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Genetic influence on enantiomeric drug disposition:

Focus on venlafaxine and citalopram

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To my beloved family,

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ABSTRACT

A molecule that is not identical to its mirror image is said to be chiral. A racemic mixture, or a racemate, is one that has equal amounts of S- and R-enantiomers of a chiral molecule. Two examples of frequently prescribed racemic drugs are the antidepressants venlafaxine (VEN) and citalopram (CIT). The R-enantiomer of VEN is a potent inhibitor of serotonin and noradrenaline reuptake, while the S-enantiomer is more selective in inhibiting serotonin reuptake. CIT is a selective serotonin reuptake inhibitor and the S-enantiomer is responsible for this effect. The R-enantiomer of CIT is therapeutically inactive, but displays other effects or side-effects. Due to the potential of different pharmacological and toxicological activities of the VEN and CIT enantiomers, it is of great interest to investigate the individual enantiomers of these drugs, concerning both pharmacokinetics and pharmacodynamics. For this purpose, it is necessary to develop stereoselective bioanalytical methods. A major clinical problem in the use of many drugs is the inter-individual variability in drug metabolism and response. Genetic variations contribute to this variability, including e.g. polymorphisms in the cytochrome P450 (CYP) enzymes. Approximately 7% of all Caucasians lack the polymorphic isoenzyme CYP2D6 and these individuals are classified as poor metabolisers. Both VEN and CIT are partly metabolised by CYP2D6. However, it is not completely known how CYP2D6 deficiency may influence the in vivo pharmacokinetics of these drugs, especially regarding the enantiomeric disposition. The overall aim of this thesis was to study the relationship between pharmacokinetics and pharmacogenetics for VEN and CIT, with emphasis on enantiomeric drug disposition in different biomatrices. In *Paper I*, a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for enantioselective determination

of VEN and its three major metabolites was developed and applied in plasma from patients and whole blood samples from forensic autopsy cases. In *Papers II and III*, the genetic influence on enantiomeric drug disposition in serum and brain following administration of racemic CIT and VEN to Sprague-Dawley and Dark Agouti rats was studied. The female Sprague-Dawley and Dark Agouti rats are considered the animal counterparts of the human extensive and poor metaboliser CYP2D6 phenotypes, respectively. Significant quantitative strain-related differences in the pharmacokinetics of CIT and VEN, and their metabolites, were observed. The results indicate that the CYP2D enzymes display a significant impact on the stereoselective metabolism of these drugs. The findings also highlight the importance of comparing different rat strains when conducting experimental pharmacokinetic studies. In *Paper IV*, the relation between *CYP2D6* genotype and the disposition of the enantiomers of VEN and its metabolites in femoral blood from forensic autopsy cases was studied. A substantial variation in the relationship between the S- and R-enantiomers of VEN, and metabolites, was found. In individuals lacking two functional *CYP2D6* alleles, a low enantiomeric S/R VEN ratio was strongly related to a high S/R ratio for the main metabolite O-desmethylvenlafaxine. Hence, by using enantioselective analysis of VEN and O-desmethylvenlafaxine, it is possible to predict if a person is a poor metaboliser genotype/phenotype for CYP2D6.

LIST OF PAPERS

This thesis is based on the following publications, referred to in the text by their designated Roman numerals (I-IV).

I. Kingbäck M, Josefsson M, Karlsson L, Ahlner J, Bengtsson F, Kugelberg FC, Carlsson B. **Stereoselective determination of venlafaxine and its three demethylated metabolites in human plasma and whole blood by liquid chromatography with electrospray tandem mass spectrometric detection and solid phase extraction.** *Journal of Pharmaceutical and Biomedical Analysis, 2010, 53(3):583-590.*

II. Kingbäck M, Carlsson B, Ahlner J, Bengtsson F, Kugelberg FC. **Cytochrome P450-dependent disposition of the enantiomers of citalopram and its metabolites: in vivo studies in Sprague-Dawley and Dark Agouti rats.** *Chirality, 2011, 23(2):172-177.*

III. Kingbäck M, Karlsson L, Carlsson B, Josefsson M, Ahlner J, Bengtsson F, Kugelberg FC. **Pharmacokinetic differences in the disposition of the enantiomers of venlafaxine and its metabolites in Sprague-Dawley and Dark Agouti rats.** *Manuscript.*

IV. Kingbäck M, Karlsson L, Zackrisson AL, Carlsson B, Josefsson M, Bengtsson F, Ahlner J, Kugelberg FC. **Influence of *CYP2D6* genotype on the disposition of venlafaxine and its three major metabolites in postmortem femoral blood.** *Forensic Science International, Epub 2011 Aug 11.*

Other publications that are not included in the thesis, but that are methodologically related:

1. Kugelberg FC, Kingbäck M, Carlsson B, Druid H. **Early-phase postmortem redistribution of the enantiomers of citalopram and its demethylated metabolites in rats.** *Journal of Analytical Toxicology, 2005, 29(4):223-228.*

2. Kugelberg FC, Alkass K, Kingbäck M, Carlsson B, Druid H. **Influence of blood loss on the pharmacokinetics of citalopram.** *Forensic Science International, 2006, 161(2-3):163-168.*

ABBREVIATIONS

CIT	Citalopram
CNS	Central nervous system
CYP	Cytochrome P-450
CYP2D6	CYP2D6 (enzyme)
CYP2D6	CYP2D6 (gene)
DA	Dark Agouti
DCIT	Demethylcitalopram
DDCIT	Didemethylcitalopram
DDV	N-, O-didemethylvenlafaxine
EM	Extensive metaboliser
ESI	Electrospray ionisation
5-HT	Serotonin
HPLC	High-performance liquid chromatography
IM	Intermediate metaboliser
IS	Internal standard
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LC-UVD	Liquid chromatography ultraviolet detection
LOD	Limit of detection
LOQ	Limit of quantification
MRM	Multiple reaction monitoring
MS	Mass spectrometry
m/z	mass-to-charge

NA	Noradrenaline
NDV	N-desmethylvenlafaxine
ODV	O-desmethylvenlafaxine
P-gp	P-glycoprotein
PM	Poor metaboliser
QC	Quality control
SD	Sprague-Dawley
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
SNRI	Serotonin and noradrenaline reuptake inhibitor
SPE	Solid-phase extraction
SSRI	Selective serotonin reuptake inhibitor
S/R ratio	Ratio between concentration of S- and R-enantiomer
t½	Half-life
VEN	Venlafaxine
UM	Ultra-rapid metaboliser
UVD	Ultraviolet detection

INTRODUCTION

Pharmacology and toxicology

Pharmacology is described as the knowledge of drugs (from Greek *pharmakon*, poison in classic Greek; drug in modern Greek). Within this area, both basic and applied research is performed. The aim of pharmacological research is to increase the knowledge of the effect of action of drugs in order to improve the use of already available drugs and to promote the development of new, more effective drugs. In clinical pharmacology, the pharmacological knowledge is applied in the clinical practice and the effect of a drug on a patient is evaluated (Rang & Dale, 2011).

Pharmacodynamics can be described as the study of the effects of drugs on the body, the mechanisms of drug action and the relationship between drug concentration and effect (Tozer & Rowland, 2006; Rang & Dale, 2011). *Pharmacokinetics* describes the time course of the various events that a drug and its metabolites undergo in the body, such as absorption, distribution, metabolism and excretion. Absorption of a drug is defined as the passage of the drug from its site of administration (e.g. oral, sublingual, injection) into the blood circulation. When reaching the plasma, the drug binds to different plasma proteins, such as albumin. The drug is then distributed within the body. The most important organs for elimination of drugs are the liver, with its drug metabolising enzymes, and the kidneys. Before the drug reaches the systemic circulation, it passes through the liver, via the portal vein, where the drug is metabolised. Drug metabolism involves an enzymatic conversion of one chemical entity to another, and can be divided into two phases which both mainly takes place in the liver. Phase I reactions involve hydrolysis, reduction and oxidation. It is catabolic and usually results in more reactive, and sometimes more toxic, products.

Phase II is a synthetic reaction and include glucuronidation, sulfation, acetylation, methylation, conjugation with glutathione and conjugation with amino acids, and often results in inactive products. The products then leave the body by either renal drug excretion in the kidneys, the hepatobiliary system or by the lungs (Tozer & Rowland, 2006; Rang & Dale, 2011). *Toxicology* is described as the knowledge of the effects of harmful substances on living organisms. Toxicological research aims to increase the knowledge of how biological systems are affected by harmful substances. These studies are of great importance for research and development of drugs.

The result of drug therapy in a population can vary due to an extensive interindividual variability in drug metabolism and drug response. Several factors can influence an individual's drug response, such as genetic factors, gender, age, nutrition, enteropatic circulation, intestinal flora and ethnic background. Genetic variations accounts for about 20-40% of the interindividual variations in the response and metabolism of many commonly used drugs, and are predominately caused by inherited differences in the nucleotide sequences in the DNA, defined as genetic polymorphism (Ingelman-Sundberg, 2004). Genetic polymorphism can be seen in both drug metabolising enzymes, drug transporters and receptors. Consequently, it can affect drug metabolism, absorption, distribution and elimination, and hence, influencing the therapeutic and toxic effects of the drug. In humans, the most common source of genetic polymorphism is single nucleotide polymorphisms (SNPs), representing 90% of all polymorphisms. SNPs can consist of a nucleotide insertion, base pair substitution or deletion. The study of genetic variations and their effects on pharmacokinetics and pharmacodynamics is referred to as *pharmacogenetics*. The newer term *pharmacogenomics* refers to the general study of all

of the many different genes that determine drug behaviour. However, the two terms are often used interchangeably. Pharmacogenetic studies investigate the influence of single genes on interindividual variations in drug metabolism. *Genotype* is referred to as all the hereditary information an individual carries within its genetic code. The genotype does not change during a lifetime. Furthermore, an individual's *phenotype* is the actual observed characteristics such as morphology, behaviour and development. Because of changes in the environment and changes associated with aging, the phenotype can change during a life span (Zackrisson, 2009; Johansson & Ingelman-Sundberg, 2011). Due to genetic polymorphism of drug metabolising enzymes, resulting in variability in phenotype, enzyme activity can be classified into four major phenotypes; (1) ultra-rapid metabolisers (UM), carrying more than two active genes, (2) extensive metabolisers (EM), with two functional genes, (3) poor metabolizers (PM) lacking functional enzymes because defective or deleted genes and (4) intermediate metabolisers (IM) with partially decreasing enzyme activity resulting in reduced but not absent enzyme activity. Due to drug overdose or therapeutic failure as a result of poor metabolism of, for example, a prodrug to the active metabolite, PMs may possess an increased risk of adverse effects (Wolf & Smith, 1999; Musshoff *et al*., 2010; Pilgrim *et al*., 2011).

The cytochrome P450 system

The cytochrome P450 (CYP) enzymes are a superfamily of phase-I enzymes involved in the oxidative activation or deactivation of both endogenous and exogenous compounds such as drugs and toxins. The CYP enzymes are found in all living organisms. In humans, the CYP enzymes account for more than 75% of all drug metabolism (Guengrich *et al*., 2008). Each CYP family member is designated by a number, each subfamily by a letter and each member of the subfamily by a second

number e.g. *CYP2D6*. The CYP enzymes are mainly hepatic, however, many of the CYPs also exist in other organs, such as the brain (Zanger *et al*., 2004). Among the CYP enzymes, CYP1A2, CYP2C19, CYP2D6 and CYP3A4 are the most important enzymes involved in the metabolism of antidepressants or in the occurrence of drug interactions (Brøsen, 1996; Dahl, 2002; Kirchheiner *et al*., 2001; Meyer *et al*., 1996; Nemeroff *et al*., 1996; Poolsup *et al*., 2000; Tanaka & Hisawa, 1999). CYP in families 1-3 mediate about 70-80% of all phase-I dependent metabolism of clinically used drugs (Evans & Relling, 1999). The majority of these enzymes are polymorphic. However, the functional importance of these variants differs, as well as the frequency of their distribution in different ethnic groups. The polymorphic enzymes, in particular CYP2C9, CYP2C19 and CYP2D6, account for about 40% of all CYP mediated drug metabolism, which makes the dosing of drugs a problem. Polymorphism in the CYP genes can cause enzyme products with abolished, reduced, altered or increased enzyme activity (Ingelman-Sundberg, 2001). Polymorphism not only affects drug disposition but can also be important in the conversion of prodrugs to their active form. Genetically determined variability in expression or function of the CYP enzymes has been shown to have a profound effect on drug efficacy (Ingelman-Sundberg, 2001; 2004; Johansson & Ingelman-Sundberg, 2011). *CYP2C19* accounts for about 3% of the total CYP content in the liver (Scordo, 2002). About 2-5% of Caucasians and 13-23% in an Oriental population lack this enzyme and can be classified as PMs (Wilkinson *et al*., 1989). Of about one dozen human CYP enzymes that catalyse biotransformation of drugs, CYP2D6 is one of the most important ones based on the number of its drug substrates (Zanger *et al*., 2004). An estimated 20-25% of all drugs in clinical use are metabolized at least in part by CYP2D6 (Evans & Relling, 1999). CYP2D6 metabolizes a number of antidepressants, antipsychotics, β-adrenoreceptor blockers, and antiarrhythmic drugs (Dahl & Sjöqvist, 2000; Otani &

Aoshima, 2000; Poolsup *et al*., 2000). CYP2D6 accounts for about 2% of the total CYP content in the liver (Scordo & Spina, 2002), hence, CYP2D6 is expressed at relatively low levels as compared to the other hepatic CYP enzymes. In addition to the liver, CYP2D6 is also expressed at lower levels in extrahepatic tissues, such as the intestine, lungs and brain (Zanger, 2001). CYP2D6 shows a very high degree of inter-individual variability. Of more than 70 allelic variances described so far for CYP2D6, approximately 15 encode non-functional enzymes, whereas others encode for enzymes with reduced, normal or increased enzyme activity. This variability is primarily due to the extensive genetic polymorphism that influences expression and function. The polymorphism of CYP2D6 is termed "the desbrisoquine polymorphism" since desbrisoquine is metabolised by CYP2D6 (Zanger *et al*., 2004). Approximately 7-10% of all Caucasians lacks the functional activity of CYP2D6 and is classified as PMs for substrates of this enzyme (Gonzalez *et al*., 1988). Consequently, several million people are thus at risk for compromised metabolism or adverse drug reactions when prescribed drugs that are CYP2D6 substrates. This lead to impaired metabolism of many centrally acting drugs such as several antidepressants including citalopram and venlafaxine. The *CYP2D6* genotypes can be assigned based on the alleles identified (Table 1).

