

**The interactions of Propafenone and its
Enantiomers with the Major Human Forms of
Cytochrome P450 in Terms of Inhibition and
Metabolic Rates.**

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A thesis submitted in partial fulfillment of the requirements for the
degree of Master of Science by research, Department of Biological
sciences, University of Central Lancashire

July 2010

Declaration

I declare that while registered as a candidate for this degree I have not been registered as a candidate for any other award from an academic institution. The work present in this thesis, except where otherwise stated, is based on my own research and has not been submitted for any other award in this or any other university

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List of Abbreviations.

| | |
|---------------|--|
| (R) PPF | R enantiomer propafenone |
| (S) PPF | S enantiomer propafenone |
| μL | micro litre |
| μM | micromoles/litre |
| AGP | alpha 1 acid glycoprotein |
| AMMC | 3-[2-(N,N-Diethyl-N-methylammonium)ethyl]-7-methoxy-4-methylcoumarin |
| ATP | Adenosine-5'-triphosphate |
| BFC | 7-Benzyloxy-4-(trifluoromethyl)couamrin |
| CEC | 3-Cyano-7-ethoxycoumarin |
| CYP | Cytochrome P450 |
| DNA | Deoxyribonucleic acid |
| E | Enzyme |
| ES | Enzyme-substrate complex |
| HFC | 7-Hydroxy-4-(trifluoromethyl)-coumarin |
| HPLC | High performance liquid chromatography |
| HSA | Human serum albumin |
| IC50 | inhibitor concentraion when inhibition is 50% |
| KM | Michaelis constant |

LC-MS Liquid chromatography- mass spectrometry

MAMC 7-Methoxy-4-(aminomethyl)-coumarin

NADPH β -Nicotinamide adenine dinucleotide 2'-phosphate reduced
tetrasodium salt hydrate

PPF propafenone

S Substrate

TCP Tranylcypromine

UV ultra violet

V_{max} maximum velocity is when the enzymes are saturated.

WL wavelength

Abstract

Propafenone is a class 1C antiarrhythmic drug used for the treatment of ventricular arrhythmias. Propafenone is a chiral compound which is normally administered as the racemate. The use of racemate therapy can be problematic as variability in both the pharmacodynamic and pharmacokinetic properties of the separate enantiomeric forms of the drug can exist.

Propafenone enantiomers have been shown to have similar pharmacological properties, but studies are limited with regards to their disposition and interaction with drug metabolising enzymes. The aim of the study conducted here was to investigate the interaction of racemic propafenone with the major cytochrome P450 isoforms (CYP2D6, CYP1A2, CYP3A4 and CYP2C19) to determine any stereospecific differences which may exist. This was conducted by measuring the *in vitro* metabolism of propafenone (racemate and enantiomers) and by developing CYP inhibition screens which could prove to be useful in providing information of potential drug interactions. In addition, stereospecific binding to human albumin was measured to investigate whether any enantiomeric differences in unbound drug exist.

For the inhibition studies propafenone (racemate and enantiomers) was incubated in 96 well plates with separate CYP isoforms in the presence of a NADPH regenerating system. CYP activity was monitored using the following fluorogenic substrates: CYP2C19/ CYP1A2 – CEC; CYP2D6 – AMMC; CYP3A4 - BFC. IC₅₀ values were calculated and compared to those of control inhibitors (CYP2C19 – Tranycylpromine; CYP2D6 – Quinidine; CYP3A4 – Ketoconazole ; CYP1A2 - Furafllyline). *In vitro* metabolism was conducted using human liver microsomes incubated with propafenone (racemate and enantiomers) and the degree of metabolism measured using hplc analysis. Protein binding was estimated for propafenone (racemate and enantiomers) by a chromatographic method utilising a chiral HSA column.

Inhibition studies showed that the lowest IC₅₀ values were obtained when propafenone was co-incubated with CYP2D6 and CYP 3A4 which is to be expected as these CYP isoforms have been shown previously to be the major ones responsible for phase 1 metabolism of racemic propafenone. There was a distinct stereospecific difference with these isoforms, with the R-

enantiomer showing a higher degree of inhibition. This would suggest that there may be merit in considering single enantiomer therapy (in this instance using the S-enantiomer) to minimise the risk of any drug-drug interactions *in vivo*. However, the *in vitro* metabolism study showed that both single enantiomers were metabolised at a higher rate than the racemic mixture. This may be explained in terms of the 2 enantiomers competing for metabolism and thus inhibiting the metabolism of each other. These results suggest a possible problem with single enantiomer therapy of propafenone as this drug has a short half life and increased metabolism would decrease this even further. Investigations into the plasma protein binding of propafenone showed that there is no difference in the binding of the separate enantiomers and therefore there would be no stereospecific differences in free drug concentration, although propafenone also binds to acid glycoprotein and so binding studies with separate enantiomers need to be conducted with this protein too.

Overall, this study shows how *in vitro* techniques can be utilised to investigate stereospecific differences in drug disposition. The work described here warrants further study on the metabolism/disposition of propafenone enantiomers *in vivo* to examine the clinical implications of enantiospecific therapy with this drug.

1.0 Introduction

1.1 Aim of the study

Propafenone is a class IC antiarrhythmic drug used for the treatment of ventricular arrhythmias. The drug contains a chiral centre and so exists in two separate enantiomeric forms although it is normally administered as the racemate (Zhong and Chen, 1999). Indeed, propafenone is one of a number of such compounds which are administered as racemates. Other antiarrhythmic drugs also administered as racemates include encainide, flecainide and verapamil (Hanada *et al*, 2008).

The aim of this study is to determine any stereospecific differences in the metabolism and distribution of the enantiomers of propafenone and to consider how this could affect the therapeutic efficacy of propafenone.

1.2 Cardiovascular disorders and drug therapy

It has been reported that diseases of the heart and circulatory system are the main cause of death in Europe; accounting for 4.3 million deaths each year (British Heart Foundation, 2008). Throughout the world we invest millions of pounds in research and preventative therapies and treatments for diseases of the heart e.g. in 2008 alone £8.4 million was given to Oxford University to aid this research. As cardiovascular disease is Britain's biggest killer it is vital that research is carried out to ultimately reduce the mortality rate of heart disease.

Certain pathophysiological disorders can lead to conditions such as tachycardia and atrial fibrillation whereby antiarrhythmic drugs may be

prescribed. It is important to know as much about these drugs as they have such a narrow therapeutic index which is an indication of the toxicity of a compound (see section 1.4). The therapeutic index compares the concentration of a drug required to be effective with that which is toxic; the lower this number, the smaller the therapeutic window i.e. the smaller the difference between an effective and toxic drug concentration (Poschel *et al*, 1985; Klotz, 2007). Antiarrhythmic drugs are subdivided according to the classification in Table 1.1. Class I drugs (including propafenone) are primarily sodium channel blockers which prevent sodium from entering the cell and therefore delay depolarization and contraction of the heart (Fuster *et al*, 2001). Class II consist of beta blockers, class III contain potassium channel blockers which delay potassium from leaving the cell and repolarization from occurring and class IV consist of calcium channel blockers (Gard, 2001).

Table 1.1 The 4 classes of antiarrhythmic drugs and examples of each (www.CVpharmacology.com)

| Class I | Class II | Class III | Class IV |
|----------------|-----------------|------------------|-----------------|
| Quinidine | Propranolol | Amiodarone | Verapamil |
| Lidocaine | Nadolol | Ibutilide | Diltiazem |
| Flecainide | penbutolol | dronedarone | Nitrendipine |

1.2.1 Propafenone Pharmacology.

Propafenone binds to and blocks the fast sodium channels which are responsible for the rapid depolarization of myocardial cells (and hence is

classed as a type IC antiarrhythmic agent). As with other drugs in this class e.g. flecainide and encainide, the dissociation of propafenone from the sodium channel is slow. In a normal heart the contraction is triggered by the depolarization of the myocardial cells originating from the sinoatrial node (phase 0) (Fig 1.1), when this occurs it allows the sodium channels to open and sodium to enter the cell and the increased permeability to sodium causes a sharp rise of phase 0 (Keynes, 2001). Phase 1 indicates the closure of the sodium channels, phase 2 indicates a plateau which is caused by calcium ions moving into the cell and the movement of potassium ions out to allow the cell to repolarize which is phase 3. Between the period of phase 0 and 3 the cell is in a refractory period where it cannot undergo stimulation (Sparks, 1987). The initial depolarization occurs when the electrical current is transferred to neighbouring cells which in turn become depolarized which causes the atria to contract; this is known as the P wave. When the P wave reaches the atrio-ventricular node the impulse is passed to the bundle of HIS and the purkinje fibres which results in depolarization of the ventricles which contract

(Garratt and Statter, 2001). When the sodium channels are blocked the slope of phase 0 decreases as a result and the amplitude of the action potential similarly decreases. The effect of reducing the rate and amplitude of depolarization by the blockage of sodium channels facilitates the reduced conduction velocity in non-nodal tissue. This causes the transmission of action potentials across the heart to reduce, which suppresses the irregular conduction that can lead to tachycardia therefore the conduction of electrical signals across the heart is slowed and the heart is less excitable

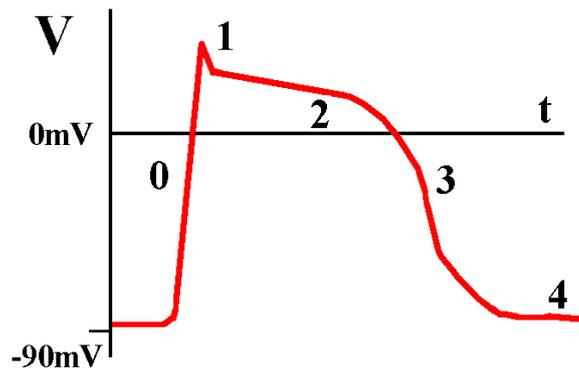


Figure 1.1. The cardiac action potential showing the different phases of depolarisation and repolarisation. (Sparks, 1987)

Propafenone also displays beta adrenoreceptor blocking activity (Zehender *et al*, 1992). Beta blockers inhibit normal sympathetic effects that act through these receptors which would increase heart rate. In addition, some beta blockers, when they bind to beta-adrenoceptors, they partially activate the receptor while preventing norepinephrine from binding to the receptor. This action provides a background of sympathetic activity whilst preventing the normal or heightened activity that would normally occur. Therefore, beta-blockers ultimately cause a decrease in heart rate, contractility, conduction velocity, and relaxation rate. Beta-blockers also possess what is referred to as membrane stabilizing activity (MSA). This effect is similar to the action of sodium-channels blockers (www.cvpharmacology.com) and hence this class of compound is also useful for antiarrhythmic effects.

Propafenone treatment is flexible as it can be used for both short and long term treatment of arrhythmias and can be administered both orally and intravenously. Intravenous administration is effective for ventricular

arrhythmias whereas an oral dose is an effective form of management of chronic re-current supraventricular tachycardia (Musto *et al*, 1988).

1.3. Racaemic drugs

As mentioned earlier propafenone is a chiral compound (Fig 1.2) which is predominantly administered as a racemate and at a dosage of between 300 - 450mg/ml (Jazwinski *et al*, 2001). When using chiral drugs it has been shown that stereoselectivity of one or more pharmacological activities may occur with the difference in activity being up to as much as 100 fold (Mehvar *et al*, 2002). Furthermore, the use of racemic antiarrhythmic drugs can be problematic with regards to their metabolism which may vary between different enantiomers. This may be especially problematic when the drug metabolism is genetically/phenotypically dependent and/or active metabolites are generated (Ha and Follath, 2004). It has been established that both enantiomers of propafenone exert similar sodium channel blocking abilities, but further research has shown that it is the (S) - enantiomer that produces greater Beta adrenoceptor blocking capabilities (Kroemer *et al*, 1994).

Research into racemate drug therapy is therefore vital as the two individual enantiomers can exert different pharmacological properties and so do not reflect the same action as the racemate. It is important to look at the individual activities of the enantiomers and the interactions between them when given as a racemic mixture as they could in fact prove to be more effective individually or potentially dangerous. Such is the case with thalidomide where the (S)-enantiomer caused the teratogenicity whilst the (R)-enantiomer acted

as a sedative (Anderson, Richardson and Alsina, 2003). Furthermore in this case, the use of separate enantiomers failed as they could interconvert in the body (Anderson, Richardson and Alsina, 2003). In fact it has been proposed that racemic drugs should be avoided altogether (Kroemer *et al*, 1994).

There are many variables that can affect a drug's efficacy; for example whether its metabolism is inhibited by its opposite enantiomer (Kroemer *et al*, 1994) or another drug, or how the drug itself inhibits the body's enzymes and the effects of the drug's metabolites. It is vital to discover these variables and the best way to minimise or eliminate them to ultimately improve a patient's health.

In particular, research into differences in drug metabolism is vital to determine possible side effects of drugs during the process of drug development. The metabolic pathway(s) for the drug needs to be fully understood as it will play an important factor in determining the pharmacological or toxicological effects (Gibson and Skett, 2001). Indeed, as will be discussed later, the activity of metabolising enzymes determines the plasma concentrations and therefore the associated effects of the drug (Botsch *et al*, 1993) and this information can be invaluable in determining the interactions of the drugs, variability and effectiveness.

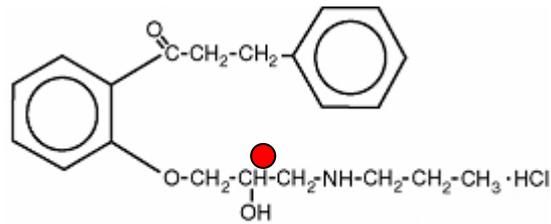


Figure 1.2 The structure of propafenone, where the red circle represents its chiral centre.

1.4. Factors that affect plasma concentration.

The pharmaceutical effectiveness of a drug is dependent upon its concentration at its site of action. The processes which determine changes in the concentration of a drug in the body can be subdivided into four discrete areas. These are the processes of absorption, distribution, metabolism and excretion which will be discussed below. It is the combined effect of these processes which determine the shape of the plasma concentration – time curve illustrated in Figure 1.4. These factors will determine how long a drug will be present in the body which is often expressed as the half life of the drug ($t_{1/2}$) which is the time taken for plasma concentration of the drug to fall by half. Propafenone has an elimination half life of approximately 2 – 10 hours increasing to as high as 32 hours in poor metabolisers. (Jogler *et al*,1998). As the work described here concentrates in particular on the drug metabolism process; this will be discussed in more detail.

The term ‘therapeutic window’ is used to describe the plasma concentration range at which a drug is effective with minimal adverse reactions. The therapeutic window lies between two regions. The lower one represents the

lack of drug and therefore its ineffectiveness. The upper region is an over exposure resulting in the therapy being effective but producing adverse effects, therefore the therapy has failed. Propafenone is one of a number of drugs for which the therapeutic window is narrow and hence relatively small changes in plasma concentration can lead to ineffectiveness/toxicity. The result of this can be seen with the situation in 2008 when Watson Pharmaceutical had to recall a batch of propafenone tablets whose size varied sufficiently to alter plasma concentration. Patients who were sensitive to small variations in dosage may have experienced serious side effects. (www.fda.gov)

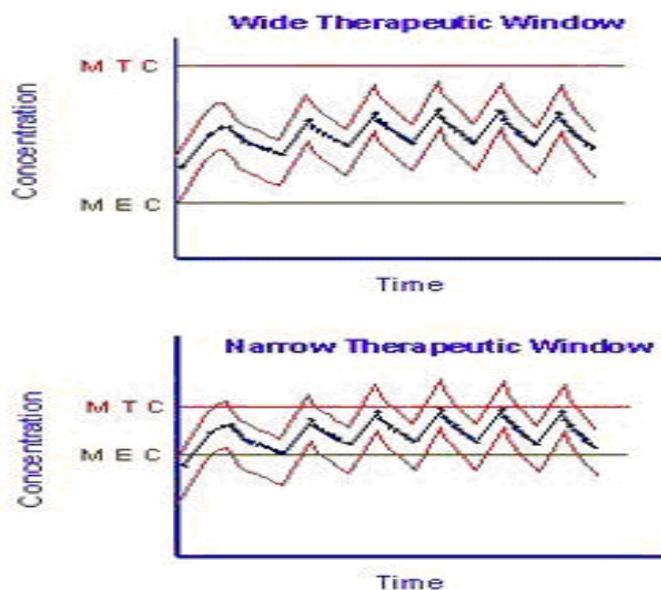


Figure 1.3 A diagrammatic representation of a wide and narrow therapeutic window. (www.vin.com).

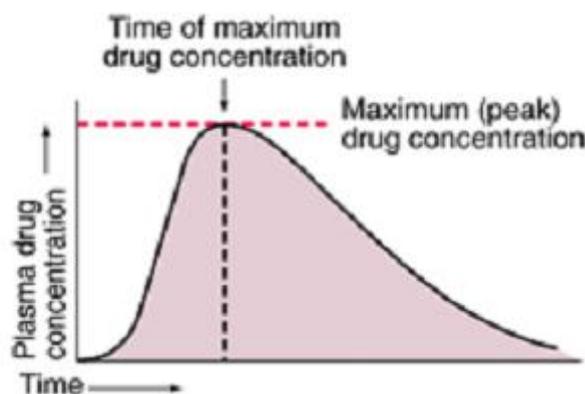


Figure 1.4. Plasma concentration–time relationship after a single oral dose of a hypothetical drug. (www.merck.com)

1.4.1. Absorption

A compound needs to be absorbed into the bloodstream before its pharmacological effects can occur. There are several factors that affect the absorption of a drug after oral administration, for instance the compounds solubility, permeability and its chemical instability. In large part absorption of a drug is determined by the compounds bioavailability. Bioavailability is a relative term used to describe the rate and extent of absorption after oral administration of a drug (Kwon, 2001). The extent of bioavailability is governed by factors such as the absorption from the gastrointestinal tract and metabolism that occurs before absorption takes place. The absorption of propafenone is extensive and rapid with a peak plasma concentration occurring after approximately 3.5 hours' (www.GSK.com). The issue of bioavailability of drugs is important in determining their therapeutic effectiveness and an estimation of bioavailability can be made using Lipinski's 'rule of five'. This indicates whether a drug is likely to be orally bioavailable. The rules specify that the drug will have more than 5 hydrogen bond donors,

no more than 10 hydrogen bond acceptors, and a molecular weight under 500 and a LogP under 5. (www.bioscreening.com) The process of absorption can occur via several routes, which include passive diffusion, facilitated diffusion and active transport. Simple diffusion of a drug across the cell membrane, it is a process driven by the random motion of the molecules. Diffusion is described by Fick's law, which states that diffusion is proportional to the concentration gradient (Washington *et al*, 2000). The difference in concentration is maintained by the continual removal of the drug from the blood to tissues. It is also dependant on the drug's lipid solubility and the degree of ionization and its size. The process of facilitated diffusion and active transport both require the presence of a carrier molecule which combines with the drug molecule and allows it to cross the bilayer. This is evident from the transport of molecules such as glucose which has low lipid solubility, and this action is selective as it will only allow the movement of molecules with a specific molecular configuration and the availability of the carriers. Facilitated diffusion does not require energy and similarly to passive diffusion, movement is dependant on a concentration gradient from high to low. An example of this is the movement of sodium into the cardiac cells via a carrier protein which exchanges sodium for calcium (Sparks, 1987). The process of active transport utilises energy in the form of ATP to pump molecules across a membrane against a concentration gradient. An example of this is Na⁺, K⁺ ATPase which expels three sodium ions for every two potassium ions that move into the cell, this movement is possible through the energy form ATP (Sparks 1987). In addition, P Glycoprotein transporters found in the gastrointestinal membrane can use the process of active transport to pump drugs back out of

the body into the gastrointestinal lumen. There is an overlap between the substrates of P Glycoprotein and CYP3A4, therefore the drugs that inhibit or induce CYP3A4 do the same to P Glycoprotein and consequently affect transportation (Troy and Beringer, 2005)

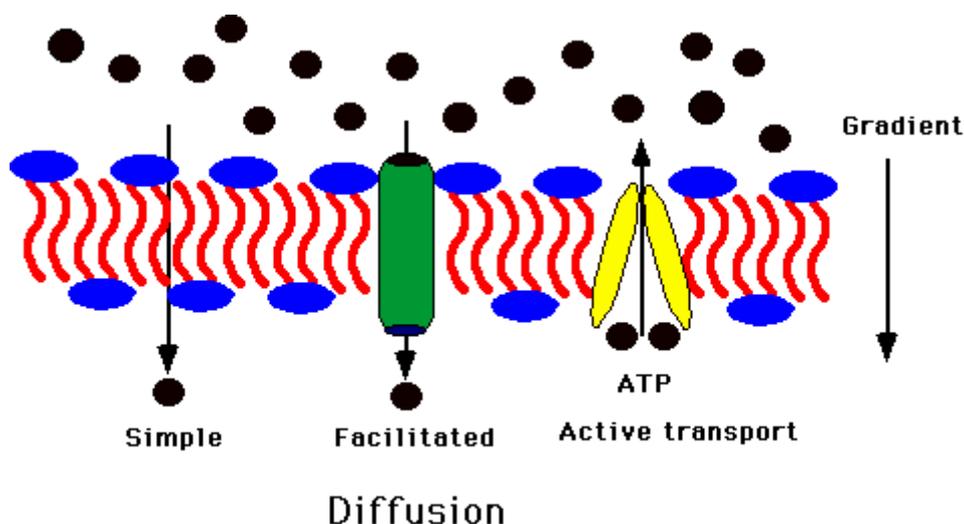


Figure 1.5. Diagram illustrating the three major processes involved in the passage of molecules across the plasma membrane lipid bilayer. (www.rpi.edu)

1.4.2 Distribution

For a drug to have its desired therapeutic effect it is necessary to be distributed in such a manner that a sufficient concentration reaches its site of action. The process of drug distribution is dependent on the physicochemical properties of the drug which can affect how drugs will partition throughout the different fluid compartments within the body. In addition, the distribution of drugs is influenced by their binding to proteins found within both the blood plasma and the tissues. An estimation of the distribution of the drugs can be made by calculating the apparent volume of distribution which is mathematical in concept defined as the distribution of a drug between the plasma and the

rest of the body. The Vd for propafenone averages between 2.5 – 4.0 L/Kg (Kerns *et al*, 1994).

1.4.2.1 Protein binding

Binding to proteins plays an essential role in the transport and distribution of drugs and it is a function of the affinity of the protein to the drug. Plasma protein binding is mainly to the protein albumin which possesses binding sites for both endogenous and exogenous compounds. The two major binding sites are known as the warfarin-azapropazonesite and the indole-benzodiazepine site (Cheng *et al*, 2004). The affinity is characterised by an association constant and because of the limited binding sites binding also depends on the concentration of the drug and protein (Rowland and Tozer 1995). Drug-protein binding may also occur with the protein alpha 1 acid glycoprotein (AGP). AGP has the ability to bind and carry many basic and neutral lipophilic drugs as well as acidic drugs such as Phenobarbital (Fournier *et al*, 2000). AGP is an acute phase plasma protein synthesized in the liver. Its concentration in blood plasma is increased in time of trauma and can be used to diagnose inflammatory conditions. The exact mechanism of inflammatory and immune responses is unknown at this time (Gunnarsson *et al*, 2007).

