PHYSICOCHEMICAL DETERMINANTS OF THE NON-SPECIFIC BINDING OF DRUGS TO HUMAN LIVER MICROSOMES

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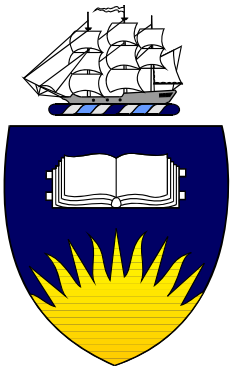

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DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university and that to the best of my knowledge and belief, it does not contain any material previously published or written by another person except where due reference is made in the text.

James A McLure

11 June 2008

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Publications

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ABSTRACT

Accurate determination of the *in vitro* kinetic parameters K_m (Michaelis constant) and K_i (inhibition constant) is critical for the quantitative prediction of *in vivo* drug clearance and the magnitude of inhibitory drug interactions. A cause of inaccuracy *in vitro* arises from the assumption that all drug added to an incubation mixture is available for metabolism or inhibition. Many drugs bind non-specifically to the membrane of the *in vitro* enzyme source.

The aims of this thesis were to: 1) investigate the comparative importance of lipophilicity (as log P), and pK_a as determinants of the non-specific binding of drugs to human liver microsomes; 2) develop and validate an ANS fluorescence technique for measuring the non-specific binding of drugs to human liver microsomes; 3) characterise the non-specific binding of a large dataset of physicochemically diverse drugs using the ANS fluorescence procedure; 4) evaluate relationships between selected physicochemical characteristics and the extent of non-specific binding of drugs to human liver microsomes and; 5) computationally model the non-specific binding of drugs to discriminate between high binding ($f_{u(mic)} < 0.5$) and low binding ($f_{u(mic)} \geq 0.5$) drugs.

The comparative binding of the basic drugs atenolol (log P = 0.1; $f_{u(mic)} = 1.00$), of propranolol (log P = 3.1; $f_{u(mic)} = 0.36 - 0.84$), and imipramine (log P = 4.8; $f_{u(mic)} = 0.42 - 0.82$) suggested that lipophilicity is a major determinant of non-specific binding. In contrast, the comparative binding of diazepam ($pK_a = 3.3$; $f_{u(mic)} = 0.69 - 0.80$), a neutral compound; and the bases propranolol ($pK_a = 9.5$; $f_{u(mic)} = 0.36 - 0.84$) and lignocaine ($pK_a = 9.5$; $f_{u(mic)} = 0.98$), indicated that pK_a was not a determinant of the extent of non-specific binding. The non-binding of lignocaine, a relatively

lipophilic base, was unexpected and confirmed by the non-binding of the structurally related compounds bupivacaine and ropivacaine. These results implicated physicochemical characteristics other than lipophilicity and charge as important for the non-specific binding of drugs to human liver microsomes.

An assay based on 1-anilinonaphthalene-8-sulfonate (ANS) fluorescence was developed using the seven drugs employed in the initial study. Non-specific binding data from equilibrium dialysis and the ANS fluorescence methods were compared and a linear correlation ($r^2 = 0.92$, $p < 0.01$) was observed at drug concentrations of 100 and 200 μM. The approach was further validated by characterising the microsomal binding of nine compounds (bupropion, chloroquine, chlorpromazine, diflunisal, flufenamic acid, meclofenamic acid, mianserine, trifluprom azine, and verapamil) using both binding methods (i.e. equilibrium dialysis and ANS fluorescence). A significant logarithmic relationship ($r^2 \geq 0.90$) was demonstrated between $f_{u(mic)}$ and the modulus of ANS fluorescence for all drugs and for basic drugs alone at concentrations of 100 and 200 μM, while the acidic/neutral drugs showed a significant linear relationship ($r^2 \geq 0.84$) at these two concentrations ($p < 0.01$). The non–binding of bupropion provided further evidence that physicochemical properties other than log P and charge were important for non-specific binding of drugs to human liver microsomes.

The ANS fluorescence technique was then used to characterise the non-specific binding of 88 physicochemically diverse compounds. In general, acids and neutrals bound to a 'low' extent ($f_{u(mic)} \geq 0.5$) whereas bases bound the full $f_{u(mic)}$ range (0.0001 – 1). Statistically significant relationships were observed between the non-specific binding of bases and log P, the number of hydrogen bond donors and hydrogen bond acceptors per molecule, and molecular mass.

Preliminary *in silico* modeling of the dataset generated by the ANS fluorescence technique, using the program ROCS, provided discrimination of all but one (itraconazole) of the 'high' binding bases. However, there were 14 false positives, resulting in low overall prediction accuracy.

Taken together, the studies conducted in this thesis provide important insights into the physicochemical factors that determine the non-specific binding of drugs to human liver microsomes.

ABBREVIATIONS

ADME	Absorption, Distribution, Metabolism, Excretion
ADMET	Absorption, Distribution, Metabolism, Excretion, Toxicology
ANS	1-anilino-8-naphthalene sulfonate
B_{max}	maximum binding capacity
Caco-2	intestinal cell line from human colorectal cancer
Chol	cholesterol
CL	clearance
C_B	concentration of bound drug
C_F	concentration of free drug
CL_H	hepatic clearance
CL_{int}	intrinsic clearance of drug metabolising enzyme(s)
C_0	initial concentration at time zero
CL_S	systemic clearance
C_{SS}	concentration of drug in plasma at steady state
C_t	drug concentration at time t after the dose
CYP	cytochrome P450
D	drug
D_F	free drug
DMSO	dimethylsulfoxide
D_N	dispersion number
E_H	hepatic extraction ratio
f_m	fraction of dose metabolised along pathway of interest
f_u	fraction of drug unbound in blood
$f_{u(inc)}$	fraction of drug unbound in an incubation

f_{um}	fraction of drug unbound in an incubation medium
$f_{u(mic)}$	fraction of drug unbound in microsomes
$f_{u(T)}$	fraction of drug unbound in tissue
HBA	hydrogen bond acceptor
HBD	hydrogen bond donor
HPLC	high performance liquid chromatography
I	inhibitor
I_u	unbound inhibitor
IND	Investigational New Drug
k	elimination rate constant
K_D	dissociation constant
K_i	inhibition constant
K_m	Michaelis constant (substrate concentration at half maximal velocity)
$K_{m(app)}$	apparent Michaelis constant
log P	log of the concentration of drug in the lipid phase / concentration of drug in the aqueous phase
M	metabolised
MM	molecular mass
MW	molecular weight
NDA	New Drug Application
NMR	nuclear magnetic resonance
NNN'N' TMED	NNN'N' tetramethylethylenediamine
NSAID	non steroidal anti-inflammatory drug
PB	potassium phosphate buffer, 0.1M, pH 7.4

PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PS	phosphatidylserine
PSA	polar surface area
Q_H	liver blood flow
QSAR	Quantitative Structure-Activity Relationship
R_c	ratio of the areas under the plasma drug concentration time curves in the presence and absence of inhibitor
ROCS	Rapid Overlay of Chemical Structures
S	substrate
SD	standard deviation
SM	sphingomyelin
t	time
$t_{1/2}$	half life of a drug dose
UGT	UDP-glucuronosyltransferase
V, v	velocity or rate of metabolite formation
V_d	volume of distribution
V_{max}	maximal velocity of a reaction at a saturating substrate concentration
V_P	plasma volume
V_T	tissue volume

CHAPTER 1
INTRODUCTION

1.1 The pharmaceutical industry

1.1.1 Historical perspective

Illness has been a part of life as long as humankind has existed. The earliest forms of cure revolved around two approaches, empiricism and magic. The empirical method involved trial and error and further application of solutions that were found to be useful in the past. Magic would be used when it was felt that natural realities had been modified as a result of mobilisation of supernatural forces thought to be capable of effecting the necessary transformation (Guillen et al. 1985).

In time, greater emphasis was placed on attempting to understand rationally the therapeutic action of substances. This was the evolution of empiricism to an order of science known as pharmacology (Guillen et al. 1985). Pharmacology can be defined as the study of the manner in which the functions of living systems are altered by drugs (Rang, Dale & Ritter 1999). The word drug is defined by the World Health Organisation as 'any substance or product that is used or intended to be used to modify or explore physiological systems or pathological states for the benefit of the recipient' (Bryant, Knights & Salerno 2003). Pharmacology is a young science, achieving independent recognition at the end of the nineteenth century, although the term pharmakon was used in Greece as early as 800BC-395AD to describe the nature of a medical remedy. Thus, the notion of administering a substance to aid in healing is thousands of years old (Guillen et al. 1985).

The late 1800s were a time when major advances in medical science evolved in tandem with dramatic developments in modern industrial technology. The

converging trends offered unprecedented opportunities to meet the needs of an emerging worldwide health care market (http://www.rocheusa.com/about/history.html). The first company was opened in 1859 (C. F. Boehringer & Soehne, in Stuttgart, Germany). Thirty-seven years later, in 1896, after gaining experience in pharmacy and the chemical trade, Fritz Hoffmann formed F. Hoffmann-La Roche & Company in Basel, Switzerland, naming it not only for himself, but also for his wife, Adéle La Roche. Hoffmann was 28 years old at the time and his goal for the company was to develop and manufacture novel drugs of uniform strength and quality and market them internationally.

Early in the twentieth century several advances lead to the establishment of the wider pharmaceutical industry. Paul Ehrlich discovered a number of arsenical compounds for treating syphilis in 1909 (Rang et al. 2003; Gensini, Conti & Lippi 2007). Following this discovery, experiments testing the biological dye methylene blue for antimalarial qualities unveiled a particular type of small molecule as a treatment for bacterial infections, the main medical problem of the time (Sorgel 2004; Gensini, Conti & Lippi 2007). Other notable developments occurred during the 1920s, including the first screening of soil samples for disease fighting agents and the serendipitous discovery of a penicillin mould inhibiting the growth of staphylococci (Abraham 1980; Hare 1982; Hunter 1997). These events, amongst others, led to the formation of an industry focused on improving world health using small molecules as therapeutic agents.

1.1.2 Drug discovery

Originally, the discovery of new drugs was by trial and error, with compounds predominantly arising from natural sources (Drews 2000). However, since the 1950s

the industry focus has been to move drug design towards a fully rational structure-based process with most therapeutic agents now being synthetic products (Rang, Dale & Ritter 1999; Schwardt, Hartmuth & Ernst 2003).

Functionally, in the majority of cases, a drug exerts a therapeutic effect by selectively binding to a biological target such as a physiological receptor, enzyme, ion channel or transporter (all of which are proteins), and either activates (agonist) or blocks (antagonist) a cellular event(s) (Drews 2000; Rang et al. 2003). Thus, the initial component of the drug discovery process comprises: 1) the identification and characterisation of a biological target, and 2) isolating a compound that binds to the biological target with the required affinity and selectivity whilst affecting a desired pharmacological response.

1.1.2.1 Identification and characterisation of a biological target

Advances in cell and molecular biology, biochemistry, recombinant DNA technology and physiology, together with the evolution of the relatively new fields of proteomics and genomics, now drive the identification of new biological targets (Hunter 1997; Egan, Walters & Murcko 2002; Schwardt, Hartmuth & Ernst 2003). Essentially, these disciplines identify altered biochemical pathways in disease or illness and/or gene sequences with levels of expression that differ between healthy and disease states highlighting potential biological targets for therapeutic intervention. Since the publication of the working draft of the human genome sequence in 2001 (Consortium 2001), the elucidation of 30,000 - 40,000 human gene sequences highlights the immense opportunity for prospective new targets for treatment (Goodnow, Guba & Haap 2003; Schwardt, Hartmuth & Ernst 2003).

Once identified, the target protein undergoes complete three-dimensional structural characterisation. The techniques employed for this include X-ray crystallography,

nuclear magnetic resonance (NMR), and computational homology modeling methods (Hunter 1997; Schwardt, Hartmuth & Ernst 2003).

1.1.2.2 Isolating active compounds

After the biological target has been identified, characterised and validated, screening for active compounds is initiated. Historically, the focus was on screening large inventories of natural products from plants, microorganisms and animals (Hunter 1997). Subsequent synthetic modifications to these molecules increased the diversity and number of compounds available for testing. Furthermore, the advent of combinatorial chemistry generated even larger libraries of synthetic chemicals to screen for biological activity (Schwardt, Hartmuth & Ernst 2003).

For a compound to be assessed as 'active' a set of specified criteria including potency, selectivity, mechanism of action, and suitable pharmacokinetic properties are tested using high throughput *in vitro* screening methods. Compounds meeting selected criteria are synthesised and secondary *in vitro* screening is routinely performed to confirm affinity and selectivity (Garrett et al. 2003). The *in vitro* systems employed for these assessments include organ baths, tissue receptor assays, whole cells, homogenised tissue, and expressed or purified recombinant proteins (Boudinot, D'Ambrosio & Jusko 1986; Evans 1992; Bowker et al. 1998; Kim et al. 2002; Wuest et al. 2005; Bowker, Noel & MacGowan 2006).

More recently, *in silico* screening has utilised pattern recognition techniques to define sets of chemical properties of compounds associated with biological activity (van de Waterbeemd 2002; van de Waterbeemd & Gifford 2003). Virtual libraries comprising millions of compounds can be applied for use *in silico*. An essential

component of this type of screening involves extracting practical size subsets for experimentation (Jamois, Lin & Waldman 2003).

1.1.2.3 Absorption, Distribution, Metabolism, Excretion and Toxicology (ADMET) in drug discovery

Previously not studied until the drug development phase, the characterisation of ADMET profiles of compounds is now undertaken early in the drug discovery process to increase the success rate of discovery programmes and to progress better candidates into drug development (Yu & Adedoyin 2003). A wide range of *in vitro* and *in silico* predictive tools are used to determine ADMET profiles (Darvas et al. 2002; Ekins et al. 2002; Clark & Grootenhuis 2003; Miners et al. 2003; Roberts 2003; Williams et al. 2003; Yu & Adedoyin 2003; Nassar & Talaat 2004). Notably, for a drug to reach the market the cost reported by TUFTS Centre for the Study of Drug Development (http://csdd.tufts.edu/) is now in excess of US $1 billion and the discovery/development process extends over 10-15 years. Of the overall cost, up to 80% can be spent on failed drug candidates (Darvas et al. 2002; Workman 2003). A significant cause of attrition is poor pharmacokinetic profiles, which are governed by ADME (Darvas et al. 2002; Roberts 2003). Thus, identifying pharmacokinetic properties of a compound at the earliest point in the drug discovery process is essential (Lin et al. 2003). This thesis addresses an aspect of preclinical pharmacokinetic assessment.

After discovering an active compound that meets initial ADMET criteria, further assessment of affinity, selectivity and ADMET occurs using *in vitro* and *in silico* models until specified lead identification requirements are met (Yu & Adedoyin 2003). Then the process of lead optimization, which utilises animal, *in vitro*, and *in situ* models for the further determination of ADMET and pharmacokinetic

properties, precedes a chosen clinical candidate. Notably, the processes of absorption, distribution, metabolism and excretion are discussed in Section 1.2.1 and Section 1.2.2.

1.1.3 Drug development

Drug development comprises five distinct phases, which together, coordinate the application of pharmacokinetic and pharmacodynamic experimentation for the eventual approval of a new drug (Lesko et al. 2000). The processes involved in drug development are depicted in Figure 1.1.

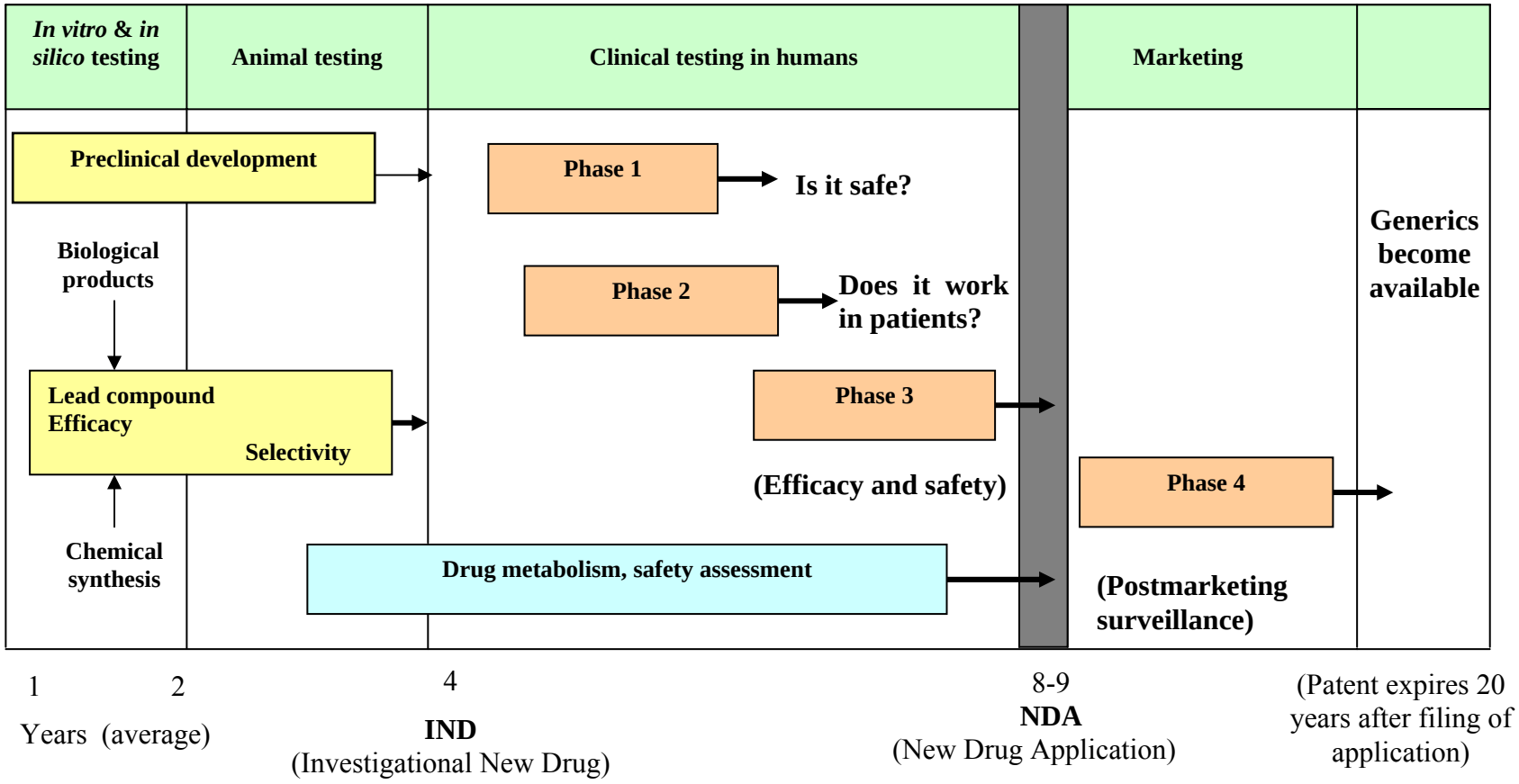


Figure 1.1: The processes of drug development

Adapted from Katzung (1998).

1.1.3.1 Preclinical development

The purpose of the preclinical phase is to assess the pharmacology and toxicity of a new compound and to narrow the candidate selection for testing in humans. Testing of efficacy, selectivity, and mechanism of action occurs in animals (Peck et al. 1992). Toxicity testing also occurs with single dosing in two species (mouse, rat/dog) at three dose levels both orally and intravenously. Subsequently, subchronic dosing (2 weeks, 1 month, 3 months) and chronic dosing (6 and 12 months) are tested in one rodent and one non-rodent species. Carcinogenic, genotoxic, teratogenic and reproductive studies are also applied in rodent species and *in vitro* (Moore 2003). *In silico,* pharmacophore models and Quantitative Structure Activity Relationships (QSAR) aid in the prediction of drug metabolism (Ekins & Obach 2000; Guner 2002; Miners et al. 2003; Smith et al. 2004). The broad goal of the preclinical phase is to integrate knowledge into the planning of the early part of clinical trials (Lesko et al. 2000).

Once the decision is made from preclinical testing that use of the new medical product appears promising and clinical testing should proceed, an IND (Investigational New Drug) must be filed to the appropriate authority (e.g. Food and Drug Administration in the United States of America; Figure 1.1).

