and (d) pharmacokinetic plasma samples collected from healthy volunteers at 2.0 h time point following oral administration of RIS along with IS.

Recovery

Liquid–liquid extraction technique gave adequate recovery and cleaner samples. The results of the evaluation of neat standards vs plasma extracted standards were estimated for OLZ, RIS and 9-OHRIS (5, 45 and 80 ng/mL) and the mean recoveries were 89.42 ± 2.89, 90.16 ± 2.06 and 90.13 ± 1.82%, respectively. The recovery of IS at 5 μg/mL was 84.25 ± 3.23%.

Linearity

The calibration curves exhibited excellent linearity with regression correlation coefficient ($r^2 > 0.998$) over the concentration range of 1.0–100ng/mL for all of the drugs in human plasma. The standard calibration curve had a consistent reproducibility over the standard concentrations across the calibration range. A typical regression equation was prepared by determining the best fit of peak-area ratio (peak area analyte/peak area IS) vs concentration, and fitted to $y = mx + c$ using a weighting factor ($1/x^2$). The lowest concentration with RSD < 20% was taken as the LLOQ and was found to be 1.0 ng/mL for all the drugs. The percentage accuracies observed for the mean of back-calculated concentration for four calibration curves for the entire drugs were within 96.10–102.50.

Accuracy and precision

The accuracy and precision of the method were evaluated as intra-day (repeatability) and inter-day (intermediate) human plasma for entire drug samples and are summarized in Table 1. The intra- and inter-day precisions were <6.96% and the accuracy ranged from 97.55 to 105.41 at all levels. All the values were within the accepted range and the method was accurate and precise.

Stability

The stability of OLZ, RIS, and 9-OHRIS in human plasma were investigated under a variety of storage and process conditions: in plasma at room temperature for 8 h; in the autosampler for 26 h at 10°C; after three freeze–thaw cycles; and after long-term storage at −80°C for 30 days. Results are summarized in Table 2 and indicated that the samples of OLZ, RIS and 9-OHRIS were stable under the conditions described.

Applicability of the validated method

We successfully validated the novel method of quantification of analytes, viz. RIS and 9-OHRIS, after a single oral dosing of 2 mg RIS (Respidon-2, Torrent Pharmaceuticals Ltd, India) tablets in healthy volunteers. The maximum concentration of RIS in our study was almost twice that of its major metabolite measured in our assay method, viz. 9-OHRIS, both compounds being quantified up to 24 h after a single oral dosing of RIS (16.48 ± 5.07 vs 10.33 ± 1.90 ng/mL). On similar lines of reference, the time taken to attain this maximum concentration in plasma for RIS was nearly one-quarter that for 9-OHRIS (0.80 ± 0.25 vs 4.0 ± 1.12 h). The AUCs of RIS from 0 to 12 h and 0 to ∞ h were 92.64 ± 27.79 and

Table 1. Intra- and inter-day precision and accuracy of olanzapine (OLZ), risperidone (RIS) and 9-hydroxyrisperidone (9-OHRIS) in human plasma *in vitro*

Nominal concentration (ng/mL)	Intra-day (n = 6)			Inter-day (n = 18)		
	Measured concentration (mean ± SD, ng/mL)	CV (%)	Accuracy (%)	Measured concentration (mean ± SD, ng/mL)	CV (%)	Accuracy (%)
RIS						
1.12	1.13 ± 0.06	5.31	100.89	1.15 ± 0.08	6.96	102.68
5.12	5.18 ± 0.13	2.51	101.17	5.28 ± 0.36	6.82	103.13
45.82	46.88 ± 0.72	1.54	102.31	48.30 ± 2.20	4.55	105.41
80.78	80.61 ± 1.62	2.01	99.79	80.12 ± 3.29	4.11	99.18
9-OHRIS						
1.09	1.07 ± 0.04	3.74	98.17	1.14 ± 0.07	6.14	104.59
5.10	5.06 ± 0.13	2.57	99.22	5.18 ± 0.31	5.98	101.57
45.21	46.72 ± 0.80	1.71	103.34	45.68 ± 2.34	5.12	101.04
81.48	80.49 ± 0.74	0.92	98.78	84.15 ± 3.44	4.09	103.28
OLZ						
1.21	1.20 ± 0.02	1.67	99.17	1.26 ± 0.07	5.56	104.13
4.98	5.15 ± 0.16	3.11	103.41	4.94 ± 0.22	4.45	99.20
44.74	46.02 ± 1.04	2.26	102.86	45.35 ± 1.94	4.28	101.36
81.32	79.33 ± 1.65	2.08	97.55	81.27 ± 2.92	3.59	99.94

