The Role of Cytochrome P450 2C19 in Flunitrazepam Pharmacokinetics and Pharmacodynamics *In Vivo*

by

Inbal Gafni

A thesis submitted in conformity with the requirements for the degree of Master of Science, Institute of Medical Science, University of Toronto

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ABSTRACT
The Role of Cytochrome P450 2C19 in Flunitrazepam Pharmacokinetics and Pharmacodynamics In Vivo
Inbal Gafni, Master of Science
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Cytochrome P450 2C19 (CYP2C19) represents an important example of a pharmacogenetic polymorphism. Flunitrazepam is a widely used benzodiazepine that is metabolised by CYP2C19 in vitro. Our objective was to examine the role of CYP2C19 activity in determining flunitrazepam kinetics and response in vivo. The omeprazole (OMP) and 5-hydroxyomeprazole (5-OH-OMP) ratio determined in the plasma 3 h following drug administration was used as a measure of CYP2C19 activity. Thirteen healthy volunteers with metabolic ratios (MR) of OMP/5-OH-OMP ranging from 0.50-28.8 received flunitrazepam 1 mg orally. With increasing MR, average levels of flunitrazepam appeared to increase (r=0.35, p=0.25), and the ratios of flunitrazepam to both N-desmethylflunitrazepam and 3-hydroxyflunitrazepam appeared to increase (r=0.44, p=0.14 and r=0.54, p=0.06, respectively). Individuals with higher MR appeared to demonstrate larger decreases in psychomotor performance (r=0.48, p=0.11). In conclusion, these data suggest that CYP2C19 contributes to the metabolism of flunitrazepam to N-desmethylflunitrazepam and 3-hydroxyflunitrazepam.
In memory of my grandparents, Sara and Elehanan Vitos, and

In honour of my grandparents, Bina and Fima Levin,

Who have enriched my life with their love and inspiration.
ACKNOWLEDGEMENTS

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>3-HF</td>
<td>3-hydroxyflunitrazepam</td>
</tr>
<tr>
<td>5-OH-OMP</td>
<td>5-hydroxomeprazole</td>
</tr>
<tr>
<td>7-AF</td>
<td>7-aminoflunitrazepam</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the concentration-time curve</td>
</tr>
<tr>
<td>CYP</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>DMF</td>
<td>N-desmethyflunitrazepam</td>
</tr>
<tr>
<td>DSM-IV</td>
<td>Diagnostic and Statistical Manual of Mental Disorders (Fourth Edition)</td>
</tr>
<tr>
<td>EC</td>
<td>enteric coated</td>
</tr>
<tr>
<td>EM</td>
<td>extensive metaboliser</td>
</tr>
<tr>
<td>FLU</td>
<td>flunitrazepam</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>IS</td>
<td>internal standard</td>
</tr>
<tr>
<td>MFO</td>
<td>mixed-function oxidase</td>
</tr>
<tr>
<td>MR</td>
<td>metabolic ratio</td>
</tr>
<tr>
<td>OMP</td>
<td>omeprazole</td>
</tr>
<tr>
<td>OMP-S</td>
<td>omeprazole sulphone</td>
</tr>
<tr>
<td>PDHQ</td>
<td>Pharmacogenetics Drug History Questionnaire</td>
</tr>
<tr>
<td>PM</td>
<td>poor metaboliser</td>
</tr>
<tr>
<td>SMS</td>
<td>Scheduled Measurement System</td>
</tr>
<tr>
<td>VAS</td>
<td>visual analogue scale</td>
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</table>
1 Introduction

1.1 Background

1.1.1 Cytochromes P450

To produce its pharmacological effect, a drug must be able to reach its intended site of action in the appropriate concentrations. The fate of a drug, that is, the route it takes and the final concentration achieved, is dependent on pharmacokinetic factors including absorption, distribution, biotransformation and excretion (Figure 1.1) (Benet et al., 1996).

![Figure 1.1. Absorption, distribution, binding, biotransformation, and excretion of a drug and its concentration at its locus of action (adapted from Benet et al., 1996).](image)

1.1.1.1 Drug Biotransformation

Drug biotransformation refers to the chemical transformation of a drug or other xenobiotic within a living organism, usually by enzyme-catalysed reactions (Riddick, 1998). This process may result in activation, maintenance of activity or inactivation of the drug. In general, biotransformation produces more hydrophilic metabolites that can be readily
excreted from the body, thereby terminating their biological activity (Benet et al., 1996). Most of the enzyme systems involved in the biotransformation of drugs are located in the liver, although other tissues have some metabolic activity, including the gastrointestinal tract, lungs, skin and kidneys (Benet et al., 1996). Following oral administration, a drug can be metabolised in the gut wall or in the liver before it reaches the systemic circulation. In addition, the drug may be transformed in the gut by gastric acid, digestive enzymes or intestinal microorganisms. This phenomenon, which is referred to as the first-pass effect, significantly limits the bioavailability of some orally administered drugs (Correia, 1995).

Drug biotransformation reactions are commonly grouped into two phases (Benet et al., 1996; Correia, 1995; Riddick, 1998). Phase I includes oxidation, reduction, and hydrolysis reactions that introduce or unmask a functional group (e.g. hydroxyl, amine, sulfhydryl) that often makes the drug more polar. This process may change the pharmacological activity of the drug quantitatively or qualitatively, or leave it intact (Correia, 1995). Phase II includes conjugation reactions that lead to the formation of a covalent linkage between a functional group on the parent compound and an endogenous substrate such as glucuronic acid, sulfuric acid, glutathione, acetic acid or an amino acid. Generally, these highly polar conjugates are inactive and are excreted rapidly in the urine and feces (Benet et al., 1996; Riddick, 1998). Many drugs undergo the sequential process of a phase I reaction followed by a phase II reaction. There are many exceptions, however.

The majority of drug biotransformation reactions in vivo are catalysed by subcellular enzymes, located in the endoplasmic reticulum, mitochondria, cytosol, and (to a limited extent) the nuclear envelope and plasma membrane (Benet et al., 1996). The enzyme
systems involved in phase I reactions are located primarily in the endoplasmic reticulum, while phase II conjugation enzyme systems are mainly cytosolic (Benet et al., 1996).

1.1.1.2 Cytochromes P450
The mixed-function oxidase (MFO) system is responsible for catalysing the phase I oxidative biotransformation of numerous and diverse endogenous and exogenous chemicals (Benet et al., 1996; Riddick, 1998). This system requires the participation of two distinct proteins that are embedded in the phospholipid bilayer of the smooth endoplasmic reticulum membranes in the liver and other tissues. The first, the cytochrome P450, is a heme-containing protein that acts as a terminal oxidase for the MFO system. The name cytochrome P450 is derived from the fact that the reduced (ferrous) form of this hemoprotein, when bound to carbon monoxide, forms a complex that has a unique absorption spectrum with a maximum at 450 nm (Correia, 1995; Riddick, 1998). This protein functions in close association with the second membrane protein, NADPH-cytochrome P450 reductase. This flavoprotein reductase contains equimolar amounts of flavin mononucleotide and flavin adenine dinucleotide and serves to transfer electrons to the cytochrome P450-drug complex (Benet et al., 1996; Correia, 1995; Riddick, 1998). There appear to be between five and twenty molecules of cytochrome P450 per molecule of reductase in the liver, and thus heme reduction is generally thought to be the rate-limiting step in drug oxidation reactions (Correia, 1995; Riddick, 1998).

The activity of these enzymes in the MFO system also requires both the reducing cofactor NADPH and molecular oxygen. In a typical reaction, one molecule of oxygen is consumed (reduced) per substrate molecule; one oxygen atom is incorporated into the product and the other contributes to the formation of water (Benet et al., 1996; Correia, 1995; Riddick, 1998). The overall balanced equation for the reaction is as follows:
In this equation, RH represents an oxidizable drug substrate and ROH represents the hydroxylated product of the reaction. A simplified scheme of the cytochrome P450-catalysed oxidative cycle is presented in Figure 1.2. This system catalyses many oxidative biotransformations including aromatic and side chain hydroxylation, deamination, dehalogenation and desulfuration, as well as some reduction reactions. This broad spectrum of reactions is attributable to the existence of multiple P450 isozymes with differing but overlapping substrate specificities (Benet et al., 1996; Riddick, 1998).

**Figure 1.2.** The catalytic cycle for a cytochrome P450-mediated hydroxylation reaction. Fe represents the heme iron, RH the substrate, and ROH the hydroxylated product (Riddick, 1998).
Over the past two billion years, the cytochrome P450 gene superfamily has evolved to metabolise an increasing number of diverse chemicals, including endogenous substances, environmental chemicals, food toxins and drugs (Guengerich, 1993). The formation of new genes can occur through DNA turnover events, including gene duplication by unequal crossing over, gene inactivations or terminal mutational events, replication slippage, insertions and gene conversions (Gonzalez & Nebert, 1990). Gonzalez and Nebert asserted that these molecular events may have been responsible for the origin and spread of most new P450 genes (Gonzalez & Nebert, 1990).

A unified nomenclature system for the P450 superfamily based on its divergent evolution has been established. Each cytochrome P450 gene or isozyme is named by the root symbol “CYP”, representing cytochrome P450, followed by an Arabic number denoting the gene family, a capital letter designating the gene subfamily and an Arabic number representing the individual gene within the subfamily (Nelson et al., 1996). The mammalian cytochrome P450 superfamily consists of at least 14 families and 26 subfamilies (Nelson et al., 1996). In general, cytochrome P450 proteins with greater than 40% sequence identity are included in the same family, and those with greater than 55% identity are included in the same subfamily (Nelson et al., 1996).

The cytochrome P450 1, 2 and 3 families (CYP1, CYP2 and CYP3) encode the enzymes that are thought to be responsible for the biotransformation of the majority of xenobiotic compounds (drugs, toxins, foods, etc.), while the gene products of the remaining cytochrome P450 families are important in the metabolism of endogenous compounds such as steroids and fatty acids (Benet et al., 1996; Gonzalez, 1992). The xenobiotic-metabolising cytochromes P450 are thought to have evolved in animals from the steroidogenic
cytochromes P450 (Gonzalez, 1992). According to one model, it was the interaction with plants, which are known to produce and secrete toxins to make them less desirable for predatory animals, that led to the selection in animals for the ability to destroy such toxins. This interaction is thought to have intensified when animals began to emerge onto land and encounter new terrestrial plants for the first time. This period of animal-plant warfare is associated with the explosion of new genes, particularly in the CYP2 family, during the past 400 million years (Gonzalez, 1992; Gonzalez & Nebert, 1990; Nebert, 1997; Nelson et al., 1996).

Table 1.1 summarises the relative proportions of the major drug metabolising cytochromes present in the human liver. CYP2C isozymes account for approximately 20% of liver cytochrome P450 proteins (Shimada et al., 1994). The genes that encode them are arranged in a cluster at chromosome 10q24 in the order CYP2C8, CYP2C9, CYP2C19 and CYP2C18 from the upstream to the downstream direction (Gray et al., 1995). The CYP2Cs are constitutively expressed in liver (Gonzalez, 1992). Recently, the detection of their mRNA has been reported in kidney, adrenal gland, brain, uterus, mammary gland, ovary, prostate, testes and duodenum, and 2C9 and 2C19 protein detected in small intestinal microsomes (Klose et al., 1999).
Table 1.1. Contents of total P-450 determined spectrally and individual P-450 forms determined immunochemically in liver microsomes from 30 Japanese and 30 Caucasian patients (Shimada et al., 1994). Values represent mean ± S.D. from 60 human samples.

<table>
<thead>
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<th>P-450 content in liver microsomes</th>
<th>nmol/mg protein</th>
<th>% of total P-450</th>
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<tr>
<td>Total P-450&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.344 ± 0.167</td>
<td></td>
</tr>
<tr>
<td>P-450 1A2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.042 ± 0.023</td>
<td>12.7 ± 6.2</td>
</tr>
<tr>
<td>P-450 2A6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.014 ± 0.013</td>
<td>4.0 ± 3.2</td>
</tr>
<tr>
<td>P-450 2B6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.001 ± 0.002</td>
<td>0.2 ± 0.3</td>
</tr>
<tr>
<td>P-450 2C&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.060 ± 0.027</td>
<td>18.2 ± 6.7</td>
</tr>
<tr>
<td>P-450 2D6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.005 ± 0.004</td>
<td>1.5 ± 1.3</td>
</tr>
<tr>
<td>P-450 2E1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.022 ± 0.012</td>
<td>6.6 ± 2.9</td>
</tr>
<tr>
<td>P-450 3A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.096 ± 0.051</td>
<td>28.8 ± 10.4</td>
</tr>
<tr>
<td>Total&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.240 ± 0.100</td>
<td>72.0 ± 15.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Determined spectrally
<sup>b</sup> Determined immunochemically
<sup>c</sup> Sum of individual forms of P-450 determined immunochemically

1.1.1.3 Metabolic Drug-Drug Interactions

A drug-drug interaction refers to one drug altering the pharmacological effects of another drug given concurrently (Lin & Lu, 1998). In the case of drug biotransformation, this event is often the result of enzyme induction and inhibition.

i. **Induction.** Certain chemicals have the ability to induce cytochromes P450 by enhancing the rate of their synthesis. For example, rifampicin is a well known inducer of several cytochromes P450, including CYP3A (Combalbert et al., 1989), CYP2C19 (Zhou et al., 1990) and CYP2C9 (Herman, 1999; Williamson et al., 1998). Many molecular mechanisms for enzyme induction have been characterised: increased gene transcription is the most common means of induction; however, post-transcriptional mechanisms such as RNA processing, mRNA stabilization, translational efficiency, and protein stabilization are also important in the regulation of expression of cytochromes P450 and may be involved in induction (Lin & Lu, 1998; Riddick, 1998). Induction results in an increased rate of biotransformation and a corresponding decrease in the availability of the
parent drug. When metabolites are less pharmacologically active than the parent drug, enzyme induction results in a reduction in pharmacological effect (Lin & Lu, 1998). In some cases, the metabolites formed during biotransformation may be clinically active, so that enzyme induction may result in increased drug action or drug-induced toxicity (Lin & Lu, 1998).

ii. Inhibition. Inhibition of drug biotransformation increases levels of the parent drug and may lead to prolonged pharmacological effects and increased incidence of drug-induced toxicity. For example, coadministration of terfenadine, an antihistamine agent metabolised by CYP3A4, and ketoconazole, a potent CYP3A4 inhibitor, led to fatal arrhythmias in some patients (Lin & Lu, 1998; Monahan et al., 1990). Inhibition can also reduce clinical efficacy if the drug is a prodrug requiring metabolic activation to achieve its effects (Lin & Lu, 1998). Inhibition can occur by several mechanisms. Competition between two or more drugs for the active site of the same enzyme (competitive inhibition) may lead to a decrease in the metabolism of one or both of these agents, depending on the relative concentration of each substrate and their relative affinities for the enzyme. An enzyme may also be irreversibly bound to a substrate, thus leading to its inactivation (Benet et al., 1996).

1.1.1.4 Non-Genetic Determinants of Individual Variation in Drug Metabolism
The dose and frequency of administration of a drug required to achieve the desired effect vary between individuals because of individual differences in pharmacodynamic and pharmacokinetic factors such as absorption, distribution, biotransformation and elimination. Physiological factors such as age and sex, disease states, diet, cigarette smoking and other environmental determinants may be involved in the regulation of drug metabolism (Benet et al., 1996; Correia, 1995; Matthews, 1995; Riddick, 1998).
1.1.1.5 Genetic Polymorphism in Drug Metabolism

Genetic factors are an important determinant of the inter-individual differences in drug metabolism. Genetic variants of drug-metabolising enzymes have been identified when individuals with atypical clinical responses were shown to have impaired metabolism of a drug (Tyndale & Sellers, 1997). Phenotypic differences in the amount of drug excreted through a polymorphically controlled pathway led to the classification of individuals as extensive (rapid) or poor (slow) metabolisers. A genetic polymorphism is commonly defined as the occurrence in a population of two or more phenotypic forms associated with different alleles of one gene (Griffiths et al., 1993), with each allele occurring with a frequency of 1% or higher (Nelson, 1999). Poor metabolisers (PMs) are individuals who possess two mutant alleles of the polymorphic gene, while extensive metabolisers (EMs) include individuals who carry two wild-type alleles and those who are heterozygous wild-type/mutant.

Many genetic polymorphisms are associated with oxidative drug metabolism and more are continually being discovered. The debrisoquine and mephenytoin polymorphisms are the best studied examples. A deficiency in debrisoquine hydroxylase activity in a subset of the population reflects one or more mutations in the CYP2D6 gene, which lead to the synthesis of CYP2D6 proteins that are either truncated or have altered enzyme activity (Kagimoto et al., 1990; Wrighton & Stevens, 1992). Affected individuals display impaired biotransformation of CYP2D6 substrates, including dextromethorphan, β-adrenoceptor antagonists such as bufuralol, antiarrythmics such as spartine, tricyclic antidepressants such as imipramine, and morphine derivatives such as codeine (Wrighton & Stevens, 1992). A polymorphism in the CYP2C19 gene has been found to account for impaired hydroxylation of the anticonvulsant mephenytoin (Goldstein et al., 1994; Wrighton et al., 1993). This polymorphism is discussed in detail below. The polymorphic expression of drug
metabolizing enzymes may in this way give rise to variability in pharmacologic and toxicologic responses.

Genetic polymorphisms also affect metabolic drug-drug interactions. Extensive metabolisers are susceptible to enzyme induction and inhibition while poor metabolisers are not (Lin & Lu, 1998). For example, rifampicin induces several CYP isoforms, including CYP2C and CYP3A. Treatment with rifampicin causes an increase in the metabolism of S-mephenytoin in EMs but not in PMs because of the absence of CYP2C19 in PMs (Zhou et al., 1990). Differential induction between PM and EM individuals can also occur as a result of the heterogeneous metabolism of the inducer (Lin & Lu, 1998). Omeprazole, for example, induces human CYP1A2 (Diaz et al., 1990) and is metabolised by the polymorphic CYP2C19 (Andersson et al., 1990; Andersson et al., 1990). After pretreatment with omeprazole, PMs (in whom omeprazole concentration is higher) experience more CYP1A2 induction than EMs, owing to their greater exposure to the drug (Rost et al., 1992). In another example, coadministration of omeprazole with diazepam, both of which are metabolised by CYP2C19, resulted in a significant increase in the AUC of diazepam in EMs but not in PMs, who have no enzyme activity for which the drugs could compete (Andersson et al., 1990).
1.1.2 The Genetically Polymorphic Cytochrome P450 2C19

1.1.2.1 Metabolism of S-Mephenytoin and the CYP2C19 Polymorphism

Mephenytoin (3-methyl-5-phenyl-5-ethylhydantoin; Mesantoin<sup>®</sup>), a 5,5-disubstituted hydantoin, was developed in the early 1940s as an antiepileptic drug (Daniel & Edeki, 1996; Wilkinson et al., 1989). It is available as a racemic mixture of S and R enantiomers. The metabolic pathways of mephenytoin consist of hydroxylation to 3-methyl-5(4-hydroxyphenyl)-5-ethylhydantoin (4'-hydroxymephenytoin) and N-demethylation to phenyl-ethylhydantoin (PEH; nivanol). In humans, the S-enantiomer is rapidly eliminated, predominantly as 4'-hydroxymephenytoin, whereas the R-enantiomer is slowly eliminated, mainly as PEH (Daniel & Edeki, 1996; Kupfer et al., 1981). This difference in metabolism is due to the stereoselective 4'-hydroxylation of the phenyl ring of the S isomer (Kupfer et al., 1981).

Investigations of the stereoselective metabolism of mephenytoin led to the discovery of a polymorphism in the 4'-hydroxylation of the S-enantiomer of mephenytoin (Kupfer et al., 1984). Typically, approximately 43% of a racemic dose of mephenytoin is recovered in a 24-hour urine sample in hydroxylated form, reflecting the almost complete hydroxylation and renal excretion of S-mephenytoin (Inaba et al., 1984). In certain individuals, however, only a few percent of the dose is excreted in the urine sample as 4'-hydroxymephenytoin (Inaba et al., 1984; Kupfer & Preisig, 1984; Wedlund et al., 1984). Thus, individuals can be characterised as either extensive or poor metabolisers of S-mephenytoin. Family studies suggested that mephenytoin 4'-hydroxylation activity is under diallelic, monogenic control, with the PM phenotype arising from the homozygous autosomal recessive genotype and the EM phenotype arising from both homozygous dominant and heterozygous genotypes (Inaba...
et al., 1986; Ward et al., 1987). No polymorphism has been reported in the metabolism of the R enantiomer.

The enzyme responsible for the 4'-hydroxylation of S-mephenytoin appeared to be a form of cytochrome P450 belonging to the 2C subfamily (Ged et al., 1988). Immunoblot analysis revealed that levels of CYP2C19 protein were correlated with microsomal S-mephenytoin 4'-hydroxylase activities in human livers (Goldstein et al., 1994; Wrighton et al., 1993). Further, Goldstein and colleagues (1994) used a yeast cDNA expression system to demonstrate that CYP2C19 has an extremely high turnover number for the 4'-hydroxylation of S-mephenytoin, approximately 100-fold higher than that of 2C9, 2C18 or 2C8 (Goldstein et al., 1994). Thus, CYP2C19 is now thought to be the principal enzyme responsible for the 4'-hydroxylation of S-mephenytoin (Goldstein et al., 1994; Wrighton et al., 1993).

1.1.2.1 Functional Characterisation of the CYP2C19 Polymorphism
The functional characterisation of CYP2C19 as a metabolic phenotype can be derived from the metabolic profiles of individuals after oral administration of racemic mephenytoin. Two analytical methods are commonly used; one expresses the trait as a hydroxylation index (HI), the other measures the ratio of the S and R enantiomers of mephenytoin in urine (S/R ratio). The HI method is based on the differential excretion of 4'-hydroxymephenytoin in EMs and PMs. The HI consists of an estimation of the molar ratio of the administered dose of S-mephenytoin to the amount of 4'-hydroxymephenytoin recovered in the urine either 8 or 12 hours post-administration (Inaba et al., 1984; Kupfer & Preisig, 1984; Wedlund et al., 1984). The difference between PM and EM traits is usually clear because in PMs very low amounts of the drug are excreted as 4'-hydroxymephenytoin, whereas in EMs, the S-enantiomer is almost completely hydroxylated (Daniel & Edeki, 1996).
The S/R ratio method relies on the observation that, while there are pronounced differences in the hydroxylation of S-mephenytoin by PMs and EMs, rates of renal clearance of the S and R enantiomers of the unchanged drug are similar (Jacqz et al., 1986; Wedlund et al., 1984). Urine samples are collected over an 8-hour period, and a second period of collection (for example, from 24 to 32 h) is often used as well (Bertilsson et al., 1992; Daniel & Edeki, 1996). In EMs, where S-mephenytoin is rapidly eliminated by hydroxylation, the S/R ratio is low (<0.9) in the first urine sample and near zero in the second (the later the urine collection, the greater the separation of the two phenotypes). In PMs, where S-mephenytoin is not hydroxylated to any great extent, this ratio will be close to unity (>0.9) in both samples (Bertilsson et al., 1992; Daniel & Edeki, 1996). Thus, the greater the ability to hydroxylate S-mephenytoin, the lower the S/R ratio (Wilkinson et al., 1989).

More recently, a third method of phenotyping CYP2C19 activity has been developed that uses omeprazole, a substrate of CYP2C19, instead of mephenytoin (Chang et al., 1995). This method is further discussed below in section 1.1.3.5.

1.1.2.3 Molecular Characterisation of CYP2C19
Two functional wild-type alleles for the CYP2C19 gene have been reported, namely CYP2C19wt1 (CYP2C19*1A) and CYP2C19wt2 (CYP2C19*1B) (Ibeanu et al., 1998; Romkes et al., 1991). CYP2C19*1B differs from CYP2C19*1A by an A991 to G substitution resulting in the amino acid change Ile331 to Val (Ibeanu et al., 1998). The molecular basis of the CYP2C19 functional polymorphism has been attributed to several mutations in the CYP2C19 gene that affect the expression or metabolic activity of CYP2C19 with regard to the hydroxylation of S-mephenytoin. The two most common mutations result in null alleles (deMorais et al., 1994; deMorais et al., 1994). The most common mutant allele, in both Caucasian and Oriental populations, has a single base pair (G→A) transition (CYP2C19m1 or
CYP2C19*2) at position 681 in exon 5 that creates an aberrant splice site. This change alters the reading frame of the mRNA starting with amino acid 215 and produces a premature stop codon 20 amino acids downstream. The resulting truncated 234-amino acid protein lacks the heme-binding region and, therefore, is catalytically inactive. This mutation was reported to account for 75% and 83% of the mutant alleles in Japanese and Caucasian subjects, respectively (deMorais et al., 1994). Subsequently, a second mutant allele (CYP2C19m2 or CYP2C19*3) was identified in Japanese PMs consisting of a G→A transition at position 636 that creates a premature stop codon in exon 4. The resulting truncated 211-amino acid CYP2C19 protein lacks the heme-binding region and the majority of the putative substrate binding regions, and is therefore inactive (deMorais et al., 1995; deMorais et al., 1994). CYP2C19*3 is found primarily in Orientals (20 to 25% of PM alleles) and rarely in Caucasians (~1% of PM alleles) (Brosen et al., 1995; deMorais et al., 1994). Together, the two defective alleles, CYP2C19*2 and CYP2C19*3, account for greater than 99% of Oriental but only 87% of Caucasian PM alleles (Brosen et al., 1995; deMorais et al., 1995; deMorais et al., 1994; deMorais et al., 1994), indicating the presence of additional mutant alleles accounting for the PM phenotype in Caucasians.

Recently, Ibeanu et al. (1998) identified a G276→C transversion in exon 2 resulting in a coding change Glu92→Asp on the CYP2C19*2 allele that also contained the known splice mutation in exon 5. This variant is termed CYP2C19*2B to distinguish it from the original variant now termed CYP2C19*2A. Fifteen percent of Caucasian CYP2C19*2 alleles were identified as CYP2C19*2B and 85% as CYP2C19*2A (Ibeanu et al., 1998).

Five additional mutant alleles have been reported. CYP2C19*4 contains an A→G transition that results in the substitution of the initiator methionine (ATG) codon for valine
(GTG) codon. The defective nature of this allele was verified by its lack of expression in a yeast cDNA expression system, the absence of translation in an *in vitro* coupled transcription-translation assay, and its presence in previously unexplained PMs of mephenytoin (Ferguson *et al*., 1998). This mutation accounted for 3% of 37 Caucasian PM alleles, increasing the sensitivity of genetic tests to 91% in Caucasian PMs (Ferguson *et al*., 1998). Garcia-Barcelo and colleagues found one CYP2C19*4 allele in one out of 21 Hong Kong Chinese subjects, giving an allele frequency of 0.004 (Garcia-Barcelo *et al*., 1999). A fourth mutation (CYP2C19*5A) was discovered in a single Chinese PM outlier belonging to the Bai ethnic group. This rare mutation contained a single C→T transition at position 1297 in exon 9, leading to an Arg133 to Trp substitution in the heme-binding region, but in all other respects was identical to CYP2C19*1A (Xiao *et al*., 1997). A second variant allele (CYP2C19*5B) containing the C→T transition at position 1297 was identified on a CYP2C19*1B allele background in one of 37 Caucasian PMs (Ibeanu *et al*., 1998). Mutagenesis experiments indicated that the single amino acid change Arg133 to Trp in the heme binding region essentially abolished activity of the recombinant CYP2C19*5A protein toward both S-mephenytoin and tolbutamide, indicating that the CYP2C19*5 alleles represent functionally deficient alleles (Ibeanu *et al*., 1998). Hence, these alleles contribute to the PM phenotype in both Chinese and Caucasian groups and their characterisation increases the maximum sensitivity of the genetic tests for identifying PMs to approximately 100% in Chinese and 92% in Caucasians (Ibeanu *et al*., 1998). CYP2C19*6 consists of a single base pair mutation (G395→A) in exon 3 and an Arg132→Gln coding change, and codes for a protein with negligible activity towards mephenytoin and tolbutamide in a cDNA expression system (Ibeanu *et al*., 1998). This mutation accounted for 1.4% of the defective
alleles in 37 Caucasian PMs (Ibeanu et al., 1998). The CYP2C19*7 allele contains a single T→A nucleotide transversion in the invariant GT at the 5' donor splice site of intron 5 and was found to contribute to the PM phenotype (Ibeanu et al., 1999). One out of 37 Danish Caucasian putative PMs subjects was found to be heterozygous for this allele (Ibeanu et al., 1999). Finally, CYP2C19*8 consists of a T138→C nucleotide transition in exon 3 that results in a Trp120→Arg substitution. Except for a silent T99→C transition in exon 1, this mutant allele is similar to the wild-type CYP2C19*1B allele (Ibeanu et al., 1999). CYP2C19*8 was found to contribute to the PM phenotype in a lung cancer case control study in French Caucasians. In this study, the frequency of the CYP2C19*8 allele was found to be 0 and 0.07 in 172 case controls and 152 individuals with lung cancer, respectively (Ibeanu et al., 1999). In a bacterial expression system, CYP2C19*8 protein exhibited a dramatic reduction of approximately 90% and 70% in the metabolism of S-mephenytoin and tolbutamine, respectively, when compared with the wild-type CYP2C19*1B protein (Ibeanu et al., 1999).

1.1.2.4 Ethnic Differences in CYP2C19-Catalysed Drug Metabolism

Differences among ethnoracial groups in the genetic determinants of the way they respond to and metabolise drugs have been described. For example, population studies indicate that 5 to 10% of Caucasians are PMs of debrisoquine (i.e. genetically deficient for the CYP2D6 enzyme) compared to approximately 1% of Asians (Nakamura et al., 1985). Considerable interethnic and geographical heterogeneity has also been reported in the distribution of the S-mephenytoin phenotype. The distribution of the PM phenotype of S-mephenytoin and the methods of determination for various ethnic groups are presented in Table 1.2.
<table>
<thead>
<tr>
<th>Population</th>
<th>PM total</th>
<th>% PM</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Asian</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Japanese</td>
<td>45/200</td>
<td>22.5</td>
<td>HI</td>
<td>(Horai et al., 1989)</td>
</tr>
<tr>
<td>Japanese (Canadian)</td>
<td>7/31</td>
<td>22.6</td>
<td>HI</td>
<td>(Jurima et al., 1985)</td>
</tr>
<tr>
<td>Japanese</td>
<td>18/100</td>
<td>18.0</td>
<td>S/R ratio</td>
<td>(Nakamura et al., 1985)</td>
</tr>
<tr>
<td>Korean</td>
<td>26/206</td>
<td>12.6</td>
<td>HI</td>
<td>(Sohn et al., 1992)</td>
</tr>
<tr>
<td>Chinese (Mainland)</td>
<td>17/98</td>
<td>17.4</td>
<td>HI</td>
<td>(Horai et al., 1989)</td>
</tr>
<tr>
<td>Chinese (Han)</td>
<td>20/137</td>
<td>14.6</td>
<td>S/R ratio</td>
<td>(Bertilsson et al., 1992)</td>
</tr>
<tr>
<td>Chinese (Han)</td>
<td>20/101</td>
<td>19.8</td>
<td>S/R ratio</td>
<td>(Xiao et al., 1997)</td>
</tr>
<tr>
<td>Chinese (Bai)</td>
<td>27/202</td>
<td>13.4</td>
<td>S/R ratio</td>
<td>(Xiao et al., 1997)</td>
</tr>
<tr>
<td>Chinese (Canadian)</td>
<td>2/39</td>
<td>5.1</td>
<td>HI</td>
<td>(Jurima et al., 1985)</td>
</tr>
<tr>
<td>Vietnamese</td>
<td>8/37</td>
<td>21.6</td>
<td>S/R ratio</td>
<td>(Brosen et al., 1995)</td>
</tr>
<tr>
<td>Indonesian</td>
<td>16/104</td>
<td>15.4</td>
<td>HI</td>
<td>(Setabudy et al., 1994)</td>
</tr>
<tr>
<td>Filipino (Saudi)</td>
<td>13/55</td>
<td>23.6</td>
<td>S/R ratio</td>
<td>(Price-Evans et al., 1995)</td>
</tr>
<tr>
<td>Indian</td>
<td>10/48</td>
<td>20.8</td>
<td>S/R ratio</td>
<td>(Doshi et al., 1990)</td>
</tr>
<tr>
<td><strong>Middle East</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saudi Arabia</td>
<td>2/102</td>
<td>2.0</td>
<td>S/R ratio</td>
<td>(Price-Evans et al., 1995)</td>
</tr>
<tr>
<td>Jewish Israeli</td>
<td>4/140</td>
<td>2.9</td>
<td>S/R ratio</td>
<td>(Sviri et al., 1999)</td>
</tr>
<tr>
<td><strong>African/African-American</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zimbabwe (Shona)</td>
<td>4/103</td>
<td>4.0</td>
<td>S/R ratio</td>
<td>(Masimirembwa et al., 1995)</td>
</tr>
<tr>
<td>Tanzanian</td>
<td>7/216</td>
<td>3.6</td>
<td>S/R ratio</td>
<td>(Bathum et al., 1999)</td>
</tr>
<tr>
<td>USA (middle TN)</td>
<td>2/191</td>
<td>3.8</td>
<td>S/R ratio</td>
<td>(Edeki et al., 1996)</td>
</tr>
<tr>
<td><strong>European</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estonian</td>
<td>6/156</td>
<td>3.9</td>
<td>S/R ratio</td>
<td>(Kiivet et al., 1993)</td>
</tr>
<tr>
<td>Danish</td>
<td>9/358</td>
<td>2.5</td>
<td>S/R ratio</td>
<td>(Drohse et al., 1989)</td>
</tr>
<tr>
<td>French</td>
<td>8/132</td>
<td>6.1</td>
<td>HI</td>
<td>(Jacqz et al., 1988)</td>
</tr>
<tr>
<td>Swedish</td>
<td>16/488</td>
<td>3.3</td>
<td>S/R ratio</td>
<td>(Bertilsson et al., 1992)</td>
</tr>
<tr>
<td>Swiss</td>
<td>12/221</td>
<td>5.4</td>
<td>HI</td>
<td>(Kupfer &amp; Preisig, 1984)</td>
</tr>
<tr>
<td>Spanish</td>
<td>5/373</td>
<td>1.3</td>
<td>S/R ratio</td>
<td>(Reviriego et al., 1993)</td>
</tr>
<tr>
<td><strong>American-European</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>USA (middle TN)</td>
<td>3/166</td>
<td>1.8</td>
<td>S/R ratio</td>
<td>(May et al., 1994)</td>
</tr>
<tr>
<td>USA (middle TN)</td>
<td>4/156</td>
<td>2.6</td>
<td>S/R ratio</td>
<td>(Wedlund et al., 1984)</td>
</tr>
<tr>
<td>USA (middle TN)</td>
<td>5/183</td>
<td>2.7</td>
<td>S/R ratio</td>
<td>(Nakamura et al., 1985)</td>
</tr>
<tr>
<td>Canadian</td>
<td>5/113</td>
<td>4.2</td>
<td>HI</td>
<td>(Jurima et al., 1985)</td>
</tr>
<tr>
<td>Canadian</td>
<td>2/83</td>
<td>2.4</td>
<td>HI</td>
<td>(Inaba et al., 1984)</td>
</tr>
</tbody>
</table>
There are striking ethnic differences in the incidence of CYP2C19 PM individuals, ~2-5% in Caucasians versus ~13-23% in Asian populations. No epidemiologic data to date exist to suggest that a particular genotype for CYP2C19 offers any survival advantage or disadvantage (Gonzalez & Nebert, 1990). The "founder effect," which is the overpropagation of a particular allele when a small group of individuals breaks off from a larger population to found a new genetically isolated colony, may provide part of the explanation for these ethnic differences, even in the absence of selection pressures (Lewis et al., 1998). Gonzalez and Nebert asserted, however, that this explanation cannot account for the large geographic differences in pharmacogenetic alleles, and that therefore some compensating advantage must be suspected (Gonzalez & Nebert, 1990; Nebert, 1997). Nebert proposed that differences in allele frequencies between ethnic groups may be due to difference in diet that may have evolved over thousands of years (Nebert, 1997). He also proposed that these differences may reflect balanced polymorphisms, where the heterozygote is conferred an advantage that offsets the disadvantage of the mutant homozygote (Nebert, 1997).