The degree of protein binding is an important factor as it is the unbound drug which is free to determine the pharmacological activity as well as undergo processes such as metabolism excretion and disposition. Propafenone is highly protein bound usually around 95%. Therefore, the higher affinity a drug has for the protein the slower the disassociation rate will be therefore lowering clearance and increasing the elimination half life (Sear, 2007). The degree of

binding to the individual enantiomers could affect their pharmacological properties by altering the amount of free drug available. Therefore quantitative determination of drug protein binding is important in drug development (Cheng *et al*, 2004).

1.4.2.2 Tissue binding

Tissue binding can also play a role in the distribution of drugs. There have been less studies of tissue binding conducted in comparison to plasma protein binding perhaps because it is more difficult to measure, and also it requires that tissue has to be disrupted and therefore loses its integrity (Rowland and Tozer, 1995). The primary effect of tissue binding is on the time period of the drug within the body, as it can increase the half life of the drug.

Furthermore, non-specific tissue binding is an important factor to be considered when conducting *in vitro* experiments (such as the drug metabolism work conducted here) as this can lead to the drug concentration to be underestimated (Austin *et al*, 2002)

1.4.3 Drug metabolism.

The process of drug metabolism is central to the excretion of drugs from the body. In general terms drug metabolites are more hydrophilic than their parent drug which decreases their reabsorption from the tubules of the kidney back into the body hence increasing their excretion. In addition, the structural changes caused by drug metabolism can, in many instances, decrease the pharmacological activity of the drug. Although this need not always be the

case with many drug metabolites showing equipotent or even greater pharmacological activity to the parent drug. Drug metabolism can be subdivided into two main phases: phase 1 and phase 2. Phase 1 involves the oxidation, reduction or hydrolysis of the drug with the majority of reactions being catalysed by the cytochrome P450 family of enzymes (Fig 1.6). This process generally makes the drug more reactive by introducing/unmasking chemically reactive groups and in doing so, may makes the drug more toxic or even more pharmacologically active (Gard, 2001). Phase 2 metabolism involves the addition of a conjugate to the molecule (Gibson and Skett, 2001) (Fig 1.6). Phase 2 metabolism requires a functional group which may be present on the original drug or is very often added or exposed during phase one metabolism (Fig 1.7). The function of phase 2 metabolism is to increase the water solubility of the compound hence increasing its excretion by decreasing the process of urinary reabsorption (Fig 1.6).

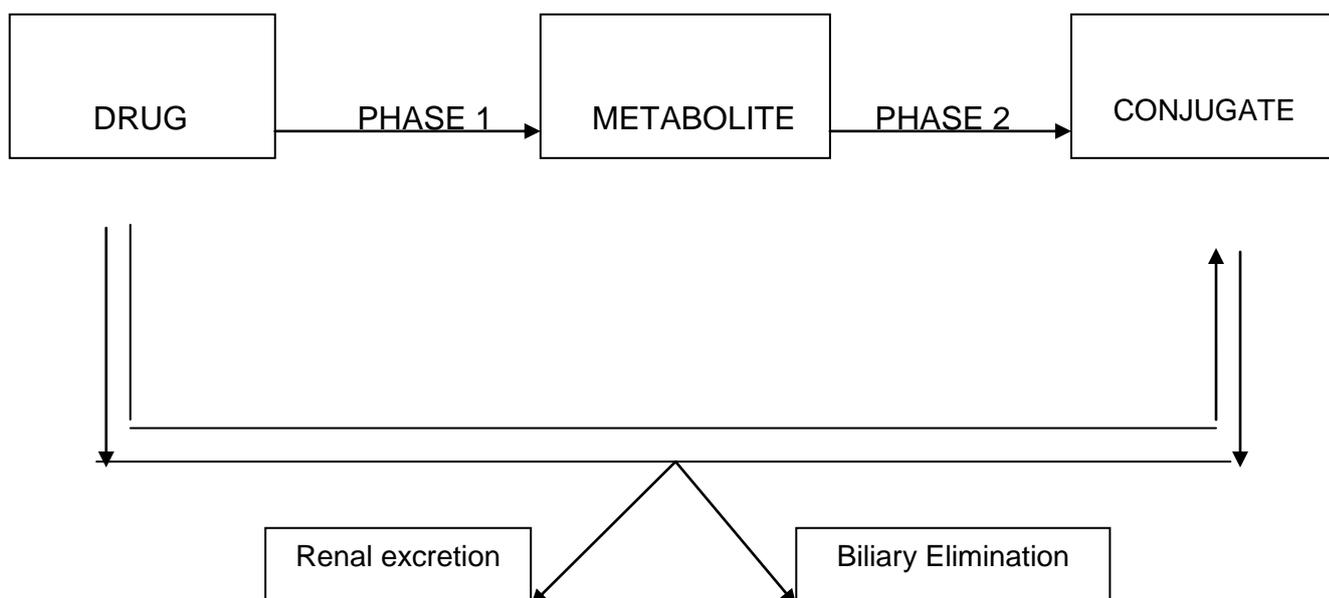


Figure 1.6. Diagrammatic representation of the metabolism of drugs via Phase I and Phase 2 processes. The overall process results in the metabolites becoming more polar than the original drug.



Aromatic Hydroxylation



Aliphatic Hydroxylation



N-Demethylation

Figure 1.7 Shows examples of phase 2 reactions

1.4.4 Excretion

Excretion is the irreversible loss of a chemically unchanged drug and occurs predominantly via the kidney with biliary excretion being the second most common route (Tozer and Rowland, 2006). Propafenone is almost entirely metabolised in the liver with only 1% of the drug being excreted unchanged (Jogler *et al*, 1998).

1.5 Cytochrome P450

The cytochrome P450 (CYP) family of enzymes are the major contributors to Phase 1 metabolism (Kwon, 2001). They are a superfamily of heme containing enzymes found in the endoplasmic reticulum of the cell. CYPs are found in high quantities in the liver although they are also found in other organs throughout the body such as the kidney, breast and gastrointestinal tract.

(Ioannides, 1996). Cytochrome P450 was first mentioned in literature in 1962 when liver microsomes (which are a suspension of the endoplasmic reticular fraction of the cell) were treated with dithionite (a reducing agent) and carbon monoxide gas which binds to the reduced ferrous heme of the Cytochrome P450 enzymes present to give a strong absorption band at 450nm. This is known as a reduced carbon monoxide difference spectrum, and it is this characteristic absorption which gives rise to the 450 element of their title, where the 'P' represents the pigment (Omura, 1999). Absorbance at 450 nm is significant as no other heme containing protein produces an absorbance at 450nm.

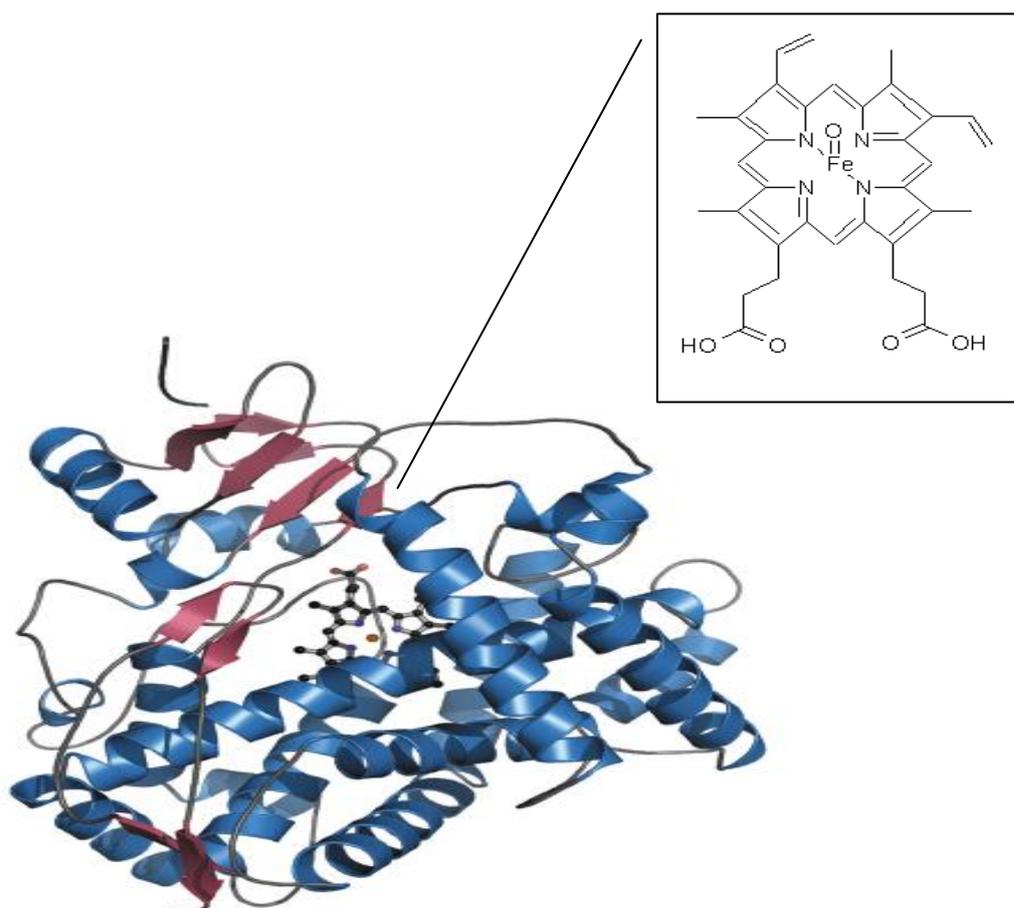


Figure 1.8. The molecular structure of Cytochrome P450 showing the heme moiety and associated Fe atom. (www.esrf.eu)

The overall process of CYP catalysed metabolism is represented in figure 1.9. The process can be summarised as CYP splitting molecular oxygen and using one atom for the generation of the metabolite and the other for the production of water. In order for Cytochrome P450 to function they need a source of electrons added to the heme iron for the catalytic cycle to occur. The electrons are donated by another protein in the endoplasmic reticulum called NADPH cytochrome P450 reductase. There is, therefore a requirement of NADPH for CYP activity both *in vivo* and *in vitro* (Mathews *et al*, 2000; Fig 1.10).

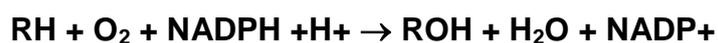


Figure 1.9 General equation for the metabolism (oxidation) of a substrate (RH) by Cytochrome P450.

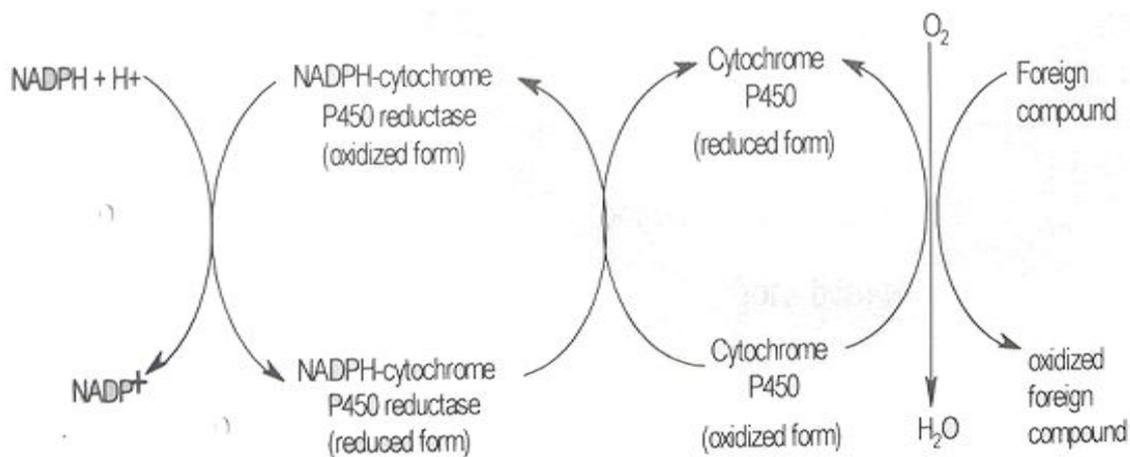


Figure 1.10 Oxidation of foreign compounds by Cytochrome P450 (Gilham *et al* 1997)

When considering the metabolism of drugs by CYP it is important to realise that a drug can be metabolised by one or multiple CYPs and that it is the affinity of the drug to the enzyme and the abundance of the enzyme in the body which affect the rate of metabolism this is important with regards to the inhibition study. (www.Cytochrome P450 Enzymes and Psychopharmacology.htm)

1.5.1 Classification of CYP

As there are so many different isoforms of P450 it is important to be able to differentiate between them. Classifying them in relation to their amino acid sequence allows this differentiation to occur (Kelly *et al*, 2006). CYP isoforms are divided into families and subfamilies with the family accounting for genes which possess at least 40% sequence homology (Ioannides, 1996). Families are symbolised with numbers whereas the subfamilies are identified using letters and must have at least 55% sequence homology (Nerbert and Gonzalez, 1987). In addition to this individual genes are identified with a number, for example CYP2D6.

To date, 57 CYP genes and 58 pseudogenes have been sequenced in humans. The most abundant form of CYP is CYP3A4 which makes up approximately 30% of the total P450 composition of tissues (Plant and Gibson, 2003). The CYP 3A family has been identified as the major cytochrome P450 expressed in the liver at all developmental stages and CYP3A4 has been proven to be responsible for up to 60% of the metabolism of pharmaceuticals (Gibson and Skett, 2001). The CYP2 family is the largest

family in humans and one third of all human P450's are in this family (www.drnelson.utmem). CYP2D6 is studied extensively as it is polymorphic and metabolises many drugs including anti-depressants and opioids (Chen and Wei- Min, 2003).

CYP1A2 is very important as it can be induced by polycyclic hydrocarbons found in cigarette smoke and it can metabolise compounds to become carcinogenic e.g. benzo (a) pyrene and has been associated with colon cancer (www.drnelson.utmem; Johns-Cupp, 1998).

As cytochrome P450 enzymes have such a central role in drug metabolism it follows that any change in CYP activity may have a profound effect on the concentration of a drug in the body, and so the pharmacological and toxicological effects. There are a number of factors which can influence CYP activity which are summarised below.

1.5.2 Factors Affecting Cytochrome P450 Activity

Genetic polymorphism is a difference in DNA sequence in a particular gene found at 1% or higher in a population. These differences lead to changes in drug metabolism when there are several metabolic phenotypes. There are usually two main phenotypes which result in the distinction between fast and slow metabolisers (Funckbrentano, 1991). This difference is subject to ethnic variability for example in caucasians the polymorphism of CYP2C19 for the poor metaboliser phenotype is only seen in 3% of the population whereas in the Asian population it can be seen in 20% (www.drnelson.utmem). Therefore

it is important to be aware of a persons race when drugs are administered that are known to be metabolised differently by different populations. It is now possible to determine what type of metaboliser a person is. In terms of progression this is an important step as CYP1A2, 2C19, 2D6 and 3A4 are responsible for oxidising more than 90% of currently used drugs.

1.5.3 Age

Drug metabolism can be affected by the age of the individual. It is generally the case that the young and the old have a lower drug metabolising capacity. (Gibson and Skett 2001). In men and women over the age of 65 the ability to metabolise drugs declines as does the amount of the relevant cytochrome P450 (Gibson and Skett, 2001). Age can have an affect on drug metabolism due to a reduction in liver volume and a reduction in the activity of some CYP's. It has been proven however that there is reduced activity of CYP1A2 and CYP2C19 with age. It has also been suggested that the inducibility of metabolising enzymes decreased with age (Kinirons and O'Mahony, 2004).

1.5.4 Drug interactions.

The risk of having an adverse reaction increases with the number of drugs taken. A drug interaction occurs when the pharmacodynamics or pharmacokinetics of one drug are altered by another. The extent of the interaction is dependant on the dose and time (Rowland and Tozer, 1995).

The use of *in vitro* studies has several advantages for example the savings on cost and time of as well as minimization of human risk due to the planning and design of clinical studies.

1.5.4.1 Induction

Induction can be defined as an increase in the transcription and hence the levels and activity of metabolising enzymes. Examples of known inducers of CYP are shown in Table 1.2. A number of drugs have been shown to increase their own metabolism as well as the metabolism of co-administered drugs via the process of induction. Due to the mechanism involved in induction, this process is not instantaneous, but has a time lag before the increase in enzyme level is observed. However, the induction of drug metabolism does have significant clinical importance as increased metabolism can lead to a decrease in plasma concentrations of drugs which may render them ineffective. For example the treatment of phenobarbitone and its effect on the muscle relaxant zoxazolamine. Zoxazolamine undergoes metabolic hydroxylation which forms an inactive metabolite. The increase in the metabolism of the muscle relaxant in turn decreases the paralysis time of the drug (Gibson and Skett, 2001).

Table 1.2 The CYPs used in this study and examples of their known inducers (www.medicine.iupui.edu)

| CYP1A2 | CYP2D6 | CYP2C19 | CYP3A4 |
|---------------|---------------|----------------|---------------|
| Omeprazole | Rifampin | Rifampin | Phenobarbital |
| Tobacco | Dexamethasone | Carbamazepine | Rifampin |
| Insulin | Piperidines | Norethindrone | Carbamazepine |
| Nafcillin | Carbamazepine | Prednisone | Barbiturates |

1.5.4.2 Inhibition

The inhibition of metabolic processes is extremely important in terms of possible therapeutic problems associated with drug-drug interactions (DDIs). In this case, the inhibition process can lead to accumulation of drug in the body which may reach toxic levels. Indeed, for cytochrome P450 enzymes, many drugs have been found to cause inhibition which has restricted their use due to the potential for severe DDIs (Li *et al*, 2007). The process of inhibition can be either competitive or non competitive in nature although in the majority of cases it is competitive inhibition which is observed. A number of clinically relevant inhibitors of cytochrome P450 are shown in Table 1.3.

An example of DDI is the interaction of felodipine with grapefruit juice. CYP3A present in enterocytes of the intestinal epithelium extensively metabolise felodipine during its absorption allowing only 30% of the administered dose to enter the portal vein. CYP3A enzymes in the liver further metabolise the drug so that only 15% of the dose is bioavailable and can exert its effect. Grapefruit juice selectively inhibits CYP3A in the enterocyte therefore increasing the oral bioavailability of felodipine by a factor of 3 (Wilkinson, 2005).

This example shows the clinical importance of identifying inhibitors of specific CYP's and the associated drug interactions in order to prevent the inability to metabolise the drug.

Table 1.3 The CYPs used in this study and examples of their known inhibitors (www.medicine.iupui.edu)

| CYP1A2 | CYP2D6 | CYP2C19 | CYP3A4 |
|---------------|---------------|----------------|---------------|
| Fluvoxamine | Fluoxetine | Omeprazole | Erythromycin |
| Cimetidine | Quinidine | Fluoxetine | Verapamil |
| Furafyline | Omeprazole | Oxcarbazepine | Ketoconazole |
| Interferon | Cimetidine | Ticlopidine | Diltiazem |

1.5.5 Metabolism of propafenone

Propafenone has been reported to be metabolised by CYP2D6 to 5-hydroxypropafenone and to N-depropyl-propafenone by CYP3A4 and 1A2; which is the minor pathway in comparison the one mediated by CYP 2D6 (Yong-Jiang and Ming-Ming, 2004) (Fig 1.11). Phase 2 metabolism is predominantly via the generation of the glucuronide. Compared to propafenone, the main metabolite, 5-hydroxypropafenone, has similar sodium and calcium channel activity, but about 10 times less beta-blocking activity (N-depropyl-propafenone has weaker sodium channel activity but equivalent affinity for beta-receptors).

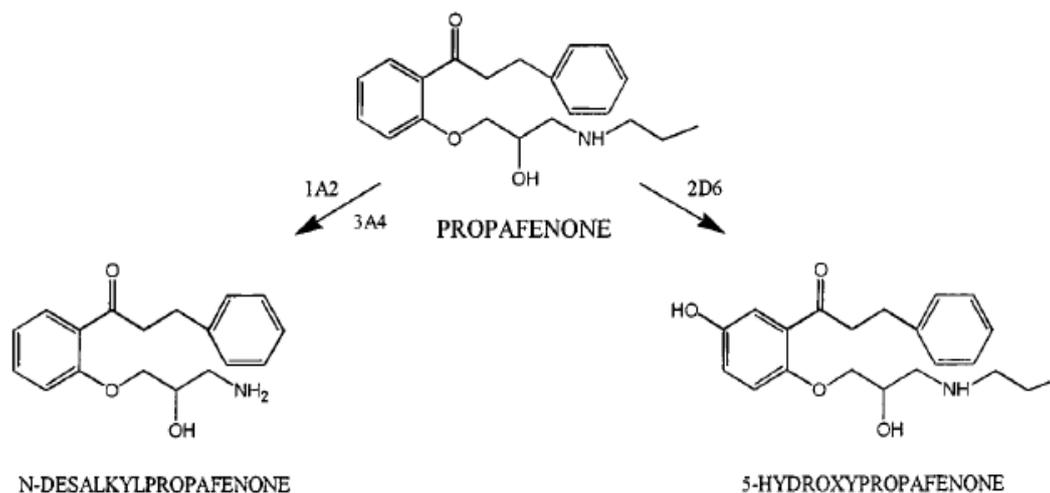


Figure 1.11 The two main metabolic pathways of propafenone and the 3 cytochrome P450's which are responsible for the catalytic process (Labbe *et al*, 2000, Munoz *et al*, 1997).

The majority of studies on the metabolism of propafenone have concentrated on the involvement of CYP2D6 with an emphasis on investigating the effect of CYP2D6 polymorphism on the therapeutic efficacy of propafenone. It has been found that there is interindividual variability in the pharmacokinetic and pharmacodynamic characteristics of propafenone resulting from the genetic polymorphism found with CYP 2D6 (Wei *et al*, 1999) (where around 7% of the white population have been shown to be poor metabolisers) (Siddoway *et al*, 1987; Dilger *et al*, 2000). It was also found that propafenone therapy was 100% effective in controlling arrhythmias in poor metabolisers and only 61% effective in extensive metabolisers (Jazwinski *et al*, 2001).

Furthermore, research on the metabolism of propafenone and its effects with the co-administration of the CYP 2D6 inhibitor quinidine has demonstrated an importance of this enzyme in the metabolism of propafenone. Indeed,

quinidine has been shown to increase the plasma concentration of propafenone in extensive metabolisers (Morike and Roden, 1994).

It has also become clear that there is significant interaction between both (R) and (S)-propafenone. With regards to their metabolism, the (R)-enantiomer can inhibit the metabolism of (S)-propafenone (Pires de Abreu *et al*, 1999). This study has indicated that the effects of racemic drug therapy cannot simply be deduced through the effects of the individual enantiomers as the beta blocking effects of (S)-propafenone were more pronounced when administered as a racemate rather than individually.

In addition when the metabolism of propafenone is reduced it creates a higher concentration of (S)-propafenone to exist. As (R)-propafenone inhibits the (S)-enantiomer which exhibits the higher beta blocking effect.