Table 2. Stability of RIS, 9-OHRIS and OLZ in human plasma *in vitro* under different conditions ($n = 6$)

Nominal concentration (ng/mL)	Bench-top (room temperature for 8 h)		Autosampler (8°C for 26 h)		Freeze–thaw cycles		Long-term stability (−80°C for 30 days)	
	Measured concentration (mean ± SD, ng/mL)	Bias (%)	Measured concentration (mean ± SD, ng/mL)	Bias (%)	Measured concentration (mean ± SD, ng/mL)	Bias (%)	Measured concentration (mean ± SD, ng/mL)	Bias (%)
RIS								
5.12	4.97 ± 0.10	−2.93	5.19 ± 0.05	1.37	4.91 ± 0.08	−4.10	5.25 ± 0.08	2.54
80.78	79.83 ± 1.03	−1.18	81.97 ± 1.32	1.47	79.91 ± 0.95	−1.08	82.57 ± 1.07	2.22
9-OHRIS								
5.10	5.25 ± 0.14	2.94	5.04 ± 0.21	−1.18	5.23 ± 0.30	2.55	4.99 ± 0.23	−2.16
81.48	82.12 ± 2.51	0.79	79.73 ± 2.15	−2.15	80.04 ± 0.72	−1.77	82.41 ± 2.18	1.14
OLZ								
4.98	4.93 ± 0.17	−1.00	5.07 ± 0.19	1.81	5.03 ± 0.16	1.00	5.02 ± 0.26	0.80
81.32	79.87 ± 1.95	−1.78	79.04 ± 2.25	−2.80	80.04 ± 0.72	−1.57	78.91 ± 1.95	−2.96

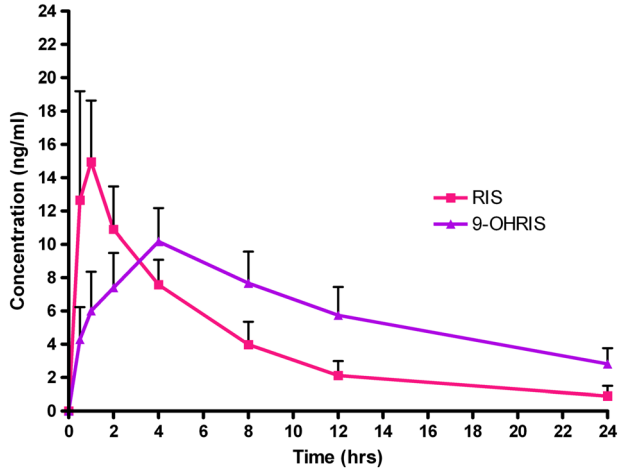

Figure 2. Mean plasma concentration–time profile of RIS and 9-OHRIS in human plasma after oral administration of 2 mg of risperidone tablet.

103.93 ± 32.38 (ng h/mL), which were less than the corresponding values for its metabolite (141. 80 ± 32.41 and 191.80 ± 49.81 ng h/mL, respectively). Moreover, the corresponding plasma half-lives for the parent and its major metabolite were 6.17 ± 1.76 and 11.54 ± 3.11 h, respectively. Finally, the first-order elimination rate constants associated with the terminal part of plasma concentration–time curves were 0.12 ± 0.04 and 0.06 ± 0.02/h, respectively. Additionally, we calculated the apparent volumes of distribution and clearance of the parent to be 2.49 ± 0.53 L/kg and 4.95 ± 1.36 mL/min kg, respectively. The corresponding values for the active metabolite of RIS in the participants were 2.56 ± 0.60 L/kg and 2.67 ± 0.79 mL/min kg. The values reported for these pharmacokinetic parameters were comparable to previously reported ones (Liu *et al*., 2013). The profile of mean plasma concentration of RIS and 9-OHRIS vs time is shown in Fig. 2.

Conclusion

A simple, sensitive and reliable UPLC method has been developed and validated for simultaneous determination of OLZ, RIS and 9-OHRIS in human plasma *in vitro*. This UPLC method has significant advantages including good resolution between peaks and adequate extraction recovery with shorter chromatographic run time. The developed method was successfully adopted to evaluate the pharmacokinetic parameters of a second-generation antipsychotic drug, RIS, and its major metabolite, 9-OHRIS, in human plasma. It can be employed in therapeutic drug monitoring practices and clinical toxicological assays. This method has similar sensitivity to other methods developed using LC-MS/MS.