There are differences between ethnoracial groups not only in the incidence of PMs of S-mephenytoin but also between EMS in the metabolism of various substrates of CYP2C19. For example, the metabolism of diazepam and mephenytoin cosegregate in Caucasian subjects, suggesting that both are metabolised by CYP2C19 (Bertilsson et al., 1989). This cosegregation does not seem to occur in Chinese, however (Zhang et al., 1990). Only a small and non-significant difference in clearance of diazepam was found between Chinese EMS and PMs of CYP2C19, with both EMS and PMs resembling Caucasian PMs (Zhang et al., 1990). Similarly, the area-under-the-concentration-time-curve (AUC) of omeprazole in
Chinese EMs was found to be almost three-fold higher than in Caucasian EMs, though both were significantly lower than their PM counterparts (Andersson et al., 1992). The over-representation of heterozygotes (who may have an intermediate phenotype) in the Chinese EM groups has been proposed as one explanation for this phenomenon (Bertilsson & Kalow, 1993; Ishizaki et al., 1994; Xie, 1997; Zhang et al., 1990). Other proposed explanations include the possibility of inter-ethnic variability in substrate specificity of CYP2C19, and the possibility of inter-ethnic variability in the frequency of unidentified alleles of reduced but not absent function (Bertilsson & Kalow, 1993; Ishizaki et al., 1994; Zhang et al., 1990).

Additional factors that may have contributed to these findings are gender and dietary differences in the studied populations (Bertilsson & Kalow, 1993). These considerations may also apply to other drugs that are metabolised by CYP2C19.

1.1.2.5 Other Substrates and Inhibitors of CYP2C19
An increasing number of therapeutically important drugs are known to be metabolised by CYP2C19. Table 1.3 lists some selected drugs that are reported to be substrates and inhibitors of CYP2C19.
### Table 1.3. Some selected substrates and inhibitors of CYP2C19

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Evidence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anticonvulsants</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mephenytoin, nirvanol</td>
<td>in vivo</td>
<td>(Kupfer &amp; Preisig, 1984)</td>
</tr>
<tr>
<td>Hexobarbital</td>
<td>in vitro</td>
<td>(Adedoyin et al., 1994; Yasumori et al., 1990)</td>
</tr>
<tr>
<td>Phenobarbitone</td>
<td>in vivo</td>
<td>(Mamiya et al., 2000)</td>
</tr>
<tr>
<td><strong>Benzodiazepines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diazepam/desmethyldiazepam</td>
<td>in vitro/in vivo</td>
<td>(Andersson et al., 1994)</td>
</tr>
<tr>
<td>Adinazolam</td>
<td>in vitro</td>
<td>(Venkatakrishnan et al., 1998)</td>
</tr>
<tr>
<td>Temazepam</td>
<td>in vitro</td>
<td>(Ono et al., 1996)</td>
</tr>
<tr>
<td><strong>Antimalarial</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proguanil</td>
<td>in vitro/in vivo</td>
<td>(Funck-Brentano et al., 1997; Somogyi et al., 1996; Wright et al., 1995)</td>
</tr>
<tr>
<td>Chlorproguanil</td>
<td>in vitro</td>
<td>(Wright et al., 1995)</td>
</tr>
<tr>
<td><strong>Proton pump inhibitors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Omeprazole/omeprazole sulphone</td>
<td>in vitro/in vivo</td>
<td>(Andersson et al., 1994; Andersson et al., 1990)</td>
</tr>
<tr>
<td>Lansoprazole</td>
<td>in vitro</td>
<td>(Pearce et al., 1996)</td>
</tr>
<tr>
<td><strong>Tricyclic Antidepressants</strong></td>
<td></td>
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</tr>
<tr>
<td>Amitriptyline</td>
<td>in vitro</td>
<td>(Olesen &amp; Linnet, 1997)</td>
</tr>
<tr>
<td>Clomipramine</td>
<td>in vitro</td>
<td>(Koyama et al., 1997; Nielsen et al., 1996)</td>
</tr>
<tr>
<td>Imipramine</td>
<td>in vitro</td>
<td>(Koyama et al., 1997; Morinobu et al., 1997)</td>
</tr>
<tr>
<td><strong>Selective serotonin reuptake inhibitors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citalopram</td>
<td>in vivo</td>
<td>(Baumann et al., 1996)</td>
</tr>
<tr>
<td>Sertraline</td>
<td>in vitro</td>
<td>(Xu et al., 1999)</td>
</tr>
<tr>
<td><strong>Skeletal muscle relaxant</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carisoprodol</td>
<td>in vivo</td>
<td>(Dalen et al., 1996)</td>
</tr>
<tr>
<td><strong>Steroid hormones</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Progesterone</td>
<td>in vitro</td>
<td>(Yamazaki &amp; Shimada, 1997)</td>
</tr>
<tr>
<td>Testosterone</td>
<td>in vitro</td>
<td>(Yamazaki &amp; Shimada, 1997)</td>
</tr>
<tr>
<td><strong>Inhibitors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>in vivo</td>
<td>(Jeppesen et al., 1996)</td>
</tr>
<tr>
<td>Fluvoxamine</td>
<td>in vivo</td>
<td>(Jeppesen et al., 1996; Jeppesen et al., 1997)</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>in vitro</td>
<td>(vonMoltke et al., 1998)</td>
</tr>
</tbody>
</table>
1.1.3 Omeprazole

Omeprazole (OMP), a substituted benzimidazole, is a specific inhibitor of the H⁺/K⁺ ATPase proton pump in gastric parietal cells (Wallmark, 1986). It is a potent long-acting inhibitor of gastric secretion that is used in the treatment of duodenal and peptic ulcers, refractory gastroesophageal reflux disease, Zollinger-Ellinson syndrome and other related hypersecretory conditions (Holt & Howden, 1991; Howden, 1991; Maton, 1991).

1.1.3.1 Pharmacodynamics

The enzyme H⁺/K⁺-ATPase is a membrane-spanning enzyme that actively secretes hydrogen ions in exchange for potassium ions. Following parietal cell activation, intracytoplasmic vesicles containing H⁺/K⁺-ATPase fuse with the apical membrane and its secretory canaliculi, exposing the enzyme to the gastric lumen (Holt & Howden, 1991).

Inhibition of H⁺/K⁺-ATPase by omeprazole blocks the final common pathway for gastric acid secretion. Omeprazole blocks the response of H⁺/K⁺-ATPase to extracellular stimuli as well as to intracellular cyclic adenosine monophosphate (cAMP), a second messenger that activates H⁺/K⁺-ATPase from within the parietal cell (Holt & Howden, 1991; Wallmark, 1986). Thus omeprazole inhibits both basal and stimulated acid secretion irrespective of the stimulus. Omeprazole also causes also a dose-dependent increase in gastrin levels (Howden, 1991).

Omeprazole is a lipophilic, weak base with a pK of 4.0 (Holt & Howden, 1991; Maton, 1991). An oral dose of omeprazole is absorbed in the intestines and reaches the parietal cells of the stomach through the bloodstream (Maton, 1991). In the intravascular and interstitial environment (where the pH is approximately 7), omeprazole is uncharged and can cross cell membranes. In the acidic environment within the secretory canaliculi of the parietal cell (where the pH is less than 2), however, omeprazole is protonated and, therefore,
becomes less lipophilic. As it is unable to diffuse back across the cell membrane, omeprazole becomes trapped in this acidic space of the secretory canaliculi. Here, omeprazole is converted to its active form, a sulfenamide, which forms a sulfhydryl bond with the membrane-bound H⁺/K⁺-ATPase on its extracellular surface. This reaction irreversibly inactivates the enzyme (Holt & Howden, 1991; Maton, 1991). The resumption of acid secretion after the administration of omeprazole therefore requires synthesis of new H⁺/K⁺-ATPase protein. The degradation half-life of H⁺/K⁺-ATPase is approximately 18 hours and this corresponds to the half-life of the pharmacologic activity of the drug (Maton, 1991). When treatment is stopped, it takes three to five days for acid secretion to resume to pretreatment levels. There is no evidence of rebound hypersecretion (Holt & Howden, 1991; Maton, 1991).

The onset of action of omeprazole is 1 hour after oral administration while its maximum effect on gastric acid secretion is achieved after approximately 6 hours (Holt & Howden, 1991; Maton, 1991). Because omeprazole is covalently linked to H⁺/K⁺-ATPase, inhibition of acid secretion lasts longer than omeprazole can be detected in the plasma. The degree of inhibition of acid secretion therefore does not correlate with the plasma concentration. In contrast, the area under the omeprazole concentration-time curve (AUC), which reflects the product of concentration and the time it is available to the parietal cells, does correlate with the degree of acid inhibition (Cederberg et al., 1989; Lind et al., 1983). Single doses produce a dose-dependent inhibition of gastric acid secretion. After one week of daily administration of 30 or 60 mg, however, there is almost total inhibition of acid secretion (Howden et al., 1984).
1.1.3.2 Pharmacokinetics
Omeprazole is acid-labile—it is degraded by gastric acid and therefore has poor bioavailability when administered orally (Howden et al., 1984; Regardh, 1986). Omeprazole is therefore usually administered in an enteric-coated formulation, which is generally dissolved at an elevated pH (~6) and absorbed in the small intestine (Andersson, 1991; Holt & Howden, 1991). Peak plasma concentrations are reached within 2 hours after oral administration and tend to increase during the first few days of treatment. Though the plasma half-life of omeprazole is approximately one hour, its pharmacological effect is much longer and so a once-daily dosing regimen is usually employed (Holt & Howden, 1991; Maton, 1991).

Initially, omeprazole is rapidly distributed to extravascular sites. The mean volume of distribution is reported to be 0.31 L/kg. Omeprazole is about 95% protein-bound, mainly to albumin and α1-acid glycoprotein (Howden, 1991).

Omeprazole is metabolized extensively in the liver (Holt & Howden, 1991). Its main metabolites are omeprazole sulphone (OMP-S), 5-hydroxyomeprazole (5-OH-OMP), and omeprazole sulfide (Regardh, 1986; Regardh et al., 1990; Renberg et al., 1989). About 80% of a given dose is excreted as metabolites in the urine, and the remainder is excreted via the bile (Cederberg et al., 1989; Regardh, 1986; Regardh et al., 1990; Renberg et al., 1989).

The pharmacokinetics of omeprazole have been studied in various patient populations. In elderly patients, the rate of elimination of omeprazole was on average slower and the bioavailability somewhat greater than in younger subjects (Landahl et al., 1992). Patients with impaired hepatic function showed a significant increase in bioavailability, a lower plasma clearance and a longer half-life than in normal subjects (Andersson et al., 1993). Thus, a reduced dose of omeprazole is recommended for elderly patients and those
with severe liver disease (AstraZeneca, 2000). The disposition of intact omeprazole was found to be unchanged in patients with impaired renal function (Naesdal et al., 1986).

1.1.3.3 Metabolism of Omeprazole In Vivo
Omeprazole concentrations in plasma show a pronounced interindividual variability, with some subjects exhibiting higher plasma concentrations and longer elimination half-lives than those with average kinetic parameters (Andersson et al., 1990; Regardh et al., 1990). Andersson and colleagues found that slow metabolisers of omeprazole are also slow metabolisers of diazepam, whose metabolism is related to the genetically determined S-mephenytoin polymorphism, suggesting that CYP2C19 was responsible for the metabolism of omeprazole (Andersson et al., 1990). Subsequently, it was shown that the metabolic pathways of omeprazole in CYP2C19 PMs are characterised by impaired 5-hydroxylation (Andersson et al., 1990). In both Caucasian and Asian subjects, omeprazole metabolism, as well as the formation of its hydroxy and the metabolism of its sulphone metabolites, cosegregates with the polymorphic metabolism of S-mephenytoin (Andersson et al., 1992; Caraco et al., 1995; Sohn et al., 1992). Thus, CYP2C19 appears to responsible for the metabolism of omeprazole to 5-hydroxyomeprazole, and perhaps also for the further metabolism (hydroxylation) of omeprazole sulphone.

Further evidence for the role of CYP2C19 in omeprazole metabolism comes from the observation that omeprazole inhibits diazepam clearance and slows its elimination in Caucasian EMs of mephenytoin in a dose-dependent fashion, presumably by competing for CYP2C19 active sites (Andersson et al., 1990; Gugler & Jensen, 1985). There is no inhibitory effect in PMs, however, presumably since there is no enzyme in these subjects for which diazepam and omeprazole could compete (Lin & Lu, 1998). Thus, omeprazole is both a substrate and a competitive inhibitor of CYP2C19.
1.1.3.4 Metabolism of Omeprazole *In Vitro*

The *in vitro* characterisation of omeprazole metabolism followed from the *in vivo* findings.

Andersson and colleagues characterised the primary and secondary metabolic pathways of omeprazole and identified the cytochrome P450 isoforms responsible for the formation of the secondary metabolites of omeprazole (Figure 1.3) (Andersson *et al*., 1994; Andersson *et al*., 1993).
Figure 1.3. Proposed scheme for metabolism of omeprazole in humans (Andersson et al., 1994; Andersson et al., 1993).
The major metabolites identified in vitro, in order of importance, appear to be 5-hydroxyomeprazole, omeprazole sulphone, 5-O-desmethylomeprazole and 3-hydroxyomeprazole. The kinetics of formation of the four metabolites studied were biphasic suggesting the involvement of multiple CYP isoforms in each case. Formation of the major metabolite, 5-OH-OMP, was significantly correlated with CYP2C19 and CYP3A4 activity and content. Inhibition studies using human recombinant P450 enzymes, human liver microsomes, and selective antibodies have shown that CYP2C19 is a major high affinity omeprazole 5-hydroxylase and CYP3A4 is a low affinity omeprazole-hydroxylating enzyme (Andersson et al., 1993; Karam et al., 1996). Other CYP2C enzymes (CYP2C8, CYP2C9, and CYP2C18) may also contribute to omeprazole hydroxylation at high substrate concentrations (Karam et al., 1996). Correlation and inhibition data for OMP-S suggest that CY3A4 is the principal enzyme responsible for the formation of this metabolite (Andersson et al., 1993; Karam et al., 1996). At high concentrations, mephenytoin caused some inhibition of OMP-S formation, possibly owing to a minor role for CYP2C19 in its formation (Andersson et al., 1993). Formation of 5-O-desmethylomeprazole was attributed mostly to CYP2C19 and to a lesser extent CYP2D6. Table 1.4 presents mean values for the Michaelis-Menton parameters for the formation of 5-OH-OMP and OMP-S, and Table 1.5 presents the mean $K_m$ values for the formation of 5-O-desmethylomeprazole and 3-hydroxyomeprazole in human liver microsomes. Estimates of relative intrinsic clearance based on the $V_{max}/K_m$ ratio, indicate that at low concentrations (comparable with those obtained in a clinical situation), 5-OH-OMP formation is four times greater than OMP-S formation (Andersson et al., 1994; Andersson et al., 1993).
Table 1.4. Mean values for the Michaelis-Menten parameters for the formation of 5-hydroxyomeprazole and omeprazole sulphone in human liver microsomes (taken from Andersson et al., 1993). $K_m$ values are expressed as $\mu$M, and $V_{\text{max}}$ values expressed as mmol mg$^{-1}$ min$^{-1}$.

<table>
<thead>
<tr>
<th></th>
<th>5-hydroxyomeprazole</th>
<th>omeprazole sulphone</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ $\mu$M</td>
<td>$K_{m1}$ $K_{m2}$</td>
<td>$K_{m1}$ $K_{m2}$</td>
</tr>
<tr>
<td>Mean</td>
<td>8.6 175</td>
<td>49 484</td>
</tr>
<tr>
<td>± s.d.</td>
<td>5.6 122</td>
<td>15 190</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>0.099 0.29</td>
<td>0.19 0.40</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>0.068 0.07</td>
<td>0.12 0.04</td>
</tr>
</tbody>
</table>

Table 1.5. Mean $K_m$ values for the formation of 5-O-desmethylomeprazole and 3-hydroxyomeprazole in human liver microsomes (taken from Andersson et al., 1993). $K_m$ values are expressed as $\mu$M.

<table>
<thead>
<tr>
<th></th>
<th>5-O-desmethylomeprazole</th>
<th>3-hydroxyomeprazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ $\mu$M</td>
<td>$K_{m1}$ $K_{m2}$</td>
<td>$K_{m1}$ $K_{m2}$</td>
</tr>
<tr>
<td>Mean</td>
<td>13.6 139</td>
<td>52 321</td>
</tr>
<tr>
<td>± s.d.</td>
<td>13.3 56</td>
<td>16 112</td>
</tr>
</tbody>
</table>

The major secondary omeprazole metabolite is thought to be hydroxyomeprazole sulphone, which was formed during incubation with both 5-OH-OMP and OMP-S. Kinetic measurements and inhibition studies indicate that the formation of hydroxyomeprazole sulphone from OMP-S is mainly mediated by CYP2C19 while the formation of the same metabolite from 5-OH-OMP is mainly CYP3A mediated. OMP-S is also metabolised to pyridine-N-oxide omeprazole sulphone principally by CYP3A (Andersson et al., 1994).

1.1.3.5 Omeprazole as a Probe Drug for CYP2C19 Activity

Ideal characteristics of a probe drug include: safety in a wide range of populations; ease of administration, sampling, and chemical analysis; reproducability of phenotype assignment; and a high degree of specificity for the enzyme of interest (Balian et al., 1995). Clearly, it is also necessary that the phenotype defined by the assay correspond to the underlying genetic polymorphism (Balian et al., 1995).

As discussed in section 1.1.2.1, mephenytoin was the original and prototypic drug used to probe the activity of the CYP2C19 polymorphism. The 4'-hydroxylation of the S-
enantiomer of mephenytoin has been used to distinguish individuals with the poor and extensive metaboliser phenotypes of this polymorphism (Kupfer & Preisig, 1984; Wedlund et al., 1984).

Balian et al. (1995) suggested that omeprazole could also be used to assess the metabolic status of CYP2C19 activity in vivo for the following reasons: (1) poor metabolisers of mephenytoin have slow omeprazole hydroxylation (Andersson et al., 1990); (2) S-mephenytoin inhibits omeprazole hydroxylation in hepatic microsomal preparations (Andersson et al., 1993); and (3) the metabolic disposition of omeprazole correlated with S-mephenytoin metabolic phenotype (Andersson et al., 1992; Sohn et al., 1992). Chang and colleagues showed that the OMP/5-OH-OMP metabolic ratio (MR), determined in plasma 3 hours after drug administration, significantly correlated with the mephenytoin S/R ratio and was in good agreement with the CYP2C19 genotype (Chang et al., 1995; Chang et al., 1995).

Thus both omeprazole and mephenytoin appear to be reliable in distinguishing between EMs and PMs of CYP2C19. Figure 1.4 compares the distribution of EMs and PMs using omeprazole and mephenytoin as the probe drug (Chang et al., 1995). There are several reasons for choosing omeprazole and not mephenytoin as a probe drug for CYP2C19. First, omeprazole is a safer drug and has fewer associated side effects. The side effects of a single dose of mephenytoin, such as sedation and drowsiness, limit its use in PMs and Asians (Balian et al., 1995; Nakamura et al., 1985). In contrast, in a study of 167 healthy volunteers given omeprazole, no subjective or objective adverse reactions were noted aside from one report of decreased libido (Balian et al., 1995). Second, omeprazole provides a more practical and convenient alternative to mephenytoin. The urinary collection period for both the hydroxylation method and the enantiomeric (S/R) ratio of mephenytoin is at least 8 hours.
Figure 1.4. Frequency distribution of the OMP/S-OH-OMP ratio (as determined in plasma drawn 3 h after a single dose of omeprazole; n=160) and of the urinary S/R mephenytoin ratio (n=141) (Chang et al., 1995).
Furthermore, it is often recommended to re-measure the enantiomeric ratio of mephenytoin during a second urine collection period several hours later to better discriminate between phenotypes. Omeprazole requires only one blood sample collected 3 hours after drug administration (Chang et al., 1995; Chang et al., 1995). Third, many individuals phenotyped with mephenytoin had an undetectable S-enantiomer (18% of the subjects in one study (Chang et al., 1995)), and therefore an unquantifiable S/R ratio (Chang et al., 1995). In contrast, the metabolic ratio of omeprazole is generally quantifiable (all 160 subject in the same study by Chang and coworkers). Thus omeprazole has the possible added benefit of identifying subjects with ultrarapid hydroxylation (Balian et al., 1995; Chang et al., 1995).

To ensure specificity, metabolic phenotype determination should not be significantly affected by the activity of alternate enzyme pathways. *In vitro* studies have shown that the hydroxylation of omeprazole is predominantly due to CYP2C19 but that it is also, to a much lesser extent, due to CYP3A4 (Andersson et al., 1993; Karam et al., 1996). In the *in vivo* study performed by Balian and colleagues (1995), approximately 70% of the variation in omeprazole hydroxylation could be explained by variation in the mephenytoin S/R ratio. That CYP2C19 is not the unique enzyme involved in the hydroxylation of omeprazole may represent a disadvantage of this drug in its use as a probe for CYP2C19 activity compared to mephenytoin.
1.1.4 Cytochrome P450 3A4 and Ketoconazole

1.1.4.1 CYP3A4
CYP3A4 is the major form of cytochrome P-450 in the human liver, comprising about 30% of total cytochromes P-450 (Shimada et al., 1994). CYP3A4 is also the most abundant cytochrome P-450 enzyme expressed in the gut, where it contributes substantially to the “first-pass” metabolism of some orally administered CYP3A4 substrates (Kolars et al., 1992). CYP3A4 complementary DNA from the liver and gut have been reported to have identical sequences, suggesting that the enzyme expressed in these two tissues is the same (Lown et al., 1998).

CYP3A4 appears to act on a broad range of substrates; it is reported to be involved in the metabolism of over half of the drugs currently being marketed (Ball et al., 1999; Bertz & Granneman, 1997; Westlind et al., 1999). CYP3A4 also appears to be particularly susceptible to induction and inhibition by many compounds (Wilkinson, 1996). Accordingly, drug-drug interactions involving CYP3A4 are common. Some of the substrates, inhibitors and inducers of CYP3A4 are listed in Table 1.6.

Table 1.6. Substrates, inhibitors and inducers of CYP3A4 (modified from (Herman, 1999))

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Inhibitors</th>
<th>Inducers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium-channel blockers (not diltiazem)</td>
<td>Calcium-channel blockers (esp. diltiazem)</td>
<td>Cyclophosphamide</td>
</tr>
<tr>
<td>Cisapride</td>
<td>Corticosteroids</td>
<td>Dexamethasone, sex steroids</td>
</tr>
<tr>
<td>Cyclosporine</td>
<td>Cyclosporine, tacrolimus</td>
<td>Erythromycin</td>
</tr>
<tr>
<td>Erythromycin, clarithromycin</td>
<td>Erythromycin, clarithromycin</td>
<td>Omeprazole, lansoprazole</td>
</tr>
<tr>
<td>Fentanyl, alfentanil, sufentanil</td>
<td>Fluvoxamine, fluoxetine, sertraline</td>
<td>Phenytoin, barbiturates</td>
</tr>
<tr>
<td>Halothane</td>
<td>Grapefruit juice</td>
<td>Rifampin</td>
</tr>
<tr>
<td>HIV protease inhibitors</td>
<td>Ketoconazole, itraconazole, fluconazole</td>
<td></td>
</tr>
<tr>
<td>Lovastatin, simvastatin, atorvastatin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Midazolam, alprazolam, triazolam, diazepam</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSRIs</td>
<td>Midazolam</td>
<td></td>
</tr>
<tr>
<td>TCAs</td>
<td>Omeprazole, lansoprazole</td>
<td>TCAs</td>
</tr>
<tr>
<td>Terfenadine, astemizole, loratadine</td>
<td>Tamoxifen</td>
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</table>
There is a large (up to 40-fold) interindividual variation in the expression and activity of CYP3A4 (Ball et al., 1999; Shimada et al., 1994; Westlind et al., 1999). The origin of this variation is not known, but it may be the result of differences in genetic, physiologic, pathologic and environmental factors, including the presence of inducers and inhibitors. For example, a 30-50% reduction in the metabolism of some substrates has been found in the elderly and those with liver dysfunction such as cirrhosis (Wilkinson, 1996). While Shimada and colleagues (1994) found no sex-related differences in the level of CYP3A4 in human liver microsomes, Hunt and colleagues reported that CYP3A4 activity in human liver microsomes was 24% higher in females than in males. In the latter study, CYP3A activity did not correlate with age, smoking status, ethanol consumption or percent ideal body weight (Hunt et al., 1992).

Transcriptional factors may play a role in interindividual variation in CYP3A4 expression. For example, receptors for hormones (e.g., pregnenolone and glucocorticoids), for drugs (e.g., nifedipine, phenytoin and rifampicin) and for xenobiotics (e.g., PCBs) have been shown to control CYP3A4 gene expression (Bertilsson et al., 1998; Blumberg et al., 1998; Ogg et al., 1999; Westlind et al., 1999). Thus, individual differences in the levels of either ligands or receptors may cause interindividual variation in gene expression (Westlind et al., 1999).

In a recent study, a novel genetic CYP3A4 variant (CYP3A4-V) was identified that contains a mutation in the 5' promotor region of the gene (Rebeck et al., 1998). The CYP3A4-V allele contains an A→G transition mutation that alters the nifedipine-specific element located -287 to -296 base pairs from the transcription start site of the gene. The frequency of the CYP3A4-V allele showed considerable interethnic variability: 4-9% in
Caucasians, 53-55% in African Americans, 9% in Hispanics and 0% in Asian populations (Amirimani et al., 1999; Ball et al., 1999). It has been suggested that individuals carrying the variant allele have lower expression of the enzyme and thus an altered disposition to substrates of CYP3A4 (Rebbeck et al., 1998). In support of this hypothesis, this polymorphism was associated with higher clinical stage and grade in men with prostate cancer, perhaps owing to increased bioavailability of testosterone leading to prostate carcinogenesis (Rebbeck et al., 1998). CYP3A4-V has also been associated with a decreased risk for treatment-related leukemia, perhaps owing to a decreased capacity for CYP3A4-mediated activation of precarcinogens (Felix et al., 1998).

The importance of this polymorphism in determining CYP3A4 phenotype is questionable, however. Westlind and co-workers evaluated the functional properties of this variant allele in individuals heterozygous for the V-allele, and found that this mutation did not significantly affect testosterone hydroxylase activity in human liver microsomes. This group also found that the binding of nuclear proteins, although having lower affinity to the V-form of the element, was unspecific in nature, suggesting that the V-allele does not influence the enzyme expression in the liver to a significant degree (Westlind et al., 1999). In vitro experiments performed by Ando and coworkers showed no significant differences in nifedipine oxidation (a CYP3A4-dependent reaction) or CYP3A4 protein levels between liver microsomes from wild-type and variant homozygote individuals (Ando et al., 1999). Similarly, Ball and co-workers reported no difference in the pharmacokinetics of nifedipine or its CYP3A4-dependent metabolite or in the rate of CYP3A4-dependent demethylation of erythromycin between individuals who were homozygous for the wild-type and variant alleles (Ball et al., 1999). Together these data suggest that the CYP3A4-V polymorphism is
not an important factor in determining the high interindividual variation in CYP3A4 expression (Ball et al., 1999; Westlind et al., 1999). Unlike the polymorphic expression of CYP2C19 and CYP2D6, in which single gene mutations account for much of the observed phenotypic variation in these enzymes, the apparent multifactorial nature of CYP3A4 expression adds to the complexity of delineating the possible role of any one factor in the observed interindividual differences (Ball et al., 1999).

1.1.4.2 Ketoconazole as a CYP3A4 Inhibitor
Ketoconazole, administered orally, is used to treat superficial and systemic fungal infections (Bennett, 1996). Ketoconazole is an antifungal imidazole derivative that acts by altering the permeability of the cell membrane by blocking the biosynthesis of fungal lipids, especially the ergosterol in fungal and yeast cell membranes. The inhibition of ergosterol synthesis is caused by the inhibition of P450-dependent 14α-demethylation of lanosterol, its precursor (Borges et al., 1983).

A dose of 2.5 mg/kg of ketoconazole produces peak plasma concentrations of about 3 to 4.5 μg/mL (5.6 to 8.5 μM). The half-life is about 1.8 hours. The absorption of ketoconazole is variable after oral administration; antacids decrease and food increases absorption. Ketoconazole is lipophilic and highly protein bound (with a free fraction of 0.01). It is widely distributed; however, concentrations in the CNS are low. In vivo, liver concentrations of ketoconazole are similar to or only slightly higher than total plasma concentrations (vonMolke et al., 1996).

Ketoconazole and many otherazole antifungal agents, including itraconazole and fluconazole, are inhibitors of human CYP3A4 in vitro and in vivo, albeit with different potencies (Maurice et al., 1992; Venkatakrishnan et al., 2000). Co-administration of these
drugs with CYP3A substrates such as cyclosporine, terfenadine, astemizole, cisapride, loratadine, warfarin, and several benzodiazepines such as diazepam and triazolam can result in clinically significant drug interactions. *In vitro* and *in vivo* studies have found that ketoconazole is a more potent inhibitor of CYP3A4 than either itraconazole or fluconazole (Greenblatt et al., 1998; vonMolke et al., 1998; vonMolke et al., 1996; vonMolke et al., 1996).

Ketoconazole inhibits CYP3A4-mediated reactions, with Ki values in the nanomolar range (~1-100 nM) (Maurice et al., 1992; vonMolke et al., 1998; vonMolke et al., 1996). It inhibits CYP2C19 mediated S-mephenytoin 4-hydroxylation as well, however, with a Ki value of 31 μM (Hall et al., 1987). Böttiger and colleagues studied the effect of ketoconazole on CYP3A4 and CYP2C19 activity *in vivo* by examining its effect on the kinetics and metabolism of omeprazole in poor and extensive metabolisers of CYP2C19 (Bottiger et al., 1997). In EMs, ketoconazole 200 mg p.o. for 4 days inhibited the formation of 5-OH-OMP in the first 2 hours, possibly by inhibiting CYP2C19. At 100 mg p.o. for 4 days, however, ketoconazole did not inhibit the formation of the CYP2C19-generated metabolite 5-OH-OMP, whereas formation of the CYP3A4-generated metabolite OMP-S was significantly inhibited, by 72% in CYP2C19 EMs and 73% in PMs. The administration of ketoconazole 50 mg p.o. for 4 days gave a less complete inhibition of OMP-S formation of 65% in EMs and 35% in PMs (Bottiger et al., 1997). It appears, therefore, that ketoconazole 100 mg can selectively inhibit CYP3A4, without significantly altering the metabolism of omeprazole by CYP2C19 *in vivo.*
1.1.5 Flunitrazepam and the Benzodiazepines

1.1.5.1 Benzodiazepine Pharmacology
Benzodiazepines are a class of structurally related psychoactive drugs that have sedative/hypnotic, anxiolytic, anti-convulsant and muscle-relaxant properties (Sigel & Buhr, 1997). All benzodiazepines are variations on the 5-aryl-1-4-benzodiazepine core (see Figure 1.5). These drugs were introduced into clinical practice 40 years ago and have become the most largely used drugs within the sedative-hypnotic class (Sigel & Buhr, 1997). They are used extensively in a variety of clinical settings, including in the treatment of anxiety, panic disorders, insomnia, alcohol withdrawal and in anaesthesia (Scott et al., 1999; Sigel & Buhr, 1997). Ten to twenty percent of the adult population in Western countries use benzodiazepines within a given year (Farre et al., 1996).

![Figure 1.5. General benzodiazepine structure (Sellers et al., 1998).](image)

Benzodiazepines exert their actions primarily by modulating the physiological activity of GABA at the GABA\(_A\) receptor (Chebib & Johnston, 2000; Scott et al., 1999; Whiting, 1999). The GABA\(_A\) receptor family is a member of the ligand gated ion channel superfamily that also includes the 5-HT\(_3\) receptor and the glycine receptor (Sieghart et al., 1999; Sigel & Buhr, 1997; Whiting, 1999). These receptors appear to be composed of five subunits, each belonging to one of eight different subunit classes for which different isoforms
exist. Six $\alpha$, four $\beta$, three $\gamma$, one $\delta$, one $\epsilon$, one $\pi$ and three $\rho$ subunits have been sequenced and cloned from the mammalian nervous system (Sieghart et al., 1999; Whiting et al., 1999). In addition, the existence of a new subunit, termed $\theta$, has been discovered (Sieghart et al., 1999; Whiting et al., 1999). Expression studies have indicated that depending on the combination of subunits, receptors with distinct pharmacological and electrophysiological properties arise (Scott et al., 1999; Sieghart et al., 1999; Whiting et al., 1999). Antibody and recombinant studies have shown that the major receptors subtypes in the mammalian brain consist of $\alpha$, $\beta$, and $\gamma$ subunits and that the co-assembly of these subunits produces receptors with properties resembling those of native receptors (Sieghart et al., 1999; Whiting, 1999; Whiting et al., 1999). Site-directed mutagenesis studies have localized the benzodiazepine binding site to the interface between the $\alpha$ and $\gamma$ subunits and have identified amino acid residues that form the pocket for modulators acting at this site (Scott et al., 1999; Sieghart et al., 1999; Sigel & Buhr, 1997; Whiting, 1999; Whiting et al., 1999).

GABA is the major inhibitory neurotransmitter in the CNS. The binding of GABA to its recognition site on the GABA$_{A}$ receptor results in a conformational change that leads to the opening of the ion channel and, in most neurones, the influx of chloride into the cell. The resulting membrane hyperpolarization in the post-synaptic cell increases its inhibitory tone by reducing the probability of generating an action potential (Scott et al., 1999; Whiting, 1999). Benzodiazepines appear to increase the efficiency of GABAergic synaptic inhibition by increasing the frequency of GABA-activated channel openings and by increasing channel conductance (Chebib & Johnston, 2000).
1.1.5.2 Ethnic Differences in Response to Benzodiazepines

Ethnic differences in prescription patterns of benzodiazepines have been reported. For example, a survey of prescriptions of psychiatrists from North America, China, Japan, Hong Kong and Taiwan who regularly treated Asian and non-Asian patients showed that the mean doses of diazepam and chlordiazepoxide prescribed to Asians were significantly lower than those prescribed to their Caucasian counterparts (Rosenblat & Tang, 1987). Other authors have indicated that Chinese patients living in Hong Kong were generally treated with substantially smaller amounts of benzodiazepines compared to Caucasians (Kumana et al., 1987). These differences between ethnoracial groups may reflect differences in response that are potentially attributable to a number of sociocultural factors, including presentation of symptoms, attitudes towards mental illness and expectations about therapy; and/or biological factors, including body weight, body fat, drug metabolism and genetic polymorphism (Matthews, 1995; Rosenblat & Tang, 1987). In support of factors that are mediated through biological effects, kinetic studies involving Asians and Caucasians have demonstrated significant differences between ethnic groups, consistently supporting a slower metabolism of benzodiazepines, in particular diazepam, in Asians (Caraco et al., 1995; Ghoneim et al., 1981; Kumana et al., 1987; Lin et al., 1988; Zhang et al., 1990). The observations that diazepam has been shown to be metabolised by the polymorphic CYP2C19 (Andersson et al., 1990; Andersson et al., 1994; Jung et al., 1997; Wan et al., 1996), and that Asians have a high frequency of mutant CYP2C19 alleles (deMorais et al., 1994; deMorais et al., 1994; Xie, 1997) implicate genetic background as an important determinant of inter-ethnic differences in response to benzodiazepines that are metabolised by this enzyme.
1.1.5.3 Benzodiazepine Abuse and Dependence

The fourth edition of the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-IV) defines substance abuse as a "maladaptive pattern of substance use manifested by recurrent and significant adverse consequences related to repeated use" (DSM-IV, 1994). Substance dependence is defined as "a cluster of cognitive, behavioural, and physiologic symptoms indicating that the individual continues use of the substance despite significant substance-related problems. There is a pattern of repeated self-administration that usually results in tolerance, withdrawal, and compulsive drug-taking behaviour" (DSM-IV, 1994). Many factors pertaining to the drug, host and environment operate simultaneously to influence drug-taking behaviour. For example, various pharmacokinetic properties (such as half-life and onset) and pharmacodynamic properties (such as potency and ability to produce positive reinforcing effects) influence drug dependence liability (Wolf & Griffiths, 1991). Dose, dosing frequency and duration of use interval may also play a role (Wolf & Griffiths, 1991). Significant host factors include genetic determinants, behavioural history, concurrent use of cross-dependent drugs, pharmacological history and family history of substance dependence (Stolerman, 1993). A number of environmental factors may also determine liability for abuse and dependence, including cost, availability, legality, peer pressure and the level of social acceptance (O'Brien & McLellan, 1996; Stolerman, 1992).