The following study will show the extent of metabolism of propafenone via these pathways but it will also include CYP2C19 which tends to be studied to a lesser degree than the other major form of cytochrome P450 (Turpeinen *et al*, 2006).

1.6 Methods Used in This Study

1.6.1 Investigation of the Activity of Cytochrome P450 *in vitro*

There are a number of techniques which can be used to investigate the activity of CYP enzymes, their interactions with different substrates and the propensity for DDIs. These are outlined in Table 1.4.

In this study, heterologous CYP expression system (cDNA expressed human drug metabolising enzymes) will be used for conducting *in vitro* inhibition studies (bactosomes, Cypex). This system is utilised rather than microsomes as the bactosomes contain only one CYP isoform as opposed to a mixture of enzymes found in hepatic microsomes. This was chosen in order to determine which CYP is inhibited to the greatest degree by propafenone and, when incubated with an individual bactosome, which one metabolises propafenone the most. By doing this the individual bactosomes can be compared and it is possible to deduce which CYP isoform propafenone (racemate and enantiomers) shows greatest interaction. The bactosomes chosen for the inhibition and metabolism studies are CYP3A4, CYP2D6, CYP2C19 and CYP1A2.

Table 1.4 The methods employed to investigate the activity of CYPs and drug metabolism (Tingle and Helsby, 2005)

| Method | Advantages | Disadvantages |
|---|---|---|
| Animal models | <ul style="list-style-type: none"> • Physiologically relevant. Can identify metabolites formed. • Provides information on clearance by multiple enzymes and organs. • Information on drugs that don't undergo hepatic clearance. | <ul style="list-style-type: none"> • Can show what is biologically possible not necessarily what is relevant for humans. • Inter-species differences in drug metabolism |
| Human liver microsomes (HLM) | <ul style="list-style-type: none"> • Allows CYP reaction phenotyping- to identify undesirable DDIs • Qualitative determination of metabolite identity • Used for prediction of <i>in vivo</i> clearance • Substrate depletion approach can be utilised. | <ul style="list-style-type: none"> • This approach is limited technically due to non-specific binding of the drug to microsomal protein. • Concerns over the appropriate drug substrate concentration • Requires prior knowledge of the metabolic fate of the drug |
| cDNA expressed human drug metabolising enzymes. | <ul style="list-style-type: none"> • Widely used to determine the individual CYP involved in the metabolism of a drug. | <ul style="list-style-type: none"> • Limited by the immunodetection and quantification of CYP in the liver • Not all CYPs are available. • Used to study a single metabolic phase and don't take into account sequential metabolic routes. |
| Hepatocytes | <ul style="list-style-type: none"> • Has the ability to examine phase 1 and 2 metabolism. • Allows identification of drug inducers and toxic metabolites. | <ul style="list-style-type: none"> • Issues concerning donor availability. • Changes in enzyme activity after isolation. • Inter individual differences between donors. • Accuracy and validity requires assessment of more compounds. |
| Liver slices | <ul style="list-style-type: none"> • Has the potential to give better qualitative/quantitative data for the metabolic fate of a drug. • More complicated system than HLM – similar predictive qualities. | <ul style="list-style-type: none"> • Limited availability • Donor variability • Require specialised equipment |

1.6.1.2 Use of inhibition studies to investigate CYP interactions *in vitro*

The importance of recognising drug-drug interactions at the level of drug metabolism to predict possible severe clinical problems has led to the development of methods to measure CYP inhibition *in vitro*. These techniques have been adopted by the pharmaceutical industry as a high throughput screen for new chemical entities at early stage development. This method has proven to be extremely useful in drug discovery, as in the 1990's guidance documents were issued from regulatory agencies in relation to the assessment of *in vitro* inhibition of the major CYP enzymes.

Therefore this method enables pharmaceutical companies to predict CYP mediated drug-drug interactions early in drug development (Yao *et al*, 2007).

The methods rely upon the use of standard enzyme substrate and their inhibition by the compounds of interest. The technique employed in this study has previously been used to determine the involvement of single or multiple enzymes in the metabolism of a drug through an inhibitory assay (Turpeinen *et al*, 2006).

The following section describes the basis of enzyme inhibition which is central to the technique used here for the investigation of propafenone interaction with different CYP isoforms.

1.6.1.3 Enzyme structure and substrate binding

Each enzyme has active sites which are three dimensional made up of groups of amino acid sequences. Substrate binding to these sites creates the enzyme

- substrate (ES) complex. In reversible reactions the substrate is bound by forces such as electrostatic, Van Der Waals or hydrogen bonds.

This mechanism was discovered by Emil Fischer and his Lock and Key system (Mathews *et al*, 2000; Fig 1.12).

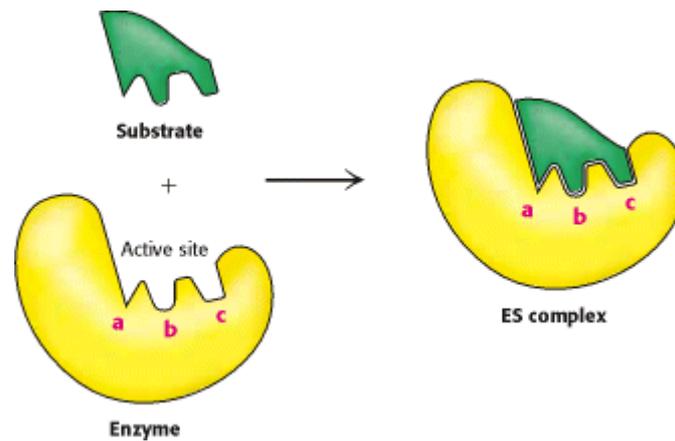


Figure 1.12. The lock and key mechanism.

(www.neurobio.drexel.edu)

Later the induced fit model was developed as shown in figure 1.13 the difference is that the enzymes active site changes to complement the substrate after it has bound (Mathews *et al*, 2000).

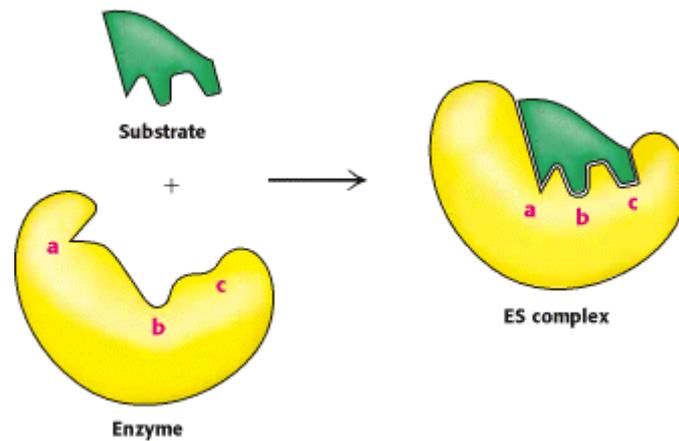


Figure 1.13 The mechanism of the induced fit model an extension of Fischer's previous idea. (www.neurobio.drexel.edu)

It is known now from the work of Leonor Michaelis and Maud Menten that the rate of catalysis (V) increases with the concentration of the substrate (S) when the concentration of the enzyme (E) is constant (www.chm.davidson.edu).

This is true when the concentration of the substrate is low, when $[S]$ becomes in excess of the enzyme concentration the situation changes where V is indirectly proportional to the concentration of the substrate.

Due to this observation Leonor Michaelis and Maud Menten deduced the following equation (Fig 1.14) which also defined the constants K_m and V_{max} also known as the Michaelis constant and is equal to the substrate concentration at which the reaction rate is half its maximal value. A high K_m indicates a weak binding, and a low K_m indicates strong binding (Mathews *et al*, 2000). Therefore the lower the K_m the stronger the bond between the substrate and the enzyme. V_{max} or maximum velocity occurs when the enzymes are saturated i.e. every enzyme molecule is occupied by substrate and carrying out the catalytic step and so an increase in substrate concentration does not increase the rate of catalysis (Mathews *et al*, 2000).

$$V = V_{\max} \frac{[S]}{[S] + K_m}$$

Figure 1.14 The Michaelis-Menten equation.

1.6.1.4 Enzyme Inhibition

This study deals with competitive inhibition, where the enzyme binds to either the substrate or the inhibitor (but not both). In general, the inhibitor exhibits a similar form to the substrate and this is why an enzyme – inhibitor (EI) complex can be established. A competitive inhibitor therefore reduces the rate of catalysis as the number of ES complexes formed decreases and EI complexes increase (Shou *et al*, 2000). This can be overcome if the concentration of the substrate increases to greater than that of the inhibitor. When competitive inhibition is occurring V_{\max} does not change as inhibition can be overcome if the substrate concentration is increased sufficiently (Mathews *et al*, 2000). The efficacy of an inhibitor can be measured through its IC_{50} which is the inhibitor concentration at which 50% of the enzyme activity is inhibited (Francke *et al*, 2002). IC_{50} values are derived by plotting the percent of activity remaining with regards to increasing inhibitor concentrations (Fig 1.15). IC_{50} values are intrinsically linked to binding affinity for competitive agonists and antagonists through the equation shown in Figure 1.16.

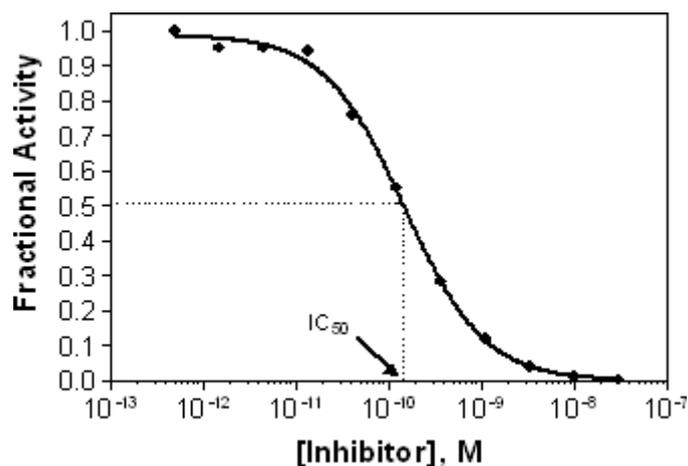


Figure 1.15 Determination of IC₅₀ values from an inhibitor concentration vs. enzyme activity plot (www.spotlite.nih.gov)

$$K_i = \frac{IC_{50}}{1 + [S]/K_m}$$

Figure 1.16 Relationship between IC₅₀ and the binding affinity of the inhibitor (K_i) (Cheng-Prusoff equation)

Determining IC₅₀ values for the interaction with CYP isoforms will therefore give information as to relative binding affinities and propensity for *in vivo* drug-drug interactions.

1.6.2 Protein binding.

The measurement of protein binding is, as has been described in section 1.4.2.1, central to understanding the disposition of drugs in the body. A protein binding study is important as it is the unbound drug that is free to exert

its pharmacological effects, therefore if it is determined that the enantiomers have a greater degree of binding than the racemate it is vital to know. Many critical pharmacokinetic parameters are a function of unbound drug fraction. Therefore quantitative determination of drug protein binding is important in drug development (Cheng *et al*, 2004).

There are a variety of techniques available for measuring protein binding including ultrafiltration and equilibrium dialysis (Kwon, 2001; Crommelin *et al*, 2002). In this study the use of a chromatographic method using a column containing Human Serum Albumin (HSA) will be used to determine and compare the protein binding of propafenone in its racemate form and its individual enantiomers. The next step will be to determine the binding of other compounds and compare to published data, as this will show that results obtained are indicative of protein binding.

1.6.3 *In vitro* metabolism.

The metabolism of compounds by CYP isoforms can be conveniently measured *in vitro*. This requires the use of a metabolising system (containing CYP) co-incubated in buffer with the compound of choice. It then remains to measure the removal of compound from the incubation and/or the production of metabolites.

To determine the extent of *in vitro* metabolism this study will utilise HPLC. (high performance liquid chromatography) to separate propafenone from its metabolites. Chromatography is characterised as a series of techniques that

are used to separate multiple components in a samples based on relative affinities of these components between the mobile phase and the stationary phase (Sadek, 2004).

HPLC involves four basic components, the injector, mobile phase, stationary phase and the detector (Fig 1.17)

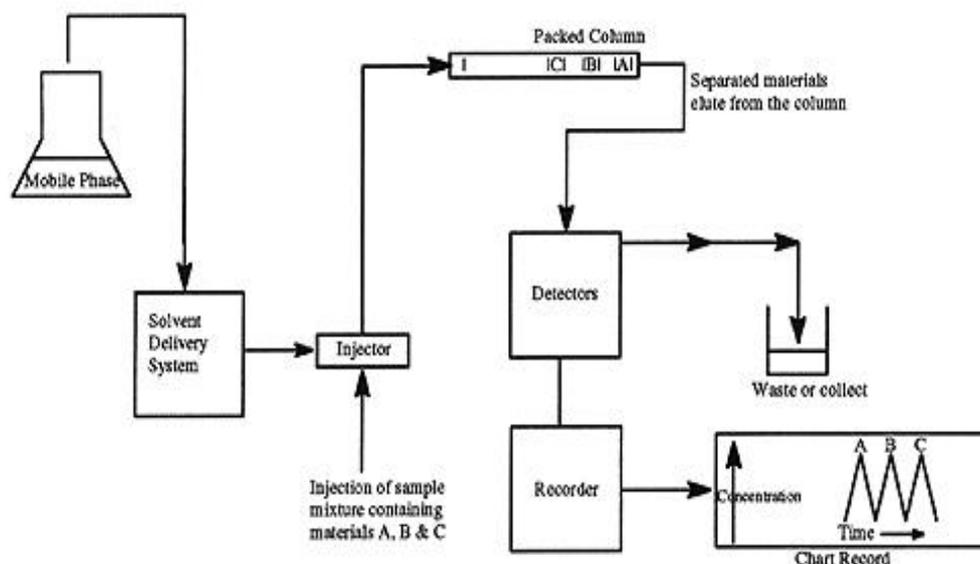


Figure 1.17 Components of a HPLC system (www.protein.iastate.edu)

The premise of HPLC is that the analyte is contained in a liquid known as the mobile phase and is pumped through a stationary phase. One phase is hydrophilic and the other lipophilic (in reversed phase HPLC it is the stationary phase which is lipophilic). The time spent interacting with the stationary phase is dependent on the analyte's polarity. This process causes a separation of the sample, the time the analyte elutes from the stationary phase (retention time) is specific. When this occurs the analyte passes

through the detector where its signal is recorded and plotted on a chromatograph (Manz *et al*, 2004).

The mobile phase used in HPLC is under high pressure which maintains a constant flow rate and in allows for reproducibility. The mechanism of separation is concerned with the relative affinity of the analyte to the mobile and stationary phases which determines the retention time of the analyte.

The percentage metabolism occurring in each test will be deduced in comparison to the control from the data collated on the chromatographs. It will determine if the enantiomers are metabolised to a greater extent or not, which is an important step in drug development. In general the metabolites are more polar than the parent compound and so reversed phase HPLC is ideal for the separation process.

By using these various techniques it is possible to gain an insight into the interactions which will determine a drug's *in vivo* disposition. As previously mentioned, this study will look at the development of these techniques to investigate stereospecific differences with the racemic antiarrhythmic drug propafenone.

2. Materials and methods.

2.1 Materials

Propafenone and its enantiomers were obtained from Knoll AG (Ludwigshafen, Germany). The bacosomes CYP 1A2, CYP3A4, CYP2C19 and CYP2D6 were purchased from Cypex (Dundee, Scotland). Human liver microsomes were obtained from Advancell (Barcelona, Spain). All chemicals used were bought from Sigma Aldrich (Gillingham, Dorset)

2.1.1 Buffers and stock Solutions

Phosphate buffer (0.5M) was prepared by dissolving 3.40 g of potassium phosphate monobasic salt and 4.35 g of potassium phosphate dibasic salt in 50 ml of deionised water. A volume of 7.5 ml was taken from the 0.5 M potassium phosphate monobasic and mixed with 35.0 ml of 0.5 M potassium phosphate dibasic to generate the final buffer with a pH of 7.4 (42.5 ml). From this solution 0.2M, 0.1M, 0.05M and 0.02M dilutions were made.

2.1.2. Preparation of cofactors.

The cofactors for CYP3A4, CYP2C19 and CYP1A2 experiments (20x stock) were made by weighing 200 mg NADP⁺ (20 mg/mL), 200 mg glucose-6-phosphate (20 mg/mL) and 133 mg magnesium chloride hexahydrate (13.3 mg/mL), these three components were then dissolved in a volume of 10 mL of deionised water. For CYP2D6 experiments (160x stock) the cofactor was made up as above using 1.60 g of NADP⁺.

2.1.3. Preparation of glucose-6-dehydrogenase

A weight of 200 mg of glucose-6-dehydrogenase was dissolved in 10 mL of deionised water.

2.1.4. Preparation of propafenone (10mM)

A weight of 37.8 mg of propafenone was dissolved in 10 mL of deionised water. It was then sonicated for 5 minutes to ensure complete dissolution.

2.1.5. Preparation of ketoconazole (0.25mM)

A weight of 0.266 mg of ketoconazole was dissolved in 20 mL of deionised water.

2.2 Preparation of the Cofactor Mixtures.

Cofactor mixtures for the production of NADPH were produced as follows.

2.2.1 CYP3A4 Assay.

A volume of 1.8 mL of 0.5 M phosphate buffer (2.1.1) was added to 90 μ L glucose-6-phosphate dehydrogenase (2.1.3), 450 μ L of cofactors (2.1.2) and 2.16 mL of deionised water.

2.2.2 CYP2C19 assay.

A volume of 450 μ L of 0.5 M phosphate buffer (2.1.1) was added to 90 μ L glucose-6-phosphate dehydrogenase (2.1.3), 450 μ L of cofactors (2.1.2) and 3.51 mL of deionised water.

2.2.3 CYP1A2 assay.

A volume of 2.0 mL of 0.5 M phosphate buffer (2.1.1) was added to 100 μ L glucose-6-phosphate dehydrogenase (2.1.3), 125 μ L of cofactors (2.1.2) and 7.78 mL of deionised water.

2.2.4 CYP2D6 assay.

A volume of 700 μ L of 0.5 M phosphate buffer (2.1.1) was added to 70 μ L glucose-6-phosphate dehydrogenase (2.1.3), 88 μ L of cofactor mixture (2.1.2) and 6.15 mL of deionised water

2.3 Measurement of NADPH Production by Regenerating System.

2.3.1 Generation of the NADPH standard curve

A stock solution of 1mM NADPH in a volume of 10 ml was made by dissolving 0.833 mg NADPH in deionised water which was diluted 1 in 5 to give a second stock of 200 μ M in 5 mL. From these solutions the following working standards were prepared. 500 μ M, 250 μ M, 125 μ M, 100 μ M, 50 μ M and 25 μ M (each incubation contained 100 μ L).

The incubation for the NADPH standard curve was measured at an absorbance of 580 nm for 10 minutes using a clear bottomed plate.

2.3.2 Measurement of NADPH production

A volume of 700 μ L 0.5M phosphate buffer (2.1.1) was added to 88 μ L of cofactor mixture (20 x solutions 2.1.2) and 6.15 mL of deionised water. 98 μ L of this mixture was pipetted into the plate and preheated in the plate reader at

37°C. Finally a further 2 µL of Glucose-6-phosphate dehydrogenase (2.1.3) was added and the absorbance was measured at 580nm for 5 minutes using the Tecan Genios Pro plate reader.

2.4. Cytochrome P450 activity and IC₅₀ of Inhibitor and Propafenone

High throughput method for determining Cytochrome P450 inhibition was adapted from BD Biosciences. (San Jose, California, USA). It was used in order to determine the IC₅₀ value of the specific CYP inhibitors and the test drug propafenone.

The Cytochrome P450 activity was determined using probe substrates for the different batosomes as shown in Table 2.1. Batosomes were used as they contain the one individual CYP rather than microsomes which contain several in different concentrations. The method was based on the principle that the metabolism of the probe substrate produces a fluorescent product.

Fluorescence was measured using a Geniospro Tecan plate reader, with excitation and emission wavelength optimised for each substrate (Table 2.1).

Each plate was run for 10 minutes and a reading of the fluorescence was taken at 1 minute intervals.

Table 2.1 The specific settings, substrates and inhibitors used for each CYP

| | CYP3A4 | CYP2C19 | CYP2D6 | CYP1A2 |
|----------------------------------|---|--|--|--|
| Inhibitor and concentration used | Ketoconazole: 5 μ M | Tranlycypromine (TCP): 5 mM | Quinidine 25 μ M | Furafylline 5mM |
| Substrate and concentration used | 7-Benzyloxy-4-(trifluoromethyl) couamrin (BFC) 10 mM | 3-Cyano-7-ethoxycoumarin (CEC) 25 μ M | 3-[2-(N,N-DiethylN-methylammonium) ethyl]-7-methoxy-4-methylcoumarin (AMMC) 10 mM | 3-Cyano-7-ethoxycoumarin (CEC) 25 μ M |
| Excitation wavelength (nM) | 410 | 409 | 390 | 410 |
| Emission wavelength (nM) | 535 | 460 | 465 | 465 |

Table 2.2 Conditions implemented when using the Genios Pro plate reader for the incubations of each bactosome preparation indicating the temperature range, wavelength the test is read at (shown for CYP1A2 here), the measurement mode and the part of the plate to be read.

| Measurement mode: | Fluorescence Top | |
|---------------------------|------------------|----|
| Excitation wavelength: | 410 | nm |
| Emission wavelength: | 465 | nm |
| Gain (Manual): | 40 | |
| Number of flashes: | 10 | |
| Lag time: | 0 | µs |
| Integration time: | 40 | µs |
| Part of the plate: | A1 - C12 | |
| Number of kinetic cycles: | 30 | |
| Kinetic interval: | 60 | s |
| Valid temperature range: | 36 - 38 | °C |
| Target Temperature: | 37 | °C |
| Current Temperature: | 37 | °C |

The settings for the Genios Pro plate reader for each incubation are shown in Table 2.2. The example here is for CYP1A2, all other experiments had the same settings except for the excitation and emission wavelengths shown in Table 2.1

2.4.1 Measurement of Cytochrome P450 activity

A volume of 100 µL of the appropriate cofactor mixture was added to 3 wells of the 96 well plate, to which 100 µL of enzyme substrate mixture was added to the 96 well plate. The final volume was therefore 200 µL. Fluorescence was

measured at wavelengths specified in Table 2.1. The experiment was conducted over 10 minutes at 37°C and values were recorded at 1 minute intervals using the Tecan Genios Pro plate reader.

For the different experiments the final CYP concentrations were as follows; CYP3A4 - 4.4pmoles/ μ L, CYP2C19 - 4.7 pmoles/ μ L, CYP2D6 - 3.0 pmoles/ μ L and CYP 1A2 - 6.0 pmoles/ μ L.

2.5 Inhibition of CYP Activity by Standard inhibitors and Propafenone

Incubation conducted the same as in 2.4 but with the addition of a specific inhibitor (see Fig 2.1)

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|----|----|----|
| A | x | x | x | x | x | x | x | x | x | x | O | O |
| B | x | x | x | x | x | x | x | x | x | x | O | O |
| C | x | x | x | x | x | x | x | x | x | x | O | O |
| D | x | x | x | x | x | x | x | x | x | x | O | O |
| E | x | x | x | x | x | x | x | x | x | x | O | O |
| F | x | x | x | x | x | x | x | x | x | x | O | O |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |

Figure 2.1. Diagram of 96 well plate indicating the contents and relative concentrations of each incubation.