1.1.3.2 Phase 1

After IND approval, trials are conducted for the first time in humans with healthy subjects, or in some cases, patients (< 100 subjects). These studies provide information on tolerability and safety, plasma concentrations, maximum safe doses, routes of metabolism and elimination, and initial estimates of the variability

associated with these measurements (Lesko et al. 2000). These data help to select optimal formulation, dose, and route of administration for patients.

1.1.3.3 Phase 2

Two separate components of Phase 2 clinical studies occur in patients (< 300). The primary component is aimed at confirmation and proof of therapeutic concept (efficacy), affirmation of acute tolerability, and maximum safe dose and plasma concentration (Workman 2003). The second component consists mainly of the exploration of dosage regimens and possible drug interactions. Importantly, genetic polymorphisms in the biological target or in drug metabolising enzymes can influence the required dose administered for therapeutic effect. For this reason the fields of genomics and proteomics are of increasing importance in designing and interpreting Phase 2 studies. Genomic studies determine changes in gene expression at the transcriptional level (Guillouzo 2001; Lundstrom 2004; Swanson et al. 2004) whereas proteomics is the study of proteins, their post translational modifications, their interactions, and in particular the changes in expression that are secondary to the effects of specific diseases or to external factors (Aebersold & Cravatt 2002; Jain 2004; Naistat & Leblanc 2004).

1.1.3.4 Phase 3

Studies using a large number of patients (up to 3000) provide complete adverse reaction profiles and estimates of variability in dose-response of different patient groups (i.e. renal or hepatic dysfunction, elderly, slow or extensive metabolisers). This marks the necessary implementation of individualised dosage regimens for these special populations (Lesko et al. 2000).

When sufficient data from preclinical and clinical studies has been collected an NDA (New Drug Application) is filed to the appropriate regulatory authorities (Figure 1.1).

1.1.3.5 Phase 4

With marketing approval obtained, post marketing surveillance for adverse effects and extension of the drug to wider patient groups including children is initiated (Lesko et al. 2000). Furthermore, pharmacoeconomic studies assessing the 'cost to benefit ratio' and 'quality of life' are conducted (Moore 2003).

1.1.4 Summary of drug discovery and development

Throughout the drug discovery and development process a pharmacological audit trail is constructed whereby all the successive stages from drug administration through to biological effects and clinical outcome can be monitored and interpreted (Workman 2003). This trail provides a logical basis for decision making on lead or candidate compounds and is designed to ensure an endpoint whereby the therapeutic agent administered provides the desired biochemical response in the safest possible manner. Testing and integrating results from *in vitro*, *in silico*, animal and *in vivo* studies helps to generate a drug with acceptable *in vivo* disposition. *In silico* methods have by far the highest throughput, followed by *in vitro* and *in vivo* approaches. Conversely, with regard to relevance and reliability of data the ranking is opposite (Darvas et al. 2002). Notably, pharmacokinetic based animal studies are quantitative while animals are also used widely for toxicity and carcinogenicity testing (Williams et al. 2003).

1.2 Pharmacokinetic background

Improvement in the predictivity of *ex vivo* experimental techniques, particularly *in vitro,* is required for minimising expenditure of pharmacokinetic profiling within industry and academia.

Pharmacokinetics is an area of pharmacology that characterises the time course of the concentration of drug within the body. Traditionally, the four physiological processes relevant to pharmacokinetics included absorption, distribution, metabolism and excretion (ADME). However, with coadministration of drugs becoming more common, drug-drug interactions also play an important role in the *in vivo* disposition of drugs. In understanding these processes and the possible inter-patient genetic variability of the biological target and/or drug metabolising enzyme(s), therapeutic doses of new drugs can be administered.

1.2.1 Absorption and distribution

1.2.1.1 Route of absorption

Drugs can be absorbed across the skin, through the nasal cavity, through the rectum, across the lungs, intramuscularly, subcutaneously, and across the conjunctiva of the eye. However, up to 90% of all drugs are administered orally and enter the bloodstream via absorption from the gastrointestinal tract (Pagliara et al. 1999). The proportion of the administered drug that reaches the portal circulation (the primary distribution medium) as intact drug represents the process of absorption (Birkett 1998; Bryant, Knights & Salerno 2003). Following absorption, the drug is carried to the liver for ‘first pass clearance’ whereby a proportion of the dose may be metabolised. The fraction of the dose that is absorbed (escaping first pass clearance)

and reaches the systemic circulation (the secondary distribution medium) is referred to as the bioavailability (Birkett 1998).

The main gastrointestinal permeation barriers to be crossed during the absorptive process are epithelial and endothelial monolayers (Jaffe 1984; Pagliara et al. 1999). Both of these specialised cell types are similar in structure and contain intercellular spaces joined by tight junctions (Jaffe 1984; Artursson 1989; Conradi et al. 1994). For a drug to reach the portal circulation (the primary distribution medium) it must move from the intestinal lumen through an unstirred water layer and a mucous coat adjacent to the epithelial cell surface and through the epithelial cell membranes themselves (Burton et al. 2002). Then the drug encounters a basement membrane, interstitial space, and mesenteric capillary wall lined by endothelia where the distribution of compounds between the blood and the interstitial fluids occurs (Pagliara et al. 1999). Any of these microenvironments can be considered a resistance to drug movement with an associated permeability coefficient (Winiwarter, Lanzner & Muller 1998; Burton et al. 2002). Notably, dietary intake can effect the gastrointestinal environment and the physicochemical state of the administered drug with variable absorption the result (Fleisher et al. 1999; Pelkonen, Boobis & Gundert-Remy 2001). The protective barriers described here and exogenous factors such as intake of food represent the considerations for the efficient delivery of a drug into the portal circulation.

1.2.1.2 Transmembranal transfer mechanisms

In drug permeation terms the process of passive diffusion, either transcellular or paracellular, is considered the most significant transmembrane transfer mechanism for the majority of drugs (Artursson 1989; Camenisch, Folkers & van de Waterbeemd 1996; Christ 2000). However, there are several other drug transfer

mechanisms described (Camenisch, Folkers & van de Waterbeemd 1996; Pagliara et al. 1999). These are shown in Figure 1.2.

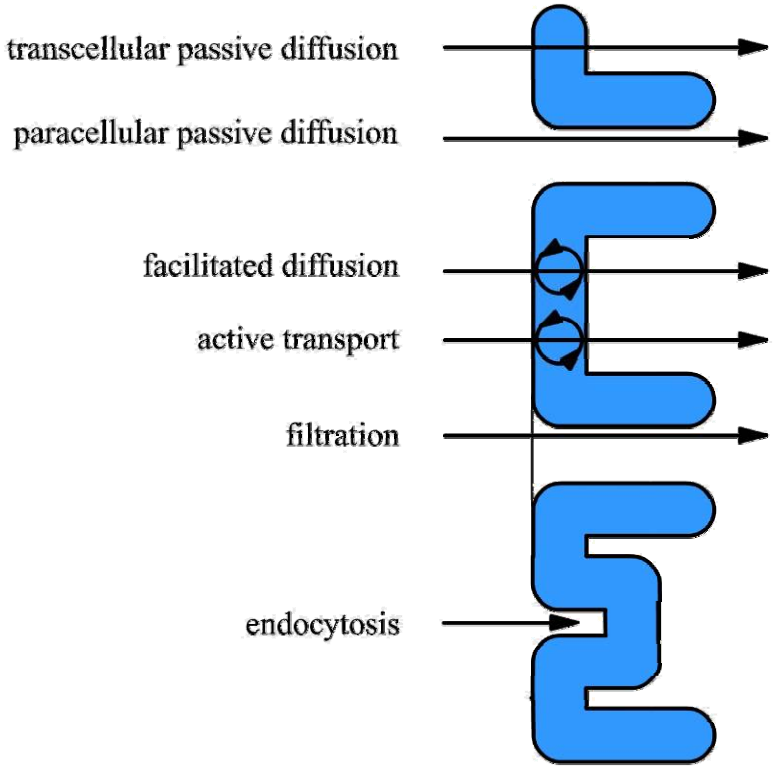


Figure 1.2: A schematic representation of the various drug-membrane transport mechanisms. This diagram was adapted from Camenisch et al. (1996).

The other transmembrane drug transport mechanisms include facilitated diffusion, active transport, filtration and endocytosis (Craig & Stitzel 1990; Camenisch, Folkers & van de Waterbeemd 1996). Facilitated diffusion carrier transport occurs when a transmembrane protein binds one or more drug molecules and releases the molecule on the opposite side of the membrane (Boon & Smith 2002). This occurs in the direction of the concentration gradient and does not require additional energy. Active transport denotes a carrier transport process that requires the consumption of energy as it often takes place against a concentration gradient (Camenisch, Folkers & van de Waterbeemd 1996). Filtration of drug molecules is dependent on the existence of a pressure gradient and on the size of the usually hydrophilic compound

relative to the size of the pore through which it is to be filtered (Schanker 1962; Hilgers, Conradi & Burton 1990). Particles are transported by endocytosis involving invagination of the membrane and subsequent vesicularisation and devesicularisation (Artursson 1989 Figure 1.2).

1.2.1.2.1 Membrane structure

Singer and Nicolson (1972) were the first to propose the now widely accepted fluid mosaic bilayer model for the gross orientation of biological membranes. Further support for their model was documented more recently (Nagle & Tristram-Nagle 2000). Other membrane related studies have demonstrated evidence for lipid domains which are suggested to be involved in the invagination process (Welti & Glaser 1994). Also, the process of 'flip flop' or translocation of certain lipids was shown (Boon & Smith 2002), while membrane rafts were identified and associated with specific signalling domains (Grassme et al. 2001).

Basically, all biological membranes are protective barriers comprised largely of phospholipids. The relative proportions of each structurally divergent phospholipid (Section 3.4.2.1; Figure 3.7, shows several phospholipid structures) varies slightly between cell types and organelles determining the fluidity of the membrane. The phospholipid bilayer has the hydrophobic portions of the lipid and cholesterol orientated toward the centre and the hydrophilic tails facing the aqueous environment with proteins and carbohydrate forming latticeworks on the surface (Figure 1.3). Biological membranes are typically 5-8 nm thick and contain an external net negative charge (Mouritsen & Jorgensen 1998).

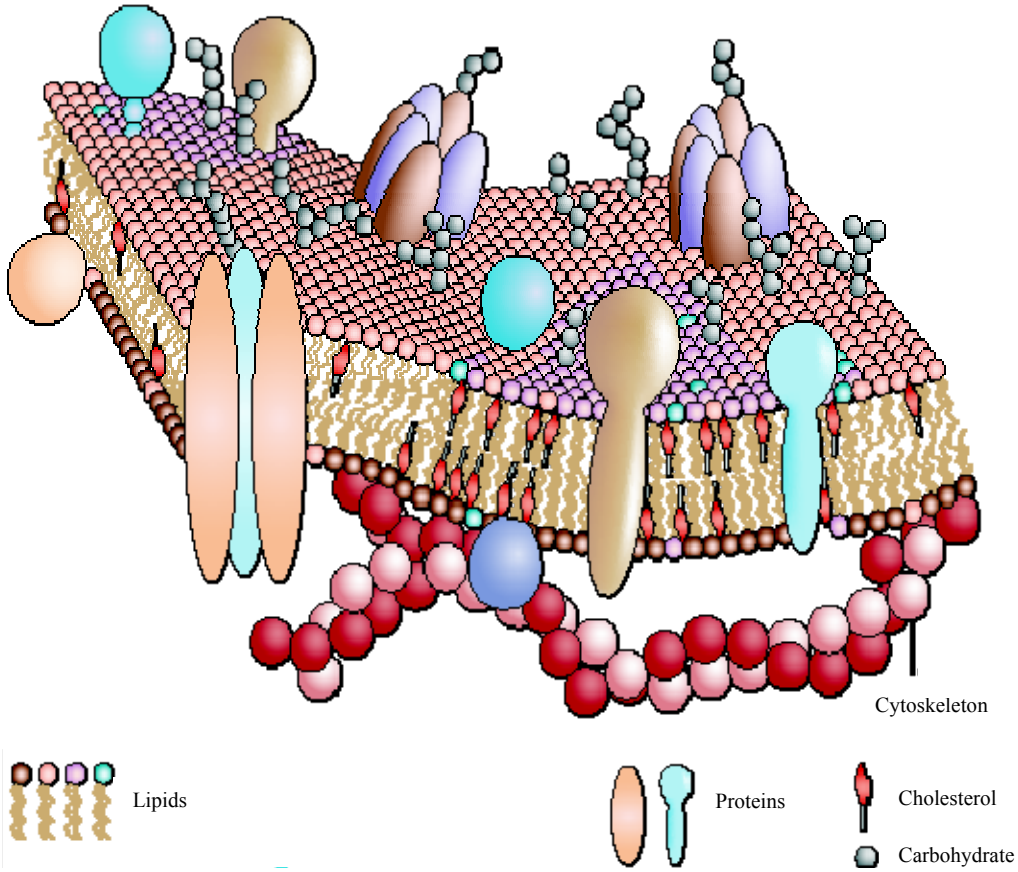


Figure 1.3: Schematic representation of the structure of a biological membrane.

This diagram was adapted from Pietzsch at
www.nature.com/horizon/livingfactor/background/pdf/membrane.pdf

1.2.1.2.2 Physicochemical determinants of drug absorption

As passive diffusion represents the most significant transcellular mechanism for drug absorption the majority of research has focussed on this aspect (Lee 2000).

In situ, in silico and in vitro techniques are utilised to aid in the prediction of drug absorption. This includes the use of isolated jejunum and colon from the rat and computational building of model monolayers. However, the most widely applied technique of the last fifteen years has been the use of the well differentiated intestinal cell line derived from human colorectal cancer, Caco-2 (Artursson 1991; Yamashita et al. 1997; Tavelin et al. 1999; Kulkarni, Han & Hopfinger 2002). This cell line

displays many of the morphological and functional properties of the intestinal epithelial cell barrier (Kulkarni, Han & Hopfinger 2002).

Traditionally, biological membranes were viewed as being freely permeable to lipophilic drugs whereas hydrophilic and particularly ionised compounds were unable to permeate (Artursson 1991; Christ 2000; Taillardat-Bertschinger et al. 2003). However, a review evaluating the prediction of drug permeation cites an increasing body of experimental evidence supporting the passive transport of ionic species (Pagliara et al. 1999).

Lipophilicity has long been linked to drug absorption because of the hydrophobic content of a biological membrane. Using Caco-2 monolayers and six drugs known to undergo passive gastrointestinal diffusion Yamashita et al. (1997) described a linear relationship (R = 0.99) between drug absorption and lipophilicity. In another study, Caco-2 cells were used to demonstrate the passive diffusion of six β-adrenoceptor antagonists with a 2000 fold range in lipophilicity (Artursson 1989). The β-blockers showed apparent permeability coefficients ranging from $41.91 \pm 4.31 \times 10^{-6}$ cm/s for the most lipophilic drug, propranolol, to $0.203 \pm 0.004 \times 10^{-6}$ cm/s for the most hydrophilic drug, atenolol. Consistent with these results was an *in situ* study in the small intestine of the rat that described the absorption of eleven drugs. The four most hydrophilic molecules showed slow absorption rates whereas the more lipophilic molecules of the series permeated far more rapidly. However, acebutolol showed anomalously slow absorption for its log P (measured as log of the octanol/water partition coefficient) value thereby implicating physicochemical characteristic(s) other than lipophilicity in the permeation of this molecule (Taylor, Pownall & Burke

1985). Taken together, these observations suggest that the rate of passive diffusion is generally faster for lipophilic drugs.

A Quantitative Structure-Permeability Relationship (QSPR) demonstrated that lipophilicity is a key, but not the sole, parameter in predicting permeability (Taillardat-Bertschinger et al. 2003). An *in vivo* study used a regional perfusion system in the proximal jejunum and twenty-two structurally diverse compounds to describe a predictive model that included the physicochemical characteristics; number of H bond donors (HBD), polar surface area (PSA), and either log $D_{5.5}$ or log $D_{6.5}$ (Winiwarter, Lanzner & Muller 1998). Another study used a combination of experimental and computational approaches to describe a set of 'rules' for permeability/absorption prediction (Lipinski et al. 1997). A database of more than two thousand compounds was used for the latter study that concluded a drug containing two or more of the following physicochemical characteristics was more likely to show poor permeation/absorption; more than 5 hydrogen bond donors (HBD), more than 10 hydrogen bond acceptors (HBA), a MW > 500 or a log P > 5. Additionally, a review of passive drug absorption models suggested that physicochemical properties of both the drug and the membrane govern transmembrane transport mechanisms (Camenisch, Folkers & van de Waterbeemd 1996). This is consistent with the reports of Burton et al. (2002) and Pelkonen et al. (2001) whereby a number of determinants of permeability were identified including various structural characteristics such as size, shape and charge, while the physiological variables, regional permeability differences, pH, luminal and mucosal enzymology, intestinal motility and the volume of intestinal fluid available were also deemed important for the permeability and/or absorption process.

1.2.1.3 Distribution

The volume of distribution is one of two primary pharmacokinetic parameters, the other being clearance. Both of these parameters can be described in terms of fundamental physiological processes. Once drug reaches the systemic circulation it can be distributed to various sites within the body (Reed 1996; Pelkonen & Turpeinen 2007). Initially drug is transported to organs which are highly perfused (e.g. brain, liver) and local concentrations may be high with redistribution to less highly perfused tissue such as muscle, fat and bone (Reed 1996; Ekins et al. 2002). The final distribution volume of the drug, also called the apparent volume of distribution (V_d), is defined as the total amount of drug in the body divided by the drug concentration in blood at any given time (Benet & Galeazzi 1979). V_d is affected by plasma and tissue volume and also the fraction of drug bound to protein and tissue (Equation 1.0).

Equation 1.0:

$$V_d = \text{plasma volume}\,(V_P) + \{\frac{\text{fraction of drug unbound in plasma}\,(f_u)}{\text{fraction of drug unbound in tissue}\,(f_{u(T)})} \times \text{tissue volume}\,(V_T)\}$$

Enhanced plasma protein binding reduces the V_d, whereas tissue binding increases the V_d (Birkett 1998). Both tissue and protein binding act as reservoirs for the drug, but only unbound drug can activate pharmacological receptors (Christ 2000).

The volume of distribution reflects the relative avidity of drug for tissue compartments compared with blood. For example warfarin has a V_d = 8L reflecting a high degree of plasma protein binding; theophylline has a V_d = 30L reflecting distribution into total body water; and chloroquine has a $V_d \approx$ 15,000L. Being very lipophilic this compound sequesters into adipose tissue. Basically, the higher the volume of distribution, the less of the drug present in the body is in blood.

1.2.2 Metabolism and excretion

Drug metabolism is important in three ways:

1. It is a major mechanism responsible for the clearance of the parent drug and therefore the dose rate at steady-state. This is subject to inter-individual variability in drug metabolising enzymes (Birkett et al. 1993). Populations are known to exhibit polymorphism in the genes encoding drug metabolising enzymes, resulting in some populations being either ultrarapid, extensive, intermediate, or poor (slow) metabolisers of certain drugs (Evans & Relling 1999; Weinshilboum 2004; Gardiner & Begg 2006).
2. Metabolism generally converts the drug into a more polar species that is less readily reabsorbed in the renal tubules of the kidney and is excreted from the body (Katzung 1998; Bryant, Knights & Salerno 2003).
3. Metabolism is generally considered a detoxification mechanism (Guengerich 1993). However, some metabolites have been shown to be pharmacologically active, in certain instances as active as the parent drug or even more so. For example, a glucuronide of morphine, morphine 6-glucuronide (M6G), has high affinity for the opioid μ-receptor (Abbott & Franklin 1991). Toxic reactive intermediates may also form, as is the case with the social drug of abuse ecstasy (Segura et al. 2001).

