The influence of the CYP2C19 and CYP2D6 genetic polymorphisms on oxidative drug metabolism

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Abstract

- The CYP2C19 and CYP2D6 genetic polymorphisms control the oxidative metabolism of many different drug classes. Populations are separated into groups of extensive metabolisers (EM), poor metabolisers (PM), and in the case of CYP2D6, ultra-rapid metabolisers (UM).

- *In vitro* studies using human liver microsomes were conducted to examine; i) the kinetics of the oxidative metabolism of flunitrazepam; and ii) which CYP450 enzymes mediate the oxidative metabolism of flunitrazepam, (S)-mephenytoin and proguanil. It was observed that the kinetics of flunitrazepam were sigmoidal, and that the formation of both the 3-hydroxy and desmethyl metabolite was mediated by CYP2C19, CYP3A4, and possibly CYP1A2. In addition, it was observed that the presence of dimethylformamide (an organic solvent) significantly inhibited the kinetics of 3-hydroxy- and desmethyl-flunitrazepam formation. The CYP450 enzymes mediating the oxidative metabolism of (S)-mephenytoin and proguanil were, CYP2C19 and CYP1A2, and CYP2C19, CYP3A4 and CYP1A2, respectively.

- The first *in vivo* study investigated the incidence of CYP2C19 PM in Caucasian and Asian populations living in Australia, by genotyping and phenotyping with proguanil. The PM incidences in Caucasians and Asians were 3.2% and 4.7%, respectively. In addition, the proguanil/cycloguanil metabolic ratio antimode of 8.61 was unable to correctly assign phenotype in two of the Caucasian subjects. Therefore, it was concluded that proguanil may not be a suitable phenotyping probe to identify CYP2C19 PM and replace mephenytoin which is no longer available.

- The second *in vivo* study investigated the incidence of CYP2D6 UM in Caucasian and Asian populations living in Australia, by genotyping and phenotyping with dextromethorphan. The incidence of UM in the Caucasian population was 8.5%.

There were no UM found in the Asian population. In addition, the log urinary dextromethorphan to total dextrorphan metabolic ratio was unable to separate UM from EM subjects. Therefore, it was concluded that the only conclusive method for identifying CYP2D6 UM is genotyping.

- The last *in vivo* pilot study investigated the changes in the metabolism of codeine in CYP2D6 UM when compared to CYP2D6 EM, by the administration of 90mg codeine and urine collection for 48hr. No changes were observed between UM and EM. It was concluded that due to underlying problems with the genotyping performed, future studies are required to re-investigate the clinical significance of changes in codeine metabolism in UM.

- The results observed indicate that genotyping is a more conclusive and accurate method for the correct identification of poor metabolisers of both the CYP2C19 and CYP2D6 genetic polymorphisms.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent for this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

24th December, 1999.

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List of Abbreviations

ACN	acetonitrile
AUC	area under the concentration time curve
CCG	chlorcycloguanil
CG	cycloguanil
C-6-G	codeine-6-glucuronide
Cl_{int}	intrinsic clearance
C_{max}	maximum concentration
Cl_o	oral clearance
DM	dextromethorphan
DMF	desmethylflunitrazepam
DMFO	dimethylformamide
DMDZ	N-desmethyldiazepam
DR	dextrorphan
DZ	diazepam
DM/DR MR	dextromethorphan/dextrorphan metabolic ratio
$EM_{d/s}$	CYP2D6 extensive metaboliser
EM_m	CYP2C19 extensive metaboliser
H-OPZ	5'-hydroxyomeprazole
IM_m	CYP2C19 intermediate metaboliser
k	elimination rate constant
K_a	absorption rate constant
K_i	affinity constant of CYP450 enzyme for inhibitor
K_m	affinity constant of CYP450 enzyme for substrate
K_s	Hill equation intrinsic dissociation constant
log DM MR	log (dextromethorphan/(total dextrorphan + 3-hydroxymorphinan)) MR
log DM/total DR MR	log (dextromethorphan/total dextrorphan) MR

M-3-G	morphine-3-glucuronide
M-6-G	morphine-6-glucuronide
3-MM	3-methoxymorphinan
MR	metabolic ratio
NC	norcodeine
3-OH-F	3-hydroxyflunitrazepam
4'-OH-meph	4-hydroxymephenytoin
3-OH-M	3-hydroxymorphinan
OPZ	omeprazole
OPZ-SFN	omeprazole sulfone
PEH	5-phenyl-5-ethylhydantoin / Nirvanol
$PM_{d/s}$	CYP2D6 poor metaboliser
PM_m	CYP2C19 poor metaboliser
PG/CG MR	proguanil/cycloguanil metabolic ratio
r	correlation coefficient
r^2	determination coefficient
r_s	Spearman rank correlation coefficient
$t_{1/2}$	half-life
t_{max}	time of maximum concentration
TMZ	3'-hydroxydiazepam / temazepam
UM	CYP2D6 ultrarapid metaboliser
V_{max}	maximum formation rate

1. Introduction

1.1 Hepatic drug metabolism

Most drugs are lipophilic enabling ready gastrointestinal absorption and delivery to the site of action within the body. Their action is often terminated by some form of metabolism at a site separate to the site of action, usually the liver. Hepatic drug metabolism is mediated by many enzymes, such as cytochrome P450s, with the objective of forming a more hydrophilic compound which is more easily excreted by the kidney, and is separated into two phases. Phase I reactions include hydrolysis, reduction, and oxidation, and involve the exposure or addition of functional groups to the parent drug creating a product which may then be readily excreted from the body, or undergo a Phase II conjugation reaction to produce a secondary metabolite, which may also be easily excreted from the body. For example, the Phase I oxidative metabolism of codeine to morphine by the cytochrome P450 enzyme CYP2D6, involves the exposure of an hydroxyl group at the 3' position. Morphine then undergoes Phase II glucuronide conjugation at the 3' or 6' positions to produce morphine-3-glucuronide or morphine-6-glucuronide, respectively (Figure 1). Phase II reactions can also produce a primary metabolite, for example if morphine is administered it is metabolised to produce morphine-3- or morphine-6-glucuronide.

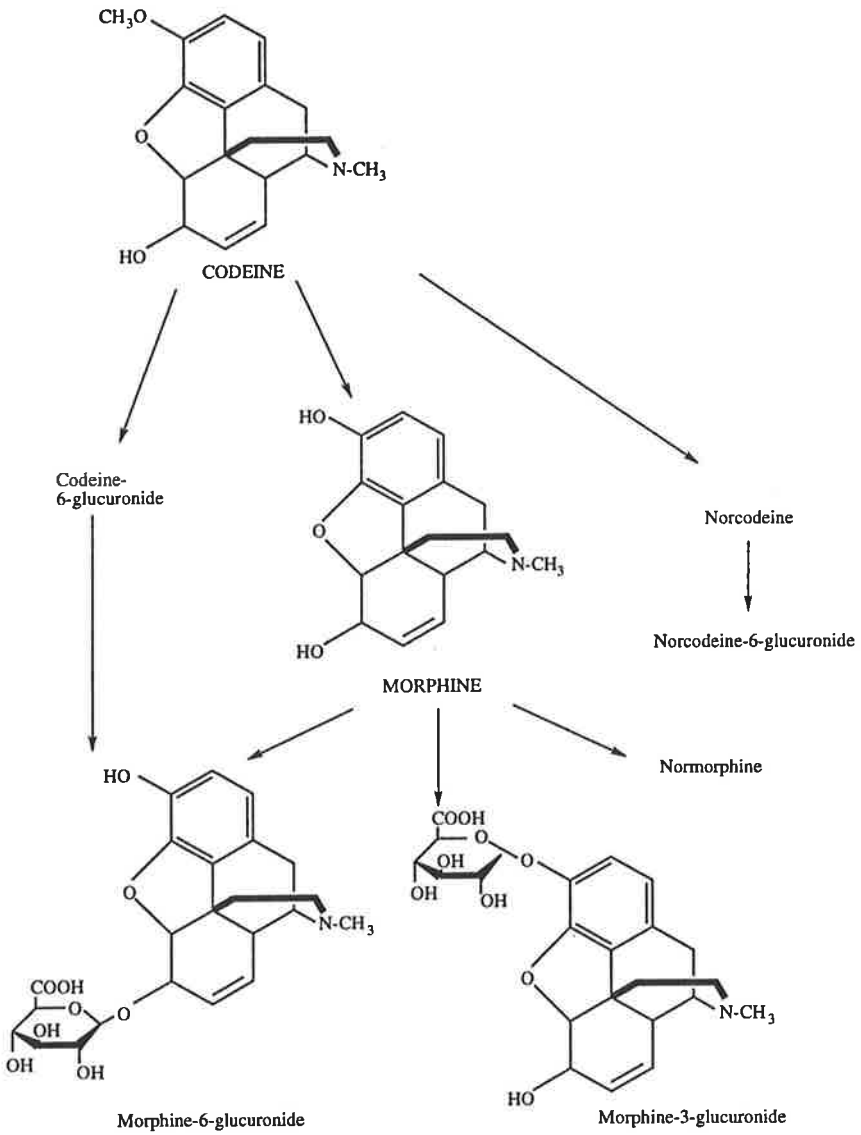


Figure 1: Hepatic metabolism of codeine

1.1.1 Cytochrome P450 System

Phase I oxidative, peroxidative and reductive reactions of both endogenous compounds, such as steroids and fatty acids, and exogenous chemicals, such as drugs and environmental pollutants, are most often mediated by the superfamily of enzymes known as the cytochrome P450 (CYP450) enzymes, also interpreted as isoforms / isoenzymes (Nelson *et al.*, 1996). The ancestral gene of this superfamily has existed for more than 3.5 billion years, which was before the prokaryote / eukaryote divergence occurred, and currently 74 gene families (alleles) have been discovered (Nelson *et al.*, 1996). Of the 14

families (alleles) found in mammals, 26 subfamilies (subsets of one allele) exist and many of these have been mapped on the human genome (Nelson *et al.*, 1996).

To distinguish between the CYP450 enzymes, a recognised nomenclature has been developed. For the gene, the italicised *CYP* denotes that it is a CYP450 enzyme; the following Arabic number denotes the family/allele, the letter denotes the subfamily (subset of the allele) where two or more exist, and the final Arabic number denotes the individual gene within the subfamily. The cDNAs, mRNAs and enzymes follow the same nomenclature but are not italicised (Nelson *et al.*, 1996). Due to the current existence of 24 subset alleles of the *CYP2D6* gene it was proposed that a standardised system of nomenclature be utilised (Daly *et al.*, 1996). This system states that an allele be denoted by CYP2D6* followed by a combination of Roman letters and Arabic numbers distinct for each allele, with a number specifying the key mutation and a letter specifying the additional mutations. This system is also recognised for the *CYP2A6*, *CYP2C9*, and *CYP2C19* genes.

The CYP450 enzymes are located in the lipid bilayer of the smooth endoplasmic reticulum together with the cytochrome P450 reductases. It is here that the metabolism of substrates takes place in a cyclic fashion. The substrate binds to the oxidised heme group (Fe^{3+}) of the CYP450 enzyme to create a complex which is then reduced by the reductases. This reduction facilitates the interaction with oxygen and an additional electron from the NAPDH-flavoprotein donor system to produce the oxidised form of the substrate, water, and regenerate the oxidised form of the CYP450 enzyme (Figure 2).

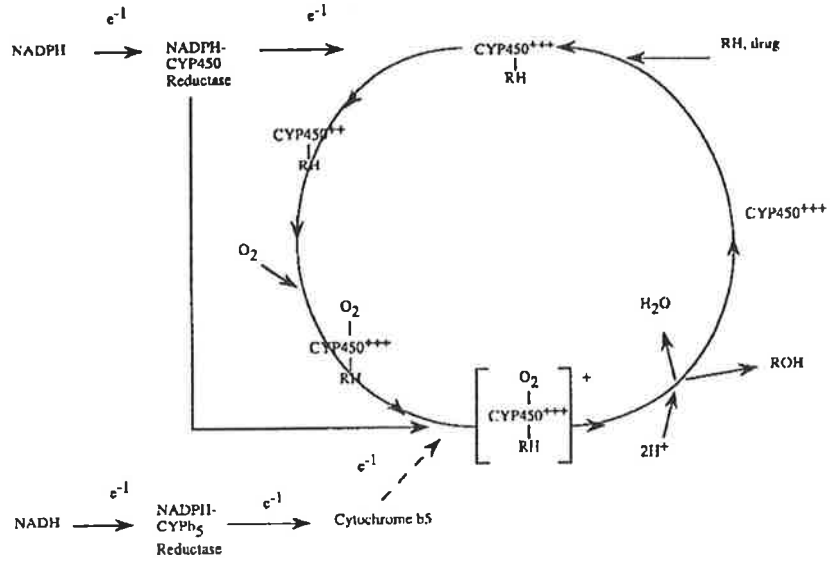


Figure 2: Metabolism of substrates by CYP450 enzymes (adapted from Klaassen *et al.*, 1986).

A substrate is known to be metabolised by CYP450 enzymes if an absolute requirement for NADPH and inhibition of metabolism by carbon monoxide and NAD is demonstrated (Meier *et al.*, 1985a). In humans 11 subfamilies have been identified; of these subfamilies 1-4 are important in xenobiotic metabolism, 5 performs thromboxane synthesis, 11 performs cholesterol side chain cleavage, and 11, 17, 19 and 21 mediate steroid synthesis (Price Evans, 1993a). The CYP450 enzymes display distinct substrate specificities, such that, for example, (S)-mephenytoin is predominantly metabolised by CYP2C19, while sparteine is metabolised by CYP2D6. Furthermore, the activity of the CYP450 enzymes is known to be influenced by environmental and disease states, interactions with other drugs (Vesell *et al.*, 1983) and genetic factors (Eichelbaum *et al.*, 1979, de Morais *et al.*, 1994a).

1.1.2 Genetic Polymorphism

Many enzymes that metabolise xenobiotics, have been shown to exhibit a large degree of interindividual variation in levels of expression. For example, an immunoblotting study conducted in the microsomal fractions of 12 human livers revealed a 16-fold and 60-fold variation in levels of CYP2D6 and CYP3A3/3A4, respectively (Forrester *et al.*, 1992). Variability in expression may alter the pharmacokinetics of the drug, resulting in either reduced drug efficacy, or increased adverse effects if the concentration of the active parent drug is above therapeutic range. These differences have also been implicated in influencing the susceptibility to toxins and carcinogens whose activation or detoxification is mediated by CYP450 enzymes, and therefore associated with an increased risk of some forms of cancer (Caporaso *et al.*, 1989). Another physiological consequence of variation in CYP450 enzyme expression is a change in the circulating steroid hormone levels, as these enzymes mediate their metabolism, and subsequent alteration in various biological processes (Forrester *et al.*, 1992).

The variability in expression of the CYP450 enzymes can be accounted for partially by genetic mutations, resulting in either a reduction in the amount of functional enzyme present or a complete deficiency of the enzyme. A genetic polymorphism is defined as "the occurrence together in the same locality of two or more discontinuous forms of a species in such proportions that the rarest of them cannot be maintained merely by a recurrent mutation" (Ford, 1971). In order for a gene to be polymorphic, the genotype of lowest frequency must be represented in greater than one per cent of the population. Below this frequency, the difference is explained by the occurrence of rare genetic mutations.

Most genetic polymorphisms have been discovered due to the occurrence of increased adverse effects to particular drugs in some subjects (later defined as poor metabolisers) when administered standard doses in pharmacokinetic studies. This was the case for the

most widely studied genetic polymorphism, that of the oxidative metabolism of debrisoquine (Eichelbaum *et al.*, 1979) and sparteine (Mahgoub *et al.*, 1977), and also for the 'newer' (S)-mephenytoin hydroxylation metabolism. Two clinical consequences of poor metabolism are toxicity for the patient if the parent drug is active, or reduced drug efficacy if the metabolite is active. For example, the lack of metabolism of mephenytoin has been reported to result in high levels of sedation (Küpfer *et al.*, 1979), a form of adverse reaction or toxicity. Reduced efficacy is illustrated in subjects where codeine is not metabolised to its active metabolite morphine, with these subjects reporting reduced analgesia during the cold pressor test when compared with subjects who formed morphine (Sindrup *et al.*, 1993a, Poulsen *et al.*, 1996). Correlations have been shown between a deficiency in metabolism and the possibility of developing adverse drug reactions due to changes in the pharmacokinetics of the drug (Smith, 1986).

1.2 Mephenytoin

Mephenytoin is a racemic mixture of the (R)- and (S)-enantiomers and the centre of chirality is located at position 5 of the hydantoin ring (Küpfer *et al.*, 1984b). It undergoes stereoselective metabolism (Figure 3) such that (S)-mephenytoin is hydroxylated at the para-4'-position of the aromatic ring by CYP2C19, forming (S)-mephenytoin arene oxide (Küpfer *et al.*, 1984b). This metabolite spontaneously rearranges to form the phenolic metabolite 4'-hydroxy-mephenytoin (4'-OH-meph), which is rapidly eliminated in urine as a glucuronide conjugate (Küpfer *et al.*, 1981). (S)-mephenytoin arene oxide can also undergo metabolism via epoxide hydrolase to form mephenytoin dihydrodiol metabolites (Küpfer *et al.*, 1984a) and N-demethylation to 5-phenyl-5-ethylhydantoin (PEH, also known as Nirvanol) which is mediated by CYP2B6 and CYP2C9 (Heyn *et al.*, 1996, Ko *et al.*, 1998). (R)-mephenytoin is less susceptible to hydroxylation, and is therefore preferentially N-demethylated to form PEH. This metabolite is pharmacologically active displaying the same anticonvulsive efficacy as racemic mephenytoin (rac-mephenytoin),

and is excreted slowly in comparison to 4'-OH-meph (Küpfer *et al.*, 1981) with an elimination half-life ($t_{1/2}$) of greater than 80hr compared with approximately 4hr for 4'-OH-meph, so that in cases of chronic dosing it was observed to accumulate, replacing mephenytoin as the major circulating hydantoin (Küpfer *et al.*, 1984c).

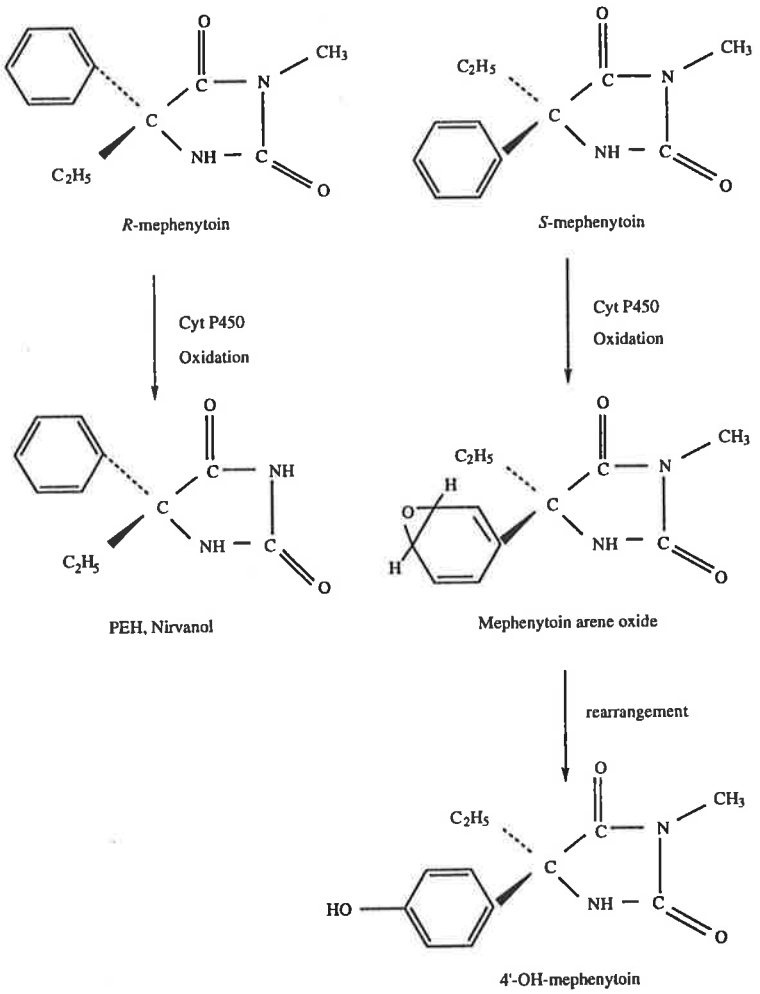


Figure 3: Metabolism of (R)- and (S)-mephenytoin.

Since the 1940's racemic mephenytoin has been marketed as an anticonvulsant. However, due to the occurrence of idiosyncratic reactions, including skin rash, adenopathy (enlargement of glands) and blood dyscrasias (Weller *et al.*, 1949, Abbott *et al.*, 1950, Deutsch *et al.*, 1950, Witkind *et al.*, 1951, Hofstatter, 1951, Abbott *et al.*, 1954, Troupin *et al.*, 1976), it was withdrawn from the Australian market, and availability is now becoming limited elsewhere. It has been noted that the bone marrow toxicity reported with chronic dosing was possibly related to the formation of the arene oxide from the (S)-enantiomer.

1.2.1 The (S)-mephenytoin hydroxylation genetic polymorphism

While Küpfer and associates were investigating differences in pharmacokinetics in normal subjects following long-term rac-mephenytoin dosing, they noticed that one subject experienced an unacceptable level of sedation. Analysis of this subject's urinary metabolic profile showed a deficiency in hydroxylation of (S)-mephenytoin (Küpfer *et al.*, 1979). To further investigate this observation, another study was conducted in which 221 healthy, unrelated Swiss subjects were given 100mg rac-mephenytoin orally, and urine was collected for the following 8hr (Küpfer *et al.*, 1984d). As the amount of unchanged mephenytoin excreted was minute, phenotypes were assigned according to the hydroxylation index (HI) which takes into account the stereoselective hydroxylation of the substrate.

$$HI = \frac{\mu mol\ mephenytoin\ dose\ ((S)\text{ - }enantiomer)}{\mu mol\ 4'\text{-}OH\text{ - }meph\ in\ urine_{(0\text{-}8hr)}}$$

A discontinuous distribution was observed in the HI of the population with 12 of 221 subjects (~5%) classified as poor metabolisers (PM_m, HI > 5.6), and 209 classified as extensive metabolisers (EM_m, HI < 5.6). By also phenotyping these subjects as PM or EM of debrisoquine (see page 40), it was observed that less than 1% of the subjects carried both defects simultaneously, therefore showing that this was a new genetic polymorphism in oxidative drug metabolism, as the two genetic polymorphisms did not co-segregate. Due to this research, it was possible for racemic mephenytoin to become a phenotypic probe for *in vivo* genetic polymorphism studies. Probe drugs are commonly used in the process of drug development, for both pharmacokinetic clinical trials and to investigate interethnic differences in drug disposition. However as indicated above, in Australia rac-mephenytoin is unavailable.

Following the discovery of this polymorphism, additional research has focused on the pharmacokinetic differences due to the mephenytoin hydroxylation deficiency. Küpfer and coworkers (Küpfer *et al.*, 1984a) administered a single oral dose of a radio labelled pseudo

racemic mixture of mephenytoin (5μCi ^{14}C-(S)-mephenytoin and 45μCi ^{3}H-(R)-mephenytoin, equivalent to 11.5μmol.kg^{-1} of each enantiomer) to 4 normal subjects and one hydroxylation deficient subject, classified as a PM_m. Following this administration, 1.4mmol of unlabelled rac-mephenytoin was given for 4 days and urine collected between the dosing intervals. It was observed that in comparison to normal subjects, there was an equal recovery of the (R)-and (S)-enantiomers of PEH in the PM_m, and PEH accumulated to concentrations approximately twice that observed in normal subjects. This indicated that the N-demethylation pathway is not stereoselective, and is not deficient in these subjects; that is the genetic polymorphism only involves the hydroxylation of (S)-mephenytoin. However, such a deficiency in the N-demethylation can only be detected by calculation of intrinsic clearance (a measure of the ability of the liver to metabolise the drug), which was not mentioned by the researchers.

1.2.2 The genetic basis of the (S)-mephenytoin hydroxylation genetic polymorphism

Two major genetic defects in the gene coding for CYP2C19, located on chromosome 10, region 10q24.1-24.3 (Goldstein *et al.*, 1994), which lead to an aberrant form of the protein have been described (de Morias *et al.*, 1994a, de Morais *et al.*, 1994b). The poor metaboliser (PM_m) phenotype results from a homozygous mutant genotype, and the extensive metaboliser (EM_m) phenotype from either a homozygous wild-type or a heterozygous wild-type / mutant genotype. The primary mutation in PM_m, representing 75% and 83% of defective alleles in Japanese and Caucasians, respectively, was previously known as m1 (de Morais *et al.*, 1994a, de Morais *et al.*, 1994b), but the introduction of new nomenclature following that used for *CYP2D6* alleles now denotes it as *CYP2C19*2* (Daly *et al.*, 1996). The *CYP2C19*2* mutation involves a single base-pair change (G → A) at position 681 in exon 5 which creates an aberrant splice site. This site is used in preference to the normal splice site in PM_m, forming a premature stop codon and a

truncated 234 amino acid protein which lacks the heme-binding region and is therefore catalytically inactive.

Another mutation previously known as m2 but now recognised as *CYP2C19*3* (Daly *et al.*, 1996), has been predominantly observed in Asian populations, and in the Japanese accounts for the remaining 15% of mutant alleles in this population (de Morais *et al.*, 1994a). It also involves a single base-pair change (G → A) at position 636 in exon 4 creating a premature stop codon yielding a truncated 211 amino acid protein which contains only the first four exons of *CYP2C19*. This defective protein not only lacks the heme-binding region, but also the proposed substrate-binding regions and is catalytically inactive. It is important to note that the *CYP2C19*3* mutation is extremely rare in Caucasian subjects (2% of defective alleles, Chang *et al.*, 1995a).

Recently additional mutations of *CYP2C19* have been identified. *CYP2C19*4* is a single base-pair change (A → G) at position 1 in exon 1 or in the initiation codon which interferes with transcription and translation of CYP2C19 causing a decrease of functional CYP2C19, and accounts for 0.6% of mutant alleles in Caucasians (Ferguson *et al.*, 1998). *CYP2C19*5A* is a single base-pair change (C → T) at position 1297 in exon 9 creating an amino acid substitution of Trp for Arg in the heme binding region abolishing CYP2C19 activity (Ibeanu *et al.*, 1998a). *CYP2C19*5B* is a variant *CYP2C19*5A* allele which carries the C → T base-pair change at position 1297, and also a second base-pair change (A → G) at position 991 of exon 7 creating another amino acid substitution of Val for (Ibeanu *et al.*, 1998a). This variant allele has no functional CYP2C19 activity, and together the *CYP2C19*5* alleles account for 0.25% and less than 0.9% of mutations in Bai Chinese and Caucasian populations, respectively. *CYP2C19*6* is a single base-pair change (G → A) at position 395 of exon 3 which causes an amino acid substitution of Gln for Arg abolishing the CYP2C19 catalytic activity (Ibeanu *et al.*, 1998b). This mutation accounts for less than 0.9% of Caucasian PM, and has only been observed in one Swiss Caucasian to date (Ibeanu *et al.*, 1998b). This study also identified a new allelic variant of *CYP2C19*2* termed *CYP2C19*2B*. This variant carries the original splice site defect and another single

base-pair change (G → C) at position 276 of exon 2 resulting in an amino acid substitution of Asp for Glu. Similar to *CYP2C19*2* (now termed *CYP2C19*2A*), this mutation creates a truncated protein with no functional activity. It was reported that 15% of *CYP2C19*2* alleles were *CYP2C19*2B* and 85% were *CYP2C19*2A* within a French Caucasian European population (Ibeanu *et al.*, 1998b). *CYP2C19*7* is a single base-pair translocation (T → A) in the splice junction donor site in intron 5, which was suggested to cause exon skipping resulting in a non-functional protein (Ibeanu *et al.*, 1999). The frequency of this allele in 172 French controls and 152 French lung cancer patients had 95% confidence intervals of 0 - 0.011 and 0 - 0.012, respectively. *CYP2C19*8* is a single base-pair change (T → C) at position 358 of exon 3 which results in an amino acid substitution of Arg for Trp, which causes a change in the putative heme binding region (Ibeanu *et al.*, 1999). The frequency of this allele in 172 French controls and 152 French lung cancer patients had 95% confidence intervals of 0 - 0.011 and 0 - 0.012 respectively.

A genetic familial study of 23 related Swiss Caucasian subjects, suggested that the *CYP2C19*2* allele is inherited in an autosomal fashion, as it was identified in both sexes (Küpfer *et al.*, 1984d). It was also postulated by the authors that PM_m appear to be homozygotes who recessively inherit the defective allele as both parents were heterozygous EM_m. The importance of the inheritance as the primary cause for this defect was highlighted with 8 PM_m identified, resulting in an incidence of 35%. This was significantly higher than the 5% reported in the unrelated Swiss Caucasian population studied by the same group (Küpfer *et al.*, 1984d).

In a similar fashion, defective alleles were observed to be inherited in an autosomal recessive mode in PM_m in a study conducted with four extended Japanese families, a total of 56 subjects (Ward *et al.*, 1987). In this study it was not reported if the PM_m possessed the *CYP2C19*2* allele or the *CYP2C19*3* allele, however the importance in inheritance causing the defect was again highlighted with a greater than two-fold increase in the PM_m incidence in related subjects (40% compared with 18% in unrelated subjects, Ward *et al.*, 1987).

1.2.3 Drugs whose metabolism cosegregates with the (S)-mephenytoin hydroxylation genetic polymorphism

Many different classes of drugs have been found to be substrates for CYP2C19. Evidence is derived from either *in vitro* studies using human liver microsomes, or *in vivo* studies in which metabolism of the substrate of interest is impaired in PM_m. The following drugs have been observed to be metabolised at least in part by CYP2C19.

1. *Hexobarbital*:

Rac-hexobarbital has been used as a probe xenobiotic to examine changes in drug oxidation which occur as a result of disease states or environmental (Knodell *et al.*, 1988). The major route of metabolism of rac-hexobarbital is 3'-hydroxylation and subsequent oxidation to a 3'-keto derivative. Metabolism to the 3'-hydroxy and 3'-keto metabolites has been observed to occur at a slower rate producing lower plasma concentrations of these metabolites in PM_m when compared to EM_m (Knodell *et al.*, 1988). Additionally, the results of an *in vitro* cDNA expression study indicate that 3'-hydroxylation of both enantiomers of hexobarbital correlates closely with 4'-hydroxylation of (S)-mephenytoin and the content of P-450 human-2 (CYP2C19) cDNA (Yasumori *et al.*, 1990).

2. *Mephobarbital*

Rac-mephobarbital is a barbiturate (N-methyl derivative of phenobarbital) that possesses phenyl, ethyl, and methyl groups similar to rac-mephenytoin (Küpfer *et al.*, 1985). It is metabolised by both aromatic hydroxylation to 4'-hydroxymephobarbital, the major metabolite, and demethylation. It has been shown that the hydroxylation of mephobarbital to the 4-hydroxy derivative is impaired in PM_m, representing less than 1% of a 90mg dose compared with $10.9 \pm 1.9\%$ of the dose in EM_m (Küpfer *et al.*, 1985). There are no data on its use as a probe for CYP2C19 phenotyping.

3. *Propranolol*

Propranolol is a racemic ß-adrenoceptor antagonist used widely to treat angina, hypertension, prevention of migraine, cardiac arrhythmias and myocardial infarction. The elimination of this drug is rapid, and occurs via hepatic glucuronidation, side chain oxidation to naphthoxylactic acid (NLA), and ring oxidation to 4'-hydroxypropranolol (Ward *et al.*, 1989a). It was concluded that CYP2C19 is partially involved in the formation of NLA as the oral clearance of propranolol to this metabolite was 55% lower in PM_m when compared with both EM_m, EM_d (extensive metabolisers of debrisoquine) and PM_d (poor metabolisers of debrisoquine) (Ward *et al.*, 1989a). Conversely, the formation of the 4'-hydroxy derivative was 75% less in PM_d compared with EM_d and PM_m, indicating the involvement of CYP2D6 in this pathway (Ward *et al.*, 1989a).

4. *Moclobemide*

Moclobemide is a reversible monoamine oxidase A (MAOA) inhibitor used as an antidepressant. It is eliminated primarily by metabolism, N-oxidation to form Ro 12-5637, C-oxidation to form Ro 12-8095, and aromatic hydroxylation (Jauch *et al.*, 1990). An *in vivo* study in 7 PM_m and 8 EM_m examined the effect of the CYP2C19 polymorphism on moclobemide metabolism, and the inhibition by moclobemide of CYP1A2-, CYP2C19- and CYP2D6- mediated metabolic reactions of caffeine, rac-mephenytoin and sparteine, respectively (Gram *et al.*, 1995). It was observed that PM_m compared to EM_m cleared moclobemide at a significantly slower rate (16.1 compared to 43.2 $L.hr^{-1}$, $P = 0.0003$), translating into a significantly longer half-life (4.0 compared to 1.8hr, $P = 0.0003$). This result was due to the formation of the C-hydroxylated metabolite, Ro 12-8095 being mediated by CYP2C19. The authors noted that this is unlikely to be of clinical significance due to the small difference; however, this depends entirely on the therapeutic index of the drug which was not discussed by the authors. It was also noted that moclobemide inhibits CYP2C19 to varying degrees in subjects, with greater inhibition observed in EM_m. Moclobemide also causes an increase in the sparteine metabolic ratio, so that inhibition of CYP2D6 oxidation was inferred, whilst no conclusion could be made as to the effect on CYP1A2 oxidation.

5. *Imipramine*

Imipramine is a tricyclic antidepressant, used widely over the past 30 years. It is cleared by N-demethylation to desipramine, an active metabolite, and by hydroxylation to 2-hydroxy-imipramine and 2-hydroxy-desipramine. Previous *in vivo* studies have demonstrated a relationship between the hydroxylation reactions and the debrisoquine / sparteine genetic polymorphism (Bertilsson *et al.*, 1983, Brøsen *et al.*, 1986). This co-segregation was confirmed by *in vitro* human liver microsomal studies which showed that the major isoform mediating the hydroxylation of imipramine is CYP2D6 (Brøsen *et al.*, 1991).

Conversely, another *in vitro* study in human liver microsomes of Japanese subjects showed N-demethylation activity correlated with 4'-hydroxylation of (S)-mephenytoin at low, but not high substrate concentrations, with the conclusion that 50% of the high affinity component of N-demethylation is mediated by CYP2C19 (Chiba *et al.*, 1994). In agreement with these results, it has been observed *in vivo* that the N-demethylation of this drug is mediated partly by CYP2C19, as this pathway is decreased by approximately 50% in PM_m (Skjelbo *et al.*, 1991). However, it has been noted that these differences are not likely to be of clinical relevance, due to the anti-depressant and toxic effects being related to the sum of the concentrations of imipramine and desipramine in the blood (Gram, 1977), with the total steady-state level of imipramine and desimipramine not changed by variation in demethylation (Skjelbo *et al.*, 1991).

Due to the unavailability of (S)-mephenytoin as a probe drug for the phenotyping of subjects for the CYP2C19 genetic polymorphism, research has particularly focussed on omeprazole, diazepam and proguanil metabolism and their relationship to this genetic polymorphism in anticipation that an alternative probe drug can be found.

1.2.3.1 Omeprazole

Omeprazole (OPZ) is a substituted benzimidazole that is a potent inhibitor of gastric acid secretion through direct interactions with the H^+- K^+- adenosine triphosphate pump in the parietal cells of the stomach, and is widely used for the treatment of gastroesophageal reflux and peptic ulcers. It is eliminated primarily by hepatic oxidation to 5-hydroxy-omeprazole (H-OPZ) and omeprazole sulfone (OPZ-SFN) (Figure 4) (Regardh *et al.*, 1985).

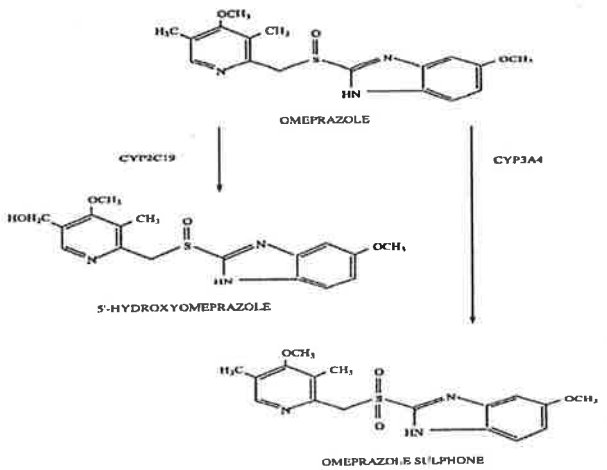


Figure 4: Human metabolism of omeprazole

In Vivo studies

There is substantial interindividual variability in the disposition of omeprazole. It has been shown that PM_m have higher maximum OPZ plasma concentrations, and a greater area under the plasma concentration-time curve (AUC) than EM_m after a week of 20mg OPZ administration daily; mean ± standard deviation 2.4 ± 0.1 compared with 0.5 ± 0.2 μM, and 9.2 ± 1.0 compared with 0.8 ± 0.2 $\mu mol.hr.L^{-1}$ (Andersson *et al.*, 1990).

In a subsequent study, 8 PM_m and 8 EM_m from a Korean population received 20mg OPZ followed by multiple blood sampling over 24hr (Sohn *et al.*, 1992b). An increase in both OPZ AUC and $t_{1/2}$, and a decrease in oral clearance of OPZ were observed to be significant

($P < 0.001$) in PM_m when compared to EM_m (Table 1). Of the metabolites, the AUC of OPZ-SFN significantly increased, and $t_{1/2}$ was significantly longer ($P < 0.001$) in PM_m compared with EM_m (Table 1), indicating partial involvement of CYP2C19 but also the involvement of other isoforms. Additionally, CYP2C19 was implicated as the main isoform involved in H-OPZ formation, as AUC and urinary excretion of this metabolite was significantly less ($P < 0.001$ and $P < 0.01$ respectively) in PM_m compared with EM_m (Table 1).

Table 1: Pharmacokinetic data of OPZ, H-OPZ and OPZ-SFN in EM_m (n=8) and PM_m (n=8) following a single oral dose of 20mg OPZ (adapted from Sohn *et al.*, 1992).

	OPZ		H-OPZ		OPZ-SFN	
	EM_m	PM_m	EM_m	PM_m	EM_m	PM_m
$t_{1/2}$ (hr)	1.4 ± 0.2	3.2 ± 0.2**	1.5 ± 0.2	3.4 ± 0.4*	2.5 ± 0.4	10.6 ± 0.9 **
AUC $(ng.(ml.hr)^{-1})$	749 ± 82.4	4480 ± 264**	491 ± 29.4	231 ± 27.3**	542 ± 91.8	2510 ± 139**
Cl_o $(ml.hr^{-1}kg^{-1})$	476 ± 64.2	59.5 ± 3.6**				

The data are mean ± S.E.; $t_{1/2}$ is half-life; AUC is area under plasma concentration-time curve of the metabolite from 0 - 10hr for OPZ and H-OPZ, and 0 - 12hr for OPZ-SFN; Cl_o is oral clearance. * indicates $P < 0.01$ compared with EM_m, ** indicates $P < 0.001$ compared with EM_m.

Similar observations were made in a study conducted with 12 Caucasian (6 PM_m, 6 EM_m) and 13 Chinese (5 PM_m, 8 EM_m) subjects who received 20mg OPZ (Andersson *et al.*, 1992) (Table 2). These data led to the conclusion that in different populations, the formation of H-OPZ and its elimination, and the elimination of OPZ-SFN co-segregate with the CYP2C19 genetic polymorphism.

Table 2: Pharmacokinetic parameters of OPZ, H-OPZ and OPZ-SFN in 12 Caucasian and 13 Chinese subjects (adapted from Andersson *et al.*, 1992).

	Caucasian			Chinese		
	EM_m	PM_m	P	EM_m	PM_m	P
OPZ						
AUC (μM.hr)	0.94 ± 0.35	11.1 ± 2.56	0.0001	2.62 ± 1.78	13.3 ± 5.59	0.005
$t_{1/2}$ (hr)	0.69 ± 0.41	2.30 ± 0.44	0.0003	0.75 ± 0.18	2.37 ± 0.21	0.0001
H-OPZ						
AUC (μM.hr)	1.52 ± 0.35	0.93 ± 0.31	0.19	1.58 ± 0.68	0.98 ± 0.21	0.16
$t_{1/2}$ (hr)	1.06 ± 0.36	3.18 ± 1.27	0.0001	1.00 ± 0.16	3.69 ± 1.00	0.0001
OPZ-SFN						
AUC (μM.hr)	0.53 ± 0.18	11.5 ± 2.24	0.0001	1.59 ± 1.12	14.3 ± 7.09	0.0001
$t_{1/2}$ (hr)	1.63 ± 0.30	7.38 ± 1.97	0.0001	2.85 ± 1.30	9.61 ± 4.01	0.002

Data is mean ± SD; AUC is area under the plasma concentration-time curve; $t_{1/2}$ is half-life.

In Vitro studies

Various *in vitro* studies have focussed on the identification of the human liver cytochrome P450 isoforms that mediate OPZ metabolism. The kinetics of formation of the major metabolites (H-OPZ and OPZ-SFN) in human liver microsomes were observed to be biphasic, indicating the involvement of at least two distinct enzymes. The high affinity component (V_{max1}) of 5-hydroxylation was found to correlate with the V_{max} of 4-hydroxylation of (S)-mephenytoin (r_s = 0.911, $P < 0.01$) (Chiba *et al.*, 1993a). Weaker correlations have also been found between the formation of H-OPZ and benzo(a)pyrene metabolism (r_s = 0.64, $P < 0.05$) and CYP3A content (r_s = 0.69, $P < 0.05$) in human liver microsomes (Andersson *et al.*, 1993). Additionally, chemical inhibition of H-OPZ formation by troleandomycin (CYP3A-specific inhibition) and racemic mephenytoin have led researchers to conclude that CYP2C19 plays the major role in the hydroxylation of OPZ, with CYP3A playing a minor role. These studies have also shown that CYP3A was the predominant isoform mediating the formation of OPZ-SFN (Andersson *et al.*, 1993).

Additionally, it must be noted that V_{max}/K_m (a measure of *in vivo* intrinsic clearance) for H-OPZ was approximately four-fold greater than that of OPZ-SFN (Andersson *et al.*, 1993). The authors suggested that these results predict that the oral clearance of OPZ *in vivo* in PM_m would be reduced, due to hydroxylation being the major pathway of clearance, which has been previously observed (Sohn *et al.*, 1992b).

Phenotyping studies using OPZ as a CYP2C19 probe

The concordance between *in vivo* and *in vitro* data for co-segregating metabolism has led researchers to suggest OPZ may be a suitable *in vivo* CYP2C19 phenotyping probe, replacing (S)-mephenytoin. A preliminary study in 14 Swedish Caucasians (5 rapid EM_m, 4 heterozygous EM_m (het EM_m) and 5 PM_m), who were given 20mg OPZ, showed that the relative mean plasma AUC of OPZ in rapid EM_m, het EM_m and PM_m were 1: 3.7: 20, which was significantly different for all groups ($P < 0.001$) (Chang *et al.*, 1995b). A significantly higher H-OPZ plasma C_{max} was observed in rapid EM_m and het EM_m compared with PM_m, 618 ± 205, 581 ± 144 and 143 ± 53nM, respectively ($P < 0.001$). Additionally, the OPZ/H-OPZ plasma concentration ratio which was determined 3 hours post-dose, correlated with the S/R mephenytoin ratio ($r_s = 0.94$, $P < 0.001$), with no overlap between the three groups. The authors concluded from this that OPZ was a suitable phenotyping probe.

A more extensive study conducted by the same researchers in 160 Swedish Caucasians of known (S)-mephenytoin phenotype involved the administration of 20mg OPZ and genotyping with respect to the most frequent mutations of *CYP2C19*, namely *CYP2C19*2* (m1) and *CYP2C19*3* (m2) (Chang *et al.*, 1995a). Observations were similar to the preliminary study, showing a significant correlation between the urinary metabolic ratio (MR) of OPZ and the urinary S/R mephenytoin ratio ($r_s = 0.63$, $P < 0.001$). Additional information from genotyping showed that EM_m heterozygous for the *CYP2C19*2* mutation had a higher OPZ MR and S/R mephenytoin ratio compared to EM_m homozygous for the wild-type allele (*CYP2C19*1*) ($P = 0.0001$). Nineteen of the 22 PM_m were homozygous

for the CYP2C19*2 mutation, and the other three were heterozygous, thus 41 of 44 mutant alleles were *CYP2C19*2*. Interestingly, one of the remaining 3 PM_m alleles was *CYP2C19*3*, previously found only in Orientals. Overall it was concluded that OPZ phenotype correlated with that of mephenytoin, and both phenotype assignments agreed well with CYP2C19 genotype assignment.

A similar study has been conducted in 27 Japanese subjects of 5 various CYP2C19 genotypes as follows (Ieiri *et al.*, 1996):

1. 10 homozygous subjects for wild-type (*CYP2C19*1/*1*)
2. 5 heterozygous subjects for *CYP2C19*2* mutation (*CYP2C19*1/*2*)
3. 5 heterozygous subjects for *CYP2C19*3* mutation (*CYP2C19*1/*3*)
4. 2 heterozygous subjects for *CYP2C19*2* and *CYP2C19*3* mutations (*CYP2C19*2/*3*)
5. 5 homozygous subjects for *CYP2C19*2* mutation (*CYP2C19*2/*2*)

Following administration of a single 20mg oral dose of OPZ, analysis of venous blood samples revealed the mean clearance of OPZ was 1370, 333, 359, 70.8, and 89.5 $ml.hr^{-1}.kg^{-1}$ for the genotypes 1, 2, 3, 4, and 5 respectively. Plasma AUC ratios of OPZ/H-OPZ were 1, 2.8, 3.4, 16, and 17.2 for the genotype classifications 1, 2, 3, 4, and 5, respectively, and this related to the OPZ/H-OPZ serum concentration ratios reported, which were 1, 3, 4, 18.8 and 20.3, respectively. Overall, the significant differences in OPZ pharmacokinetic parameters observed between subjects with at least one wild-type allele (genotypes 1, 2, and 3) and subjects with two mutated alleles (genotypes 4 and 5) indicate that OPZ metabolism is impaired in subjects without functional CYP2C19. The authors stated that impairment in OPZ metabolism was explained by differences in (S)-mephenytoin metabolic capacity, and was indicative of a CYP2C19 gene-dose effect in OPZ metabolism.

More recently, two studies have also investigated the correlation between the hydroxylation of OPZ and other CYP2C19 probe drugs (Kortunay *et al.*, 1997, Herrlin *et al.*, 1998). Kortunay and colleagues phenotyped 116 Turkish subjects by administration of

20mg omeprazole followed by a single blood sample 3hr later. PM_m were classified as those with log OPZ/H-OPZ ≥ 0.8. Thirty-one subjects of this group (28 EM_m and 3 PM_m) were also phenotyped with both rac-mephenytoin (100mg) and proguanil (200mg) one week later. Following drug administration, 8hr urine samples were collected and analysed for the S/R mephenytoin ratio and proguanil/cycloguanil (PG/CG) ratio. It was observed that there was a statistically significant, although not a strong, correlation between the OPZ/H-OPZ ratio and both the S/R mephenytoin ($r_s = 0.38$, $P < 0.03$), and PG/CG ratios ($r_s = 0.35$, $P < 0.05$), respectively. Additionally, all 3 PM_m identified with the OPZ/H-OPZ ratio were also phenotypic PM_m with S/R mephenytoin and PG/CG. The authors noted that no clear antimode of OPZ/H-OPZ ratio could be identified in these subjects, and suggested that this may be due to different CYP2C19 activity in the Turkish population, or due to the influence of CYP3A4 on the metabolic ratio. Thus it was concluded that further combination phenotyping and genotyping studies were required to investigate the appropriate antimode when using OPZ as a probe.

The second study investigated a correlation between OPZ/H-OPZ ratios and S/R mephenytoin ratios in Bantu Tanzanians (Herrlin *et al.*, 1998). Sixty-two subjects were phenotyped on separate occasions with both 100mg racemic mephenytoin and 20mg OPZ. The S/R mephenytoin and OPZ/H-OPZ ratios were calculated following analysis of an 8hr urine sample and a single blood sample, respectively. PM_m were classified as those having a S/R ratio of greater than 0.8 and OPZ/H-OPZ greater than 7. It was observed that the correlation between the two ratios was significant ($r_s = 0.59$, $P < 0.01$), and all PM_m identified by the S/R ratio were also PM_m when phenotyped using OPZ. Genotyping for *CYP2C19*1*, **2* and **3* mutations revealed that of those subjects classified as phenotypic PM_m with OPZ, only 3 of the 4 were genotypic PM_m. Additionally, of the 8 phenotypic PM_m identified with racemic mephenytoin, only 3 were genotypic PM_m. Thus, the authors concluded that the correlation between phenotype and genotype was poor. Several explanations for these observations were offered; firstly, another *CYP2C19* mutation not tested for could be present in this population. Thus, apparent wild-type alleles could carry *CYP2C19*4*, **5* or **6* mutations, which although rare in other populations studied could

occur with a higher incidence in Tanzanians. Furthermore, the hydroxylation of OPZ and oxidation to OPZ-SFN is also mediated by CYP3A4 (Yamazaki *et al.*, 1997). The influence of this isoform has not been assessed, and it was suggested that dietary influences, for example aflatoxins in African nuts which have been shown *in vitro* to be partially metabolised by CYP3A4 (Gallagher *et al.*, 1996), could cause the discrepancy through interaction with CYP3A4. It was therefore concluded that further research was required in this population, particularly to identify the incidence of other *CYP2C19* mutations and applicable antimodes for the separation of EM_m and PM_m groups.

The first study to use OPZ as a probe following collection of an 8hr urine samples was in 100 North Indian subjects (Lamba *et al.*, 1998a). The measure used to assign phenotype was the log hydroxylation index, defined as the logarithm of the ratio of administered OPZ (20mg, 57.9 µmol) to H-OPZ (µmol) excreted in urine. This calculation was deemed appropriate due to the lack of detectable OPZ in urine over 8hr. The antimode separating EM_m and PM_m in this population was observed to be a log hydroxylation index of 1.7. Eleven subjects (11%) were identified as PM_m. This incidence was much lower than the 20% previously observed in a Bombay Indian population phenotyped with racemic mephenytoin (Doshi *et al.*, 1990). The difference in the incidence of PM_m between the populations could be explained by the different ethnic groups in India, and is similar to the differences observed between Kenyan and Tanzanian black populations in Africa. It does not indicate that the log hydroxylation index in urine is inappropriate for CYP2C19 phenotyping. However, genotyping was not conducted as part of this study; therefore, the correlation between phenotype and genotype has not been investigated. This is required to establish a more appropriate statistical evaluation of the antimode using this phenotypic measure.

The above evidence suggests that in both Caucasian and Oriental populations, OPZ could replace (S)-mephenytoin as a phenotyping probe. However, a potential problem of routine phenotyping exists, as blood samples are required to establish the metabolic ratio of OPZ. This is an invasive technique in comparison to a 8hr urine sample usually utilised.

Additionally, this sample must be taken at 3hr post-administration, as at earlier or later times levels of H-OPZ or OPZ, respectively, are too low to be measured. The possibility of using H-OPZ concentrations in an 8hr urine sample exists, however requires validation via investigation of the correlation between the genotype and phenotype. Thus, a more appropriate replacement probe is yet be found.

1.2.3.2 Diazepam

Diazepam (DZ) is a benzodiazepine used widely as a muscle relaxant, sedative, anxiolytic and anticonvulsant. It has a low hepatic clearance to two primary metabolites, 3'-hydroxy-diazepam (temazepam, TMZ) and N-desmethyldiazepam (nordiazepam, DMDZ) (Figure 5). DMDZ is pharmacologically active, and is hydroxylated at the 3' position to form oxazepam. The clearance of DZ has been found to be dependent on age, gender, smoking, liver disease, concomitant medication and also genetic variability (Greenblatt *et al.*, 1980).

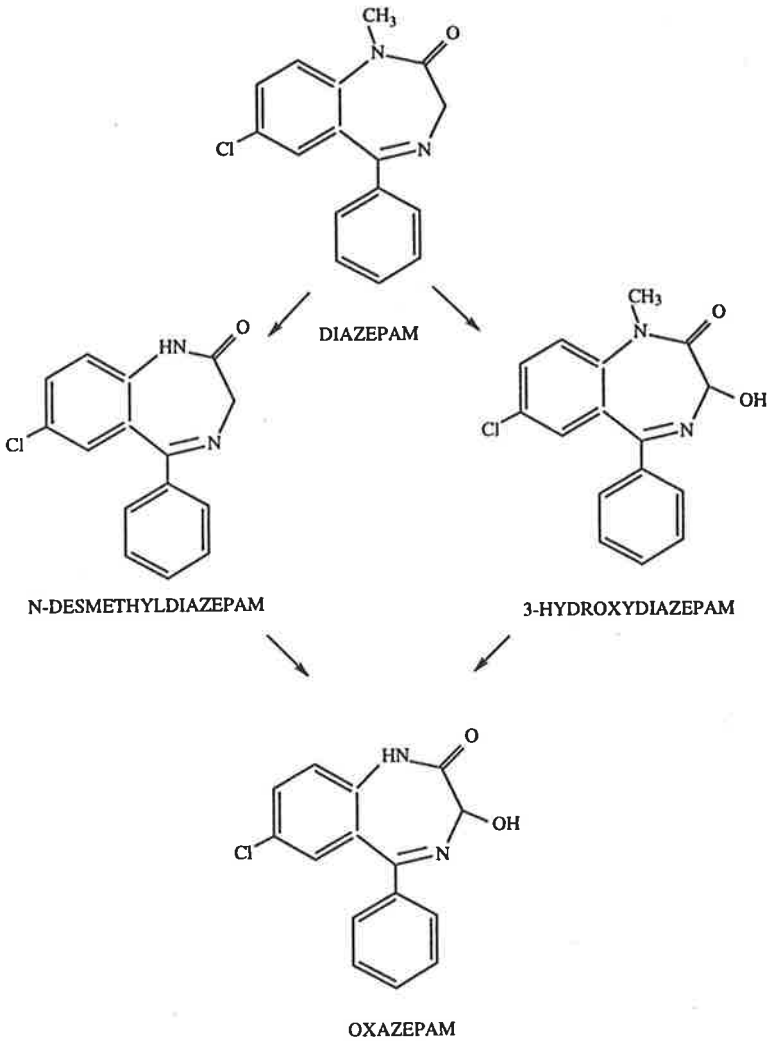


Figure 5: Metabolism of diazepam in humans (Jung *et al.*, 1997)

In the early 1980's, a study examined DZ kinetics in 12 Caucasian and 13 Oriental subjects after a 0.2mg.kg^{-1} intravenous dose (Ghoneim *et al.*, 1981). Of the kinetic parameters studied, no difference between racial groups was observed in the unbound fraction of DZ in plasma, elimination half-life and volume of distribution (corrected for body weight) at steady-state. Conversely, total body clearance was significantly higher in Caucasians than Orientals, 0.40 ± 0.03 versus 0.29 ± 0.03 ml.min^{-1}kg^{-1} ($P < 0.01$), and DMDZ concentrations were significantly higher in Orientals versus Caucasians ($P < 0.05$). It was from these preliminary findings that researchers then investigated the hepatic metabolism of DZ and how it differs in various populations.

Bertilsson and colleagues conducted a study in 16 Swedish Caucasians, which included 4 PM of debrisoquine (PM_d) and 3 PM_m, to examine the metabolism of DZ in relationship to either the debrisoquine or the (S)-mephenytoin genetic polymorphisms (Bertilsson *et al.*, 1989). The study involved separate oral administration of 10mg DZ and 10mg DMDZ and collection of blood samples for up to 18 and 28 days post-dose for DMDZ and DZ, respectively. No significant differences were observed between DZ and DMDZ kinetics in EM_d and PM_d. However in PM_m, the plasma clearances of both DZ and DMDZ were significantly less than in EM_m (mean ± SD, DZ: 12.0 ± 1.1 compared with 25.7 ± 5.4 $ml.min^{-1}$, P = 0.0008; DMDZ: 5.0 ± 0.9 compared with 11.0 ± 1.8 $ml.min^{-1}$, P = 0.0001). The decrease in plasma clearance translated into significantly longer plasma half-lives (DZ: 88.3 ± 17.2 compared with 40.8 ± 14.0hr, P = 0.0002; DMDZ: 128 ± 23 compared with 59.0 ± 16.8hr, P = 0001). It was therefore concluded that the metabolism of DZ via N-demethylation (to DMDZ) and of DMDZ via hydroxylation (to oxazepam) is related to the (S)-mephenytoin genetic polymorphism. These authors additionally reported anecdotal evidence that "many Hong Kong physicians routinely prescribe smaller diazepam doses for Chinese than for white Caucasians". This observation coincides with a higher incidence of PM_m in Oriental populations.

A similar study was conducted in 8 EM_m and 8 PM_m from a Chinese Han population (Zhang *et al.*, 1990). In contrast to the above findings of Bertilsson and colleagues, the disposition of DZ was found to be similar in EM_m and PM_m; however, the plasma concentration of DMDZ at 7, 14 and 24 days was significantly higher in PM_m compared with EM_m, as was the $t_{1/2}$ of DMDZ ($P < 0.02$). Additionally, $t_{1/2}$ of DZ for both phenotypic groups was similar to Caucasian PM_m and more than twice those in EM_m (the data of Caucasians came from (Bertilsson *et al.*, 1989). The authors concluded that these data demonstrate that the disposition of DZ, and thus the oral plasma clearance, is slower in the Chinese population, and is also independent of the (S)-mephenytoin hydroxylation phenotype. It was postulated that it is possible that the (S)-mephenytoin hydroxylase enzyme present in Chinese EM_m has a different substrate specificity in comparison to

Caucasians, so that it is unable to metabolise DZ, or only able to mediate metabolism at a slower rate.

An additional study in 9 Chinese subjects previously phenotyped with racemic mephenytoin and genotyped for *CYP2C19*2* and *CYP2C19*3* investigated the elimination of diazepam (Wan *et al.*, 1996). Similar to the results of Zhang and colleagues, the plasma elimination $t_{1/2}$ of DZ and DMDZ in phenotypic PM_m was significantly longer than EM_m, 101 ± 32.3 versus 34.7 ± 23.0hr (P = 0.001), and 220 ± 62.7 versus 103 ± 27.5hr (P = 0.05), respectively. Additionally, the clearance of DZ was significantly lower in phenotypic PM_m than in EM_m, 2.7 ± 0.9 versus 11.7 ± 6.5ml.min^{-1} (P = 0.0001).

In an effort to further classify which CYP450 isoforms are involved in DZ metabolism, *in vitro* studies in liver microsomes have been completed. Beischlag and associates showed that DMDZ and TMZ were the major oxidative metabolites of DZ in human livers (n=7), and that (S)-mephenytoin competitively inhibited the formation of DMDZ in rat livers with a K_i = 400μM but no inhibition was observed in human livers (Beischlag *et al.*, 1992). A more systematic *in vitro* investigation reporting the kinetics of formation of TMZ and DMDZ, and inhibition by chemicals and antibodies has been conducted (Andersson *et al.*, 1994). The kinetics of formation of both metabolites were described as "atypical and consistent with substrate activation". Additionally, activation by α-naphthoflavone (a CYP3A activator) was observed with a greater effect on TMZ, when compared with DMDZ formation. This indicated CYP3A was only partially involved in DMDZ formation. Chemical and antibody inhibition studies revealed that TMZ is formed mainly by CYP3A isoforms, whilst DMDZ is mediated by both CYP2C19 and CYP3A. However, the authors failed to pre-incubate diethyldithiocarbamate (DDC), a mechanism-based CYP2E1 inhibitor. Thus, the involvement of this isoform can not be ruled out.

Data from another *in vitro* human liver microsomal study in EM_m (n=3) and PM_m (n=3) reinforce the notion that the metabolism of DZ to DMDZ is complex (Yasumori *et al.*, 1994). No difference was observed in the formation of TMZ from DZ, but PM_m showed

significantly lower DMDZ formation from 20μM DZ when compared to EM_m ($P < 0.001$). The Lineweaver-Burk and Eadie-Hofstee plots for DMDZ formation in microsomes prepared from EM_m livers indicated that at least two CYP450 isoforms were involved in this metabolic pathway. However, from PM_m, these plots indicated involvement of a single isoform with a single component V_{max} and K_m. Another difference was noted when (S)-mephenytoin and anti-human CYP2C antibody inhibited DMDZ formation by 69% and 80%, respectively in a concentration dependent manner in EM_m, but not PM_m. Conversely, the formation of TMZ was not different between EM_m and PM_m, could not be inhibited by anti-human CYP2C antibody, but could be by anti-human CYP3A antibody. Significant correlations were observed between (S)-mephenytoin 4'-hydroxylation and DMDZ formation ($r = 0.95$, $P < 0.001$) and, the amount of CYP3A catalysing testosterone 6ß-hydroxylation and TMZ formation ($r = 0.81$). The researchers concluded that different CYP450 isoforms mediate DMDZ formation in EM_m and PM_m liver microsomes, with the low affinity enzyme lacking in PM_m, whilst CYP3A isoforms in both phenotypic groups mediate TMZ formation.

In summary, although the above research has shown that the N-demethylation of DZ to DMDZ is largely mediated by CYP2C19, other isoforms are also involved, such as CYP3A, making this metabolic pathway complex. For this reason, DZ may not be a suitable phenotyping probe to replace (S)-mephenytoin. However, this segregation of DZ metabolism with the (S)-mephenytoin genotype remains of clinical importance when prescribing this drug to patients of different phenotypic and/or genotypic groups.

1.2.3.3 Proguanil

Proguanil is an arylbiguanide which has been used since the 1940's as an prophylactic antimalarial agent. It is a prodrug, requiring aromatic cyclization by cytochrome P450 isoforms to produce the active dihydrotriazine metabolite cycloguanil (CG) (Figure 6). CG

inhibits plasmodial dihydrofolate reductase (Carrington *et al.*, 1951). This enzyme is responsible for regenerating tetrahydrofolate in the synthesis cycle of deoxythymidylate (dTMP) which is essential for the synthesis of DNA, and subsequently the life cycle of the malarial parasite (*Plasmodium falciparum*). Proguanil is also N-dealkylated to form 4-chlorphenylbiguanide (Figure 6); however, quantitatively this is a minor pathway. Approximately 60% of the total plasma clearance of proguanil remains unaccounted for, with 11.2 ± 4.2% excreted in urine as CG, 25% excreted in urine, and 10% excreted in faeces (Edstein *et al.*, 1988), whilst the overall urinary recovery of both proguanil and CG has been reported to range from 54 - 77% of the administered dose (Somogyi *et al.*, 1996).