Alleles not carrying any of the determined polymorphisms are classified as **1* (wild-type). The outcomes of the genotype can be categorized into four groups: individuals carrying no active gene (i.e. carrier of only the **3*, **4*, **5* or **6* alleles, also known as PMs), individuals carrying one active gene (i.e. carrier of **1* in combination with one of the alleles **3*, **4*, **5* or **6*, also known as IMs) individuals with two active genes (i.e. carrier of two **1* alleles, also known as EMs) and individuals carrying more than two active genes (UMs).

Table 1. *CYP2D6* genetic alleles variants.

Allele	Nucleotide change, cDNA	RefSNP ID	Effect on protein	Enzyme activity
*CYP2D6*1*	wild-type			normal
*CYP2D6*1xN*	wild-type and gene duplication		xN active genes	increased
*CYP2D6*3*	2549delA	rs35742686	frameshift	none
*CYP2D6*4*	1846G>A	rs3892097	splicing defect	none
*CYP2D6*4xN*	1846G>A and gene duplication		xN inactive genes	none
*CYP2D6*5*	gene duplication		CYP2D6 deleted	none
*CYP2D6*6*	1707delT	rs5030655	frameshift	none

Animal models

Although experiments in isolated *in vitro* systems are important contributors to understanding the underlying mechanisms of drug action and disposition, *in vivo* studies in animal models are necessary in order to investigate the influence of a substance on the whole body system. An advantage with using animal models is that a unique knowledge can be received concerning the events of psychoactive substances at the site of action i.e. in the brain, which is more difficult to study in humans. Hence, when investigating pharmacokinetics, pharmacodynamics and pharmacogenetics of CNS acting drugs, different animal models are valuable complements to human studies (Kraemer *et al.*, 2004; Kugelberg *et al.*, 2001; 2002). Toxicological testing in animals is used to define the upper limits of exposure to be tested in human studies. In addition, the results from pharmacodynamic studies are used to identify concentration ranges where optimal therapeutic effects are likely to be observed (Gill *et al.*, 1989; Mashimoto & Serikawa, 2009; Amore *et al.*, 2010). A wide range of species are used as animal models in scientific research. It is often a combination of previously done research using that type of animal, scientific relevance, availability and the feasibility of the experiment that decides which type of

animal is chosen for various studies. If the purpose is to use the animals as a model for a function or a disease that affects humans, it can be suitable to select an animal that resembles the human in that respect. Rats are used nearly universally for pharmacokinetic studies and have been used extensively for a long time in research. Hence, the physiology and functions of rats are very well known. For instance, the various centres in the brain are charted and nominal values for the kidneys, liver and heart are available in the literature. The Sprague-Dawley (SD) rat for example, is an outbred multipurpose breed of albino rat used extensively in medical research and is considered a general model for the study of human health and disease. The rat is used as a model for toxicology, reproduction, pharmacology, and behavioural research (Kugelberg *et al*., 2003; 2005; 2006; Shima *et al*., 2011). One of the major differences in pharmacokinetics between animals and humans is that the rate of drug elimination is faster in animals. This is especially true in small rodents (Fredricson Overø, 1982a; Howell *et al*., 1994).

Suitable animal models for *CYP2D6* polymorphism are of considerable interest, since the implications of the polymorphism for exogenous compounds can be difficult to study in humans. Female Dark Agouti (DA) rats have been shown to be a model of the human PM phenotype, since they show impaired metabolism for a number of CYP2D6 substrates. Both male and female SD rats are used as a model for the EM phenotype in respect to CYP2D6 (Al-Dabbagh *et al*., 1981; Gonzalez *et al*., 1987; Schultz-Utermoehl *et al*., 1999). The *CYP2D* subfamily has evolved differently in humans and rats. Isoenzymes of the human *CYP2D* subfamily are encoded by one active *CYP2D6* gene and two pseudogenes, while in the rat, six genes, *CYP2D1-5* and *CYP2D18*, have been identified (Gonzalez *et al*., 1988; Matsunaga *et al*., 1990). It is still unclear which of these six genes that is/are homologous to the human *CYP2D6*. It has

long been assumed that *CYP2D1* corresponds well with the human *CYP2D6* (Barham *et al*., 1994; Miksys *et al*., 2000). However, another study has shown that also *CYP2D2* corresponds well (Schultz-Utermoehl *et al*., 1999).

Forensic toxicology

Forensic toxicology comprises different fields such as toxicology, pharmacology and analytical chemistry. The purpose of forensic toxicology is to aid medical or legal investigation of death, poisoning and drug use. Accordingly, forensic toxicological analysis is performed in both living and deceased individuals, and typically involves measuring the concentrations of alcohol, licit and illicit drugs in the blood or urine of the subject, followed by a scientific interpretation of the results. Postmortem drug analysis, where measurements are performed on a deceased person, can be useful when trying to determine the circumstances of the fatality. A suspected intoxication can be verified, and a driver responsible for a traffic accident can be analysed for possible drug abuse. Postmortem analysis presents special challenges to the forensic toxicologist, the information present is often incomplete or ambiguous, which requires extra careful selection of methods and the inherent uncertainty must be considered when drawing conclusions based on the results. Interpretations may be aided by adding information regarding the metabolic capacity of the investigated individual (Drummer, 2007; Pilgrim *et al*., 2011). Pharmacogenetics can markedly influence an individual´s response to a drug, ultimately increasing the risk of fatal drug toxicity. Pharmacogenetic studies are therefore relevant in forensic toxicology and can be of value in the interpretation of drug related deaths, particularly in unintentional drug poisonings where the cause of death is unclear. Due to pharmacogenetic investigations, additional information about an individual´s

metabolic capacity and potential drug response may be obtained (Druid *et al.*, 1999; Wong *et al.*, 2003; Musshoff *et al.*, 2010; Sajantila *et al.*, 2010; Pilgrim *et al.*, 2011).

Chirality in pharmacology and toxicology

A molecule that is not identical to its mirror image is said to be chiral (Greek *cheir*, "hand"). A chiral molecule is one that does not contain a plane of symmetry. The usual cause of chirality is the presence of a tetrahedral carbon atom which is bound to four different groups, generating a so-called stereocenter. Chiral compounds can exist as a pair of mirror image stereoisomers called enantiomers, denoted S- (sinister) or R- (rectus) configuration, which are related to each other as a right hand is related to a left hand (Figure 1) (McMurry, 1998). A racemic mixture, or a racemate, is one that has equal amounts of S- and R-enantiomers of a chiral molecule. A single enantiomers is optically active while the racemate is optically inactive, which means that there is no net rotation of plane-polarized light. The reason for this is that the two enantiomers rotate plane-polarized light in opposite directions.

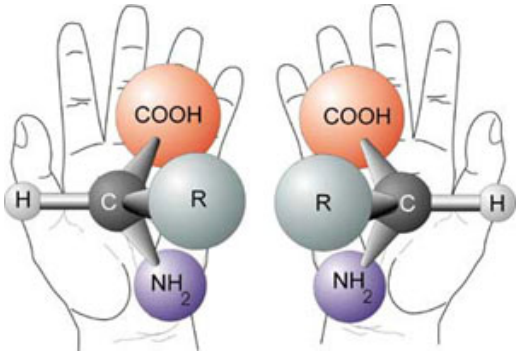

Figure 1. A pair of enantiomers, illustrating how they are related to each other as the right hand is related to the left (adapted from Reis *et al.*, 2006).

One important difference between two enantiomers is their interactions with other chiral molecules or substrates. Most major drug targets are chiral, including proteins, metabolic enzymes, receptor sites, lipids and steroids. If either the substrate or its binding site is chiral, the biological reaction is said to be stereoselective (Testa, 1986). As a result, enantiomers of a racemic drug often differ markedly in their pharmacokinetics, therapeutic efficacy, toxicology and other biological properties, and this incomplete picture has called for further attention. During the last 20 years, a great progress has been made concerning stereoselective chemical analysis and synthesis (Maier *et al*., 2001; Scriba, 2002). This has raised the importance of stereochemistry for the effect of drugs. Today, new chiral drugs are introduced as pure enantiomers, and already clinically established racemic drugs have been evaluated in order to investigate if one of the enantiomers has a clinical significant advantage as compared to the racemate.

In forensic toxicology, chiral bioanalysis is used to estimate illicit drug preparations and biological specimens. For example, it has been estimated that more than 50% of illicit drugs possess at least one chiral centre. Hence, chiral analysis has the potential to assist in determination of cause of death and help in correct interpretation of substance abuse (Smith *et al*., 2009). In 2001, Knowles, Noyori and Sharpless were awarded with the Noble Prize for the development of methods that synthesize only one of the stereochemic forms of a chiral molecule (for further details, see http://nobelprize.org/chemistry/laureates/2001).

Pharmacodynamics

Biological systems are chiral entities, and in this chiral environment enantiomers can experience stereoselective absorption, protein binding, transport, enzyme interactions, metabolism, receptor interactions and DNA-binding. For example, the drug efflux transporter P-glycoprotein (P-gp), which participates in drug absorption, distribution and excretion, is regulated stereospecifically (Uhr *et al*., 2003; Choong *et al*., 2010). Also, the property of protein binding may be influenced by chirality, albumin for example has stereospecific binding preferences (Chuang & Otagiri, 2006; Smith *et al*., 2009). The receptors are the major sites of drug action, and there are multiple examples of varied receptor types with chiral dependence. However, the magnitude of the differences between a pair of enantiomers in their pharmacokinetic parameters tends to be relatively modest in comparison to their pharmacodynamic properties (Hutt, 2007). Enantiomers of racemic drugs might, as already mentioned, possess different pharmacokinetic, pharmacodynamic, therapeutic, and adverse effect profiles (Figure 2).

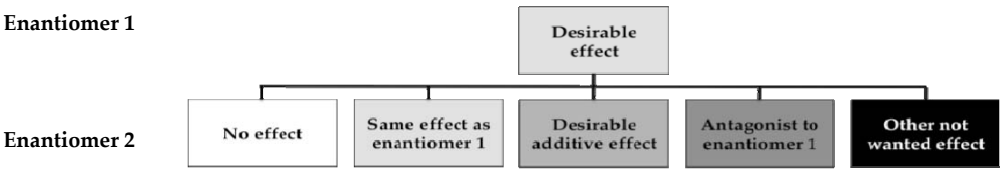

Figure 2. Enantiomeric interaction possibilities of enantiomer 1 and enantiomer 2.

The analgetic drug methadone is one example of a racemate with different pharmacodynamic properties in the separate enantiomers. The opioid activity of methadone resides in the R-enantiomer. Methadone is primarily metabolized by the CYP enzymes CYP3A4, CYP2B6, and CYP2C19, and to a lesser extent by CYP2C9

and CYP2D6 (Gerber *et al*., 2004; Totah *et al*., 2007). Genetic polymorphism, coupled with dose-dependent stereochemistry, might underlie the clinical toxicity seen following administration with methadone. Studies have shown that CYP2B6 displays stereo-preference for S-methadone, and PMs of CYP2B6 have been associated with a reduced ability to metabolize S-methadone, and an increased risk of prolonged QTc interval (Eap *et al*., 2007). Another example is thalidomide which was introduced as a sedative drug in the late 1950s. However, in 1961, it was withdrawn from the market due to teratogenicity and neuropathy, resulting in birth defects (Moghe *et al*., 2008). Studies showed that the R-enantiomer was responsible for the sedative effects (Höglund *et al*., 1998; Eriksson *et al*., 2000), whereas the S-enantiomer and its derivatives were reported to be teratogenic (Heger *et al*., 1994).