For the last 25 years, benzodiazepine abuse and dependence have been a focus for public concern. Patients who are prescribed benzodiazepines for therapeutic use rarely abuse them or escalate their prescribed dose (Romach *et al.*, 1992), although some patients may become physically dependent on them (Busto *et al.*, 1986; Woods *et al.*, 1992; Woods & Winger, 1995). The abuse of benzodiazepines, however, is commonly seen as a part of polydrug use worldwide, particularly by people who abuse sedatives and opioids (Woods *et
al., 1992; Woods & Winger, 1997; Woods & Winger, 1995). Human experimental studies have shown that benzodiazepines have a potential for abuse, but their abuse liability is lower than that of barbiturates, heroin or amphetamines (deWit & Griffiths, 1991; Woods et al., 1992; Woods & Winger, 1995). It has also been reported that there are differences between individual benzodiazepines with respect to abuse liability (Busto et al., 1994; Griffiths & Wolf, 1990; Wolf & Griffiths, 1991).

1.1.5.4 Flunitrazepam: Patterns of Use and Abuse

Flunitrazepam—marketed under the trade name Rohypnol by Hoffman-La Roche—is manufactured worldwide. For the past 10 years, flunitrazepam has been one of the most widely used sedative-hypnotics internationally, accounting for an average of 6 to 7% of the market for sedative-hypnotics in 20 countries in Europe, Latin America, Asia, Australia, Africa and the Middle East (Woods & Winger, 1997). In Western Europe, flunitrazepam is the most widely prescribed sedative-hypnotic (CEWG, 1995). Flunitrazepam has never been manufactured or sold legally in North America and its possession is currently illegal. In recent years, however, it has become available illicitly, and reports of abuse have attracted a great deal of scrutiny. It is reportedly one of the biggest drug problems in South Florida and Texas, and its use appears to be growing widely across the USA (CEWG, 1995).

Flunitrazepam intoxication is characterised by sedation, extreme disinhibition, severe memory impairment or amnesia, muscle relaxation, gastrointestinal disturbances, visual impairment, and slowing of psychomotor performance. When flunitrazepam is combined with other sedatives, antidepressants, analgesics, or tranquillisers, the sedative effect is intensified (Saum & Inciardi, 1997). Paradoxically, although the drug is classified as a depressant, flunitrazepam can induce excitability or aggressive behaviour in some users.
Even though flunitrazepam taken alone rarely leads to death, its combination with alcohol reduces its safety margin and increases the likelihood of death due to enhanced central nervous system depression. In the context of self-intoxication, this combination can lead to "blackouts" that last 8-24 hours, depending on the dose (Schechter, 1998).

Most illicit users of flunitrazepam use it in combination with other drugs (DEA, 1995; Farre et al., 1996). Heroin addicts use flunitrazepam to enhance low-quality heroin (Smith et al., 1996), and to self-medicate withdrawal symptoms from heroin or methadone (Barnas et al., 1992). It is also used to reduce the anxiety and agitation from a crack or cocaine binge (Smith et al., 1996). Flunitrazepam tablets are usually taken by mouth, although some people crush the tablets and snort or smoke the powder, or dissolve it for injection (Smith et al., 1996). In some areas, flunitrazepam is associated with gang activity; in others, it is known as a "club drug" (Smith et al., 1996). In North America, flunitrazepam is used most frequently in conjunction with alcohol, with which it seems to have synergistic effects, as an "alcohol extender" and disinhibitory agent (CEWG, 1995). This latter effect of the combination of flunitrazepam and alcohol has led to a pattern of men slipping flunitrazepam into women's drinks to facilitate sexual relations. Thus, flunitrazepam has received notoriety as the "date-rape drug" in the lay media and provoked the concern of governments and federal legislators (Saum & Inciardi, 1997).

1.1.5.5 Flunitrazepam Pharmacokinetics
Flunitrazepam (5-(2-flurophenyl)-1,3-dihydro-1-methyl-7-nitro-2H-1,4-benzodiazepin-2-one) has an absolute oral bioavailability of 80% to 90% (Cano et al., 1977: Mattila & Larni, 1980). It has a large volume of distribution of 3.3 ± 0.6 L/kg and is 77-79% bound to plasma proteins. Plasma concentrations of flunitrazepam are dose-related. Peak plasma
concentrations, reached within 2 h of drug administration, are in the order of 10-15 ng/ml after a single oral dose of flunitrazepam 2 mg.

There is a striking discrepancy between the overall half-life, which is approximately 25 hours (Grahnen et al., 1991; Jochemsen et al., 1983; Kangas et al., 1982; Wickstrom et al., 1980), and the duration of clinical effect, which is approximately 8 hours. To explain this discrepancy, Cano and colleagues proposed a three-compartment model that describes the time-course of the plasma concentrations (Cano et al., 1977). In this model, the central and the second compartments are approximately equal in size, and rapid exchange takes place between them. The third compartment is approximately four times as large as the central one and flunitrazepam moves in and out of it slowly. The clinical effects of flunitrazepam are observed during the distribution phase, in which the majority of active agent is found in the central compartment and in the easily accessible second compartment. The fall of flunitrazepam plasma concentrations that occurs in this phase is predominantly due to distribution, and only to a small extent to elimination. However, Cano and colleagues note that elimination from the body during this phase is determined by the rate of metabolism, and that therefore the action of flunitrazepam may be somewhat stronger and more prolonged in patients with severely impaired hepatic function. In contrast, by the beginning of the equilibrium phase, most of the drug in the body is in the third compartment. During this phase, the overall elimination rate of the drug from the body is determined by the rate of reflux from the third compartment (Cano et al., 1977). This model provides one explanation for the discrepancy between flunitrazepam’s short duration of action and long overall half-life by proposing that flunitrazepam is first rapidly redistributed from the plasma, where it is active pharmacologically, to peripheral compartments. Slow reflux from these
compartments then maintains flunitrazepam in the plasma at a low concentration for a long period of time. Another possible explanation for this discrepancy is the development of acute tolerance.

Flunitrazepam is very lipophilic, and it therefore enters the brain and takes effect very rapidly (Arendt et al., 1987). For example, sedation occurs 20-30 minutes after an oral dose and peaks within 1-2 hours (Simmons & Cupp, 1998). Psychomotor impairment peaks at 1-1.5 hours following oral administration (Ingum et al., 1992). Flunitrazepam has a slightly more rapid clinical effect after intramuscular injection than when given by mouth (McGowan et al., 1980). Bond and coworkers demonstrated that flunitrazepam produces subjective effects within 5 minutes after intranasal administration in normal subjects (Bond et al., 1994).

Compared to other benzodiazepines, flunitrazepam has an intermediate duration of action (Mattila & Larni, 1980; Scharf et al., 1979; Woods & Winger, 1997). In general, the sedative effect of flunitrazepam persists for 8 hours following a 2 mg dose, while psychomotor impairment may last for as long as 12 hours (Simmons & Cupp, 1998).

1.1.5.6 Flunitrazepam Metabolism In Vitro and In Vivo
Flunitrazepam (FLU) is eliminated almost exclusively by metabolism; that is, less than 1% is excreted as unchanged drug. Most of the metabolised drug is excreted by the kidneys, with about 10% excreted in the feces. Approximately 20 metabolites have been identified in the urine. The main metabolic pathways involve reductive metabolism to 7-aminoflunitrazepam (7-AF) and oxidative metabolism to 3-hydroxyflunitrazepam (3-HF) and N-desmethyflunitrazepam (DMF) (Figure 1.6) (Wendt, 1976). The parent compound is thought to be primarily responsible for the hypnotic effects and probably for the anxiolytic, sedative, and subjective “liking” effects (Wendt, 1976; Wickstrom et al., 1980), though some
authors have asserted that the metabolites, in particular DMF, may also have some activity (Dorow et al., 1982; Drouet-Coassolo et al., 1990).

Flunitrazepam is similar in structure to diazepam (Figure 1.7). Like diazepam, flunitrazepam is metabolised in the liver and follows similar catabolic pathways. Diazepam undergoes oxidative metabolism to 3'-hydroxydiazepam and dethmethyldiazepam, whose formation is catalysed by CYP3A4 and CYP2C19/3A4 respectively (Andersson et al., 1994).
Introduction

Flunitrazepam (FLU)  
7-Aminoflunitrazepam (7-AF)

N-Desmethylflunitrazepam (DMF)  
3-Hydroxyflunitrazepam (3-HF)

Figure 1.6. Proposed scheme for metabolism of flunitrazepam.

Figure 1.7. Chemical structure of diazepam.
In vitro studies in our lab using cDNA expressed CYP microsomes and human liver microsomes revealed that CYP2C19 and CYP3A4 are the principal cytochrome P450 enzymes involved in the metabolism of FLU to DMF and 3-HF (Kilicarslan et al., 2000). In cDNA expressed microsomes from a baclovirus expression system, CYP2C19 and CYP3A4 mediated the formation of DMF with high and low affinity, respectively. CYP3A4 mediated the formation of 3-HF with high affinity, while CYP2C19 also mediated this reaction, but with very low affinity (Table 1.7). The formation of both metabolites in human liver microsomes followed biphasic kinetics, indicating that there were two enzymes involved in each metabolic pathway (Table 1.7). Using the kinetic constants derived from human liver microsomes, it was estimated that at therapeutic concentrations of flunitrazepam (0.03 µM) the relative contribution of CYP2C19 and CYP3A4 to the formation of DMF was approximately 60% and 40%. Chemical inhibition (Table 1.8) and immunoinhibition (Table 1.9) data support the involvement of CYP2C19 and CYP3A4 in the metabolism of FLU to DMF and 3-HF (Kilicarslan et al., 2000). Taken together, these data strongly suggest that CYP2C19 and CYP3A4 are important enzymes involved in the in vitro metabolism of FLU to DMF and that CYP3A4 is the major contributor to the formation of 3-HF with some contribution from CYP2C19.
Table 1.7. Formation of desmethylflunitrazepam and 3-hydroxyflunitrazepam from flunitrazepam by human liver and cDNA expressed microsomes.

<table>
<thead>
<tr>
<th></th>
<th>cDNA expressed</th>
<th>Human liver microsomes (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CYP2C19</td>
<td>CYP3A4</td>
</tr>
<tr>
<td><strong>Desmethylflunitrazepam</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High affinity:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{\text{max}}$ (nmol/mg/min)</td>
<td>0.095</td>
<td>0.08 ± 0.03</td>
</tr>
<tr>
<td>$K_m$ (µM)</td>
<td>11.1</td>
<td>42 ± 17</td>
</tr>
<tr>
<td>$V_{\text{max}}/K_m \times 10^{-3}$</td>
<td>8.56</td>
<td>1.9</td>
</tr>
<tr>
<td>Low affinity:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{\text{max}}$ (nmol/mg/min)</td>
<td>1.27</td>
<td>0.17 ± 0.10</td>
</tr>
<tr>
<td>$K_m$ (µM)</td>
<td>108</td>
<td>165 ± 100</td>
</tr>
<tr>
<td>$V_{\text{max}}/K_m \times 10^{-3}$</td>
<td>11.76</td>
<td>1.0</td>
</tr>
<tr>
<td><strong>3-Hydroxyflunitrazepam</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High affinity:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{\text{max}}$ (nmol/mg/min)</td>
<td>0.420</td>
<td>0.126 ± 0.031</td>
</tr>
<tr>
<td>$K_m$ (µM)</td>
<td>34.0</td>
<td>87 ± 20</td>
</tr>
<tr>
<td>$V_{\text{max}}/K_m \times 10^{-3}$</td>
<td>12.35</td>
<td>1.4</td>
</tr>
<tr>
<td>Low affinity:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{\text{max}}$ (nmol/mg/min)</td>
<td>0.861</td>
<td>0.499 ± 0.158</td>
</tr>
<tr>
<td>$K_m$ (µM)</td>
<td>642</td>
<td>289 ± 85</td>
</tr>
<tr>
<td>$V_{\text{max}}/K_m \times 10^{-3}$</td>
<td>1.34</td>
<td>1.7</td>
</tr>
</tbody>
</table>
Introduction

Table 1.8. Results of chemical inhibition studies. Flunitrazepam metabolism was inhibited in human liver microsomes using isozyme specific inhibitors ketoconazole (CYP3A inhibitor), sulphenazole (CYP2C9 inhibitor), omeprazole (CYP2C19 and CYP3A4 substrate), S-mephenytoin (CYP2C19 substrate) and α-naphthoflavone (CYP1A2 inhibitor). Inhibition was evaluated at flunitrazepam concentrations equivalent to the formation Km for the particular set of human liver microsomes being used.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Desmethylflunitrazepam</th>
<th>3-Hydroxyflunitrazepam</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ketoconazole (0.5 µM)</td>
<td>78%</td>
<td>94%</td>
</tr>
<tr>
<td>S-Mephenytoin (400 µM)</td>
<td>31%</td>
<td>18%</td>
</tr>
<tr>
<td>Omeprazole (80 µM)</td>
<td>24%</td>
<td>41%</td>
</tr>
<tr>
<td>Sulfaphenazole (400 µM)</td>
<td>&lt; 10%</td>
<td>No inhibition</td>
</tr>
<tr>
<td>α-naphthoflavone (400 µM)</td>
<td>&lt; 10%</td>
<td>No inhibition</td>
</tr>
</tbody>
</table>

Table 1.9. Results from immunoinhibition studies. Experiments were carried out in human liver microsomes with antibodies to CYP3A4 (monoclonal), CYP2C19 (monoclonal), CYP2C13 (polyclonal), CYP2A6 (monoclonal), CYP2B1 (polyclonal) and CYP2D6 (polyclonal).

<table>
<thead>
<tr>
<th></th>
<th>Desmethylflunitrazepam</th>
<th>3-Hydroxyflunitrazepam</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CYP3A4 antibodies</td>
<td>45%</td>
<td>80%</td>
</tr>
<tr>
<td>Anti-CYP2C19 serum</td>
<td>26%</td>
<td>No inhibition</td>
</tr>
<tr>
<td>Anti-CYP2C13 antibodies</td>
<td>13%</td>
<td>No inhibition</td>
</tr>
<tr>
<td>Anti-CYP1A antibodies</td>
<td>&lt; 10%</td>
<td>&lt; 10%</td>
</tr>
<tr>
<td>Anti-CYP2A6 antibodies</td>
<td>&lt; 10%</td>
<td>&lt; 10%</td>
</tr>
<tr>
<td>Anti-CYP2B1 antibodies</td>
<td>&lt; 10%</td>
<td>&lt; 10%</td>
</tr>
<tr>
<td>Anti-CYP2D6 antibodies</td>
<td>&lt; 10%</td>
<td>&lt; 10%</td>
</tr>
</tbody>
</table>
Coller and colleagues (1999) studied the *in vitro* kinetics of the oxidative metabolism of FLU to 3-HF and DMF using human liver microsomes when FLU was dissolved in dimethylformamide and acetonitrile. They found that the kinetics of the formation of 3-HF and DMF were non-linear and best estimated using the Hill equation. Inhibition of their formation was also studied in an expressed enzyme system using specific chemical inhibitors and antibodies. CYP2C19, CYP3A4 and CYP1A2 were reported to mediate the formation of both 3-HF and DMF (Coller *et al.*, 1999).

In addition to these *in vitro* data, some studies of the *in vivo* metabolism of flunitrazepam have also been conducted. Luurila and coworkers studied the interaction between erythromycin and flunitrazepam. They found that erythromycin 500 mg t.i.d. administered for four days caused a 25% increase in the AUC of flunitrazepam 1 mg and a significant increase in its half-life (Luurila *et al.*, 1996), suggesting that CYP3A4 is involved in flunitrazepam’s metabolism *in vivo*.

A clinical pilot-study was conducted by our group to examine the kinetic and subjective effects of flunitrazepam in one Chinese EM (CYP2C19*1/*1) and two Chinese PMs of CYP2C19 (both CYP2C19*2/*3) (Sellers *et al.*, 1998). Each subject received a 1 mg oral dose of flunitrazepam. Plasma concentrations, subjective effects and psychomotor impairment were measured before and up to 24 h after drug administration. The individuals who were PMs for CYP2C19 experienced more sedation and “spacey feeling” and exhibited greater psychomotor impairment and higher plasma flunitrazepam concentrations compared to the EM (Figure 1.8). These data suggest that CYP2C19, in addition to having an *in vitro* role, is also involved in the metabolism of flunitrazepam *in vivo*. 
Figure 1.8. Flunitrazepam plasma concentration profiles for 3 human subjects.
1.1.5.7 Flunitrazepam Pharmacodynamics

Flunitrazepam, like other benzodiazepines, exerts its effects by binding to the GABA<sub>A</sub> receptor. Flunitrazepam's pharmacological potency correlates well with its affinity (defined as the binding strength of a drug to its receptor (Stryer, 1995)) and intrinsic efficacy (defined as the inherent ability of a ligand to induce conformational change of the receptor that is required to transduce the event of signal recognition into a physiologically relevant response (Facklam et al., 1992)) at the GABA<sub>A</sub>. Flunitrazepam has been shown to have much greater affinity at the benzodiazepine receptor than clobazam, somewhat greater affinity than diazepam, oxazepam, and alprazolam, and less affinity than midazolam and triazolam. Studies comparing the intrinsic efficacy of flunitrazepam with that of other benzodiazepines have indicated that, in measures of electroencephalographic (EEG) changes and blockade of seizures, flunitrazepam has a greater efficacy than midazolam, oxazepam or clobazam (Woods & Winger, 1997). The high receptor affinity and efficacy of flunitrazepam combine to make it one of the most potent benzodiazepines: on average approximately 10 times more potent than diazepam, although there is wide variation in this potency coefficient among different effects (Mattila & Lami, 1980). For example, Ingum and coworkers showed that flunitrazepam was approximately 20-fold more potent than diazepam with respect to psychomotor impairment (Ingum et al., 1994).

Flunitrazepam possesses all the main pharmacologic activities of the benzodiazepines, such as anxiolytic, anticonvulsant, muscle relaxant, and central sedative and hypnotic effects. The sedative/hypnotic effect of this drug is potentiated by the presence of a nitro group and a fluorine atom in the molecular structure (Mattila & Lami, 1980). Plasma concentrations of flunitrazepam after oral and intramuscular administration correlate
with the sedative effects of the drug (Clarke et al., 1980; Grahnen et al., 1991; Mattila & Larni, 1980). The plasma concentration needed to produce an effect equal to 50% of the maximum effect for sedation (EC<sub>50</sub>) is approximately 7 ng/ml. The concentration-effect relationship for sedation is steep, indicating a virtually 'all or none' effect, with a slope factor of approximately 14 (Grahnen et al., 1991).

Flunitrazepam causes a dose- and time-dependent anterograde amnesic effect (Kangas et al., 1982; Mattila & Larni, 1980). Bareggi and colleagues reported that 2-mg and 4-mg doses significantly reduced explicit memory, which they defined as declarative memory that is accessible by conscious awareness and can be declared—brought to mind—verbally (as a proposition) or non-verbally (as an image). This form of memory includes facts and general information as well as events personally experienced. The EC<sub>50</sub> values for memory impairment were 6.1 ng/ml and 6.4 ng/ml for prose and trigrams delayed recall, respectively. Higher concentrations (EC<sub>50</sub>=13.2 ng/ml) were needed to impair attention (Bareggi et al., 1998).

Flunitrazepam also produces pronounced psychomotor impairment at a dose of 1 and 2 mg (Ingum et al., 1992) and impaired balance at a dose of 2 mg (Farre et al., 1996).

A 2 mg dose of flunitrazepam produced an increase in a variety of subjective measures that could be related with a positive mood, such as “high,” “good effects” or “liking” (Bond et al., 1994; Farre et al., 1996). It also produced some feelings suggestive of some degree of intoxication (reported sensations of “drunkenness” or “bad effects”) and an increased score for dysphoria (Farre et al., 1996).

A characteristic feature of flunitrazepam on the cardiovascular system is peripheral vasodilation, perhaps due to its direct relaxing effect on vascular smooth muscle. This effect
combined with a decrease in cardiac output results in a decrease in blood pressure with flunitrazepam intake (Mattila & Larni, 1980). In addition, flunitrazepam is reported to have a depressive influence on respiration (Mattila & Larni, 1980).

In clinical use, flunitrazepam is very well tolerated and has few side effects, although sedation, drowsiness, dizziness and tiredness are reported by approximately 10-20% of people using it. These side effects are dose-related (Bond et al., 1994).

1.1.5.8 Therapeutic Uses of Flunitrazepam
Although illegal in Canada and the USA, flunitrazepam is used elsewhere in the treatment of insomnia and as a preanaesthetic medication (Mattila & Larni, 1980; Simmons & Cupp, 1998). Its fast onset of action, high efficacy, and intermediate duration of action make it a useful therapeutic agent (Woods & Winger, 1997).

The hypnotic effects of flunitrazepam have lead to its use in the treatment of insomnia. After a 2-mg dose, flunitrazepam has been shown to decrease sleep latency, decrease the number and amounts of awake periods during sleep and prolong periods of deep sleep (Bixler et al., 1977; Mattila & Larni, 1980). This dose of the drug also increases the time period to the first rapid eye movement (REM) sleep and decreases the amount of slow wave and REM sleep during the night (Bixler et al., 1977; Mattila & Larni, 1980). The dosage recommendation for flunitrazepam in the treatment of insomnia is 1-2 mg. A 2-mg regimen, however, is likely to produce residual psychomotor effects (for example with driving the next day) and anxiolytic effects, which may be desirable or undesirable depending on the patient’s daily activity and requirements (Mattila & Larni, 1980; Woods & Winger, 1997). The data with regard to residual subjective and objective effects after 8 hours following a single 1-mg dose is divergent. Several investigators have suggested a 0.5-mg
dose when impaired performance the next day is to be avoided (Grahnen et al., 1991; Nicholson & Stone, 1980).

Flunitrazepam's anterograde amnesic effects make it useful as a preoperative sedative. The amnesia that occurs with flunitrazepam begins within 20-30 minutes (Kangas et al., 1982). A reduction in explicit memory (Bareggi et al., 1998) following flunitrazepam administration is beneficial as it prevents patients from recalling unpleasant experiences during different stages of anaesthesia and operation. In addition, the patient becomes sleepy and anxiety is minimised (Mattila & Larni, 1980).

1.1.5.9 Abuse Liability of Flunitrazepam
The abuse liability of a compound can be defined as "its capacity to produce psychologic dependence . . . or physiologic dependence, in conjunction with the capacity to alter behaviour in a manner that is detrimental to the individual or his or her social environment" (Woods & Winger, 1997). Flunitrazepam is a benzodiazepine with an intermediate duration of action, high receptor affinity and intrinsic efficacy, relatively high lipid solubility, rapid onset of effects, pleasurable subjective effects and rapid bioavailability (Woods & Winger, 1997). This profile, which contributes to its utility in therapeutics, leads to a high theoretical probability that flunitrazepam will be abused by drug abusers who have access to it. This idea is supported by surveys of people who were dependent on heroin (San et al., 1993) and who were enrolled in a methadone-maintenance program (Barnas et al., 1992), who reported a distinct preference for flunitrazepam over other benzodiazepines.

Experimental studies of the abuse liability of flunitrazepam are limited, but existing data suggest that flunitrazepam may be similar to other benzodiazepines in its ability to produce drug-seeking or drug-taking behaviour, physiologic dependence and adverse or toxic
effects (Woods & Winger, 1997). Epidemiological studies of patient populations, however, suggest that flunitrazepam is generally used appropriately and conservatively, apparently with low liability of abuse, as is characteristic of benzodiazepines. With respect to abuse liability, flunitrazepam seems to be distinguished from other benzodiazepines in its popularity among opioid abusers (Woods & Winger, 1997). In addition, flunitrazepam is the principal benzodiazepine abused among Taiwanese-Chinese, despite the availability of other benzodiazepines in Taiwan (Dr. Lien Wen SU, Taipei City Psychiatric Centre, personal communication).
1.2 General Rationale
The liver enzyme cytochrome P450 2C19 (CYP2C19) represents an important example of a pharmacogenetic polymorphism. Inheritable mutations in the CYP2C19 gene prevent the production of active enzyme, resulting in the poor metabolism of its substrates. Fifteen to seventeen percent of the Chinese population are CYP2C19 poor metabolisers compared to 3-5% of Caucasians, reflecting the higher frequency of the CYP2C19 mutant alleles in the Chinese compared to the Caucasian population (Bertilsson et al., 1992; Nakamura et al., 1985). CYP2C19 is responsible for the metabolism of many therapeutically important drugs, including some benzodiazepines. Chinese are reported to be more sensitive to benzodiazepines (in particular diazepam) than Caucasians (Matthews, 1995; Rosenblat & Tang, 1987). In addition, flunitrazepam appears to be a preferred drug of abuse among Taiwanese-Chinese, despite the availability of other benzodiazepines (Dr. Lien Wen SU, Taipei City Psychiatric Centre, personal communication). The explanation for these observations is unknown, but the higher frequency of CYP2C19 mutant alleles among ethnic Chinese may play a part.

Flunitrazepam is metabolized by CYP2C19 in vitro. The in vivo roles of CYP2C19 and of its polymorphic expression in flunitrazepam metabolism are not known, however. A preliminary study with two poor metabolisers of CYP2C19 and one extensive metaboliser showed that the PMs, compared to the EM, had higher plasma flunitrazepam concentrations, experienced more sedation and "spacey feeling" and exhibited greater psychomotor impairment, suggesting that CYP2C19 may play a role in the metabolism of flunitrazepam in vivo.
1.3 **General Objective**
To examine the role of cytochrome P450 2C19 activity in determining flunitrazepam kinetics and response *in vivo.*

1.4 **General Hypothesis**
CYP2C19 is important in the metabolism of flunitrazepam *in vivo,* and this role results in differences in flunitrazepam kinetics and response among people with variable CYP2C19 activity.

1.5 **Research Plan**
Our general objective was to examine the role of cytochrome P450 2C19 activity in determining flunitrazepam kinetics and response *in vivo.*

The most elegant way to approach this question was to compare CYP2C19 EMs to PMs for flunitrazepam kinetics and response. A comparison of wt/wt and mut/mut groups would yield the best opportunity to test our general hypothesis, as the potentially confounding effect of heterozygotes would be excluded. A comparison of genotypes should also control for the confounding effects of other sources of differences, and an ideal study would therefore be stratified by genotype, ethnoracial group (with a comparison of Caucasians and Chinese populations) and sex, and it would control for smoking status and other drug use. In addition, to rule out discordance between genotype and true CYP2C19 activity (owing, for example, to a previously-unidentified allele that genotypes identically to the wild-type allele on existing tests), each subject's phenotype would also be considered.

Although to our knowledge there is no systematic data specific to Chinese, published research suggests that the recruitment of subjects from ethnoracial minorities can be
problematic. Before proceeding, therefore, we determined if recruitment of people of Chinese ancestry was feasible (Study I).

Another approach to achieve our general objective was to selectively inhibit flunitrazepam's CYP2C19-mediated metabolic pathways in CYP2C19 EMs. This approach stems from an experimental method where enzyme inhibitors are used to create a phenotype that resembles that produced by a mutation; the result is known as a "phenocopy". We chose to use omeprazole as the CYP2C19 inhibitor because it is widely and safely used. However, omeprazole is also a competitive inhibitor of CYP3A4, which may also be involved in the metabolism of flunitrazepam. Ketoconazole is a very potent and selective inhibitor of CYP3A4 in vivo. Therefore, by examining the effects of these two inhibitors (omeprazole and ketoconazole) on flunitrazepam kinetics, the metabolic profile of flunitrazepam and the role of CYP2C19 could potentially be established in vivo.

First, however, we wanted to determine the minimum number of days of pretreatment of ketoconazole required to achieve maximum inhibition of CYP3A4 without inhibiting CYP2C19. In addition, we wanted to examine the feasibility of creating a CYP2C19 PM phenocopy with these inhibitors (Study II).

The third approach to our general objective was to compare flunitrazepam kinetics and response in CYP2C19 EMs to PMs in the general population—that is, without controlling for ethnicity (Study III). We decided to use CYP2C19 activity as the basis for comparison because this approach enabled us to control for potentially confounding variables that might have interfered with a comparison between genotypes.

Having approached our general objective from these three perspectives, we sought to characterise our determination of the omeprazole metabolic ratio as a tool for distinguishing
CYP2C19 EMs and PMs, and to examine the relationship between CYP2C19 genotype, phenotype and ethnoracial background in the context of our sample of Toronto residents (Study IV).
2 General Methods

2.1 Drugs
Losec® (omeprazole magnesium, 10 mg delayed release tablets) was generously provided by Astra Pharma Inc. (Mississauga, Ontario). Rohypnol® (flunitrazepam, 1 mg scored tablets) was provided by F. Hoffmann-La Roche Ltd. (Basel, Switzerland).

2.2 Ethical and regulatory considerations

2.2.1 Ethical Approval
The study protocols were approved by the Research Ethics Board for Sunnybrook & Women’s College Health Sciences Centre (App # 9768 and # 9943) as well as the Human Subject Review Committee at the University of Toronto (protocol reference #4318).

2.2.2 Consent
All subjects in this study were volunteer participants. The studies were explained in detail to subjects, who were then given ample opportunity to ask questions. Voluntary consent was then obtained in writing using the IRB-approved consent form in the presence of a witness. Each subject was given a copy of the consent form.

2.2.3 Procedures to Minimise Risk and Monitor Subjects
Subjects were fully informed about the possible risks of the drugs used in these studies, which were documented in the IRB-approved consent. The study was conducted by a highly experienced treatment and research team. In this context, any emergent events could have been detected quickly and rapidly. In addition, every subject was given an after-hours contact number.
2.2.4 Confidentiality
The consent form states that: "The data I will provide will be kept strictly confidential and secure and only available to the researchers and treatment staff involved in the conduct of the study. Neither my name nor any pieces of identifying information will be kept together with the other data that I may provide. My records will be afforded all the same confidentiality afforded hospital records." These measures were strictly adhered to.

2.2.5 Discontinuation and Drop-Out Criteria for the Subjects
Discontinuation and drop-out criteria were:

a) subject request (subjects were told that they may withdraw from the study at any time and that this will not prejudice their right to current or future treatment at the participating institutions);

b) non-compliance:

c) if it would be to the subject's detriment for them to continue (e.g. adverse event).

2.3 Recruitment
We recruited individuals of an appropriate genotype, as determined from analysis of their stored blood samples, who had previously consented to being contacted regarding future experimental studies. In addition, we recruited subjects in the Metropolitan Toronto area by posting ethics-approved notices and through word of mouth.

2.4 Inclusion Criteria
Subjects were required to meet the following criteria to be accepted into any of the studies:

1. Age 18-65 years

2. A willingness and capacity to give written informed consent
3. Sufficient literacy in English to fully understand the consent form and to communicate with study staff

4. A fixed, non-institutional address

2.5 Exclusion Criteria
Subjects were excluded from the study if they met any of the following criteria:

1. Any medical condition that requires investigation or treatment (e.g. presence of clinically important cardiovascular, respiratory, hepatic or renal condition)

2. Any psychiatric condition requiring investigation or treatment (e.g. depression, suicidal ideation)

3. Current continuous use of psychoactive medication

4. The meeting of DSM-IV criteria for psychoactive substance abuse or dependence within the past 6 months

5. Known allergic or idiosyncratic reactions to benzodiazepines

6. History of violent behaviour under the influence of anxiolytics

7. Known allergic or idiosyncratic reactions to omeprazole or related drugs

8. Females taking oral contraceptives

9. Pregnancy or lactation

10. Sexual practices presenting a risk of pregnancy

11. Treatment with an investigational drug within 30 days prior to participation in this study

12. Non-compliance or unreliability

13. Any physical or psychological condition which, in the opinion of the investigator, is likely to interfere with collection of the measures required
2.6 Pre-Study Assessment Day Procedures

After consent was obtained (Appendices A and B), subjects underwent the following procedures to ensure their suitability for this study and that there were no contraindications to their participation.

1. Breathalyser test for alcohol
2. Pharmacogenetics Drug Use History Questionnaire (PDHQ; if not already completed)
3. CYP2C19 Activity Test (Phenotyping)
4. CYP2C19 Genotype Test (if not already completed)
5. Practice SMS
6. Medical history and physical examination including an ECG and blood sample
   [hemogram, liver function tests (GGT, AST, ALT), pregnancy test (if female)]

2.6.1 Pharmacogenetics Drug History Questionnaire (PDHQ)

The Pharmacogenetics Drug History Questionnaire (PDHQ) is a comprehensive, standardised questionnaire that was used to document past and/or current psychoactive drug use. A trained member of our team administered the PDHQ through an interactive computer program. This questionnaire was designed to obtain information on the demographics of each subject (birth date, sex, eye and hair colour, education and ethnoracial background of their grandparents), history of any psychoactive drug use and pattern of use. Separate modules for each class of psychoactive agents (that is, ethanol, nicotine, cannabis, sedative-hypnotics, stimulants, opiates, inhalants, hallucinogens and antidepressants) were used to assess the use of the agents in that class of drugs. Drug dependence or abuse was diagnosed using DSM-IV criteria (DSM-IV, 1994).
2.6.2 CYP2C19 Activity Test (Phenotyping)
Omeprazole was used as the probe drug for CYP2C19. Subjects were required to fast from midnight the night before the assessment until the time of the assessment. In addition, subjects were asked to not consume any grapefruit juice or alcohol for 24 hours prior to the assessment. In the morning of the assessment, each subject received omeprazole 20-mg p.o., and a blood sample was taken 3 hours later. Omeprazole (OMP) and its metabolites, 5-hydroxyomeprazole (5-OH-OMP) and omeprazole sulphone (OMP-S), were assayed using HPLC as described in section 2.8.2 below. A standardised breakfast was served 1 hour post drug administration.

CYP2C19 phenotype was assigned based on the subjects’ ability to convert OMP to 5-OH-OMP. The metabolic ratio (MR) between the plasma concentrations of OMP and 5-OH-OMP was determined 3 hours post drug administration. Poor metaboliser (PM) and extensive metaboliser (EM) phenotypes were established according to the analysis described in Study IV. The EM and PM phenotypes were defined by ratios of OMP to 5-OH-OMP of 0.5 to 9.4 and 11.7 to 34.4, respectively.

