Identification of Cytochrome P450 Isoforms Involved in the Metabolism of Oxycodone

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Abstract

Oxycodone (OC) has been in use as an analgesic drug since 1917. Its analgesic potency is similar to that of morphine, and it has been recommended for use in the treatment of moderate to severe pain. The elimination of OC in humans is mainly hepatic, and metabolism by both phase I and phase II reactions is involved. Cytochrome P450 dependent N- and O-demethylation are the most important phase I metabolic pathways, and the metabolites are noroxycodone (NOC) and oxymorphone (OM), respectively.

The enzymes involved in the metabolism of OC are poorly described, and therefore the aim of this study was to determine the enzyme kinetics for the formation of NOC and OM, and to identify which liver P450 isoform(s) are involved in these two metabolic reactions.

Enzyme kinetics were obtained for 6 CYP2D6 extensive metabolizers (EM) and 1 CYP2D6 poor metabolizer (PM) liver. OM formation exhibited two enzyme kinetics in both the EMs and the PM. K_M values for the EMs were 143±84μM and 29.3±34.1mM (mean±SD), while the PM had K_M values of 217μM and 26.7mM. NOC formation had single enzyme kinetics in most livers, and the K_M was 4.07±3.92mM (mean±SD) with a range of 1.52-12.9mM. Substantial variability in the intrinsic clearance (V_{max}/K_M) through the NOC pathway was observed (55-724 μl/hr/mg protein).

Using specific chemical and antibody inhibitors and CYP expressed enzymes, it was shown that NOC formation is mainly dependent on CYP3A4, while CYP2D6 is the main isoform involved in OM formation. However, at least one other enzyme is responsible for 4.1±3.3% (mean±SD) of OM formation, and a minor CYP2C19 involvement cannot be excluded from NOC formation. Environmental (CYP3A4) and genetic (CYP2D6) factors are likely to contribute to variability in the analgesic response to oxycodone in patients with pain.

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 is made in the text.

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

Adelaide, December 2000

Ingvild Rasmussen

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List of Abbreviations

ACN	Acetonitrile
Chlor	Chlorzoxazone
Cl_{int}	Intrinsic clearance
CO	Carbon monoxide
Coum	Coumarin
CYP450	Cytochrome P450
CYP/*CYP*	Cytochrome protein/gene
%CV	Coefficient of variation (%)
DDC	Diethyldithiocarbamate
Dex	Dextromethorphan
DMSO	Dimethylsulfoxide
EDTA	Ethylene diamine tetraacetic acid (edetate disodium)
EM	Extensive metabolizer
Fura	Furafylline
HLM	Human liver microsomes
HM	Hydromorphone
HPLC	High performance liquid chromatography
hr	Hour(s)
IS	Internal standard
Keto	Ketoconazole
K_i	Inhibition constant
K_M	Michaelis Menten dissociation constant
LKM1	Liver-kidney microsomal type 1 (antibodies)
LOQ	Limit of quantification
LQC	Low quality control
µl	Microliter(s)
µg	Microgram(s)
µM	Micromolar

MeOH	Methanol
mg	Milligram(s)
min	Minutes
ml (or mL)	Milliliter(s)
mM	Millimolar
MQC	Medium quality control
n	Number of replicates
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
nmol	nanomole(s)
NOC	Noroxycodone
NOM	Noroxymorphone
OC	Oxycodone
OM	Oxymorphone
p	Probability
PM	Poor metabolizer
QC	Quality control
Quin	Quinidine
r^2	Coefficient of determination
S (or s)	Substrate concentration
SD	Standard deviation
S-meph	S-mephenytoin
Std.	Standard (for calibration curve)
Sulph	Sulphaphenazole
TOA	Troleandomycin
UV	Ultraviolet
v	Velocity
V_{max}	Maximal velocity (of enzyme catalyzed reaction)

1. Introduction

1.1 Drug metabolism

The human body is constantly exposed to foreign substances (xenobiotics), which may have various effects on physiological functions. Xenobiotics can be man-made or from natural sources, and include drugs as well as unwanted chemicals such as toxins from plants and animals, industrial chemicals and pollutants (Parkinson, 1996).

1.1.1 The role of biotransformation

Many xenobiotics entering the body are lipophilic, a feature that enables them to cross bilayered lipid membranes such as the surfaces of the gastrointestinal and respiratory tracts. However, these substances are also subject to reabsorption by the kidneys, which allows them to remain in the body for an extended period of time. Consequently changes in chemical properties are required to facilitate the elimination of these substances. Xenobiotics are converted to forms that are more readily excreted by the kidneys; a process referred to as biotransformation or metabolism (Parkinson, 1996; Correia, 1998).

1.1.2 Phase I and phase II reactions

Xenobiotic biotransformations are generally divided into phase I and phase II reactions.
In phase I reactions a functional group is exposed (e.g. -OH, $-NH_2$ or SH) by hydrolysis, reduction or oxidation to leave the molecule more hydrophilic. This is sometimes sufficient to make the metabolite excretable by the kidneys, but most commonly they are metabolised further by a phase II reaction.
The phase II reactions are often referred to as conjugations since they involve binding (via a transferase enzyme) of endogenous compounds to the newly formed functional groups. Phase II reactions include glucuronidation, sulfation, acetylation, methylation, and conjugation with glutathione or amino acids. Of these reactions, glucuronidation is the

most common. Some drugs possess functional groups that already make them suitable for conjugation (-OH, -COOH), in which case the phase II reactions may occur without the preceding phase I reaction. The products of phase II reactions are highly polar, a property that makes them suitable for renal excretion (Parkinson, 1996; Correia, 1998).

1.1.3 Anatomical distribution of biotransforming enzymes

The enzymes involved in biotransformation of drugs are located in several tissues, such as gastrointestinal epithelium, skin, nasal mucosa, lungs, kidneys, brain and other tissues, although the liver contains the largest amounts of the common xenobiotic biotransforming enzymes. Within the liver cells (and cells in most other organs), the enzymes are found mainly in the endoplasmic reticulum or in the cytosol, but smaller amounts are also found in mitochondria, nuclei and lysosomes (Parkinson, 1996).

Homogenization and differential centrifugation of liver tissue disrupts the endoplasmic reticulum, which then forms microvesicles referred to as liver microsomes. Liver microsomes possess high concentrations of xenobiotic-biotransforming enzymes, and are therefore commonly used to study the metabolism of drugs *in vitro* (Parkinson, 1996).

1.1.4 Cytochrome P450

Most phase I reactions are catalysed by the mixed function oxygenase system, which is located in the endoplasmic reticulum of the cells (e.g. hepatocytes). Cytochrome P450 enzymes are the main components of this system, and they have many important biological functions (e.g. steroid synthesis). These enzymes are also involved in the biotransformation of xenobiotics. Cytochrome P450 can be found in bacteria, plants, yeast and animals, a fact indicating that the ancestral gene coding for this protein family has existed since early forms of life developed more than 3.5 billion years ago (Nelson et al., 1996).

Each individual cytochrome P450 isoenzyme consists of a single protein and a haem group as a prosthetic moiety (Evans, 1993; Chang and Kam, 1999). In its reduced form

cytochrome P450 binds CO, a complex that has a maximum light absorbance at 450nm. Hence the derivation of the name of the enzyme (Correia, 1998).

The cytochrome P450 drug oxidation cycle (Figure 1) begins with the binding of a substrate (e.g. drug) to the oxidized form of cytochrome P450 (Fe^{3+}). This is then reduced when an electron is received from NADPH reductase. The reduced complex of cytochrome P450 (Fe^{2+}) and substrate binds molecular oxygen and then receives another electron from the NADPH reductase. The end products of the cycle are an oxidized substrate, cytochrome P450 in its original oxidized form and water (Correia, 1998).

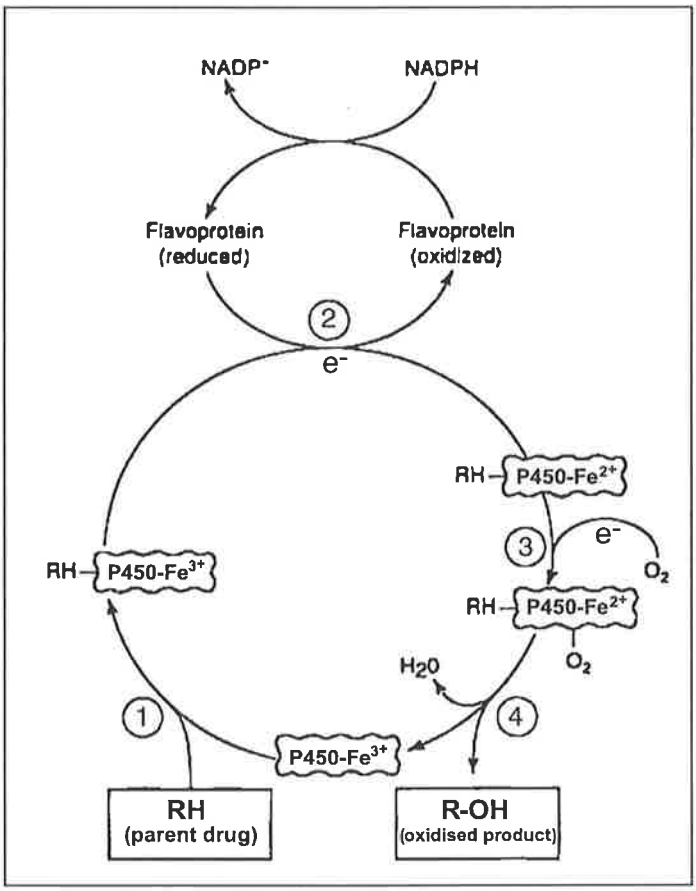

Figure 1 The catalytic cycle of drug oxidation by cytochrome P450 (Correia, 1998).

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1.1.4.1 Cytochrome P450 families and subfamilies

There are 17 known cytochrome P450 families in humans, which form 39 subfamilies (Nelson, 2000). This is an area that is currently subject to extensive research, and new discoveries are frequent.

The general nomenclature for the cytochrome P450 enzymes is based on the sequence similarities within the families and subfamilies. The prefix CYP designates cytochrome P450 in all species (CY for cytochrome and P for pigment). P450 isoforms with more than 40% amino acid homology are in the same family, which is designated by an Arabic numeral (e.g. CYP2). Each family may consist of subfamilies, which have at least 55% amino acid homology and are denoted by a capital letter (e.g. CYP2D). The individual enzyme is denoted with an Arabic numeral at the end (e.g. CYP2D6), and is referred to as an isoenzyme of CYP450. The genes associated with the enzymes are denoted after the same system, but in italic (e.g. *CYP2D6)* (Nelson, 2000; Chang and Kam, 1999).

CYP1, CYP2 and CYP3 constitute more than 70% of the total hepatic P450s, and are responsible for most xenobiotic metabolism (Wrighton and Stevens, 1992).
Table 1 shows the function of the various CYP families that have been identified in humans, and it is based on the reviews by Nelson et al. (1996) and (Nelson, 2000). Some of the P450 isoenzymes important in drug metabolism will be discussed further in section 1.1.6.

1.1.5 Factors regulating cytochrome P450 metabolic capacity

CYP metabolic capacity may need to be considered when deciding on the drug dose and the frequency of administration, in order to achieve the desired therapeutic effect. The capacity of metabolism shows substantial inter-individual variability, and this can be due to genetic and physiological factors, as well as environmental factors.

Table 1 Cytochrome P450 families and functions in human (Nelson, 2000).

CYP1, CYP2, CYP3, CYP4	Important in xenobiotic metabolism
CYP5	Thromboxane synthesis
CYP7A	Cholesterol hydroxylase (bile acid biosynthesis)
CYP7B	Brain specific cholesterol hydroxylase
CYP8A	Prostaglandin synthesis
CYP8B	Involved in bile acid synthesis
CYP11, CYP17, CYP19, CYP21	Steroid biosynthesis
CYP24	Mitochondrial enzyme involved in vit. D degradation
CYP26	Retinoic acid hydroxylase important in development
CYP27	Mitochondrial steroid synthesis
CYP39	Unknown function
CYP46	Cholesterol hydroxylase
CYP51	Cholesterol biosynthesis

1.1.5.1 Environmental factors

Drugs can inhibit or induce the activity of CYP isoforms. This can lead to clinically significant drug-drug interactions when multi-drug treatment is necessary.

CYP inhibition can be competitive or non-competitive. A competitive inhibitor binds to the same site on the CYP as the substrate, and may or may not be metabolised by the enzyme it inhibits. Non-competitive inhibitors bind to a different binding site on the CYP. Reduced drug clearance and an increased elimination half-life are the direct pharmacokinetic consequences of inhibition of drug metabolism. The clinical consequences of CYP inhibition depend on the type of inhibition, as well as the activity and toxicity of both the parent drug and its metabolite(s). If a drug with a narrow therapeutic window accumulates in the blood stream due to such an interaction, toxicity may occur (e.g. warfarin). Drugs with active metabolites may show reduced effect if its metabolism is inhibited (e.g. codeine). Conversely, drug interactions can sometimes be used intentionally in medical treatment. An example of this is the co-administration of CYP3A4 inhibitors,

ketoconazole and/or erythromycin, with cyclosporine. Cyclosporine is an immunosuppressant metabolised by CYP3A4, and its use is very costly. CYP3A4 inhibitors will increase the bioavailability and prolong the elimination half-life of this drug, and therefore reduce the dosage and frequency of administration (Parkinson, 1996; Touw, 1997).

Inducers of cytochrome P450s increase the rate of xenobiotic metabolism. The result is an increased clearance and reduced elimination half-life, which is a problem clinically as increased drug doses and more frequent drug administration are needed (Parkinson, 1996). Great care must be taken when multi-drug treatment regimens are necessary due to the potential drug-drug interactions.

There are also examples of CYP450 activity being modulated by dietary factors. Grapefruit juice inhibits the activity of CYP3A4 along the gastrointestinal tract (Bailey et al., 1994; Lilja et al., 2000). The result is a significantly increased bioavailability when CYP3A4 dependent drugs with high first metabolism are administrated orally. Cruciferous vegetables (e.g. cabbage) cause induction of CYP1A2 activity and inhibition of CYP2E1 activity (Yang et al., 1992).

Other lifestyle dependent factors include cigarette smoking and alcohol. Cigarette smoking induces the activity of CYP1A2, whilst chronic alcohol consume increases the activity of CYP2E1 (Glue and Clement, 1999).

1.1.5.2 Genetic polymorphisms

Genetic polymorphism is a genetic variation that can cause impairment of, for example, enzymatic activity in certain members of the population. In order to call it a polymorphism, the difference in DNA sequence has to be found in at least 1% of the population (Chang and Kam, 1999). Such polymorphisms are caused by genetic alterations, and the result may be either a complete deletion of the enzyme or an alteration (usually reduction) in metabolising capacity (Touw, 1997). This variability in enzyme expression may alter the pharmacokinetics of a drug. Lack of metabolic activity may lead to accumulation of parent drug and less formation of the metabolite(s). The consequence of this depends on the activity of the parent drug and the metabolite(s). Accumulation of a drug that is active in its

original form can raise the plasma concentration to a level higher than the therapeutic range, which in turn can increase the incidence of side effects and toxicity (e.g. sparteine). If a metabolite is active and of importance for the drug's mechanism of action (prodrug), reduced enzymatic activity will lead to insufficient effect of the drug (e.g. codeine) (Kroemer and Eichelbaum, 1995; Touw, 1997; Chang and Kam, 1999). Attention may therefore need to be paid to the patient's genetic state when administering a drug.

The genetic polymorphism of drug metabolising pathways was first discovered due to increased incidence of adverse effects in some patients when administering the drug at standard recommended doses (Mahgoub et al., 1977; Edeki, 1996). The most studied genetic polymorphism involving cytochrome P450 is the debrisoquine/sparteine polymorphism, which was discovered about 25 years ago (Mahgoub et al., 1977; Eichelbaum et al., 1975). Several cytochrome P450s have now been shown to be subject to polymorphisms, and the individual polymorphisms involved in drug metabolism will be discussed in section 1.1.6.

Genotyping and phenotyping make it possible to group individuals with respect to the various drug metabolising genetic polymorphisms. Genotype is usually assessed using a method based on polymerase chain reaction (PCR), which involves amplification of the DNA region encoding for the enzyme mutation by use of complementary primers. Phenotype is determined by estimating enzyme activity for a selected probe substrate that is metabolised by the particular enzyme. Both methods have advantages and disadvantages. Genotyping is increasingly popular since the results provided are accurate and detailed, but the method is costly and the genotype does not always reflect function. Phenotyping gives a functional assessment of the enzymatic activity, taking environmental factors influencing enzyme activity into account (e.g. drug interactions). However, the use of a probe drug introduces the potential of adverse effects and concerns with regard to invasiveness and inconvenience. Furthermore, the genotype remains uncertain (Nebert, 2000; Kroemer and Eichelbaum, 1995; Streetman et al., 2000).

Individuals are characterized as poor metabolizers (PM) or extensive metabolizers (EM) with respect to the polymorphically expressed enzyme (e.g. CYP2D6). Another group, ultra rapid metabolizers (URM), has also been discovered for CYP2D6 (Touw, 1997).

Substantial differences in genetic polymorphisms have been found between inter-ethnic populations. For example, only 1% of a Japanese population is PM with respect to CYP2D6, while the percentage in a Caucasian population is 5-10 (Daly et al., 1996; Edeki, 1996). Another typical example is the CYP2C19 polymorphism. Poor metabolizers for this enzyme represent only 2-5% in Caucasian populations, whereas the frequency for Asian populations is as high as 23% (Bertilsson, 1995; Chang and Kam, 1999). Many known genetic polymorphisms have been the subject of population studies, and knowledge in this area is increasing rapidly.

Genetic polymorphisms may play a role in the development of various disease states, such as cancer (e.g.CYP2E1), adrenal hyperplasia (CYP1A2) and Parkinson's disease (e.g.CYP2D6). The evidence for these relations is not conclusive and further investigation is needed to support current research (Chang and Kam, 1999). Knowledge about the association between disease and CYP genotypes may help to prevent many fatal diseases, since chemical exposure sometimes contributes to the disease onset.

1.1.5.3 Gender, age and disease states

Gender-related differences in CYP450 metabolic capacity can be found in several species. This is particularly prominent in rats, where male animals have higher rates of xenobiotic metabolism than females (Mugford and Kedderis, 1998). The difference between genders is less prominent in humans. When a difference is present, females are generally found with higher plasma concentrations of the drug than males after equivalent doses, although little consistency is seen between studies in this area. For example, several studies have indicated that CYP2C19, CYP2D6, and CYP2E1 have higher metabolic capacity in men, whereas drugs metabolised by CYP3A4 seem to be eliminated faster by women (Mugford and Kedderis, 1998; Tanaka, 1999a). However, these gender-related differences cannot be related directly to differences in CYP450 activity. Several *in vitro* studies have shown that expression of CYP450 isoforms relevant for xenobiotic metabolism is the same in both genders (George et al., 1995; Shimada et al., 1994). The aetiology of gender-related differences in xenobiotic metabolism is probably due to CYP induction and down-

regulation, and includes differences in environmental exposure (e.g. alcohol consumption and smoking) as well as the influence of androgenic hormones (Touw, 1997; Tanaka, 1999a). Other pharmacokinetic parameters than metabolism (e.g. absorption and protein binding) will also alter the fate of a drug in the body. It is therefore difficult to correlate gender-related differences in drug pharmacokinetics to metabolism alone (Tanaka, 1999a; Mugford and Kedderis, 1998).

In vivo studies using probe drugs for various CYP450 isoforms indicate that metabolic capacity changes between the various stages of life. Generally the activity is low at birth, increases until it peaks in young adults, and then decreases with old age (Tanaka, 1998). The aging pattern for different enzymes varies. It has been shown in population studies that CYP1A2 capacity decreases above the age of 55, while CYP2D6 capacity decreases above the age of 65. These differences indicates that age-related changes in metabolic capacity can be correlated directly to the specific CYP450 isoforms, although other factors may also contribute to pharmacokinetic changes with age (e.g. hepatic blood flow) (Touw, 1997; Tanaka, 1998).

Several disease states can influence drug metabolising capacity. Fat accumulation in the liver, alcoholic, viral- or drug-induced hepatitis, alcoholic cirrhosis and biliary cirrhosis are some examples. These conditions impair the function of drug metabolising enzymes, which in turn affects drug elimination. Liver cancer is also associated with reduced metabolic capacity. This is mainly caused by reduced CYP450 content. Poisoning with certain heavy metals (e.g. lead) has been shown to impair enzyme activity or cause defective formation of enzymes. In both situations the consequence will be reduced metabolic capacity (Correia, 1998).

1.1.6 Cytochrome P450 enzymes of relevance for human drug metabolism

The majority of commonly used drugs are metabolised by a limited number of cytochrome P450 isoenzymes (Touw, 1997). These are described below.

1.1.6.1 CYP2D6

CYP2 constitutes about 35% of total hepatic P450, and it is the family that contains the highest number of subfamilies (Lewis, 1998). CYP2D6 is an important isoenzyme from the CYP2 family since it has been related to the metabolism of more than 60 different drugs, including several tricyclic antidepressants, selective serotonin reuptake inhibitors, analgesic drugs, anti-arrhythmic drugs, beta-blockers, anti-hypertensives and others. CYP2D6 accounts for approximately 2% of total hepatic P450 (Touw, 1997; Lewis, 1998; Chang and Kam, 1999; Nebert et al., 1999).

CYP2D6 is subject to genetic polymorphism, and drugs metabolised by CYP2D6 are therefore at high risk of adverse effects. This particular polymorphism has been well studied since many drugs are metabolised by CYP2D6, and reduced metabolism can be manifested in 5-10% of Caucasians (Kroemer and Eichelbaum, 1995; Daly et al., 1996). Codeine is one commonly used drug that is highly influenced by the CYP2D6 polymorphism. This drug exerts its effect via an active metabolite (morphine), and the pathway is mediated by CYP2D6 (Chen et al., 1988). Lack of an antinociceptive effect of codeine is seen in PM with respect to CYP2D6 (Sindrup and Brøsen, 1995).

The gene encoding for CYP2D6 is located on chromosome 22 (Eichelbaum et al., 1987), and many mutations of this gene have been discovered. Although five alleles represent over 95% of the PM variants (Dalén et al., 1999), 70 allelic variations had been described up to June 2000 (Oscarson, 2000). *CYP2D6*1* is representing the normal functioning enzyme, and this allele is also referred to as the "wild type" (Sachse et al., 1997). The most frequent alleles representing PM with respect to CYP2D6 are *CYP2D6*3* (frame shift mutation), *CYP2D6*4* (splicing mutation), *CYP2D6*5* (gene deletion), *CYP2D6*7* (base pair change mutation) and *CYP2D6*6* (frame shift mutation) (Daly et al., 1996), with *CYP2D6*4* counting for about 75% of the PMs in Caucasians (Bertilsson, 1995). The overall result from the various mutations is no function or decreased effect of the enzyme in homozygotes (Daly et al., 1996). The mutated genes are recessive, and the PMs are homozygous for any two defective alleles. EMs can be either heterozygous or homozygous for the dominant functioning allele (Touw, 1997).

Another allele, *CYP2D6*10,* represented 38% of *CYP2D6* alleles in a Japanese population, which is similar to that found in other studies on Asian populations (Nishida et al., 2000). This allele has been reported to represent only about 2% in Caucasians (Griese et al., 1998). *CYP2D6*10* results in reduced enzyme activity. Consequently the average EM in Asian populations has lower CYP2D6 activity than the average EM from a Caucasian population (Bertilsson, 1995; Nishida et al., 2000).

Gene duplication or amplification can occur for the CYP2D6 gene, resulting in individuals with ultrarapid metabolism. Up to twelve extra copies of the *CYP2D6*2* allele have been described and related to high CYP2D6 activity. About 1-2% of Caucasians are classified as ultra rapid metabolizers (URM) (Dalén et al., 1999), but substantial interethnic variation has been reported. Aklillu et al. (1996) found a high frequency (29%) of individuals with duplicated or multi-duplicated CYP2D6 genes in an Ethiopian population of 122 subjects. Phenotyping of CYP2D6 is usually carried out with dextromethorphan as a probe drug since this drug has a wide safety margin. CYP2D6 mediated O-demethylation is the rate limiting step in dextromethorphan metabolism, and since dextromethorphan and its metabolites are excreted in the urine, CYP2D6 phenotype can be identified by calculating the urinary metabolic ratio (dextromethorphan/dextrorphan at 8 hours). Several other drugs have also been used to phenotype for CYP2D6 activity, for example debrisoquine and sparteine, although their availability and safety have limited their use (Streetman et al., 2000).

1.1.6.2 CYP3A4

CYP3A accounts for about 30% of the total hepatic P450 and 70% of the gut wall P450 in humans. Four genes, *CYP3A3*, *CYP3A4*, *CYP3A5*, and *CYP3A7* control this family, but the latter is only present *in utero*. *CYP3A3* and *CYP3A4* have an amino acid similarity of 97%, and are therefore difficult to separate. In spite of this, CYP3A4 has shown a greater expression than CYP3A3 in the human liver, and it is generally accepted that CYP3A3 is included when referring to CYP3A4. CYP3A5 is only expressed in about 25-30% of liver samples, and is generally enhancing the metabolism of drugs that are metabolised by CYP3A4. (Wrighton and Stevens, 1992; Chang and Kam, 1999).

CYP3A4 metabolises more than 50 % of all drugs (e.g. several opioid analgesics, erythromycin, warfarin, diazepam), as well as several endogenous compounds (e.g. testosterone, oestradiol, progesterone). This CYP isoform has therefore been subject to extensive research (Benet et al., 1996; Nelson, 2000).

Substantial inter-individual variability in metabolising capacity is seen for CYP3A4 (20-40 fold - *in vitro* studies) (Ball et al., 1999), but the mechanisms behind the variation remains to be fully clarified. The expression of CYP3A4 is highly inducible by a wide range of xenobiotics, and this is believed to be the main reason for the inter-individual variability. Recent research on CYP3A4 regulation has shown that nuclear hormone receptors, named pregnane X receptors, are of major importance in the induction of CYP3A4. These receptors bind to xenobiotic response elements in the *CYP3A4* promoter region, and are activated by known CYP3A4 inducers (Jones et al., 2000). No evidence of a functionally important allelic variability is found in the coding region of the *CYP3A4* gene, and the population distribution after phenotyping is unimodal (Guengerich, 1999). A genetic variation has been found in the 5'promotor region of the *CYP3A4* gene, and there is a clear inter-racial difference in the frequency of this variation. However, there are no functional differences between the two genotypes (Ball et al., 1999; Wandel et al., 2000).

Many probes have been used to phenotype CYP3A activity. CYP3A plays a major role in the 6β-hydroxylation of endogenous cortisol, and the ratio of 6β-hydroxycortisol to free cortisol in urine has therefore been used as a measure of CYP3A activity. However, the validity of this test has been questioned since the correlation is poor between this method and other accepted phenotyping methods. Another approach to CYP3A phenotyping is the erythromycin breath test. CYP3A4 selectively N-demethylates erythromycin. When [^{14}C]-,N-methyl erythromycin is used, the cleaved carbon of the methyl group is expired as $^{14}CO_2$, and the extent of CYP3A4 activity can be measured as radioactivity. The erythromycin breath test provides a rapid approach to CYP3A4 phenotyping, but CYP3A5 activity is not measured, and the test only reflects the hepatic CYP3A4 activity since the probe is intravenously administered. The most reliable of all reported phenotyping methods for CYP3A is the use of oral midazolam. Midazolam can be safely administrated orally and intravenously, and it is metabolised by CYP3A4 as well as CYP3A5. CYP3A activity can be measured as an hydroxylation ratio or total plasma clearance. The disadvantage of this

method is the inconvenience of multiple intravenous blood samples (Thummel and Wilkinson, 1998; Streetman et al., 2000).

1.1.6.3 CYP2C9/CYP2C19

The CYP2C subfamily comprises about 20% of the total hepatic CYP450, making it the largest subfamily within the CYP2 group (Lewis, 1998). There are four known members of this family in humans; CYP2C8, CYP2C9, CYP2C18 and CYP2C19 (Nelson et al., 1996). CYP2C8 and CYP2C18 have an amino acid homology of 76%, while the homology between CYP2C9 and CYP2C19 is 91% (Richardson and Johnson, 1996). Within the CYP2C family, CYP2C9 and CYP2C19 are the most important. The CYP2C family metabolises a wide range of drugs, but despite the high sequence similarity little overlap in substrate specificity is seen between the CYP2C isoforms (Miners and Birkett, 1998).

CYP2C9 is considered to be one of the most important drug-metabolising enzymes in humans. Examples of drugs metabolised by CYP2C9 are tolbutamide, fluoxetine, phenytoin and warfarin. CYP2C9 is induced and inhibited by many drugs. Rifampicin and barbiturates are examples of CYP2C9 inducers, while sulphaphenazole is a potent inhibitor. The latter is used *in vitro* and *in vivo* to determine CYP2C9 involvement in metabolism of drugs (Miners and Birkett, 1998). Three different alleles of CYP2C9 have been found, two of which result in decreased metabolism (Oscarson, 2000). The PM phenotype has shown to be less than 1% in Caucasian populations (Miners and Birkett, 1998).

CYP2C19 is involved in the metabolism of S-mephenytoin, proguanil, diazepam and many other commonly used drugs. This CYP2C isoform has been well studied since it is subject to a genetic polymorphism that impairs 4'-hydroxylation of S-mephenytoin. The PMs with respect to this enzyme represent about 2-5% of Caucasians (Nebert et al., 1999), while the incidence in Asian populations has been reported to be up to 23% (Bertilsson, 1995). Seven different mutations that can cause this polymorphism have been registered by February 2000, all of them resulting in no enzymatic activity *in vivo*. The two most common alleles responsible for impaired enzyme activity are *CYP2C19*2* (splicing mutation) and

*CYP2C19*3* (stop codon) (Oscarson, 2000). The latter has only been found in Asian populations (Nebert et al.,1999).

Mephenytoin has been the standard drug in phenotyping for CYP2C19 (urinary ratio between S- and R- enantiomers), but adverse effects and problems with long-term sample stability have reduced its use. Omeprazole has been tested as an alternative to mephenytoin. CYP2C19 phenotype is given by the omeprazole hydroxylation index, which is the ratio between omeprazole and 5'hydroxyomeprazole in serum 2-3 hours after an oral dose of omeprazole. However, the reliability of omeprazole phenotyping in non-healthy individuals, as well as the optimal omeprazole dosing has not been fully investigated. Therefore it remains unclear which drug is the preferred probe for CYP2C19 phenotyping (Streetman et al., 2000).

1.1.6.4 CYP1A2

CYP1 is also involved in xenobiotic metabolism, and the most studied isoform, CYP1A2, accounts for approximately 13% of the total hepatic P450. This isoform of CYP450 is only found in the liver, and its endogenous functions are unclear. Xenobiotics metabolised by CYP1A2 include caffeine, tertiary amines (e.g. amitriptyline, imipramine) and clozapine. It has also been related to the metabolism of polycyclic aromatic compounds, a process associated with cancer (Touw, 1997; Chang and Kam, 1999).

CYP1A2 is highly sensitive to induction (e.g. cigarette smoke). Consequently inter-individual variation in metabolic capacity is seen, but no genetic polymorphisms have been reported (Touw, 1997; Chang and Kam, 1999). Caffeine has been used as a probe substrate to assess metabolic capacity of CYP1A2 (Streetman et al., 2000).

1.1.6.5 CYP2E1

CYP2E1 accounts for 7% of total hepatic P450, and it is involved in the metabolism of many organic solvents and alcohols as well as several structurally diverse drugs. These include paracetamol, many volatile anaesthetics (e.g. diethyl ether) and chlorzoxazone.

CYP2E1 is also toxicologically important since it appears to activate a large number of suspected carcinogens (Chang and Kam, 1999; Touw, 1997; Wrighton and Stevens, 1992).

CYP2E1 activity varies considerably between individuals. One reason for this is induction, and ethanol is known to be a potent inducer. Genetic polymorphism of CYP2E1 has also been reported, and the mutated alleles correlate with increased risk of diseases associated with xenobiotic exposure, such as nasopharyngeal cancer (Daly et al., 1998; Nelson, 2000). The allele frequency within different ethnic populations is yet to be determined (Tanaka, 1999b). Chlorzoxazone is used as a probe to assess CYP2E1 activity, although its use can be questioned since other enzymes may be involved in the metabolism of this drug (Streetman et al., 2000)

1.1.6.6 Substrate specificity and structure-activity relationship

Studies have shown that there are relationships between substrate molecular structure and CYP isoform involvement. It is therefore possible to predict CYP isoform involvement if the molecular structure of a substance is known.
CYP2D6 metabolises drugs with a basic nitrogen atom, which usually contains an aromatic ring. The distance between the basic nitrogen and site of oxidation is 5, 7 or 10 Å. Substrates of CYP2D6 are also relatively hydrophilic (de Groot et al., 1999; Lewis et al., 2000). CYP3A4 substrates are structurally diverse. They generally have a high molecular volume, and are relatively lipophilic. CYP1A2 metabolises planar molecules with medium molecular volume. CYP2E1 substrates are also relatively planar, but a low structural volume characterizes these substrates. CYP2C9 metabolises substrates that are weakly acid and lipophilic, while substrates to CYP2C19 are neutral or weakly basic and moderately lipophilic (Lewis et al., 2000).

1.1.7 Determination of CYP450s

Knowledge about the enzymes involved in a drug's metabolism is important in clinical pharmacology. Interactions with other drugs (i.e. drug-drug interactions) metabolised by

the same enzyme must be avoided if possible, and multi-drug treatment programs are common clinically. The influence of genetic polymorphisms may also need to be taken into account if the involved enzymes are known. Thus severe adverse effects from drugs may be avoided.