Pharmacokinetics

Many of the processes involved in pharmacokinetics involve a direct interaction with chiral biological macromolecules, such as transporters, membrane lipids and enzymes. Hence, following administration of a racemic drug, the individual enantiomers rarely exist in a 50:50 ratio in the body, also, they often exhibit different pharmacokinetic profiles. For example, one enantiomer of a racemic mixture may demonstrate the therapeutic activity of interest, while the second may contribute to adverse events or complicate assessments of absorption, distribution, metabolism and excretion (Ott & Giacomini, 1993; Hutt, 2007). Factors that influence the stereoselectivity of drug disposition are; formulation and route of administration, chemical and enzymatic *in vivo* stereochemical stability, drug interactions (both enantiomeric and with a second drug), disease state, age, gender, race and pharmacogenetics (Hutt, 2007). The anticoagulant drug warfarin is one example of a drug with stereoselective metabolism that shows a significant interpatient

metabolism and dosing requirements (Lindh *et al.*, 2009; Rane & Lindh, 2010). Warfarin is a racemic mixture, although the S-isoform is significantly more potent (Scott, 1993). The metabolism of the S-enantiomer occurs via CYP2C9, CYP3A4 and ketoreductase, whereas CYP1A2, CYP2C19, CYP3A4 and ketoreductase are responsible for the metabolism of the R-enantiomer. Thus, the possible influence of concomitant drugs on the various CYP enzymes involved can affect the clearance of both enantiomers. In addition, since the R-enantiomer inhibits the metabolism of the S-enantiomer at CYP2C9, impaired metabolism of R-warfarin may cause increased levels of the active S-isoform. CYP2C9 poor metabolizers have shown reduced activity and require lower warfarin doses (Rettie & Tai, 2002; Osman *et al.*, 2007; Au & Rettie, 2008).

Chiral antidepressant drugs

Many antidepressants, as well as their metabolites, are racemic mixtures, such as citalopram, venlafaxine, reboxetine, mirtazapine and fluoxetine. Selective serotonin reuptake inhibitors (SSRIs) have during the last 15-20 years become the preferable choice for the treatment of depression. Citalopram (Cipramil) is one example of the transition of a racemic drug to its pure active enantiomer, escitalopram (Cipralex) (Montgomery *et al.*, 2001; 2011; Garnock-Jones & McCormack, 2010). This type of transition is called "chiral switching" (Tucker, 2000; Núñez *et al.*, 2009). Venlafaxine (Effexor) is another widely used racemic antidepressant drug. In 2008, desvenlafaxine, the racemic succinate salt of the major active metabolite of venlafaxine, formed by the action of CYP2D6 on the parent compound to O-desmethylvenlafaxine, was approved for the treatment of depression by the Food and Drug Administration in the USA (Lourenco & Kennedy, 2009; Perry & Cassagnol, 2009).

Venlafaxine

The racemic drug venlafaxine (VEN) belongs to the pharmacodynamic class of dual serotonin and noradrenaline reuptake inhibitors (SNRIs) and is used for the treatment of psychiatric disorders (Holliday & Benfield, 1995). VEN is a bicyclic phenylethylamine compound and has a chiral centre which gives a racemic mixture of two enantiomers; S-(+)-venlafaxine (S-VEN) and R-(-)-venlafaxine (R-VEN) (Ellingrod & Perry, 1994).

Pharmacodynamics and pharmacokinetics

At lower doses, VEN is a potent serotonin (5-HT) reuptake inhibitor, and at higher doses, it is also a potent inhibitor of noradrenaline (NA) reuptake (Harvey *et al*., 2000). VEN has no affinity for adrenergic, serotoninergic, muscarinic or histaminergic receptors (Muth *et al*., 1986; Holliday & Benfield, 1995), but is to a lesser extent an inhibitor of presynaptic reuptake of dopamine (Muth *et al*., 1986). Both S- and R-VEN exhibit pharmacological activity. While the R-enantiomer is a potent inhibitor of both 5-HT and NA reuptake, the S-enantiomer is more selective in inhibiting primary 5-HT reuptake (Holliday & Benfield, 1995).

VEN is phase-I metabolized in the liver, mainly by the CYP system. The known major pathway for the metabolism of VEN is illustrated in Figure 3. In humans, VEN is metabolized by CYP2D6 to its main metabolite O-desmethylvenlafaxine (ODV) and by CYP3A4 to N-desmethylvenlafaxine (NDV). NDV is then further metabolised to N,O-didesmethylvenlafaxine (DDV), possibly by CYP2D6 (Muth *et al*., 1986; 1991). Further, some studies have showed support for a possible involvement of CYP2C9 and CYP2C19 in the metabolism (Fogelman *et al*., 1999; McAlpine, 2011). ODV contributes to the overall pharmacological effects of VEN since it exhibits a

pharmacological profile similar to that of VEN. NDV and DDV display less potent effects on 5-HT and NA reuptake compared with VEN and ODV (Muth *et al*., 1986; 1991; Otton *et al*., 1996; Fogelman *et al*., 1999). In humans, ODV is present at higher plasma concentrations than VEN itself after VEN administration (Howell *et al*., 1993). Time to peak plasma/serum levels of VEN is 0.5-1 h in rats and 1-2 h in humans (Howell *et al*., 1993; 1994). Corresponding levels for ODV are 0.5-1 h in rats and 4-5 h in humans, respectively. In humans, the mean half life (t½) of VEN and ODV is 5 h and 11 h, respectively. In rats, the t½ of VEN and ODV is 1 h (Howell *et al*., 1994).

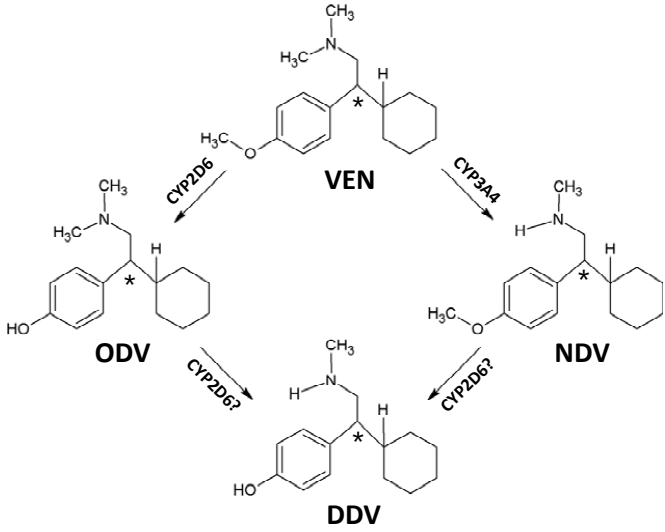

Figure 3. The metabolism of venlafaxine (VEN) to its main metabolites O-desmethylvenlafaxine (ODV), N-desmethylvenlafaxine (NDV) and N, O-didesmethylvenlafaxine (DDV). * = chiral center.

Toxicology

Many of the newer generations of antidepressants, including SSRIs and SNRIs, are known to have a low toxicity profile, and have been shown to be safer when overdosed as compared to the older tricyclic antidepressants (Henry *et al*., 1997;

Pacher *et al*., 1999). However, serious side effects have been observed after administration of higher doses (Grundemar *et al*., 1997). Finnish postmortem data suggests that VEN has a higher toxicity as compared to SSRIs (Koski *et al*., 2005). Overdoses with VEN have been associated with several adverse effects such as sedation, tachycardia, seizures, hypertension and serotonin syndrome (Schweizer *et al*., 1994; Ereshefsky *et al*., 1996) and fatal overdoses have been reported for VEN alone or in combination with other compounds (Settle *et al*., 1998; Mazur *et al*., 2003). Furthermore, it has been suggested that VEN may be more toxic in *CYP2D6* PMs (Lessard *et al*., 1999; Langford *et al*., 2002). Consequently, subjects who are *CYP2D6* poor metabolisers or who are taking interacting drugs may achieve drug concentrations similar to those found in overdose. Notably, no *in vivo* data describing the pharmacological effects of the VEN enantiomers have been reported so far.

Citalopram

Citalopram (CIT) belongs to the pharmacodynamic class of selective serotonin reuptake inhibitors (SSRIs) and is used for the treatment of psychiatric disorders (Hyttel & Larsen, 1985; Milne & Goa, 1991). CIT is a racemic bicyclic phthalane derivative and has a chiral center which gives a racemic mixture of two enantiomers; S-(+)-citalopram (S-CIT) and R-(-)-citalopram (R-CIT).

Pharmacodynamics and pharmacokinetics

CIT binds to the 5-HT transporter protein and thereby inhibit transport or uptake of 5-HT into serotonergic neurons. The inhibited transport or uptake of 5-HT into the serotonergic neurons results in an increased availability of 5-HT in the synaptic cleft. The S-enantiomer of CIT is the pharmacologically active component of racemic CIT

and mediates the antidepressant effect (Hyttel *et al*., 1992; Baumann & Eap, 2001; Baumann *et al*., 2002). Since a decade the S-enantiomer of CIT (escitalopram) is available as a separate SSRI (Montgomery *et al*., 2001). Compared with CIT, the metabolites are weaker and less selective 5-HT reuptake inhibitors, and are not considered to play a major role for the SSRI effect, with the exception of S-DCIT that possesses some activity (Milne & Goa, 1991). The metabolites are less lipophilic than the parent compound, hence, they enter the brain less readily than the parent compound. CIT, unlike tricyclic antidepressants, appears to have little effect on NA or dopamine systems. The known major pathway for metabolism of CIT is illustrated in Figure 4. In humans, racemic CIT is demethylated to demethylcitalopram (DCIT) by the CYP isoenzymes CYP3A4, CYP2C19 and CYP2D6 (Baumann *et al*., 2002). DCIT is then further demethylated by CYP2D6 to didemethylcitalopram (DDCIT). CIT and its metabolites are also oxidated by monoamine oxidase A (MAO-A) and MAO-B to citalopram propionic acid derivate and citalopram-N-oxide in both human liver and brain (Kosel *et al*., 2001; Rochat *et al*., 1998).

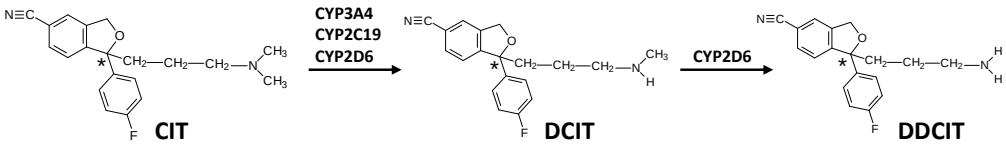

Figure 4. The metabolism of citalopram (CIT) to its main metabolites demethylcitalopram (DCIT) and didemethylcitalopram (DDCIT) by the cytochrome P450 enzymes. * = chiral center.

As CIT, the metabolites DCIT and DDCIT are chiral compounds and exist as enantiomers. CIT is bound to plasma protein to 80%, while the protein binding of the demethylated metabolite is 74%. It is widely distributed among peripheral tissues, with a volume of distribution estimated to 14 L/kg (Fredricson Overø, 1982; Joffe *et*

al., 1998; Kragh-Sorensen *et al*., 1981). The absorption is not affected by food, and its oral bioavailability is reported to be about 80 % (Joffe *et al*., 1998). There are no major qualitative differences in the route of metabolism between animals and humans, however, quantitative differences is seen (Baumann & Larsen, 1995). Time to peak plasma/serum levels of CIT is 0.5 h in rats and 2-5 h in humans. In humans, the mean t½ of CIT is 30-38 h, whereas the mean t½ for DCIT and DDCIT is 51 and 108 h, respectively (Fredricson Overø, 1982b; Sidhu *et al*., 1997). The t½ in rats is 3-7 h (Fredricson Overø, 1982a). The elimination of the S-enantiomer of CIT and its metabolites is faster than the elimination of the R-enantiomer (Sidhu *et al*., 1997; Kugelberg *et al*., 2001; 2003).

Toxicology

A study involving 469 cases of SSRI overdoses showed that SSRIs were relatively safe in overdose, nevertheless, seizures and coma occurred in several cases and serotonin syndrome was reported in 14% of the cases (Ibister *et al*., 2004). Overdoses with CIT have been associated to a risk of developing serious adverse effects such as electrocardiogram abnormalities and convulsions (Grundemar *et al*., 1997). Fatalities with CIT occur more frequently when it is combined with other drugs (Dams *et al*., 2001). However, the SSRIs appear to present a low risk of fatal poisoning when taken alone or in combination with alcohol (Koski *et al*., 2005). The disposition of the enantiomers of CIT and metabolites, in relation to *CYP2D6* and *CYP2C19* genotype distributions, has been reported in forensic autopsy cases (Holmgren *et al*., 2004; Carlsson *et al*., 2009).

Chiral bioanalysis

Conventional bioanalytical methods for drug analysis do not often differentiate the enantiomers of racemic drugs. However, due to the potential of different pharmacological and toxicological activities of the enantiomers of racemic drugs, there is of great interest to study the individual enantiomers of such drugs more profound, concerning both pharmacodynamic and pharmacokinetic properties. For this purpose, development of stereoselective bioanalytical methods is necessary. Today, chiral separation and bioanalysis has become one of the most active areas of analytical chemistry and the advances within chiral separation techniques have made the measurement of the concentrations of the individual enantiomers in biological fluids possible (Carlsson, 2003; Misl'anová & Hutta, 2003; Lämmerhofer, 2010).