2.6.3 CYP2C19 Genotyping
Genotyping analysis was done by members of Dr. R. F. Tyndale’s laboratory (Department of Pharmacology, University of Toronto). Genomic deoxyribonucleic acid (DNA) was extracted from blood samples and analysed for the CYP2C19*1 wild-type allele, and the CYP2C19*2 and CYP2C19*3 null mutant alleles using polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) analysis, using a method adapted from deMorais and colleagues (deMorais et al., 1994; deMorais et al., 1994).
2.6.3.1 CYP2C19*2
As described in section 1.1.2.3, the CYP2C19*2 variant is characterised by a single base (G→A) substitution in exon 5, producing an aberrant splice site (deMorais et al., 1994). The determination of this allele was first described by deMorais and colleagues (1994). Briefly, exon 5 was amplified using intron 4- and exon 5-specific primers for CYP2C19, and the PCR products were digested with Smal and analysed by gel electrophoresis. In the wild-type gene, the 169-bp PCR products are cut to yield fragments of 120 and 49 bp. In individuals who are homozygous for CYP2C19*2, the Smal site is destroyed and the 169-bp fragment is not cut. In heterozygous individuals, all three bands (49 bp, 120 bp and 169 bp) are observed.

Analysis of the CYP2C19*2 DNA sequence in exon 5 has revealed that the G to A mutation in exon 5 causes the formation of a BstNI digestion site that is not found in the wild-type CYP2C19 sequence (Nowak, 1997). Thus, the PCR product was also digested with BstNI to provide a mutant-positive digestion in addition to the wild-type-positive digestion produced by Smal.

2.6.3.2 CYP2C19*3
The CYP2C19*3 variant is characterised by a G→A transition in exon 4 of the CYP2C19 gene (deMorais et al., 1994). The detection of this mutant allele was done using a PCR restriction enzyme analysis adapted from deMorais and her colleagues (1994). Briefly, exon 4 was amplified using primers that anneal to introns 3 and 4, and the PCR product was digested with BamHI and analysed by gel electrophoresis. In the wild-type gene, the 329-bp PCR products are cut to yield fragments of 233 and 96 bp. In individuals who are homozygous for CYP2C19*3, the BamHI site is destroyed and the 329-bp fragment is not
cut. Accordingly, wild-type homozygotes, wt/CYP2C19*3 heterozygotes, and CYP2C19*3 homozygotes yielded banding patterns of 233/96 bp, 329/233/96 bp and 329 bp, respectively.

2.6.3.3 CYP2C19*4 and CYP2C19*5
Genotyping analysis for CYP2C19*4 and CYP2C19*5 alleles was done by Dr. Andrea Gaedigk (The Children's Mercy Hospital, Section of Clinical Pharmacology & Experimental Therapeutics, Kansas City, MO) for selected samples.

2.6.4 SMS: Scheduled Measurement System
The acute effects of the drug, including subjective and objective measures, were collected through a computer program called SMS (the Scheduled Measurement System) (Kaplan, 1995). The measures were organised into test cycles, where each cycle consisted of a number of questions and a manual tracking test. There was one cycle at baseline and then at set intervals after drug was administered.

2.6.4.1 Subjective Measures
The subjective measures included 35 self-rated visual analogue scales (VAS) (Appendix C). The first 27 VAS items were taken from the Tufts University Benzodiazepine Effects Scales (TUBS), an instrument supplied by Dr. David Greenblatt of the Tufts-New England Medical Centre, where it is used in studies of the subjective effects of benzodiazepines. The first 12 items form a sedation scale and the other 15 items measure other subjective perceptions of various mood states, each scored individually. Eight additional VAS items determine drug effects (any drug effects, drug liking, good drug effect, bad drug effect, feeling in body, feeling in mind, high feeling and sick feeling). These measures have been used extensively to examine subjective perceptions of sedation and mood states in previous studies with various substances (Greenblatt et al., 1991; Kaplan et al., 1997; Lapierre et al., 1990; Scavone et al., 1998), and have been demonstrated to be sensitive to depressant effects of
benzodiazepines, administered at typical therapeutic doses (Greenblatt et al., 1988; Greenblatt et al., 1989; Greenblatt et al., 1994; Greenblatt et al., 1993).

2.6.4.2 Objective Performance Measures
We used a manual tracking task as an objective measure for psychomotor performance. Using a joystick, a subject attempts to keep the image of an airplane centered over the image of an oscillating road as the latter moves down a computer monitor. This test has been used to measure the pharmacodynamic effects of benzodiazepines as well as other depressants and anxiolytics (Busto et al., 1994; Busto et al., 1999; Sellers et al., 1992), and has been used successfully to detect a difference in psychomotor performance with dextromethorphan between EMs and PMs of CYP2D6 (Zawertailo et al., 1998).

2.7 Sample Collection
Blood was collected by means of an indwelling canula in a forearm vein or by separate venipunctures. Blood samples were collected in 10 cc heparinized vacutainer tubes. All samples were kept on ice and processed together at the end of each study day. Blood processing involved centrifuging the samples at 2500 rpm for 15 min. in a Centra-CL3 Centrifuge set at approximately 10°C. The plasma layer was removed and stored at -20°C until analysed.

Urine samples were kept refrigerated throughout the study day. The volume and pH of each sample was measured. An approximate 20 ml aliquot of each sample was saved and stored at -20°C until analysed.
2.8 Analytical Methods
Chromatography is a method for separating, identifying and quantifying the various components of a solution. High performance liquid chromatography (HPLC) uses a mechanical pump to drive a mobile phase containing the sample through a column composed of small particles. The differences between the components in their interaction with the stationary phase (adsorption), determined by polarity and size, result in different mobilities as the sample passes through the column. This effect leads to the separation of the sample into its components. Usually, strong polar interactions and Van der Waals attractions assist in this adsorption, causing longer retention times. We used a reversed phase system, however, in which the most polar compound elutes first. The compounds were detected using ultraviolet spectrometry. The UV light absorption data is converted into a chromatogram. The area under each peak on the chromatogram is proportional to the amount of each component in the mixture.

2.8.1 Drugs and Chemicals
Omeprazole, 5-hydroxyomeprazole, omeprazole sulphone, and the internal standard, H153 (C\textsubscript{16}H\textsubscript{16}N\textsubscript{3}O\textsubscript{1}S), were supplied by Astra (Dr. Andersson, Hässle AB, Sweden). Clonazepam, flunitrazepam, 7-aminoflunitrazepam, 3-hydroxyflunitrazepam and desmethyflunitrazepam were obtained from Hoffmann-La Roche Ltd. (Vaudreuil, PQ, Canada). Acetonitrile, methanol (Fisher Scientific, Fair Lawn, NJ, USA), dichloromethane (DCM), tetrahydrofuran (THF) and hexane (Caledon Laboratories Ltd., Georgetown, ON, Canada) were of HPLC grade. All other chemicals were of analytical reagent grade. All solutions were prepared with Milli-Q de-ionized water (Millipore Corporation, Bedford, MA).

Carbonate buffer (100 nM) was prepared by dissolving 2.12g Na\textsubscript{2}CO\textsubscript{3} (Fisher Scientific Company, Fair Lawn, NJ, USA) in 200 ml water, and brought down to a pH of 9.3
with phosphoric acid. Phosphate buffer (1M) was prepared by dissolving 13.8g NaH₂PO₄·H₂O (ACP, Montreal, PQ) in 100 ml water, and brought up to a pH of 7.0 with 5N KOH. β-glucuronidase from *E. Coli* (Sigma) was dissolved in 1M phosphate buffer to make 5.5×10⁻³ Fisher U/ml. Na₂CO₃-NaHCO₃ buffer was prepared by dissolving 2.65g of sodium carbonate (Na₂CO₃) and 2.1g of sodium hydroxycarbonate (NaHCO₃) in 250 ml water and brought to a pH of 10.0 using phosphoric acid.

### 2.8.2 Omeprazole Assay

The concentrations of omeprazole (OMP) and its metabolites, 5-hydroxyomeprazole (5-OH/OMP) and omeprazole sulphone (OMP-S), were determined in plasma and urine using reversed-phase HPLC, using an adaptation of a published method (Lagerstrom & Persson, 1984). The author worked with Helma Nolte and Jenny Yanhua Zhang to perform the plasma assay; Jenny Yanhua Zhang performed the urine assay.

#### 2.8.2.1 Omeprazole Standard Solutions

Stock standards of 5-hydroxyomeprazole (550 μM), omeprazole sulphone (550 μM) and omeprazole (550 μM) were prepared by dissolving 0.994, 0.994 and 0.949 mg of each compound, respectively, in 1 ml methanol and then adding carbonate buffer to a total of 5 ml. Seven combination standards were prepared using these stock standard solutions. Table 2.1 presents the final concentration of each compound in the combination standard solutions.

<table>
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<tr>
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<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-OH-OMP</td>
<td>0.56</td>
<td>0.11</td>
<td>0.44</td>
<td>2.2</td>
<td>5.5</td>
<td>11</td>
<td>22</td>
</tr>
<tr>
<td>OMP-S</td>
<td>0.56</td>
<td>0.11</td>
<td>0.44</td>
<td>2.2</td>
<td>5.5</td>
<td>11</td>
<td>22</td>
</tr>
<tr>
<td>OMP</td>
<td>0.56</td>
<td>0.11</td>
<td>0.22</td>
<td>1.1</td>
<td>2.75</td>
<td>5.5</td>
<td>11</td>
</tr>
</tbody>
</table>
A stock standard of H153 (100 μg/ml) was prepared by dissolving 2 mg in 4 ml methanol and then adjusting the volume to 20 ml with the carbonate buffer. A working standard of H135 (25 μg/ml for plasma and 10 μg/ml for urine) was also prepared.

Omeprazole and its metabolites are unstable in light and in an acidic medium. Thus, the stock standard was made in alkaline solution and stored in a dark freezer at -20°C. From our experience, it appears that all the working standards are stable for at least 3 weeks in the refrigerator (4°C).

A 100 μl aliquot of each combination standard was added to tubes containing 500 μl blank plasma. The plasma used had been previously determined to be free of interference peaks at the retention time of the drugs of interest including the internal standard. These tubes were extracted daily and injected along with the other samples in order to generate a standard curve.

Similarly, a standard curve was set up by adding 50 μl of each combination standard to a tube containing blank urine. The urine had been previously determined to be free of interference peaks at the retention time of the drugs of interest including the internal standard. Like the plasma samples, these standards were always extracted along with the other samples.

Unextracted standards were also prepared by adding 100 μl combination standard, 50 μl H153 working standard and 100μl 1M phosphate buffer pH 7.0.

2.8.2.2 Omeprazole Plasma Assay

2.8.2.2.1 Extraction

Prior to separation by HPLC, omeprazole and its metabolites were extracted from the plasma.

Plasma samples were thawed, mixed and spun down at 3000rpm for 10 min. in a Western
Scientific Silencer Centrifuge. Five hundred microliters of each plasma sample (including a blank) was pipetted into a 10 ml conical polypropylene tube. One hundred microliters of 100 nM carbonate buffer (pH 9.3) was then added to the samples and the blank. Fifty microliters of H153 and 100 μl 1 M phosphate buffer (pH 7.0) were added to each tube (including standards) and mixed by vortex. 4.5 ml DCM was added as the extraction solution. The samples were then shaken vigorously in an Eberbach Horizontal Shaker set on high for 10 min., followed by centrifugation at 3000 rpm for 10 min. The aqueous (top) phase was then aspirated, and 3 ml organic phase was transferred to a second set of tubes. The samples were evaporated to just-dryness under nitrogen at 45°C in a Pierce Reacti-Therm III Heating Module. After a maximum of 20 min, 250 μl mobile phase were added to the tubes. The samples were then mixed by vortex and transferred to an Eppendorf test-tube. Eighty-five microliters of each sample was injected into the HPLC apparatus.

2.8.2.2 HPLC
The liquid chromatography system was composed of an Hewlett Packard (HP) 1050 series autosampler and a Waters pump. A HP C18, 5 μm, 125 x 4-mm column was used to separate the sample into its components. The elute was monitored by a HP 1050 series UV detector at a wavelength of 302 nm. A HP 3396 Series II integrator was then used to integrate the resulting peaks. The mobile phase consisted of 20 mM potassium phosphate buffer [20 mM KH2PO4 from Mallinckrodt (Paris, Kentucky, USA), brought up to a pH of 7.5 with 5N KOH] and acetonitrile (72:28 v/v), which was filtered with Millipore type FH filter 0.5 μM prior to use. The flow rate through the system was set at 0.7 ml/min. The retention times for 5-hydroxyomeprazole, omeprazole sulphone, omeprazole and the internal standard (H153) were approximately 4.1, 7.1, 11.4 and 16.5 minutes (Figure 2.1).
Figure 2.1. A typical HPLC chromatogram showing 5-hydroxyomeprazole (4.1 min.), omeprazole sulphone (7.1 min.), omeprazole (11.4 min.) and the internal standard (H153; 16.5 min.). The integrated areas for each peak are presented below the curve.
UV absorbance data was generated as a chromatogram with integrated peaks. The four peaks of interest were 5-hydroxyomeprazole, omeprazole sulphone, omeprazole and H153 (internal standard). Peak area ratios were determined by dividing each of the peak areas for 5-hydroxyomeprazole, omeprazole sulphone and omeprazole by the peak area for the internal standard (H153). Concentrations for each compound were calculated by multiplying the peak area ratio by the mean value for (peak area ratio)/(known concentration) for the standards. This method of calibration was used because it confers equal weight to each standard sample and results in less error for low concentrations than the traditional linear regression method of deriving standard curves (Figure 2.2).

The minimum quantifiable limit (sensitivity), defined as the concentration at which the coefficient of variation for the integrated peak (repeated 7 to 10 times) is less than or equal to 15%, was approximately 0.05 nmol/ml for all compounds.

The percent recovery, established by comparing the extracted to unextracted samples, was 83-97%, 89-104%, 91-106% and 86-101% for 5-hydroxyomeprazole (0.056-22 nmol/ml), omeprazole sulphone (0.056-22 nmol/ml), omeprazole (0.056-11 nmol/ml) and the internal standard (H153, 1250 nmol/ml), respectively.

Within-day variation in this assay was calculated for combination standard #3, combination standard #6 and one sample (Subject 4, Day 1, Sample 6). For each sample, six extractions were assayed separately. The results were used to calculate the percent-coefficient of variation for each compound (Table 2.2), which in all nine cases was less than 10%. Between-day variation was calculated for combination standards #3 and #6 for the first seven assay days of the experiment (Table 2.2). The percent-coefficient of variation was less than or equal to 15% in all six cases.
Figure 2.2. A typical standard curve used to quantify omeprazole. Peak area ratio is the ratio of the peak area of omeprazole to that of the internal standard (H153). The trend line was forced through (0,0), and the slope was determined as the mean value for (peak area ratio/known concentration) for the standards.
Table 2.2. Summary of within-day and between-day variation for detection of 5-hydroxyomeprazole, omeprazole sulphone and omeprazole.

### Within-day variation

<table>
<thead>
<tr>
<th>5-Hydroxyomeprazole</th>
<th>Std. #3</th>
<th>Std. #6</th>
<th>Sample 1004-01-06</th>
</tr>
</thead>
<tbody>
<tr>
<td>nmoles/ml</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>0.353 11.947</td>
<td>0.484</td>
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<tr>
<td>0.343 12.377</td>
<td>0.503</td>
<td></td>
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<tr>
<td>0.357 12.286</td>
<td>0.531</td>
<td></td>
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</tr>
<tr>
<td>0.401 11.956</td>
<td>0.536</td>
<td></td>
<td></td>
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<tr>
<td>0.366 12.474</td>
<td>0.556</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.382 12.407</td>
<td>0.520</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>0.367</td>
<td>0.522</td>
<td></td>
</tr>
<tr>
<td>S.D.</td>
<td>0.021</td>
<td>0.025</td>
<td></td>
</tr>
<tr>
<td>% C.V.</td>
<td>5.8</td>
<td>4.9</td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
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</table>

### Between-day variation

<table>
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<tr>
<th>5-Hydroxyomeprazole</th>
<th>Std. #3</th>
<th>Std. #6</th>
</tr>
</thead>
<tbody>
<tr>
<td>nmoles/ml</td>
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<td></td>
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<tr>
<td>0.329 11.253</td>
<td>0.496</td>
<td></td>
</tr>
<tr>
<td>0.383 11.441</td>
<td>0.456</td>
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<tr>
<td>0.496 12.314</td>
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<tr>
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<tr>
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### Omeprazole sulphone

<table>
<thead>
<tr>
<th>nmoles/ml</th>
<th>Std. #3</th>
<th>Std. #6</th>
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<tr>
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### Omeprazole sulphone

<table>
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### Omeprazole

<table>
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<tr>
<td>0.138 6.071</td>
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2.8.2.3 Omeprazole Urine Assay

2.8.2.3.1 Extraction
Urine samples were thawed, mixed and centrifuged for 10 min. at 3000 rpm in a Western Scientific Silencer Centrifuge. Two hundred and fifty microliters of each sample (including one blank) was pipetted into a 10 ml conical polypropylene tube. One hundred microliters of β-glucoronidase was then added to all tubes (including standards). The tubes were then incubated for 30 min. at 37°C. 50 µl of carbonate buffer was added to the plasma samples and the blank. Fifty microliters of H153 and 100 µl 1 M phosphate buffer pH 7.0 were then added to all tubes (including standards) and mixed by vortex. 3.5 ml DCM was added to each tube as the extraction solution. The samples were then shaken vigorously in an Eberbach Horizontal Shaker set on high for 10 min., followed by centrifugation at 3000 rpm for 10 min. The aqueous (top) phase was then aspirated and 2.5 ml organic phase was transferred to a second set of tubes. The samples were evaporated to just-dryness under nitrogen at 45°C in a Pierce Reacti-Therm III Heating Module. After a maximum of 20 min., 250 µl mobile phase was added to the samples, which were then mixed by vortex and transferred to an Eppendorf test-tube. These tubes were centrifuged for 4 min. Fifty microliters of each sample was injected into the HPLC apparatus.

2.8.2.3.2 HPLC
The liquid chromatography apparatus used for the urine assay was similar to that used to determine omeprazole plasma concentrations. The same column, HP C18, 5 µm, 125 x 4-mm column, was used to separate the sample into its components. The mobile phase consisted of 20 mM potassium phosphate buffer (20 mM KH₂PO₄ brought up to a pH of 7.88 with 5N KOH) and acetonitrile (74:26 v/v). The flow rate through the system was set at 0.7 ml/min. The retention times for 5-hydroxyomeprazole, omeprazole sulphone, omeprazole
and the internal standard (H153) were approximately 4.15, 6.11, 11.33 and 16.90 for the urine assay. The percent recoveries for 5-hydroxyomeprazole, omeprazole sulphone and omeprazole were 83 ± 4, 119 ± 22 and 104 ± 14 (mean ± SD), respectively. The percent coefficients of within-day variation for 5-hydroxyomeprazole, omeprazole sulphone and omeprazole in the urine assay were 4.7, 6.3 and 5.9, respectively, and of between-day variation were 3.7, 3.7, and 6.7, respectively.

2.8.3 *Flunitrazepam Plasma Assay*

The flunitrazepam plasma assay was developed and performed by Dr. Wenjiang Zhang in our laboratory.

2.8.3.1 Standards

Extracted and unextracted standard solutions of 7-aminoflunitrazepam (7-AF), 3-hydroxyflunitrazepam (3-HF), desmethylflunitrazepam (DMF), flunitrazepam (FLU) and clonazepam were prepared daily. The concentrations ranged from 0-11.8 ng/ml, 0-13.72 ng/ml, 0-18.21 ng/ml and 0-13.05 ng/ml for 7-aminoflunitrazepam, 3-hydroxyflunitrazepam, desmethylflunitrazepam and flunitrazepam, respectively. The standards to be extracted were added to plasma that had previously been determined to be free of interference peaks at the retention time of the drugs of interest including the internal standard. These samples were extracted with the other samples. The extracted samples were used to make standard curves for the determination of the concentration of flunitrazepam (Figure 2.3) and its metabolites in the plasma samples. Recovery rates were calculated by comparing results from extracted and unextracted samples.
Figure 2.3. A typical standard curve used to quantify flunitrazepam. Peak area ratio is the ratio of the peak area of flunitrazepam to that of the internal standard (clonazepam). The trend line was forced through (0,0), and the slope was determined by linear regression.
2.8.3.2 Extraction
Plasma samples were thawed at room temperature, vortexed and centrifuged for 5 min. at 3000 rpm at 20°C in a Western Scientific Silencer Centrifuge. 1.5 ml of the supernatant was transferred into 10 ml conical polypropylene tubes containing 50 μl clonazepam (internal standard, 5.0 μM). Three hundred microliters of Na₂CO₃-NaHCO₃ buffer (pH=10.0) followed by 6 ml hexane/DCM (75:25:v/v) were added to each tube. The tubes were vortexed for 30 min. and then centrifuged for 15 min. at 3000 rpm at 20°C. The organic (top) layer was transferred to 10 ml conical polypropylene tubes and evaporated to just-dryness under nitrogen gas at room temperature. The residue was reconstituted in 120 μl mobile phase, mixed by vortex for 10 seconds and transferred to an Eppendorf test-tube. Fifty microliters of each sample were then injected into the HPLC apparatus.

2.8.3.3 HPLC
The liquid chromatography system was composed of a HP 1100 Series autosampler and a HP 1100 Series Quaternary Pump. A Waters Spherisorb S5 ODS2, 5 μm, 150 × 4.6 mm column was used. A HP 1100 Series Variable Wavelength Detector monitored the elute at a wavelength of 250 nm. An HP Chem Station for LC and LC/MS System integrated the resulting peaks. The mobile phase consisted of acetonitrile: potassium phosphate buffer (KH₂PO₄; 10 μM; pH=3.2):methanol:THF at a ratio of 22.5:67:9:0.5 (v/v/v/v). The flow rate through the system was set at 1.0 ml/min.

The retention times for 7-aminoflunitrazepam, 3-hydroxyflunitrazepam, desmethylflunitrazepam, clonazepam and flunitrazepam were approximately 5.26, 12.89, 14.51, 19.08 and 22.75 minutes (Figure 2.4). The minimum quantifiable limit (sensitivity) for all compounds was 1 ng/ml. The percent recovery for 7-aminoflunitrazepam, 3-
hydroxyflunitrazepam, desmethyflunitrazepam, flunitrazepam and clonazepam were 54.6-66.0, 98.4-111.6, 43.7-58.4, 99.0-111.5 and 62.1-70.6, respectively. The percent within-day variation for 7-aminoflunitrazepam, 3-hydroxyflunitrazepam, desmethyflunitrazepam and flunitrazepam were 2.1-7.7, 0.9-4.5, 1.5-7.7 and 3.7-4.0, respectively.
Figure 2.4. A typical chromatogram of 7-aminoflunitrazepam (5.26 min.), 3-hydroxyflunitrazepam (12.89 min.), desmethylflunitrazepam (14.51 min.), the internal standard (clonazepam; 19.08 min.) and flunitrazepam (22.75 min.).
3 Study I. Recruitment of Chinese Subjects

3.1 Rationale
In Toronto, people of Chinese ancestry represent the largest minority ethnoracial group (numbering approximately 400,000 in the Greater Toronto Area according to the 1996 Census). Chinese people have a large frequency (40%) of CYP2C19 mutant alleles. This group therefore represents a highly suitable population from which to draw subjects. The literature suggests that the recruitment of subjects from ethnoracial minorities can be problematic, though no specific information is available about ethnic Chinese. In addition, our understanding of the specific values and belief systems of people of Chinese ancestry (e.g. with regards to the drawing of blood and taking drugs) prompted a concern that it may conflict with the design of our relatively invasive study of kinetics.

3.2 Objective
To determine the likelihood of recruiting Chinese subjects for a study of flunitrazepam kinetics and response.

3.3 Materials and Methods
3.3.1 Subjects
The inclusion and exclusion criteria were as specified in the General Methods with the following additional inclusion criteria:
1. Four grandparents of Chinese primary racial identification
2. Pre-menopausal (if female)
Subjects were recruited from October 1998 to April 1999 using the strategies outlined in Table 3.1. Our goal was to recruit 32 subjects to participate in the study, 8 each CYP2C19 wt/wt males and females and 8 each CYP2C19 mut/mut males and females.
### Table 3.1. Recruitment strategies

Targeted telephone solicitations of previously genotyped individuals

**Posters**
- University and college campuses
  - University of Toronto, St. George Campus (weekly)
  - University of Toronto at Scarborough (twice)
  - Humber College (once)
  - Ryerson Polytechnic University (four times)
- Hospitals and health care providers
  - Sunnybrook & Women's College Health Sciences Centre, Women's College Campus (weekly)
  - Toronto General Hospital (Western Division) (twice)
  - Centre for Addiction and Mental Health (monthly)
- Spadina and College area (bulletin boards, library, coin laundry, grocery stores, etc.; biweekly)
- Community and cultural centres (once each)
  - Eastview Neighbourhood Community Centre
  - St. Christopher House
  - St. Stephen's Community House
  - University Settlement Recreation Centre
  - Cecil Community Centre
  - Chinese Canadian National Council, Toronto Chapter
  - Chinese Information and Community Services
  - South East Asian Services Association
  - Woodgreen Community Centre of Toronto
  - Asian Community Centre of Toronto
  - Community Information Centre of Metropolitan Toronto
- Advertisements (once each)
  - The Toronto Sun
  - Sing Tao Newspaper
  - The Sing Wah News
  - SWCHSC newsletter (December, 1998)
- Email listserves (once each)
  - University of Toronto Chinese Christian Fellowship
  - University of Toronto Chinese Student Association
- Educational/recruiting initiatives at the University of Toronto
  - First year pharmacy class (February 1999)
  - Third year pharmacology class PCL360 (March 1999)
- Others
  - Centre for Addiction and Mental Health Information Centre
  - Word of mouth
3.3.2 Study Design
People who responded with interest were initially screened for inclusion and exclusion criteria by telephone. Information about the study, including its objectives and rationale, was conveyed in simple, non-technical language. Respondents were explained that participants were required to come in for an initial assessment of 3.5 h duration. This pre-study assessment required a breathalyser test for alcohol, administration of omeprazole and a blood sample. The indications and clinical use of omeprazole were described. Each subject would be asked a number of questions about their drug use history (PDHQ). In addition, each subject would be required to complete a computerised motor co-ordination test and a questionnaire about how she or he was feeling (collected through the Scheduled Measurement System). Subjects received monetary compensation for their participation in this assessment. Initially, the compensation was $15. This value was increased in mid-November to $50, and the two subjects who had already participated were offered the difference.

Respondents were told that if they met the strict requirements set out for this study, they would be asked to participate. Prior to the study each would be required to undergo a short medical examination that included a blood test.

Originally, the study was designed to include three study day sessions, each approximately 9 h long, and three brief follow-up visits after each study day. Respondents were told that on each study day, they would be given a medication (flunitrazepam or midazolam). A total of 13 blood samples (7 ml each) would be collected immediately before and up to 3 days after drug administration. Urine would be collected in 24 h collections until 72 h. Subjective effects and motor co-ordination would be measured throughout the study day. Subjects would be compensated $300 for the inconvenience and their time.
This design was modified in mid-November (after two subjects were screened) to include only one study day with two brief follow-ups. Respondents were told that they would receive 1 mg of flunitrazepam orally on the study day. A blood sample would be taken immediately before and up to 48 hours following drug administration (12 blood samples in total). Urine would be collected for 8 hours following drug administration. Subjective effects and motor co-ordination would be measured throughout the study day. Subjects would receive a monetary compensation of $130 for the inconvenience and their time.

An opportunity was then given for respondents to ask questions, which were answered fully. Only those who were interested in participating in the study itself were recruited to take part in the pre-study assessment day.

Eighteen subjects participated in the pre-study assessment day as described in Section 2.6 (General Methods). Subjects who were eligible based on their genotype were then asked to participate in the study proper. They were given more details about flunitrazepam, including its indications, expected effects and possible side effects.

3.4 Results
A flow chart representing the various stages of recruitment is shown in Figure 3.1.

Approximately 75% of respondents were eligible to participate in the study, but only approximately 25% of the eligible respondents were interested in participating in the study. The reasons stated for not wanting to participate in the study included: (1) subject had insufficient available time, (2) subject was unwilling to take medication, (3) protocol was too invasive, particularly with respect to blood being drawn, (4) monetary compensation was insufficient, and (5) possible side effects of the drug were undesirable.
Figure 3.1. Recruitment of Chinese subjects.
Eighteen subjects participated in the pre-assessment day. Their demographic information is presented in Table 3.2. These statistics probably under-represent the level of education of this group of people, as many subjects were still completing various stages of education. For example, all four subjects who reported having completed high school were currently enrolled in bachelor’s degree programs; similarly, at least four individuals who reported having completed a bachelor’s degree were currently enrolled in graduate studies. Eleven samples were genotyped in time for the corresponding subjects to be contacted within the time frame of this study. Of these, six had eligible genotypes. Only one of these subjects, however, agreed to participate in the study, yielding a withdrawal rate of 83%. Three subjects cited reasons of insufficient time, and one was also concerned about taking a psychoactive medication. One subject was unreachable.

**Table 3.2. Demographic information**

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<tr>
<td>Males</td>
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3.5 Discussion
In a review of recruitment methods, Swanson and Ward noted that "the practice of recruiting subjects into clinical trials is embryonic at best" (Swanson & Ward, 1995). Although recruitment is critical to clinical trials, it appears that researchers have only recently begun to appreciate the time, energy, and substantial financial resources required to successfully recruit and enrol study participants (McCabe et al., 1994; Swanson & Ward, 1995; Wilcox et al., 1996). Swanson and Ward assert that recruitment is a problem in most clinical trials, and that the complexity and extent of recruitment are often underestimated (Swanson & Ward, 1995). They suggest that, in general, the initial approach to recruitment is rarely successful, the recruitment phase takes longer than investigators initially plan, the pool of eligible persons is often overestimated, and fewer participants are produced than planned. Investigators generally do not carefully and consistently report their recruitment experiences (Swanson & Ward, 1995), which limits opportunities for increasing knowledge and improving methods. In addition, much of the literature is centred around recruiting patients for preventative or treatment studies, as opposed to recruiting healthy volunteers as research subjects who are paid for their participation in the study (Wilcox et al., 1996). Moreover, insights gained into the recruiting process for varying projects and participants cannot easily be generalised (Wilcox et al., 1996). Swanson and Ward proposed that pilot studies of the recruitment process are useful in determining feasibility and identifying the most effective methods (Swanson & Ward, 1995).

The involvement of racial or ethnic minorities in clinical trials is relatively new (Swanson & Ward, 1995). Until recently, the predominant population group in clinical trials has been well-educated, middle-class, married Caucasian males (Swanson & Ward, 1995). It is now recognised that conducting trials in various populations is important, as their
responses to drugs may differ (Matthews, 1995). Therefore, factors that determine the participation of diverse ethnoracial, cultural, socio-economic, sex and age groups need to be understood. Unfortunately, little has been documented about the participation of subjects from minority groups, and what is available is largely anecdotal (Roberson, 1994) and does not seem to address the Chinese population specifically.

In the present study, we employed multiple simultaneous strategies to recruit subjects of Chinese ancestry. This tactic of combining strategies was recommended by Swanson and Ward, who reported that in studies that have formally evaluated their recruitment strategies, no single strategy was successful in meeting recruitment goals (Swanson & Ward, 1995). Unfortunately, our data with respect to the relative efficacy of each recruitment strategy are limited, and a more formal system of evaluating their success would provide an opportunity to determine the best overall approach to recruitment. Nonetheless, our data do enable us to make a number of observations and conclusions about recruitment.

The flow chart in Figure 3.1 suggests that barriers to subject recruitment and retention operated at two principal stages. First, most eligible respondents (approximately 75%) were not interested in participating in the study. The reasons that they reported for this preference appear to be commonly held; Swanson and Ward noted: “The most foreboding of these [barriers to patient participation] seem to be time and effort involved, particularly waiting time and travel time, interventions or side effects that are seen as unpleasant” (Swanson & Ward, 1995). From our discussions with subjects and other investigators, it appears that some of these factors (notably the invasiveness of blood-taking and, to a lesser extent, drug administration) may be particularly relevant to individuals within the Chinese population. Partly in response to subjects’ feedback and partly based on our intuition that time and
monetary considerations were probably important, we modified the study design and recruitment strategy to substantially reduce the time commitment required from subjects and to offer a higher rate of compensation. Our data do not permit an analysis of the extent to which these changes were successful at increasing recruitment and retention rates, however.

Second, most subjects (5 out of 6) who had an eligible genotype withdrew their participation. The withdrawal of subjects at this late stage represented a large investment lost, and it is therefore particularly important to understand its causes with the view of improving retention. This withdrawal was probably not due in most cases to the subject learning new information, as the details of the study were discussed with each prospective subject prior to the pre-study assessment day. Instead, withdrawal was probably related to a change in attitude and/or perception of the inconvenience, risks and benefits of participation, and this hypothesis was supported by the stated reasons for withdrawal (insufficient time and unwillingness to take medication). The time gap between pre-study assessment and scheduling for the study, which in some cases was as long as two months, may have been one predisposing factor for this shift. Ideally, this gap would have been minimised, but in this case it was unavoidable owing to the late availability of flunitrazepam. Another factor that may have contributed to the shift in willingness to participate is subjects’ experience of the pre-study assessment day. Though we went to great lengths to make the subjects’ experiences pleasant and comfortable, it is possible that their experience was different than what they had expected.

Language was another obstacle to recruitment. Because the study was equipped to involve English-speaking subjects only, we included only people who had sufficient literacy in English to fully understand the consent form and to communicate with study staff. We
therefore deliberately selected recruitment strategies that would attract English-speaking
Chinese people (such as English language advertisements), and this probably led to the
further exclusion of non-English speakers who did not respond to our recruiting efforts.

Probably there were additional underlying factors that determined why some people
participated in the study and others did not. An understanding of these factors may shed light
on the difficulties with retention discussed above and may also address the question of how
to improve the initial response rate. For example, factors that have been reported as general
barriers to patient recruitment and participation in clinical trials include: negative images of
scientific research and clinical drug trials presented by mass media (Wilcox et al., 1996), fear
of being a "guinea pig" for clinical research (Swanson & Ward, 1995), the belief that
investigators are more interested in research than in the well-being of subjects (Swanson &
Ward, 1995), and negative family perceptions of drug trials (Swanson & Ward, 1995). We
observed the latter concern directly, as some subjects asked that their participation not be
disclosed to family members. Other barriers that apply specifically to recruiting members of
an ethnoracial minority include: language (Swanson & Ward, 1995), differences in health
beliefs and behaviours that stem from religious and cultural beliefs and the primacy of non-
Western medicine (Roberson, 1994), perceived and actual racial discrimination within the
context of medical research (the Tuskegee Syphilis experiment, for example) and elsewhere
in society (El-Sadr & Capps, 1992; Roberson, 1994; Swanson & Ward, 1995), and fear and
mistrust of health care professionals and the health care system (Roberson, 1994; Swanson &
Ward, 1995).

The demographics of subjects who participated in the pre-study assessment indicate
that we recruited primarily young and educated individuals from the Chinese population. To
the extent that this population more resembles mainstream Canadian society than do others within this ethnoracial group, this observation suggests that the barriers to recruitment that stem from ethnoracial differences were indeed at work in the present study. A more comprehensive recruitment strategy would therefore seek to also include other segments of the Chinese population. These demographics also indicate that we recruited more men than women. The reasons for this difference may include the exclusion criterion of oral contraceptive use, cultural differences in gender roles and the added responsibilities of women related, for example, to child and parent care. Since the study's objectives required an equal number of men and women, a successful recruiting strategy would therefore include special efforts to target women.