Several approaches are used to determine which P450s are involved in the metabolism of a drug. These include *in vitro* studies as well as clinical assessment methods. Standardized *in vitro* techniques for the determination of CYP450 involvement include:

1) correlating the formation rate of the test drug with that of enzyme-specific substrates in well-characterized tissue (for example in a panel of human liver microsomes)
2) selective inhibition or stimulation in enzyme containing tissue (chemical inhibitors or inducers with selectivity for specific enzymes; e.g. ketoconazole inhibits CYP3A4 activity)
3) immunoinhibition (enzyme-specific antibodies used for inhibitory studies)
4) expressed or purified enzymes (individual CYP450 isoforms cloned from yeast, bacterial or insect cells, or purified from human liver cells)

There are advantages and disadvantages in the use of all these methods, and a combined approach is recommended (Halpert et al., 1994; Glue and Clement, 1999).
It is essential to use concentrations of the chemical inhibitors that provide specificity in action, since chemical inhibitors generally lose their specificity at increasing concentrations (Newton et al., 1995). Chemical inhibitors are often used initially, followed by the use of antibodies and expressed enzymes. The latter two provide more accurate information on CYP450 involvement, while an initial chemical inhibition study can help in the selection of antibodies and expressed enzymes. This will be the approach adopted in this project.

Co-administration of known enzyme specific inhibitors can provide clinical information on CYP450 involvement (Glue and Clement, 1999). It has been proposed that clinically significant changes in drug concentrations only occur if the inhibited enzyme is responsible

for elimination of more than 50% of the drug. This does not apply if the therapeutic window of the drug is narrow (Rowland, 1975).

1.2 Opioid pharmacology

1.2.1 Opioids – definition and classification

Opioids are any substance that produces effects similar to that of morphine, and that are blocked by the nonspecific opioid receptor antagonist, naloxone. This includes naturally existing opium alkaloids extracted from the opium poppy (*Papaver Somniferum*), semi-synthetic derivatives structurally related to these (e.g. oxycodone), pharmacologically similar synthetic surrogates (e.g. methadone) and various neuropeptides (e.g. enkephalin) (Rang et al., 1995b; Way et al., 1998).

Opioid drugs include full agonists (e.g. morphine), partial agonists (e.g. pentazocine), antagonists (e.g. naloxone) and mixed agonist-antagonists (e.g. buprenorphine). The latter have different effects depending on the receptor to which they bind.

The main chemical structural group within the opioid class is that with a similar structure to morphine; the phenanthrenes. Besides morphine, this group includes several of the strong and weak opioid agonists commercially used for treatment of pain (e.g. codeine and oxycodone) as well as several opioid antagonists (e.g. naloxone and naltrexone) (Way et al., 1998).

1.2.2 Mechanism of action - opioid receptors

Opioid agonists exert their effects on target proteins referred to as opioid receptors. These receptors are mainly located in the central nervous system (CNS), but a smaller fraction can also be found in certain peripheral tissues. Central effects of opioids are mediated at multiple sites within the CNS. The receptors have been located in the dorsal horn of the spinal cord, in ventral caudal thalamus of the diencephalon, in the periaqueductal grey of the midbrain, as well as in the rostral ventral medulla. Opioids directly influence the transmission of pain and indirectly modulate pain transmission via descending inhibitory pathways (Way et al., 1998).

Three types of opioid receptors are known to exist; μ(mu), δ(delta) and κ(kappa) (IUPHAR classification of opioid receptors). These are themselves heterogeneous, with each of them divided into subtypes (Dhawan et al., 1996).

Opioid receptors are G-protein coupled receptors. Following binding of an opioid agonist to the receptor, adenylate cyclase, a membrane protein that converts intracellular AMP to cAMP (cyclic adenosine-3'5'-monophosphate), is inhibited. This in turn reduces the intracellular levels of cAMP, which either activates K^+-channels or closes voltage-gated Ca^{2+}-channel. Opening of K^+-channels hyperpolarize the neuron and stops the impulse transmission, while suppression of Ca^{2+}-currents reduces neurotransmitter release in presynaptic nerve terminals. Both mechanisms are inhibitory to the transmission of nerve signals, and can affect afferent neurons directly or indirectly. The latter involves an increase of activity in descending inhibitory pathways via interneurons in the brain (Reisine and Pasternak, 1996; Bourne, 1998; Way et al., 1998)

Physiological effects from an opioid or an endogenous opioid peptide depend on their affinity for the different receptors. The antinociceptive effect of the opioids is mediated mainly through the opioid μ-receptor, and the affinity to this receptor is usually related to the analgesic potency of the drug. κ- and δ- receptors can also be involved in analgesia, but to a lesser extent than the μ-receptor and mainly at the spinal level (Rang et al., 1995b). No clinically available drugs act exclusively on the κ- and δ- receptors.

1.2.3 Opioid effects and clinical use

Analgesia, euphoria, sedation and respiratory depression are the principal effects of opioids (Way et al., 1998). These are all central nervous system effects, although the respiratory depression has been proposed to have a peripheral component (Way et al., 1998; Yeadon and Kitchen, 1989; Willette and Sapru, 1982).

Other CNS-mediated effects include nausea, miosis and suppression of the cough reflex. Nausea (often followed by vomiting) is mediated by activation of brain stem chemoreceptors, and it is one of the most common and troublesome side effects after intake

of opioids. Miosis (constriction of the pupils) is an important diagnostic parameter in opioid misuse and overdose.

The most important peripheral effect of opioids is reduction of gastro-intestinal (GI) motility, which results in constipation. This is due to high levels of opioid receptors in the GI tract, but central mechanisms are also involved (Way et al., 1998)

Opioids are primarily used in the treatment of pain, and according to WHO (World Health Organization) recommendations they should be used in pain treatment when non-opioid drugs no longer control the pain adequately (WHO, 1996). Table 2 lists some of the major opioids in use.

Compound (generic name)	Analgesic potency
Morphine	High
Fentanyl	High
Hydromorphone	High
Oxymorphone	High
Methadone	High
Levorphanol	High
Oxycodone	Moderate to high
Pethidine	Moderate
Codeine	Low
Propoxyphene	Low

Table 2 Common opioid analgesics (WHO, 1996; Way et al., 1998)

Morphine is the most commonly used opioid for moderate to severe pain, and as a result of extensive experience with this particular drug it is often used as a reference drug when assessing other analgesic drugs. Previously reported studies on the analgesic activity of opioid agonists (e.g. oxycodone) have usually been comparative studies against morphine.

Opioid drugs have been used for recreational purposes for thousands of years (opium), and misuse remains a problem in modern society. The opioid misuse is reinforced by the high addictive potential of the opioid drugs, and withdrawal syndrome becomes evident in the absence of opioids in addicted individuals. Several opioid drugs (e.g. methadone) are used in the treatment of opioid withdrawal. This therapy is referred to as opioid substitution, and in addition to removing the withdrawal symptoms, it aims to bring the subjects back to a more settled life-style (Ward el al., 1992).

Opioid drugs are also used as cough suppressants (e.g. codeine) and to control diarrhoea (e.g. diphenoxylate) (Way et al., 1998).

1.2.4 Tolerance to opioids and opioid rotation

Variability in patient response to opioid analgesics is common. Occurrence of adverse effects and tolerance is also frequently seen when opioid drugs are used in the treatment of pain (Ashby et al., 1999). Tolerance can occur towards the desired effects (analgesia) as well as the adverse effects; the latter being a favourable situation as the therapeutic window is widened. Analgesic tolerance is apparent when increased doses are needed to maintain the same analgesic potency over time. Escalating doses may result in an increase of side effects and toxic potential. This situation often expedites a change in treatment regimen (Mercadante, 1999).

There is substantial evidence for cross-tolerance between opioids. This is when tolerance develops to one opioid agonist and concomitantly causes tolerance to other opioids (Mercadante, 1999). However, clinical data show that patients can be successfully transferred from one opioid to another, a situation referred to as opioid rotation or opioid substitution (Ashby et al., 1999; MacDonald et al., 1993; Sjøgren et al., 1994; Mercadante, 1999). Opioid rotation can be successful since the cross-tolerance is incomplete.

In conclusion, clinicians need therapeutic options for the management of pain, and another opioid can be an alternative.

1.3 Oxycodone

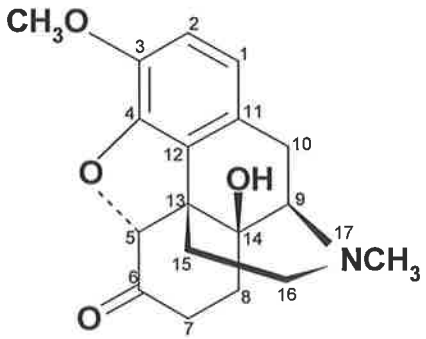
1.3.1 Oxycodone – history and use

Oxycodone (OC) is a semi-synthetic derivative of thebaine, which is a naturally occurring opium alkaloid. Oxycodone has been used since 1917 (Kalso and Vainio, 1990), and it is available both in combination with other analgesics, for example paracetamol (e.g. Percocet ®) or aspirin (e.g. Percodan ®), and as a separate drug (e.g. Endone ®) (Martindale, 1999; MIMS Annual, 2000). It has been recommended as a drug for use in moderate to severe pain (Pöihiä et al., 1993; WHO, 1996).

Oxycodone is most frequently administered in tablet form, but rectal suppositories (e.g. Proladone ®) are also available. A controlled-release formulation of oxycodone (e.g. OxyContin ®) has recently been introduced to the market in Australia. This provides a longer lasting effect and greater administration intervals, which is particularly advantageous for patients with chronic pain (MIMS Annual, 2000; Hagen and Babul, 1997; Kaplan et al., 1998). Parenteral formulations of oxycodone are not available on the market (Martindale, 1999).

1.3.2 Structure and Physicochemical properties

Oxycodone (Figure 2) is usually derived from thebaine, but it can also be synthesized by modification of morphine (Glare and Walsh, 1993).



Oxycodone

Figure 2 Chemical structure of oxycodone (C-atoms numbered)

The chemical name of oxycodone is 4,5α-epoxy-14-hydroxy-3-methoxy-17-methylmorphinan-6-one (Weinstein and Gaylord 1979), but it is also referred to as 7,8-dihydro-14-hydroxycodeinone (Martindale, 1999).
Oxycodone has the formula $C_{18}H_{21}NO_4$ (M_{Wt}=315.36). It is a weak base (pKa=8.5) that exists in two tautomeric forms. Oxycodone is usually administered as the hydrochloride salt, which has the formula $C_{18}H_{22}ClNO_4$ (M_{Wt}=351.82). Oxycodone has also been used as the terephthalate salt and as a pectinate salt in pharmaceutical formulations. Oxycodone in its free base form is soluble in alcohol, chloroform and dichloromethane, but almost insoluble in ether, water and sodium hydroxide (Budavari et al. 1989; Pöihiä et al., 1993; Brauer and Ralfkiær, 1998).

A drug's lipid solubility is an important determinant of its pharmacokinetic characteristics. Rate and extent of absorption from the gut, penetration of the blood-brain barrier and penetration into other organs can all be predicted from the drugs lipid solubility. This parameter is also an important determinant of how the drug should be formulated (oral versus parenteral).
Different approaches have been used to assess the lipid solubility of oxycodone. Pöyhiä and Seppälä (1994) compared the lipid solubility of oxycodone to other opioid drugs using three previously described *in vitro* methods; the shake-flask method for partition of drug between tris-buffer and n-octanol, solubility in human fat, and reversed phase high-performance liquid chromatography (van de Waterbeemd and Testa, 1987; Leo et al., 1971; Rosenberg et al., 1986). All three methods gave the same lipid solubility for oxycodone compared to the other drugs tested. The lipid solubility of oxycodone was shown to be relatively low, and close to that of morphine with the apparent partition coefficients (P_{app}) being 0.7±0.3 and 0.5±0.2, respectively. This is in accordance with Roy and Flynn (1988) and Mahjour et al. (1989) who both found the P_{app} of morphine to be 0.7. Plummer et al. (1990) found the P_{app} of oxycodone and morphine to be 1.7 and 1.0, respectively.

Several other opioids with similar structures to oxycodone (Figure 3) have been well investigated, and predictions about oxycodone pharmacokinetic and pharmacodynamic properties have frequently been made on data from these studies.

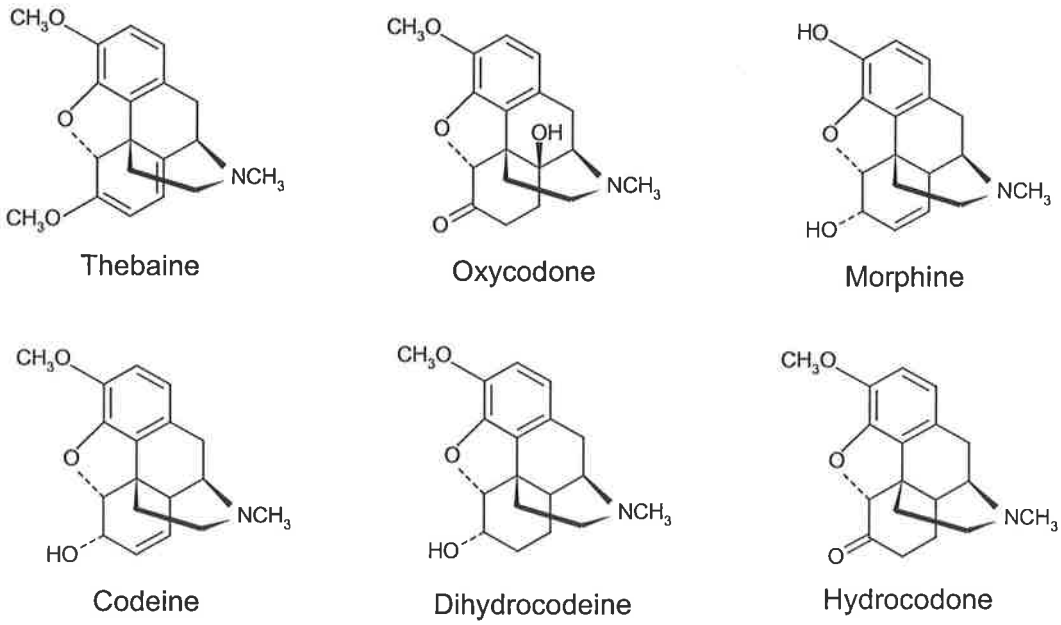


Figure 3 Chemical structures of oxycodone and closely related phenanthrene opioids.

Codeine and morphine are the most studied of these drugs. Codeine has an active metabolite (morphine) that is responsible for the effect of the drug, and a similar mechanism of action has been proposed for oxycodone due to the structural similarities (Sindrup and Brøsen, 1995; Cleary et al., 1994). Details about this will be discussed later in this chapter. Dihydrocodeine and hydrocodone are other opioids with closely related structures. Hydrocodone only differs from oxycodone in the lack of an hydroxyl group at C-14 (Rang et al., 1995b; Reisine and Pasternak, 1996).

1.3.3 Pharmacology of oxycodone

1.3.3.1 Receptor binding

The analgesic effect of opioid drugs has been related to their opioid μ–receptor affinity (see section 1.2.2).

The binding affinity of oxycodone to the opioid μ–receptor has been studied in radio receptor assays. Kalso et al. (1990) found that the μ–receptor affinity of oxycodone is 1/10

compared to that of morphine when ^{3}H-dihydromorphine and ^{3}H-naloxone were used as radio-ligands. Oxycodone μ–receptor affinity compared to that of morphine was only 1/40 in a study by Chen et al. (1991a). They used ^{3}H-DAMGO as the radio-ligand.
Oxycodone binding affinity to δ- and κ-receptors has not been well described in the literature.

1.3.3.2 Animal antinociception

Ross and Smith (1997) showed that the antinociceptive effect of oxycodone in rats involve binding of oxycodone to κ-receptors. Maximum antinociception was seen after 5-7 minutes when oxycodone was administered intracerebroventricularly. In this initial phase the antinociception could be related directly to oxycodone, since first-pass metabolism, and thereby formation of active metabolites, was avoided. The antinociceptive effect of oxycodone was attenuated completely in rats if the specific κ-receptor-antagonist nor-binaltorphimine was pre-administered. In contrast, when the μ–receptor selective antagonist naloxonazine was administered prior to oxycodone, the antinociceptive effect of oxycodone was not attenuated until after the initial phase (15 minutes), and the duration of action was only shortened from 90 to 60 minutes. Interestingly the antinociceptive effect of morphine was unaffected by the κ-receptor-antagonist, whereas complete attenuation of nociception was seen with the μ–receptor antagonist. These results suggest that oxycodone has an affinity to the opioid κ-receptor in rats, and that the μ–receptor binding may not be the only determinant of analgesia. However, the results cannot necessarily be related to humans, and further studies are therefore needed to investigate the relationship between κ-receptor binding and oxycodone antinociception in humans.

1.3.3.3 Physiological effects of oxycodone in man

The effects of oxycodone are the same as for opioids in general (section 1.2.3), with an analgesic potency equivalent to that of morphine (Kalso et al., 1991; Beaver et al., 1978b). The analgesic potency of oxycodone is discussed in detail in section 1.3.5.

Adverse effects of oxycodone seem to be similar, but milder, to those of morphine and other comparable opioids. Leow et al. (1992a/b) found that drowsiness and light-headedness are the most frequently reported side effects. Nausea, vomiting, abdominal pain, urinary retention, dry mouth, headache, nervousness, paresthesia, ear discharge, hypotension and pruritus have also been noted (Leow et al., 1992a/b; Benziger et al., 1997). Curtis et al. (1999) showed that patients receiving oxycodone had a higher incidence of headache comparing with patients on morphine. Another study indicated that constipation might be more frequently seen with oxycodone compared to morphine, and that there is no significant difference in incidence of nausea between the two drugs (Heiskanen and Kalso, 1997). In contrast to this, Pöihiä et al. (1993) found that nausea is not commonly observed. They also reported an equivalent addictive potential to morphine. Furthermore, less delirium is experienced with oxycodone compared to morphine (Maddocks et al., 1996).

1.3.4 Pharmacokinetics of oxycodone

The fate of a drug in the body has an impact on drug effect. It is therefore important to have knowledge about the pharmacokinetic properties of a drug.

1.3.4.1 Bioavailability

One advantage of using oxycodone is its high oral bioavailability. Several studies have investigated the bioavailability of oxycodone after oral and rectal administration.
Beaver et al. (1978a) showed that oxycodone retained at least half of its analgesic potency when it was administered orally compared to equivalent intramuscular injections.
Kalso and Vainio (1990) studied the efficacy of oxycodone and morphine in patients with cancer pain (n=19), and found the assumed bioavailabilities (ratio of oral and intravenous dosing) of oxycodone and morphine to be 58 (38-77) % and 38 (17-54) %, respectively. The latter is in accordance with Osborne et al. (1990) who found that morphine had an oral bioavailability of 19-30 %. Another study, comparing single intramuscular and oral doses of oxycodone, showed that the bioavailability of oxycodone was 60±20 % (Pöihiä et al., 1992). In this study the bioavailability data were based on oxycodone plasma

concentrations. Leow et al. (1992a) also determined bioavailability of oxycodone on the basis of oxycodone plasma concentrations. They found a mean oral bioavailability of 87% in cancer patients (n=12), the highest oral bioavailability reported for oxycodone, but the results showed a substantial inter-individual variability (28-139 %). Finally, a study comparing oral and rectal administration routes to intravenous administration of oxycodone in post-operative patients (n=12) showed a bioavailability of 42±7.5 % and 47±12.0 % for the oral and rectal routes, respectively (Leow et al., 1992b). These results were also based on oxycodone plasma concentrations.

Wide variation is seen in the results from the bioavailability studies on oxycodone. One explanation for this might be that the oral administration is compared to different parenteral routes in the various studies. Intravenous administration provides 100% availability of the dose, while the intramuscular route may deviate from the assumed 100%. Another possible explanation is that different parameters have been used to calculate bioavailability. The most reliable method is to calculate the bioavailability from blood sample data (plasma concentrations). However, some studies base their calculations on subjective reports from the patients (Beaver et al., 1978a) or dose requirements (oral contra parenteral administration) (Kalso and Vaino, 1990). It is unlikely that studies using different parameters will give the same results in the calculation of oral bioavailability.

In summary it can be concluded that the bioavailability of oxycodone is markedly higher than that of morphine. Glucuronidation at C-3 in morphine is important in its first-pass metabolism. It has been suggested that the higher bioavailability for oxycodone is due to methylation at C-3 (Figure 2), a feature that may prevent the molecule from extensive first-pass metabolism (Beaver et al., 1978a).

1.3.4.2 Protein binding and volume of distribution

Leow et al. (1993) studied the serum protein binding of oxycodone and morphine in human plasma using ultrafiltration. They found that albumin was the major binding protein for both drugs, and that the binding was dependent on protein concentration. Moreover they found that the affinity for AAG (α1-acid glycoprotein), an acute phase reactant protein, was higher than for albumin for both drugs. However, the concentration of AAG in serum of a healthy person is much lower than albumin. The binding percentages of oxycodone to

albumin and AAG were calculated to be 31-39 % and 5-10 %, respectively. The total serum protein binding for oxycodone in this study was 45.1±0.4 % at physiological pH and temperature. The drug concentration at therapeutic range (5 – 100 ng/ml) did not influence the protein binding significantly. A decrease in albumin concentration is seen in patients with renal and hepatic failure, while the AAG concentration will be elevated after trauma, surgery and certain disease states. In both cases, an alteration in protein binding of oxycodone would be expected, since the protein binding of oxycodone is dependent on the protein concentration in plasma. Temperature and pH changes appear to have a considerable effect on the serum protein binding. An increase in protein binding could be detected after decreasing the temperature, while lowering the pH resulted in a significantly reduced serum protein binding. The pharmacological action of a drug is related to the unbound fraction of the drug in plasma. However, since the protein binding of oxycodone is considered relatively low (<50%), notable alterations in the pharmacological effects of oxycodone and its pharmacokinetics are not expected in spite of alterations in the degree of protein binding.

Pöyhiä et al. (1991) studied the pharmacokinetics of intravenously administered oxycodone (0.07mg/kg) in post-operative patients (n=9). Volume of distribution at steady state was 2.6±0.5 L/kg (mean±SD). Similarly, Leow et al. (1992b) found the volume of distribution of oxycodone (0.08mg/kg) at steady state to be 2.5±0.8 L/kg after intravenous administration to post-operative patients (n=48). These studies indicate that oxycodone is highly distributed into tissues. This is also the case for morphine that has a volume of distribution between 1 and 6 L/kg in various studies and a protein binding similar to oxycodone (Glare and Walsh, 1991).

1.3.4.3 Clearance and elimination

The elimination of oxycodone is mainly hepatic (Tallgren et al., 1997). Only 8-14% of a single dose is excreted unchanged by the kidneys during the first 24 hours (Pöyhiä et al., 1992). The plasma clearance of oxycodone was found to be 0.78±0.33 L/min in post-operative patients (n=9), which is in accordance with findings in cancer patients (n=12) where the plasma clearance was found to be 0.81±0.44 L/min (Pöyhiä et al., 1991;

Leow et al. 1992a). The clearance of oxycodone is lower than morphine, which has a clearance of 1.2-2.0 L/min (Milne et al., 1996). The renal clearances for oxycodone and morphine are 0.08 and 0.10 L/min, respectively (Pöyhiä et al., 1992; Glare and Walsh, 1991).

There are several studies that have estimated the half-life of oxycodone. Pöyhiä et al. (1992) found the half-life after intramuscular injection of oxycodone in nine healthy volunteers to be 4.89±0.77 (mean±SD) hours, while the half-life after oral administration in the same subject group was 5.12±1.65 hours. Leow et al. (1992b) studied the pharmacokinetics of oxycodone in post-operative patients (n=12 in each group) and found the half-life to be 5.45±1.43 hours after intravenous injection, while for IR (immediate-release)-tablets and rectal suppositories it was 5.65±1.13 and 5.40±1.19 hours, respectively. The pharmacokinetics of oxycodone in cancer patients was investigated in another study by Leow et al. (1992a). The mean intravenous half-life was 3.01±1.37 hours, while the mean oral half-life was 3.51±1.43 hours. Substantial inter-individual variation is seen in all the studies. The half-life also seems to vary slightly between the different administration forms after a single dose, and between healthy subjects, cancer patients and post-operative patients. These variations may be caused by differences in distribution and clearance rates, which are altered by disease, surgery and general anaesthesia (Leow et al., 1992a). In general, dosing is required every 4 hours when oxycodone is administered as IR-tablets, rectal suppositories or intravenous injections. (Leow et al., 1992a; Pöyhiä et al., 1991; Pöyhiä et al., 1992). Table 3a summarizes the studies described above. The half-life of controlled-release (CR) oxycodone is similar to that in immediate release oral formulations, but dosing every 12 hours provides adequate pain control since delayed and higher C_{max} (due to larger dose magnitude) sustain the plasma concentration within the therapeutic range longer (Benziger et al., 1997; Hagen and Babul, 1997; Citron et al., 1998; Kaplan et al., 1998; Bruera et al., 1998).

Table 3a Summary of studies that determine the half-life of oxycodone.

	Intravenous	Intramuscular	Oral (IR)
Healthy voluntaries (n=9)		4.89±0.77 hours	5.12±1.65 hours
(Pöihiä et al., 1992)		(ranges were not	presented)
Post-operative patients (n=12)	5.45±1.43 hours		5.65±1.13 hours
(Leow et al., 1992b)	range: 4.54-6.36		range: 4.93-6.37
Cancer patients (n=?)	3.01±1.37 hours		3.51±1.43 hours
(Leow et al., 1992a)	range: 1.48-5.86		range: 1.87-6.58

1.3.4.4 Metabolism of oxycodone

Oxycodone has several metabolic pathways, although these have not been well described in the literature. Figure 4 shows the structure of oxycodone and its main metabolites.

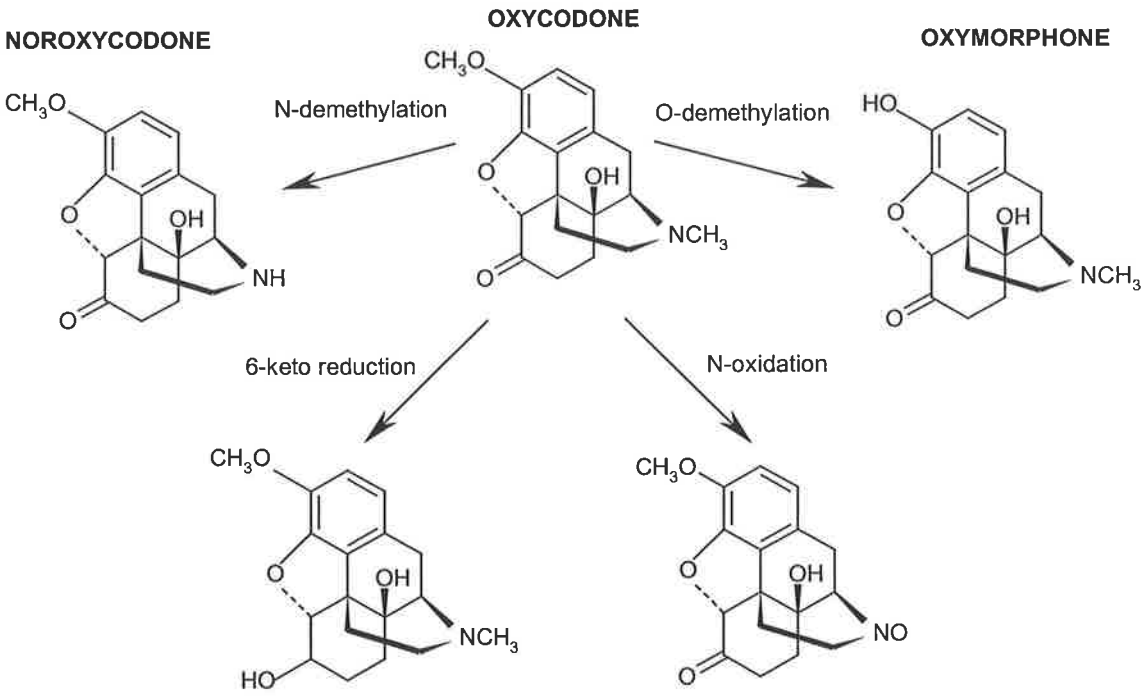


Figure 4 The metabolism of oxycodone

A quantitatively important metabolite of oxycodone is noroxycodone (NOC). Noroxycodone is formed by N-demethylation of oxycodone, and it is important in the first-pass metabolism of oxycodone (Ishida et al., 1979; Ishida et al., 1982; Pöihiä et al., 1992; Pöihiä et al., 1993). Maximum plasma concentrations after a single oral controlled-release dose of oxycodone (20 mg) were found to be 15.2±4.5 ng/ml (Kaiko et al., 1996). Noroxycodone is considered unimportant for the analgesic effect of oxycodone. The antinociceptive potency in rats has been shown to be 0.17 for noroxycodone relative to morphine, while the potency of oxycodone relative to morphine was 0.44 (Leow and Smith, 1994). This indicates that the potency of noroxycodone is less than that of oxycodone itself, and it is therefore unlikely to contribute to the effects of oxycodone.

Another metabolite of great interest is oxymorphone (OM), formed by O-demethylation of oxycodone (Ishida et al., 1979; Ishida et al., 1982; Pöihiä et al., 1993). The amount of oxymorphone formed is small compared to that of noroxycodone. Kaiko et al. (1996) found a maximum plasma concentration of 0.82±0.85 ng/ml when 20 mg controlled-release oxycodone was administered to healthy volunteers. In other studies, the amount of oxymorphone formed was below the limit of quantification (Pöihiä et al., 1991; Pöihiä et al., 1992). Even though the plasma concentration of oxymorphone is low after administration of oxycodone, its presence is considered important. Oxymorphone has high antinociceptive activity and may therefore contribute to oxycodone's analgesic effect. This possible contribution will be discussed in depth in section 1.3.5.

Oxycodone also undergoes N-oxidation and 6-keto-reduction, and finally conjugation of both oxycodone and the metabolites (Ishida et al., 1979; Pöihiä et al., 1993). These pathways are very poorly described in the literature. Pöihiä et al. (1992) found that noroxycodone is mainly excreted as the unconjugated metabolite, while oxymorphone was found mostly as the conjugated form in the urine. The total dose of oxycodone has not been recovered after administration in humans. Studies in other mammalian species indicate significant interspecies differences in urinary and faecal recovery of oxycodone and its metabolites (Ishida et al., 1982), and conclusions on human metabolism and excretion can therefore not be made on the basis of these studies.

1.3.4.5 Enzymes involved in the hepatic metabolism of oxycodone

The enzymes involved in the oxidative metabolism of oxycodone in humans are poorly described. CYP2D6 is involved in the O-demethylation of codeine, dihydrocodeine and hydrocodone, drugs that are structurally similar to oxycodone (Mortimer et al., 1990; Kirkwood et al., 1997b; Hutchinson, 1999). Several studies suggest that CYP2D6 also is of major importance in the formation of oxymorphone from oxycodone (Otton et al., 1993; Heiskanen et al., 1998; Brauer and Ralfkiær, 1998). However, it is not clear whether the O-demethylation of oxycodone is due to CYP2D6 alone, or if there are other enzymes involved.

Otton et al. (1993) used specific and non-specific CYP2D6 inhibitors to investigate the enzyme involvement in the O-demethylation of oxycodone in human liver microsomes. Quinidine, a CYP2D6 specific inhibitor, inhibited the formation of oxymorphone in a concentration-dependent manner when oxycodone was incubated in microsomes from a CYP2D6 EM liver. Fluoxetine, which is a non-selective inhibitor of CYP2D6, showed similar inhibitory effect to quinidine in the same liver. Formation of oxymorphone was also evident in microsomes from a CYP2D6 PM, but the formation was five fold lower than that of the EM. Quinidine had no inhibitory effect on the formation of oxymorphone in this liver. However, they found that fluoxetine inhibited oxymorphone formation in the same PM liver in a similar manner to that seen for the EM. This study showed that CYP2D6 is involved in the O-demethylation of oxycodone. The presence of OM in the PM and the lack of inhibition by quinidine in the PM indicates that enzymes other than CYP2D6 are involved in the formation of oxymorphone in addition to CYP2D6. This activity is inhibited by fluoxetine. Support from studies in other PM livers is needed, since there are intra-phenotype differences in metabolic activity.

Heiskanen et al. (1998) conducted a double-blind crossover study where the plasma oxymorphone concentration was measured over time after administration of 20mg CR-oxycodone with quinidine (200mg) or placebo premedication. Ten healthy volunteers were included in the study, and all the subjects were EM with respect to CYP2D6. Quinidine effectively blocked the formation of oxymorphone, and since the concentration of oxymorphone was unmeasurable in 8 of 10 subjects after quinidine premedication it was concluded that CYP2D6 is the only enzyme involved in oxymorphone formation. The validity of the conclusion can be questioned since only a few values in the presented plasma oxymorphone concentration curve for the placebo group were above the limit of quantification (0.2 ng/ml).

Noroxycodone formation may be dependent on CYP3A4; an assumption made on the basis of studies conducted on structurally similar phenanthrenes. N-demethylation of codeine, dihydrocodeine and hydrocodone are dependent on CYP3A4 (Mortimer et al., 1990; Kirkwood et al., 1997b; Hutchinson, 1999). Brauer and Ralfkiær (1998) also indicated this involvement in preliminary studies on oxycodone.

1.3.5 The analgesic potency of oxycodone

Since the analgesic effect of opioids has traditionally been related to the degree of μ–receptor binding, oxycodone should be less potent than morphine (section 1.3.3.1). However, the analgesic potency of oxycodone has been shown to be the same as for morphine in the treatment of acute postoperative pain, and 0.7 the potency in cancer pain (Kalso et al., 1991; Beaver et al., 1978b). The suggested explanations for this have forked into two main theories. The first theory involves other receptors as responsible for oxycodone's analgesic effect (Ross and Smith, 1997), while the second, and the most supported theory, suggests that oxycodone acts as a prodrug (Cleary et al., 1994).