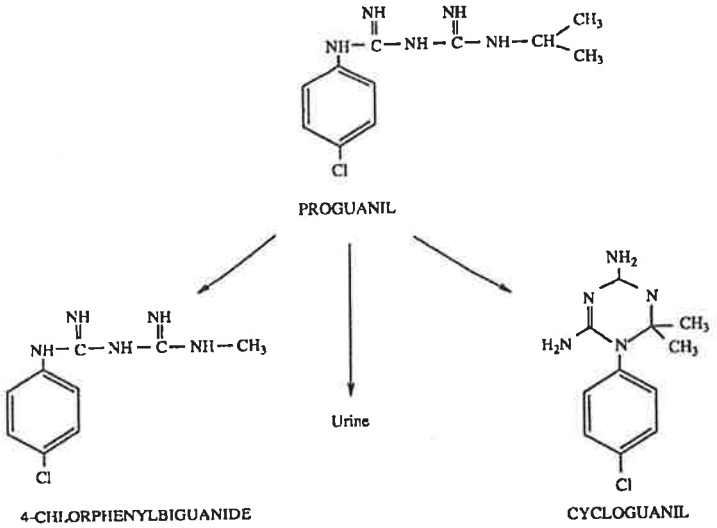


Figure 6: Metabolism of Proguanil (Birkett *et al.*, 1994)

Following observations that prophylaxis and treatment with proguanil can be effective in people with a reduced ability to metabolise proguanil to CG, it has been suggested that proguanil itself has antimalarial properties (Ward *et al.*, 1989b, Mberu *et al.*, 1995). This has been conclusively shown in a recent study in CYP2C19 EM_m (n=33) and PM_m (n=62) patients treated with proguanil in Vanuatu who had *Plasmodium falciparum* or *Plasmodium vivax* infections (Kaneko *et al.*, 1999). Therapeutic efficacy was assessed in terms of parasite density following treatment for 3 days with either 300 or 500mg proguanil, with no significant difference between EM_m and PM_m groups ($P > 0.5$). This

indicated that although PM_m had reduced blood concentrations of CG, the malarial infections were reduced to a similar degree in EM_m. Therefore, proguanil was concluded to have antimalarial activity. Its mode of action remains unknown, but it has been shown to be distinct from the inhibition of plasmodial dihydrofolate reductase that is inhibited by CG (Fidock *et al.*, 1998). Furthermore, 4-chlorphenylbiguanide has been shown to have poor antimalarial activity, such that the antimalarial activity of proguanil can not be attributed to formation of this metabolite (Watkins *et al.*, 1984, Eriksson *et al.*, 1989).

A genetic polymorphism in the enzyme mediating the activation of proguanil to CG was suggested when daily doses of proguanil failed to be prophylactic in some patients. A study of 135 subjects by Ward and associates observed a marked difference in the plasma concentrations of CG, such that following a single oral dose of 200mg proguanil while at steady-state, 10% of subjects formed less CG, 14ng.ml^{-1} compared with 86ng.ml^{-1}. These subjects had urinary PG/CG metabolic ratios of greater than 10, which indicates impairment of PG metabolism (Ward *et al.*, 1989b). Variable drug absorption was discounted as the cause of this difference in metabolic ratios, as similar summed concentrations of proguanil and CG were observed for all subjects, leading to the possibility of a genetic deficiency in metabolism. Similar findings were obtained in the following studies:

(i) A study in which a single oral dose of 2.5mg/kg proguanil was administered to 10 subjects, with 2 subjects (1 Kenyan, 1 European Caucasian) having significantly lower CG plasma concentrations, ranging from 21 - 62nM and 21 - 84 nM,when compared to the other 8 subjects, 21 - 220nM, ($P < 0.01$) (Watkins *et al.*, 1987).

(ii) A study in which 65 black Kenyans were given a single oral dose of 200mg proguanil, with 23 subjects (35%) having CG plasma concentrations too low to be quantified (less than 1ng.ml^{-1}) (Watkins *et al.*, 1990).

(iii) A study in which a single oral dose of 200mg proguanil was administered to 10 Caucasian subjects, 2 of whom had significantly lower plasma CG concentrations ($P < 0.05$) (Helsby *et al.*, 1990a).

In an endeavour to discover which CYP450 enzymes were mediating the metabolism of proguanil to CG, Ward and his colleagues conducted a panel study in eighteen subjects who had been previously phenotyped with reference to the (S)-mephenytoin (EM_m or PM_m) and sparteine (EM_s or PM_s) genetic polymorphisms (Ward *et al.*, 1991). This included four separate study groups who each received a single oral dose of 200mg proguanil;

I. 5 subjects were PM_m /EM_s.

II. 1 subject was PM_m /PM_s.

III. 3 subjects were EM_m /EM_s.

IV. 6 subjects were EM_m /PM_s.

It was observed that subjects in group I who were PM_m had significantly higher urinary PG/CG ratios than EM_m in group III, PG/CG range: 9.69 - 48.2, and 0.04 - 4.52, respectively, $P = 0.0043$. This suggested that the activation of proguanil was deficient in these subjects, and furthermore, that the formation of CG was mediated by CYP2C19. CYP2D6 was concluded to not play a role in CG formation since no difference in metabolic ratios was observed between PM_s and EM_s. However, the authors failed to exclude the possible role of other CYP450 enzymes.

Funck-Brentano and colleagues conducted a similar study, determining the hydroxylation index (HI) of rac-mephenytoin, the proguanil metabolic ratio (PG/CG), and the dextromethorphan metabolic ratio (a marker of CYP2D6 activity) in 25 subjects (Funck-Brentano *et al.*, 1992). Two subjects classified as PM_m and one subject classified as an intermediate metaboliser (IM_m) had urinary PG/CG ratios of greater than 50, while the remaining subjects who were designated EM_m had urinary PG/CG ratios of less than 10. It must be noted that larger studies are needed to further investigate this observation. No relationship was observed with the dextromethorphan metabolic ratio. Additionally, no correlation was observed between the urinary PG/CG ratio and the HI of rac-mephenytoin. Therefore, it was concluded that although the formation of CG co-segregates with genetically determined (S)-mephenytoin hydroxylation, these indices exhibit differences in variability, so that there is no direct correlation.

Another study has demonstrated co-segregation of metabolism of proguanil and (S)-mephenytoin (Brøsen *et al.*, 1993). It involved administration of a single oral dose of 100mg proguanil to 35 Vietnamese subjects who had been phenotyped previously with 100mg rac-mephenytoin and 100mg sparteine. The recoveries of CG and 4-chlorphenylbiguanide as a percentage of the dose were 1.6 and 0.4% respectively in the 8 PM_m of (S)-mephenytoin, and 5.8 and 1.9% respectively in the 26 EM_m ($P < 0.001$). No statistical relationship existed between the indices of proguanil metabolism and the sparteine metabolic ratios in the subjects. It is important to note that PM_m are still able to form small amounts of CG when administered proguanil; therefore, it has been implied that other CYP450 enzymes other than CYP2C19 mediate the metabolism of proguanil (Wright *et al.*, 1994). This will be discussed more fully below.

1.2.4 HPLC assay artifacts, assignment of antimodes separating EM_m and PM_m, and the incidence of proguanil and (S)-mephenytoin genetic polymorphisms

To further investigate the co-segregation of the proguanil and (S)-mephenytoin genetic polymorphism, many studies have been conducted to observe the incidence of both polymorphisms in various populations. Some problems arise with these studies due to the use of different urinary metabolic ratio "cut-off" points for assigning a proguanil PM_m, as no clear consensus on the antimode currently exists. Most phenotyping studies conducted with proguanil use an antimode of 10 to separate PM_m from EM_m (refer to table 3). However, the basis for this has never been substantiated, and therefore, further research is needed in a larger group of subjects, which should include greater numbers of PM_m, in order to determine the exact relationship between the number of mutant alleles and the proguanil metabolic ratio. When rac-mephenytoin is utilised as the probe, either the urinary S/R mephenytoin ratio or the hydroxylation index (HI, described on page 8) can

assign phenotypic status. However, caution must be taken when using the S/R mephenytoin ratio, as incorrect phenotyping can result due to the formation and excretion of an acid-labile metabolite in EM_m which can be converted back to (S)-mephenytoin after acid treatment of urine prior to analysis (Wedlund *et al.*, 1987).

Zhang and associates studied the reproducibility of the S/R mephenytoin ratio under conditions commonly used to handle urine samples (Zhang *et al.*, 1991). They observed that the S/R mephenytoin ratio in EM_m increased significantly after storage of the urine at -20°C. After 1, 3, 6, and 24 months the mean ratio increased by 6.9, 20, 177, and 216%, respectively. There was no change in the ratio of PM_m after 24 months, and there was no relationship between the pH of the urine and the percentage change in the S/R ratio. The stability of the ratio varied between EM_m leading the authors to suggest that there may be a difference in the extent to which this acid-labile metabolite is produced and its stability. They concluded that overestimation of the frequency of PM_m due to the interference of this metabolite can be avoided by researchers taking the following precautions. If the S/R mephenytoin ratio cannot be measured within 1 month of collection, extraction of the samples with dichloromethane should be performed immediately. This organic extract, which does not contain the polar acid-labile metabolite, can later be analysed. Alternatively, clarification of the phenotype assigned can be performed by treating the urine with 12M hydrochloric acid as the ratio will not increase in the true PM_m.

Another problem faced by researchers in assigning the frequency of a phenotype is the often low numbers of subjects used in studies. This means that different frequencies may be observed when a larger population is studied. On account of these factors it is only possible to observe general trends from data of these studies (Table 3). Firstly, the proguanil PM_m incidence is much higher in Thai, Kenyan and Khmer subjects than in the Caucasian population. The incidence in the New Zealand population was observed to be intermediate, which the authors suggested could be explained by the large degree of inter-racial marriage, as 97% of Maori subjects reported European ancestry (Wanwimolruk *et al.*, 1995a). Nevertheless, due to the prevalence of malaria in the countries where high

incidence of PM_m is observed, a greater possibility of clinical treatment failure with proguanil may exist. Similar to proguanil, the PM_m incidence when phenotyped with (S)-mephenytoin is higher in Asian populations when compared to Caucasians, reinforcing the suggestion of similar genetic control of the polymorphisms. It is interesting that the black Shona population of Zimbabwe exhibited similar PM_m incidence to Caucasians, using (S)-mephenytoin as a probe, but different PM_m incidence than that of a black Kenyan population (Watkins *et al.*, 1990), when proguanil was used as a probe. Additionally, a study phenotyping different Chinese populations with rac-mephenytoin found a difference between the incidence of PM_m, although this difference was not statistically significant (Xiao *et al.*, 1997). Therefore, it seems necessary and mandatory to specify the ethnic origin of the population.

Table 3 : The incidence of proguanil and (S)-mephenytoin PM_m phenotype in various populations.

Population	PG/CG antimode	Proguanil PM incidence	(S)-meph HI or S/R ratio	(S)-meph PM incidence	Reference
Kenyan	PM >10	35% (n = 65)			(Watkins *et al.*, 1990)
Tanzanians			S/R > 0.9	4.6% (n = 216)	(Skjelbo *et al.*, 1996)
Tanzanians			S/R > 0.8	7.5% (n = 106)	(Herrlin *et al.*, 1998)
Tanzanians			S/R > 0.9	3.6% (n = 195)	(Bathum *et al.*, 1999)
Shona Zimbabwe			S/R > 0.9	4% (n = 103)	(Masimirembwa *et al.*, 1995)
New Zealand Maori	PM > 10	7% (n = 43)			(Wanwimolruk *et al.*, 1995a)
South Pacific Polynesian	PM > 10	13.6% (n = 59)			(Wanwimolruk *et al.*, 1998)
Estonian			S/R > 0.9	3.9% (n = 156)	(Kiivet *et al.*, 1993)
Russian				2.3% (n = 218)	(Marandi *et al.*, 1997)
East Greenlanders			S/R > 0.9	9.3% (n = 272)	(Clasen *et al.*, 1991)
West Greenlanders			S/R > 0.9	2.9% (n = 166)	(Clasen *et al.*, 1991)
Khmer	PM > 10	18% (n = 87)			(Wanwimolruk *et al.*, 1995b)
Japanese			S/R = 0.95	18 - 25% (n = 100)	(Nakamura *et al.*, 1985)
Chinese			S/R > 1.00	15% (n = 137)	(Bertilsson *et al.*, 1992)
Chinese Han			S/R > 0.76	19.8% (n = 101)	(Xiao *et al.*, 1997)
Chinese Bai				13.4% (n = 202)	
Vietnamese			S/R = 0.79	22% (n = 37)	(Brøsen *et al.*, 1993)

Korean			HI > 2.90	13% (n = 206)	(Sohn *et al.*, 1992a)
Indian			S/R > 0.9	20.8% (n = 48)	(Doshi *et al.*, 1990)
Thai	PM > 10	18% (n = 170)			(Edstein *et al.*, 1994)
Caucasian			Not available	3.5% (n = 2209)	(Reviriego *et al.*, 1993)
Caucasian	PM > 9.0	3% (n = 99)			(Hoskins *et al.*, 1998)
British	PM > 9.0	10% (n = 135)			(Ward *et al.*, 1989b)
French	PM > 10	8.3% (n = 24)	HI > 1.49	8.3% (n = 24)	(Funck-Brentano *et al.*, 1992)
American (Tennessee)			S/R > 0.6	2.6% (n = 156)	(Wedlund *et al.*, 1984)
Jewish (Israeli)			S/R > 0.8	2.9% (n = 140)	(Sviri *et al.*, 1999)

1.2.5 Enzymic Bases of proguanil and (S)-mephenytoin genetic polymorphisms

1.2.5.1 Pharmacokinetic Studies

PM_m of either (S)-mephenytoin or proguanil display different pharmacokinetics due to catalytically inactive enzymes. Helsby and associates performed a pharmacokinetic study of proguanil in 10 healthy volunteers and observed that in 3 PM_m, the oral clearance of proguanil was decreased when compared to the other subjects, 443.7 ± 172.3 versus 858 ± 482ml.min^{-1}, and the half-life of proguanil was increased from 17.6 to 31.4hr. Due to the decrease in oral clearance of proguanil, the maximum plasma concentration of CG was observed to decrease from 141 ± 45.2 to 44.2 ± 15.1ng.ml^{-1} (Helsby *et al.*, 1990a). Another study involved the oral administration of 100mg proguanil to 9 subjects with

multiple blood sampling over 72hr, and up to 96hr in 2 subjects with low PG/CG metabolic ratios (Somogyi *et al.*, 1996). It was observed that the partial clearance of proguanil to CG ranged from 0.41 to 10.1L.hr^{-1}, and was almost abolished in some subjects. The authors concluded that the difference in clearance to CG was the predominant factor causing a three-fold range in AUC values of proguanil (3.3 - 9.5 mg.L^{-1}hr), as there was little observable variation in renal excretory clearance of proguanil (6.6 - 10.3L.hr^{-1}). Additionally, it was shown that a relationship exists between the partial metabolic clearance of proguanil to CG and the urinary metabolic ratio ($r^2 = 0.96$, $P < 0.0001$), indicating that this ratio is a valid index from which to quantitate the clearance of proguanil to CG.

An investigation of the pharmacokinetics of mephenytoin after repeated doses in 4 normal subjects and one PM_m showed that the urinary recovery of 4'-OH-meph over 5 days was lower in the PM_m, 88 μmol compared with 3211 ± 336 μmol in the normal subjects (Küpfer *et al.*, 1984a). Another study of (S)-mephenytoin pharmacokinetics was conducted in 8 EM_m and 6 PM_m subjects (Wedlund *et al.*, 1985). For EM_m and Pm_m, the elimination half-life of (S)-mephenytoin was 2.1 ± 0.9 and 63 ± 33 hr, and the oral clearance was 4.7 ± 2.8 and 0.029 ± 0.007 L.min^{-1}, respectively. These data demonstrate that the disposition of mephenytoin is altered significantly ($P < 0.05$) due to the (S)-mephenytoin genetic polymorphism.

The differences in pharmacokinetics of mephenytoin in PM_m when compared with EM_m are attributable to different liver enzyme kinetics as seen with *in vitro* research. Meier and his associates conducted an *in vitro* study of (S)-mephenytoin kinetics in human liver microsomes of PM_m (n=2) and EM_m (n=8) They found that in the PM_m the K_m was higher (150.6 and 180.6 compared with 37.8 ± 9.6μM (S)-mephenytoin), while the V_{max} was lower (0.76 and 0.69 compared with 4.85 ± 1.65nmol 4'-OH-meph.mg protein^{-1}hr^{-1}) (Meier *et al.*, 1985a). It is interesting to note that if the hydroxylation of (S)-mephenytoin was catalysed entirely by CYP2C19, then microsomes of PM_m which contain the aberrant form

of this enzyme, which is catalytically inactive, would be unable to form any 4'-OH-mephenytoin. Therefore, these data indicate that other CYP450 enzymes are involved.

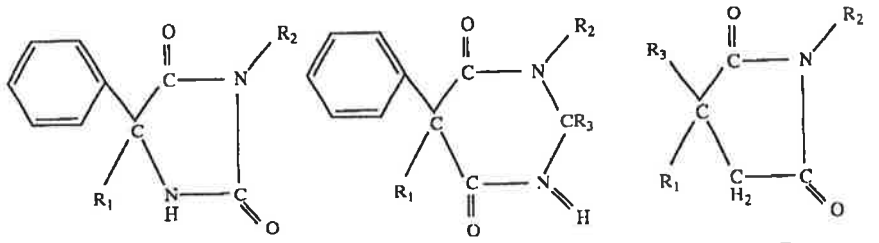
Similar *in vitro* studies of proguanil kinetics have been conducted with human liver microsomes (n=4) (Birkett *et al.*, 1994). The observed K_m ranged from 35 - 183 μM (mean ± SD, 96 ± 52) and the V_{max} ranged from 241 - 1772 pmol.mg protein^{-1}.hr^{-1} (770 ± 538). Another *in vitro* study of proguanil metabolism in microsomes from three human livers revealed the K_m to be relatively constant (21.8 - 29.6 μM), whilst the V_{max} was variable (90 - 492 pmol.mg protein^{-1}.hr^{-1}) (Helsby *et al.*, 1990b).

Another indication as to whether the *in vitro* metabolism is mediated by single or multiple enzymes is an Eadie-Hofstee plot (V_{max} / initial substrate concentration versus V_{max}). If this plot is linear it is deduced that one enzyme is involved. However, it must be remembered that if two enzymes mediating a reaction have similar K_m values, the Eadie-Hofstee plot would appear to be linear. Therefore, simple consideration of linear plots obtained for proguanil (Birkett *et al.* 1994) and (S)-mephenytoin (Chiba *et al.* 1993b) does not rule out the involvement of two enzymes.

1.2.5.2 Chemical and Immunological Inhibition Studies

Chemical and immunological inhibition studies have been conducted to observe the effect of common CYP450 enzyme specific substrates on the metabolism of proguanil and (S)-mephenytoin. With reference to the metabolism of (S)-mephenytoin, this area has not been well investigated with only one major study being conducted (Hall *et al.*, 1987). Barbiturates, hydantoins and succinimides which contain a similar chiral carbon to (S)-mephenytoin were observed to have varying ability to cause inhibition of (S)-mephenytoin metabolism. In figure 7, the drugs which have solid underlines were classified as strong inhibitors, those with dashed underlines were weaker and those with no underlines did not

inhibit. The degree of inhibition was related to the substitution of different atoms at the R_2 locus. For example, primidone, phenobarbital and phenytoin, which have a hydrogen atom at this position were not inhibitors of (S)-mephenytoin metabolism. However, if an ethyl or methyl derivative (also known as an aryl residue) was substituted for the hydrogen, competitive inhibition of some form was established. The authors concluded that in the case of these drugs, a necessary requirement for inhibition of the CYP2C19 enzyme is an "aryl residue α to the carbonyl carbon of an N-alkyl lactam of a five or six membered ring" (Hall *et al.*, 1987). It is important to note that this requirement is not able to be extrapolated to other drugs which are structurally unrelated.



HYDANTION

	R_1	R_2
Mephenytoin	C_2H_5	CH_3
Ethotoin	H	C_2H_5
PEH	C_2H_5	H
Phenytoin	C_6H_5	H
N_3-Methylphenytoin	C_4H_5	CH_3

BARBITURATE

	R_1	R_2	R_3
Mephobarbital	C_2H_5	CH_3	=O
N^4-Ethylphenobarbital	C_2H_5	C_2H_5	=O
N^1-Methylprimidone	C_2H_5	CH_3	H
N^1-Ethylprimidone	C_2H_5	C_2H_5	H
Phenobarbital	C_2H_5	H	=O
Primidone	C_2H_5	H	H

SUCCINIMIDE

	R_1	R_2	R_3
Methsuximide	C_6H_5	CH_3	CH_3
Phensuximide	C_6H_5	CH_3	H
Ethosuximide	C_2H_5	H	CH_3

Figure 7: Chemical Structures of drugs related to (S)-mephenytoin (Hall *et al.*, 1987).

Among other drugs studied, diazepam gave partial competitive inhibition (Hall *et al.*, 1987) indicating that a substrate-enzyme-inhibitor complex formed which retained some catalytic activity. This fits well with *in vivo* observations which found that the N-demethylation of DZ and the hydroxylation of DMDZ is related to the (S)-mephenytoin genetic polymorphism (Bertilsson *et al.*, 1989, as described on p 23). Additionally ketoconazole (an anti-fungal agent, CYP3A4 inhibitor), which directly interacts with the heme group of the enzyme showed linear mixed inhibition, so that the substrate-enzyme-inhibitor complex retained no catalytic activity. Sparteine failed to inhibit 4'-OH-meph

formation, indicating that CYP2D6 does not mediate the reaction. PEH also caused no inhibition. However, Hall stated that an interaction of PEH with CYP2C19, and subsequent 4-hydroxylation of PEH, is possible if this drug is preferentially available to CYP2C19. Warfarin was concluded to be a competitive inhibitor of (S)-mephenytoin metabolism *in vitro*, but Hall pointed out that the high K_i value in relation to the K_m for 4'-OH-meph formation (1183 and 213 μM, respectively) suggests that warfarin is not a substrate for the CYP450 enzyme(s) involved in (S)-mephenytoin metabolism.

Another chemical inhibition study reported that proguanil competitively inhibited 4'-OH-meph formation, providing more evidence that the metabolism of proguanil and (S)-mephenytoin co-segregate (Meier *et al.*, 1985a). A smaller study conducted by Inaba and associates focused on the inhibition observed with a range of pharmacological agents. These chemicals included sedatives, mono-amine oxidase inhibitors and ß-adrenoceptor blockers, and they showed that from these respective classes, diazepam, tranylcypromine and propranalol were inhibitors of 4'-OH-meph formation, with K_i values of 100μM, 8μM and 112μM, respectively (Inaba *et al.*, 1985).

The major immunological study investigating (S)-mephenytoin *in vitro* metabolism involved incubating antibodies directed to the CYP3A and CYP2C enzymes in Japanese human liver microsomes (Yasumori *et al.*, 1993). It was observed that anti-P450 human 2-IgG inhibited 4'-OH-meph formation in microsomes from EM_m by up to 80%, confirming that the enzyme involved belonged to the CYP2C subfamily. The fact that this inhibition was not complete may indicate that another isoform mediates the metabolism of (S)-mephenytoin, or may be due to non-specific interaction of the antibody with CYP2C19, so that some enzyme remains free to metabolise substrates. CYP3A was not involved, as the antibody directed towards this enzyme caused no inhibition. Therefore, it was concluded that the formation of 4'-OH-meph is mediated by CYP2C19.

Similar studies investigating the chemical inhibition of proguanil metabolism *in vitro* have been conducted. Helsby and associates observed that rac-mephenytoin was a competitive

inhibitor, mean K_i = 22μM (range 2.4 - 48.7) (Helsby *et al.*, 1990b). They noted that the K_i values were similar to the K_m of (S)-mephenytoin, 37.8 ± 9.6μM, previously observed (Meier *et al.*, 1985a), which is implied by the definition of a competitive inhibitor. This suggests co-segregating routes of metabolism. They also observed that sparteine (metabolised by CYP2D6) and tolbutamide (metabolised by CYP2C9) failed to inhibit the metabolism of (S)-mephenytoin.

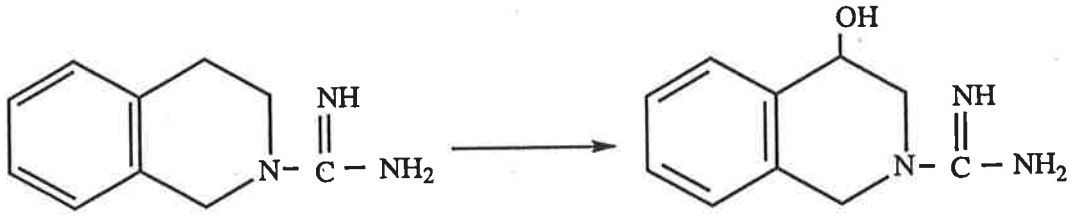
Birkett and colleagues conducted inhibition studies and correlations of proguanil activation with various chemicals and antibodies directed towards CYP450 enzymes (Birkett *et al.*, 1994). In doing so, they investigated the role of CYP3A in the pathway, finding that troleandomycin, whose metabolism is mediated by this enzyme, was a competitive inhibitor. Up to 70% inhibition of proguanil metabolism was observed in those livers with high CYP3A content, while in those with a low content, a maximum of 25% inhibition was found. This indicated that CYP3A is involved, but the degree of involvement seems to be dependent on the amount of the enzyme within each liver. Significant correlations of CG formation with CYP3A activity, omeprazole hydroxylation, benzo(a)pyrene, and caffeine 8-oxidation (r = 0.643, 0.882, 0.642 and 0.659, respectively) have been observed (Birkett *et al.*, 1994). These chemicals / drugs have their metabolism mediated wholly or substantially by CYP3A. Therefore at least *in vitro*, proguanil metabolism is likely to be mediated by CYP2C19, as shown by competitive inhibition by mephenytoin. This pathway must also involve other CYP450 enzymes including CYP3A, hence co-segregation with (S)-mephenytoin hydroxylation may occur, but not in a 1:1 ratio; that is all the enzymes involved in proguanil metabolism are not necessary all involved in the 4'-hydroxylation of (S)-mephenytoin.

Following consideration of the research reported above regarding the co-segregating metabolism of proguanil and (S)-mephenytoin, several gaps exist. Firstly, the *in vitro* evidence states that proguanil metabolism is mediated by CYP2C19 and CYP3A4, whilst little information regarding metabolism of (S)-mephenytoin by CYP450 enzymes other than CYP2C19 has been reported. In order for proguanil to have a potential role as a

CYP2C19 phenotypic probe *in vivo*, further investigation of the similarities in CYP450 enzyme metabolism is required. In addition to the *in vitro* studies, although *in vivo* studies have shown that PM identified with administration of rac-mephenytoin are also PM when identified with proguanil, no direct correlation between the two metabolic ratios has been shown. This indicates that factors other than CYP2C19 activity influence the metabolism of either or both proguanil and (S)-mephenytoin. Furthermore, no statistical evaluation of the appropriate PG/CG MR antimode to assign CYP2C19 phenotypic status has been reported, rather visual inspection of histograms has been used. Both of these issues need further investigation with a combined phenotyping, using proguanil as the probe drug, and CYP2C19 genotyping study.

1.3 Debrisoquine / Sparteine

Debrisoquine is a drug commonly used as a phenotypic probe for the CYP2D6 genetic polymorphism. Clinically it has been used as an antihypertensive due to its adrenergic receptor blocking qualities. It is metabolised primarily via ring hydroxylation to form the 4-hydroxy derivative (Figure 8) (Mahgoub *et al.*, 1977). This metabolism is mediated by CYP2D6, and as such, the formation of 4-hydroxy-debrisoquine is under polygenic control, and is reduced substantially in the PM_d, 2% of the debrisoquine dose compared with 30 - 40% in EM_d (Idle *et al.*, 1979).



DEBRISOQUINE 4-HYDROXYDEBRISOQUINE

Figure 8: Metabolism of Debrisoquine (Mahgoub *et al.*, 1977)

Sparteine is a quinolizidine alkaloid which has been used as an antiarrhythmic and uterine stimulating drug (Eichelbaum *et al.*, 1986). When administered via oral or intravenous routes, two main metabolites are found in the urine, 2- and 5-dehydrosparteine (Figure 9) (Eichelbaum *et al.*, 1979). In a study of 38 Caucasian (Swedish and German) subjects it was observed that there was a relationship between the ability of a subject to form these metabolites and the ability to form the 4-hydroxy metabolite of debrisoquine, $r_s = 0.91$, $P < 0.001$ (Eichelbaum *et al.*, 1982). Therefore, it was concluded that the metabolism of the drugs was under the control of a genetic polymorphism. The enzyme mediating the metabolism of debrisoquine and sparteine was identified as debrisoquine hydroxylase, later known as CYP2D6. Sparteine has been and remains a phenotypic probe to separate subjects classified as either EM_d or PM_d, the latter having a reduction in CYP2D6 metabolic capacity.

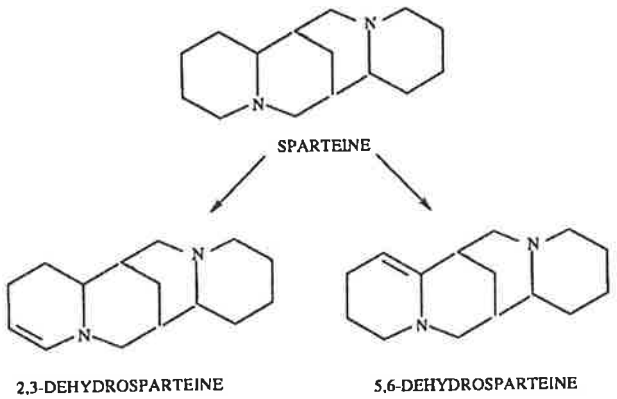


Figure 9: Metabolism of sparteine (adapted from Eichelbaum *et al.*, 1979)

Dextromethorphan is the other commonly used CYP2D6 phenotypic probe. Clinically, it is used as an antitussive. Following oral administration it is both O-demethylated to dextrorphan which is then N-demethylated to 3-hydroxymorphinan, and N-demethylated to 3-methoxymorphinan which is then O-demethylated to 3-hydroxymorphinan (Figure 10, Kerry *et al.*, 1994). The O-demethylation reactions have been observed to be cosegregated with debrisoquine hydroxylation (Schmid *et al.*, 1985, Küpfer *et al.*, 1986), and therefore it has been concluded that these reactions are mediated by CYP2D6.

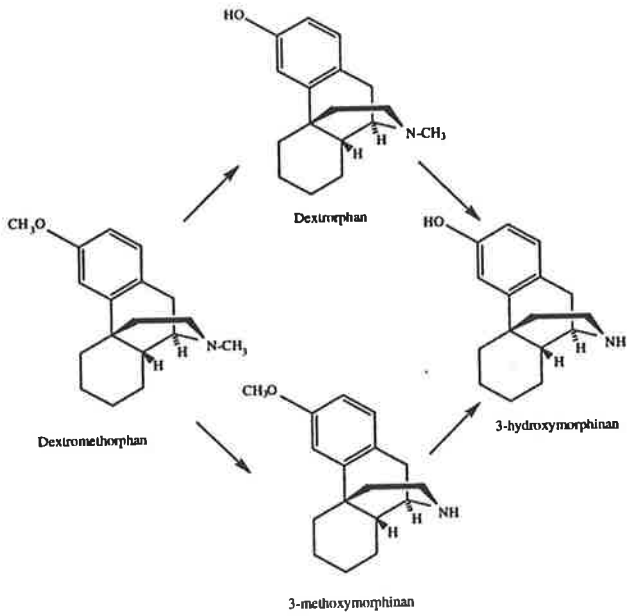


Figure10: Metabolism of dextromethorphan in humans.

1.3.1 The Debrisoquine / Sparteine oxidation genetic polymorphism

Similar to the discovery of the (S)-mephenytoin genetic polymorphism, it was through clinical observations that the oxidation of debrisoquine and sparteine were observed to be polymorphic. Newton and colleagues reported that in 6.8% of women in labour administered 100 - 200mg sparteine severe side-effects were experienced, including tetanic contractions of the uterus and placental abruptions which sometimes eventuated in the death of the baby (Newton *et al.*, 1966). Additionally, it was reported that during a study of the pharmacokinetics of a slow-release formulation of sparteine, two subjects experienced diplopia, blurred vision, dizziness and headache (Eichelbaum *et al.*, 1986). This was found to be due to reduced metabolism of sparteine so that the plasma concentrations were 3 - 4 fold higher in these subjects. In the case of debrisoquine, a study examined its metabolism in 94 subjects after a single 10mg dose (Mahgoub *et al.*, 1977). A metabolic ratio (MR) was calculated as follows;

$$MR = \frac{\%\text{ dose excreted in 8hr as unchanged debrisoquine}}{\%\text{ dose excreted in 8hr as 4 - hydroxydebrisoquine}}$$

It was observed that the 91 subjects had MR ranging from 0 - 8, these subjects were classified as EM_d, while the remaining 3 subjects had a MR above 21 leading to the classification of Pm_d.

Debrisoquine is a strongly basic drug (pK_a 12.5), and it has been shown that many other basic drugs are metabolised by CYP2D6, and hence are affected by the CYP2D6 genetic polymorphism (see section 1.3.3). Additionally, through panel-type studies it has been observed that drugs with different molecular structures and pharmacological actions are also affected (Price Evans, 1993b). Therefore, the clinical consequences of the CYP2D6 genetic polymorphism have made this area of research large.

1.3.2 The genetic basis of the Debrisoquine / Sparteine genetic polymorphism

Since the early 1980's the genetic basis of this polymorphism has been the focus of much research. The gene encoding for the CYP2D6 enzyme is located on the long arm of chromosome 22, region 22q11.2 - 12.2. As of October 1999, close to 30 allelic variations in the *CYP2D6* gene have been discovered. These include base pair changes / deletions, frameshifts, splicing defects, creation of stop codons and deletion of the entire gene, which result in either decreased or total loss of activity of the enzyme (Table 4). The most common mutations are *CYP2D6*3* (previously known as CYP2D6A), *CYP2D6*4A/B* (CYP2D6B), *CYP2D6*5* (CYP2D6D), *CYP2D6*7* (CYP2D6E), and *CYP2D6*6A/B* (CYP2D6T) (Daly *et al.*, 1996).

Table 4: Allelic variations of the *CYP2D6* gene ((Daly *et al.*, 1996) except where indicated)

Allele	Nucleotide changes	Trivial name	Effect	CYP2D6 activity
*CYP2D6*2*	$G_{1749}C$; $C_{2938}T$; $G_{4268}C$	CYP2D6L	Base pair changes	Decreased
*CYP2D6*2B* (Marez *et al.*, 1997)	$G_{119}A$; $G_{1749}C$; $C_{2938}T$; $G_{4268}C$		Basepair changes	Decreased
*CYP2D6*2C* (Marez *et al.*, 1997)	$C_{1127}T$; $G_{1749}C$; $C_{2938}T$; $G_{4268}C$		Basepair changes	Decreased
*CYP2D6*2D* (Marez *et al.*, 1997)	$G_{1749}C$; $T_{2558}C$; $C_{2938}T$; $G_{4268}C$		Basepair changes	Decreased
*CYP2D6*3*	A_{2937} deletion	CYP2D6A	Frameshift	None
*CYP2D6*3B* (Marez *et al.*, 1997)	$A_{1837}G$; A_{2937} deletion		Frameshift	None
*CYP2D6*4A*	$C_{188}T$; $C_{1062}A$; $A_{1072}G$; $C_{1085}G$; $G_{1749}C$; $G_{1934}A$; $G_{4258}C$	CYP2D6B	Splicing defect	None
*CYP2D6*4B*	$C_{188}T$; $C_{1062}A$; $A_{1072}G$; $C_{1085}G$; $G_{1934}A$; $G_{4258}C$		Splicing defect	None
*CYP2D6*4D* (Marez *et al.*, 1997)	$C_{188}T$; $C_{1127}T$; $G_{1749}C$; $G_{1934}A$; $G_{4268}C$		Splicing defect	None
*CYP2D6*4E* (Marez *et al.*, 1997)	$C_{188}T$; $G_{1749}C$; $G_{1934}A$; $G_{4268}C$		Splicing defect	None
*CYP2D6*4F* (Marez *et al.*, 1997)	$C_{188}T$; $C_{1062}A$; $A_{1072}G$; $C_{1085}G$; $G_{1749}C$; $G_{1934}A$; $C_{1946}T$; $G_{4258}C$		Splicing defect	None
*CYP2D6*4G* (Marez *et al.*, 1997)	$C_{188}T$; $C_{1062}A$; $A_{1072}G$; $C_{1085}G$; $G_{1749}C$; $G_{1934}A$; $C_{3026}T$; $G_{4258}C$		Splicing defect	None
*CYP2D6*4H* (Marez *et al.*, 1997)	$C_{188}T$; $C_{1062}A$; $A_{1072}G$; $C_{1085}G$; $G_{1749}C$; $G_{1934}A$; $G_{3965}C$; $G_{4258}C$		Splicing defect	None

*CYP2D6*41* (Marez *et al.*, 1997)	$C_{188}T$; $C_{1062}A$; $A_{1072}G$; $C_{1085}G$; $G_{1749}C$; $G_{1934}A$;		Splicing defect	None
*CYP2D6*5*	*CYP2D6* deleted	CYP2D6D	Gene deletion	None
*CYP2D6*6A*	T_{1795} deletion	CYP2D6T	Frameshift	None
*CYP2D6*6B*	T_{1795} deletion; $G_{2064}A$		Frameshift	None
*CYP2D6*6C* (Marez *et al.*, 1997)	T_{1795} deletion; $G_{2064}A$; $G_{4258}C$		Frameshift	None
*CYP2D6*6D* (Marez *et al.*, 1997)	T_{1795} deletion; $G_{3376}A$		Frameshift	None
*CYP2D6*7*	$A_{3023}C$	CYP2D6E	Base pair change	None
*CYP2D6*8*	$G_{1846}T$	CYP2D6G	Stop codon	None
*CYP2D6*9*	A_{2701} - A_{2703} or G_{2702} - A_{2704} deleted	CYP2D6C	Base pair deleted	Decreased
*CYP2D6*10A*	$C_{188}T$; $G_{1749}C$; $G_{4258}C$	CYP2D6J	Base pair change	Decreased
*CYP2D6*10B*	$C_{188}T$; $C_{1127}T$; $G_{1749}C$; $G_{4258}C$	CYP2D6Ch1	Base pair change	Decreased
*CYP2D6*10D* (Marez *et al.*, 1997)	$C_{188}T$; $C_{1127}T$; $G_{1749}C$; $G_{2031}A$; $G_{4258}C$	CYP2D6Ch2	Base pair change	Decreased
*CYP2D6*11*	$G_{971}C$; $G_{1749}C$; $C_{2938}T$; $G_{4268}C$	CYP2D6F	Splicing defect	None
*CYP2D6*12*	$G_{212}A$; $G_{1749}C$; $C_{2938}T$; $G_{4268}C$		Base pair change	None
*CYP2D6*13*	CYP2D7P/CYP2D6 hybrid (exon 1 of CYP2D7, exons 2-9 of CYP2D6)		Frameshift	None
*CYP2D6*14*	$G_{1846}A$		Base pair change	None
*CYP2D6*15*	T_{226} insertion			None
*CYP2D6*16*	CYP2D7P/CYP2D6 hybrid (exons 1 -7 of CYP2D7P, exons 8-9 of CYP2D6)	CYP2D6D2	Frameshift	None
*CYP2D6*17*	$C_{1111}T$; $G_{1726}C$; $C_{2938}T$; $G_{4268}C$	CYP2D6Z	Base pair change	Decreased
*CYP2D6*18* (Yokoi *et al.*, 1996)	9-base insertion (TCACCCGTG) in exon 9, position 4213		Base pair changes	Decreased
*CYP2D6*19* (Marez *et al.*, 1997)	$G_{1749}C$; A_{2627} - T_{2630} deleted; $C_{2938}T$; $G_{4268}C$			
*CYP2D6*20* (Marez-Allorge *et al.*, 1999)	single base insertion (C) (exon 4, position 2061); $C_{2066}T$; $T_{2067}C$; $C_{2938}T$		Frameshift	None

*CYP2D6*21* (Chida *et al.*, 1999)	single base insertion (C) (exon 5, position 2661); $G_{1749}C$; $C_{2938}T$; $G_{4268}C$		Base pair changes	Decreased

Similar to the (S)-mephenytoin genetic polymorphism, family studies have indicated that PM_d are homozygous for the autosomal recessive mutant alleles, with this oxidation polymorphism being controlled by a single gene (Eichelbaum *et al.*, 1982, Mahgoub *et al.*, 1977).

1.3.3 Drugs whose metabolism cosegregates with the debrisoquine / sparteine genetic polymorphism

Comparable to the (S)-mephenytoin genetic polymorphism, there are many classes of drugs whose metabolism has been found to cosegregate with the debrisoquine / sparteine genetic polymorphism. Evidence from both *in vivo* and *in vitro* studies indicates that the following drugs are metabolised in part by CYP2D6, and therefore, are affected by the polymorphism (Price Evans, 1993b, except where indicated);

1. *Antiarrhythmics*: flecainide, encainide, mexiletine, propafenone, N-propylajmaline.
2. *Antianginal*: perhexiline, diltiazem
3. *Antihypertensive*: guanoxan, indoramin
4. *Antipsychotics*: zuclopenthixol, thioridazine, haloperidol
5. *Analgesics*: codeine, oxycodone (Heiskanen *et al.*, 1998), hydrocodone (Otton *et al.*, 1993), ethylmorphine (Liu *et al.*, 1995)
6. *ß-Adrenoceptor antagonists*: alprenolol, bufuralol, metoprolol, propranolol and timolol.
7. *Tricyclic antidepressants*: imipramine, amitriptyline, clomipramine, fluoxetine, paroxetine, nortriptyline, venlafaxine (Otton *et al.*, 1996), citalopram (Sindrup *et al.*, 1993b), sertraline (Kobayashi *et al.*, 1999)
8. *Bronchodilator*: methoxyphenamine
9. *Antitussive*: dextromethorphan

10. *Hallucinogen*; 4-methoxyamphetamine

1.3.3.1 Codeine

Codeine and its O-demethylated metabolite morphine are natural components of the opium poppy, *Papaver somniferum*. (Sindrup *et al.*, 1996). Studies have shown that the main metabolite of codeine is the 6-glucuronide (70 - 80% of the dose), with the formation of morphine (less than 10% of the dose) and N-demethylation derivative norcodeine (10% of the dose) representing minor pathways, whilst only 10% of the dose is excreted unchanged (Figure 10) (Adler *et al.*, 1955, Chen *et al.*, 1988, Chen *et al.*, 1991b). It has been reported that codeine is one tenth as potent as morphine when used as an analgesic, however when it is given as a larger dose (180mg) a hypoalgesic effect is observed equivalent to that observed with 24mg of morphine (Wallenstein *et al.*, 1961). It has been postulated and seems logical, that this effect is due to the formation of morphine, as codeine has a substantially lower affinity for the μ-opioid receptor when compared with morphine, 20,000 nM versus 7nM (Pert *et al.*, 1973). A study by Chen and colleagues supported this report with a finding that only morphine exhibits high affinity when binding to the μ receptor, while codeine and its other metabolites have 1/200th the affinity (Chen *et al.*, 1991a).

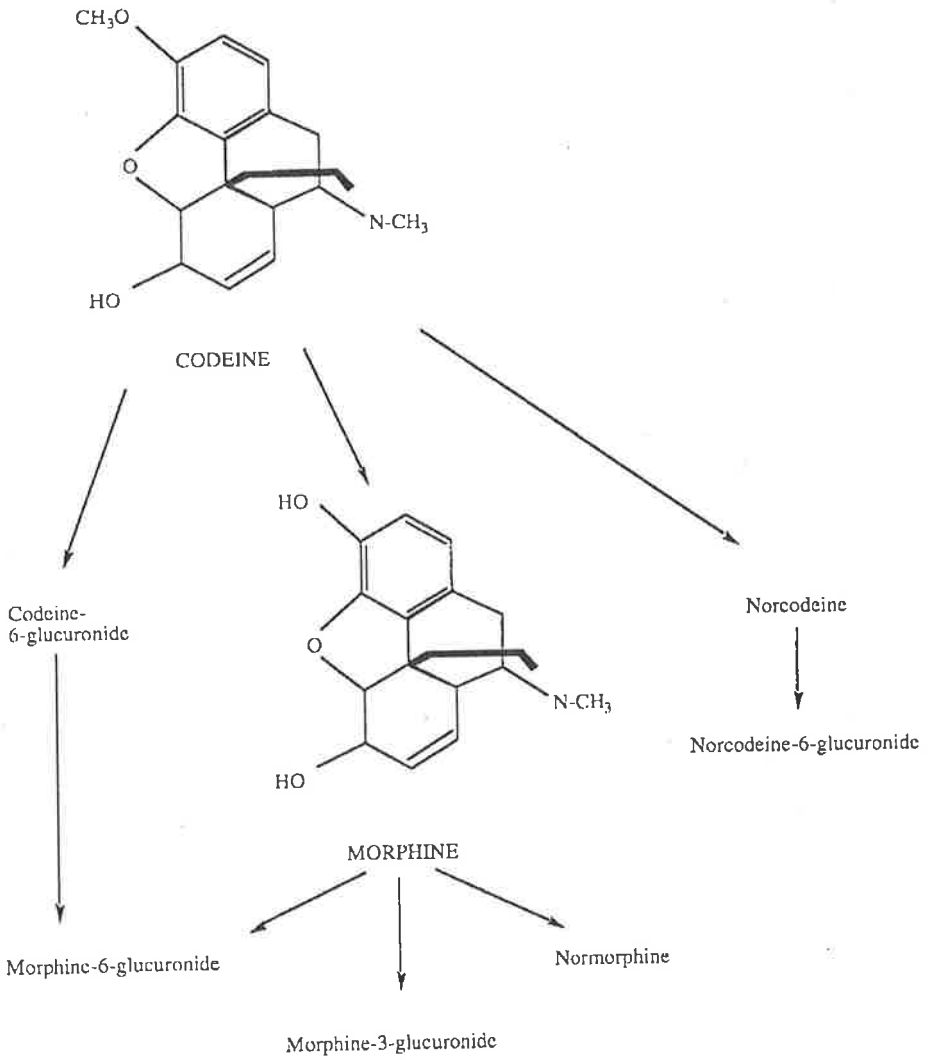


Figure 11: Metabolism of Codeine in humans (Sindrup *et al.*, 1996)

From the late 1970's it was observed that the formation of morphine in some subjects was lower in comparison to others, suggesting that the O-demethylation of codeine to morphine might be under the control of a genetic polymorphism. A study by Findlay and colleagues in 12 male subjects investigated plasma codeine and morphine concentrations after doses of combination codeine analgesics (Findlay *et al.*, 1978). These were two tablets of aspirin-codeine phosphate, containing 325mg aspirin and 30mg codeine, and two tablets of acetaminophen (300mg total) and codeine phosphate (30mg total). Following administration of either preparation, blood samples were taken over 24hr, allowing analysis of codeine and morphine concentrations and various pharmacokinetic parameters.

It was observed that there was intersubject variability in the codeine and morphine concentrations (Table 5).

Table 5: A) Plasma codeine and morphine plasma concentrations and B) pharmacokinetic parameters following administration of aspirin-codeine or acetaminophen-codeine, all values are mean ± standard error of the mean (SEM) (range) (Findlay *et al.*, 1978).

A)

Aspirin-codeine	30min	1hr	8hr	24hr
codeine ($ng.ml^{-1}$)	67.8 ± 23.1 (1.1 - 226.5)	120.3 ± 11.6 (20.2 - 174)	23.2 ± 1.6 (13 - 31.5)	1.4 ± 0.1 (0.7 - 2.1)
morphine ($ng.ml^{-1}$)	3.0 ± 1.3 (0 - 11.5)	5.3 ± 1.1 (0.2 - 12.0)	2.3 ± 0.4 (0 - 4.7)	1.0 ± 0.2 (0 - 2.0)
acetaminophen - codeine				
codeine ($ng.ml^{-1}$)	78.0 ± 11.1 (9.7 - 210)	133.6 ± 9.3 (65 - 210)	21.6 ± 1.7 (10.8 - 37)	1.6 ± 0.2 (0.3 - 2.9)
morphine ($ng.ml^{-1}$)	4.6 ± 0.8 (0.6 - 11.7)	7.3 ± 0.9 (1.7 - 16.3)	2.8 ± 0.2 (0.9 - 4.8)	1.2 ± 0.1 (0.4 - 2.4)

B)

	codeine C_{max} ($ng.ml^{-1}$)	codeine K_a (hr^{-1})	codeine AUC ($ng.ml^{-1}hr$)	morphine AUC ($ng.ml^{-1}hr$)
aspirin-codeine	158.8 ± 10.8	8.4 ± 2.4	614.0 ± 29.2	56.0 ± 10.4
acetaminophen-codeine	137 ± 8.8	5.7 ± 1.2	590.0 ± 30.2	66.3 ± 5.6

Another study investigated the disposition of codeine in smokers (n=11) when compared to nonsmokers (n=12) following administration of either 60mg codeine sulfate orally or 60mg codeine phosphate intramuscularly and blood sampling for 12hr (Rogers *et al.*, 1982). Although they found little difference in the pharmacokinetic parameters between smokers and nonsmokers, they did report similar interindividual variability in these parameters (see table 6). Additionally, they noted that the plasma morphine AUC values for six subjects were very low in comparison to the other subjects. For example, the unconjugated morphine AUC in one subject was 1.33ng.hr.ml^{-1} whilst another subject's was 166ng.hr. ml^{-1}, suggesting a lower level of codeine O-demethylation in the first subject.

Table 6: Codeine pharmacokinetic parameters in smokers and nonsmokers following administration of 60mg codeine sulfate orally, all values are mean ± SEM (Rogers *et al.*, 1982).

	Smokers	Nonsmokers
AUC ($ng.hr.ml^{-1}$)	466 ± 53	467 ± 24
C_{max} ($ng.ml^{-1}$)	145 ± 35	93 ± 5
t_{max} (hr)	0.88 ± 0.10	1.16 ± 0.14
$t_{1/2}$ (hr)	2.8 ± 0.2	2.7 ± 0.1

Another study investigated the plasma concentrations of codeine and morphine after repeated oral administration of 60mg codeine as 7 consecutive doses over 3 days to 12 subjects (Quiding *et al.*, 1986). These researchers had similar findings in terms of interindividual variability in pharmacokinetic parameters of both morphine and codeine (table 7), with one subject showing no detectable amount of morphine after any doses.

Table 7: Pharmacokinetic parameters of morphine and codeine after seven doses of 60mg codeine, all values are mean ± SD (Quiding *et al.*, 1986).

	C_{max} ($ng.ml^{-1}$)	t_{max} (hr)	k (hr)	$t_{1/2}$ (hr)	AUC_{0-8} (hr.ng.m)
codeine (n=12)	149 ± 60	1.0 ± 0.5	0.28 ± 0.03	2.5 ± 0.3	473 ± 130
morphine (n=11)	3.8 ± 2.4	1.0 ± 0.4	0.20 ± 0.08	4.2 ± 2.6	12.4 ± 7.0

Some years later, Dayer and colleagues noted during an *in vitro* study that in liver microsomes from a PM_d, the O-demethylation reaction had a higher K_m and a lower V_{max} than in those from an EM_d, 1000 versus 149μM, and 1.6 versus 17.6 nmol.mg $protein^{-1}.hr^{-1}$, respectively (Dayer *et al.*, 1988). This study also showed that the O-demethylation of codeine was inhibited by quinidine (a CYP2D6 inhibitor, K_i = 15nM), and correlated with the O-demethylation of dextromethorphan (r_s = 1.00, $P < 0.01$), which is CYP2D6-mediated. Therefore, it was concluded that CYP2D6 mediated the formation of morphine.

In vivo studies have confirmed results found in the *in vitro* study mentioned above. For example in 50 subjects, including 3 PM_d, a strong correlation was observed between

morphine formation following codeine administration and the O-demethylation of dextromethorphan (Chen *et al.*, 1988). Differences in codeine pharmacokinetics have also been noted in PM_d. In one study of 11 EM_d and 12 PM_d, morphine could not be quantified in the plasma of the latter group taken at time = 0, 90, 150 and 210min, (limit of quantification = 4nM) (Sindrup *et al.*, 1990). In contrast, no difference was seen between the groups when comparing the other metabolic pathways.

These observations were confirmed by research conducted by Yue and colleagues in 132 Swedish Caucasians phenotyped with debrisoquine (Yue *et al.*, 1989b). Following administration of 25mg codeine phosphate orally and 8hr urine collection, O-demethylation (codeine/morphine + morphine-3- and -6-glucuronide), N-demethylation (codeine/(norcodeine + norcodeine glucuronide + normorphine)) and glucuronidation (codeine/(codeine-6-glucuronide)) metabolic ratios (MR) were calculated, and the relationship between these metabolic ratios and the debrisoquine/4-hydroxydebrisoquine ratio (DB MR) was investigated. Of the codeine MRs studied the only significant correlation was observed between the log O-demethylation MR and the DB MR, $r_s = 0.77$, $P < 0.001$, with PM_d and EM_d having O-demethylation MR ranging from 8.3 - 55.1, and 0.4 - 5.5, respectively. Additionally, PM_d when compared with EM_d excreted significantly less morphine (mean ± SD of % urinary recovery, 0.02 ± 0.03 compared with 0.1 ± 0.008, $P < 0.001$), morphine-3- (0.2 ± 0.1 compared with 2.6 ± 1.5, $P < 0.001$) and -6-glucuronide (0.003 ± 0.02 compared with 0.5 ± 0.2, $P < 0.001$) and normorphine (0.03 ± 0.02 compared with 0.8 ± 0.4, $P < 0.001$), and significantly more codeine-6-glucuronide (64.7 ± 3.8 compared with 62.2 ± 3.2, $P < 0.05$) and norcodeine (3.7 ± 2.1 compared with 2.2 ± 0.7, $P < 0.001$). There were no significant differences between the % urinary recovery of codeine or norcodeine glucuronide when PM_d were compared with EM_d. These authors concluded that the O-demethylation of codeine co-segregated with debrisoquine hydroxylation.

Perhaps of most importance in confirming that the formation of morphine *in vivo* is mediated by CYP2D6 is a study which showed that this metabolism can be completely blocked with a 50mg dose of quinidine (Desmeules *et al.*, 1991). Seven EM_d and 1 PM_d

were given placebo / placebo, placebo / 100mg codeine, or 50mg quinidine / 100mg codeine in a randomised double-blind crossover design, and blood samples were collected over 2.5hr following codeine administration. In the presence of quinidine, morphine C_{max} and AUC in EM_d were significantly decreased, 1.5 ± 1.0 versus 17.9 ± 8.8 nM ($P < 0.001$), and 2.9 ± 2.0 versus 30.5 ± 12.3 nM.hr ($P < 0.001$), respectively. There was no significant difference in morphine t_{max} of EM_d, nor were there any significant differences in morphine C_{max}, AUC or t_{max} of PM_d. From these data it was concluded that codeine metabolism to morphine is mediated by CYP2D6, as quinidine significantly reduced this pathway in EM_d, whilst in PM_d there was no significant difference, as genetically there is little CYP2D6 activity to abolish.

Recently, another *in vivo* study has shown the effect of quinidine co-administration on the disposition and pharmacodynamics of codeine in 10 Caucasian and 8 Chinese subjects who were all EM_d (Caraco *et al.*, 1999). The study was a randomised, double-blind crossover design with administration of placebo / 120mg codeine, and 100mg quinidine / 120mg codeine, followed by blood sampling over 24hr and urine sampling over 48hr. When codeine was administered with placebo, it was observed that the plasma codeine AUC was significantly lower, and the concentrations of normorphine and norcodeine glucuronide were significantly higher in Caucasian subjects compared to the Chinese subjects (Table 8). Additionally, clearance of codeine via O-demethylation was significantly higher in Caucasian subjects versus Chinese subjects, whilst there were no significant differences in clearance of codeine via N-demethylation or glucuronidation. Quinidine reduced O-demethylation of codeine regardless of ethnic origin; however, the absolute reduction in this clearance was higher in Caucasian subjects than in Chinese subjects (Table 8). Conversely, the N-demethylation and glucuronidation of codeine as absolute values were not affected by quinidine, however, the $t_{1/2}$ and AUC of norcodeine significantly increased in Caucasians, and the urinary recovery of norcodeine increased significantly in both groups (Table 8). Pharmacodynamic effects of codeine as measured by ventilation, psychomotor function and pupil diameter were not changed by quinidine co-administration in Chinese, but were significantly reduced in Caucasians. From these data it was

concluded that Chinese may experience less analgesia when administered codeine due to less production of morphine, and that effects of quinidine on codeine disposition and pharmacodynamics are dependent on ethnic origin.

Table 8: Pharmacokinetics of codeine metabolites after administration of placebo / 120mg codeine and 100mg quinidine / 120mg codeine in 10 Caucasian (Ca) and 8 Chinese (Ch) subjects (Caraco *et al.*, 1999).

Compound	$t_{1/2}$ (hr)		AUC (nM.hr)		Recovery % dose	
	Ca	Ch	Ca	Ch	Ca	Ch
After placebo						
Normorphine	9.24	5.65 *	606	327*	2.23	1.66
Norcodeine-glucuronide	5.24	2.47*	2164	950*	2.84	1.73
Codeine-6-glucuronide	2.87	3.16	38853	40166	53.0	71.5*
Codeine	2.55	3.10	3160	5159*	4.52	7.29*
After quinidine						
Norcodeine	5.24#	4.51	994##	1159	5.04##	4.47##
Norcodeine-glucuronide	7.51	3.51	2359	943	3.85	1.65
Codeine-6-glucuronide	3.09	3.66	47909	40583	70.3	73.2
Codeine	2.86	2.72	4604	5405	5.05	6.35

All data are mean; * indicates $P < 0.02$ between Caucasian and Chinese, # indicates $P < 0.03$ versus placebo, ## indicates $P < 0.01$ versus placebo.

1.3.3.2 Dextromethorphan

As mentioned in section 1.3 dextromethorphan has been used as a CYP2D6 phenotyping probe. The O-demethylation of dextromethorphan to dextrorphan is mediated by CYP2D6, as is the O-demethylation of 3-methoxymorphinan, the N-demethylated metabolite of dextromethorphan, to produce 3-hydroxymorphinan (refer to section 1.3,

figure 10). From the mid-1980's it was reported that *in vivo*, the O-demethylation of dextromethorphan was polymorphic. A study of 268 Swiss subjects administered 25mg dextromethorphan observed that 23 subjects (9%) had a dextromethorphan/dextrorphan (DM/DR) MR of > 0.3, and a reduction in the dextrorphan production over 8hr when compared to EM (n=245), 1.2 ± 0.91 versus 35 ± 9.3 μM, which indicated that they were PM (Schmid *et al.*, 1985). In addition, it was observed that there was a significant difference between the dextromethorphan recovered over 8hr between male and female EM, 0.34 ± 0.45 (n=127) versus 0.20 ± 0.23 (n=118), $P < 0.05$. Consequently, a significant difference in the DM/DR MR was reported between male and female EM, 0.013 ± 0.028 (n=127) versus 0.069 ± 0.011 (n=118), $P < 0.05$. These differences were not observed when comparing male and female PM, therefore, this indicated that there are gender differences in the metabolism of dextromethorphan. However, these can not be explained by an interaction with oral contraceptives, as the authors reported that there was "no significant influence of oral contraceptive use" in female EM. These researchers also investigated the relationship between the DM/DR MR and the debrisoquine/4-hydroxydebrisoquine MR in EM (n=40) and PM (n=22), observing a correlation between the two MRs, $r_s = 0.78$, $P < 0.0001$, which indicated that the metabolism of debrisoquine and dextromethorphan co-segregates.

Another study has investigated the polymorphic distribution of dextromethorphan O-demethylation in a French population (n=103, 61 male, 42 female) (Larrey *et al.*, 1987). Following administration of 40mg dextromethorphan, urine was collected for 10hr and the DM/DR MR calculated. Similar to reports in the Swiss population, it was observed that 4 subjects (3.9%) were PM, with mean ± SD (range) DM/DR MR of 3.4 ± 0.7 (0.3 - 4.3), compared with the 99 EM who had DM/DR MR of 0.016 ± 0.018 (0.0009 - 0.090). Additionally, the recovery of dextrorphan was lower in PM when compared with EM, 8.7 ± 6.9 (3.4 - 18.8) versus 48.7 ± 20.9 (20.5 - 89.2)μM. In contrast to the study in Swiss subjects, there was no report any gender differences in dextromethorphan metabolism.

Further investigation of factors influencing the DM/DR MR, was performed in Swiss subjects previously phenotyped with both debrisoquine and rac-mephenytoin (Küpfer *et al.*, 1986). Following administration of the 25mg capsule, the DM/DR MR of PM (n=22) and EM (n=41), previously identified with debrisoquine, was significantly related to the debrisoquine MR, $r_s = 0.86$, $P < 0.001$. In contrast, there was no significant relationship between the DM/DR MR and the (S)-mephenytoin hydroxylation index. The influence of urinary pH was evaluated in PM (n=23) subjects with urine pH varying from 5 to 7, with no relationship observed between urine pH and DM/DR MR. Furthermore, in one EM and one PM of dextromethorphan it was observed that the important urine collection times in the subjects were the first 4hr and the entire 8hr, respectively, such that collection for an 8hr period is especially important in PM. There was no relationship between the urinary recovery of dextrorphan and initial dextromethorphan dose when studied in one EM, doses ranging from 5 to 440μmole (20mg of dextromethorphan = 71μmole), in either solid (capsule or pure dextromethorphan) or liquid (syrup preparation containing 25mg dextromethorphan, 180mg ammonium chloride and 100mg dexpanthenol per 10ml) form. Furthermore, from this investigation on dose dependency, it was shown that the additional constituents of the liquid form did not alter the urinary recovery of dextrorphan. The final section of this *in vivo* study addressed the repeatability of the DM/DR MR, where 8 PM and 14 EM had the original dextromethorphan phenotyping repeated, with 12 weeks separating the study days. It was observed that there was a correlation between the DM/DR MR on the separate occasions, $r = 0.97$, suggesting that this index of enzyme activity produces repeatable results.