- 3 replications are performed for each assay
 - The shaded area represents the presence of inhibitor. The decrease in shading represents the dilution of the inhibitor
 - X symbolises the wells that contain CYP and Substrate
 - O symbolises the presence of the substrate.
- Well 11 contains the maximum concentration of inhibitor and the substrate. Well 12 contains only the substrate

The incubation mixture contained the individual bactosome at the CYP concentrations specified in section 2.4. The specific inhibitor for each bactosome, the fluorescent probe substrate used at concentrations as stated in Table 2.1 and the appropriate cofactor mixture (2.2).

The final volume was 200 μ L with the maximum well concentrations of the inhibitors furafylline, 100 μ M, TCP, 200 μ M, quinidine, 0.5 μ M, ketoconazole, 5 μ M and propafenone at 200 μ M.

The inhibitors were diluted threefold between each well. After the serial dilutions and prior to the addition of the enzyme- substrate mixture the plate was preheated for 10 minutes in the Genios Pro Tecan plate reader at 37°C.

2.5.1 Enzyme – Substrate mixture for CYP 3A4

The enzyme-substrate mixture for CYP 3A4 contained a volume of 8.0 mL 200 mM phosphate buffer (2.1.1) and 80 μ L of BFC (10 mM). 100 μ L of this mixture was pipetted into wells 11 and 12. To the remaining substrate mixture 15.5 μ L of CYP 3A4 was added. 100 μ L of this was pipetted into wells 1 – 10.

2.5.2 Enzyme – Substrate mixture for CYP 2C19

The enzyme-substrate mixture for CYP 2C19 contained a volume of 8.0 mL 50 mM phosphate buffer (2.1.1) and 20 μ L of CEC (25 μ M). 100 μ L of this mixture was pipetted into wells 11 and 12. To the remaining substrate mixture 21.7 μ L of CYP 2C19 was added. 100 μ L of this was pipetted into wells 1 – 10.

2.5.3 Enzyme – Substrate mixture for CYP 1A2

The enzyme-substrate mixture for CYP 1A2 contained a volume of 1.6 mL 50 mM phosphate buffer (2.1.1), 6.4 mL deionised water and 40 μ L of CEC (25 μ M). 100 μ L of this mixture was pipetted into wells 11 and 12. To the remaining Substrate mixture 2.4 μ L of CYP1A2 was added and then 100 μ L of this was pipetted into wells 1 – 10.

2.5.4 Enzyme – Substrate mixture for CYP 2D6

The enzyme-substrate mixture for CYP 2D6 contained a volume of 6.97 mL 50 mM phosphate buffer (2.1.1) and 2.1 μ L of AMMC (10 mM). 100 μ L of this mixture was pipetted into wells 11 and 12. To the remaining Substrate mixture 24.5 μ L of CYP 2D6 was added and then 100 μ L of this was pipetted into wells 1 – 10.

Well 11 contained the maximum concentration of the specific inhibitor and substrate. Well 12 contained the substrate. This process was replicated 3 times for each incubation. Wells 9 and 10 contained only the specific CYP its substrate and cofactor. Fluorescence produced in these wells was indicative of maximum metabolism of the fluorescent substrate in the absence of an inhibitor.

2.6 In Vitro Metabolism of Propafenone and its Enantiomers using Human Liver Microsomes

2.6.1 Conditions for HPLC analysis of propafenone.

Reversed phase HPLC analysis of samples containing propafenone was conducted under the following conditions. Isocratic HPLC was conducted using an ODS Hypersil 4.6 mm x 250 mm column with a mobile phase consisting of water, acetonitrile and methanol in the following proportion (25:30:45 respectively), plus 0.03% v/v of triethylamine and 0.05% v/v phosphoric acid. The mobile phase was pumped at a rate of 1 mL/ min and the eluent was measured at a wavelength of 210 nm. The injection volume was 20 µL.

2.6.2 Generation of standard curve for propafenone and its enantiomers

The concentration of propranolol was constant at 0.5mM and the concentration of propafenone varied to produce a standard curve and allow quantification of propafenone. The concentrations of propafenone used were 0.5 mM, 0.2 mM, 0.1 mM, 50 µM, 20 µM.

2.6.2.1 Stock solution of propafenone (racemate and its enantiomers).

A stock solution of 5 mM propafenone in a volume of 10mL was made by dissolving 18.9 mg of propafenone in deionised water. A second stock of 1mM propafenone was made from the above solution. A 5 mM stock solution of the internal standard propranolol was made in a volume of 10mL by dissolving 14.8 mg in water.

2.6.3 Method for the determination of the percentage metabolism of propafenone and its enantiomers

1 mM propafenone(final concentration 0.05mM) (Anzenbacherova *et al*, 2003) stock (25 μ L) was preincubated for 5 minutes at 37° C with 6 μ L of human liver microsomes (40 mg/ mL) in 369 μ L 0.1 M phosphate buffer (pH 7.4) (2.1.1)

A volume of 100 μ L 1 mM NADPH was then added to the incubation mixture, giving a final volume was 500 μ L.

The control incubations did not contain NADPH; the mixtures were incubated for 30 minutes at 37°C.

To terminate the reaction 500 μ L of acetonitrile was added together with the internal standard propranolol (10 μ L of 5 mM). The incubation mixtures were centrifuged at 13,000 rpm for 10 minutes.

A 10 μ L aliquot of the supernatant was taken and added to 90 μ L of the mobile phase making a final volume of 100 μ L. This was done as it produced good separation of peaks.

This solution was then analysed using HPLC as detailed in section 2.6.1.

2.7 Protein Binding Study

2.7.1 HPLC analysis

Isocratic HPLC analysis was conducted using a chiral human serum albumin (HSA) column (50 X 3.0mm) purchased from Chromtech (Apple valley, MN).

Under the following conditions: flow rate: 0.5 mL/min, detection: UV 210 nm.
Mobile phase consisted of 5% propan-2-ol in 20mM potassium phosphate pH 7.0.

2.7.2 Preparation of working solutions

1 mM stock solutions were made up for the following propafenone, carbamazepine, verapamil, diltazem, desipramine, propranolol, nortriptyline and ketoconazole. From these solutions 0.01 mM working solutions were made.

20 μ L of 0.01mM test solutions (2.7.2) was injected into the HPLC. This was repeated 3 times.

2.8 Statistical analysis

The student T test was used to compare data in this study. With $P < 0.05$ taken as a significant difference between the two groups of data.

3.0 Results

3.1 NADPH production by the regeneration system

The initial aim was to determine the validity of the regeneration system. A NADPH standard curve was produced to allow quantification of the NADPH being produced (Fig 3.1). Using the regeneration system for CYP 3A4, CYP1A2 and CYP 2C19 the rate of production of NADPH was at its greatest for the first 60 seconds of incubation and that the production begins to plateau by 5 minutes (Fig 3.2). It is evident that the regenerating system does produce NADPH.

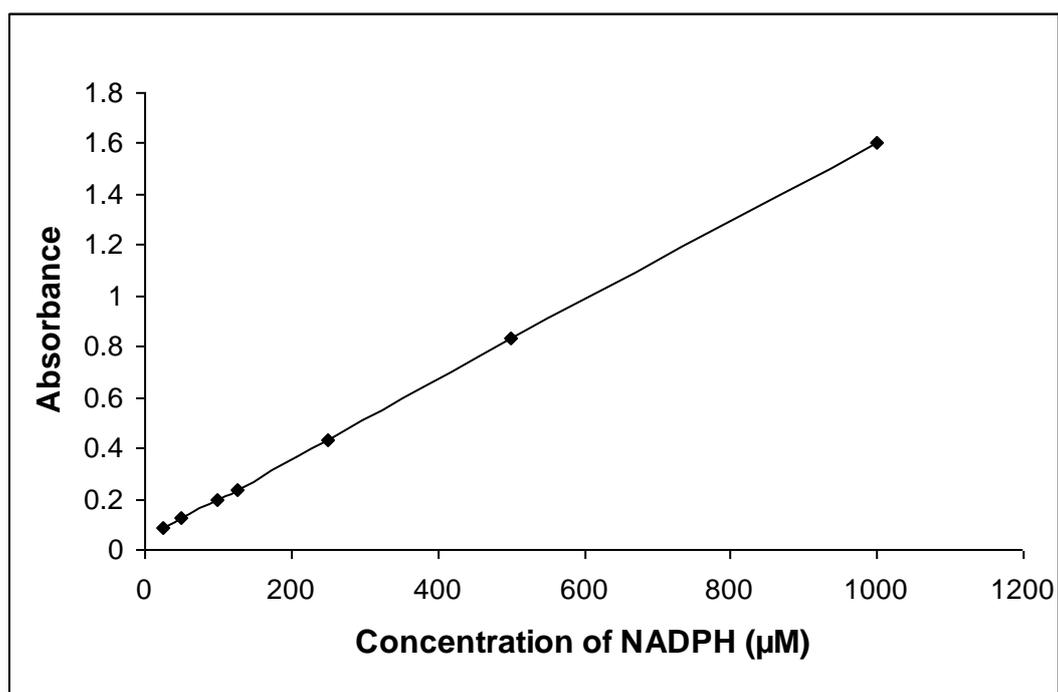


Figure 3.1 NADPH standard curve measured at 340 nm (n=1)

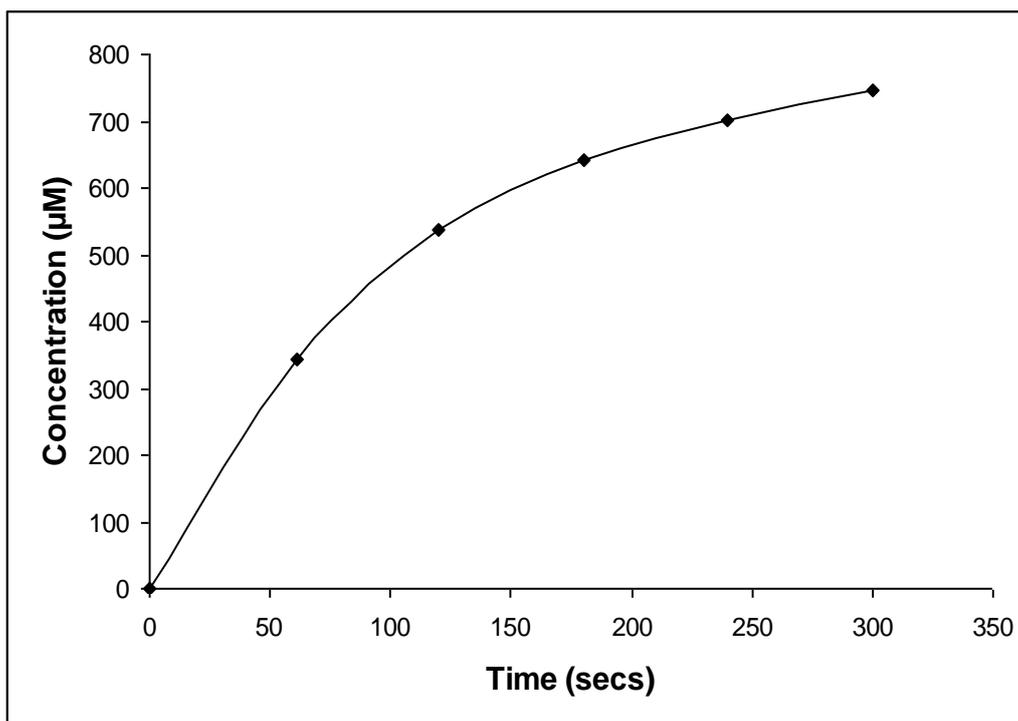


Figure 3.2 Production of NADPH over a period of 5 minutes (n=1) Production of NADPH using the NADPH regenerating system.

3.2 Measurement of CYP Activity

Figures 3.3 to 3.6 show typical graphs for the metabolism of the fluorescent substrate in the presence of the respective CYP. This was determined prior to any further investigation. Metabolism occurs in a linear fashion over the time course measured for all of the CYPs.

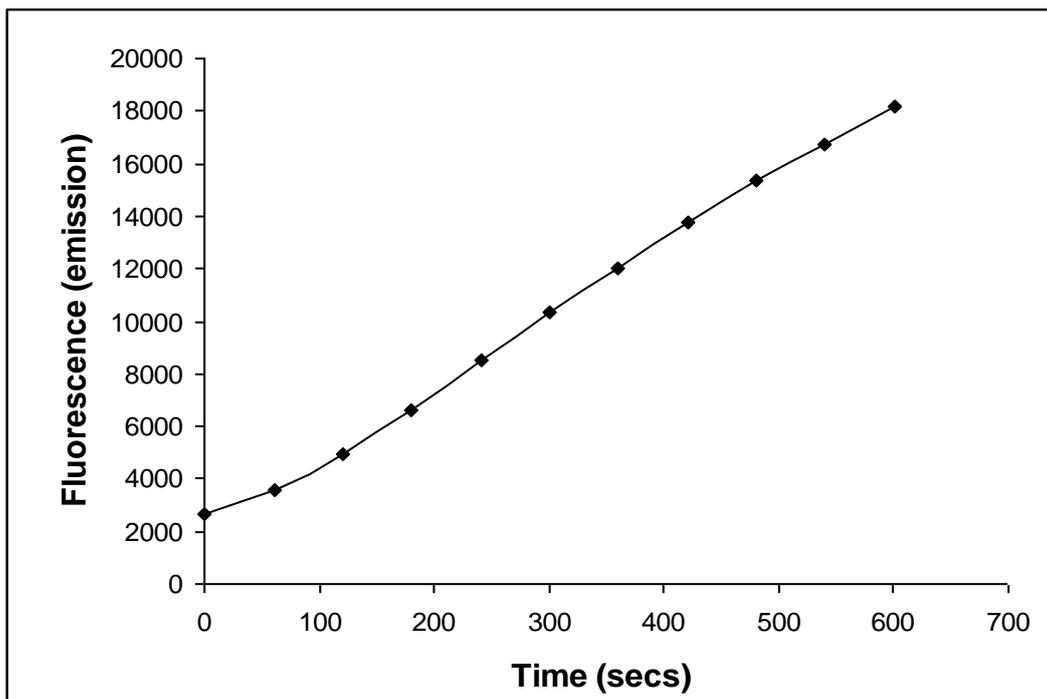


Figure 3.3. The metabolism of BFC to a fluorescent product by CYP 3A4 containing bacosomes (n =1)

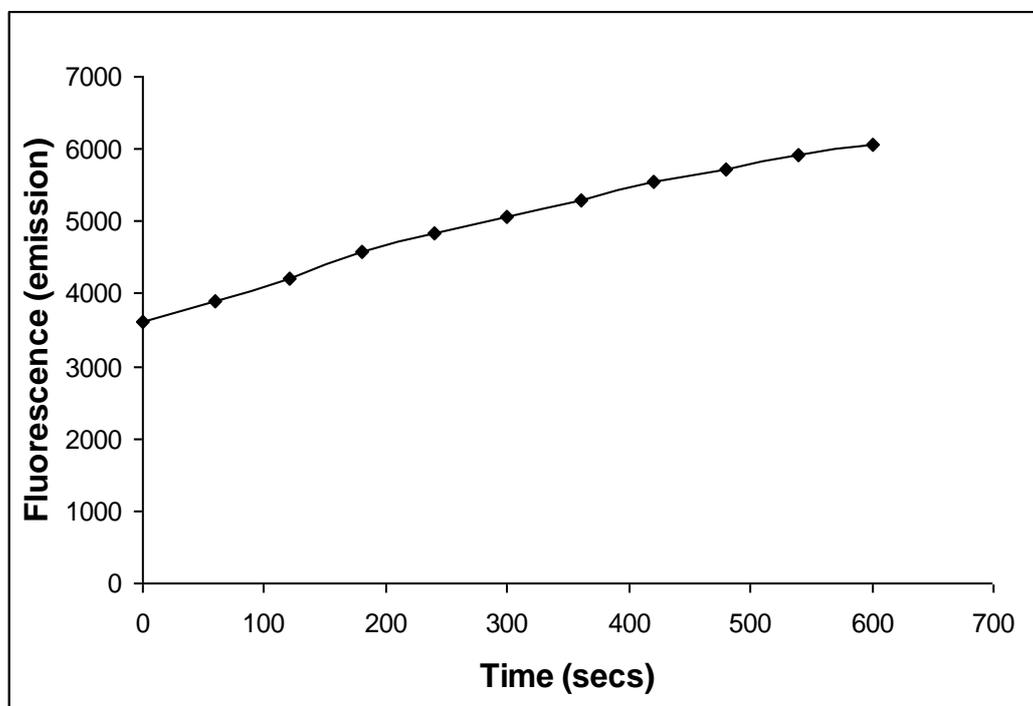


Figure 3.4 The metabolism of CEC to a fluorescent product by 2C19 containing bacosomes (n=1).

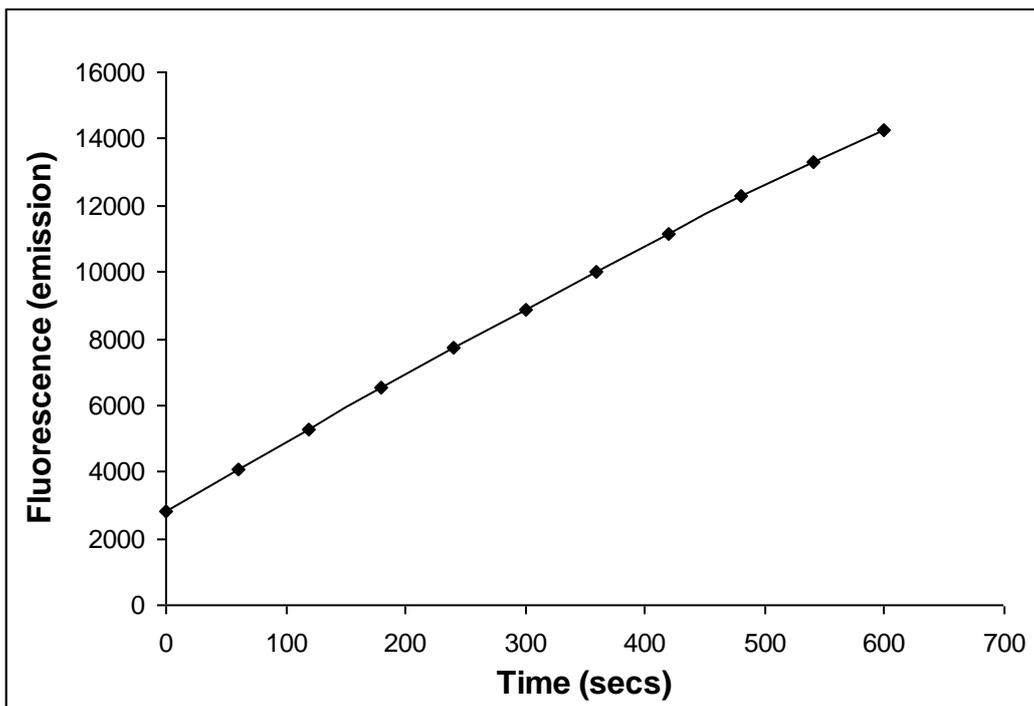


Figure 3.5 The metabolism of CEC to a fluorescent product by CYP1A2 containing bactosomes (n=1).

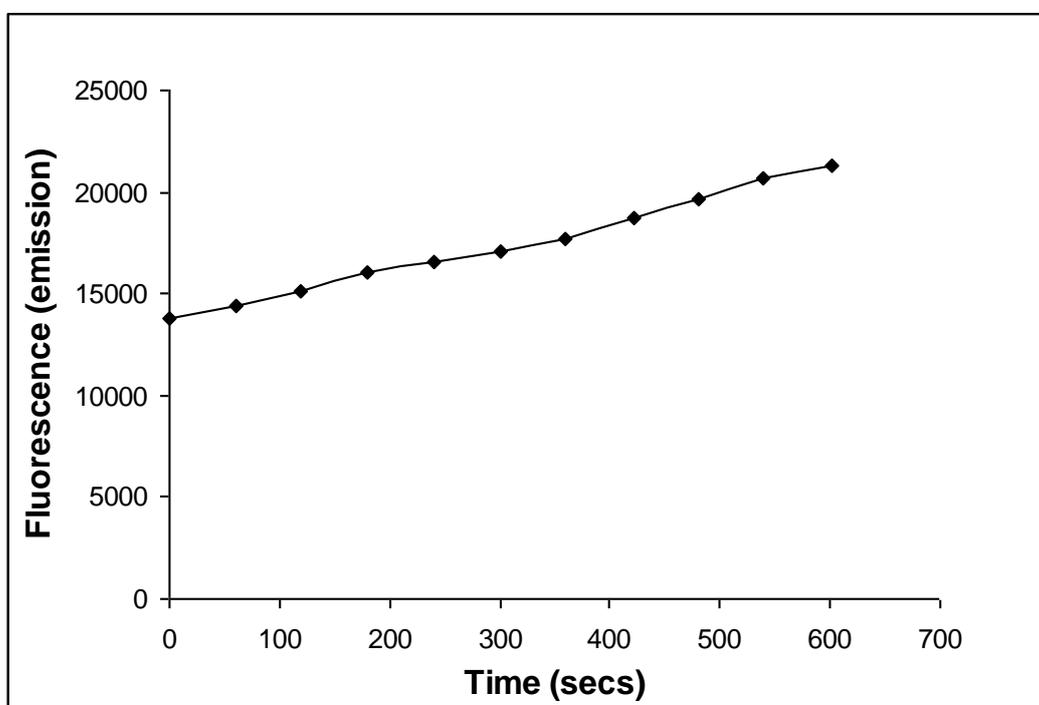


Figure 3.6 The metabolism of AMMC to a fluorescent product by CYP2D6 containing bactosomes (n=1).