1.2.2.1 Clearance

Clearance is a critical parameter since, for any desired response (the target concentration in blood), clearance determines the maintenance dose rate of a drug at steady-state. Clearance describes the efficiency of irreversible elimination of a drug from the body (Birkett 1998; Bryant, Knights & Salerno 2003). Elimination in this context refers either to the excretion of the unchanged drug into urine, gut contents,

expired air, or sweat, or to the metabolic conversion of the drug into a different compound(s) (i.e. biotransformation; Birkett 1998). Most therapeutic small molecules are reasonably lipophilic and require metabolic conversion to be eliminated from the body. After metabolism, the parent drug is 'cleared' from the systemic circulation, even though the metabolite is still in the body. The metabolite is usually a more polar species that is excreted from the body, not reabsorbed in the renal tubules, as is the case with highly lipophilic parent compounds (Birkett 1998). Uptake of the parent drug into tissues is not regarded as clearance if the unchanged drug eventually re-distributes out of the tissue, however slowly this process occurs (Birkett 1998).

Clearance is defined as 'the volume of blood cleared of drug per unit time' and the units are thus volume per time, usually litres per hour or mL per minute. Clearance can be by a particular metabolic pathway (intrinsic clearance of an enzyme = CL_{int}), a particular organ (i.e. hepatic clearance = CL_H), or by the whole body (systemic clearance = CL_S). Total body clearance is the sum of all the different clearance processes occurring for a given drug (Birkett 1998).

Hepatic metabolism is the major clearance mechanism for most prescribed drugs in humans, although metabolic conversion can occur in other organs, such as the gut, kidney and lung (Birkett 1998; Miners et al. 2003). The drug-metabolising enzymes responsible for the majority of metabolic clearance are the cytochromes P450 (CYP) and the UDP-glucuronosyltransferases (UGT). These exist as gene superfamilies (Nebert et al. 1991; Miners et al. 2004) with approximately 60% of all marketed drugs primarily being cleared by cytochrome P450 (CYP) catalysed metabolism (McGinnity & Riley 2001). Within the liver, CYP and UGT isoforms localise on the

smooth endoplasmic reticulum in hepatocytes (Brown & Black 1989; Edwards et al. 1991; Bossuyt & Blanckaert 1997).

1.2.2.1.1 Classification of drug metabolism

Drug metabolism reactions are generally classified as one of two types. These are functionalisation (Phase 1) reactions that involve the introduction or 'unmasking' of a polar functional group into the molecule (performed by CYP and some other enzymes; Guengerich 1993) and conjugation (Phase 2) reactions which involve the covalent linking of a suitable functional group present in the molecule with a polar endogenous compound (performed for example by UGT; King et al. 2000). With each interaction the xenobiotic is generally converted into a more polar species aiding in the elimination of the compound from the body (Bryant, Knights & Salerno 2003). It should be noted, however, that the functionalization/phase 1 and conjugation/phase 2 classification has limitations, and a nomenclature system based on mechanistic considerations has been proposed (Josephy, Guengerich & Miners 2005).

1.2.2.1.2 Extraction ratio

Once the drug is absorbed into the bloodstream from the gut lumen (Section 1.2.1), it is taken via the portal vein to the liver where initial elimination of drug can occur. This process is known as the first past extraction and is described by the expression:

Equation 1.1

First pass extraction = $1 - E_H$

The hepatic extraction ratio (E_H) is a function of hepatic blood flow (Q_H), the fraction of drug unbound in blood (f_u), as only unbound drug is able to be metabolised, and the intrinsic clearance of the drug metabolising enzymes (CL_{int}).

This can be expressed in terms of the 'well stirred' model of hepatic clearance (see Equation 1.10) as:

Equation 1.2

$$E_H = \frac{f_u \times CL_{int}}{Q_H + f_u \times CL_{int}}$$

The value of liver blood flow (Q_H) used is generally 90 litres per hour (Obach 1997; Ito et al. 1998a; Obach 1999), while the fraction of drug unbound in blood (f_u) ranges from 0 to 1. Drug is bound to proteins, and other constituents, in blood to a variable extent. In general, acidic drugs bind to albumin and basic drugs to α1-acid glycoprotein, with the extent of binding depending on the affinity of the drug for the protein and the protein concentration. Some drugs are very highly bound (e.g. the weak acid warfarin is 99.9% bound to albumin in blood and thus has an $f_u = 0.001$). This is a reversible process, with bound and unbound drug in equilibrium (Protein + Drug ⇔ Drug-Protein). For drugs that exhibit Michaelis-Menten or substrate inhibition kinetics (see Section 1.2.2.2) the intrinsic clearance is described by the expression:

Equation 1.3

$$CL_{int} = \frac{V_{max}}{K_m}$$

The parameter V_{max} is the maximal velocity of the reaction at a saturating substrate concentration, whilst the K_m is the Michaelis constant which is determined as the substrate concentration at half-maximal velocity.

1.2.2.1.2.1 Bioavailability

The fraction of the dose escaping first pass extraction by the liver is $1-E_H$ (Equation 1.1). The extent of absorption from the gut lumen and the first pass hepatic extraction determine the fraction of the dose that reaches the systemic circulation as intact drug (the bioavailability; Birkett 1998). Once in the systemic circulation the drug is distributed throughout the body and is able to access the designated therapeutic target.

1.2.2.1.3 Steady state concentration and half-life

During chronic dosing, the drug accumulates in the body until a steady state is reached (after 3-5 half-lives) at which point the rate of administration equals the rate of elimination. At this point the concentration of drug in plasma at steady state (C_{SS}) is determined only by the administration rate and the clearance (Equation 1.4).

Equation 1.4

Rate of administration = CL × Css.

Clearance is also a determinant of the half-life ($t_{1/2}$) of a drug, which is the time taken for the plasma concentration of drug to fall by one half. The derivation of the half-life follows from Equation 1.5 and Equation 1.6. The fall in concentration in plasma after a single dose is an exponential (logarithmic) function of the time after the dose for a drug exhibiting first order kinetics:

Equation 1.5

$$C_t = C_0 \times e^{-kt}$$

where C_t is the drug concentration at time t after the dose, C_0 is the initial concentration at time zero and k is the elimination rate constant. Solving Equation

1.4 when $C_t = C_0 \times 0.5$, when the drug concentration in plasma has fallen by half, results in:

Equation 1.6

$$k = \frac{0.69}{t_{1/2}}$$

The elimination rate constant k expresses the proportion of drug eliminated per unit time. Half-life is a reciprocal function of the elimination rate constant (Birkett 1998). The numerator in Equation 1.6 is the natural logarithm of 2.0. It can also be shown that $k = V_D / CL_s$. Thus:

Equation 1.7

$$t_{1/2} = \frac{0.69 V_d}{CL_S}$$

The half-life of a drug dose determines the duration of action, the time taken to reach steady state (3-5 half-lives), and the frequency of dosing.

1.2.2.1.4 Total body clearance

The total clearance from the body is made up of individual clearances as shown by Equation 1.8.

Equation 1.8

$$CL_S = CL_{Hepatic} + CL_{Gut} + CL_{Renal} + CL_{Other}$$

While hepatic clearance is the principal elimination mechanism for the majority of drugs, the gut, kidney, lung, adrenals and skin can also be sites of metabolism. However, rarely are these sites considered quantitatively important. As such, the remainder of this thesis focuses solely on hepatic drug clearance.

1.2.2.1.4.1 Hepatic clearance

The hepatic clearance (CL_H) is a function of the extraction ratio (E_H) and liver blood flow (Q_H) and is expressed by:

Equation 1.9

$$CL_H = Q_H \times E_H$$

Thus, the expression for hepatic clearance of an administered therapeutic may be expressed in terms of the well-stirred model of hepatic clearance (see Equation 1.2):

Equation 1.10

$$CL_H = Q_H \times \frac{f_u \times CL_{int}}{Q_H + f_u \times CL_{int}}$$

Importantly, the intrinsic clearance denoted in Equation 1.10 relates to the total intrinsic clearances of the drug metabolising enzymes in a whole liver (Section 1.3.1).

Hepatic clearance is further simplified to two limiting situations where the metabolising enzyme either has low or high activity toward the drug.

1.2.2.1.4.1.1 Low hepatic clearance

Instances occur where the intrinsic clearance of the substrate is much lower than liver blood flow and the capacity of the liver to metabolise the drug is 'limited'. When $CL_{int} << Q_H$, equation 1.10 simplifies to: $CL_H \approx f_u \times CL_{int}$. Thus, the determinants of CL_H for a low hepatic clearance drug are f_u and CL_{int}.

1.2.2.1.4.1.2 High hepatic clearance

Conversely, when CL_{int} is high in relation to Q_H (i.e. $CL_{int} >> Q_H$) equation 1.10 simplifies to $CL_H \approx Q_H$. Thus, the principal determinant of drug hepatic clearance becomes liver blood flow.

1.2.2.2 Michaelis-Menten Enzyme Kinetics

Most, but not all, reactions catalysed by cytochrome P450 and UDP-glucuronosyltransferase follow Michaelis-Menten kinetics (Equation 1.11). Thus, when $K_m >> [S]$, the rate of elimination increases in proportion with plasma concentration (or dose) and clearance (v/S) is a constant (Figure 1.4).

Equation 1.11

$$v = \frac{V_{max} \times S}{K_m + S}$$

where:

S = substrate concentration

v = velocity or rate of metabolite formation

V_{max} = maximal rate of metabolite(s) formation

K_m = the Michaelis constant (substrate concentration at 50% of V_{max})

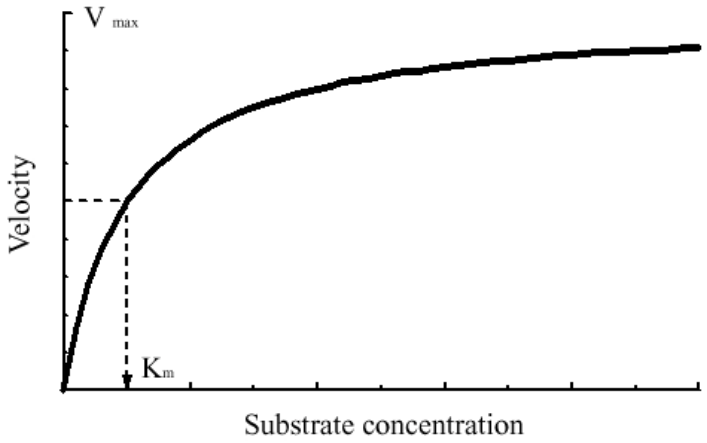


Figure 1.4: Plot of velocity versus substrate concentration for a metabolic reaction exhibiting hyperbolic or Michaelis-Menten kinetics

As was noted in Equation 1.3, the ratio of V_{max} to K_m (Figure 1.4) represents the intrinsic clearance (CL_{int}). Intrinsic clearance is the cornerstone of the *in vitro - in vivo* extrapolation (Section 1.3.1) of hepatic drug clearance (Houston 1994).

1.2.3 Drug-drug interactions

Drugs are often co-administered to enhance therapeutic effect or to treat more than one ailment. Consequently, drug-drug interactions can occur at a number of different sites that may alter the pharmacokinetic profiles of one or both drugs. The possible sites of drug-drug interactions include: 1) gastrointestinal absorption, 2) plasma and/or tissue protein binding, 3) carrier-mediated transport across biological membranes (e.g. renal and biliary excretion), and 4) drug metabolising enzymes. Pharmacodynamic interactions such as antagonism or agonism at the receptor may also increase or decrease the effects of a drug (Ito et al. 1998b).

Particular attention has been paid to drug-drug interactions involving altered metabolic clearance over the last fifteen years since these may result in the loss of efficacy or toxicity. For example, co-administration of ketoconazole (an antifungal) and terfenadine (an antihistamine) was reported to cause potentially life threatening

ventricular arrhythmias (Monahan et al. 1990). Inhibition of CYP-mediated drug metabolism by a concomitantly administered drug is one of the major causes of drug-drug interactions in humans that at worst can result in death (Ito, Brown & Houston 2004). As with the *in vitro-in vivo* prediction of clearance, *in vitro-in vivo* prediction of drug inhibition is an important consideration in the development of safe therapeutic agents.

1.3 *In vitro* systems

Understanding and defining the interactions occurring *in vitro* is essential for the interpretation and generation of meaningful pharmacokinetic data. This thesis aims to improve the understanding of interactions between a drug and the *in vitro* matrix in order to improve the accuracy of prediction of *in vitro* intrinsic clearance and inhibition, and consequently the extrapolated *in vivo* clearance and changes in this parameter.

In vitro systems are used widely to predict drug disposition in humans throughout the drug discovery and development processes (Birkett et al. 1993; Iwatsubo et al. 1997a; Iwatsubo et al. 1997b; Obach et al. 1997; Drews 2000; Ekins et al. 2002; Soars, Burchell & Riley 2002; Bleicher et al. 2003; Lomardino & Lowe 2004). Prediction of the pharmacokinetics and possible drug interactions from *in vitro* data has become something of a 'holy grail' in drug development, since accurate prediction minimises the potential for unfavourable ADME characteristics or a life threatening drug interaction after human exposure (Tracy 2003).

Studies on absorption aside, the major use of *in vitro* procedures in the drug discovery process involves the successful prediction of clearance and hepatic extraction (Williams et al. 2003). Most *in vitro* drug metabolism studies use liver to

provide the enzyme source including hepatic microsomes, liver slices, and hepatocytes. Recombinant enzymes expressed in cell culture (e.g. *E. coli* or baculoviral directed expression in insect cells) have also been utilised.

Sources of liver include humans, rats, rabbits, dogs, mice, guinea pigs and monkeys (Schwab, Raucy & Johnson 1988; Gemzik, Halvorson & Parkinson 1990; Dahl, Nordin & Bertilsson 1991; Fabre et al. 1993; Ashforth et al. 1995; St-Pierre & Pang 1995; Zomorodi & Houston 1996; Ball et al. 1997; Ghahramani et al. 1997; Iwatsubo et al. 1997a; Ludden et al. 1997; Tan et al. 1997; Yamazaki et al. 1997). Importantly, the main drug metabolising enzymes (cytochrome P450 and UDP-glucuronosyltransferase) are anchored to some form of biological membrane in the various *in vitro* systems. *In vitro* drug metabolism experiments, like the inhibition studies described in Section 1.3.2, consist of a known quantity of enzyme and substrate in a physiologically based medium containing all the cofactors required for metabolism. Metabolite formation over a designated period of time is quantified using chromatographic or other procedures, with rate of metabolite formation plotted against substrate concentration (Figure 1.4). These data are used to determine K_m and V_{max} (by model fitting) for the reaction and in turn can be used to calculate an *in vitro* intrinsic clearance which can be scaled up to an *in vivo* clearance (Section 1.3.1).

1.3.1 *In vitro-in vivo* extrapolation of hepatic clearance

1.3.1.1 Scaling of the *in vitro* intrinsic clearance to predict *in vivo* intrinsic clearance

1) Calculate the *in vitro* intrinsic clearance

$$CL_{int} = \frac{V_{max}}{K_m}$$

pmol/min/mg × L/μmol

$\Rightarrow$ μL/min/mg microsomal protein

2) Extrapolate to 1 gm of liver (i.e. 32 mg of microsomal protein per 1 gm liver; Barter et al. 2007)

Multiply by 32

$\Rightarrow$ μL/min/g of liver

3) Extrapolate to whole liver

Multiply by 1500g and by 10^{-6}

$\Rightarrow$ L/min/liver (i.e. per 70 kg person)

This intrinsic clearance value per liver can then be inserted into one of four equations that describe physiological models of hepatic clearance to complete the calculation of *in vivo* clearance. These expressions incorporate drug-protein binding in the blood, liver blood flow, the intrinsic enzyme activity (CL_{int}), and the anatomical arrangement of hepatic circulation (Wilkinson & Shand 1975). The four physiological models of hepatic clearance differ in the nature of the hepatic blood circulation. The simplest and most frequently used model is identical to that described by fundamental physiological processes (Equation 1.10) and is known as the well-stirred model of hepatic clearance.

The other models of hepatic clearance include the parallel tube model, the distributed sinusoidal perfusion model, and the dispersion model. All models are described in Section 1.3.1.2.

1.3.1.2 Models for hepatic clearance

1.3.1.2.1 The well stirred model of hepatic clearance

The well stirred model of hepatic clearance assumes that all drug which passes through the portal vein or hepatic artery is rapidly stirred in the sinusoid (periportal → centrilobular) and that there is no concentration gradient of drug in that direction. The unbound drug concentration in the hepatic vein is therefore assumed to be the same as the unbound concentration in the sinusoids (Pang & Rowland 1977; Iwatsubo et al. 1996).

1.3.1.2.2 The parallel tube model

The parallel tube model has each sinusoid as a cylinder and it is assumed that cylinders are of the same size and have the same intrinsic clearance and the same blood flow. There is always a concentration gradient from the periportal side to the centrilobular side assuming no diffusion or dispersion occurs along the blood flow path in the sinusoid (Pang & Rowland 1977). The equation for the parallel tube model is:

Equation 1.12

$$CL_H = Q_H(1 - e^{\frac{-CL_{int} \times f_u}{Q_H}})$$

where terms have been described previously.

1.3.1.2.3 The distributed sinusoidal perfusion model

The distributed sinusoidal perfusion model extends the parallel tube model by assuming that the blood flow rate and intrinsic clearance of each sinusoid exhibit some variation, more closely resembling the physiological situation. The sinusoids in intact whole liver are divided into n groups and each of these consists of sinusoids

that have the same intrinsic clearance and blood flow (Bass, Roberts & Robinson 1987).

1.3.1.2.4 The dispersion model

The dispersion model is regarded as a cylinder in which dispersion along the flow path occurs. The term "dispersion number" (D_N) was introduced to reflect the extent of this dispersion (Roberts & Rowland 1986; Houston 1994).

1.3.2 Predicting *in vivo* inhibition of metabolism from *in vitro* systems

The *in vitro* techniques used to predict *in vivo* drug inhibition employ the enzyme sources described previously for clearance extrapolation (e.g. hepatic microsomes, hepatocytes, and recombinant enzymes). The magnitude of an *in vivo* drug-drug interaction arising from the inhibition of metabolic clearance for a high or low hepatic drug given orally can be predicted using the ratio of inhibitor concentration [I] *in vivo* to the inhibition constant (K_i) determined *in vitro* according to Equation 1.13 (Ito et al. 1998b; Yao & Levy 2002; Ito, Brown & Houston 2004).

Equation 1.13

Ratio of the areas under the plasma drug concentration time curves in the presence and absence of inhibitor: $(R_c) = \dfrac{1}{f_m \times \{1/(1 + I_u / K_i)\} + 1 - f_m}$

Where f_m is the fraction of the dose metabolized along the pathway of interest

When $f_m = 1$, $R_c = 1 + \dfrac{[I]}{K_i}$, (Ito et al. 1998b)

Inhibition data may be represented graphically. For example the reciprocal of the rate of metabolite formation as 1/V (y-axis) is plotted against the added inhibitor

concentration [I] (x-axis), which is known as a Dixon plot. An example of a Dixon plot is shown in Figure 1.5.

K_i values can be determined from the Dixon plot, or more correctly, by computationally fitting experimental data to the expressions for competitive, non-competitive, and mixed inhibition. Goodness of fit to kinetic and inhibition equations can be assessed statistically.

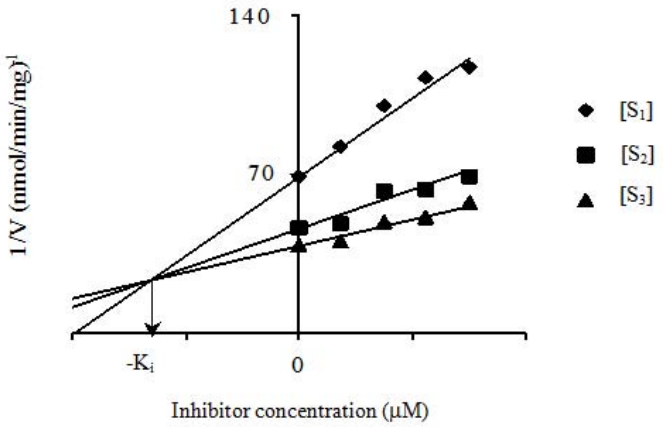


Figure 1.5: A diagram of a Dixon plot showing competitive or mixed drug-drug inhibition

1.3.3 Assessing the reliability of *in vitro* based predictions of *in vivo* pharmacokinetics

Since their inception, *in vitro* systems have proved to be useful for identifying metabolic pathways and the enzymes involved in these reactions (Rane, Wilkinson & Shand 1977; Schwab, Raucy & Johnson 1988; Dahl, Nordin & Bertilsson 1991; Fabre et al. 1993; Ashforth et al. 1995; Zomorodi & Houston 1996; Ball et al. 1997; Breyer-Pfaff, Fischer & Winne 1997; Ghahramani et al. 1997). Moreover, there is a high attrition rate of candidate compounds in drug discovery and development, with a major reason being unacceptable pharmacokinetic properties (Section 1.1.2.3). Thus, a major aim of *in vitro-in vivo* extrapolation is to accurately predict (i.e. within

10 - 20% of actual values) human pharmacokinetic parameters, especially CL_{int}, CL_H, and E_H (Obach et al. 1997; McGinnity & Riley 2001).