It has been suggested that oxycodone has an intrinsic antinociceptive effect, and that this is mediated by CNS κ-receptors. Ross and Smith (1997) supported this theory with studies in rats, which have been fully described in section 1.3.3.2. A recent study showed that co-administration of sub-analgesic doses of oxycodone and morphine in rats resulted in marked antinociceptive synergy, and that adverse behavioural effects (e.g. sedation) were reduced compared to equipotent doses of either opioid alone (Ross et al., 2000). Pre-administration of the κ-receptor antagonist nor-binaltorphimine reduced the synergistic antinociceptive effect of oxycodone (40nmol) and morphine (15nmol) (intracerebroventricular dosing) to a level equivalent to that of morphine (15nmol) alone (no more than 20% of the maximum possible effect). No antinociception was found for oxycodone alone at 40nmol, and pre-administration of the μ-receptor antagonist naloxonazine completely abolished the synergistic antinociception of morphine and oxycodone. These data further support the involvement of κ-receptors in oxycodone nociception. However, no studies have investigated κ-receptor involvement in oxycodone analgesia in humans.

When a drug elicits its effect through its metabolite(s) it is referred to as a prodrug. A low receptor affinity of the original drug molecule may therefore not indicate the magnitude of the drug's effects *in vivo*.

Codeine, an opioid recommended for mild to moderate pain, has been used as an example of a prodrug. Codeine has a very low affinity (Ki=248nM) for the μ-receptor, but still induces analgesic effect in most subjects (Chen et al., 1991a; Chen et al., 1991b; Sindrup and Brøsen, 1995). It has been shown that about 10 % of the administered codeine is metabolised into morphine by O-demethylation, and that morphine has a high μ–receptor affinity (Ki=1.2nM) (Adler et al., 1955; Chen et al., 1991a). Therefore it was originally believed that the analgesic effect of codeine is due to formation of morphine. Lack of morphine formation from codeine in subjects that are poor metabolizers with respect to CYP2D6 shows that O-demethylation of codeine is dependent on CYP2D6 (Mortimer et al., 1990; Chen et al., 1991b). Studies in humans have revealed that codeine has no effect on experimental pain in CYP2D6 PMs and CYP2D6 EMs pre-treated with quinidine (CYP2D6 inhibitor) (Sindrup et al., 1990; Caraco et al., 1996a; Sindrup et al., 1992), thus supporting the theory of codeine as a prodrug with morphine as the active metabolite.
Oxycodone is closely related to codeine in chemical structure, and oxymorphone has the same structural similarity to morphine as oxycodone has to codeine (Figure 5).

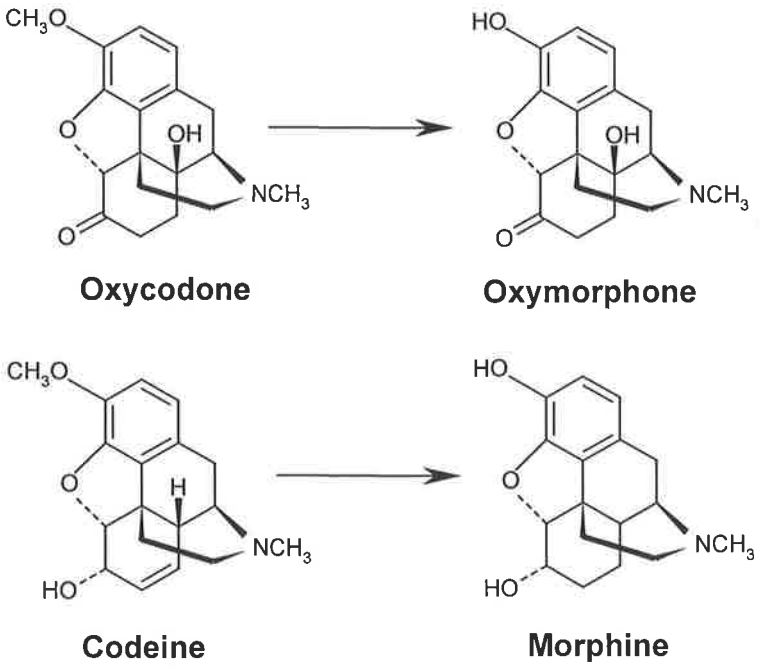


Figure 5 O-demethylation of oxycodone and codeine

A similar relationship to that of codeine and morphine has, as a result of the structural resemblance, been suggested for oxycodone and its O-demethylated metabolite oxymorphone (Cleary et al., 1994). This could be the explanation for the substantial analgesic potency of oxycodone.

The concentration of oxymorphone in plasma after administration of oxycodone is low, but oxymorphone has a μ–receptor affinity 20 times that of morphine and 60 times that of oxycodone (Beaver et al., 1977; Chen et al., 1991a). Oxymorphone might therefore be responsible for the analgesic effect despite the small amount formed.

Several studies in animals and humans have aimed to evaluate the role of oxymorphone in mediating the opioid effects of oxycodone, and some of these studies are described below.

Cleary et al. (1994) studied the analgesic effect of oxycodone and codeine in a rat *in vivo* model. Female Dark Agouti (DA) rats, which lack the CYP2D1 enzyme (the rat's analogue to human CYP2D6), represented the PM, while the Sprague-Dawley (SD) rats having the enzyme present, represented the EM. The analgesic effect of oxycodone and codeine was measured by the "tail-flick-latency-time test". DA rats, and SD rats injected with quinine, a specific inhibitor of CYP2D1, had the same maximum response to oxycodone as SD rats (without quinine), but the effect was prolonged. This indicated that oxycodone itself, rather than oxymorphone, is responsible for the effect of oxycodone. In contrast, codeine proved to be a prodrug dependent on the CYP2D1 pathway since the DA and quinine treated SD rats had a significantly reduced analgesic effect compared to the SD rats (untreated). It was implied that even very low oxymorphone formation could have been enough to produce an analgesic effect after oxycodone treatment due to the high μ–receptor affinity of oxymorphone, but this does not explain the prolonged action of oxycodone in the CYP2D1 impaired rats.

Heiskanen et al. (1998) concluded that the drug effect of oxycodone is probably not mediated by oxymorphone, since pre-administration of the CYP2D6 inhibitor (quinidine) in 10 healthy CYP2D6 EMs did not change drug effects as measured by subjective symptoms, psychomotor function and specific drug effect questionnaire ratings. However, this study only assessed drug effects other than analgesia, and since pain was not present in the subjects the study does not explain the high analgesic potency of oxycodone.

Maddocks et al. (1996) studied the extent of delirium in oxycodone and morphine in 13 cancer patients. It was found that the only CYP2D6 PM in the study did not achieve adequate pain-control after administration of oxycodone (Table 3), and the patient also needed higher breakthrough doses. This indicated that a CYP2D6 dependent pathway is responsible for the analgesic effect of oxycodone. Studies in more patients are needed to decide whether the phenotype of the patient was the reason for lack of analgesic effect. Only one PM was included in the study, and substantial inter-individual variability was seen amongst the EMs.

Table 3b Visual Analogue Scale scores for pain in CYP2D6 EMs and PM (Maddocks et al., 1996).

	Baseline	Steady state	End point (day 6)
Pain EM mean±SD (n=12)	3.0±3.2	1.43±1.65	1.62±1.68
Pain PM (n=1)	1.0	3.25	3.46

Studies in rats showed that the concentration of morphine in the brain was significantly higher than in plasma after administration of codeine (Chen et al., 1990). Similarly, it has been shown in humans that morphine concentration in cerebrospinal fluid is higher than in plasma after codeine intake (Sindrup et al., 1996). This may be due to O-demethylation of codeine in the brain, in close relation to opioid receptors, and it has therefore been suggested that the plasma concentration of morphine after codeine administration may be irrelevant for the analgesic effect of codeine (Chen et al., 1990; Sindrup and Brøsen, 1995; Sindrup et al., 1996). Similar studies with oxycodone have not been published.
In conclusion, the reason for the high analgesic potency of oxycodone in humans is yet not known, and disagreements are seen in the current research.

1.3.6 Summary

Oxycodone appears to have several clinical advantages compared to other similar opioids (e.g. morphine), such as diminished side effects and a high oral bioavailability. Alternatives are needed when selecting analgesics in the treatment of pain, and the advantages provided by oxycodone certainly make it worthy of consideration as a viable alternative to morphine.

The metabolism of oxycodone is poorly described, and the involvement of different P450 isoforms has not yet been systematically tested. This is an area of great interest since pharmacokinetic and pharmacodynamic properties of drugs are altered due to modulation of enzyme activity. First, the influence of genetically or environmentally determined differences in expression of various cytochrome P450 enzymes on oxycodone cannot be predicted without knowledge of which enzymes are involved in oxycodone metabolism. Second, knowledge of enzyme involvement is also needed in order to prevent interactions with other drugs metabolised by the same enzyme(s). And finally, further knowledge about enzyme involvement in oxycodone metabolism may benefit future research on the importance of oxymorphone in mediating oxycodone analgesia.

1.4 Scope of present research

1.4.1 Hypotheses

The following hypotheses were made on the basis of preliminary research on oxycodone and results from research on drugs of similar structure:

- CYP2D6 is the major CYP enzyme involved in the oxidative metabolism of oxycodone to oxymorphone
- CYP3A4 is the major CYP enzyme involved in the oxidative metabolism of oxycodone to noroxycodone

1.4.2 Aims

The aims of the research described in this thesis were:

- to investigate the enzyme kinetics for the formation of noroxycodone and oxymorphone from oxycodone in human liver microsomes
- to identify which liver P450 isoenzyme(s) are involved in these two metabolic reactions

Human liver microsomes from 6 CYP2D6 EMs and 1 CYP2D6 PM were used to study the oxidative metabolism of oxycodone. Formation of oxymorphone and noroxycodone was measured using high performance liquid chromatography (HPLC). Kinetic data were obtained by fitting the Michaelis Menten equation to the metabolite(s) formation data. Chemical inhibitors, antibody inhibitors and expressed enzymes were used when investigating the involvement of specific liver P450 enzymes.

2. Materials and Methods

2.1 Chemicals

Noroxycodone and oxymorphone hydrochloride were obtained from Du Pont Pharmaceuticals, (Wilmington, Delaware, USA). Oxycodone hydrochloride, bovine serum albumin - fraction V (BSA), folin-ciocalteau reagent, diethyldithiocarbamate (DDC), DL-isocitric acid Na_3, isocitrate dehydrogenase (NADP, type IV), sulphaphenazole, troleandomycin (TOA), coumarin, chlorzoxazone, hydromorphone hydrochloride and sodium perchlorate ($NaClO_4$) were purchased from Sigma Chemical Company (St Louis, Missouri, USA). Furafylline and S-mephenytoin were purchased from Ultrafine Chemicals (Manchester, England). Ketoconazole was purchased from Janssen Pharmaceuticals (Beerse, Belgium). Dextromethorphan was obtained from Roche Products Pty Ltd (Sydney, New South Wales, Australia). Quinidine sulfate and NADP-Na_2 were purchased from Merck (Darmstadt, Germany). Sodium carbonate (Na_2CO_3) and potassium di-hydrogen orthophosphate (KH_2PO_4) were obtained from Merck Pty Ltd (Kilsyth, Victoria, Australia). Copper sulfate ($CuSO_4$), dimethyl sulfoxide (DMSO), magnesium chloride ($MgCl_2$), di-sodium hydrogen orthophosphate (Na_2HPO_4), potassium chloride (KCl), sodium hydroxide (NaOH), potassium-sodium tartrate (K^+-Na^+-tartrate), orthophosphoric acid and hydrochloric acid (HCl) were obtained from Ajax Chemicals (Sydney, New South Wales, Australia). Acetonitrile (ACN), dichloromethane (DCM) and methanol came from BDH Laboratory Supplies (Poole, England). Ethylenediaminetetra-acetic acid (EDTA) was obtained from BDH Chemicals Australia (Kilsyth, Victoria, Australia). Carbon monoxide (CO) was obtained from CGI (Adelaide, South Australia, Australia). Sodium dithionate was obtained from May & Baker Ltd. (Dagenham, England). MilliQ water was used in all experiments.

2.2 Human liver microsomes

2.2.1 Liver tissue

Samples of livers had been collected from patients with hepatic tumors who underwent liver resection at the Royal Adelaide Hospital. Ethical approval had been obtained from the Research Ethics Committees of the Royal Adelaide Hospital and the University of Adelaide, and the patients had given their written informed consent for the liver tissue to be studied. Clinical details, demographic data and CYP2D6 genotype/phenotype are tabulated in **Appendix A.**

Seven livers were used in this project (internal numbers: #5, #11, #21, #23, #24, #31 and #35). Samples from the livers used were CYP2C19 genotyped (tested for *CYP2C19*2* and *CYP2C19*3* alleles) in this laboratory using polymerase chain reaction (PCR) (Coller et al., 1997), while CYP2D6 genotype had been determined (tested for *CYP2D6*3* and *CYP2D6*4A/B* alleles) for all the livers by the Institute of Medical and Veterinary Science (Adelaide, Australia) according to an established PCR method (Heim and Meyer, 1990).

2.2.2 Preparation method

Human liver microsomes were prepared after the method described by Zanger et al. (1988). In order to maintain the enzymatic activity, the liver samples had been stored at -80°C, and all preparation steps were carried out at 4°C. Storage on ice was used at all times, and the preparations were always transported on ice.

Frozen liver was cut into small pieces and preparation buffer (1mM EDTA and 0.15M KCl, pH adjusted to 7.3) was added. A mechanical homogenizer (Ultra Turax, Staufen i. Breisgau, Germany) was used to homogenize the liver/preparation buffer mix, and a uniform suspension was obtained after approximately 15 seconds. The suspension was filtered through gauze, and the filtrate was transferred to tubes fitting the rotor head of the centrifuge (Beckman J2-21, Beckman Instruments, Inc., Palo Alto, California, USA). After 15 minutes of centrifugation at 12 000 *g*, a pellet of waste (membranes etc.) was formed at

the bottom of the tube, and fat layer was floating on the top of the supernatant. The supernatant (without the fat) was transferred to new tubes, and centrifuged again for 15 minutes at 27 000 *g* (Beckman J2-21). The supernatant of this was carefully transferred to tubes made for the ultracentrifuge (Beckman L7-55, Beckman Instruments, Inc.), and centrifuged for 60 minutes at 105 000 *g*. This centrifugation splits the cytosol from the membranous parts of the cell, leaving the latter as a pellet at the bottom of the tubes. The pellets were resuspended in wash buffer (0.1M phosphate buffer with 1.0 mM EDTA, adjusted to pH 7.25), and recentrifuged at 105 000 *g* for 60 minutes. The resulting microsomal pellet was resuspended in storage solution (0.1M phosphate buffer with 1.0 mM EDTA, adjusted to pH 7.4). Approximately 1 ml storage solution per 2 grams original tissue weight was used. The microsomes were stored in aliquots of 0.5 ml at -80°C and thawed only once prior to use.

2.2.3 Determination of protein concentration

The protein concentration of the microsomal suspensions was determined after the method described by Lowry et al. (1951).

Six standards of bovine serum albumin (BSA) were prepared in duplicate over a protein concentration range of 0 to 800 µg/ml. The microsome samples were also prepared in duplicate at 1/25 and 1/50 dilutions. The unknown samples and standards were treated the same as follows:

Two milliliters of Solution A (0.5ml 2% $CuSO_4$, 0.5ml 4% K^+-Na^+-tartrate, 49ml 3% Na_2CO_3/0.4% NaOH) were added. The tubes were vortexed and placed on icc for 10 minutes. Two hundred microlitres of Solution B (3ml millipore water, 1.5ml folin-ciocalteau reagent) were added and the samples were then placed on ice in the dark for another 30 minutes. The ultraviolet absorbance of the standards and unknown samples was read at 550nm using a variable wavelength spectrophotometer (Hitachi U-2000, Hitachi Ltd, Tokyo, Japan). The protein concentration of the unknown samples was determined from the standard curve formed from the BSA samples.

2.2.4 Determination of cytochrome P450 content

The cytochrome P450 contents of the microsomal samples were determined after the method of Omura and Sato (1964).

Microsomal suspensions were diluted 1 in 7.5 with 0.1M potassium buffer (pH=7.4), and then bubbled with carbon monoxide at a rate of 1 bubble/second for 30 seconds. Each sample was divided in two cuvettes, and a background scan from 400 to 500nm was performed by the use of a double beam spectrophotometer (Hitachi, model U-2000, Hitachi Ltd., Tokyo, Japan). A few crystals (1-2mg) of fresh sodium dithionite were added to one of the cuvettes. The cuvette was inverted gently to mix before it was replaced in the spectrophotometer and scanned at wavelengths from 400-500nm. Cytochrome P450 content was calculated by using the following formula:

$$Total \cdot CYP450 \cdot content = \frac{(A_{450} - A_{490})after \cdot Na_2S_2O_4 - (A_{450} - A_{490})baseline}{0.106}$$

(0.106 = molar extinction coefficient for CYP450, A= absorption)

Correction was made for dilution and measured protein concentration (2.2.3) to achieve a final value in nmol CYP450/mg protein.

2.3 Oxycodone metabolism studies

2.3.1 Method development

The incubation method used in this study was developed in preliminary studies by Brauer and Ralfkiær (1998). They tested an incubation procedure for oxycodone in human liver microsomes, and a method for extracting oxycodone and its metabolites from the incubation mixture. The methods are similar to previous studies on oxycodone by Otton et al. (1993) and Cleary et al. in this laboratory (unpublished). Kirkwood et al. (1997a) and Foster et al. (1999) also used similar methods for the incubation of dihydrocodeine and methadone in human liver microsomes.

2.3.2 General procedures for microsome incubation

The total volume of the incubations was generally 250µl, except in some of the high substrate concentration samples for the kinetic studies. The incubations were carried out in 10ml flat bottom plastic tubes.

The microsomes were diluted in storage buffer (1mM EDTA, 0.1M Na_2HPO_4, pH=7.4) to a protein concentration of 2.5 mg/ml (50µl in incubation mixture → 0.5 mg/ml). This had been determined in a protein-dependency study (section 2.7.1).

Oxycodone was diluted in incubation buffer (0.1M Na_2HPO_4, pH=7.4) since the enzymatic activity of the microsomes proved to be pH-sensitive in preliminary studies (section 2.7.3).

NADPH regenerating system was made up from isocitrate dehydrogenase (1 mg/ml), NADP-Na_2^+ (10mM), DL-isocitric acid-Na_3 (50mM) and $MgCl_6x6H_2O$ (50mM). MilliQ water was used to dissolve the components. The solution was stored at -20°C in aliquots.

2.3.2.1 Terminating metabolite formation

An alkaline solution (20% Na_2CO_3) was used to stop the reaction. Alkalinisation causes oxidation of the opioid amine moiety, a reaction that is necessary for the opioids to extract into the organic solvent (dichloromethane) (Kirkwood et al., 1997a). Brauer and Ralfkiær (1998) tested the efficiency of 20% Na_2CO_3 in stopping the metabolite formation from oxycodone in human liver microsomes and found that no difference was seen in metabolite formation after the samples had been left for 10 and 30 minutes on ice.

2.3.2.2 Internal standard

Hydromorphone hydrochloride had been selected as the internal standard in preliminary studies by Brauer and Ralfkiær (1998). It did not interfere with any of the peaks, and the recovery was similar to oxymorphone and noroxycodone in the selected organic extraction solvent (dichloromethane). The internal standard was made up as a 10 µg/ml solution in MilliQ water.

2.3.3 Kinetic studies

13 different oxycodone concentrations were incubated with the microsomes in duplicate to determine their Michaelis Menten kinetics.

The samples contained:

- 25µl NADPH regenerating system
- 50µl microsome suspension (2.5 mg/ml protein)
- 175µl substrate

The oxycodone concentrations used ranged between 50µM - 20000µM, which made the corrected concentration in the incubation mixture between 35µM – 14000µM.

2.3.3.1 Incubation method

- 175 µl substrate and 25µl NADPH regenerating system were pipetted into 10ml plastic tubes. The reaction was started by adding 50 µl of the microsome suspension to the tubes
- The tubes were placed in shaking water bath (Julabo SW-20C, Julabo Labortechnik GMBH, Seelbach, Germany) at 37°C for 60 minutes (based on time dependency study – section 2.7.2). The reaction was then stopped by adding 300 µl 20% Na_2CO_3, and the tubes were placed on ice for ten minutes. Internal standard (70 µl) was added separately just after the stop solution.

Exceeding the highest calibration standard for noroxycodone formation made the noroxycodone quantification difficult in three livers. The problem was solved by repeating these particular samples with only half the volumes of all components in the incubation mixture. Another approach to the problem was to half the incubation time. However, the results were better with reduced volumes due to better separation of the peaks when less substrate was present.

2.3.3.2 Extraction method

- 4ml dichloromethane were added to each tube. The tubes were rotary mixed for 5 minutes and then centrifuged at 1700g for 5 minutes
- The aqueous upper layer was removed, since the drug and metabolites now had been extracted into the organic solvent. The organic phase was transferred to new 10ml tubes containing 200µl 0.1M HCl. The tubes were rotary mixed for 5 minutes and then centrifuged at 1700g for 5 minutes.
- The analytes had now been back-extracted into the acid, and 150µl of the acidic layer (appearing as a bubble on top) was transferred to auto-sampler vials.

2.3.3.3 Standards & Quality Controls

The metabolite formation needed to be quantified for Michaelis Menten kinetic determination. A standard curve was therefore needed, and quality controls (QC) in duplicate were made to verify the accuracy and precision of the standard curve.
The standard/QC samples contained:

- 50µl microsome suspension (2.5 mg/ml protein)
- 150µl incubation buffer
- 50µl standard/QC

These samples were incubated and extracted using the same procedure as the unknown incubation samples, except that NADPH was excluded in order to save on this expensive chemical. Brauer and Ralfkiær (1998) showed that omitting NADPH from the standard incubations did not alter the slope for the oxymorphone standard curves significantly. However, a statistically significant alteration of the slope (~11%) was seen for the noroxycodone standard curve when NADPH was excluded from the incubation mixture. In spite of this minor alteration, it had been decided to exclude NADPH since the main focus of the project was qualitative rather than strictly quantitative, and due to the high cost of the NADPH regenerative system. These decisions were also valid for the current project.

Preliminary studies by Brauer and Ralfkiær (1998) indicated that the formation ratio between oxymorphone and noroxycodone is approximately 1:10. From this it could be concluded that the oxymorphone concentration in standards and QCs had to be about 10 times lower than the noroxycodone concentration.
Stock solutions of oxymorphone and noroxycodone for the standards were made up by diluting the hydrochloride salt of oxymorphone in water and the free base of noroxycodone in methanol, to concentrations of 2 mM and 3 mM respectively. The standards were prepared from the fresh stock solutions in a concentration range from 0.334 – 50 µM for oxymorphone and 5 – 500 µM for noroxycodone. Since the standards were diluted by one in five in the incubation mixture, the final concentration ranges were 0.134 – 10 µM for oxymorphone and 1 – 100 µM for noroxycodone, and the assays comprised 8 standard samples. Another higher standard was later added due to excessive formation of

noroxycodone for some livers. This standard was made by doubling the volume of the top standard in the incubation mixture (keeping the total volume the same), and the final concentrations of metabolites were 20μM for oxymorphone and 200μM for noroxycodone.

Quality controls (QCs) were made up from stock solutions prepared in 1998 by PhD student David Foster. These dilutions had been made up from individual weightings, and the concentrations used are listed in Table 4.

	Concentration OM/NOC (μM)
HQC	5/50
MQC	1/10
LQC	0.25/2.5

Table 4 Quality controls (QC) used with calibration curve: concentration of QC in incubation mixture
(HQC=high quality control, MQC=middle quality control, LQC= low quality control)

An analytical run comprised 8-9 standards, 6 QCs (duplicates of HQC, MQC, LQC), and the unknown samples.

2.3.4 Chemical inhibitor experiments

Chemical inhibitors with known CYP specificity were incubated concomitantly with oxycodone in microsomes from three extensive metabolizer livers (HLM#11, #31 and #35) and one poor metabolizer liver (HLM#24) to investigate which CYP isoenzymes are involved in the metabolism of oxycodone. The inhibitors were tested at a fixed oxycodone concentration that had to be in the vicinity of K_M for each of the two metabolic pathways. The rate of formation is linear up to the K_M substrate concentration, and the detection of inhibition or induction will therefore be more accurate. Since NOC and OM formation had highly different K_M values, the experiments had to be carried out twice for each liver. OM and NOC formation were compared to the formation in control samples containing the same solvent concentration as that in the inhibitor samples. All incubations were carried out in duplicate. The inhibitors used are listed in Table 5.

Inhibitor	Abbrev.	Isoenzyme(s)	Concentration (μM)	Solvent
Furafylline	Fura	CYP1A2	100, 25	ACN
Coumarin	Coum	CYP2A6	100	H_2O
Sulphaphenazole	Sulph	CYP2C9	100	DMSO
S-mephenytoin	S-meph	CYP2C19	100	MeOH
Dextromethorphan	Dex	CYP2D6	100	H_2O
Quinidine	Quin	CYP2D6	100, 10, 1, 0.1	H_2O
Diethyldithiocarbamate	DDC	CYP2E1	100	H_2O
Troleandomycin	TOA	CYP3A4	10	MeOH
Chlorzoxazone	Chlor	CYP2E1	100	MeOH
Ketoconazole	Keto	CYP3A4	100, 10, 1, 0.1	MeOH

Table 5 Chemical inhibitors used in the characterisation of CYP450 isoforms involved in OC metabolism. The solvents used to dilute the inhibitors are listed in the right column.

The inhibitors were incubated without OC to determine if the inhibitors themselves or possible metabolites were extracted, and whether they caused any interfering HPLC peaks. DDC and quinidine each had a late eluting peak at about 180 minutes, which made it necessary to have longer HPLC run time for these samples. One inhibitor (omeprazole) had to be excluded due to multiple interfering peaks.

The incubation mixture for the inhibitor studies contained:

- 125μl substrate
- 25μl NADPH
- 50μl inhibitor or 50μl solvent to substitute the inhibitor
- 50μl microsome suspension

The procedures for incubation and extraction were the same as for the kinetic studies, except that furafylline, TOA and DDC had to be preincubated with the microsomes in the presence of NADPH for 15 minutes due to their mechanism of action. These three inhibitors inactivate the enzymes only if they can complex with the isoenzyme in the presence of NADPH (Newton et al., 1995). In these samples the reaction was started with the addition of substrate.

Hickman et al. (1998) and Chauret et al. (1998) have shown that different solvents that are commonly used to dissolve chemical inhibitors can affect CYP mediated metabolic reactions. The control samples were therefore compared to a duplicate that only had incubation buffer in addition to substrate, NADPH and microsomes.

2.3.5 Antibody inhibition studies

Microsomes from three extensive metabolizer livers (HLM#21, #31 and #35) were used to examine the inhibition by monoclonal antibodies on oxycodone metabolism. Monoclonal antibodies to CYP2D6, CYP3A4, CYP1A2 and CYP2E1 (selected on the basis of the chemical inhibitor studies) were obtained from GENTEST Corporation (Massachusetts, USA), and stored at -20°C as recommended by the manufacturer. The protein content of the monoclonal antibodies was 10mg/ml, and the amount used in the incubations ranged from 2.4-8µl/100µg microsomal protein. This was in accordance with the manufacturer's recommendations.

LKM1 (liver/kidney microsomal antibody type 1) positive serum was also examined in all three livers. The main autoantigen for LKM1 is CYP2D6 (Manns et al., 1989), and the serum can therefore be used in inhibitory studies to investigate CYP2D6 involvement in metabolic reactions. The serum was obtained from a patient suffering from autoimmune chronic hepatitis, and the antibody titre in the serum was 64. The amount of serum used in the incubations ranged from 0.2-2µl/50µg microsomal protein.

Oxycodone was incubated at a concentration near the oxymorphone K_M value for formation of the respective liver sample. Oxymorphone and noroxycodone formation were compared to that of a control containing the antibody solvent (Tris buffer for the monoclonal antibodies and control serum from a healthy volunteer for the LKM1 antibodies). All incubations were carried out in duplicate.

2.3.5.1 Incubation procedure for the antibodies

The samples were prepared in Eppendorf tubes due to the small volumes used at the initial stages of the procedure.

- The antibodies were pre-incubated with 50µl microsomes on ice for 10 minutes
- 25µl NADPH regenerating system and necessary incubation buffer were added (total volume of incubation mixture after addition of substrate was 250µl)
- The reaction was started with the addition of 125µl oxycodone (samples shaken softly by hand), and incubation time was as usual 60 minutes
- The reaction was stopped with 300µl 20% Na_2CO_3, and 60µl internal standard was

added before the tubes were vortexed and placed on ice for 10 minutes

- The total volume of the samples was then transferred to 10ml tubes

The extraction procedure was the same as for the kinetic studies (section 2.3.3.2).

2.3.6 Expressed CYP enzymes

Microsomes from human lymphoblastoid cells containing CYP2D6 and CYP3A4, and insect cell supersomes containing CYP2D6, CYP1A2 and CYP2C19 were obtained from GENTEST Corporation (Massachusetts, USA). The enzymes were stored at -80°C according to the manufacturer's recommendations. The enzymes were incubated with oxycodone in duplicate and screened for oxymorphone and noroxycodone formation. Since oxymorphone and noroxycodone formation have different K_M values, the substrate concentration was fixed between the two K_M concentrations at 1000μM. It was decided to keep the amount of CYP450 in the incubation at a constant level (6pmol P450/incubation), although this involved a slight variation in the protein concentrations between the enzymes. This assay was run with a calibration curve and quality controls in order to quantify the metabolite formation.

2.3.6.1 Incubation procedure for the expressed enzymes

- The expressed enzymes were thawed quickly and kept on ice until use
- 125μl oxycodone, 25μl NADPH regenerating system and incubation buffer (at a volume that would make the final volume of incubation mixture 250μl) were pipetted into Eppendorf tubes and pre-warmed in a shaking water bath for ten minutes
- The reaction was started with the addition of expressed enzymes (samples shaken softly by hand)

The rest of the procedure was the same as previously described in the antibody inhibitor studies (section 2.3.5.1).

2.3.6.2 Kinetic studies in expressed enzymes

Kinetic studies of the metabolites formation by CYP3A4 and CYP2D6 lymphoblastoid microsomes and CYP2C19 supersomes were conducted for the purpose of comparing the K_M values for the expressed enzymes with those obtained in the human microsome kinetic studies. The microsomes and supersomes were incubated with varying concentrations of oxycodone (35-525μM for CYP2D6, 525-7000μM for CYP3A4 and CYP2C19), and the protocol was followed as above (section 2.3.2 and 2.3.3).

2.4 High Performance Liquid Chromatography (HPLC) assay for noroxycodone and oxymorphone in microsomes

Reversed phase HPLC was used to quantify oxymorphone and noroxycodone formation. The HPLC method used in this study was a modification of the method used by Hutchinson (1999) to study the oxidative metabolism of hydrocodone.

The HPLC system consisted of a Shimadzu LC-6A liquid chromatograph pump (Shimadzu Corporation, Kyoto, Japan), a Shimadzu SIL-9A autosampler, a Shimadzu C-R6A chromatopac integrator and a Jasco UVIDEC-100-V UV spectrophotometer (Japan Spectroscopic Co. Ltd., Tokyo, Japan).

The stationary phase consisted of a 53.mm x 7mm Platinum EPS C18 100A3U Rocket column and 10mm ALLTIMA C18 5U precolumn (Alltech Associates, Inc, Deerfield, Illinois, USA).

The mobile phase was composed of 1 % acetonitrile, 20 mM KH_2PO_4 and 10 mM $NaClO_4$, and the pH was adjusted to 3.0 with ortho phosphoric acid (10M). The mobile phase was filtered and then degassed by sonication for at least 15 minutes.

The flow rate was 3.0 ml/min and the absorbance was measured at 210nm.
A typical HPLC chromatograph after incubation of oxycodone with human liver microsomes is shown in figure 6.

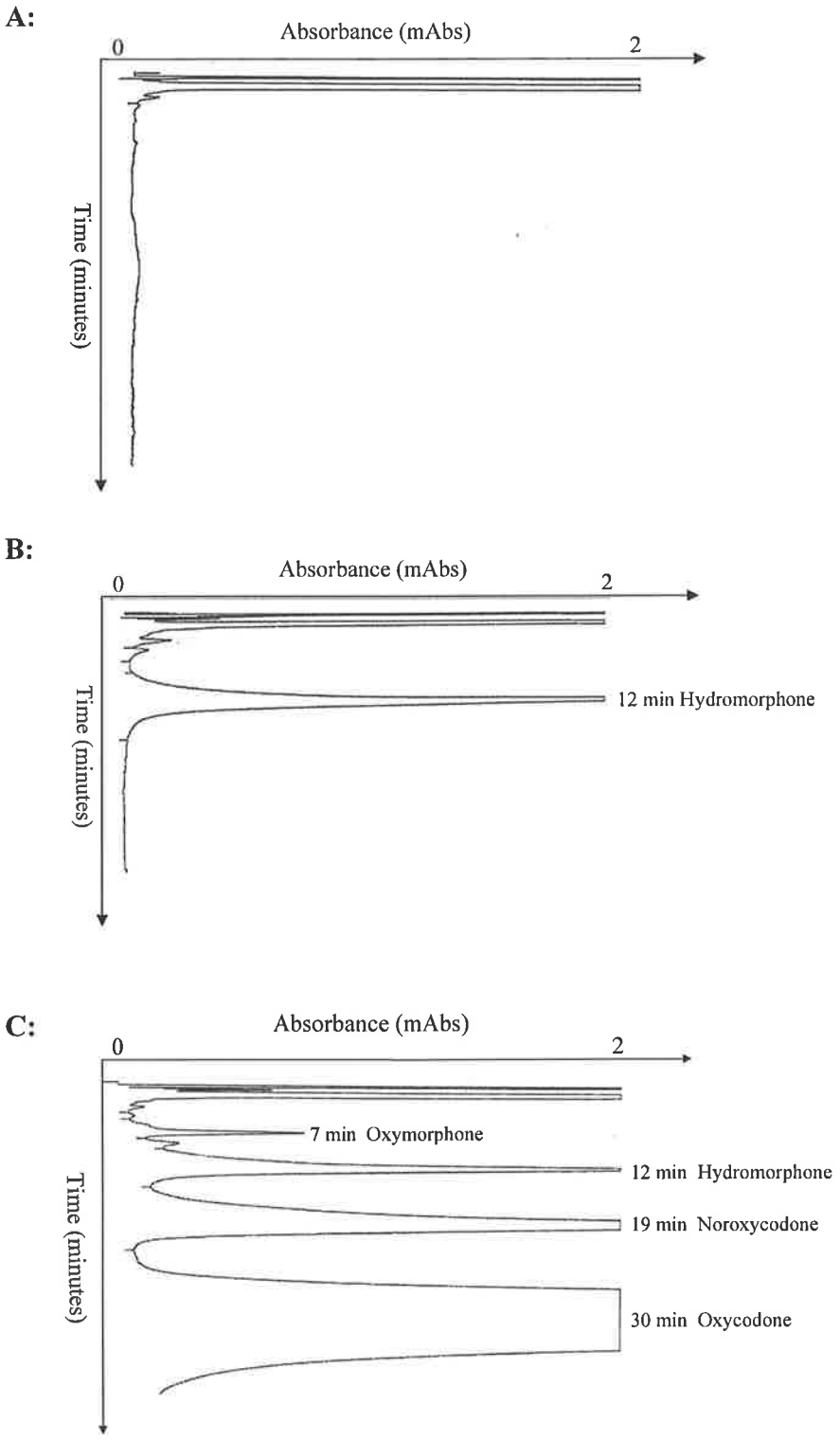


Figure 6 A: HPLC chromatograph from blank HLM extract. B: HPLC chromatograph from extract only containing internal standard. C: Typical HPLC chromatograph following incubation of oxycodone in HLM. OM (7 min), internal standard – HM (12 min), NOC (19 min) and OC (30 min)

2.5 Data analysis

2.5.1 Assay calibration curve

Peak heights obtained from the chromatograms were used for data analysis. Peak height ratios of metabolites to internal standard were plotted against the known metabolite concentration of the standards in order to determine the calibration curves. The slope, intercept and r^2 of the standard curves were then calculated using linear regression analysis (Regression, Blackwell Scientific Publications Ltd., Oxford, England) with 1/y weighting. Percent deviation of standards from line of best fit was calculated using Excel 97 spreadsheet.