Following the above *in vivo* evidence showing a co-segregation between dextromethorphan and debrisoquine metabolism, *in vitro* studies were published regarding the involvement of CYP2D6 in dextromethorphan metabolism in human liver microsomes. A study of human liver microsomes of 6EM, 1IM and 1PM investigated the *in vitro* kinetic values, and inhibition of the O-demethylation of dextromethorphan (Dayer *et al.*, 1989). The K_m values for EM (mean ± SD), IM and PM were 3.4 ± 1.0, 8.7 and 48μM, respectively, and the V_{max} values were 10.2 ± 5.3, 2.9 and 2.2nmol.mg^{-1}.hr^{-1}, respectively.

Dextromethorphan O-demethylation was inhibited by quinidine, sparteine, and debrisoquine, with K_i values of 15nM, 45 and 25μM, respectively. Therefore, it was concluded that the metabolism of dextromethorphan to dextrorphan was mediated by cytochrome P450 db_1 (CYP2D6). A similar K_i value (25 ± 8nM) for the inhibition of dextromethorphan O-demethylation by quinidine in human liver microsomes had been reported previously (Broly *et al.*, 1989).

Another *in vitro* study of dextromethorphan O-demethylation showed that the Eadie-Hofstee plots for the formation of dextrorphan in EM (n=3) indicated a two site enzyme model of metabolism, whereas the Eadie-Hofstee plots for formation of dextrorphan in PM (n=2), and for the formation of 3-hydroxymorphinan from 3-methoxymorphinan in both EM and PM indicated a one site enzyme model of metabolism (Kerry *et al.*, 1994). Furthermore, it was observed that when compared to the EM, the PM had higher K_m, and lower V_{max} and Cl_{int} for both dextromethorphan and 3-methoxymorphinan O-demethylation (Table 9). However, there were no differences between the K_m, V_{max} and Cl_{int} values in EM and PM for the N-demethylation of both dextromethorphan and dextrorphan (Table 9). Although the K_m values for dextromethorphan O-demethylation were much higher than those reported by (Dayer *et al.*, 1989), the authors suggested that this was due to the limited dextromethorphan concentrations used in the earlier study, 0.5 – 20μM, compared to that used in this study, 0.6 – 3000μM.

Table 9: *In vitro* kinetic values of dextromethorphan metabolism in EM (n=3) and PM (n=2) human liver microsomes (Kerry *et al.*, 1994).

	K_m (μM)	V_{max} (nmol.hr^{-1}.mg^{-1})	Cl_{int} (ml. hr^{-1}.mg^{-1})
Dextromethorphan O-demethylation			
EM	133 ± 151	15.3 ± 3.51	0.26 ± 0.021
PM	359 ± 285	8.95 ± 1.48	0.035 ± 0.021
3-methoxymorphinan O-demethylation			
EM	7.96 ± 1.44	15.7 ± 10.9	2.00 ± 1.56
PM	260 ± 66.5	2.75 ± 2.19	0.012 ± 0.011
Dextromethorphan N-demethylation			
EM	846 ± 187	26.7 ± 0.57	0.033 ± 0.006
PM	710 ± 46.0	13.5 ± 2.12	0.020 ± 0.000
Dextrorphan N-demethylation			
EM	2567 ± 1343	59.3 ± 12.3	0.027 ± 0.006
PM	3400 ± 1273	21.5 ± 3.54	0.010 ± 0.000

Incorporated into the above *in vitro* kinetic study were some experiments investigating inhibition of dextromethorphan metabolism, including both chemicals and LKM1 antibodies (specific for CYP2D6) (Kerry *et al.*, 1994). It was reported that quinidine competitively inhibited the O-demethylation of dextromethorphan, with a K_i value of 0.1μM, whilst the effect of quinidine on O-demethylation of 3-methoxymorphinan was not reported. In addition, LKM1 antibodies inhibited the O-demethylation of both dextromethorphan and 3-methoxymorphinan, with no inhibition of the N-demethylation of either dextromethorphan or dextrorphan observed. From consideration of both the kinetic and inhibition data, it was concluded that CYP2D6 mediates the formation of the O-demethylated metabolites of both dextromethorphan and 3-methoxymorphinan, whilst the formation of the N-demethylated metabolites of dextromethorphan and dextrorphan is not mediated by CYP2D6. This agrees with additional studies which have shown that the N-demethylation of dextromethorphan is mediated by CYP3A (Jacqz-Aigrain *et al.*, 1993, Gorski *et al.*, 1994).

More recently, it has been shown *in vivo* that concomitant administration of quinidine has an effect on the pharmacokinetics of dextromethorphan (Capon *et al.*, 1996). In this study 6 EM (previously phenotyped with dextromethorphan) were administered in a randomised cross-over design either placebo plus 30mg dextromethorphan 1hr later, or 50mg quinidine plus 30mg dextromethorphan 1hr later. Plasma from pre-dose, then 0.5, 1, 1.5, 2, 3, 4, 8, 12, 24, 48, 72, 96, 120, 144 and 168hr after dose, and urine from 0 - 12hr, 12 - 24hr, and then every 24hr until 168hr after dose, was collected. It was observed that in comparison with placebo, dosing with quinidine significantly increased the C_{max} and plasma AUC for dextromethorphan ($P < 0.001$); significantly decreased and increased, the C_{max} and $t_{1/2}$ ($P < 0.001$), respectively, for dextrorphan; significantly decreased, and increased, the C_{max} ($P < 0.001$), plasma AUC ($P < 0.01$) and $t_{1/2}$ (P, 0.001), respectively, for 3-hydroxymorphinan; and significantly increased the C_{max} and plasma AUC ($P < 0.01$), for 3-methoxymorphinan (Table 10). In addition, it was reported that the renal clearance of dextromethorphan was significantly reduced when quinidine was given, 2.8 versus 9.0L.hr^{-1}, $P < 0.001$, as was the intrinsic clearance of dextromethorphan to dextrorphan, 79 versus 4737L.hr^{-1}, $P < 0.001$). In conclusion, these data showed that in the presence of quinidine, which is a potent inhibitor of CYP2D6, the metabolism of dextromethorphan was inhibited.

Table 10: Pharmacokinetic parameters in 6EM following administration of either placebo plus dextromethorphan (DM) or quinidine plus dextromethorphan; data are median (range) (Capon *et al.*, 1996).

	C_{max} ($mg.l^{-1}$)	AUC ($mg.(l.hr)^{-1}$)	$t_{1/2}$ (hr)
Dextromethorphan			
Placebo + DM	1.4 (0.7 – 13.7)	9.0 (1.2 – 160)	2.4 (1.3 – 4.0)
Quinidine + DM	24.9 (16.6 – 40.9)	383 (200 – 1214)	5.6 (3.8 – 6.6)
Dextrorphan			
Placebo + DM	396 (250 – 722)	1701 (1420 – 5650)	1.4 (1.2 – 1.9)
Quinidine + DM	113 (37 – 129)	1669 (1242 – 5852)	6.6 (3.6 – 13.6)
3-hydroxymorphinan			
Placebo + DM	104 (83 – 135)	648 (621 – 984)	2.0 (1.1 – 3.3)
Quinidine + DM	50 (19 – 97)	873 (693 – 1092)	6.4 (3.7 – 17.1)
3-methoxymorphinan			
Placebo + DM	0 (0 – 0.5)	0 (0 – 2.9)	
Quinidine + DM	0.8 (0 – 1.3)	13.8 (0 – 69)	

1.3.4 Enzymic bases of the debrisoquine / sparteine genetic polymorphism

1.3.4.1 *In vivo* pharmacokinetic studies

A study led by Eichelbaum observed differences in the pharmacokinetics of 100mg sparteine when administered orally to EM_s and PM_s (Eichelbaum *et al.*, 1982). Both groups of subjects absorbed sparteine to the same extent, as the total recoveries of the parent drug and the metabolites were similar. However, the mean $t_{1/2}$ and total Cl were 3-fold higher and 3-fold lower, respectively in PM_s. It was also observed that in the PM_s, the clearance of sparteine was almost totally via renal excretion and not metabolism.

1.3.4.2 *In vivo* kinetic studies

It has been shown that the formation of the 2-dehydro metabolite of sparteine has a greater than 40-fold difference in K_m in EM_s and PM_s liver microsomes (51 ± 15 compared with 1938 ± 1113 μM), but similar V_{max} values (104 ± 57 compared with 87 ± 54 $pmol.min^{-1}.mg^{-1}$), leading to the conclusion that the difference in metabolism is due to a deficient enzyme rather than a decreased quantity of the enzyme (Eichelbaum *et al.*, 1986).

1.3.4.3 Chemical inhibition studies

The most widely studied chemical inhibitor of the CYP2D6 enzyme is quinidine, a dextrorotatory stereoisomer of the antimalarial drug quinine, used clinically as an antiarrhythmic. An *in vitro* study by Otton showed that among 18 chemicals tested, quinidine was the most potent inhibitor, with a K_i of 60nM (Otton *et al.*, 1984). Confirming this result were the observations of an *in vivo* study conducted in 8 patients with supraventricular arrhythmias (5 male, 3 female Caucasians) (Brinn *et al.*, 1986). It was observed that after a treatment period of one week, during which 600 - 800mg sparteine was given per day, the formation of the 2- and 5-dehydro derivatives was abolished. This inhibition of metabolism was explained by the fact that quinidine binds to the enzyme (Otton *et al.*, 1988) causing subjects normally classified as EM_s to exhibit the phenotype of a PM_s for the period of administration of quinidine.

Researchers have also investigated the effect of quinidine on the 4-hydroxylation of debrisoquine. An *in vivo* study of 5 male EM and 1 female EM, previously identified with debrisoquine phenotyping, showed that administration of 50mg quinidine increased the debrisoquine MR from; 0.3 to >50, 0.6 to >50, 0.2 to >50, 1.1 to 25, 0.2 to 7.4, and 0.05 to 3.2, at 8hr after quinidine administration (Ayesh *et al.*, 1991). Furthermore, it was observed that the MR returned to original values by 192hr after quinidine administration.

It was concluded that quinidine did inhibit the metabolism of debrisoquine to produce phenocopy in these subjects, which is the phenomenon whereby subjects become PM in the presence of the inhibitor. This agreed with an earlier *in vivo* study, which reported that quinidine administration in EM (n=8) caused a mean increase in debrisoquine MR of 26-fold (Speirs *et al.*, 1986).

1.3.5 Incidence of the debrisoquine / sparteine polymorphism

Poor metabolisers of debrisoquine / sparteine ($PM_{s/d}$) are identified by decreased metabolism of debrisoquine, sparteine or dextromethorphan. Therefore, a urinary metabolic ratio (MR) is most commonly used;

$$MR = \frac{\text{amount of parent drug excreted}}{\text{amount of metabolite excreted}_{(0\text{ - }8\text{hr urine})}}$$

It has been stated that if the MR is greater than either 12.3 or 20, for debrisoquine and sparteine, respectively, subjects are assigned with the phenotype of $PM_{s/d}$ (Eichelbaum *et al.*, 1982). Researchers have found that in contrast to the (S)-mephenytoin genetic polymorphism, the incidence of $PM_{s/d}$ in Orientals is 0 - 5%, whilst in Caucasians it is 3 - 10% (Table 11).

Table 11: Incidence of $PM_{s/d}$ phenotype in various populations

Population	Number of subjects	Frequency of $PM_{s/d}$ (%)	Reference
Caucasians			
British	258	9	(Evans *et al.*, 1980)
	94	3	(Mahgoub *et al.*, 1977)
German	360	5	(Eichelbaum *et al.*, 1979)
Danish	301	7	(Brøsen *et al.*, 1985)
	358	9.2	(Drohse *et al.*, 1989)
Swiss	268	9	(Schmid *et al.*, 1985)
Swedish	205	8.8	(Sanz *et al.*, 1989)
	757	5.4	(Steiner *et al.*, 1988)
Finish	107	6	(Syvalahti *et al.*, 1986)
Spanish	124	10	(Henthorne *et al.*, 1989)
Turkish	109	3.8	(Basci *et al.*, 1994)
Russian	218	7.8	(Marandi *et al.*, 1997)
Estonian	156	4.5	(Kiivet *et al.*, 1993)
Canadian	83	7	(Inaba *et al.*, 1984)
Australian	100	6	(Peart *et al.*, 1986)
West Greenlander	174	2.3	(Clasen *et al.*, 1991)
East Greenlander	309	3.3	(Clasen *et al.*, 1991)
Canadian native Indian	95	1.1	(Nowak *et al.*, 1997)
North Indian	100	3	(Lamba *et al.*, 1998b)
South Pacific Polynesian	100	0	(Wanwimolruk *et al.*, 1998)
African American	104	7.7	(Leathart *et al.*, 1998)
Black Tanzanian	216	0.5	(Bathum *et al.*, 1999)
Zimbabwean	114	0	(Masimirembwa *et al.*, 1993)
Black Zimbabwean	103	1.9	(Masimirembwa *et al.*, 1996)
Gabonese	154	1.9	(Panseret *et al.*, 1999)
Saudi Arabian	102	2	(Price Evans, 1993c)
Sinhalese	111	0	(Weerasuriya *et al.*, 1994)
Filipino	55	0	(Price Evans, 1993c)
Korean	152	0	(Roh *et al.*, 1996)
Chinese	269	0.7	(Lou *et al.*, 1987)
Japanese	100	0	(Nakamura *et al.*, 1985)

1.3.6 Occurrence of ultrarapid metabolisers of CYP2D6

The clinical consequences of loss of metabolism of drugs effected by the debrisoquine / sparteine genetic polymorphism are well documented. More unusual however, is the occurrence of ultrarapid metabolism in some subjects who carry multiple copies of the *CYP2D6* functional gene (ultrarapid metabolisers, UM). This phenomenon of gene amplification is rare in normal human cells and as such only limited examples have been reported (Prody *et al.*, 1989, Srivastava *et al.*, 1985, Turner *et al.*, 1985). If the gene that is amplified encodes for an enzyme that metabolises a particular drug or groups of drugs as is the case of CYP2D6, drug resistance may be observed (Stark *et al.*, 1984). Mechanisms by which the *CYP2D6* gene is duplicated or multiduplicated have recently been proposed (Lundqvist *et al.*, 1999). Specifically, the arrangement of the *CYP2D* genes (*CYP2D6, 7* and *8*), and sequence analysis of the regions surrounding the multiple *CYP2D6* genes, in subjects carrying more than one copy of *CYP2D6* were studied. It was concluded that if subjects carried alleles with up to 5 copies of *CYP2D6* the mechanism was that of an unequal crossover of alleles at a specific point of the surrounding 3' region of the *CYP2D6* gene which has a specific repetitive sequence. Additionally, unequal segregation, replication of DNA encoding for *CYP2D6* separate from chromosomal replication, and incorporation of these additional *CYP2D6* genes into the original chromosomal DNA by homologous recombination was suggested as a mechanism for creation of alleles with 13 copies of *CYP2D6*.

Two anecdotal cases have been described (Bertilsson *et al.*, 1993);

Case 1: a woman whose debrisoquine MR was 0.07 required 500mg nortriptyline daily, which was 3 - 5 times the recommended dose, in order to reach therapeutic levels required to treat her depression. Note that a MR of less than 12.3 classifies a subject as an EM, whilst above this antimode of 12.3 a subject is classified as a PM_d.

Case 2: a psychiatric patient required 300mg clomipramine daily to control agoraphobia, normal dose range is 25 - 150mg.

Following genetic analysis, it was found that these subjects had a 12-fold amplification of the functional *CYP2D6* gene (*CYP2D6*2xN*), identified by the presence of a 12.1kb fragment following *Eco* RI restriction endonuclease analysis of the gene. These additional *CYP2D6* copies cause more of the functional enzyme to be expressed, and therefore, increase the rate of metabolism. Of clinical note is that conventionally patients who have not responded to one antidepressant are switched to another antidepressant. However, if the lack of response is due to rapid metabolism, this switch would not necessarily be effective due to most antidepressants and serotonin uptake inhibitors being metabolised by CYP2D6. Thus, it seems that identification of patients with multiple copies of *CYP2D6* could help avoid therapeutic failure when administering drugs metabolised by this enzyme.

Recent studies have investigated the prevalence of multiple copies of *CYP2D6* in various populations. It was observed that following the administration of 10mg debrisoquine and quantification of the MR, that 7% (n=217) of Spanish subjects had multiple copies of *CYP2D6* (Agundez *et al.*, 1995). This was confirmed by genotyping which revealed the presence of the 12.1kb *Eco* RI digestion fragment, indicative of multiple copies of *CYP2D6*. It was noted that the prevalence of multiple copies of *CYP2D6* was identical to that of the PM_d. Additionally, the drug metabolic activity of these subjects was found to increase with the number of copies of the gene; that is there is a gene dosage effect where subjects with a single copy metabolised 35 - 40% of debrisoquine, those with two copies metabolised an additional 37% of the remaining debrisoquine and so on. Other studies have reported incidences of *CYP2D6* duplication of 29% in Ethiopians (Aklillu *et al.*, 1996), 21% in Saudi Arabians (McLellan *et al.*, 1997), 9% in black Tanzanians (Bathum *et al.*, 1999), 3.8% in African Americans (Leathart *et al.*, 1998), 0.8% in Danish (Bathum *et al.*, 1998), 1% in Swedish (Dahl *et al.*, 1995), and 2.6% in Germans (Sachse *et al.*, 1997).

In reference to the CYP2D6 genetic polymorphism, it is apparent that the prevalence and clinical implications of the PM phenotype / genotype have been widely investigated. In comparison, there are substantial gaps in the literature regarding the prevalence and clinical repercussions of the UM phenotype / genotype. For example, no studies have been

conducted to observe the prevalence of multiple copies of *CYP2D6* in Caucasian population living in Australia or Asian populations using both phenotyping and genotyping techniques. In addition, the MR antimode to separate EM from UM, and the ability of the MR to successfully identify all UM has not been scientifically investigated with a combined phenotyping and genotyping study. Finally, the clinical implications of the presence of multiple copies of *CYP2D6* have been addressed only in anecdotal evidence (see above), with no substantial work researching changes in drug metabolism in UM when compared to EM.

1.4 Diagnostic analysis of patients to identify genetic polymorphisms

1.4.1 Phenotyping

In large scale population studies, phenotyping via the urinary metabolic ratio is most commonly used to identify poor metabolisers. This involves the administration of a probe drug, for example debrisoquine or more commonly dextromethorphan for the CYP2D6 polymorphism, and collection of urine for a set period of time, usually 8hr. The parent drug and its metabolite (the one whose formation is affected by the polymorphism), for example debrisoquine and 4-hydroxydebrisoquine, are then quantified to give a metabolic ratio (MR);

$$MR = \frac{\text{parent drug excreted}}{\text{metabolite excreted}}$$

This ratio is a reflection of the intrinsic clearance of the parent drug to its metabolite, and thus reveals if the subject is a PM or an EM in respect to a CYP450 enzyme.

Often cocktail phenotyping is undertaken. This is when two or more probe drugs, for example dextromethorphan and (S)-mephenytoin are co-administered to assess two separate polymorphisms at one time. A study was conducted to investigate whether this

co-administration alters either MR (Guttendorf *et al.*, 1990), and as neither MR was changed, it was concluded that this cocktail phenotyping is an appropriate method.

Problems can arise with the use of phenotyping to identify these polymorphisms as was mentioned previously (see section 1.2.4). Other complications can arise due to the occurrence of adverse drug reactions in PM, and incorrect assignment of phenotypes due to interference of co-administered drugs or renal / liver impairment. Therefore, if another easier, less complex, and more efficient method of identifying these polymorphisms is used, both time and cost can be cut. This may be represented by genotyping.

1.4.2 Genotyping

It has been noted that the interindividual variability in metabolism of these probe drugs observed with a genetic polymorphism is due to the presence of variant alleles of the genes encoding for the CYP450 enzymes. Therefore, through the identification of these alleles it is possible to establish if a subject is a PM or an EM. Most often, this process involves the use of molecular biology techniques such as the polymerase chain reaction (PCR), an *in vitro* reaction which amplifies a portion of the genomic DNA containing the region encoding for a mutation of a particular enzyme. In more detail, annealing of two specific primers complementary to the sequence of the gene required for investigation allows synthesis of the new strand of DNA in opposite directions via DNA polymerase enzymes (Figure 12) (Taylor, 1991).

There are several requirements for this reaction to take place:

1. deoxynucleotides (dNTPs): these provide energy for the reaction and the nucleosides to be incorporated in the synthesis of the new strands of DNA.
2. DNA polymerase: this is the enzyme required to synthesise the new strands of DNA.

3. specific primers: these anneal to the DNA template, allowing a portion of the gene to be targeted.

4. DNA template: isolated from subject's blood or tissue samples; it is from this that the synthesis of the new DNA occurs.

5. reaction buffer containing magnesium (Mg^{2+}): this metal ion is required for efficient enzyme activity, as it creates a complex with the primers, the DNA template and the DNA polymerase and therefore allows synthesis of the new DNA to occur.

It is important to note that both the dNTPs and primers are present in large excess so that the synthesis can occur over a number of cycles of heating and cooling of the reaction mixture. Most often 25 to 35 cycles of heating and cooling is sufficient to produce 100ng - 1μg of DNA from a starting concentration of 50ng genomic DNA (Taylor, 1991).

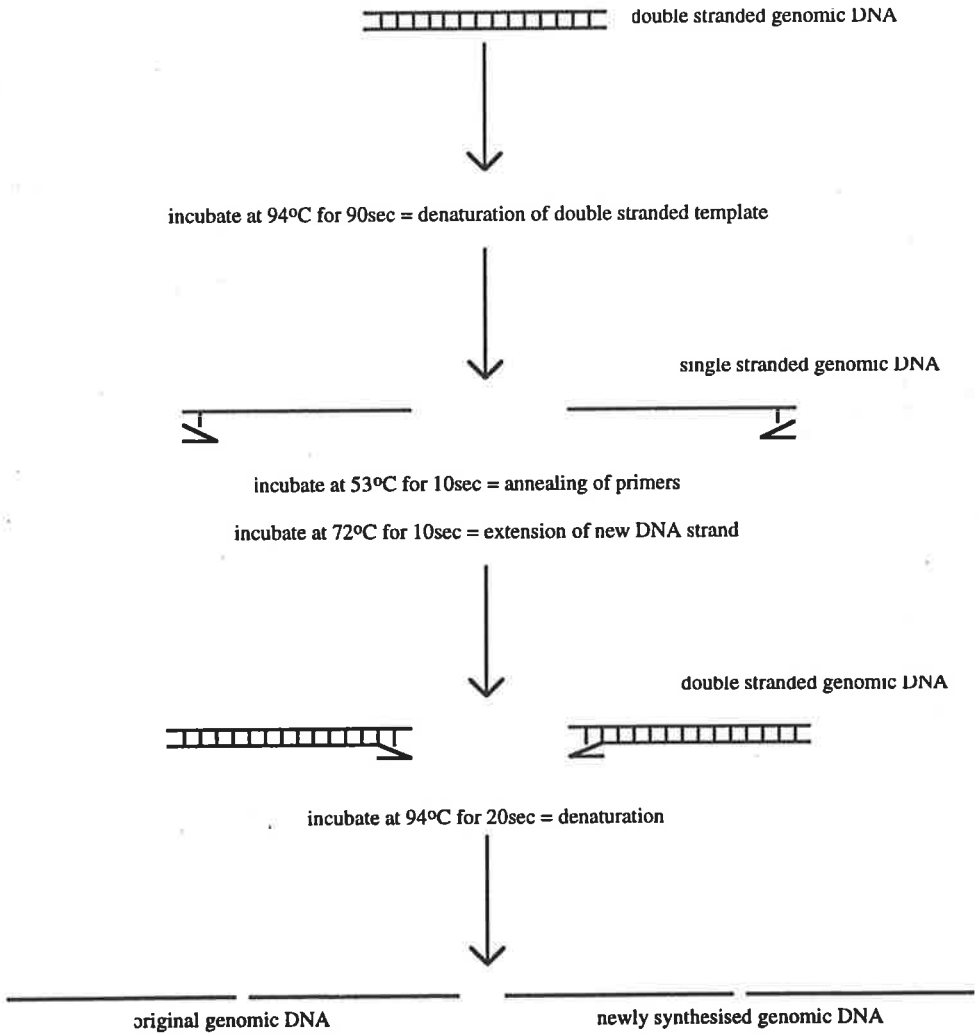


Figure 12: Schematic representation of PCR amplification

1.5 Thesis Overview, Hypotheses and Aims

In relation to the CYP2C19 genetic polymorphism, the suitability of proguanil to replace mephenytoin (which is no longer available for human use) as a phenotypic probe depends to a large extent on whether or not the same CYP450 enzyme(s) mediate the oxidative metabolism of these two drugs. Previous *in vitro* data showed that proguanil is oxidatively metabolised by both CYP2C19 and CYP3A4, whilst there is no *in vitro* evidence to show which CYP450 enzymes, apart from CYP2C19, mediate the oxidative metabolism of (S)-mephenytoin. In addition, although poor metabolisers (PM) identified by phenotyping with (S)-mephenytoin have also been shown to be PM when phenotyped with proguanil, *in vivo* the (S)-mephenytoin and proguanil metabolic ratios did not significantly correlate. This would suggest that factors other that CYP2C19 activity influence the oxidative metabolism of either or both (S)-mephenytoin and proguanil.

Hypothesis 1: using human liver microsomes, proguanil oxidative metabolism to CG is mediated by CYP2C19 and CYP3A4, whilst (S)-mephenytoin metabolism to 4'-OH-meph is mediated exclusively by CYP2C19.

Aim 1: The aim of the *in vitro* study is to investigate the oxidative metabolism of (S)-mephenytoin and proguanil to 4'-OH-meph and CG, respectively, in order to identify which CYP450 enzymes mediate these reactions. This will be addressed in chapter 2.

As discussed above, *in vivo* pharmacokinetic changes in the metabolism of (S)-mephenytoin can be attributed to changes in enzyme activity, shown *in vitro* by changes in kinetic values of (S)-mephenytoin metabolism in human liver microsomes. Therefore, from *in vitro* studies identifying which CYP450 enzymes are involved in the oxidative metabolism of (S)-mephenytoin and proguanil, it is possible to predict *in vivo* similarities in metabolism, and subsequently evaluate the appropriateness of proguanil as a

replacement probe for (S)-mephenytoin. This will be addressed in an *in vivo* study chapter 3.

The other *in vitro* study in this thesis was conducted as part of a major research strategy in my department, that of investigating the metabolism of drugs of abuse and dependence in order to determine the source of variability in their clinical response. Flunitrazepam is a drug of abuse with similar structure to another benzodiazepine, diazepam. The *in vitro* oxidative metabolism of flunitrazepam to 3'-hydroxyflunitrazepam and desmethylflunitrazepam has not been investigated previously, particularly the identification of which CYP450 enzymes are involved.

Hypothesis 2: The N-demethylation of flunitrazepam to desmethylflunitrazepam would be mediated by CYP2C19 and CYP3A4, whereas the 3'-hydroxylation of flunitrazepam to 3'-hydroxyflunitrazepam would be mediated by CYP3A4.

Aim 2: The aim of this *in vitro* study is to determine, the *in vitro* kinetics of, and which CYP450 enzymes mediate the oxidative metabolism of flunitrazepam.

Aim 3: To develop an HPLC assay for the quantification of flunitrazepam and its metabolites in human liver microsomes. These aims (2 and 3) will be addressed in chapter 2.

Similar to the *in vitro* studies of the oxidative metabolism of (S)-mephenytoin and proguanil, identification of the CYP450 enzymes oxidizing flunitrazepam could be used to predict; i) *in vivo* drug-drug interactions between flunitrazepam and other drugs which are metabolised by the same CYP450 enzymes; ii) variability in metabolism of flunitrazepam especially if CYP2C19, CYP2D6 or CYP3A4 are involved; iii) interethnic differences in the metabolism of flunitrazepam, as it has been shown previously that the incidences of both CYP2C19 and CYP2D6 PM vary between different ethnic groups.

Based on the *in vitro* studies of metabolism of (S)-mephenytoin and proguanil (chapter 2), *in vivo* studies correlating the CYP2C19 genotype and phenotype assigned by the proguanil metabolic ratio (PG/CG MR) are needed. Previously, there has been no study of the incidence of genotypic CYP2C19 PM in Caucasian and Asian populations living in Australia. In contrast to genotype assignment, phenotype as assessed by the PG/CG MR may be altered in populations living in Australia when compared to other Caucasian and Asian populations previously studied, due to the influence of environmental factors on proguanil metabolism (CYP3A4 activity) and excretion (renal clearance). In addition, there has been no statistical evaluation of the proguanil urinary metabolic ratio antimode used to separate CYP2C19 EM and PM groups.

Hypothesis 3: There will be a lack of concordance between the CYP2C19 genotype and phenotype assigned by PG/CG MR.

Hypothesis 4: The PM incidences in the Caucasian and Asian populations in this study would range between 2.6 - 8%, and 15 - 25%, respectively.

Aims 4 and 5: The aims of the *in vivo* study are to: i) investigate the concordance between the CYP2C19 genotype (based on *CYP2C19*2* and *CYP2C19*3* mutations), and phenotype assignment using the PG/CG MR in Caucasian and Asian populations living in Australia; and, ii) use three graphical methods (histogram, probit plot, normal test variable plot) to evaluate the PG/CG MR antimode separating the CYP2C19 genotypic EM and PM groups. These aims will be addressed in chapter 3.

In reference to the CYP2D6 genetic polymorphism, no study has reported the incidence of CYP2D6 ultra-rapid metabolisers (UM), identified as carriers of multiple copies of CYP2D6 in a Caucasian population living in Australia or any Asian population. In addition, it has been shown previously that CYP2D6 UM and EM can not be successfully separated on the basis on MR when any of the commonly used probe drugs for CYP2D6 (sparteine, debrisoquine and dextromethorphan) are administered.

Hypothesis 5: The incidence of CYP2D6 UM in the Australian Caucasian population would be similar to other Caucasian populations (0.8 - 2.6%).

Aims 6 and 7: The aims of this *in vivo* study are to: i) use genotyping and phenotyping with dextromethorphan to investigate the incidence of CYP2D6 UM in Caucasian and Asian populations living in Australia; and, ii) evaluate the ability of the log dextromethorphan MR to separate genotypic UM from EM. These aims will be addressed in chapter 4.

In contrast to the many studies illustrating differences in the metabolism of drugs by CYP2D6 in PM when compared with EM, there has been only a small number of drug metabolism studies in CYP2D6 UM.

Hypothesis 6: The codeine to morphine urinary metabolic ratio would decrease, and a smaller fraction of the codeine dose would be metabolised to the other metabolites (codeine-6-glucuronide, norcodeine) in CYP2D6 UM when compared to EM.

Aim 8: The aim of this *in vivo* pilot study is to investigate the metabolism of codeine in CYP2D6 UM (n=2) and EM (n=2) via administration of codeine followed by urine collection for 48hr. This aim will be addressed in chapter 5.

In summary, the overall aim of this thesis is to investigate the influence of the CYP2C19 and CYP2D6 genetic polymorphisms on oxidative drug metabolism, using *in vitro* and *in vivo* studies.

2. *In vitro* studies of oxidative drug metabolism in human liver microsomes

2.1 Introduction

In terms of oxidative drug metabolism involving the CYP450 enzymes, it is common to study the metabolism *in vitro* in a human liver microsomal system which being a subcellular fraction of human liver tissue contains all the CYP450 enzymes (Wrighton *et al.*, 1993). From studying the formation of oxidative metabolites from the parent drug, it is possible to obtain kinetic parameters such as K_m (affinity constant of the CYP450 enzyme(s) for the drug), V_{max} (maximum formation rate of metabolites by CYP450 enzymes), and Cl_{int} (intrinsic clearance, the hepatic metabolic clearance of the parent drug to its metabolite). It is also possible to characterise which CYP450 enzymes are mediating the formation of the metabolites via the use of three methods, these being specific chemical inhibition studies, inhibition by monoclonal antibodies directed towards specific CYP450 enzymes, and metabolism by expressed CYP450 enzymes. Using the data obtained from kinetic studies and identification of CYP450 enzymes involved (CYP characterisation), it is then possible to predict the likelihood of changes to pharmacokinetic parameters in *in vivo* drug metabolism. For example, if a drug is metabolised by CYP2C19 it becomes possible that patients falling within the PM group may have altered clearance. Examples of this, as mentioned in chapter 1, include the metabolism of tricyclic anti-depressants (imipramine, Skjelbo *et al.*, 1991), antimalarials (proguanil, Ward *et al.*, 1991), and benzodiazepines (diazepam, Bertilsson *et al.*, 1989). Additionally, if two drugs are concomitantly administered, and both are metabolised by the same CYP450 enzyme, it is possible that reduced metabolism of one or both the drugs could occur, depending on relative affinity constants and plasma concentrations. The possibility of these *in vivo* metabolism situations may necessitate the need for clinicians to change the dosage of drugs to attain a therapeutic concentration of the drug or prevent adverse effects.

Flunitrazepam (Rohypnol®) is a 7-nitro benzodiazepine, and one of the mostly widely abused drugs in Europe, America and Australia (Darke *et al.*, 1995). A study of heroin addicts admitted for detoxification revealed that 68.5% also consumed flunitrazepam, whilst 12.4% also consumed diazepam (San *et al.*, 1993). Flunitrazepam is structurally similar to diazepam and possesses the typical activities of benzodiazepines, it is an anxiolytic, anticonvulsant, muscle relaxant, central sedative and hypnotic, leading to its clinical use as an anaesthetic and night-time hypnotic (Mattila *et al.*, 1980). Additionally, it has been reported that flunitrazepam is ten times as potent as diazepam weight for weight (Mattila *et al.*, 1980). A pharmacokinetic comparison of flunitrazepam and diazepam revealed that flunitrazepam had a shorter elimination half-life, 15 -35hr compared with 20 - 60hr (Cano, 1983), a larger volume of distribution, 6.7 ± 1.7 (Drouet-Coassolo *et al.*, 1990) compared with 1 $L.kg^{-1}$, and a larger total plasma clearance (45 compared with 235$ml.min^{-1}$) (Cano, 1983). These pharmacokinetic parameters are influenced by age, sex. liver function and protein binding (flunitrazepam is 80% bound in plasma) (Cano, 1983).

Flunitrazepam undergoes both oxidative and reductive metabolism, to produce three metabolites; 7-aminoflunitrazepam, 3-hydroxyflunitrazepam (3-OH-F) and desmethylflunitrazepam (DMF) (Figure 13).

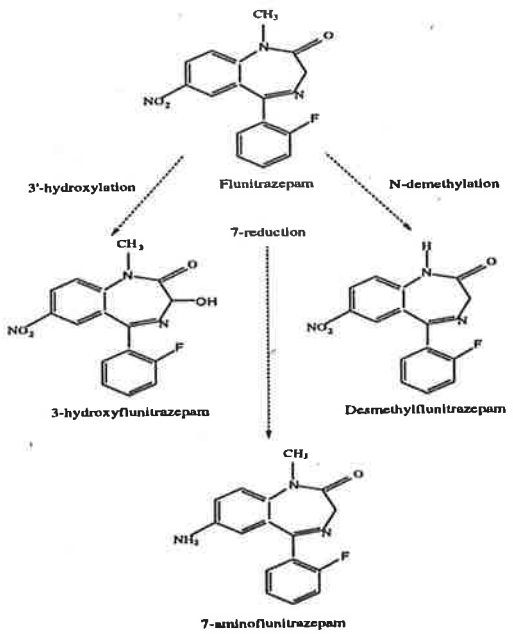


Figure 13: Metabolism of flunitrazepam in humans

There is little information about the oxidative metabolism of flunitrazepam *in vitro* in respect to both kinetics (V_{max}, K_m) and identification of CYP450 enzyme(s) involved. Diazepam is metabolised by both CYP2C19 and CYP3A4 (Andersson *et al.*, 1993, refer to section 1.2.3.2).

Proguanil has been suggested and accepted by some researchers (refer to section 1.2.4) as a replacement probe drug for the CYP2C19 genetic polymorphism. This would only be possible if proguanil and (S)-mephenytoin (withdrawn from the Australian market due to adverse effects) shared a common oxidative metabolic pathway. A preliminary investigation of which CYP450 enzyme(s) are involved in the metabolism of both proguanil and (S)-mephenytoin using specific chemical inhibitors has been reported in my honour's thesis (Coller, 1995).

The following hypotheses and subsequent aims will be addressed in this chapter:

Hypothesis 1: using human liver microsomes, proguanil oxidative metabolism to CG is mediated by CYP2C19 and CYP3A4, whilst (S)-mephenytoin metabolism to 4'-OH-meph is mediated exclusively by CYP2C19.

Aim 1: The aim of the *in vitro* study is to investigate the oxidative metabolism of (S)-mephenytoin and proguanil to 4'-OH-meph and CG, respectively, in order to identify which CYP450 enzymes mediate these reactions.

Hypothesis 2: The N-demethylation of flunitrazepam to desmethylflunitrazepam would be mediated by CYP2C19 and CYP3A4, whereas the 3'-hydroxylation of flunitrazepam to 3-hydroxyflunitrazepam would be mediated by CYP3A4.

Aim 2: The aim of this *in vitro* study is to determine, the *in vitro* kinetics of, and which CYP450 enzymes mediate the oxidative metabolism of flunitrazepam.

Aim 3: To develop an HPLC assay for the quantification of flunitrazepam and its metabolites in human liver microsomes.

2.2 Methods

2.2.1 Chemicals

Flunitrazepam, 3-OH-F and DMF were obtained from Roche Products Pty Ltd (Dee Why, Australia). (±)-4'-OH-meph and (S)-mephenytoin were purchased from Ultrafine Chemicals (Manchester, UK). Chlorcycloguanil (CCG), cycloguanil (CG) and proguanil were obtained from ICI Pharmaceuticals (Macclesfield, England). Lorazepam was obtained from Wyeth Laboratories (Sydney, Australia). Furafylline was a kind donation from Professor Wolfgang Pfleiderer (Chemogen, University of Konstanz, Germany). Other materials were obtained from the following sources: bovine serum albumin (fraction V), butylated hydroxytoluene (BHT), diethyldithiocarbamate (DDC), DL-isocitric acid (trisodium salt), Folin-Ciocalteau reagent, isocitrate dehydrogenase (NADP, type IV), α-naphthoflavone, sulphaphenazole, triethylamine (TEA), and troleandomycin (TAO) from Sigma Chemical Company (St. Louis, MO, USA); omeprazole from Astra Pharmaceuticals Pty Ltd (Sydney, Australia); nicotinamide adenine dinucleotide phosphate disodium salt (NADP-Na_2) from Merck (Darmstadt, Germany); copper sulphate ($CuSO_4$), dimethyl sulfoxide (DMSO), magnesium chloride ($MgCl_2$), orthophosphoric acid, potassium chloride (KCl), potassium-sodium-tartrate (K^+-Na^+-tartrate), sodium dihydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$), sodium hydroxide (NaOH), and sodium pyrophosphate decahydrate ($Na_4P_2O_7.10H_2O$) from Ajax Chemicals (Auburn, Australia); sodium dithionite ($Na_2S_2O_4$) from M & B Chemicals (England); diethyl ether, dimethylformamide (DMFO), sodium dihydrogen orthophosphate monohydrate ($NaH_2PO_4.H_2O$), and sodium carbonate (Na_2CO_3) from Merck Pty Limited (Kilsyth, Australia); acetonitrile (ACN), ethylenediaminetetraacetic acid (EDTA), and methanol (MeOH) from BDH Chemicals (Poole, England); carbon monoxide (CO) from CIG (Adelaide, Australia); phenobarbitone sodium from Faulding (Adelaide, Australia). Human CYP2E1 and CYP3A4 monoclonal antibodies and microsomes from human lymphoblastoid cells containing expressed

CYP2D6, CYP2C19, and CYP3A4, and CYP2C19 supersomes (baculovirus infected insect cells) were obtained from Gentest Corporation (Woburn, MA, USA).

2.2.2 Patients

Patients of the Royal Adelaide Hospital gave their written informed consent for their liver tissue to be removed and studied following partial hepatectomy. This was approved by the Committee on the Ethics of Human Experimentation of the University of Adelaide and the Human Ethics Committee of the Royal Adelaide Hospital. Refer to Appendix A for select patient demographic information. Following liver removal, liver tissue was cut into small pieces, snap frozen in liquid nitrogen and stored at -80°C.

2.2.3 Genotyping procedure

The human liver samples (HLS) were genotyped for the two genetic mutations of the *CYP2C19* gene, namely *CYP2C19*2* and *CYP2C19*3*. This was performed via polymerase chain reaction (PCR) amplifications and restriction endonuclease digestions of PCR products as previously described and detailed below (Coller *et al.*, 1997). Genotyping for two mutations of the *CYP2D6* gene, namely *CYP2D6*3* and *CYP2D6*4A/B*, was carried out in the Institute of Medical and Veterinary Science (Adelaide, Australia), according to an established method (Heim *et al.*, 1990).

2.2.3.1 Isolation and quantification of genomic DNA

Genomic DNA was isolated from liver tissue using a QIAamp® tissue kit (QIAGEN Pty Ltd, Clifton Hill, Victoria, Australia). The principle behind isolation of genomic DNA was based on lysis of liver cells and then centrifugation. Twenty-five mg of liver tissue was cut into small pieces and placed in a 1.5ml eppendorf tube. 20µl of QIAGEN Protease (17.8mg.ml^{-1}) and 180µl of Buffer ATL were added, samples were mixed immediately by vortexing and incubated on a heating block set at 55°C until tissue was completely lysed (between 1 and 3hr). Following incubation, 200µl Buffer AL was added and mixing occurred by immediate vortexing for 15sec. This was followed by a 10min incubation on a heating block set at 70°C. 210µl of absolute ethanol was then added, samples vortexed and then loaded onto the QIAamp spin column. Centrifugation occurred at 5200g for 1min after which the filtrate was discarded. Two successive washes of the DNA, using 500µl of Buffer AW and centrifugation at 5200g for 1min, were performed in order to remove cellular debris and proteins. Following this, genomic DNA was eluted from the spin column via addition of 200µl of 10mM Tris-HCl, pH 9.0 preheated to 70°C, and centrifugation at 5200g for 1min.

Quantification of the yield and purity of isolated genomic DNA was achieved by performing a wavelength scan of the eluate from 220 - 350nm. Absorbances at 260 and 280nm are of particular importance as pure DNA has a $A_{260/280}$ ratio of 1.7 - 1.9. The concentration of genomic DNA was calculated as follows;

concentration of genomic DNA = A_{260} x 50µg.ml^{-1} x dilution factor

50µg.ml^{-1} is the conversion factor used, as one optical density unit at 260nm is equivalent to 50µg.ml^{-1} of genomic DNA.

2.2.3.2 Polymerase Chain Reaction (PCR) reagents for CYP2C19 genotyping

PCR reactions for the *CYP2C19* genotyping contained 100ng of genomic DNA, 1x Taq reaction buffer (67mM Tris-HCl, pH 8.8, 16.6mM $(NH_4)_2SO_4$, 0.2mg.ml^{-1} gelatin, 0.45% Trixton X-100), 50µM of dNTPS (dATP, dCTP, dGTP, and dTTP), 2mM $MgCl_2$, 0.25µM PCR primers (detailed below), and 2.5 units of Taq DNA Polymerase. All reactions took place in 0.5ml microcentrifuge tubes (Astral Scientific, Gymea, Australia).

2.2.3.3 PCR primers for CYP2C19 genotyping

The primers used to isolate *CYP2C19*2* and *CYP2C19*3* PCR products were identical to those used by (de Morais *et al.*, 1995), and were custom synthesised at the Institute of Medical and Veterinary Science (Adelaide, Australia). They had the following sequences;

A. Forward primer for *CYP2C19*2*,

5'- AAT TAC AAC CAG AGC TTG GC -3'

B. Reverse primer for *CYP2C19*2*,

5'- TAT CAC TTT CCA TAA AAG CAA G -3'

C. Forward primer for *CYP2C19*3*,

5'- AAA TTG TTT CCA ATC ATT TAG CT -3'

D. Reverse primer for *CYP2C19*3*,

5'- ACT TCA GGG CTT GGT CAA TA -3'

2.2.3.4 PCR Cycling conditions for CYP2C19 genotyping

For detection of mutations of the *CYP2C19* gene, uniform thermal cycling conditions were utilised. Initial denaturation of the genomic DNA occurred at 94°C for 5min, followed by

35 cycles of 94°C for 20sec, 53°C for 10sec, and 72°C for 10sec. The final extension step occurred at 72°C for 5min, after which samples were cooled to 4°C. Thermal cyclers used were PE Cetus thermal cycler (Perkin-Elmer Ltd., Beaconsfield, England) and PTC-100™ Programmable Thermal Controller (MJ Research Inc., MA, USA).

2.2.3.5 Agarose gel electrophoresis for CYP2C19 genotyping

CYP2C19 PCR products were separated using a 4% NuSieve® 3:1 agarose gel. This solution was prepared via dissolution of 8g of powder in 200ml of 1 x TBE buffer (10.8g Tris base, 5.5g boric acid, and 0.74g EDTA). Gels were set in trays, and mounted in a submerged gel electrophoresis tank (Plaztek Scientific, Upper Beaconsfield, Australia). Tanks were filled with 1 x TBE buffer and sample mixtures loaded. PCR products prior to loading on the gel were mixed with a 1x gel loading buffer (0.04% bromophenol blue, 6.7% sucrose) in the ratio 10:2. In order to estimate length of PCR products a DNA marker was also loaded on to the gel, this was pUC19 DNA restricted with *Hpa* II to give fragments of 500, 489, 404, 331, 242, 190, 147, 111, 110, 67, 34, and 26 base pairs in length. Bands on the gel corresponding to DNA fragments of 169bp for *CYP2C19*2* and 329bp for *CYP2C19*3* were visualised after staining the gel with ethidium bromide and viewing with UV transilluminator.

2.2.3.6 Restriction endonuclease digestion of PCR products for CYP2C19 genotyping

Following confirmation that PCR products were of correct length with agarose gel electrophoresis, restriction endonuclease digestion was performed on these products to allow observation of possible mutations. For *CYP2C19* genotyping, *CYP2C19*2* and

*CYP2C19*3* fragments were digested by *Sma* I and *Bam* HI restriction endonucleases, respectively. To 12µl of *CYP2C19*2* PCR product, 8.2µl of digestion mix (2µl 10 x restriction enzyme buffer for *Sma* I [33mM Tris-acetate, pH 7.9, 10mM magnesium-acetate, and 66mM potassium-acetate], 0.2µl of 15U.µl^{-1} *Sma* I, and 6µl of autoclaved water) was added. Digestions occurred at 25°C on a heating block overnight (approximately 16hr), and were stopped by placing reaction tubes on ice. Agarose gel electrophoresis and staining were performed as described previously, allowing observation of 120bp and 49bp products after the wild-type allele was digested. If the PCR product contained a *CYP2C19*2* mutation, digestion was not observed.

To detect the *CYP2C19*3* mutation, to 12µl of the PCR product, 8.3µl of digestion mix (2µl 10 x restriction enzyme buffer for *Bam* HI [10mM Tris-HCl, pH 7.5, 10mM $MgCl_2$ and 150mM NaCl], 0.3µl of 10U.µl^{-1} *Bam* HI, and 6µl of autoclaved water) was added. Digestions occurred at 37°C on a heating block for 1hr, and were terminated by placing reaction tubes on ice. Agarose gel electrophoresis and staining revealed fragments of 233bp and 96bp after the wildtype allele was digested. If the PCR product contained a *CYP2C19*3* mutation, digestion was not observed.

2.2.3.7 Sequencing Analysis of *CYP2C19*1*, and *CYP2C19*2* PCR products

Sequencing of genomic DNA, *CYP2C19*1*, and *CYP2C19*2* PCR products (n=1 of each) confirmed genotyping results. It utilised dye termination reactions and was performed in the Department of Microbiology and Immunology, of the University of Adelaide (Adelaide, Australia) and in the FMC Sequencing Centre, Flinders Medical Centre (Bedford Park, Australia). The sequence of *CYP2C19*1* (wild-type) PCR product was then compared with the sequence previously published (de Morais *et al.*, 1994b), to ensure *CYP2C19*1* alleles identified in the present study were identical, and the sequences of *CYP2C19*2* PCR products were compared with *CYP2C19*1* for known mutations.

2.2.4 Microsome Preparation

Microsomes were prepared based on a method of differential centrifugation of liver homogenate (Zanger *et al.*, 1988). This method involved cutting a known weight of liver into small pieces and placing then into 2-4ml of microsomal preparation solution (1.0mM EDTA, 0.15M KCl, pH 7.3) per gram of liver. This mixture was homogenised with the large blade of a mechanical homogeniser (Thyristor Regler, John Morris Scientific Instruments Pty Ltd, Sydney, Australia), filtered through gauze pads, then homogenised with a teflon plunger (0.2mm clearance) in a large glass potter. Aliquots of this homogenate were centrifuged at 12,000g at 4°C for 15min (Beckman model J2-21 Centrifuge, JA 20 Rotor, Beckman, CA, USA). The pellets were discarded and the supernatant centrifuged at 27,000g at 4°C for 15min (Beckman model J2-21 Centrifuge, JA 20 Rotor, Comp -4 (corrects the temperature for the speed of the rotor)). The pellets were again discarded and the supernatant centrifuged further at 105,000g at 4°C for 60min (Beckman model L7-55 Ultracentrifuge, 70.1 Ti Rotor, Beckman, Fullerton, CA, USA). Then the cytosol was discarded and the pellets rehomogenised in 2-3ml of microsomal wash buffer (1.0mM EDTA, 0.1M $Na_4P_2O_7.10H_2O$, pH 7.25) in a glass/glass potter. Aliquots were recentrifuged at 105,000g at 4°C for 60min (Beckman model L7-55 Ultracentrifuge, 70.1 Ti Rotor). The cytosol was discarded and the pellets were rehomogenised in the glass/glass potter with 1ml of microsomal storage buffer (1.0mM EDTA, 0.1M Na_2HPO_4, pH 7.40) per 2g of original liver. 200µl aliquots were placed in 1.5ml Eppendorf tubes and stored at -80°C.

2.2.5 Microsomal Protein Assay

Microsomal protein concentrations were determined via the method of Lowry *et al.*, 1951. In duplicate, six standard protein concentrations (0, 50, 100, 200, 400, and 800 $\mu g.ml^{-1}$) of bovine serum albumin (fraction V) were prepared, along with a 1/25 and 1/50 dilution of

the microsomal suspension. 2ml of solution 1 (consisting of 0.5ml 2% $CuSO_4$, 0.5ml 4% K^+-Na^+-tartrate, and 49ml 3% Na_2CO_3, 0.4% NaOH) were added to each standard and sample. After vortexing, these were left to stand for 10min, then 200µl of solution 2 (consisting of 1.5ml Folin-Ciocalteau reagent and 3ml water) were added and standards and samples were left to stand for 20 - 30min in the dark. The absorbance at 550nm was read using a double-beam spectrophotometer (Hitachi, model U-2000, Hitachi Ltd., Tokyo, Japan) to construct a standard curve of concentration of bovine serum albumin versus absorbance, from which the unknown microsomal protein concentrations were calculated.

2.2.6 Total CYP450 Assay

Total CYP450 content of the microsomes was determined via the method of Omura *et al.*, 1964. A 1 in 10 dilution of microsomal suspension, made with the microsomal storage buffer (1.0mM EDTA, 0.1M Na_2HPO_4, pH 7.40), was bubbled with CO for 30sec (approximately 1 bubble.sec^{-1}) and the solution separated equally into two cuvettes. These were placed in a double-beam spectrophotometer (Hitachi, model U-2000, Hitachi Ltd., Tokyo, Japan) and a running baseline obtained between 400 and 500nm. 1-2g of $Na_2S_2O_4$ was added to the sample cuvette, inverted a few times, and a reduced-CO versus oxidised-CO difference spectrum was obtained by scanning from 400 to 500nm. The CYP450 concentration was calculated using the following equation;

$$\text{Total CYP450 content (pmol.ml}^{-1}) = \frac{(A_{450} - A_{490})_{\text{after Na2S2O4}} - (A_{450} - A_{490})_{\text{baseline}}}{0.106 \text{ (extinction coefficient)}}$$

A final value of pmol CYP450.mg protein^{-1} was obtained after correcting for the dilution and the concentration of protein originally obtained.

2.2.7 Flunitrazepam Solubility

Flunitrazepam was not soluble in water, MeOH, ethanol, or DMSO. It was soluble in ACN for up to two months at a concentration of 42mM (13.2mg.ml^{-1}, 100% ACN) at room temperature (judged by the appearance of particulate matter in the solution which could not be re-dissolved), and soluble in DMFO for up to three months at a concentration of 100mM (31.4mgml^{-1}, 100% DMFO) at room temperature.

2.2.8 Determination of kinetic parameters

A: Flunitrazepam dissolved in DMFO

Incubations with microsomes from HLS# 5, 18, 21, 22, 24 and 31 were performed in duplicate at 37°C (SW - 20C Julabo waterbath, John Morris Scientific Pty Ltd, Chatswood, Australia) for 45min. The final volume of 250µl contained 25µl NADPH generating system (1mM NADP-Na_2, 5mM DL-isocitric acid-Na_3, 5mM $MgCl_2.6H_2O$ and 1 unit NADP), microsomal incubation buffer (0.1M Na_2PO_4, pH 7.40), flunitrazepam (25 - 600µM, n=11) and 50µl of 4mg.ml^{-1} (0.2mg) microsomal protein. All incubations, including controls contained equivalent amounts of DMFO (final incubation concentration of 1% v/v), the solvent for flunitrazepam. The incubations were initiated by the addition of microsomal protein, and under these conditions, linearity of 3-OH-F and DMF formation from 50 and 450µM flunitrazepam was observed up to 60min and with 1mg.ml^{-1} microsomal protein (Figures 14 and 15). Incubations were stopped with the addition of 250µl of 1M sodium carbonate, and samples were prepared as described below (see section 2.2.9).

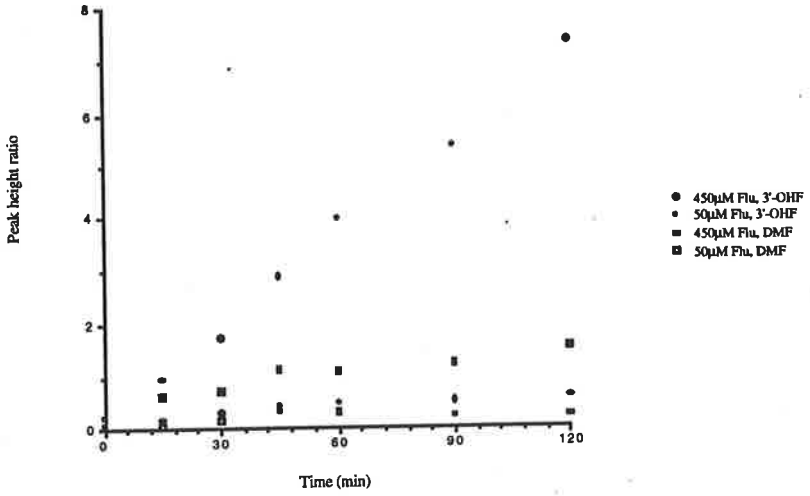


Figure 14: Relationship between 3-OH-F and DMF formation (peak height ratio) from flunitrazepam (50, 450µM), and incubation time (min) in human liver microsomes.

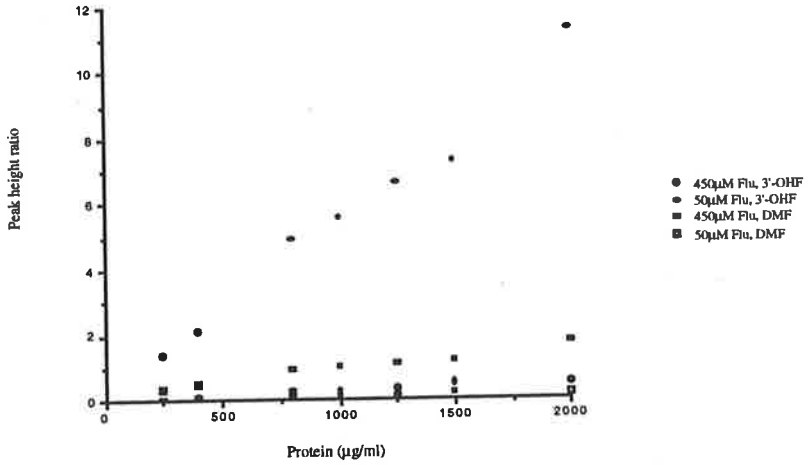


Figure 15: Relationship between 3-OH-F and DMF formation (peak height ratio) from flunitrazepam (50, 450µM), and microsomal protein content (µg/ml) in human liver microsomes.

Varying concentrations of α-naphthoflavone (0, 25, 50, 75, 100µM) were added to incubations of HLS #22 containing 320µM flunitrazepam to determine maximum activation of metabolism to 3-OH-F. Additionally, the kinetics of 3-OH-F formation were

again studied in microsomes (n=1, HLS #22) in the presence of 50μM α-naphthoflavone (the concentration where maximum activation was observed). Conditions of the incubations and sample processing did not alter from the previous kinetic study. The activation of DMF in the presence of α-naphthoflavone could not be investigated due to chromatographic interference.

B: Flunitrazepam dissolved in ACN

In HLS #5, 18, 21, 22 and 31, incubation conditions and components were identical to those used in part A of the study, the only exception being a reduced flunitrazepam concentration range (25, 50, 75, 100, 150, 200, 250, 400μM) due to reduced solubility in ACN. Similar to part A of the study, a constant concentration of ACN was added to each incubation (final incubation concentration was 1% v/v). Incubations were stopped by the addition of 250μl 1M sodium carbonate and samples were prepared as described below (see section 2.2.9).

2.2.9 Assay Methods

A, B: Flunitrazepam

50μl of 10μM lorazepam (internal standard, dissolved in 0.16% methanol) and 3ml of extraction solvent (hexane:diethyl ether, 50%:50%, v/v) were added to glass tubes containing the incubates and sodium carbonate. Each was mixed on a rotary mixer for 10min and then centrifuged at 1,500g for 10min. The organic phase was removed using glass pasteur pipettes into clean 5ml glass tubes and evaporated to dryness at 55°C under vacuum. The residue was resuspended in 250μl mobile phase and 25μl injected onto the HPLC system.

C: (S)-mephenytoin, D: Proguanil

Microsomal incubations (refer to section 2.2.12 below) were centrifuged (Eppendorf 5415C centrifuge, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) at 15,800g for 10min and a 50µl aliquot of supernatant was injected on to the HPLC system.

2.2.10 Instrumentation

A reversed phase HPLC system was utilised for the separation and quantification (metabolites only) of: A,B Flunitrazepam, 3-OH-F, DMF and lorazepam (Coller *et al.*, 1998), C (S)-mephenytoin, 4'-OH-meph and phenobarbitone (method modified from Meier *et al.*, 1985b) and D proguanil, CG and CCG (method modified from Birkett *et al.*, 1994).

The HPLC system consisted of a LC-6A pump (Shimadzu, Kyoto, Japan) with a cut-off pressure of 4kgf.cm^{-2}, a Wisp autoinjector (Model 710B, Waters, Milford, MA, USA), a variable wavelength UV absorbance detector (Model 875-UV/VIS detector, Jasco, Japan) and a C-R6A Chromatopac integrator (Shimadzu, Kyoto, Japan). Compounds were separated by a Pellicular ODS C_{18} pre-column (1cm) and a stainless-steel column (15cm x 4.6mm) packed with C_{18} 5µm ODS-2 packing material (Spherisorb; Phase Separations, Queensferry, UK). The UV detector wavelength was set at 210 and 238nm, for the flunitrazepam and (S)-mephenytoin assay, and the proguanil assay, respectively.

2.2.11 Assay Validation

2.2.11.1 Mobile Phase modifications

The best separation of 3-OH-F, DMF, lorazepam and flunitrazepam was achieved with a mobile phase at a flow rate of 1.5ml.min^{-1} consisting of 26% ACN and 50mM NaH_2PO_4 in water, adjusted to pH 4 with orthophosphoric acid (Figure 16).

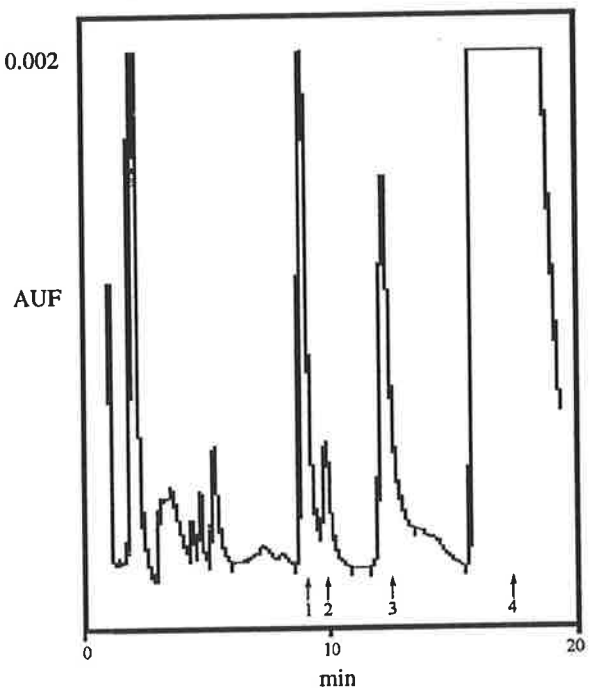


Figure 16: Representative HPLC chromatogram of 3-OH-F (1, 1.9μM), DMF (2, 0.2μM), lorazepam (3, 1.6μM) and flunitrazepam (4, 500μM).

The best separation of 4'-OH-meph, phenobarbitone, and (S)-mephenytoin was achieved with a mobile phase at a flow rate of 1.5ml.min^{-1} consisting of 20% ACN, 10mM TEA, and 5.5mM NaH_2PO_4 in water, adjusted to pH 5.5 with orthophosphoric acid (Figure 17).