Discussion of the theoretical aspects important for extrapolating *in vitro* data commenced in 1975 (Wilkinson & Shand 1975). Subsequently, a good correlation reported between predicted and observed hepatic extraction ratios of seven drugs by isolated livers provided proof of the potential of the *in vitro - in vivo* extrapolation (Rane, Wilkinson & Shand 1977). However, more recent studies have shown that human hepatic clearance is generally only predicted within a 2-10 fold error (Iwatsubo et al. 1997a; Lave et al. 1997; Ludden et al. 1997; Lave, Coassolo & Reigner 1999; Shibata, Takahashi & Ishii 2000; Kalvass et al. 2001; Sjoberg et al. 2001; Boase & Miners 2002). *In vitro* estimations of *in vivo* inhibition, although widely used, have similarly not yielded accurate predictions (Yao & Levy 2002; Hutzler, Messing & Wienkers 2005).

In regards to clearance prediction the actual clearance is almost invariably higher than the predicted clearance. Possible factors contributing to these disparate values include metabolism by extrahepatic tissues, incorrect assumptions about the equilibrium of drug between blood and the hepatocyte (Figure 1.6), incorrect fraction of drug unbound used in the *in vivo* clearance model, a role for uptake and efflux transporters, or incorrect determination of V_{max} and/or K_m due to an invalid assumption that all of the substrate molecules added to an incubation are available to bind the catalytic enzyme (Obach 1996; McLure, Miners & Birkett 2000; Yao & Levy 2002). Similarly, underestimates of inhibition have been reported with the factors preventing consistent quantitative prediction of inhibition of drug metabolism including concurrent induction of CYP, interactions involving transporters, and

unknown inhibitor concentration available to the enzyme site *in vitro* and *in vivo* (Yao & Levy 2002).

1.4 Towards reliable quantitative prediction of *in vivo* pharmacokinetic parameters from *in vitro* data

Accurate determination of K_m and K_i is critical for the quantitative extrapolation of *in vivo* clearance and inhibitory drug interactions, respectively (Houston 1994; Iwatsubo et al. 1997a; Ito et al. 1998b; Lave, Coassolo & Reigner 1999; McLure, Miners & Birkett 2000; Yao & Levy 2002). A common suggested cause for inaccurate predictions of both *in vivo* clearance and inhibition is that not all of the drug added to *in vitro* incubations is available to the enzyme for either catalysis or inhibition. This is the specific area of research that this thesis addresses.

1.4.1 Non-specific binding of drugs to *in vitro* matrices

Despite numerous non-specific binding studies throughout the 1960's and 1970's (Balzer et al. 1968; Bickel & Steele 1974; Schafer 1974; Bickel et al. 1975; Bickel & Weder 1976; Di Francesco & Bickel 1977), the earliest of which demonstrated the binding of drugs to subcellular fractions *in vitro* and inferred its impact on the prediction of *in vivo* kinetics (Gillette 1963), most publications based on *in vitro* metabolism have assumed that all added drug molecules in an incubation are available to the enzyme. This is not necessarily the case as many drugs have been shown to bind non-specifically to the lipid-protein milieu of *in vitro* matrices after addition to the incubation medium. For instance, reserpine, chlorpromazine, prenylamine and imipramine were shown to bind to vesicles isolated from the sarcoplasmic reticulum of rabbit striated muscle cells as well as to the lipids extracted from vesicular preparations (Balzer et al. 1968). Furthermore the binding

of 14-deoxy-14-[(2'-diethylamino-ethyl)-mercaptoacetoxy]-dihydromutilin, a diterpenoid drug, to rat liver microsomes was also noted (Schuster, Fleschurz & Helm 1975).

More recently, numerous reports have demonstrated that many drugs, especially lipophilic amines, bind extensively to subcellular fractions including human and rat liver microsomes (Baarnhielm, Dahlback & Skanberg 1986; Obach 1996, 1997; McLure, Miners & Birkett 2000; Austin et al. 2002). In fact, most drugs are lipid soluble organic compounds that are predicted to bind any biological membrane to some extent (McLure, Miners & Birkett 2000).

In vivo, drug bound to protein in blood is in equilibrium with free drug and protein; the free drug in blood (D_F) is in equilibrium across the plasma-cell interface and may possibly bind the cell membrane and slowly enter into the cell; the free drug in the hepatocyte is in equilibrium with drug bound to tissue and is free to be metabolised (M) or to inhibit a cytochrome P450 (CYP) or UDP-glucuronosyltransferase (UGT) enzymes in the endoplasmic reticulum (Figure 1.6).

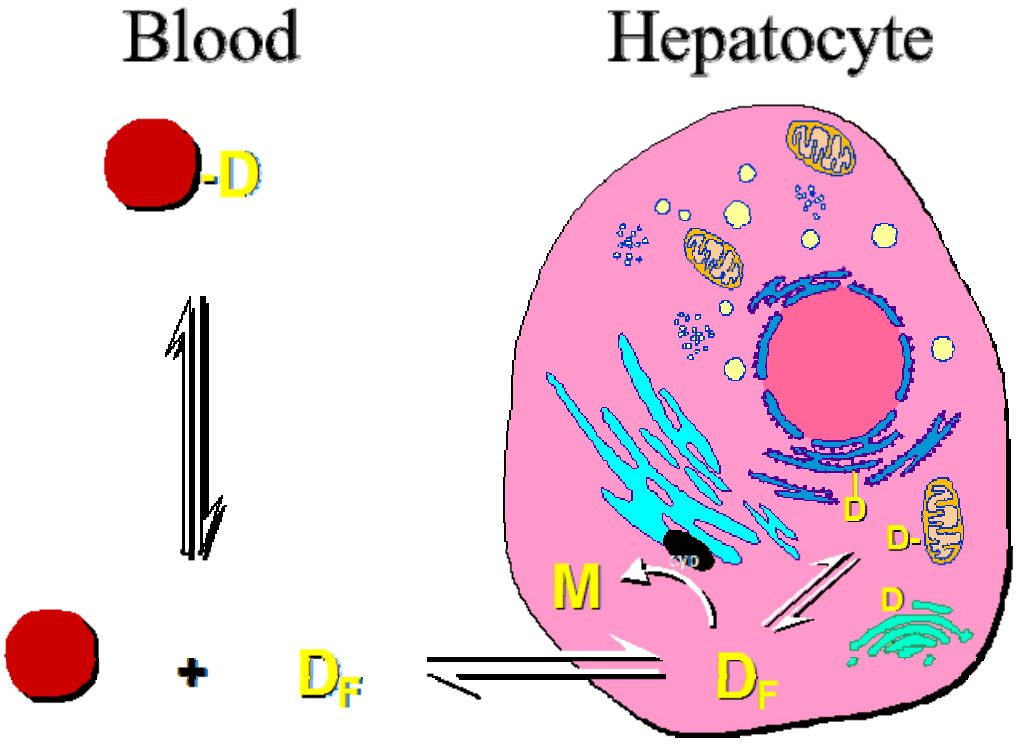


Figure 1.6: Equilibrium relationship *in vivo* between drug in blood plasma and that in the hepatocyte.

Extrapolating this relationship to that which occurs in *in vitro* incubations is not necessarily valid. Drug that is unavailable for metabolism or inhibition as a result of being bound to the *in vit*ro matrix (Figure 1.7) must be accounted for in the calculation of K_m (Figure 1.8) or K_i.

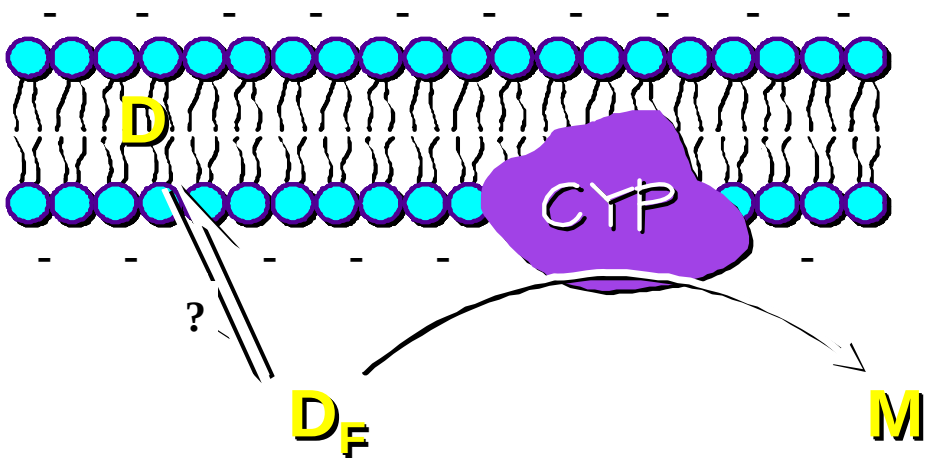


Figure 1.7: Relationship between free drug in an incubation, *in vitro* membrane binding, and cytochrome P450 metabolism.

The relationship between drug bound to the membrane of an *in vitro* matrix and free drug in an incubation is not yet fully understood (Figure 1.7), but drug bound non-specifically to a biological membrane is categorised as being unable to access the drug metabolising enzyme for either metabolism or inhibition. Accounting for the free and not total added drug concentration has resulted in significant improvement to the prediction of clearance and inhibition (Section 1.4.1.1; Obach 2000; Margolis & Obach 2003; Riley, McGinnity & Austin 2005; Grime & Riley 2006).

1.4.1.1 Accounting for non-specific binding of drugs to in vitro matrices: impact on the in vitro – in vivo extrapolation of clearance and inhibition

As was noted by Obach (1996) most *in vitro* studies conducted up until the mid 1990's assumed that all drug molecules added to an incubation mixture were freely available for metabolism or inhibition. However, Baarnhielm et al. (1986) were the first to measure the amount of drug bound to the *in vitro* matrix and incorporate a correction factor for free drug in the incubation into the calculation of intrinsic clearance. This group found that the lipophilic calcium channel blocker, felodipine, was extensively bound to the incubation matrix (liver microsomes). When felodipine

clearance was predicted using the expression describing the well stirred model of hepatic clearance (Equation 1.12) without correcting for the microsomal membrane binding in the *in vitro* incubation, the *in vivo* clearance was drastically underpredicted. However, when K_m was corrected for the free fraction of drug in the incubation medium (Equation 1.14 and Figure 1.7), a highly significant improvement was noted in the extrapolation of hepatic clearance.

Equation 1.14

$$CL_{int'} = \frac{V_{max}}{K_m \times f_{um}}$$

Where:

f_{um} = free fraction of drug in the incubation medium

and other terms have been described previously.

Subsequently, Obach (1997) undertook an examination of the non-specific binding of three drugs to liver microsomes; warfarin (an organic acid), imipramine, and propranolol (lipophilic organic bases). These three drugs were chosen because they represented compounds with low free fractions in plasma ($f_u \leq 0.01$), as is the case for felodipine. Warfarin bound to microsomes minimally whereas the two lipophilic bases imipramine and propranolol bound the microsomal membrane extensively. Incorporating the free fraction of drug into the expression for the well-stirred model of hepatic clearance improved the prediction of clearance by four and two fold for imipramine and propranolol respectively, whereas only a slight improvement in clearance prediction was noted for warfarin. Unlike the study of Baarnhielm et al. (1986) in this instance, the free fraction of drug was incorporated directly into the well-stirred model of hepatic clearance.

This is described by the expression:

Equation 1.15

$$CL_H = \frac{Q_H \times f_u \times (\frac{CL_{int}}{f_{u(mic)}})}{Q_H + f_u \times (\frac{CL_{int}}{f_{u(mic)}})}$$

Where:

$f_{u(mic)}$ = fraction of unbound drug in the microsomal incubation

and other terms have been described previously.

In the Obach (1997) study, hepatic microsomal preparations from rat, dog, human, and monkey were shown to bind drugs to a similar extent. The binding of propranolol, warfarin and imipramine varied only marginally across the animal species.

Two studies further demonstrated the impact of including the free fraction of drug in an incubation on the prediction of clearance. Obach (1999) assessed the microsomal binding of twenty-nine compounds comprising of a range of basic, neutral and acidic drugs. In twenty-three of the twenty-nine cases, human hepatic clearance was improved markedly when the free fraction of drug was accounted for and included in the scale up of hepatic clearance (Table 1.1). Work in this laboratory, (McLure 1998), also found recalculation of *in vivo* clearance accounting for the free fraction of drug in an incubation made a significant difference to clearance prediction, especially to the extensively bound amiodarone. The human hepatic clearance of amiodarone, when corrected for non-specific binding, improved eighty-fold. The corrected *in vitro* predictions of nortriptyline and amitriptyline also showed marked improvement compared to the uncorrected predictions (McLure 1998; Table 1.1).

Table 1.1: Effect of incorporating microsomal binding data into the well-stirred model of hepatic clearance (Equation 1.15)

Drug	Observed clearance *in vivo* (ml/min/kg)	Predicted clearance uncorrected (ml/min/kg)	Predicted clearance corrected for non-specific binding (ml/min/kg)	Reference
Bases				
Amiodarone*	140-450	2.2	167	(McLure 1998)
Amitriptyline*	850	136	333	(McLure 1998)
Amitriptyline	12	0.8	4.2	(Obach 1999)
Chlorpromazine	11	1.5	8.6	(Obach 1999)
Clozapine	2.9	0.3	1.9	(Obach 1999)
Desipramine	12	2.8	8.8	(Obach 1999)
Diltiazem	12	2.9	3.6	(Obach 1999)
Diphenhydramine	9.5	0.7	2.2	(Obach 1999)
Imipramine	14	2.2	8.6	(Obach 1996)
Imipramine	12	1.6	6.6	(Obach 1999)
Ketamine	20	12	15	(Obach 1999)
Lorcainide	18	6.7	9.9	(Obach 1999)
Nortriptyline*	660	1.9	47.1	(McLure 1998)
Propafenone	19	6.5	13	(Obach 1999)
Propranolol	16	4.8	9.2	(Obach 1996)
Quinidine	2.7	0.5	1.4	(Obach 1999)
Verapamil	19	9	13	(Obach 1999)
Neutrals				
Diazepam	0.6	0.04	0.2	(Obach 1999)
Methoxsalen	18	4.3	4.5	(Obach 1999)
Prednisone	4.9	0.8	3.4	(Obach 1999)
Triazolam	4.7	2.7	3.3	(Obach 1999)
Zolpidem	5.7	0.3	0.5	(Obach 1999)
Acids				
Amobarbital	0.35	0.24	0.32	(Obach 1999)
Hexobarbital	3.6	1.2	1.4	(Obach 1999)
Methohexital	16	9.9	11	(Obach 1999)
Tenidap	0.10	0.01	0.02	(Obach 1999)
Tenoxicam	0.03	0.02	0.03	(Obach 1999)
Warfarin	0.081	0.017	0.023	(Obach 1997)
Warfarin	0.08	0.01	0.02	(Obach 1999)

* Clearance values are expressed in ml/min

Clearly, large differences in *in vitro* based predictions of human hepatic clearance were observed for particular basic compounds (i.e. amiodarone, amitriptyline and nortriptyline) and to a lesser extent neutral (diazepam and prednisone) and acidic drugs (amobarbital and warfarin) when the free fraction of drug was included in the calculation of hepatic clearance (Table 1.1). Although the drugs shown in Table 1.1

showed marked improvement in the prediction of clearance, only three of the twenty-nine drugs listed were within 10 - 20% of the observed clearance values.

In vitro inhibition studies are less advanced than clearance studies, yet the investigation of Yao & Levy (2002) suggested that $K_{i,\ unbound}$ is a better estimate of inhibitor concentration available to the enzyme in human liver microsomes and when accounted for increased the accuracy of calculated K_i values. Furthermore, it was stated that the extent of non-specific binding should be systematically evaluated especially for potent lipophilic inhibitors (Yao & Levy 2002).

1.4.1.2 Methodology for incorporating the free fraction of drug into calculations of in vitro – in vivo hepatic clearance

The two separate methods of correcting for non-specific binding by Baarnhielm et al. (1986), whereby a correction factor was applied to the K_m (Equation 1.14) and Obach (1997), where this correction factor was incorporated directly into the well-stirred model of hepatic clearance (Equation 1.15) are potentially incorrect. In both drug metabolism and inhibition studies, experiments are performed over a range of concentrations and the available drug must be measured and accounted for at each individual drug concentration tested. Numerous compounds have been shown to bind to *in vitro* matrices in a concentration dependent manner (Obach 1997; McLure, Miners & Birkett 2000) and consequently available rather than added substrate at each concentration investigated should be used to calculate kinetic constants. The effect of non-specific binding on the calculation of K_m is shown in Figure 1.8.

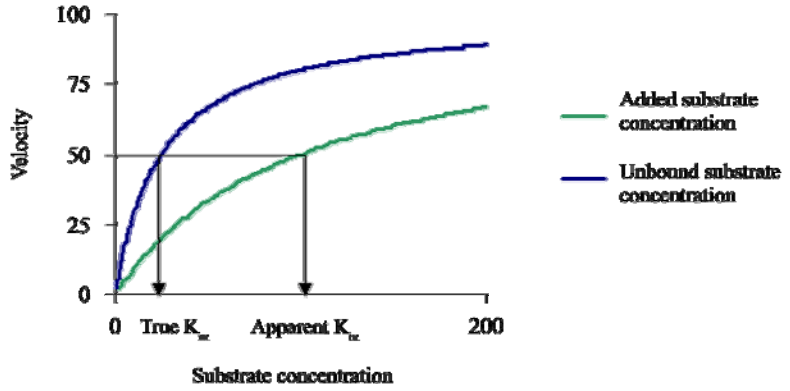


Figure 1.8: Rate versus substrate concentration plot showing overestimation of K_m (the apparent K_m) when uncorrected substrate concentration is employed.

The overestimation of K_m when total added substrate is used (Figure 1.8), which also occurs for K_i, results in an underestimation of *in vitro* clearance (Equation 1.3) and inhibition (Equation 1.13). When bound substrate at each tested concentration is accounted for and available, rather than total drug concentration added is applied to the x-axis, the true K_m and K_i value is determined.

1.4.1.3 Techniques for calculating the free fraction of drug in in vitro matrices

1.4.1.3.1 Equilibrium dialysis

The 'gold standard' technique employed for measuring non-specific binding of drugs to *in vitro* matrices, in most cases to hepatic microsomes, has been equilibrium dialysis (Lindup 1987). Experiments consist of a suspension of microsomes on one side of the dialysis cell and a physiologically based buffer on the other side of the cell. Binding studies are initiated by the addition of drug to one side of the cell. The complete assembly of dialysis cells is immersed in water at 37°C and is rotated until equilibrium is reached.

The unbound fraction of drug in microsomes, $f_{u(mic)}$, is calculated using the equilibrium concentration of free drug (i.e. the concentration in the buffer

compartment) as a proportion of the total concentration (i.e. concentration in the microsome compartment). This is expressed as:

Equation 1.16:

$$f_{u(mic)} = \frac{C_{buffer}}{C_{total}} = \frac{C_{free}}{C_{free} + C_{bound}}$$

Importantly, the non-specific binding of drug to any *in vitro* matrix can be measured using this protocol.