Chiral separation by HPLC

During the last decades, several fundamental problems concerning separation of enantiomers have been solved and a number of analytical tools have been established. In the field of bioanalysis, high-performance liquid chromatography (HPLC) has been established as the major technique for enantioseparation (Maier *et al*., 2001; Scriba, 2002; Misl'anová & Hutta, 2003; Lämmerhofer, 2010). Two different strategies for separation of enantiomers can be used; i.e. indirect or direct separation. The indirect method is based on the formation of a pair of diastereoisomers of the racemic mixtures by derivitisation with a chiral reagent and separation with conventional HPLC. The direct approach, however, utilizes chiral discrimination achieved by a chiral selector. The basic principle for the direct separation of the enantiomers is the temporary diasteromeric complexes that are formed. The chiral selector may be a mobile-phase additive or the stationary phase in the

chromatographic column. A variety of chiral stationary phases are now available for the separation of enantiomers by HPLC, and they have been shown to be very useful in the chromatographic resolution of a wide range of racemic mixtures, i.e. drugs and metabolites (Lämmerhofer, 2010). There are several types or classes of chiral stationary phases available. Phases based on macrocyclic antibiotics and cyclodextrins are commercially available and have, since their introduction, become popular and have proven useful as chiral stationary phases in HPLC due to their abilities to handle a large spectrum of analytes (Armstrong, 1994; Armstrong & Zhang, 2001). Macrocyclic antibiotics possess a great number of stereogenic centres and functional groups which allows multiple interactions with chiral molecules (Ward & Farris, 2001). In HPLC these columns are mainly used in reversed phase modes but normal phase mode and polar organic mode have also been used (Bressolle *et al.*, 1996; Berthod *et al.*, 2004; Desai & Armstrong, 2004; Bosakova *et al.*, 2005; Berthod, 2009). The macrocyclic antibiotics vancomycin (Figure 5), ristocetin A, teicoplanin, avoparcin, rifamycin B and thiostrepton have been used for chiral separations (Ward & Farris, 2001).

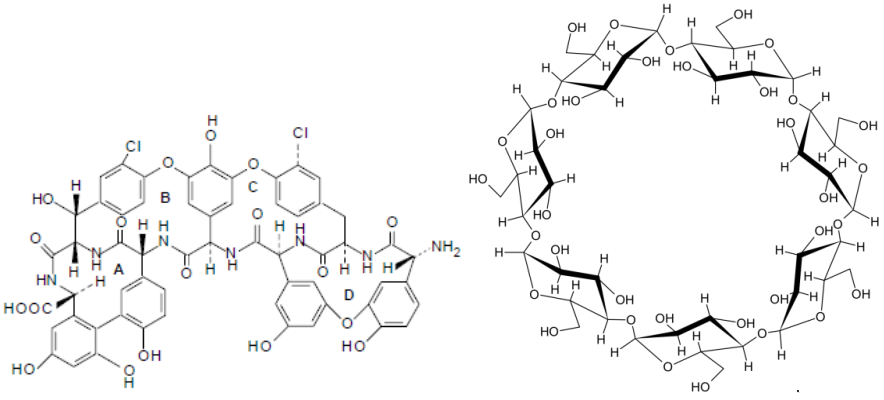

Figure 5. Chemical structures for vancomycin (left) and cyclodextrin (right) (adapted from Carlsson, 2003).

Cyclodextrin based materials (Figure 5) are bonded to a support such as silica and are prepared using similar techniques to those for making conventional reverse phases. The three most characterized cyclodextrins, denoted α, β and γ, contain six, seven and eight glucose units, respectively, resulting in different sized cavities. Moreover, different derivatives of cyclodextrins are available (e.g. acetylated) (Han, 1997).

Detection

Different sorts of detectors display different levels of sensitivity, dynamic range and specificity. Hence, due to the different properties of detectors, it is of great importance that the detector chosen for a specific method is compatible with the chromatography.

Spectrometry

A widely used more general detector for drug analysis and other applications has been the ultraviolet absorption detector (UVD), although for very sensitive applications, the fluorescence detector is also very popular. The limits of detection for particular components can be extended by using a variable wavelength UVD. Fluorescence measurements have provided some of the highest sensitivities available in HPLC. Some drugs (e.g. VEN and CIT) have native fluorescence, and if the parent drug is fluorescent, its metabolites are also probably fluorescent. Detection techniques based on fluorescence affords greater sensitivity to sample concentration, but less sensitivity to instrument instability. This is due to the fluorescent light being measured against a very low light background (Lindsey, 1992).

Mass spectrometric detection

During the last decades, mass spectrometry (MS) has emerged as an indispensable analytical technique. Today, liquid chromatography (LC) coupled to MS is frequently used in routine qualitative and quantitative analysis. A LC/MS instrument consists of three major components: an ion source that generates ions at atmospheric pressure, one or multiple mass analysers, which filters ions, and a detector that detects ions (Figure 6). The analytes eluted from the chromatographic column are ionized in the ion source, charged molecules are produced and the mobile phase is removed. Once the ions are created in atmospheric pressure, they are extracted from the ion source and transferred to the high vacuum region in the mass spectrometer (Moberg, 2006). The ions are extracted into the analyser region of the mass spectrometer where they are separated according to their mass-to-charge ratios (m/z). The extracted ions are detected and this signal sent to a data system where the m/z ratios are registered together with their relative abundance for presentation in the format of a m/z spectrum. In tandem MS ions are separated in two dimensions. A schematic picture of a LC-MS/MS instrument is displayed in Figure 6. MS-MS can be used in order to produce structural information about a compound by fragmenting specific sample ions inside the instrument and identifying the resulting fragment ions. The ions are fragmented by collision with a gas, a method termed collision-induced dissociation. The most common ionization methods at atmospheric pressure are chemical ionization (APCI) and electrospray ionization (ESI). Briefly, ionization using APCI takes place in the gas phase, whereas in ESI it is mainly considered to take place in the liquid phase. Several examples of the utility of chiral HPLC-ESI-MS/MS can be found in the literature (Kammerer *et al*., 2004; Coles *et al*., 2007).

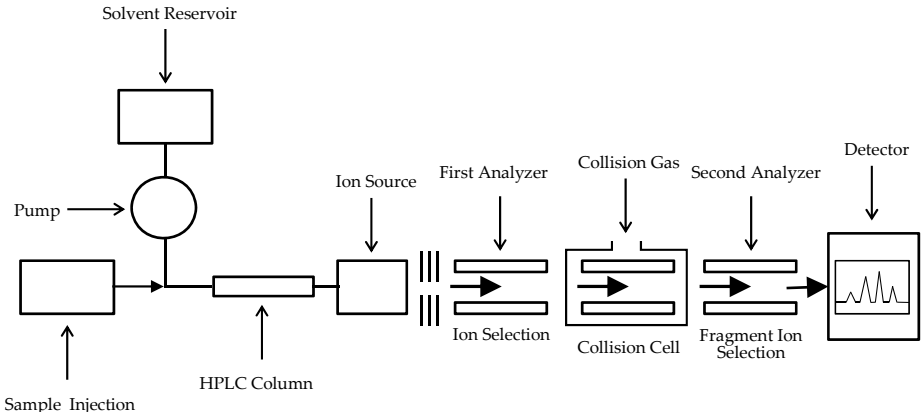

Figure 6. A schematic illustration of the LC-MS/MS instrumentation.

Sample preparation

Sample preparation is an important pre-analytical step in drug analysis, and includes isolation, cleanup and concentration (or occasionally dilution) of samples. The purpose of sample preparation is to enhance assay selectivity and sensitivity, and to reduce amounts of interfering matrix components. The extent of sample pre-treatment depends on the complexity of the sample, and has great importance when drugs in biological matrices such as plasma, urine and tissue homogenates are analysed (Misl'anová & Hutta, 2003). There are several different strategies for sample preparation such as liquid liquid extraction and solid phase extraction. In bioanalysis, several different matrices often are of interest for analysis. Urine, plasma and whole blood samples are commonly used; however, alternative matrices such as brain, liver, bile, hair, nails, bone, fat or muscle can also be of interest (Verplaetse & Tytgat, 2011).

Solid-phase extraction

In solid-phase extraction (SPE), the analytes to be extracted are partitioned between a solid and a liquid phase (Wille & Lambert, 2007). For extraction, the analytes must have a greater affinity for the solid phase than for the sample matrix. Interfering compounds are rinsed off the stationary phase by one or several washing steps and then the analytes are desorbed with a solvent. The principles for separation involve intermolecular forces (i.e. hydrophobic interaction and ion-ion forces) between the analyte, active sites on the adsorbent and in the liquid phase or sample matrix. Extraction can be performed in reversed-phase or normal-phase mode. Reversed-phase partitions solutes from a polar phase to a non-polar phase, which may be in the form of a hydrocarbon chain or polymeric sorbent. In normal phase SPE, polar compounds dissolved in a non-polar solvent are extracted by adsorption to a polar sorbent. The most common sorbents used are chemically bonded silica phases (Wille & Lambert, 2007). In bioanalysis normally the reversed phase mode is used as the substances of interest most often is dissolved in an aqueous phase such as plasma or blood.

Matrix effects

The biological matrix can have a considerable effect on the way an analysis is conducted and the quality of the results obtained, these effects are called matrix effects. Ion suppression/enhancement is a type of matrix effects and a well known phenomenon in LC-MS/MS analysis and may affect detection and quantification of the analytes, and reproducibility and accuracy of the method. It is especially observed when using ESI, and depends mainly on the sample matrix constitution, sample preparation procedure, quality of chromatographic separation, mobile phase

additives and ionisation mode. To eliminate sample matrix components and reduce matrix effects, liquid-liquid extraction or SPE are commonly used (Levine, 2006).

Method development and validation

Reliable data is a prerequisite for correct interpretation of analytical findings and analytical methods must be fully validated in order to demonstrate their applicability for the intended use. Accurate analytical methods with high precision for the quantitative evaluation of drugs and their metabolites in biological matrices are mandatory for pharmacological and toxicological studies. The choice of analytical method is dependent of the specific needs and purposes. The best suited chromatographic method and the best suited type of detection have to be taken into consideration. In toxicology it is desirable to have access to analytical methods that cover both therapeutic and toxic concentrations of drugs, hence a wide concentration range is needed. If both the parent drug as well as its metabolites and/or their enantiomers are supposed to be analysed in the same run, a selective method is required. Before separation and detection, the sample often needs to be prepared by sample pre-treatment. The extent of sample pre-treatment usually depends on the complexity of the sample. Different types of matrices have their own characteristics, causing differences between the matrices, such as protein, sugar and lipid contents. In forensic toxicology, both ante- and postmortem samples may contain a variety of drugs and their metabolites in a wide concentration range. In addition, the sample matrices are often complex, hence, effective sample preparation is essential (Peters & Maurer, 2002; Peters *et al.,* 2007). Method validation includes procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix is reliable and reproducible for the intended use (Peters & Maurer, 2002; Peters *et al.,* 2007). Other factors, such as appropriate calibration

model, stability (short- and longterm) and matrix effects are investigated. If the method is intended to quantify more than one analyte, each analyte should be tested. When developing a new bioanalytical method, a full validation is necessary. The fundamental parameters for method validation include accuracy, precision, selectivity, sensitivity, reproducibility, and stability. Also, the limit of detection (LOD) and the limit of quantification (LOQ) have to be determined. The validation of a chiral bioanalytical method is similar to any bioanalytical method. However, since the pharmacodynamic as well as pharmacokinetic properties may differ between enantiomers, the parameters in the method validation have to be determined for each individual enantiomer (Ducharme *et al*., 1996). Due to the increased number of analytes to be separated when analysing a chiral compound and its metabolites, the run time can be quite long in order to achieve an acceptable separation. As a consequence of the long analysis time, a limited number of samples could be processed each day. Hence, the extent of validation of the method may be hampered. In such cases, historical calibration from the analysis of multiple standard samples could be used. To verify correct quantitations, freshly prepared quality control (QC) samples are included consecutively in each run. To further improve and verify precision and accuracy of a chiral analytical method, it is valuable to use QC´s with different amount of each enantiomer (Carlsson *et al*., 2001; Holmgren *et al*., 2004). Unfortunately, not all enantiomers are available commercially.

AIMS

The overall aim of the present thesis was to study the relationship between pharmacokinetics and pharmacogenetics for two chiral antidepressant drugs, venlafaxine and citalopram, with emphasis on enantiomeric drug disposition in different biomatrices.

Specific aims:

1. To develop and validate a bioanalytical method for the enantioselective determination of venlafaxine and its three demethylated metabolites in human plasma and whole blood by using liquid chromatography-tandem mass spectrometric detection and solid phase extraction (Paper I).

2. To study the genetic influence on enantiomeric drug disposition in serum and brain following administration of racemic citalopram and venlafaxine to Sprague-Dawley and Dark Agouti rats (Papers II and III).

3. To study the relation between *CYP2D6* genotype and the disposition of the enantiomers of venlafaxine and its metabolites in femoral blood from forensic autopsy cases (Paper IV).