3.3 Inhibition Studies using CYP3A4

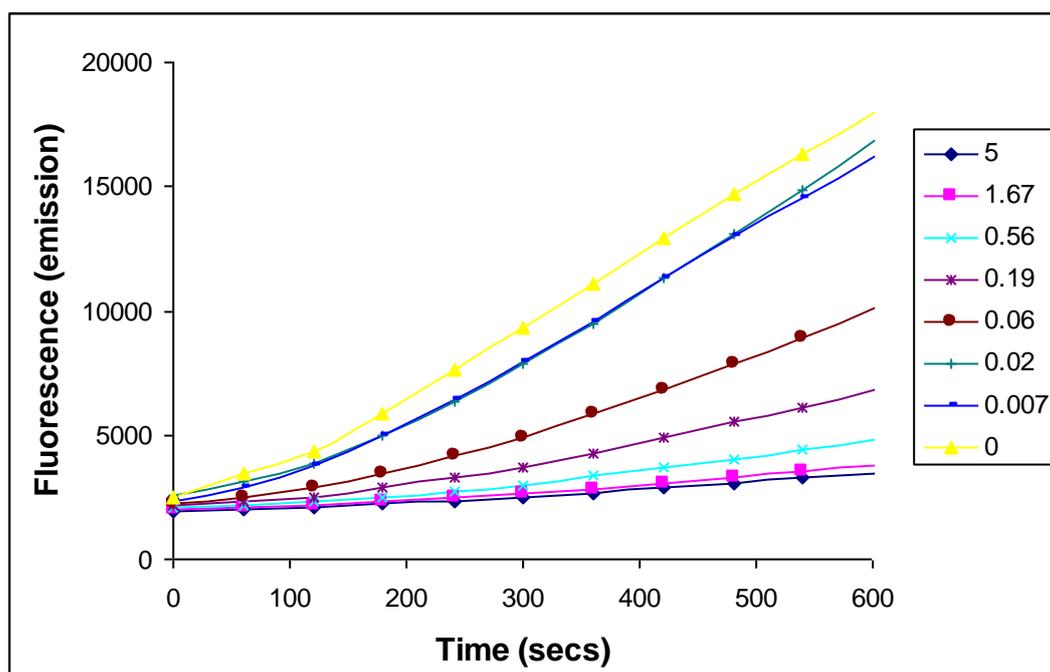


Figure 3.7 The effect of ketoconazole on the metabolism of BFC by CYP3A4. Concentrations (μM) of ketoconazole are shown on the graph, values are mean of 4 measurements

Figure 3.7 shows the metabolism of BFC to a fluorescent product by CYP3A4 is decreased by the co-incubation with ketoconazole (CYP3A4 inhibitor) in a dose dependent manner. Similarly the metabolism is decreased when co-incubated with racemic propafenone (Fig 3.8) and its separate enantiomers (Fig 3.9 and 3.10)

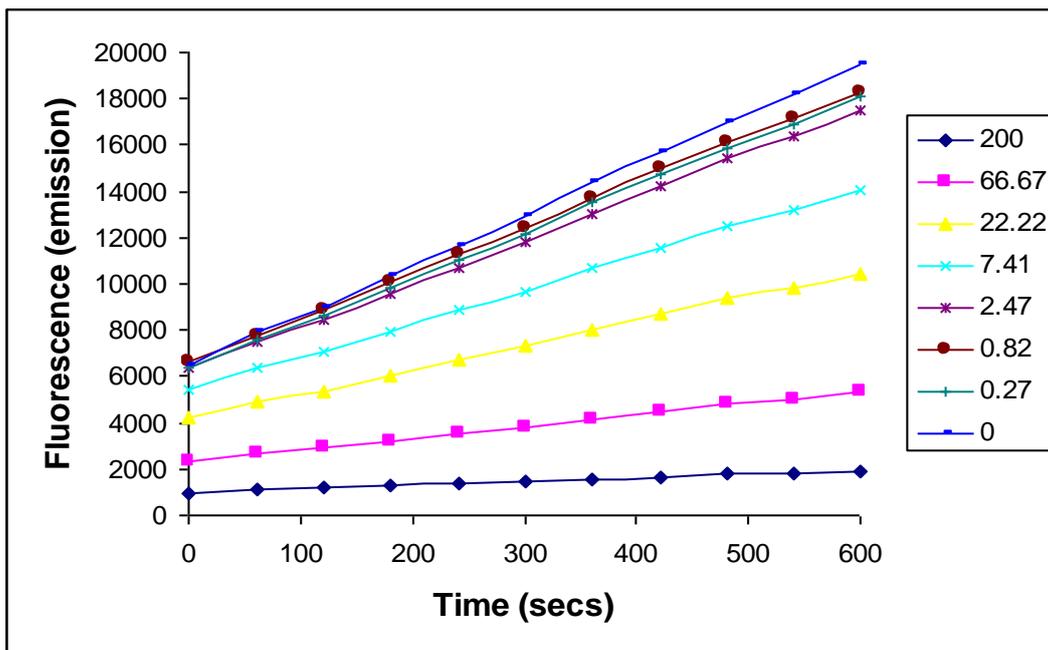


Figure 3.8. The effect of racemic propafenone on the metabolism of BFC by CYP3A4. Concentrations (μM) of racemic propafenone are shown on the graph values are mean of 4 measurements

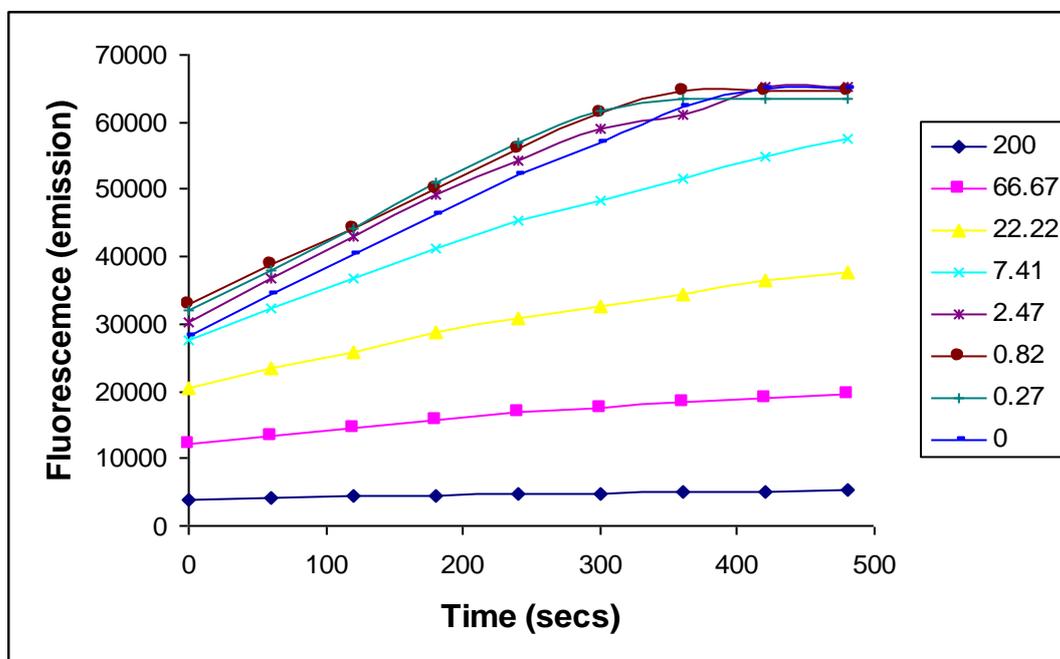


Figure 3.9 The effect of (R)-propafenone on the metabolism of BFC by CYP3A4. Concentrations (μM) of (R)-propafenone are shown on the graph, values are mean of 4 measurements

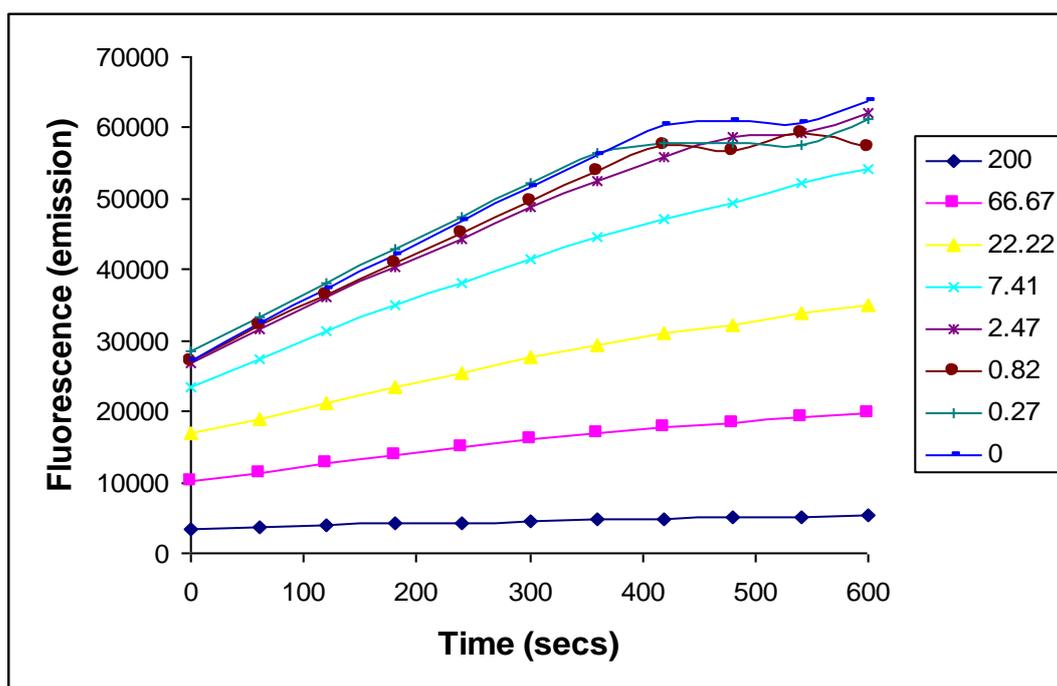


Figure 3.10 The effect of (S)-propafenone on the metabolism of BFC by CYP3A4. Concentrations (μM) of (S)-propafenone are shown on the graph, values are mean of 4 measurements

Figure 3.8 illustrates the inhibition of CYP3A4 with racemic propafenone. As the concentration of inhibitor increases, the rate of metabolism (fluorescence) decreases. This effect is more predominant at the higher concentrations of racemic propafenone, for example $22.22\mu\text{M}$ to $200\mu\text{M}$.

Figure 3.9 shows CYP activity when inhibited by (R)-propafenone. The activity is only shown up to 8 minutes despite the incubation continuing for a further 2 minutes as there was a lack of consistency with measurements after the 8 minute period.

Figure 3.10 shows the metabolism of BFC when inhibited by (S)-propafenone. Over a period of 10 minutes it follows a similar trend to (R)-propafenone.

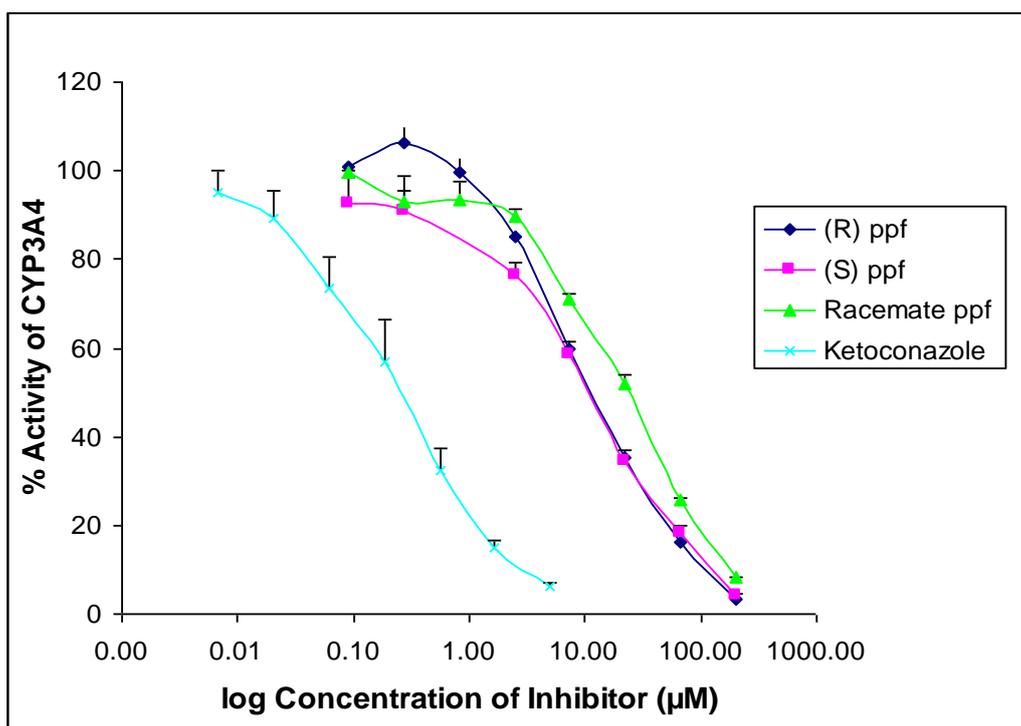


Figure 3.11 The percentage activity of CYP3A4 when incubated with ketoconazole, racaemic propafenone and its enantiomers, values are mean of 4 measurements \pm S.E.

Figure 3.11 compares the percentage activity of CYP3A4 when incubated by racaemic propafenone and its enantiomers. This figure illustrates the similarity in the effect of both enantiomers which is evident from the calculated IC_{50} values (Table 3.1). The percentage activity of CYP3A4 remains higher at lower (R)-propafenone concentration in comparison to its opposite enantiomer.

The racemate follows a similar trend but the IC_{50} is significantly higher than (R)- propafenone ($P < 0.005$) (Table 3.1). The figure shows a difference between propafenone and CYP3A4's known inhibitor ketoconazole.

Ketoconazole inhibits CYP3A4 to a greater extent its IC_{50} value is significantly lower ($P < 0.005$).

3.4 Inhibition studies using CYP2C19.

Figure 3.12 shows the metabolism of CEC to a fluorescent product by CYP2C19 is decreased by co-incubation with TCP (CYP2C19 inhibitor) in a dose dependent manner. Similarly the metabolism is decreased when co-incubated with racaemic propafenone (Fig 3.13) and its separate enantiomers (Fig 3.14 and 3.15)

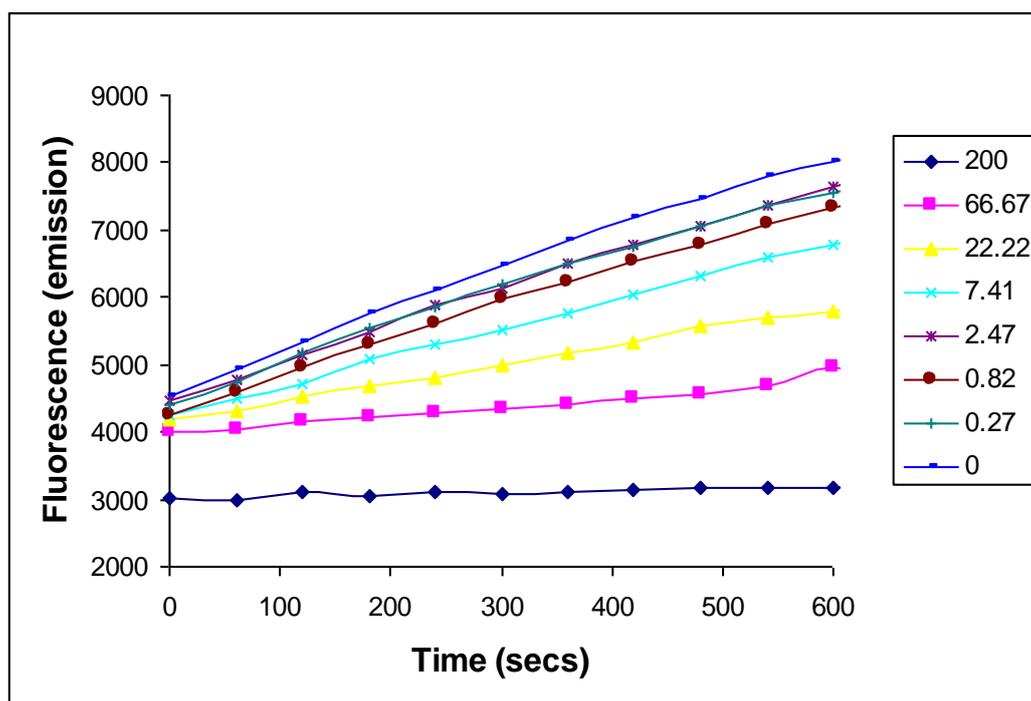


Figure 3.12 The effect of TCP on the metabolism of CEC by 2C19. Concentrations (μM) of TCP are shown on the graph, values are mean of 4 measurements

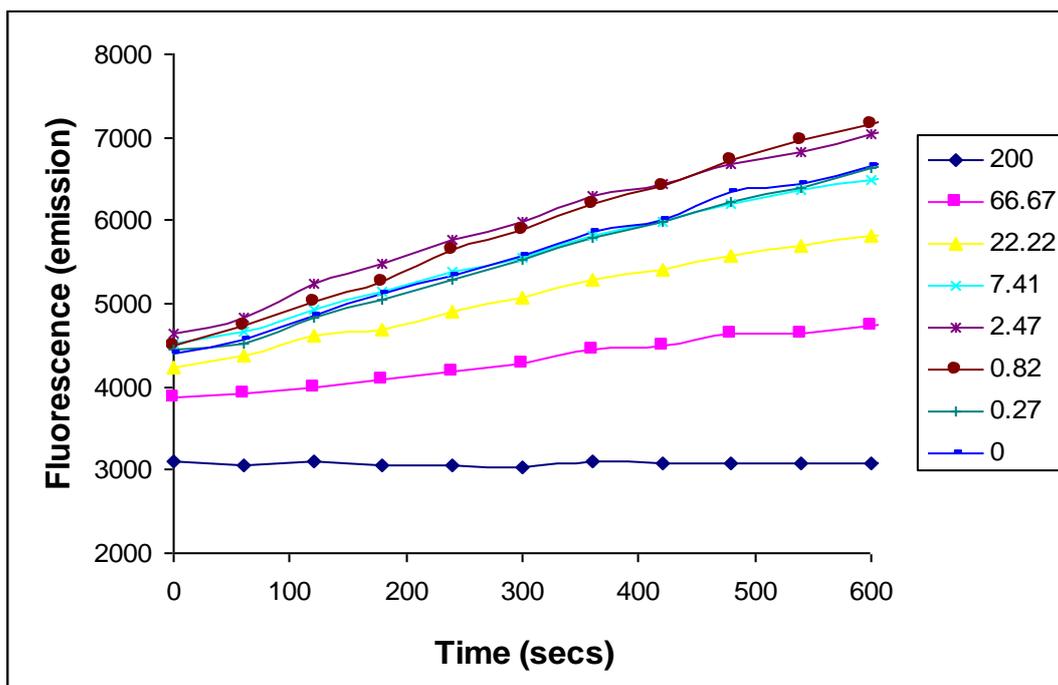


Figure 3.13 The effect of racemic propafenone on the metabolism of CEC by 2C19. Concentrations (μM) of propafenone are shown on the graph, values are mean of 4 measurements

Figure 3.12 shows that at the highest inhibitor concentration $200\mu\text{M}$ there is complete inhibition of metabolism as seen by no distinct change in fluorescence over time. This was also evident with racemic propafenone with less inhibition occurring at the lower concentrations (Fig 3.13).

From figures 3.14 and 3.15 it can be seen that the degree of inhibition by the two enantiomers is similar which is evident in their IC_{50} values (Table 3.1).

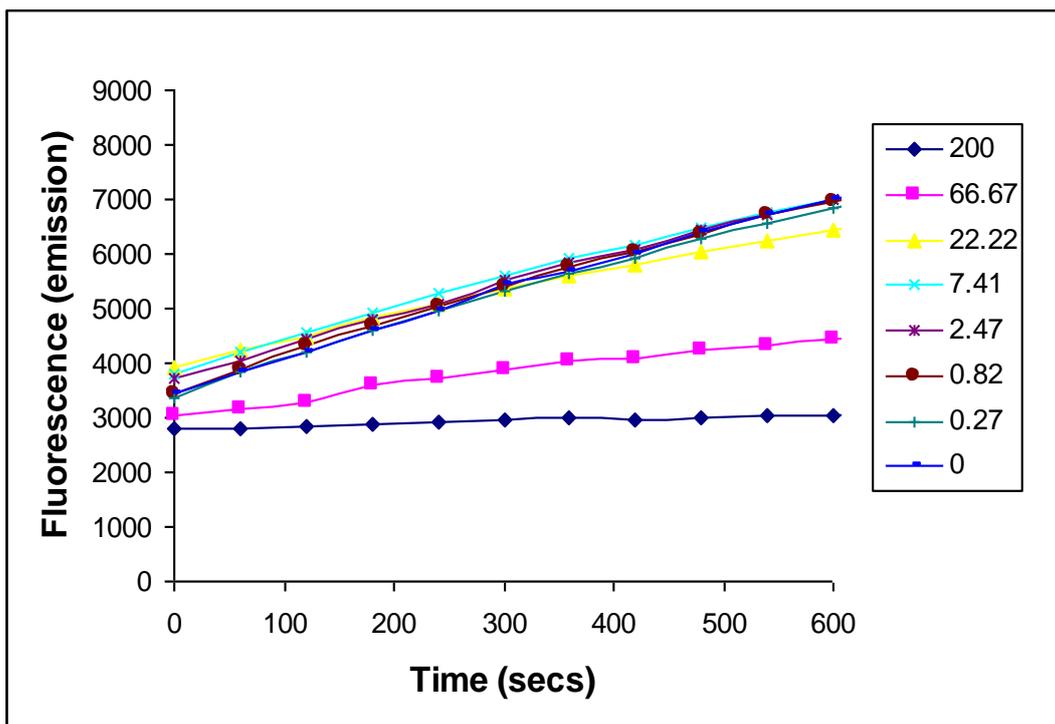


Figure 3.14 The effect of (R)-propafenone on the metabolism of CEC by 2C19. Concentrations (μM) of (R)-propafenone are shown in the graph, values are mean of 4 measurements

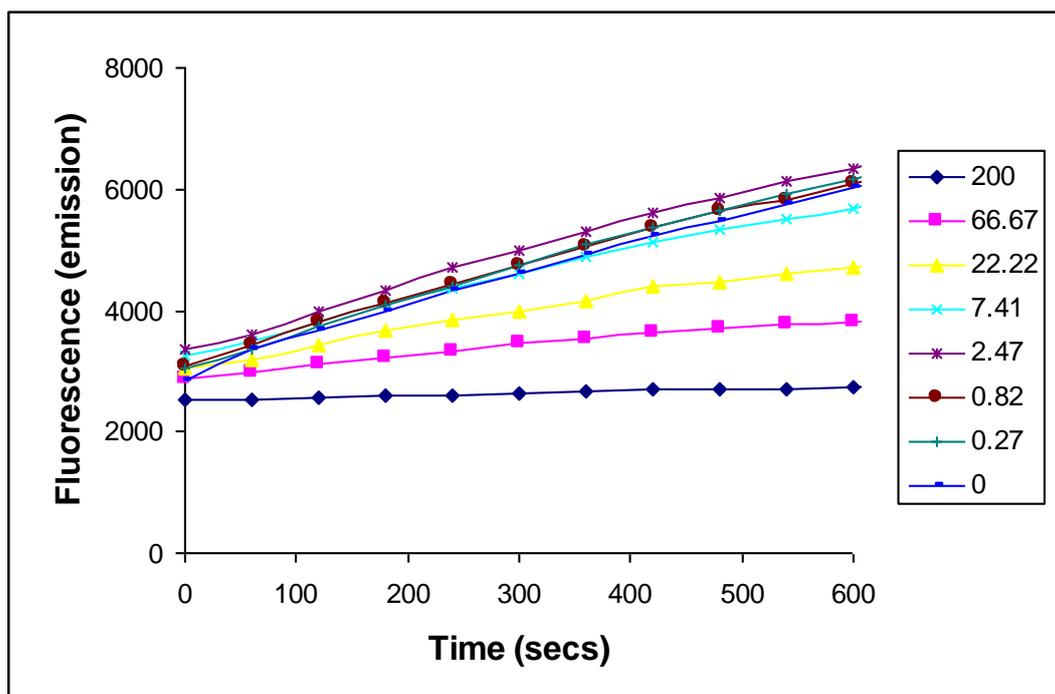


Figure 3.15 The effect of (S)-propafenone on the metabolism of CEC by 2C19. Concentrations (μM) of (S)-propafenone are shown in the graph, values are mean of 4 measurements.