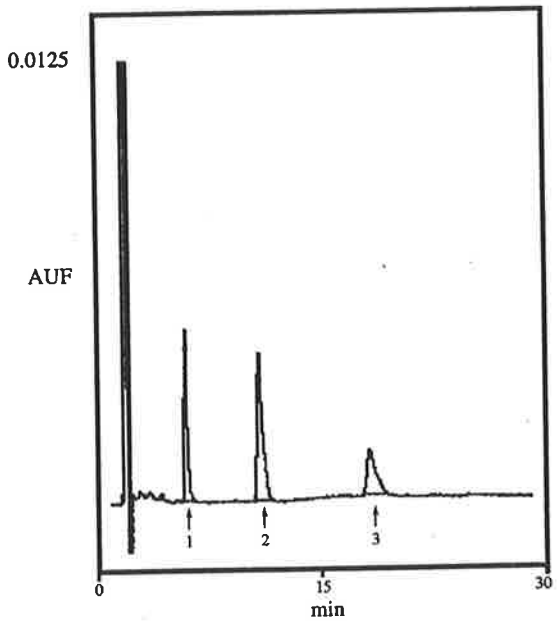


Figure 17: Representative HPLC chromatogram of reference mixture of 4'-OH-meph (1, 1.5µM), phenobarbitone (2, 1.5µM), and (S)-mephenytoin (3, 1.5µM).

The best separation of CG, CCG, and proguanil was achieved with a mobile phase at a flow rate of 1.5ml.min^{-1} consisting of 20% ACN, 10mM triethylamine, 5.5mM NaH_2PO_4 in water, adjusted to pH 2.2 with orthophosphoric acid (Figure 18).

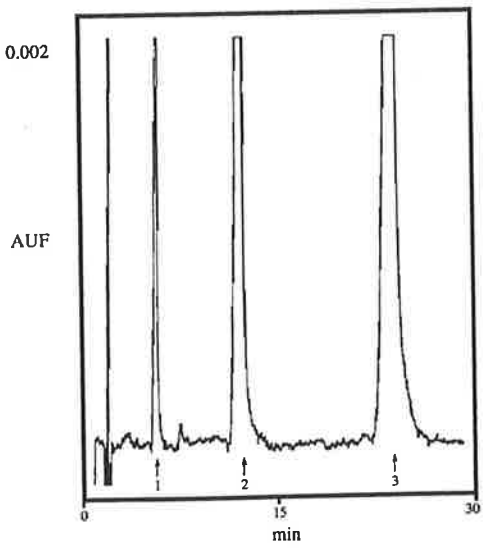


Figure 18: Representative HPLC chromatogram of reference mixture of CG (1, 0.12mM), CCG (2, 0.1mM) and proguanil (3, 0.25mM).

2.2.11.2 Calibration curves, precision, inaccuracy, limit of quantification and extraction efficiency

A, B: Flunitrazepam

Calibration curves were constructed for 3-OH-F and DMF, with 8 final concentrations ranging from 0.2-10μM. Low, medium and high quality control (QC) samples were also prepared, with final concentrations of 0.5, 2.5 and 6μM for both compounds. Standards and QC samples containing both metabolites were prepared identically to the microsomal incubations (excluding the addition of flunitrazepam) in 10ml glass tubes and placed at 37°C (SW - 20C Julabo waterbath) for 30min. Following this, 250μl of 1M sodium carbonate was added and sample preparation as described above (section 2.2.9) was performed. The limit of quantification for both metabolites was 200nM, approximately 66ng.ml^{-1} 3-OH-F and approximately 99ng.ml^{-1} DMF.

The method was validated by assaying 15 QC samples (5 each of low, medium and high concentrations) and 5 of the lowest standard (0.2μM) on a single assay day to determine the intra-day inaccuracy and precision. Inter-day inaccuracy and precision were determined by analysis of 6 QC samples (2 each of low, medium and high concentrations) and the lowest standard (0.2μM) on seven different assay days (Table 12).

Extraction efficiency was analysed for both metabolites at each QC concentration and for the internal standard (lorazepam). The peak heights of 3-OH-F, DMF and lorazepam extracted from the QC samples were compared to those obtained by direct injections of solution of these compounds in DMFO. Extraction efficiency was not determined when ACN was used as the solvent for flunitrazepam.

Peak heights of each metabolite were converted into peak height ratios using the peak height of the internal standard, and linear regression analysis of peak height ratios against

nominal concentrations provided an estimate of slope, intercept and coefficient of determination (r^2). An assay was accepted if the r^2 was greater than 0.990, and inaccuracy of all standard and QC samples in comparison to nominal values was less than ±15%.

Table 12: Summary of intra- and inter-assay precision and inaccuracy for 3-OH-F and DMF in human liver microsomes.

Nominal Conc. = nominal concentration, Cal. Conc. = calculated concentration, CV = coefficient of variation, n = sample size, L.O.Q. = limit of quantification

	Nominal Conc. (μM)	Cal. Conc. (mean ± SD)	% Inaccuracy	% CV	n
Intra-assay			L.O.Q.		
3-OH-F	0.2	0.19 ± 0.01	- 9.7	3.3	5
DMF	0.2	0.20 ± 0.01	- 0.2	5.9	5
			QCs		
3-OH-F	0.5	0.54 ± 0.05	+ 8.4	9.5	5
	2.5	2.60 ± 0.35	+ 3.9	13.5	5
	6	6.92 ± 0.96	+15	13.8	5
DMF	0.5	0.50 ± 0.02	+0.5	3.7	5
	2.5	2.42 ± 0.11	- 3.2	4.4	5
	6	5.61 ± 0.74	-6.5	13.1	4
Inter-assay	(4 or 5 different assay days)		L.O.Q.		
3-OH-F	0.2	0.19 ± 0.01	- 3.5	5.2	4
DMF	0.2	0.19 ± 0.01	- 5.5	2.6	5
			QCs		
3-OH-F	0.5	0.55 ± 0.02	+ 9.5	3.6	4
	2.5	2.43 ± 0.05	- 2.9	2.2	4
	6	6.81 ± 0.41	+ 13.4	6.0	4
DMF	0.5	0.52 ± 0.02	+ 3.4	3.6	5
	2.5	2.52 ± 0.07	+ 0.9	2.7	5
	6	5.96 ± 0.16	- 0.6	2.7	5

C: (S)-mephenytoin

The method was validated in 1995 and data reported in my honours thesis (Coller, 1995). In summary, intra-assay precision and inaccuracy for quantification of 4'-OH-meph were determined within one assay of 6 replicates of 4 standard curve samples (0.5, 5, 10 and 30μM) and each QC sample (1.5, 4 and 7.5μM). Inter-assay precision and inaccuracy were determined over 6 assays with duplicate values of each standard curve (0.5, 1, 2, 5, 8, 10, 15 and 30μM) and QC sample. All precision and inaccuracy values were less than or equal to 11%. The limit of quantification of 4'-OH-meph was 500nM, (approximately 117ng.ml^{-1}).

D: Proguanil

The method was validated in 1995 and data reported in my honours thesis (Coller, 1995). In summary, intra-assay precision and inaccuracy for quantification CG were determined within one assay of 6 replicates of 3 standard curve samples (0.2, 0.8 and 1.5μM) and each QC sample (0.5, 0.75 and 1.2μM). Inter-assay precision and inaccuracy were determined over 6 assays with duplicate values of each standard curve (0.2, 0.4, 0.6, 0.8, 1, 1.25 and 1.5μM) and QC sample. All precision and inaccuracy values were less than or equal to 14%. The limit of quantification was 0.2μM CG, approximately 58ng. ml^{-1}.

2.2.12 CYP450 enzyme characterisation experiments

2.2.12.1 Chemical Inhibition Studies

A: Flunitrazepam dissolved in DMFO

Microsomes from HLS #5, 18, 21, 22, 24 were used to examine the inhibition of 3-OH-F and DMF formation. Fixed concentrations of flunitrazepam based on the K_s values (intrinsic dissociation constant, see section 2.2.13) were incubated in a total volume of 250µl with the NADPH generating system, microsomal protein, microsomal incubation buffer and varying concentrations (10 - 100µM) of inhibitors. The chemical inhibitors considered to be specific for various CYP450 enzymes were: furafylline (CYP1A2, 25 and 100µM), coumarin (CYP2A6, 100µM), sulphaphenazole (CYP2C9, 100µM) and troleandomycin (CYP3A4, 10 and 100µM) (Newton *et al.*, 1995). Inhibition by diethyldithiocarbamate (CYP2E1/3A, 10 and 100µM), (S)-mephenytoin (CYP2C19, 100µM), and omeprazole (CYP2C19/3A4, 100µM) were also studied. Conditions of incubations did not alter from the kinetic study (see section 2.2.8), except that furafylline, diethyldithiocarbamate and troleandomycin required 15min pre-incubation prior to the addition of the substrate, as their inhibition is mechanism-based (Newton *et al.*, 1995) and NADPH-dependent complexion was needed.

Inhibitor stocks were made in various solvents due to differences in solubility. Of those not made up in water, 2% dimethyl sulphoxide (DMSO) (0.5% final incubation concentration) was used to dissolve furafylline and sulphaphenazole, 2% methanol (0.5% final incubation concentration) was used to dissolve troleandomycin, 1% methanol (0.25% final incubation concentration) was used to dissolve (S)-mephenytoin. Omeprazole was dissolved in a 2% solution of MeOH, pH 10.5 to prevent degradation as its stability is highly pH dependent (Mathew *et al.*, 1995). Incubations containing equivalent amounts of DMSO and MeOH were used as controls. Of the solvents used, 2% DMSO caused 45 and

24% inhibition (mean, n=4), 2% MeOH caused 18 and 11% inhibition (mean, n=3), 2% MeOH pH 10.5 caused 28 and 25% inhibition (mean, n=3), and 1% MeOH caused 26 and 23% inhibition (mean, n=3) of formation of 3-OH-F and DMF, respectively when compared to aqueous controls.

B: Flunitrazepam dissolved in ACN

Microsomes from HLS#5, 22, and 31 were used to re-examine the inhibition of 3-OH-F and DMF formation. Fixed concentrations of flunitrazepam based on the K_s values were incubated in a total volume of 250µl with the NADPH generating system, microsomal protein, microsomal incubation buffer and varying concentrations of inhibitors (10 - 100µM). The following chemical inhibitors were re-investigated; furafylline (CYP1A2, 25µM), troleandomycin (CYP3A4, 10µM) and (S)-mephenytoin (CYP2C19, 100µM). Additionally, combination inhibition of 3-OH-F and DMF formation by furafylline (25µM) plus troleandomycin (10µM), and by troleandomycin (10µM) plus (S)-mephenytoin (100µM) was investigated. Conditions of incubations did not alter from the kinetic study (see section 2.2.8), except that furafylline and troleandomycin required 15min pre-incubation prior to the addition of the substrate. Inhibitor stocks were identical to those as in part A except 2% ACN (final incubation concentration was 0.5% v/v) was used to dissolve furafylline. Incubations containing equivalent amounts of ACN and MeOH were used as controls.

C: (S)-mephenytoin

The inhibition of 4'-OH-meph formation was studied in human liver microsomes from HLS #5, 15, 16, 21, 22 and 31. A fixed concentration, of (S)-mephenytoin, 50µM, was chosen on the basis of the K_m previously observed in Michaelis-Menten kinetic studies (Coller, 1995). This was incubated in a total volume of 250µl with 25µl NADPH generating system, 62.5µl of 4mg.ml^{-1} microsomal protein (0.25mg), microsomal incubation buffer and various chemical inhibitors as described above. Incubations were

performed in duplicate at 37°C (SW - 20C Julabo waterbath) for 2hr. Incubations were stopped with the addition of 150µl of ACN containing 5µM phenobarbitone sodium or 10µM CG which acted as internal standards. Two internal standards were used due to interference in chromatography with some chemical inhibitors. However, phenobarbitone sodium was used when inhibition by proguanil was being studied.

Inhibitor stocks were made in varying solvents due to differences in solubility. Of those not made up in water, 2% dimethyl sulphoxide (DMSO) (0.5% final incubation concentration) was used to dissolve sulphaphenazole, 2% MeOH (0.5% final incubation concentration) was used to dissolve troleandomycin and diazepam, 11% MeOH (0.3% final incubation concentration) was used to dissolve dextromethorphan (CYP2D6 specific), and 2% ACN was used to dissolve furafylline. Omeprazole was dissolved in a 2% solution of MeOH, pH 10.5 to prevent degradation as its stability is highly pH dependent (Mathew *et al.*, 1995). Incubations containing equivalent amounts of DMSO, MeOH and ACN were used as controls. Of the solvents used, 2% DMSO caused 48% inhibition (mean, n=5), 2% MeOH caused 15% inhibition (mean, n=5), 2% MeOH pH 10.5 caused 22% inhibition (mean, n=5), and 2% ACN caused 35% inhibition (mean, n=3) of formation of 4'-OH-meph.

D: Proguanil

The inhibition of CG formation was studied in human liver microsomes from HLS #5, 16, 22 and 31. A fixed concentration of proguanil, 88, 55, 62 and 64µM for HLS #5, 16, 22 and 31, respectively, was chosen based on the K_m values obtained from the Michaelis-Menten kinetic studies (Coller, 1995). This was incubated with 25µl NADPH generating system, 50µl of 4mg.ml^{-1} microsomal protein (0.2mg), microsomal incubation buffer and various chemical inhibitors in a final incubation volume of 250µl as described above. Incubations were performed in duplicate at 37°C (SW - 20C Julabo waterbath) for 45min. Incubations were stopped with the addition of 150µl of ACN containing 15µg.ml^{-1} of chlorcycloguanil which acted as an internal standard. The chemical inhibitors were

dissolved in the same organic solvents as used in inhibition study of (S)-mephenytoin (see above). Of the solvents used, 2% DMSO caused 38% activation (mean, n=4), 2% MeOH caused 9% inhibition (mean, n=4), and 2% MeOH pH 10.5 caused 5% activation (mean, n=4) of formation of CG.

2.2.12.2 Inhibition by monoclonal antibodies

A: Flunitrazepam dissolved in DMFO

Microsomes from HLS #5 and 22 were used to examine the inhibition of 3-OH-F and DMF formation. Fixed concentrations of flunitrazepam based on the K_s values were incubated in a total volume of 250µl with the NADPH generating system, microsomal protein and microsomal incubation buffer, in the presence of human CYP2E1 and CYP3A4 monoclonal antibodies. Antibodies (at concentrations of $2\mu l.100\mu g^{-1}$ and $8\mu l.100\mu g^{-1}$ microsomal protein) were pre-incubated with microsomal protein for 15min on ice prior to the addition of remaining incubation constituents. Conditions of incubations did not alter from the kinetic study, nor did the extraction or sample processing (see section 2.2.8 and 2.2.9).

C: (S)-mephenytoin

Microsomes from HLS #5 were used to examine the inhibition of 4'-OH-meph formation. A fixed concentration of (S)-mephenytoin, 50µM, based on the K_m values was incubated in a total volume of 250µl with the NADPH generating system, microsomal protein, microsomal incubation buffer, in the presence of human CYP2E1 and CYP3A4 monoclonal antibodies. Antibodies (at concentrations of $2\mu l.100\mu g^{-1}$ and $8\mu l.100\mu g^{-1}$ microsomal protein) were pre-incubated with microsomal protein for 15min on ice prior to

the addition of remaining incubate constituents. Conditions of incubations and sample processing did not alter from the chemical inhibition study (see section 2.2.12.1).

D: Proguanil

Microsomes from HLS #5, 16 and 21, and from HLS #5 were used to examine the inhibition of CG formation by human CYP3A4 and CYP2E1 monoclonal antibodies, respectively. Fixed concentrations of proguanil, 88, 55 and 52μM for HLS #5, 16 and 21, respectively, based on the K_m values were incubated in a total volume of 250μl with the NADPH generating system, microsomal protein, microsomal incubation buffer, in the presence of human CYP2E1 and CYP3A4 monoclonal antibodies. Antibodies (at concentrations of $2\mu l.100\mu g^{-1}$ and $8\mu l.100\mu g^{-1}$ microsomal protein) were pre-incubated with microsomal protein for 15min on ice prior to the addition of remaining incubate constituents. Conditions of incubations and the sample processing did not alter from the chemical inhibition study (see section 2.2.12.1).

2.2.12.3 Formation of 3-OH-F, DMF, 4'-OH-meph and CG by expressed CYP450 enzymes

The oxidative metabolism of flunitrazepam to 3-OH-F and DMF (when flunitrazepam was dissolved in either DMFO or ACN), of (S)-mephenytoin to 4'-OH-meph, and of proguanil to CG was studied with the use of expressed CYP450 enzymes. Microsomes from human lymphoblastoid cells containing expressed CYP2D6, CYP2C19 and CYP3A4, and CYP2C19 supersomes were incubated separately with 320μM flunitrazepam, 50μM (S)-mephenytoin or 88μM proguanil in a total volume of 250μl with the NADPH generating system, and microsomal incubation buffer. These concentrations were used to ensure that formation of metabolites would be evident. Conditions of incubations and assays did not alter from the kinetic and chemical inhibition studies. Additionally, the CYP2C19

supersome-mediated oxidative metabolism of (S)-mephenytoin to 4'-OH-meph, and of proguanil to CG was studied to obtain kinetic estimates (K_m and V_{max}). CYP2C19 supersomes were incubated with varying concentrations of either (S)-mephenytoin (5, 10, 25, 50, 75 and 200μM) or proguanil (5, 10, 15, 25, 50, 75, 100, 200 and 300μM), NADPH regenerating system and microsomal incubation buffer in a total volume of 250μl. Incubation conditions and sample processing did not alter from the chemical inhibition studies (see section 2.2.12.1) except that incubation time was 1hr compared with previous 2hr for (S)-mephenytoin and 45min for proguanil.

2.2.13 Data Analysis

All raw data were transcribed to Microsoft® Excel (Version 5.0, Microsoft Corporation, WA, USA) spreadsheets, and calibration curves of peak height ratio versus concentrationwere constructed by linear regression. From the slope and intercept of the calibration curves, accuracy of the standard curve and QC concentrations, and concentrations of 3-OH-F, DMF, 4'-OH-meph and CG were determined. The rate (V) of 3-OH-F and DMF formation was calculated as number of nanomoles formed per milligram of microsomal protein per hour ($nmol.mg^{-1}.hr^{-1}$). After extraction of the unincubated microsomal mixture spiked with flunitrazepam, a small peak corresponding to the retention time of DMF was observed. The size of this peak was concentration dependent, and represented 12.7 ± 2.9% (n=5), and 6.1 ± 2.0% (n=4) of total DMF peak height when flunitrazepam was dissolved in DMFO and ACN, respectively. This peak was subtracted from the total DMF peak before kinetic values were calculated. Eadie-Hofstee plots of formation rates (V) of 3-OH-F and DMF versus concentrations of flunitrazepam (S) were constructed. Both the single-enzyme Michaelis-Menten equation (1) and the Hill equation (2) were fitted to the flunitrazepam data using non-linear least-squares regression analysis (Regression; Blackwell Scientific Software, Blackwell Scientific Publications, Oxford, UK).

Single-enzyme Michaelis-Menten model: $V = \frac{V_{max}.S}{K_m + S}$ (1)

Hill Equation: $V = \frac{V_{max}.S^n}{K_s^{\ n} + S^n}$ (2)

V_{max} = maximum formation rate

n = slope factor

K_s = intrinsic dissociation constant

S = flunitrazepam concentration

This provided an estimate of the K_s, V_{max} and n values. Maximum intrinsic clearance of flunitrazepam (Cl_{max}) was estimated from plots of flunitrazepam concentration (S) versus V/S (personal communication with Dr B Houston, Manchester, UK).

Mann Whitney U-tests were performed to determine if; i) the estimated kinetic parameters of formation of 3-OH-F were significantly different from those of DMF formation when flunitrazepam was dissolved in either DMFO or ACN, and ii) if the kinetic parameters estimated for either 3-OH-F or DMF formation when flunitrazepam was dissolved in DMFO were significantly different from those estimated when ACN was the organic solvent. Inhibition data for all three assays was expressed as a percentage of the corresponding controls and the one-tailed t-test was used to determine if inhibition was significantly different from controls. All data are tabulated as mean ± standard deviation (%CV).

2.3 Results

2.3.1 CYP2C19 / CYP2D6 Genotyping

Genotyping for *CYP2C19* revealed the presence of *CYP2C19*1* (wildtype) and *CYP2C19*2* (mutation m1) alleles, but no *CYP2C19*3* (mutation m2) alleles were found. Overall, genotyping indicated that all livers were wildtype, except HLS #5 which was heterozygote *CYP2C19*1/*2*. Genotyping for *CYP2D6* revealed the presence of *CYP2D6*1* (wildtype) and *CYP2D6*4* (mutation B) alleles, but no *CYP2D6*3* (mutation A) alleles were found. Overall genotyping indicated that HLS #5 and 31 were wildtype, HLS #18, 21 and 22 were heterozygote *CYP2D6*1/*4*, and HLS #24 was a homozygote *CYP2D6*4/*4*, and hence a genotypic poor metaboliser.

2.3.2 Flunitrazepam kinetic parameters

2.3.2.1 Flunitrazepam dissolved in DMFO

3-OH-F formation

The Eadie-Hofstee plots obtained for the formation of 3-OH-F were non-linear (refer to appendix B). Both the single enzyme Michaelis-Menten model and the Hill equation were used to provide estimates of the derived kinetic parameters. The Hill equation was observed to provide the best estimates (appendix B, Table 31, determined largely on sum of squares valus, determination coefficients and kinetic estimates provided), shown in Table 13. There was a 2-fold variation in the K_s values (range 165 - 338μM), and an almost 4-fold variation in the V_{max} values (range 21.5 - 81.3nmol.mg protein^{-1}hr^{-1}). In one liver, HLS #31, 3-OH-F formation was extremely low and the Hill equation constants (K_s, V_{max}, n) could not be quantified. However, at 200μM flunitrazepam, the 3-OH-F formation

rate was 2.7 ± 0.2nmol.mg^{-1}hr^{-1} in HLS #31, and in the other five livers (HLS #5, 18, 21, 22 and 24) at 200μM flunitrazepam the values were 11.5, 10.5, 16.6, 13.7 and 16.8nmol.mg protein^{-1}hr^{-1}. There was an almost 3-fold variation in the maximum intrinsic clearance of flunitrazepam to 3-OH-F (9.3 ± 4.4μl.mg protein^{-1}hr^{-1}, range 6.1 - 16.9μl.mg protein^{-1}hr^{-1}).

DMF formation

The Eadie-Hofstee plots obtained for the formation of DMF were non-linear (see appendix C). Both the single enzyme Michaelis-Menten model and the Hill equation were used to provide estimates of the derived kinetic parameters. The Hill equation was observed to provide the best estimates (appendix C, Table 32), shown in Table 13. There was an almost 2-fold variation in the K_s values (range 178.6 - 390.7μM), and an almost three-fold variation in the V_{max} values (range 3.4 - 10.1nmol.mg protein^{-1}hr^{-1}). In one liver, HLS #31, DMF formation was extremely low and the Hill equation constants (K_s, V_{max}, n) could not be quantified. However, at 200μM flunitrazepam, the DMF formation rate was 1.2 ± 0.1nmol.mg protein^{-1}hr^{-1} in HLS #31, and in the other five livers (HLS #5, 18, 21, 22 and 24) at 200μM flunitrazepam the values were 1.9, 1.2, 2.5, 2.4 and 1.8nmol.mg protein^{-1}hr^{-1}. There was a 2-fold variation in the maximum intrinsic clearance of flunitrazepam to DMF (1.3 ± 0.4μl.mg protein^{-1}.hr^{-1}, range 0.85 - 1.85μl.mg protein^{-1}.hr^{-1}). There was a significant difference in the V_{max} and the maximum intrinsic clearance of flunitrazepam to 3-OH-F when compared to DMF (P = 0.008, and P= 0.004, respectively).

Activation by α-Naphthoflavone

50μM α-naphthoflavone activated the formation of 3-OH-F 160%. This was the maximum percentage activation observed over 25-100μM concentration range of α-naphthoflavone. In the presence of 50μM α-naphthoflavone, the kinetics of 3-OH-F formation from flunitrazepam were not substantially different (n=1, HLS #22). The Eadie-Hofstee plot remained non linear and the kinetic parameters were best predicted using the Hill equation (Appendix D). The V_{max} increased slightly in the presence of 50μM α-naphthoflavone, (9.7 ± 2.3 versus 6.7 ± 1.9nmol.mg protein^{-1}.hr^{-1}).

2.3.2.2 Flunitrazepam dissolved in ACN

3-OH-F formation

The Eadie-Hofstee plots obtained for the formation of 3-OH-F were non-linear (see appendix E), thus the derived kinetic parameters were best estimated by the Hill equation (appendix E, Table 32) and are summarised in Table 13. There was a 1.3-fold variation in the K_s values (range 173 - 231μM), and an almost 6-fold variation in the V_{max} values (range 35 - 198nmol.mg protein^{-1}.hr^{-1}). In one liver, HLS #21, 3-OH-F formation was extremely low and the Hill equation constants (K_s, V_{max}, n) could not be quantified. The maximum intrinsic clearance of flunitrazepam to 3-OH-F varied 3.5-fold (28 ± 15μl.mg protein^{-1}.hr^{-1}, range 13 - 47μl.mg protein^{-1}.hr^{-1}).

DMF formation

The Eadie-Hofstee plots obtained for the formation of DMF were non-linear (see appendix F), thus the derived kinetic parameters were best estimated by the Hill equation (appendix F, Table 33) and are summarised in Table 13. There was an 8-fold variation in the K_s values (range 74 - 597μM), and an almost 18-fold variation in the V_{max} values (range 3 - 48nmol.mg protein^{-1}.hr^{-1}). In one liver, HLS #21, DMF formation was extremely low and the Hill equation constants (K_s, V_{max}, n) could not be quantified. The maximum intrinsic clearance of flunitrazepam to DMF varied 9.5-fold (3.6 ± 2.5μl.mg protein^{-1}.hr^{-1}, range 0.65 - 6.3μl.mg protein^{-1}.hr^{-1}). There were no significant differences between the kinetic parameter estimates obtained for the formation of 3-OH-F and DMF (V_{max}: P = 0.057; K_s: P = 1.000; n: P = 0.486; CL_{max}: P = 0.095). Additionally, there were no statistically significant differences (P > 0.05) between the kinetic estimates obtained for the formation of either metabolite when flunitrazepam was dissolved in DMFO or in ACN.

Table 13: Kinetic parameters derived from the Hill equation for 3-OH-F (A) and DMF (B) formation from flunitrazepam in human liver microsomes, when flunitrazepam was dissolved in DMFO compared with ACN. K_s - intrinsic dissociation constant, V_{max} - maximum formation rate, n - slope factor, HLS - human liver sample, NQ = not quantifiable.

A: 3-OH-F

HLS #	K_s (μM)		V_{max} (nmol.mg^{-1} hr^{-1})		n	
	DMFO	ACN	DMFO	ACN	DMFO	ACN
5	194	231	21.5	198	3.6	1.8
18	338	173	46.5	63.5	2.3	3.6
21	269	NQ	41.1	NQ	2.4	NQ
22	165	215	21.6	107	2.5	3.3
24**	279	-	81.3	-	2.6	-
31	NQ	183	NQ	34.5	NQ	1.5
mean	249	201	42.4	101	2.7	2.55
S.D.	69.5	27.1	24.5	71.4	0.5	1.05
CV (%)	27.9	13.5	57.6	70.7	18.5	41.2

B: DMF

HLS #	K_s (μM)		V_{max} (nmol.mg^{-1} hr^{-1})		n	
	DMFO	ACN	DMFO	ACN	DMFO	ACN
5	179	597	3.4	48.1	2.6	1.08
18	391	154	5.2	10.3	1.8	2.67
21	297	NQ	6.5	NQ	2.1	NQ
22	199	428	4.3	26.1	1.6	1.57
24**	365	-	10.1	-	1.7	-
31	NQ	74	NQ	2.69	NQ	1.83
mean	286	313	5.9*	21.8	1.96	1.79
S.D.	95.4	242	2.6	20.1	0.40	0.67
CV (%)	33.4	77.3	44.1	92.2	20.4	37.4

$P > 0.05$: K_s, V_{max}, n, DMFO versus ACN, * $P = 0.008$, V_{max} 3-OH-F (DMFO) versus V_{max} DMF (DMFO), ** indicates CYP2D6 genotypic PM, - indicates liver was not studied.

2.3.3 CYP450 enzyme characterisation studies

2.3.3.1 Chemical Inhibition studies

A: Flunitrazepam dissolved in DMFO

3-OH-F formation was significantly inhibited by 100μM omeprazole, 10μM and 100μM troleandomycin, 10μM and 100μM diethyldithiocarbamate, and 25μM and 100μM furafylline. Mean differences from control were -26 ± 13% (P = 0.009), -78 ± 13% (P = 0.0004), -88 ± 9% (P < 0.0001), -74 ± 19% (P = 0.004), -80 ± 14% (P = 0.0002), -78 ± 13% (P = 0.0013), and -79 ± 17% (P = 0.0005), respectively (all n=5). Additionally, in the presence of both 10μM troleandomycin and 25μM furafylline, 3-OH-F formation was significantly inhibited with mean difference from control of -85 ± 3% ($P < 0.0001$). This was not significantly different (P > 0.05) from the mean difference in the presence of either 10μM troleandomycin or 25μM furafylline alone. (S)-mephenytoin and sulphaphenazole did not significantly inhibit 3-OH-F formation (P = 0.94, P = 0.18) (Figure 19).

DMF formation was significantly inhibited by 10μM and 100μM troleandomycin, 10μM and 100μM diethyldithiocarbamate, and 25μM and 100μM furafylline. Mean differences from control were - 63 ± 6% (P = 0.0003), -58 ± 28% (P = 0.009), -56 ± 20% (P = 0.012), - 65 ± 12% (P = 0.0003), -50 ± 2% (P = 0.0007), and -63 ± 13% (P = 0.0004), respectively (all n=5). Additionally, in the presence of both 10μM troleandomycin and 25μM furafylline, DMF formation was significantly inhibited with a mean difference from control of -72 ± 3.5% (P < 0.0001). This was significantly different from the mean difference obtained in the presence of either 10μM troleandomycin (P = 0.049) or 25μM furafylline (P = 0.0002) alone. (S)-mephenytoin, omeprazole and sulphaphenazole did not significantly inhibit DMF formation (P > 0.5) (Figure 19).

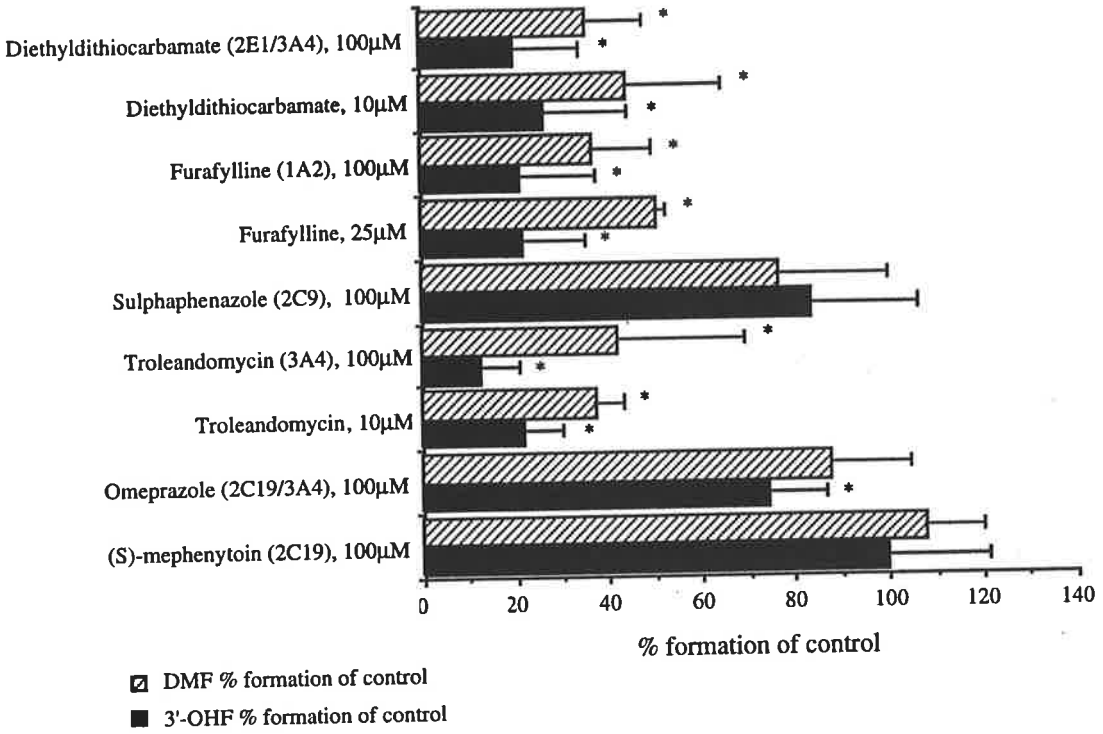


Figure 19: Chemical inhibition of 3-OH-F and DMF formation from flunitrazepam in human liver microsomes (n=4) when flunitrazepam was dissolved in DMFO, * indicates statistical significance ($P < 0.05$) compared with control, and the error bars indicate SD.

B: Flunitrazepam dissolved in ACN

3-OH-F formation was significantly inhibited by 25µM furafylline and 10µM troleandomycin. Mean differences from control were; -53 ± 13% ($P = 0.018$), and -34 ± 13% ($P = 0.045$), whilst there was no inhibition by (S)-mephenytoin, 23 ± 22% ($P = 0.13$) (Figure 20). Additionally, in the presence of 10µM troleandomycin plus 25µM furafylline, 3-OH-F formation was significantly inhibited with mean difference from control of -64 ± 14% ($P = 0.016$), and 100µM (S)-mephenytoin plus 10µM troleandomycin significantly inhibited 3-OH-F formation with mean difference from the control of -49 ± 11.5% ($P = 0.018$) (Figure 20). This was significantly different from the mean difference obtained in the presence of 100µM (S)-mephenytoin alone ($P = 0.004$), but not significantly different from that observed in the presence of 10µM troleandomycin alone ($P = 0.226$).

DMF formation was significantly inhibited by 25μM furafylline with a mean difference from control of -38 ± 11% (P = 0.028), whilst 10μM troleandomycin and 100μM S-mephenytoin had no effect, with mean differences from control of -21 ± 13% (P = 0.105), and 12 ± 23% (P = 0.364), respectively (Figure 20). Additionally, in the presence of 10μM troleandomycin plus 25μM furafylline, and 10μM troleandomycin plus 100μM (S)-mephenytoin, desmethylflunitrazepam formation was significantly inhibited with mean differences from control of -48 ± 13% (P = 0.022), and -38 ± 15% (P = 0.048) respectively (Figure 20). The latter was significantly different from the mean difference obtained in the presence of 100μM (S)-mephenytoin alone (P = 0.02).

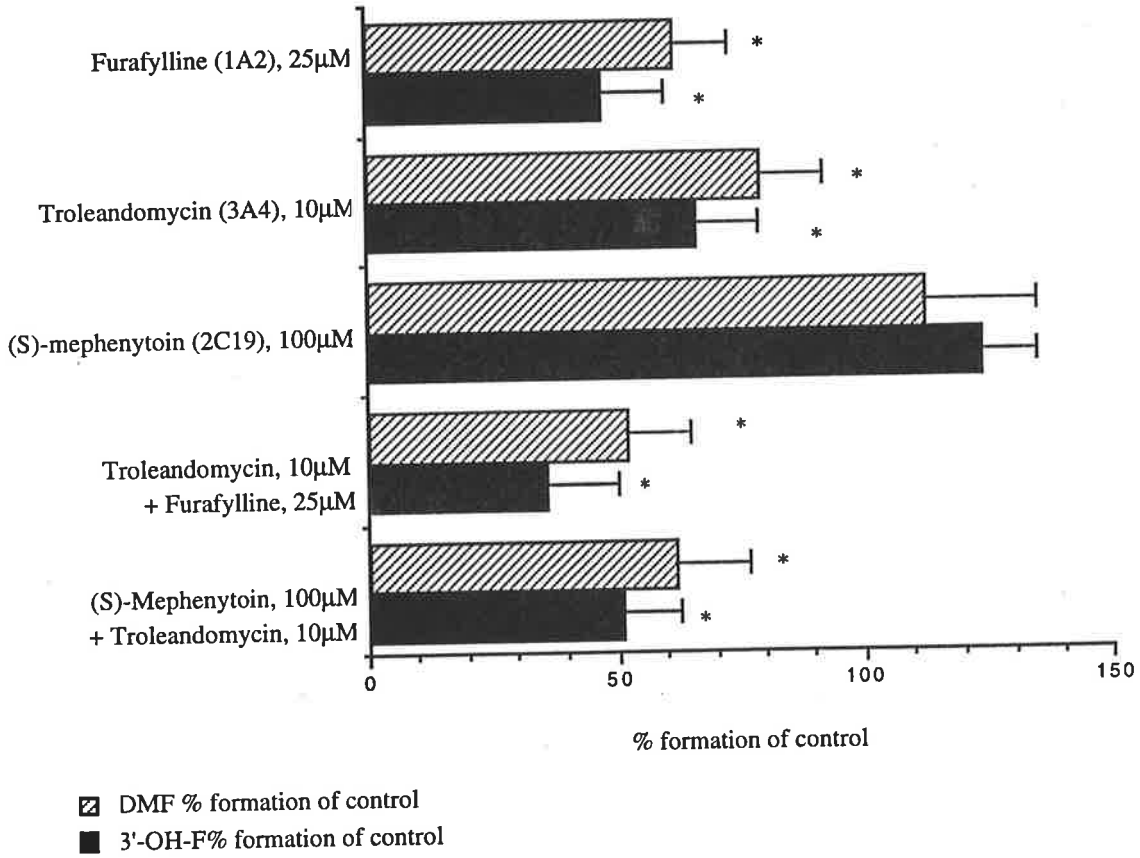


Figure 20: Chemical inhibition of 3-OH-F and DMF formation from flunitrazepam in human liver microsomes (n=4) when flunitrazepam was dissolved in ACN, * indicates statistical significance (P < 0.05) compared with control, and the error bars indicate SD.

C: S-mephenytoin

4'-OH-meph formation was significantly inhibited by omeprazole, diethyldithiocarbamate, proguanil, 25μM and 100μM furafylline, diazepam, 100μM troleandomycin, and sulphaphenazole with mean differences from control of -98 ($P < 0.0001$), -90 ± 10% ($P < 0.0005$), -36 ± 9 ($P < 0.005$), -33 ± 4% ($P < 0.001$), -36 ± 6% ($P = 0.005$), -25 ± 2% ($P < 0.0001$), -20 ± 12% ($P < 0.05$), and -12 ± 2% ($P < 0.001$); 10μM troleandomycin and 10μM dextromethorphan did not significantly inhibit 4'-OH-meph formation ($P > 0.05$) (Figure 21).

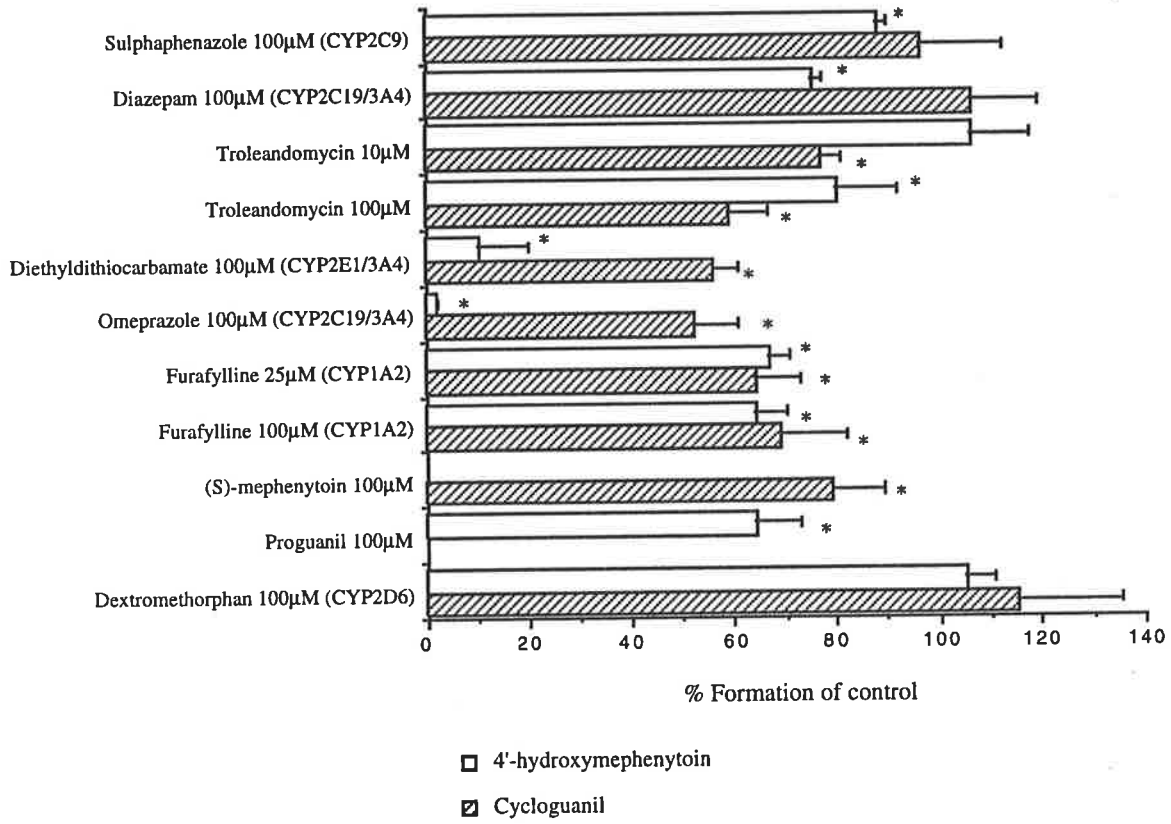


Figure 21: Chemical inhibition of 4'-OH-M and CG formation from (S)-mephenytoin and proguanil, respectively, in human liver microsomes (n=4), * indicates statistical significance ($P < 0.05$) compared with control, and the error bars indicate SD.

D: Proguanil

CG formation was significantly inhibited by omeprazole, 10 and 100μM troleandomycin, diethyldithiocarbamate, 25μM and 100μM furafylline, and (S)-mephenytoin with mean differences from control of -48 ± 9% ($P < 0.01$), -23 ± 4% ($P < 0.05$), -41 ± 8% ($P < 0.001$) -44 ± 5% ($P < 0.0005$), -36 ± 9% ($P < 0.005$), -31 ± 13% ($P < 0.01$), and -21 ± 10% ($P = 0.01$), respectively (Figure 21). Inhibition of CG formation by 10μM troleandomycin plus 25μM furafylline plus 100μM (S)-mephenytoin was not 100%, with mean difference from control of -46 ± 10% ($P < 0.02$). This was not significantly different from the mean difference observed in the presence of 10μM troleandomycin alone ($P = 0.114$) or 25μM furafylline alone ($P = 1.000$). Diazepam, dextromethorphan and sulphaphenazole did not significantly inhibit CG formation (Figure 21).

2.3.3.2 Inhibition by monoclonal antibodies

A: Flunitrazepam dissolved in DMFO

Human CYP3A4 monoclonal antibodies inhibited the formation of 3-OH-F and DMF from flunitrazepam. The mean differences from control values were -50 ± 7% and -38 ± 6%, respectively (2μl CYP3A4.100μg microsomal protein^{-1}), and - 71 ± 6% and - 46 ± 0%, respectively (8μl CYP3A4.100μg microsomal protein^{-1}). Human CYP2E1 monoclonal antibodies did not inhibit the formation of 3-OH-F and DMF from flunitrazepam. The mean differences from control values were -5 ± 8% and -4 ± 1%, respectively (2μl CYP3A4.100μg microsomal protein^{-1}), and -6 ± 8% and -12 ± 10%, respectively (8μl CYP3A4.100μg microsomal protein^{-1}). No statistical analysis was performed due to low sample size (n=2).

C: (S)-mephenytoin

Human CYP2E1 and CYP3A4 monoclonal antibodies did not inhibit the formation of 4'-OH-meph from (S)-mephenytoin. The mean differences from control values in the presence of 2µl and 8µl CYP2E1.100µg microsomal protein^{-1} were 1 ± 15% and 4 ± 14%, respectively, and in the presence on 2µl and 8µl CYP3A4.100µg microsomal protein^{-1} were 10 ± 4% and 24 ± 5%, respectively.

D: Proguanil

Human CYP2E1 and CYP3A4 monoclonal antibodies did not inhibit the formation of CG from proguanil. The mean differences from control values in the presence of 2µl and 8µl CYP2E1.100µg microsomal protein^{-1} were 27 ± 10% and 8 ± 10%, respectively, and in the presence on 2µl and 8µl CYP3A4.100µg microsomal protein^{-1} were 27 ± 2% and -9 ± 8%, respectively.

2.3.3.3 Formation of 3-OH-F, DMF, 4'-OH-meph and CG by expressed CYP450 enzymes

A: Flunitrazepam dissolved in DMFO

Microsomes from human lymphoblastoid cells containing expressed CYP2D6 and CYP2C19 did not metabolise flunitrazepam to either of its oxidative metabolites. However, microsomes from human lymphoblastoid cells containing expressed CYP3A4 metabolised flunitrazepam to both 3-OH-F and DMF. Table 14 shows the formation rates of both metabolites by expressed CYP3A4 compared to those in microsomes from human liver samples (n=3).

B: Flunitrazepam dissolved in ACN

Microsomes from human lymphoblastoid cells containing expressed CYP2C19 and CYP3A4, and CYP2C19 supersomes metabolised flunitrazepam to both oxidative metabolites. The formation rates of both metabolites by expressed CYP2C19 and CYP3A4, and CYP2C19 supersomes were compared to those in microsomes from HLS #5 (Table 14). The Vmax and Km kinetic parameters were not estimated with expressed CYP2C19 and CYP3A4 due to limited amount of these enzymes available.

Table 14: Formation rates (per picomole P450 in incubation) of 3-OH-F and DMF by expressed CYP2C19, CYP2D6, CYP3A4 and CYP2C19 supersomes in comparison with that of human liver microsomes (HLS n=3), when 320µM flunitrazepam was dissolved in DMFO and ACN. < L.O.Q. - less than the limit of quantification.

	3-OH-F formation ($pmol.mg^{-1}.hr^{-1}$)/pmol P450	DMF formation ($pmol.mg^{-1}.hr^{-1}$)/pmol P450
	DMFO	DMFO
HLS (n=3)	563 - 3618*	19 - 256*
Expressed CYP3A4	657	57
Expressed CYP2C19	< L.O.Q.	< L.O.Q.
Expressed CYP2D6	< L.O.Q.	< L.O.Q.
CYP2C19 Supersomes	< L.O.Q.	< L.O.Q.
	ACN	ACN
HLS #5	1811	253
Expressed CYP3A4	91	43
Expressed CYP2C19	59	216
CYP2C19 Supersomes	37	120

* range (n=3)

C: S-mephenytoin

Microsomes from human lymphoblastoid cells containing expressed CYP2D6 and CYP3A4 did not form 4'-OH-meph from (S)-mephenytoin. Conversely, microsomes from

human lymphoblastoid cells containing expressed CYP2C19 and CYP2C19 supersomes were able to form 4'-OH-meph. The formation rates by the expressed enzymes were compared to those in microsomes from HLS #5 (Table 16). The formation kinetics of 4'-OH-meph by CYP2C19 supersomes revealed a linear Eadie-Hofstee plot, and kinetic estimates were obtained from a single Michaelis-Menten enzyme model. The K_m, V_{max} and coefficient of determination (r^2) for the fit of the model to the data for 4'-OH-meph were 70μM, 55 x 10^{-2} nmol.pmol $P450^{-1}.hr^{-1}$, and 0.99 (Figure 22).

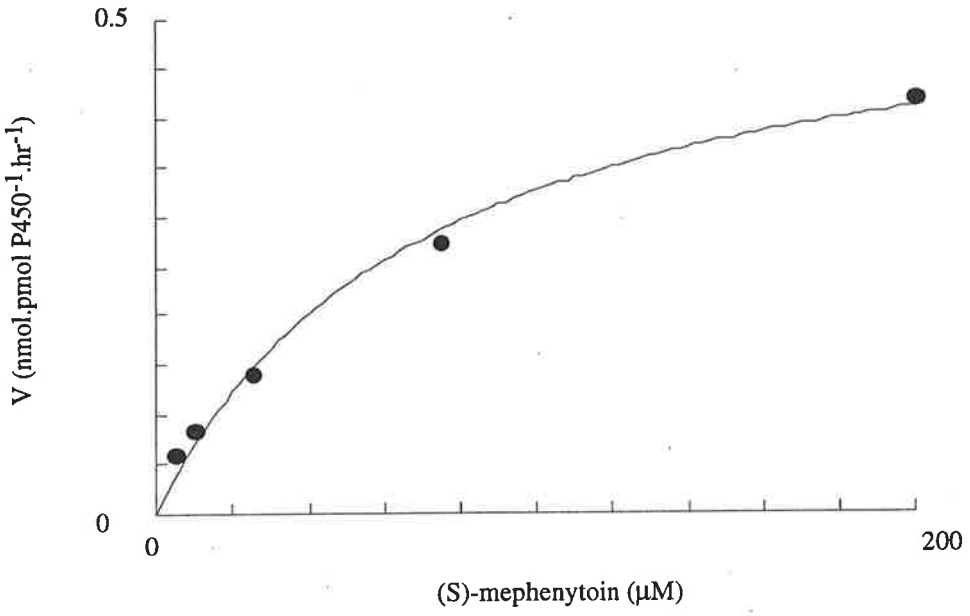


Figure 22: Formation of 4'-OH-meph from (S)-mephenytoin by CYP2C19 supersomes.

D: Proguanil

Microsomes from human lymphoblastoid cells containing expressed CYP2D6 did not form CG from proguanil. Conversely, microsomes from human lymphoblastoid cells containing expressed CYP3A4 and CYP2C19 supersomes formed CG, but formation of CG by expressed CYP2C19 only was below the limit of quantification of the assay. The formation rates of CG by the expressed enzymes were compared to those in microsomes from HLS #16 (Table 16). The formation kinetics of CG by CYP2C19 supersomes revealed a linear Eadie-Hofstee plot, and kinetic estimates were obtained from a single Michaelis-Menten enzyme model. The K_m, V_{max} and coefficient of determination (r^2) for

the fit of the model to the data for CG were 7.7μM, 2.5 x 10^{2} nmol.pmol $P450^{-1}.hr^{-1}$, and 0.95 (Figure 23).

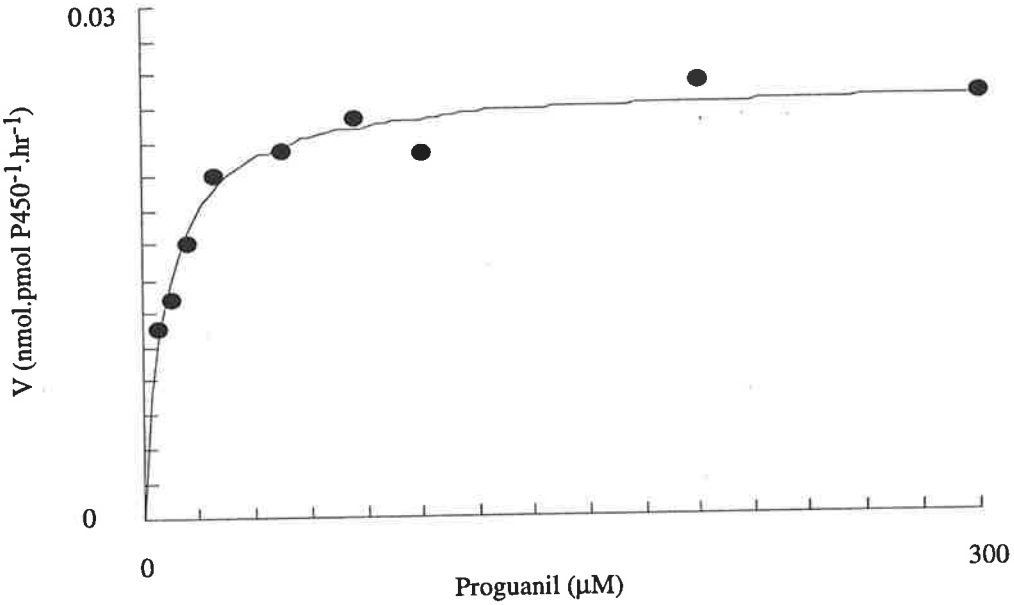


Figure 23: Formation of CG from proguanil by CYP2C19 supersomes.

Table 16: Formation rates of CG and 4'-OH-meph from 88μM proguanil and 50μM (S)-mephenytoin, respectively, by expressed CYP2C19 and CYP3A4, and CYP2C19 supersomes in comparison with that of human liver microsomes (HLS #16 for CG, or #5 for 4'-OH-meph).

	CG formation nmol.pmol $P450^{-1}.hr^{-1}$	4'-OH-meph formation nmol.pmol $P450^{-1}.hr^{-1}$
HLS #16 or #5	1.92 x 10^{-3}	1.37 x 10^{-2}
Expressed CYP3A4	1.33 x 10^{-3}	< L.O.Q.
Expressed CYP2C19	< L.O.Q.	8.56 x 10^{-2}
CYP2C19 Supersomes	24.6 x 10^{-3}	18.8 x 10^{-2}

2.4 Discussion

Previous investigators have demonstrated that the choice of organic solvent for dissolving substrates in *in vitro* CYP450 studies can have a profound effect on the results and their interpretation (Hickman *et al.*, 1998, Chauret *et al.*, 1998). For example, CYP2C19 activity is inhibited up to 90% by DMFO, but remains unchanged by ACN. It therefore seems reasonable to assume that kinetic estimates and CYP450 enzyme characterisation for any substrate could differ according to which organic solvent is chosen to dissolve the substrate. This study of *in vitro* flunitrazepam kinetics and CYP450 characterisation in the presence of DMFO and ACN examined this assumption.

The kinetic parameters for the formation of both 3-OH-F and DMF in the 5 livers were best estimated using the Hill equation, regardless of the organic solvent used to dissolve flunitrazepam. The unidentified peak corresponding to the retention time of DMF was found to be substrate-concentration dependent and is likely to be a small contaminant of DMF in the pure flunitrazepam sample. The non-linearity of the Eadie-Hofstee plots and the sigmoidicity of the formation rates suggest that the kinetics of the oxidative metabolism of flunitrazepam are complex and one possible explanation is that of cooperativity in oxidation (Ueng *et al.*, 1997). This could occur when the formed metabolite, either 3-OH-F or DMF, binds to a site on the CYP450 enzyme(s) and induces a conformational change which activates oxidative metabolism of the remaining flunitrazepam. This hypothesis conforms to the observation of Andersson and colleagues, who studied the oxidative metabolism of diazepam, which undergoes similar metabolism to flunitrazepam in human liver microsomes (Andersson *et al.*, 1994). Sigmoidal kinetics have been reported for other CYP3A substrates, such as caffeine (Tassaneeyakul *et al.*, 1992) and progesterone (Schwab *et al.*, 1988) suggesting that CYP3A may be involved in the oxidative metabolism of flunitrazepam to 3-OH-F and DMF.

The oxidative metabolism of flunitrazepam was inhibited in the presence of the organic solvent, DMFO. This was deduced from the observation that the V_{max} values obtained for the formation of 3-OH-F and DMF increased (non-statistical significance) when flunitrazepam was dissolved in ACN compared with those rates when DMFO was the organic solvent, range: 34.5 - 199 and 2.69 - 48.1nmol.mg protein^{-1}.hr^{-1}, versus 21.5 - 81.3 and 3.4 - 10.1nmol.mg protein^{-1}.hr^{-1}. Inhibition of flunitrazepam metabolism by DMFO is also reflected in the increase (non-statistical significance) in the maximum intrinsic clearance of flunitrazepam to its oxidative metabolites when ACN was used as the organic solvent. Perhaps these increases may have been significant if the present study had included larger sample sizes (n=6 used in the present study). Conversely, the K_s values obtained for the formation of 3-OH-F and DMF when flunitrazepam was dissolved in DMFO, were similar to those obtained when ACN was used as the organic solvent, range: 165 - 337μM, and 179 - 391μM, versus 173 - 231μM and 73.9 - 597μM, respectively. It has been shown previously that DMFO inhibits CYP2C19 mediated metabolism by a larger degree than CYP3A4, 90% versus 10% (Hickman *et al.*, 1998); therefore, it is likely that DMFO inhibited the CYP2C19-mediated formation of both oxidative metabolites but not the formation mediated by CYP3A4. The fact that the K_s values were similar regardless of the organic solvent suggests that CYP2C19 and CYP3A4 have similar affinity for flunitrazepam.

Identification of the CYP450 enzymes mediating the oxidative metabolism of flunitrazepam was deduced from three observations. The involvement of CYP3A4 was concluded, firstly by the use of enzyme selective chemical inhibitors, as significant inhibition of the formation of 3-OH-F and DMF was observed with troleandomycin. Secondly, human monoclonal antibodies directed against CYP3A4 inhibited the formation of both metabolites, and finally, the use of expressed CYP3A4 provided further confirmation of the involvement of this enzyme in the oxidative metabolism of flunitrazepam. The observation of sigmoidal kinetics for the formation of 3-OH-F and DMF also indicates involvement of CYP3A4, as this is common for substrates of this enzyme (Ueng *et al.*, 1997).

When flunitrazepam was dissolved in DMFO, it was concluded that CYP2C19 was not involved, as (S)-mephenytoin did not inhibit the formation of both metabolites, and expressed CYP2C19 could not form either metabolite. This false conclusion was due to the significant inhibition by DMFO of CYP2C19 activity (Hickman *et al.*, 1998). In contrast, when flunitrazepam was dissolved in ACN, CYP2C19 was confirmed as an isoform mediating the oxidative metabolism of flunitrazepam to both metabolites, based on the results obtained using expressed CYP2C19 and CYP2C19 supersomes. The formation rates were similar to and 3-fold higher than those catalysed by expressed CYP3A4 for 3-OH-F and DMF, respectively. Additionally, the fact that DMSO, an organic solvent used to dissolve some chemical inhibitors, reduced the formation of both oxidative metabolites by a mean value of 35% further supports the involvement of CYP2C19, as this CYP450 enzyme is inhibited up to 70% by DMSO (Hickman *et al.*, 1998).

(S)-mephenytoin alone did not significantly inhibit the formation of either metabolite, but appeared to activate the formation of 3-OH-F and DMF when ACN was used as the organic solvent, mean differences from control of 23 ± 22% and 12 ± 23%, respectively. However, in combination with troleandomycin, significant inhibition of the formation of DMF was observed. One possible explanation could be that the additional ACN used in these incubations, as (S)-mephenytoin was dissolved in ACN, caused activation of the oxidative metabolism of flunitrazepam via another enzyme, for example CYP1A2, which has been shown previously to be activated up to 120% by ACN (Hickman *et al.*, 1998). Therefore, (S)-mephenytoin could be inhibiting both reactions, however the level of inhibition is less than that of the activation by ACN, so that overall, no inhibition was observed.

Other CYP450 enzymes were either confirmed or discounted as being involved using similar observations. CYP1A2 was involved, as furafylline (highly specific for CYP1A2 at 25μM) produced significant inhibition of 3-OH-F and DMF formation. CYP2D6 was discounted, as expressed CYP2D6 could not form either metabolite, and there was no difference in the kinetics of the microsomes from the CYP2D6 poor metaboliser.

Investigation of chemical inhibition by dextromethorphan (CYP2D6 specific) was deemed unnecessary due to the results obtained with the expressed CYP2D6 enzyme. Similarly, CYP2E1 was discounted, as the human monoclonal antibody directed against CYP2E1 could not produce significant inhibition of formation of either metabolite. This observation again highlights the caution needed when drawing conclusions from inhibition studies utilising diethyldithiocarbamate, which has been shown recently to be a non-specific chemical inhibitor of CYP2E1, with 80% and 60% inhibition of CYP2E1 and CYP3A4, respectively (Eagling *et al.*, 1998). Finally CYP2C9 was discounted, as sulphaphenazole did not produce significant inhibition of the formation of either metabolite.

Similarities between the *in vitro* oxidative metabolism of flunitrazepam and diazepam (Andersson *et al.*, 1994) can be noted. Based on V_{max} values, for both substrates, the formation of the 3'-hydroxy- metabolite is greater than that of the desmethyl metabolite (2-fold for flunitrazepam, and 5-fold for diazepam). The kinetics of the formation of both metabolites are sigmoidal, so that the Hill equation provides the best estimate of kinetic parameters. However, α-naphthoflavone was unable to revert this sigmoidicity to simple Michaelis-Menten kinetics (via prevention of substrate activation) in the case of flunitrazepam, in contrast to that of diazepam. This may indicate that the cooperativity involved in the oxidative metabolism of flunitrazepam is more complex than for diazepam. The other similarity relates to the isoforms mediating the formation of the 3'-hydroxy and desmethyl metabolites. For both flunitrazepam and diazepam, CYP2C19 and CYP3A4 mediate the formation of the desmethyl metabolite, whilst the formation of the 3'-hydroxy metabolite of flunitrazepam is also mediated by CYP2C19 and CYP3A4, but only CYP3A4 is involved in the formation of 3'-hydroxy-diazepam (temazepam). It is interesting to note that Andersson and colleagues (Andersson *et al.*, 1994) did not investigate the involvement of CYP1A2 in the oxidative metabolism of diazepam, which was concluded to be involved in the present study with flunitrazepam, although further confirmation is needed via utilisation of CYP1A2 antibody and CYP1A2 expressed

enzymes. Thus, it is not possible to make any further conclusions as to the similarities or otherwise in the oxidative metabolism of diazepam and flunitrazepam.

As indicated in the introduction, proguanil would be an appropriate replacement probe drug for (S)-mephenytoin if similarities in oxidative metabolism of these drugs can be shown. Identification of CYP450 enzyme(s) mediating the formation of 4'-OH-meph and CG was deduced in a similar manner to those mediating the formation of 3-OH-F. CYP2C19 and 3A4 involvement in the formation of 4'-OH-meph was concluded firstly by the use of chemical inhibition data, as significant inhibition of 4'-OH-meph formation by omeprazole (98%), diazepam (25%) and 100μM troleandomycin (20%) was observed. However, the small degree of inhibition by 100μM troleandomycin, although statistically significant, suggests that the 4'-hydroxylation of (S)-mephenytoin is not predominantly via CYP3A4, and that the inhibition by omeprazole and diazepam was observed mainly due to inhibition of CYP2C19, with minor CYP3A4 inhibition. It was reported previously that diazepam was a partial competitive inhibitor of CYP2C19 (K_i= 12μM) (Hall *et al.*, 1987), and a reversible inhibitor of CYP3A4 (Iribarne *et al.*, 1996). Another study has shown that omeprazole competitively inhibits metabolism of (S)-mephenytoin by CYP2C19 with a K_i value of 3.1 ± 2.2μM, and noncompetitively inhibits metabolism by dextromethorphan by CYP3A4 with a K_i value of 84.4 ± 4.0μM (Ko *et al.*, 1997). The difference in these K_i values support the larger degree of CYP2C19 inhibition by omeprazole when compared to diazepam. The role of CYP3A4 was further discounted due to the lack of inhibition by human CYP3A4 monoclonal antibodies, and the inability of expressed CYP3A4 to form 4'-OH-meph.