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FAST AND SENSITIVE VALIDATED METHOD FOR QUANTITATION OF HALOPERIDOL IN HUMAN PLASMA USING ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY (UPLC) WITH DIODE ARRAY DETECTION

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ABSTRACT

A simple, sensitive and rapid ultra-performance liquid chromatography (UPLC) method has been developed and validated for estimation of haloperidol (HAL) in human plasma in vitro. Sample was prepared by simple liquid-liquid extraction technique. Chromatographic separation of HAL and IS was achieved by using Acquity UPLC BEH shield RP18 column and maintained at 35ºC temperature. The mobile phase consist of a mixture of 12 mM ammonium acetate buffer pH 3.5 adjusted with acetic acid (80%, v/v) and acetonitrile (20%, v/v) at a flow rate of 0.4 mL/min. Quantification was carried out on a photodiode array detector set at 240 nm. HAL and IS retention time were found to be 1.8 and 1.4 respectively. The total run time was 2.5 min. The method was validated for specificity, selectivity, recovery, accuracy, precision, recovery and stability. The calibration curve was linear the concentration range of 1 to 100 ng/mL. The method can be employed in therapeutic drug monitoring practices and clinical toxicological assays.

KEYWORDS: UPLC; Haloperidol; Therapeutic drug monitoring; human plasma.

INTRODUCTION

Antipsychotic agents are used to manage the psychotic episodes and behavioural symptoms in psychiatric patients. First generation Antipsychotics (haloperidol and chlorpromazine) are popular for the treatment of schizophrenia.[1] Haloperidol (HAL) is a known dopamine antagonist and has been widely used in the treatment of psychosis.[2] In addition, the drug has established that the notable value for determining poor compliance of patients in the Therapeutic Drug Monitoring (TDM) studies. Therefore, highly selective, sensitive and accurate bio-analytical methods are essential since most of these drugs are presents in low concentration in plasma.[3]

Several analytical methods have been described for determination of antipsychotics using HPLC; hence these methods have various disadvantages in terms of expensive solvent, complex sample extraction and run time. In previous studies, different High Performance Liquid Chromatography (HPLC) methods with UV detection[4,5], caplillary gas-liquid chromatography[6], coulometric[7,8] and fluorescence detection[9,10] were reported to detect and quantify the antipsychotic medications. Recently numerous studies have developed methods base on Liquid chromatography coupled with mass spectrometry (LC-MS/MS) to have increased sensitivity in detection with less run time.[11,12] Although LC-MS/MS methods are beneficial in many ways, the costly instrumentation and less accessibility to regular hospital practice and laboratories are major limitations.

The aim of the study is to establish a rapid, simple and accurate UPLC method to detect and quantify the HAL in plasma. In this view, a simple, sensitive and reproducible Ultra High Performance Liquid Chromatography (UPLC) with Diode Array Detection (DAD) for HAL was developed and validated. The method has less run time, can able to transfer to LC-MS/MS if needed as compatible buffer and chromatographic conditions were used in determination. It can be employed in therapeutic drug monitoring and clinical toxicological studies.

MATERIALS AND METHODS

Chemicals and reagents

Haloperidol (HAL) and clozapine (IS) were purchased from Sigma-Aldrich, India. Acetic acid and Methyl tert-butyl ether of HPLC grade were obtained from Himedia, India. The HPLC grade acetonitrile and ammonium acetate were purchased from Sigma-Aldrich, India.

Human blank plasma was obtained from blood bank at PSG Hospitals, Coimbatore. Ultrapure water was obtained using a milli-Q system from Millipore (Milford, USA).

Instrumentation and Chromatography conditions

Analysis was performed on an UPLC system that consisted of a Waters Acquity H class UPLC system equipped with a quaternary pump and 96-vial autosampler coupled with a diode array UV detector (Waters, Milford, MA, USA) set at 240 nm. The analysis was carried out on an Acquity UPLC BEH C18 column from Waters (2.1 mm × 100mm; 1.7µm) and the column temperature maintained at 35ºC and the autosampler was kept at 8ºC. The mobile phase consisted of a mixture of 12 mM ammonium acetate buffer pH 3.5 adjusted with acetic acid (80%, v/v) and acetonitrile (20%, v/v) at a flow rate of 0.4 mL/min. The total run time was 2.5 min and the injection volume was 5µL.