2.5.2 Michaelis Menten kinetics

Oxymorphone and noroxycodone concentrations of unknown samples were calculated from the calibration curves using Excel 97 spreadsheet. Velocity of metabolite formation (v) was calculated as a function of metabolite formation (mol), microsomal protein content of incubation mixture and incubation time using the same software.

Eadie-Hofstee plots (v versus v/S) were made using Cricket Graph or Prism 3.02. Visual inspection of these plots was used to assess whether to fit a one or two enzyme model for the Michaelis Menten kinetics.

The Michaelis Menten equation (Regression) was fitted to the kinetic data, and values for V_{max} and K_M could be obtained.

One enzyme model:
$$v = \frac{V_{max} \times [S]}{K_M + [S]}$$

Two enzyme model:
$$v = \left(\frac{V_{max\,1} \times [S]}{K_{M1} + [S]}\right) + \left(\frac{V_{max\,2} \times [S]}{K_{M2} + [S]}\right)$$

v = velocity of metabolite formation
[S] = substrate concentration
V_{max} = maximum velocity of metabolite formation
K_M = Michaelis Menten constant (a measure of the enzyme's affinity for the drug (oxycodone))

Weighting of the data used was not the same in all parts of the study (no weighting, 1/y or 1/y*y weighting). The criteria used to determine the weighting is discussed under the results of the kinetic studies (section 3.3.1 and 3.3.2).

Intrinsic clearance (Cl_{int}) was calculated by the following formula:

$$Cl_{int} = \frac{V_{max}}{K_M}$$

or

$$Cl_{int} = \frac{V_{max1}}{K_{M1}} + \frac{V_{max2}}{K_{M2}}$$

The intrinsic clearance is the liver's ability to metabolise a drug in the absence of restricting factors (liver blood flow and plasma protein binding). Cl_{int} is directly related to the metabolic capacity of the involved enzymes.

2.5.3 Chemical and antibody inhibitor studies

The formation of metabolites in the chemical inhibitor and antibody inhibitor assays was evaluated as percent formation of the respective control samples. The percent variability within the duplicates was calculated, and the data were expressed as the mean±SD of the three livers. Statistical significance of inhibition or induction was tested using a paired, two tailed Student t-test with significance limit set at $p<0.05$.

2.5.4 Expressed enzyme studies

Standard curves as described above were used in the expressed enzyme studies to quantify the metabolite formation. The Michaelis Menten equation was used to generate kinetic profiles and to determine the K_M for the enzymes that showed involvement in an initial screening study. The K_M for the individual enzymes could then be compared to that determined in the microsome kinetic studies. All data are presented as mean±SD, and in some cases median and range. Percent coefficient of variation (%CV) was calculated by dividing the SD by mean.

2.6 Assay validation

2.6.1 Calibration curves

In order to set the acceptance criteria for an analytical run, the method had to be validated prior to the kinetic studies. The standard procedure in the laboratory is to run 6 separate calibration curves with quality controls (QC – 2 each of HQC, MQC, LQC), of which one is more extensive with 6 of each QC and 6 of the lowest standard. The latter was used to calculate the intra-assay variability (precision and accuracy) and limit of quantification (LOQ), while the inter-assay variability (precision and accuracy) was determined from all 6 calibration curves. The following formulae were used to calculate precision (as %CV) and accuracy (%accuracy):

$$\%CV = \left(\frac{SD}{mean \cdot concentration} \right) \times 100$$

$$\%accuracy = \left(\frac{mean \cdot concentration}{nom. \cdot concentration} \right) \times 100$$

(mean·concentration = mean estimated concentration, nom·concentration = nominal concentration)

2.6.1.1 Inter assay validation results

The percent coefficient of variation (%CV) was well below 10 percent for the slope in all calibration curves obtained during the validation. Percent accuracies were lower than 10 percent for of all the QC concentrations (Table 6 and 7). Figure 7 shows examples of calibration curves for noroxycodone (NOC) and oxymorphone (OM) obtained in the assay validation.

Table 6 Inter-assay validation for noroxycodone in human liver microsomes

	Slope	r^2
Mean	0.136	0.997
SD	0.008	0.003
%CV	5.57	0.34
n =	6	6

Nominal conc.	HQC 50μM	MQC 10μM	LQC 2.5μM
Mean conc.	52.7	9.95	2.32
SD	3.43	0.54	0.18
%CV	6.50	5.42	7.62
Mean %accuracy	105.4	99.5	92.8
n =	12	12	12

Table 7 Inter-assay validation for oxymorphone in human liver microsomes

	Slope	r^2
Mean	0.348	0.996
SD	0.016	0.003
%CV	4.60	0.27
n =	6	6

Nominal conc.	HQC 5μM	MQC 1μM	LQC 0.25μM
Mean conc.	5.13	0.99	0.23
SD	0.40	0.07	0.02
%CV	7.72	7.25	9.19
Mean %accuracy	102.6	98.6	91.7
n =	12	12	12

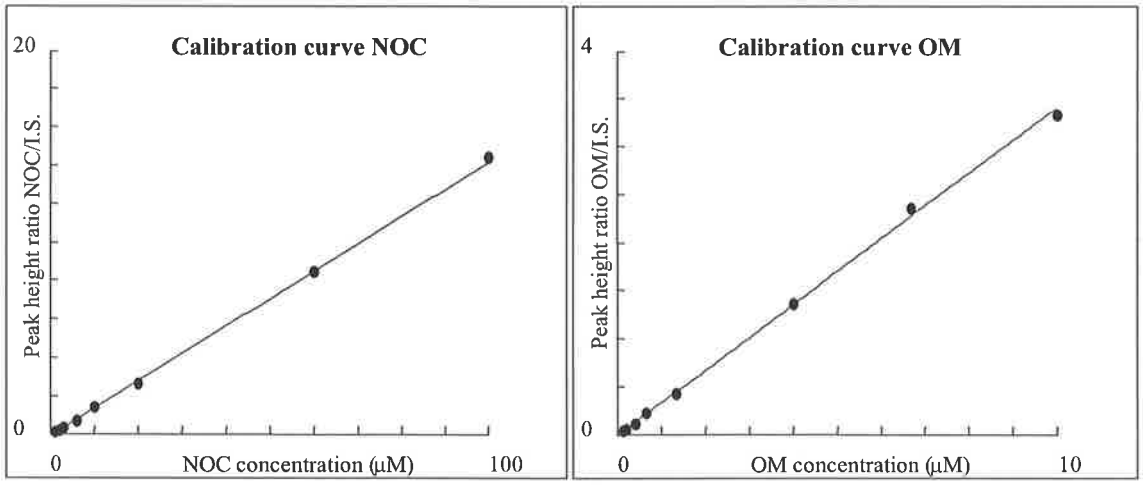


Figure 7 Examples of calibration curves for NOC and OM obtained in the assay validation.

2.6.1.2 Intra assay validation results

All QC concentrations resulted in percent accuracies within 15% (Table 8 and 9).

Table 8 Intra-assay validation for noroxycodone in human liver microsomes

Nominal conc.	HQC 50μM	MQC 10μM	LQC 2.5μM
Mean conc.	55.92	10.29	2.50
SD	2.84	0.35	0.10
%CV	5.08	3.43	4.06
Mean %accuracy	111.8	102.9	99.9
n =	6	6	6

Table 9 Intra-assay validation for oxymorphone in human liver microsomes

Nominal conc.	HQC 5μM	MQC 1μM	LQC 0.25μM
Mean conc.	5.34	1.02	0.26
SD	0.31	0.04	0.04
%CV	5.75	3.64	14.46
Mean %accuracy	106.8	102.2	102.7
n =	6	6	6

2.6.1.3 Limit of quantification (LOQ)

The limit of quantification (LOQ), defined as the lowest standard for each metabolite, showed an inaccuracy of 15.5 and 14.6 % for NOC and 11.8 and 18.5% for OM in the inter-assay and intra-assay validation. The precision was less than 10% for both analytes (Table 10 and 11).

Table 10 Limit of quantification of noroxycodone

Nominal conc.	Interassay Std.8 1μM	Intraassay
Mean conc.	1.16	1.15
SD	0.06	0.08
%CV	5.21	7.09
Mean %accuracy	115.5	114.6
n =	6	6

Table 11 Limit of quantification of oxymorphone

	Interassay	Intraassay
	Std.7	
Nominal conc.	0.134µM	
Mean conc.	0.15	0.16
SD	0.01	0.01
%CV	9.89	8.33
Mean %accuracy	111.8	118.5
n =	6	6

2.6.1.4 Assay acceptance criteria

The assay acceptance criteria were set as a result of the validation, and were in accordance with the standard procedures for the laboratory. To be accepted the calibration curves should have:

- Less than 10 % deviation from line of best fit for standards with exception of the lower two which should be respectively less than 15 % and 20 %. At least 7 of the standards have to be within these criteria.
- Less than 10 % deviation from nominal concentration for HQC and MQC. Less than 20% deviation for LQC. One of each QC has to meet these criteria, and a minimum of 4 QCs in total have to be valid.
- $r^2>0.99$

2.6.2 Recovery study

A recovery study was carried out as part of one extensive calibration curve in the validation study (see section 2.6.1). For all QCs a known volume (3ml, which equals 75 % of the total volume) of the organic solvent was transferred during the extraction procedure. The unextracted QCs were prepared to contain the same concentration as the theoretical concentration of the given volume extracted QCs (75%). The peak heights of the extracted and the unextracted samples could then be compared to determine the percent recovery of each metabolite and the internal standard.

The recovery of noroxycodone and oxymorphone was more than 90%, and did not appear

to be different over the concentration range studied. The recovery of hydromorphone (HM) was lower (mean 75%) than that obtained for noroxycodone and oxymorphone, and was identical to that previously reported by Hutchinson (1999) when using the same extraction method in the study of hydrocodone metabolism. Tables 12-14 show the recoveries obtained for noroxycodone, oxymorphone and internal standard (HM).

Table 12 Percent recovery for Noroxycodone after extraction.

	HQC 50µM	MQC 10µM	LQC 2.5µM	All QCs
Mean	96.4	97.3	88.0	**93.9**
SD	3.34	3.03	3.84	5.41
%CV	3.46	3.12	4.36	5.76
n =	6	6	6	18

Table 13 Percent recovery for Oxymorphone after extraction.

	HQC 5µM	MQC 1µM	LQC 0.25µM	All QCs
Mean	94.2	97.3	92.9	**94.8**
SD	2.46	2.48	14.4	8.23
%CV	2.61	2.55	15.4	8.68
n =	6	6	6	18

Table 14 Percent recovery for internal standard (hydromorphone) after extraction.

	HQC	MQC/LQC	All QCs
	60µl of a 10µg/ml solution		
Mean	76.1	74.7	**75.1**
SD	5.44	2.82	3.78
%CV	7.16	3.77	5.03
n =	6	12	18

HQC samples were injected at a different volume to MQC and LQC, and consequently the peak height was different for the internal standard (hydromorphone) between HQC and MQC/LQC. These are therefore presented in two different columns even though the concentration of internal standard (HM) in the sample should be the same (Table 14).

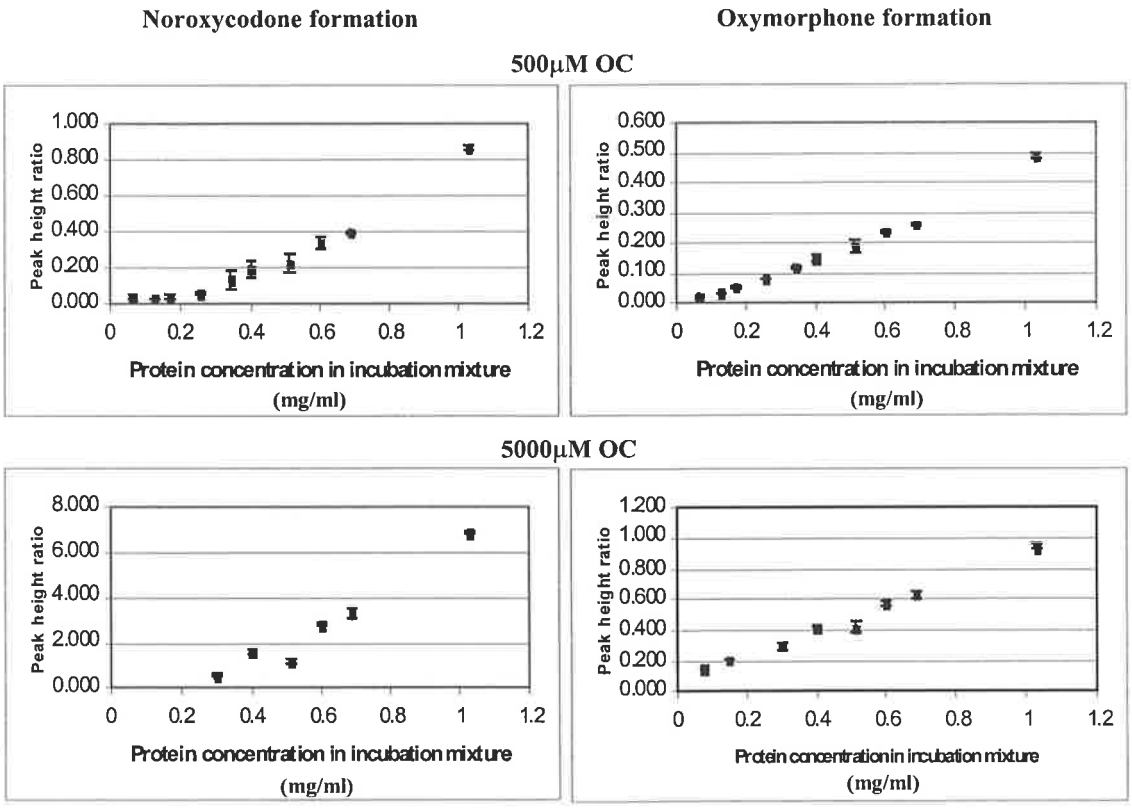
2.7 Kinetic validation

2.7.1 Protein-dependency study

Two different oxycodone concentrations (500 and 5000μM) were incubated with microsomes with protein concentrations varying from 0.064 to 1.026 mg/ml (conc. in incubation mixture). The study was performed in duplicate.

As a result of the protein dependency study 125μg protein/incubation (0.5mg protein/ml) was selected as the optimal protein concentration for further studies. This protein concentration was in the linear portion of the curve relating metabolite formation with protein concentration, and both metabolites were formed at levels that could be quantified accurately from a calibration curve (Figure 8).

Figure 8 Metabolite formation at various microsomal protein concentrations (mg/ml) at two different OC concentrations. Each data point represents mean±SD (n=2).

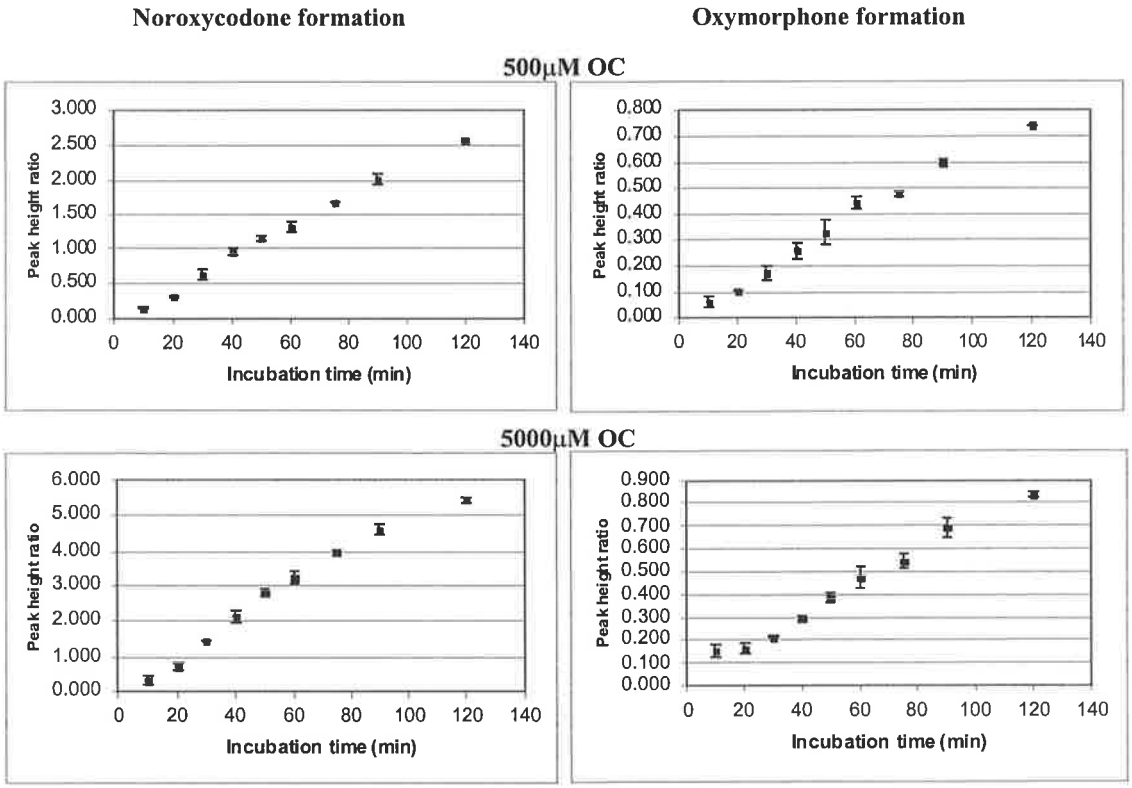


2.7.2 Incubation time-dependency study

Two different oxycodone concentrations (500 and 5000μM) were incubated with microsomes and the incubation time ranged from 10 to 120 minutes. The study was performed in duplicate.

Figure 9 shows the relationship between incubation time and metabolite formation from 10 to 120 minutes. The curve did not clearly plateau before 120 minutes. The incubation time for further incubations was set at 60 minutes since this was well within the linear portion of the curve, and since both metabolites were formed at levels that could be quantified accurately from a calibration curve.

Figure 9 Metabolite formation in human liver microsomes at various incubation times at two different OC concentrations. Each data point represents mean±SD (n=2).

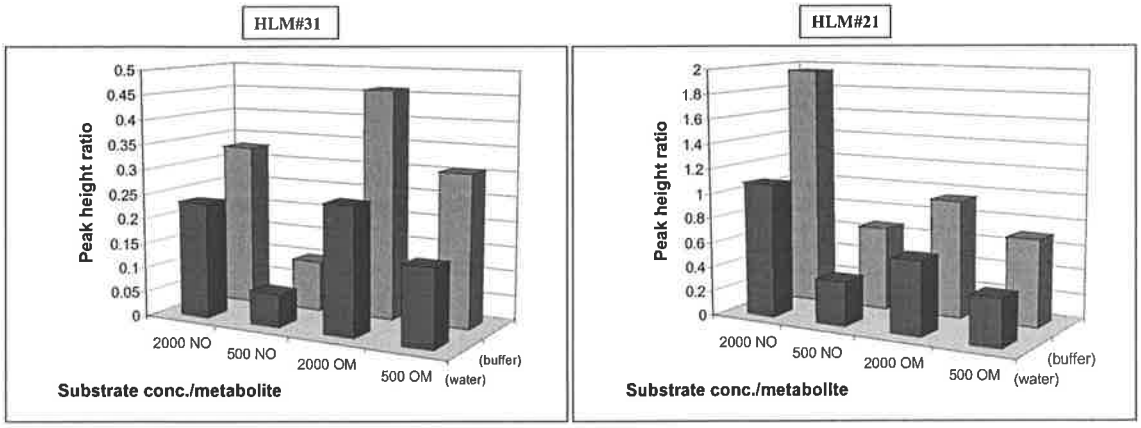


2.7.3 Influence on metabolite formation by substrate solvent pH

Oxycodone was diluted in incubation buffer and in MilliQ water. Both dilutions were incubated with two livers (HLM#21 and #31) at two different oxycodone concentrations (500 and 2000μM) in order to compare the extent of metabolite formation.

Higher formation of both metabolites was observed when oxycodone was diluted in incubation buffer as compared to oxycodone diluted in water (Figure 10).

Figure 10 Comparison of metabolite formation when incubation buffer (pH=7.6) and water (pH=5.7) were used as solvents for substrate (OC). Metabolite formation is expressed as peak height ratio of metabolite to internal standard. Each column represents the mean of duplicate. Microsomes from two different livers were incubated with two OC concentrations.



The pH in both the incubation buffer dilution and in the water dilution was measured, and it was found to be 7.6 and 5.7 respectively. Since the formation of metabolites appear sensitive to substrate solvent pH, it was decided to use oxycodone diluted in buffer since this should protect against any pH changes during the experiment, and since the pH was close to physiological pH.

3. Results

3.1 Assay performance during kinetic studies

It was necessary to increase the volume of internal standard in the kinetic and expressed enzyme studies compared to the validation (70µl versus 60µl). This alteration was made to facilitate internal standard peak detection in the samples that contained high OC concentration during the kinetic and expressed enzyme studies where the volumes injected onto the HPLC system were low (15µl). As a consequence of this internal standard volume change, the mean slope of the standard curves changed to a different value (NOC - 0.089 and OM – 0.251) for the kinetic and expressed enzyme studies compared to the validation data (NOC - 0.136 and OM – 0.348) (2.6.1.1), but the slope changed less than 10 % within these studies. The variability of QC data as precision and accuracy was less than 10 %. In contrast, the lowest NOC standard showed substantial variability, and this standard had to be excluded in four of the assays since it did not meet the assay criteria. However, the formation of NOC was normally well above the lowest standard, and therefore excluding this standard did not prevent quantification of any of the samples. Table 15 and 16 summarize the ongoing assay performance.

Table 15 On going assay performance of noroxycodone

	Slope	r^2
Mean	0.089	0.998
SD	0.007	0.002
%CV	7.85	0.16
n =	10	10

	HQC 50µM	MQC 10µM	LQC 2.5µM
Mean	49.3	9.12	2.42
SD	1.24	0.56	0.19
%CV	2.51	6.14	7.73
%Accuracy	98.7	91.3	96.8
n =	20	20	20

Table 16 On going assay performance of oxymorphone

	Slope	r^2
Mean	0.251	0.999
SD	0.015	0.002
%CV	6.06	0.15
n =	10	10

	HQC 5μM	MQC 1μM	LQC 0.25μM
Mean	4.97	1.02	0.23
SD	0.15	0.04	0.02
%CV	2.92	4.01	8.22
%Accuracy	99.4	102.3	93.3
n =	20	20	20

3.2 Microsome properties and genotype data

All of the prepared microsomes had acceptable protein and P450 concentrations. Table 17 summarizes the properties and the CYP2D6 and CYP2C19 genotype data of the microsomes used in this project. The patient data are also presented individually in Appendix A.

Table 17 Human liver microsome data.

HLM internal no.	CYP2D6 genotype	CYP2C19 genotype	Protein content ± SD (mg/ml)	P450 content (pmolP450/mg protein)
#5	*1/*1(EM)	*1/*2(EM)	4.97±0.24	598
#11	*1/*1(EM)	*1/*2(EM)	6.29±0.08	540
#21	*1/*4(EM)	*1/*1(EM)	3.71±0.23	305
#23	*1/*1(EM)	*1/*1(EM)	3.68±0.07	154
#24	*4/*4(PM)	*1/*1(EM)	15.10±0.04	335
#31	*1/*1(EM)	*1/*1(EM)	10.26±0.73	221
#35	*1/*1(EM)	*1/*1(EM)	7.15±0.15	211

(phenotype in brackets).

3.3 Kinetic studies

The kinetics of NOC and OM formation were determined for 6 CYP2D6 extensive metabolizers and 1 CYP2D6 poor metabolizer (Table 17). The kinetic profiles and Eadie-Hofstee plots for all livers are shown in Appendix B.

3.3.1 Oxymorphone formation

The Eadie-Hofstee plots were curvilinear for all livers (including the PM liver), indicating involvement of two or more enzymes (Figure 11 and 12). The two enzyme Michaelis Menten model were therefore fitted to the data. The data from HLM#11 and #23 only fitted the Michaelis Menten model with 1/y*y weighting. This weighting was therefore chosen for all the OM kinetic data for consistency through the results. Sum of squares and coefficient of determination (r^2) in the remaining HLM did not change substantially with the use of 1/y*y compared to the results obtained with 1/y weighting.

The Michaelis Menten plots did not plateau in all livers, indicating that OM formation had not saturated within the OC concentration range used (e.g. PM in Figure 12). Several of the values for K_{M2} were also above the top OC concentration (20mM) (Table 18), which makes these estimates inaccurate. Ideally higher OC concentrations should be used to achieve saturation of OM formation, but this was not possible since the OM and NOC peaks obtained in the HPLC analysis could not be resolved at OC concentrations higher than 20mM.

It was evident from the mean intrinsic clearance values obtained from the EMs that the high affinity enzyme involved in the formation of OM was responsible for 94-98% of the overall OM formation in the EMs.

K_M values obtained for liver #24 (PM) were within the range of those from the EMs. However, the maximum velocity of metabolite formation (V_{max1}) and intrinsic clearance (Cl_{int1}) for the high affinity enzyme were substantially lower in the PM (Table 18).

Figure 11 shows a typical kinetic profile and Eadie-Hofstee plot of OM formation in an EM, while Figure 12 shows the results from the PM. Table 18 summarizes the kinetic data for OM formation for all livers.

Figure 11 Typical kinetic profile and Eadie-Hofstee plot of OM formation in CYP2D6 EM liver.

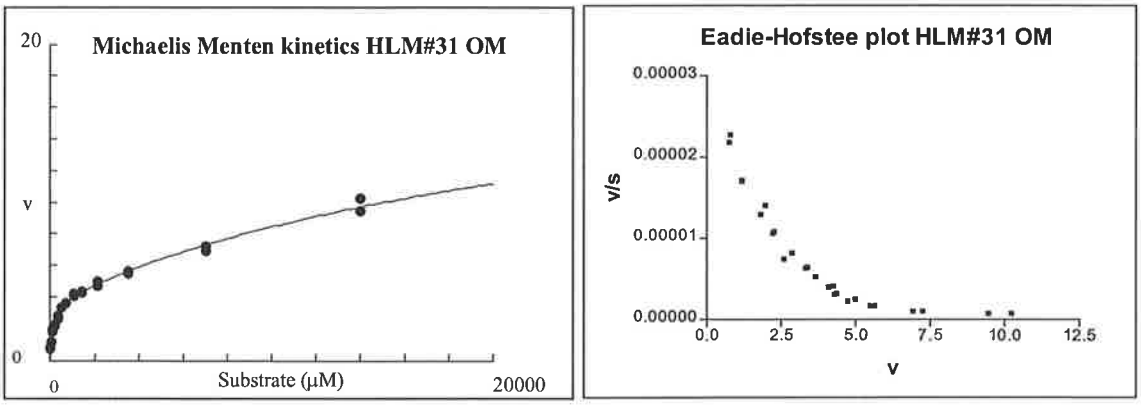


Figure 12 Kinetic profile and Eadie-Hofstee plot of OM formation in CYP2D6 PM liver.

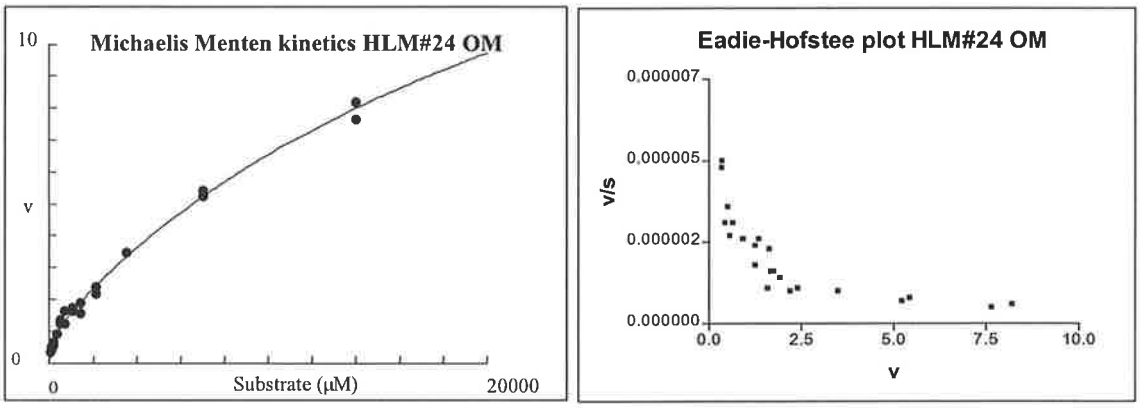


Table 18 Michaelis Menten kinetics of Oxymorphone formation in 6 EM and 1 PM livers.

HLM	V_{max1} (nmol/mg/hr)	K_{M1} (μM)	Cl_{int1} (μl/hr/mg)	V_{max2} (nmol/mg/hr)	K_{M2} (mM)	Cl_{int2} (μl/hr/mg)
#5	3.99	83.7	47.7	12.3	3.81	3.22
#11	6.46	258	25.1	48.9	46.1	1.06
#21	5.30	79.0	67.1	12.8	3.99	3.21
#23	11.4	228	50.0	81.9	89.6	0.91
#31	3.79	153	24.8	17.4	27.1	0.64
#35	2.42	57.2	42.3	12.8	5.17	2.47
Mean	5.56	143	42.8	31.0	29.3	1.92
±SD	3.17	84.1	16.1	28.7	34.1	1.19
%CV	57.1	58.9	37.7	92.4	116	61.9
#24-	1.03	217	4.73	20.3	26.8	0.76

3.3.2 Noroxycodone formation

The Eadie-Hofstee plots were linear for most livers, indicating involvement of only one enzyme. The one enzyme Michaelis Menten model was therefore fitted to the data. Liver #24 (PM) gave results that were within the range for the EMs. Liver #23 and #31 had Eadie-Hofstee plots that were slightly curvilinear, and for these two livers both the one and the two enzyme model fitted the data adequately. The one enzyme model was used since there was no substantial difference in the fit. Liver #35 appeared to have an Eadie-Hofstee plot that curved back on the v/s axis, but no other model than the one enzyme Michaelis Menten model fitted the data.

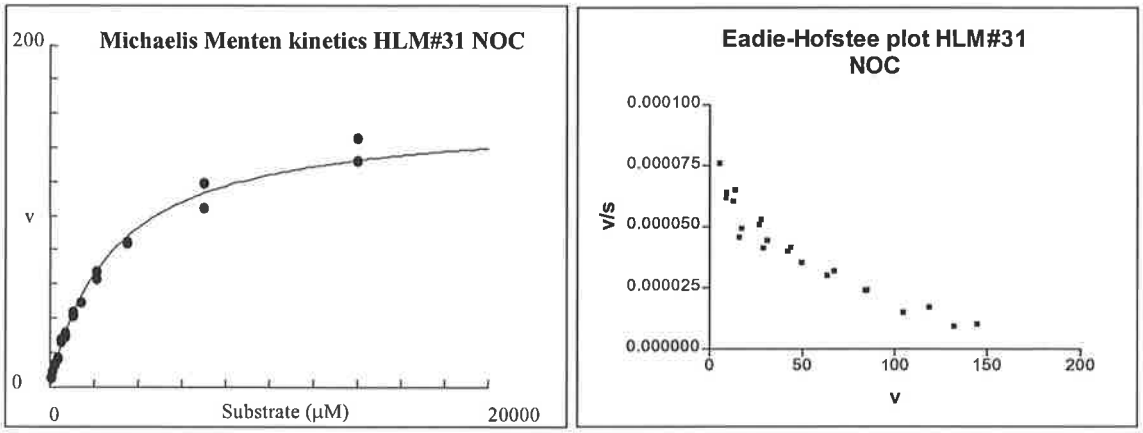
Table 19 summarizes the kinetic data of NOC formation for all livers, while Figure 13 A shows a typical kinetic profile and Eadie-Hofstee plot of NOC formation.

Table 19 Michaelis Menten kinetics of Noroxycodone formation in 6 EM livers and 1 PM liver.