The role of CYP2C19 in (S)-mephenytoin metabolism was further confirmed as CYP2C19 supersomes formed the 4'-hydroxy metabolite. The K_m estimate obtained (70μM) was similar to that obtained in human liver microsomes (51μM, Coller, 1995), indicating that the CYP450 enzyme mediating this reaction is predominantly CYP2C19. Other CYP450 enzymes were either confirmed or discounted as being involved in the formation of 4'-OH-meph using similar observations. CYP1A2 was involved as furafylline significantly

inhibited 4'-OH-meph. This is consistent with the findings of Chiba and associates who showed that (S)-mephenytoin metabolism is inhibited in human liver microsomes by imipramine, whose N-demethylation pathway is mediated by CYP2C19, 1A2 and 3A (Chiba *et al.*, 1994). Further investigations using expressed CYP1A2 are needed to confirm these results. CYP2C9 and CYP2D6 could be discounted due to lack of inhibition by sulphaphenazole and dextromethorphan, respectively. CYP2E1 was discounted as being involved due to lack of inhibition by human CYP2E1 monoclonal antibodies. This result again highlights the need for caution when drawing conclusions from inhibition studies utilising diethyldithiocarbamate, which is a non-specific inhibitor of CYP2E1 (Eagling *et al.*, 1998). Therefore, from these data it can be concluded that the CYP450 enzymes mediating the formation of 4'-OH-meph is predominantly CYP2C19, with the role of 1A2 yet to be confirmed.

CYP2C19 and CYP3A4 were confirmed as isoforms mediating CG metabolism firstly due to the observation of significant inhibition by omeprazole and troleandomycin. It is unclear why diazepam did not produce significant inhibition in a similar manner to omeprazole. However, this may be explained by the differences in the types of inhibition of CYP2C19, partial competitive for diazepam with K_i of 12μM (Hall *et al.*, 1987) compared with competitive for omeprazole with K_i of 3.1 ± 2.2μM (Ko *et al.*, 1997). Therefore, omeprazole when compared to diazepam would have a greater ability to inhibit the involvement of CYP2C19 in CG formation. Additionally, it is possible that inhibition of the CYP3A4 mediated formation of CG is greater in the presence of omeprazole when compared to diazepam due to different types of inhibition; noncompetitive inhibition by omeprazole with a K_i of 84.4 ± 4.0μM (Ko *et al.*, 1997), versus reversible inhibition by diazepam (a K_i value has not been reported). It is unlikely that this difference in inhibition can be explained by the presence of different organic solvents used to dissolve omeprazole and diazepam as the only difference was that of the pH of the methanolic solution. Overall, these data indicate that CYP2C19 plays a minor role in the metabolism of proguanil.

The conclusion that CYP2C19 plays a minor role in the metabolism of proguanil agrees with the observation that expressed CYP3A4 was able to form CG at a rate similar to that seen in human liver microsomes, but expressed CYP2C19 did not form CG. The higher formation rate of CG by CYP2C19 supersomes than that seen with expressed CYP3A4 is likely to be due to reduced catalytic activity of CYP3A4 when compared with the CYP2C19 supersomes. Furthermore, the K_m estimate obtained when CG was formed by CYP2C19 supersomes was much lower than that obtained with human liver microsomes, 7.7μM versus 43 - 76μM, indicating the involvement of more than one CYP450 enzyme.

It remains unknown why 100% inhibition of CG formation was not obtained in the presence of 10μM troleandomycin plus 25μM furafylline plus 100μM (S)-mephenytoin. It could be speculated from these data that other CYP450 enzymes, not inhibited by these chemicals, are involved in the formation of CG, although all the other CYP450 characterisation data do not lend support to this conclusion. CYP1A2 involvement was also evident by significant inhibition of CG by furafylline. This contrasts with previous findings (Birkett *et al.*, 1994). However, these investigators did not preincubate furafylline with NADPH and microsomes, which is essential as inhibition is mechanism-based (Halpert *et al.*, 1994). The potential role of CYP1A2 in CG formation needs further investigation with expressed CYP1A2 enzyme. CYP2E1 was discounted, as although diethyldithiocarbamate produced significant inhibition, human CYP2E1 monoclonal antibodies did not inhibit CG formation. CYP2D6 and CYP2C9 were discounted due to lack of inhibition of proguanil metabolism by dextromethorphan and expressed CYP2D6, and sulphaphenazole, respectively.

The inhibition of proguanil metabolism by (S)-mephenytoin was small (although significant), which agrees with previous reports noting that the degree of inhibition is dependent on the CYP3A content of human liver microsomes (Birkett *et al.*, 1994). Therefore, it is possible that if the livers used in the present study have a high CYP3A content, as evident by a high degree of inhibition by troleandomycin, a smaller degree of inhibition by (S)-mephenytoin would be evident.

Implications of the results obtained from the study of flunitrazepam oxidative metabolism include the possibility of drug-drug interactions between flunitrazepam and other CYP2C19 and/or CYP3A4, and possibly CYP1A2, substrates. This possibility has already been reported in the case of erythromycin, a known substrate and mechanism-based inhibitor of CYP3A4 (Zhang *et al.*, 1996). Lurrila and colleagues found that pretreatment of subjects with erythromycin (500mg, once daily for 3 days) caused a 25% increase in the area under the flunitrazepam plasma concentration-time curve (AUC, 0-42hr) and a 56% increase in half-life following a single 1mg oral dose of flunitrazepam (Luurila *et al.*, 1996). Additionally, the involvement of CYP2C19 may indicate the possibility of inter-ethnic differences in the oxidative metabolism of flunitrazepam, especially if a patient is a CYP2C19 PM, as the percentage of different ethnic populations within this metabolic group varies markedly (refer to section 1.2.4). Furthermore, the data should serve as a reminder to researchers about the caution needed when using organic solvents in *in vitro* systems such as human liver microsomes. In addition, when predicting *in vivo* metabolism by a particular CYP450 enzyme from *in vitro* results, it should be noted that the involvement of any particular CYP450 enzyme can vary due to inter-individual variability in the expression.

In conclusion, this study has demonstrated that the *in vitro* formation of 3-OH-F and DMF is mediated by CYP2C19, 3A and possibly 1A2. Additionally, the Michaelis-Menten kinetic parameters of flunitrazepam are sigmoidal, indicating the involvement of cooperativity in metabolism. It has also been observed that similarities do exist between the *in vitro* metabolism of flunitrazepam and diazepam, such that common CYP450 enzymes mediate the hydroxylation and demethylation reactions.

The data obtained in the study of (S)-mephenytoin and proguanil oxidative metabolism in human liver microsomes suggest that *in vitro*, metabolism is catalysed by two common CYP450 enzymes, CYP2C19 and 1A2, although the extent to which each contributes to the overall oxidation differs, and CYP3A4 is also substantially involved in the formation of proguanil. Comparisons between population based phenotyping using proguanil as the

in vivo phenotyping probe and CYP2C19 genotyping analysis may help in clarifying the role of proguanil as a probe for the CYP2C19 genetic polymorphism. This will be addressed in the chapter 3.

3. *In vivo* CYP2C19 phenotyping and genotyping in human subjects

3.1 Introduction

In vivo studies using probe drugs are often used to identify people who have a reduced or increased capacity to metabolise drugs by a particular CYP450 enzyme, such as CYP2C19 or CYP2D6, resulting in a particular phenotype status. Racemic-mephenytoin was commonly used, and still remains used in many European countries, to assign CYP2C19 phenotype status until the reports of severe adverse effects led to its withdrawal from the drug market and the need to find an appropriate replacement probe drug. Proguanil, which is metabolised by CYP2C19 and CYP3A4 to form an active metabolite cycloguanil (CG), has been suggested and used by several researchers to phenotype for CYP2C19 (Watkins *et al.*, 1990, Funck-Brentano *et al.*, 1992, Edstein *et al.*, 1994, Wanwimolruk *et al.*, 1995, Hoskins *et al.*, 1998). It is logical to conclude that the reliability of the phenotype assigned following the administration of a probe drug depends on the degree to which the probe drug used is metabolised by the particular CYP450 enzyme. Therefore, the appropriateness of using proguanil to assign CYP2C19 phenotypic status needs to be addressed, particularly due to the involvement of CYP3A4, and possibly CYP1A2 in cycloguanil formation (see chapter 2). Additionally, the antimode of the proguanil / cycloguanil metabolic ratio (PG/CG MR) used to assign CYP2C19 phenotype has previously been chosen by researchers by visual inspection of histograms, with no quantitative statistical evaluation to explain the value of 10 (refer to section 1.2.4).

The following hypotheses and subsequent aims will be addressed in this chapter:

Hypothesis 3: There will be a lack of concordance between the CYP2C19 genotype and phenotype assigned by PG/CG MR.

Hypothesis 4: The PM incidences in the Caucasian and Asian populations in this study would range between 2.6 - 8%, and 15 - 25%, respectively.

Aims 4 and 5: The aims of the *in vivo* study are to: i) investigate the concordance between the CYP2C19 genotype (based on *CYP2C19*2* and *CYP2C19*3* mutations), and phenotype assignment using the PG/CG MR in Caucasian and Asian populations living in Australia; and, ii) use three graphical methods (histogram, probit plot, normal test variable plot) to evaluate the PG/CG MR antimode separating the CYP2C19 genotypic EM and PM groups.

3.2 Methods

3.2.1 Chemicals

Chlorcycloguanil (CCG), cycloguanil (CG) and proguanil were obtained from ICI Pharmaceuticals (Macclesfield, England). Other materials were obtained from the following sources: Taq-DNA Polymerase, Taq reaction buffer, 250mM magnesium chloride ($MgCl_2$), dNTP's, *Sma* I and *Bam* HI restriction endonucleases and reaction buffers, and pUC19 DNA restricted with *Hpa* II DNA marker came from GeneWorks (Thebarton, Australia); NuSieve® 3:1 Agarose from FMC (distributed by Adelab Scientific, Norwood, Australia); bromophenol blue (sodium salt), ethidium bromide, triethylamine (TEA), Trizma® Base (Tris-base), Trizma® hydrochloride (Tris-HCl) from Sigma Chemical Company (St. Louis, MO, USA); boric acid, hydrochloric acid (HCl), orthophosphoric acid, sodium dihydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$), sodium hydroxide (NaOH) from Ajax Chemicals (Auburn, Australia); acetonitrile (ACN), chloroform, ethylenediaminetetraacetic acid (EDTA), and sucrose from BDH Chemicals (Poole, England).

3.2.2 Subjects

Ethics approval for the study was obtained from the Royal Adelaide Hospital Ethics Committee. Subjects recruited included 61 Caucasians, 21 Asians (Chinese, Malaysian, Japanese) and 1 African. The genetic heritage of these subjects was known back to the second generation, in order to exclude subjects with mixed race backgrounds. Only healthy adults aged between 18 and 52 yr were allowed to participate in the study, and exclusion occurred if there was history of pregnancy or lactation, known medical conditions including epilepsy, respiratory conditions such as asthma, renal impairment or

kidney disease, and concomitant medication excluding the oral contraceptive. Prior to commencing the study subjects gave their written informed consent, and any adverse reactions were self-reported. Subject demographic data is shown in Table 17. Sixteen Caucasians and one Asian subject reported concomitant oral contraceptive use, one Caucasian reported use of marijuana, one Caucasian reported use of budesonide and one Caucasian reported occasional use of salbutamol.

Table 17: Subject demographic data. EM = CYP2C19 genotypic extensive metaboliser, PM = CYP2C19 genotypic poor metaboliser, all data are mean ± SD.

	Caucasian EM (n=59)	Caucasian PM (n=2)	Asian EM (n=20)	Asian PM (n=1)	African EM (n=1)
Gender	25 male, 34 female	2 female	9 male, 11 female	female	male
Age (years)	26.2 ± 7.3	19, 20	21.9 ± 3.3	20	34
Weight (kg)	70 ± 13.4	63, 53	60 ± 10	58	88
Height (cm)	172 ± 9.2	169, 155	167 ± 9.4	163	180
Tobacco (cigarettes/day)	1.6 ± 4.8	0, 0	0.8 ± 3.4	0	0
Alcohol (standard drinks/week)	6.4 ± 10.7	8, 1	0.7 ± 1.3	0	0
Caffeine (cups/day)	2.5 ± 2.0	1, 0	0.3 ± 0.5	0	4

3.2.3 Study Protocol

After emptying their bladders, subjects self administered concomitantly 100mg proguanil hydrochloride (one Paludrine® tablet, ICI Australia Operations Pty Ltd) and 30mg dextromethorphan hydrobromide (one capsule prepared by the Royal Adelaide Hospital Pharmacy Department, Adelaide, Australia) and collected urine for the following 8hr. No restriction was placed on food or drink consumed, or cigarette smoking; the only restriction was that no medication except the oral contraceptive was to be taken during the

period of urine collection. The pH and volume of urine were recorded and a 20ml aliquot was stored at -20°C for analysis. Subjects were also required to rinse their mouths with 15ml of saline which was collected for isolation of genomic DNA from buccal cells. For maximum cell collection this was performed as least 1hr after eating, drinking or brushing teeth. The mouthwash samples were stored at -20°C for analysis.

3.2.4 Isolation and quantification of genomic DNA

Genomic DNA was isolated from buccal cell samples using a QIAamp® blood kit (QIAGEN Inc., CA, USA). The principle behind isolation of genomic DNA was based on lysis of buccal cells and then centrifugation. 10ml of the mouthwash sample was centrifuged at 1,600g for 10min to obtain a buccal cell pellet, the supernatant mouthwash was removed and the buccal cell pellet resuspended in 200µl of remaining mouthwash. These buccal cells were mixed with 25µl of QIAGEN Protease (17.8mg.ml^{-1}) and 200µl Buffer AL followed by immediate vortexing for 15sec. This was followed by a 10min incubation on a heating block set at 70°C. The pH of the sample was then checked. It was required to be acidic in order to ensure maximum binding of the DNA to the silica membrane of the spin column, which was achieved with drop wise addition of 0.1M acetic acid. 210µl of absolute ethanol was then added, samples vortexed and then loaded onto the QIAamp spin column. Centrifugation occurred at 5,200g for 1min after which the filtrate was discarded. Two successive washes of the DNA, using 500µl of Buffer AW and centrifugation at 5,200g for 1min, were performed in order to remove cellular debris and proteins. Following this, genomic DNA was eluted from the spin column via addition of 200µl of 10mM Tris-HCl, pH 9.0 preheated to 70°C, and centrifugation at 5,200g for 1min.

Quantification of the yield and purity of genomic DNA was performed as described previously (see section 2.2.3.1).

3.2.5 Genotyping Analysis

Genomic DNA isolated from buccal cell samples was genotyped to identify *CYP2C19*2* and *CYP2C19*3* mutations as described previously (see sections 2.2.3.2 - 2.2.3.5)

3.2.6 Phenotyping Analysis

3.2.6.1 Extraction of urine

200µl subject urine was extracted following alkalisation with 250µl 1M NaOH and addition of 100µl 0.5mM (144µg.ml^{-1}) CCG, which acted as the internal standard for HPLC quantification, with 3ml of chloroform washed previously with 1M HCl. Samples were rotary mixed for 10min and centrifuged at 1,600g for 10min. The aqueous layer was aspirated to waste and the organic layer transferred to a 10ml plastic tube containing 200µl 0.02M orthophosphoric acid. Samples were rotary mixed and centrifuged again, and 50µl of the acid bubble was removed for injection on to the HPLC system. The volume of urine extracted was increased if concentrations of either CG or proguanil were below the limit of quantification.

3.2.6.2 HPLC system

A reversed phase HPLC system was utilised for the separation of CG, CCG and proguanil, and quantification of CG and proguanil. The HPLC system consisted of a LC-6A pump (Shimadzu, Kyoto, Japan) with a cut-off pressure of 4kgf.cm^{-2}, a SIL-10A autoinjector (Shimadzu, Kyoto, Japan), a variable wavelength UV absorbance detector (Model 875-UV/VIS detector, Jasco, Japan) set at 238nm, with computer integration (DP 800 Data

Interface and DP 800 Chromatographic software, ICI Instruments, Australia). Compounds were separated by a Pellicular ODS C_{18} pre-column (1cm) and a stainless-steel column (15cm x 4.6mm) packed with C_{18} 5µm ODS-2 packing material (Spherisorb; Phase Separations, Queensferry, UK).

The mobile phase at a flow rate of 1.5ml.min^{-1} that gave best separation of CG, CCG and proguanil was 17% ACN, 10mM TEA, and 5.5mM NaH_2PO_4 in water, adjusted to a pH of 2.5 with orthophosphoric acid. Separation of chromatographic peaks representing CG, CCG and proguanil was identical to that shown in chapter 2 (see figure 18).

3.2.6.3 Assay Validation

Calibration, precision, inaccuracy, extraction efficiency and limit of quantification

Calibration curves were constructed for CG and proguanil with 7 final concentrations ranging from 1 to 40µg.ml^{-1}, and from 2 to 80µg.ml^{-1}, respectively. Low, medium and high quality control (QC) samples were also prepared, with final concentrations of 4, 15, 25µg.ml^{-1} and 8, 30, 50µg.ml^{-1}, respectively. Calibration curve and QC samples containing both CG and proguanil were prepared as 1 in 10 dilutions in blank urine and extracted identically to subject urine samples.

Peak areas of CG and proguanil were converted into peak area ratios using the peak area of CCG, and unweighted linear regression analysis of peak area ratios against nominal concentrations provided an estimate of slope, intercept and coefficient of determination (r^2). An assay was accepted if the r^2 was greater than 0.990, and all standard and QC samples had inccuracy in comparison to nominal values less than 15%.

The method was validated by assaying 18 QC samples (6 each of low, medium and high concentrations for CG and proguanil) on a single assay day to determine the intra-day inaccuracy and precision. Inter-day inaccuracy and precision were determined by analysis of 6 QC samples (2 each of low, medium and high concentrations for CG and proguanil) on 4 different assay days (Table 18).

Table 18: Summary of Intra- and Inter-assay precision and inaccuracy validation for CG and proguanil (PG) in urine.

Nominal conc. = nominal concentration, Cal. conc. = calculated concentration, CV = coefficient of variation, n = sample size.

	Nominal conc. ($\mu g.ml^{-1}$)	Cal. conc. (mean ± SD)	% Inaccuracy	% CV	n
Intra-assay					
CG	4	4.2 ± 0.1	+ 5.7	2.1	6
	15	15.5 ± 0.3	+ 3.1	1.7	6
	25	24.8 ± 0.9	- 0.6	3.4	6
PG	8	9.2 ± 0.4	+ 14.9	4.2	6
	30	32.2 ± 0.7	+ 7.3	2.3	6
	50	49.5 ± 1.4	- 1.0	2.7	6
Inter-assay	(4 different assay days)				
CG	4	4.2 ± 0.1	+ 4.5	2.1	8
	15	15.7 ± 0.2	+ 4.5	1.5	8
	25	25.9 ± 0.5	+ 3.4	1.9	8
PG	8	8.6 ± 0.4	+ 7.1	4.9	8
	30	32.4 ± 0.8	+ 8.1	2.3	8
	50	51.8 ± 2.0	+ 3.6	3.8	8

Extraction efficiency was evaluated for CG and proguanil at each QC concentration and for CCG, the internal standard. The peak heights of CG, proguanil and CCG extracted from the QC samples were compared to those obtained by direct injections of aqueous solutions of the QC samples and internal standard (Table 19).

Table 19: Summary of extraction recovery of cycloguanil, chlorcycloguanil and proguanil from urine

Concentration ($\mu g.ml^{-1}$)	Aqueous peak height (mean ± SD, n=4)	Extracted peak height (mean ± SD, n=4)	Recovery
CG: 4	40492.5 ± 221.8	34621.8 ± 1148.8	85.5%
CG: 15	143945.5 ± 1724.4	127228.5 ± 2865.8	88.4%
CG: 25	243559.3 ± 4057.7	205325.8 ± 5513.3	84.3%
CCG: 144*	60427.5 ± 1590.9	56665.8 ± 1360.9	93.7%
PG: 8	18832.8 ± 286.4	17251.5 ± 803.8	91.6%
PG: 30	62715.5 ± 416.9	60362.8 ± 1596.9	96.2%
PG: 50	99328 ± 1098.8	92395.8 ± 2979.8	93.0%

* internal standard concentration, n=12

The limits of quantification, for CG and proguanil were $1\mu g.ml^{-1}$ and $2\mu g.ml^{-1}$, respectively.

3.2.7 Data Analysis

All raw data were transcribed to Microsoft® Excel (Version 5.0, Microsoft Corporation, WA, USA), and calibration curves were constructed by linear regression (Regression; Blackwell Scientific Software, Blackwell Scientific Publications, Oxford, UK). From the slope and intercept, concentrations of CG and proguanil were calculated and accuracy of the standard curve and QC concentrations ascertained via comparison with nominal concentrations. Concentrations of CG and proguanil in subject urine were determined similarly. Proguanil metabolic ratios (PG/CG) were calculated by division of amount of proguanil excreted by amount of CG excreted over the study period. Probit scores were calculated using Microsoft® Excel as follows;

Probability = (subject number - 0.5) / total subject number

Probit score = [NORMINV[a](probability, mean = 0, standard deviation = 1)] + 5

[a] NORMINV is the function which returns the inverse of the normal cumulative distribution (Jackson *et al.*, 1989).

NTV (normal test variable) plots were constructed after the following conversion of the raw data (Endrenyi *et al.*, 1991);

1. PG/CG MR converted to logarithmic form: L = log PG/CG MR
2. The median (mL) and standard deviation (sL) of the log PG/CG MR were calculated.
3. The cumulative frequency, F, was calculated: F = n/N, where n = cumulative number of subjects with a particular PG/CG MR, and N = total number of subjects.
4. The secondary measure of $Z = F - (1 - F)e^{1.6x}$ was calculated.
5. NTV was calculated: NTV = -Z if $F \leq 0.5$ or $NTV = Z/e^{1.6x}$ if $F \geq 0.5$.

The NTV scores were then plotted against F.

Unpaired t-tests (GraphPad PRISM, Version 2.0, GraphPad Software, Inc., San Diego, CA, USA) were used to determine: i) if mean PG/CG MR of CYP2C19 genotypic EM were significantly different to that of genotypic PM within either the Caucasian or Asian populations; ii) if differences exist between the PG/CG MR of Caucasian genotypic EM or PM and Asian genotypic EM or PM; and iii) if gender differences in PG/CG MR occur in either population. Spearman rank correlation tests were used to determine if there were relationships between PG/CG MR and the following: i) urine pH or volume, ii) age, iii) subject weight, iv) subject height, v) cigarette consumption, vi) alcohol consumption, and vii) caffeine consumption. All data are tabulated as mean ± SD (95% confidence intervals).

3.3 Results

3.3.1 CYP2C19 Genotype

Genotyping for *CYP2C19* revealed the presence of *CYP2C19*1*, *CYP2C19*2* and *CYP2C19*3* alleles. Fifty-two subjects were wildtype EM (*CYP2C19*1/*1*), of whom 42 were Caucasian and 10 were Asian. Twenty-six were heterozygote EM for the *CYP2C19*2* mutation (*CYP2C19*1/*2*), of whom 16 were Caucasian, 9 were Asian, and one was African. One Caucasian and one Asian subject were heterozygote EM for the *CYP2C19*3* mutation (*CYP2C19*1/*3*). The incidence of the *CYP2C19*2* mutation among Caucasians and Asians was 26% and 43%, respectively, whilst the incidence of the *CYP2C19*3* mutation was 1.6% and 4.7% in Caucasian and Asians, respectively. Three subjects, 2 Caucasian and 1 Asian, were homozygote PM (*CYP2C19*2/*2*). The incidence of genotypic PM in the Caucasian population was 3.2% and in the Asian population was 4.7%.

3.3.2 CYP2C19 Phenotype

The proguanil metabolic ratio (PG/CG MR) ranged from 0.72 to 20.3. The histogram of PG/CG MR (Figure 24) shows the majority of subjects, regardless of ethnic origin, had PG/CG MR less than 8. Except for one Caucasian heterozygote EM (*CYP2C19*1/*2*) who had a PG/CG MR of 19.30, and one Caucasian homozygote PM (*CYP2C19*2/*2*) who had a PC/CG MR of 5.61, other phenotypes and genotypes were concordant (Figure 25).

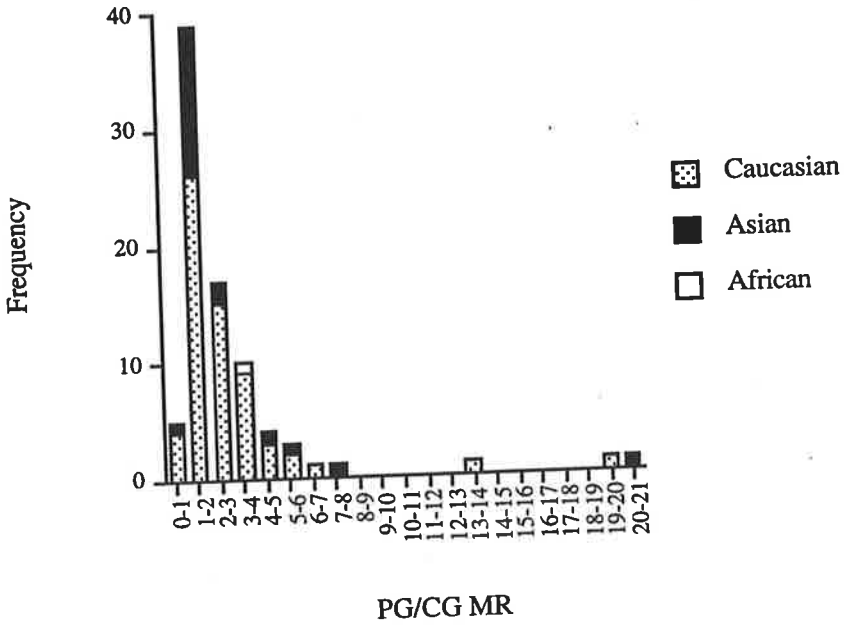


Figure 24: Histogram showing the frequency of various PG/CG metabolic ratios of Caucasian (n=62), Asian (n=21) and African (n=1) subjects.

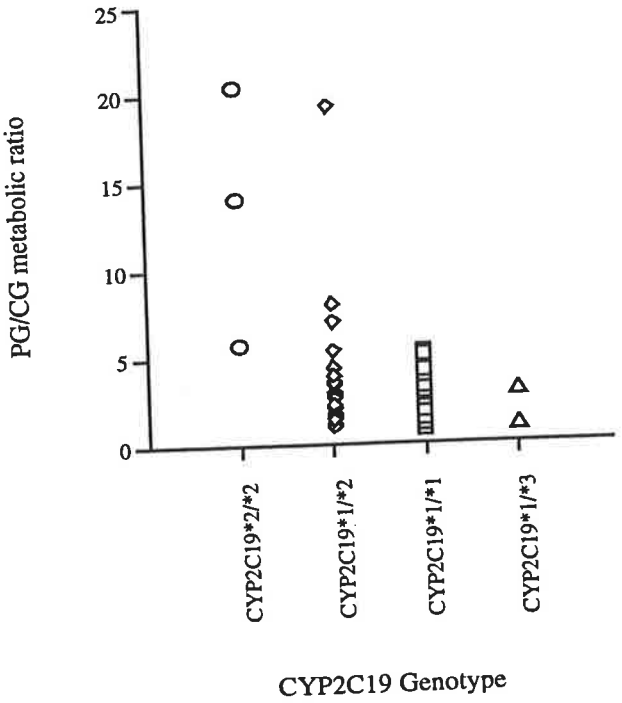


Figure 25: PG/CG metabolic ratios of subjects (n=83) with various CYP2C19 genotypes.

The mean ± SD (95% confidence intervals) PG/CG MR of Caucasian genotypic EM (n=60) was not significantly different in comparison to that of the Asian genotypic EM (n=19), 2.57 ± 2.52 (1.92 - 3.22) versus 2.34 ± 1.75 (1.5 - 3.18), (P = 0.71). The mean ± SD PG/CG MR of Caucasian genotypic PM was 9.78 ± 5.89. This was significantly different to the mean PG/CG MR of Caucasian genotypic EM, 2.57 versus 9.78 (P = 0.0003). Due to small subject numbers, no statistical analysis was performed to compare the PG/CG MR between Asian genotypic EM and PM. Within the Caucasian genotypic EM subjects, there was a significant difference between the PG/CG MR of homozygote subjects (*CYP2C19*1/*1*, n=42), and the PG/CG MR of heterozygote subjects (*CYP2C19*1/*2*, n=16), 2.11 ± 1.12 versus 3.98 ± 4.3 (P = 0.01). There was no significant difference observed in the Asian genotypic EM population, however heterozygote subjects (n=10) did have a higher mean PG/CG MR in comparison to homozygote subjects (n=9), 2.77 compared to 1.78. These observations suggest a CYP2C19 gene dose effect and are similar to previous findings (Hoskins *et al.*, 1998). There were no significant differences found between the PG/CG MR of Caucasian *CYP2C19*1/*1* subjects when compared to Asian *CYP2C19*1/*1* (P = 0.40), or between Caucasian *CYP2C19*1/*2* subjects when compared to Asian *CYP2C19*1/*2* (P = 0.44). Additionally, no gender differences in PG/CG MR were observed within either genotypic EM population; Caucasian, males (n=25) compared with females (n=34), 2.03 ± 1.05 versus 3.02 ± 3.17, P = 0.14; and Asian, males (n=9) compared with females (n=11), 1.82 ± 0.69 versus 2.67 ± 2.21, P = 0.28.

Using linear regression of the probit plot (Figures 26a), the antimode separating the genotypic EM and PM Caucasians was ascertained as 8.61. As only one genotypic PM was found within the Asian population studied it was not feasible to use graphical analysis, either probit or normal test variable (NTV) plots (Figures 26b and c) to ascertain an antimode. Additionally, the NTV plot did not clearly separate genotypic EM from PM in the Caucasian population studied (Figure 26d). Probit plot analysis was repeated after the inclusion of data from a previous study in this laboratory (Coller *et al.*, 1996), which included Caucasian subjects with the following PG/CG MR (CYP2C19 genotype) (Figure

26e); 0.93 (CYP2C19*1/*1), 1.08 (CYP2C19*1/*1), 1.73 (CYP2C19*1/*2), 2.62 (CYP2C19*1/*2), 3.44 (CYP2C19*1/*2), and 14.7 (CYP2C19*2/*2). The antimode separating genotypic EM and PM decreased to 8.55.

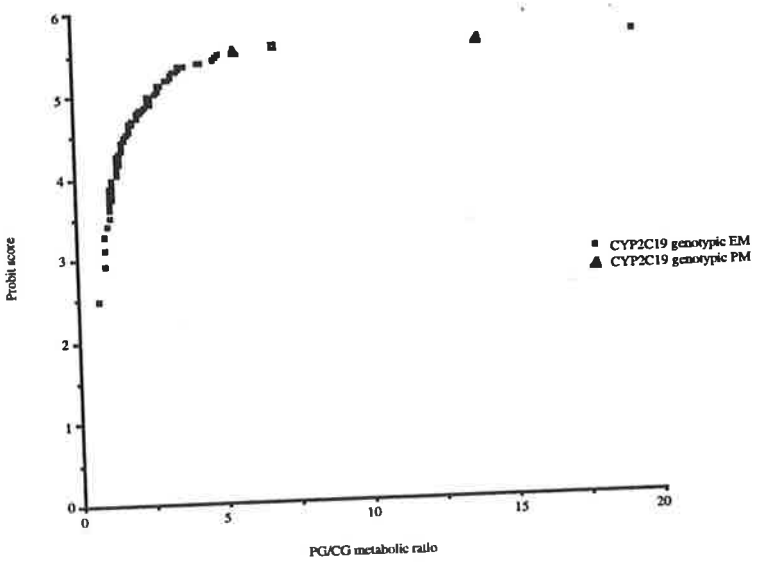


Figure 26a: Probit plot of PG/CG metabolic ratio in 8hr urine of Caucasian CYP2C19 genotypic EM (n=59) and PM (n=2).

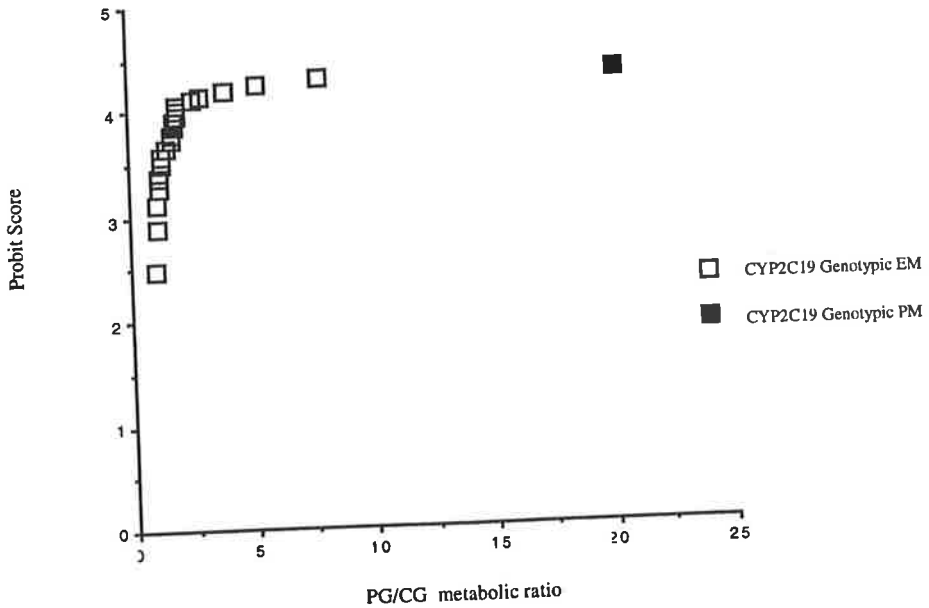


Figure 26b: Probit plot of PG/CG metabolic ratio in 8hr urine of Asian CYP2C19 genotypic EM (n=20) and PM (n=1).

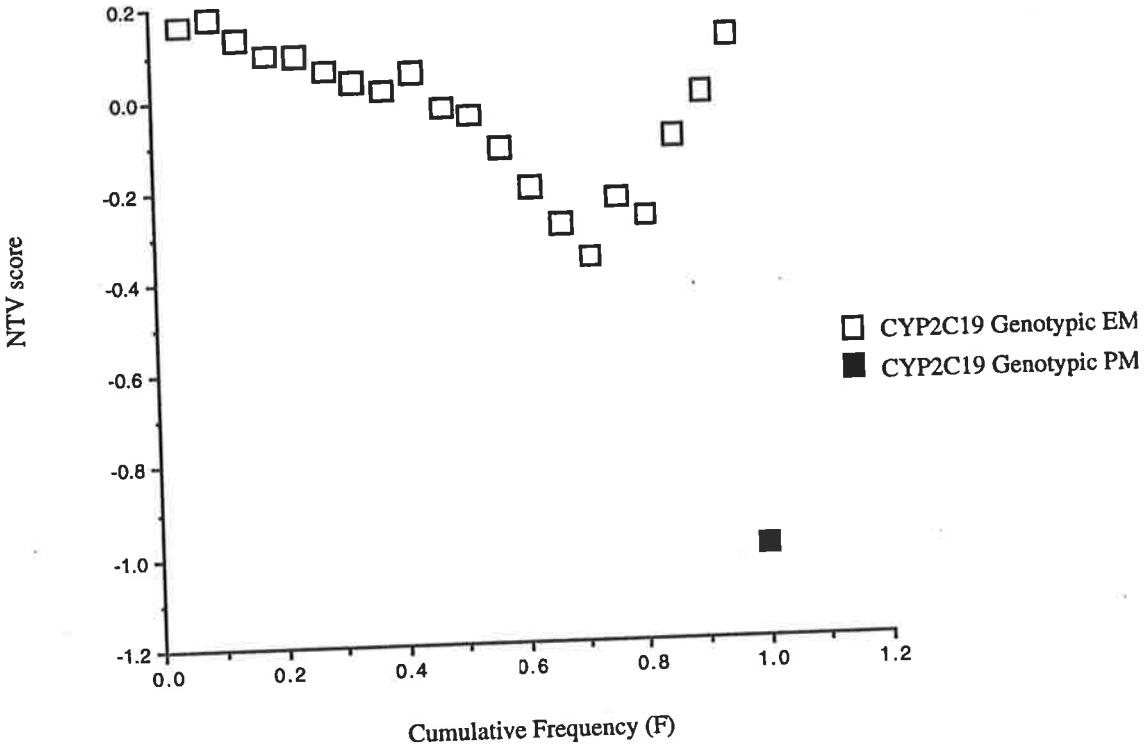


Figure 26c: Normal test variable (NTV) plot of Asian CYP2C19 genotypic EM (n=20) and PM (n=1).

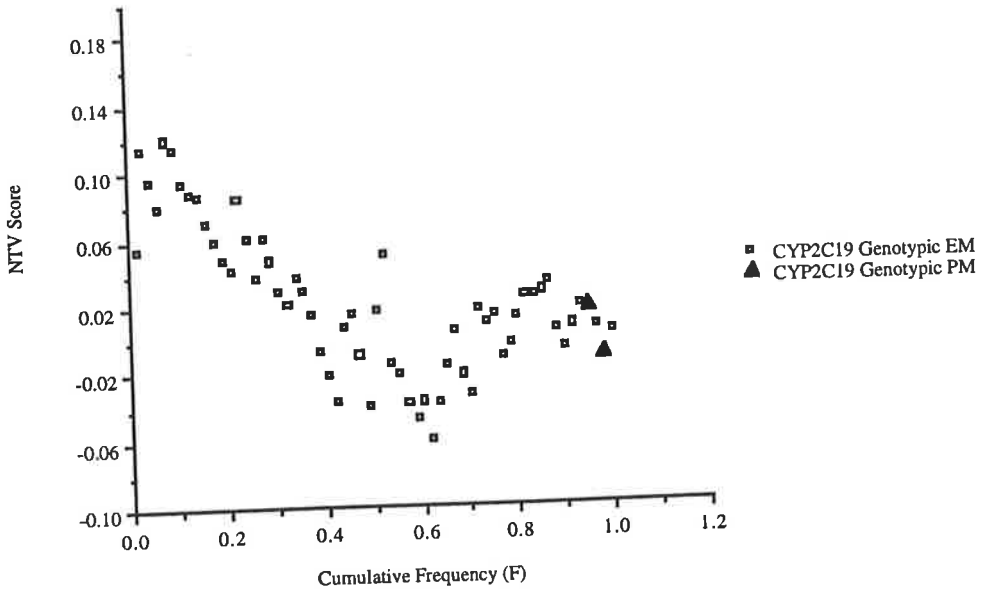


Figure 26d: Normal test variable (NTV) plot of Caucasian CYP2C19 genotypic EM (n=59) and PM (n=2).

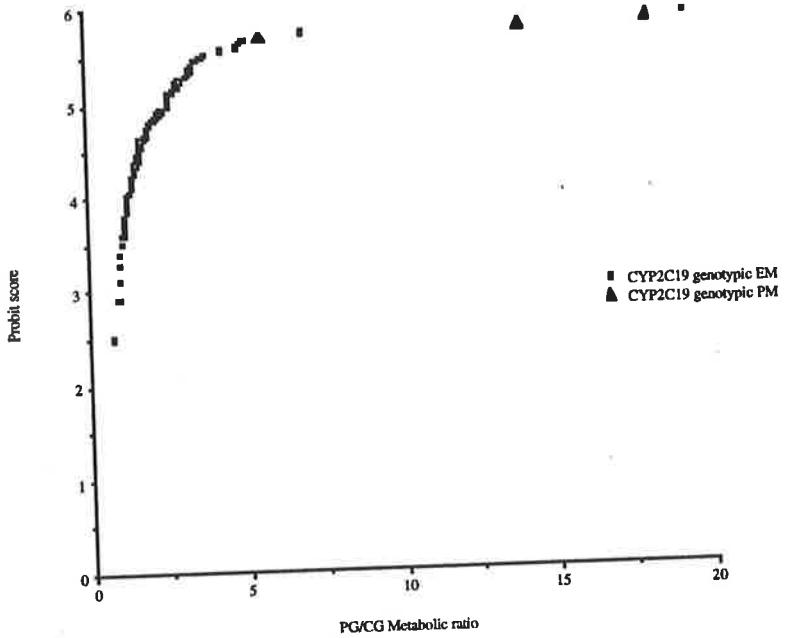


Figure 26e: Combined probit plot of PG/CG metabolic ratio in 8hr urine of Caucasian CYP2C19 genotypic EM (n=64) and PM (n=3).

There was no significant correlation between PG/CG MR and; i) urine pH or urine volume (r_s = 0.197, P = 0.08, and r_s = 0.138, P = 0.22, respectively, refer to appendix G for individual subject data); ii) age (r_s = -0.101, P = 0.36); iii) subject weight (r_s = -0.027, P = 0.81); iv) subject height (r_s = -0.116, P = 0.29); v) cigarette consumption (r_s = 0.034, P = 0.76); vi) alcohol consumption (r_s = -0.174, P = 0.64); vii) caffeine consumption (r_s = -0.021, P = 0.85).

3.3.3 Adverse Effects

28 subjects (34%) self-reported adverse effects (Table 20). Of those reported, headache, dizziness, nausea, abdominal pain and a dry mouth are the most common for proguanil (Caswell, 1999).

Table 20: Incidence of adverse effects reported by 83 subjects.

Subject	Ethnic origin	PG/CG MR	CYP2C19 genotype	Self-reported adverse effects
#2	Caucasian	3.43	*CYP2C19*1/*2*	wheezy, coughing, increased heart rate, light-headed
#7	Caucasian	4.96	*CYP2C19*1/*1*	mild headache
#8	Caucasian	1.49	*CYP2C19*1/*1*	slight nausea
#10	Caucasian	2.94	*CYP2C19*1/*1*	dry mouth
#11	Caucasian	2.63	*CYP2C19*1/*1*	nausea, "fuzzy-headedness"
#19	Caucasian	1.55	*CYP2C19*1/*1*	mild headache
#21	Caucasian	3.27	*CYP2C19*1/*2*	slight dizziness, slight headache
#24	Caucasian	1.70	*CYP2C19*1/*1*	slight feeling of gastric reflux
#27	Caucasian	1.51	*CYP2C19*1/*1*	dry mouth
#28	Caucasian	2.33	*CYP2C19*1/*1*	headache, slight dizziness, dry mouth
#33	Caucasian	1.11	*CYP2C19*1/*1*	slight headache
#34	Caucasian	3.03	*CYP2C19*1/*1*	slight headache
#44	Caucasian	2.80	*CYP2C19*1/*2*	dry mouth
#46	Asian	1.70	*CYP2C19*1/*2*	mild abdominal discomfort, headache, dry mouth
#49	Asian	1.96	*CYP2C19*1/*2*	dry mouth, mild nausea
#53	Asian	1.08	*CYP2C19*1/*2*	dry throat
#54	Caucasian	2.00	*CYP2C19*1/*2*	headache, insomnia
#56	Asian	1.85	*CYP2C19*1/*1*	headache, insomnia
#57	Caucasian	1.21	*CYP2C19*1/*1*	dizziness
#62	Asian	1.25	*CYP2C19*1/*1*	dizziness
#67	Asian	1.50	*CYP2C19*1/*1*	drowsiness
#68	African	3.87	*CYP2C19*1/*2*	dry mouth, headache
#69	Asian	1.05	*CYP2C19*1/*1*	slight tightness of chest
#73	Caucasian	1.84	*CYP2C19*1/*1*	slight headache
#74	Caucasian	0.97	*CYP2C19*1/*1*	giddiness, nausea
#75	Caucasian	1.41	*CYP2C19*1/*1*	slightly pale
#77	Asian	1.92	*CYP2C19*1/*1*	drowsiness
#80	Caucasian	2.23	*CYP2C19*1/*2*	mild nausea, mild headache

3.4 Discussion

Genotyping results indicate the incidence of CYP2C19 PM in Caucasians and Asians of this study population was 3.2% and 4.7% respectively. No 95% confidence intervals were able to be reported due to the small numbers of genotypic PM in either population (n=2 Caucasian, n=1 Asian). Previous studies in Caucasian populations using proguanil as the probe drug have found that the incidence ranges from 3% (Caucasian population living in Australia, n=99, Hoskins *et al.*, 1998) to 10% (British, n=135, Ward *et al.*, 1989b). When racemic-mephenytoin was used as a probe drug in a Caucasian population in America (n=156) an incidence of 2.6% was observed (Wedlund *et al.*, 1984). Therefore, it seems that the incidence reported by this study is similar to previous observations in Caucasian populations, and is almost identical to that of another Caucasian population living in Australia, 3.2% versus 3% (Hoskins *et al.*, 1998).

Of more interest is the lower incidence of CYP2C19 PM in Asians of our study population (4.7%). Using racemic-mephenytoin as a probe, researchers have reported an incidence as high as 25% (Nakamura *et al.*, 1985). Subjects were recruited to participate in the present study on the condition that both their biological mother and father had the same ethnicity, whether Asian or Caucasian. This was done to avoid problems of mixed-racial backgrounds, and therefore, the lower incidence can not be explained by the possibility of Asian subjects with either a Caucasian mother or father. In fact the majority of the Asian subjects (67%) in the present study stated their specific background as Chinese, and consequently a PM incidence of 15% was expected, as found by Bertilsson and colleagues from a study of 137 Chinese subjects (Bertilsson *et al.*, 1992). Perhaps the decreased incidence may be explained by the very low number of Asian subjects (n=21) in the study, so that if the subject number were increased, the number of PM may also increase.

Several graphical methods are used to determine if the study population shows a bimodal distribution; these being histograms, probit plots and NTV plots. Problems in

interpretation of data using these methods can arise. In the case of histograms, the shape of the profile has been shown to be dependent on the position of the column division (Jackson *et al.*, 1989), so that different divisions of the index being studied, for example a metabolic ratio, can change the shape of the distribution. When using probit plot analysis, small deviations from a normal distribution are difficult to detect, and the antimodes which separate two groups within a population have been shown not to coincide with inflections of the plots, so that using probit plot analysis to select the antimode separating groups within a population has given invalid results (Jackson *et al.*, 1989). Conversely, the NTV plot is a sensitive method for detecting small changes in the index being studied which cause the population to be distributed bimodally, as the NTV becomes negative (Endrenyi *et al.*, 1991). However, an antimode from NTV plots, which generally is indicated by the minimum of the plot (Endrenyi *et al.*, 1991), is often difficult to ascertain. In order to obtain an accurate estimate of the antimode it is usual to combine results obtained from all three graphical methods (Endrenyi *et al.*, 1991). The difficulty in interpreting these methods can explain the use of visual inspection used by many researchers to designate an antimode.

In the present study, a bimodal distribution in PG/CG MR was observed in Caucasian and Asian study populations, as seen with the histogram, the probit plot and the NTV plot. The antimode separating Caucasian CYP2C19 genotypic EM and PM was observed to be 8.61, whilst the number of genotypic PM in the Asian population made assignment of the antimode impossible. The antimode of 8.61 was derived from calculation of the PG/CG MR at the point of intersection of the lines of best fit of the CYP2C19 genotypic EM and PM probit plots, and is similar to the antimode of 10 used by the majority of researchers (Ward *et al.*, 1989a, Watkins *et al.*, 1990, Helsby *et al.*, 1990a, Funck-Brentano *et al.*, 1992, Wanwilmolruk *et al.*, 1995a, and Wanwimolruk *et al.*, 1995b). The NTV plot did not give any extra information regarding the assignment of this antimode, which was presumably due to the small number of genotypic PM in the Caucasian population studied. It must also be noted that a change in the number of genotypic PM included in the probit plot analysis could possibly alter the assignment of this antimode. This was observed by

the decrease to 8.55 following inclusion of data from a previous study (Coller *et al.*, 1997) and subsequent probit plot re-analysis.

It was observed that the PG/CG MR antimode did not satisfactorily separate genotypic PM and EM Caucasian groups in the present study. Two discrepancies were found between the phenotype and genotype, those being a homozygote PM (*CYP2C19*2/*2*) with a PG/CG MR of 5.61 and a heterozygote EM (*CYP2C19*1/*2*) with a PG/CG MR of 19.30. Both of these subjects were Caucasian and concomitantly taking the oral contraceptive (Trifeme 28) at the time of participation of the study. Previous research has shown that the metabolism of several CYP2C19 substrates is inhibited by oral contraceptives. Examples include propranalol (Walle *et al.*, 1996), diazepam (Abernethy *et al.*, 1982), and imipramine (Abernethy *et al.*, 1984). Furthermore, *in vitro* inhibition of hepatic CYP2C19 metabolism of racemic mephenytoin by oestradiol and ethinyloestradiol has been reported, with K_i values of 200 and 104μM, respectively. Trifeme 28 is an oral contraceptive with combination tablets containing ethinyloestradiol and levonorgestrel (Caswell, 1999). Therefore, it is possible that this oral contraceptive may have inhibited the metabolism of proguanil to CG, so that a higher PG/CG MR would have resulted in the CYP2C19 genotypic EM. However, this inhibition of proguanil metabolism by Trifeme 28 does not explain the low PG/CG MR in the genotypic PM, nor does it explain why this interaction did not change the metabolism of proguanil in other female subjects (n=17) concomitantly taking an oral contraceptive. It must be noted that the concomitant administration of dextromethorphan is unlikely to have influenced the PG/CG MR, as previous studies have shown that the PG/CG MR remains unchanged in this situation (Funck-Brentano *et al.*, 1992, Foster *et al.*, 1994). One explanation is the involvement of other CYP450 enzymes in the formation of CG, especially CYP3A4 (as discussed in chapter 2). CYP3A4 hepatic content is known to vary between individuals by up to 60-fold (Forrester *et al.*, 1992), therefore, if the genotypic PM had a high hepatic CYP3A4 content, the metabolism of proguanil to cycloguanil may not have been altered compared to an EM with low hepatic CYP3A4 content.

The incidence of adverse effects reported by subjects was large (34%) in comparison to other phenotyping studies who have reported no adverse effects (Wanwimolruk *et al.*, 1995a, Basci *et al.*, 1996). All subjects were EM (either *CYP2C19*1/*1* or *CYP2C19*1/*2*), therefore, the incidence of adverse effects does not appear to be due to the inability to metabolise proguanil. It has not been previously reported whether CG itself causes adverse effects. Consequently, it is not possible to conclude whether the effects are due to the presence of proguanil or CG. The dose used in this study was half of that normally used to phenotype subjects (Wanwimolruk *et al.*, 1995a, Basci *et al.*, 1996). Therefore, it seems unlikely that the dosage can explain the difference. Some side effects could be attributed to the presence of dextromethorphan which was taken concomitantly, for example dizziness, dry mouth and changes in breathing (Caswell, 1999). Finally, it is possible that the method of reporting of adverse effects between this study and others may differ. Subjects in this study were asked to complete an information sheet which asked specifically about adverse effects which may have prompted them to report all effects, whilst if subjects were just verbally questioned about how they felt after participating, adverse event reporting may decrease.

In conclusion, the results of this *in vivo* study have shown that proguanil can assign the correct phenotypic status in the majority of subjects regardless of ethnic origin. However, in agreement with the original hypothesis, the fact that some discrepancies exist between phenotypic and genotypic status highlights the need for further investigation of proguanil as a probe drug in larger Caucasian populations ($n > 200$), followed by review of its potential to replace (S)-mephenytoin. Furthermore, caution is required by researchers when assigning phenotypes using PG/CG MR, and these data indicate that genotyping of subjects / patients is required to fully confirm phenotype status.

This completes the studies investigating the CYP2C19 genetic polymorphism. The next chapter will discuss the CYP2D6 genetic polymorphism, in particular, the incidence of UM in Caucasian and Asian populations living in Australia.

4. *In vivo* CYP2D6 phenotyping and genotyping in Caucasians and Asian subjects, the incidence of CYP2D6 ultra-rapid metabolisers

4.1 Introduction

Much research has focussed on the incidence of the mutations of *CYP2D6* which lead to the polymorphic poor metaboliser group in different ethnic populations. Of equal clinical and pharmacogenetic importance is the occurrence of multiple copies of *CYP2D6*, where people carry more than two copies of the functional gene (see section 1.3.6). Currently, studies have been conducted in Swedish, Danish, German, Spanish, Ethiopian, Saudi Arabians, African Americans and Zimbabwean populations. However, the incidence of this genetic phenomenon has not been investigated in a Caucasian population living in Australia or Asian populations.

The following hypothesis and subsequent aims will be addressed in this chapter:

Hypothesis 5: The incidence of CYP2D6 UM in the Australian Caucasian population would be similar to other Caucasian populations (0.8 - 2.6%).

Aims 6 and 7: The aims of this *in vivo* study are to: i) use genotyping and phenotyping with dextromethorphan to investigate the incidence of CYP2D6 UM in Caucasian and Asian populations living in Australia; and, ii) evaluate the ability of the dextromethorphan MR to separate genotypic UM from EM.

4.2 Methods

4.2.1 Chemicals

Dextromethorphan hydrobromide (DM), dextrorphan hydrobromide (DR), 3-methoxymorphinan hydrobromide (3-MM), and 3-hydroxymorphinan hydrobromide (3-OH-M) were obtained from Roche Products Pty Ltd (Sydney, Australia). Pholcodine was obtained from F.H.Faulding & Co Limited (Adelaide, Australia). Other materials were obtained from the following sources: Expand™ Long Template PCR system from Boehringer Mannheim Australia Pty. Ltd. (Rose Park, Australia); Hi-Lo mixed DNA marker came from GeneWorks (Thebarton, Australia); AquaPor® LE Agarose from National Diagnostics (distributed by Adelab Scientific, Norwood, Australia); bromophenol blue (sodium salt), ethidium bromide, ß-glucuronidase (Type B3, from Bovine liver), triethylamine (TEA), Trizma® Base (Tris-base), Trizma® hydrochloride (Tris-HCl) from Sigma Chemical Company (St. Louis, MO, USA); boric acid, hydrochloric acid (HCl), orthophosphoric acid, sodium dihydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$), sodium hydroxide (NaOH) from Ajax Chemicals (Auburn, Australia); acetonitrile (ACN), chloroform, diethyl ether, ethylenediaminetetraacetic acid (EDTA), propan-2-ol and sucrose from BDH Chemicals (Poole, England).

4.2.2 Subjects

Subjects were identical to those recruited in chapter 3 (see section 3.2.2).

4.2.3 Study Protocol

Study protocol was identical to that in chapter 3 (see section 3.2.3).

4.2.4 Isolation and quantification of genomic DNA

Genomic DNA was isolated from buccal cell samples using a QIAamp® blood kit (QIAGEN Inc., CA, USA), as detailed in chapter 3, and quantification of the yield and purity did not alter (see section 3.2.4).

4.2.5 Genotyping Analysis (based on Griese *et al.*, 1998)

4.2.5.1 Polymerase Chain Reaction (PCR) reagents

PCR reactions for the *CYP2D6* genotyping utilised the Expand™ Long Template PCR System (Boehringer Mannheim Australia Pty. Ltd., Rose Park, Australia) according to the manufacturer's specifications. Reactions contained 400ng of genomic DNA, buffer 3 (1x PCR buffer with 2.25mM $MgCl_2$ and detergents), 2.5mM of dNTPS (dATP, dCTP, dGTP, and dTTP), 2mM $MgCl_2$, 0.3μM PCR primers (detailed below), and 0.75μl of Taq and Pwo DNA Polymerase mix (1.5% final volume). All reactions were conducted in 0.5ml microcentrifuge tubes (Astral Scientific, Gymea, Australia).

4.2.5.2 PCR primers

The primers used to isolate the *CYP2D6*2xN* PCR product were custom synthesised at GeneWorks (Thebarton, Australia). They had the following sequences;

A. Forward primer for *CYP2D6*2xN*,

5'- TGG TGT CTT TGC TTT CCT GGT GAC -3'

B. Reverse primer for *CYP2D6*2xN*,

5'- GTG GTG GGG CAT CCT CAG G -3'

4.2.5.3 PCR thermal cycling conditions

For detection of *CYP2D6*2xN*, the following thermal cycling conditions were utilised. Initial denaturation of the genomic DNA occurred at 93°C for 2min, followed by 30 cycles of 93°C for 30sec, 66°C for 1.5min, and 68°C for 7min. The final extension step occurred at 68°C for 7min, after which samples were cooled to 4°C. Thermal cyclers used were PE GenAmp 9600 thermal cycler (Perkin Elmer, Buckinghamshire, UK) and PTC-100™ Programmable Thermal Controller (MJ Research Inc., MA, USA).

4.2.5.4 Agarose gel electrophoresis

*CYP2D6*2xN* PCR products were separated using a 1% AquaPor™ LE agarose gel. This solution was prepared by dissolution of 1g of powder in 100ml 1 x TBE buffer (10.8g Tris base, 5.5g boric acid, and 0.74g EDTA). Gels were set in trays, and mounted in a submerged gel electrophoresis tank (Plaztek Scientific, Upper Beaconsfield, Australia). Tanks were filled with 1 x TBE buffer and sample mixtures loaded. PCR products were mixed with a 1x gel loading buffer (0.04% bromophenol blue, 6.7% sucrose) in the ratio

10:2 prior to loading on the gel. In order to estimate the length of PCR products, a Hi-Lo mixed DNA marker was also loaded on to the gel. Bands on the gel corresponding to DNA fragments of 7.8kb for *CYP2D6*2xN* were visualised after staining the gel with ethidium bromide and viewing with UV transilluminator.

Positive control DNA samples of *CYP2D6*2xN* subjects previously confirmed via endonuclease digestion and Southern blotting were supplied by Dr Ulrich Griese (Dr Margarete-Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart, Germany) and used as part of every PCR assay.

4.2.6 Phenotyping Analysis

4.2.6.1 Extraction of urine (based on Chen *et al.*, 1990)

500µl urine (calibration curve samples, quality control samples and subjects' samples) was extracted following alkalisation with 200µl 1M NaOH and addition of 50µl 50µg.ml^{-1} pholcodine (which acted as the internal standard for HPLC quantification) with 4ml of extraction solvent (20:9:1 diethyl ether : chloroform : propan-2-ol). Samples were rotary mixed for 10min and centrifuged at 750g for 10min. The organic layer was transferred to a tube containing 200µl 0.1M orthophosphoric acid. Samples were again rotary mixed and centrifuged, the organic layer aspirated to waste and the remaining aqueous layer removed for injection on to the HPLC system. The volume of subject urine extracted was increased if concentrations of dextromethorphan or its metabolites were below the limit of quantification (see section 4.2.6.4 below).

4.2.6.2 Hydrolysis of 3-hydroxymorphinan-conjugates and dextrorphan-conjugates

100µl subject urine was mixed with 400µl water and 100µl 10,000 $U.ml^{-1}$ ß-glucuronidase. This amount of ß-glucuronidase was found to be the optimum amount needed for hydrolysis of conjugates, most likely glucuronides, following the results of hydrolysis with amounts of 100µl of 2,500, 5,000, 10,000, 15,000 and 20,000 $U.ml^{-1}$. A blank sample containing 500µl blank urine and 100µl 10,000 $U.ml^{-1}$ ß-glucuronidase, and standards and QC samples containing 50µl of standard or QC aqueous solution, 450µl blank urine and 100µl 10,000 $U.ml^{-1}$ ß -glucuronidase were also prepared. Pholcodine (50µl, 50$\mu g.ml^{-1}$) was added to all samples and they were then placed in a shaking waterbath at 37°C overnight (approximately 16hr).

4.2.6.3 HPLC system

A reversed phase HPLC system was utilised for the separation and quantification of 3-OH-M, 3-MM, DR and DM. The HPLC system consisted of a LC-6A pump (Shimadzu, Kyoto, Japan) with a cut-off pressure of $4kgf.cm^{-2}$, a SIL-10A autoinjector (Shimadzu, Kyoto, Japan), a fluorescence detector (Model LC-240, Perkin Elmer, Buckinghamshire, UK) with excitation and emission wavelengths of 225 and 320nm respectively, with computer integration (DP 800 Data Interface and DP 800 Chromatographic software, ICI Instruments, Melbourne, Australia). Compounds were separated by a Pellicular ODS C_{18} pre-column (1cm) and a stainless-steel column (15cm x 4.6mm) packed with 5µm cyano packing material (Spherisorb S5CN; Phase Separations, Queensferry, UK).

The mobile phase at a flow rate of 1ml.min^{-1} that gave best separation of the 3-OH-M, 3-MM, DR and DM was 19% ACN and 0.065% TEA in water, adjusted to pH 3.0 with orthophosphoric acid (Figure 27).

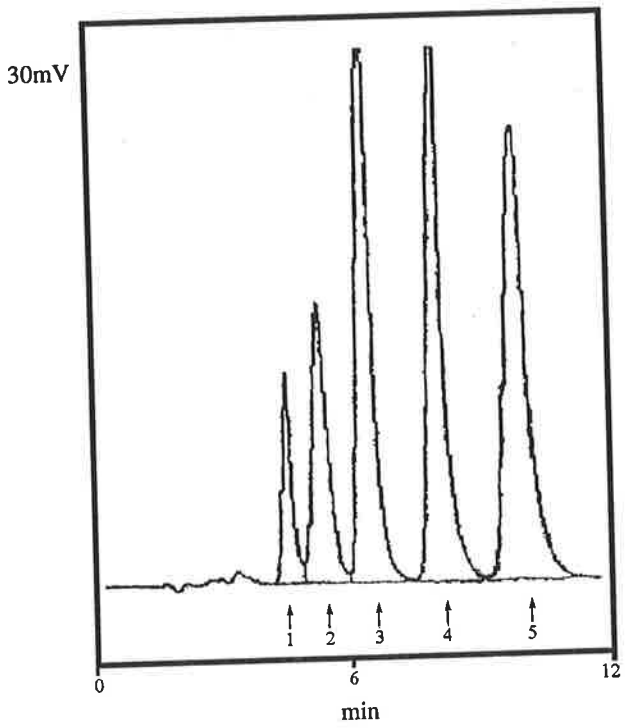


Figure 27: Representative HPLC chromatogram of calibration standard of 3-OH-M (1, 7.5µg.ml^{-1}), DR (2, 7.5µg.ml^{-1}), pholcodine (3, 4µg.ml^{-1}), 3-MM (4, 7.5µg.ml^{-1}) and DM (5, 7.5µg.ml^{-1}) after extraction from blank urine.