MATERIALS AND METHODS

Chiral bioanalysis of venlafaxine (Paper I)

Solid-phase extraction of plasma samples

Before the determination of the concentrations, solid-phase extraction was performed in order to clean and concentrate the samples. Prior to extraction, the plasma samples were centrifuged. The extraction was performed with Isolute C8 columns 100 mg (International Sorbent Technology, Hengoed, UK). Initially, columns were activated with 1 ml methanol and 1 ml ultrapure water. Thereafter, 0.2 ml of sample was added to the columns followed by 20 μl internal standard (Mexiletine, 5 mmol/l). The columns were then washed with 1 ml ultrapure water, followed by 2 ml methanol:ultrapure water (50:50; v/v) and 2 ml acetonitrile. Thereafter, the columns were dried for 1 min. VEN and its metabolites were then eluated with 1.5 ml acetonitrile with 10 mM trifluoric acid and evaporated with nitrogen at 50°C in a block thermostat (Grant QBT2; Grant Instruments (Cambridge) Ltd, UK). The analytes were reconstituted in 50 μl mobile phase consisted of tetrahydrofuran: 10 mM ammonium acetate pH 6 (10:90; v/v) and transferred to a vial. The samples were then placed in the autosampler and 5 μl of each sample was injected onto the chiral column for analysis.

Solid-phase extraction of whole blood samples

For whole blood, an especially designed extraction method was used. The samples were extracted according to the method described for plasma above, but with some modifications due to the higher viscosity and the expected higher concentrations in

blood as compared to plasma. Briefly, 20 µl of internal standard (Mexiletine, 50 mmol/l) was added to 0.2 ml whole blood and diluted with 3 ml ultrapure water. After vortex mixing and sonification for 5 minutes, the solution was centrifuged. After conditioning of the extraction columns with methanol and ultrapure water, the centrifuged samples were poured on to the columns and thereafter extracted according to the original procedure described for plasma above. After evaporation, the samples were reconstituted in 100 µl mobile phase and 2 µl of each sample was injected onto the chiral column. Hence, the whole blood samples were reconstituted in a greater volume, but a smaller volume was injected.

Standards and quality control samples

Plasma standard solutions were prepared by diluting the working solutions in human drug-free plasma. Seven different standards were prepared ranging between 1-1000 nmol/l (for each enantiomer of VEN and ODV) and 0.5-500 nmol/l (for each enantiomer of NDV and DDV). Plasma QCs were prepared with concentrations of 2 and 500 nmol/l (for the enantiomers of VEN and NDV) and 1 and 250 nmol/l (for the enantiomers of ODV and DDV). For whole blood, standards were prepared in drug-free human whole blood, ranging between 10-4000 nmol/l (for each enantiomer of VEN and ODV) and 5-2000 nmol/l (for each enantiomer of NDV and DDV). Whole blood QCs were prepared with concentrations of 15, 300 and 3000 nmol/l (for the enantiomers of VEN and ODV) and 7.5, 150 and 1500 nmol/l (for the enantiomers of NDV and DDV).

Determination of the enantiomers of venlafaxine and metabolites

The concentrations of the S- and R-enantiomers of VEN, ODV, NDV and DDV in human plasma and whole blood were determined by using enantioselective liquid

chromatography with tandem mass spectrometric detection (LC-MS/MS). A representative chromatogram for the enantiomers of VEN and its metabolites is shown in Figure 7.

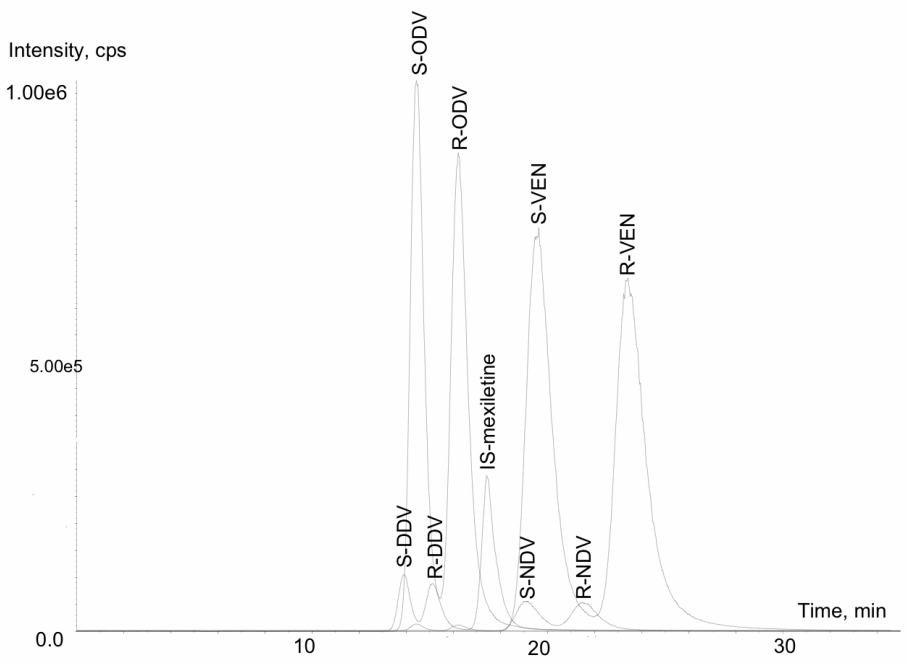

Figure 7. Representative chromatogram of a plasma quality control sample prepared with concentrations of 500 nmol/l for the enantiomers of venlafaxine (VEN) and O-desmethylvenlafaxine (ODV), and 250 nmol/l for N-desmethylvenlafaxine (NDV) and N,O- didesmethylvenlafaxine (DDV). Internal standard mexiletine.

The chromatographic system consisted of an Acquity LC-system (Waters, Milford, MA, USA) and a Sciex API 4000 tandem mass detector equipped with an ESI ion source (PE Sciex; Ontario, Canada). Chromatographic separation was performed on a

Chirobiotic-V column (5 μm particle size, 250 x 2.1 mm; Sigma-Aldrich) with a 5 μm in-line filter (VICI AB International, Switzerland). The column temperature was kept at 10°C using a Jones Chromatography Model 7955 column chiller/heater (Hengoed, UK). The mobile phase used consisted of tetrahydrofuran:ammonium acetate (10 mM) pH 6.0 (10:90; v/v) with a flow rate of 0.2 ml/min. A solution composed of 0.05% formic acid in acetonitrile, delivered by a Gynkotek 480 pump (Dionex; Sunnyvale, CA, USA), was added post column at a flow rate of 0.2 ml/min in order to lower the pH.

Table 2. The LC-MS/MS parameters and retention times for the enantiomers of venlafaxine (VEN), O-desmethylvenlafaxine (ODV), N-desmethylvenlafaxine (NDV), N,O-didesmethylvenlafaxine (DDV) and for the internal standard mexiletine. Relative intensities calculated with respect to the S-enantiomer of VEN. (N.C. = Not Calculated)

Compound	Transition (Q1/Q3)	Collision energy (V)	Relative area intensity (%)		Retention time (min)	
			S-enantiomer	R-enantiomer	S-enantiomer	R-enantiomer
VEN	278/58 278/260	45 20	100	102	19.6	23.4
ODV	246/58 246/264	40 20	98.1	104	14.4	16.2
NDV	246/121 246/264	35 20	49.2	54.2	19.1	21.6
DDV	250/107 250/232	40 18	52.7	57.8	13.9	15.2
Mexiletine	180/58 180/105	22 27	N.C.	N.C.	N.C.	N.C.

The compounds were ionized in electrospray positive mode at 5000 V. Nitrogen was used as nebulizer, auxiliary, curtain and collision gas and was set at 50, 70 and 30 psi and a value of 5. The auxiliary gas temperature was set at 600°C. Multiple reaction monitoring (MRM) mode was used for quantification. The two most abundant transitions originating from product ions, of the protonated molecular ions for VEN, its metabolites and the internal standard were used. General parameters for all transitions were as follows; declustering potential (DP): 50 V, entrance potential (EP): 12 V and collision cell exit potential: 20 V. The remaining parameters and the retention times are shown in Table 2. Data acquisition and peak integration, recording the area of the peaks, were performed using Analyst 1.4 software (PE Sciex; Ontario, Canada).

Method validation

To evaluate linearity, calibration curves were prepared and analysed in duplicates. The inter-day variation of the method was evaluated in plasma and whole blood by determining QC samples at different concentration levels in five replicates at five different days. Five replicates at each concentration were used for the intra-day variation. Matrix effects were evaluated according to Matuszewski *et al*., 2003, i.e. by comparing the concentrations found of known amounts of working standards with those measured in control plasma and whole blood spiked with the same amount of analytes before or after extraction. Furthermore, the influence of matrix components on ESI was studied using MS scanning for phospholipids and proteins in both plasma and whole blood samples (m/z 400-750 and 1200-1800, respectively). The stability of VEN and its metabolites in reconstituted samples was evaluated by analysing QC samples exposed to different time and temperature conditions. The extraction recoveries in plasma and whole blood were determined by comparing

extracted spiked blank samples with unextracted reference samples prepared at the same concentrations. Samples at two different concentration levels in triplets were analysed.

Experimental studies (Papers II and III)

Animals

Aged-matched 8-week old female Sprague-Dawley (SD) rats and Dark Agouti (DA) rats were obtained from Scanbur BK AB, Sollentuna, Sweden. Animals had free access to tap water *ad libitum* and standard laboratory pelleted chow containing 14.5% crude protein (R70; Latamin AB, Vadstena, Sweden). The rats were kept in groups of 2-3 in macrolone cages with sawdust bedding under climate-controlled conditions for normal indoor temperature and humidity. The animals were kept in a constant 12:12 h light:dark cycle synchronous with daylight. The rats were allowed to recuperate for at least one week from transported-induced stress before the beginning of the experiments. The studies were approved by the Animal Ethics Committee, Linköping (No. 10-04).

Drug administration and sample collection

Chronic administration of citalopram

Citalopram HBr (H. Lundbeck A/S, Copenhagen-Valby, Denmark) was dissolved in a mixture of 0.9% NaCl and propylene glycol (40:60; v/v) and administered subcutaneously. SD (n=9) and DA (n=9) rats were administered a bodyweight-adjusted chronic drug treatment regimen (15 mg/kg daily) for 13 days by using osmotic pumps (ALZET® model 2ML2; Scanbur BK AB, Sollentuna, Sweden). Osmotic pumps are miniature pumps that continuously deliver test agents at controlled rates into laboratory animals (Figure 8). When implanted intraperitoneally or subcutaneously, the pumps serve as a constant source for prolonged drug delivery

and are an alternative method to repeated animal dosing that reduces animal handling and stress. For further and more complete description of the osmotic pumps see www.alzet.com.

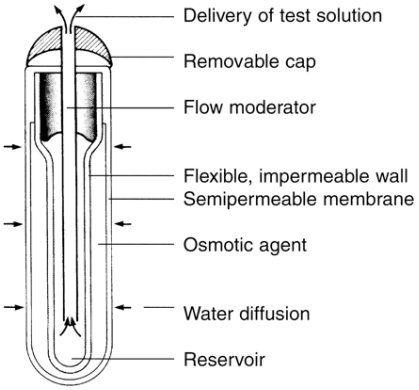

Figure 8. Schematic figure of an osmotic pump (adapted from Wamberg & Tauson, 1998).

The osmotic pumps were filled with 2 ml of a drug solution corresponding to the CIT dose 15 mg/kg daily. The concentration of the drug solution was adjusted to allow delivery of a similar dose/kg body weight to both rat strains at a rate of 5 µl/h for 13 days. The rats were shaved and a minor skin incision was made between the scapulae during halothane (Fluothane, Zeneca Ltd., Macclesfield Cheshire, U.K.) anesthesia. A subcutaneous pocket was formed by blunt dissection of the connective tissues, and the osmotic pumps were inserted. The skin incision was closed with sutures (Ethilon®II 3/0, Ethicon®; Johnson & Johnson AB, Sollentuna, Sweden). The pumps were left in place during the entire study (i.e. for 13 days), hence, there was no washout period before the rats were sacrificed. After the sacrifice, the residual amounts in the pumps were assessed by aspirating with a graduated syringe for checking the delivery profile of the pumps. The rats where weighed five times

during the study. At the time of the sacrifice (i.e. day 13), the rats were decapitated with a guillotine under halothane anesthesia and mixed arterio-venous blood was collected from the neck wound. The blood samples were left for 30 min to allow clotting of the blood, followed by centrifugation for collection of the supernatant serum. After collection of blood samples, the brain was removed from the scull and dissected. First, the corpus pineale was removed and the cortex and hippocampus were then peeled off. Thereafter, the basal ganglia, cerebellum and diencephalon were removed. The mesencephalon–pons was left as the last brain region. The neocortical hemisphere and the mesencephalon-pons tissue samples were weighed and homogenized in 2 ml Milli-Q water (Millipore AB, Stockholm, Sweden) by the use of a sonifier (Sonics Vibra-Cell VC 130; Chemical Instruments AB, Lidingö, Sweden) and thereafter centrifuged. All samples were stored at -70°C until analysis.