Preparation of stock and standard solution

Stock solution of HAL and IS were prepared at a concentration of 1 mg/mL. The stock solution of HAL and IS were stored at 4ºC and were found to be stable for one month. Further dilution was made in methanol: water (50:50. v/v) to produce working stock solution for the calibration standards and quality control (QC) standards. The IS working stock solution of 250 µg/mL was prepared in methanol-water (50:50 v/v) by dilution of the stock solutions. Calibration samples were prepared by spiking 240 µL of blank human plasma with the appropriate working solution of HAL (10µL) on the day of analysis. All the samples were stored at -80 ± 10°C until analysis.

Sample preparation

A liquid-liquid extraction method was carried out for extraction of HAL from human plasma. To an aliquot of 250µL of plasma, IS solution (25µL of 5µg/mL) and 12mM ammonium acetate (pH-9.0) were added and mixed for 30 seconds on a spinix vortex shaker (Tarsons, India). After the addition of 3.0 mL of tert-butyl methyl ether, the mixture was vortexed for 5 min and followed by centrifuged at 10,000 rpm for 5 min at 4ºC on an eppendrof 5810R centrifuge (Eppendrof AG, Hamburg, Germany). The clear organic layer (2.5mL) was separated into 5mL polypropylene tubes and evaporated to dryness at 40ºC using nitrogen evaporator (Turebovap®, Biotage, USA). The residue was reconstituted in 200µL of the mobile phase, vortex mixed for 3.0 min and 5µL were injected onto the UPLC system for analysis.

METHOD VALIDATION

The present method was validated for specificity, linearity, accuracy, precision, recovery and stability using Food and Drug Administration (FDA) guidelines[13] for the assay in human plasma.

Specificity

Specificity of the method was determination by analysing six replicates of blank plasma obtained from six different donors and investigating the possible interference at the LC peak region for HAL and IS. The acceptance criterion was that at least five out of six lots should have response less than five times the lower limit of quantitation (LLOQ) level in the same matrix.

Linearity

Linearity was assessed by linear regression analysis with the use of working standard solutions and spiked biological samples containing the drugs of interest at different concentrations within the range of 1.0 to 100 ng/mL. The calibration curve were constructing by plotting the ratio of the peak area of each analyte to the peak area of the IS versus the normal drug concentration. The slopes, intercept and coefficients of determination were calculated with least square linear regression analysis of the data with the use of a $1/x^2$ (x-concentration) weighting factor. The acceptance criteria for each back calculated standard concentration were ±15% deviation from the nominal value except at LLOQ which was set at ±20%.[13]

Accuracy and precision

Accuracy and precision for intra- and inter-day assay samples were estimated by analysing six replicates containing HAL at three different QC levels, that is, 5, 50 and 85ng/mL. Accuracy, which represents the closeness of agreement between the mean values obtained from the series of measurements by the method to the actual value. The accuracy should be within 85-115% of the nominal value, except at LLOQ, where it should not deviate more than 20 %.[13]

Recovery

The efficiency of HAL and IS extraction from the human plasma was determined by comparing the responses of the analyte extracted from replicate QC samples (n=6) with the response of analyte from the neat standards at equivalent concentration by a liquid-liquid

extraction process. HAL recovery was assessed by analytes of the three various concentration (5, 50, 85 ng/mL) while the recovery of the IS was determined at a single concentration of 2 µg/mL.

Stability

Stability assessment was conducted to evaluate the stability of HAL in human plasma samples under different conditions. Freeze-thaw (three cycles) and long-term stability was assessed by QC samples stored at 30 days at -80ºC. Bench-top stability study evaluate the stability of analyte in plasma at room temperature was determined at low (5 ng/mL) and high (85 ng/mL) QC concentration for 8 h.

RESULTS AND DISCUSSION

Specificity

Specificity of the developed method was determined by comparing the chromatogram of blank plasma, blank plasma spiked with IS and with analyte (1ng/mL). As shown in Fig. 1 and Fig. 2, no interfering peaks from endogenous compounds were observed at the retention time of analyte and IS. The total chromatographic run time was 2.5 min.