4.2.6.4 Assay Validation

Calibration, precision, inaccuracy, extraction efficiency and limit of quantification

Calibration curves were constructed for 3-OH-M, 3-MM, DR and DM with 10 final concentrations ranging from 0.025 to 25µg.ml^{-1}. Low, medium, high and very high quality control (QC) samples were also prepared, with final concentrations of 0.125, 0.750, 2.5 and 8.5µg.ml^{-1}, respectively. Standard curve and QC samples containing 3-OH-M, 3-MM,

DR and DM were prepared as 1 in 10 dilutions in blank urine and extracted identically to subject urine samples.

Peak areas of 3-OH-M, 3-MM, DR and DM were converted into peak area ratios using the peak area of the internal standard, and unweighted linear regression analysis of peak area ratios against nominal concentrations provided an estimate of slope, intercept and coefficient of determination (r^2).

The method was validated by assaying 10 QC samples (5 each of low and very high concentrations of 3-OH-M, 3-MM, DR and DM) and 5 calibration curve samples at the proposed limit of quantification on a single assay day to determine the intra-day inaccuracy and precision. Inter-day inaccuracy and precision were determined by analysis of QC samples (one each of low and very high concentrations of 3-OH-M, 3-MM, DR and DM) and a calibration sample at the limit of quantification on 5 different assay days (Table 21).

The extraction recoveries from plasma have been reported previously at a concentration of 0.2$\mu g.ml^{-1}$ of DM, DR, 3-OH-M, 3-MM and pholcodeine, respectively, as 57 ± 2%, 77 ± 2%, 69 ± 4%, 60 ± 2% and 63 ± 4% (Chen *et al.*, 1990). The extraction recoveries from urine were not assessed.

The limit of quantification for all compounds of interest (3-OH-M, 3-MM, DR and DM) was 0.025$\mu g.ml^{-1}$.

An assay was accepted if the r^2 was greater than 0.990, and all standard and QC samples in comparison to nominal values had inaccuracy of less than 15%.

Table 21: Summary of Intra- and Inter-assay precision and inaccuracy validation for 3-OH-M, 3-MM, DR and DM in urine.

Nominal conc. = nominal concentration, Cal. conc. = calculated concentration, CV = coefficient of variation, n = sample size.

	Nominal conc. ($\mu g.ml^{-1}$)	Cal. conc. (mean ± SD)	% Inaccuracy	% CV	n
Intra-assay					
3-OH-M	0.025	0.028 ± 0.0005	13.1	1.93	5
	0.125	0.099 ± 0.007	-20.5	7.67	5
	8.5	9.11 ± 0.17	7.22	1.87	5
3-MM	0.025	0.021 ± 0.001	-14.5	4.84	5
	0.125	0.110 ± 0.0016	11.6	1.53	5
	8.5	8.45 ± 0.083	-0.59	0.98	5
DR	0.025	0.025 ± 0.0013	1.38	5.19	5
	0.125	0.106 ± 0.0016	15.5	1.50	5
	8.5	8.54 ± 0.111	0.49	1.30	5
DM	0.025	0.023 ± 0.0011	-7.54	4.64	5
	0.125	0.117 ± 0.0027	6.18	2.35	5
	8.5	8.68 ± 0.072	2.10	0.83	5
Inter-assay	(5 different assay days)				
3-OH-M	0.025	0.0268 ± 0.0043	7.2	16.1	5
	0.125	0.128 ± 0.0124	2.24	9.73	5
	8.5	8.46 ± 0.490	-0.51	5.85	5
3-MM	0.025	0.027 ± 0.0019	8.00	6.93	5
	0.125	0.124 ± 0.0110	-0.48	8.81	5
	8.5	8.72 ± 0.44	2.57	5.10	5
DR	0.025	0.025 ± 0.0027	0.00	11.0	5
	0.125	0.124 ± 0.0058	-0.64	4.68	5
	8.5	8.40 ± 0.280	-1.16	3.36	5
DM	0.025	0.0248 ± 0.0015	-0.80	5.98	5
	0.125	0.131 ± 0.0055	4.48	4.24	5
	8.5	8.63 ± 0.490	1.58	5.71	5

4.2.7 Data Analysis

All raw data were transcribed to Microsoft® Excel (Version 5.0, Microsoft Corporation, WA, USA) and calibration curves were constructed by linear regression (Regression; Blackwell Scientific Software, Blackwell Scientific Publications, Oxford, UK). From the slope and intercept, concentrations of 3-OH-M, 3-MM, DR and DM were calculated and accuracy of the standard curve and QC concentrations ascertained by comparison with nominal concentrations. Concentrations of 3-OH-M, 3-MM, DR and DM in subject urine were found similarly. The amount of 3-OH-M, 3-MM, DR and DM excreted over 8hr was calculated as the concentration ($\mu g.ml^{-1}$) multiplied by the urine volume collected (ml), and then converted to mg by a division of 1000. Log dextromethorphan metabolic ratios (Log DM MR) were calculated via logarithm of the amount of dextromethorphan excreted divided by the amount of total 3-OH-M plus total DR excreted over the study period. Similarly, the log (DM / total DR) MR was also calculated to allow comparison with other studies who have not included the total 3-OH-M in the calculation of MR. Probit scores were calculated using Microsoft® Excel as described above (see section 3.2.7). Unpaired t-tests (GraphPad PRISM, Version 2.0, GraphPad Software, Inc., San Diego, CA, USA) was used to determine if there were significant differences between; i) the mean log DM MR of CYP2D6 non-UM Caucasians and UM, ii) the mean log (DM/total DR) MR of CYP2D6 non-UM Caucasians and UM, iii) the mean log DM MR of CYP2D6 non-UM Caucasians and Asian, iv) the mean log (DM/total DR) MR of CYP2D6 non-UM Caucasians and Asian, and v) the mean log DM MR and the mean log (DM/total DR) MR in all subjects. Spearman rank correlation tests (GraphPad PRISM, Version 2.0, GraphPad Software, Inc., San Diego, CA, USA) were used to determine if there were relationships between either log DM MR or log (DM/total DR) MR and the following: i) urine pH or volume, ii) age, iii) subject weight, iv) subject height, v) cigarette consumption, vi) alcohol consumption, and vii) caffeine consumption. All data are reported as mean ± SD (95% confidence levels).

4.3 Results

4.3.1 Genotyping

Genotyping for *CYP2D6*2xN* revealed 5 subjects who carried multiple copies of the *CYP2D6* gene on at least one allele. Three subjects were not able to be genotyped due to insufficient DNA being isolated from their buccal cell sample. These subjects were excluded from results of CYP2D6 phenotyping in this study, so that the total subject number was 80. All subjects that carried multiple *CYP2D6* copies were Caucasian, therefore, the alleleic incidence of CYP2D6 UM in this population was 8.5%. No Asian subjects or the African subject carried multiple copies of *CYP2D6*.

4.3.2 Phenotyping

Using an antimode of log MR of -0.5 (Schmid *et al.*, 1985), the incidences of phenotypic PM in Caucasian and Asian populations were 5 (n=3) and 10% (n=2), respectively. These subjects had DM MR of 0.19, 1.01, -0.36, and 0.12, -0.28, respectively. Excluding these phenotypic PM, the log DM MR in non-*CYP2D6*2xN* Caucasian (n=51) and Asian (n=18) subjects, were -2.20 ± 0.58 (-2.36 to -2.03), and -1.76 ± 0.49 (-2.01 to -1.52), respectively. The log DM MR of the one African subject was -2.25. The histogram of the log DM MR shows that the majority of subjects had log DM MR less than -1 (Figure 27), which is also illustrated in the probit plot (Figure 28, 29). Excluding the phenotypic PM, the log (DM/total DR) MR in non-*CYP2D6*2xN* Caucasian and Asian subjects were -2.02 ± 0.57 (-2.18 to -1.85) and -1.59 ± 0.49 (-1.83 to -1.34), respectively. There were significant differences between non-*CYP2D6*2xN* Caucasian and Asian subjects in both the log DM MR or the log (DM/total DR) MR, P = 0.006, and P = 0.008, respectively. In both non-*CYP2D6*2xN* Caucasian and Asian populations studied there were no significant

differences between the log DM MR and the log (DM/total DR) MR, P = 0.125 and P = 0.301, respectively.

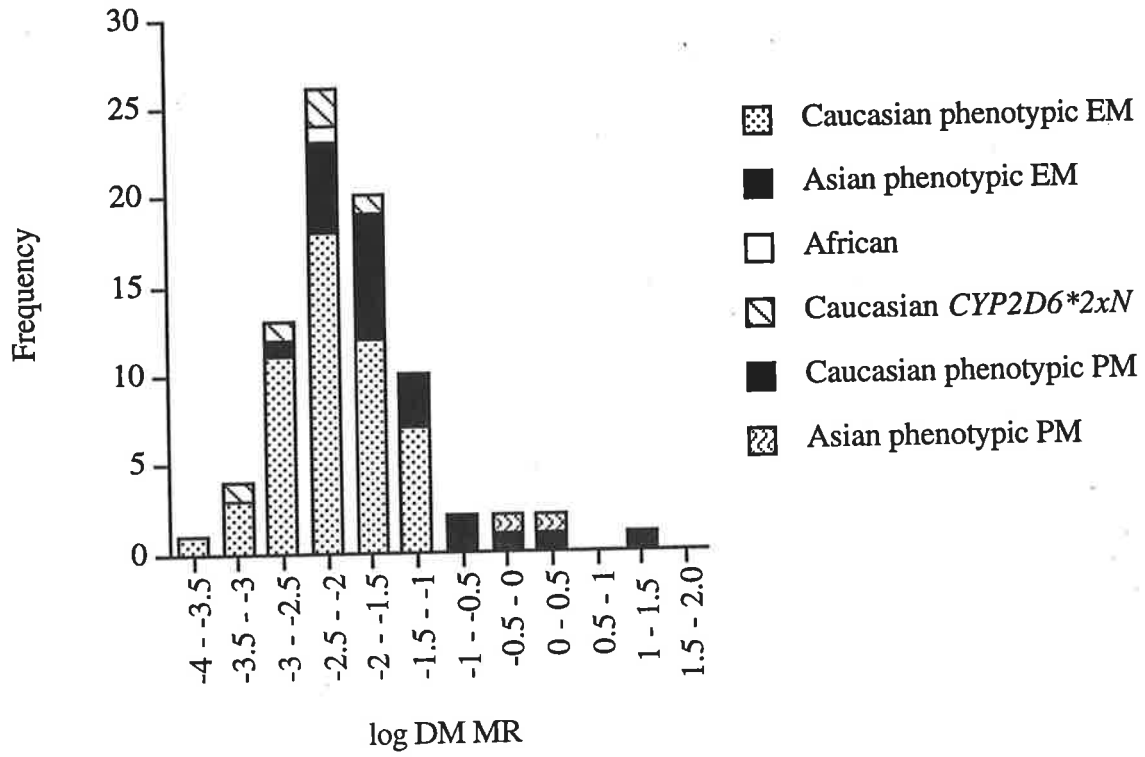


Figure 27: Histogram showing the frequency of various log DM metabolic ratios of Caucasian phenotypic EM (n=51), Caucasian phenotypic PM (n=3), Caucasian *CYP2D6*2xN* (n=5), Asian phenotypic EM (n=18), Asian phenotypic PM (n=2), and African (n=1) subjects.

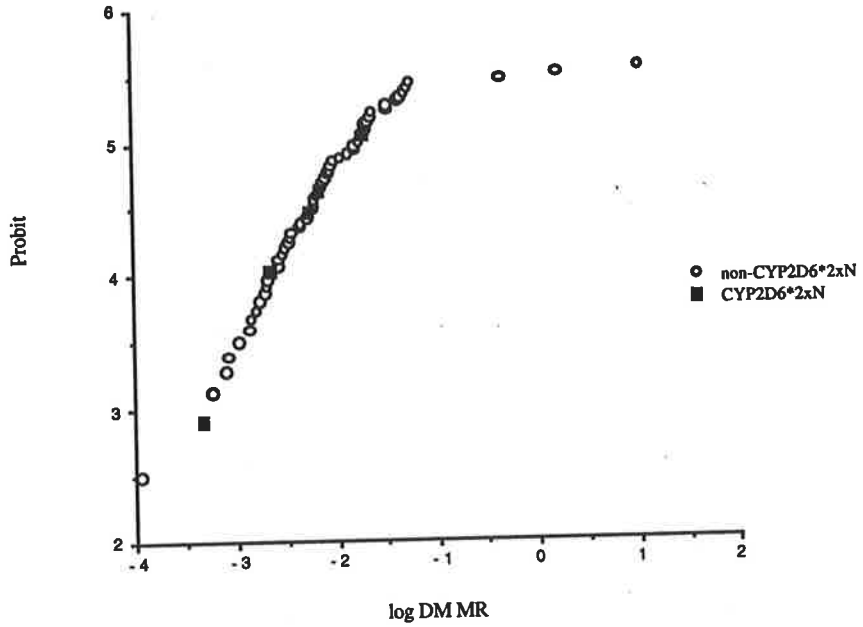


Figure 28: Probit plot of log DM metabolic ratio in 8hr urine of Caucasian subjects with CYP2D6 genotype, *CYP2D6*2xN* (n=5) and non-*CYP2D6*2xN* (n=54).

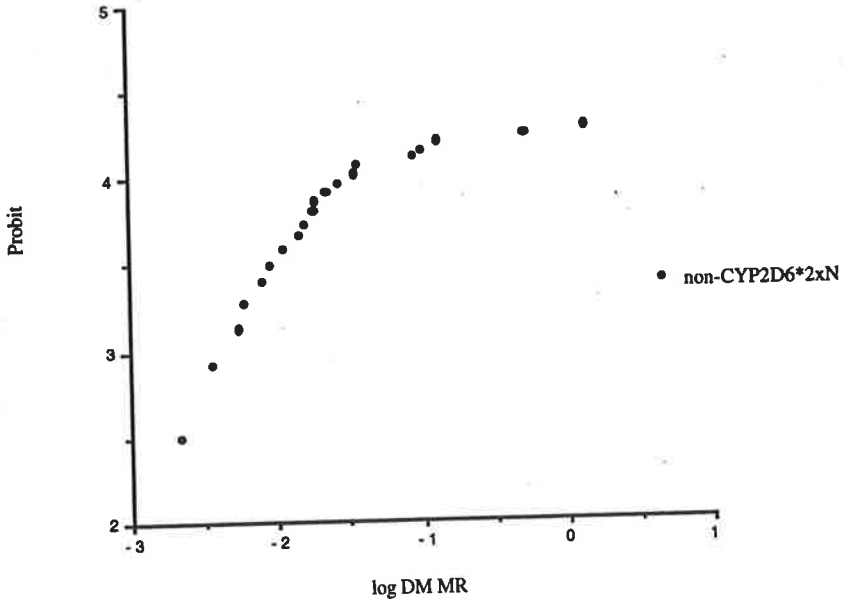


Figure 29: Probit plot of log DM metabolic ratio in 8hr urine of Asian subjects (n=20), with CYP2D6 genotype non-*CYP2D6*2xN*.

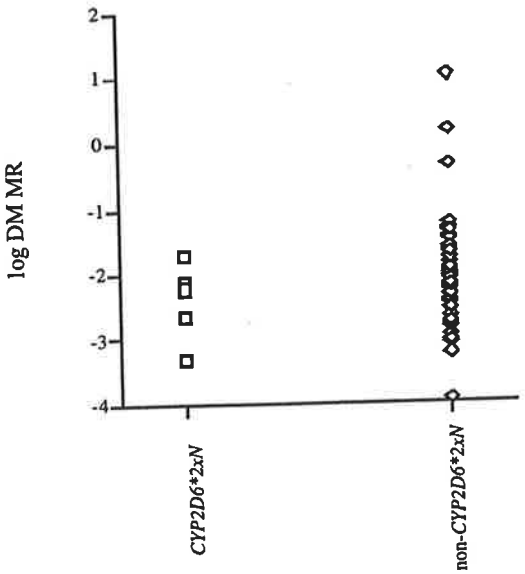


Figure 30: log DM metabolic ratios of Caucasian subjects (n=59) with *CYP2D6*2xN* and non-*CYP2D6*2xN* genotypes.

The log DM MR of Caucasian subjects (n=5) that carried multiple copies of *CYP2D6* (CYP2D6 UM), -2.45 ± 0.56 (-3.15 to -1.75), was not significantly different when compared with the remaining non-PM Caucasian subjects (n=51), -2.20 ± 0.58 (-2.36 to -2.03), P = 0.410 (Figure 30). Similarly, the log (DM/total DR) MR of CYP2D6 UM, was not significantly different when compared with the remaining Caucasian subjects, -2.25 ± 0.61 (-3.01 to -1.49) versus -2.02 ± 0.57 (-2.18 to -1.85), P = 0.405. There was no significant difference between the log DM MR and the log (DM/total DR) MR in CYP2D6 UM, P = 0.665.

Significant relationships between the log DM MR and urine pH and the log (DM/total DR) MR and urine pH were observed for non-CYP2D6*2xN Caucasian subjects, r_s = -0.550, P < 0.0001, and r_s = -0.569, P < 0.0001, respectively. There was a significant relationship between the log (DM/total DR) MR and urine pH in Asian subjects, r_s = -0.447, P = 0.048, but there was no relationship between the log DM MR and urine pH in these subjects, r_s = -0.410, P = 0.073. There were no significant relationships between either the log DM MR or the log (DM/total DR) MR and urine pH in Caucasian CYP2D6 UM, r_s = -0.62, P = 0.350, and r_s = -0.36, P = 0.517, respectively. Additionally, there were no significant relationships between either the log DM MR or the log (DM/total DR) MR and urine volume in any groups of subjects (refer to appendix H for individual subject data). There were no significant correlations between either the log DM MR or the log (DM/total DR) MR and subject weight, height, age, cigarette consumption, alcohol consumption and caffeine consumption.

4.3.3 Adverse Effects

26 subjects (32.5%) self-reported adverse effects (Table 22). Of those reported, dizziness, dry mouth, and altered breathing are most commonly seen with dextromethorphan administration (Caswell, 1999).

Table 22: Incidence of self-reported adverse effects reported by 80 subjects (Note: subjects were also taking proguanil).

Subject	Ethnic origin	log DM MR	CYP2D6 genotype	Self-reported adverse effects
#2	Caucasian	-2.43	non-*CYP2D6*2xN*	wheezy, coughing, increased heart rate, light-headed
#7	Caucasian	-2.15	*CYP2D6*2xN*	mild headache
#8	Caucasian	-2.35	non-*CYP2D6*2xN*	slight nausea
#10	Caucasian	-3.32	*CYP2D6*2xN*	dry mouth
#11	Caucasian	0.15	non-*CYP2D6*2xN*	nausea, "fuzzy-headedness"
#19	Caucasian	-1.85	non-*CYP2D6*2xN*	mild headache
#21	Caucasian	-3.10	non-*CYP2D6*2xN*	slight dizziness, slight headache
#24	Caucasian	-1.75	non-*CYP2D6*2xN*	slight feeling of gastric reflux
#27	Caucasian	-1.33	non-*CYP2D6*2xN*	dry mouth
#28	Caucasian	-2.88	non-*CYP2D6*2xN*	headache, slight dizziness, dry mouth
#33	Caucasian	-2.19	non-*CYP2D6*2xN*	slight headache
#34	Caucasian	-2.81	non-*CYP2D6*2xN*	slight headache
#44	Caucasian	-2.70	non-*CYP2D6*2xN*	dry mouth
#46	Asian	-1.56	non-*CYP2D6*2xN*	mild abdominal discomfort, headache, dry mouth
#49	Asian	-1.80	non-*CYP2D6*2xN*	dry mouth, mild nausea
#53	Asian	-2.43	non-*CYP2D6*2xN*	dry throat
#54	Caucasian	-2.24	*CYP2D6*2xN*	headache, insomnia
#56	Asian	-1.94	non-*CYP2D6*2xN*	headache, insomnia
#62	Asian	-1.72	non-*CYP2D6*2xN*	dizziness
#68	African	-2.25	non-*CYP2D6*2xN*	dry mouth, headache
#69	Asian	-2.67	non-*CYP2D6*2xN*	slight tightness of chest
#73	Caucasian	-1.73	non-*CYP2D6*2xN*	slight headache
#74	Caucasian	-1.27	non-*CYP2D6*2xN*	giddiness, nausea
#75	Caucasian	-1.94	non-*CYP2D6*2xN*	slightly pale
#77	Asian	-1.73	non-*CYP2D6*2xN*	drowsiness
#80	Caucasian	-2.18	non-*CYP2D6*2xN*	mild nausea, mild headache

4.4 Discussion

The incidence of CYP2D6 UM in the Caucasian population within the present study was observed to be 8.5%. This is similar to the finding in a Spanish population whose incidence was 7% (Agundez *et al.*, 1995), but higher than the incidence found in Swedish and German populations, 4.7% (Dahl *et al.*, 1995) and 3.6% (Sachse *et al.*, 1997), respectively. Additionally, the incidence of CYP2D6 UM in this study is lower than in Ethiopians (29%, Aklillu *et al.*, 1996) and Saudi Arabians (20%, McLellan *et al.*, 1997). The number of subjects participating in the Spanish, Swedish and German studies was 217, 320, and 589, respectively, whilst the number of Caucasians who participated in the present study was only 60. It is possible that the incidence of genotypic CYP2D6 UM identified in the present Caucasian population could be artificially high due to the smaller number of subjects. Therefore, further studies with larger subject numbers are necessary to confirm or discount the original hypothesis of a similar incidence of CYP2D6 UM when compared to Danish, Swedish and German populations. No Asian subjects genotyped carried *CYP2D6*2xN*, and the incidence of this genotype in Asians has not been reported previously. However, due to small number of Asians in this study there is a possibility that gene duplication of *CYP2D6* occurs in this population, which may be revealed with larger studies.

The log DM MR of Caucasian subjects with *CYP2D6*2xN* genotype was -2.4 ± 0.6. This was not significantly different to the ratio in Caucasian subjects who did not carry *CYP2D6*2xN*, -2.0 ± 0.8, P = 0.32. Due to genotyping in the present study being limited to identification of *CYP2D6* duplication, it remains possible that the subjects identified genotypically as CYP2D6 UM carry either wildtype *CYP2D6* or mutations of *CYP2D6* on the other allele, so that overall their CYP2D6 metabolic activity is similar to the remaining subjects. This has been shown previously in a Gabonese population phenotyped with dextromethorphan, where 2 subjects with genotype *CYP2D6*1/*2x2* had log (DM/DR) MR of -2.2 and -1.9, compared with 5 subjects with genotype *CYP2D6*1/*1* who had log

(DM/DR) MR of -2.9 ± 0.3 (Panseret *et al.*, 1999). Another study of 456 Germans phenotyped with dextromethorphan showed there was no difference between the mean log (DM/DR) MR of subjects with 2 or 3 active copies of CYP2D6, -2.52 and -2.70, respectively (Sachse *et al.*, 1997).

The question then arises as to which phenotypic probe of CYP2D6 can separate CYP2D6 UM from EM. The researchers of the previously mentioned study in the German population also phenotyped 133 Germans with debrisoquine in order to compare the abilities of the probes to separate these genotypic groups (Sachse *et al.*, 1997). Of those phenotyped with dextromethorphan, 12 carried 3 active genes and had a mean (range) log (DM/DR) MR of -2.70 (-3 to -2.3), whilst 246 subjects carried 2 active genes and had a mean (range) log (DM/DR) MR of -2.52 (-2.70 to -2.52), with no significant difference between the mean MR. Conversely, of those phenotyped with debrisoquine, 5 carried 3 active genes and had a range of log debrisoquine MR from -1.02 to -0.52. This was significantly different to the MR in subjects with 2 active genes (n=66) whose mean log debrisoquine MR ranged from -0.40 to -0.07, $P < 0.01$. The authors concluded that dextromethorphan was unable to distinguish between UM and EM groups as effectively as debrisoquine due to the extensive metabolism of dextromethorphan in both UM and EM. Another study has found that although the median log debrisoquine MR was lower in subjects with gene duplication (-1.15, n=15), some subjects without gene duplication had similar MR (Dahl *et al.*, 1995). This distribution of CYP2D6 MR of UM and EM were again observed in an Ethiopian population phenotyped with debrisoquine (Aklillu *et al.*, 1996). 33 subjects with more than 2 active genes had median MR of 0.13 - 0.33, whilst those with 1 - 2 active genes had median MR of 0.25 - 1.29, so that separation of these groups was incomplete. Finally, it has also been shown that sparteine can not effectively separate CYP2D6 UM from EM in a Danish population (Bathum *et al.*, 1998). It was reported that 9 subjects with *CYP2D6* duplication and 51 subjects without gene duplication had a metabolic ratio below 0.14. These authors suggested that the sparteine MR is unable to separate subjects with high metabolic activity after a single dose, but that after repeated dosing a difference in the metabolic ratio may be evident.

In contrast to the above observations, a study in Spaniards has shown a significant difference between the debrisoquine MR in subjects with gene duplication, 0.33 (n=9, 95% confidence limit 0.22 - 0.45), when compared to those with wildtype CYP2D6, 1.50 (n=12, 95% confidence limit 0.88 - 2.14, $P < 0.002$; Agundez *et al.*, 1995). Perhaps the ability to separate these groups depends on the contribution of renal clearance to the total clearance. This may change under the influence of other environmental factors, such as diet, which alter renal clearance.

The finding that there was a higher (not significant) log DM MR when Asian subjects were compared to Caucasian subjects (P=0.015) is not surprising. A previous study of Swedish (n=1011) and Chinese (n=695) subjects phenotyped with 10mg debrisoquine reported a shift to the right of the histogram of distribution of debrisoquine/4-hydroxydebrisoquine metabolic ratios in Chinese when compared to that of the Caucasian population, so that the Chinese subjects had a higher debrisoquine/4-hydroxydebrisoquine metabolic ratio (Bertilsson *et al.*, 1992). The researchers stated that this loss of CYP2D6 activity, and hence higher metabolic ratios, is due to a gene insertion reducing the translation of the *CYP2D6* gene to functional enzyme. This gene insertion is present in 17% of Chinese subjects (Yue *et al.*, 1989a, Johansson *et al.*, 1991), and therefore, it seems that the result of a higher mean log DM MR in this study may be due to mutations of *CYP2D6*, especially *CYP2D6*10*, (present in 20% of Japanese, Tateishi *et al.*, 1999), which causes an decrease in the production of functional CYP2D6, but not to such a level that subjects become PM. It has recently been shown that the *CYP2D6*10* mutation lowers metabolic activity of CYP2D6 by 50% (Tateishi *et al.*, 1999).

The observations that the log DM MR was significantly associated with urine pH are unexpected. This may indicate that the renal clearance of dextromethorphan is variable, due to interindividual "differences in the efficiency of secretory mechanisms or as a result of dietary habits as they affect urine pH and hence tubular secretion" (Jackson *et al.*, 1986). This variation in renal clearance would subsequently influence the MR.

Overall, it was observed in the present study that the incidence of CYP2D6 gene duplication in a Caucasian population living in Australia was 8.5%, but these subjects could not be separated from the remaining Caucasian subjects on the basis of log DM MR. It must be concluded from the present study and others conducted in different populations, that phenotyping with any of the 3 commonly used CYP2D6 probe drugs (dextromethorphan, debrisoquine and sparteine) is not able to consistently separate subjects with or without gene duplication. Therefore, the identification of subjects carrying more than two active CYP2D6 genes must be achieved by genotyping.

The next chapter will investigate *in vivo* the changes in metabolism of codeine (a known substrate of CYP2D6) in CYP2D6 UM compared with EM.

5. Effect of multiple copies of the *CYP2D6* gene on codeine metabolism: a pilot study

5.1 Introduction

The clinical implications of the genetic polymorphism of the CYP2D6 enzyme in terms of the influence of mutations of *CYP2D6* leading to reduced metabolism has been studied extensively. The more recent discovery of people carrying duplicate or multiple copies of *CYP2D6* was initially reported through anecdotal evidence (Bertilsson *et al.*, 1993). This occurrence of gene duplication has opposite clinical implications to the occurrence of gene mutation, especially when the drug, which is metabolised by CYP2D6, has a narrow therapeutic index. In the case of codeine it is established that the metabolism to morphine is mediated by CYP2D6 (see section 1.3.3.1). The increased formation of morphine and subsequent glucuronide conjugates would then have clinical implications, especially in terms of the dose able to be administered to these people without the experience of adverse effects, whilst still obtaining analgesia.

The following hypothesis and subsequent aims will be addressed in this chapter:

Hypothesis 6: The codeine to morphine urinary metabolic ratio would decrease, and a smaller amount of codeine would be metabolised to the other metabolites (codeine-6-glucuronide, norcodeine) in CYP2D6 UM when compared to EM.

Aim 8: The aim of this *in vivo* pilot study is to investigate the metabolism of codeine in CYP2D6 UM (n=2) and EM (n=2) via administration of codeine followed by urine collection for 48hr.

5.2 Methods

5.2.1 Chemicals

Codeine phosphate and morphine sulphate, morphine-3-glucuronide (M-3-G) and hydromorphone hydrochloride, and norcodeine (NC) were obtained from F.H.Faulding (Adelaide, Australia), Sigma Chemical Company (St Louis, MO, USA), and Eli Lilly (Indianapolis, IN, USA), respectively. Dihydrocodeine bitartrate came from Knoll (Ludwigshafen, Germany). Codeine-6-glucuronide (C-6-G) and morphine-6-glucuronide (M-6-G) were synthesised by Dr Geoffery Reynolds (School of Chemical Technology, South Australian Institute of Technology, Adelaide, Australia, 1989). Other materials were obtained from the following sources: triethylamine (TEA), and lauryl sulphate sodium salt (sodium dodecyl sulfate, SDS) from Sigma Chemical Company (St. Louis, MO, USA); hydrochloric acid (HCl), orthophosphoric acid, sodium dihydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$), sodium hydroxide (NaOH) from Ajax Chemicals (Auburn, Australia); acetonitrile (ACN), and chloroform from BDH Chemicals (Poole, England).

5.2.2 Subjects

Four Caucasian subjects (3 males, 1 female) previously identified as either carriers of multiples copies of *CYP2D6* (n=2) or not carriers of multiple copies of *CYP2D6* (n=2) from the large scale phenotyping/genotyping study described in chapter 4 were recruited. See appendix I for subject data.

5.2.3 Study Protocol

Ethics approval for the study was obtained from the Royal Adelaide Hospital Research Ethics Committee. Subjects self-administered 90mg codeine phosphate (capsule made up at the Royal Adelaide Hospital Pharmacy Department, Adelaide) orally after emptying their bladders. Urine was then collected over a 48hr period. The pH and volume of urine collected was recorded and a 20ml aliquot stored at -20°C for analysis.

5.2.4 Quantification of codeine and metabolites in urine

5.2.4.1 Codeine, Morphine and Norcodeine (based on Chen *et al.*, 1989a)

5.2.4.1.1 Extraction of urine

200µl subject urine was extracted following addition of 200µl 1M Na_2CO_3 buffer pH 9.6 and 80µl 10µg.ml^{-1} dihydrocodeine (the internal standard for HPLC quantification) with 5ml of chloroform previously washed with 0.1M HCl. Samples were rotary mixed for 10min and centrifuged at 2,250g for 10min. The aqueous layer was aspirated and the organic layer was transferred to a tube containing 200µl 0.1M HCl. Samples were rotary mixed and centrifuged again, and a 150µl aliquot of the acid bubble was removed for injection on to the HPLC system. Extraction of morphine, norcodeine, codeine and dihydrocodeine using this method has been reported to be greater than or equal to 76% (Chen *et al.*, 1989a). The volume of urine extracted was increased if concentrations of codeine, morphine or norcodeine were below the limit of quantification.

5.2.4.1.2 HPLC System

A reversed phase HPLC system was utilised for the separation and quantification of morphine, norcodeine, and codeine. The HPLC system consisted of a LC-6A pump (Shimadzu, Kyoto, Japan) with a cut-off pressure of 4kgf.cm^{-2}, a SIL-10A autoinjector (Shimadzu, Kyoto, Japan), a fluorescence detector (Model LC-240, Perkin Elmer, Buckinghamshire, UK) excitation and emission wavelengths of 230 and 350nm respectively, with computer integration (DP 800 Data Interface and DP 800 Chromatographic software, ICI Instruments, Melbourne, Australia). Compounds were separated by a Pellicular ODS cyano pre-column (1cm) and a stainless-steel column (15cm x 4.6mm) packed with 5µm cyano packing material (Spherisorb S5CN; Phase Separations, Queensferry, UK).

The mobile phase at a flow rate of 1ml.min^{-1} that gave best separation of morphine, norcodeine, and codeine was 4% ACN, and 0.1% TEA in water, adjusted to a pH of 3.1 with orthophosphoric acid (Figure 31).

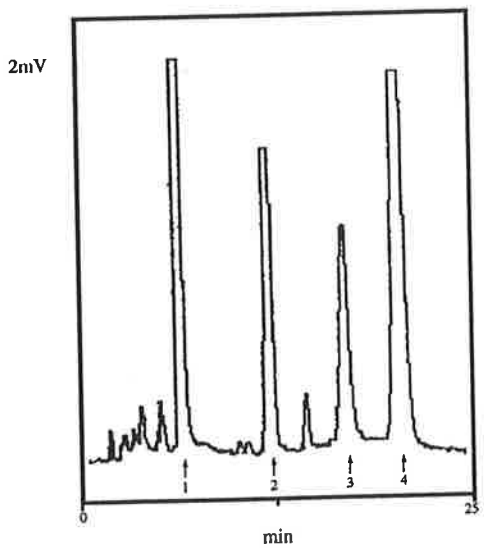


Figure 31: Representative chromatogram of calibration standard of morphine (1, 25µg.ml^{-1}), norcodeine (2, 10µg.ml^{-1}), dihydrocodeine (3, 4µg.ml^{-1}) and codeine (4, 5µg.ml^{-1}) following extraction from blank urine.

5.2.4.1.3 Assay Validation

Calibration curves, precision, inaccuracy, extraction efficiency and limit of quantification

Calibration curves were constructed for morphine, norcodeine and codeine (free-base) with 6 final concentrations ranging from 0.05 to 25$\mu g.ml^{-1}$ for morphine, 0.25 to 10$\mu g.ml^{-1}$ for norcodeine, and 0.25 to 5$\mu g.ml^{-1}$ for codeine. Low, medium, and high quality control (QC) samples were also prepared, with final concentrations of 7.5, 15, and 22.5$\mu g.ml^{-1}$ for morphine, 0.75, 2 and 7.5$\mu g.ml^{-1}$ for norcodeine, and 0.6, 1.5 and 3$\mu g.ml^{-1}$ for codeine. Calibration curve and QC samples containing morphine, norcodeine and codeine were prepared as 1 in 10 dilutions in blank urine and extracted identically to subject urine samples.

Peak areas of morphine, norcodeine and codeine were converted into peak area ratios using the peak area of the internal standard, and unweighted linear regression analysis of peak area ratios against nominal concentrations provided an estimate of slope, intercept and coefficient of determination (r^2).

The method was validated by assaying 18 QC samples (6 each of low, medium and high concentrations for morphine, norcodeine and codeine) and 6 standard samples at the limit of quantification on a single assay day to determine the intra-day inaccuracy and precision. Inter-day inaccuracy and precision were determined by analysis of 3 QC samples (one each of low, medium and high concentrations for morphine, norcodeine and codeine) and 4 standard samples at the limit of quantification on 4 different assay days (Table 23).

The limits of quantification for morphine, norcodeine and codeine, were 0.05$\mu g.ml^{-1}$, 2.5$\mu g.ml^{-1}$ and 0.25$\mu g.ml^{-1}$, respectively.

An assay was accepted if the coefficient of variation (r^2) was greater than 0.990, and all standard and QC samples had inaccuracy in comparison to nominal values of less than 15%.

Table 23: Summary of Intra- and Inter-assay precision and inaccuracy validation for morphine, norcodeine, and codeine in urine.

Nominal conc = nominal concentration, Cal conc = calculated concentration, CV = coefficient of variation, n = sample size.

	Nominal conc ($\mu g.ml^{-1}$)	Cal conc (mean ± SD)	% Inaccuracy	% CV	n
Intra-assay					
morphine	2.5	2.66 ± 0.04	6.32	1.40	6
	7.5	8.59 ± 0.24	14.58	3.23	6
	15	15.9 ± 0.42	6.18	2.63	6
	22.5	25.4 ± 0.97	12.74	3.84	6
norcodeine	0.25	0.25 ± 0.03	1.33	10.2	3
	0.75	0.74 ± 0.05	1.09	6.13	4
	2	1.92 ± 0.14	3.81	7.07	4
	7.5	7.11 ± 0.70	5.17	9.86	4
Codeine	0.188	0.184 ± 0.016	-2.43	8.92	6
	0.6	0.604 ± 0.023	0.64	3.88	6
	1.5	1.50 ± 0.641	0.13	4.27	6
	3	2.90 ± 0.688	-3.30	2.37	6
Inter-assay	(4 different assay days)				
Morphine	2.5	2.65 ± 0.15	5.90	5.60	4
	7.5	8.23 ± 0.21	9.73	2.55	4
	15	14.8 ± 0.52	-1.07	3.50	4
	22.5	23.8 ± 0.65	5.74	2.74	4
NC	0.25	0.25 ± 0.02	-1.00	8.33	4
	0.75	0.74 ± 0.04	-2.00	5.03	4
	2	1.92 ± 0.13	-4.00	6.92	4
	7.5	7.11 ± 0.70	-5.20	9.89	4
Codeine	0.188	0.185 ± 0.014	-1.84	7.47	4
	0.6	0.608± 0.030	1.27	5.06	4
	1.5	1.44 ± 0.056	-4.26	3.94	4
	3	2.86 ± 0.072	-4.83	2.52	4

5.2.4.2 Morphine-3-Glucuronide (M-3-G) and Morphine-6-Glucuronide (M-6-G) (based on Milne *et al.*, 1991)

5.2.4.2.1 Extraction of urine

100µl subject urine was extracted following dilution to 500µl with blank urine, addition of 3ml 500mM bicarbonate buffer pH 9.3 and 50µl 1mg.ml^{-1} hydromorphone (the internal standard for HPLC quantification) using Sep-Pak® C_{18} 5µm cartridges (Waters Chromatography Division, Millipore Corporation, Milford, MA, USA). Cartridges were conditioned consecutively with 10ml 100% MeOH, 5ml eluant (25% ACN, 10mM NaH_2PO_4 in water, adjusted to pH 2.3 with addition of orthophosphoric acid), and 10ml water. Samples were loaded on to the cartridges from syringes with a Techicon® autoanalyzer proportioning pump (Techicon Ltd., Dublin, Ireland). Cartridges were washed consecutively with 5mM bicarbonate buffer pH 2.3 (20ml), water (500µl) and eluant (300µl). Compounds were eluted from the cartridges using 500µl eluant, which was collected in 0.5ml eppendorf tubes, and 100µl was injected on to HPLC. Extraction recovery of M-3-G, M-6-G and hydromorphone was greater than or equal to 86%. The volume of urine extracted was increased if concentrations of M-3-G or M-6-G were below the limit of quantification.

5.2.4.2.2 HPLC System

A reversed phase HPLC system was utilised for the separation and quantification of M-3-G, M-6-G and hydromorphone. The HPLC system consisted of a LC-6A pump (Shimadzu, Kyoto, Japan) with a cut-off pressure of 4kgf.cm^{-2}, a SIL-10A autoinjector (Shimadzu, Kyoto, Japan), a UV detector (Model UVIDEC-100-V, Jasco, Japan Spectroscopic Co., Ltd., Tokyo, Japan) at a fixed wavelength of 210nm, with computer

integration (DP 800 Data Interface and DP 800 Chromatographic software, ICI Instruments, Melbourne, Australia). Compounds were separated by a Pellicular ODS C_{18} pre-column (1cm) and a C_{18} 5µm cartridge (Waters, MA, USA) in a RCM module.

The mobile phase at a flow rate of 1.5ml.min^{-1} that gave best separation of M-3-G, M-6-G and hydromorphone was 23% ACN, 10mM NaH_2PO_4 and 0.230g/L SDS in water, adjusted to pH 2.3 with orthophosphoric acid (Figure 32).

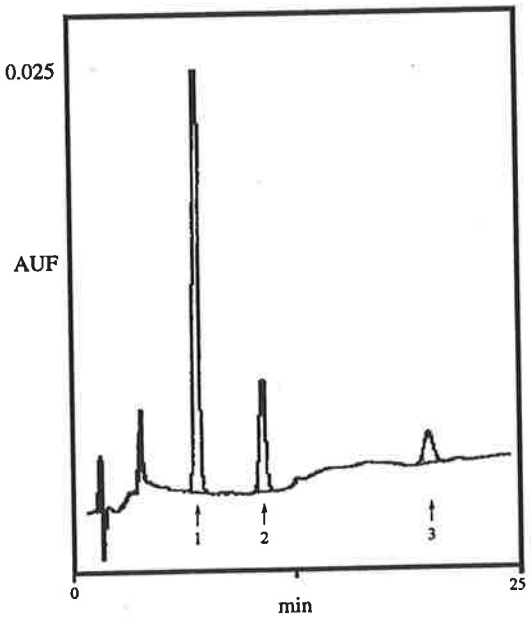


Figure 32: Representative chromatogram of calibration standard of M-3-G (1, 2mg.L^{-1}), M-6-G (2, 0.5mg.L^{-1}) and hydromorphone (3, 0.1mg.ml^{-1}) following extraction from blank urine.

5.2.4.2.3 Assay Validation

Calibration curves, precision, inaccuracy, extraction efficiency and limit of quantification

Calibration curves were constructed for M-3-G and M-6-G with 6 final concentrations ranging from 250 to 5000$\mu g.L^{-1}$ for M-3-G, and 125 to 1250$\mu g.L^{-1}$ for M-6-G. Low and high QC samples were also prepared, with final concentrations of 250 and 3000$\mu g.L^{-1}$ for M-3-G, and 125 and 875$\mu g.L^{-1}$ for M-6-G, respectively. Calibration curve and QC samples containing M-3-G and M-6-G were prepared as 1 in 20 dilutions in blank urine and extracted identically to subjects' urine samples.

Peak areas of M-3-G and M-6-G were converted into peak area ratios using the peak area of the internal standard, and unweighted linear regression analysis of peak area ratios against nominal concentrations provided an estimate of slope, intercept and coefficient of determination (r^2).

The method was validated by assaying 4 QC samples (4 each of the high concentration for M-3-G and M-6-G) and 4 standard samples at the limit of quantification on a single assay day to determine the intra-day inaccuracy and precision. Inter-day inaccuracy and precision were determined by analysis of one QC sample (one high concentration for M-3-G and M-6-G) and one standard sample at the limit of quantification on 4 different assay days (Table 24).

The limits of quantification for M-3-G and M-6-G were respectively, 250$\mu g.L^{-1}$ and 125$\mu g.L^{-1}$.

An assay was accepted if the coefficient of variation (r^2) was greater than 0.990, and all standard and QC samples had inaccuracy in comparison to nominal values of less than 25%.

Table 24: Summary of Intra- and Inter-assay precision and inaccuracy validation M-3-G and M-6-G in urine.

Nominal conc = nominal concentration, Cal conc = calculated concentration, CV = coefficient of variation, n = sample size.

	Nominal conc ($\mu g.ml^{-1}$)	Cal conc (mean ± SD)	% Inaccuracy	% CV	n
Intra-assay					
M-3-G	250	269 ± 25.0	7.58	9.31	4
	3000	2276 ± 88.6	-24.1	3.89	4
M-6-G	125	130 ± 31.1	4.11	23.9	4
	875	784 ± 34.8	-10.4	4.44	4
Inter-assay	(3 or 4 different assay days)				
M-3-G	250	253 ± 13.9	-1.30	5.51	4
	3000	2424 ± 208	19.2	8.57	4
M-6-G	125	143 ± 11.2	-14.1	7.84	3
	875	732 ± 40.6	16.4	5.55	4

5.2.4.3 Codeine-6-Glucuronide (C-6-G) (based on Chen *et al.*, 1989b)

5.2.4.3.1 Processing of urine

50µl subject urine was diluted to 500µl with water, and 50µl was injected on to HPLC. The volume of subject urine injected was increased if the concentration of C-6-G was below the limit of quantification.

5.2.4.3.2 HPLC System

A reversed phase HPLC system was utilised for the quantification of C-6-G. The HPLC system consisted of a LC-6A pump (Shimadzu, Kyoto, Japan) with a cut-off pressure of 4kgf.cm^{-2}, a SIL-10A autoinjector (Shimadzu, Kyoto, Japan), a fluorescence detector (Model LC-240, Perkin Elmer, Buckinghamshire, UK) excitation and emission wavelengths of 235 and 340nm respectively, with computer integration (DP 800 Data Interface and DP 800 Chromatographic software, ICI Instruments, Melbourne, Australia). Compounds were separated by a Pellicular ODS cyano pre-column (1cm) and a stainless-steel column (25cm x 4.6mm) packed with 5µm cyano material (Waters, MA, USA).

The mobile phase at a flow rate of 1ml.min^{-1} that gave best chromatographic quantification of C-6-G was 5% ACN, 10mM NaH_2PO_4 and 0.03% TEA in water, adjusted to pH 3.0 with orthophosphoric acid (Figure 33).

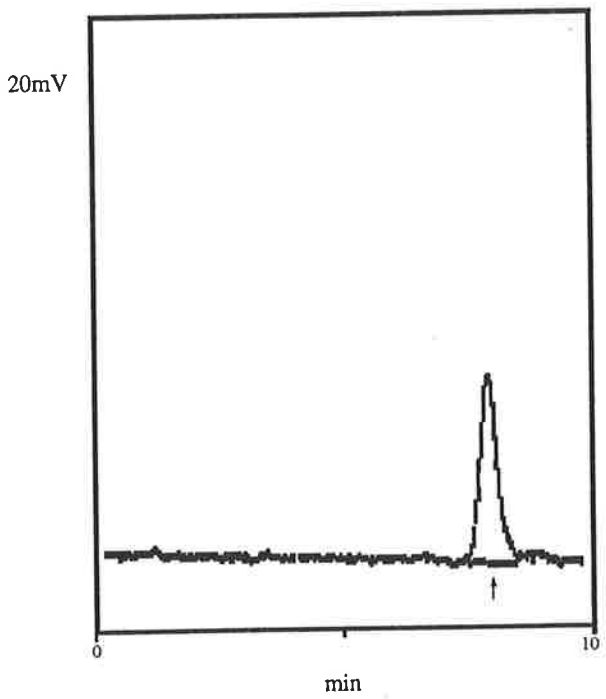


Figure 33: Representative HPLC chromatogram of calibration standard of C-6-G (5µg. ml^{-1}) from blank urine.

5.2.4.3.3 Assay Validation

Calibration curve, precision, inaccuracy, extraction efficiency and limit of quantification

Calibration curves were constructed for C-6-G with 7 final concentrations ranging from 1 to 25μg.ml^{-1}. Low, medium and high quality control (QC) samples were also prepared, with final concentrations of 4, 8 and 12μg.ml^{-1}. Calibration curve and QC samples containing C-6-G were prepared as 1 in 10 dilutions in blank urine and water (50μl standard / QC, 50μl blank urine and 400μl water) and varying volumes were injected on to HPLC system.

Unweighted linear regression analysis of peak areas of C-6-G against nominal concentrations provided an estimate of slope, intercept and coefficient of determination (r^2).

The method was validated by assaying 12 QC samples (4 each of low, medium and high concentration) and 4 standard samples at the limit of quantification on a single assay day to determine the intra-day inaccuracy and precision. Inter-day inaccuracy and precision were determined by analysis of 3 QC samples (one low, medium and high concentration) and one standard sample at the limit of quantification on four different assay days (Table 25).

The limit of quantification for C-6-G was 1μg.ml^{-1}.

An assay was accepted if the coefficient of variation (r^2) was greater than 0.990, and all standard and QC samples had inaccuracy in comparison to nominal values of less than 15%.

Table 25: Summary of Intra- and Inter-assay precision and inaccuracy validation for C-6-G in urine.

Nominal conc = nominal concentration, Cal conc = calculated concentration, CV = coefficient of variation, n = sample size.

	Nominal conc ($\mu g.ml^{-1}$)	Cal conc (mean ± SD)	% Inaccuracy	% CV	n
Intra-assay					
C-6-G	1.0	1.05 ± 0.01	4.91	0.94	4
	4.0	3.84 ± 0.03	-3.97	0.74	4
	8.0	7.62 ± 0.25	-4.74	3.34	4
	12.0	11.66 ± 0.19	-2.85	1.61	4
Inter-assay	(4 different assay days)				
C-6-G	1.0	1.02 ± 0.12	1.95	11.30	4
	4.0	3.85 ± 0.09	-3.86	2.39	4
	8.0	7.74 ± 0.05	-3.24	0.71	4
	12.0	11.40 ± 0.18	-5.00	1.59	4

5.2.5 Data Analysis

All raw data were transcribed to Microsoft® Excel (Version 5.0, Microsoft Corporation, MA, USA) and calibration curves of peak area ratio (morphine, NC, codeine, M-3-G and M-6-G) or peak area (C-6-G) were constructed by linear regression (Regression; Blackwell Scientific Software, Blackwell Scientific Publications, Oxford, UK). From the slope and intercept, concentrations of morphine, NC, codeine, M-3-G, M-6-G, and C-6-G were calculated, and accuracy of the standard curve and QC concentrations ascertained via comparison with nominal concentrations. Concentrations of morphine, NC, codeine, M-3-G, M-6-G, and C-6-G in subject urine were found similarly. Amounts of morphine, NC, codeine, M-3-G, M-6-G, and C-6-G recovered in urine (µmole) were calculated by multiplying concentration by urine volume, and then converted to percentage of the initial dose given (90mg = 226.49µmole). The ratios of the amount of codeine recovered

(μmole) to total morphine (morphine, M-3-G and M-6-G (μmole)), to norcodeine (μmole), and to C-6-G (μmole) were also calculated. All data are reported as mean ± SD.

5.3 Results

5.3.1 Amount of codeine, morphine, norcodeine, M-3-G, M-6-G and C-6-G recovered

Table 26 shows the amounts of codeine, morphine, norcodeine, M-3-G, M-6-G and C-6-G recovered in the urine of subjects over the 48hr period. The total percentage recovery of the 90mg dose was 73.5 ± 10.9%. Of this, up to 76%, 1.96%, 0.15%, 1.84%, and 0.68% was as C-6-G, norcodeine, morphine, M-3-G, and M-6-G, respectively, while up to 5.75% was unchanged codeine.

Table 26: Amount of codeine, norcodeine (NC), morphine, M-3-G, M-6-G and C-6-G recovered in the urine of four subjects following a 90mg codeine phosphate dose (226.5µmole free base).

	Subject #1 non-UM	Subject #2 non-UM	Subject #3 UM	Subject #4 UM
Codeine (µmole)	7.35	13.0	7.17	9.48
C-6-G (µmole)	116	154	157	172
NC (µmole)	4.45	3.11	2.76	3.88
Morphine (µmole)	0.26	0.09	0.33	NQ
M-3-G (µmole)	1.97	3.19	4.17	0.91
M-6-G	0.82	1.25	1.54	0.64
Total morphine (µmole)	3.04	4.53	6.04	1.55
Total recovery (µmole)	130	175	173	187
log DM MR	-2.85	-2.46	-2.66	-2.15

NQ = not quantifiable

Table 27: Amount of codeine, norcodeine (NC), morphine, M-3-G, M-6-G and C-6-G recovered in the urine of four subjects as percentages (%) of 90mg codeine phosphate dose (226.5µmole free base).

	Subject #1 non-UM	Subject #2 non-UM	Subject #3 UM	Subject #4 UM
Codeine	3.25	5.75	3.16	4.19
C-6-G	51.0	68.1	69.5	76.1
NC	1.96	1.37	1.22	1.71
Morphine	0.12	0.04	0.15	-
M-3-G	0.87	1.41	1.84	0.40
M-6-G	0.36	0.55	0.68	0.28
Total morphine	1.34	2.00	2.67	0.68
Total recovery	57.8	77.2	76.5	82.7

In terms of the amount of morphine produced, either as morphine or as M-3-G and M-6-G, subjects #1, and 2 who do not carry multiple copies of *CYP2D6* produced more than subject #4 who has been genotyped as *CYP2D6*2xN*, 3.04 and 4.53 versus 1.55µmole. Additionally, the amount of morphine recovered over 48hr from subject #4 was below the limit of quantification of the assay and remained so even after assaying undiluted urine, which was less than 0.01µg.ml^{-1} (15µmole) once urine volume had been accounted for. Conversely, subject #3 also genotyped as *CYP2D6*2xN* produced the highest amount of total morphine, 6.04µmole. There was no correlation between the amount of total morphine recovered and the log DM metabolic ratio (log DM MR).

The amount of norcodeine recovered over 48hr was unrelated to the occurrence of multiple copies of *CYP2D6*, as non-*CYP2D6*2xN* subjects (#1 and 2) had similar levels as *CYP2D6*2xN* subjects (#3 and 4). The amount of C-6-G recovered over 48hr was marginally greater in the urine of *CYP2D6*2xN* subjects (#3 and 4) when compared with the remaining subjects (#1 and 2), 157 and 172.34 versus 116 and 154µmole.

The activity of CYP2D6 was observed to be the highest in subject #3 and lowest in subject #4, codeine / total morphine urinary metabolic ratio of 1.19 versus 6.12 (Table 28).

Subject #2 and 1 showed the lowest and highest CYP3A activity, respectively, codeine / norcodeine urinary metabolic ratio of 4.19 versus 1.65 (Table 28). The highest level of glucuronidation was seen from subject #3, and the lowest from subject #1, codeine / C-6-G urinary metabolic ratio of 0.046 and 0.085, respectively (Table 28). Note that the urine pH of subjects #1 – 4 were not markedly different, ranging from 5.67 to 6.28. In contrast there was a difference in the urine volume, ranging from 2010 to 4300L (refer to appendix I).

Table 28: Urinary metabolic ratios of codeine to total morphine (morphine, M-3-G and M-6-G), norcodeine, and C-6-G in urine of four subjects following a 90mg codeine phosphate dose (226.5µmole free base).

	Subject #1	Subject #2	Subject #3	Subject #4
Codeine / total morphine (CYP2D6)	2.41	2.88	1.19	6.12
Codeine / norcodeine (CYP3A4)	1.65	4.19	2.60	2.44
Codeine / C-6-G (UGT2D2)	0.064	0.085	0.046	0.055

5.3.2 Adverse Effects

All subjects reported adverse effects following the 90mg codeine phosphate dose irrespective of their clearance of codeine to total morphine and genotype (Table 29). These effects are commonly experienced when an opioid is administered (Caswell, 1999).

Table 29: Self-reported adverse effects reported by subjects (n=4) of differing CYP2D6 genotypes

Subject	Codeine / Total morphine	CYP2D6 genotype	Self-reported adverse effects
#1	2.41	non-*CYP2D6*2xN*	light-headed, lack of concentration
#2	2.88	non-*CYP2D6*2xN*	stomach cramps, dry mouth, lack of concentration
#3	1.19	*CYP2D6*2xN*	light-headed, dry mouth
#4	6.12	*CYP2D6*2xN*	mild stomach cramps, headache, dry mouth

5.4 Discussion

No apparent differences between the UM and non-UM in the CYP2D6 mediated metabolism of codeine were observed. Interestingly, a slight increase in total morphine recovered was observed in one UM (subject #3), whilst the other UM (subject #4) had the lowest recovery of total morphine. The variation in total morphine recovered was also noted once the variable absorption of codeine was taken into account, as seen by the lowest ratio of 1.19 for subject #3 and the highest ratio of 6.12 for subject #4. The data from subject #4 is in contrast to the original hypothesis, that the recovery of total morphine would be higher than in a non-UM, and is also contradictory to the only other study of codeine metabolism in subjects with more than one copy of *CYP2D6* (Yue *et al.*, 1997). In addition, it was observed that even though the data from subject #3 agreed with the original hypothesis, this change was not as distinct as the increase in the O-demethylation pathway reported by (Yue *et al.*, 1997). These investigators reported that the O-demethylation pathway of codeine to morphine, morphine-6-glucuronide, morphine-3-glucuronide, and normorphine increased in UM, representing up to 15.3% of the total recovery of codeine, compared with 1.7 - 8.7% in EM and 0.34% in PM.

Several differences exist between the present pilot study and that of Yue and colleagues (Yue *et al.*, 1997). The dose of codeine phosphate was 90mg in this study compared with 25mg, and the period of urine collection was 48hr compared with 8hr. The larger dose was chosen following ethics approval previously granted for an analgesia study in healthy volunteers conducted in our laboratory. In more detail, codeine phosphate doses of 60, 90 and 120mg were approved, and it was decided that administration of 90mg in the present study would increase the total amount of morphine formed in comparison to the 60mg dose (allowing for ease of assay for morphine, M-3-G and M-6-G), and may also decrease the incidence of adverse effects experienced by the subjects in comparison to the 120mg dose. It is unlikely that the difference in codeine dose can explain the difference in total morphine recovered, for although there have been no reports concerning variation in

morphine formation from codeine due to dose, it has been shown previously that the percentage of a 30mg codeine dose recovered as morphine, M-3-G or M-6-G did not change significantly after chronic administration of the dose over 56hr, in CYP2D6 EM (n=8) (Chen *et al.*, 1991b). This indicates that in EM subjects, CYP2D6 mediated metabolism to morphine and its subsequent glucuronidation is unlikely to become saturated with a 90mg dose. Furthermore, additional studies of codeine disposition in CYP2D6 EM and PM have used oral codeine doses of 170mg (Eckhardt *et al.*, 1998) and 120mg (Caraco *et al.*, 1999).

It is not known how different the CYP2D6 metabolic capacities are between the UM subjects in this pilot study when compared to those in the study by (Yue *et al.*, 1997). The UM in the latter study were not genotyped, but phenotyped with debrisoquine and reported as UM if the debrisoquine metabolic ratio was less than or equal to 0.11, whilst the UM in this study were phenotyped with dextromethorphan, making comparison difficult. As discussed in chapter 4 (see page 161), a number of studies have been unable to successfully use phenotyping with either debrisoquine, dextromethorphan or sparteine to separate subjects with and without multiple copies of CYP2D6.

It remains possible that the UM in this study may also carry mutations of *CYP2D6*. In this situation, even though the subjects carry more than one copy of *CYP2D6*, the fact that a mutation exists on the other allele may cause the level of functional CYP2D6 to be equivalent to a CYP2D6 EM. Therefore, no change in the metabolism of codeine to morphine would be observed. This has been observed in a previous study of a Swedish population which identified one subject of genotype *CYP2D6*2xN/*4* with a debrisoquine MR of 0.54, versus the median of 38 subjects with genotype *CYP2D6*1/*1* which was 0.17 (Dahl *et al.*, 1995). It has also been shown in a study of German Caucasians which identified 4 subjects with genotype of either *CYP2D6*2x2/*4* or *CYP2D6*2x2/*6* with a mean log DM MR of -2.30, versus the mean of subjects with genotype *CYP2D6*1/*1* (n=62), which was -2.70 (Sachse *et al.*, 1997). Therefore, due to lack of CYP2D6 genotyping of subjects for identification of the most common mutations of *CYP2D6* which

lead to non-functional enzyme in the present study, it seems plausible that the lack of expected results may be explained by the presence of mutations which overall caused a loss of CYP2D6 activity.

Another possible explanation may be that an error occurred in the original *CYP2D6*2xN* genotyping (results reported in chapter 4), so that subjects #3 and 4 may not carry multiple copies of the gene. Retrospective genotyping analysis performed by Dr Griese (Dr Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart Germany) in an effort to confirm the genotype of subjects #3 and 4 led to conflicting results. These were that subject #3 had genotype *CYP2D6*1/*1*, whilst the genotype of subject #4 could not be confirmed due to unsuccessful PCR amplification from the genomic DNA. It should be noted that positive controls used in the present study were provided by Dr Griese and had been shown to be *CYP2D6*2xN* by analysis with restriction enzymes and Southern blotting. Therefore, even though positive controls were used in both assays to establish if PCR amplification had been successful, the genotyping method used to detect *CYP2D6*2xN* did not produce consistent results. In fact, it was observed in the present study that the PCR varied in success from assay to assay, so that on some occasions PCR needed to be repeated to gain a result. This could be due to the quality of the subject DNA, for if there is contamination of the sample with cellular RNA, PCR would not be successful. Additionally, contamination of any constituent of the PCR reaction can cause failure. Consequently, it can only be assumed that because the positive controls were successful on the day of assay of the DNA of both subjects #3 and 4, that the results of genotype *CYP2D6*2xN* are valid. Unfortunately, as discussed previously, the log DM MR can neither confirm nor discount this possibility due to the inability of this value to separate the two genotypic groups.

Other observations that the N-demethylation and glucuronide pathways did not change between UM and non-UM, with mean ratios of 2.92 versus 2.52, and 0.075 versus 0.051, respectively, were similar to reports of Yue and colleagues. They showed that for CYP2D6 UM, 6.9 and 79.4% of the codeine dose was recovered via the N-demethylation

and glucuronide pathways, respectively, versus that for CYP2D6 EM 6.8 and 82.4% (Yue *et al.*, 1997). These data were expected as CYP2D6 does not mediate the metabolism of codeine to these metabolites.

The fact that the morphine concentration for subject #4 was below the limit of quantification for the assay does not indicate that codeine metabolism to morphine did not occur, rather that all the morphine formed could have undergone glucuronidation. Other studies have previously reported similar observations, so that for some subjects no detectable morphine is found (Quiding *et al.*, 1986, Shah *et al.*, 1990).

The clinical implications for drug therapy for patients who carry multiple copies of the *CYP2D6* gene have been reported previously in anecdotal form (Bertilsson *et al.*, 1993). Usually a dose of drug is required that is several times above the normal therapeutic dose due to increased metabolism via the CYP2D6 pathway. For these patients, the clinical consequences of carrying multiple *CYP2D6* have not been widely studied, unlike the clinical consequences of patients who have no functional CYP2D6 due to mutations in *CYP2D6*. This pilot study of the metabolism of codeine was conducted to investigate if the metabolism of codeine via CYP2D6 would increase in subjects who carry multiple copies of the *CYP2D6* gene (CYP2D6 UM), in comparison to those who did not (CYP2D6 non-UM). The total recovery of the codeine dose in urine over 48 hours ranged from 58 to 83% which is similar to a previous report which stated that it ranges from 60 to 90% (Adler *et al.*, 1955). Codeine-6-glucuronide represented the highest percentage of the codeine dose (51 - 76), whilst clearance to norcodeine, morphine and morphine-3- and morphine-6-glucuronide represented minor pathways (up to 2%). This is in agreement with previous studies (Chen *et al.*, 1991b, Caraco *et al.*, 1999). The adverse effects reported are consistent with those experienced when codeine is administered with no difference observed between UM and non-UM.