Acute administration of venlafaxine

DA (n=10) and SD (n=10) rats were administered a single subcutaneous injection of racemic VEN (15 mg/kg bodyweight) during a short halothane (Fluothane; Zeneca Ltd, Maccesfield Cheshire, UK) anaesthesia. The rats were decapitated under halothane anaesthesia three hours following drug administration and mixed arterio-venous blood was collected from the neck wound. The blood samples were then left for 30 min to allow clotting. Thereafter, the blood samples were centrifuged and the supernatant serum was collected. After collection of blood samples, the brain was removed from the scull and the neocortical hemisphere (frontal cortex) and the mesencephalon-pons regions were dissected out (for details see "Chronic administration of citalopram"). The dissected brain tissue samples were weighed and

homogenized in 2 ml Milli-Q water by the using a sonifier and thereafter centrifuged. All samples were stored at -70°C until analysis.

Chiral determination of drugs

Determination of the enantiomers of citalopram and metabolites

The concentrations of the enantiomers of CIT and its metabolites in serum and brain homogenate supernatant were determined by using enantioselective HPLC with fluorescence detection according to a previously described procedure (Rochat *et al*., 1995) with some modifications (Carlsson *et al*., 2001; Kugelberg *et al*., 2001).

The extraction of the samples was carried out with solid-phase extraction according to a previously described method (Carlsson *et al*., 2001; Kugelberg *et al*., 2001). After elution and evaporation, the dried samples were redissolved in 100 µl of methanol:100 mmol/l citrate triethylamine buffer, pH 6.3 (55:45; v/v). A volume of 50 µl sample was injected on to a Cyclobond I 2000 Ac 250 x 4.6 mm column (Astec, Whippany, NJ, U.S.A.) with a Gynkotek Gina 50 autosampler (Dionex, Sunnyvale, CA, U.S.A.). The mobile phase was delivered through a Gynkotek 480 pump (Dionex) at a flowrate of 0.8 ml/min. Detection was performed using a Waters 474 fluorescence detector (Waters Corporation, Milford, MA, U.S.A.) at an excitation wavelength of 240 nm and an emission wavelength of 300 nm. The temperature of the column was set to 30°C using a Jones Chromatography Model 7955 column chiller/heater (Hengoed, U.K.). The detection signals were recorded and processed using Chromeleon (Version 6.40; Dionex, Sunnyvale, CA, U.S.A.). The limits of detection for the enantiomers of CIT and its metabolites were 2 nmol/l, respectively. The extraction recoveries were ranging between 87% and 110% (Carlsson *et al*., 2001).

A representative chromatogram for the enantiomers of CIT and its metabolites is shown in Figure 9.

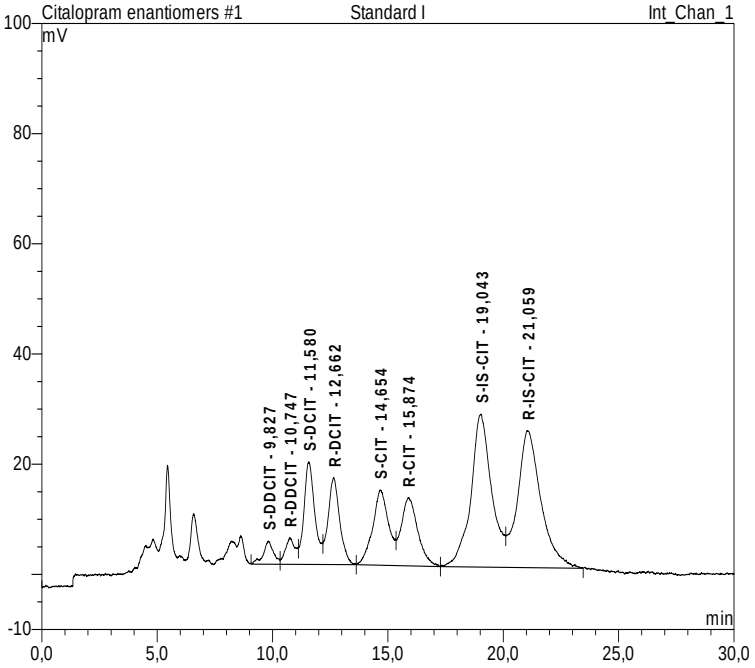

Figure 9. Representative chromatogram of a plasma standard sample prepared with concentrations of 31 nmol/l for the enantiomers of citalopram (CIT), 16 nmol/l for the enantiomers of demethyl-citalopram (DCIT), and 3.5 nmol/l for the enantiomers of didemethylcitalopram (DDCIT).

Determination of the enantiomers of venlafaxine and metabolites

The brain samples were weighed and homogenised in 2 ml ultrapure water (Millipore AB, Stockholm, Sweden) by a sonifer (Sonics VibraCell VC 130; Chemical Instruments AB, Lidingö, Sweden) followed by centrifugation. All samples were then stored at -70°C until analysis. The extraction of brain and serum was performed by using solid-phase extraction according to the procedure used for human. The

concentrations of the enantiomers of VEN and its metabolites in serum and brain homogenate were determined by using enantioselective LC-MS/MS. For details, see pp. 35 “Chiral bioanalysis of venlafaxine”.

Statistical analysis

Data were expressed as means ± standard error of the mean (SEM). A probability of <5% ($p<0.05$) was considered statistically significant. When two dependent groups were compared, a two-tailed Student’s t-test for paired observations was applied. When two independent groups were compared, a two-tailed Student’s t-test for unpaired observations was used. All statistical analyses were performed using StatView for Windows Version 5.0 (SAS® Institute Inc., Cary, NC, U.S.A.).

Forensic toxicological study (Paper IV)

Experimental design

The cases included in the present study were selected from all forensic autopsies that were performed in Sweden during 2008. After exclusion of badly decomposed samples and cases with too little blood available for drug analysis, 56 cases were found positive for VEN. Enantioselective analysis of VEN and its metabolites, along with genotyping for *CYP2D6*, were performed in femoral blood from the selected cases (for details, see below). Seven of the cases were found positive for CIT and these were re-analysed with an enantioselective HPLC method as described previously. A wide range of prescription drugs were determined in femoral blood by using gas chromatography (GC) fitted with a nitrogen-phosphorus detector (Druid & Holmgren, 1997). Ethanol was determined in blood by head-space GC according to a previously described method (Jones & Schuberth, 1989). Urine and/or blood samples were also analysed for different classes of illicit drugs after a direct request by the responsible forensic pathologist. Information related to each of the cases (age, gender, other found drugs, cause and manner of death) was provided from the forensic pathology and toxicology databases at the Swedish National Board of Forensic Medicine. The study was approved by the Regional Ethical Review Board in Linköping, Sweden (No. M87-08).

Determination of the enantiomers of venlafaxine and its metabolites

The concentrations of the S- and R-enantiomers of VEN and its metabolites ODV, NDV and DDV in postmortem femoral blood were determined by using the LC-MS/MS method as described previously.

Genotyping for *CYP2D6*

Genomic DNA was extracted using King Fischer ML (Thermo Scientific, Vantaa, Finland). Three single nucleotide polymorphisms (SNPs), **3*, **4* and **6*, were included in the genotyping of *CYP2D6* and identified by PCR and pyrosequencing (Zackrisson *et al*., 2003) with some minor modifications (Zackrisson *et al*., 2010). Also, the copy number variation (CNV) of *CYP2D6* was determined in order to identify carriers of whole gene deletion (*CYP2D6*5*) or multiple gene copies (*CYP2D6xN*). For determination of the gene copy number, the pseudogene *CYP2D8* was co-amplified with the *CYP2D6* gene in the PCR as described previously (Söderbäck *et al*., 2005) with some minor modifications (Zackrisson *et al*., 2010). Identification of the genes that was multiplied in samples with more than two gene copies was determined using peak pattern recognition of programs from the SNP analysis (Zackrisson *et al*., 2010).

Statistical analysis

Descriptive statistics of mean, median and range were used to describe the data. Mean and standard deviation were calculated for the enantiomeric (S/R) concentration ratios and the metabolite/parent drug (M/P) concentration ratios. Statistical comparisons between the groups were analysed by the Mann-Whitney test. A p-value less than 0.05 were considered statistically significant. All statistical analyses were performed using StatView® (SAS® Institute, Cary, NC, version 5.0).

RESULTS AND DISCUSSION

Chiral bioanalysis of venlafaxine (Paper I)

When using achiral analysis for racemic drugs, the total (S+R) concentrations of the enantiomers are determined. However, no information about the concentrations of the individual enantiomers or S/R ratios is given. Several studies have shown that enantioselective analysis of parent drug and metabolites can be of importance when interpreting data in therapeutic drug monitoring and forensic toxicology (Eap *et al*., 2000; Baumann *et al*., 2002; Holmgren *et al*., 2004; Carlsson *et al*., 2001; 2009). Hence, development of bioanalytical methods for studies of the metabolism and pharmacokinetics of the separate enantiomers of racemic drugs is of great importance in clinical pharmacology and toxicology (Brocks, 2006; Hutt, 2007; Lu, 2007). In Paper I, a sensitive stereoselective method for simultaneous analysis of the enantiomers of VEN and its three demethylated metabolites in human plasma and whole blood using LC-MS/MS was developed and validated.

By using a chiral stationary phase (Vancomycin) the individual enantiomers were separated. However, due to a limited selectivity between the enantiomeric pairs of VEN and its metabolites, some of the peaks were overlapping. Since the overlapping peaks could not be detected by conventional LC-UVD, a LC-MS/MS method was developed. Compared to the most frequently used enantioselective LC-UVD methods, LC-MS/MS exhibits a higher specificity and sensitivity (Desai & Armstrong, 2004). LC-MS/MS features unique transitions for determination of the individual compounds, which enables separate detection for the overlapping enantiomers. For each compound, the same transition was used for the S- and R-

enantiomer. Due to chromatographic separation, NDV and ODV could be determined individually even if the same transitions were used. The enantiomers of VEN and metabolites were not completely baseline separated, and the resolution for the enantiomers of each compound was: VEN 2.49, ODV 1.91, NDV 1.31 and DDV 1.13. However, the separation was considered sufficient for quantification of the separate enantiomers. Large differences in the concentrations between enantiomers in an enantiomeric pair may however influence the accuracy of the quantification. Inaccurate results are more likely at extreme enantiomeric ratios if the resolutions of the peaks are not complete (Meyer, 1995). In order to further improve and verify the precision and accuracy, controls with different amount of each enantiomer are needed (Carlsson *et al*., 2001; Holmgren *et al*., 2004). However, since not all enantiomers of VEN and metabolites are commercially available, the QCs had to be made from racemic mixtures. To achieve an enantiomeric separation of the selected analytes within reasonable time, the final run time was about 35 minutes in Paper I. The long analysis time resulted in that a limited number of samples could be processed each day. The extent of validation of the method was limited due to the long analysis time. In the method, historical calibration from the analysis of multiple standard samples was used. By reanalysis of standards within a week, the method was proven stable. The intra- and inter-day precision was evaluated in plasma and whole blood by analysing five replicates of three different concentrations on the same day and during five consecutive days. The intra-day precision was <6.3% and the inter-day precision was <9.9% for plasma and <15% and <19% for whole blood, respectively. The mobile phase consisted of tetrahydrofuran and ammonium acetate buffer and the most successful chromatographic separation was achieved at pH 6.0. The selected pH for the mobile phase was unfavourable for electrospray ionisation in positive mode, demonstrating that what is optimal for chromatography is not

necessarily optimal for electrospray ionisation and mass detection. To increase the sensitivity, 0.05% formic acid in acetonitrile was added to the mobile phase post column. With this modification, the sensitivity increased markedly (2-, 6-, 4- and 7-fold for VEN, ODV, NDV and DDV, respectively). Hence, by adding a post column additive consisting of formic acid in acetonitrile, the pH could be lowered giving a more effective ionisation and evaporation resulting in improved detector response and increased peak area for all target analytes. The calibration curves were linear within a limited concentration range. However, at the high end of the curves, some saturation was observed. Therefore a quadratic regression with inverse x (1/x) weighting was used for quantification. The calibration curves showed a correlation with a correlation coefficient of >0.999. Furthermore, the lower limit of quantification was 0.5 nmol/l for the enantiomers of VEN and ODV, and 0.25 nmol/l for NDV and DDV. No interfering peaks were found in the LC-MS/MS chromatograms of blank plasma or whole blood. Due to the ability to concentrate the samples reversed phase SPE was used for pre-treatment of the samples. The samples were concentrated two fold for whole blood and four fold for plasma. The extraction recoveries in plasma and whole blood ranged between 75-110% and 68-107%, respectively. Due to the high sensitivity of the method small sample volumes could be used. Especially for whole blood, small sample volumes are favourable for the SPE procedure, since the columns then do not clog up as easily. The extraction procedure resulted in clean extracts and neither extracted plasma nor whole blood samples showed any severe matrix effects in the LC-MS/MS analysis, proven by qualitative and quantitative results. The matrix effect data for VEN and its metabolites ranged between 100 and 108% in plasma and between 90.1 and 97.0% in whole blood. No presence of phospholipids or proteins in the sample matrix was found. The lack of matrix effects was also evidenced by the fact that the precision of the method was high even at

lower concentrations. However, a difference of about 20% was shown for the extraction recovery for VEN and NDV between low and high concentrations.