HLM	V_{max} (nmol/mg/hr)	K_M (mM)	Cl_{int} (µl/hr/mg)
#5	7412	12.9	575
#11	1825	2.52	724
#21	939	2.67	352
#23	280	2.98	94
#31	160	2.88	55
#35	432	3.05	142
#24-PM	493	1.52	325
Mean	1649	4.07	324
±SD	2603	3.92	252
%CV	157	96.4	78.0
Median	493	2.88	325
Range	160-7412	1.52-12.9	55-724

Due to problems with substantially higher formation of NOC in some of the livers, the weighting of the data when performing the regression analysis varied. In HLM #5, 11 and 21 no weighting was better than 1/y and 1/y*y since the top six concentrations of OC had been incubated with alterations to the method (section 2.3.3.1), and in different assays than the lower concentrations. The fitting of the data for these livers was not as accurate as the data obtained from the livers where the procedures were kept consistent and within one assay for all OC concentrations, although they met the assay validation criteria.

A:



B:

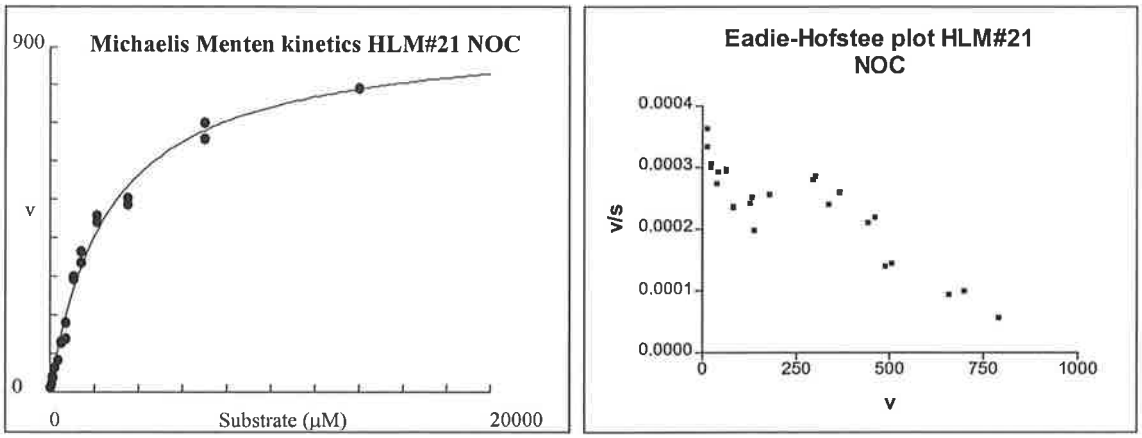


Figure 13

A: Typical kinetic profile and Eadie-Hofstee plot of NOC formation.

B: Kinetic profile and Eadie-Hofstee plot from liver where the top 6 OC concentrations had been incubated in separate assay.

A discontinuity was seen where the curve transferred from data obtained from two different assays, and the error was magnified in the Eadie-Hofstee plots (Figure 13 B). However, the error appeared as a fixed error since the slopes of the Eadie-Hofstee plots were consistent between the two halves of the curves. The kinetic studies in HLM #23, 31, 35 and 24 had been carried out with no alterations to the method, and for these livers 1/y weighting of the data resulted in the best fit.

The results from HLM#5 were inconsistent with the results obtained from the rest of the livers. The K_M value was more than four times higher for HLM#5 compared to the highest K_M calculated for the other livers.

3.3.3 Total intrinsic Clearance

Substantial variability was seen for the total intrinsic clearance of OC when the individual Cl_{int} values for OM and NOC were summed (Table 20).

Table 20 Total intrinsic clearance values of OC to OM and NOC (µl/hr/mg). OM high = high affinity enzyme involved in OM formation. OM low = low affinity enzyme involved in OM formation.

Pathway	#5	#11	#21	#23	#31	#35	#24	Mean±SD
NOC	575	724	352	94.1	55.4	142	325	
OM high.	47.7	25.1	67.1	50.0	24.8	42.3	4.73	
OM low	3.22	1.06	3.21	0.91	0.64	2.47	0.76	
Total Cl_{int}	626	750	422	145	81	187	330	363±252

NOC formation contributed more to the total Cl_{int} than OM formation (Table 21). Contribution to the total intrinsic clearance of oxycodone from the individual O- and N-demethylation pathways are listed in Table 21 and illustrated in Figure 14.

Table 21 Percent contribution to the total oxidative metabolism by the NOC and OM (high and low affinity enzyme) pathways.

Pathway	#5	#11	#21	#23	#31	#35	#24	Mean EM
NOC	91.9	96.5	83.3	64.9	68.5	75.9	98.3	87.9
OM low	0.5	0.1	0.8	0.6	0.8	1.3	0.2	0.5
OM high	7.6	3.3	15.9	34.5	30.7	22.7	1.4	11.6

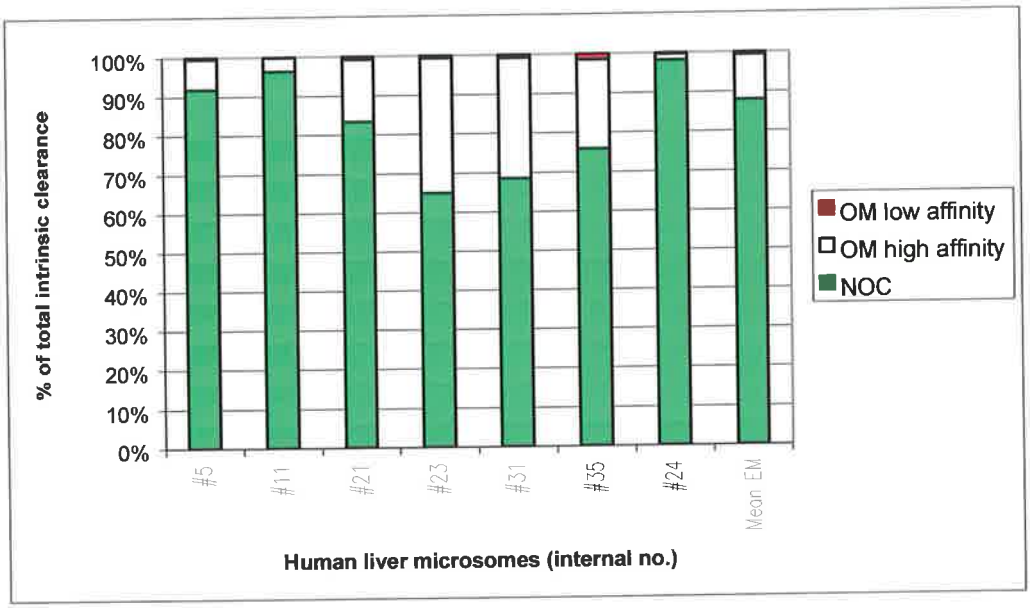


Figure 14 Extent of involvement of different metabolic pathways in OC oxidative metabolism.

3.4 Chemical inhibition studies

3.4.1 CYP isoform specific chemical inhibitiors

3.4.1.1 Oxymorphone formation

Figure 15 and Table 22 show the results from the chemical inhibitor studies in three CYP2D6 EMs when the OC concentration was similar to the K_{M1} of OM. Each bar in Figure 15 represents three livers (mean±SD). The variation within all duplicates was less than 10%.

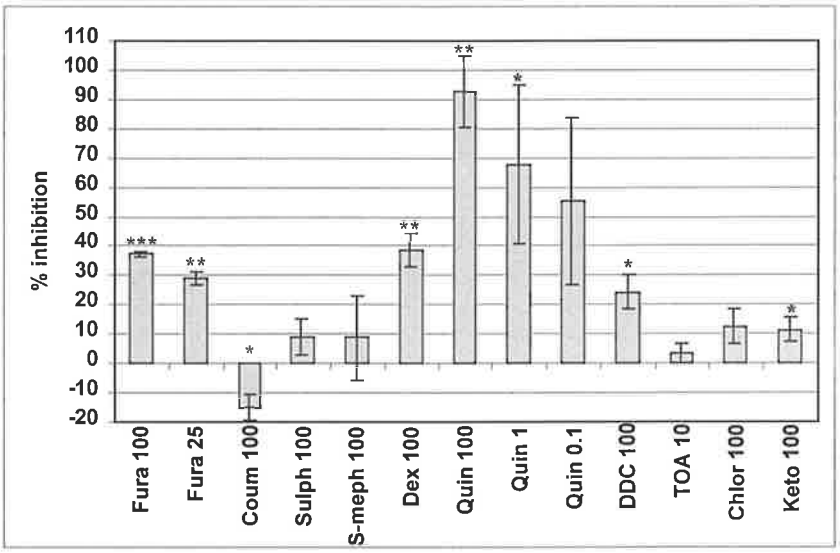


Figure 15 Percent inhibition of OM formation by CYP isoform specific chemical inhibitors (compared to control samples) in CYP2D6 EMs (n=3). Each bar represents the mean inhibition in three livers, while the error bars refer to SD. Statistical significance is indicated with * for p<0.05, ** for p<0.01 and * for p<0.001.**

Sulphaphenazole (p=0.129), chlorzoxazone (p=0.065), TOA (p=0.227) and S-mephenytoin (p=0.393) did not inhibit OM formation (p>0.05).

OM formation was inhibited by quinidine (CYP2D6 inhibitor) in a concentration-dependent manner, although the SD between the three livers was large. The variability was mainly due to substantially less inhibition in HLM#11 (Table 22). Dextromethorphan, another CYP2D6 inhibitor, was also responsible for a significant (p=0.007) inhibition of OM formation (38%).

Table 22 Percent inhibition of OM formation by CYP isoform specific inhibitors in the individual livers tested.

	% Inhibition				
Inhibitor μM	HLM#11	HLM#31	HLM#35	Mean	±SD
Furafylline 100	+38.1	+36.6	+37.0	+37.2	0.78
Furafylline 25	+26.5	+31.0	+29.2	+28.9	2.28
Coumarin 100	-11.2	-13.9	-19.7	-14.9	4.34
Sulphaphenazole100	+14.8	+2.42	+9.81	+9.00	6.20
S-mephenytoin 100	+24.9	+1.63	-0.30	+8.74	14.0
Dextromethorphan 100	+32.0	+42.6	+40.8	+38.5	5.69
Quinidine 100	+78.7	+100	+100	+92.9	12.3
Quinidine 1	+37.4	+76.5	+89.2	+67.7	27.0
Quinidine 0.1	+22.3	+70.0	+73.3	+55.2	28.5
DDC 100	+27.1	+28.2	+17.4	+24.3	5.94
TOA 10	+4.76	-0.48	+5.94	+3.41	3.42
Chlorzoxazone 100	+9.05	+19.2	+9.19	+12.5	5.81
Ketoconazole 100	+14.1	+13.6	+6.53	+11.4	4.22

Furafylline, which is known to inhibit CYP1A2, inhibited formation of OM by 37% in the EMs when the inhibitor concentration was 100μM ($p=0.0001$). The inhibition of OM formation was still around 29% when the furafylline concentration was reduced to 25μM ($p=0.002$). At this concentration, furafylline is highly specific towards CYP1A2.

DDC (100μM) inhibited OM formation by 24%, and the inhibition was significant ($p=0.019$). The formation of OM was also inhibited by ketoconazole. Although the inhibition was low (11%), statistical significance was seen ($p=0.043$). Induction of OM formation was seen with coumarin (15%), and the effect was statistically significant with $p=0.027$.

The chemical inhibitor studies were also carried out at a concentration near the K_M for NOC for the purpose of investigating NOC formation. The results for OM from these studies are supportive of the results described in this section, and can be found in Appendix C.

The results from the chemical inhibitor study in the CYP2D6 PM were inconclusive. OM formation was very low, and it was therefore difficult to detect the difference between the control and the inhibitor samples accurately. Substantial variability within the duplicates was also noted. The results are presented in Appedix C.

3.4.1.2 Noroxycodone formation

Figure 16 and Table 23 show the percent inhibition of NOC formation in the chemical inhibitor studies when the OC concentration was similar to the K_M of NOC. Each bar in Figure 16 represents three CYP2D6 EM livers (mean±SD), except for quinidine 10μM and ketoconazole 10μM where only two livers were used. The variation within all duplicates was less than 10%.

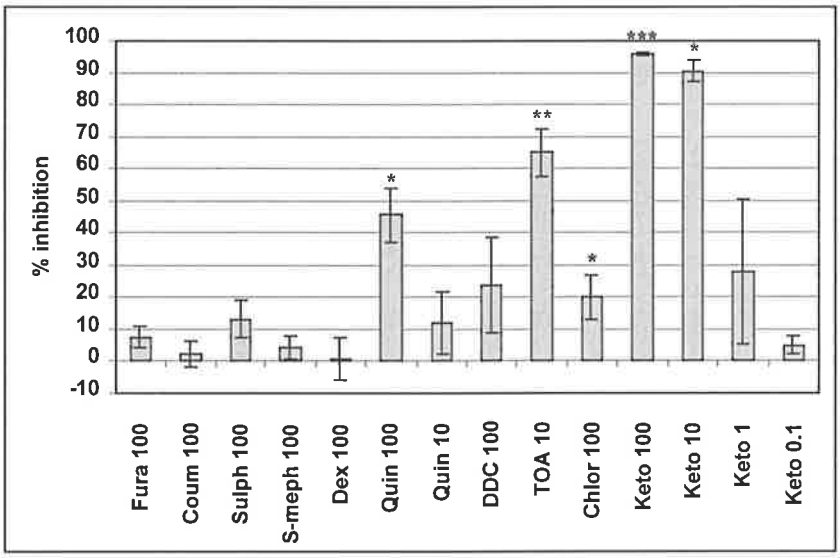


Figure 16 Percent inhibition of NOC formation by CYP isoform specific chemical inhibitors (compared to control samples) in CYP2D6 EMs (n=3, Quin 10μM and Keto 10μM n=2). Each bar represents the mean inhibition in three(/two) livers, while the error bars refer to SD. Statistical significance is indicated with * for p<0.05, ** for p<0.01 and * for p<0.001.**

Furafylline (p=0.061), coumarin (p=0.422), sulphaphenazole (p=0.063), S-mephenytoin (p=0.189), dextromethorphan (p=0.848) and quinidine at 10μM (p=0.328) did not significantly inhibit NOC formation (p>0.05).

Ketoconazole, a potent and selective CYP3A4 inhibitor, inhibited NOC formation in a concentration-dependent fashion. At an inhibitor concentration of 100μM, NOC formation was almost nonexistent. TOA, another CYP3A4 inhibitor, resulted in 65% inhibition at an inhibitor concentration of 10μM. Quinidine reduced NOC formation to 55% of the control at an inhibitor concentration of 100μM, while the formation at quinidine 10μM was almost

90 %. Chlorzoxazole also inhibited NOC formation significantly (20±7 % inhibition, p=0.038).

Table 23 Percent inhibition of NOC formation by CYP isoform specific inhibitors in the individual livers tested.

	% Inhibition				
Inhibitor μM	HLM#11	HLM#31	HLM#35	Mean	±SD
Furafylline 100	+7.74	+3.93	+10.5	+7.40	3.32
Coumarin 100	+6.54	-1.47	+1.90	+2.32	4.02
Sulphaphenazole100	+7.06	+13.3	+19.1	+13.2	6.00
S-mephenytoin 100	+8.38	+1.01	+3.39	+4.26	3.76
Dextromethorphan 100	-6.83	+4.09	+5.25	+0.84	6.66
Quinidine 100	+37.0	+46.0	+53.9	+45.7	8.43
Quinidine 10	-----	+18.7	+5.21	+12.0	9.57
DDC 100	+22.5	+9.85	+39.2	+23.8	14.7
TOA 10	+70.6	+56.7	+68.2	+65.2	7.42
Chlorzoxazone 100	+27.9	+15.0	+17.0	+19.94	6.92
Ketoconazole 100	+95.2	+96.2	+95.9	+95.8	0.51
Ketoconazole 10	-----	+88.2	+92.8	+90.5	3.28
Ketoconazole 1	+4.59	+29.2	+49.4	+27.7	22.4
Ketoconazole 0.1	+2.48	+7.95	+4.37	+4.93	2.78

The NOC data obtained from the chemical inhibitor studies where OC was incubated at a concentration near the K_{MI} of OM were also interpreted. These results can be found in Appendix C. They are supportive of the results described in this section and also include one CYP2D6 PM.

3.4.2 Solvent inhibition

3.4.2.1 Oxymorphone formation

Figure 17 shows the effects of solvents used in the chemical inhibitor studies on OM formation when the OC concentration was similar to the K_{MI} of OM. Each bar in Figure 17 represents the mean of three CYP2D6 EM livers, while the error bars represent the SD between the livers. Percent variability within the solvent duplicates was less than 10%.
Methanol (MeOH) inhibited the formation of OM significantly ($p<0.05$), while induction of OM formation was observed in the presence of 0.83 % DMSO ($p<0.01$). ACN showed significant ($p<0.05$) induction at a concentration of 2.5 %, but insignificant induction and

substantial variability was seen for ACN at 10 %. Appendix C holds the results from the study where OC concentration was near the K_M of NOC. These results were similar, but DMSO induction could not be seen.

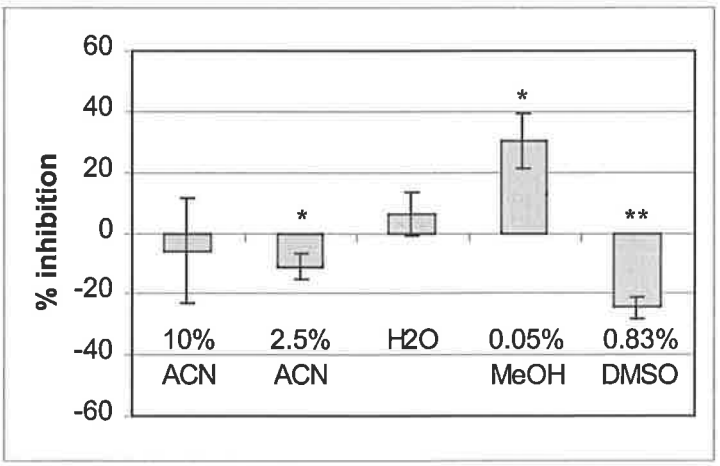


Figure 17 Inhibition of OM formation by solvents used in the chemical inhibitor studies (n=3).

3.4.2.2 Noroxycodone formation

Figure 18 shows the effects of solvents used in the chemical inhibitor studies on NOC formation when the OC concentration was similar to the K_M of NOC. Each bar in Figure 18 represents three CYP2D6 EM livers (mean±SD). Percent variability within all the solvent duplicates was less than 10 %.

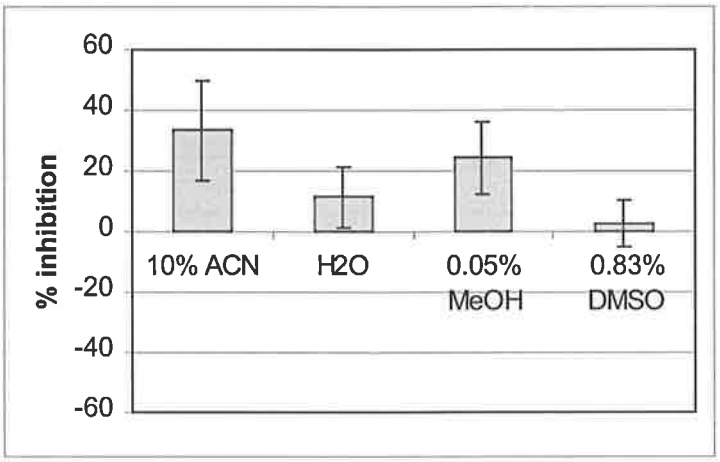


Figure 18 Inhibition of NOC formation by solvents used in the chemical inhibitor studies (n=3).

ACN and MeOH inhibited the formation of NOC by 33±16 and 24±12 percent respectively, while DMSO and water had little effect. However, the inhibition was not statistically significant for any of the solvents. The results from the study where OC concentration was close to the K_M of OM can be found in Appendix C. These results were similar to the results presented in Figure 18.

3.5 Antibody inhibition studies

3.5.1 Monoclonal antibodies

3.5.1.1 Oxymorphone formation

Figure 19 shows the effect of various CYP isoform monoclonal antibodies on OM formation when the OC concentration was similar to the K_{M1} of OM. Each bar represents three CYP2D6 EM livers (mean±SD).

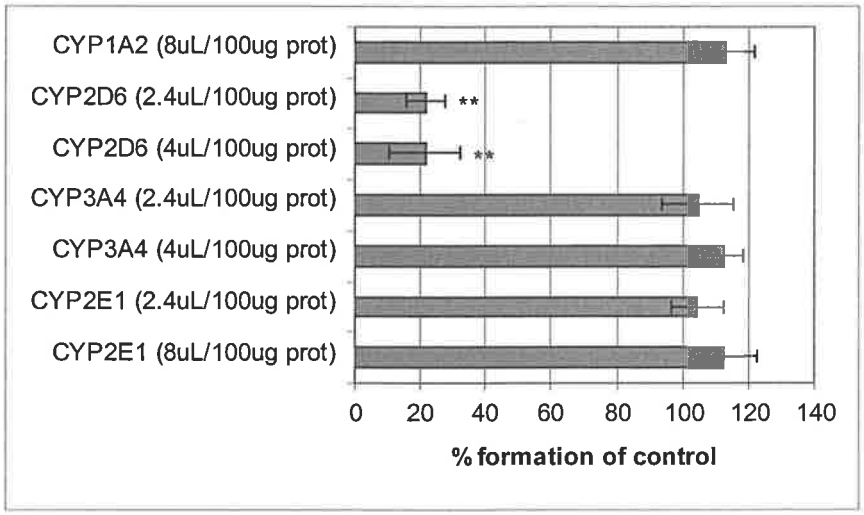


Figure 19 Percent OM formation compared to control samples in 3 CYP2D6 EM livers (mean±SD) in the presence of monoclonal CYP antibodies. Statistical significance is indicated with ** for p<0.01. (prot= protein, uL=µl, ug=µg)

Monoclonal antibodies against CYP2D6 inhibited OM formation, but the inhibition was not antibody-concentration dependent. The difference between the two antibody concentrations used was rather small, but the assay was not repeated at other antibody concentrations due to the expense of using antibodies. No other antibodies (CYP1A2, CYP2E1, CYP3A4) inhibited OM formation.

3.5.1.2 Noroxycodone formation

Figure 20 shows the effect of various CYP isoform monoclonal antibodies on NOC formation when the OC concentration was similar to the K_{M1} of OM. Due to the expense of using antibodies the study was not carried out at a concentration similar to the K_M of NOC. Each bar in Figure 20 represents three CYP2D6 EM livers (mean±SD).

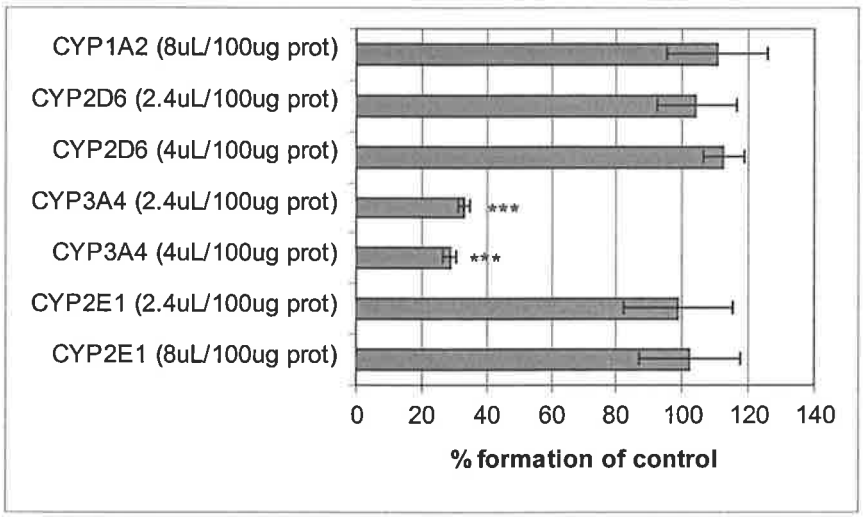


Figure 20 Percent NOC formation compared to control samples in 3 CYP2D6 EM livers (mean±SD) in the presence of monoclonal CYP antibodies. Statistical significance is indicated with * for p<0.001. (prot= protein, uL=µl, ug=µg)**

Antibodies towards CYP3A4 inhibited NOC formation in an antibody concentration-dependent manner. The other antibodies tested did not have any significant effect on NOC formation ($p>0.05$).

3.5.2 LKM1 antibodies (CYP2D6)

3.5.2.1 Oxymorphone formation

Concentration dependent inhibition was seen for OM formation when LKM1 antibodies directed against CYP2D6 was included in the incubation mixture (Figure 21). The maximum inhibition seen with the highest antibody concentration (2µl/50µg protein) was

50 %, and the inhibition was statistically significant for all the antibody concentrations. Variability was within 10 % for the duplicate incubations except in one case.

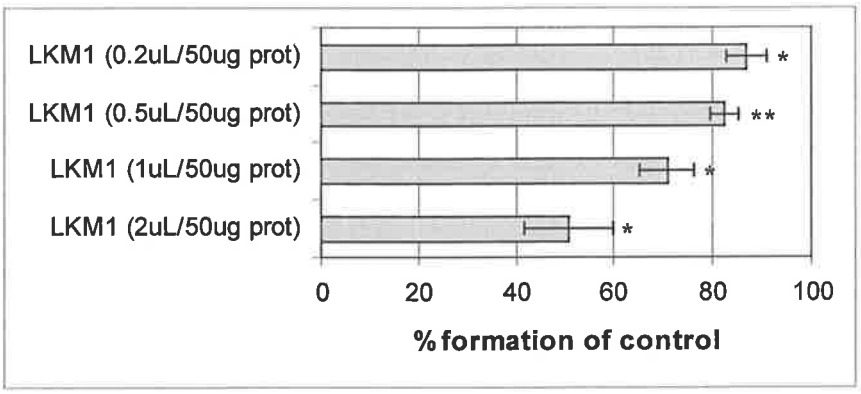


Figure 21 Oxymorphone formation in 3 livers (mean±SD) in the presence of LKM1 antibodies. Statistical significance is indicated with * for p<0.05 and ** for p<0.01.

3.5.2.2 Noroxycodone formation

NOC formation was similar to 90 % of the control samples when LKM1 antibodies directed against CYP2D6 were included in the incubation mixture (Figure 22). Although the inhibition observed was statistically significant, no antibody-concentration dependent difference was seen. Percent variability was above 10 % for 6 of the 12 duplicates.

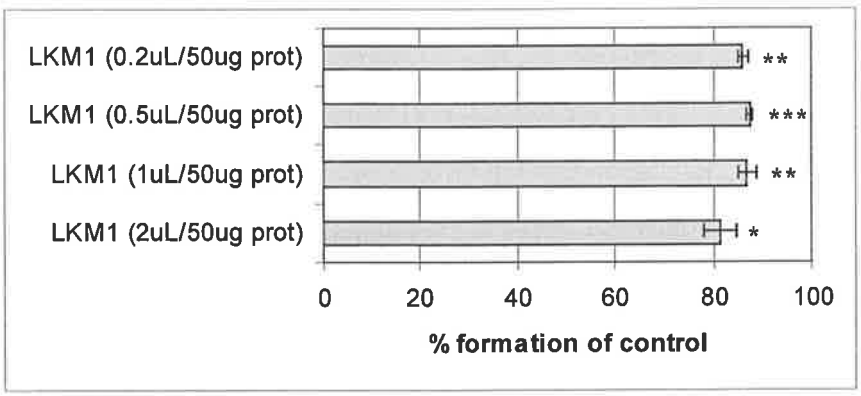


Figure 22 Noroxycodone formation in 3 livers (mean±SD) in the presence of LKM1 antibodies. Statistical significance is indicated with * for p<0.05, ** for p<0.01 and * for p<0.001.**

3.6 Expressed CYP enzymes

3.6.1 Metabolite formation from OC in a panel of expressed enzymes

Figure 23 shows NOC and OM formation when OC was incubated with various expressed enzymes. Each bar represents a duplicate incubation (mean±SD), except for OM formation in CYP3A4 expressed enzymes where only one single sample showed a visible peak in the chromatogram.

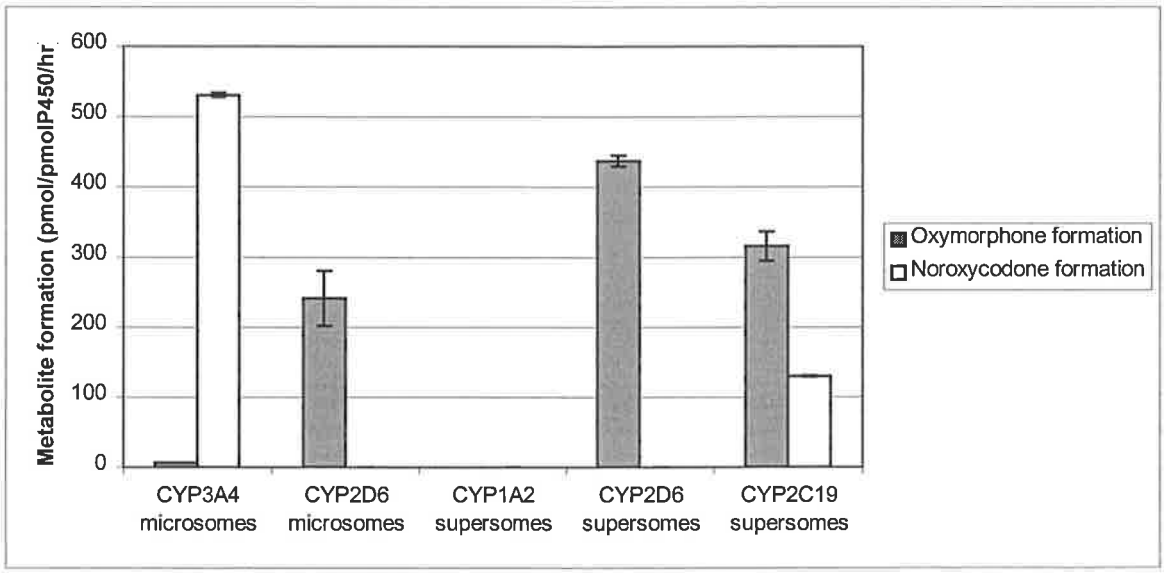


Figure 23 Formation of OM and NOC in the presence of expressed CYP enzymes.

3.6.1.1 Oxymorphone formation

Formation of OM was found with CYP2D6 microsomes and supersomes, as well as with the CYP2C19 supersomes (Figure 23). One single sample had detectable formation of OM with the presence of CYP3A4 microsomes, but OM formation was not seen in further studies with CYP3A4 expressed enzymes.

3.6.1.2 Noroxycodone formation

NOC formation was seen when OC was incubated with CYP3A4 microsomes, and also to a lesser extent with CYP2C19 supersomes (Figure 23).

3.6.2 Kinetic studies in expressed enzymes

Kinetic profiles were obtained for OM formation in CYP2D6 microsomes and NOC formation in CYP3A4 microsomes. A range of OC concentrations were also incubated with CYP2C19 supersomes, but no kinetic model fitted the formation data for either of the metabolites.

3.6.2.1 CYP2D6 microsomes

A kinetic profile of OM formation by CYP2D6 microsomes was obtained using eight concentrations of OC (35-525 μM). The data fitted the Michaelis Menten one enzyme model (Figure 24) with a K_M of 57.8 μM and Vmax of 8.63 nmol/mg/hr. The K_M was at the lower end of the range that was found in the kinetic studies for the human liver microsomes (K_{M1} 57.2-258 μM). The Eadie-Hofstee plot formed a straight line as expected (Figure 24) since only one enzyme was present.

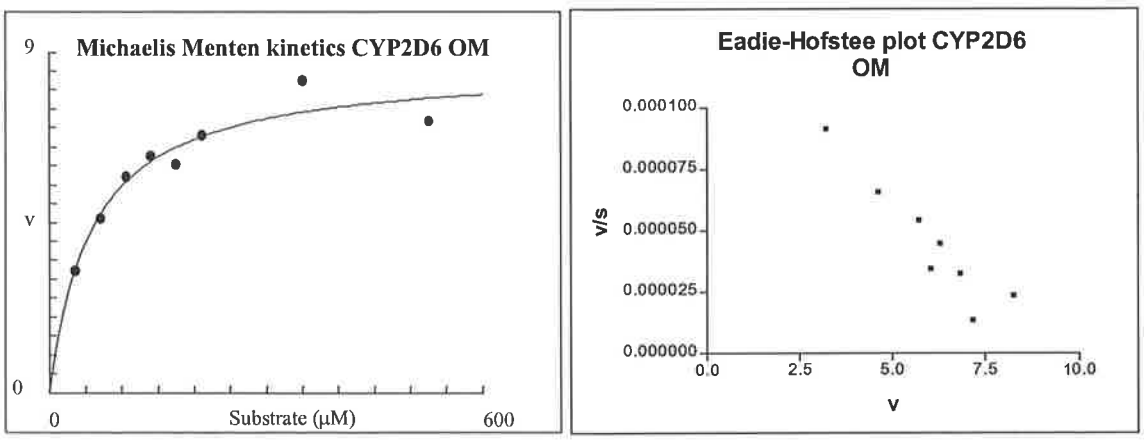


Figure 24 Kinetic profile and Eadie-Hofstee plot for OM formation in CYP2D6 expressed microsomes.

3.6.2.2 CYP3A4 microsomes

Six concentrations of OC (525-7000 μM) were used to produce a kinetic profile of NOC formation in CYP3A4 expressed microsomes. The data obtained fitted the Michaelis Menten one enzyme model (Figure 25) with a K_M of 2.4 mM and Vmax of 240.3 nmol/mg/hr. The K_M obtained was within the range (1.52-12.9 mM) and close to the median K_M value (2.9 mM) obtained in the human liver microsome kinetic studies. The Eadie-Hofstee plot indicated a straight line (Figure 25), which confirms the presence of a single enzyme only.