In summary, several limitations of the present study, including the small numbers of participating subjects, genotyping assay problems and confounding influences of possible

CYP2D6 mutations which were not investigated, makes it difficult to assess the potential of CYP2D6 duplicated genes on the metabolism of codeine. As previous studies have shown, multiple copies of *CYP2D6* influences drug metabolism and therefore clinical implications do exist concerning both therapeutic failure and the possibility of patients experiencing adverse effects, especially with narrow therapeutic index drugs. Therefore, future research should consider these implications on a similar scale to that devoted to the study of the influence of *CYP2D6* mutations (that is poor metabolisers) on drug therapy. Additionally, future studies investigating the CYP2D6 metabolism of codeine and other drugs need to be conducted in other populations to determine if results are independent of racial background, especially considering the high prevalence of multiple *CYP2D6* in some populations (29% of Ethiopians, Aklillu *et al.*, 1996).

6. Summary

This thesis presents the results of studies in humans investigating aspects of the CYP2C19 and CYP2D6 genetic polymorphisms. The major findings of this study are divided into two groups, *in vitro* and *in vivo* observations.

Using human liver microsomes *in vitro*, the kinetics of the oxidative metabolism of flunitrazepam to 3'-hydroxyflunitrazepam and desmethylflunitrazepam were found to be sigmoidal, suggesting co-operative metabolism, and hence estimates of K_m and V_{max} for these reactions were best estimated by the Hill equation. It has been shown previously that the kinetics of the oxidative metabolism of diazepam (a structurally related benzodiazepine) are also sigmoidal, indicating similarity in CYP450 enzymes mediating flunitrazepam and diazepam metabolism.

Additionally, it was observed that the kinetic values were significantly altered in the presence of two different organic solvents, dimethylformamide (DMFO) and acetonitrile (ACN), used to dissolve flunitrazepam which is completely water insoluble. In agreement with previous studies on the effect of organic solvents on *in vitro* microsomal metabolism, I concluded that DMFO inhibited the oxidative metabolism of flunitrazepam, as the V_{max} values for both 3-OH-F and DMF formation in the presence of acetonitrile were higher. This highlights the need for researchers to be cautious when analysing *in vitro* oxidative metabolism data when the substrate is dissolved in an organic solvent. This also has an impact on assignment of CYP450 enzyme(s) mediating oxidative metabolism, as different CYP450 enzymes are inhibited to varying degrees by one particular organic solvent.

Investigation of which CYP450 enzymes mediate the oxidative metabolism of flunitrazepam to both 3-OH-F and DMF revealed that CYP2C19, CYP3A4 and possibly CYP1A2 are involved. Previous research has identified CYP2C19 and CYP3A4

involvement in the oxidative metabolism of diazepam, again leading to the conclusion that the oxidative metabolism of flunitrazepam *in vitro* is similar to that of diazepam. Future studies are required to investigate in more detail the involvement of CYP1A2 in both diazepam and flunitrazepam metabolism, as previous research of diazepam did not identify this CYP450 enzyme. It is suggested that the use of expressed CYP1A2 enzyme would address the involvement of CYP1A2 in the formation of both 3-OH-F and DMF conclusively. My research has identified another drug whose metabolism involves the polymorphic CYP2C19 enzyme.

Additional *in vitro* experiments studied the CYP450 enzymes mediating the oxidative metabolism of proguanil and (S)-mephenytoin. It was observed that CYP2C19 and CYP1A2 are involved in the metabolism of both substrates, and that CYP3A4 is also substantially involved in the formation of proguanil from cycloguanil. Therefore, although there are some similarities in the metabolism of proguanil and (S)-mephenytoin, the fact that CYP3A4 content can vary up to 60-fold between individuals indicates that proguanil may not be a suitable phenotyping probe for the CYP2C19 genetic polymorphism. This issue was addressed in the first *in vivo* study conducted.

A combined phenotyping (using proguanil as the probe drug) and genotyping study of CYP2C19 in a Caucasian and Asian population living in Australia, revealed that the prevalences of genotypic PM were 3.2 and 4.7%, respectively. The PM incidence in the Caucasian population was similar to that reported in another study of Caucasians living in Australia. However, the PM incidence in the Asian population was observed to be much lower than that previously reported. It was concluded that this is probably due to the low number of Asian subjects studied, and therefore the observation must be interpreted with caution.

Previously the PG/CG MR antimode used to separate CYP2C19 EM and PM had no statistical basis. In this study, three types of graphical analysis were used to determine the PG/CG MR antimode that separates genotypic EM from PM in the Caucasian population

studied. These analyses were histograms, probit plots, and normal test variable plots. It was noted that a statistical evaluation of the antimode in the Asian population was not possible due to the low number of genotypic PM identified. Using linear regression of the probit plot, an antimode of 8.61 was determined, which did not successfully separate the genotypic EM and PM groups, whilst the histogram and NTV plot did not add any further information in regard to the antimode. In addition, it was observed that following inclusion of other data from a previous study in our laboratory, and then repeat probit plot analysis, the antimode observed decreased to 8.55, indicating that the antimode is dependent on the number of subjects in a study.

Nonetheless, using the antimode of 8.61, two discrepancies were observed between the CYP2C19 genotype and PG phenotype. One CYP2C19 genotypic PM had a PG/CG MR of 5.61 and one CYP2C19 heterozygote genotypic EM had a PG/CG MR of 19.30. Although genotyping was not performed to identify all CYP2C19 mutations, it has been reported previously that *CYP2C19*2* and *CYP2C19*3* represent up to 85% of mutated alleles in the Caucasian population. However, the failure to test for the other CYP2C19 mutations does not explain why a CYP2C19 genotypic PM had a PG/CG MR of 5.61. Therefore, in agreement with the original hypothesis, it was concluded that factors other than CYP2C19 activity influence the PG/CG MR, for example, CYP3A4 activity in addition to variability in the renal clearance of PG.

This is the first study to report discrepancies between the phenotypic status assigned by the PG/CG MR and the genotypic status. Therefore, it was concluded that further studies are required, with larger subject numbers (including a larger number of PM), to investigate not only the ability of the PG/CG MR to separate CYP2C19 genotypic EM from PM, but also the appropriate antimode.

The second *in vivo* study investigated the prevalence of subjects carrying multiple copies of the *CYP2D6* gene in Caucasian and Asian populations living in Australia. It was observed that the incidence in the Caucasian population was 8.5%. However, on the basis

of the log DM MR these subjects could not be separated from the remaining Caucasian subjects. This observation must be considered in the light of the limitation that no genotyping was performed to identify CYP2D6 PM. Therefore, subjects identified as carrying multiple copies of *CYP2D6* (CYP2D6 UM) may also carry *CYP2D6* mutations so that overall, their CYP2D6 metabolic activity may be similar to genotypic EM.

The observation that log DM MR could not distinguish between CYP2D6 UM and EM agrees with previous studies in Caucasian populations, which have reported that none of the three commonly used CYP2D6 probe drugs (debrisoquine, sparteine and dextromethorphan) is able to successfully separate CYP2D6 UM from EM. The 8.5% incidence of CYP2D6 UM was much higher than that previously reported in other Caucasian populations (0.8 – 4%). However, due to the relatively small number of subjects in the Caucasian population studied (n=59), this may be artificially high.

It was also observed that no Asian subject carried multiple copies of CYP2D6. This is the first study investigating this phenomenon in Asians, but due to the low subject number (n=20) this conclusion must be accepted with caution, as subjects carrying multiple copies of CYP2D6 may be found in a larger sample size of this population. Another observation of interest is that non-UM Caucasian subjects had lower log DM/DR MR than the Asian subjects studied. This agrees with previous studies which have shown that a gene insertion in 17% of Asians, and the high prevalence of another *CYP2D6* mutation, *CYP2D6*10* (present in up to 50% of the Japanese population), leads to a decrease in the metabolic activity of CYP2D6.

The observation that the log DM MR significantly correlated with urine pH had not been reported previously by studies using dextromethorphan as a CYP2D6 phenotypic probe. It was concluded that this indicates that the renal clearance of dextromethorphan is variable due to dietary influences altering urine pH.

In summary, it was concluded that further investigation of the prevalence of subjects carrying multiple copies of the *CYP2D6* gene in a Caucasian population living in Australia and any Asian population with large numbers of subjects are needed to confirm the results of this study. Furthermore, the fact that the log DM MR was unable to separate the CYP2D6 UM from the remaining population suggests that, as with sparteine and debrisoquine, it is not adequate to identify these subjects with phenotyping, and that the only conclusive results can come from genotyping for *CYP2D6*2xN*.

The final *in vivo* study was a pilot study to investigate the difference in codeine metabolism in Caucasian subjects carrying multiple copies of *CYP2D6* (n=2) when compared with those without multiple copies of *CYP2D6* (n=2). No significant differences between the two groups of subjects in the metabolism of codeine to morphine (mediated by CYP2D6) and then to M-3-G and M-6-G were found. It must be stated however, that similarly to the study investigating the prevalence of *CYP2D6* multiple copies, no additional genotyping to identify the common mutations of *CYP2D6* was performed. Therefore, if the CYP2D6 UM subjects also carried a *CYP2D6* mutation, then their CYP2D6 mediated metabolism would not be different from the remaining subjects.

In addition, problems with confirmation of genotyping for *CYP2D6*2xN* were experienced; Dr Griese (Stuttgart, Germany) was unable to identify this allele in the genomic DNA of the subjects in this study. Due to the fact that identical positive controls were used in both laboratories, and were successful on the days that these subjects' DNA were genotyped in my laboratory, it was concluded that the results reported in chapter 4 would be accepted without confirmation. Furthermore, it was observed that the genotyping assay was not successful on 100% of assay days, which could be due to contamination of PCR constituents, or the poor quality of template DNA. This discrepancy between the genotyping results from the two laboratories and inability of the method to produce a result 100% of the time may indicate that there are underlying problems with the current *CYP2D6*2xN* genotyping method, which should be reviewed.

Considering the small number of subjects of my study and the problems experienced with the genotyping method, it was impossible to conclude whether or not there were differences in codeine metabolism in subjects carrying multiple copies of *CYP2D6*. Therefore, future research studying subjects with multiple copies of *CYP2D6* on a similar scale to that currently devoted to the study of effects of *CYP2D6* mutations (PM) on drug metabolism is required. Furthermore, this needs to be investigated in different ethnic populations due to the known variation in prevalence of CYP2D6 UM, since the impact on CYP2D6 drug metabolism would be greatest in populations where a large percentage of subjects are CYP2D6 UM.

Future research and significance of results

The results from the *in vitro* studies of the oxidative metabolism of (S)-mephenytoin, proguanil and flunitrazepam highlight the importance of identifying which CYP450 enzymes mediate the metabolism of a drug. This information is of great importance in the area of drug development, as it can be used to predict *in vivo* drug-drug interactions, and inter-individual variability and interethnic differences in the metabolism of a particular drug of interest. In addition, it must be noted that this prediction may change due to influences of organic solvents used for substrate dissolution. This is the first study to identify which CYP450 enzymes mediate the oxidative metabolism of flunitrazepam. The results are of clinical significance, as the involvement of CYP2C19 and CYP3A4 would explain inter-individual and interethnic variability in the metabolism of flunitrazepam which have not been studied, and allow prediction of possible drug-drug interactions between flunitrazepam and other CYP2C19 and CYP3A4 substrates.

The results from the*in vivo* study involving CYP2C19 genotyping and phenotyping showed that the PG/CG MR is influenced by factors other than CYP2C19 activity. Until these factors are elucidated further, genotyping to identify the common mutations of CYP2C19 can be the only conclusive method for identification of PM. In addition, future

studies should investigate the suitability of other drugs metabolised exclusively by CYP2C19 to use as a replacement phenotyping probe. Nonetheless, it remains of clinical relevance to identify CYP2C19 PM due to the increasing number of drugs metabolised by this enzyme (one of which I identified - flunitrazepam), and in the area of clinical trials concerned with drug development.

There were limitations to the *in vivo* study investigating the incidence of CYP2D6 UM which limited the conclusions of the study. These limitations include the small number of subjects, particularly in the Asian population, and the lack of complete genotyping. Therefore, future studies should ensure that genotyping to identify both multiple copies of *CYP2D6* and common *CYP2D6* mutations is performed to provide as complete as possible CYP2D6 genotype. In addition, studies with larger subject numbers are needed to further determine the incidence of CYP2D6 UM in Caucasian and Asian populations living in Australia. Based on the incidence of CYP2D6 UM metabolisers in previous Caucasian populations studied, I would suggest that > 500 subjects are required; however, more Asian subjects may be required following an initial study, if the incidence of UM is low. The identification of CYP2D6 UM can only be conclusively achieved by genotyping, as the phenotypic MRs are unable to separate UM from EM. In terms of clinical implications, it is of great importance to identify these subjects in order for therapeutic success to be achieved with the avoidance of toxicity (if metabolite is active).

The limitations in the pilot study of *in vivo* codeine metabolism were identical to those of the first CYP2D6 *in vivo* study. Therefore, it can not be concluded that the results of this study suggest there are no differences in the metabolism of codeine between CYP2D6 UM and EM. Future studies should include genotyping for the common mutations of *CYP2D6*, to provide complete genotype, and include a larger number of subjects in both UM and EM groups. These numbers can be predicted by calculating the power of the study based on differences in codeine/total morphine urinary metabolic ratio found in the study of Yue and colleagues. This could be best achieved through a multi-center collaborative trial, provided the subjects of either population are of known ethnicity. In addition, further

development of the CYP2D6 genotyping assay to identify multiple copies of *CYP2D6* is required, particularly addressing the reliability of results obtained.

The study of differences in CYP2D6 mediated drug metabolism in UM is of equal importance to the vast number of studies already conducted in PM. This presents a huge area of future researchin terms of studying one drug in different ethnic populations of CYP2D6 UM compared to EM.

In this thesis, I have reported findings of investigations of the influence of genetic polymorphisms on the CYP450 enzyme mediated oxidative metabolism of particular drugs. The conclusions drawn from this research allow clinical prediction of inter-individual and interethnic variability in the metabolism of drugs by the CYP2C19 and CYP2D6 enzymes, and the possibility of drug-drug interactions with other substrates of these enzymes. It has also highlighted future research to be conducted in this area, with the overall aim of improving clinical outcomes for patients.

Appendix A: Liver Patient Demographics

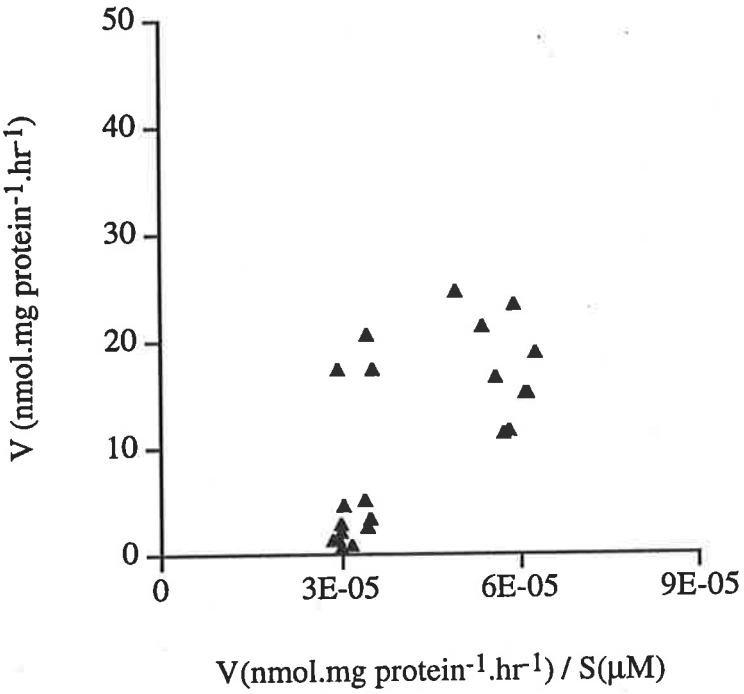
HLS #	Age	Sex	Alcohol (g/week)	Smoking (/day)	Caffeine (/day)	Liver Enzymes: Total P450	GGT	ALP	LD	AST	ALT	Medication: Regular / Premedication	Medication: During surgery
5	66	F	nil	nil	1 tea/1-4 coffee	233	14	64	192	25	-	nil / pethidine, metoclopramide	gentamicin, amoxycillin, metronidazole, ephedrine, neostigmine/atropine, bupivacaine, suxamethonium, vecuronium,fentanyl, thiopentone, lignocaine
15	51	M	< 10	nil	9 tea	157	51	447	207	43	122	cephalexin, "panadeine" / nil	thiopentone, fentanyl, atracurium, morphine, bupivacaine, gentamicin, metronidazole, amoxycillin
16	25	F	20	nil	2 coffee	300	-	-	-	-	-	diazepam, paracetamol, "mersyndol"(codeine, paracetamol, doxylamine) / temazepam	fentanyl, pancuronium, vecuronium, propofol
18	65	F	nil	nil	-	227	38	118	179	14	23	diazepam, thyroxine, temazepam	bupivacaine, adrenaline, morphine, thiopentone, atracurium, gentamicin, amoxycillin, fentanyl
21	70	M	< 10	nil	2 coffee	163	29	93	219	11	11	nil / temazepam, heparin	thiopentone, fentanyl, vecuronium, amoxycillin, gentamicin, metronidazole
22	54	F	< 10	nil	3 coffee	482	-	98	179	12	37	pyridoxine, microlax, metronidazole, cephalothin, gentamicin, hydrocortisone, promethozone / temazepam	thiopentone, fentanyl, atracurium, bupivacaine, adrenaline, fentanyl
23	62	M	300	nil	1 coffee	201	28	76	188	45	21	prednisolone / temazepam	thiopentone, fentanyl, vercuronium, morphine, hydrocortisone, amoxycillin, gentamicin, metronidazole
24	42	F	70	nil	3-4 tea / <1 coffee	192	32	101	187	15	21	temazepam	thiopentone, fentanyl, atracurium, morphine, amoxycillin, gentamicin, metronidazole
31	73	M	< 10	nil	-	169	21	105	-	-	10	frusemide / temazepam	fentanyl, streptomicin, atracurium, bupivacaine, gentamicin, amoxycillin, metronidazole

Normal range of liver enzymes: GGT 5-60U/L; ALP 30-95U/L; LD 110-230U/L; AST 13-45U/L; ALT 0-45U/L
Total P450 = total P450 content of microsomes (pmol P450.mg protein^{-1})

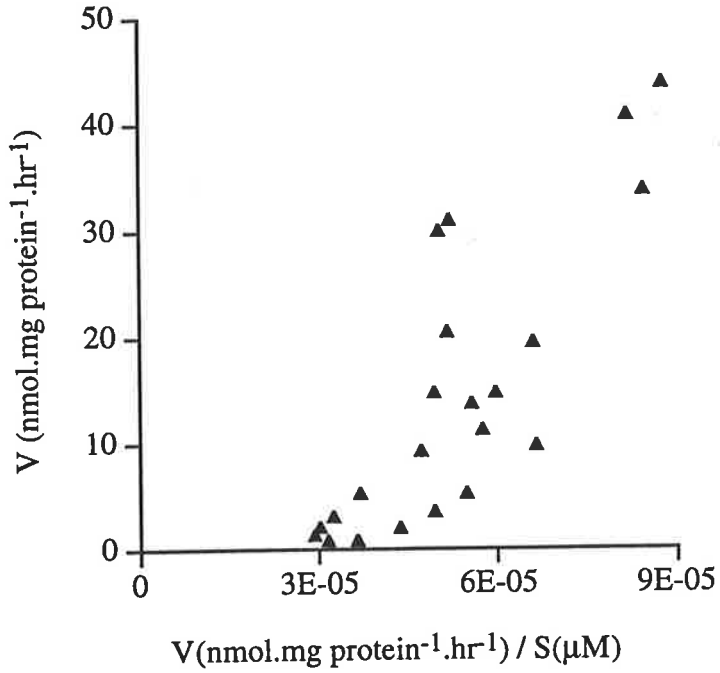
Appendix B

Eadie-Hofstee plots of 3-OH-F formation from flunitrazepam dissolved in DMFO in human liver microsomes.

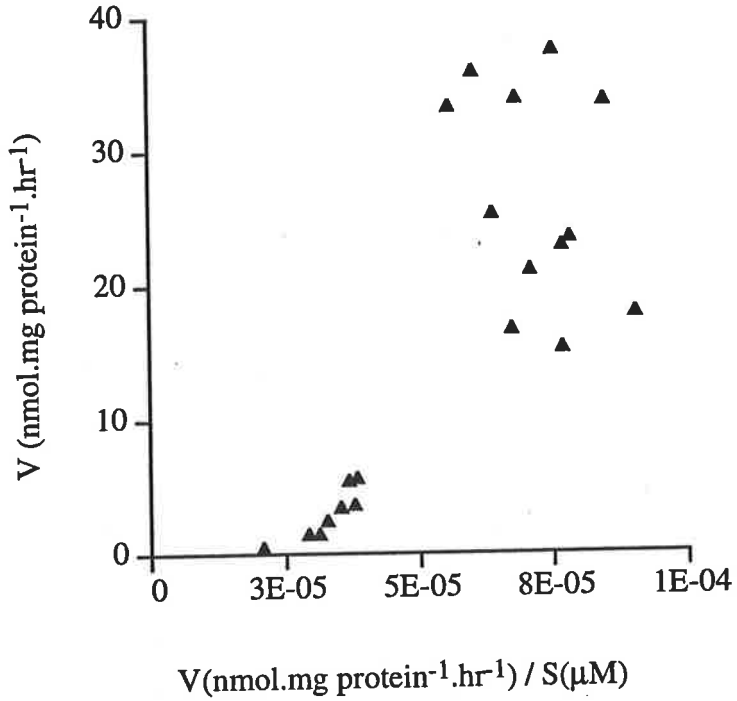
HLS #5



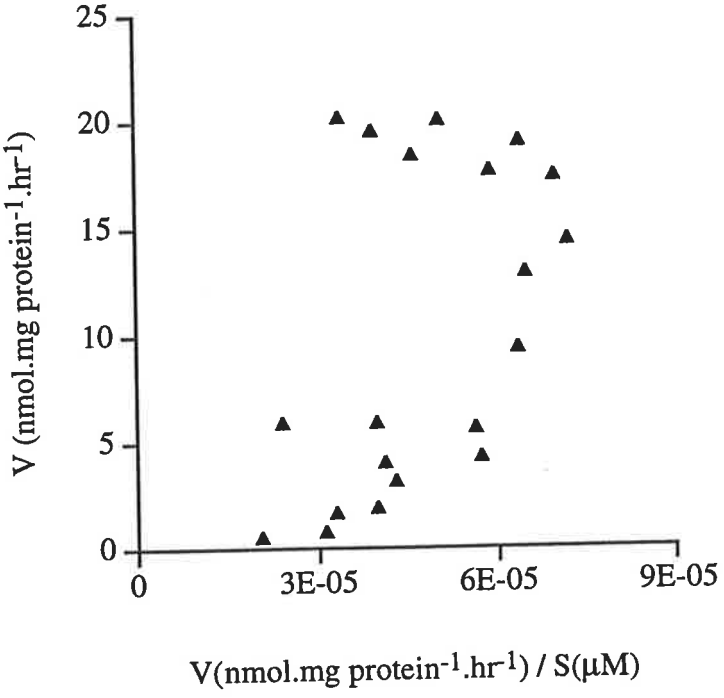
HLS #18

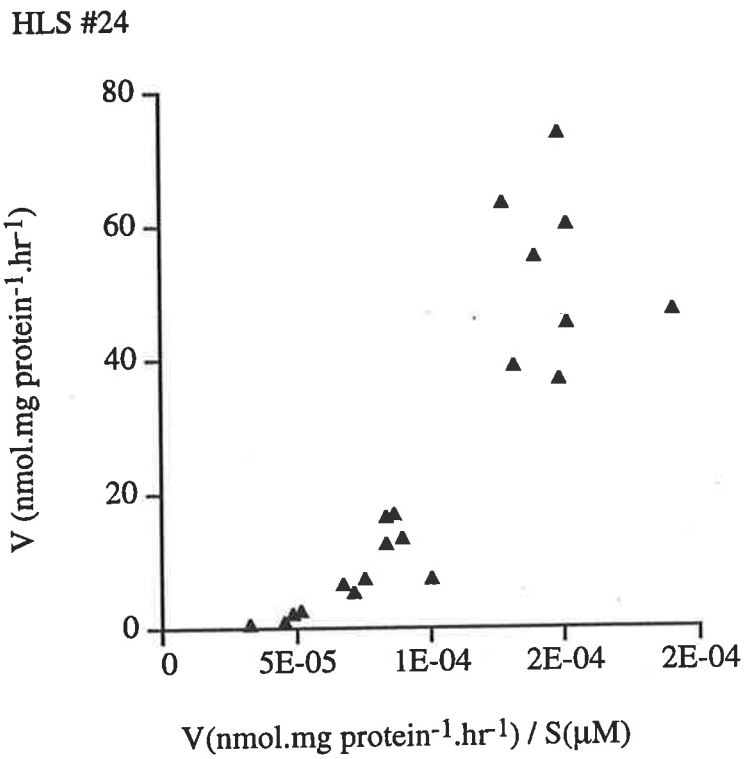


HLS #21

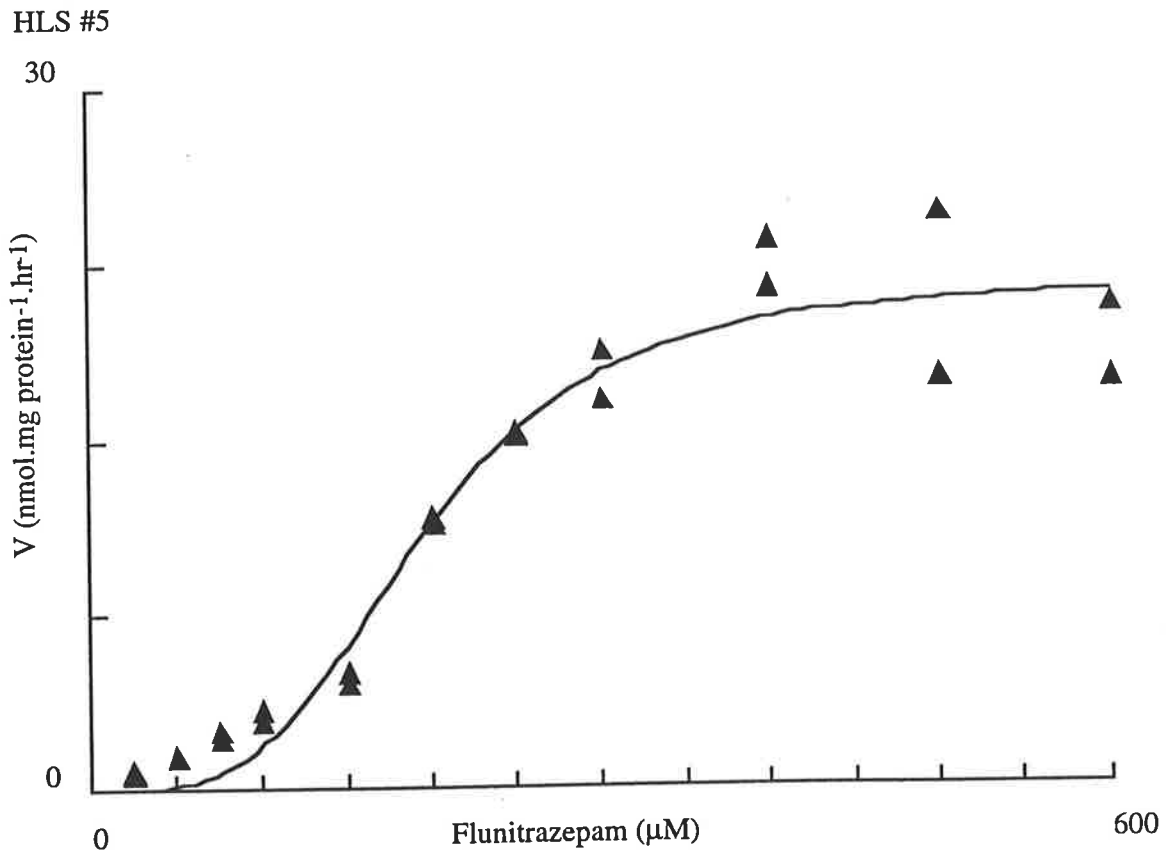

40
30
20
10
0
V (nmol.mg protein-1.hr-1)
0
3E-05
5E-05
8E-05
1E-04
V(nmol.mg protein-1.hr-1) / S(μM)

HLS #22

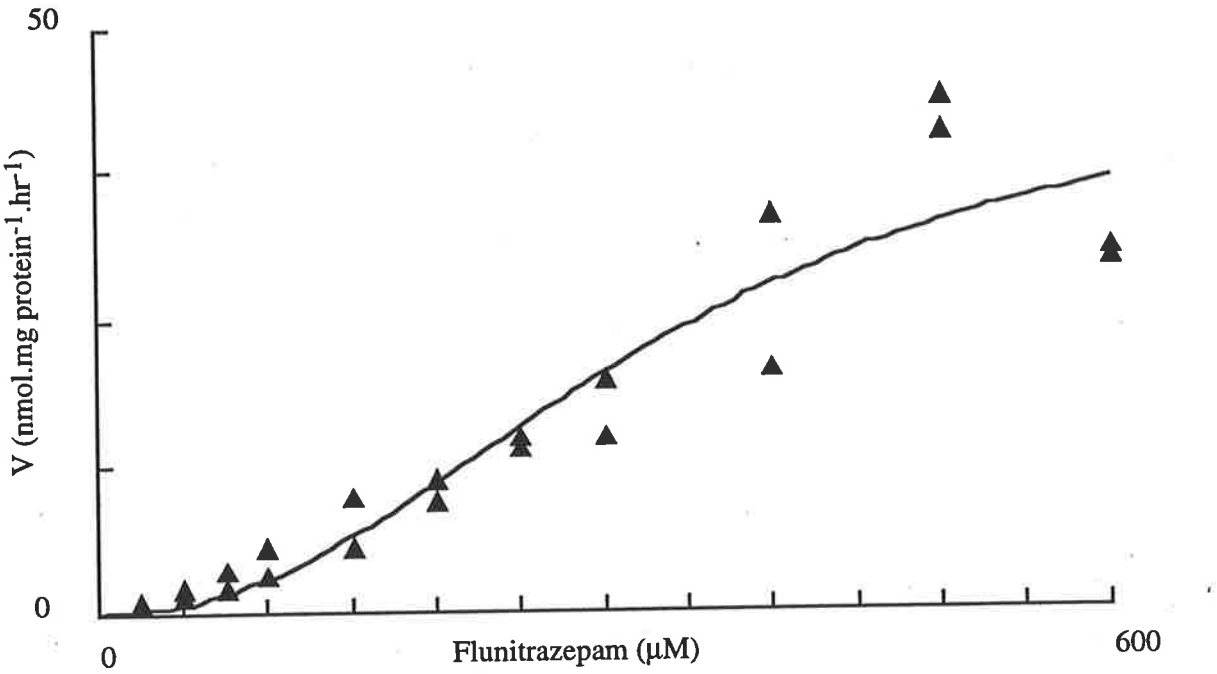

25
20
15
10
5
0
V (nmol.mg protein-1.hr-1)
0
3E-05
6E-05
9E-05
V(nmol.mg protein-1.hr-1) / S(μM)



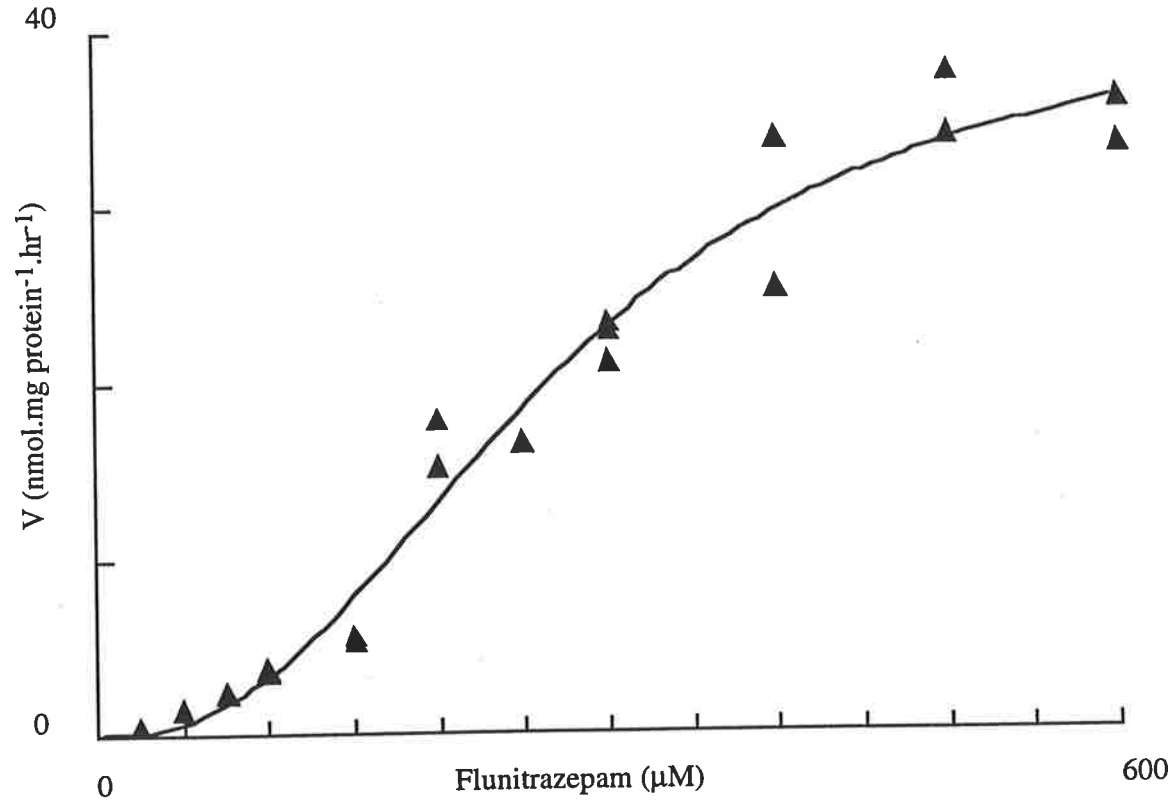
3-OH-F formation from flunitrazepam dissolved in DMFO in human liver microsomes, line of best fit from the Hill equation.



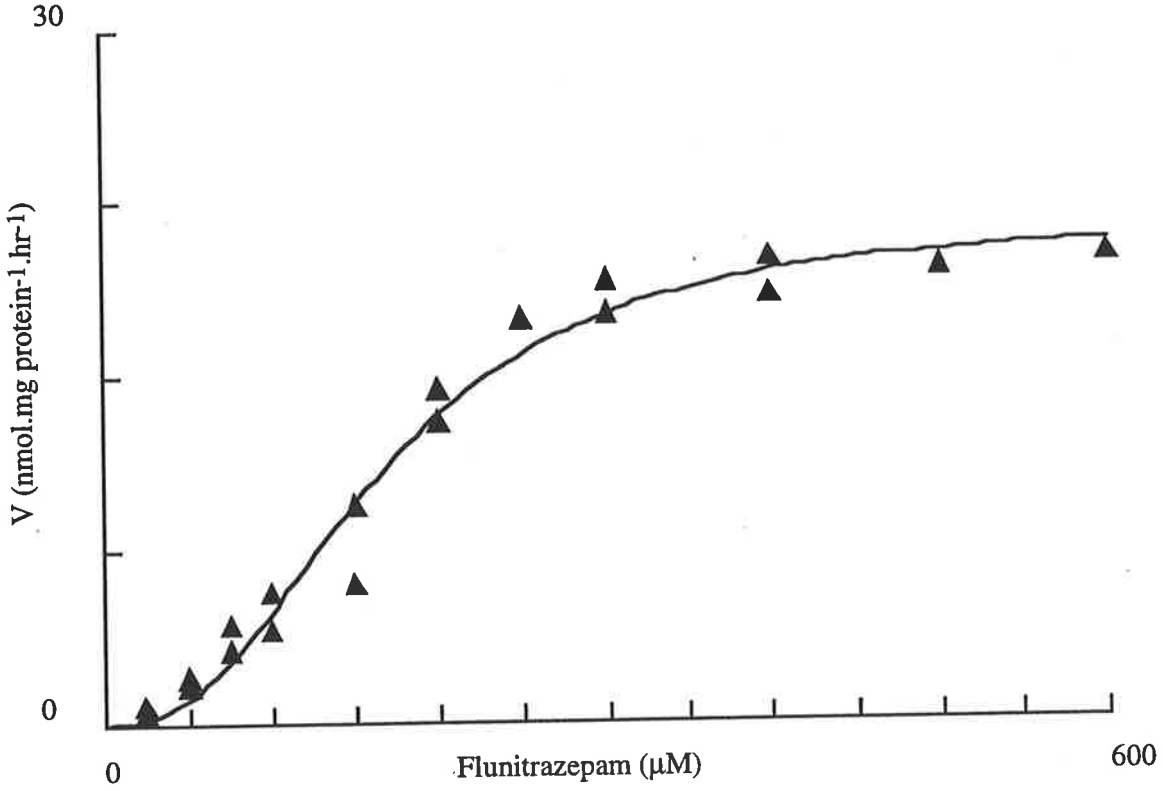
HLS #18


50
V (nmol.mg protein-1.hr-1)
0
0
Flunitrazepam (µM)
600

HLS #21


40
V (nmol.mg protein-1.hr-1)
0
0
Flunitrazepam (µM)
600

HLS #22


30
0
V (nmol.mg protein-1.hr-1)
0
Flunitrazepam (μM)
600

HLS #24

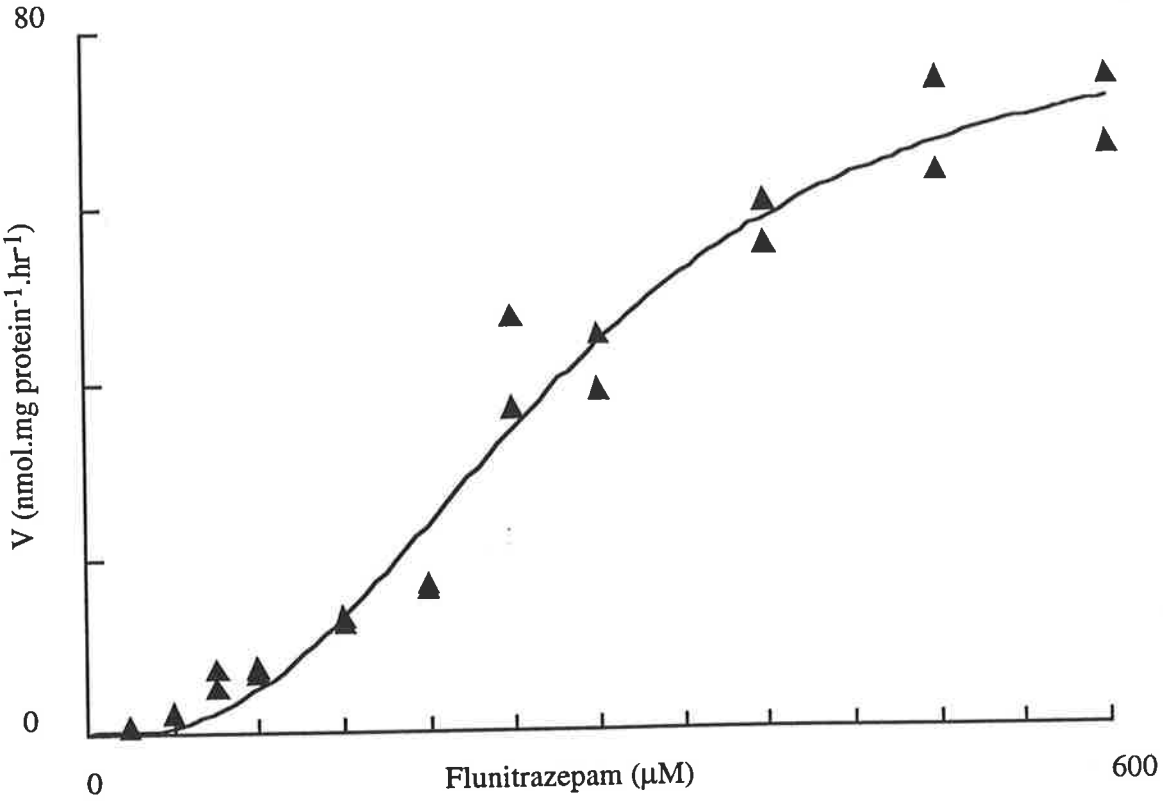

80
0
V (nmol.mg protein-1.hr-1)
0
Flunitrazepam (μM)
600

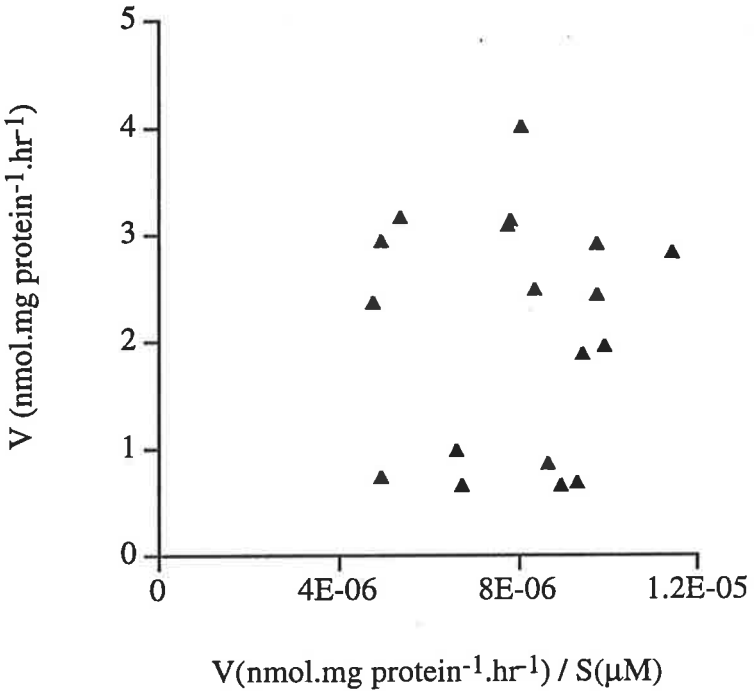
Table 30: Summary data to demonstrate fit of either the Hill equation or the single-enzyme Michaelis-Menten equation to the 3-OH-F formation data.

Hill Equation	HLS #5	HLS #18	HLS #21	HLS #22	HLS #24
Sum of squares	73	403	103	26	450
Standard deviation from equation	1.9	4.6	2.5	1.3	4.9
Determination coefficient	0.95	0.89	0.97	0.97	0.97
K_s (μM)	194	337	269	165	279
V_{max} (nmol.mg^{-1}.hr^{-1})	21.5	46.5	41	21.6	81.3
Single-enzyme Michaelis-Menten					
Sum of squares	199	508	226	87	986
Standard deviation from equation	3.2	5.0	3.6	2.3	7.0
Determination coefficient	0.87	0.87	0.93	0.91	0.93
K_m (μM)	750	1.3×10^{7}	4241	542	17077
V_{max} (nmol.mg^{-1}.hr^{-1})	52	7.9×10^{5}	310	43.9	2261

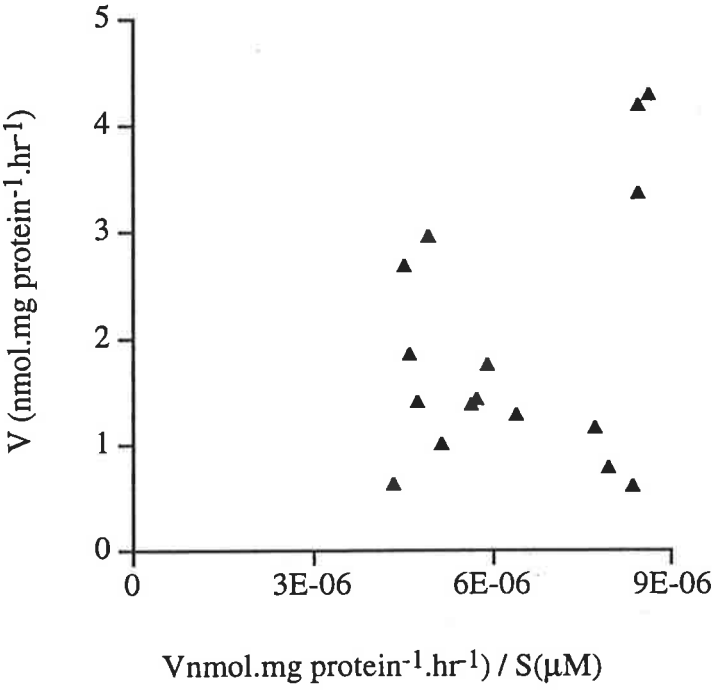
Appendix C

Eadie-Hofstee plots of DMF formation from flunitrazepam dissolved in DMFO in human liver microsomes.

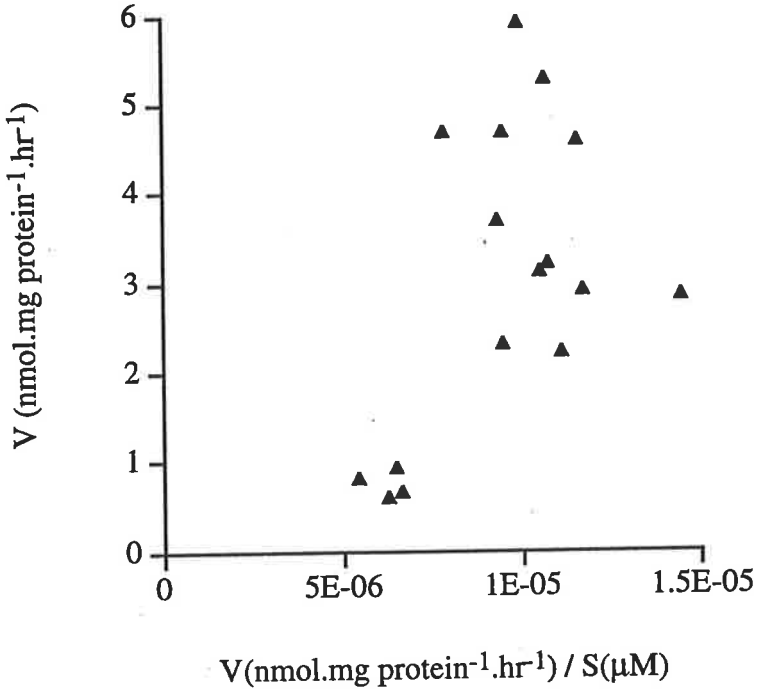
HLS #5



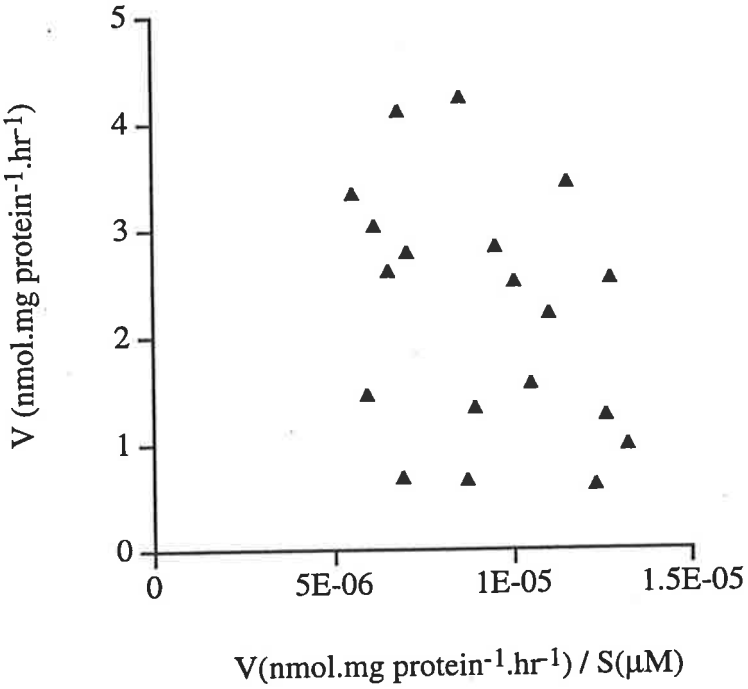
HLS #18

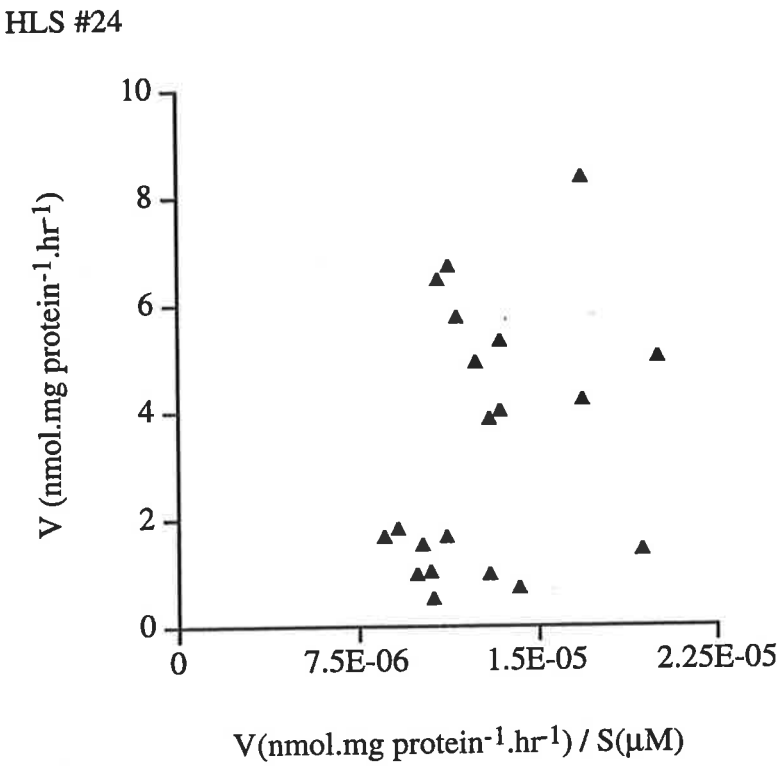


HLS #21

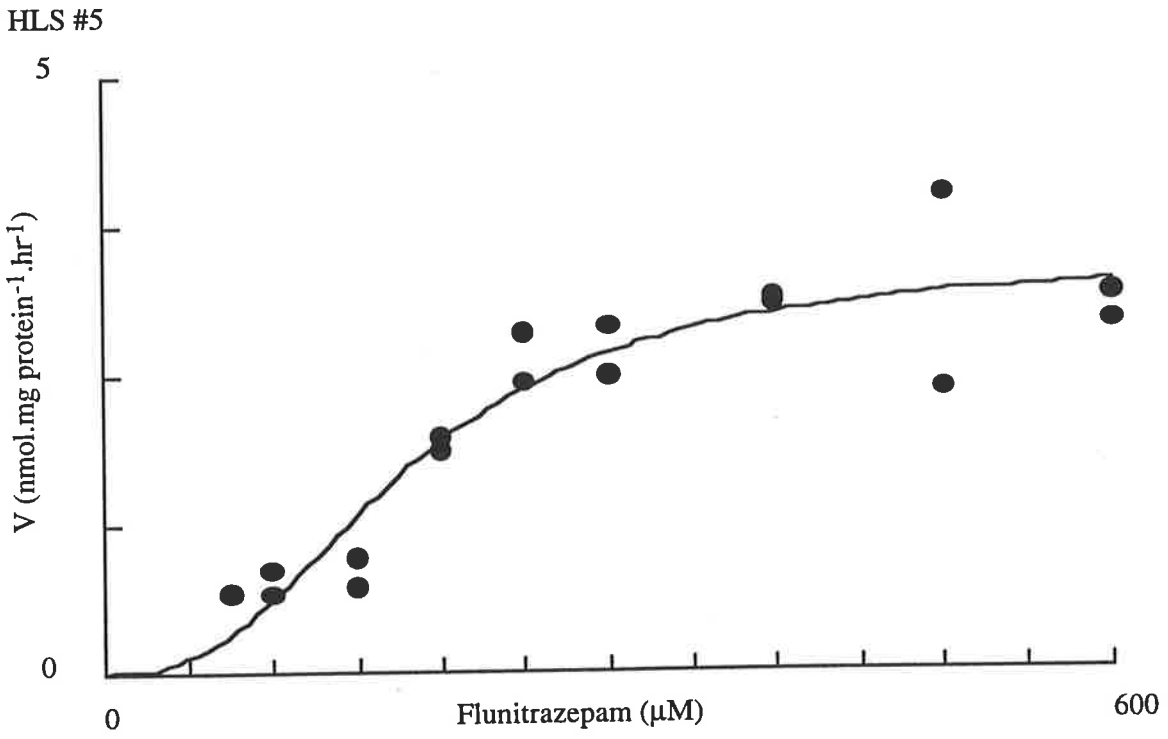

6
5
4
3
2
1
0
V (nmol.mg protein-1.hr-1)
0
5E-06
1E-05
1.5E-05
V(nmol.mg protein-1.hr-1) / S(μM)

HLS #22

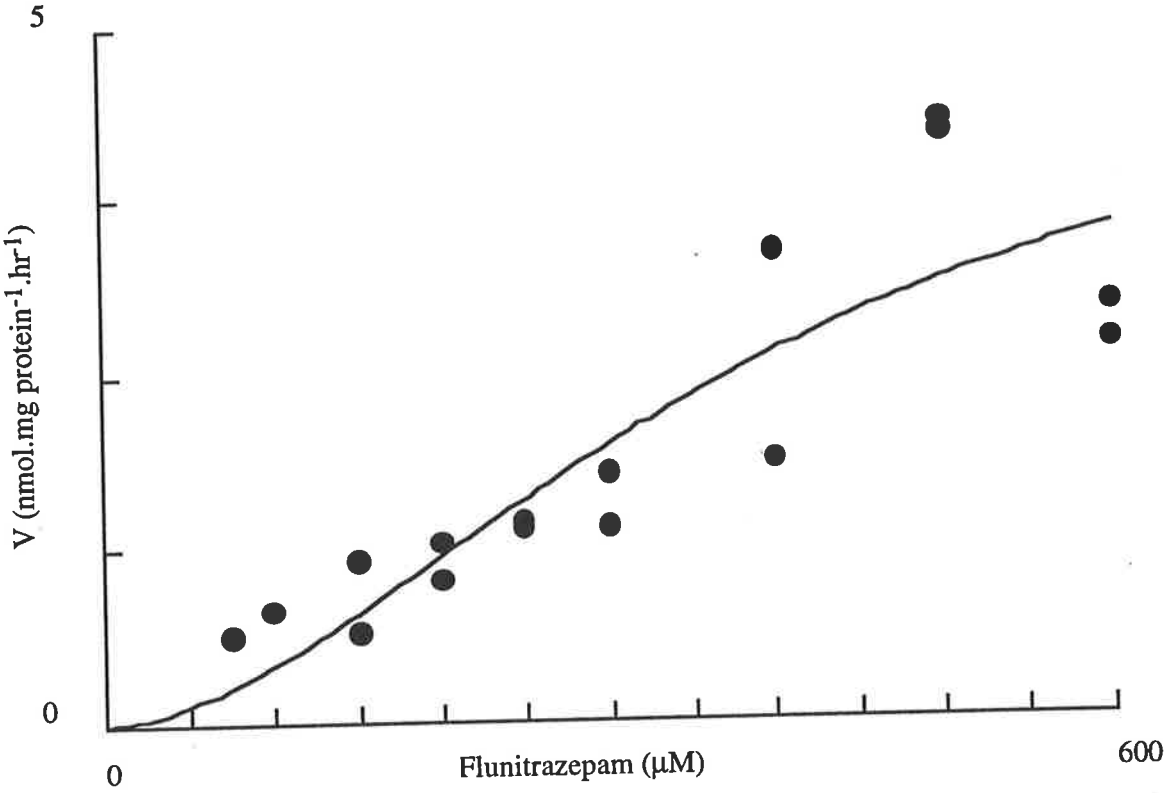

5
4
3
2
1
0
V (nmol.mg protein-1.hr-1)
0
5E-06
1E-05
1.5E-05
V(nmol.mg protein-1.hr-1) / S(μM)



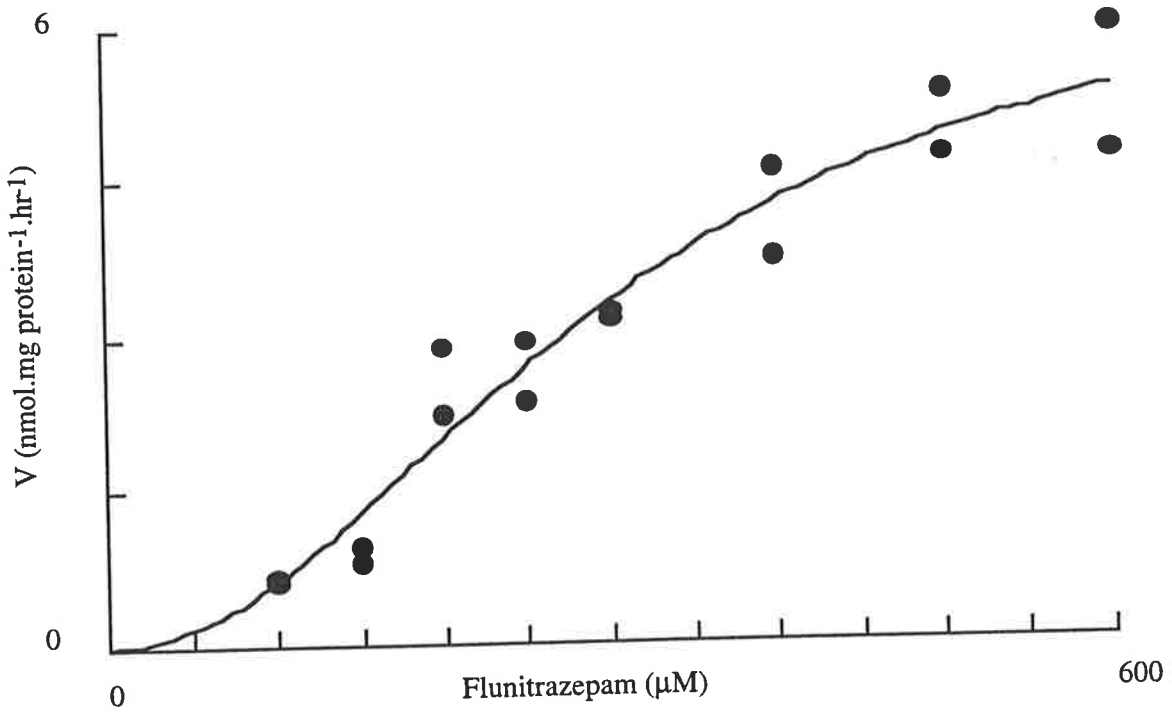
DMF formation from flunitrazepam dissolved in DMFO in human liver microsomes, line of best fit from the Hill equation.