In toxicology it is desirable to have access of analytical methods which cover both therapeutic and toxic concentrations of drugs. The results for application of the method in different matrices showed generally higher concentrations in postmortem blood as compared to patient plasma (Table 3). Compared to the calibration curve used for the plasma samples a different calibration curve with a wider concentration range was used for the postmortem blood samples. Moreover, in some cases it was even necessary to re-run samples if the concentrations exceeded the high end of the calibration curve. This was carried out by diluting the samples or by using a smaller sample volume for the extraction.

Table 3. Total (S+R) concentrations (nmol/l) and enantiomeric ratios (S/R) of venlafaxine (VEN), O-desmethylvenlafaxine (ODV), N-desmethylvenlafaxine (NDV) and N,O-didesmethylvenlafaxine (DDV) in patient plasma and postmortem blood.

Matrix	Sample	Total (S+R) concentration				S/R ratios			
		VEN	ODV	NDV	DDV	VEN	ODV	NDV	DDV
Patient plasma	1	119	1171	5.69	36.1	4.33	0.87	N.C.	0.47
	2	506	1007	16.7	45.9	2.89	0.87	2.91	0.42
	3	84.6	755	50.0	280	1.01	0.87	1.40	0.94
	4	91.3	884	34.6	266	1.31	0.89	1.35	1.03
	5	264	659	57.9	205	1.34	0.98	1.24	1.18
Postmortem blood	1	4651	3368	562	537	1.08	0.78	1.05	1.03
	2	912	1967	307	561	0.81	0.84	1.26	1.00
	3	4465	376	1676	133	0.59	12.3	0.46	1.99
	4	1221	2495	165	474	1.11	0.7	1.53	1.12
	5	1855	1793	159	763	0.82	1.17	0.73	0.90

As shown in Table 3, the concentrations of the S- and R-enantiomers of VEN and its metabolites in the five patient plasma and postmortem samples varied. The total drug concentrations were found to be higher in the postmortem blood samples as compared to the patient plasma samples, also, differences in the S/R ratios were seen. Interestingly, compared to the other samples, case 3 in whole blood shows a great difference regarding the S/R ratios for both VEN and ODV. These results indicate that enantioselective analysis may be valuable when interpreting the concentrations of VEN and its metabolites.

Experimental studies (Papers II and III)

To the author's knowledge, these are the first studies that show significant quantitative strain-related differences in the pharmacokinetics of CIT and VEN, and their respective metabolites, in female DA rats as compared to female SD rats. The rat models mimic the pharmacokinetic features seen in human PMs of CYP2D6 (DA) and EMs of CYP2D6 (SD). Furthermore, marked differences in the disposition of the S- and R-enantiomers of the drugs and their metabolites were observed between the different rat strains. This indicates a role of CYP2D6 in the metabolism of these antidepressants and their metabolites. The rats administered CIT had a starting weight of 222-251 g (SD) and 123-140 g (DA) and were weighed five times during the study (Figure 10). Although the DA rats had a lower weight as compared to the SD rats, both strains followed the same weight curve. The rats administered a single-dose of VEN had a starting weight of 221-251 g (SD) and 123-144 g (DA).

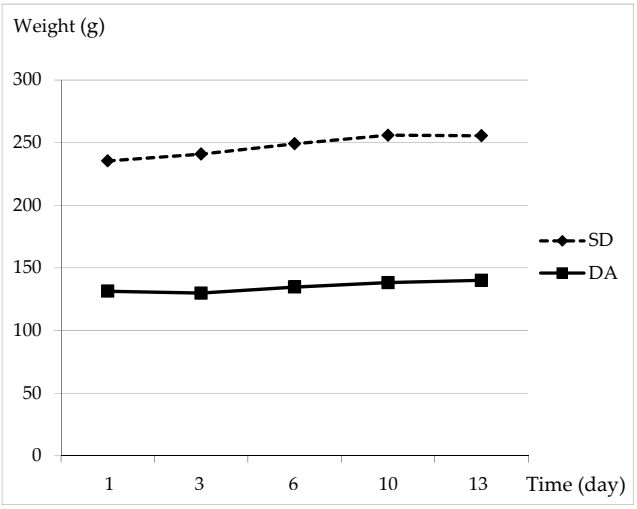

Figure 10. Mean weight curves for Sprague-Dawley (SD) and Dark Agouti (DA) rats during chronic administration of citalopram.

In Paper II, the strain-related differences were evidenced by higher levels of CIT and DCIT but lower levels of DDCIT in both serum and brain in the DA rats as compared to the SD rats (Table 4).

Table 4. Total (S+R) concentrations (nmol/l) and S/R ratios of citalopram (CIT), demethylcitalopram (DCIT) and didemethylcitalopram (DDCIT) in Sprague-Dawley (SD) and Dark Agouti (DA) rats. Ctx = cortex. Mes = mesencephalon-pons.

Matrix		Total (S+R) concentration			S/R ratio		
		CIT	DCIT	DDCIT	CIT	DCIT	DDCIT
Serum	SD	296	151	142	0.80	0.28	0.12
	DA	647	305	78.0	0.53	33.0	0.19
Ctx	SD	1812	225	130	0.85	0.17	0.21
	DA	2037	260	77,2	0.36	0.26	0.41
Mes	SD	1770	239	102	0.88	0.19	0.21
	DA	2518	280	51.0	0.50	0.26	0.50

Both serum and brain levels of R-CIT and R-DCIT were markedly higher in DA rats than in SD rats, resulting in lower S/R ratios of CIT in DA rats as compared to the SD, possibly by different rate of metabolism. The concentrations of R-DDCIT were lower in the DA rats than in the SD rats. Consequently, the S/R ratios of DDCIT were higher in DA rats as compared to SD rats. The levels of CIT were 3-6 times higher in the brain in both rat strains as compared to serum. No significant differences in concentrations could be found between the brain regions. In relation to CIT, less metabolite concentrations were found in the brain than in serum.

In vitro studies in human liver microsomes have shown that CYP2C19, CYP2D6 and CYP3A4 favor a more rapid demethylation of S-CIT compared to R-CIT (Olesen & Linnet, 1999; von Moltke *et al*., 2001). In contrast, CYP2D6 has been shown to favor R-DCIT over S-DCIT in the second demethylation step (Olesen and Linnet, 1999). The present results indicate that the metabolism of the R-enantiomeric forms of CIT and its metabolites was more affected by the CYP2D deficiency than the S-enantiomeric forms. One explanation for this observation might be that one, or several, of the CYP2D enzymes has different substrate affinity for the enantiomers of CIT. Interestingly, previous research has shown that R-CIT counteracts the serotonin enhancing properties of S-CIT (Mørk *et al*., 2003; Sánchez *et al*., 2003; 2004; Mansari *et al*., 2007; Mnie-Fiali *et al*., 2007). The finding of a negative effect of R-CIT emphasizes the relevance of the results from the present study. Hence, the present data tend to suggest that the pharmacodynamic SSRI effect might be less pronounced in the CYP2D-deficient DA rats as compared to the CYP2D-replete SD rats. Given the limitations of extrapolating animal data to the clinical situation, the findings indicate that a dose reduction to PMs of CYP2D6 might not be enough to ''normalize'' the pharmacokinetics and pharmacodynamics of CIT. This also highlights the importance of enantioselective drug analysis, especially in patients not responding to drug treatment.

For VEN, the quantitative strain-related differences in the pharmacokinetics was evidenced by the observation of higher concentrations of VEN in the DA rats as compared to the SD rats in both serum and brain (Table 5). The levels of the enantiomers in serum and brain, especially S-VEN, were markedly higher in DA rats than in SD rats. Consequently, the S/R ratio of VEN was higher in the DA rats as compared to SD rats. The total concentrations of ODV in serum and brain were, also

found to be higher in the DA rats as compared to the SD rats. Furthermore, the concentrations of S-ODV in serum and brain were higher in DA rats as compared to SD rats. Hence, the S/R ratio of ODV was higher in the DA rats in both serum and brain regions. The levels of NDV were markedly higher in the DA rats than in the SD rats. However, no significant differences in the NDV S/R ratio between the strains were found.

Table 5. Total (S+R) concentrations (nmol/l) and S/R ratios of venlafaxine (VEN), O-desmethylvenlafaxine, N-desmethylvenlafaxine and N,O-didesmethylvenlafaxine in Sprague-Dawley (SD) and Dark Agouti (DA) rats. N.D.= not detected. Ctx = cortex. Mes = mesencephalon-pons.

Matrix		Total (S+R) concentration				S/R ratio			
		VEN	ODV	NDV	DDV	VEN	ODV	NDV	DDV
Serum	SD	637	65.0	183	3.30	0.96	9.11	0.12	0.48
	DA	1153	153	1123	1.08	1.94	42.8	0.11	0.77
Ctx	SD	3530	151	402	N.D.	1.08	8.63	0.14	N.D.
	DA	7005	296	1295	N.D.	2.07	26.6	0.12	N.D.
Mes	SD	3069	103	330	N.D.	1.05	11.0	0.17	N.D.
	DA	5579	198	1239	N.D.	2.08	2.08	0.13	N.D.

In general, the levels of the metabolites of VEN were found to be lower than the parent drug levels in both SD and DA rats. In relation to the parent compound, less metabolite concentrations were found in the brain compared to serum. The serum P/M ratios for ODV and NDV were 10.5 and 3.5 in the SD rats, and 7.6 and 1.0 in the DA rats, respectively. These results are in agreement with previous studies in humans, where PMs of *CYP2D6* showed higher plasma levels of VEN and NDV compared to EMs (Eap *et al*., 2003; Grasmäder *et al*., 2004). The observation of

markedly higher levels of VEN and NDV in the *CYP2D* deficient DA rats indicates that the formation of ODV is impaired. The finding of higher concentrations of NDV is in agreement with a study by Shams *et al*., 2006, where higher levels of NDV were found in PMs, which is explained by an increased metabolism by CYP3A4 as a consequence of impaired activity of CYP2D6 (Shams *et al*., 2006). However, the levels of ODV were not found to be lower in the DA rats compared to the SD rats. This may be explained by the markedly higher concentrations found in the DA rats. According to several studies, alternative enzymes are, at higher concentrations, available for the metabolism, which makes the differences between the two strains less evident (Barham *et al*., 1994). Hence, interstrain differences in the metabolism are more marked at lower substrate concentrations, at which the CYP2D enzymes make a major contribution to the metabolism.

Differences were also observed between the strains with respect to the levels of the S- and R-enantiomers of VEN and its three metabolites. The present finding can be compared with a human study performed by Eap *et al* including PMs and EMs of CYP2D6 (Eap *et al*., 2003). Eap *et al* reported that both of the enantiomers of VEN were higher in PMs. This is in accordance with the present results. In addition, Eap et al reported that PMs displayed a lower S/R ratio of VEN compared to EMs. These results suggest that CYP2D6 displays a marked stereoselectivity towards the R-enantiomer.

The concentrations of CIT and VEN and their respective metabolites were measured in two different brain regions; the frontal cortex, which is an important projection area where the serotonergic synapses are present, and in the mesencephalon-pons, the site for serotonergic cell bodies. In both brain regions, higher concentrations of

drugs were seen compared to in serum, which is in agreement with previous studies (Kugelberg *et al.*, 2001; 2003). Further, the S/R ratios were found to be similar in serum and brain. The CYP enzymes, including CYP2D, are except from the liver also present in the brain in both humans and rats. The highest expression of CYP2D in brain has been observed in the cerebral cortex, hippocampus, cerebellum and the brainstem (Miksys *et al.*, 2000; Miksys & Tyndale, 2002). However, the level of the CYP enzymes is too low to significantly affect the overall pharmacokinetics of drugs in the body (Hedlund *et al.*, 2001).

Taken together, Papers II and III emphasize the importance of investigating the pharmacokinetics in not only one, but different rat strains, especially for racemic drugs. A study performed by Saito *et al.* (2004) showed that the pharmacokinetics of diazepam differed among different rat strains due to genetic polymorphism. Because of these differences, the major metabolites may be depending on the rat strain, and hence, may cause significant differences in pharmacokinetics of the drug (Saito *et al.*, 2004). Furthermore, by comparing the results with similar studies in mice, significant differences concerning the kinetics can be seen between rats and mice (Karlsson *et al.*, 2010; 2011). Karlsson *et al.* (2010) reported the following S/R ratios for wild-type mice (3 h after administration); VEN 1.24, ODV 1.69 and NDV 2.77. These results can be compared with the S/R ratios in SD rats found in Paper III; VEN 0.96, ODV 9.11 and NDV 0.12. Further, in humans, a S/R ratio of 1.3 in plasma was reported following a single dose of racemic VEN (Wang *et al.*, 1992). Gex-Fabry and co-workers have reported S/R ratios of 0.4-2.5 after chronic administration of VEN (Gex-Fabry *et al.*, 2002; 2004). A case report published by Eap *et al.* (2000) reported a S/R VEN ratio of 1.9 in serum. Hence, marked differences can be seen between different species. By

comparing the data for mice and rats with the human S/R ratios in plasma presented in Paper I (VEN 1.01-4.22, ODV 0.87-0.98 and NDV 1.24-2.91), it can be concluded that mice, compared to rats, seem to be more similar to humans with respect to the metabolism of VEN (Karlsson *et al.,* 2010; 2011).