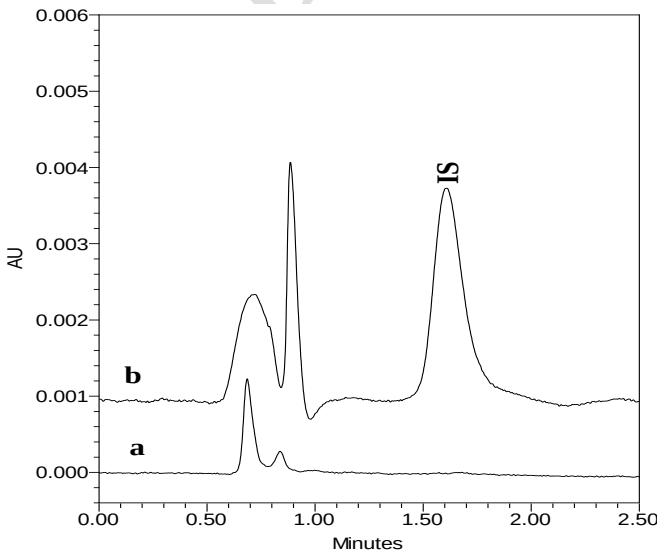

Fig. 1. Representative UPLC chromatograms of (a) blank plasma sample; (b) blank plasma sample spiked with IS.

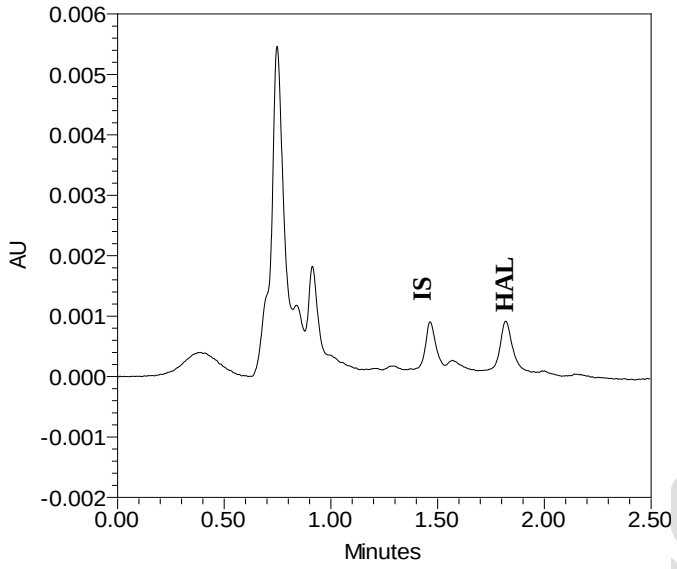

Fig. 2. Representative UPLC chromatograms of blank plasma spiked with analyte (1ng/mL).

Linearity

The quantification of HAL was based on the area ratio of the analyte over the IS vs HAL concentration. The plasma calibration curve was constructed using eight calibration standards (viz., 1-100 ng/mL) in human plasma and showed excellent linearity with regression coefficient ($r^2 > 0.991$). The standard curve had a consistent reproducibility over the standard concentration across the calibration range. The calibration curve was prepared by determining the best fit of peak-area ratio (peak area analyte / peak area IS) vs concentration and fitted to the y=mx+c using weighting factor ($1/x^2$). The lowest concentration with the RSD < 20% was taken as the LLOQ and was found to be 1.0 ng/mL for HAL. The percentage accuracy observed for the mean of back-calculated concentration for four calibration curves for the HAL was within 92.72-106.57.

Accuracy and precision

Accuracy and precision data for intra- and inter-day human plasma samples are presented in Table 1. The assay values on both the occurrences (intra- and inter-day) were found to be within the accepted variable limits.

Table 1. Intra- and inter-day precision and accuracy of HAL in human plasma *in vitro*.

Nominal concentration (ng/mL)	Intra-day (n = 6)			Inter-day (n = 18)		
	Measured concentration (mean ± SD),	CV (%)	Accuracy (%)	Measured concentration (mean ± SD),	CV (%)	Accuracy (%)
HAL						
4.94	4.85 ± 0.13	2.68	98.18	4.82 ± 0.14	2.90	97.57
50.16	48.76 ± 0.67	1.37	97.21	49.34 ± 1.37	2.78	98.37
84.92	84.55 ± 2.69	3.18	99.56	83.80 ± 2.22	2.65	98.68

Recovery

Liquid-liquid extraction method gave adequate recovery and cleaner samples. Comparison of neat standards vs human plasma-extracted standards were estimated for HAL (5, 50, 85 ng/mL) and the mean recovery was ranged from 81.51 ± 3.51, 78.91 ± 1.70 and 81.18 ± 1.86%, respectively. The recovery of IS at 2 µg/mL was 77.82 ± 1.61%.