In conclusion, respondents who met the inclusion and exclusion criteria were recruited at a rate of approximately 25%, and subjects who were found to be eligible by genotyping were retained at a rate of 17%, yielding an overall rate of recruitment, retention and eligibility of 4%. Thus, current methods were insufficient to enable the recruitment of 32 Chinese subjects of the appropriate genotype and sex within the given time frame. To improve the recruitment process, we recommend a multi-pronged approach to increase the number of respondents, the rate of recruitment and the rate of retention. Such an approach could be developed with the help of qualitative methods; for example focus groups of participants and non-participants could be held to identify barriers to participation of the Chinese population at all levels. Changes to the recruitment process and to the design of the study could then be implemented to reduce modifiable barriers. This approach could also include efforts to foster a closer relationship to the Chinese community, for example by implementing educational and co-operative initiatives at churches, community centres,
community events and in health care settings. Third, it might include involving a member of the Chinese community in the recruitment and study team who would help establish connections to the Chinese community, who could help develop trust among potential participants in the team and who could help bridge any existing cultural and linguistic gaps.
4 Study II. Feasibility of Creating a CYP2C19 Phenocopy Using Omeprazole and Ketoconazole

4.1 Rationale
A phenocopy is an environmentally induced phenotype that resembles the phenotype produced by a mutation. We sought to create CYP2C19 poor metaboliser (PM) phenocopies with omeprazole, a CYP2C19 inhibitor. This approach would enable us to study the influence of CYP2C19 on flunitrazepam kinetics and response by comparing extensive metabolisers (EMs) to individuals with low CYP2C19 activity (CYP2C19 PM phenocopies). We chose omeprazole as the CYP2C19 inhibitor because it is a well-studied inhibitor of the CYP2C19 system, has been widely and safely used, and is well tolerated in humans. Omeprazole, however, is also a competitive inhibitor of CYP3A4, which has been found to play an important role in the metabolism of flunitrazepam to desmethylflunitrazepam and 3-hydroxyflunitrazepam in vitro (Kilicarslan et al., 2000). We therefore chose to inhibit CYP3A4 with ketoconazole, a selective and specific inhibitor of CYP3A4, to resolve the roles of CYP2C19 and CYP3A4 in the metabolism of flunitrazepam in vivo. We expected that the differences in the metabolism and clinical effects of flunitrazepam with and without ketoconazole would reflect the relative contribution of CYP3A4, whereas the differences with and without the addition of omeprazole to ketoconazole would reflect the relative contribution of CYP2C19.

Before we could proceed with this study, however, some methodological issues had to be resolved. In this design, it is important to use a highly specific inhibitor of CYP3A4 at the lowest possible doses that will achieve the desired effects to minimise the risk of non-selective inhibition of other CYP enzymes. Böttiger and colleagues showed that
ketocanazole 100 mg p.o. can selectively inhibit CYP3A4 without inhibiting CYP2C19 (Bottiger et al., 1997). They administered ketocanazole daily for 4 days, however, because they did not know whether to expect a reversible or irreversible inhibition effect. Indeed, the nature of ketocanazole’s inhibition of CYP3A4 is not well understood (Venkatakrishnan et al., 2000). Various dosing schedules for ketocanazole have been used to inhibit CYP3A4 by different investigators, ranging from pretreatment for 2 days twice a day (Greenblatt et al., 1998) to 5 daily doses (Olkkola et al., 1994; Varhe et al., 1994). These studies did not look at the differences in selectivity and potency at different dosing frequencies.

We sought to establish, therefore, the minimum number of daily pre-treatments of ketocanazole required to achieve selective and maximum inhibition of CYP3A4. In addition, the complexity of the metabolism of omeprazole, which is thought to involve several competing and multi-step pathways that are mediated by several enzymes (some of which participate in multiple pathways), suggested that it would be worthwhile to test our model of the interaction between ketocanazole and omeprazole. These methodological considerations led us to examine the feasibility of creating a CYP2C19 PM phenocopy using omeprazole and ketocanazole.

4.2 Objectives
To determine the duration of daily ketocanazole dosing required to selectively inhibit CYP3A4 without inhibiting CYP2C19, and

To determine the feasibility of creating a CYP2C19 PM phenocopy by inhibiting both CYP2C19 with omeprazole and CYP3A4 with ketocanazole
4.3 Hypothesis
Ketoconazole selectively inhibits CYP3A4, resulting in a proportional reduction in the sulfoxidation of omeprazole (but not in its hydroxylation), and reaches maximum inhibition within four days of administration.

4.4 Study Design
4.4.1 Subjects
Nine subjects who were CYP2C19 EMs (wt/wt or wt/mut) were recruited for the study, five females and four males. The inclusion and exclusion criteria were as specified earlier with the following additions: subjects were excluded from the study if they met either of the following criteria:

1. Known allergic or idiosyncratic reactions to ketoconazole or related drugs
2. Concurrent use of terfenadine, astemizole, cisapride, or itraconazole

Three subjects participated in a pre-study assessment day, as detailed in Section 2.6 (General Methods). The rest underwent these initial procedures during the first study day. Written consent (Appendix D) was obtained according to Section 2.2 (General Method).

4.4.2 Test Day Schedule
Subjects were instructed to not consume grapefruit juice or alcoholic beverages for 24 hours prior to and during the study. Subjects were also asked to fast (to not consume any food or drink) from midnight before each study day. Each subject received a breathalyser test on arrival for testing at approximately 8:00 a.m., and a blood alcohol level of zero was required for the subject to continue to participate in the study. On each study day, subjects received omeprazole 40 mg p.o. On days 2 through 5, subjects also received ketoconazole 100 mg p.o. concomitantly. Approximately 30 minutes before the drug was given, an intravenous catheter was inserted into a forearm vein of one arm for blood sampling. A 7 ml blood
sample was taken immediately before drug administration and at 30, 60, 120, 180, 240, 300, and 360 minutes afterward to determine kinetic parameters. The bladder was emptied prior to drug administration, and urine was collected in measured volumes as voided in 3 h pooled samples post drug administration. Subjects were provided a light breakfast (cereal and fruit), a standardised lunch (sandwich and vegetables) and non-caffeinated beverages. Subjects were assessed and discharged by a physician (see Summary of Study Day Procedures, Appendix E).

4.4.3 Data Analysis
Plasma samples were analysed for omeprazole, 5-hydroxyomeprazole and omeprazole-sulphone by UV-HPLC as described in Section 2.8.2.2 (General Methods). The area under the concentration-time curve (AUC) from 0 to 6 hours was calculated as the sum of the products of each measured concentration and its corresponding time interval. Because the level of the three drugs at time zero was undetectable or near-undetectable for each day, the effects of accumulation of drug between days did not need to be accounted in the AUC calculations. Metabolic ratios of concentrations were calculated from AUCs. We chose the metabolic ratios of metabolite to parent drug as a way of representing enzyme activity down that metabolic pathway. The LS-means, which take into account missing data, and standard errors of these pharmacokinetic parameters were calculated using SAS. The GLM procedure in SAS was used to compare kinetic parameters between days. Statistical significance between days was determined using the logarithms of AUC and metabolic ratio.

We used the OMP/5-OH-OMP ratio at 3 h following drug administration as a measure of CYP2C19 activity and as a criterion to divide subjects into slow and fast metabolisers. For subjects 1001 to 1006, this ratio was determined following a 40 mg dose on study day 1. Because subjects 1007 to 1009 were also phenotyped with omeprazole 20
mg on a separate occasion during the Pre-Study Assessment Day (see Section 2.6.2), the omeprazole metabolic ratio for these individuals was calculated as the mean of the two determinations to achieve a more accurate estimate.

Urine data were analysed for omeprazole and its metabolites by UV-HPLC as described in Section 2.8.2.3 (General Methods). Omeprazole sulphone was undetectable, an observation that has been made by others (Sohn et al., 1992). The percent of dose recovered was calculated for omeprazole and 5-hydroxyomeprazole for the cumulative 6 hour period. Omeprazole and its metabolites are unstable in acidic conditions, and Lagerstrom and Pearson recommended that the collection of urine be done in an alkaline buffered solution (Lagerstrom & Persson, 1984). Because of this methodological consideration (urine was not buffered in the present study), and because the absence of omeprazole sulphone made it difficult to make conclusions about ketoconazole’s effect on CYP3A4 activity, urine data were not considered in the discussion below. The urine results are reported, however, in Appendix F for completeness.

4.5 Results
Nine subjects were recruited for this study. As one subject was discontinued at the beginning of day 2 due to high blood pressure, eight subjects completed the study as per protocol (4 females and 4 males). The mean age ± sample standard deviation for these eight subjects was 37.6 ± 15.4. The mean weight ± sample standard deviation was 82.0 ± 15.2 kg. All subjects were non-smokers and medication-free. Table 4.1 summarises the demographic information of all nine subjects.
Study II. Feasibility of Creating a CYP2C19 Phenocopy

Table 4.1. Demographic information

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>Age</th>
<th>Weight (kg)</th>
<th>Ethnoracial background</th>
<th>Genotype</th>
<th>OMP/5-OH ratio at 3h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1001</td>
<td>F</td>
<td>31</td>
<td>73.2</td>
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<td>*1/*1</td>
<td>2.07</td>
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<tr>
<td>1002</td>
<td>M</td>
<td>41</td>
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<tr>
<td>1003</td>
<td>F</td>
<td>25</td>
<td>98.4</td>
<td>Indian/Spanish</td>
<td>*1/*2</td>
<td>0.67</td>
</tr>
<tr>
<td>1004*</td>
<td>F</td>
<td>61</td>
<td>55.4</td>
<td>Caucasian</td>
<td>*1/*1</td>
<td>2.13</td>
</tr>
<tr>
<td>1005</td>
<td>M</td>
<td>59</td>
<td>72.0</td>
<td>Unknown</td>
<td>*1/*2</td>
<td>3.39</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(apparently Caucasian)</td>
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<td></td>
</tr>
<tr>
<td>1006</td>
<td>M</td>
<td>36</td>
<td>67.4</td>
<td>Caucasian</td>
<td>*1/*1</td>
<td>0.82</td>
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<tr>
<td>1007</td>
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<td>82.5</td>
<td>Caucasian</td>
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</tr>
<tr>
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<td>102.3</td>
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<tr>
<td>1009</td>
<td>M</td>
<td>62</td>
<td>96.4</td>
<td>Caucasian</td>
<td>*1/*1</td>
<td>1.01</td>
</tr>
</tbody>
</table>

* Subject discontinued after day 1

Examples of plasma concentration versus time profiles for omeprazole (OMP) and its metabolites, 5-hydroxyomeprazole (5-OH-OMP) and omeprazole sulphone (OMP-S), for each of the five study days for two subjects are given in Figure 4.1. The plasma concentration profiles from day 3 of subjects 2 and 3 suggested grossly delayed absorption of the drug, perhaps owing to non-compliance with the protocol with respect to fasting. These data were therefore excluded from the analysis.
Figure 4.1. Plasma concentration versus time profiles for omeprazole and its metabolites, 5-hydroxyomeprazole and omeprazole sulphone, for each of the five study days for subject 1001 (fast metaboliser, OMP/5-OH-OMP=2.07) and subject 1008 (slow metaboliser, OMP/5-OH-OMP=8.87).
Figure 4.2 shows the mean AUCs for all subjects from 0 to 6 hours for OMP and its metabolites over the 5 study days. On day one, the mean AUCs for OMP-S and 5-OH-OMP were similar (2.53 nmoles·h/ml and 2.68 nmoles·h/ml, respectively), and the mean $AUC_{OMP}$ was about 3.5 times greater than that of its metabolites (9.28 nmoles·h/ml). On day 5, $AUC_{OMP}$ was increased by 89% ($p<0.05$) relative to day 1, $AUC_{OMP-S}$ was increased by 41% ($p<0.05$) and there was no significant change in $AUC_{5-OH-OMP}$.

Figure 4.3 shows the mean metabolic ratios of the AUCs of each metabolite to OMP for each day. Both the OMP-S/OMP and 5-OH-OMP/OMP ratios decreased significantly over the duration of the study, by 40% and 75%, respectively. There was a significant difference in the OMP-S/OMP ratio on the first day compared to the other days, which were not significantly different from each other. There was also a significant difference in the 5-OH-OMP/OMP ratio on the first day compared to the others; in this case, however, the second day was also significantly different than days 4 and 5.
Figure 4.2. All subjects. Mean area under the concentration versus time curve (AUC) from 0 to 6 hours for omeprazole (OMP) and its metabolites, omeprazole sulfone (OMP-S) and 5-hydroxyomeprazole (5-OH-OMP), over the 5 study days. The values shown are the least-squares-mean ± standard error. The numbers above the bars indicate the days that are statistically different from that day.
Figure 4.3. All subjects. Mean metabolic ratios of AUC_{metabolite} to AUC_{OMP} over the 5 study days. The values shown are the least-squares-mean ± standard error. The numbers above the bars indicate the days that are statistically different from that day.
Although all subjects genotyped as EMs of CYP2C19, we also divided them into two groups according to their CYP2C19 enzyme activity to determine its impact on the outcome of repeated omeprazole and ketoconazole administration. The subjects exhibited a range of CYP2C19 activity, with OMP/5-OH-OMP metabolic ratios ranging from 0.67 to 8.87, with an anti-mode between 3.4 and 7.8. Accordingly, the subjects with high CYP2C19 activity, whose OMP/5-OH-OMP ratios was less than 3.4, were classified as fast metabolisers (n=6) and those with low CYP2C19 activity, whose ratio was greater than 7.8, were classified as slow metabolisers (n=2).

The trends observed with the group of fast metabolisers of CYP2C19 were similar to those seen with all subjects. Figures 4.4 and 4.5 show the mean AUCs from 0 to 6 h for omeprazole and its metabolites and the mean ratios of each metabolite to omeprazole, respectively, for fast metabolisers. On day 1, the AUCs for OMP, OMP-S and 5-OH-OMP were 5.89 nmoles·h/ml, 1.98 nmoles·h/ml and 2.78 nmoles·h/ml, respectively. On day 5, AUC_{OMP} was increased by 156% (p<0.05) relative to day 1, AUC_{OMP-S} was increased by 41% (p<0.05) and there was no significant change in AUC_{5-OH-OMP}. The OMP-S/OMP and 5-OH-OMP/OMP ratios decreased significantly over the duration of the study, by 50% and 76%, respectively.
Figure 4.4. Subjects with high CYP2C19 activity (fast metabolisers). Mean area under the concentration versus time curve (AUC) from 0 to 6 hours for omeprazole (OMP) and its metabolites, omeprazole sulfone (OMP-S) and 5-hydroxyomeprazole (5-OH-OMP), over the 5 study days. The values shown are the least-squares-mean ± standard error. The numbers above the bars indicate the days that are statistically different from that day.
Figure 4.5. Subjects with high CYP2C19 activity (fast metabolisers). Mean metabolic ratios of $\text{AUC}_{\text{metabolite}}$ to $\text{AUC}_{\text{OMP}}$ over the 5 study days. The values shown are the least-squares-mean ± standard error. The numbers above the bars indicate the days that are statistically different from that day.
Figures 4.6 and 4.7 show the mean AUCs from 0 to 6 h for omeprazole and its metabolites and the mean ratios of each metabolite to omeprazole, respectively, for the slow metabolisers. On day 1, the AUCs for OMP, OMP-S and 5-OH-OMP were 19.44 nmoles·h/ml, 3.97 nmoles·h/ml and 2.38 nmoles·h/ml, respectively. Compared to the fast metabolisers of CYP2C19, slow metabolisers had more OMP, more OMP-S and a similar amount of 5-OH-OMP on the first day. There was no significant change in the AUCs of OMP or its metabolites from day 1 to day 5. The 5-OH-OMP/OMP ratio decreased significantly over the duration of the study, however, by 41%.

Table 4.2 summarises the values for mean AUCs and mean metabolic ratios for days 1 and 5 and their respective percent differences for all subjects, fast metabolisers and slow metabolisers.
Figure 4.6. Subjects with low CYP2C19 activity (slow metabolisers). Mean area under the concentration versus time curve (AUC) from 0 to 6 hours for omeprazole (OMP) and its metabolites, omeprazole sulfoe (OMP-S) and 5-hydroxyomeprazole (5-OH-OMP), over the 5 study days. The values shown are the least-squares-mean ± standard error. None of the values were statistically different between days.
Figure 4.7. Subjects with low CYP2C19 activity (slow metabolisers). Mean metabolic ratios of AUC$_{\text{metabolite}}$ to AUC$_{\text{OMP}}$ over the 5 study days. The values shown are the least-squares-mean ± standard error. The numbers above the bars indicate the days that are statistically different from that day.
Table 4.2. AUC\textsubscript{0-4h} (nmol·h/ml) for omeprazole, 5-hydroxyomeprazole and omeprazole sulphone, and metabolic ratios determined on the first and fifth days.

<table>
<thead>
<tr>
<th></th>
<th>All Subjects (n=8)</th>
<th>Fast Metabolisers (n=6)</th>
<th>Slow Metabolisers (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1\textsuperscript{st} Day</td>
<td>5\textsuperscript{th} Day</td>
<td>Δ (%)</td>
</tr>
<tr>
<td>OMP AUC</td>
<td>9.28</td>
<td>17.51</td>
<td>+89*</td>
</tr>
<tr>
<td>OMP-S AUC</td>
<td>2.48</td>
<td>3.50</td>
<td>+41*</td>
</tr>
<tr>
<td>OMP-S/OMP</td>
<td>0.31</td>
<td>0.19</td>
<td>-40*</td>
</tr>
<tr>
<td>5-OH-OMP AUC</td>
<td>2.68</td>
<td>2.53</td>
<td>-6</td>
</tr>
<tr>
<td>5-OH-OMP/OMP</td>
<td>0.69</td>
<td>0.17</td>
<td>-75*</td>
</tr>
</tbody>
</table>

* p<0.05

Table 4.3. AUC (nmol·h/ml) for omeprazole, 5-hydroxyomeprazole and omeprazole sulphone, and metabolic ratios determined on the first and fifth days of repeated administration of omeprazole EC granules 20mg in CYP2C19 extensive and poor metabolisers (adapted from Andersson (1991)).

<table>
<thead>
<tr>
<th></th>
<th>Extensive Metabolisers (n=6)</th>
<th>Poor Metabolisers\textsuperscript{†}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1\textsuperscript{st} Day</td>
<td>5\textsuperscript{th} Day</td>
</tr>
<tr>
<td>OMP AUC</td>
<td>0.47</td>
<td>1.00</td>
</tr>
<tr>
<td>OMP-S AUC</td>
<td>0.31</td>
<td>0.67</td>
</tr>
<tr>
<td>5-OH-OMP AUC</td>
<td>1.02</td>
<td>1.19</td>
</tr>
<tr>
<td>5-OH-OMP/OMP</td>
<td>2.45</td>
<td>1.46</td>
</tr>
</tbody>
</table>

\textsuperscript{†} n=4 for omeprazole and n=2 for the metabolites
4.6 Discussion

Omeprazole appears to have the unusual quality that its bioavailability increases with repeated dosing (Prichard et al., 1985). Omeprazole plasma AUC has been shown to increase during the first 4 days of repeated administration, with the most pronounced increase observed for the highest dose (Andersson, 1991; Andersson et al., 1990; Andersson et al., 1991; Howden et al., 1984; Kovacs et al., 1999; Prichard et al., 1985). One explanation for this increase in AUC after repeated dosing is an increase in absorption due to a decreased effect of gastric acidity on the formulation during continuous treatment with omeprazole (Andersson, 1991; Andersson et al., 1990; Andersson et al., 1991; Prichard et al., 1985). Omeprazole is acid labile, and therefore acid-resistant enteric-coated (EC) formulations have been developed. Nonetheless, it is possible that the first dose, despite its acid resistant enteric coating, may be degraded in the stomach by gastric acids and therefore have a reduced systemic availability. The profound and persistent decrease in intragastric acidity caused by the drug (Lind et al., 1983) may stabilise the EC granules and in turn lead to increased bioavailability on subsequent days. In support of this hypothesis, Howden and colleagues reported a marked increase in the absorption rate constant with seven daily doses of 30 and 60 mg of enteric-coated formulation of omeprazole, suggesting that the increased availability is secondary to enhanced absorption (Howden et al., 1984). Andersson and coworkers specifically investigated the potential contribution of reduced acidity to the increase in AUC from the first dose to steady-state. In contrast, they showed no statistically significant difference in the AUC of omeprazole between subjects under untreated and acid suppressed conditions, thus providing counter evidence for the hypothesis that gastric acidity is a major contributing factor to increased AUC with repeated omeprazole administration.
(Andersson et al., 1991). One author has noted, however, that this study included only eight subjects and that the 20% increase in mean AUC under acid suppressed conditions (p=0.07) may have reached statistical significance if more subjects had been included (Thomson et al., 1997).

Another explanation for the increase in omeprazole AUC observed with repeated dosing is a decrease in elimination of the drug. Andersson and colleagues gave an intravenous tracer dose of [14C]-omeprazole simultaneously with the first and last oral dose of a 7 day repeated daily administration of omeprazole 20 mg. They found no change in the kinetics of the tracer dose, indicating that the increased AUC of the 20 mg repeated oral dose may have been a result of decreased first-pass metabolism (Andersson, 1991; Andersson et al., 1990). With higher doses of omeprazole, however, a decreased systemic clearance may also contribute to the effect, as clearance was reduced by 47% during repeated intravenous dosing of 40 mg of omeprazole (Andersson, 1991; Cederberg et al., 1992). Several investigators have suggested that the decrease in elimination during repeated omeprazole dosing might be explained by omeprazole acting as an inhibitor of its own metabolism (Andersson et al., 1990; Rost & Roots, 1996). Competitive inhibition seems unlikely in light of omeprazole's short half-life and the fact that it is not detected in the plasma by the next daily dose (Andersson et al., 1990; Rost & Roots, 1996), but it has been suggested that omeprazole may exert an inhibitory effect through an inactivation of CYP2C19 by omeprazole that persists beyond the presence of omeprazole in the plasma, or through competitive inhibition of CYP2C19 by OMP-S (Andersson et al., 1990; Rost & Roots, 1996). Omeprazole-sulphone is eliminated more slowly from the plasma than omeprazole itself (low concentrations of this metabolite were detected in plasma 24 h after dosing in this study and those of others
(Andersson et al., 1990; Chang et al., 1995; Rost & Roots, 1996)) and has been also shown to inhibit the cytochrome P-450 system in vitro (Gugler & Jensen, 1985).

Andersson investigated the metabolism of omeprazole in 6 EMs of CYP2C19 (determined by phenotyping with S-mephenytoin and omeprazole) and 2 PMs. Each subject received omeprazole 20 mg as EC granules for one week. The plasma levels of OMP, 5-OH-OMP, and OMP-S were determined on the first and last day of treatment. The results of this study are shown in Table 4.3 (page 112). The author concluded that the increased AUC\textsubscript{OMP} observed during repeated administration of this drug in EMs was probably the result of partial inhibition of CYP2C19, which plays a major role in the metabolism of omeprazole in these individuals (Andersson, 1991). It follows that there was no increase in the AUC of 5-OH-OMP with repeated dosing of omeprazole because its formation, which is mediated primarily by CYP2C19, was inhibited. In contrast, AUC\textsubscript{OMP-S}, whose formation is mediated primarily by CYP3A4, appeared to increase with increasing AUC\textsubscript{OMP}. Furthermore, according to the author’s hypothesis, PMs did not display an increase in AUC\textsubscript{OMP} during repeated dosing because these individuals lacked the CYP2C19 enzyme (Andersson, 1991). Accordingly, 5-OH-OMP exhibited unchanged AUC values while the AUC\textsubscript{OMP-S} increased only slightly in the group of slow metabolisers. Chang and colleagues also investigated the effects of eight daily doses of omeprazole 20 mg (enteric-coated granules) on the pharmacokinetics of omeprazole and its metabolites in 9 EMs and 5 PMs of CYP2C19 (Chang et al., 1995). They found results similar to those reported by Andersson (Andersson, 1991)—in EMs, AUC\textsubscript{OMP} and AUC\textsubscript{OMP-S} increased and AUC\textsubscript{5-OH-OMP} was unchanged from first to eighth dose, whereas in PMs, AUC\textsubscript{OMP} and AUC\textsubscript{5-OH-OMP} did not change with repeated
omeprazole administration—except for AUC_{omp}-s in PMs, which significantly increased with repeated dosing (Chang et al., 1995).

The formulation of omeprazole used in the studies described above may have influenced its kinetic properties. Several acid-resistant enteric-coated formulas of omeprazole are currently available on the market. For example, AstraZeneca manufactures Antra® and Antra MUPS® in Europe and Losec® in Canada, while Mepha uses the tradename Gasec-40 Gastrocaps® for its product. Antra is a hard gelatin capsule containing individually enteric-coated omeprazole pellets. Antra MUPS tablets, recently introduced, is a multiple units tablet that contains approximately 1000 individually enteric-coated omeprazole magnesium pellets per tablet of 20 mg omeprazole. The Losec delayed-release tablets used in our studies are formulated with omeprazole magnesium salt and covered with a highly acid-resistant coating (Pitre, 2000). Thomson and colleagues compared the pharmacodynamics and pharmacokinetics of omeprazole 20 mg enteric-coated tablet and capsule. Although the formulations were shown to be equipotent, subjects who received tablets had a significantly higher AUC, greater C_{max} and longer t_{max} (Thomson et al., 1997). The difference observed in these pharmacokinetic variables may be due to different stomach emptying properties of these formulations. The omeprazole capsule (as a multiple unit formulation) is gradually emptied from the stomach into the intestine, while the tablet (as a single unit formulation) is emptied from the stomach and dissolved as one unit (AstraZeneca, 2000; Thomson et al., 1997). Consequently, the tablet formulation of omeprazole remains in the stomach for a longer period, which may result in prolonged exposure to acid and degradation under single dose conditions (Thomson et al., 1997). In addition, as the absorption and first-pass metabolism of omeprazole tablets occurs in a shorter period of time
(AstraZeneca, 2000; Thomson et al., 1997), this formulation may be more susceptible to a reduced first-pass effect. In sum, caution should be exercised when making comparisons of pharmacokinetic variables between studies that use different formulations of omeprazole.

In the present study, omeprazole 40 mg was administered daily for five days and ketoconazole 100 mg was administered concomitantly on days 2 to 5. Given the above considerations, the observed increase in omeprazole AUC$_{(0-4h)}$ by 89% from days 1 to 5 was likely due to the combined effects of increased AUC with repeated administration of omeprazole and inhibition of CYP3A4 by ketoconazole. Two previous studies have investigated the change in AUC$_{OMP}$ exhibited following daily administration of omeprazole for 5 days specifically at a 40-mg dose. Prichard and colleagues observed an increase of 90% in the AUC$_{(0-12h)}$ of OMP in eight healthy male subjects with repeated administration encapsulated enteric-coated granulate of omeprazole in 5 daily morning doses (Prichard et al., 1985). Andersson and colleagues observed an increase of 182% in AUC$_{(0-\infty)}$ of omeprazole administered as enteric-coated granules (Andersson et al., 1991). We expected, therefore, that the increase in AUC observed in this study would have been larger, given the additional inhibitory effects of ketoconazole: this discrepancy might be attributable, however, to differences in CYP2C19 activity among tested subjects (see below), other inter-individual differences, differences in formulation between studies, and differences in the measured AUC time frame.

With repeated administration of omeprazole 40 mg for 5 days, Prichard and colleagues observed an increase of 110 % in the AUC$_{(0-12h)}$ of OMP-S from day 1 to day 5 (Prichard et al., 1985). Similarly, Andersson found that the AUC of OMP-S increased by 114% during repeated dosing of OMP 20 mg in EMs of CYP2C19 (Andersson, 1991). We
found that the AUC for OMP-S increased by 41% with 4 days of ketoconazole administration. That this increase was much smaller than the increases reported by Prichard and Andersson suggests that CYP3A4 was indeed inhibited by ketoconazole.

Andersson found that the AUC of 5-OH-OMP was unchanged during repeated omeprazole dosing (Andersson, 1991). Similarly, we found no significant change in AUC_{5-OH-OMP} over the 5 days despite the availability of more parent drug, suggesting that the 5-OH pathway was inhibited, most likely via the effects of repeated administration of omeprazole on CYP2C19. We cannot, however, exclude the possibility that ketoconazole was also inhibiting this pathway, either through inhibition of CYP3A4 or non-selective inhibition of CYP2C19. The former possibility is bolstered by the finding that the role of CYP3A4 in the hydroxylation pathway increases at higher doses of omeprazole due to saturation of CYP2C19 (Andersson & Regardh, 1990; Rost & Roots, 1996; Yamazaki et al., 1997).

We determined the metabolic ratios of metabolite to parent drug as a way of representing enzyme activity down each metabolic pathway. The OMP-S/OMP ratio decreased significantly with ketoconazole administration (by approximately 40%), confirming that ketoconazole inhibited CYP3A4 in the sulfoxidation pathway. Böttiger and coworkers found that CYP3A4 was inhibited by 72% with five days of ketoconazole 100 mg administration, as defined by the increase in OMP-S AUC (Bottiger et al., 1997). Since OMP-S AUC is subject to the effects of repeated omeprazole administration, it is not useful as an index of CYP3A4 inhibition in this study, and direct comparison of the degrees of enzyme inhibition is therefore difficult. The decrease in metabolic ratio occurred primarily on the first day of ketoconazole administration, which suggests that only one day of
ketoconazole was required to achieve most of its inhibitory effect. Inhibition of the CYP2C19-mediated metabolism of OMP-S with repeated administration of omeprazole and/or accumulation in the plasma of OMP-S between days, however, may have caused an increase in the metabolic ratio with days, potentially partially masking the inhibitory effect of ketoconazole. The duration of ketoconazole pretreatment required to achieve its full inhibitory effect therefore cannot be determined conclusively.

Andersson's results with repeated dosing of omeprazole show that the metabolic ratio of 5-OH-OMP/OMP decreased by 40% (Andersson, 1991). These results are consistent with those of Kovacs and colleagues, who also found that the 5-OH-OMP/OMP ratio in EMs of CYP2C19 decreased with repeated administration of omeprazole 40 mg (Kovacs et al., 1999). We found a marked and statistically significant decrease in 5-OH-OMP/OMP ratio of 75%. As with AUC_{5-4}^{14}OMP, we believe that the most likely explanation for the decrease in this metabolic ratio is that the repeated administration of omeprazole led to inhibition of CYP2C19. Again, however, it is also possible that ketoconazole had an inhibitory effect on this pathway.

Although all the subjects in this study had the EM genotype for CYP2C19, two of the subjects had a much lower CYP2C19 activity than the rest. Thus, subjects were divided into two groups according to their CYP2C19 activity to control for the confounding effect of variable CYP2C19 activity. The group of fast metabolisers of CYP2C19 exhibited the same general trends as all subjects. Notably, however, the increase in AUC_{OMP} in the fast metabolisers (156%) was much greater than that observed with all subjects (86%), probably because the fast metabolisers had more CYP2C19 activity initially available for inhibition. The greater change in AUC_{OMP} in this group lends credence to our hypothesis that inhibition
of both CYP2C19 (by repeated omeprazole administration) and CYP3A4 (with ketoconazole) caused the observed changes in omeprazole levels.

We expected that slow metabolisers of CYP2C19 would exhibit a blunted increase in AUC_{OMP} with days (primarily due to CYP3A4 inhibition by ketoconazole), a decrease in AUC_{OMP-S} (also due to CYP3A4 inhibition), and no change in AUC_{\text{H-Omp}}. There were no significant differences in any of these measures with days in the two slow metabolisers. The small number of people in this group, however, limited our power of detecting changes, and it is therefore difficult to draw conclusions from these results.

Some additional considerations warrant further discussion. First, an acidic environment is thought to be important for the dissolution and absorption of ketoconazole (Bennett, 1996; Chin et al., 1995). With repeated administration of omeprazole and the subsequent increase in gastric pH, therefore, the bioavailability of ketoconazole may have diminished. This potentially confounding drug-drug interaction may have had the effect of reducing the inhibitory effect of ketoconazole with days.

Second, we observed considerably higher AUCs for omeprazole and omeprazole sulphone than did other investigators. For example, our mean AUC_{OMP} after a single dose was approximately 5-fold greater than that reported by Andersson and colleagues (Andersson et al., 1991), 10-fold greater than that reported elsewhere by Andersson (using the double of his result following a 20 mg dose as an estimate; see Table 4.2), and 3-fold greater than that reported by Prichard (Prichard et al., 1985). These discrepancies may be due to a decreased first-pass effect in our study as a result of differences in formulation. In support of this hypothesis, we note that the discrepancy diminishes when we compare our slow metabolisers to Andersson’s PMs (to 1.3-fold; see Table 4.2), in whom CYP2C19 activity is less or
absent, suggesting that at least part of the discrepancy may have been due to inhibition of CYP2C19 during first-pass metabolism in our subjects.

Third, this study lacked an opportunity for direct comparison of kinetic parameters between subjects who had been administered repeated omeprazole with and without ketoconazole. We therefore used as controls data from other studies (Andersson, 1991; Andersson et al., 1991; Chang et al., 1995; Prichard et al., 1985), but this comparison is limited by differences that may have existed between, for example, drug formulations and subjects. A cross-over design would have been advantageous in this regard and represents a possible avenue for further work.

In conclusion, although in the present study one day of ketoconazole administration appeared to be sufficient to achieve its full inhibitory effect of approximately 40%, we cannot determine conclusively the duration of ketoconazole pretreatment required to achieve full inhibition of CYP3A4. Furthermore, because the repeated administration of omeprazole does not allow the roles of CYP2C19 and CYP3A4 in the metabolism of omeprazole to be resolved with ketoconazole, we cannot confirm the reported finding that ketoconazole is selective for CYP3A4 at this dose. Consequently, the feasibility of phenocopying CYP2C19 poor metabolisers with omeprazole and ketoconazole remains confounded by these issues, indicating that results from such an experiment would be difficult to interpret.
5 Study III. The Influence of CYP2C19 Activity on Flunitrazepam Kinetics and Response In Vivo

5.1 Rationale
Since the recruitment of Chinese subjects was not feasible compared to the relatively easy recruitment of Caucasians, a study was conducted with individuals with known CYP2C19 phenotype and genotype of any ethnoracial background. Despite the relatively lower frequency of CYP2C19 null alleles in individuals from the non-Asian population, their greater availability and willingness to participate allowed us to examine the role of CYP2C19 activity in determining flunitrazepam kinetics and response in individuals with a range of CYP2C19 activity. If CYP2C19 is important in the \textit{in vivo} metabolism of flunitrazepam, then inter-individual variation in CYP2C19 activity should explain differences in flunitrazepam kinetics and response.

5.2 Objective
To determine the influence of CYP2C19 activity on flunitrazepam kinetics and response \textit{in vivo}.

5.3 Hypothesis
Individuals who have lower CYP2C19 activity will exhibit a higher concentration of flunitrazepam in the plasma and greater psychomotor impairment and will report greater subjective effects.
5.4 Study Design

5.4.1 Subjects
Thirteen subjects were recruited for the study, six females and seven males. All subjects participated in the pre-study assessment day (procedures as outlined in Section 2.6 (General Methods)) prior to participating in this study, during which time their genotype and phenotype were determined and they were given an opportunity to practise the SMS module. The inclusion and exclusion criteria were as specified in Sections 2.4 and 2.5 (General Methods) with the following modification: subjects who smoked were not excluded from this study. Written consent (Appendix G) was obtained according to section 2.2 (General Methods).

5.4.2 Test day schedule
Subjects were instructed to not consume grapefruit juice or alcoholic beverages for 24 hours prior to and during the study. Subjects were also asked to fast (to not consume any food or drink) from midnight before each study day. Each subject received a breathalyser test on arrival for testing at approximately 8:00 a.m., and a blood alcohol level of zero was required for the subject to continue to participate in the study. On each study day, each subject received flunitrazepam 1 mg p.o. A 7 ml blood sample was taken immediately before and at 0.67, 1.33, 2, 3, 4, 5, 6, 7, 8, 24 and 48 hours post drug administration. Psychomotor performance and self-rated subjective measures of sedation and mood states were recorded before and 0.67, 1.33, 2, 3, 5 and 8 hours after drug administration. Subjects were provided a light breakfast (cereal and fruit), a standardized lunch (sandwich and vegetables) and non-caffeinated beverages. Smoking was not allowed during the first 8 hours following drug administration. Subjects were assessed and discharged by a physician (see Summary of Study Day Procedures, Appendix H).
5.4.3 Data Analysis

All blood samples were genotyped for the CYP2C19*2 and *3 allele variants. In addition, blood samples from subjects 1001, 1005, 1006, 1007 and 10013 were genotyped for the CYP*4 and *5 allele variants and found to contain neither mutation.