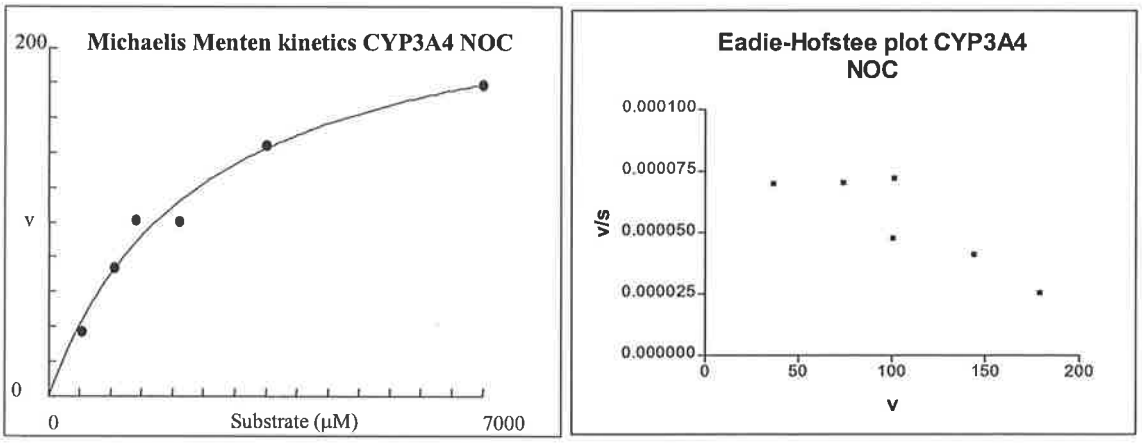


Figure 25 Kinetic profile and Eadie-Hofstee plot for NOC formation in CYP3A4 expressed microsomes.

3.6.3 Inhibition of metabolite formation by S-mephenytoin in CYP2C19 expressed enzymes

Formation of NOC and OM was only inhibited by 20-30% when S-mephenytoin (CYP2C19 chemical inhibitor) at 100μM was incubated concomitantly with OC with the CYP2C19 supersomes (Figure 26). This test was performed since the chemical inhibitor studies did not support involvement of CYP2C19 for either of the metabolites in contrast to the expressed enzymes studies.

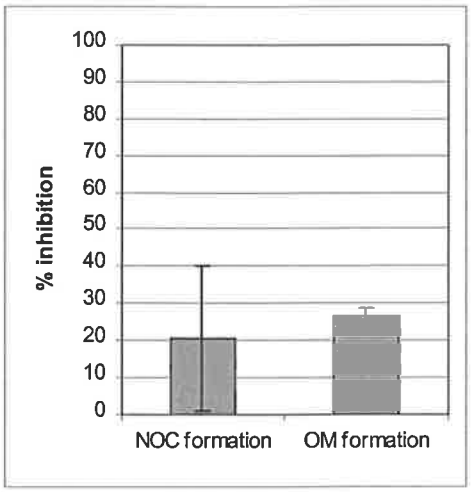


Figure 26 Percent inhibition of OM and NOC formation when S-mephenytoin (100μM) was incubated concomitantly to OC in CYP2C19 expressed enzymes.

3.7 Unidentified peak

An unidentified peak with a retention time of about 8 minutes was seen after incubation of OC with human liver microsomes (Figure 27). This peak was negligible in Figure 6 (Chapter 2) since its appearance becomes increasingly prominent with higher substrate concentrations. The chromatograph presented in Figure 6 shows incubation with only 300μM substrate, while the substrate concentration in Figure 27 was 500μM. Noroxymorphone, a metabolite of OM, could be excluded as the suspected compound since it had a retention time of about 5 minutes when injected onto the HPLC.

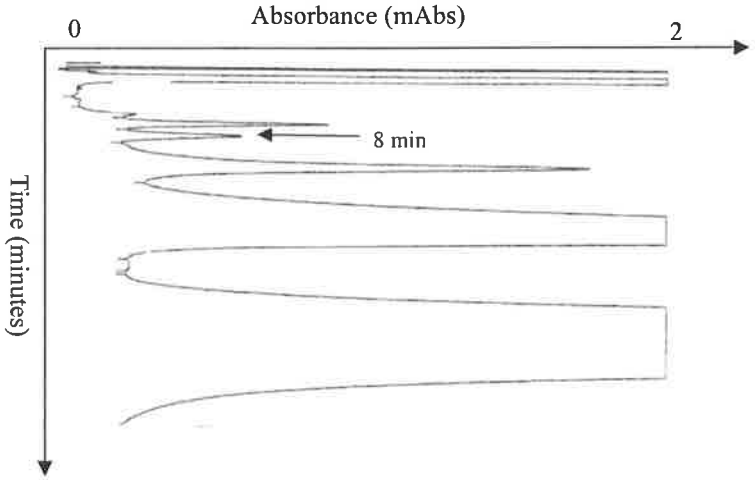


Figure 27 HPLC chromatograph following incubation of 500μM OC in HLM#11. Unidentified peak indicated with arrow (see Figure 6, Chapter 2).

Formation of the unidentified peak was evident in the chemical inhibitor studies where the OC concentration was near the K_M of NOC formation (relatively high substrate concentration), and it was possible to detect inhibition of its formation by some of the inhibitors. Therefore some conclusions on CYP450 involvement could be made. The peak was also present in the expressed enzyme studies. No formation of the unidentified compound was seen in the antibody studies, and this was probably due to the low OC concentration used (~K_{MI} of OM formation). Antibody inhibition could therefore not be determined.

3.7.1 Kinetic data

Quantification of formation was not possible for the unidentified metabolite, therefore velocity (v) was expressed as peak height ratio (PHR)/mg protein/hr. K_M could then be determined by plotting this v value against substrate concentration in the Michaelis Menten regression. The Eadie-Hofstee plots were in most cases slightly curved, while for HLM#35 it was curving back at the v/s axis. This was also seen for NOC formation in this liver. The one enzyme model could be fitted to the data from all the liver microsomes, but for 5 of 7 livers the two enzyme model also fitted the data with a better fit. The K_M was 5.96±2.00mM (mean±SD) when the data were fitted to the one enzyme model. The two enzyme model resulted in K_M values of 1.90±3.02mM and 17.6±15.9mM (n=5).

3.7.2 Chemical inhibitors

Formation of the unidentified peak was inhibited significantly ($p<0.05$) by quinidine (100μM), DDC (100μM), TOA (10μM), chlorzoxazone (100μM) and ketoconazole (100 and 10μM) (Figure 28).

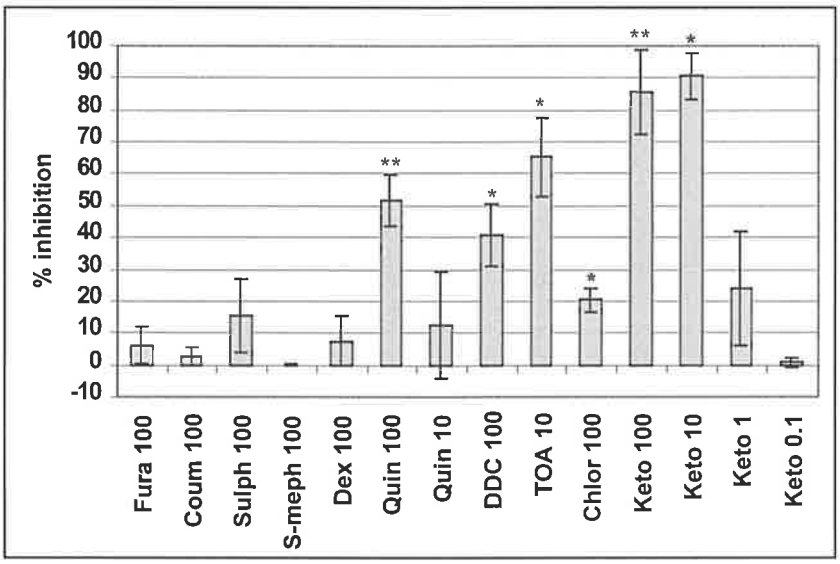


Figure 28 Percent inhibition of unidentified metabolite by CYP isoform specific chemical inhibitors in CYP2D6 EMs (n=3). Each bar represents the mean inhibition in three livers, while the error bars refer to SD. Statistical significance is indicated with * for $p<0.05$ and ** for $p<0.01$.

3.7.3 Expressed enzymes

Formation of the unidentified metabolite was seen with all the expressed enzymes tested. Each bar in Figure 29 represents a duplicate (mean±SD).

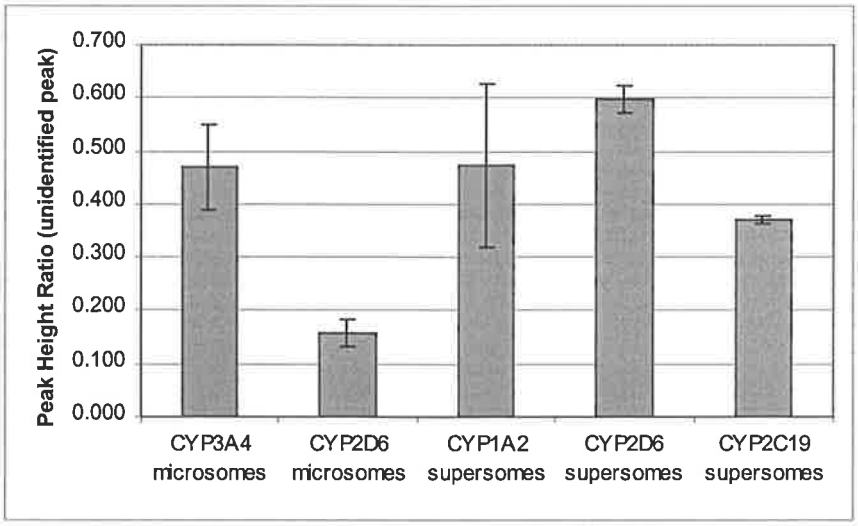


Figure 29 Formation of unidentified peak in the presence of expressed enzymes.

4. Discussion

The aims of this study were to investigate the enzyme kinetics of the formation of noroxycodone and oxymorphone from oxycodone in human liver microsomes, and to identify the P450 isoform(s) involved in these two metabolic reactions. It was hypothesized that CYP3A4 is the major isoform involved in formation of noroxycodone, and that CYP2D6 plays a major role in oxymorphone formation. In order to satisfy the aims of the study it was important to use an accurate and precise method for quantification of noroxycodone and oxymorphone in human liver microsomal preparations.

Previous methods for quantification of oxycodone and its metabolites include HPLC with electrochemical detection (Smith et al., 1991-oxycodone; Otton et al., 1993-oxymorphone) and gas chromatography/mass spectrometry (Kaiko et al., 1996-oxycodone/noroxycodone/oxymorphone). A new HPLC method with UV detection was developed for quantification of the O- and N-demethylated metabolites of hydrocodone in this laboratory (Hutchinson, 1999). This method combined the use of sodium perchlorate as an ion pairing agent and a C18 rocket column to allow separation of hydrocodone and the metabolites even at high substrate concentrations. Due to the structural similarity of oxycodone and hydrocodone (Figure 3) it was decided to try this assay method for the quantification of noroxycodone and oxymorphone, and only minor changes to the method were needed. The only major problem was separation of noroxycodone from oxycodone at substrate concentrations above 3500μM. This problem was dealt with by reducing the volume injected onto the HPLC, and, for some of the livers, by reducing the incubation volume for the respective samples. The HPLC run time was long (60 minutes), and most of the assay-runs lasted for up to 2 days. In spite of this, high precision and accuracy were obtained for inter- and intra-day variability, for both the standard curves and the quality controls, in all the assays performed.

Preliminary studies by Brauer and Ralfkiær (1998) demonstrated that the extraction of oxycodone and its metabolites, noroxycodone and oxymorphone, from the incubated

samples was necessary since unextracted samples caused interfering peaks and noisy baseline in the chromatographs, as well as repeated blockage of the HPLC prefilter. Brauer and Ralfkiær (1998) also tested various solvents for extraction and found that dichloromethane resulted in the best recovery of noroxycodone, oxymorphone and hydromorphone (internal standard). Their method for extraction was used in the present study, and a recovery of more than 90% was found for both oxymorphone and noroxycodone.

Enzyme kinetic studies for the metabolism of oxycodone have not been published in international journals previously, but very preliminary kinetic studies have been conducted by Brauer and Ralfkiær (1998) in this laboratory. Kinetic studies are needed in order to determine the extent of the different metabolic pathways using the intrinsic clearance values (V_{max}/K_M). It is also necessary to conduct kinetic studies prior to CYP inhibition studies in order to obtain an estimate of which substrate concentration(s) should be used in the incubations (should be similar to K_M). Different K_M and V_{max} values were obtained for oxymorphone and noroxycodone formation, indicating that different enzymes catalyse O- and N-demethylation of oxycodone. The two pathways will therefore be discussed separately.

The Eadie-Hofstee plots obtained for oxymorphone formation were curved, and the two enzyme Michaelis Menten model fitted the data for all the livers (6 EMs and 1 PM). This finding indicates that there are at least two enzymes involved in the O-demethylation of oxycodone. The mean values obtained for V_{max} in the EMs were 5.56±3.17 (V_{max1}) and 31.0±28.7 (V_{max2}) nmol/hr/mg protein, while the K_M values obtained were 143±84 μM (K_{M1}-high affinity) and 29.3±34.1 mM (K_{M2}-low affinity), respectively. The K_{M1} is slightly lower than the K_M value obtained (262±81μM, n=4 EM) from an unpublished study conducted in this laboratory (Brauer and Ralfkiær, 1998), but they obtained their kinetic parameters by fitting the one enzyme Michaelis Menten model to the data rather than the two enzyme model.

The K_{M2} obtained from 4 of 7 livers, as well as the mean for the EMs, was above the highest oxycodone concentration used. This suggests that oxymorphone formation in the

respective livers was not saturated at the substrate concentrations used, and the corresponding Michaelis Menten plots further supported this, as the curves did not plateau. It can therefore be concluded that the estimations of the kinetic parameters obtained for the low affinity enzyme are rather inaccurate.

Intrinsic clearance values were a mean of 42.8±16.1 µl/hr/mg for the high affinity enzyme (Cl_{int1}) in the EMs, while the values obtained for the low affinity enzyme was only 1.92±1.19 µl/hr/mg (Cl_{int2}). These data demonstrate that the low affinity enzyme in EMs only contributes about 4.3 % to the total oxymorphone formation, and that the inaccuracy of the K_{M2} values was not likely to be important.

Previous studies have associated CYP2D6 with the O-demethylation of oxycodone (Otton et al., 1993; Heiskanen et al., 1998). The CYP2D6 PM was therefore expected to have one less enzyme involved in oxymorphone formation compared to the EMs. However, the two enzyme Michaelis Menten model fitted the data from the PM better than a one enzyme model, and the Eadie-Hofstee plot was clearly curvilinear. The K_{M1} value obtained for the PM was in the same range as the K_{M1} obtained for the EMs, while the intrinsic clearance for this high affinity enzyme was substantially lower in the PM. The PM liver used in this study had been genotyped as *CYP2D6*4* (homozygous), which corresponds with no CYP2D6 activity (Daly et al., 1996). Thus, the high affinity enzyme seen in the PM was not CYP2D6 with decreased activity, although the K_M value indicated this. It is possible that more than two high affinity enzymes are involved in oxymorphone formation for EMs. Fitting of a three enzyme Michaelis Menten model to the EM data was attempted without success. However, if K_{M3} is similar to one of the other K_M values the model will not be able to determine its value. Such a similarity was suggested in the K_{M1} value obtained for the PM.

The kinetic studies for N-demethylation were not conclusive to whether one or more enzymes are involved. Linear Eadie-Hofstee plots were obtained for most of the livers, but the Eadie-Hofstee plots for two of the livers curved slightly. Both the one and the two enzyme Michaelis Menten model fitted the data from these two livers, but the improvement in fit was not substantial. Eadie-Hofstee plot that curved back on the v/s axis was obtained

for one single liver, but this liver only fitted the one enzyme Michaelis Menten model. It was decided to use the one enzyme Michaelis Menten model, since this fitted the data for all the livers, and since the statistical law of parsimony states that the simplest model should be used when several options of equal value are available. The median value obtained for V_{max} was 493 nmol/hr/mg protein (range: 160-7412), while the median K_M value obtained was 2.88 mM (range: 1.52-12.9). The mean values obtained for V_{max} and K_M were not representative since the kinetic parameters obtained from one liver (HLM#5) were highly untypical. Extensive and poor metabolizers with respect to CYP2D6 had similar enzyme kinetics for noroxycodone formation. In comparison to the present studies, Brauer and Ralfkiær (1998) characterized the formation of noroxycodone by a V_{max} of 1598±347 nmol/hr/mg protein and a K_M of 1.60±0.22 mM in 4 livers.

Both the K_M and the V_{max} values obtained for HLM#5 were 4 times higher than the second highest obtained K_M and V_{max} in the present kinetic studies for noroxycodone. HLM#5 was one of the livers with high noroxycodone formation, requiring adjustments in the incubation method as described in section 2.3.3.1, and a higher V_{max} was therefore expected. However, the K_M value should not differ substantially from the other livers if the same enzyme catalyses the reaction. For example, the K_M obtained from HLM#11 was close to the median although the intrinsic clearance for HLM#11 was the highest of all the obtained intrinsic clearance values. It is difficult to explain why oxycodone kinetics in HLM#5 deviated from the kinetics in the other livers. The patient was not receiving any enzyme inducing drugs. Moreover, studies on hydrocodone and methadone in the same liver did not show unusual kinetics, even though the same enzyme (CYP3A4) is involved in the N-demethylation of these two drugs (Hutchinson, 1999; Foster et al., 1999). The reason for this finding therefore remains unknown.

Cooperativity in oxidation has been reported for CYP3A4. This is, when binding of a substrate in a particular conformation can facilitate oxygen activation and thereby enhance catalytic activity (Ueng et al., 1997). One feature of this model is the sigmoidal curve for the formation rate, with corresponding Eadie-Hofstee plot that shifts back on the v/s axis (to the left when v/s is x axis) (non-linear). The cooperativity model can therefore be an explanation for the non-linear Eadie-Hofstee plot obtained for noroxycodone formation in

one liver (HLM#35). The Eadie-Hofstee plot obtained for the unidentified peak (section 3.7.1) in the same liver also reflected sigmoidal kinetics, and from the CYP identification studies this substance appeared to be CYP3A4 dependent. It has been shown that lipid soluble organic compounds, particularly bases, bind non-specifically to microsomal membranes. If the substrate concentration in a microsomal incubation is in the saturable range, and if the non-specific binding is not corrected for, sigmoidal formation kinetics may occur (McLure et al., 2000). This could be another explanation for the non-linear Eadie-Hofstee plot for HLM#35.

The total intrinsic clearance of oxycodone to noroxycodone and oxymorphone was highly variable (range: 81-750 µl/hr/mg protein), and much of this variability could be accounted for by the variable contribution from the N-demethylation pathway (range: 55-724 µl/hr/mg protein). The variability in intrinsic clearance was similar when excluding HLM#5.

Information on the enzymes involved in a drug's metabolism is now required during drug development, since knowledge from *in vitro* enzyme kinetics and inhibition studies can provide a guide to which *in vivo* drug interaction studies need to be conducted. However, no previous studies have systematically tested the enzyme(s) involved in the oxidative metabolism of oxycodone, and the present study therefore aimed to identify which cytochrome P450 enzymes are involved in the primary oxidative metabolism of oxycodone.

Otton et al. (1993) studied oxycodone metabolism *in vitro* using human liver microsomes from 1 CYP2D6 EM and 1 CYP2D6 PM. They found that oxymorphone formation was significantly inhibited by quinidine (a potent CYP2D6 inhibitor) in the CYP2D6 EM, and that oxymorphone formation was substantially smaller (about 1/5) in the PM compared to the EM (K_M and V_{max} values were not reported). It was therefore concluded that CYP2D6 is important in oxycodone O-demethylation. Heiskanen et al. (1998) supported this involvement as inhibition of CYP2D6, using quinidine, effectively blocked the formation of oxymorphone after administration of oxycodone in 10 healthy CYP2D6 EMs. CYP2D6 involvement in oxycodone O-demethylation is further supported by studies on structurally

similar drugs (codeine, dihydrocodeine, hydrocodone), where CYP2D6 involvement has been documented (Mortimer et al., 1990; Kirkwood et al., 1997b; Hutchinson, 1999). Based on these previous studies, it was hypothesized prior to the present study that CYP2D6 is involved in the O-demethylation of oxycodone. This involvement was confirmed in the present study using CYP specific chemical inhibitors, antibody inhibitors, CYP expressed enzymes, and comparing the kinetic studies from CYP2D6 EMs and a PM.

Strong evidence for CYP2D6 mediated O-demethylation of oxycodone was found in the CYP specific chemical inhibitor studies, as significant inhibition was observed with quinidine and dextromethorphan. Dextromethorphan and quinidine are both CYP2D6 inhibitors, and therefore useful tools in the determination of CYP2D6 involvement in metabolic reactions (Schmider et al., 1997; von Moltke et al., 1998; Newton et al., 1995; Sai et al., 2000).
Quinidine potently inhibited oxymorphone formation in a concentration-dependent manner, with almost complete inhibition at an inhibitor concentration of 100μM. Potent inhibition was also seen in HLM#31 and HLM#35 at inhibitor concentrations of 1μM (77 and 89 %) and 0.1μM (70 and 73 %). However, the variability in quinidine inhibition was large for the three livers due to less potent inhibition in HLM#11 (1μM – 37 % and 0.1μM – 22 %). Dextromethorphan inhibition was also lower for this particular liver. Therefore, the inhibition studies suggest that HLM#11 is less dependent on the CYP2D6 pathway compared to HLM#31 and HLM#35. This is not in accordance with the kinetic studies for oxymorphone formation, as HLM#31 and HLM#11 had similar clearance values for the high affinity enzyme.

The studies of the effect of solvents on oxymorphone formation lend further support to the involvement of CYP2D6 since DMSO significantly induced oxymorphone formation by 25 %. According to Hickman et al. (1998) CYP2D6 is the only CYP450 isoform induced by DMSO.

Monoclonal antibodies directed against CYP2D6 and LKM1 antibodies inhibited oxymorphone formation significantly. The monoclonal antibodies inhibited oxymorphone formation by 78 % at two different antibody-concentrations (2.4 and 4μl/100μg protein).

The lack of concentration dependency was probably due to the small difference between the two antibody concentrations used. The maximum inhibition with LKM1 antibody positive serum was 50 % (with addition of 2µl LKM1 positive serum per 50 µg microsomal protein), and the inhibition was antibody-concentration dependent. Kirkwood et al. (1997b) used LKM1 antibodies to identify CYP2D6 involvement in the O-demethylation of dihydrocodeine, and almost complete inhibition (94 %) of metabolite formation was found after addition of 1 µl LKM1 positive serum per 50 µg microsomal protein. However, the serum in this study had an antibody titre of 640, while the titre in the present study was only 64.

CYP2D6 microsomes and supersomes showed substantial oxymorphone formation. Furthermore, the one enzyme Michealis Menten model fitted the data obtained from the kinetic study in the CYP2D6 microsomes, and the K_M value (57.8µM) was within the range of the K_{M1} values obtained from the human liver microsome kinetic studies (57.2-258µM). This strongly indicates that CYP2D6 is one of the high affinity enzymes involved in oxymorphone formation.

CYP2D6 involvement in oxymorphone formation was further supported by the results from the human liver microsome kinetic studies since the clearance for the CYP2D6 PM via the oxymorphone pathway was only 5.49 µl/hr/mg as compared to 44.75 µl/hr/mg (mean) for the CYP2D6 EMs.

Furafylline at 25µM significantly inhibited oxymorphone formation by 29 %. A mechanism-based mode of action for furafylline CYP1A2 inhibition has been shown, as furafylline causes loss of CYP1A2 activity depending on pre-incubation time and presence of cofactor (Kunze and Trager, 1993; Tassaneeyakul et al. 1994). Several studies have shown that furafylline selectively inhibits CYP1A2 *in vitro* at concentrations up to 100µM if furafylline is pre-incubated with NADPH and microsomes for at least 10 minutes prior to addition of substrate (Tassaneeyakul et al. 1994; Newton et al., 1995; Sai et al.,2000). The present chemical inhibitor study therefore suggests that CYP1A2 is involved in the formation of oxymorphone. Induction of oxymorphone formation by acetonitrile in the

solvent study could be an indication of CYP1A2 involvement as CYP1A2 is the only CYP isoform subject to potent induction by acetonitrile (Hickman et al., 1998), although the induction seen in this study was rather inconsistent. However, CYP1A2 antibodies and expressed CYP1A2 enzymes did not support the involvement of CYP1A2. Studies with monoclonal antibodies and expressed enzymes are more reliable methods for CYP identification than the use of chemical inhibitors (Shou et al., 2000; Sai et al., 2000). An involvement of CYP1A2 in O-demethylation of oxycodone is therefore not likely.

Significant inhibition (24%) of oxymorphone formation was seen with 100μM DDC. This inhibitor is acting on CYP2E1, but several studies indicate that its selectivity is poor since it also has been shown to inhibit CYP1A2/1A1, CYP2A6, CYP2B6, CYP2C8 and CYP3A3/4 (Eagling et al., 1998; Chang et al., 1994). CYP2E1 antibodies did not support CYP2E1 involvement, and chlorzoxazone (CYP2E1 inhibitor) inhibition was insignificant. It has been shown that CYP2E1 metabolises molecules with a low structural volume (Lewis et al., 2000). It is unlikely that CYP2E1 is involved in oxymorphone formation since oxycodone has a large molecular volume.

Involvement of CYP2C19 in the formation of oxymorphone was only supported by the studies in expressed enzymes. However, antibodies towards CYP2C19 were not available, and the chemical inhibitor studies did not include a potent CYP2C19 inhibitor. The potency of S-mephenytoin as an inhibitor of CYP2C19 was tested in the expressed enzymes, and it was shown that it only inhibited oxymorphone formation by 27 %. Interestingly, Brauer and Ralfkiær (1998) found 60 % inhibition of oxymorphone formation with S-mephenytoin in a CYP2D6 PM. Omeprazole, which is frequently used as an inhibitor of CYP2C19 *in vitro*, had to be excluded in the present study due to peak interferences in the HPLC chromatographs. Consequently S-mephenytoin was the only CYP2C19 specific inhibitor used in the chemical inhibitor studies, and involvement of CYP2C19 in oxymorphone formation could not be excluded on the basis of the inhibitor studies.

Ketoconazole (100μM) statistically significantly inhibited oxymorphone formation, but only by a mean of 11 %. Although ketoconazole is a potent CYP3A4 inhibitor, it has been shown that its CYP specificity is low at concentrations above 1-2μM (Sai et al., 2000).

CYP2D6, CYP2C9, CYP2C19 and CYP1A1/2 are all inhibited by ketoconazole at 100μM when tested with known CYP450 substrates (Newton et al., 1995; Sai et al., 2000). CYP3A4 involvement in the O-demethylation of oxycodone was not supported by the antibody studies, and only one sample in the expressed enzyme studies with CYP3A4 had detectable oxymorphone formation.

Coumarin induced the formation of oxymorphone significantly (15%). Coumarin is a selective inhibitor of CYP2A6 (Pelkonen et al., 2000), but no published research describes CYP specific induction by coumarin. Conclusions can therefore not be drawn from this observation.

CYP3A4 has been related to the N-demethylation of many opioids structurally related to oxycodone. It has been shown that CYP3A4 is involved in the metabolism of codeine to norcodeine (Caraco et al., 1996b; Yue and Säwe, 1997), and the same relationship has been described for dihydrocodeine (Kirkwood et al., 1997b) and hydrocodone (Hutchinson, 1999). Due to the structural resemblance between these drugs and oxycodone, it was hypothesized that CYP3A4 is involved in oxycodone N-demethylation. However, no previously published studies have attempted to identify its involvement. It was evident in the present study that CYP3A4 catalyses oxycodone N-demethylation, and the involvement was supported by all the CYP identification methods.

Several chemical inhibitors directed against CYP3A4 were used. TOA (10μM) inhibited noroxycodone formation by 65%. Chang et al. (1994) demonstrated that TOA is highly selective for CYP3A if the correct experimental conditions (pre-incubation) are applied. Moreover, a study by Newton and co-workers (1995) showed that TOA has high CYP3A4 selectivity in human liver microsomes at concentrations up to 400μM. Ketoconazole at 100μM almost completely abolished the formation of noroxycodone, and the inhibition was also substantial (90%) when the inhibitor concentration was reduced to 10μM. However, the inhibitory effect of ketoconazole was highly variable and insignificant at 1 and 0.1μM. Several studies have shown that ketoconazole is a potent inhibitor of CYP3A4-catalysed metabolism of many known CYP3A4 probe drugs at inhibitor concentrations as

low as 1 and 0.1μM (Newton et al., 1995; Baldwin et al., 1995; Sai et al., 2000), although CYP3A4-mediated diazepam oxidation is barely affected by ketoconazole concentrations under 2μM (Sai et al, 2000). It has been shown that ketoconazole at low concentrations (0.1-1μM) is highly specific for CYP3A4, whereas higher concentrations can inhibit other CYP450s (e.g. 5μM – CYP1A1) (Sai et al., 2000). Thus it is important to supplement ketoconazole inhibition data with other identification approaches when the role of CYP3A4 is investigated in a metabolic reaction.

Quinidine is a selective CYP2D6 inhibitor, but it has been shown that at higher concentrations it also inhibits CYP3A4 activity (Newton et al., 1995; Sai et al., 2000). Since neither the antibody studies nor the expressed enzyme studies supported the involvement of CYP2D6, and due to the lack of quinidine inhibition at 10μM, it can be concluded that the inhibition seen with quinidine at 100μM supports the involvement of CYP3A4.

CYP3A4 involvement in noroxycodone formation was further supported by the inhibition seen in the presence of monoclonal antibodies directed against CYP3A4. The inhibition was highly significant ($p<0.001$), and antibody-concentration dependency was found.

CYP3A4 expressed microsomes showed substantial noroxycodone formation, and the one enzyme Michealis Menten model could be fitted to the data obtained from a kinetic study in these microsomes. The K_M value (2.4mM) was within the range (1.52-12.9mM) and close to the median K_M value (2.9mM) obtained for noroxycodone formation in the human liver microsome kinetic studies, suggesting that CYP3A4 is the major enzyme involved in liver noroxycodone formation. Furthermore, CYP3A4 involvement was supported by the human liver microsome kinetic studies since no difference was seen in K_M for the PM compared to the EMs. Mortimer et al. (1990) showed that CYP3A4 activity is the same in CYP2D6 poor and extensive metabolizers.

Expressed CYP2C19 supersomes were able to form noroxycodone, and the formation was more than 20 % of that observed for noroxycodone formation in CYP3A4 microsomes.

Involvement of CYP2C19 was not supported by the chemical inhibitor studies as the only inhibitor used against this isoform, S-mephenytoin, did not alter noroxycodone formation significantly. However, as described previously, it was shown in CYP2C19 supersomes that S-mephenytoin is not a very potent inhibitor of CYP2C19. Hutchinson (1999) found that CYP2C19 is involved in the N-demethylation of hydrocodone. Similar to this study, S-mephenytoin did not have an effect on the N-demethylation reaction, while omeprazole, another CYP2C19 inhibitor, inhibited the pathway significantly (20%). Since omeprazole caused peak interferences in the HPLC chromatographs in the present study it could not be used to determine CYP2C19 involvement in noroxycodone formation.

Chlorzoxazone (100μM) inhibited noroxycodone formation by 20 %. Lucas et al. (1999) showed that this inhibitor is highly specific for CYP2E1, although earlier studies have suggested that CYP3A4 also is involved in chlorzoxazone metabolism (Gorski et al., 1997; Shimada et al., 1999). DDC (100μM) inhibition of noroxycodone formation was highly variable and statistically insignificant. DDC has been used as an *in vitro* probe for CYP2E1, but several studies demonstrate that DDC has a low level of selectivity for CYP2E1, and that DDC at 100μM also inhibits the activity of various other CYP isoforms (Chang et al., 1994; Eagling et al., 1998; Sai et al., 2000). Since the CYP2E1 antibodies did not inhibit noroxycodone formation it was concluded that the inhibition observed with chlorzoxazone and insignificantly with DDC was due to CYP3A4 involvement.

The results of the solvent study were inconclusive for noroxycodone formation since the inhibition seen for all the solvents was statistically insignificant, and since a wide range of CYP450s are inhibited by all the used solvents.

Functional CYP isoforms are necessary for inhibition to be seen with specific chemical and antibody inhibitors. It is assumed in the antibody and chemical inhibitor studies that all CYP isoforms are functional in the microsomes. This assumption is reasonable for CYP2D6 and CYP2C19 since all the livers had been tested for the respective activities. However, the intrinsic isoform activities for the remaining isoforms are unknown. To by-pass this problem it would be necessary to incubate the microsomes with known CYP specific substrates to confirm the isoform activity prior to the inhibitor studies. This

involves many difficulties, such as obtaining probes and metabolites, as well as providing the assays. The time frame of this study did not allow these comprehensive preliminary studies.

The current study focused on oxymorphone and noroxycodone formation. However, another unidentified peak was observed in the chromatographs. The retention time of this compound was 8 minutes, which was slightly longer, but resolved from oxymorphone (7 minutes). The chemical inhibitor studies showed that CYP3A4 was involved in the formation of this unidentified compound, but involvement of other enzymes than CYP3A4 could not be excluded since the peak was detected after oxycodone incubation with all the tested expressed enzymes (CYP3A4, CYP2D6, CYP1A2, CYP2C19). Ishida et al. (1979) characterized the metabolic pathways of oxycodone, and found that the oxidative metabolites of oxycodone include noroxycodone, oxymorphone and noroxymorphone. The chemical structure of oxycodone has no other readily oxidisable sites than that accounted for with these three metabolites. It was confirmed that the peak was not noroxymorphone, since noroxymorphone had a retention time of 4 minutes. It is suggested that the unidentified peak is a metabolite of a contaminant in the oxycodone stock rather than a metabolite of oxycodone itself, and that this contaminant elutes at a similar retention time to oxycodone. To further characterise the unidentified peak it is necessary to investigate the molecular structure of the compound. This could be carried out by using Mass Spectrometry (MS) analysis after collecting the peak from the HPLC elution.