1.4.1.3.2 ANS fluorescence

The introduction of a fluorescence technique for measuring the extent of microsomal membrane binding of drugs occurred in the early 1970's (Diaugustine, Eling & Fouts 1970; Dallner & Azzi 1972; Hawkins & Freedman 1973; Birkett 1974). The fluorophore employed for measuring microsomal binding of drugs in these studies was 1-anilino-8-napthalene sulphonate (ANS). This compound fluoresces in the phospholipid bilayer of the membrane at excitation and emission wavelengths of 375 nm and 470 nm, respectively. On addition of certain drugs, changes in ANS fluorescence were noted. These drug induced changes in ANS fluorescence were initially correlated to changes in the structure of the membrane (Diaugustine, Eling & Fouts 1970), however alternate evidence suggested that the changes in fluorescence reflected a change in the net charge on the membrane as a result of the binding of drug (Birkett 1974). Irrespective of the exact mechanism, this technique was proposed as a screening method for measuring the microsomal binding of drugs (Birkett 1974).

1.4.2 Qualitative prediction of the microsomal binding of drugs

1.4.2.1 The effect of charge

Obach (1996 and 1997) compiled microsomal binding data on eight drugs, six of which were bases that bound the membrane extensively. Results were consistent with previous data that demonstrated that weak bases bound extensively to microsomes whereas weak acids bound minimally or not at all (Balzer et al. 1968; Bickel & Steele 1974; Francesco & Bickel 1977). A further twenty-nine compounds were tested, twelve bases, eight neutrals and nine acids (Obach 1999). Overall, binding appeared to be dependent on charge; bases > neutrals > acids. The importance of charge on the extent of binding was supported by several other studies (Carlile et al. 1999; McLure, Miners & Birkett 2000; Obach 2000; Venkatakrishnan et al. 2000).

The microsomal membrane contains a net negative charge resulting from the overall negative charge associated with the polar heads of phospholipids such as phosphatidylserine and phosphatidylinositol (Chapter 3, Figure 3.7). Thus, an attractive electrostatic interaction between basic compounds, positively charged at pH 7.4, and the microsomal membrane, and a repulsive electrostatic interaction for acidic compounds, negatively charged at pH 7.4, is predicted (Birkett 1974; McLure, Miners & Birkett 2000). That neutral and weak acidic molecules have been shown to bind to biological matrices highlights determinants other than charge as important for the non-specific binding of a drug to microsomes.

1.4.2.2 The effect of lipophilicity

Early data on hepatic microsomal binding demonstrated extensive binding of lipophilic bases (Balzer et al. 1968; Bickel & Steele 1974; Birkett 1974; Francesco & Bickel 1977). More recently, felodipine and amiodarone, two highly lipophilic bases (log P > 5), were reported to bind extensively to microsomes. In both instances

greater than 90% of the tested drug concentration(s) bound the lipid-protein milieu (Baarnhielm, Dahlback & Skanberg 1986; McLure, Miners & Birkett 2000). Furthermore, the lipophilic basic compounds imipramine, propranolol, amitriptyline, nortriptyline, and chlorpromazine were shown to be extensively membrane bound (Balzer et al. 1968; Francesco & Bickel 1977; Obach 1997; McLure, Miners & Birkett 2000).

Biological membranes are composed of a phospholipid bilayer as shown in Figure 1.3, while the lipophilicity is defined by the International Union of Pure and Applied Chemistry (IUPAC) as the affinity of a molecule or moiety for a lipophilic environment (Waterhouse 2003). Thus, extent of microsomal membrane binding is predicted to increase with lipophilicity.

1.4.2.3 A predictive unified binding model of microsomal binding of drugs

A non-specific binding model was proposed from the saturable binding of two lipophilic basic compounds, namely amitriptyline and nortriptyline (McLure, Miners & Birkett 2000). Binding data were fitted to a standard binding model to determine the maximum binding capacity B_{max}, and the dissociation constant K_D, which is the relative affinity of the drug for the membrane**.** Saturable binding of nortriptyline (20 – 1000 μM) to the microsomal membrane, at a human liver microsomal concentration of 1 mg/ml, was demonstrated (Figure 1.9).

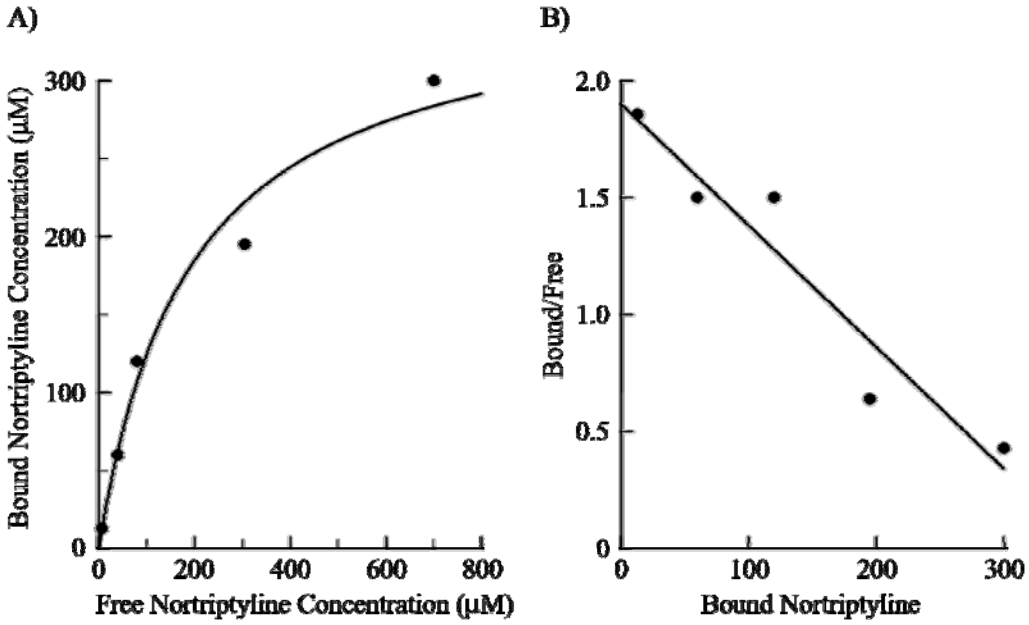


Figure 1.9: Binding of nortriptyline to human liver microsomes (1 mg/ml): A) Binding plot of free (x-axis) versus bound (y-axis) drug concentration and; B) Scatchard plot with bound/free drug on the y-axis and bound drug on the x-axis.

Saturable binding of a drug to a membrane is likely to apply to other lipophilic bases (McLure, Miners & Birkett 2000). The B_{max} and K_D values for nortriptyline were determined at varying microsome concentrations. The B_{max} varied proportionately with microsome concentration, whereas K_D remained constant (McLure, Miners & Birkett 2000).

McLure, Miners & Birkett (2000) proposed that two situations occur in relation to correcting CL_{int} values for microsomal membrane binding. The first is where the K_m, and therefore the substrate concentration used in an *in vitro* kinetic study, is much less than the K_D for microsomal membrane binding of the substrate. In this situation the $f_{u(mic)}$ is independent of the substrate concentration but will still be dependent on the microsomal protein concentration. The apparent K_m can then be corrected to the 'true' K_m by multiplying by the fraction of unbound drug at the microsomal protein concentration used in the *in vitro* study:

'True' $K_m = K_{m(app)} \times f_{u(mic)}$

The second case is where the substrate concentration range used in an *in vitro* study is similar to or higher than the K_D for microsomal membrane binding. The fraction of substrate in the incubation mixture will then vary across the substrate concentration used. This precludes a simple proportional correction of apparent K_m for the membrane binding of a drug. In this situation $f_{u(mic)}$ varies with both substrate and microsome concentrations and sigmoidal kinetics is predicted (Figure 1.10).

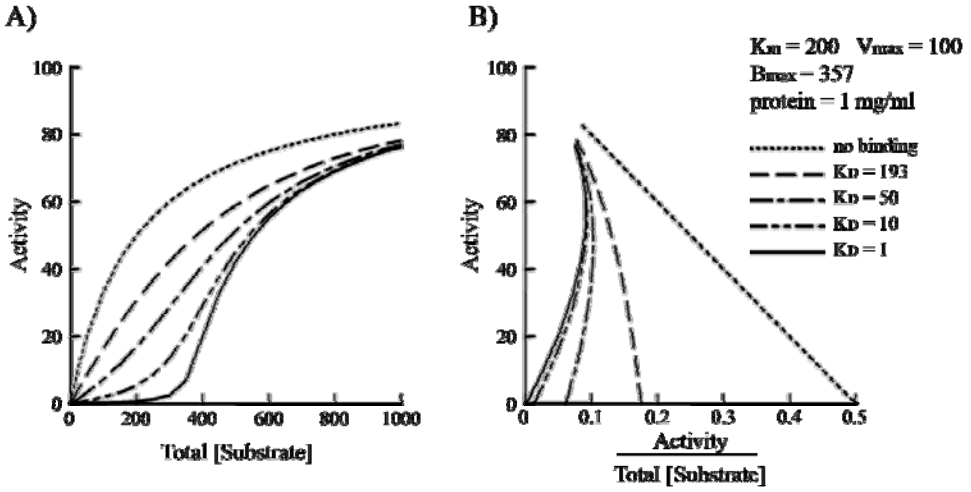


Figure 1.10: Simulation showing the effect of: A) Non-specific binding with varying K_D on Michaelis-Menten kinetics and B) Non-specific binding on an Eadie Hofstee plot. The plots use total (added) substrate concentration, while the reaction velocities were calculated using free (unbound) substrate concentration.

Sigmoidal *in vitro* kinetics were previously reported for several CYP and UGT enzymes and interpreted as autoactivation due to substrate binding at more than one site or to the simultaneous binding of two molecules of substrate at the active site (Venkatakrishnan et al. 1995; Ekins et al. 1997; Ueng et al. 1997; Ekins et al. 1998; Korzekwa et al. 1998). The simulation shown in Figure 1.10 proposes that sigmoidal kinetics can be predicted to occur in some circumstances as a result of non-specific binding of the substrate to the microsomal membrane (McLure, Miners & Birkett

2000). This is further evidence of the importance of identifying and defining the interactions occurring *in vitro* for the generation of meaningful kinetic data.

1.5 Aims of current research

The overall aim of this work was to characterise the physicochemical determinants of drugs that influence the extent of non-specific binding to human liver microsomes.

The specific aims were to:

- investigate the comparative importance of lipophilicity (as log P), and pK_a (at a constant molecular mass) as determinants of the non-specific binding of drugs to human liver microsomes
- develop and validate an ANS fluorescence technique for measuring the non-specific binding of drugs to human liver microsomes
- characterise the non-specific binding of a large dataset of physicochemically diverse drugs using the ANS fluorescence procedure
- evaluate relationships between selected physicochemical characteristics (previously identified as important in permeability studies) and the extent of non-specific binding of drugs to human liver microsomes
- computationally model the non-specific binding of drugs to discriminate between highly bound drugs ($f_{u(mic)} < 0.5$) and those drugs bound to a lesser extent ($f_{u(mic)} \geq 0.5$).

CHAPTER 2
MATERIALS AND METHODS

2.1 Chemicals and reagents

2.1.1 Chemicals and reagents for analytical, fluorescence and dialysis procedures

Chemicals and reagents used in the HPLC procedures, and equilibrium dialysis and fluorescence techniques are listed in Table 2.1.

Table 2.1: Chemicals and reagents used in fluorescence and dialysis procedures

Chemicals and Reagents	Supplier
Acetonitrile	Asia Pacific Speciality Chemicals Ltd, NSW, Australia
1-Acetyl-2-phenylhydrazine	Sigma Aldrich, Sydney, Australia
Albendazole	Sigma Aldrich, Sydney, Australia
Alclofenac	Continental Pharma, Belgium
Amiodarone hydrochloride	Sigma Aldrich, Sydney, Australia
Amitriptyline hydrochloride	Sigma Aldrich, Sydney, Australia
Amodiaquine hydrochloride	Sigma Aldrich, Sydney, Australia
8-Anilino-1-naphthalene sulfonic acid	Sigma Aldrich, Sydney, Australia
Antazoline hydrochloride	Sigma Aldrich, Sydney, Australia
Atenolol	AstraZeneca Pty Ltd, Sydney, Australia
Benzydamine hydrochloride	Sigma Aldrich, Sydney, Australia
(S)-(-)-(N) Benzyl-α-methylbenzylamine	Sigma Aldrich, Sydney, Australia
Budesonide	Sigma Aldrich, Sydney, Australia
Bupivacaine hydrochloride	Astra Pharmaceuticals, Sydney, Australia
Bupropion hydrochloride	Sigma Aldrich, Sydney, Australia
Caffeine	Sigma Aldrich, Sydney, Australia
Carbamazepine	Sigma Aldrich, Sydney, Australia
Carbazole	Sigma Aldrich, Sydney, Australia
Chloroquine diphosphate	Sigma Aldrich, Sydney, Australia
Chlorpheniramine maleate	Sigma Aldrich, Sydney, Australia
Chlorphentermine hydrochloride	Parke Davis & Co, Sydney, Australia
Chlorpromazine hydrochloride	Sigma Aldrich, Sydney, Australia
Cicloprofen	E.R Squibb & Sons Inc., NJ, USA
Cinnoline hydrochloride	Sigma Aldrich, Sydney, Australia
Clenbuterol hydrochloride	Sigma Aldrich, Sydney, Australia
Clonidine hydrochloride	Sigma Aldrich, Sydney, Australia
Coniine	Sigma Aldrich, Sydney, Australia
Copper sulfate	BDH Chemicals, Kilsyth, Australia

Chemicals and Reagents	Supplier
Desipramine hydrochloride	Sigma Aldrich, Sydney, Australia
Desmethyl nortriptyline	Roche Pharmaceuticals, Australia
Dextropropoxyphene hydrochloride	Fawns & McAllan, Sydney, Australia
Diazepam	Roche Pharmaceuticals, Basle, Switzerland
Diclofenac	Sigma Aldrich, Sydney, Australia
Diflunisal	Merck, Sharp & Dohme Pty Ltd, Australia
Diltiazem hydrochloride	Sigma Aldrich, Sydney, Australia
Dimethylamine	Fluka Chemie, Buchs, Switzerland
Diphenhydramine hydrochloride	Sigma Aldrich, Sydney, Australia
Dipotassium hydrogen orthophosphate	Asia Pacific Speciality Chemicals Ltd, NSW, Australia
4,4'Dipyridyl dihydrochloride	Sigma Aldrich, Sydney, Australia
Disopyramide phosphate	Pharmacia Pty Ltd, Sydney, Australia
Doxepin hydrochloride	Sigma Aldrich, Sydney, Australia
Econazole nitrate	Bristol-Myers Squibb Pharmaceuticals, Melbourne, Australia
Ethanol	Chem Supply, Adelaide, Australia
9-Ethoxycarbazole	Sigma Aldrich, Sydney, Australia
Fenoprofen	Eli Lilly Pty Ltd, Sydney, Australia
Flufenamic acid	Parke Davis & Co, Sydney, Australia
Fluoxetine hydrochloride	Sigma Aldrich, Sydney, Australia
Fluphenazine dihydrochloride	Sigma Aldrich, Sydney, Australia
Flurbiprofen	Sigma Aldrich, Sydney, Australia
Fluvoxamine maleate	Sigma Aldrich, Sydney, Australia
Gliclazide	Servier Laboratories Pty Ltd, Melbourne, Australia
Hydralazine hydrochloride	Sigma Aldrich, Sydney, Australia
3-Hydroxytyramine	Sigma Aldrich, Sydney, Australia
Ibuprofen	Sigma Aldrich, Sydney, Australia
Imipramine hydrochloride	Sigma Aldrich, Sydney, Australia
Isoniazid	Sigma Aldrich, Sydney, Australia
Itraconazole	Janssen-Cilag Pty Ltd, Sydney, Australia
Labetolol hydrochloride	Sigma Aldrich, Sydney, Australia
Lamotrigine	Wellcome Foundation, London, UK
Lignocaine hydrocholoride monohydrate	Sigma Aldrich, Sydney, Australia
Meclofenamic acid	Parke Davis & Co, Australia
Mephentermine hemisulfate	Sigma Aldrich, Sydney, Australia
Metyrapone	Sigma Aldrich, Sydney, Australia
Mianserine hydrochloride	Organon Pty Ltd, Sydney, Australia
N,N-Dimethylbenzylamine	Sigma Aldrich, Sydney, Australia
S-Naproxen	Syntex Research, CA, USA
N-Benzylmethylamine	Sigma Aldrich, Sydney, Australia
N-Di-desmethylimipramine hydrochloride	Ciba-Geigy Ltd, Basle, Switzerland

Chemicals and Reagents	Supplier
Niflumic acid	E.R Squibb & Sons Inc, NJ, USA
NNN'N' Tetramethylene diamine	Sigma Aldrich, Sydney, Australia
Nortriptyline hydrochloride	Sigma Aldrich, Sydney, Australia
1-Octanol	Reidel-de Haen AG, Germany
Orthophosphoric acid	BDH Laboratory Supplies, UK
Pentanesulfonic acid	Eastman Kodak Co., NY, USA
Perhexiline maleate	Sigma Aldrich, Sydney, Australia
Phenacetin	Sigma Aldrich, Sydney, Australia
Phenelzine sulfate	Sigma Aldrich, Sydney, Australia
β-Phenylethylamine	Sigma Aldrich, Sydney, Australia
Phenylpropanolamine hydrochloride	Sigma Aldrich, Sydney, Australia
Phentolamine hydrochloride	Sigma Aldrich, Sydney, Australia
4-Phenylbutylamine	Sigma Aldrich, Sydney, Australia
4-Phenylpyridine	Fluka Chemie, Buchs, Switzerland
Phenytoin	Parke Davis & Co, Sydney, Australia
Pindolol	Sigma Aldrich, Sydney, Australia
Potassium dihydrogen orthophosphate	Merck Pty Ltd, Victoria, Australia
Probenecid	Sigma Aldrich, Sydney, Australia
Propofol	Sigma Aldrich, Sydney, Australia
Propranolol hydrochloride	Sigma Aldrich, Sydney, Australia
Protriptyline hydrochloride	Sigma Aldrich, Sydney, Australia
Quinidine hydrochloride	Sigma Aldrich, Sydney, Australia
Quinine hemisulfate	Sigma Aldrich, Sydney, Australia
Quipazine maleate	Sigma Aldrich, Sydney, Australia
R,S- Warfarin	Sigma Aldrich, Sydney, Australia
Ropivacaine hydrochloride	Astra Pharmaceuticals, Sydney, Australia
Sodium (+) - tartrate	Ajax Chemicals Ltd, Melbourne, Australia
Sodium carbonate	BDH Chemicals, Kilsyth, Australia
Sodium hydroxide	BDH Chemicals, Kilsyth, Australia
Sodium salicylate	Sigma Aldrich, Sydney, Australia
Sotalol hydrochloride	Sigma Aldrich, Sydney, Australia
Spermidine	Sigma Aldrich, Sydney, Australia
Spermine	Sigma Aldrich, Sydney, Australia
Terbutaline hemisulfate	Sigma Aldrich, Sydney, Australia
Tetrahydrozoline hydrochloride	Sigma Aldrich, Sydney, Australia
Thioridazine hydrochloride	Sigma Aldrich, Sydney, Australia
Thiothixene hydrochloride	Sigma Aldrich, Sydney, Australia
Tolbutamide	Hoechst Australia Ltd, Melbourne, Australia
Trans-2-phenylcyclopropylamine hydrochloride	Sigma Aldrich, Sydney, Australia
Tranylcypromine hydrochloride	Sigma Aldrich, Sydney, Australia
Triethylamine	BDH Laboratory Supplies, UK
Trifluoperazine hydrochloride	Sigma Aldrich, Sydney, Australia
Triflupromazine hydrochloride	Sigma Aldrich, Sydney, Australia
Verapamil hydrochloride	Knoll Australia Pty Ltd, Sydney

2.2 Human liver microsomes

2.2.1 Human livers

Non-specific binding experiments were performed with pooled microsomes from two human livers. Donors of the respective livers were a 49 year old female and a 22 year old male. Neither were cigarette smokers, however both received dopamine prior to death. Approval was obtained from the Flinders Medical Centre Committee on Clinical Investigation to use human liver tissue for drug disposition studies *in vitro*.