The percentage activity of CYP2C19 follows a similar trend when incubated with TCP, racemic and (R)-propafenone, however there is a significant difference between the calculated IC_{50} for racemic propafenone and (R)-propafenone ($P < 0.05$, Table 2.1). There are no significant differences between the activity of the known inhibitor TCP and propafenone and its enantiomers. In all instances, inhibition was only evident at concentrations greater than $10 \mu\text{M}$. (Fig 3.16)

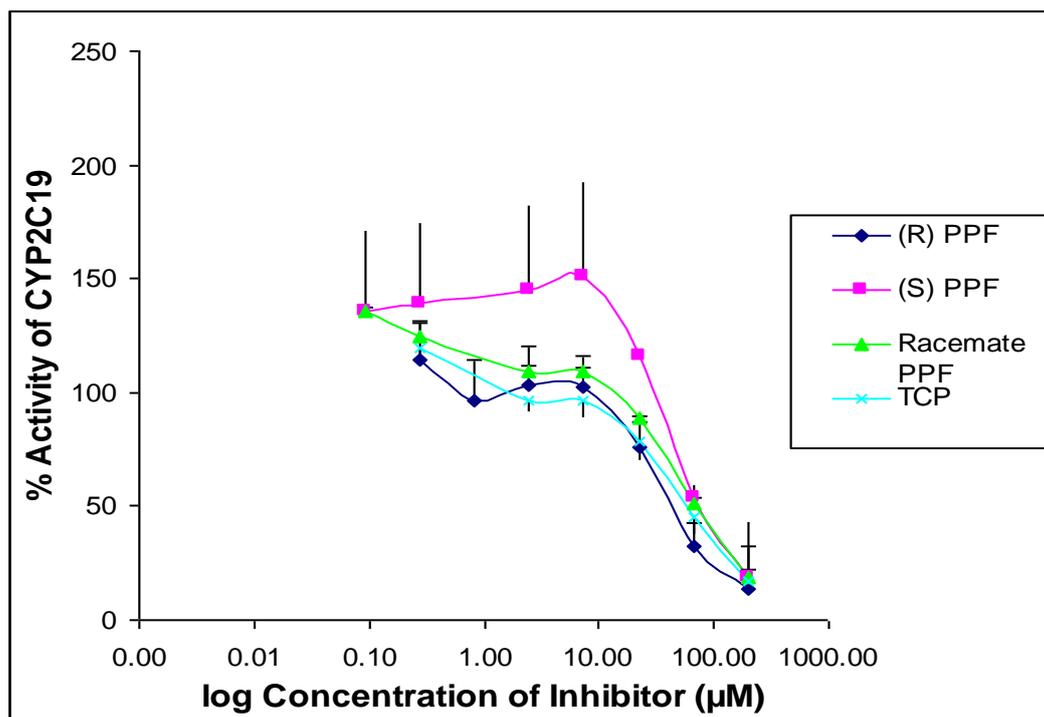


Figure 3.16 The percentage activity of CYP2C19 when incubated with TCP, racaemic propafenone and its enantiomers. Values are mean of 4 measurements \pm S.E.

3.5 Inhibition studies using CYP1A2

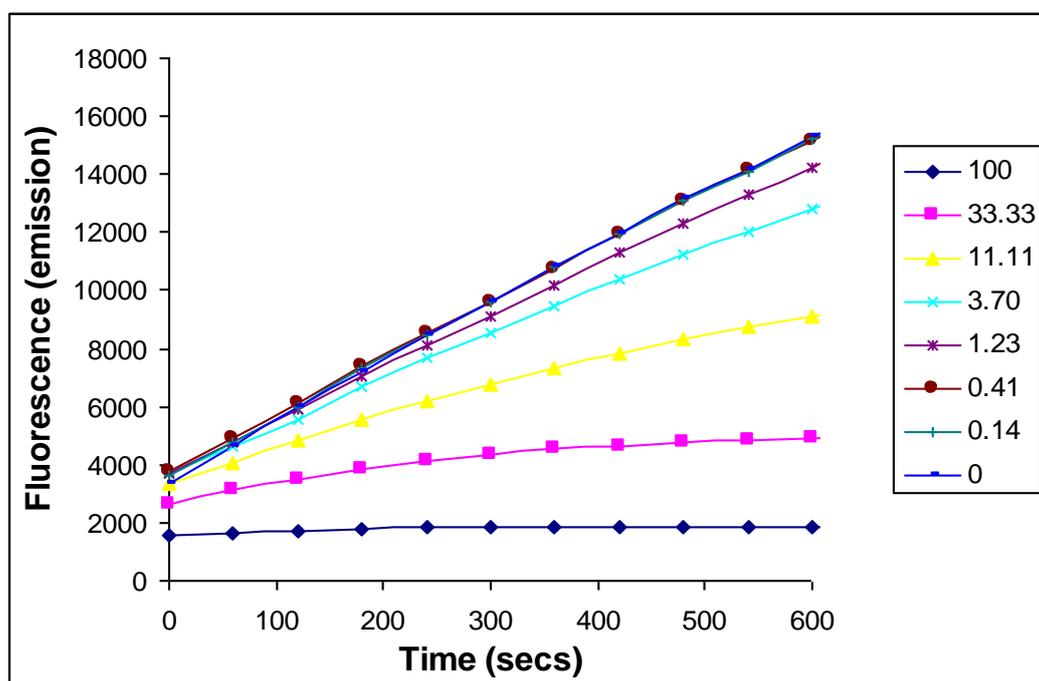


Figure 3.17 The effect of furafylline on the metabolism of CEC by CYP1A2. Concentrations (μM) of furafylline are shown in the graph. Values are mean of 4 measurements

Figure 3.17 shows that the metabolism of CEC to a fluorescent product by CYP1A2 is decreased by co-incubation with furafylline (CYP1A2 inhibitor) in a dose-dependent manner. Similarly, the metabolism of CEC is decreased in a dose dependent manner when co-incubated with racemic propafenone (Fig 3.18) and its separate enantiomers (Fig 3.19 and 3.20)

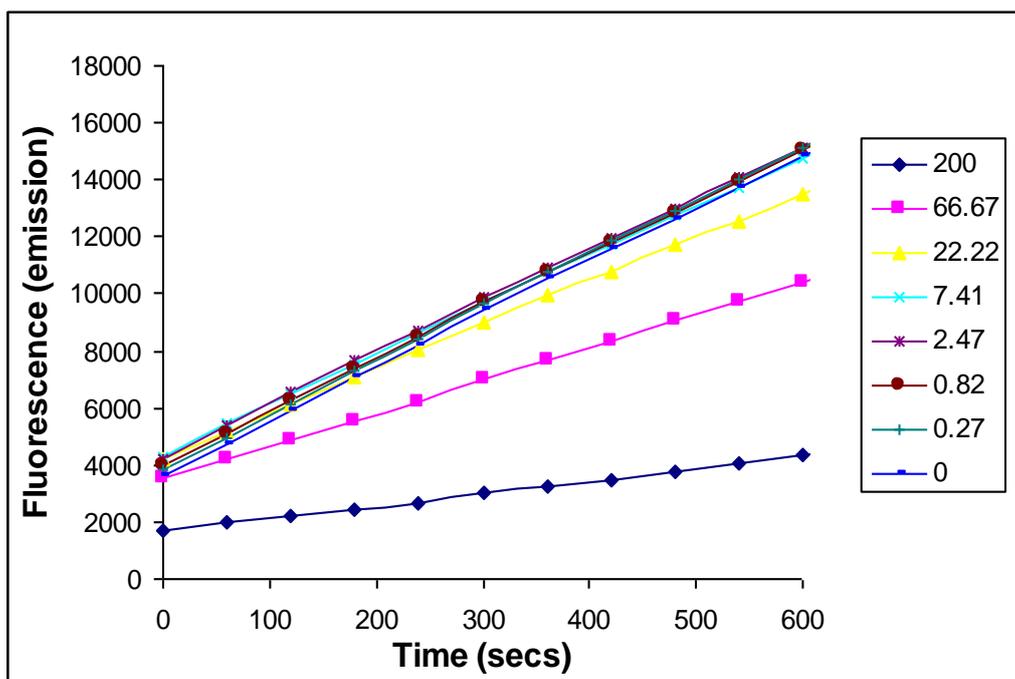


Figure 3.18 The effect of racemic propafenone on the metabolism of CEC by CYP1A2. Concentrations (μM) of racemic propafenone are shown in the graph. Values are mean of 4 measurements

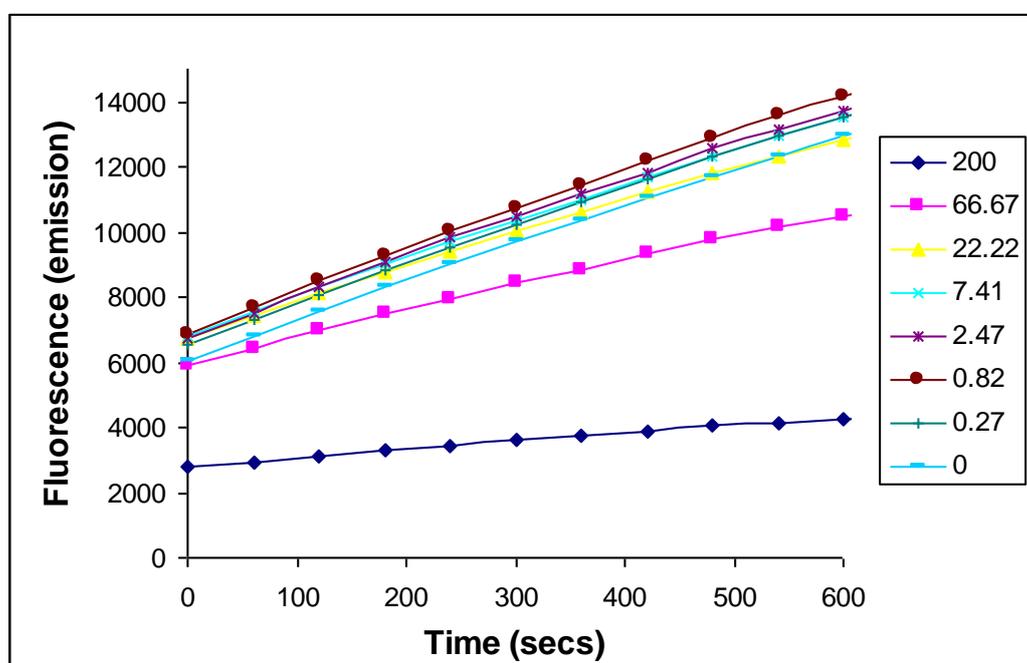


Figure 3.19 The effect of (R)-propafenone on the metabolism of CEC by CYP1A2. Concentrations (μM) of (R)-propafenone are shown in the graph. Values are mean of 4 measurements

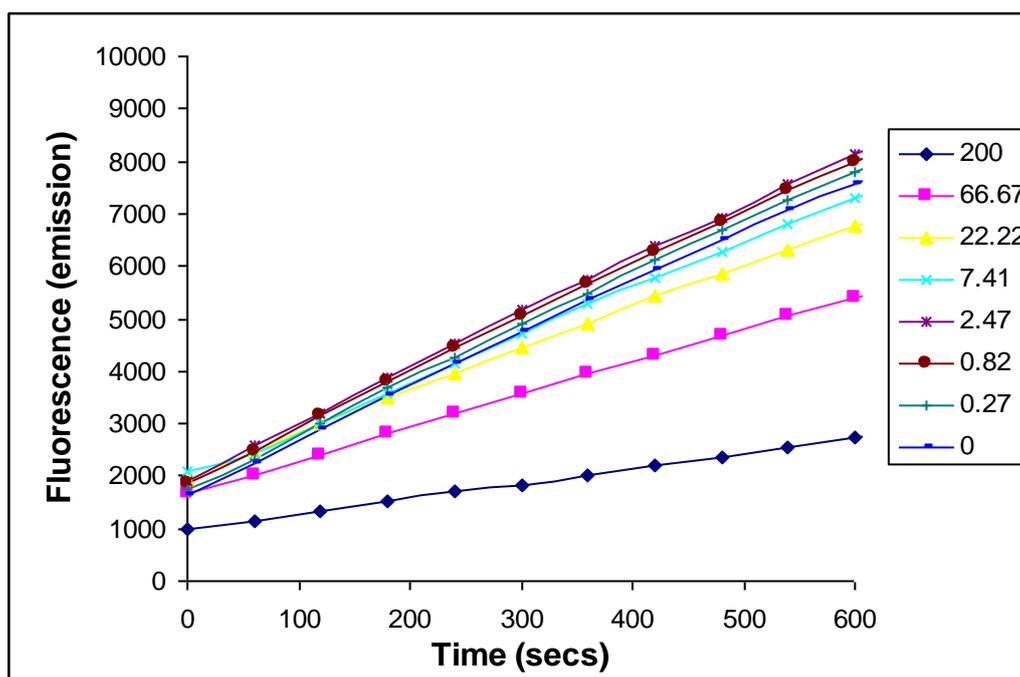


Figure 3.20 The effect of (S)-propafenone on the metabolism of CEC by CYP1A2. Concentrations (μM) of (S)-propafenone are shown in the graph. Values are mean of 4 measurements

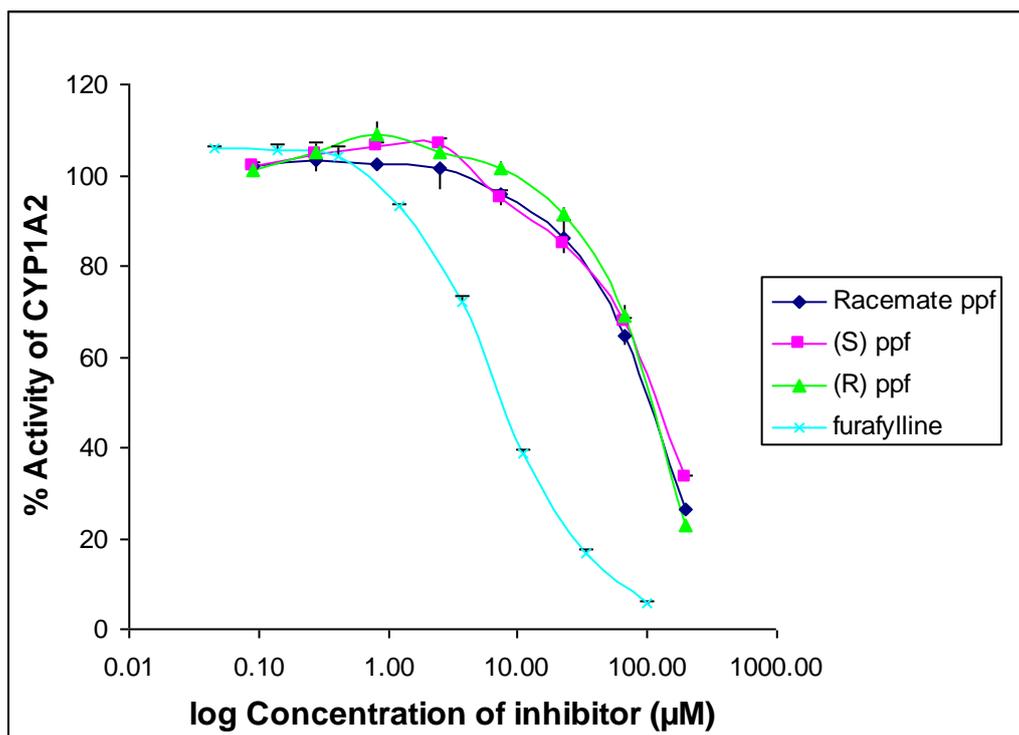


Figure 3.21 The percentage activity of CYP1A2 when incubated with furafylline, racaemic propafenone and its enantiomers. Values are mean of (n=4) measurements \pm S.E.

There is no significant difference in the IC_{50} values of propafenone and its enantiomers which are within 10 μ M of each other. However, there is a significant difference between the inhibitory effects of furafylline and propafenone and its enantiomers ($P < 0.005$; Table 3.1).

3.6 Inhibition studies using CYP2D6

The incubations with CYP2D6 showed the most variability in results of all of the CYPs used and consequently the results for racaemic propafenone are not shown as they were too inconsistent.

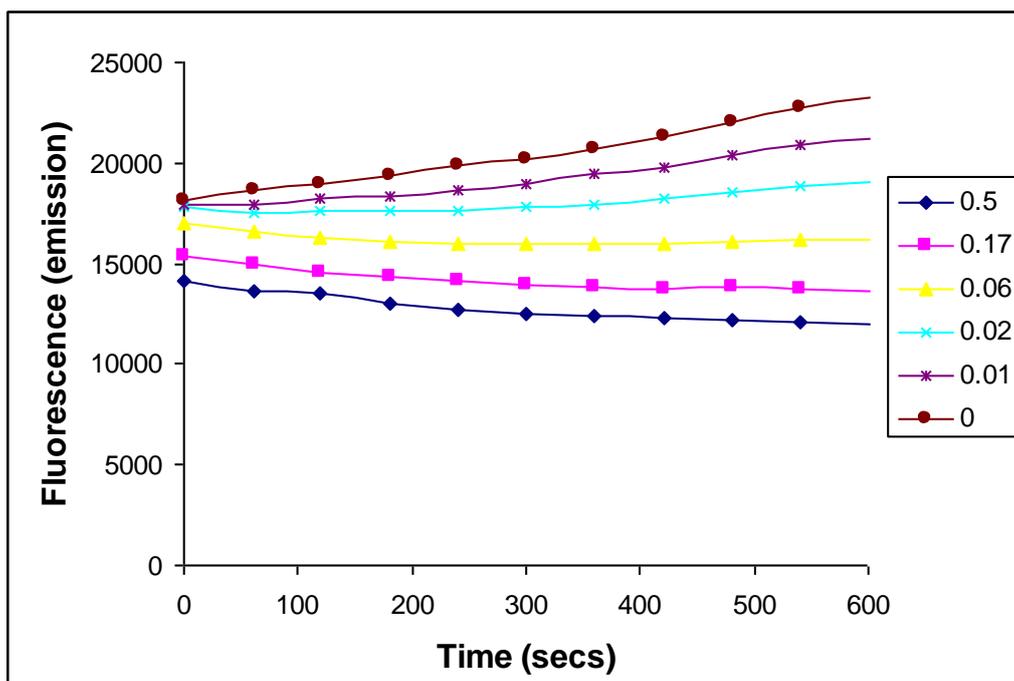


Figure 3.22 The effect of quinidine on the metabolism of AMMC by CYP2D6. Concentrations of quinidine are shown in the graph (n=4).

When CYP2D6 was incubated with quinidine (CYP2D6 inhibitor) the rate of metabolism was decreased with increasing concentration of quinidine. Figure 3.22 shows that at concentrations higher than 0.02 μ M quinidine inhibition is complete with no activity observed.

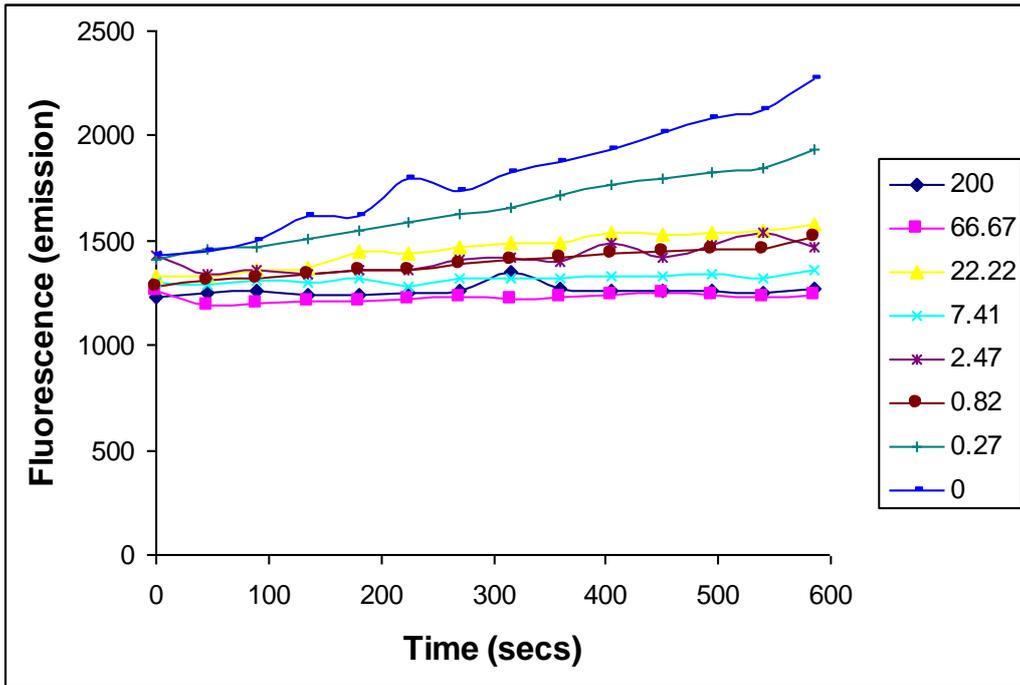


Figure 3.23 The effect of (R)-Propafenone on the metabolism of AMMC by CYP2D6. Concentrations (μM) of (R)-Propafenone are shown in the graph. Values are mean of 4 measurements

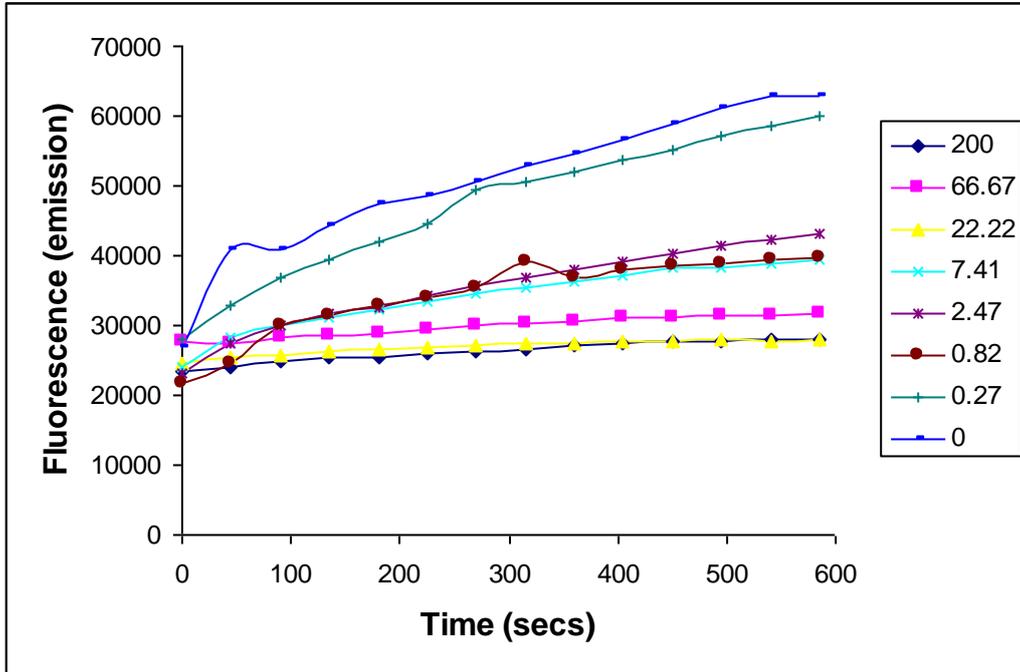


Figure 3.24 The effect of (S)-propafenone on the metabolism of AMMC by CYP2D6. Concentrations (μM) of (S)-propafenone are shown in the graph. Values are mean of 4 measurements

Figures 3.23 to 3.24 show the metabolism of AMMC in the presence of (R) and (S)-propafenone. Figure 3.23 shows that at the highest inhibitor concentration 200 μ M there is complete inhibition of metabolism as seen by no distinct change in fluorescence over time. This was also evident with (S)-propafenone with complete inhibition occurring at the highest concentration (Fig 3.24).