Samples were analysed for flunitrazepam (FLU) and its metabolites, 7-aminoflunitrazepam (7-AF), N-desmethyflunitrazepam (DMF) and 3-hydroxyflunitrazepam (3-HF), by UV-HPLC as described in Section 2.8.3 (General Methods). The area under the concentration-time curve (AUC) was calculated as the sum of the products of each measured concentration and its corresponding time interval. Average concentrations were calculated by dividing AUCs by the total time interval, yielding a time-weighted average. Ratios of concentrations were calculated from average concentrations.

For pharmacodynamic measures, average responses were calculated as the time-weighted average response minus pre-drug response (baseline). Peak response (delta min or max) values were calculated as the minimum or maximum score minus baseline.

We examined correlations between omeprazole metabolic ratios (MRs) and various flunitrazepam pharmacokinetic and pharmacodynamic parameters. The omeprazole to 5-hydroxyomeprazole (OMP/5-OH-OMP) ratio was used as a measure of CYP2C19 activity, and the omeprazole to omeprazole sulphone (OMP/OMP-S) ratio was used as a measure of CYP3A4 activity; increasing ratios represent decreasing enzyme activity in both cases. We used Spearman’s rank correlation, which is a non-parametric test that ranks both variables to be correlated, because the omeprazole metabolic ratio could not be assumed to be normally distributed. Two variables were considered potentially of interest if p<0.50. Three subjects had no quantifiable 3-HF and three subjects had no quantifiable DMF. These subjects were
assigned the lowest rank for AUC and average concentration for the corresponding metabolite, and were assigned equal ranks for ratios.

Pearson correlation coefficients and p-values were determined for the relationship between average flunitrazepam concentration for 4 and 8 hours and pharmacodynamic effects.

To rule out sex, ethnoracial background, age and weight as potential confounders, we used separate GLM procedures in SAS for each of these variables to determine whether there was a significant difference between subjects in omeprazole metabolic ratio and flunitrazepam pharmacokinetic parameters. We compared males and females, Caucasian subjects and non-Caucasians, subjects older than 49 and those younger than 49, and subjects heavier than 95 kg and those lighter than 95 kg. These numerical boundaries were set to correspond with antimodes in the data. To test the hypothesis that CYP2C19 genotype and phenotype were associated, we also compared the omeprazole metabolic ratios and flunitrazepam kinetic parameters of genotypic EMs (wt/wt and wt/mut) and the genotypic PM (mut/mut).

5.5 Results

Demographics

Table 5.1 presents demographic data for all subjects. Thirteen subjects participated in the study (6 females and 7 males). The mean age ± sample standard deviation was 37 ± 14 years. The mean weight ± sample standard deviation was 75 ± 18 kg (80 ± 18 kg for females and 70 ± 17 kg for males). The subjects came from various ethnoracial backgrounds: Caucasians made up the largest group (n=7), followed by Chinese (n= 3).
Table 5.1. Demographic information

<table>
<thead>
<tr>
<th>Metabolic Ratio (OMP/5-OH) after 3 h</th>
<th>Subject</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>Weight (kg)</th>
<th>Ethnicity</th>
<th>DSM-IV Drug Abuse and Dependence</th>
<th>Current Medications</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>High CYP2C19 Activity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.50</td>
<td>1004</td>
<td>F</td>
<td>41</td>
<td>75</td>
<td>Native/Caucasian</td>
<td>Past abuse of ethanol, cannabis and sedative-hypnotics</td>
<td>Paroxetine, Oxycodeone/Acetaminophen</td>
<td>*1/*1</td>
</tr>
<tr>
<td>0.67</td>
<td>1012</td>
<td>F</td>
<td>26</td>
<td>97</td>
<td>Indian/Spanish</td>
<td>None</td>
<td>None</td>
<td>*1/*2</td>
</tr>
<tr>
<td>0.82</td>
<td>1003</td>
<td>M</td>
<td>36</td>
<td>68</td>
<td>Caucasian</td>
<td>Past abuse of ethanol</td>
<td>None</td>
<td>*1/*1</td>
</tr>
<tr>
<td>0.83</td>
<td>1008</td>
<td>M</td>
<td>26</td>
<td>57</td>
<td>Chinese</td>
<td>Lifetime abuse of ethanol</td>
<td>None</td>
<td>*1/*1</td>
</tr>
<tr>
<td>0.98</td>
<td>1009</td>
<td>F</td>
<td>62</td>
<td>76</td>
<td>Caucasian</td>
<td>Past abuse of alcohol</td>
<td>Celebrex</td>
<td>*1/*2</td>
</tr>
<tr>
<td>1.09</td>
<td>1002</td>
<td>M</td>
<td>35</td>
<td>108</td>
<td>Caucasian</td>
<td>Past dependence on opiates</td>
<td>None</td>
<td>*1/*1</td>
</tr>
<tr>
<td>2.40</td>
<td>1001</td>
<td>F</td>
<td>30</td>
<td>70</td>
<td>Caucasian</td>
<td>None</td>
<td>None</td>
<td>*1/*1</td>
</tr>
<tr>
<td>3.39</td>
<td>1010</td>
<td>M</td>
<td>60</td>
<td>63</td>
<td>Unknown (apparently Caucasian)</td>
<td>None</td>
<td>None</td>
<td>*1/*2</td>
</tr>
<tr>
<td>3.50</td>
<td>1013</td>
<td>F</td>
<td>49</td>
<td>56</td>
<td>Caucasian</td>
<td>Past abuse of ethanol</td>
<td>Premarin, Famotidine, Robaxacet</td>
<td>*1/*2</td>
</tr>
<tr>
<td>4.24</td>
<td>1007</td>
<td>M</td>
<td>23</td>
<td>66</td>
<td>Chinese</td>
<td>History of nicotine abuse</td>
<td>None</td>
<td>*1/*3</td>
</tr>
<tr>
<td>7.83</td>
<td>1005</td>
<td>M</td>
<td>49</td>
<td>65</td>
<td>Indian</td>
<td>None</td>
<td>None</td>
<td>*1/*3</td>
</tr>
<tr>
<td>8.87</td>
<td>1006</td>
<td>F</td>
<td>24</td>
<td>106</td>
<td>Caucasian</td>
<td>None</td>
<td>None</td>
<td>*1/*2</td>
</tr>
<tr>
<td>28.8</td>
<td>1011</td>
<td>M</td>
<td>23</td>
<td>64</td>
<td>Chinese</td>
<td>History of nicotine abuse</td>
<td>None</td>
<td>*2/*3</td>
</tr>
</tbody>
</table>

Low CYP2C19 Activity
**CYP2C19 phenotype and genotype**

The subjects exhibited a range of CYP2C19 activity, with OMP/5-OH metabolic ratios ranging from 0.50 to 28.8. There was only one genotypic PM (*2/*3): he had the lowest CYP2C19 activity (MR=28.8). The rest of the group consisted of six homozygous (wt/wt) EMs and six heterozygous EMs (five *2/wt individuals and one *3/wt individual), with metabolic ratios ranging from 0.50 to 8.87.

**Pharmacokinetics**

A summary of selected pharmacokinetic and pharmacodynamic findings is presented at the end of the section in Table 5.12 for the reader’s convenience.

Examples of concentration-time curves for flunitrazepam and its metabolites are given in Figure 5.1 for subject 1009 (a genotypic EM), who exhibited high CYP2C19 activity, and subject 1011 (a genotypic PM), who exhibited low CYP2C19 activity. Although the maximum concentration and time to maximum concentration could not be precisely determined because samples were measured 40 min. apart during the early phase of the study, the apparent mean maximum concentration of flunitrazepam for all subjects was 9.9 ± 3.1 ng/ml and the apparent time to maximum concentration of flunitrazepam for all subjects occurred at 40 min. or 80 min. Tables 5.2, 5.3, 5.4 and 5.5 present the various kinetic parameters for all subjects at 3 h, for 0-4 h, 0-6.5 h and 0-48 h, respectively. The 3 h time point was selected for more detailed analysis because this was the time point at which omeprazole metabolic ratios were determined. 3-HF and DMF could be quantified until at least 4 and 6.5 hours, respectively, for all subjects except those who had no detectable metabolite at any time. Thus the 0-4 h and 0-6.5 h intervals were selected because they were deemed to encompass the most accurate representation of the pharmacokinetic parameters for these metabolites. The 0-48 h interval represented the full duration of the study.
Figure 5.1. Plasma concentration versus time curves for flunitrazepam (FLU) and its metabolites, desmethylflunitrazepam (DMF), 3-hydroxyflunitrazepam (3-HF) and 7-aminoflunitrazepam (7-AF), for subject 1009 (OMP/5-OH-OMP=0.98, high CYP2C19 activity; top) and subject 1011 (OMP/5-OH-OMP=28.8, low CYP2C19 activity; bottom).
Table 5.2. Average plasma concentrations and ratios at 3 hours post drug administration. Subjects are listed in order of decreasing CYP2C19 activity as measured by omeprazole phenotyping.

<table>
<thead>
<tr>
<th>Subject #</th>
<th>OMP/5-OH-OMP (CYP2C19)</th>
<th>OMP/OMP-S (CYP3A4)</th>
<th>FLU</th>
<th>DMF</th>
<th>FLU/DMF</th>
<th>3-HF</th>
<th>FLU/3-HF</th>
<th>7-AF</th>
<th>FLU/7-AF</th>
<th>7-AF/(DMF+3-HF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1004</td>
<td>0.50</td>
<td>3.85</td>
<td>2.75</td>
<td>0.22</td>
<td>12.29</td>
<td>0.24</td>
<td>11.29</td>
<td>1.36</td>
<td>2.03</td>
<td>2.91</td>
</tr>
<tr>
<td>1012</td>
<td>0.67</td>
<td>2.62</td>
<td>3.16</td>
<td>0.45</td>
<td>6.96</td>
<td>0.94</td>
<td>14.86</td>
<td>1.75</td>
<td>3.74</td>
<td>1.98</td>
</tr>
<tr>
<td>1003</td>
<td>0.82</td>
<td>2.28</td>
<td>6.53</td>
<td>0.44</td>
<td>14.75</td>
<td>0.44</td>
<td>10.05</td>
<td>2.43</td>
<td>2.29</td>
<td>0.91</td>
</tr>
<tr>
<td>1008</td>
<td>0.83</td>
<td>3.32</td>
<td>5.56</td>
<td>0.71</td>
<td>7.84</td>
<td>0.55</td>
<td>2.70</td>
<td>1.37</td>
<td>2.00</td>
<td>0.91</td>
</tr>
<tr>
<td>1009</td>
<td>0.98</td>
<td>1.19</td>
<td>2.73</td>
<td>0.49</td>
<td>5.54</td>
<td>1.01</td>
<td>2.19</td>
<td>1.34</td>
<td>3.50</td>
<td>2.06</td>
</tr>
<tr>
<td>1002</td>
<td>1.09</td>
<td>4.58</td>
<td>2.04</td>
<td>0.76</td>
<td>2.68</td>
<td>0.07</td>
<td>27.92</td>
<td>2.59</td>
<td>0.78</td>
<td>3.11</td>
</tr>
<tr>
<td>1001</td>
<td>2.40</td>
<td>4.18</td>
<td>3.45</td>
<td>0.58</td>
<td>5.97</td>
<td>0.16</td>
<td>21.98</td>
<td>2.57</td>
<td>1.34</td>
<td>3.50</td>
</tr>
<tr>
<td>1010</td>
<td>3.39</td>
<td>2.48</td>
<td>4.54</td>
<td>0.47</td>
<td>9.57</td>
<td>0.28</td>
<td>16.22</td>
<td>1.56</td>
<td>2.92</td>
<td>2.06</td>
</tr>
<tr>
<td>1013</td>
<td>3.50</td>
<td>3.31</td>
<td>6.42</td>
<td>0.62</td>
<td>11.33</td>
<td>0.10</td>
<td>72.58</td>
<td>1.06</td>
<td>6.67</td>
<td>1.47</td>
</tr>
<tr>
<td>1007</td>
<td>4.24</td>
<td>10.88</td>
<td>7.04</td>
<td>0.62</td>
<td>11.33</td>
<td>0.10</td>
<td>72.58</td>
<td>1.06</td>
<td>6.67</td>
<td>1.47</td>
</tr>
<tr>
<td>1005</td>
<td>7.83</td>
<td>5.15</td>
<td>6.39</td>
<td>0.58</td>
<td>5.97</td>
<td>0.16</td>
<td>21.98</td>
<td>2.57</td>
<td>1.34</td>
<td>3.50</td>
</tr>
<tr>
<td>1006</td>
<td>8.87</td>
<td>8.69</td>
<td>3.82</td>
<td>0.14</td>
<td>26.62</td>
<td>2.46</td>
<td>1.56</td>
<td>2.06</td>
<td></td>
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</tr>
<tr>
<td>1011</td>
<td>28.78</td>
<td>4.47</td>
<td>5.97</td>
<td>0.55</td>
<td>10.91</td>
<td>0.10</td>
<td>59.13</td>
<td>2.09</td>
<td>2.86</td>
<td>3.22</td>
</tr>
</tbody>
</table>

Table 5.3. Average plasma concentrations and ratios for the 0-4 hour period post drug administration. Subjects are listed in order of decreasing CYP2C19 activity as measured by omeprazole phenotyping.

<table>
<thead>
<tr>
<th>Subject #</th>
<th>OMP/5-OH-OMP (CYP2C19)</th>
<th>OMP/OMP-S (CYP3A4)</th>
<th>FLU</th>
<th>DMF</th>
<th>FLU/DMF</th>
<th>3-HF</th>
<th>FLU/3-HF</th>
<th>7-AF</th>
<th>FLU/7-AF</th>
<th>7-AF/(DMF+3-HF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1004</td>
<td>0.50</td>
<td>3.85</td>
<td>4.56</td>
<td>0.31</td>
<td>14.64</td>
<td>0.28</td>
<td>16.30</td>
<td>1.02</td>
<td>4.45</td>
<td>1.73</td>
</tr>
<tr>
<td>1012</td>
<td>0.67</td>
<td>2.62</td>
<td>3.38</td>
<td>0.28</td>
<td>12.07</td>
<td>0.01</td>
<td>16.18</td>
<td>1.22</td>
<td>5.16</td>
<td>1.61</td>
</tr>
<tr>
<td>1003</td>
<td>0.82</td>
<td>2.28</td>
<td>6.30</td>
<td>0.37</td>
<td>17.08</td>
<td>0.39</td>
<td>16.18</td>
<td>1.22</td>
<td>5.16</td>
<td>1.61</td>
</tr>
<tr>
<td>1008</td>
<td>0.83</td>
<td>3.32</td>
<td>7.99</td>
<td>0.73</td>
<td>10.76</td>
<td>0.64</td>
<td>12.34</td>
<td>2.03</td>
<td>3.88</td>
<td>1.48</td>
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<td>0.98</td>
<td>1.19</td>
<td>4.32</td>
<td>0.49</td>
<td>8.82</td>
<td>0.83</td>
<td>5.21</td>
<td>1.20</td>
<td>3.61</td>
<td>0.91</td>
</tr>
<tr>
<td>1002</td>
<td>1.09</td>
<td>4.58</td>
<td>3.59</td>
<td>0.57</td>
<td>6.25</td>
<td>0.08</td>
<td>46.15</td>
<td>2.49</td>
<td>1.44</td>
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<td>10.55</td>
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</tr>
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<td>10.88</td>
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<td>0.15</td>
<td>53.49</td>
<td>0.95</td>
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<td>1.82</td>
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<td>3.45</td>
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</tr>
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</tr>
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<td>13.82</td>
<td>0.14</td>
<td>43.28</td>
<td>1.64</td>
<td>3.61</td>
<td>2.90</td>
</tr>
</tbody>
</table>
**Table 5.4.** Average plasma concentrations and ratios for the 0-6.5 hour period post drug administration. Subjects are listed in order of decreasing CYP2C19 activity as measured by omeprazole phenotyping.

<table>
<thead>
<tr>
<th>Subject #</th>
<th>OMP/5-OH-OMP (CYP2C19)</th>
<th>OMP/OMP-S (CYP3A4)</th>
<th>FLU</th>
<th>DMF</th>
<th>FLU/DMF</th>
<th>7-AF</th>
<th>FLU/7-AF</th>
<th>7-AF/ (DMF+3-HF)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1004</td>
<td>0.50</td>
<td>3.85</td>
<td>3.40</td>
<td>0.28</td>
<td>11.98</td>
<td>0.92</td>
<td>3.69</td>
<td>1.56</td>
</tr>
<tr>
<td>1012</td>
<td>0.67</td>
<td>2.62</td>
<td>2.84</td>
<td>0.24</td>
<td>11.92</td>
<td>0.70</td>
<td>4.06</td>
<td></td>
</tr>
<tr>
<td>1003</td>
<td>0.82</td>
<td>2.28</td>
<td>5.65</td>
<td>0.42</td>
<td>13.44</td>
<td>1.54</td>
<td>3.67</td>
<td>2.03</td>
</tr>
<tr>
<td>1008</td>
<td>0.83</td>
<td>3.32</td>
<td>6.18</td>
<td>0.73</td>
<td>8.52</td>
<td>2.14</td>
<td>2.89</td>
<td>1.56</td>
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<td>1009</td>
<td>0.98</td>
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<td>6.47</td>
<td>1.28</td>
<td>2.63</td>
<td>0.97</td>
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<td>2.64</td>
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<td>2.44</td>
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<td>8.99</td>
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<td>3.12</td>
<td>2.33</td>
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<td>6.33</td>
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<td>3.54</td>
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<td></td>
<td>1.52</td>
</tr>
<tr>
<td>1011</td>
<td>28.78</td>
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<td>0.41</td>
<td>12.78</td>
<td>2.01</td>
<td>2.62</td>
<td>3.55</td>
</tr>
</tbody>
</table>

* DMF and 3-HF levels from 0-6.5 h and 0-4 h, respectively.

**Table 5.5.** Average plasma concentrations and ratios for the full study duration (0-48 h). Subjects are listed in order of decreasing CYP2C19 activity as measured by omeprazole phenotyping.

<table>
<thead>
<tr>
<th>Subject #</th>
<th>OMP/5-OH-OMP (CYP2C19)</th>
<th>OMP/OMP-S (CYP3A4)</th>
<th>FLU</th>
<th>FLU/DMF*</th>
<th>FLU/3-HF*</th>
<th>7-AF</th>
<th>FLU/7-AF</th>
<th>7-AF/ (DMF+3-HF)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1004</td>
<td>0.50</td>
<td>3.85</td>
<td>0.96</td>
<td>3.39</td>
<td>3.44</td>
<td>0.52</td>
<td>1.85</td>
<td>0.88</td>
</tr>
<tr>
<td>1012</td>
<td>0.67</td>
<td>2.62</td>
<td>0.94</td>
<td>3.94</td>
<td>1.21</td>
<td>0.77</td>
<td>1.21</td>
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</tr>
<tr>
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<td>1.82</td>
<td>4.33</td>
<td>4.67</td>
<td>1.40</td>
<td>1.30</td>
<td>1.85</td>
</tr>
<tr>
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<td>2.59</td>
<td>1.19</td>
<td>1.39</td>
<td>0.87</td>
</tr>
<tr>
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<td>1.19</td>
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<td>1.05</td>
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<td>1.08</td>
<td>0.55</td>
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<tr>
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<td>1.40</td>
<td>2.96</td>
<td>7.12</td>
<td>0.93</td>
<td>1.52</td>
<td>1.58</td>
</tr>
<tr>
<td>1013</td>
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<td>1.82</td>
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<td></td>
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<tr>
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<td>1.84</td>
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<td>12.03</td>
<td>0.95</td>
<td>1.94</td>
<td>1.37</td>
</tr>
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<td>1.45</td>
<td></td>
<td>1.73</td>
<td></td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td>1006</td>
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<td>8.69</td>
<td>0.82</td>
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<td>13.28</td>
<td>1.48</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
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<td>28.78</td>
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<td>2.17</td>
<td>5.28</td>
<td>15.90</td>
<td>1.18</td>
<td>1.83</td>
<td>2.10</td>
</tr>
</tbody>
</table>

* DMF and 3-HF levels from 0-6.5 h and 0-4 h, respectively.
CYP2C19 activity, as measured by the OMP/5-OH-OMP metabolic ratio, appeared to be related to plasma concentrations of flunitrazepam and its metabolites. Figure 5.2 illustrates the relationship between OMP/5-OH-OMP and several pharmacokinetic measures, namely FLU, FLU/DMF and FLU/3-HF. Table 5.6 summarises the Spearman's correlations between OMP/5-OH-OMP metabolic ratio (CYP2C19 pathway) and a number of pharmacokinetic parameters. A trend of increasing FLU average concentration with decreasing CYP2C19 activity was observed. This correlation appeared to be strongest at 3 hours (r=0.42, p=0.15), but it was also observed at all other time points, including over the full study duration (r=0.35 and p=0.25). Average concentration of DMF showed a weak trend of decreasing metabolite level with decreasing CYP2C19 activity (for 0-6.5 h, r=-0.28 and p=0.36). The metabolic ratio of FLU (0-48 h) to DMF (0-6.5 h) increased with decreasing CYP2C19 activity (r=0.44, p=0.14). A relatively strong trend of decreasing 3-HF average concentration with decreasing activity was observed (for 0-4 h, r=-0.46 and p=0.12). The metabolic ratio of FLU (0-48 h) to 3-HF (0-4 h) showed a strong trend of increasing ratio with decreasing CYP2C19 activity (r=0.54, p=0.06). 7-AF, which was detectable and quantifiable for 48 hours in all subjects, showed a strong trend of increasing average concentration with decreasing CYP2C19 activity (for 0-48 h, when the correlation was strongest, r=0.49 and p=0.09). The metabolic ratio of FLU to 7-AF showed a trend of decreasing ratio with decreasing CYP2C19 activity, a trend that was weak for 0-48 h but stronger for 0-4 and 0-6.5 hours. We also calculated the ratio of 7-AF to 3-HF+DMF as a measure of the intrinsic activities of the non-CYP mediated pathway relative to the CYP mediated pathways. This ratio showed a strong trend of increasing ratio with decreasing CYP2C19 activity (for 0-4 h, for example r=0.53 and p=0.06).
Figure 5.2. Several pharmacokinetic measures versus OMP/5-OH-OMP: (A) FLU plasma concentration (ng/ml), (B) FLU / DMF and (C) FLU / 3-HF.
### Table 5.6. Spearman's Correlations between OMP/5-OH-OMP metabolic ratio (CYP2C19 pathway) and pharmacokinetic parameters

<table>
<thead>
<tr>
<th>Kinetic parameter</th>
<th>at 3 h</th>
<th>0-4 h</th>
<th>0-6.5 h</th>
<th>0-48 h</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Spearman r</td>
<td>p-value</td>
<td>Spearman r</td>
<td>p-value</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FLU</td>
<td>0.42</td>
<td>0.15</td>
<td>0.29</td>
<td>0.34</td>
</tr>
<tr>
<td>DMF</td>
<td>-0.13</td>
<td>0.68</td>
<td>-0.18</td>
<td>0.56</td>
</tr>
<tr>
<td>FLU / DMF</td>
<td>0.39</td>
<td>0.19</td>
<td>0.43</td>
<td>0.15</td>
</tr>
<tr>
<td>3-HF</td>
<td>-0.37</td>
<td>0.21</td>
<td>-0.46</td>
<td>0.12</td>
</tr>
<tr>
<td>FLU / 3-HF</td>
<td>0.48</td>
<td>0.10</td>
<td>0.46</td>
<td>0.11</td>
</tr>
<tr>
<td>7-AF</td>
<td>0.37</td>
<td>0.22</td>
<td>0.31</td>
<td>0.30</td>
</tr>
<tr>
<td>FLU / 7-AF</td>
<td>-0.06</td>
<td>0.84</td>
<td>-0.42</td>
<td>0.15</td>
</tr>
<tr>
<td>7-AF / (3-HF+DMF)</td>
<td>0.55</td>
<td>0.05</td>
<td>0.53</td>
<td>0.06</td>
</tr>
</tbody>
</table>

*p-value

*a* FLU (0-48h) / DMF (0-6.5h)

*b* FLU (0-48h) / 3-HF (0-4h)

*c* 7-AF (0-6.5h) / [3HF (0-4h) + DMF (0-4h)]

*d* 7-AF (0-48h) / [3HF (0-4h) + DMF (0-4h)]

### Table 5.7. Spearman's Correlations between OMP/OMP-S metabolic ratio (CYP3A4 pathway) and pharmacokinetic parameters

<table>
<thead>
<tr>
<th>Kinetic parameter</th>
<th>at 3 h</th>
<th>0-4 h</th>
<th>0-6.5 h</th>
<th>0-48 h</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Spearman r</td>
<td>p-value</td>
<td>Spearman r</td>
<td>p-value</td>
</tr>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>FLU</td>
<td>0.18</td>
<td>0.57</td>
<td>0.09</td>
<td>0.76</td>
</tr>
<tr>
<td>DMF</td>
<td>0.09</td>
<td>0.77</td>
<td>-0.01</td>
<td>0.98</td>
</tr>
<tr>
<td>FLU / DMF</td>
<td>0.27</td>
<td>0.37</td>
<td>0.29</td>
<td>0.33</td>
</tr>
<tr>
<td>3-HF</td>
<td>-0.49</td>
<td>0.09</td>
<td>-0.48</td>
<td>0.10</td>
</tr>
<tr>
<td>FLU / 3-HF</td>
<td>0.46</td>
<td>0.12</td>
<td>0.51</td>
<td>0.07</td>
</tr>
<tr>
<td>7-AF</td>
<td>0.24</td>
<td>0.44</td>
<td>0.20</td>
<td>0.52</td>
</tr>
<tr>
<td>FLU / 7-AF</td>
<td>-0.12</td>
<td>0.71</td>
<td>-0.30</td>
<td>0.31</td>
</tr>
<tr>
<td>7-AF / (3-HF+DMF)</td>
<td>0.44</td>
<td>0.13</td>
<td>0.42</td>
<td>0.15</td>
</tr>
</tbody>
</table>

*p-value

*a* FLU (0-48h) / DMF (0-6.5h)

*b* FLU (0-48h) / 3-HF (0-4h)

*c* 7-AF (0-6.5h) / [3HF (0-4h) + DMF (0-4h)]

*d* 7-AF (0-48h) / [3HF (0-4h) + DMF (0-4h)]
A similar analysis was performed with the OMP/OMP-S ratio to examine the relationship between CYP3A4 activity and flunitrazepam kinetics (Table 5.7). The thirteen subjects in this study had OMP/OMP-S metabolic ratios ranging from 1.19 to 10.88 (Table 5.2). Average concentrations of FLU and DMF were not related to CYP3A4 activity. In the 4-hour period following drug administration, there was a weak trend of increasing FLU/DMF with decreasing CYP3A4 activity. In contrast, average concentration of 3-HF decreased with decreasing CYP3A4 activity (at 0-4 h, r=-0.48 and p=0.10), and the metabolic ratio of FLU/3-HF increased with decreasing CYP3A4 activity (r=0.514, p=0.07). There were weak trends of increasing average concentration of 7-AF and decreasing FLU/7-AF ratio with decreasing CYP3A4 activity. The 7-AF/(3-HF+DMF) ratio appeared to increase with decreasing enzyme activity.

The relative importance of CYP2C19 and CYP3A4 activities in the CYP-mediated pathways was estimated by examining the relationship between the ratio of 5-OH-OMP/OMP-S and both average metabolite concentrations and FLU/metabolite ratios. With increasing 5-OH-OMP/OMP-S, DMF concentration increased (for 0-6.5 h, r=0.45 and p=0.13), FLU(0-48 h)/DMF(0-6.5 h) decreased (r=-0.54 and p=0.06), 3-HF concentration increased (for 0-4 h, r=0.33 and p=0.28), and FLU(0-48 h)/3-HF(0-4 h) decreased (r=-0.48 and p=0.10).

Pharmacodynamics
Psychomotor performance and subjective perception of sedation and mood states were measured during the acute 8-hour period following drug administration. Because the onset of effects of flunitrazepam is rapid and its effects peak within the first few hours after a 1 mg oral dose, we conducted our analysis over the first 4 hours as well as over the full 8 hours.
Tables 5.8 and 5.9 present the average responses (minus baseline) for each subject for 0-4 hours and 0-8 hours, respectively. Minimum level (trough minus baseline) is also reported for "% over road mean," our measure of psychomotor performance. Only measures that have been reported as typical effects of flunitrazepam and for which responses were consistent with a drug response (i.e., followed a plasma level profile shape) are included. For example, Figures 5.3 and 5.4 show the responses for two measures, psychomotor performance and sedation item mean, that met these selection criteria. All the reported measures gave convex-up curves, except for "% over-road mean" and "thinking speeded up," which gave convex-down curves, and "feeling in body" and "feeling in mind," for which some subjects gave convex-up curves while others gave convex-down curves. "Anxious" was one measure that did not meet our selection criteria as responses were highly variable, and its response-time curves are presented as an example in Figure 5.5. The relationships between enzyme activities and flunitrazepam concentration with pharmacodynamic effects over 4 hours and 8 hours are presented in Tables 5.10 and 5.11, respectively.
Table 5.8. Average responses (minus baseline) for 0-4 hours post drug administration. Subjects are listed in order of decreasing CYP2C19 activity as measured by omeprazole phenotyping.

<table>
<thead>
<tr>
<th>Subject #</th>
<th>% Over road mean (Min levels)</th>
<th>% Over road mean (Ave levels)</th>
<th>Sedation item mean</th>
<th>Fatigued</th>
<th>Thinking speeded up</th>
<th>Any drug effect</th>
<th>Spacey</th>
<th>High</th>
<th>Good effect</th>
<th>Bad effect</th>
<th>Feeling in body</th>
<th>Feeling in mind</th>
</tr>
</thead>
<tbody>
<tr>
<td>1004</td>
<td>8.3</td>
<td>0.6</td>
<td>3.4</td>
<td>13.0</td>
<td>-27.1</td>
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</table>

Table 5.9. Average responses (minus baseline) for 0-8 hours post drug administration. Subjects are listed in order of decreasing CYP2C19 activity as measured by omeprazole phenotyping.

<table>
<thead>
<tr>
<th>Subject #</th>
<th>% Over road mean (Min levels)</th>
<th>% Over road mean (Ave levels)</th>
<th>Sedation item mean</th>
<th>Fatigued</th>
<th>Thinking speeded up</th>
<th>Any drug effect</th>
<th>Spacey</th>
<th>High</th>
<th>Good effect</th>
<th>Bad effect</th>
<th>Feeling in body</th>
<th>Feeling in mind</th>
</tr>
</thead>
<tbody>
<tr>
<td>1004</td>
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<td>0.6</td>
<td>-21.8</td>
<td>-14.5</td>
<td>-13.6</td>
<td>46.7</td>
<td>37.2</td>
<td>25.7</td>
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<td>33.5</td>
</tr>
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<td>-29.8</td>
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<td>1010</td>
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<td>40.5</td>
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<td>36.2</td>
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<td>7.1</td>
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<td>8.6</td>
<td>14.6</td>
<td>-5.8</td>
<td>-17.4</td>
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</table>
Figure 5.3. Psychomotor performance (mean % over road). Subjects are arranged in order of increasing OMP/5-OH-OMP ratio (decreasing CYP2C19 activity).
Figure 5.3. continued
Figure 5.4. Sedation (mean score for 12 VASs). Subjects are arranged in order of increasing OMP/5-OH-OMP ratio (decreasing CYP2C19 activity).
Subject 1013  OMP/5-OH-OMP: 3.50

Subject 1006  OMP/5-OH-OMP: 8.87

Subject 1007  OMP/5-OH-OMP: 4.24

Subject 1011  OMP/5-OH-OMP: 28.78

Average

Figure 5.4. continued
Figure 5.5. Anxious VAS (Calm ... Anxious). Subjects are arranged in order of increasing OMP/5-OH-OMP ratio (decreasing CYP2C19 activity).
Figure 5.5. continued
Table 5.10. Correlations of OMP/5-OH-OMP (CYP2C19), OMP/OMP-S (CYP3A4) and average flunitrazepam concentration with pharmacodynamic effects over 4 hours (n=13 for all measures except % over road mean, for which n=12)

<table>
<thead>
<tr>
<th>Measure</th>
<th>OMP/5-OH-OMP (CYP2C19)</th>
<th>OMP/OMP-S (CYP3A4)</th>
<th>Flunitrazepam (4 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ave conc</td>
<td>Delta min</td>
<td>Delta max</td>
</tr>
<tr>
<td></td>
<td>r</td>
<td>r</td>
<td>r</td>
</tr>
<tr>
<td>% over road mean</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.26</td>
<td>0.21</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>0.39</td>
<td>0.49</td>
<td>0.75</td>
</tr>
<tr>
<td>Sedation item mean</td>
<td>0.40</td>
<td>0.38</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>0.18</td>
<td>0.19</td>
<td>0.49</td>
</tr>
<tr>
<td>Fatigued</td>
<td>0.24</td>
<td>0.21</td>
<td>-0.05</td>
</tr>
<tr>
<td>Thinking speeded up</td>
<td>0.44</td>
<td>0.48</td>
<td>0.87</td>
</tr>
<tr>
<td>Any drug effect</td>
<td>-0.35</td>
<td>-0.29</td>
<td>-0.08</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>0.33</td>
<td>0.79</td>
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<tr>
<td>Spacey</td>
<td>0.10</td>
<td>0.18</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>0.73</td>
<td>0.56</td>
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<td>High</td>
<td>-0.05</td>
<td>-0.03</td>
<td>-0.05</td>
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<td>0.87</td>
<td>0.91</td>
<td>0.86</td>
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<td>Good effect</td>
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<td>-0.29</td>
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</tr>
<tr>
<td></td>
<td>0.45</td>
<td>0.33</td>
<td>0.62</td>
</tr>
<tr>
<td>Bad effect</td>
<td>0.30</td>
<td>0.45</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>0.32</td>
<td>0.12</td>
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<tr>
<td></td>
<td>0.39</td>
<td>0.19</td>
<td>0.19</td>
</tr>
<tr>
<td>Feeling in mind</td>
<td>-0.57</td>
<td>-0.34</td>
<td>-0.34</td>
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<td>0.04</td>
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Table 5.11. Correlation of OMP/5-OH-OMP (CYP2C19), OMP/OMP-S (CYP3A4) and average flunitrazepam concentration with pharmacodynamic effects over 8 hours (n=13 for all measures except % over road mean, for which n=12)

<table>
<thead>
<tr>
<th>Measure</th>
<th>OMP/5-OH-OMP (CYP2C19)</th>
<th>OMP/OMP-S (CYP3A4)</th>
<th>Flunitrazepam (8 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ave conc r</td>
<td>Delta min r</td>
<td>Delta max r</td>
</tr>
<tr>
<td>% over road mean</td>
<td>-0.48 p-value</td>
<td>0.11 p-value</td>
<td>-0.25 p-value</td>
</tr>
<tr>
<td>Sedation item mean</td>
<td>0.08 r</td>
<td>0.21 r</td>
<td>-0.25 r</td>
</tr>
<tr>
<td>Fatigued</td>
<td>0.38 r</td>
<td>0.38 r</td>
<td>0.18 r</td>
</tr>
<tr>
<td>Thinking speeded up</td>
<td>0.05 r</td>
<td>0.21 r</td>
<td>-0.21 r</td>
</tr>
<tr>
<td>Any drug effect</td>
<td>0.04 r</td>
<td>-0.15 r</td>
<td>0.17 r</td>
</tr>
<tr>
<td>Spacey</td>
<td>0.17 r</td>
<td>0.18 r</td>
<td>0.25 r</td>
</tr>
<tr>
<td>High</td>
<td>0.02 r</td>
<td>-0.03 r</td>
<td>-0.04 r</td>
</tr>
<tr>
<td>Good effect</td>
<td>-0.08 r</td>
<td>-0.17 r</td>
<td>-0.01 r</td>
</tr>
<tr>
<td>Bad effect</td>
<td>0.79 r</td>
<td>0.59 r</td>
<td>0.97 r</td>
</tr>
<tr>
<td>Feeling in body</td>
<td>0.31 r</td>
<td>0.46 r</td>
<td>0.31 r</td>
</tr>
<tr>
<td>Feeling in mind</td>
<td>0.30 r</td>
<td>0.11 r</td>
<td>0.30 r</td>
</tr>
<tr>
<td>Feeling in body</td>
<td>0.59 r</td>
<td>0.22 r</td>
<td>-0.37 r</td>
</tr>
<tr>
<td>Feeling in mind</td>
<td>-0.50 r</td>
<td>0.21 r</td>
<td>-0.37 r</td>
</tr>
</tbody>
</table>
The relationship between OMP/5-OH-OMP metabolic ratio and our objective measure for psychomotor performance, "% over road mean," over 4 h is illustrated in Figure 5.6. There was a strong negative correlation between the change in psychomotor performance (% over road mean) and flunitrazepam concentration over 4 and 8 hours (r = -0.76 and p < 0.005, and r = -0.69 and p = 0.01, respectively). Further, there was a greater decrease in psychomotor performance with decreasing CYP2C19 activity (r = -0.48 and p = 0.11 over 4 and 8 h). Figure 5.3 and Figure 5.7 present response-time curves and hystereses, respectively, for the mean "% over road" scores for all subjects. For this measure, we report correlations only with minimum response because it appeared that performance at later times was better than baseline, perhaps owing to the effects of practise. We believe that these correlations capture the portion of the response that is linearly predictable by the metabolic ratio.
Figure 5.6. Change in psychomotor performance (reported as delta % over road mean) over 4 h versus OMP/5-OH-OMP metabolic ratio.
Figure 5.7. Psychomotor performance (mean % over road). Subjects are arranged in order of increasing OMP/5-OH-OMP ratio (decreasing CYP2C19 activity). Effect is plotted against flunitrazepam plasma concentration and time. Most curves show a counterclockwise time orientation.
Figure 5.7. continued
Subjective measures gave varied results. “Sedation item mean,” a composite score of 12 separate visual analogue scales, showed a weak trend of increasing sedation with decreasing CYP2C19 activity but was weakly negatively correlated to flunitrazepam concentration. Its response-time curves are presented in Figure 5.4. “Fatigue” showed a stronger trend of increasing response with decreasing CYP2C19 activity but was not related to flunitrazepam concentration. “Thinking speeded up” showed a weak trend of increasing response with decreasing CYP2C19 activity and similarly had a strong positive correlation to flunitrazepam concentration (though its responses were convex-down). Also unexpectedly, “any drug effect” showed a trend of decreasing response with decreasing CYP2C19 activity and a negative correlation with flunitrazepam concentration. “Spacey” and “high” did not appear to correlate with CYP2C19 activity, though there was a negative correlation between “spacey” and flunitrazepam concentration. “Good effect,” “feeling in body” and “feeling in mind” showed a negative correlation with OMP/5-OH-OMP metabolic ratio, while “bad effect” showed a positive correlation. None of these measures, however, appeared to relate to flunitrazepam concentration.