Forensic toxicological study (Paper IV)

This study shows the influence of the *CYP2D6* genotype on the enantiomeric disposition of VEN and its three main metabolites in femoral blood from 56 forensic autopsy cases with different causes of death.

The cases were divided into three groups related to *CYP2D6* genotype: cases with 2 active genes (EM), cases with 1 active allele (IM) and cases without any active alleles (PM). No cases with more than 2 active genes (UM) were found. The total (S+R) concentrations and S/R ratios for the respective groups (0, 1 and 2 active genes) are shown in Table 6.

Table 6. The total number of active genes and the total (S+R) concentrations (μg/g) and S/R ratios of venlafaxine (VEN), O-desmethylvenlafaxine (ODV), N-desmethylvenlafaxine (NDV) and N,O-didesmethylvenlafaxine (DDV).

Number of active genes	Total (S+R) concentration				S/R ratio			
	VEN	ODV	NDV	DDV	VEN	ODV	NDV	DDV
0 (n=6)	1.15	0.17	0.66	0.10	0.50	11.9	0.51	1.78
1 (n=24)	1.14	0.76	0.24	0.15	1.07	1.27	0.77	1.00
2 (n=23)	0.75	0.66	0.16	0.15	1.01	0.99	1.12	1.02

In six cases, a low S/R VEN ratio was associated with a high S/R ODV ratio. Genotyping showed that these six individuals carried two inactive *CYP2D6* genes. The metabolite/parent drug (M/P) concentration ratios for the cases with 0 active genes were 0.23 ± 0.13 for ODV/VEN, 0.74 ± 0.46 for NDV/VEN and 0.14 ± 0.14 for DDV/VEN. The M/P ratios for the groups with 1 and 2 active genes were 1.26 ± 1.35

and 2.01 ± 1.36 for ODV/VEN, 0.21 ± 0.20 and 0.22 ± 0.17 for NDV/VEN, and 0.25 ± 0.29 and 0.42 ± 0.44 for DDV/VEN, respectively.

According to several studies (Schweizer *et al*., 1994; Ereshefsky *et al*., 1996; Koski *et al*., 2005), VEN has a relatively higher fatal toxicity compared to SSRIs and other newer antidepressants. This finding has been explained by for example, a high prevalence of drug interactions and possible differences in prescribing practice of VEN compared with SSRIs. With regard to VEN toxicity, possible physiological drug effects have also to be considered. Decreased *CYP2D6* activity has been associated with cardiovascular toxicity following VEN treatment. Therefore, a possible link between this finding and the observed differences in enantiomeric disposition of VEN and metabolites in EMs and PMs cannot be excluded. The enantiomers of VEN display partly different activities on serotonin and noradrenalin reuptake, however, for the metabolites, only few data have been published regarding the pharmacological profiles. To the author's knowledge, no data are available describing the effects of the separate enantiomers of the VEN metabolites. The high number of fatalities associated with VEN underscores the importance of correct interpretation of toxicological results in forensic cases. Many of the other drugs found in the cases are metabolized by the same enzymes as VEN, some are also known inhibitors. These interactions may affect the pharmacokinetics of the drugs. Patients who are CYP2D6 PMs, or are taking interacting drugs, may receive concentrations in the range of those found in overdose when taking therapeutic doses. Hence, drug interactions involving VEN may have occurred in several of the cases included in the present study. The combination of tramadol and VEN was found in five cases and the risk of serotonin syndrome cannot be negligible. Other drugs found in the cases are inducers or inhibitors of CYP enzymes, e.g.

carbamazapine and dextropropoxyphene. Case 17 was classified as an IM (*1/*4) for *CYP2D6* but had a low ODV/VEN ratio of 0.35 indicating a PM metabolic ratio. In addition, the S/R ratios for VEN and ODV were 0.28 and 5.92, respectively, also in line with the enantiomeric ratios found in PMs. Interestingly, the CYP3A4 inducer carbamazepine was present in this case leading to the conclusion that more NDV than expected was formed. The contribution of a CYP inducer is evident when comparing two cases, 17 and 14, with and without concominant carbamazepine intake (Table 8).

Table 8. The total (S+R) concentrations (μg/g) and S/R ratios of venlafaxine (VEN), O-desmethylvenlafaxine (ODV), N-desmethylvenlafaxine (NDV) and N,O-didesmethylvenlafaxine (DDV) as well as presence of other drugs and *CYP2D6* genotype for case 14 and 17.

Case	Total (S+R) concentration				S/R ratio				Other drugs	*CYP2D6*
	VEN	ODV	NDV	DDV	VEN	ODV	NDV	DDV		
14	1.42	0.98	0.16	0.15	1.08	0.78	1.06	1.02	Ethanol	*1/*4
17	1.19	0.42	1.01	0.17	0.28	5.92	0.42	0.95	Carbamazepine Alimemazine Dm-alimemazine	*1/*4

These cases had the same genotype (*1/*4) and displayed similar VEN levels (about 1.2-1.4 μg/g) but the distribution of the enantiomers and metabolites was clearly different. Accordingly, an individual with a *CYP2D6 *1/*4* genotype may turn into a CYP2D6 PM phenotype with respect to VEN metabolism when carbamazepine is also present in blood. These two cases emphasize the importance of enantioselective analysis and/or CYP genotyping when the toxicological results are difficult to interpret.

A comprehensive toxicological analysis is essential when fatal drug intoxications are investigated. Paper IV shows that information about the disposition of the enantiomers of VEN and its metabolites could assist in the interpretation of forensic toxicological results. A low S/R VEN ratio was strongly related to a high S/R ODV ratio in individuals lacking two functional *CYP2D6* alleles. It is concluded that enantioselective analysis of VEN and ODV can predict if a person is a PM genotype/phenotype for *CYP2D6*. Knowledge of the relationship between the S- and R-enantiomers of this antidepressant drug and its active metabolite is also important since the enantiomers display different pharmacodynamic profiles.

CONCLUDING REMARKS

Chiral bioanalysis of venlafaxine (Paper I)

- A validated LC-MS/MS method for enantioselective determination of VEN and its three metabolites was developed and applied in plasma from patients and whole blood samples from forensic autopsy cases.
- The developed method covers a wide concentration range and the enantiomers of VEN and metabolites could be analyzed within a single run.
- By using a post column additive, the sensitivity of the method was markedly increased.
- No significant matrix effects were found for VEN or its metabolites. Furthermore, there were no findings of phospholipids or proteins in the sample extracts.

Experimental studies (Papers II and III)

- Using the Sprague-Dawley/Dark Agouti rat model, significant quantitative strain-related differences in the pharmacokinetics of CIT and VEN, and their metabolites, were observed.
- The results indicate that the CYP2D enzymes display a significant impact on the stereoselective metabolism of CIT and VEN, and their metabolites, in these two rat strains.
- The present findings may give hints for possible differences in the stereoselective pharmacokinetics of CIT and VEN in human CYP2D6 EMs and PMs.
- The two studies highlight the importance of comparing different rat strains when conducting experimental pharmacokinetic studies.

Forensic toxicological study (Paper IV)

- The results from this study show how the *CYP2D6* genotype influences the disposition of the enantiomers of VEN and its three main metabolites, in femoral blood from forensic autopsy cases.
- A substantial variation in the relationship between the S- and R-enantiomers of VEN, and metabolites, was found. In individuals lacking two functional *CYP2D6* alleles, a low S/R VEN ratio was strongly related to a high S/R ODV ratio.
- By using enantioselective analysis of VEN and ODV, it is possible to predict if a person is a PM genotype/phenotype for CYP2D6.
- The results show that information about the disposition of the enantiomers of VEN and its metabolites could assist in the interpretation of forensic toxicological results.

FUTURE ASPECTS

Bioanalysis. At the time when the enantioselective method (Paper I) was developed, no deuterated internal standard (IS) for VEN was commercially available; however, present both deuterated VEN and ODV are available. By using a deuterated IS some saturation effects can be compensated and variation of matrix effects between individuals reduced. Hence, it would be interesting to use a deuterated analogue as an IS in the present method. The enantioselective analysis of VEN is fairly time consuming. This is partly due to the long retention times in the bioanalysis of the enantiomers of VEN, but also due to the extensive SPE procedures needed before analysis and detection. By substitute the manual extraction with automated SPE, the method could be more time and labour effective.

Animal models. In order to assess the relationship between drug concentrations and clinical effect of antidepressant drugs, as well as the potential drug toxicity, the drugs were analysed in both animal models and human cases. The experimental studies performed in Paper II and III were based on an established animal model. This rat model mimic the pharmacokinetic features seen in human PMs of CYP2D6 (female Dark Agouti rats) and EMs of CYP2D6 (female Sprague-Dawley rats). This model is still valuable for pharmacokinetic studies of new drugs, for which the metabolic pathways are unknown. However, as mentioned previously the *CYP2D* subfamily has evolved differently in humans and rats. To overcome this issue of species differences in *CYP2D* isoforms between humans and rodents, a transgenic mouse line expressing the human *CYP2D6* gene has been generated (Corchero *et al*., 2001). The *CYP2D6* transgenic mice exhibits enhanced metabolism and disposition of debrisoquine and is, together with its wild-type control, models for human extensive

metabolisers and poor phenotypes, respectively. Human *CYP2D6* has been detected in the liver, kidneys, and intestine of these animals. Hence, the *CYP2D6* transgenic mouse has a broad application in studies of *CYP2D6* polymorphism in drug discovery and development, and in clinical practice toward individualized drug therapy (Corchero *et al*., 2001).

P-glycoprotein. Another factor responsible for the variability of drug response is the active transport of endogenous and exogenous substances over biological barriers such as the blood-brain-barrier (BBB). The transport protein P-glycoprotein (P-gp) is part of the BBB and protects the brain through an active transport of substances back to the blood stream. Studies suggest that P-gp may limit the ability of several antidepressants to cross the BBB, thus resulting in inadequate brain concentrations, contributing to the poor success rate of current antidepressant therapies (Uhr *et al*., 2008). Low expression of P-gp can increase molecules transfer through BBB, hence, a high expression of P-gp can inhibit drugs to reach the effect compartment, CNS, in therapeutic doses. A knock-out mouse has been introduced as an animal model to study the importance of P-gp in drug distribution (Schinkel *et al*., 1994). While P-gp is encoded by a single gene in humans (*ABCB1*), its functions are performed by two homologues in mice: the *abcb1a* and *abcb1b* genes. By comparing brain/plasma ratios between these P-gp-deficient knockout mice to those of wild-type controls, it has been possible to identify drugs as P-gp substrates *in vivo*. Studies performed by our research group, using P-gp knockout mice, have showed that P-gp plays an important role in limiting brain entry of the enantiomers of VEN and its metabolites after both acute and chronic dosing, offering the possibility that the expression of P-gp in patients may be a contributing factor for limited treatment response (Karlsson *et al*., 2010; 2011). Hence, it would be of great interest to perform studies on the

disposition of the enantiomers of CIT and VEN in relation to *ABCB1* genotype polymorphisms in materials comprising both patients and forensic autopsy cases.

Polymorphism in CYP2C19. CYP2D6 has been considered the major enzyme involved in the metabolism of VEN, and genetic variations of *CYP2D6* have been shown to be associated with differences in drug concentrations and clinical response. Recently, McAlpine *et al*., (2011), investigated the relation between concentrations of VEN and its active metabolite ODV and the genetic variants of both *CYP2D6* and *CYP2C19* in humans. Similar to *CYP2D6*, *CYP2C19* is also polymorphic and possesses several different gene variants which can result in abnormal metabolism. The study showed that the metabolism of VEN and ODV is not only influenced by *CYP2D6* but also by *CYP2C19*. Furthermore, the total concentrations of VEN+ODV were found to be more strongly associated with *CYP2C19* than with *CYP2D6*. Since both VEN and ODV are pharmacologically active the total concentration of these two compounds achieved during treatment is expected to affect the therapeutic response. Hence, McAlpine and co-workers suggest that polymorphisms in both genes are likely to have an impact on clinical outcome (McAlpine *et al*., 2011). In the future, it would be valuable with further studies, in patients as well as in forensic autopsy cases, to unravel the impact of CYP2C19 on the metabolism of VEN and its major metabolites.

Other racemic drugs. In 2008, desvenlafaxine, the racemic major active metabolite of venlafaxine, was approved for treatment of depression by the Food and Drug Administration (FDA) in the USA (Lourenco & Kennedy, 2009; Perry & Cassagnol, 2009). However, to the knowledge of the author, no pharmacokinetic nor pharmacodynamic data on the racemate or the separate enantiomers have been presented. Obviously, it is of great importance to perform experimental, clinical and

postmortem studies on the enantiomers of desvenlafaxine together with genotyping. Further, also other racemic drugs, such as mirtazapine and tramadol, may be of interest to study more profound with focus on their enantiomers.

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