Stability

The expected concentration of HAL at 5 and 85 ng/mL samples deviated within ±15% of the nominal concentrations in a series of stability test, viz. Bench-top (8 h), three freeze-thaw cycles and long-term stability at -80ºC for 30 days (Table 2). The results were found to be within the assay variability limits during the entire process.

Table 2. Stability of HAL in human plasma *in vitro* under different conditions (n=6).

Nominal concentration (ng/mL)	Bench –top (room temperature for 8 h)		Autosampler (8°C for 26 h)		Freeze-thaw cycles		Long-term stability (-80°C for 30 days)	
	Measured concentration (mean ± SD, ng/mL)	Bias (%)	Measured concentration (mean ± SD, ng/mL)	Bias (%)	Measured concentration (mean ± SD, ng/mL)	Bias (%)	Measured concentration (mean ± SD, ng/mL)	Bias (%)
HAL								
4.94	4.89 ± 0.16	-1.02	4.91 ± 0.17	-0.61	4.95 ± 0.17	0.20	5.01 ± 0.12	1.40
84.92	85.05 ± 1.82	0.15	83.28 ± 1.99	-1.97	83.53 ± 0.56	-1.66	83.36 ± 1.91	-1.87

CONCLUSION

This paper describes a rapid, sensitive and specific UPLC-DAD method to quantify the HAL and its validation for the analysis of HAL in human plasma. The UPLC-DAD method has significant advantages over other techniques including the simplicity of shorter

chromatographic run time, sharper peaks and higher extraction recovery. It can be employed in therapeutic drug monitoring practices and clinical toxicological assays. This method provides similar sensitivity like LC-MS/MS and can be used as an alternative method with a highly cost effective and sensitive analytes yield.

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plasma by high-performance liquid chromatography with coulometric detection. Ther Drug Monit., 1988; 10: 177-183.

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Ethical Committee Certificate

PSG Institute of Medical Sciences & Research
Institutional Human Ethics Committee

POST BOX NO. 1674, PEELAMEDU, COIMBATORE 641 004, TAMIL NADU, INDIA
Phone : 91 422 - 2598822, 2570170, Fax : 91 422 - 2594400, Email : psgethics2005@yahoo.co.in

Proposal Number	:	**12/082**
Project Title	:	**Population pharmacokinetic analysis of centrally acting drugs metabolized by CYP2D6**
Investigator(s)	:	**Dr M Ramanathan [1]** **Dr S Ramalingam [2]** **Mr M Siva Selvakumar [1]** **Dr K Ramadoss [2]** **Dr G Raghuithaman [2]**
Institution	:	**PSG College of Pharmacy [1]** **PSGIMS & R [2]**
Waiver of Consent	:	**No**
Review Type	:	**Exempt**
Date of the Meeting	:	**N/A**
Decision	:	**Approved**
Approval Date	:	**11.05.2012**
Validity of the Approval	:	**One year**

Approval for this study is given under the following terms and conditions:

1. Non-adherence to the Standard Operating Procedures (SOP) of the Institutional Human Ethics Committee (IHEC) and national and international ethical guidelines shall result in withdrawal of approval (suspension or termination of the study). SOP will be revised from time to time and revisions are applicable prospectively to ongoing studies approved prior to such revisions.
2. PIs are required to send progress reports (in the form of an extended abstract with publications if any) to the IHEC every six months (and a month before expiry of approval date, if renewal of approval is being sought).
3. Request for renewal must be made at least a month ahead of the expiry of validity along with a copy of the progress report.

11/05/2012

Dr Y S Sivan
Member - Secretary

SECRETARY
PSG IMS & R
COIMBATORE-641 004
INSTITUTIONAL HUMAN ETHICS COMMITTEE

PSG Institute of Medical Sciences & Research
Institutional Human Ethics Committee

POST BOX NO. 1674, PEELAMEDU, COIMBATORE 641 004, TAMIL NADU, INDIA
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April 19, 2013

To
Dr M Ramanathan
Principal
PSG College of Pharmacy
Coimbatore

The Institutional Human Ethics Committee, PSG IMS & R, Coimbatore -4, has reviewed your proposal on 18th April, 2013 in its expedited review meeting held at College Council Room, PSG IMS&R, between 1.30 pm and 2.30 pm, and discussed your application to renew the study entitled:

"Population pharmacokinetic analysis of centrally acting drugs metabolized by CYP2D6"

The following documents were received for review:

1. Application for renewal
2. Status report of the study

After due consideration, the Committee has decided to renew the approval for the above study.