We also analysed the relationships between the sum of FLU and DMF average concentrations and the pharmacodynamic effects to examine the additive effect of this potentially active metabolite. The results yielded trends similar to those observed for flunitrazepam alone.

To enable an analysis of the possible influence of CYP3A4 on pharmacodynamic effects, we conducted a similar analysis using the OMP/OMP-S ratio (Tables 5.10 and 5.11). Psychomotor performance decreased with decreasing CYP3A4 activity. Other subjective effects had only a very weak or absent relationship to enzyme activity, except for “good
effect”, “bad effect”, “feeling in body” and “feeling in mind.” These four effects were related to CYP3A4 activity in a way that was similar to their relationship to CYP2C19 activity.

Table 5.12 provides a summary of important findings.

**Table 5.12. Summary of important findings.** Correlations between OMP/5-OH-OMP metabolic ratio (CYP2C19 pathway) and selected pharmacokinetic measures (average plasma concentrations) and pharmacodynamic effects (average responses).

<table>
<thead>
<tr>
<th>Pharmacokinetic Measures</th>
<th>Spearman r</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLU (0-48 h)</td>
<td>0.35</td>
<td>0.25</td>
</tr>
<tr>
<td>DMF (0-6.5 h)</td>
<td>-0.28</td>
<td>0.36</td>
</tr>
<tr>
<td>FLU (0-48 h) / DMF (0-6.5 h)</td>
<td>0.44</td>
<td>0.14</td>
</tr>
<tr>
<td>3-HF (0-4 h)</td>
<td>-0.50</td>
<td>0.12</td>
</tr>
<tr>
<td>FLU (0-48 h) / 3-HF (0-4 h)</td>
<td>0.54</td>
<td>0.06</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pharmacodynamic Effects (0-4 h)</th>
<th>Spearman r</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>% over road mean*</td>
<td>-0.48</td>
<td>0.11</td>
</tr>
<tr>
<td>Sedation item mean</td>
<td>0.26</td>
<td>0.39</td>
</tr>
<tr>
<td>Fatigue</td>
<td>0.40</td>
<td>0.18</td>
</tr>
<tr>
<td>Good effect</td>
<td>-0.23</td>
<td>0.45</td>
</tr>
<tr>
<td>Bad effect</td>
<td>0.30</td>
<td>0.32</td>
</tr>
<tr>
<td>Feeling in body</td>
<td>-0.26</td>
<td>0.39</td>
</tr>
<tr>
<td>Feeling in mind</td>
<td>-0.57</td>
<td>0.04</td>
</tr>
</tbody>
</table>

* The correlation with % over road mean was calculated using the delta minimum value (minimum response minus baseline).
5.6 Discussion
In this study, we examined the influence of CYP2C19 activity on flunitrazepam kinetics and response in vivo. We found preliminary evidence that suggests (1) CYP2C19 contributes to the metabolism of flunitrazepam to desmethylflunitrazepam and 3-hydroxyflunitrazepam in vivo, and (2) individuals with lower CYP2C19 activity experience greater psychomotor impairment.

*CYP2C19 phenotype and genotype*
Our index of CYP2C19 activity was the OMP/5-OH-OMP plasma concentration ratio, determined 3 hours after drug administration. This metabolic ratio has been shown to correspond to CYP2C19 phenotype as determined with S-mephenytoin (the original polymorphism-defining drug) and to CYP2C19 genotype (Chang et al., 1995; Chang et al., 1995). Twelve subjects had metabolic ratios that ranged from 0.50 to 8.87. These subjects consisted of both heterozygous and homozygous EMs, with no apparent distinction between these groups. One subject had a much lower measured CYP2C19 activity (OMP/5-OH-OMP=28.8) than the others. This individual was the only genotypic PM in the group. This separation between EMs and PM is consistent with our findings from other subjects (see Section 6.5, Study IV) and is similar to the results reported by Chang and coworkers (Chang et al., 1995).

Although the OMP/5-OH-OMP plasma concentration ratio has been mainly used to characterise individuals as EMs or PMs of CYP2C19, we believe that it is also a useful measure of the relative activity of this enzyme. The value of this ratio has been shown to correlate with the mephenytoin S/R ratio \( r^2=0.63; P<0.001; n=141 \) (Chang et al., 1995), \( r^2=0.681; P<0.001; n=40 \) (Balian et al., 1995)] which describes a pathway that is essentially
exclusively CYP2C19 mediated (Goldstein et al., 1994; Wrighton et al., 1993). In addition, in vitro studies have shown that at low substrate concentrations, CYP2C19 is the most important enzyme mediating the metabolism of OMP to 5-OH-OMP (Karam et al., 1996). This method of quantifying CYP2C19 activity does suffer, however, from the limitation that CYP3A4 is also involved in this pathway, and variation in CYP3A4 activity may therefore confound its relationship to CYP2C19 activity.

**Pharmacokinetics**

The plasma concentrations of flunitrazepam and its metabolites, 7-aminoflunitrazepam and desmethylflunitrazepam, produced profiles that were similar to those reported in the literature (Wickstrom et al., 1980). The profile of flunitrazepam was characterised by a rapid absorption phase (Jochemsen et al., 1983; Mattila & Larri, 1980) peaking before 2 h and a steeply declining distribution phase with a second small peak at 5 hours (which has been reported by McGowan and coworkers, who suggest that it is probably the result of entero-hepatic recirculation (McGowan et al., 1980)). After 8 hours, flunitrazepam entered into a slow elimination phase. Flunitrazepam was detectable in the plasma for the full 48 hours.

7-Aminoflunitrazepam was quantitatively the most important metabolite, followed by desmethylflunitrazepam, as has been reported in the literature (Singlas, 1979; Wickstrom et al., 1980). The concentrations of desmethylflunitrazepam and 3-hydroxyflunitrazepam were lower than the minimum quantifiable limit (sensitivity) of the assay at most time points. The shapes of the plasma concentration profiles of these metabolites, however, were typical of plasma concentration profiles and were grossly similar between individuals.

Though the correlations between CYP2C19 activity and many of the pharmacokinetic parameters were, individually, not statistically significantly different from zero, taken together they create a consistent, predictable pattern that implicates CYP2C19 in the
metabolism of flunitrazepam. The negative correlation between average FLU concentration and CYP2C19 activity points to the involvement of CYP2C19 in the metabolism of the drug. The positive correlations between DMF and 3-HF average concentrations and CYP2C19 activity and the negative correlations between the metabolic ratio of FLU to each of these metabolites and CYP2C19 activity suggest that CYP2C19 was involved in the DMF and 3-HF pathways. Concordantly, the negative correlation between 7-AF and CYP2C19 activity suggests that in individuals with lower CYP2C19 activity, more FLU was converted to 7-AF. The positive correlation between the metabolic ratio of FLU to 7-AF and CYP2C19 activity suggests that FLU may have been shunted down the 7-AF pathway in subjects with lower CYP2C19 activity. Last, the negative correlation between the ratio of 7-AF to 3-HF + DMF and CYP2C19 activity suggests that relatively more FLU was converted to 7-AF than to DMF and 3-HF in those individuals with lower CYP2C19 activity. Though these correlations are not statistically significant, it is interesting to note that of the 28 p-values determined for the correlations between CYP2C19 activity and pharmacokinetic parameters (see Table 5.3), 21 were less than or equal to 0.25, and the directions of all correlations were consistent with CYP2C19 playing a role in the hydroxylation and desmethylation metabolic pathways of flunitrazepam. For reference, the correlation between flunitrazepam average plasma levels between 0 and 48 h and OMP/5-OH-OMP would have been achieved with statistical significance at the p=0.05 level with the same number of subjects (n=13) if the correlation coefficient were 0.56, or with the observed correlation coefficient (r=0.35) and a total of 32 subjects. Alternatively, in modelling the data, the addition of two hypothetical subjects with optimal ranks near the upper end of the observed range of metabolic ratios resulted in a statistically significant correlation.
These results are consistent with the \textit{in vitro} data presented by both Kilicarslan (2000) and Coller (1999), who found that CYP2C19 was involved in the formation of DMF and 3-HF. Kilicarslan and coworkers suggested that CYP2C19 may play a more important role in the metabolism of flunitrazepam to DMF than to 3-HF \textit{in vitro} (Kilicarslan \textit{et al.}, 2000). Our data suggest that \textit{in vivo} the enzyme may have a role at least as an important in flunitrazepam’s metabolism to 3-HF as DMF, as its activity correlated to both FLU/3-HF ratio ($r=0.54$) and FLU/DMF ($r=0.44$). Our observation that CYP2C19 may be involved in the metabolism of flunitrazepam is also consistent with its known role in the metabolism \textit{in vivo} of diazepam (Bertilsson \textit{et al.}, 1989; Wan \textit{et al.}, 1996), a structurally related benzodiazepine that shares many kinetic characteristics with flunitrazepam (Cano \& Sumirtapura, 1981).

There are a number of factors that influence the plasma concentration of a drug and the activity of enzymes other than the genetic determinants of metabolism. For example, sex, age, ethnoracial background, weight, liver size and function, circadian rhythms, diet, and environmental factors such as concurrent exposure to metabolic inducers or inhibitors all determine the pharmacokinetics of a drug through metabolic enzyme activity as well as other processes, such as absorption, distribution and elimination. Sex-dependent differences have been reported in the CYP450 metabolism of a number of drugs, for example, although for CYP2C19 the evidence is somewhat controversial (Kupfer \& Preisig, 1984; Xie \textit{et al.}, 1997). Studying CYP2C19 activity (rather than its genotype) enables us to account for inter-individual differences that result in variation in enzyme activity. To confirm, however, that such differences do not better explain the variation in enzyme activity than does genotype, we tested whether or not CYP2C19 activity was different between groups for a variety of
non-pharmacogenetic factors (old and young, Caucasian and non-Caucasian, male and female, and low and high weight). There were no significant differences (p>0.05) between groups, while there was a significant difference between genotypic EMs and PM (p<0.0001), suggesting that none of the non-genetic factors by themselves could explain the variation in CYP2C19 activity. We also sought to determine whether or not pharmacokinetic parameters differed between these same groups and found that there were no significant differences (p>0.05) between groups, with two exceptions: Caucasians had lower FLU/7-AF ratios than non-Caucasians, and low weight individuals had higher average FLU concentrations and higher FLU/7-AF for 0-48 hours than high weight individuals. Our finding that age had no relationship with pharmacokinetics is consistent with the results reported by Kanto and colleagues (Kanto et al., 1981). We conclude that none of these factors singly, therefore, could convincingly explain the variation in pharmacokinetic parameters.

Pharmacodynamics
The relationship between CYP2C19 activity and pharmacodynamic effects was less clear than with pharmacokinetic effects. It was, however, somewhat stronger over the first four hours compared to the full eight hour period, perhaps owing to flunitrazepam's rapid onset and time to peak effects.

Our objective measure of pharmacodynamic effect, psychomotor impairment, has been previously identified as an effect of flunitrazepam (Farre et al., 1996; Ingum et al., 1992). In the present study, CYP2C19 activity appears to have been associated with the extent to which subjects experienced psychomotor impairment, although the correlation was not statistically significant—suggesting that individuals with lower activity were more impaired.
Study III. Influence of CYP2C19 Activity on Flunitrazepam

Twenty-four self-rated subjective measures were also used to characterise the effects of flunitrazepam. The responses for 13 of these measures were consistent with a drug response. Of those, three ("seclusive," "elated" and "sick") have not been reported as typical effects of flunitrazepam and were excluded from our analysis. "Sedation" and "fatigue" (Clarke et al., 1980; Grahnen et al., 1991; McGowan et al., 1980), "any drug effect" (Farre et al., 1996), "high" (Farre et al., 1996), "good effect" (Bond et al., 1994; Farre et al., 1996) and "bad effect" (Farre et al., 1996) exhibited responses that were consistent with previously reported effects of flunitrazepam. "Spacey" (Greenblatt et al., 1989; Greenblatt et al., 1994; Greenblatt et al., 1993) and "thinking speeded up" (Greenblatt et al., 1988; Greenblatt et al., 1989) showed trends that have been previously reported for other benzodiazepines.

"Anxious," "tense" and "nervous" measures did not meet our selection criteria, although we had expected them to give responses based on the known pharmacodynamic properties of the drug. The reasons for this discrepancy may be that, although anxiolytic effects have been found in normal populations (Bond & Lader, 1975; File et al., 1982), other investigators were unable to detect such effects in non-anxious individuals (Bond et al., 1994). It is also possible that the environment of a constrained laboratory setting may have acted to distort subjects' responses.

With six measures ("sedation item mean," "fatigued," "thinking speeded up," "any drug effect," "spacey" and "high"), we expected a more pronounced effect with decreasing CYP2C19 activity. This trend was observed for "fatigued" and, to a lesser extent, for "sedation item mean" (although neither were statistically significant), suggesting that CYP2C19 activity may have determined the sedative effects of the drug. "Thinking speeded up" and "any drug effect" appeared to correlate weakly with CYP2C19 activity but in the
direction opposite to what was expected (and without statistical significance); as CYP2C19 activity decreased, the response for "thinking speeded up" increased and the response for "any drug effect" decreased. "Spacey" and "high" did not appear to be associated with CYP2C19 activity.

The "good effect", "bad effect", "feeling in mind" and "feeling in body" measures allowed us to examine the quality of the drug response in individuals with varying CYP2C19 activities. Although the correlations were not statistically significant, the observations that "good effect", "feeling in body" and "feeling in mind" appeared to decrease as CYP2C19 activity decreased, while "bad effect" increased with decreasing activity, together suggest that individuals with low CYP2C19 activity may have had a more negative experience of the drug.

We posit that variation in CYP2C19 activity results in variation in flunitrazepam plasma concentration, ultimately leading to variation in the experience of the drug. The strength of pharmacodynamic effects of the drug should then correlate to both CYP2C19 activity and flunitrazepam plasma concentration. The objective measure of flunitrazepam effect, psychomotor performance, appeared to demonstrate these relationships, as "%-over-road mean" varied both with CYP2C19 activity (although without statistical significance) and strikingly with average flunitrazepam concentrations over 4 and 8 hours. It appears, therefore, that CYP2C19 activity may have determined the extent to which subjects experienced the psychomotor impairment effect of flunitrazepam. With the subjective effects, however, there was little or no relationship between pharmacodynamic effect and flunitrazepam concentration (and in some instances there was even a weak trend that implied lesser effect with greater concentration), although there were some correlations to CYP2C19
activity. These observations imply that the relationship between enzyme activity and subjective effects must be interpreted with caution. It is not surprising, perhaps, that the pharmacodynamic effects were not significantly correlated to CYP2C19 activity since the relationship between CYP2C19 activity and flunitrazepam plasma concentration was itself not statistically significant.

If the metabolites of flunitrazepam are pharmacologically active, the effects of the drug will be determined not only by the concentration of flunitrazepam but also by those of its metabolites. There is some debate in the literature as to whether this is the case or not. Some authors report that desmethylflunitrazepam may have some activity (Dorow et al., 1982; Drouet-Coassolo et al., 1990). Others hold that the parent drug is the compound chiefly responsible for the hypnotic effects (Wendt, 1976; Wickstrom et al., 1980). Singlas (1979) has argued that the metabolites of flunitrazepam do not contribute to the pharmacological activity of the drug since no increase in effects is observed after repeated administration of flunitrazepam, during which time there is an accumulation of metabolites (Singlas, 1979). To allow for the potential activity of desmethylflunitrazepam, we examined the relationship between the sum of average FLU and DMF concentrations and pharmacodynamic effects and found similar results compared to FLU alone.

There are a number of limitations inherent in the study of subjective effects that may explain why, in general, their relationships to CYP2C19 activity and flunitrazepam concentration were weak. First, there is intra- and inter-individual variability in the dose-response relationship for flunitrazepam. For sedation, for example, the coefficient of variation for EC50 within and between 20 healthy subjects that received 1 mg of flunitrazepam on two occasions was reported to be 27% and 39%, respectively (Grahnen et
al., 1991). It has also been reported that elderly patients (individuals over 60) experienced a greater sedative effect of flunitrazepam (0.015 mg/kg administered i.v.) without any difference in pharmacokinetics (Kanto et al., 1981). Second, there is intra- and inter-individual variability of mood states, personality, interactions with the environment, and interpretation and understanding of the descriptors used in measuring subjective effects, all of which are other determinants of subjective responses. Finally, owing to the absence of placebo-controls in the design, we cannot exclude the possibility that the subjective responses were, at least in part, determined by the subjects’ expectations rather than the true pharmacodynamic properties of the drug. These considerations, together with our finding that part of the excess flunitrazepam that results from poor CYP2C19 activity may have been shunted down the 7-AF pathway, lead to the possibility that differences in flunitrazepam concentration owing to variation in enzyme activity may have been insufficient to cause observable differences in subjective effects.

CYP3A4
We also sought to examine the role of CYP3A4 in the metabolism of flunitrazepam. Because CYP3A4 is the principal enzyme responsible for the metabolism of omeprazole to omeprazole sulphone (Karam et al., 1996), we used the OMP/OMP-S plasma concentration ratio as an index of CYP3A4 activity. We chose the 3 h time point because it corresponded to the time point at which the OMP/5-OH-OMP metabolic ratio was determined. As with the OMP/5-OH ratio, whose pathway is mediated by more than one enzyme, this index has the limitation that CYP2C19 also plays a minor role in the sulfoxidation of omeprazole (Andersson et al., 1993). Indeed, there appears to be a correlation between OMP/5-OH-OMP and OMP/OMP-S ($R^2 = 0.36$), which may be due to the imperfect specificity of these
indices and/or the possibility that some individuals have overall lower metabolic activity owing to lower liver function, for example.

Although there were no significant correlations between OMP/OMP-S and the pharmacokinetic parameters, the observed trends suggest that CYP3A4 may be involved in the metabolism of flunitrazepam. The positive correlation observed between 3-HF average concentration and CYP3A4 activity and the negative correlation observed between metabolic ratio of FLU to 3-HF and CYP3A4 activity suggest that CYP3A4 was involved in the 3-HF pathway. The weak correlations between CYP3A4 activity and 7-AF and the FLU to 7-AF ratio suggest that in subjects with lower CYP3A4 activity, more FLU may have been converted to 7-AF. The negative correlation between the ratio of 7-AF to 3-HF + DMF and CYP3A4 activity suggests that relatively more FLU was converted to 7-AF than to DMF and 3-HF in individuals with lower CYP3A4 activity. Because there was only a 9-fold variation in the activity of CYP3A4 using this index, it is difficult to interpret from these findings the extent to which CYP3A4 may have been involved in the metabolism of flunitrazepam. Taken together, however, these findings suggest that it may play a role, particularly in the hydroxylation pathway.

We used the ratio 5-OH-OMP/OMP-S as an index of the relative activities of CYP2C19 and CYP3A4. That this index was positively correlated to both DMF and 3HF and negatively correlated to FLU/DMF and FLU/3HF suggests that variation in CYP2C19 activity was more important than CYP3A4 activity in determining the variation in both desmethylation and hydroxylation pathways (the former somewhat more strongly than the latter). This finding may be interpreted as meaning that CYP2C19 was the more important
enzyme in both pathways, but this interpretation should be tempered by the fact that in this study there was quantitatively more variation in CYP2C19 activity than in CYP3A4 activity.

One major limitation to this study that warrants consideration is the limited number of subjects (n=13), especially those with poor CYP2C19 activity. Although there was a 58-fold variation in the activity of CYP2C19 among all subjects, twelve of the subjects had only an 18-fold variation in enzyme activity. The one outlier whose omeprazole metabolic ratio was much higher than the rest was the only genotypic PM. A larger sample with a broader spectrum of CYP2C19 activities and more genotypic poor metabolisers would enable us to more sensitively determine the influence of CYP2C19 activity on flunitrazepam kinetics and response and also to characterise the role of its polymorphic expression. Further research in this direction will help to resolve what quantitative effect the CYP2C19 poor metaboliser phenotype has on flunitrazepam concentration and will therefore provide a better understanding of the clinical ramifications of this polymorphism.

These data provide preliminary in vivo evidence that suggests CYP2C19 contributes to the metabolism of flunitrazepam to N-desmethylflunitrazepam and 3-hydroxyflunitrazepam. Our results also suggest that in individuals with lower CYP2C19 activity, flunitrazepam may reach a higher concentration and may be shunted down the 7-aminoflunitrazepam pathway. They also suggest that individuals with lower CYP2C19 activity may experience greater psychomotor impairment. Although the observed correlations were not statistically significant, we believe that their strength and consistency enable us to view them as preliminary evidence in support of our hypotheses. The involvement of CYP2C19 and CYP3A4 in flunitrazepam’s metabolism implies that this drug
may interact with other substrates and inhibitors of these enzymes. The implications of CYP2C19 involvement also includes the possibility that individuals with low CYP2C19 activity experience greater toxicity and may require less flunitrazepam to achieve the same effects than those with higher CYP2C19 activity.
6 Study IV. Study of CYP2C19 Phenotype and Genotype Relationship

6.1 Rationale

The literature supports the use of omeprazole as a probe drug for CYP2C19 activity because the ratio between the plasma concentrations of omeprazole and 5-hydroxyomeprazole at 3 hours post-administration correlates well with CYP2C19 genotype. Researchers have found a bimodal non-overlapping distribution of phenotype, with extensive metabolisers (EMs) genotyping as wt/wt or wt/mut and poor metabolisers (PMs) genotyping as mut/mut (Chang et al., 1995; leiiri et al., 1996). We sought to confirm this relationship with our analytical methods and our sample of Toronto residents.

6.2 Objectives

1. To characterise our determination of the omeprazole metabolic ratio as a tool for distinguishing CYP2C19 EMs and PMs
2. To examine the relationship between CYP2C19 phenotype and genotype
3. To examine the relationship between CYP2C19 phenotype and ethnoracial background

6.3 Hypotheses

1. Using omeprazole as a probe drug, a bimodal distribution exists of CYP2C19 activity, with phenotypic EMs having either the wt/wt or wt/mut genotype and phenotypic PMs having the mut/mut genotype.
2. Within EMs, individuals with wt/mut genotype have a higher mean metabolic ratio of omeprazole to 5-hydroxyomeprazole than those with wt/wt genotype.
3. Individuals with the same genotype have similar metabolic ratios regardless of their ethnoracial background.

6.4 Methods

6.4.1 Subjects
All thirty-eight subjects who had completed the pre-study assessment day procedures were included in the present study. These individuals therefore represented subjects from studies I, II, and III, in addition to others who were recruited but did not participate in any studies. The inclusion and exclusion criteria were mixed: each subject met the criteria of the particular study for which she or he was recruited.

6.4.2 Data Analysis
The phenotype and genotype of each subject were determined according to the methods described in Sections 2.6.2 and 2.6.3 (General Methods). The omeprazole metabolic ratio (MR), determined as the ratio of the plasma concentrations of omeprazole to 5-hydroxyomeprazole (OMP/5-OH-OMP) 3 h after drug intake, was used to determine CYP2C19 phenotype. Blood samples were genotyped for the CYP2C19*2 and *3 allele variants. In addition, 5 blood samples from subjects of Study III were also genotyped for the CYP*4 and *5 allele variants (and were found to contain neither mutation). The NPAR1WAY procedure in SAS was used to compare omeprazole metabolic ratios between groups. We used the Wilcoxon Rank Sum Test, a non-parametric test that compares two samples drawn from independent populations, because the omeprazole metabolic ratio could not be assumed to be normally distributed (with equal variances) within genotype. In addition, to rule out sex and age as potential confounders, we used the SAS GLM procedure
and Type I sums of squares to model omeprazole metabolic ratio with genotype and each of these variables.

6.5 Results
Thirty-eight subjects were included in the present study, 18 females and 20 males. Subjects ranged in age from 20 to 62, and their mean age ± sample standard deviation was 35.1 ± 12.6. The subjects came from various ethnoracial backgrounds; individuals of Chinese ancestry made up the largest group (n=16), followed by Caucasians (n=14). The distribution of CYP2C19 genotypes (wt/wt, wt/mut, and mut/mut) and the frequencies of each allele (*1, *2 and *3) in the populations studied are shown in Table 6.1 and Table 6.2, respectively.

<table>
<thead>
<tr>
<th>Table 6.1. Distribution of CYP2C19 genotypes</th>
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<tbody>
<tr>
<td>Extensive metabolisers (EMs)</td>
</tr>
<tr>
<td>Wt/wt</td>
</tr>
<tr>
<td>Chinese</td>
</tr>
<tr>
<td>Caucasians</td>
</tr>
<tr>
<td>Others</td>
</tr>
<tr>
<td>Total</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 6.2. Frequency of CYP2C19*1, *2 and *3 alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>*1</td>
</tr>
<tr>
<td>Chinese</td>
</tr>
<tr>
<td>Caucasians*</td>
</tr>
<tr>
<td>Others</td>
</tr>
</tbody>
</table>

* These frequencies include two individuals who were specifically recruited on the basis of their genotype. Excluding them, the frequencies of *1, *2 and *3 alleles were 0.79, 0.21 and 0.

The distribution of omeprazole metabolic ratios is represented in Figure 6.1 with a frequency distribution histogram of the log(OMP/5-OH-OMP). The individual and mean OMP/5-OH-OMP ratios, grouped according to genotype, are presented in Figure 6.2.
Figure 6.1. Frequency histogram of the log(OMP/5-OH-OMP) for all subjects. Values shown on the x-axis are the midpoint of each bin.
Figure 6.2. Omeprazole/5-hydroxyomeprazole metabolic ratios for individuals who were wt/wt (*1/*1), wt/mut (*1/*2 or *1/*3) and mut/mut (*2/*2 or *2/*3) for the CYP2C19 gene. The mean values for each group are indicated by a bar.
The metabolic ratio ranged from 0.5 to 7.8 in the wt/wt group, from 0.7 to 9.4 in the wt/mut group, and from 11.7 to 34.4 in the mut/mut group. No overlap was observed between genotypic EMs and PMs. Although a bimodal distribution of EMs and PMs is not apparent in the histogram, an antimode between metabolic ratios of 9.4 and 11.6 can be observed in Figure 6.2. The homozygote and heterozygote groups of EMs were largely overlapping and demonstrated no apparent bimodality. The mean (± sample standard deviation) OMP/5-OH-OMP metabolic ratios for individuals who were CYP2C19 wt/wt, wt/mut and mut/mut were 2.1 ± 2.3, 4.0 ± 3.0 and 22.8 ± 9.0, respectively. The mean MRs for individuals who were wt/wt and wt/mut were each significantly different from the mean MR for those who were mut/mut (p<0.0005), and were also significantly different from each other (p<0.03). Among heterozygous EMs, the mean MR did not differ significantly between individuals who were *1/*3 and *1/*3. Likewise, among PMs, the mean MR did not differ significantly between individuals who were *2/*2 and *2/*3.

To examine the relationship between CYP2C19 phenotype and ethnoracial background, we studied the omeprazole MRs in Chinese and Caucasian individuals, the two largest ethnoracial groups in our population. There was no significant difference in MR between Chinese and Caucasian EMs. Because one individual in this comparison was specifically recruited on the basis of their genotype, this comparison was also restricted to subjects who were recruited through advertisements and who could therefore be considered part of a random sample with respect to their allele frequencies. No significant difference between ethnoracial groups was observed. We also compared the mean MR of all Chinese and Caucasians in each genotypic group (Table 6.3) and detected no difference between ethnoracial groups among wt/wt, wt/mut, and mut/mut genotypes. In Chinese subjects, there
were significant differences (p ≤ 0.05) between the three genotypic groups. In Caucasian subjects, the differences were not statistically significant, although a comparison of wt/wt and wt/mut groups yielded a p-value of 0.07.

Table 6.3. Mean (± standard deviation) omeprazole metabolic ratio (OMP/5-OH-OMP) for each of the three genotypic groups in Chinese and Caucasian subjects

<table>
<thead>
<tr>
<th></th>
<th>Extensive metabolisers (EMs)</th>
<th>Poor metabolisers (PMs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wt/wt</td>
<td>wt/mut</td>
</tr>
<tr>
<td>Chinese</td>
<td>1.5 (± 2.0)</td>
<td>5.1 (± 3.2)</td>
</tr>
<tr>
<td>Caucasians</td>
<td>1.5 (± 0.6)</td>
<td>4.3 (± 3.4)</td>
</tr>
</tbody>
</table>

6.6 Discussion
In this study, we sought to determine the utility of the omeprazole metabolic ratio, as determined by our omeprazole assay, as a probe for distinguishing CYP2C19 EMS and PMs, and we examined the relationships between CYP2C19 phenotype, genotype and ethnoracial background in our study population. Our major findings are that: (1) there was no overlap in the omeprazole metabolic ratios of genotypic EMS and PMs, with EMS phenotyping in the range of 0.5 to 9.4 and PMs phenotyping in the range of 11.7 to 34.4; (2) within the EM group, heterozygotes (wt/mut) had a significantly higher mean metabolic ratio than homozygotes (wt/wt); and (3) there were no differences in mean metabolic ratios between Chinese and Caucasian individuals of the same genotype.

The omeprazole hydroxylation ratio, expressed as the ratio of plasma concentrations of omeprazole to 5-hydroxyomeprazole determined 3 h after drug intake, allows populations to be divided into EMS and PMs (Chang et al., 1995; Chang et al., 1995; Ieiri et al., 1996; Kimura et al., 1999). Chang and coworkers studied 160 healthy Swedish subjects and found that the metabolic ratio of EMS of S-mephenytoin ranged from 0.1 to 4.9, while that of PMs ranged from 7.1 to 23.8 (Chang et al., 1995). This classification was in good agreement with
their genotypic results for the *2 and *3 mutant alleles. Ieiri and coworkers studied 27 healthy Japanese subjects and found that genotypic EMs and PMs had metabolic ratios that ranged from 0.04 to 5.0, and from 6.6 to 12.2, respectively (Ieiri et al., 1996). Most recently, Kimura and coworkers studied 78 healthy Japanese subjects and found metabolic ratios from 0.02 to 5.0 in individuals who genotyped as EMs, and from 5.1 to 12.3 in those who genotyped as PMs (Kimura et al., 1999).

In the present population of 38 individuals, subjects who genotyped as EMs of CYP2C19 had lower MRs of omeprazole (0.5-9.4) than those who genotyped as PMs (11.7-34.4), with no overlap between groups. Though Chang and colleagues noted that the log(OMP/5-OH-OMP) of omeprazole of 144 wt/wt EMs was “fairly normally distributed” (Chang et al., 1995), the nature of the distribution (e.g. normal, bimodal, etc.) in our sample was not apparent, probably owing to the smaller number of subjects. Nonetheless, an apparent antimode was observed between 9.4 and 11.6. Because there was no overlap in the metabolic ratios of the two groups, our assay yields complete concordance between genotype and phenotype. Although its utility in predicting the genotype of individuals whose phenotype falls near the antimode is limited by the relatively small “buffer zone” between groups, this assay may nonetheless be a useful tool for distinguishing CYP2C19 EMs and PMs.

The observed ranges of metabolic ratios appear to be higher than those reported in the literature, reflecting the fact that, after a 40 mg dose, we observed larger values for AUCOMP than did other investigators. As was discussed in Study II, this discrepancy may be the result of a decreased first-pass effect due to the particular drug formulation used in the present study.
The mean metabolic ratio for omeprazole differed significantly between the three genotypic groups. Thus, there appears to be a gene dose effect with the mutant CYP2C19 alleles, an observation made by other investigators with regard to the metabolism of omeprazole (Chang et al., 1995; Ieiri et al., 1996) and diazepam (Qin et al., 1999).

Differences have been reported in the literature between CYP2C19 EMs of different ethnoracial groups in the metabolism of various substrates of CYP2C19 (Andersson et al., 1992; Caraco et al., 1996; Zhang et al., 1990). For example, in one study, the AUC of omeprazole was significantly lower and its oral clearance significantly higher in Caucasian EMs than in Chinese EM subjects (Caraco et al., 1996). One explanation for the altered drug disposition in Chinese versus Caucasian subjects is that the higher proportion of heterozygous EMs in the Chinese population may result in a lower mean CYP2C19 activity for this group (Bertilsson & Kalow, 1993; Caraco et al., 1996; Ishizaki et al., 1994; Xie, 1997; Zhang et al., 1990). We did not detect any differences in the omeprazole metabolic ratio between Caucasian and Chinese EMs, but this may have been due to the fact that, unlike in the general populations, there were more Caucasian than Chinese heterozygotes in these groups. Our finding that heterozygous EMs had a higher omeprazole hydroxylation ratio (lower CYP2C19 activity) than homozygous EMs (comparing Chinese subjects as well as all subjects) corroborates the hypothesis that inter-ethnic differences in CYP2C19 activity are due to the over-representation of heterozygous EMs in the Chinese population. Our finding that there was no difference in the MR between Caucasian and Chinese homozygous wt/wt EMs provides further corroborating evidence for this hypothesis.