Cytochrome P450 is involved in the metabolism of many drugs, including oxycodone. Knowledge of which CYP isoforms are involved in the metabolism of a drug is therefore important in order to predict drug-drug interactions. Inter-individual variability in metabolic activity is common for many CYP450 isoforms. Such variability can be due to genetic or environmentally related differences in enzyme expression, and can influence the clinical outcome of drugs metabolized by these CYP450 isoforms.

If the CYP isoforms involved in a drug's metabolism are characterized, it is possible to estimate the likelihood for inter-individual variability in drug elimination. CYP3A4 is subject to substantial inter-individual variability (Ball et al., 1999). This study showed that

CYP3A4 was the main enzyme catalysing oxycodone N-demethylation, and a 13-fold variability in noroxycodone formation was seen. These *in vitro* studies indicate that substantial variability in clearance and half-life of oxycodone may occur *in vivo* since the N-demethylation pathway was important for the total intrinsic clearance of oxycodone. However, the importance of N-demethylation for elimination of oxycodone *in vivo* has not been fully investigated for humans. Further studies are therefore needed to determine the percent contribution of different metabolic pathways for oxycodone *in vivo*.

There is considerable speculation on whether the O-demethylation of oxycodone to oxymorphone is important for the analgesic effect of oxycodone. The present study has shown that CYP2D6 is responsible for most of the oxymorphone formation in CYP2D6 EMs, and that oxymorphone formation in a PM was almost 1/10 of that in EMs. It has been shown for codeine that CYP2D6 PMs have less potent analgesic effect compared to CYP2D6 EM (Sindrup et al., 1990). CYP2D6 PMs will have less potent analgesic effect of oxycodone than EMs, if oxymorphone formation is contributing in mediating the analgesic effect of oxycodone. Further research is needed to investigate the relationship between CYP2D6 genotype and analgesic potency of oxycodone in patients with pain.

5. Conclusions

CYP2D6 proved to be the major enzyme involved in the O-demethylation of oxycodone, but at least one other enzyme was responsible for a small fraction (~4%) of oxymorphone formation. CYP1A2, CYP2C19 or/and CYP2E1 may be involved, but discrepancies were seen between the different approaches for CYP identification. Other enzymes, not tested for in this study, may contribute to the formation of oxymorphone.

Alterations in oxymorphone formation may influence the analgesic effect of oxycodone if oxycodone acts as a prodrug with oxymorphone as the active metabolite. CYP2D6 variability will alter oxymorphone formation significantly, and may therefore influence oxycodone's effect. Variability in enzyme expression for the other CYP isoform(s) involved in oxycodone O-demethylation is not expected to influence oxycodone effect since the contribution to oxymorphone formation is minor. O-demethylation of oxycodone is not important for its total clearance, and variability in activity of the enzyme(s) involved in this pathway will therefore not influence the elimination of oxycodone *in vivo*.

The implications of oxycodone N-demethylation have been shaded by the interest in oxycodone O-demethylation. CYP3A4 involvement in N-demethylation of oxycodone was confirmed in this study, and it was shown that noroxycodone formation is of major importance for total intrinsic clearance of oxycodone. Although CYP3A4 was responsible for the majority of oxycodone N-demethylation, a minor involvement of CYP2C19 cannot be excluded. However, if CYP2C19 is able to form noroxycodone, its contribution is very small, and it is not likely to affect the clearance of oxycodone since CYP2C19 only comprise 1 % of the total liver CYP450 (Lewis et al., 1998).

The clearance via the CYP3A4 pathway varied 13-fold, and the variability in noroxycodone formation between the different livers caused a high variability of the total intrinsic clearance. Thus, variation in CYP3A4 expression is expected to influence oxycodone clearance *in vivo*.

CYP3A4 is involved in the metabolism of more than 50 % of all drugs (Benet et al., 1996). Drug-drug interactions may therefore be important for oxycodone due to the importance of CYP3A4 mediated N-demethylation in the total clearance.

Some discrepancies were found between the different CYP identification studies. For example, the chemical inhibition studies strongly supported CYP1A2 involvement, while the antibody studies and the studies in expressed enzymes showed no CYP1A2 involvement. This study has therefore demonstrated the need for several approaches when determining CYP isoform involvement.

Oxymorphone formation was mainly catalysed by CYP2D6. Genetic polymorphism of CYP2D6 is not likely to influence the clearance of oxycodone since the O-demethylation pathway of oxycodone is minor. However, if oxymorphone formation is important for the effect of oxycodone, a significant variability in analgesic response to oxycodone within a population can be expected.

This study showed that CYP3A4 is the main enzyme involved in the N-demethylation of oxycodone. It was also demonstrated that N-demethylation is the quantitatively most important pathway in the oxidative metabolism of oxycodone. Inter-individual variability in CYP3A4 expression may therefore affect oxycodone elimination *in vivo*, and due to the frequent involvement of CYP3A4 in xenobiotic metabolism, drug-drug interactions may be important in oxycodone treatment.

Appendix A

Clinical and Demographic data for the liver donors

NAME: | **DATE:** 24.5.90

HLS# 5 | **U.R. NUMBER:** 606 831

AGE: 66 | **HEIGHT:** 170 cm. | **ALCOHOL:** 0 g/week

SEX: FEMALE | **WEIGHT:** 73 kg. | **TOBACCO:** 0 /day

CAFFEINE: 1 T, 1/4 C /day

ALLERGIES: nil known

HISTORY : Ca. L liver lobe - hemihepatectomy
Ca. small intestine - resection
cholecystectomy

MISC:

PHENO: DM - EM | GENO: 2D6-W/W

LIVER WT: 170 g. | USED: 107 g.

REMAINING: 63 g.

MEDICATIONS:

REGULAR : nil

PREMED: pethidine 75mg.
metaclopramide 7.5mg

THEATRE:
gentamycin 120mg.
amoxycillin 1g.
metronidazole 500mg.
ephedrine 12mg.
neostygmine/atropine 2.5/1.2mg.
bupivacaine 0.375% total 6ml.
suxamethonium 100mg.
vecuronium total 12mg.
fentanyl total 150mg.
thiopentone total 300mg.
lignocaine spray to vocal cords

MICROSOME RESULTS:

BIOCHEMISTRY / HAEMATOLOGY RESULTS

Na: 143 K: 3.7 Cl: 104 bicarb: 32 anion gap: 11 osmolarity: 279 glucose: 4.6

urea: 3.5 creatinine: 0.06 urate: 0.24 PO4: 1.34 total Ca: 2.27 **ionised Ca.:**

albumin: 42 **globulins:** **cholesterol:** **conj. bili.:** total bili: 5

GGT: 14 ALP: 64 LD: 192 AST: 25 ALT:

Hb: 12.9 RBC: 4.21 PCV: 37.8 MCV: 89.8 MCH: 30.6 MCHC: 34.1

platelets: 236

WBC: 3.8 neut.: 63 % lymph.: 29 % mono.: 8 % eosin.: % baso.: %

other: APTT 27sec. prothrombin ratio 1.0
globulins 24 cholesterol 5.6 conj. bili. 1

NAME: DATE: 22.8.91

HLS# 11 U.R. NUMBER: 617 600

AGE: 41 HEIGHT: 160 cm. ALCOHOL: g/week

SEX: FEMALE WEIGHT: 60 kg. TOBACCO: /day

CAFFEINE: /day

ALLERGIES:

HISTORY : liver metastases - resection
Ca. colon - L. hemicolectomy

MISC:

PHENO: GENO: 2D6-W/W

LIVER WT: 65 g. USED: g.

REMAINING: 65 g.

MEDICATIONS:

REGULAR : digesic 2, 19.8
temazepam 10mg. (20mg. 21.8)

PREMED: diazepam 20mg.
omnopon/scopolamine 20/0.4mg.

THEATRE: thiopentone 225mg.
fentanyl 350mg.
vecuronium 4mg.
pancuronium 3mg.
t-d-curare 15mg.
ampicillin 1g.
gentamicin 80mg.
metronidazole 500mg.
metoclopramide 10mg.

MICROSOME RESULTS:

BIOCHEMISTRY / HAEMATOLOGY RESULTS

Na: 136 K: 4.4 Cl: 102 bicarb: 29 anion gap: 9 osmolarity: 270 glucose: 6.7

urea: 3.2 creatinine: 0.07 urate: 0.3 PO4: 0.95 total Ca: 2.27 **ionised Ca.:** 1.19

albumin: 42 **globulins:** 34 **cholesterol:** **conj. bili.:** 1 total bili: 6

GGT: 17 ALP: 84 LD: 192 AST: 21 ALT:

Hb: 16.4 RBC: 5.34 PCV: 48.3 MCV: 90.5 MCH: 30.7 MCHC: 33.9

platelets: 220

WBC: 21.3 neut.: 93 % lymph.: 5 % mono.: 2 % eosin.: % baso.: %

other: APTT 24
prothrombin ratio 1.4

NAME: DATE: 28.10.93

HLS# 21 U.R. NUMBER: 637 671

AGE: 70 HEIGHT: 172 cm. ALCOHOL: <10 g/week

SEX: MALE WEIGHT: 75 kg. TOBACCO: 0 /day

CAFFEINE: 2 C /day

ALLERGIES: nil known

HISTORY : liver metastases - resection
Ca. rectum - resection

MISC: liver weight includes alfoil

PHENO: DM GENO: 2D6-W/B

LIVER WT: 186.8 g. USED: 19 g.

REMAINING: 167.8 g.

MEDICATIONS:

REGULAR : nil

THEATRE: thiopentone 250mg.
fentanyl 200mg.
vecuronium 1mg.
amoxycillin 1g.
gentamicin 80mg.
metronidazole 1g.

PREMED: temazepam 20mg.
heparin 5000u

MICROSOME RESULTS:

BIOCHEMISTRY / HAEMATOLOGY RESULTS

Na: 140 K: 4 Cl: 102 bicarb: 30 anion gap: osmolarity: glucose:

urea: 7.6 creatinine: 0.09 urate: PO4: total Ca: **ionised Ca.:**

albumin: 42 **globulins:** 32 **cholesterol:** **conj. bili.:** total bili: 13

GGT: 29 ALP: 93 LD: 219 AST: 11 ALT: 11

Hb: 14.2 RBC: PCV: MCV: MCH: MCHC:

platelets: 205

WBC: 9.3 neut.: % lymph.: % mono.: % eosin.: % baso.: %

other: APTT 25sec.
prothrombin ratio 1.1

NAME: | **DATE:** 30.11.93

HLS# 23 | **U.R. NUMBER:** 676 071

AGE: 62 | **HEIGHT:** ? cm. | **ALCOHOL:** 300 g/week

SEX: MALE | **WEIGHT:** 77 kg. | **TOBACCO:** 0 /day

CAFFEINE: 1 C /day

ALLERGIES: nil known

HISTORY:
liver metastases - resection
Ca. colon - resection
hypertension

MISC:

PHENO: | **GENO:** 2D6-W/W

LIVER WT: 102.2 g. | **USED:** 78.2 g.

REMAINING: 24 g.

MEDICATIONS:

REGULAR:
prednisolone 3mg. mane
'Golytely' 2L 29.11

PREMED: teamzepam 20mg.

THEATRE:
thiopentone 250mg.
fentanyl 300mg.
vecuronium 18mg.
morphine 15mg.
hydrocortisone 100mg.
amoxycillin 1g.
gentamicin 80mg.
metronidazole 500mg..

MICROSOME RESULTS:

BIOCHEMISTRY / HAEMATOLOGY RESULTS

Na: 140 K: 3.7 Cl: 101 bicarb: 30 anion gap: 13 osmolarity: 275 glucose: 5.0

urea: 4.2 creatinine: 0.07 urate: PO4: total Ca: **ionised Ca.:**

albumin: 38 **globulins:** 31 **cholesterol:** **conj. bili.:** total bili: 19

GGT: 28 ALP: 76 LD: 188 AST: 45 ALT: 21

Hb: 13.1 RBC: 4.40 PCV: 38.1 MCV: 86.7 MCH: 29.8 MCHC: 34.4

platelets: 158

WBC: 14.2 neut.: 93 % lymph.: 2 % mono.: 5 % eosin.: % baso.: %

other: total protein 69

NAME: | DATE: 9.12.93

HLS# 24 | U.R. NUMBER: 708 503

AGE: 42 | HEIGHT: 165 cm. | ALCOHOL: 70 g/week

SEX: FEMALE | WEIGHT: 53 kg. | TOBACCO: 0 /day

CAFFEINE: 3-4T, <1C /day

ALLERGIES: nil known

HISTORY: hepatic tumour - resection
nil else significant

MISC:

PHENO: DM- GENO: 2D6-B/B

LIVER WT: 216 g. USED: 10.9 g.

REMAINING: 205.1 g.

MEDICATIONS:

REGULAR: temazepam 20mg. nocte 8.12

PREMED: temazepam 20mg.

THEATRE: thiopentone 300mg.
fentanyl total 300mg.
atracuronium total 145mg.
morphine 15mg.
amoxycillin 1g.
gentamicin 80mg.
metronidazole 500mg.

MICROSOME RESULTS:

BIOCHEMISTRY / HAEMATOLOGY RESULTS

Na: 137 K: 3.9 Cl: 104 bicarb: 31 anion gap: 6 osmolarity: 271 glucose: 5.5

urea: 5.0 creatinine: 0.08 urate: 0.3 PO4: 0.9 total Ca: 2.34 **ionised Ca.:** 1.19

albumin: 43 **globulins:** 30 **cholesterol:** **conj. bili.:** total bili: 10

GGT: 32 ALP: 101 LD: 187 AST: 15 ALT: 21

Hb: 13.8 RBC: 4.78 PCV: 41.4 MCV: 86.6 MCH: 29.0 MCHC: 33.4

platelets: 361

WBC: 7.4 neut.: 65 % lymph.: 27 % mono.: 6 % eosin.: 2 % baso.: 1 %

other: total protein 73 amylase 111
APTT 23 prothrombin ratio 1.0
ESR 20mm

NAME: | **DATE:** 15.12.94

HLS# 31 | **U.R. NUMBER:**

AGE: 73 | **HEIGHT:** | cm. **ALCOHOL:** "occational" g/week

SEX: MALE | **WEIGHT:** 85 kg. **TOBACCO:** Nil /day

CAFFEINE: /day

ALLERGIES: nil known

HISTORY : Metastasis right liver lobe > colorectal primary (anterior resection 1990)

MISC:

PHENO: | GENO: 2D6-W/W

LIVER WT: 233.8 g. USED: 66.9 g.

REMAINING: 166.9 g.

MEDICATIONS:

REGULAR : Frusemide 40mg oral mane (given 15.12.94)

PREMED: Temazepam 10mg oral 07:00

THEATRE:
Epidural
Fentanyl 400mg
STP 175 mg
ATC 125 mg
Marcaine 0.25% 20ml
Fentanyl 200µg
Gentamycin 80mg all in IV
Amoxycillin 1g
09:00
Metronidazole 500mg

MICROSOME RESULTS:

BIOCHEMISTRY / HAEMATOLOGY RESULTS

Na: 140 K: 4.3 Cl: 104 bicarb: 23 anion gap: 17 osmolarity: 278 glucose: 4.8

urea: 6.0 creatinine: 0.08 urate: | PO4: 1.23 total Ca: 2.11 **ionised Ca.:** 1.14

albumin: 33 **globulins:** 27 **cholesterol:** | **conj. bili.:** | total bili: 0

GGT: 21 ALP: 105 LD: | AST: | ALT: 10

Hb: 10.6 RBC: 4.12 PCV: 34.1 MCV: 82.8 MCH: 25.8 MCHC: 31.2

platelets: 270

WBC: 17.8 neut.: 84 % lymph.: 8 % mono.: 8 % eosin.: % baso.: %

other: prothrombin ratio 1.0
APTT 22 secs
Pre-op.: Hb 9.7, platelets 555, WBC 10. Note that haematology results were obtained 16.12.94

NAME: | **DATE:** 28.3.96

HLS# 35 | **U.R. NUMBER:**

AGE: 65 | **HEIGHT:** 170 cm. | **ALCOHOL:** 140 - 210 g/week

SEX: MALE | **WEIGHT:** 74 kg. | **TOBACCO:** /day

CAFFEINE: 4C, 4T /day

ALLERGIES: nil known

HISTORY :
Dukes Ca Rectum - anterior resection (10/95)
- chemoRx
hepatic resection

MISC:

PHENO: | GENO: 2D6 -W/W

LIVER WT: 218.5 g. | USED: g.

REMAINING: 218.5 g.

MEDICATIONS:

REGULAR : Cimetidine, 400mg twice day

PREMED: Temazepam, 20mg

THEATRE:
Midazolam, 2mg
Atracurium, 50mg
Ephedrine, 30mg
Thiopentone (sodium), total 350mg
Fentanyl, 200mg
Morphine, 6mg
Gentamycin, 120mg
Amoxl, 1g
Flagyl, 500mg
Epidural - Marcaine 25% with Adren 34, Fentanyl 50 + 50

MICROSOME RESULTS:

BIOCHEMISTRY / HAEMATOLOGY RESULTS

Na: 143 K: 3.9 Cl: 102 bicarb: 26 anion gap: 19 osmolarity: 281 glucose: 4.5

urea: 4.5 creatinine: 0.09 urate: - PO4: - total Ca: 2.24 **ionised Ca.:** 1.12

albumin: 42 **globulins:** 27 **cholesterol:** **conj. bili.:** total bili: 15

GGT: 32 ALP: 107 LD: 139 AST: ALT: 9

Hb: 13.1 RBC: 3.94 PCV: 37.5 MCV: 95.1 MCH: 33.2 MCHC: 34.9

platelets: 144

WBC: 3.6 neut.: 64 % lymph.: 25 % mono.: 8 % eosin.: 2 % baso.: 1 %

other:

Appendix B

Michaelis Menten kinetics

&

Eadie-Hofstee plots

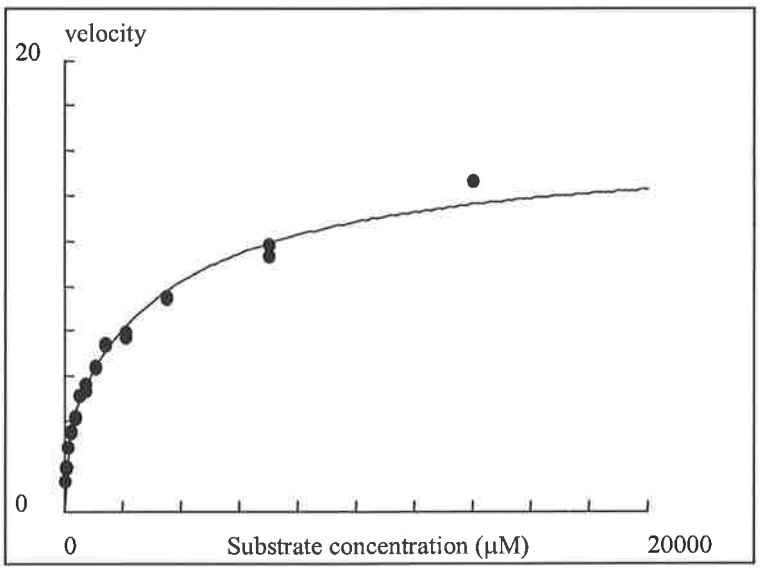
for

Oxymorphone and Noroxycodone formation

in seven liver microsome preparations

Oxymorphone formation HLM#5

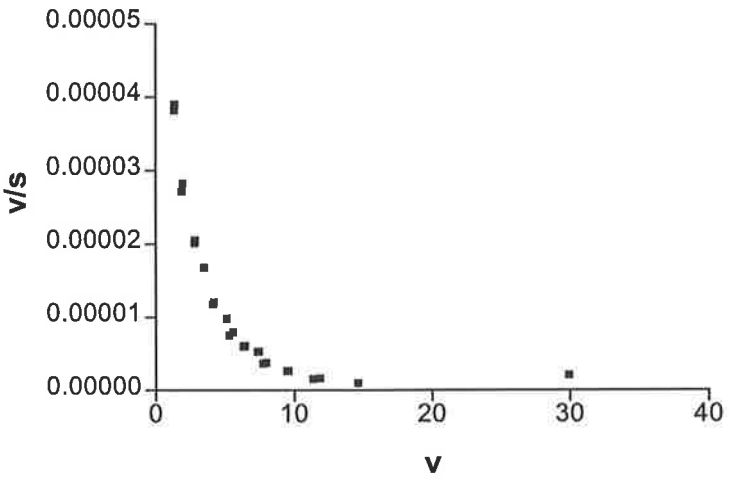
Michaelis Menten plot



Michaelis-Menten two enzyme kinetics (1/y*y weighting)
velocity=((Vmax1*substrate)/(Km1+substrate))+((Vmax2*substrate)/(Km2+substrate))
Iteration count = 109
Sum of squares = 2.10165
Standard deviation = 0.316352
Determination coef. = 0.992762

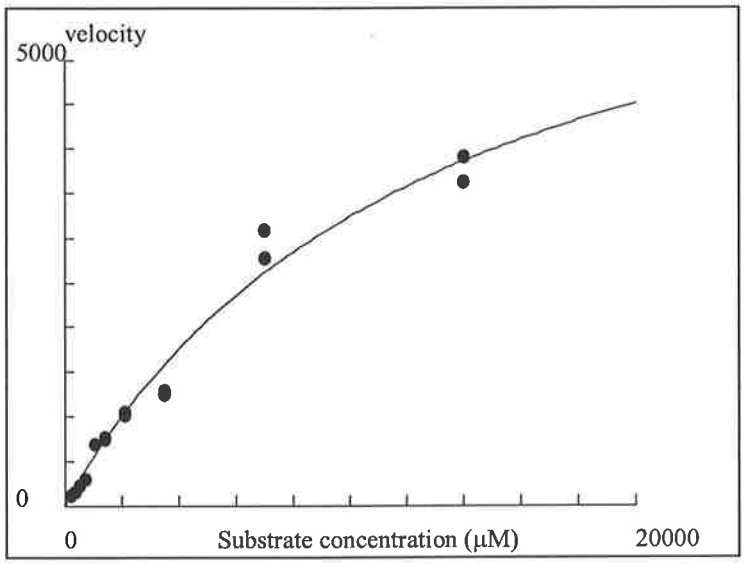
Vmax1	3.98622
Km1	83.7423
Vmax2	12.2599
Km2	3811.01

Eadie-Hofstee plot



Noroxycodone formation HLM#5

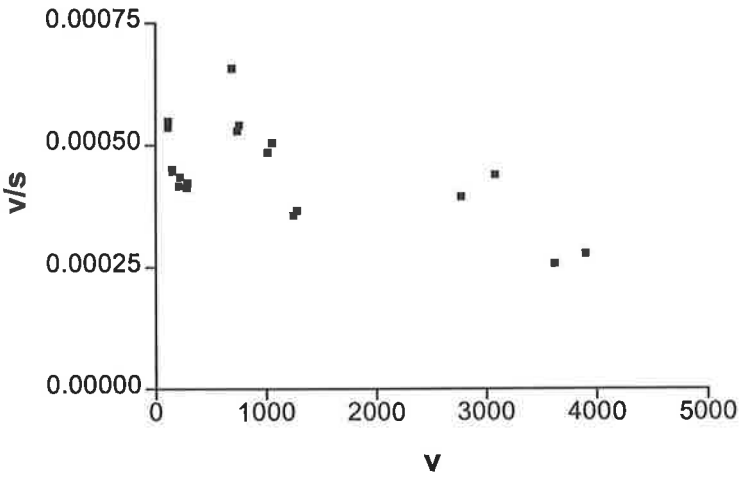
Michaelis Menten plot



Michaelis-Menten enzyme kinetics (no weighting)
velocity=Vmax*substrate/(Km+substrate)
Iteration count = 28
Sum of squares = 573079
Standard deviation = 178.431
Determination coef. = 0.979635

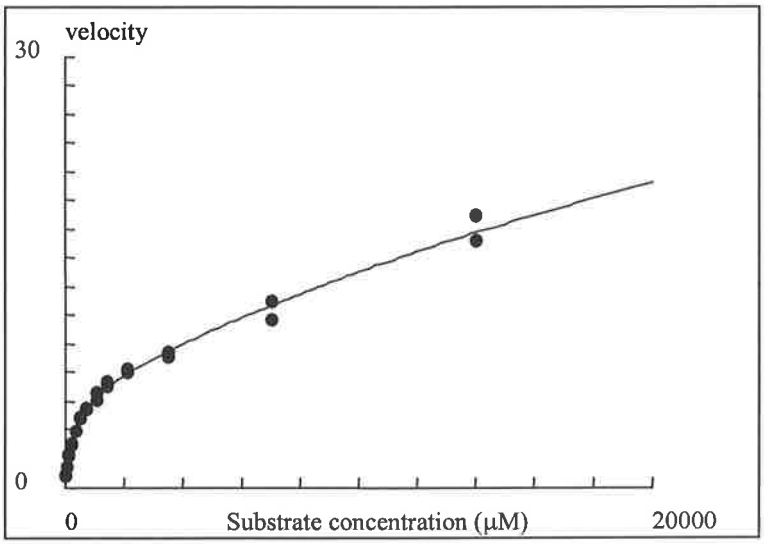
Vmax	7411.48
Km	12892.9

Eadie-Hofstee plot



Oxymorphone formation HLM#11

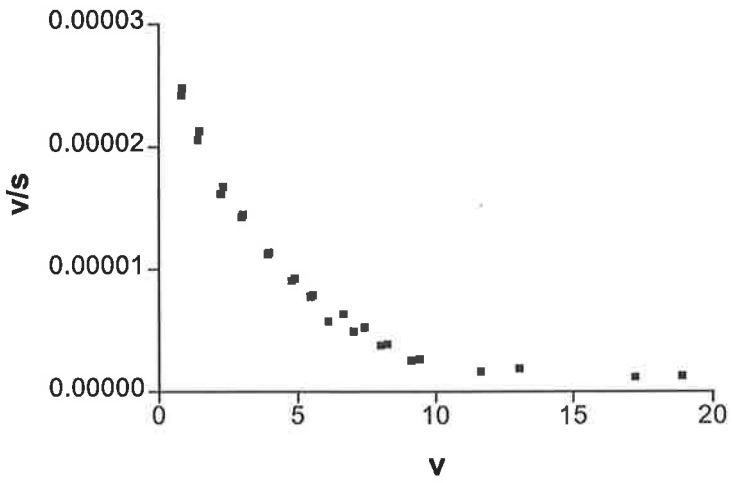
Michaelis Menten plot



Michaelis-Menten two enzyme kinetics (1/y*y weighting)
velocity=((Vmax1*substrate)/(Km1+substrate))+((Vmax2*substrate)/(Km2+substrate))
Iteration count = 107
Sum of squares = 3.68047
Standard deviation = 0.409016
Determination coef. = 0.993353

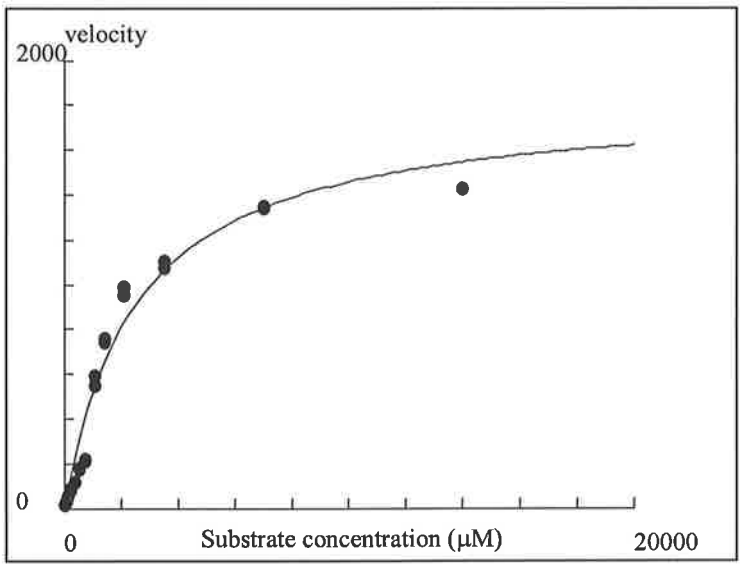
Vmax1	6.45571
Km1	257.77
Vmax2	48.8531
Km2	46098.6

Eadie-Hofstee plot



Noroxycodone formation HLM#11

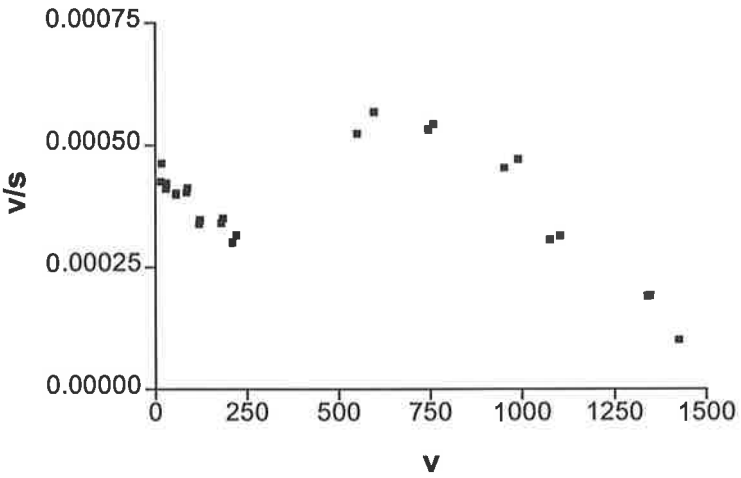
Michaelis Menten plot



Michaelis-Menten one enzyme kinetics (no weighting)
velocity=Vmax*substrate/(Km+substrate)
Iteration count = 1001
Sum of squares = 213455
Standard deviation = 96.3362
Determination coef. = 0.963602

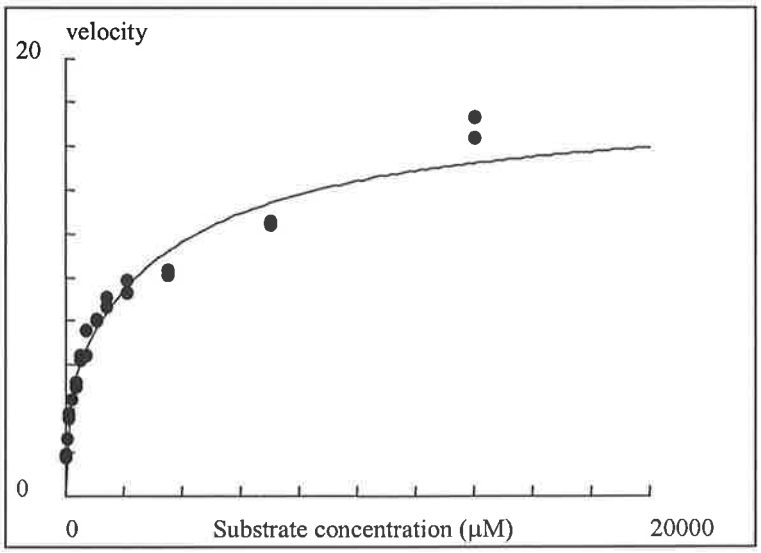
Vmax	1824.59
Km	2523.42

Eadie-Hofstee plot



Oxymorphone formation HLM#21

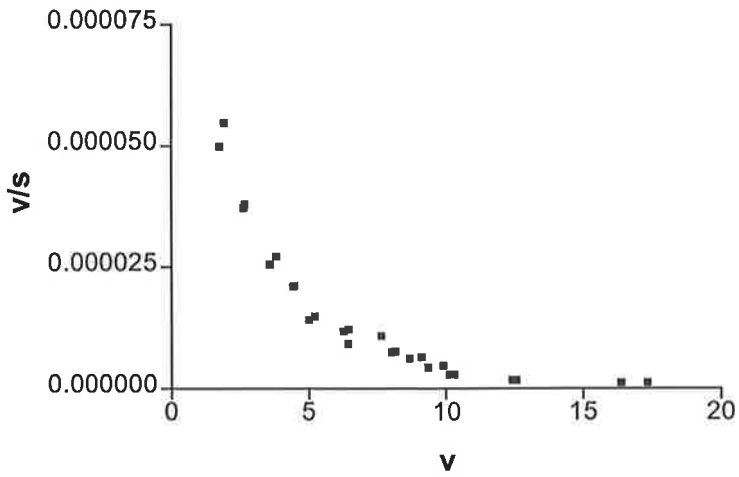
Michaelis Menten plot



Michaelis-Menten two enzyme kinetics (1/y*y weighting)
velocity=((Vmax1*substrate)/(Km1+substrate))+((Vmax2*substrate)/(Km2+substrate))
Iteration count = 112
Sum of squares = 11.8449
Standard deviation = 0.73376
Determination coef. = 0.97234

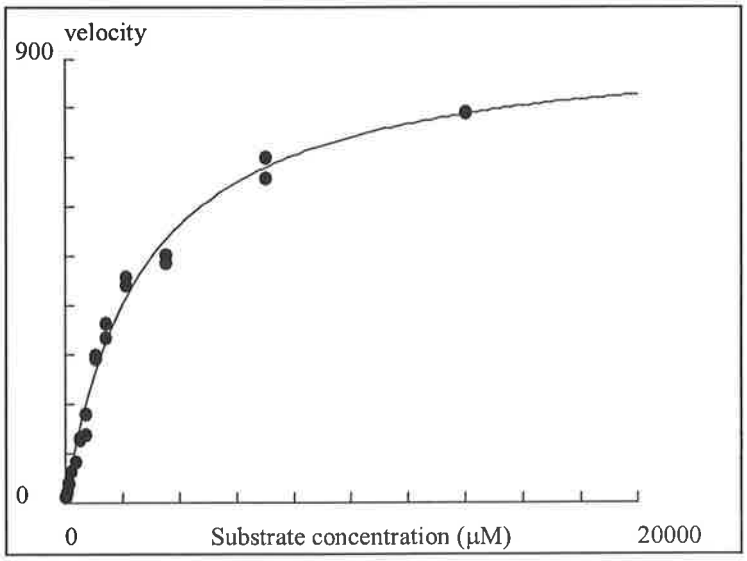
Vmax1	5.29921
Km1	79.0152
Vmax2	12.793
Km2	3986.04