The members who attended the meeting held on at which your proposal was discussed, are listed below:

Name	Qualification	Responsibility in IHEC	Gender	Affiliation to the Institution Yes/No	Present at the meeting Yes/No
Dr P Sathyan	DO, DNB	Clinician, Chairperson	Male	No	Yes
Dr S Bhuvaneshwari	M.D	Clinical Pharmacologist Member – Secretary	Female	Yes	Yes
Dr Sudha Ramalingam	M.D	Epidemiologist Alt. Member – Secretary	Female	Yes	Yes
Dr Y S Sivan	Ph D	Member – Social Scientist	Male	Yes	Yes
Dr D Vijaya	Ph D	Member – Basic Scientist	Female	Yes	Yes

The renewal is valid for one year (From 11.05.2013 to 10.05.2014).

This Ethics Committee is organized and operates according to Good Clinical Practice and Schedule Y requirements.

Non-adherence to the Standard Operating Procedures (SOP) of the Institutional Human Ethics Committee (IHEC) and national and international ethical guidelines shall result in withdrawal of approval (suspension or termination of the study). SOP will be revised from time to time and revisions are applicable prospectively to ongoing studies approved prior to such revisions.

Yours truly,

19.4.13

Dr S Bhuvaneshwari
Member - Secretary

SECRETARY
PSG IMS & R
COIMBATORE-641 004
INSTITUTIONAL HUMAN ETHICS COMMITTEE

Proposal No. 12/082

PSG Institute of Medical Sciences & Research
Institutional Human Ethics Committee

Recognized by The Strategic Initiative for Developing Capacity in Ethical Review (SIDCER)
POST BOX NO. 1674, PEELAMEDU, COIMBATORE 641 004, TAMIL NADU, INDIA
Phone : 91 422 - 2598822, 2570170, Fax : 91 422 - 2594400, Email : ihec@psgimsr.ac.in

April 11, 2014

To
Dr M Ramanathan
Principal
PSG College of Pharmacy
Coimbatore

The Institutional Human Ethics Committee, PSG IMS & R, Coimbatore -4, has reviewed your proposal on 11th April, 2014 in its expedited review meeting held at IHEC Secretariat, PSG IMS&R, between 10.00 am and 11.00 am, and discussed your application to renew the study entitled:

" Population pharmacokinetic analysis of centrally acting drugs metabolized by CYP2D6"

The following documents were received for review:

1. Your letter dated 10.04.2014
2. Application for renewal
3. Status report of the study
4. Amendment approval letter dated 08.04.2014

After due consideration, the Committee has decided to renew the approval for the above study.

The members who attended the meeting held on at which your proposal was discussed, are listed below:

Name	Qualification	Responsibility in IHEC	Gender	Affiliation to the Institution Yes/No	Present at the meeting Yes/No
Dr P Sathyan	DO, DNB	Clinician, Chairperson	Male	No	Yes
Dr S Bhuvaneshwari	M.D	Clinical Pharmacologist Member - Secretary	Female	Yes	Yes
Dr Sudha Ramalingam	M.D	Epidemiologist Alt. Member - Secretary	Female	Yes	Yes
Dr Y S Sivan	Ph D	Member –Social Scientist	Male	Yes	Yes

The renewal is valid for one year (From 11.05.2014 to 10.05.2015).

This Ethics Committee is organized and operates according to Good Clinical Practice and Schedule Y requirements.

Non-adherence to the Standard Operating Procedures (SOP) of the Institutional Human Ethics Committee (IHEC) and national and international ethical guidelines shall result in withdrawal of approval (suspension or termination of the study). SOP will be revised from time to time and revisions are applicable prospectively to ongoing studies approved prior to such revisions.

Kindly note this approval is subject to ratification in the forthcoming full board review meeting of the IHEC.

Yours truly,

11.4.14

SECRETARY
PSG IMS&R
COIMBATORE-641004.
INSTITUTIONAL HUMAN ETHICS COMMITTEE

Dr S Bhuvaneshwari
Member – Secretary