We observed frequencies of the *1, *2 and *3 alleles in individuals recruited randomly from the Chinese population in Toronto of 0.56, 0.28 and 0.16; in comparison,
deMorais reported frequencies of 0.289 and 0.044 for the *2 and *3 allele in 75 Chinese subjects (deMorais et al., 1995). In our sample of randomly recruited Caucasians, the frequencies of the *1 and *2 alleles were 0.79 and 0.21, respectively. Using the Hardy-Weinberg equation, Brosen and coworkers calculated a frequency of mutant alleles in Caucasians of 0.18 (Brosen et al., 1995). Since the CYP2C19*2 allele in Caucasians has been reported to account for 86.5% of alleles in PMs in Caucasians (Brosen et al., 1995; deMorais et al., 1994; deMorais et al., 1994), we estimate an overall *2 allele frequency in Caucasians of 0.16 (0.865×0.18). Our observed allele frequencies for both Chinese and Caucasians are therefore in reasonable agreement with what has been reported in the literature. Though our analysis of five individuals, some with borderline phenotype, revealed that none contained a CYP2C19*4 or *5 allele, we cannot exclude the possibility that these mutations were present in other individuals or that rarer mutations were present in any of our sample. It has been reported, however, that the CYP2C19*2 and *3 mutant alleles together account for greater than 99% of Chinese and 87% of Caucasian PM alleles (Brosen et al., 1995; deMorais et al., 1995; deMorais et al., 1994; deMorais et al., 1994).

Sex (Xie et al., 1997) and age (Landahl et al., 1992) both affect the metabolism of omeprazole and therefore were potential confounders of the observed relationship between genotype and phenotype. We modelled the effect of each of these variables together with genotype and phenotype. Neither sex nor age were found to significantly contribute to the phenotype.

In conclusion, it appears that with this assay there is concordance between CYP2C19 genotype and phenotype, with EMs and PMs having metabolic ratios in the non-overlapping ranges of 0.5 to 9.4, and 11.7 to 34.4, respectively. Thus, this assay appears to be useful in
distinguishing EMs and PMs, and a blinded study of a larger number of subjects would enable the determination of its sensitivity and specificity. Although the genotype gives unambiguous assignment of CYP2C19 PM and EM status, the large variation in metabolic ratios within genotypic groups suggests that more information can be gained by also determining CYP2C19 activity with phenotyping. This assay therefore may be useful in quantifying CYP2C19 activity.

Our results suggest that CYP2C19 activity may be determined by a gene dosage effect, with heterozygotes having an intermediate phenotype between wt/wt EMs and mut/mut PMs. Although our power may have been limited by a relatively small sample size, the results also suggest that Chinese and Caucasian individuals with the same genotype have no differences in phenotype. Together these findings support the hypothesis that the reported differences between Chinese and Caucasians in the metabolism of CYP2C19-mediated substrates is due to the higher proportion of heterozygotes in the Chinese population.
7 General Discussion

Our general objective was to examine the role of cytochrome P450 2C19 activity in determining flunitrazepam kinetics and response in vivo. With this series of studies, we have developed a better understanding of both methodology and the underlying question.

Our assay for determining the omeprazole metabolic ratio yielded no overlap between genotypic EMs and PMs, with EMs phenotyping in the range of 0.5 to 9.4 and PMs phenotyping in the range of 11.7 to 34.4. We therefore conclude that this assay is useful in distinguishing EMs and PMs. While genotyping has the advantage of offering an unequivocal assignment of CYP2C19 status, phenotyping gives a quantitative measure of CYP2C19 activity. A combination of genotype and phenotype may therefore be required to fully characterise an individual’s CYP2C19 behaviour.

Another methodological consideration concerned the recruitment of sufficient subjects from the Chinese population, whose members have a high frequency of the mutant CYP2C19 alleles and make up the largest ethnoracial minority in Toronto. Our findings indicate that recruiting these subjects within a reasonable time frame using our strategy was unfeasible. Nonetheless, our observations and recommendations may lead to a future, more successful recruitment process. More complete reporting, in general, of recruitment successes and failures within and across diverse populations will facilitate this process in the future.

We were unable to determine the duration of daily ketoconazole dosing required to selectively inhibit CYP3A4 without inhibiting CYP2C19, a third methodological consideration. Because of omeprazole’s complex kinetics with repeated administration, we were unable to determine the extent or selectivity of CYP3A4 inhibition by ketoconazole,
and we therefore conclude that the feasibility of creating a CYP2C19 PM phenocopy by inhibiting CYP2C19 with omeprazole and CYP3A4 with ketoconazole remains undetermined.

In this dissertation, we provide preliminary evidence that suggests CYP2C19 is involved in the metabolism of flunitrazepam to N-desmethylflunitrazepam and 3-hydroxyflunitrazepam in vivo. We also provide data that suggests individuals with lower CYP2C19 activity may experience greater psychomotor impairment with flunitrazepam. Although the observed correlations were not statistically significant, we believe that their strength and consistency enable us to make these tentative conclusions. The implications of these results include the possibility that individuals with low CYP2C19 activity experience greater toxicity and may require less flunitrazepam to achieve the same effects than those with higher CYP2C19 activity. The involvement of CYP2C19 in flunitrazepam’s metabolism also implies that this drug may interact with other CYP2C19 substrates and inhibitors.

The frequency of mutant CYP2C19 alleles in our sample of Toronto residents resembled the frequencies reported in the literature, with individuals of Chinese ancestry having a higher frequency (40%) than Caucasians (21%). Further, our study of phenotype and genotype supports the notion that CYP2C19 activity is determined by a gene dose effect, with heterozygous EMs (wt/mut) exhibiting an intermediate phenotype between high-activity homozygous EMs (wt/wt) and PMs (mut/mut). Moreover, our results suggest that Chinese and Caucasian individuals with the same genotype have no differences in phenotype. Together with our finding that individuals with lower CYP2C19 activity may exhibit higher plasma levels of flunitrazepam and greater psychomotor impairment and sedation, these
results support the hypothesis that Chinese are more sensitive to some benzodiazepines due to the higher frequency of CYP2C19 mutant alleles in this population. They also yield the prediction that Chinese are more sensitive to flunitrazepam in particular. It is interesting to note that although in the present study poor metabolisers of CYP2C19 appeared to have a more negative experience of flunitrazepam, the drug appears to be a preferred drug of abuse among Taiwanese-Chinese (Dr. Wen Su, personal communication).

To extend these findings, the next step is to determine the influence of the polymorphic expression of CYP2C19 on flunitrazepam—that is, to determine the extent to which the CYP2C19 poor metaboliser phenotype affects flunitrazepam kinetics and response. A larger sample size with more poor metabolisers would be helpful in this regard. Samples of both Caucasian and Chinese populations would enable us to test our predictions that Chinese are more sensitive to flunitrazepam (by comparing EMs from both ethnoracial groups), and that this difference is solely due to the higher frequency of mutant alleles among individuals of Chinese ancestry (by comparing subjects with the same genotype from both ethnoracial groups). Of course, to recruit enough Chinese subjects the recruitment process would have to be refined, perhaps with the help of the observations and recommendations made here. If the influence of the polymorphic expression of CYP2C19 on flunitrazepam kinetics and response proves to be of sufficient magnitude to be clinically relevant, epidemiological studies could be conducted to determine its effect on patterns of use and abuse.
8 REFERENCES


9 APPENDICES

9.1 Appendix A. Study Procedure and Consent Form – “Genetic Test”

STUDY PROCEDURE AND CONSENT FORM - “GENETICS TEST”

I. ______________________________, hereby consent to participate in the “Pharmacogenetics” research project being conducted at the University of Toronto and the Psychopharmacology and Dependence Research Unit of Women’s College Hospital under the direction of Edward M. Sellers, M.D., Ph.D. and Rachel F. Tyndale, Ph.D. The purpose of this research, the procedures to be followed and possible risks of this research have been explained to me by ______________________________. In consenting to participate, I understand that:

PURPOSE

1. The purpose of this test is to determine which form of particular genes I possess. These genes are responsible for directing how the body processes drugs.

PROCEDURES

1. As part of the study, a blood sample (20 ml; or about 1 tablespoon) will be taken from a vein in my arm for analysis of my DNA. DNA is the body “chemical” which carries information on human inheritance and the structure of all body chemicals and components. Analysis by special techniques of my DNA can determine my drug metabolizing capacity. The person collecting the blood will be experienced in the procedure and I can expect little and brief pain associated with inserting the needle. Afterward, there is some chance of slight bruising or inflammation, but this is a routine procedure that presents very low risk to me.
THERAPEUTIC BENEFITS

1. I can expect no therapeutic benefit from participating in this study.

CONFIDENTIALITY

1. The data I will provide will be kept strictly confidential and secure, available only to the researchers involved in this study and for the purpose set out in this consent form. Neither my name nor any piece of identifying information will be kept together with the other data that I may provide. My records will be treated with the same confidentiality afforded my medical records.

2. The results of this study may be published and if so will be published in such a manner that I will not be identifiable. Published reports will refer to group data and not to a particular individual. Published reports may separate data by racial group.

3. I understand and give my permission that a sample of DNA will be taken in this study. I understand that:
   a) my DNA will be used as part of this study, but it may also be used as part of future studies;
   b) any future use will be ethically and scientifically approved by a University or Hospital Ethics Review Board;
   c) all information obtained will be held in confidence, unavailable for identification;
   d) my DNA will be anonymously banked and cannot and will not be used for my own benefit and that no results, even if unfavourable results are found, will be forwarded to me;
   e) my DNA sample will be stored for a period of no less than ten (10) years; and
   f) I may request, in writing at any time, that my DNA sample be destroyed.
4. I understand that my DNA sample will be stored in a lockable freezer and only my subject number is on the vial. There is no linkage between my name and my DNA sample in the laboratory.

COMPENSATION

1. In consideration of the inconvenience and time involved in this study, I will receive $25. If I am also participating in the CYP Activity Study, I will receive this payment at the same time as I receive payment for the other study.

GENERAL CONDITIONS

1. I may decline to answer any particular questions asked of me. If this refusal makes my participation in this study of no scientific value, my participation can be terminated.

2. I understand that I may be approached in the future and asked to participate in related research projects. I am entirely at liberty to decline and will not be subject to any coercion.

3. As part of this study, I will provide information about myself including medical history, current medication use and non-prescription drug use.

4. I understand that participation in this study is voluntary and that I may withdraw at any time and for any reason. If I should withdraw from the study, I will not lose any benefits (i.e. I will not jeopardize my right to present or future treatment at Women's College Hospital).
RIGHTS OF SUBJECTS

1. This research project has been reviewed by the Research Ethics Board at Women's College Hospital.

2. Patients or study volunteers having questions or concerns about their rights in this research project should contact the Research Ethics Board Coordinator at (416) 351-3733.

MY CONSENT

1. I have had an opportunity to ask questions, and my questions have been satisfactorily answered.

2. I will be given a copy of the consent form at the time I sign it.

Dated at Toronto this __________________________ day of __________________________ 19 ______

__________________________________________  ______________________________________
Signature                                                                                     

__________________________________________  ______________________________________
Signature                                                                                     

__________________________________________  ______________________________________
Print Name                                                                                     Address

__________________________________________
Witness's Signature

__________________________________________
Print Name
9.2 Appendix B. Study Procedure and Consent Form – "Omeprazole Test"

**STUDY PROCEDURE AND CONSENT FORM - "OMEPRAZOLE TEST"**

I. ____________________________, hereby consent to participate in the "Pharmacogenetics" research project being conducted at the University of Toronto and the Psychopharmacology and Dependence Research Unit of Women's College Hospital under the direction of Edward M. Sellers, M.D., Ph.D. and Rachel F. Tyndale, Ph.D. The purpose of this research, the procedures to be followed and possible risks of this research have been explained to me by ____________________________. In consenting to participate, I understand that:

**PURPOSE**

1. The purpose of this test is to determine my pattern of metabolizing a substance called omeprazole (Losec®). This drug will permit the investigators to learn the relative activity of a drug metabolizing enzyme (a natural chemical in the body that processes or breaks down drugs and other chemicals) in my body called "CYP2C19".

**MEDICATION**

1. Omeprazole (Losec®) is a widely used and well tolerated medication used to treat stomach ulcers. Omeprazole has been given safely in the dose used in this study to millions of individuals without risk or hazard. At the single dose I will receive (20 mg), the side effects that might occur are a slight degree of nausea and/or diarrhea.
PROCEDURES

1. I will take a 20 mg omeprazole tablet with 1 cup of water.

2. Before and after taking the drug, I will complete a computerized motor coordination activity and a questionnaire about how I am feeling.

3. Three hours after taking the drug, a blood sample (20 ml or about 1 tablespoon) will be taken from a vein in my arm. The person collecting the blood will be experienced in this procedure and I can expect little or brief pain associated with inserting the needle. Afterward, there is some chance of slight bruising or inflammation, but this is a routine procedure that presents very low risk to me.

THERAPEUTIC BENEFITS

1. I can expect no therapeutic benefit from participating in this study.

CONFIDENTIALITY

1. The data I will provide will be kept strictly confidential and secure, available only to the researchers involved in this study and for the purpose set out in this consent form. Neither my name nor any piece of identifying information will be kept together with the other data that I may provide. My records will be treated with the same confidentiality afforded my medical records.

2. The results of this study may be published and if so will be published in such a manner that I will not be identifiable. Published reports will refer to group data and not to a particular individual. Published reports may separate data by racial group.
COMPENSATION

1. In consideration of the inconvenience and time involved in this study, I will receive $25.

GENERAL CONDITIONS

1. I may decline to answer any particular questions asked of me. If this refusal makes my participation in this study of no scientific value, my participation can be terminated.

2. I understand that I may be approached in the future and asked to participate in related research projects. I am entirely at liberty to decline and will not be subject to any coercion.

3. As part of this study, I will provide information about myself including medical history, current medication use and non-prescription drug use.

4. I can reach study personnel (after working hours) in case of an emergency (e.g. side effects) by phoning an emergency number which I will be provided with on a separate card.

5. I understand that participation in this study is voluntary and that I may withdraw at any time and for any reason. If I should withdraw from the study, I will not lose any benefits (i.e. I will not jeopardize my right to present or future treatment at Women’s College Hospital).
RIGHTS OF SUBJECTS

1. This research project has been reviewed and approved by the Research Ethics Board at Women's College Hospital.

2. Patients or study volunteers having questions or concerns about their rights in this research project should contact the Research Ethics Board Coordinator at (416) 351-3733.

MY CONSENT

1. I have had an opportunity to ask questions, and my questions have been satisfactorily answered.

2. I will be given a copy of the consent form at the time I sign it.

Dated at Toronto this ____________________ day of ____________________, 19____

________________________________________  __________________________________________
Signature                                      Address

________________________________________  __________________________________________
Print Name                                     Witness's Signature

________________________________________
Print Name
9.3 Appendix C. Subjective Measures

Tufts University Benzodiazepines Effects Scales
The following items are displayed as visual analogue scales, where the endpoints are labelled “A little” and “A lot”. The mean response is used as a measure of sedation.
1. How do you feel now? drowsy
2. How do you feel now? slowed down
3. How do you feel now? sleepy
4. How do you feel now? sedated
5. How do you feel now? tired
6. How do you feel now? worn out
7. How do you feel now? listless
8. How do you feel now? fatigued
9. How do you feel now? exhausted
10. How do you feel now? sluggish
11. How do you feel now? weary
12. How do you feel now? bushed

The following sets of endpoints for visual analogue scales are all displayed (one at a time) as responses to the question “How do you feel now? I feel ....”. The individual items are scored separately, not combined into a single scale.
13. Calm ... Anxious
14. Normal ... Bloated
15. Normal ... Sad
16. Energetic ... Fatigued
17. Thinking slowed down ... Thinking speeded up
18. Peaceful ... Tense
19. Normal ... Spacey
20. Friendly ... Seclusive
21. Normal ... Elated
22. Unhungry ... Hungry
23. Unpleasant ... Pleasant
24. At ease ... Nervous
25. Relaxed ... Excited
26. Normal ... Easily irritated
27. Discontented ... Contented
28. I feel a drug effect ... certainly not ... certainly
29. I like the drug dis dislike intensely ... like intensely
30. I feel the drug’s good effects ... very detectable
31. I feel the drug’s bad effects ... very detectable
32. The feeling in my body is ... very unpleasant ... very pleasant
33. The feeling in my mind is ... very unpleasant ... very pleasant
34. I feel this high ... absolutely normal ... very high
35. I feel this sick ... very well ... very sick
9.4 Appendix D. Consent Form (Study II)

CONSENT FORM (Study II)

I, ____________________________, hereby consent to participate in the research study entitled “Duration of Ketoconazole Pretreatment” being conducted on the Psychopharmacology and Dependence Research Unit of Sunnybrook and Women’s College Health Sciences Centre – Women’s College Campus under the direction of Edward M. Sellers, M.D., Ph.D., Inbal Gafni, B.Sc.H., Howard L. Kaplan, Ph.D. and Usoa E. Busto, Pharm.D. The purpose of the research, the procedures to be followed, and the possible risks of this research have been explained to me by ___________________________. In consenting to participate, I understand that:

PURPOSE

1. The purpose of this study is to determine the duration of ketoconazole administration in inhibiting the enzyme CYP3A4.

OVERVIEW

1. Once the study begins, I will participate in five visits on five consecutive days, each six hours long.

2. On each of the five study day sessions, I will receive (by mouth) a dose of omeprazole 40 mg. On days 2 through 5, I will also receive ketoconazole 100 mg at the same time. In the succeeding hours I will be required to provide blood and urine samples for laboratory analysis (see attached Procedures Flow Sheet).
MEDICATIONS

1. Omeprazole (Losec®) is a widely used and well-tolerated medication used to treat peptic and duodenal ulcer disease. Omeprazole has been given safely in the dose used in this study to millions of individuals without risk or hazard. Reported side effects of chronic administration include nausea, vomiting, diarrhea, abdominal pain and headache. These effects occur in less than 3% of subjects taking the drug. In previous studies in 20 individuals using omeprazole 20 mg, no side effects were reported.

2. Ketoconazole (Nizoral®) is an antifungal medication used in the therapy of superficial and systemic fungal infections. Reported side effects during chronic use include nausea, vomiting, skin rashes, chemical and liver changes. These side effects are dose-related and temporary. Common side effects at the doses I will receive in this study are mild transient nausea.

3. In the unlikely event that I suffer clinically significant side effects related to the above-noted medications, such as excessive nausea, my participation in the study may be terminated.

PRE-STUDY ASSESSMENT

1. Prior to participation in the study, I will undergo an assessment and medical examination. I will be checked for any medical condition that might prevent my participation. On this day, a urine sample will be obtained and blood samples will be taken from a vein in my arm. The person collecting the blood will be experienced the procedure and I can expect little and brief pain associated with inserting the needle. Afterwards there is a small chance of slight bruising or inflammation but this is a routine procedure that presents very low risk to me.
2. I understand that, if female, I must agree to a pregnancy test, and the result must be negative prior to entry into the study. There will be no cost to me for this test. I understand that I am advised not to become pregnant during the study or for one month after my participation is terminated.

STUDY DAY PROCEDURES

1. If I am eligible and I agree to volunteer, I will participate in five study day sessions, approximately 6 hours long.

2. On the evening before each study day session, I will not eat any food or take any psychoactive drugs (including alcohol, cannabis) after midnight until such time that the study procedures allow me to eat.

3. At approximately 9:00 a.m. I will receive an oral dose of omeprazole (40 mg).

4. On days 2 to 5, I will also receive an oral dose of ketoconazole (100 mg) at the same time.

5. On the study day, a fine plastic tube will be inserted in my forearm vein, and a blood sample (7 ml or two teaspoons) will be taken before taking the medication and at different intervals after I receive the study medication. A total of eight (8) blood samples will be taken that day. The person collecting the blood will be experienced in the procedure and I can expect a little pain associated with inserting the fine plastic tube. Afterwards there is some chance of slight bruising or inflammation but this is a routine procedure that presents very low risk to me. The fine plastic tube will be removed at the end of the study day.

6. On each study day, I will empty my bladder prior to receiving drug and will collect all of my urine after receiving drug for the next 6 hours (in 3-hour collection bottles).
RISKS AND BENEFITS

1. There are no significant risks expected with completion of the study procedures. I may become bored. I will have access to reading material, a TV, and a video VCR player for entertainment. I may experience side effects from the drug, which has been previously detailed in this consent form under the subheading “Medications”. Nursing or other trained staff will carefully monitor me throughout my participation to minimize the change of any serious risk.

2. There are no direct benefits to me by participating in this study.

3. The benefits of this research will relate primarily to the general scientific value of gaining a better understanding about the interaction between ketoconazole and omeprazole at different durations of ketoconazole pretreatment.

CONFIDENTIALITY

1. The data I will provide will be kept strictly confidential and secure, available only to the investigators and for the purposes set out in the purpose section above. Neither my name nor any pieces of identifying information will be kept together with the data that I may provide. My records will be treated with the same confidentiality afforded to medical records.

2. The results of this study may be published and if so will be published in such a manner that I will not be identifiable. Published reports will refer to group data and not to a particular individual.
COMPENSATION

1. In consideration of the inconvenience and the time involved in this study, I will receive compensation of $310. If I withdraw or my participation is terminated, I will receive partial payments. Payments will be as outlined in the attached schedule. I understand the study payment schedule and agree to the compensation rules.

GENERAL CONDITIONS

1. I agree not to drink caffeinated beverages between arriving and completing the first 6 hours of blood collection on the study day.

2. I will not take any psychoactive drugs or alcohol on the day prior to or for the duration of the study.

3. I will not drink or drink anything prior to arriving for the study day. I understand that I will receive a light breakfast, a light lunch, and juice throughout the day.

4. If I experience any unusual signs or symptoms during the course of this study, I will immediately report them to study personnel.

5. I can reach study personnel (after working hours) in case of an emergency (e.g. side effects) by phoning an emergency number which I will be provided with on a separate card.

6. If I become ill as a result of my taking part in the study, I understand that Dr. E.M. Sellers or his designate will arrange medical treatment.
7. During the study, I may decline to answer any particular questions asked of me or refuse to participate in specific procedures requested of me. If this refusal makes my participation in this study of no scientific value, then my participation can be terminated.

8. I may withdraw from the study at any time and for any reason. If I withdraw from the study this will in no way alter my right to present or future treatment at Sunnybrook and Women's College Health Sciences Centre – Women's College Campus.

9. The investigators may stop my involvement in the study at any time (e.g. medical reasons, non-compliance with protocol or my behaviour towards staff during the study).

10. I may be invited to participate in other studies for which I will sign a separate consent.

11. By signing this form I agree that I am not at present, or within the past six months, dependent on alcohol or any drug.

RIGHTS OF SUBJECTS

1. This research project has been reviewed by the Research Ethics Board at Sunnybrook and Women's College Health Sciences Centre – Women's College Campus.

2. Patients or study volunteers having questions or concerns about their rights in this research project should contact the Research Ethics Board Coordinator at (416) 351-3733.

MY CONSENT

1. I have had the opportunity to ask questions about the study, and my questions have been satisfactorily answered.

2. I will be given a copy of my consent form at the time I sign it.
This consent form was read in my presence by ________________________, who has informed me that he or she has carefully considered and understood each point above. I hereby confirm that the study will be conducted in accordance with the conditions and procedures set out above.
STUDY 1-1. DURATION OF KETOCONAZOLE PRETREATMENT

PAYMENT SCHEDULE

Subjects may be dropped from the study due to inappropriate conduct, non-compliance, or violation of study protocol. Such undesirable behavior results in wasted resources, disrupts the study timetable and may compromise the safety of staff and other participants. Subjects dropped due to the above reasons will receive a token fee of $25 for participating in the study day plus $40 for each completed study day. See table below.

Subjects who complete the study compliant to all rules or subjects who are dropped due to circumstances beyond their control (i.e. medical reasons, technical problems) will be rewarded for their time and effort by a fee of $60 for each day plus a $10 completion bonus (MAX of $310). See table below. Subjects who display a good track record of compliance will be more than welcome to participate in future studies if they qualify.

<table>
<thead>
<tr>
<th>Study Day Completed</th>
<th>Non-Compliant Subjects (in $)</th>
<th>Compliant Subjects (in $)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$25</td>
<td>$60</td>
</tr>
<tr>
<td>2</td>
<td>$65</td>
<td>$120</td>
</tr>
<tr>
<td>3</td>
<td>$105</td>
<td>$180</td>
</tr>
<tr>
<td>4</td>
<td>$145</td>
<td>$240</td>
</tr>
<tr>
<td>5</td>
<td>$185</td>
<td>$310 ($300 + $10)</td>
</tr>
</tbody>
</table>

SUBJECT’S CONSENT

I have read and understood the conditions in the “Payment Schedule” above. The research staff has answered any questions I had pertaining to it. I agree to comply with the above conditions.

__________________________________________  _________________________
Signature                                                                 Date
## Appendix E. Summary of Study Day Procedures (Study II)

<table>
<thead>
<tr>
<th>Time</th>
<th>Activity/Procedure/Test</th>
</tr>
</thead>
</table>
| - 01 00 h| - Subject arrives for testing  
- Blood alcohol level determination  
- Baseline vitals (BP, T, P, R) |
| - 00 30 h| - Intravenous catheter inserted into forearm vein  
- Baseline blood samples extracted  
- Subject voids to empty bladder |
| 00 00 h  | Day 1: Omeprazole 40 mg administered p.o.  
Days 2-5: Ketoconazole 100 mg + omeprazole 40 mg administered p.o. |
| 00 30 h  | - Blood sample extracted  
- Breakfast |
| 01 00 h  | - Blood sample extracted |
| 02 00 h  | - Blood sample extracted |
| 03 00 h  | - Blood sample extracted  
- Urine sample collected  
- Vitals |
| 04 00 h  | - Blood sample extracted  
- Lunch |
| 05 00 h  | - Blood sample extracted |
| 06 00 h  | - Blood sample extracted  
- Urine sample collected  
- Vitals |
9.6 Appendix F. Urine data

Mean percent dose recovered from urine for all subjects over the 5 study days. The values shown are the least-squares-mean with the error bars reflecting the standard error.
CONSENT FORM (Study III)

I, ____________________________, hereby consent to participate in the research study entitled “Influence of Race, Sex and Genotype” being conducted on the Psychopharmacology and Dependence Research Unit of Sunnybrook and Women’s College Health Sciences Centre – Women’s College Campus under the direction of Edward M. Sellers, M.D., Ph.D., Howard L. Kaplan, Ph.D. and Usoa E. Busto, Pharm.D. The purpose of the research, the procedures to be followed and the possible risks of this research have been explained to me by ____________________________ In consenting to participate, I understand that:

PURPOSE

1. The purpose of this study is to determine my pattern of metabolizing (processing or breaking down) a medication called flunitrazepam.

2. I understand that individuals have different forms of the enzyme (natural processing chemical) called CYP2C19. “Extensive metabolizers” of this enzyme refer to individuals in which this enzyme is active while “poor metabolizers” lack the activity of this enzyme.

3. By signing this form I agree that I am not at present, or within the past six months, physically dependent on alcohol or any drug.
OVERVIEW

1. On the test day, I will receive flunitrazepam 1 mg as an oral capsule at approximately 8:00 a.m. In the succeeding hours I will be required to provide blood and urine samples for laboratory analysis (see attached Procedures Flow Sheet).

MEDICATIONS

1. Flunitrazepam is marketed as a sleeping medication in many countries and has been used by thousands of patients. Common side effects at the doses I will receive in this study are drowsiness, sedation, tiredness and memory impairment. Some patients have also reported difficulty concentrating and staying awake, the feeling of slowing of their thought process and difficulties with balance. All these effects are temporary and reversible and they disappear as the drug is eliminated from the body. Many patients do not experience any side effects to this medication.

2. In the unlikely event that I suffer clinically significant side effects related to the above-noted medications, such as excessive sedation, my participation in the study may be terminated.

PRE-STUDY ASSESSMENT

1. Prior to participation in the study I will undergo an assessment and medical examination. I will be checked for any medical condition which might prevent my participation. On this day, a urine sample will be obtained and blood samples will be taken from a vein in my arm. The person collecting the blood will be experienced the procedure and I can expect little and brief pain associated with inserting the needle. Afterwards there is a small chance of slight bruising or inflammation but this is a routine procedure that presents very low risk to me.
2. I understand that, if female, I must agree to a pregnancy test, and the result must be negative prior to entry into the study. There will be no cost to me for this test. I understand that I am advised not to become pregnant during the study or for one month after my participation is terminated.

**STUDY DAY PROCEDURES**

1. If I am eligible and I agree to volunteer, I will participate in one study day session, approximately 9 hours long.

2. On the evening before the study day session, I will not eat any food or take any psychoactive drugs (including alcohol, cannabis) after midnight until such time that the study procedures allow me to eat.

3. At approximately 8:00 a.m. I will receive my oral dose of flunitrazepam (1 mg).

4. On the study day, a fine plastic tube will be inserted in my forearm vein, and a blood sample (10 ml or two teaspoons) will be taken before taking the medication and at different intervals after I receive the study medication. A total of eight (8) blood samples will be taken that day. The person collecting the blood will be experienced in the procedure and I can expect a little pain associated with inserting the fine plastic tube. Afterwards there is some chance of slight bruising or inflammation but this is a routine procedure that present very low risk to me. The fine plastic tube will be removed at the end of the study day.

5. My urine will be collected prior to drug administration and as a pooled sample for the first 8 hours post-drug administration.
6. I will return to the study site for two days following each study day, per the attached schedule, at which time one blood sample will be collected.

RISKS AND BENEFITS

1. There are no significant risks associated with completion of the study procedures. I may become bored. I may experience side effects from the drug, which has been previously detailed in this consent form under the subheading "Medications." I will be carefully monitored by nursing or other trained staff throughout my participation to minimize the change of any serious risk.

2. The benefits of this research relate primarily to the general scientific value of gaining a better understanding about differences in patterns of metabolizing (processing or breaking down) flunitrazepam.

CONFIDENTIALITY

1. The data I will provide will be kept strictly confidential and secure, available only to the investigators and for the purposes set out in the purpose section above. Neither my name nor any pieces of identifying information will be kept together with the data that I may provide. My records will be treated with the same confidentiality afforded to medical records.

2. The results of this study may be published and if so will be published in such a manner that I will not be identifiable. Published reports will refer to group data and not to a particular individual.
COMPENSATION

1. In consideration of the inconvenience and the time involved in this study, I will receive compensation of $130. If I withdraw or my participation is terminated, I will receive partial payments. Payments will be as outlined in the attached schedule. I understand the study payment schedule and agree to the compensation rules.

GENERAL CONDITIONS

1. I agree not to drink caffeinated beverages between arriving and completing the first 6 hours of blood collection on the study day.

2. I will not take any psychoactive drugs or alcohol on the day prior to or the same day of the study.

3. I will not drink or drink anything prior to arriving for the study day. I understand that I will receive a light breakfast prior to receiving drug, juice throughout the day and a light lunch.

4. If I become ill as a result of my taking part in the study, I understand that medical treatment will be arranged by Dr. E.M. Sellers or his designate.

5. During the study I may decline to answer any particular questions asked of me or refuse to participate in specific procedures requested of me. If this refusal makes my participation in this study of no scientific value, then my participation can be terminated.

6. I may withdraw from the study at any time and for any reason. If I withdraw from the study this will in no way jeopardize my right to present or future treatment at Sunnybrook and Women’s College Health Sciences Centre.
7. The investigators may terminate my involvement in the study at any time (e.g. medical reasons, non-compliance with protocol or my behaviour towards staff during the study).

8. I may be invited to participate in a similar study for which I will sign a separate consent.

RIGHTS OF SUBJECTS

1. This research project has been reviewed by the Research Ethics Board at Sunnybrook and Women’s College Health Sciences Centre.

2. Patients or study volunteers having questions or concerns about their rights in this research project should contact the Research Ethics Board Coordinator at (416) 480-4276.

MY CONSENT

1. I have had the opportunity to ask questions about the study, and my questions have been satisfactorily answered.

2. I will be given a copy of my consent form at the time I sign it.
Dated at Toronto this _________________ day of ______________________, 19 ___.

______________________________
Signature

______________________________  ______________________________
Print Name

______________________________
Address

______________________________
Witness's Signature

This consent form was read in my presence by ________________________, who has informed me that he or she has carefully considered and understood each point above. I hereby confirm that the study will be conducted in accordance with the conditions and procedures set out above.

______________________________
Signature

______________________________
Print Name
STUDY 5-A. INFLUENCE OF RACE, SEX AND GENOTYPE

PAYMENT SCHEDULE

Subjects may be dropped from the study due to inappropriate conduct, non-compliance, or violation of study protocol. Such undesirable behavior results in wasted resources, disrupts the study timetable and may compromise the safety of staff and other participants. Subjects dropped due to the above reasons will receive a token fee of $25 for participating in the testing day plus $5 for each completed follow-up visit. See table below.

Subjects who complete the study compliant to all rules or subjects who are dropped due to circumstances beyond their control (i.e. medical reasons, technical problems) will be rewarded for their time and effort by a fee of $90 for the testing day plus $5 for each follow-up visit and a $30 completion bonus (MAX of $130). See table below. Subjects who display a good track record of compliance will be more than welcome to participate in future studies if they qualify.

<table>
<thead>
<tr>
<th>Testing Day Completed</th>
<th>Follow-Up Visits Post-Testing Day</th>
<th>Non-Compliant Subjects</th>
<th>Compliant Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>$25</td>
<td>$75</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>$30</td>
<td>$95</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>$35</td>
<td>$130 ($100 + $30)</td>
</tr>
</tbody>
</table>

SUBJECT'S CONSENT

I have read and understood the conditions in the "Payment Schedule" above. Any questions pertaining to it have been answered by the research staff. I agree to comply with the above conditions.

______________________________  _______________________
Signature                    Date
### 9.8 Appendix H. Summary of Flunitrazepam Study Day Procedures

<table>
<thead>
<tr>
<th>Study Day</th>
<th>Time</th>
<th>Activity/Procedure/Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>- 01 00 h</td>
<td>Subject arrives for testing</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Blood alcohol level determination</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Baseline vitals (BP, T, P, R)</td>
</tr>
<tr>
<td></td>
<td>- 00 45 h</td>
<td>Intravenous catheter inserted into forearm vein</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Baseline blood samples extracted</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Baseline SMS</td>
</tr>
<tr>
<td></td>
<td>00 00 h</td>
<td>Flunitrazepam 1 mg given p.o.</td>
</tr>
<tr>
<td></td>
<td>00 40 h</td>
<td>Blood sample extracted</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SMS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Breakfast</td>
</tr>
<tr>
<td></td>
<td>01 20 h</td>
<td>Blood sample extracted</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SMS</td>
</tr>
<tr>
<td></td>
<td>02 00 h</td>
<td>Blood sample extracted</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vitals</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SMS</td>
</tr>
<tr>
<td></td>
<td>03 00 h</td>
<td>Blood sample extracted</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vitals</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SMS</td>
</tr>
<tr>
<td></td>
<td>04 00 h</td>
<td>Blood sample extracted</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lunch</td>
</tr>
<tr>
<td></td>
<td>05 00 h</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>SMS</td>
</tr>
<tr>
<td></td>
<td>06 00 h</td>
<td>Blood sample extracted</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Snack</td>
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<tr>
<td></td>
<td>07 00 h</td>
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<tr>
<td></td>
<td>07 40 h</td>
<td>SMS</td>
</tr>
<tr>
<td></td>
<td>08 00 h</td>
<td>Blood sample extracted</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intravenous catheter removed</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vitals</td>
</tr>
<tr>
<td>Day 2</td>
<td>24 00 h</td>
<td>Blood sample extracted</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vitals</td>
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<tr>
<td>Day 3</td>
<td>48 00 h</td>
<td>Blood sample extracted</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vitals</td>
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</tbody>
</table>