2.2.2 Preparation of human liver microsomes

Microsomes were prepared using differential centrifugation following a modified procedure of Robson et al. (1987). All samples and solutions used during the preparation of human liver microsomes were stored and used on ice. Liver portions in 0.1M phosphate buffer (PB; pH 7.4) containing 1.15% w/v potassium chloride were homogenised sequentially with a Janke and Kunkle Ultra Turax at 24,000 rpm and a Potter-Elvehjem homogeniser with mechanical drive at 1480 rpm. The homogenate was centrifuged at 700 g for 10 min and then at 13,250 g for 10 min at 4°C (JA-20 Beckman Coulter rotor). The supernatant fraction was aspirated and centrifuged at 105,000 g for 60 min at 4°C (50.2 Ti Beckman rotor). The resulting pellet was resuspended in 0.1M PB (pH 7.4) containing 1.15% w/v potassium chloride and centrifuged at 105,000 g (50.2 Ti Beckman Coulter rotor) for 60 min at 4°C. The final microsomal pellet was suspended in 0.1M PB (pH 7.4) containing 20% glycerol and stored at -80°C until required.

Microsomes prepared from the two livers were pooled and used for the equilibrium dialysis and fluorescence experiments described in Chapters 3, 4, 5 and 6.

2.2.2 Measurement of microsomal protein concentration

The protein concentration of human liver microsomes was determined using the technique of Lowry et al. (1951) with bovine serum albumin as the standard.

2.3 Equilibrium dialysis procedures

2.3.1 Equilibrium dialysis

Direct measurement of the microsomal membrane binding of drugs was performed with an equilibrium dialysis apparatus (Dianorm, Munich, Germany) containing teflon dialysis cells of 1.2 ml capacity per side, using a working volume of 1 ml. Spectrapor #4 dialysis membrane (molecular mass cut off 12,000 –14,000 Da) was purchased from Spectrum Medical Industries Inc. (Los Angeles, CA, USA) and was prepared by soaking overnight in PB (0.1M, pH 7.4) at 4°C. It has been demonstrated previously that there is no significant binding of acidic, basic or neutral drugs to this membrane (Wanwimolruk 1983).

Each of the drugs was diluted 1:100 upon addition to the dialysis cell to give the concentration required (Chapters 3 and 5). PB (0.99 ml; 0.1M, pH 7.4) and a 0.01 ml aliquot of the test drug was loaded into one side of each dialysis cell. A suspension of human liver microsomes in PB (1 ml: protein concentration 1 mg/ml) was added to the other side of the dialysis cell. The dialysis cell assembly was immersed in a water bath maintained at 37°C by a Tecarri TE 7 Tempette heater and rotated at 12 rpm for 3 hr. After dialysis each side of the cell was unloaded by expelling the contents into a 5 ml glass test tube by positive pressure applied with an Eppendorf pipette.

A negative control and microsome / microsome and PB / PB controls were employed in each dialysis experiment. The negative control comprised PB (1 ml; 0.1M, pH 7.4)

without drug on one side of the dialysis cell and a suspension of human liver microsomes in PB (1 ml; 1 mg/ml protein concentration) on the other side. Microsome/microsome controls consisted of a suspension of human liver microsomes (0.99 ml: protein concentration 1 mg/ml) and a 0.01 ml aliquot of the test drug at a "mid" concentration of the range tested with a suspension of human liver microsomes (1 ml: protein concentration 1 mg/ml) in the other side of the dialysis cell. PB was substituted for the human liver microsomal suspension to prepare the PB/PB controls.

Drugs utilised in dialysis experiments were separated into two groups. Initially, seven drugs (propranolol, imipramine, atenolol, diazepam, lignocaine, bupivacaine and ropivacaine) were dialysed to systematically investigate the importance of log P and pK_a as determinants of non-specific microsomal binding (Chapter 3). A further nine drugs were dialysed to generate sufficient data for equilibrium dialysis and fluorescence correlations for acidic and basic compounds (Chapter 5).

2.3.2 Determination of drug concentrations by high performance liquid chromatography (HPLC)

2.3.2.1 HPLC Equipment and conditions

The HPLC system used for the quantification of bupivacaine, imipramine, diazepam, chloroquine, atenolol, propranolol, lignocaine, ropivacaine and verapamil comprised a Rheodyne 7725 manual injector, an LC 1110 HPLC solvent delivery system, an LC 1200 variable wavelength UV-VIS detector (all ICI, Melbourne, Australia), and a BBC Goetz Metrawatt SE 120 chart recorder. An Agilent 1100 Series HPLC system (Agilent Technologies Australia Pty Ltd, Sydney, Australia), comprising an on-line degasser, a quaternary gradient pump, auto sampler and a variable wavelength UV-VIS detector was used for measurement of meclofenamic acid, diflunisal,

chlorpromazine, bupropion, mianserine, triflupromazine and flufenamic acid concentrations. Mobile phase composition, flow rate, detector wavelength, column type, and retention times of analytes and internal standards are given in Table 2.3.

2.3.2.2 Preparation of samples

Concentrations of drugs in the buffer and microsome compartments of the equilibrium dialysis apparatus were measured by HPLC. All samples were treated with acetonitrile or mixtures containing acetonitrile (plus internal standard with the exception of imipramine) to precipitate microsomal protein. After dilution, samples were vortex mixed for 10 sec and then centrifuged at 2000 g for 10 min at 4°C. An aliquot of the supernatant fraction was injected onto the HPLC column. Sample preparation for each drug is shown in Table 2.2.

Table 2.2: Equilibrium dialysis sample preparation prior to HPLC analysis

Drug	Dilutions of dialysis samples and controls
Atenolol	1:10 with 60/40 acetonitrile - water then 1:1 with PB
Bupivacaine	1:10 with mobile phase (Table 2.3)
Bupropion	2:3 with acetonitrile
Chloroquine	1:10 with 72/28 acetonitrile - water then 1:1 with PB
Chlorpromazine	2:3 with acetonitrile
Diazepam	1:10 with mobile phase (Table 2.3)
Diflunisal	2:3 with acetonitrile then 1:1 with PB
Flufenamic acid	1:2 with acetonitrile then 1:1 with water
Imipramine	1:10 with mobile phase (Table 2.3)
Lignocaine	1:10 with mobile phase (Table 2.3)
Meclofenamic acid	2:3 with acetonitrile then 1:1 with water
Mianserine	2:3 with acetonitrile
Propranolol	As for atenolol
Ropivacaine	1:10 with mobile phase (Table 2.3)
Triflupromazine	2:3 with acetonitrile
Verapamil	1:10 with mobile phase (Table 2.3)

2.3.2.2.1 HPLC and chromatography conditions for individual drug assays

High performance liquid chromatography assays were developed for each drug investigated in dialysis experiments. The mobile phase, column type, flow rate, detector wavelength, and retention time of the analyte and the internal standard are shown in Table 2.3.

Table 2.3: HPLC conditions for drug assays

Drug	Mobile phase	HPLC column	Mobile phase flow rate (ml/min)	Detector wavelength (nm)	Retention time of analyte (min' sec")	Internal standard / Retention time (min' sec")	
Atenolol	80% water containing 5.2 mM pentanesulfonic acid and 0.3 mM NNN'N' TMED / 20% acetonitrile (adjusted to pH 2.6 with orthophosphoric acid)	C-8	1	230	7' 26"	sotalol	10’30”
Bupivacaine	85% 100 mM phosphate buffer pH 5.9 / 15% acetonitrile containing 0.1% dimethylamine (adjusted to pH 3.0 with orthophosphoric acid)	C-18	1	210	13' 17"	lignocaine	3’30”
Bupropion	60% PB (50 mM, pH 7.4) containing 10 mM triethylamine and 7 mM heptanesulfonic acid / 40% acetonitrile	**C-8	1	214	2' 24"	verapamil	4’48”
Chloroquine	64% water containing 5.2 mM pentanesulfonic acid and 0.3mM NNN'N' TMED / 36% acetonitrile (adjusted to pH 2.6 with orthophosphoric acid)	C-8	1	220	4' 48"	ropivacaine	7’30”
Chlorpromazine	44% sodium acetate buffer (10 mM, pH 4.3) / 56% acetonitrile	*C-8	1	254	4' 00"	thioridazine	3’12”
Diazepam	46% water containing 5.2 mM, pentanesulfonic acid and 0.3 mM NNN'N' TMED / 54% acetonitrile (adjusted to pH 2.6 with orthophosphoric acid)	C-18	1	230	6' 08"	desmethyl-diazepam	4' 15"
Diflunisal	70% PB (25mM, pH 7.4) containing 7.2 mM triethylamine / 30% acetontitrile	C-18	1	240	2' 45"	mefenamic acid	3’12”

Drug	Mobile phase	HPLC column	Mobile phase flow rate (ml/min)	Detector wavelength (nm)	Retention time of analyte (min' sec")	Internal standard / Retention time (min' sec")	
Flufenamic acid	70% PB (25 mM, pH 7.4) containing 7.2 mM triethylamine / 30% acetonitrile	C-18	1.5	270	4' 10"	diclofenac	2’15”
Imipramine	as for diazepam	C-18	1	259	6' 00"		
Lignocaine	as for bupivacaine	C-18	1	210	3' 30"	ropivacaine	6' 47"
Meclofenamic acid	as for diflunisal	C-18	1	240	4' 01"	diflunisal	3’06”
Mianserine	45% PB (25 mM, pH 7.4) containing 7.2 mM triethylamine / 55% acetonitrile	**C-8	1	254	3' 36"	doxepin	2’06”
Propranalol	as for chloroquine	C-8	1	220	9' 09"	pindalol	4’25”
Ropivacaine	as for lignocaine	C-18	1	210	6' 47"	lignocaine	3’30”
Triflupromazine	47.5% sodium acetate buffer (10 mM, pH 4.3) / 42.5% acetonitrile	**C-8	1	254	4' 06"	chlorpromazine	3’12”
Verapamil	as for diazepam	C-8	1	220	5' 50"	ropivacaine	3’50”

C-18: Waters, Nova Pak, particle size 4 micron, 3.9 (id) x 150 mm C8: Beckman Ultrasphere (Octyl), particle size 5 micron, 4.6 (id) x 250 mm

*C8: Develosil, particle size 5 micron, 4.6 x 150 mm **C-8: Agilent Zorbax Eclipse XDB, particle size 5 micron, 4.6 x 150 mm

2.3.2.3 Standard curves

Standards for each drug investigated in equilibrium dialysis experiments were prepared in both PB (0.1M, pH 7.4) and suspensions of human liver microsomes in PB (1 mg/ml). Standards were treated in the same manner as experimental samples (Section 2.3.2.2). Drug was added to samples to provide concentrations in the desired range. The concentration range used spanned the concentrations in the corresponding equilibrium dialysis experiment. Unknown concentrations for all drugs were determined by comparison of drug to internal standard peak area ratios relative to the standard curve. Internal standards were employed in all assays, with the exception of imipramine.

Standard curves for all drugs were linear over the concentration ranges used (at least five points). R^2 values ranged from 0.990 - 0.999. Typical standard curves are shown in Chapter 3, Figure 3.1**.**

2.3.2.4 Reproducibility of the sample preparation and HPLC procedures

The overall reproducibility of the sample preparation and HPLC procedures was assessed for three different concentrations of each of the drugs investigated by equilibrium dialysis. The 'high', 'medium' and 'low' concentrations selected spanned the concentration range employed in the corresponding equilibrium dialysis experiment. Samples were prepared in PB (0.1M, pH 7.4) and in a suspension of human liver microsomes in PB (1 mg/ml), and subsequently analysed in the same manner as samples obtained from dialysis cells. The samples were analysed in quadruplicate (without the dialysis step) as described in Section 2.3.2.2.

2.3.3 Calculation of $f_{u(mic)}$

The binding of drugs to human liver microsomes is expressed as the unbound fraction of drug in the microsomal suspension (i.e. $f_{u(mic)}$), giving a value between 0 and 1. Thus, $f_{u(mic)}$ was calculated as the proportion of free drug (i.e. concentration in the buffer compartment) to free plus bound drug (i.e. concentration in the microsome compartment) according to the expression:

Equation 2.1

$$f_{u(mic)} = \frac{C_{buffer}}{C_{total}} = \frac{C_{free}}{C_{free} + C_{bound}}$$

CHAPTER 3
AN INVESTIGATION OF THE PHYSICOCHEMICAL DETERMINANTS OF THE NON-SPECIFIC BINDING OF DRUGS TO HUMAN LIVER MICROSOMES USING EQUILIBRIUM DIALYSIS

3.1 Introduction

The binding of drugs to subcellular fractions was first studied in the 1960's, when binding of reserpine, chlorpromazine and prenylamine to the membrane of the sarcoplasmic reticulum was reported by Balzer et al. (1968). Soon after, the non-specific binding of the basic compounds chlorpromazine and imipramine to kidney, liver, lung, brain and skeletal muscle microsomes was demonstrated (Bickel & Steele 1974). Binding varied little across the range of microsomal preparations. The microsomal binding of four acidic compounds was also investigated and all showed very weak or undetectable binding to liver microsomes (Bickel & Steele 1974). Chlorpromazine was further employed as the test drug to analyse membrane binding to rat liver microsomes, mitochondria, mitochondrial membranes, brain synaptosomes, myelin vesicles, red blood cell membranes, protein free liposomes and pure egg lethicin (Di Francesco & Bickel 1977). Affinity and binding capacity values were not significantly different in all cases. A more recent study investigated the microsomal binding of propranolol, warfarin and imipramine to liver microsomes from a number of mammalian species (Obach 1997). No significant difference was found between the binding of these three drugs to rat, dog, monkey and human liver microsomes. Taken together, these studies suggested that non-specific binding of drugs tends not to vary to a major extent between subcellular fractions or species.

Following the initial studies performed in the 1960's and 1970's, the non-specific microsomal binding of drugs was largely ignored for twenty years. The intense interest in *in vitro-in vivo* extrapolation for the prediction of human pharmacokinetic parameters that occurred in the 1990's resulted in re-evaluation of the importance of non-specific microsomal binding. In particular, it was recognised that non-specific binding could potentially alter the kinetic parameters K_m, CL_{int}, and K_i for drugs determined using *in vitro* systems, primarily incubations of human liver microsomes, leading to erroneous prediction of *in vivo* pharmacokinetics.

Initial studies conducted in this laboratory (McLure, Miners & Birkett 2000) and by Obach (Obach 1997, 1999) investigated the microsomal binding of a range of acidic, neutral and basic drugs in an attempt to establish the effect of charge on microsomal membrane binding. Using an equilibrium dialysis technique, it was shown that the lipophilic organic bases imipramine and propranolol bind human liver microsomes extensively, displaying $f_{u(mic)}$ values < 0.5 (Obach 1997). Furthermore, the binding of the organic bases amitriptyline and nortriptyline was shown to be saturable (McLure, Miners & Birkett 2000). The microsomal membrane binding of nortriptyline was tested at three different microsomal protein concentrations (0.5, 1.0 and 2.0 mg/ml) and the data were fitted to a standard binding model to determine the maximum binding capacity, B_{max}, and the dissociation constant, K_D. The B_{max} varied approximately proportionately with microsome concentration, whereas the K_D remained constant. In contrast, warfarin and phenytoin, both moderately lipophilic acidic compounds, bound human liver microsomes to a relatively minor extent with respective $f_{u(mic)}$ values of 0.85 and 0.89 at a drug concentration of 20 μM and a human liver microsomal protein concentration of 1 mg/ml (Obach 1997; McLure, Miners & Birkett 2000). The extent of microsomal binding of the basic and acidic

compounds described is largely representative of the data reported to the commencement of this thesis (Bickel & Steele 1974; Birkett 1974; Francesco & Bickel 1977; Obach 1997, 1999; McLure, Miners & Birkett 2000).

Obach (1999) also investigated binding of the neutral drugs midazolam and triazolam to human liver microsomes (1 mg/ml microsomal protein concentration). Both midazolam and triazolam are lipophilic compounds with log P values > 3. The unbound fractions of midazolam and triazolam were 0.88 and 0.78, respectively. These initial data indicate that lipophilic neutral compounds may bind human liver microsomes, although possibly to a lesser extent than lipophilic basic compounds. Notably, the binding of neutral compounds to the microsomal membrane immediately implicates physicochemical characteristics other than charge as being important for drug-microsome binding. Along with molecular mass, lipophilicity and charge have also been proposed as determinants of drug membrane permeability in relation to absorption (Artursson 1991; Barton et al. 1997; Lipinski et al. 1997; Wang, Fu & Lai 1997; Yamashita et al. 1997; Winiwarter, Lanzner & Muller 1998; Pagliara et al. 1999; Tavelin et al. 1999). Thus, studies were undertaken here to systematically characterise the relationships between the non-specific binding of drugs to human liver microsomes and pK_a and log P at constant molecular mass.

Atenolol, imipramine and propranolol, all organic bases, were investigated to determine the effects of log P at constant pK_a and molecular mass (Table 3.1). Propranolol, lignocaine (both organic bases) and diazepam (a very weak organic acid) comprised the second group of drugs which explored the effects of pK_a, and hence percentage ionisation at pH 7.4, at constant log P and molecular mass (Table 3.1). Bupivacaine and ropivacaine, structurally similar to lignocaine, were

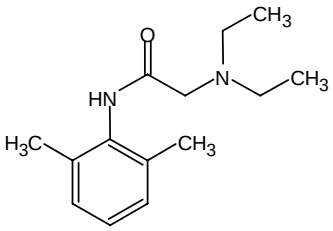
additionally investigated after the unexpected result from the lignocaine dialysis experiment (Figure 3.1).

Table 3.1: Physicochemical characteristics of test drugs

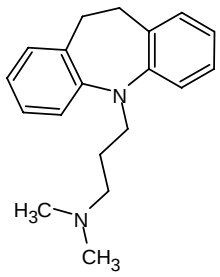
		% ionisation		
Compound	**pK_a**	**pH 7.4**	**log P**	**Molecular mass**
Group 1				
Atenolol	9.6	99.4	*0.1*	266.3
Propranolol	9.5	99.2	*3.1*	259.3
Imipramine	9.5	99.2	*4.8*	280.4
Group 2				
Diazepam	*3.3*	*0*	2.9	284.8
Lignocaine	*7.9*	*69*	2.4	244.4
Propranolol	*9.5*	*99.2*	3.1	280.4
Group 3				
Bupivacaine	8.1	83.4	3.6	288.4
Ropivacaine	8.1	83.4	3.1	329

Experimental pK_a values for all drugs except ropivacaine, were obtained from Analytical Profiles of Drug Substances (Vol 1,4,13,14,15,19), and from the Handbook of Pharmacokinetic Data (Jack 1992). The log P values for all drugs and the pK_a value for ropivacaine were determined using SciFinder Solaris, Advanced Chemistry Software V4.64. The percentage ionisation at pH 7.4 was calculated using the Henderson-Hasselbach equation.

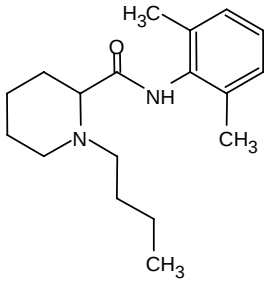
Equilibrium dialysis was employed to measure the binding of drugs to human liver microsomes. This technique is used widely in studies investigating the binding of drugs and other compounds to subcellular fractions (Bickel & Steele 1974; Bickel et al. 1975; Francesco & Bickel 1977; Obach 1997, 1999; McLure, Miners & Birkett 2000; Obach 2000; Venkatakrishnan et al. 2000). Following dialysis, analysis of drug present in both compartments of the equilibrium dialysis apparatus permits calculation of the fraction of drug unbound in microsomes, $f_{u(mic)}$, (Chapter 2, Section 2.3.3), the maximal binding capacity (B_{max}), and the dissociation constant (K_D) for drugs that exhibit saturable binding (Chapter 1; Section 1.4.1.4).



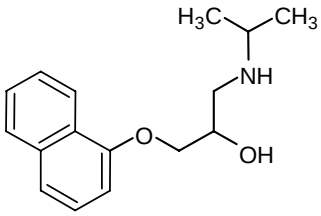
Lignocaine



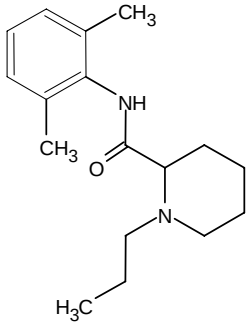
Imipramine



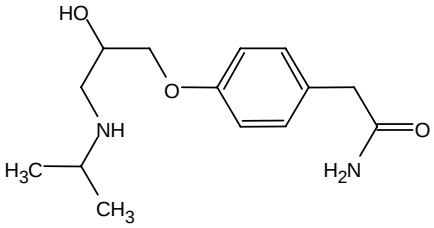
Bupivacaine



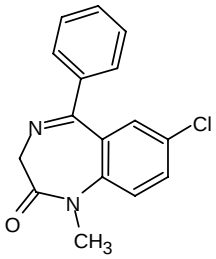
Propranolol



Ropivacaine



Atenolol



Diazepam

Figure 3.1: Chemical structures of investigational compounds.