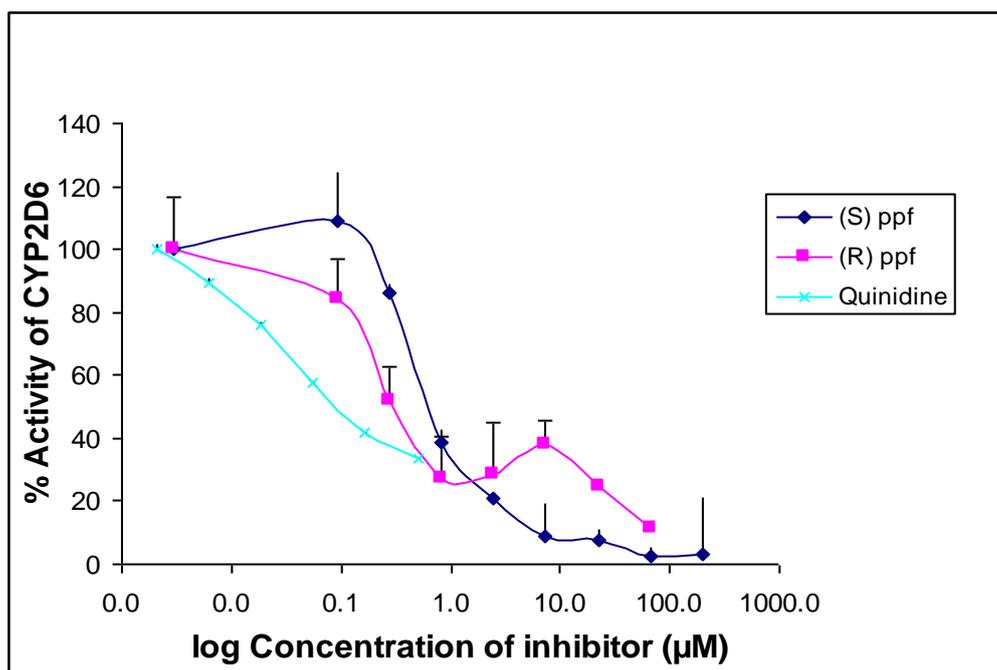


Figure 3.25 The percentage activity of CYP2D6 when incubated with quinidine, racemic Propafenone and its enantiomers. Values are mean of 4 measurements \pm S.E.

Quinidine is a more effective inhibitor of CYP2D6 than the propafenone enantiomers (Fig 3.25). It was not possible to conduct statistical tests on this data, however the (R)-enantiomer showed greater inhibition than the (S)-enantiomer (Table 3.1).

3.7 IC₅₀ Values

Table 3.1 The mean IC₅₀ values for propafenone, its individual enantiomers and the CYP known inhibitors. Values are expressed as mean ± standard deviation (n=4).

| Bactosomes | IC ₅₀ (µM) | | | |
|-----------------|--|-----------------------|----------------------------|----------------------------|
| | Known inhibitor | ppf racemate 10 mM | (R) ppf enantiomer 10mM | (S) ppf enantiomer 10mM |
| CYP 1A2 | furafylline (F) (5mM) 8.44 ± 0.44☼ | 121.60 ± 3.86 | 122.46 ± 6.85 | 127.02 ± 10.71 |
| CYP 3A4 | ketoconazole (K) (0.25mM) 0.19 ± 0.18*×△ | 28.57 ± 3.61× | 12.49 ± 1.70 | 17.74 ± 10.42 |
| CYP 2D6 | quinidine (Q) (25uM) 0.04 ± 0.002 | Not Measured | 0.33 ± 0.1 | 0.57 ± 0.14 |
| CYP 2C19 | TCP (10mM) 60.84 ± 24.75 | 59.54 ± 2.72† | 42.78 ± 10.41 | 76.23 ± 35.50 |

*P<0.05. IC₅₀ values are significantly different from racemic ppf and its enantiomers.

† P<0.05. IC₅₀ values are significantly different from (R) ppf.

☼P< 0.005. IC₅₀ values are significantly different from racemic ppf and its enantiomers.

× P< 0.005. IC₅₀ values are significantly different from (R) ppf

△P< 0.005. IC₅₀ values are significantly different from racemic ppf

In summary, the data in Table 3.1 indicates that the IC₅₀ value for racemic propafenone was significantly different to (R)-propafenone when incubated with CYP3A4 and CYP2C19. The IC₅₀ values for the known inhibitors of

CYP1A2 and CYP3A4 were significantly different from propafenone and its enantiomers.

The CYP2C19 assays show a significant difference in IC_{50} values of racemic propafenone and (R)-propafenone yet when comparing (R)-propafenone and the racemate to (S)-propafenone they are statistically similar.

3.8 In Vitro Metabolism of Propafenone and its Enantiomers with Human Liver microsomes

Figures 3.26 and 3.27 are examples of the chromatographs produced following HPLC analysis of samples from the incubations conducted to measure metabolism. In these examples incubations containing the (S)-enantiomer are shown. The control (Fig 3.26) shows a large first peak at 4.9 minutes which is the internal standard propranolol and a smaller second peak at 5.6 minutes which is (S)-propafenone. Figure 3.27 shows the results from test incubation where the cofactor NADPH was added. There is a large first peak at 5 minutes which is the internal standard and the second peak at 5.6 minutes which has a smaller peak area than the corresponding one in figure 3.26.

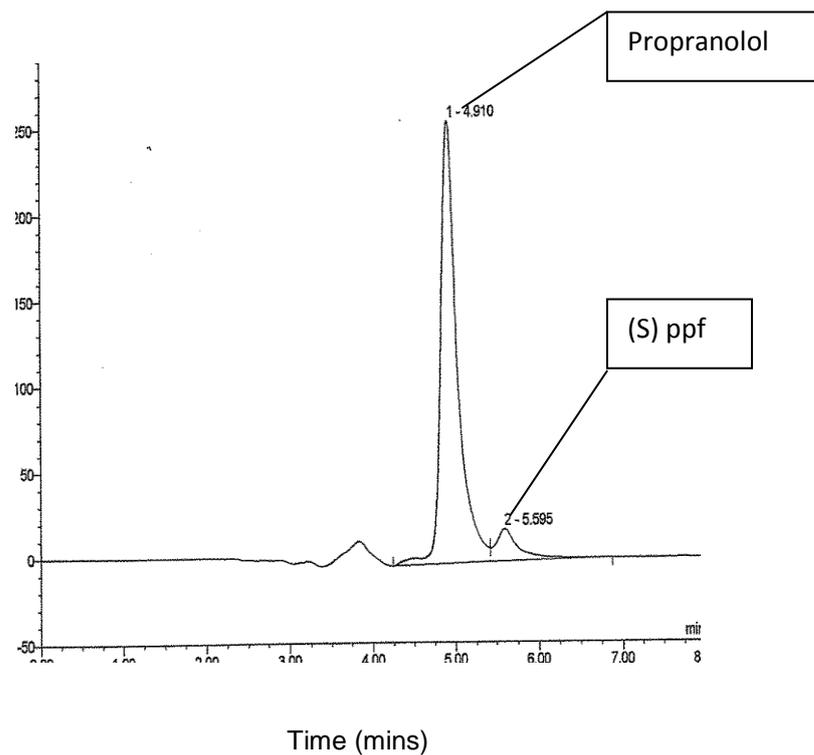


Figure 3.26 Typical HPLC trace for (S)-propafenone (peak at 5.6 minutes) in control incubations (-NADPH) along with the internal standard propranolol (peak at 4.9 minutes).

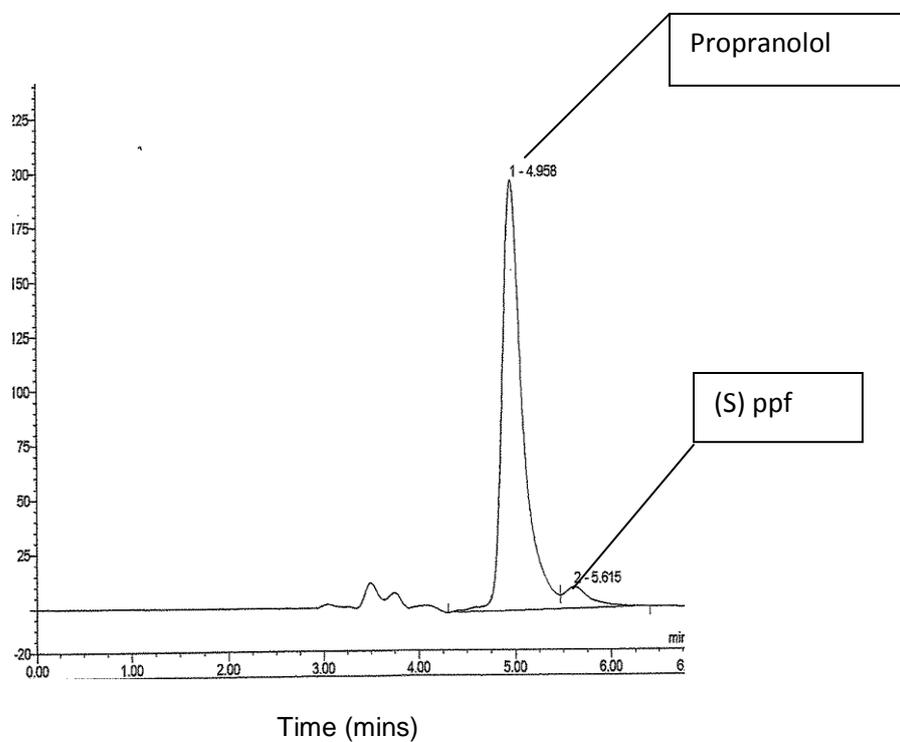


Figure 3.27 Typical HPLC trace for (S)-propafenone (peak at 5.6 minutes) in a test incubation (+ NADPH) along with internal standard propranolol (peak at 4.9 minutes).

Figures 3.28 to 3.30 show the peak area ratios for propafenone and the internal standard propranolol at varying propafenone concentrations which produce standard curves allowing quantification of metabolism.

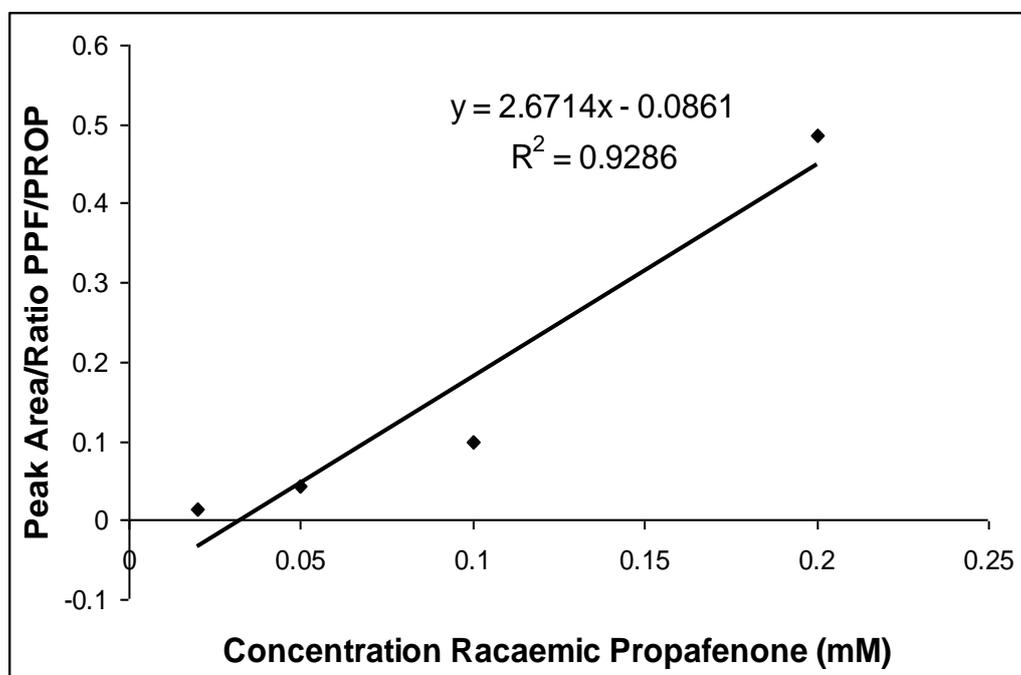


Figure 3.28 Standard curve for racemic propafenone when incubated with the internal standard propranolol (n=1).

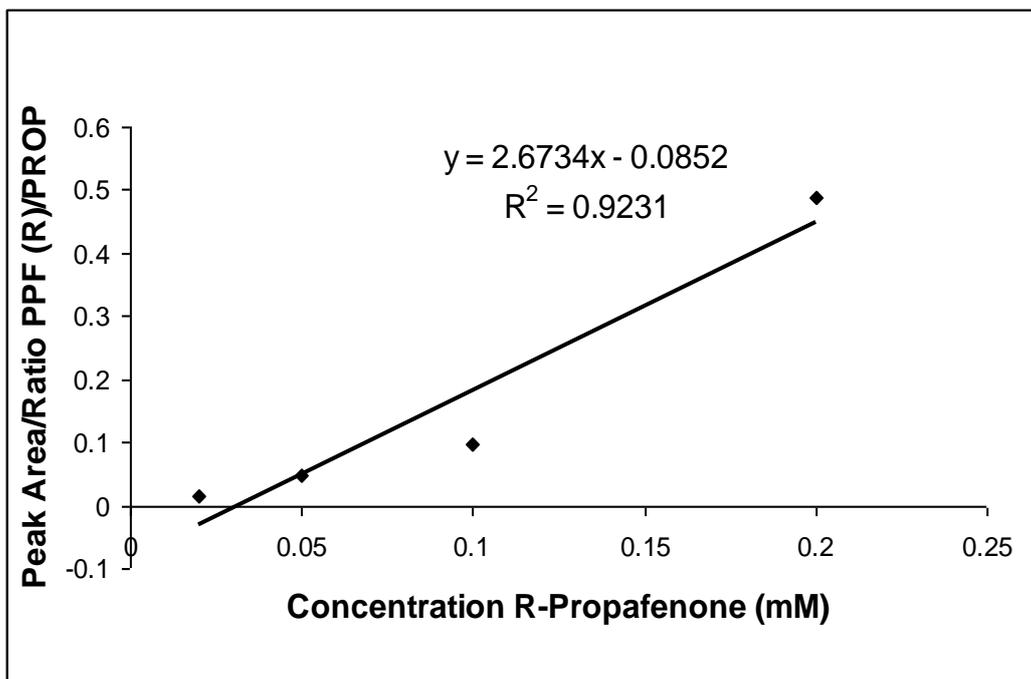


Figure 3.29 Standard curve for the (R)-enantiomer when incubated with the internal standard propranolol (n=1).

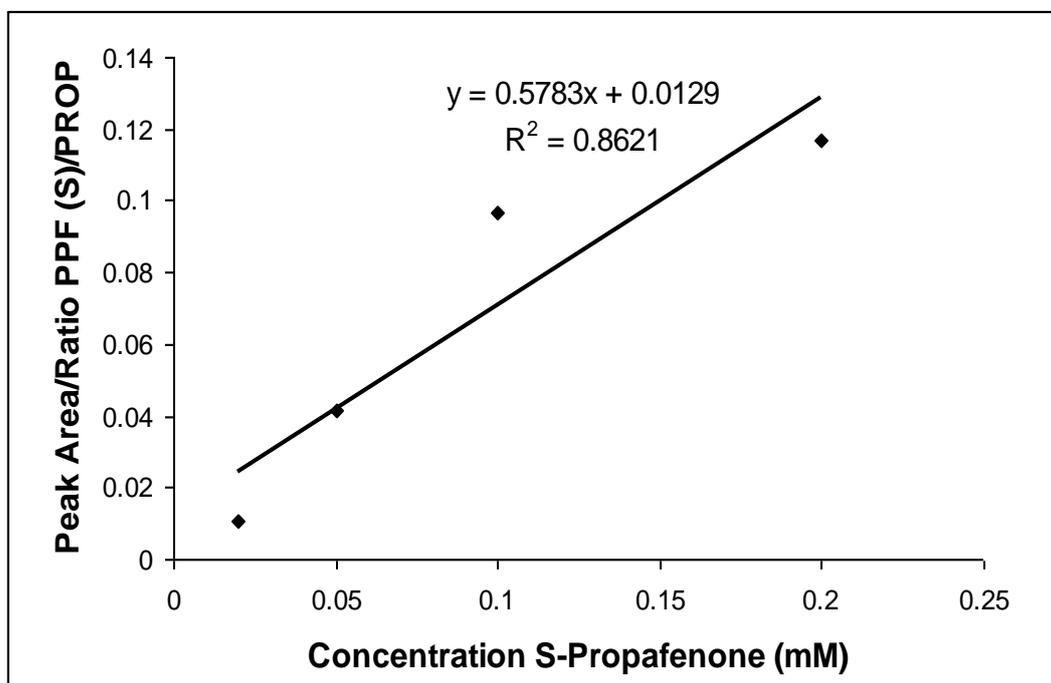


Figure 3.30 Standard curve for the (S)-Enantiomer when incubated with the internal standard propranolol (n=1)

Table 3.2 shows that NADPH dependent metabolism by human liver microsomes occurred for both racemic propafenone and its individual enantiomers. However, the extent of metabolism was significantly less ($p < 0.001$) for racemic propafenone than for either of the individual enantiomers. There was no significant difference in the extent of metabolism of the individual enantiomers.

Table 3.2 Percentage metabolism of racemic propafenone and its enantiomers. Mean \pm standard deviation (n=4)

| | Racemic ppf | (R)-ppf | (S)-ppf |
|--------------|------------------|----------------|----------------|
| % metabolism | 16.0 \pm 2.3 * | 23.7 \pm 8.2 | 27.3 \pm 7.2 |

* $P < 0.001$, percentage metabolism is significantly lower compared to the enantiomers

3.9 Protein Binding

The results for protein binding for the standard drugs used are shown in Figure 3.31. Protein binding levels are similar, although there is a consistent overestimate for protein binding when compared with that found in the literature (www.chromtech.se). There was no difference in protein binding for the two separate enantiomers of propafenone when determined in this manner. When the racemic form of propafenone was used both, enantiomers co-eluted from the column. The amount of protein binding

calculated by this method was 88.6%. Typical HPLC traces for propafenone and also for propranolol are shown in figures 3.32 and 3.33.

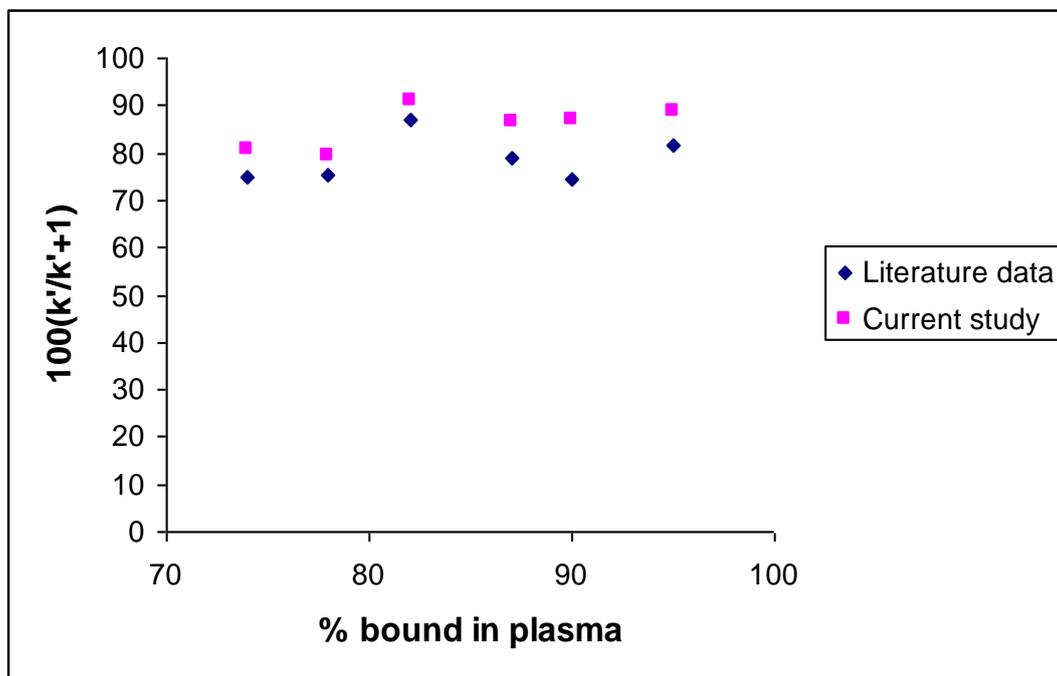


Figure 3.31 Comparison of published data (chromtech. com) to data compiled in this study. The percentage protein binding of different compounds is represented the graph shows the compounds in the following order; carbamazepine, diltiazem, desipramine, propranolol, verapamil and propafenone.