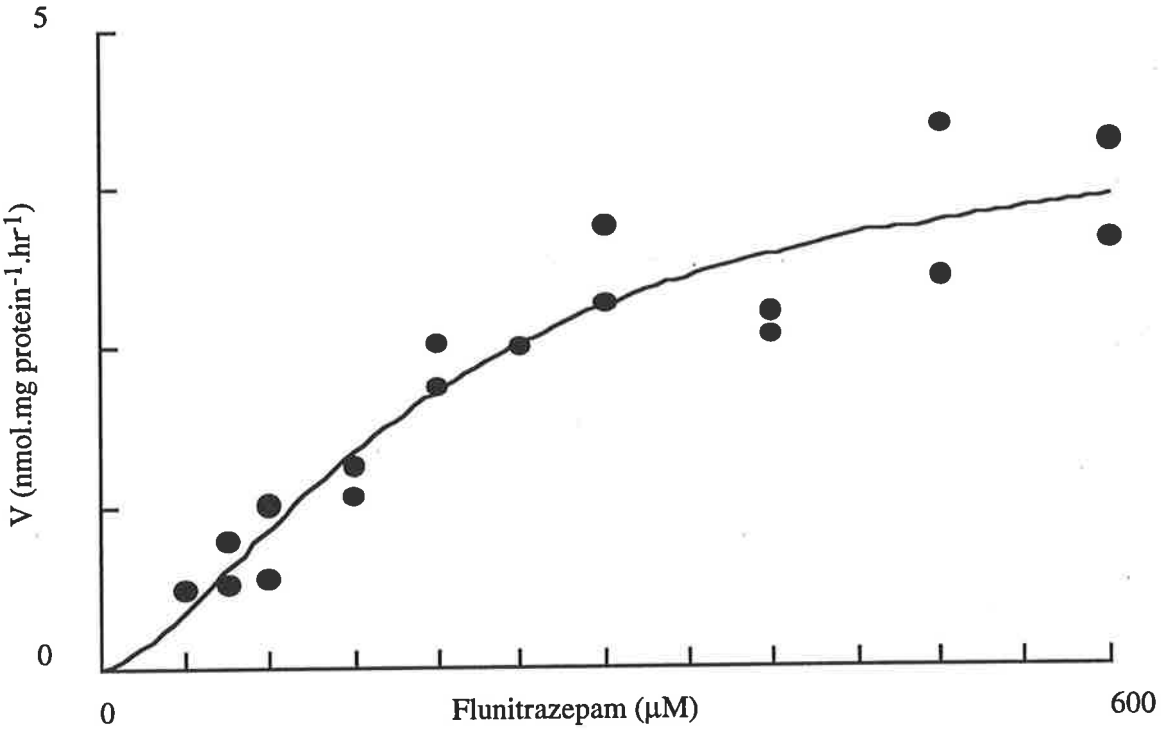
HLS #18


5
V (nmol.mg protein-1.hr-1)
0
0
Flunitrazepam (μM)
600

HLS #21


6
V (nmol.mg protein-1.hr-1)
0
0
Flunitrazepam (μM)
600

HLS #22


5
0
V (nmol.mg protein-1.hr-1)
0
Flunitrazepam (μM)
600

HLS #24

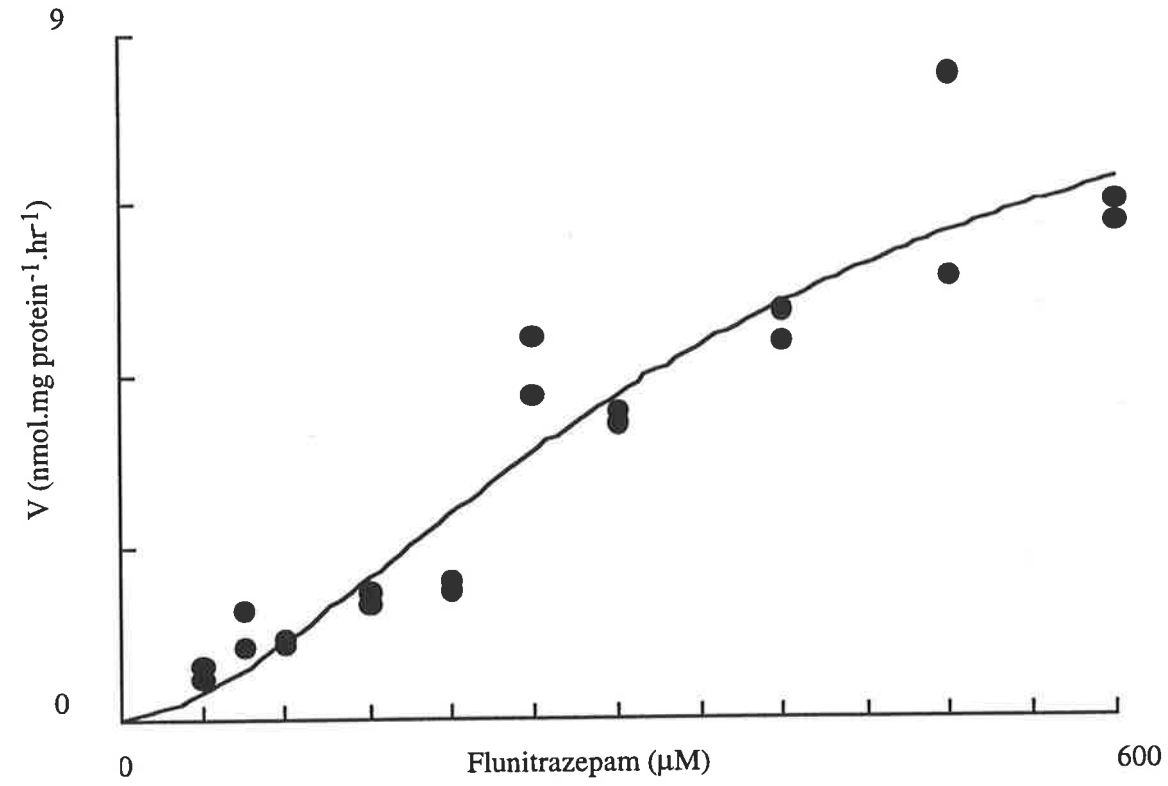
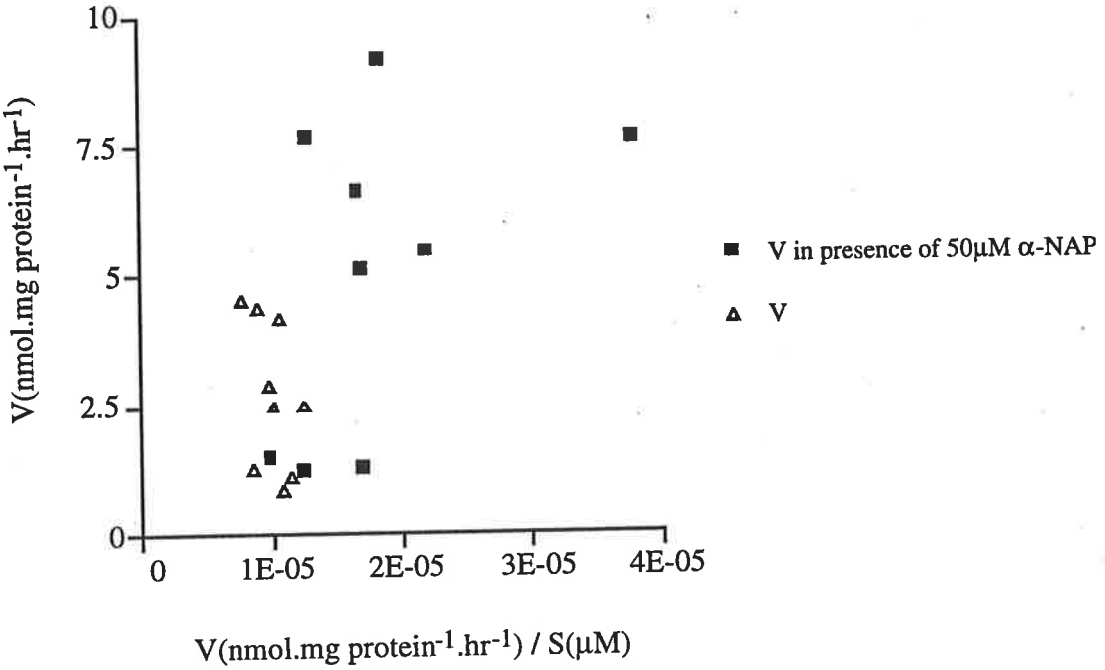

9
0
V (nmol.mg protein-1.hr-1)
0
Flunitrazepam (μM)
600

Table 31: Summary data to demonstrate fit of either the Hill equation or single-enzyme Michaelis-Menten equation to the DMF formation data.

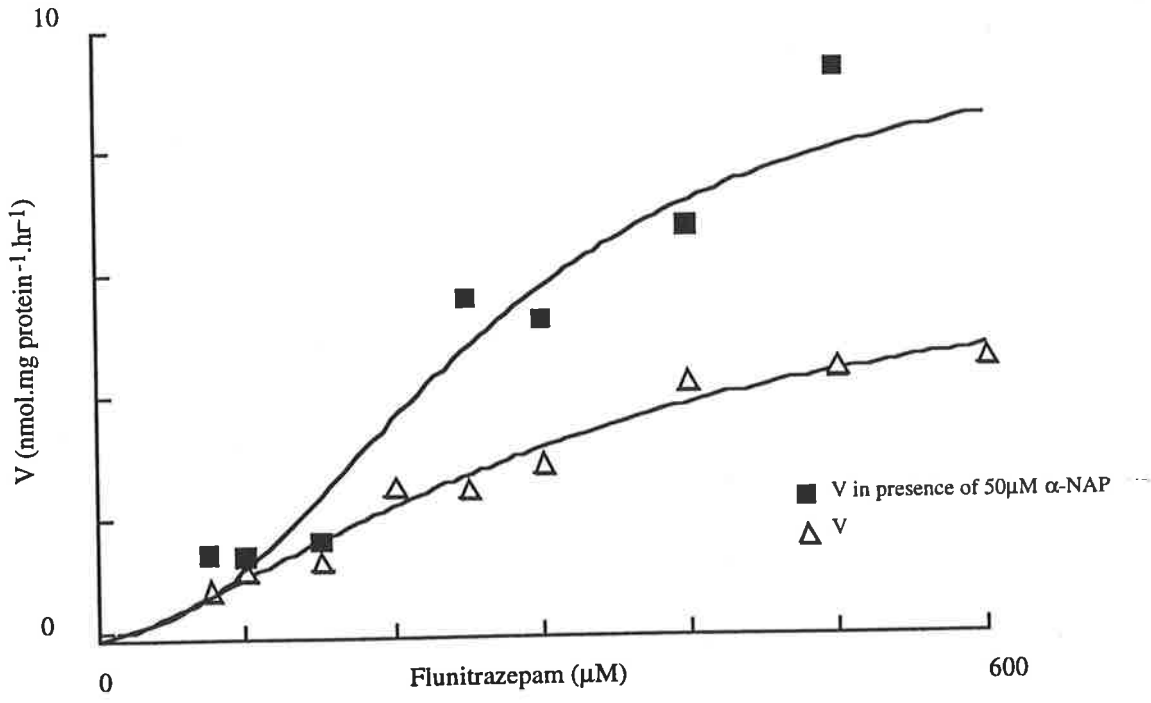
Hill	HLS #5	HLS #18	HLS #21	HLS #22	HLS #24
Sum of squares	2.5	5.5	2.7	2.6	10.9
Standard deviation from equation	0.41	0.65	0.46	0.41	0.80
Determination coefficient	0.87	0.75	0.94	0.89	0.90
K_s (μM)	179	391	297	199	365
V_{max} (nmol.mg^{-1}.hr^{-1})	3.4	5.2	6.5	4.3	10
Single-enzyme Michaelis-Menten					
Sum of squares	3.8	5.8	3.9	2.9	12.2
Standard deviation from equation	0.49	0.64	0.53	0.42	0.82
Determination coefficient	0.80	0.73	0.91	0.88	0.89
K_m (μM)	477	3972	2972	442	2772
V_{max} (nmol.mg^{-1}.hr^{-1})	6.2	27	33	6.6	41

Appendix D

Eadie-Hofstee plots of 3-OH-F formation from flunitrazepam dissolved in DMFO with and without 50μM α-naphthoflavone (α-NAP) in human liver microsomes (HLS #22).



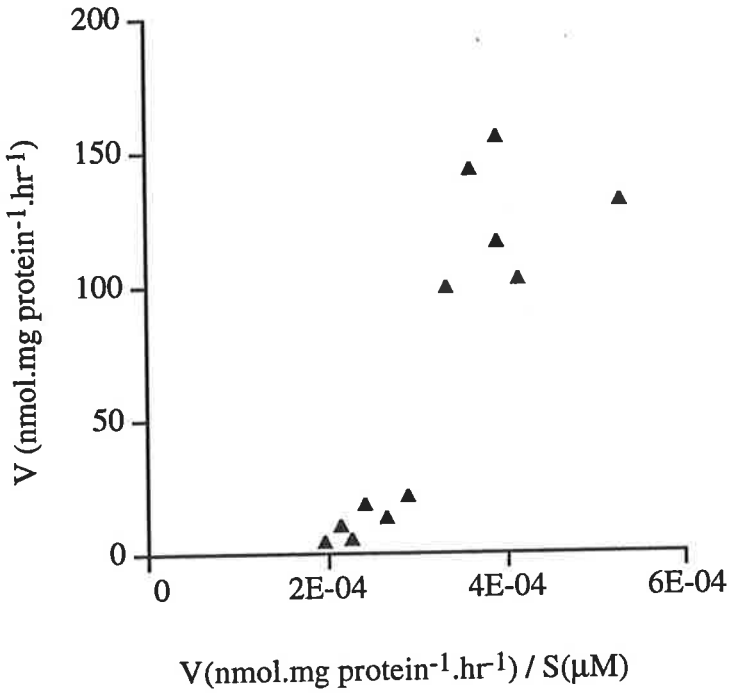
Formation of 3-OH-F from flunitrazepam dissolved in DMFO with and without 50μM α-naphthoflavone (α-NAP) in human liver microsomes (HLS #22), line of best fit from the Hill equation.



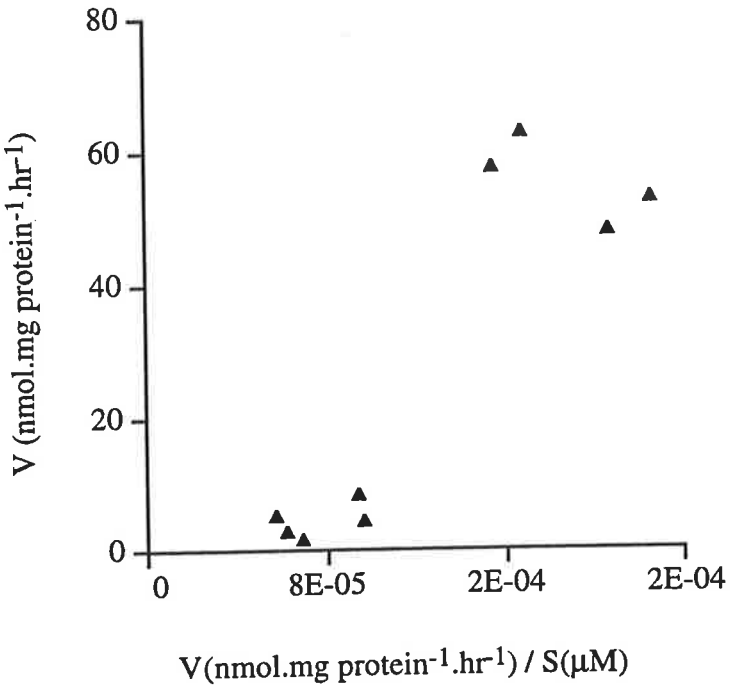
Appendix E

Eadie-Hofstee plots of 3-OH-F formation from flunitrazepam dissolved in ACN in human liver microsomes.

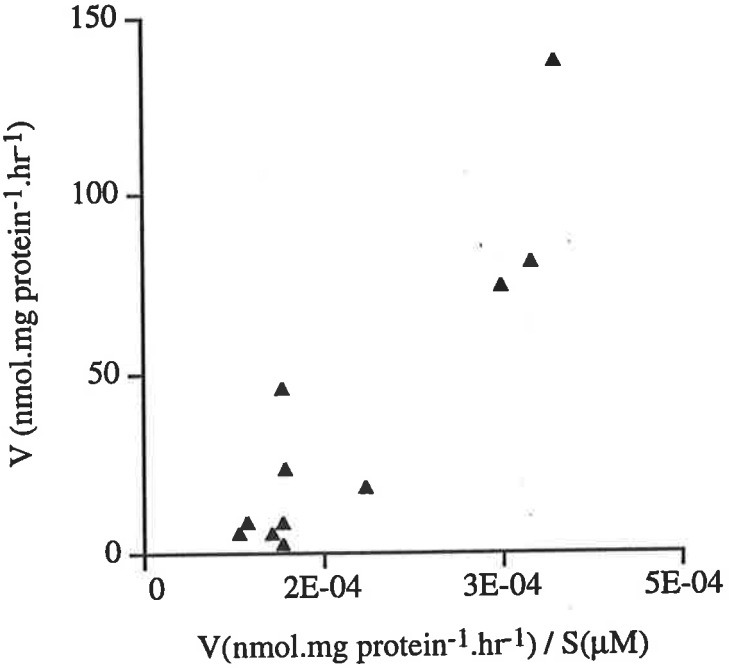
HLS #5



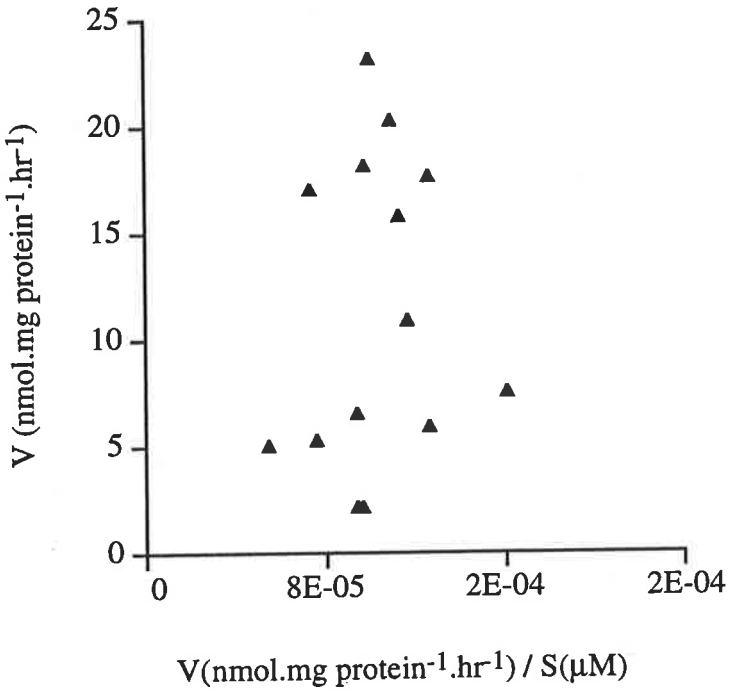
HLS #18



HLS #22

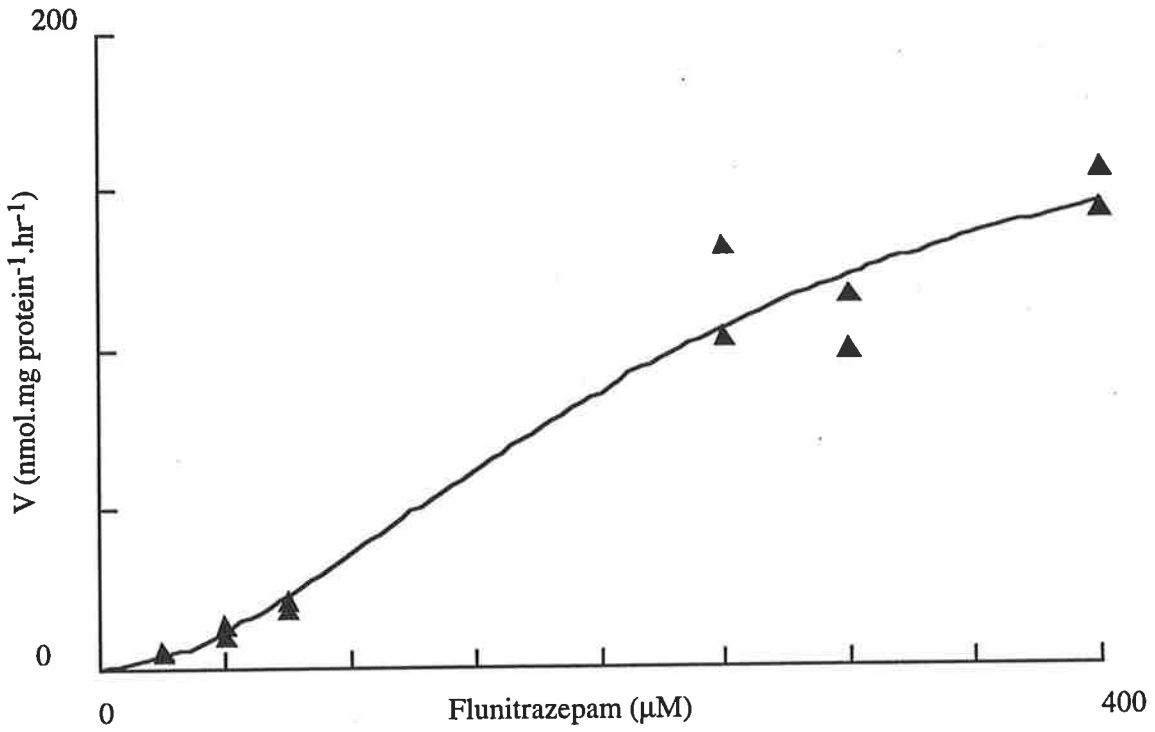

150
100
50
0
V (nmol.mg protein-1.hr-1)
0
2E-04
3E-04
5E-04
V(nmol.mg protein-1.hr-1) / S(µM)

HLS #31

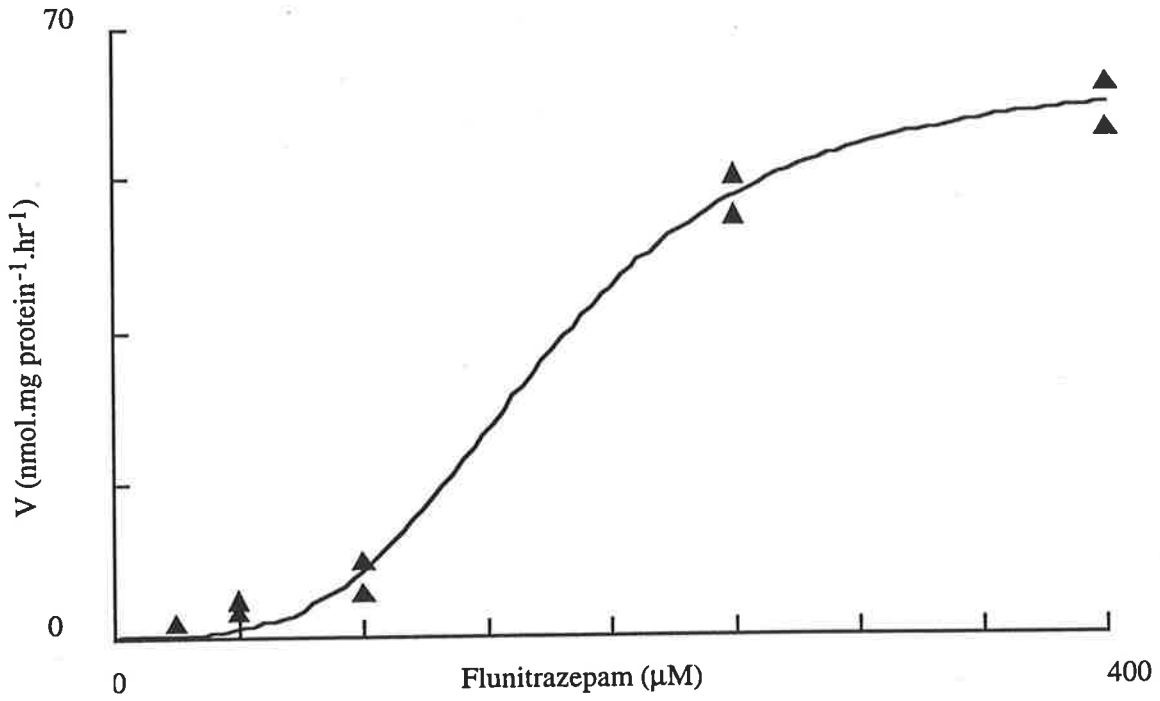

25
20
15
10
5
0
V (nmol.mg protein-1.hr-1)
0
8E-05
2E-04
2E-04
V(nmol.mg protein-1.hr-1) / S(µM)

3-OH-F formation from flunitrazepam dissolved in ACN in human liver microsomes, line of best fit from the Hill equation.

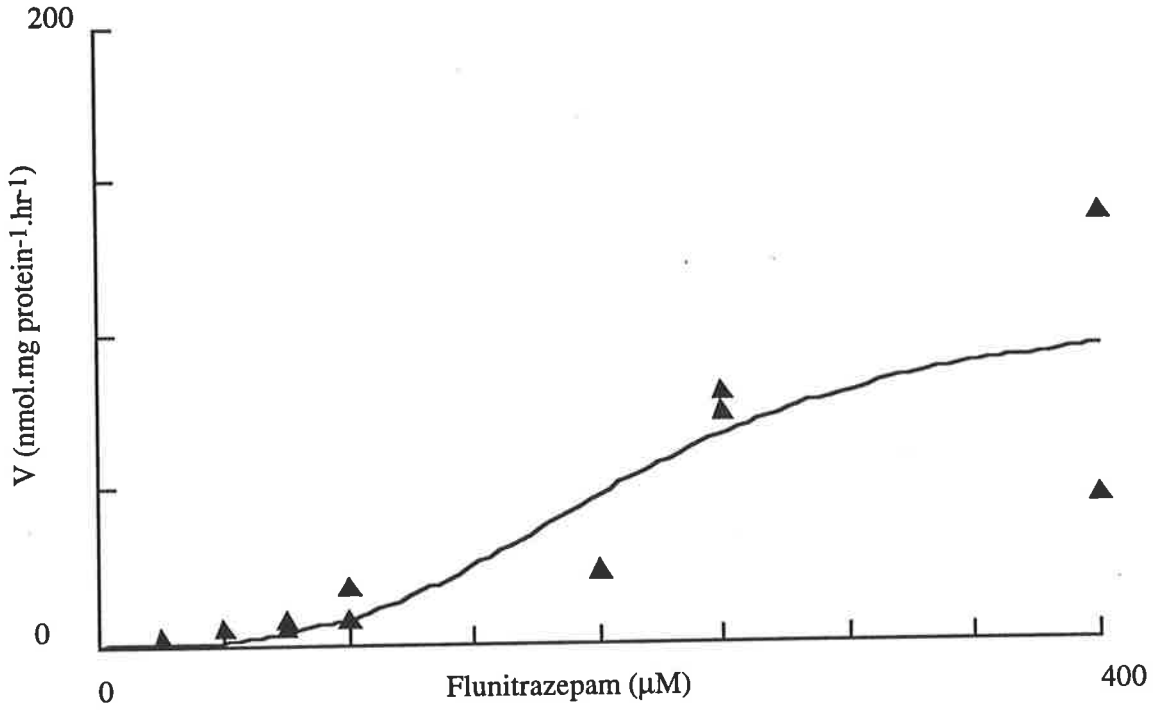
HLS #5



HLS #18



HLS #22


200
V (nmol.mg protein-1.hr-1)
0
0
Flunitrazepam (μM)
400

HLS #31

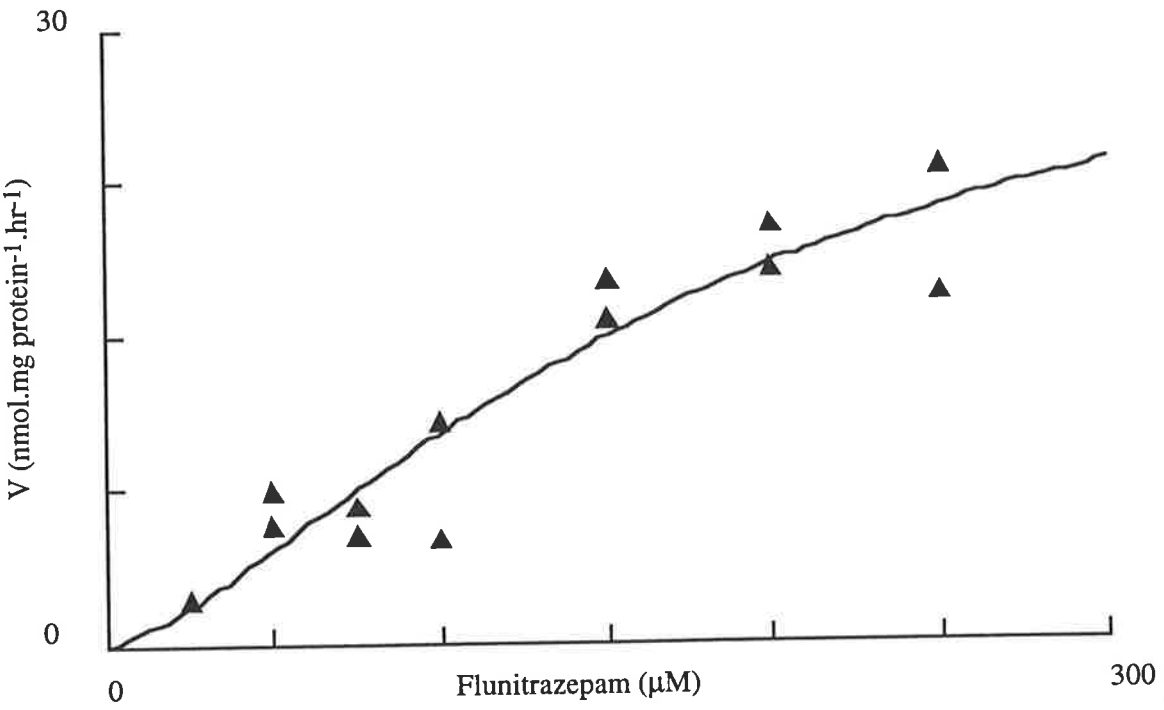

30
V (nmol.mg protein-1.hr-1)
0
0
Flunitrazepam (μM)
300

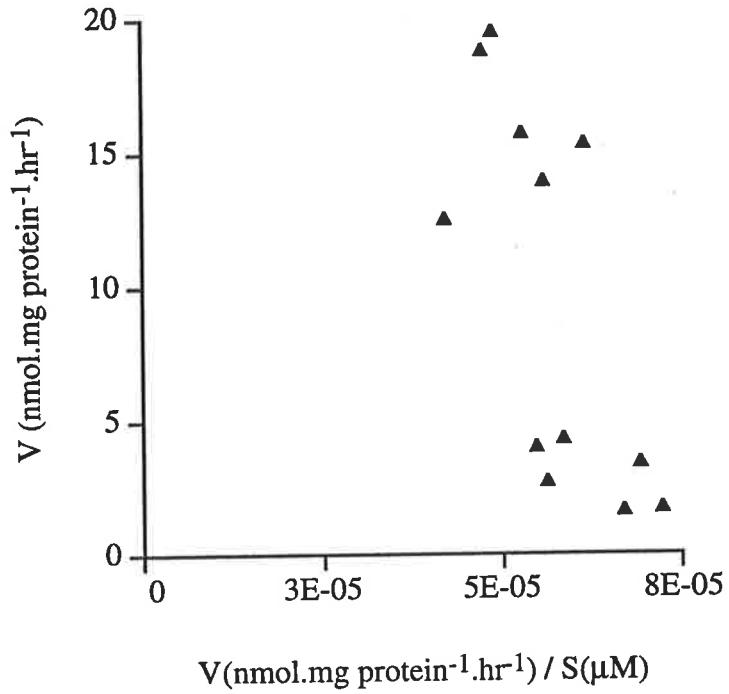
Table 32: Summary data to demonstrate fit of either the Hill equation or single-enzyme Michaelis-Menten equation to the 3-OH-F formation data.

Hill	HLS #5	HLS #18	HLS #22	HLS #31
Sum of squares	1357	5.8	5246	76.2
Standard deviation from equation	12.3	0.98	25.6	2.63
Determination coefficient	0.97	0.95	0.72	0.89
K_s (μM)	231	152	215	183
V_{max} (nmol.mg^{-1}.hr^{-1})	198	10	107	34
Single-enzyme Michaelis-Menten				
Sum of squares	1977	11.4	6056	80.3
Standard deviation from equation	14.1	1.27	25.9	2.58
Determination coefficient	0.95	0.91	0.68	0.88
K_m (μM)	4786	977	$1.5x10^8$	789
V_{max} (nmol.mg^{-1}.hr^{-1})	1973	34	$3.5x10^7$	89

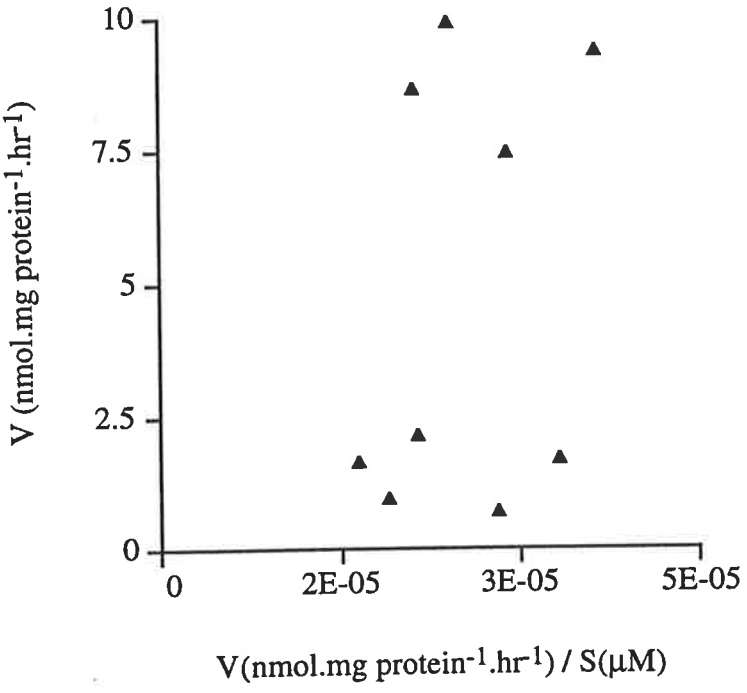
Appendix F

Eadie-Hofstee plots of DMF formation from flunitrazepam dissolved in ACN in human liver microsomes.

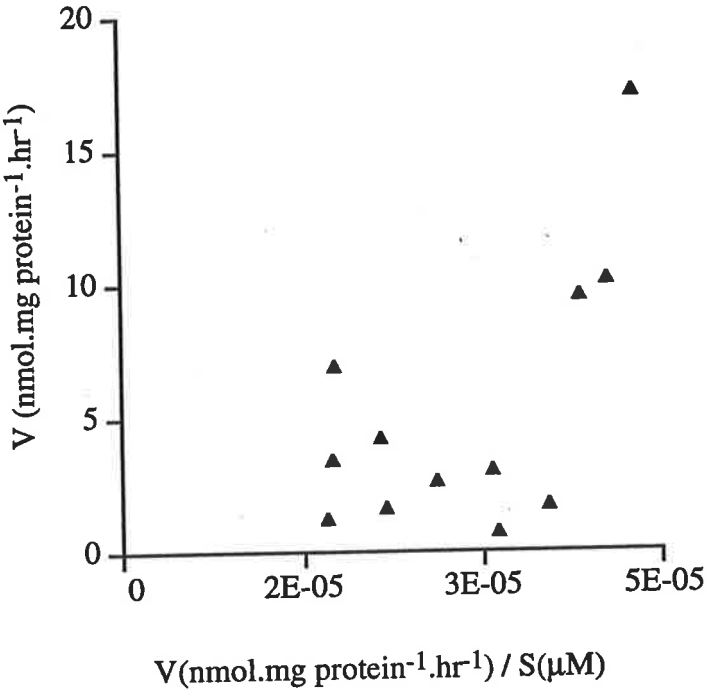
HLS #5



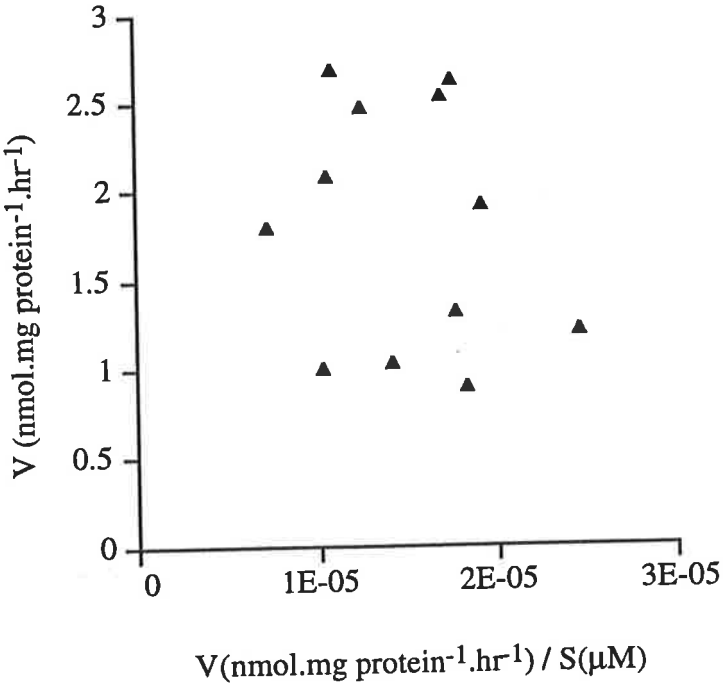
HLS #18



HLS #22

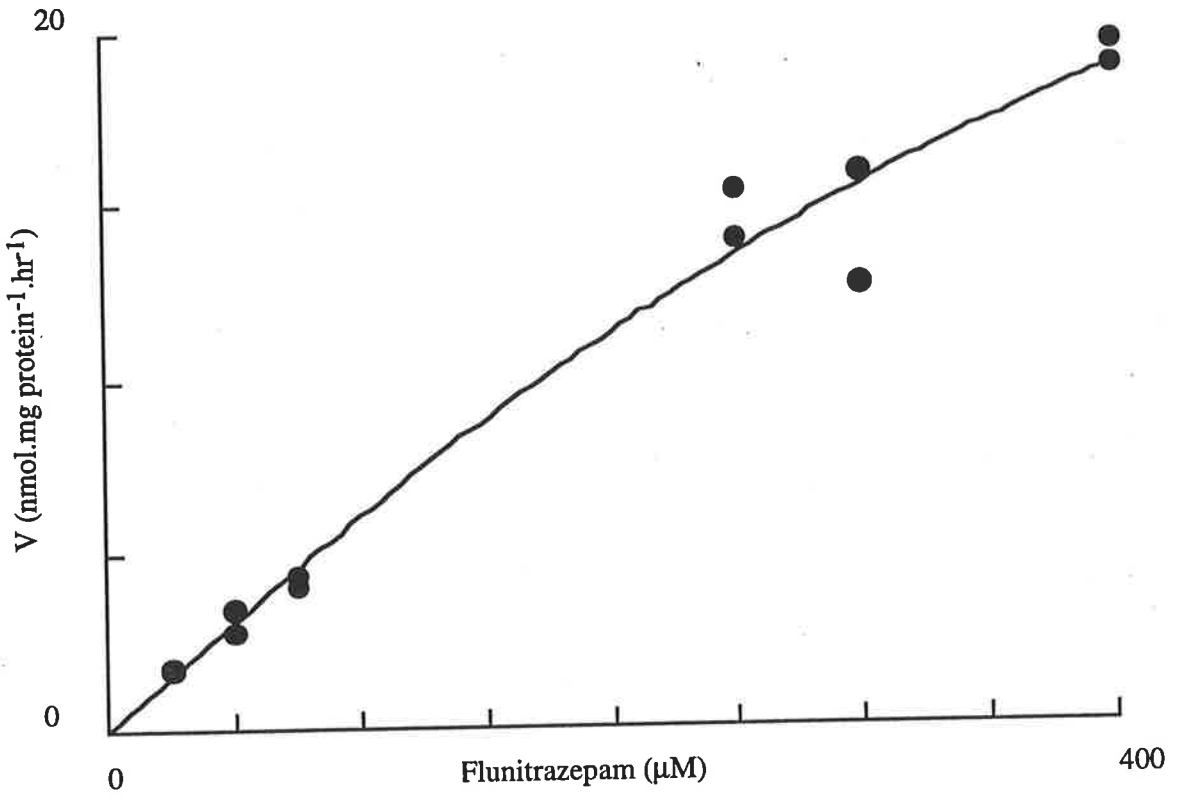

20
15
10
5
0
V (nmol.mg protein-1.hr-1)
0
2E-05
3E-05
5E-05
V(nmol.mg protein-1.hr-1) / S(µM)

HLS #31

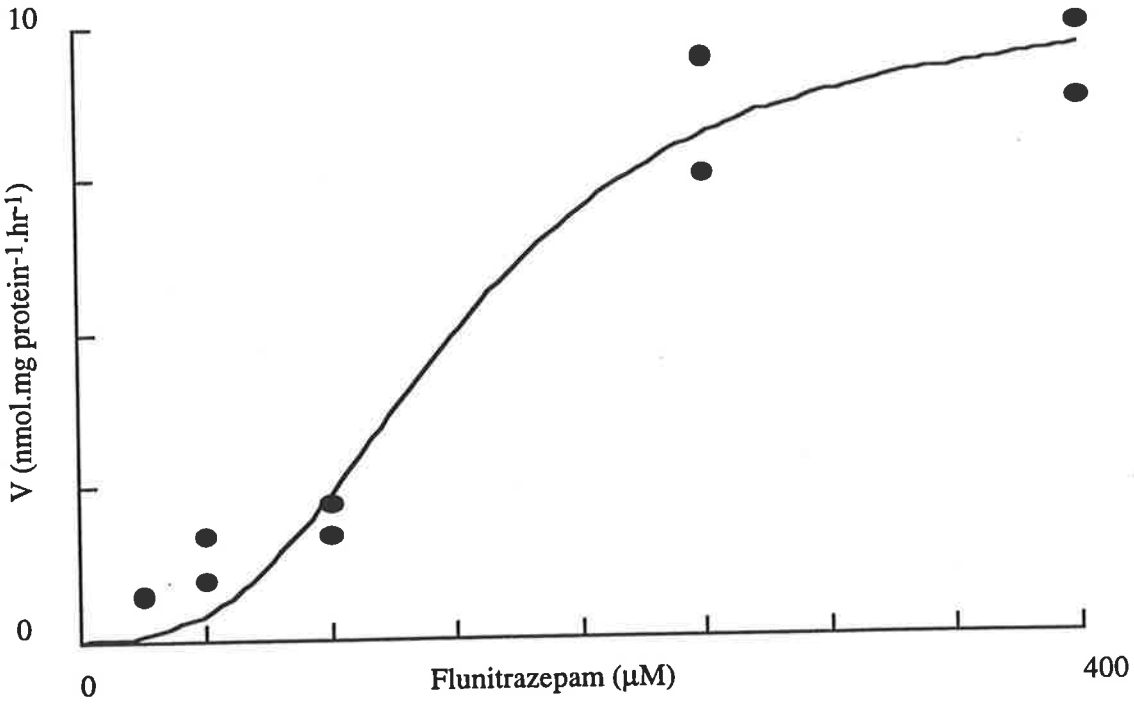

3
2.5
2
1.5
1
0.5
0
V (nmol.mg protein-1.hr-1)
0
1E-05
2E-05
3E-05
V(nmol.mg protein-1.hr-1) / S(µM)

DMF formation from flunitrazepam dissolved in ACN in human liver microsomes, line of best fit from the Hill equation.

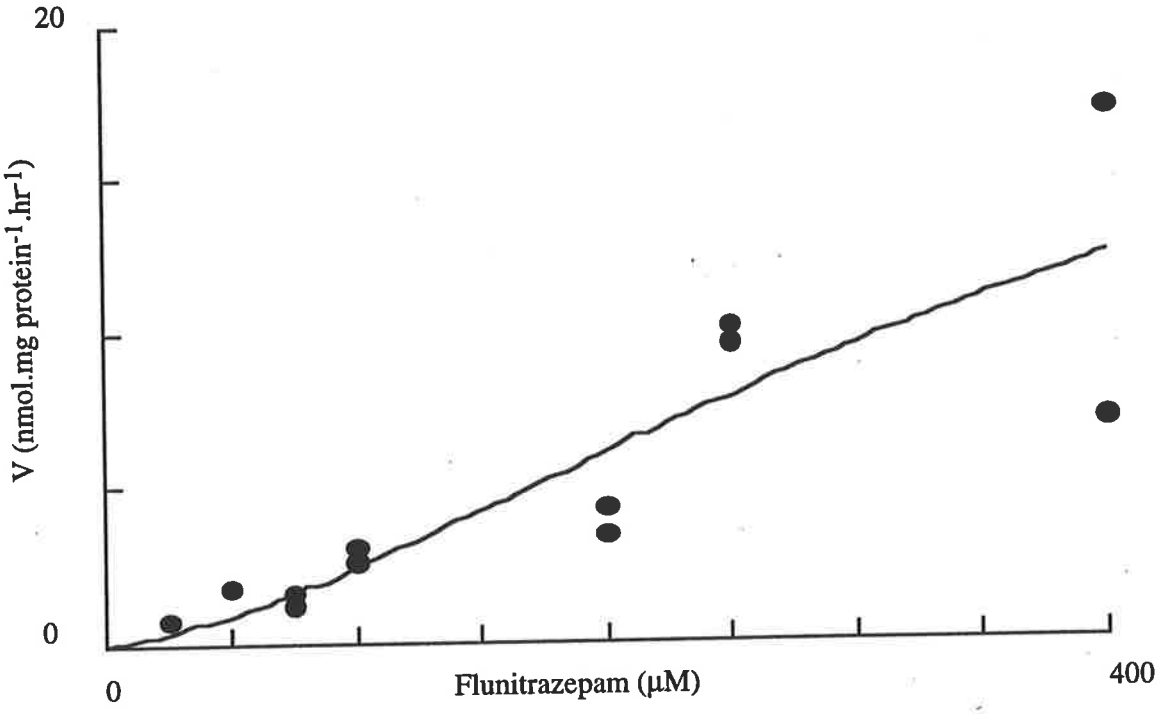
HLS #5



HLS #18



HLS #22


20
V (nmol.mg protein-1.hr-1)
0
0
Flunitrazepam (μM)
400

HLS #31

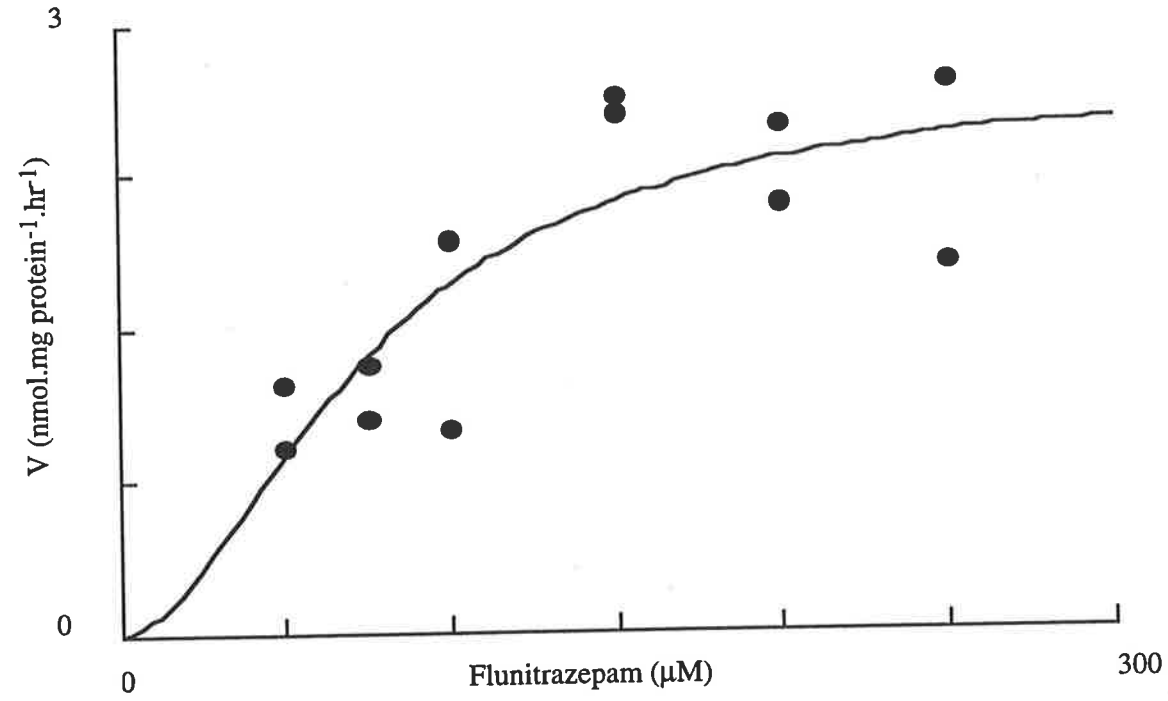

3
V (nmol.mg protein-1.hr-1)
0
0
Flunitrazepam (μM)
300

Table 33: Summary data to demonstrate fit of either the Hill equation or single-enzyme Michaelis-Menten equation to the DMF formation data.

Hill	HLS #5	HLS #18	HLS #22	HLS #31
Sum of squares	13.0	5.6	71.1	1.7
Standard deviation from equation	1.2	0.98	2.8	0.44
Determination coefficient	0.97	0.95	0.73	0.66
K_s (μM)	600	151	428	74
V_{max} (nmol.mg^{-1}.hr^{-1})	48	10	26	2.7
Single-enzyme Michaelis-Menten				
Sum of squares	13.2	11.4	73.3	1.8
Standard deviation from equation	1.1	1.27	2.7	0.43
Determination coefficient	0.97	0.91	0.72	0.64
K_m (μM)	969	977	2.1×10^6	130
V_{max} (nmol.mg^{-1}.hr^{-1})	65	34	63772	3.8

Appendix G

Summary data for each subject of CYP2C19 phenotyping / genotyping study

Subject Number	Racial Background	CYP2C19 genotype	PG/CG	Urine pH	Urine volume (ml)
1	Caucasian	*CYP2C19*1/*1*	1.17	6.23	870
2	Caucasian	*CYP2C19*1/*2*	3.43	6.86	930
3	Caucasian	*CYP2C19*1/*1*	2.95	6.88	470
4	Caucasian	*CYP2C19*1/*1*	1.65	6.24	680
5	Caucasian	*CYP2C19*1/*1*	3.71	7.14	800
6	Caucasian	*CYP2C19*1/*1*	1.62	5.52	630
7	Caucasian	*CYP2C19*1/*1*	4.96	6.77	730
8	Caucasian	*CYP2C19*1/*1*	1.49	6.70	610
9	Caucasian	*CYP2C19*1/*1*	1.22	5.98	540
10	Caucasian	*CYP2C19*1/*1*	2.94	7.03	910
11	Caucasian	*CYP2C19*1/*1*	2.63	5.34	195
12	Caucasian	*CYP2C19*1/*1*	1.24	6.14	320
13	Caucasian	*CYP2C19*1/*1*	5.10	5.86	420
14	Caucasian	*CYP2C19*1/*1*	1.52	5.61	330
15	Caucasian	*CYP2C19*1/*1*	1.72	5.96	460
16	Caucasian	*CYP2C19*1/*1*	1.14	6.04	390
17	Caucasian	*CYP2C19*1/*1*	0.96	6.20	1390
18	Caucasian	*CYP2C19*1/*1*	3.83	5.46	970
19	Caucasian	*CYP2C19*1/*1*	1.55	5.62	460
20	Caucasian	*CYP2C19*1/*1*	3.57	7.06	1010
21	Caucasian	*CYP2C19*1/*2*	3.27	6.46	380
22	Caucasian	*CYP2C19*1/*2*	6.99	5.58	430
23	Caucasian	*CYP2C19*1/*1*	2.16	6.11	160
24	Caucasian	*CYP2C19*1/*1*	1.70	5.64	420
25	Caucasian	*CYP2C19*1/*1*	1.68	5.88	225
26	Caucasian	*CYP2C19*1/*1*	4.88	5.90	335
27	Caucasian	*CYP2C19*1/*1*	1.51	5.35	160
28	Caucasian	*CYP2C19*1/*1*	2.33	6.88	620
29	Caucasian	*CYP2C19*1/*2*	3.35	7.72	530
30	Caucasian	*CYP2C19*1/*1*	1.67	6.21	515
31	Asian	*CYP2C19*1/*2*	0.91	5.88	495

32	Caucasian	*CYP2C19*1/*1*	1.91	5.76	735
33	Caucasian	*CYP2C19*1/*1*	1.11	5.95	380
34	Caucasian	*CYP2C19*1/*1*	3.03	6.09	240
35	Asian	*CYP2C19*2/*2*	20.31	6.73	850
36	Asian	*CYP2C19*1/*1*	1.13	5.68	270
37	Caucasian	*CYP2C19*2/*2*	5.61	5.68	240
38	Asian	*CYP2C19*1/*2*	5.30	5.48	410
39	Caucasian	*CYP2C19*1/*2*	2.62	6.71	810
40	Asian	*CYP2C19*1/*1*	4.05	5.96	200
41	Caucasian	*CYP2C19*1/*1*	2.46	6.39	600
42	Caucasian	*CYP2C19*1/*1*	0.72	5.39	200
43	Caucasian	*CYP2C19*1/*1*	1.14	5.68	190
44	Caucasian	*CYP2C19*1/*2*	2.80	7.18	325
45	Caucasian	*CYP2C19*1/*2*	1.41	5.18	410
46	Asian	*CYP2C19*1/*2*	1.70	6.00	1180
47	Asian	*CYP2C19*1/*2*	2.69	6.08	280
48	Asian	*CYP2C19*1/*2*	1.39	5.75	690
49	Asian	*CYP2C19*1/*2*	1.96	6.47	1250
50	Asian	*CYP2C19*1/*2*	1.90	5.62	490
51	Caucasian	*CYP2C19*1/*2*	3.38	6.48	720
52	Caucasian	*CYP2C19*1/*2*	4.36	5.38	320
53	Asian	*CYP2C19*1/*2*	1.08	6.76	1240
54	Caucasian	*CYP2C19*1/*2*	2.00	7.24	350
55	Caucasian	*CYP2C19*1/*1*	2.94	5.88	740
56	Asian	*CYP2C19*1/*1*	1.85	5.73	450
57	Caucasian	*CYP2C19*1/*1*	1.21	5.85	220
58	Caucasian	*CYP2C19*1/*1*	1.41	6.11	290
59	Caucasian	*CYP2C19*2/*2*	13.94	7.32	340
60	Caucasian	*CYP2C19*1/*1*	1.91	5.49	200
61	Caucasian	*CYP2C19*1/*1*	1.90	5.87	120
62	Asian	*CYP2C19*1/*1*	1.25	5.32	85
63	Caucasian	*CYP2C19*1/*1*	1.26	5.30	190
64	Caucasian	*CYP2C19*1/*1*	2.42	5.94	320
65	Caucasian	*CYP2C19*1/*2*	2.61	6.12	360
66	Asian	*CYP2C19*1/*1*	2.05	6.30	410
67	Asian	*CYP2C19*1/*1*	1.50	6.40	415
68	African	*CYP2C19*1/*2*	3.87	5.72	790
69	Asian	*CYP2C19*1/*1*	1.05	6.83	270

70	Caucasian	*CYP2C19*1/*2*	2.00	6.12	1050
71	Asian	*CYP2C19*1/*3*	3.00	6.04	1100
72	Caucasian	*CYP2C19*1/*2*	19.30	6.57	330
73	Caucasian	*CYP2C19*1/*1*	1.84	6.25	350
74	Caucasian	*CYP2C19*1/*1*	0.97	5.60	250
75	Caucasian	*CYP2C19*1/*1*	1.41	6.29	360
76	Asian	*CYP2C19*1/*2*	7.96	6.60	320
77	Asian	*CYP2C19*1/*1*	1.92	5.76	120
78	Caucasian	*CYP2C19*1/*2*	2.60	5.46	275
79	Caucasian	*CYP2C19*1/*2*	1.29	6.78	295
80	Caucasian	*CYP2C19*1/*2*	2.23	5.80	790
81	Caucasian	*CYP2C19*1/*3*	0.96	7.29	1050
82	Asian	*CYP2C19*1/*1*	1.31	5.87	8
83	Asian	*CYP2C19*1/*1*	1.72	5.79	240

Appendix H

Summary data for subjects of CYP2D6 phenotyping / genotyping study

CYP2D6 gene duplication - "√" indicates more than two copies of *CYP2D6*, log DM MR = log dextromethorphan metabolic ratio

Subject Number	Racial Background	CYP2D6 gene duplication	log DM MR	Urine pH	Urine volume (ml)
1	Caucasian		-2.66	6.23	870
2	Caucasian		-2.43	6.86	930
3	Caucasian		-2.56	6.88	470
4	Caucasian		-2.56	6.24	680
5	Caucasian		-2.20	7.14	800
6	Caucasian		-1.67	5.52	630
7	Caucasian	√	-2.15	6.77	730
8	Caucasian		-2.35	6.70	610
9	Caucasian		-2.46	5.98	540
10	Caucasian	√	-3.32	7.03	910
11	Caucasian		0.19	5.34	195
12	Caucasian		-1.68	6.14	320
13	Caucasian	√	-1.72	5.86	420
14	Caucasian		-2.5	5.61	330
15	Caucasian		-2.85	5.96	460
16	Caucasian		-2.65	6.04	390
17	Caucasian		-2.07	6.20	1390
18	Caucasian		-2.02	5.46	970
19	Caucasian		-1.85	5.62	460
20	Caucasian		-2.04	7.06	1010
21	Caucasian		-3.10	6.46	380
22	Caucasian		-1.79	5.58	430
24	Caucasian		-1.75	5.64	420
25	Caucasian		-2.96	5.88	225
26	Caucasian		-2.44	5.90	335
27	Caucasian		-1.33	5.35	160
28	Caucasian		-2.28	6.88	620
29	Caucasian		-3.95	7.72	530

30	Caucasian		-1.47	6.21	515
31	Asian		-1.64	5.88	495
32	Caucasian		-1.79	5.76	735
33	Caucasian		-2.19	5.95	380
34	Caucasian		-2.84	6.09	240
35	Asian		-2.22	6.73	850
36	Asian		-1.73	5.68	270
37	Caucasian		-3.24	5.68	240
38	Asian		-0.98	5.48	410
39	Caucasian		-2.78	6.71	810
40	Asian		-1.84	5.96	200
41	Caucasian		-2.13	6.39	600
42	Caucasian		-1.24	5.39	200
43	Caucasian		-1.63	5.68	190
44	Caucasian		-2.70	7.18	325
45	Caucasian		1.01	5.18	410
46	Asian		-1.56	6.00	1180
47	Asian		0.13	6.08	280
48	Asian		-2.26	5.75	690
49	Asian		-1.80	6.47	1250
50	Asian		-0.28	5.62	490
51	Caucasian		-2.06	6.48	720
52	Caucasian		-1.48	5.38	320
53	Asian		-2.43	6.76	1240
54	Caucasian	√	-2.24	7.24	350
55	Caucasian		-2.33	5.88	740
56	Asian		-1.94	5.73	450
58	Caucasian		-1.62	6.11	290
59	Caucasian		-3.06	7.32	340
60	Caucasian		-1.31	5.49	200
61	Caucasian		-2.66	5.87	120
62	Asian		-1.72	5.32	85
63	Caucasian		-1.71	5.30	190
64	Caucasian		-2.21	5.94	320
65	Caucasian		-2.03	6.12	360
66	Asian		-2.03	6.30	410
68	African		-2.25	5.72	790
69	Asian		-2.67	6.83	270

70	Caucasian		-2.12	6.12	1050
71	Asian		-2.09	6.04	1100
72	Caucasian		-0.36	6.57	330
73	Caucasian		-1.69	6.25	350
74	Caucasian		-1.27	5.60	250
75	Caucasian		-1.94	6.29	360
76	Asian		-0.88	6.60	320
77	Asian		-1.43	5.76	120
78	Caucasian		-1.37	5.46	275
79	Caucasian		-2.54	6.78	295
80	Caucasian		-2.18	5.80	790
81	Caucasian		-2.75	7.29	1050
82	Asian		-1.44	5.87	8
83	Asian		-1.04	5.79	240

Appendix I

Summary data of subjects in codeine pilot study

Subject, sex	Age (yr)	CYP2D6 gene duplication	Urine pH	Urine volume (ml)	log DM MR
#1, female	25		6.11	3730	-2.85
#2, male	26		5.67	2010	-2.46
#3, male	49	√	6.28	3900	-2.66
#4. male	30	√	6.04	4300	-2.15

Appendix J

Papers published in support of thesis

Coller, J. K., Somogyi, A.A. & Bochner, F. (1997) Association between CYP2C19 genotype and proguanil oxodative polymorphism.
British Journal of Clinical Pharmacology, v. 43(6), pp. 659-660

Coller, J. K., Somogyi, A.A. & Bochner, F. (1998) Quantification of flunitrazepam's oxidative metabolites, 3-hydroxyflunitrazepam and desmethylflunitrazepam, in hepatic microsomal incubations by high-performance liquid chromatography. *Journal of Chromatography B, v. 719(1-2), pp. 87-92*

Coller, J. K., Somogyi, A.A. & Bochner, F. (1999) Comparison of (S)-mephenytoin and proguanil oxidation in vitro: contribution of several CYP isoforms.
British Journal of Clinical Pharmacology, v. 48(2), pp. 158-167

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Amendments

As a result of the comments made by the examiners of this thesis, the following amendments were made, (note that negative line numbers indicate counting up from the foot of the page):

- page i, lines 11-12; the specific kinetic reactions were described.
- page ii, lines 1-2; the metabolic ratio was specifically defined.
- page v, line 20; changed from disposition rate constant to elimination rate constant.
- page v, line 21; changed from elimination rate constant to absorption rate constant.
- page 5, line 3; 'discovered to be due to' changed to 'discovered due to'.
- page 5, line 11; 'is changes' changed to 'is a change'.
- page 7; figure 3 was changed to show complete correct structures.
- page 10, line 11; reference citation Chang *et al.* (1995) changed to Chang *et al.* (1995a).
- page 14, line -1; 'that a alternative' changed to 'that an alternative'.
- page 19, line -2; 'Kotunay' changed to 'Kortunay'
- page 25, line 18; 'napthoflavone' changed to 'naphthoflavone'.
- page 26, line -7; 'may be a suitable' changed to 'may not be a suitable'.
- page 27, line -6; reference citation Ward *et al.* (1989) changed to Ward *et al.* (1989b).
- page 36, line -8; 'affect' changed to 'effect'.
- page 43, line 9; 'has' changed to 'have'.
- page 52, line 11; 'affect' changed to 'effect'.
- page 53, line 2; 'affects' changed to 'effects'.
- page 54, line -2; 'of Swiss subject' changed to 'in Swiss subjects'.
- page 60, line 8; 'Otten' changed to 'Otton'.
- page 63, line 12; 'copy' changed to 'more than one copy'.

- page 64, line 16; 'occurrence' was deleted.
- page 64, line -3; 'has' changed to 'have'.
- page 73, line -2 and subsequently throughout; '3'-hydroxyflunitrazepam' and '3'-OH-F' were changed to '3-hydroxyflunitrazepam' and '3-OH-F', respectively.
- page 76, line 9; 'folin-ciocalteau' changed to 'Folin-Ciocalteau'.
- page 78, 82, 87, 126, 127, 147, 165; all 'rpm' values changed to equivalent 'g' values.
- page 81, line 15; 'wass' changed to 'was'.
- page 88, 89, 149, 166, 170, 173; all figures changed to give concentrations of compounds in chromatograms and a vertical scale.
- page 98, line 9, page 130, lines -5 and -13, page 152, lines 1 and -13, page 175, line -11; 'Microsoft excel' changed to 'Microsoft® Excel'.
- page 117, line 3; 'appropirate' changed to 'appropriate'.
- page 122, line 5; 'statue' changed to 'status'.
- page 125; table 17 changed to contain the two individual values for Caucasian PM rather than the mean and SD.
- page 142, line -11; 'successfully' was deleted.
- page 148, line 7; '50µl 50µg.ml^{-1} pholcodeine' changed to 'Pholcodine (50µl; 50µg. ml^{-1})'.
- page 149, line -1; space was inserted between curve/and.
- page 165, line 2; space was inserted between Research/Ethics.
- page 189, line 9; 'are' changed to 'is'.
- page 191, line -8; 'CY2C19' changed to 'CYP2C19'.
- page 193, line 7; sentence changed from 'The thesis has investigated' to 'In this thesis, I have reported findings of investigations'.
- bibliography; a sentence was inserted to explain the style of the referencing used.

In addition, a comment from one examiner was made regarding the future of the phenotyping technique. For clarification the following was added to the abstract, page ii:

'The results observed indicate that genotyping is a more conclusive and accurate method for the correct identification of poor metabolisers of both the CYP2C19 and CYP2D6 genetic polymorphisms.'

One examiner posed the valid question of whether a table could be included containing information on page 10-11. This was not amended as the information was clearly and extensively provided in the text, and therefore, a table was deemed unnecessary in this case.

Finally, a comment was made which addressed the potential of mephobarbital to become a replacement probe for the CYP2C19 genetic polymorphism. As discussed on page 12, the metabolism of this drug cosegregates with the S-mephenytoin genetic polymorphism. However, no studies have been published demonstrating its use as a phenotypic probe. Therefore, the following sentence was added on page 12 for clarification: 'There are no data on its use as a probe for CYP2C19 phenotyping.'