3.2 Equilibrium dialysis

Details for the equilibrium dialysis procedure are given in Chapter 2, Section 2.3.

3.2.1 Drug concentration ranges

Concentration ranges employed for atenolol, diazepam, imipramine, lignocaine and propranolol were chosen to permit characterisation of potential saturable binding to human liver microsomes (McLure, Miners & Birkett 2000). Ten to sixteen concentrations were investigated for each drug. Bupivacaine and ropivacaine were investigated at only two concentrations to further explore the unexpected result from the lignocaine binding studies. Concentration ranges for the drugs investigated are listed in Table 3.2.

Table 3.2: Concentration ranges of drugs employed in dialysis experiments

Drug	Concentration range (μM)
Atenolol	100 - 1000
Propranolol	50 - 1000
Imipramine	50 - 1000
Diazepam	50 - 1000
Lignocaine	50 - 1000
Bupivacaine	50 and 500
Ropivacaine	50 and 500

Diazepam and atenolol were dissolved in methanol, whereas lignocaine, bupivacaine, ropivacaine, propranolol and imipramine (all available as salts) were dissolved in water. Drug concentrations were 100–fold higher than those required for the dialysis experiments, as solutions were diluted 1:100 upon addition to the dialysis cells.

Experience in this laboratory indicates that 1% v/v aqueous solutions of methanol (final diluted concentration) does not perturb binding to human liver microsomes.

3.2.2 Derivation of human liver microsomal binding parameters of drugs

Maximal binding capacity, B_{max}, and the dissociation constant, K_D, were calculated by fitting experimental data to Equation 3.1 (McLure, Miners & Birkett 2000) using the program EnzFitter (Biosoft, Cambridge, UK). Simple weighting was applied to all data points and curves were fitted using the Marquardt-Levenberg algorithm.

Equation 3.1

$$C_B = \frac{B_{\max} \times C_F}{K_D + C_F}$$

Where:
C_B = concentration of bound drug
B_{max} = maximum binding capacity
K_D = dissociation constant
C_F = concentration of free drug

3.3 Results

3.3.1 Chromatography and standard curve linearity

Under the chromatographic conditions employed (Table 2.3), chromatograms were free from interfering peaks. Representative chromatograms (for diazepam) are shown in Figure 3.2. With the exception of bupivacaine and the internal standard for the atenolol assay (sotalol), retention times for all analytes and internal standards were less than ten minutes (Chapter 2, Table 2.3). Standard curves were linear ($r^2 > 0.990$) over the concentration ranges investigated (Figure 3.3) and were stable for the duration of each drug assay. The ratios of slopes of the standard curves prepared in

suspensions of human liver microsomes to those prepared in PB were within the range 0.98 - 1.13, indicating essentially equivalent recovery from each matrix.

Figure 3.2: Chromatograms from a dialysis experiment with diazepam (200 μM). A) Sample from PB compartment of the dialysis cell; and B) Sample from the microsome compartment of the dialysis cell. DIAZ = diazepam, IS = internal standard (desmethyldiazepam).

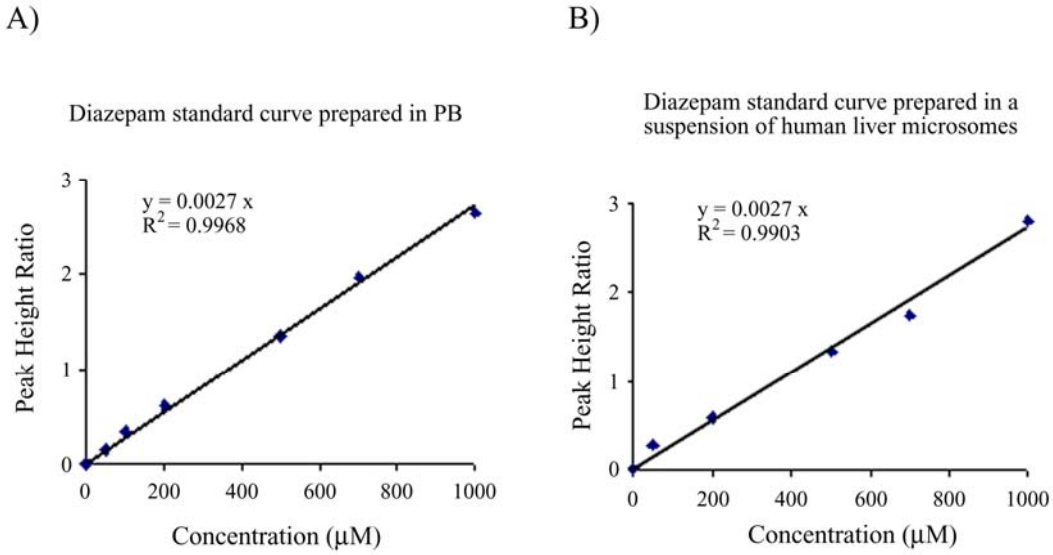


Figure 3.3: Representative standard curves for diazepam prepared in: A) PB and B) a suspension of human liver microsomes in PB (1 mg/ml). Points are experimentally determined values and the solid lines are the linear regression lines of best fit.

3.3.2 Validation of the drug assay and equilibrium dialysis methods

3.3.2.1 Drug assay validation

Overall between day assay imprecision and inaccuracy for the individual assay procedures was determined from quadruplicate determinations of dialysis samples, as outlined in Chapter 2, Section 2.3.2.4. Imprecision and inaccuracy were calculated at a 'low', 'medium' and 'high' concentration within the range of concentrations investigated for each drug according to Equation 3.2 and Equation 3.3, respectively. Data are shown in Table 3.3 and Table 3.4.

Equation 3.2:

$$\text{Imprecision} = \frac{\text{SD of all measured sample concentrations}}{\text{Mean of all sample concentrations}} \times 100$$

Table 3.3: Overall assay imprecision for drug standards prepared in a suspension of human liver microsomes in PB (1 mg/ml) and in PB alone

Imprecision (%)						
Drug	**Human liver microsomes**			**Phosphate buffer**		
	Low	**Med**	**High**	**Low**	**Med**	**High**
Atenolol	9.7	7.4	3.0	2.7	1.0	2.1
Propranolol	2.4	3.3	2.2	3.8	3.2	1.3
Imipramine	1.8	1.8	1.8	2.4	1.5	1.9
Diazepam	9.1	1.2	0.7	0	1.5	2.7
Lignocaine	2.8	4.1	2.9	2.4	1.0	5.1
Bupivacaine	8.4	5.8	2.5	8.4	0	0
Ropivacaine	3.4	4.8	4.5	5.1	0.9	1.5

Equation 3.3:

$$Y = \frac{\text{Mean of all measured sample concentrations}}{\text{Known added drug concentration}} \times 100$$

Inaccuracy (%) = 100 - Y

Table 3.4: Overall assay inaccuracy for drug standards prepared in a suspension of human liver microsomes in PB (1 mg/ml) and in PB alone

Inaccuracy (%)						
Drug	**Human liver microsomes**			**Phosphate buffer**		
	Low	**Med**	**High**	**Low**	**Med**	**High**
Atenolol	10.0	4.0	6.3	9.0	12.0	1.6
Propranolol	9.0	9.9	14.0	9.2	11.7	10.8
Imipramine	0.8	5.7	13.7	2.8	4.3	9.3
Diazepam	6.4	13.0	0.5	10.0	5.8	8.4
Lignocaine	8.0	1.5	8.7	10.2	7.5	6.4
Bupivacaine	6.0	0.9	3.5	6.0	4.5	3.6
Ropivacaine	6.4	0.9	3.4	8.0	1.7	5.1

3.3.2.2 Equilibrium dialysis validation

3.3.2.2.1 Equilibrium dialysis reproducibility

Binding experiments were performed in singlicate to maximise the number of concentrations that could be investigated for each drug on each day. However, to increase the number of data points for the calculation of binding constants, experiments with each drug were repeated on at least three different days. Between day precision of the microsomal binding of each drug was assessed from the repeated measurement of $f_{u(mic)}$ at two different substrate concentrations. Results are

summarised in Table 3.5, which shows the fraction of unbound drug in microsomes and the standard deviation determined from at least three experiments. Standard deviations were less than or equal to 10% of the $f_{u(mic)}$ range at both the high and low concentrations.

Table 3.5: Variation in the determination of $f_{u(mic)}$ from dialysis experiments, using 1 mg/ml human liver microsomes and drug concentrations of 100 and 500 μM (unless otherwise stated)

Drug	**$f_{u(mic)}$ ± SD**	
	100 μM	**500 μM**
Atenolol	1.02 ± 0.07	1.00 ± 0.00
Propranolol	0.48 ± 0.05	0.59 ± 0.06[b]
Imipramine	0.48 ± 0.01	0.69 ± 0.04
Diazepam	0.76 ± 0.06	0.76 ± 0.03
Lignocaine	1.08 ± 0.10[a]	1.04 ± 0.06
Bupivacaine	0.96 ± 0.06	0.97 ± 0.03
Ropivacaine	0.94 ± 0.03[a]	0.97 ± 0.03

[a] $f_{u(mic)}$ measured at 50 μM

[b] $f_{u(mic)}$ measured at 400 μM

3.3.3 Human liver microsomal binding of drugs

3.3.3.1 Effect of varying log P on the binding of drugs to human liver microsomes

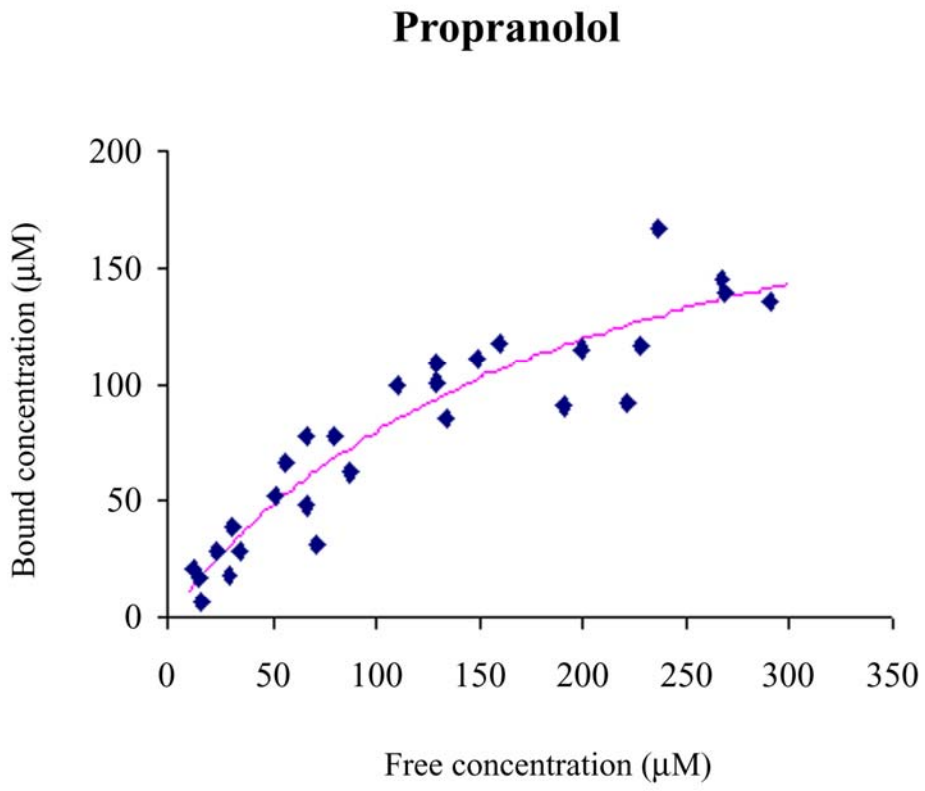
As noted in Section 3.1, atenolol, propranolol and imipramine were selected to systematically test the effect of varying log P, at a constant pK_a and molecular mass (Table 3.1, Group 1), on the non-specific binding of drugs to human liver microsomes. Values of $f_{u(mic)}$ for this group of drugs are shown in Table 3.6.

Table 3.6: Binding of propranolol, atenolol and imipramine to human liver microsomes

Drug	**$f_{u(mic)}$ range**
Atenolol	1.00
Propranolol	0.36 – 0.84
Imipramine	0.42 – 0.82

Atenolol did not bind to human liver microsomes. However, propranolol and imipramine bound in a concentration dependent manner. Binding plots for these two drugs are shown in Figure 3.4.

A)



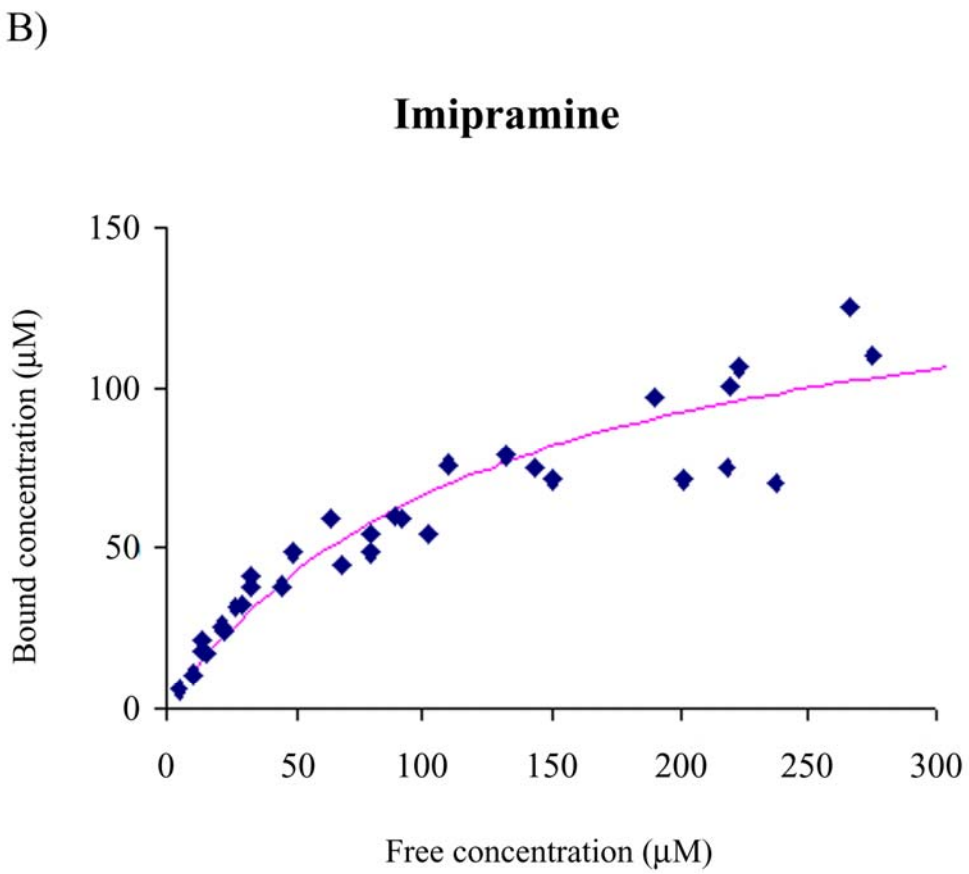


Figure 3.4: Binding plots for A) propranolol and B) imipramine. Points show experimental data while curves were obtained from fitting to Equation 3.1.

Derived K_D (± parameter SE) values for propranolol and imipramine were 200 ± 59 and 303 ± 52 µM, respectively. The respective B_{max} (± parameter SE) values were 238 ± 38 and 186 ± 16 nmol/mg microsomal protein.

3.3.3.2 Effect of varying pK_a on the binding of drugs to human liver microsomes

Propranolol, diazepam and lignocaine were chosen to investigate the effect of varying pK_a (and consequently % ionisation at pH 7.4), while maintaining log P and molecular mass constant, on the non-specific binding of drugs to human liver microsomes (Table 3.1). Drug-microsome binding data are shown in Table 3.7.

Table 3.7: Microsomal binding of drugs with varying pK_a values

Drug	**$f_{u(mic)}$ range**
Diazepam	0.69 - 0.80
Lignocaine	0.98
Propranolol	0.36 - 0.84

As shown in Section 3.3.3.1, propranolol binds extensively to human liver microsomes in a concentration dependent manner. The neutral drug diazepam bound to human liver microsomes to a moderate extent (20 - 31%) over the concentration range tested. Like imipramine and propranolol, the binding of diazepam to human liver microsomes was saturable (Figure 3.5), with K_D and B_{max} values of 933 ± 435 μM and 339 ± 128 nmol/mg microsomal protein, respectively.

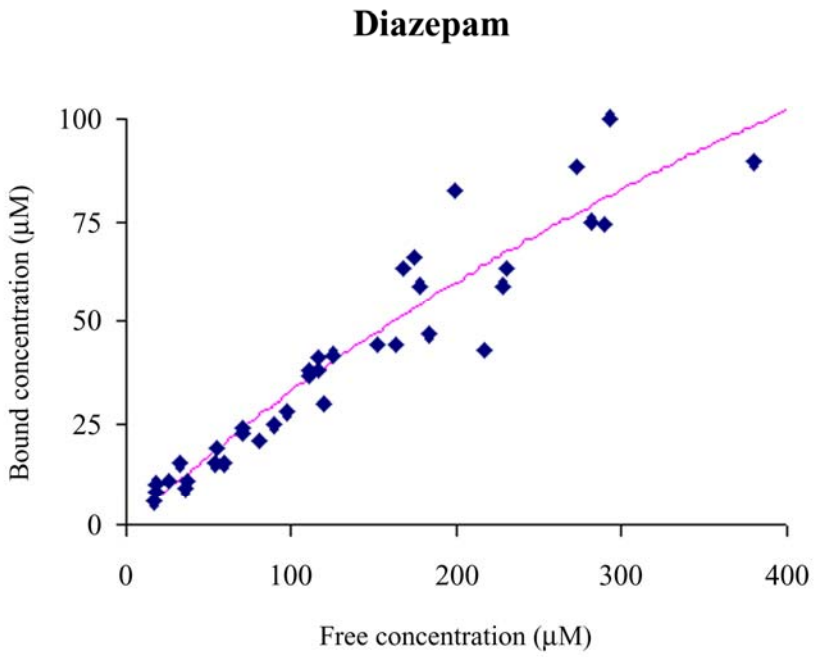


Figure 3.5: Binding plot of diazepam to human liver microsomes. Points show experimental data while curves were obtained from fitting the data to Equation 3.1.

Lignocaine did not bind to human liver microsomes. Over the concentration range tested, $f_{u(mic)}$ was essentially unity. Lignocaine data from the initial dialysis is shown in Figure 3.6.

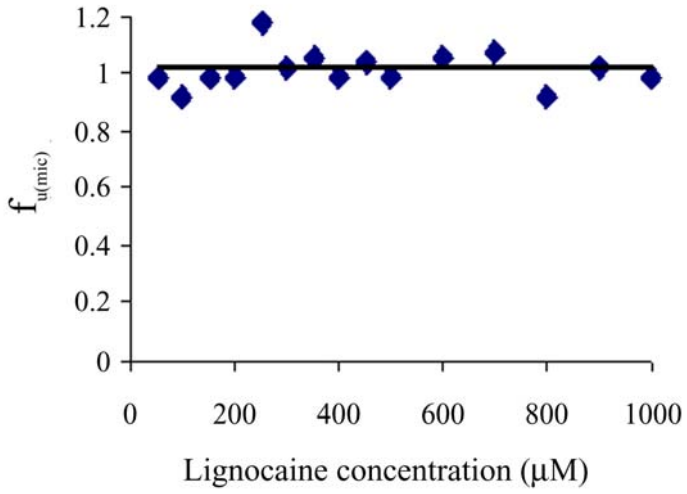


Figure 3.6: Plot of the microsomal binding of lignocaine. The drug concentration range tested was 50 - 1000 μM at a human liver microsomal concentration of 1 mg/ml.