Eadie-Hofstee plot



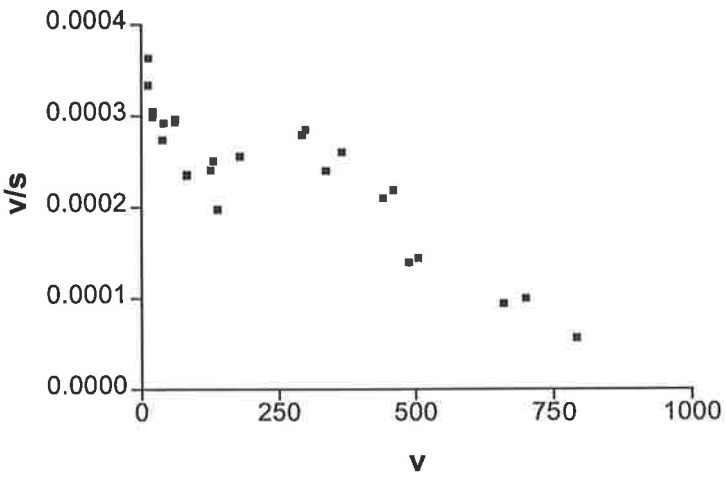
Noroxycodone formation HLM#21

Michaelis Menten plot



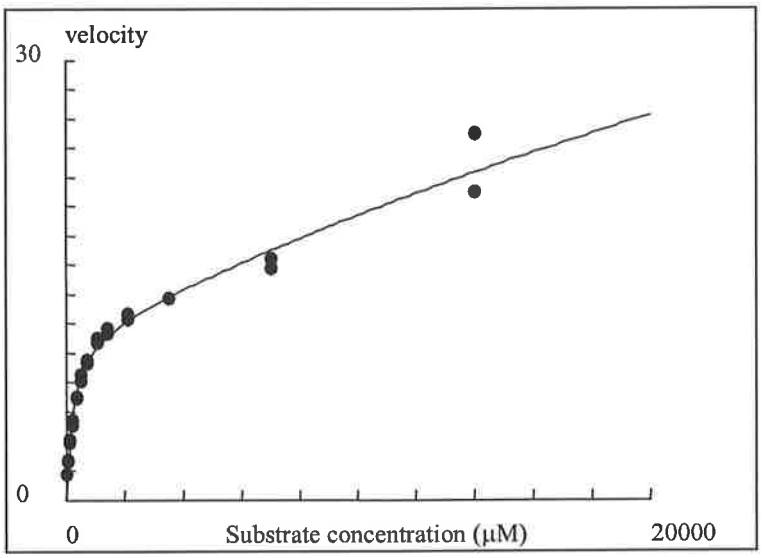
Michaelis-Menten enzyme kinetics (no weighting)
velocity=Vmax*substrate/(Km+substrate)
Iteration count = 35
Sum of squares = 16680.3
Standard deviation = 26.3631
Determination coef. = 0.98986
Vmax 938.997
Km 2673.26

Eadie-Hofstee plot



Oxymorphone formation HLM#23

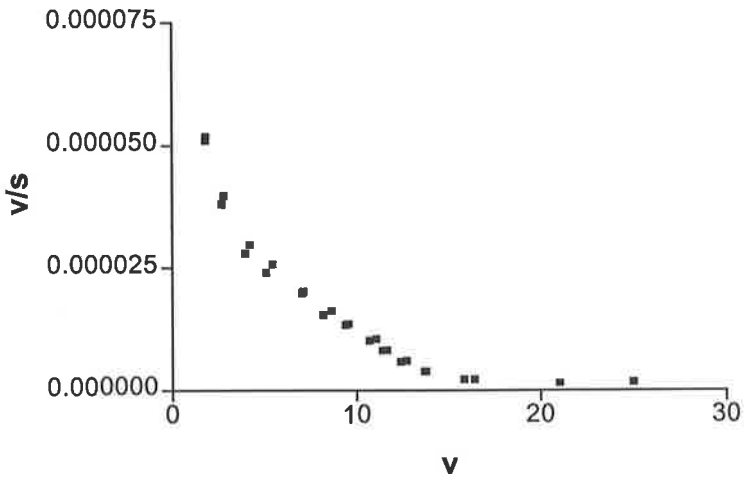
Michaelis Menten plot



Michaelis-Menten two enzyme kinetics (1/y*y weighting)
velocity=((Vmax1*substrate)/(Km1+substrate))+((Vmax2*substrate)/(Km2+substrate))
Iteration count = 118
Sum of squares = 13.2435
Standard deviation = 0.775871
Determination coef. = 0.984357

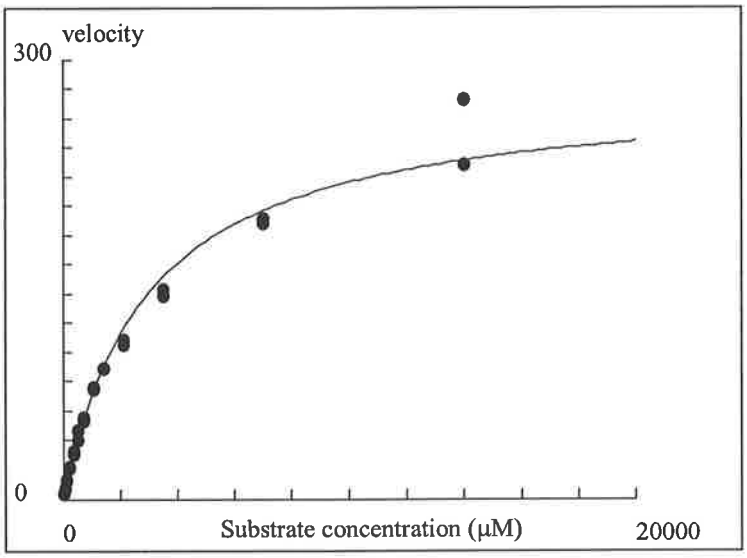
Vmax1	11.3791
Km1	227.606
Vmax2	81.944
Km2	89606.1

Eadie-Hofstee plot



Noroxycodone formation HLM#23

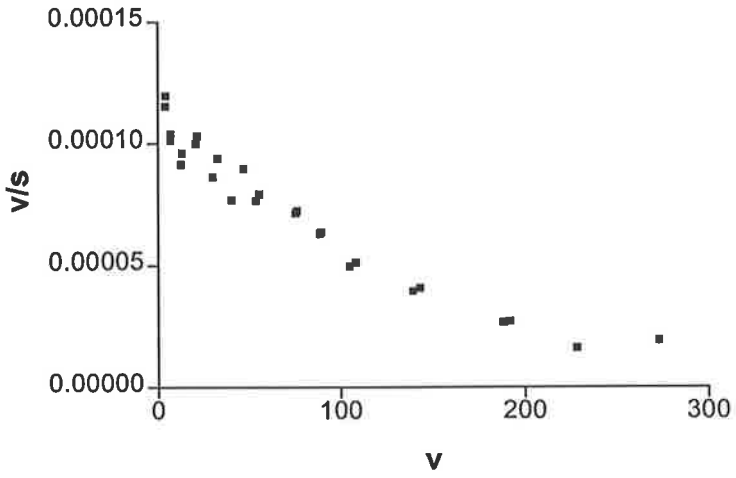
Michaelis Menten plot



Michaelis-Menten enzyme kinetics (1/y weighting)
velocity=Vmax*substrate/(Km+substrate)
Iteration count = 35
Sum of squares = 2310.84
Standard deviation = 9.8125
Determination coef. = 0.98338

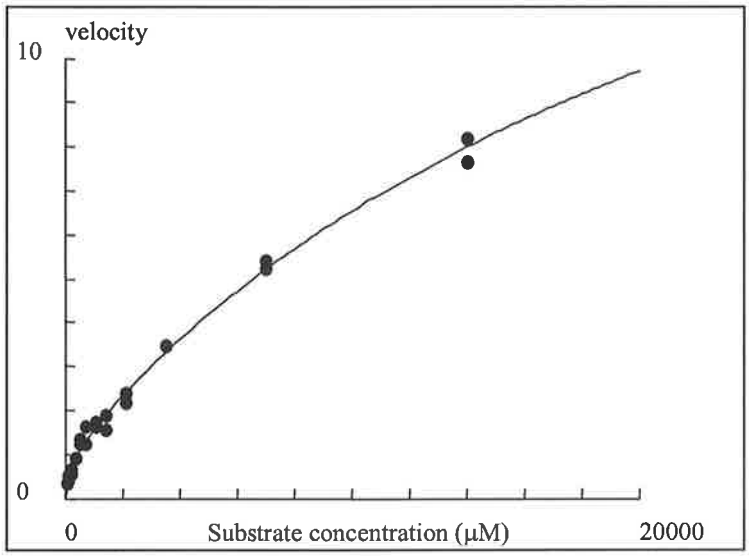
Vmax	280.384
Km	2982.26

Eadie-Hofstee plot



Oxymorphone formation HLM#24

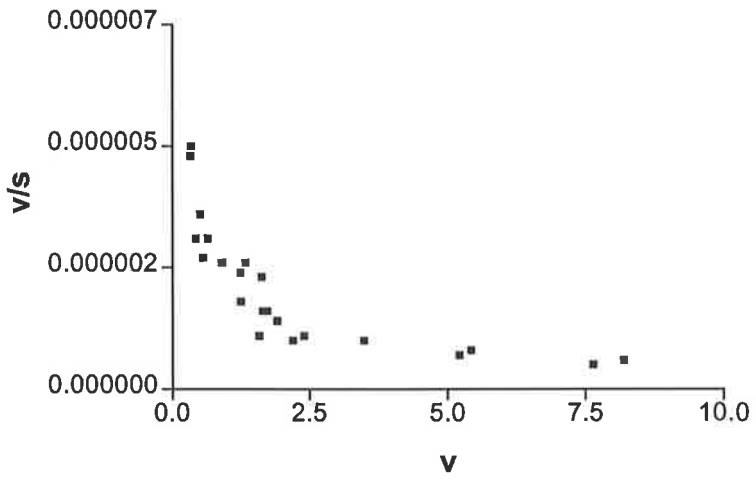
Michaelis Menten plot



Michaelis-Menten two enzyme kinetics (1/y*y weighting)
velocity=((Vmax1*substrate)/(Km1+substrate))+((Vmax2*substrate)/(Km2+substrate))
Iteration count = 262
Sum of squares = 0.621101
Standard deviation = 0.176224
Determination coef. = 0.994521

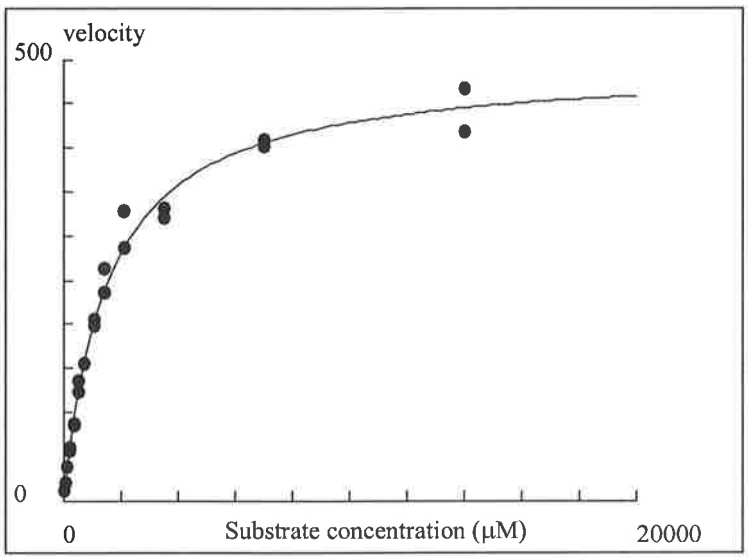
Vmax1	1.02489
Km1	217.712
Vmax2	20.3171
Km2	26749.9

Eadie-Hofstee plot



Noroxycodone formation HLM#24

Michaelis Menten plot

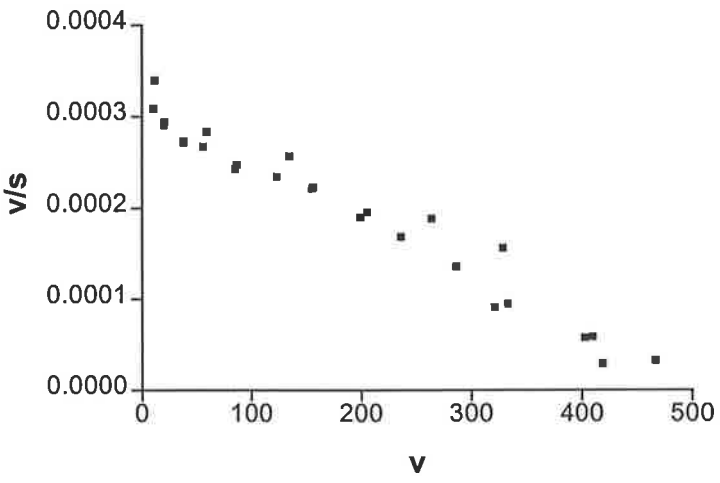


Michaelis-Menten enzyme kinetics (1/y weighting)
velocity=Vmax*substrate/(Km+substrate)

Iteration count = 40
Sum of squares = 4633.85
Standard deviation = 13.8952
Determination coef. = 0.99123

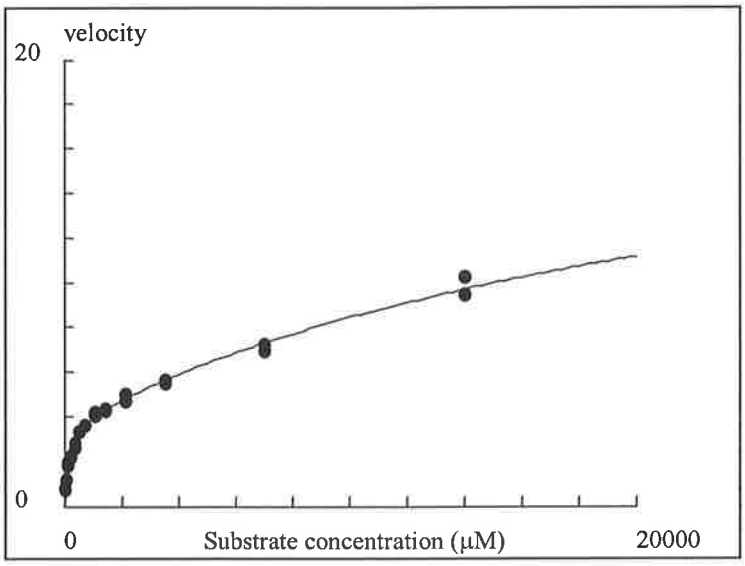
Vmax	493.421
Km	1524.89

Eadie-Hofstee plot



Oxymorphone formation HLM#31

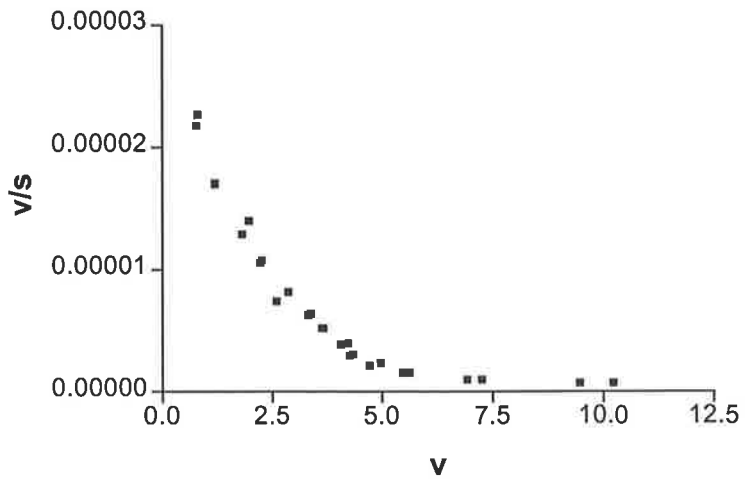
Michaelis Menten plot



Michaelis-Menten two enzyme kinetics (1/y*y weighting)
velocity=((Vmax1*substrate)/(Km1+substrate))+((Vmax2*substrate)/(Km2+substrate))
Iteration count = 105
Sum of squares = 0.777792
Standard deviation = 0.188027
Determination coef. = 0.994823

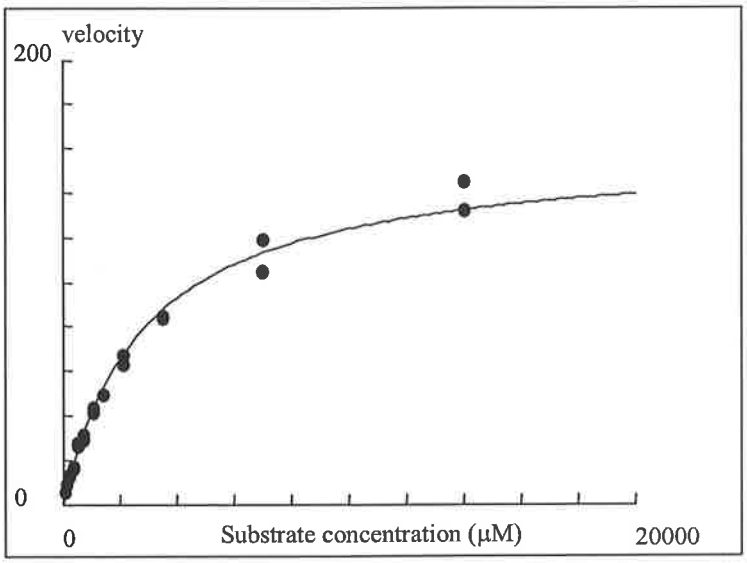
Vmax1	3.78772
Km1	152.751
Vmax2	17.4037
Km2	27088.5

Eadie-Hofstee plot



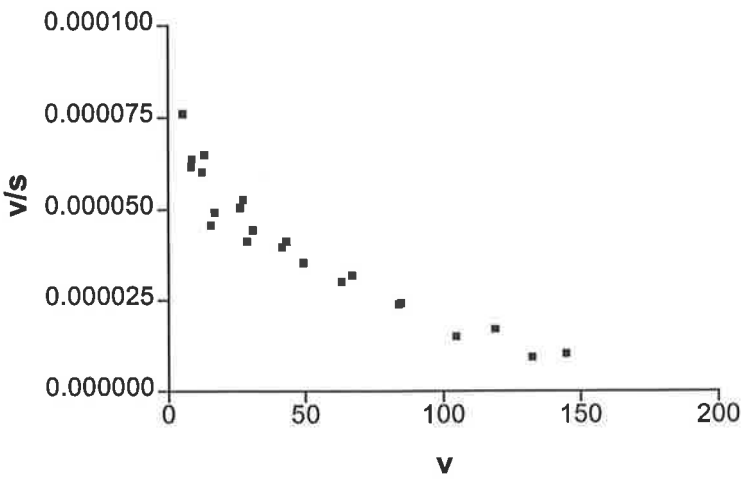
Noroxycodone formation HLM#31

Michaelis Menten plot



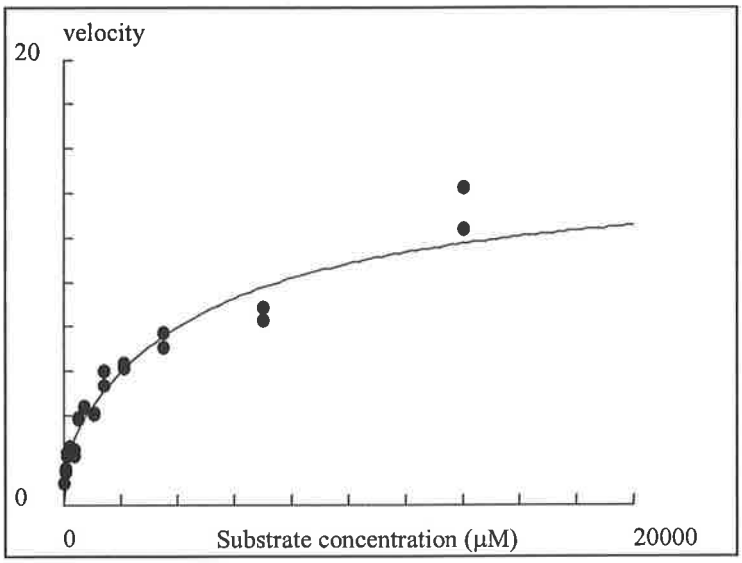
Michaelis-Menten enzyme kinetics (1/y weighting)
velocity=Vmax*substrate/(Km+substrate)
Iteration count = 35
Sum of squares = 346.672
Standard deviation = 3.96961
Determination coef. = 0.991444
Vmax 159.45
Km 2881.6

Eadie-Hofstee plot



Oxymorphone formation HLM#35

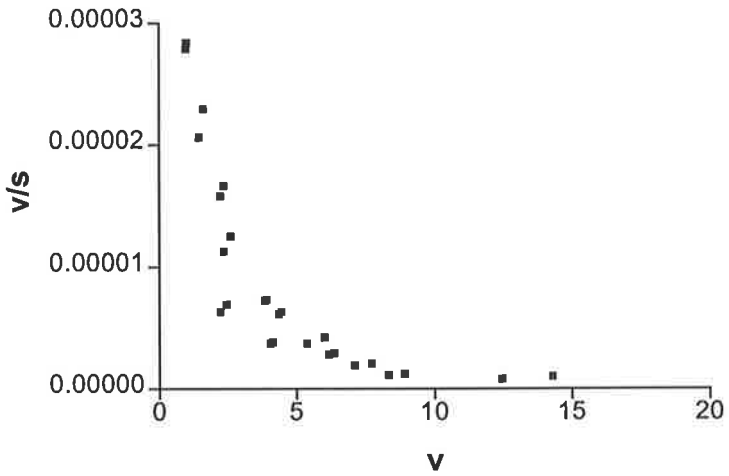
Michaelis Menten plot



Michaelis-Menten two enzyme kinetics (1/y*y weighting)
velocity=((Vmax1*substrate)/(Km1+substrate))+((Vmax2*substrate)/(Km2+substrate))
Iteration count = 103
Sum of squares = 13.5277
Standard deviation = 0.784152
Determination coef. = 0.952736

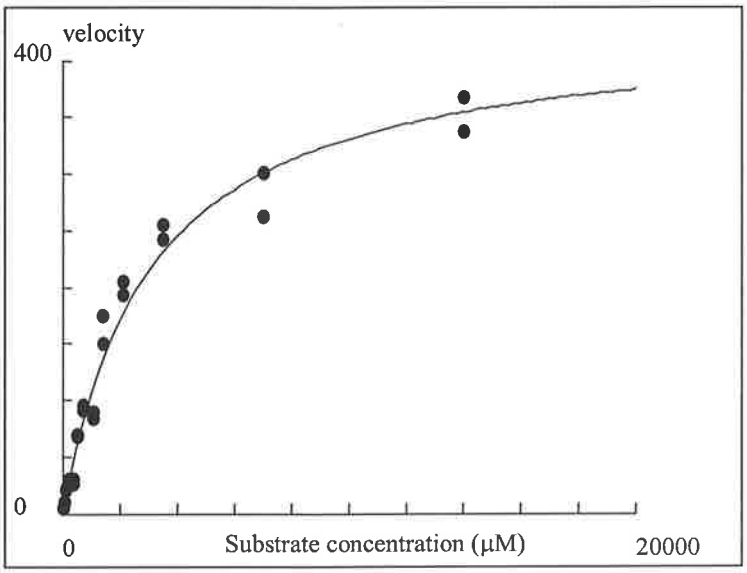
Vmax1	2.42385
Km1	57.1585
Vmax2	12.759
Km2	5171.55

Eadie-Hofstee plot



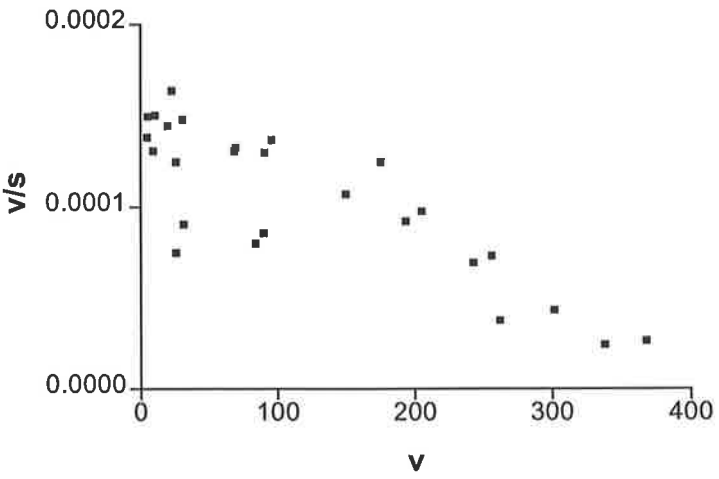
Noroxycodone formation HLM#35

Michaelis Menten plot



Michaelis-Menten enzyme kinetics (1/y weighting)
velocity=Vmax*substrate/(Km+substrate)
Iteration count = 34
Sum of squares = 7684.02
Standard deviation = 17.8932
Determination coef. = 0.976289
Vmax 431.649
Km 3050.02

Eadie-Hofstee plot

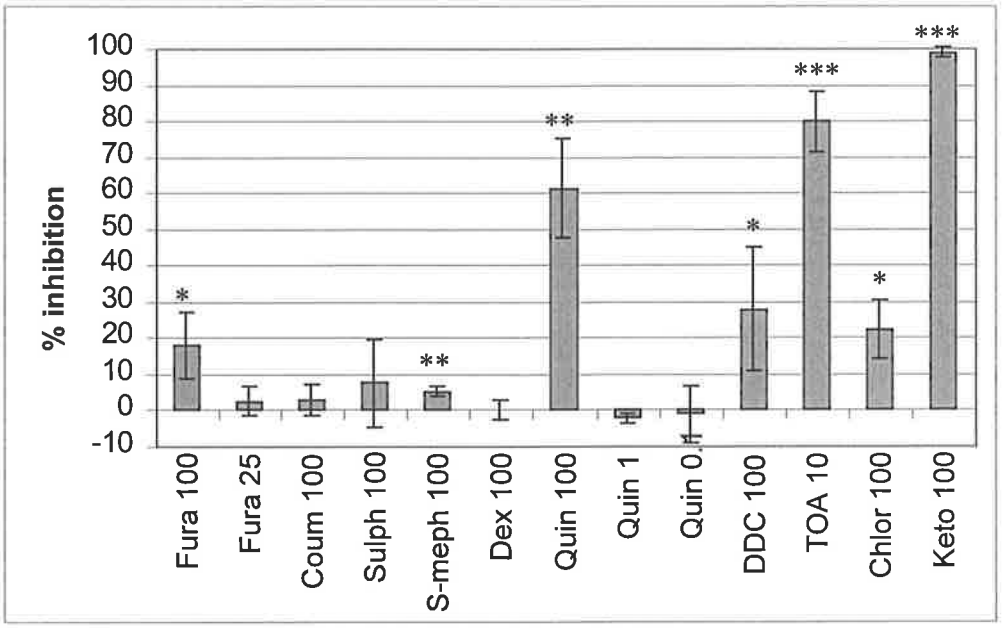


Appendix C

Additional data for Chemical inhibition studies

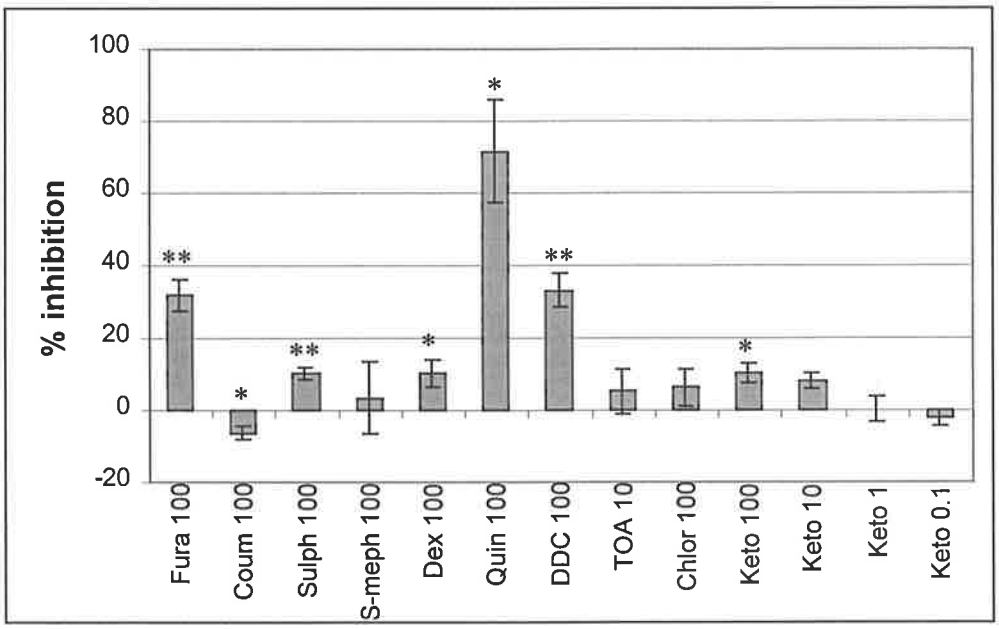
CYP isoform specific chemical inhibitors

Percent inhibition of NOC formation when OC concentration was similar to K_M of OM:



n = 4 (3 CYP2D6 EM and 1 PM)

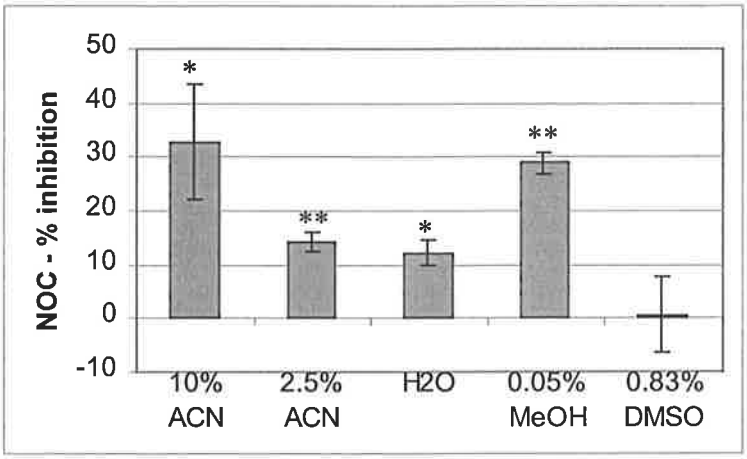
Percent inhibition of OM formation when OC concentration was similar to K_M of NOC:



n = 3 (3 CYP2D6 EM), Keto 10μM – n = 2

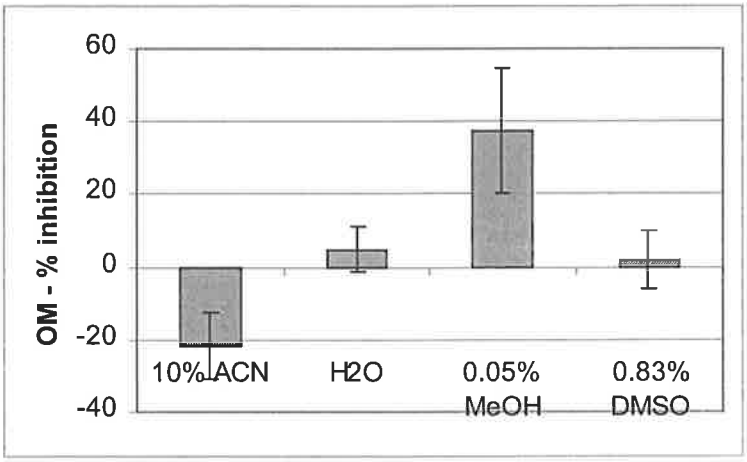
Solvent inhibition

Percent inhibition of NOC formation when OC concentration was similar to K_M of OM:



(n = 3 CYP2D6 EM)

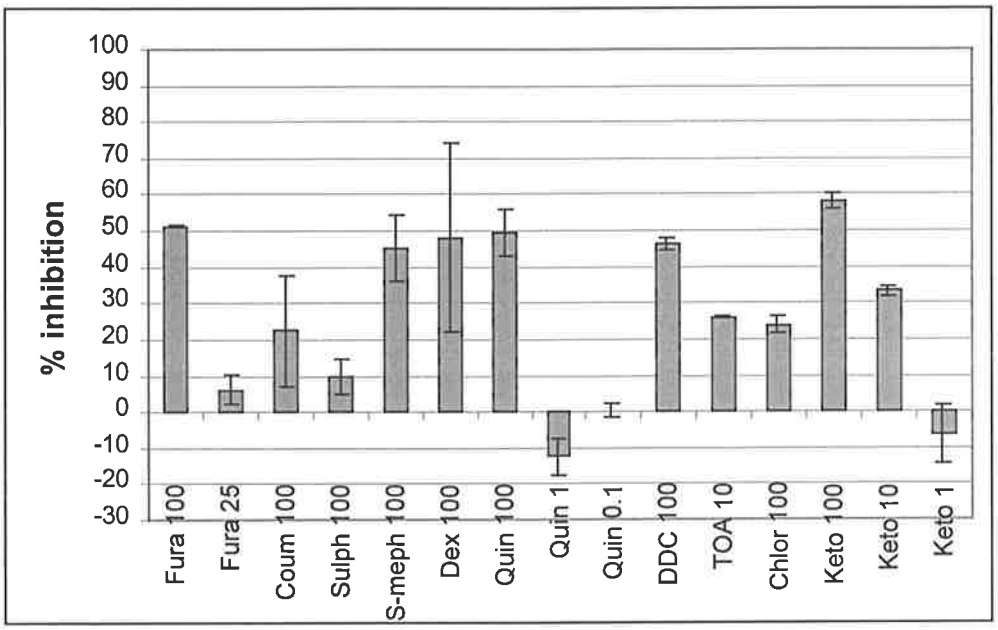
Percent inhibition of OM formation when OC concentration was similar to K_M of NOC:



(n = 3 CYP2D6 EM)

CYP isoform specific chemical inhibitiors: OM formation for a CYP2D6 PM

Percent inhibition of OM formation in a PM:



Each bar represents the mean of a duplicate. Error bars show SD.

OC concentration was similar to K_M of OM

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