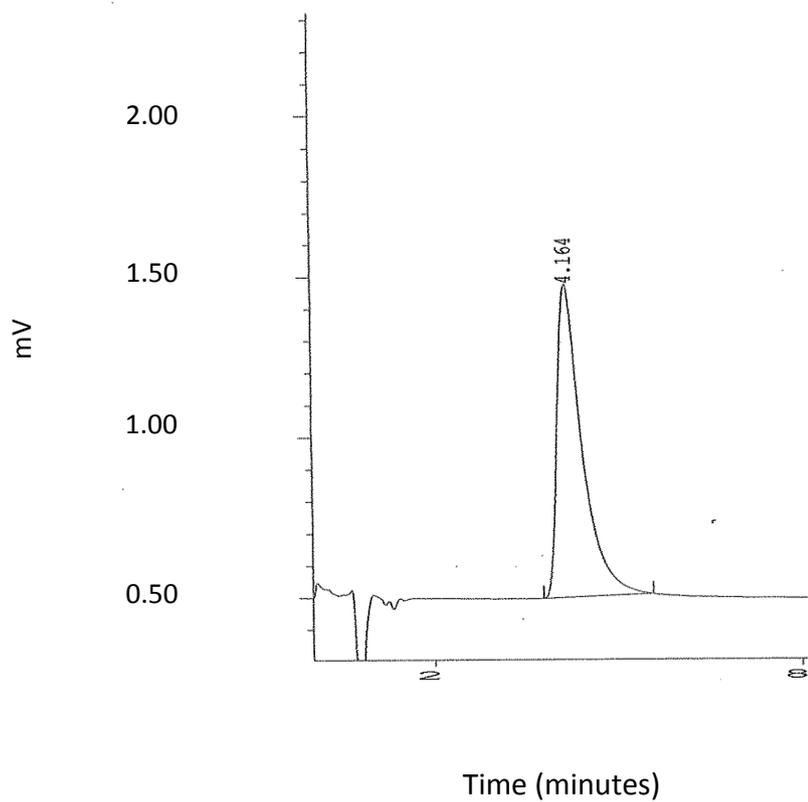


Figure 3.32 Chromatograph of 0.01 M propafenone on the HSA column and its retention time of 4.16 minutes.

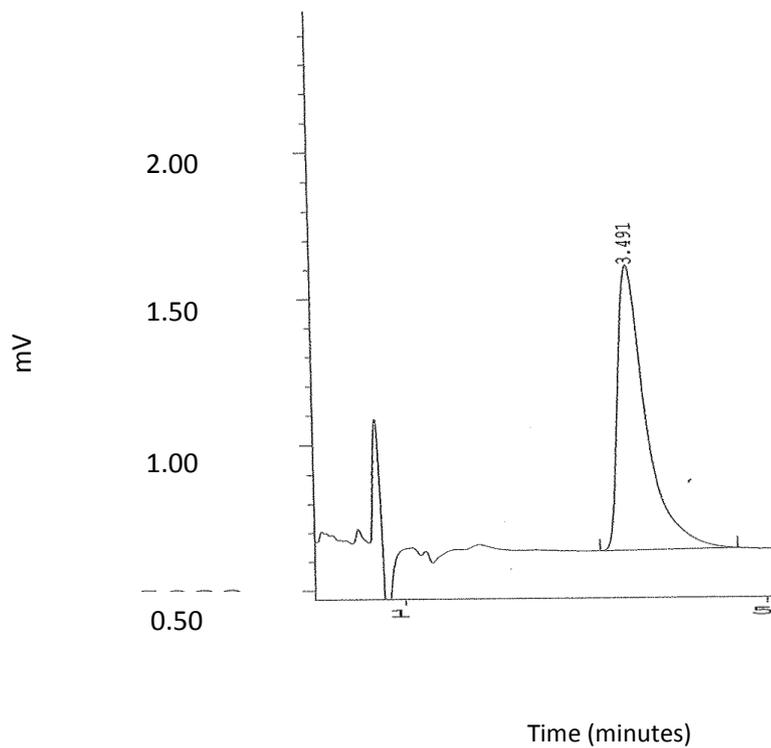


Figure 3.33 Chromatograph of 0.01 M propranolol on the HSA column showing its retention time of 3.49 minutes.

4.0 Discussion

4.1 Background

The aim of this study was to assess the differences in the disposition and metabolism of propafenone enantiomers in particular with regard to the interaction with the major human forms of cytochrome P450. This was primarily done through analysis of racemic propafenone and its individual enantiomers' inhibitory effects and their metabolism by cytochrome p450. As previously described, propafenone is a class IC antiarrhythmic drug and its therapeutic use is flexible as it can be used for both short and long term treatment of arrhythmias. It can be administered both orally and intravenously. Each course has its benefits, for example; intravenous administration is effective for ventricular arrhythmias whereas an oral dose is an effective form of management of chronic re-current supraventricular tachycardia (www.patft.uspto.gov). Propafenone is a chiral compound and it has been established that both enantiomers exert similar sodium channel blocking abilities, but further research has shown that it is the (S)- enantiomer that produces greater beta adrenoreceptor blocking capabilities which is important as beta blockers are the only cardiovascular drugs clearly effective in preventing sudden cardiac death in patients with prior heart attacks (Kroemer *et al*, 1994). As propafenone is normally administered as a racemate it is important to investigate the efficacy of the drug to determine if it is more efficient to administer one enantiomer as opposed to the racemate. This study focuses on the metabolism and distribution of the enantiomers and if there are any stereospecific differences which affect the therapeutic efficacy of propafenone. The aim of the study was to look at each enantiomer

individually. Acquiring samples of the individual enantiomers was difficult; it was time consuming and so during the interim period other means to separate the racemate drug were explored. For example using a chiral column (Zhong and Chen, 1999), A reverse phase ODS column (Yong-Jiang and Ming-Ming, 2004), or using diastereomeric salt formation (Kroemer *et al*, 1994). Whilst these methods were viable options the availability of individual enantiomers from Knoll AG allowed the study to progress without having to resort to their use.

4.2 Inhibition studies.

The inhibition studies measured the interactions between propafenone, its enantiomers and the human CYP isozymes CYP1A2, CYP 2C19, CYP 2D6 and CYP3A4. These studies were based on a method using a fluorescent probe substrate. The individual CYP metabolises the probe substrate to its fluorescent product which can be measured to determine enzyme activity. For example, for the incubations of CYP3A4, the substrate BFC is metabolised to its fluorescent product HFC. To allow higher throughput, the method was scaled down to be conducted in a 96 well plate and fluorescence was measured using a plate reader. Automated assays using this type of technology have proved to be an advantage as they relieve human burden and reduce error involved with manual operations (Yao *et al*, 2007). Inhibition occurs following the co-incubation of the different CYP substrates with another compound (in this instance the standard CYP inhibitor and propafenone) resulting in a decrease in fluorescence. The degree of inhibition was quantified by determining the IC_{50} (i.e. amount of drug causing 50%

inhibition of enzyme activity) which can be related to the drug's affinity for the enzyme (Barile, 2004). This high throughput screening method is used regularly by the pharmaceutical industry to estimate the involvement of single or multiple enzymes involved in drug metabolism and to screen the inhibitory potency of drugs under development through calculation of their IC₅₀ values (Yamamoto *et al*, 2003; Turpeinen *et al*, 2006).

The actual method developed for this study was adapted from one described by BD Biosciences which utilised a NADPH regenerating system. This system was chosen rather than a single addition of NADPH to ensure a constant supply of co-factor to maintain the reaction for the duration of the incubation. It was found that the NADPH regenerating system was effective in generating and maintaining NADPH levels throughout the reaction time (Fig 3.2). The results for the metabolism of the test substrates by the different CYP isoforms show that there was a constant rate of metabolism as seen by a linear production of metabolite (fluorescence) with time. The presence of an inhibitor was found to decrease the reaction rate in a dose dependent manner as observed by a decrease in fluorescence. This allowed the production of inhibition curves which were used to calculate IC₅₀ values for standard inhibitors and propafenone (Table 3.1). Whilst the results for CYP1A2, CYP3A4 and CYP2C19 showed a high degree of reproducibility, this was not the case with CYP2D6 which, despite numerous attempts it did not display the same level of consistency.

Overall the IC₅₀ values calculated in this study are higher than those found in the literature. For example it has been suggested that the IC₅₀ of propafenone is 20 µM for the inhibition of CYP1A2 (Zhou *et al*, 2003) and 0.02 µM when

incubated with CYP2D6 (McGinnity *et al*, 2008). BD Biosciences state the IC₅₀ values for ketoconazole, quinidine, TCP and furafylline to be 0.05 µM, 0.014 µM, 0.75 µM and 1.3 µM respectively. The IC₅₀ values calculated in this study for the known inhibitors of the individual CYP's are all consistently higher. This result was unexpected and so further investigation would help to determine why there is such a difference and the difference in *in vitro* systems used could be factor one worth investigating. Indeed, it has been suggested that factors such as non-specific binding resulting from the high protein content of incubations used by some laboratories may result in erroneous results in these types of studies (Austin *et al*, 2002).

4.2.1 Inhibition with CYP 3A4.

The incubations with CYP3A4 show that (R) and (S)-propafenone have similar inhibitory properties. The IC₅₀ values for propafenone racemate and enantiomers are all significantly greater than those for the standard inhibitor ketoconazole. However, both enantiomers show mean IC₅₀ values which are lower than for the racemate although only the (R)-enantiomer and the racemate are significantly different (p< 0.005) (Table 3.1).

These findings indicate that (R)- propafenone is the more potent inhibitor showing a stereospecific difference in interaction of propafenone enantiomers with CYP3A4. This is important clinically as CYP3A4 is the major human form of P450 responsible for the metabolism of a high number of drugs and so inhibition of this enzyme has a high potential for drug-drug interactions.

4.2.2 Inhibition with CYP2C19.

Propafenone and its enantiomers had statistically similar IC₅₀ values as CYP2C19's known inhibitor TCP. It was only (R)-propafenone which was significantly different from the racemate. In this experiment, greater variability was found with the enantiomers of propafenone as opposed to the racemate. The IC₅₀ values were relatively high when compared to the standard inhibitor (TCP). Therefore, it would be expected that inhibition of CYP2C19 would be minor and indeed no evidence has been reported for this type of interaction. The IC₅₀ value for (S)-propafenone is higher than that of the (R)-propafenone and the racemate; therefore it is indicative of a lower affinity for the enzyme than is seen with the (R)-enantiomer and the racemate (Fig 3.16 and Table 3.1).

4.2.3 Inhibition with CYP1A2.

Analysis of CYP1A2 showed that there were no significant differences in the IC₅₀ values for propafenone and its enantiomers. However, the results do show that as an inhibitor of CYP1A2, racemic propafenone and its enantiomers are relatively poor inhibitors of CYP1A2 when compared to furafylline (CYP1A2's known inhibitor) which had an IC₅₀ value of 8.44 μM. There are conflicting reports for the IC₅₀ of propafenone on CYP1A2, with values ranging from 20 μM to 0.225 mM (Zhou *et al*, 2003). However, in a study on the co-administration of propafenone with caffeine it has been reported that propafenone causes significant inhibition of CYP1A2 activity *in vivo* (Michaud *et al*, 2006). In the case of CYP1A2 there appeared to be less

variability than with the other CYPs. There is no significant difference in the inhibitory effects of propafenone or its enantiomers on the activity of CYP1A2 (Fig 3.21) indicating that there are no stereospecific differences in propafenone metabolism by CYP1A2. The results do tally with expected involvement of the different CYPs i.e. the main metabolic pathways for propafenone involve CYP2D6 and CYP3A4 rather than CYP1A2.

4.2.4 Inhibition with CYP2D6.

The assays involving CYP2D6 were more difficult to perform as there was considerable variability between the inhibitory effects of propafenone and its enantiomers (Fig 3.25 and Table 3.1) which resulted in no IC₅₀ value being calculated for the racemate in this instance. In comparison to CYP2D6's known inhibitor quinidine, the IC₅₀ values for the enantiomers was approximately greater by a factor of tenfold. The IC₅₀ value calculated in this study for quinidine was 0.037 µM which is more than double that stated in the literature (BD Biosciences.com). This could be because, when performing the assay it proved the most difficult to reproduce. As a result of the problems in obtaining data it was not possible to conduct any statistical tests, although the (R)-enantiomer exhibited a greater inhibitory effect than the (S)-enantiomer. The potential for propafenone and its enantiomers to cause significant drug-drug interactions is highlighted by the low IC₅₀ values determined in this study. Furthermore, individuals displaying a slow metaboliser phenotype for CYP2D6 would show even greater effects as it is possible that metabolism could be inhibited to an even greater extent in this case. The findings for CYP2D6

mirror those of CYP3A4 where (R)-propafenone proves to be the more potent inhibitor. In comparison to the other bacosomes used in this study, propafenone did prove to be the most potent inhibitor when incubated with CYP2D6. This is important as previously discussed CYP2D6 is responsible for the metabolism of more than 40 drugs, therefore the inhibitory effect of propafenone on CYP2D6 could reduce the effectiveness of other drugs when co-administered (Hemeryck *et al*, 2000). Further investigation into refining this method could therefore be warranted.

Overall, the IC₅₀ results (Table 3.1) show that propafenone is the most potent inhibitor of CYP2D6 in comparison to the other CYPs tested. The results also indicate that propafenone is least effective as an inhibitor when incubated with CYP1A2. A major finding from these studies has been that (R)-propafenone shows the greatest inhibitor effect for each CYP except CYP1A2. Further studies are required to investigate the *in vivo* effect of propafenone and its enantiomers with regards to CYP interaction and inhibition. It is apparent that the CYP isoforms which have been shown to be the major ones responsible for the Phase 1 metabolism of propafenone display the greatest amount of inhibition (namely CYP2D6 and CYP3A4). This is not unexpected as it is with these CYP isoforms that the greatest binding affinity/interaction would be predicted. Therefore, it may be expected that the (R)-enantiomer would undergo more metabolism especially with CYP3A4 and CYP2D6. In fact this is not the case, although the individual enantiomers display more metabolism than the racemate. This may be due to relative amounts of CYP in the microsomal fraction used in the metabolism studies, but it could also suggest that the inhibition assays are not always able to predict stereoselective

differences. Future investigation could include the repetition of the inhibition study using human liver microsomes rather than the individual bacosomes as this is what exists in the body naturally. This can be an issue as an increase in protein concentration can change the non-specific protein binding. Further investigation could determine if there are any similarities between the individual CYPs used throughout this study.

4.3 In Vitro Metabolism of Propafenone and its Enantiomers with Human Liver microsomes

The metabolism study was performed using human liver microsomes which, unlike the bacosomes used in the inhibition study, contain a mixture of CYP isoforms. This, of course, is the situation found *in vivo*, but it can make the data more difficult to analyse. Ideally, the metabolism study was to be carried out using chromatographic separation with mass spectrometer analysis (LC-MS) to detect the presence of propafenone and metabolites, but unfortunately this was not possible and so the study was performed using HPLC with diode array detection. This method yielded accurate results and enabled quantification of metabolism by measuring how much parent propafenone remained at the end of the incubation.

Consideration was given to various methodologies to extract the parent drug and metabolites from the incubation samples prior to HPLC analysis. These included the use of solid phase extraction as has previously been described for the phase 1 metabolites 5-hydroxypropafenone and N-despropylpropafenone (Hoffman *et al*, 2000). Another option was the use of dichloromethane as an agent to carry out the extraction and concentration of

the samples (Pires de Abreu *et al*, 1999). Ultimately the method used in this study (refer to section 2.6.5) was chosen due to its simplicity, speed and not having to carry out extensive sample preparation.

Whilst the use of HPLC methodology is an extremely powerful one for the separation and quantification of drugs and their metabolites, it was not without its problems. The technique was not always reliable with equipment failure being the greatest problem which limited the number of studies to be conducted. In developing the method, the original mobile phase chosen contained water, acetonitrile and methanol at the following proportions (56:16:28 v/v/v). This mixture also contained 0.03% triethylamine and 0.05% phosphoric acid and was based on a method described by Zhou *et al* (2001). The concentrations of the individual components were altered as the retention time was significantly longer than anticipated. The organic element of the mixture was increased because the column is lipophilic therefore increasing the lipophilic part of the solvent meant that the compounds were eluted quicker. The final mobile phase contained water, acetonitrile and methanol (25:30:45 v/v/v). This alteration now ensured that the propafenone and internal standard propranolol peaks were produced at around 6-7 minutes whilst still retaining the separation of propafenone from the internal standard and metabolites.

4.3.1 Percentage metabolism

From the chromatographs (Fig 3.26 and 3.27) it is clear that adequate separation was achieved to allow quantification of propafenone levels. The results are interesting in that the percentage metabolism of the racemate was

considerably lower than that of the enantiomers. It has been reported that, when incubated together, (R)-propafenone will inhibit the metabolism of (S)-propafenone (Zhou *et al*, 2003). This may explain the decreased metabolism observed with the racemate as opposed to the individual isomers. When comparing the metabolism of the individual enantiomers the difference is not significant, although the (S)-enantiomer experiences more metabolism than its counterpart which is in agreement with previous work (Zhou *et al*, 2003). As mentioned earlier in this discussion, the results of the inhibition studies may suggest that the (R)-enantiomer would experience more metabolism as it had a greater affinity to CYP3A4 and CYP2D6 in comparison to the racemate and (S)-propafenone which is not the case. It would have been fascinating to confirm which CYP specifically was involved in the metabolism of propafenone by HLM as this would also allow a better comparison between the inhibition studies. This could be achieved by using specific CYP inhibitors in incubations with HLM. This is a point for future research in this area.

Bringing together the results of all of the studies, they show that overall the (S)-enantiomer had the greatest percentage metabolism in comparison to the racemate and the (R)-propafenone which was unexpected as the (S)-propafenone showed a lower affinity to all of the CYPs used in the inhibition study compared to the (R)-enantiomer and the racemate in particular with CYP2C19 and CYP1A2. Yet the inhibition study proved that it was not significantly different to the racemate in its inhibitory effects. Although there were differences in the activity of the CYPs used in the study, the metabolism study has shown that the individual enantiomers are metabolised to a greater extent than the racemate which could be problematic when using only one

enantiomer for therapy rather than the racemate. The half life for propafenone is short which necessitates regular dosing. Decreasing the half life even further could be unacceptable for the use of the drug *in vivo*. This is in contrast to the inhibition study which suggests administering the (S)-enantiomer would be the best option as (R)-propafenone shows the greatest inhibitory effect and is metabolised and cleared at a greater rate than its opposite enantiomer. Therefore, further study both *in vitro* and *in vivo* is warranted to investigate the feasibility of enantioselective therapy of propafenone.

4.4 Protein Binding

Protein binding is an important factor as many critical pharmacokinetic parameters such as metabolism rate, renal excretion rate and steady-state distribution volume are the effect of the unbound drug fraction (Cheng *et al*, 2004). Therefore, the higher affinity a drug has for the protein the slower the disassociation rate will be, creating lower clearance rate and increasing the elimination half life (Sear 2007). HSA was used as it is the most abundant protein found in human blood and plasma (Cheng *et al*, 2004). It has two major binding sites known as the warfarin-azapropazone and the indole-benzodiazepine site. The methods primarily utilised to determine drug-protein binding are equilibrium dialysis (Kwon, 2001) and ultrafiltration (Crommelin *et al*, 2002). There are advantages and disadvantages to both of these methods. For example equilibrium dialysis is temperature controlled and known as the standard procedure yet it takes a long time to reach equilibrium. Ultrafiltration

is a fast process and requires a small amount of sample but the volume of ultrafiltrate may not be suitable for drugs assays (Kwon, 2001).

This study employs a novel and faster method of analysis. The sample preparation was simple and so experimental duration was significantly shorter.

The protein binding study showed the difference in distribution of propafenone compared to other compounds. The HSA column is chiral and is able to separate racemic compounds directly. The results show that the racemic propafenone did not separate and therefore the enantiomers eluted at the same time as the racemate. This means that both enantiomers exhibit similar protein binding to the racemate on HSA and that there is no stereospecific variation in the binding of the different enantiomers. Therefore there is no influence on disposition *in vivo* and so the enantiomers have the same amount of unbound drug free to undergo biological processes such as metabolism and elimination. This data is in agreement with that published by Hong *et al* (2009) who also found that binding to HSA was not enantioselective. In addition figure 3.21 shows propafenone has one of the highest percentage protein binding of 88.6% second only to desipramine at 91.2% in this experiment. This study is in agreement with published data indicating that propafenone exhibits a high protein binding of 95% (Ijaz and Khan, 2001). However, propafenone has also been shown to bind to alpha 1 acid glycoprotein in an enantioselective manner and so further studies in this area would need to take this binding into account (Hong *et al*, 2009).

4.5 Scope for Future work

It is known that CYP2D6 and CYP3A4 are the main CYPs to metabolise propafenone. In the future more work should be done to concentrate on CYP2C19 as it appears to be understood far less than any of the other CYPs in this study. The inhibition studies yielded interesting results in that it proved CYP2C19 was inhibited by propafenone to a greater extent than CYP1A2. A study of its metabolism properties would be interesting to see. Future studies should also include the use of LC-MS to look at the individual metabolites being produced and to quantify rate of metabolism. Are certain metabolites being produced in greater concentrations than others and which pathway is favoured?

4.6 Problems and Developments

Throughout this study there were several obstacles to overcome for example, the inhibition study involving CYP2D6. Concordant results were difficult to achieve in comparison to the other CYPs, it took several replications to achieve the results (Fig 3.23 and 3.24). When performing this test the well containing the blank was consistently high and some instances it was higher than the test samples. This observation was unexpected as the blank only contained the substrate. The substrate was frozen to allow use over several sessions and so this could have affected its efficacy, therefore it was chosen to make it up fresh and the results were more consistent. Due to this the reliability of the CYP2D6 results are questionable. This test was performed under the same conditions in terms of the preparation stages and incubation time. The only major difference was the use of different cofactors. The

cofactor used for CYP2D6 was eight times more concentrated. With more time the incubations could be performed using the other cofactor mix to see if there was any change in CYP2D6's behaviour. Also it would be interesting to see the results using the other substrate MAMC.

The metabolism study used human liver microsomes rather than the individual bacosomes. This was done as neither the time or the money was available to invest in new sets of bacosomes and so it was chosen to use human liver microsomes for the metabolism incubations.

The original method for the metabolism study (refer to 2.6.3) was developed as there was no propafenone peak visible when the samples were analysed. There was definitely propafenone in the system and so this was unexpected. Therefore after checking the injector and the column by running 10 μ L of 1mm propafenone dissolved in water, a peak was produced. In order to investigate this occurrence further the sample was dissolved in the mobile phase. A 10 μ L aliquot of the sample mixture was added to 190 μ L of mobile phase and ran. A peak was now visible but the optimum conditions to produce good separation and resolution of the peaks needed to be ascertained. It is believed that the phosphate buffer was interacting with the sample solution and having a negative effect on the sample mixture and causing no peak to be recorded. Different ratios of the sample and mobile phase were tested to determine the one that produced the best separation and resolution. A 1 in 10, 1 in 20, 1 in 50 and 1 in 100 were tested and ultimately it was decided that the 1 in 10 dilution yielded the best results. The flow rate was reduced from 1 ml/minute to 0.5 ml/ minute as the peaks were now coming off at around 3 minutes which interfered with the solvent front. 6 minutes was the desired run time.

The samples were diluted to 1 in 10 with the mobile phase and then run from that point onwards; therefore the results obtained in section 3.8 were done so using this alteration. This change proved to work well which is visible from the chromatographs (Fig 3.26 and 3.27)

In conclusion there are many factors which affect a drugs efficacy.

Investigating racemate therapy is difficult. Treatment with the individual enantiomers or racemic propafenone have advantages and disadvantages, which are indicated in this study. Each CYP analysed in this study is inhibited by propafenone to varying degrees but there is room for further investigation especially in terms of the inhibition of HLM as that is how it naturally occurs in the body. This study has exposed the interactions of propafenone and its enantiomers with the major human forms of cytochrome P450, yet it cannot conclusively say that therapy using individual enantiomers is the best form of treatment in this case. Work in this area needs to be continued.

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