For confirmation, further lignocaine dialysis experiments were performed in triplicate at 50, 500 and 1000 μM. Results were consistent with the data shown in Figure 3.6.

3.3.3.3 Human liver microsomal binding of bupivacaine and ropivacaine

Given the unexpected result for lignocaine, the structurally related compounds bupivacaine and ropivacaine, which have similar log P, pK_a and molecular mass to lignocaine (Table 3.1), were investigated. Equilibrium dialysis experiments were performed in triplicate at two substrate concentrations. Human liver microsomal binding data for these two drugs are shown in Table 3.8. As was observed for lignocaine, neither bupivacaine nor ropivacaine bound to human liver microsomes (Table 3.8).

Table 3.8: Binding of bupivacaine and ropivacaine to human liver microsomes

Drug	$f_{u(mic)} \pm SD$	
	50μM	**500μM**
Bupivacaine	0.99 ± 0.06	0.95 ± 0.02
Ropivacaine	0.97 ± 0.03	0.98 ± 0.03

3.4 Discussion

3.4.1 Human liver microsomal binding of the investigational drugs

The studies performed here systematically investigated the effect of lipophilicity (as log P) and percent ionisation on the non-specific binding of drugs to human liver microsomes using equilibrium dialysis. To achieve this, it was necessary to establish HPLC assays for the seven drugs investigated. The assays developed were suitably specific, accurate and precise for their experimental application.

The organic bases atenolol, propranolol and imipramine are all greater than 99% ionised at pH 7.4 (Table 3.1), but exhibit varying degrees of lipophilicity. Atenolol (log P = 0.1) did not bind to human liver microsomes. Propranolol (log P = 3.1) and imipramine (log P = 4.8) bound to a similar extent. Moreover, binding of both compounds was saturable and the respective derived K_D and B_{max} values were of similar order. Conversely, diazepam, lignocaine and propranolol have similar log P values (2.4 – 3.1), but percent ionisation at pH 7.4 varies from 0 to 99.2%. Diazepam, un-ionised at pH 7.4, bound to human liver microsomes to a lesser extent ($f_{u(mic)}$ = 0.69 - 0.8) than the extensively ionised base propranolol ($f_{u(mic)}$ = 0.36 – 0.84). As observed for propranolol and imipramine, binding of diazepam was concentration dependent, although the derived B_{max} and K_D values were higher than

those of propranolol and imipramine. In contrast, the organic base lignocaine (log P = 2.4, ionisation at pH 7.4 = 69%) did not bind to human liver microsomes. To explore this observation further, binding of the structurally related compounds bupivacaine (log P = 3.6, ionisation at pH 7.4 = 83.4%) and ropivacaine (log P = 3.1, ionisation at pH 7.4 = 83.4%) was investigated. Neither compound bound to human liver microsomes.

Atenolol (log P = 0.1) is a very slightly hydrophobic, near fully ionised base at pH 7.4. This compound did not bind to human liver microsomes. Propranolol and imipramine bound to human liver microsomes to a similar extent to two previously studied tricyclic antidepressants, amitriptyline and nortriptyline (McLure, Miners & Birkett 2000). Imipramine, which is also a tricyclic antidepressant, is structurally related to amitriptyline and nortriptyline. All four drugs exhibit comparable pK_a and log P values. At a microsomal protein concentration of 1 mg/ml and a drug concentration of 100 μM, respective $f_{u(mic)}$ values for nortriptyline, amitriptyline, propranolol and imipramine were 0.40, 0.42, 0.48, and 0.47 (present study and McLure, Miners & Birkett 2000). Furthermore, all four drugs exhibited saturable binding to human liver microsomes at a human liver microsomal protein concentration of 1 mg/ml. Respective B_{max} and K_D values (± SD) for nortriptyline, amitriptyline, propranolol and imipramine are: 382 ± 54 nmol/mg/microsomal protein, 375 ± 23 nmol/mg/microsomal protein, 238 ± 38 nmol/mg/microsomal protein and 186 ± 16 nmol/mg/microsomal protein; and 147 ± 44 μM, 178 ± 33 μM, 200 ± 59 μM, and 303 ± 52 μM. In a previously published investigation the free fractions of imipramine and propranolol at a drug concentration of 100 μM and 1 mg/ml human liver microsomes were reported as 0.22 and 0.44, respectively (Obach

1997). The discrepancy in the lower $f_{u(mic)}$ for imipramine reported by Obach (1997) is unclear, although methods vary slightly between laboratories. Obach's studies included 3.3 mM magnesium chloride in the buffer side of the dialysis cell, used radiolabelled drug and a different drug analysis method. However, the $f_{u(mic)}$ for imipramine observed here was close in value to that reported for the structurally related compound amitriptyline.

Diazepam, the only neutral drug investigated here, bound saturably to human liver microsomes, with $f_{u(mic)}$ values of 0.69 - 0.8 over the drug concentration range tested. In a previous study, the structurally related compounds midazolam and triazolam were dialysed against 1 mg/ml human liver microsomes at a drug concentration of 1 μM (Obach 1999). Consistent with the observations for diazepam, ($f_{u(mic)}$ = 0.76 at 100 μM), midazolam and triazolam $f_{u(mic)}$ values were 0.88 and 0.78, respectively.

The lack of binding of lignocaine to human liver microsomes was unexpected. Lignocaine is a moderately lipophilic compound. All previous studies, and other observations reported in this chapter, indicated that basic organic drugs with experimental log P values > 1.3 bind significantly to human or rat liver microsomes (Obach 1997, 1999; McLure, Miners & Birkett 2000; Hemeryck, De Vriendt & Belpaire 2001; Kalvass et al. 2001; Austin et al. 2002; Margolis & Obach 2003). The lignocaine result is the first observation of a reasonably lipophilic basic drug not binding to human liver microsomes. The non-binding of lignocaine to human liver microsomes was confirmed in multiple experiments. It was further shown that the structurally related compounds bupivacaine (log P = 3.4) and ropivacaine (log P = 2.2) did not bind to human liver microsomes.

3.4.2 Physicochemical determinants of a drug binding to human liver microsomes

Binding of drugs to the membranes of subcellular fractions has been recognised for decades, however the physicochemical determinants responsible for this type of binding are not fully understood. Most publications consider the microsomal binding of basic compounds as a function of charge and lipophilicity (Bickel & Steele 1974; Di Francesco & Bickel 1977; Obach 1997; McLure, Miners & Birkett 2000; Hemeryck, De Vriendt & Belpaire 2001). In particular, these reports implicitly assume that lipophilic basic compounds bind the microsomal milieu extensively. Clearly, however, exceptions occur. The lipophilic bases lignocaine, bupivacaine and ropivacaine do not bind to human liver microsomes (present study). Published data for acidic compounds suggest lesser or no binding to microsomes (Bickel & Steele 1974; Birkett 1974; Obach 1997; Carlile et al. 1999; McLure, Miners & Birkett 2000; Austin et al. 2002), although the acidic compound tenidap exhibits significant non-specific binding ($f_{u(mic)}$ value of 0.32 at a microsomal protein concentration of 3 mg/ml; Obach 1999). Less work has been performed on the microsomal binding of neutral compounds, but available data suggests an intermediate level of binding compared to basic and acidic compounds (Obach 1999; Austin et al. 2002).

3.4.2.1 Microsomal membrane structure

The non-specific binding of drugs to human liver microsomes requires consideration of the physicochemical characteristics of both the drug and the microsomal membrane.

All eukaryotic membranes are comprised of phospholipids, cholesterol, and proteins, the relative proportions of which vary between the membranes of specialised cells and subcellular components (Yeagle 1993). Membrane lipids generally have an

amphipathic structure, where one end of the molecule has a polar group (the 'head') whilst the remainder of the molecule is a hydrophobic hydrocarbon chain (the 'tail'). The lipids organise in an aqueous environment forming a phospholipid bilayer with the polar head facing the aqueous environment and the hydrocarbon chains facing inward creating a hydrophobic core. Phosphatidylcholine (PC), phosphatidyl-ethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylinositol (PI), and sphingomyelin (SM) are the primary lipids of cell membranes. The lipid composition of membrane preparations of human and rat liver microsomes is given in Table 3.9.

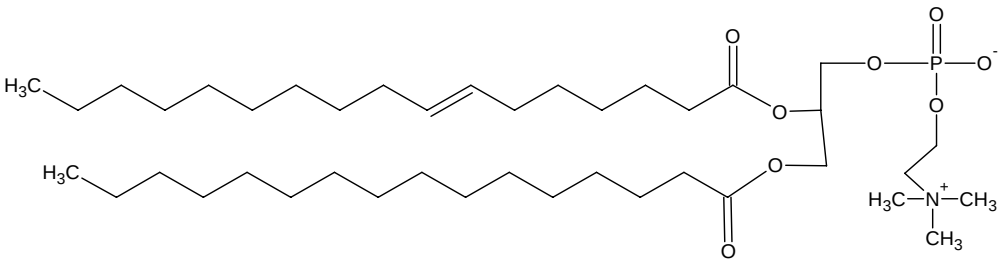
Table 3.9: Major phospholipid composition of liver microsomal membranes from adults of the human and the rat. Adapted from (Kapitulnik et al. 1987).

	Percent of total phospholipids				
Source	**PC***	**PE**	**PI**	**PS**	**SM**
Human	42	31	12	5	4
Rat	58	19	7	8	3

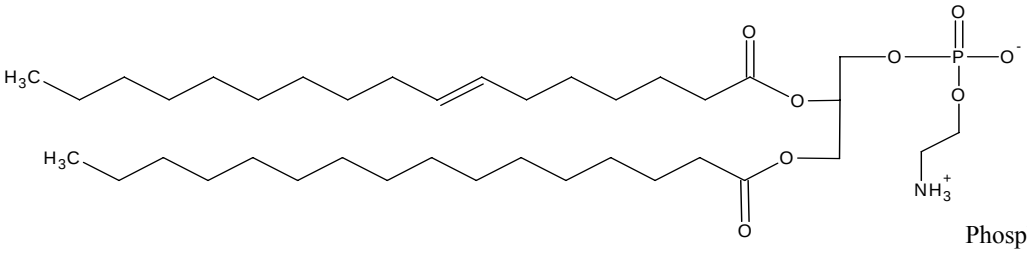
* see text above for abbreviations

The chemical structure of the polar head of the lipid determines the surface charge exposed to the aqueous environment. Phosphatidylcholine carries a full negative charge on the phosphate and a full positive charge on the quaternary ammonium at physiological pH (Figure 3.7). Thus, phosphatidylcholine is zwitterionic and electrically neutral. This is also the case for phosphatidylethanolamine and sphingomyelin. Phosphatidylinositol carries a negative charge at pH 7.4 and it is possible that the hydroxyl groups of inositol may additionally form ester bonds with phosphate. Phosphatidylserine contains three charged groups; a negatively charged phosphate, a positively charge amino, and a negatively charge carboxyl (Figure 3.7), thus exhibiting an overall negative charge. Hence, the microsomal membrane carries a degree of negative surface charge at pH 7.4.

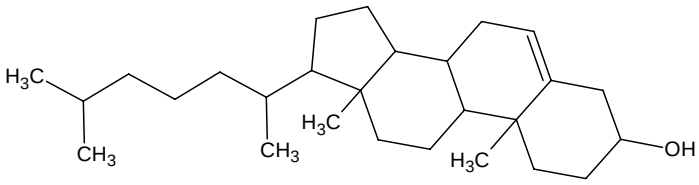
Neutron diffraction and calorimetric studies suggest that drugs may associate with both the hydrocarbon core and polar head group region of synthetic and native biological membranes. In particular, the naphthalene ring of propranolol (a β-adrenoceptor antagonist) associates with the first few methylene groups of the fatty acid acyl chain (about 10 Å from the bilayer surface), while the charged amine of the side chain appears to position close to the phosphate moiety of the phospholipid head group (Herbette, Katz & Sturtevant 1983; Herbette, Chester & Rhodes 1986). Nimodipine (a dihydropyrimidine calcium channel blocker) also appeared to bind to membranes at the interface of the head groups and hydrocarbon core, with more of the drug within the inner region of the monolayer (Herbette, Katz & Sturtevant 1983). Timolol is less lipophilic than propranolol, with potentially charged groups at either end of the molecule; the amino group has a pK_a of 9.4 and the nitrogen on the morpholino ring has a pK_a of 6.9. Herbette et al. (1983) proposed that the additional charge on timolol reduced its penetration into the bilayer, with most of the molecule residing in the polar head group region. While these observations implicate charge and lipophilicity as key determinants of non-specific binding, relationships are not clear cut.



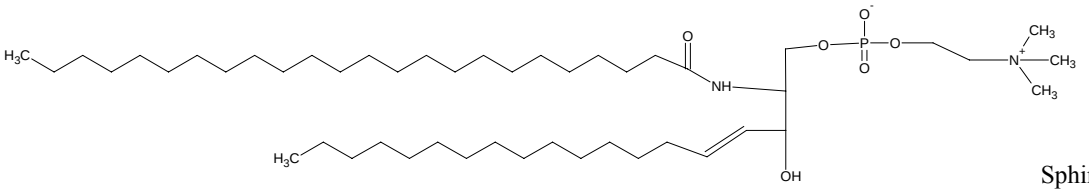
Phosphatidylcholine



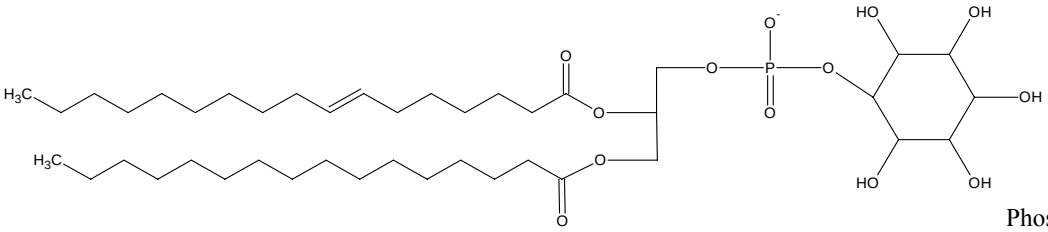
Phosphatidylethanolamine



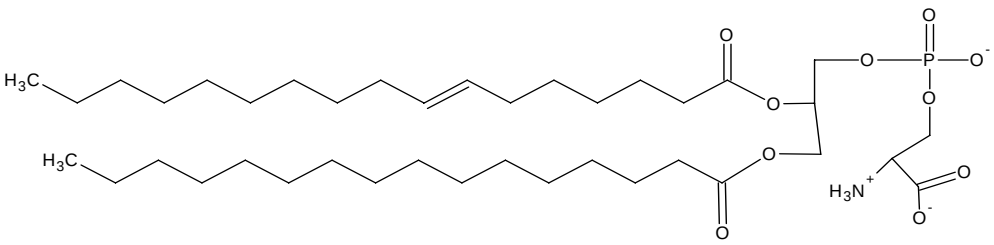
Cholesterol



Sphingomyelin



Phosphatidylinositol



Phosphatidylserine

Figure 3.7: Chemical structures of microsomal membrane lipids

3.4.2.2 Charge and extent of ionisation

As discussed previously, binding to human liver microsomes has been reported for organic bases, organic acids, and neutral organic compounds. In general, the extent of binding of bases tends to be greater than that observed for acids and neutral drugs. Nevertheless, these observations suggest that the presence of a basic or acidic function is not obligatory for binding. Similarly, binding is not restricted to just one type of charge, although in general basicity is associated with greater binding.

Further exploration of drug charge as a determinant of microsomal binding requires an examination of the extent of ionisation at the experimental pH. Previous studies have also investigated the microsomal binding of a range of basic, neutral and acidic drugs that display a varying extent of ionisation at the experimental pH, typically 7.4 or 7.5 (Obach 1999; McLure, Miners & Birkett 2000; Austin et al. 2002). Data for bases are summarised in Table 3.10. There is no clear relationship between extent of ionisation of basic drugs at the experimental pH and extent of binding to microsomes. For instance, in Table 3.10 the bases predicted to be ≥ 99 percent ionised exhibit $f_{u(mic)}$ values ranging from 0.009 to 1.

Table 3.10: Basic drugs predicted ionisation state and free fraction in microsomes

Drug	Predicted % ionisation	$f_{u(mic)}$	Reference
Atenolol	99.4	1	Present study
Desipramine	99.4	0.12	(Obach 1999)
Propafenone	99.4	0.15	(Austin et al. 2002)
Nortriptyline	99.3	0.35	(McLure, Miners & Birkett 2000)
Propranolol	99.2	0.44, 0.48	(Obach 1999), Present study
Imipramine	99.2	0.16, 0.48	(Obach 1999), Present study
Thioridazine	99.2	0.009	(Austin et al. 2002)
Betaxolol	99	0.62	(Austin et al. 2002)

Drug	Predicted % ionisation	$f_{u(mic)}$	Reference
Clomipramine	99	0.038	(Austin et al. 2002)
Amitriptyline	99	0.35	(McLure, Miners & Birkett 2000)
Chlorpromazine	98.8	0.11	(Obach 1999)
Trimeprazine	98.4	0.08	(Austin et al. 2002)
Promethazine	98	0.11	(Austin et al. 2002)
Diphenhydramine	97.6	0.71	(Austin et al. 2002)
Verapamil	96.9	0.37	(Austin et al. 2002)
Tamoxifen	95.2	0.003	(Austin et al. 2002)
Pimozide	94.1	0.007	(Austin et al. 2002)
Quinidine	94.1	0.7	(Obach 1999)
Astemizole	90.9	0.012	(Austin et al. 2002)
Bupivacaine	83.4	0.96	Present study
Ropivacaine	83.4	0.94	Present study
Clozapine	79.9	0.26	(Austin et al. 2002)
Lignocaine	69	1.08	Present study
Diltiazem	66.6	0.86	(Obach 1999)
Ketamine	55.7	0.49	(Obach 1999)
Amiodarone	12.5	< 0.01	(McLure, Miners & Birkett 2000)

All studies used a microsomal protein concentration of 1 mg/ml. Obach (1999) and Austin et al. (2002) data were generated at a drug concentration of 1 μM, whereas McLure, Miners & Birkett (2000) reported $f_{u(mic)}$ values for nortriptyline and amitriptyline at 20 μM. Results for amiodarone and those of the present study results were obtained using a drug concentration of 100 μM.

The effect that the extent of ionisation of acidic drugs has on microsomal binding is not clear either. Seventeen acids, each ≥ 92% ionised under experimental conditions, exhibited $f_{u(mic)}$ values ranging from 0.65 – 0.99 at 1 mg/ml rat liver microsomal protein concentration and a drug concentration of 1 μM (Austin et al. 2002). Notably, the $f_{u(mic)}$ range is less than that of basic compounds. The repulsive electrostatic interaction with the net negatively charged microsomal membrane has been suggested as the reason for the lower extent of non-specific binding of acidic

compounds (Birkett 1974; McLure, Miners & Birkett 2000). The non-specific binding of nineteen neutral drugs highlights the intermediate level of binding of this class of compound. Observed $f_{u(mic)}$ values ranged from 0.34 for isradipine to 0.98 for 2-ethoxybenzamide (Austin et al. 2002).

3.4.2.3 Lipophilicity

An early study by Bickel et al. (1975) reported the uptake of lipophilic drugs into hepatocytes and subsequent binding to the endoplasmic reticulum. This study, together with two recent reports (Austin et al. 2002; Margolis & Obach 2003), implicate lipophilicity as the major, if not sole, determinant of the non-specific membrane binding of drugs.

A recent study using rat liver microsomes (1 mg/ml microsomal protein concentration) reported that the most extensive binders were the highly lipophilic compounds amiodarone, pimozide, tamoxifen and thioridazine (Austin et al. 2002). All of these compounds have log P values greater than 5.6 and exhibit a free fraction in microsomes of less than 0.01 (Austin et al. 2002). It is noteworthy that all four compounds are bases. Conversely, the hydrophilic (cinoxacin) and near hydrophilic (atenolol log P = 0.1) compounds, which have log P values ≤ 0.1, do not bind non-specifically to liver microsomes (McLure, Miners & Birkett 2000; Austin et al. 2002). It therefore appears that the two extremes of drug binding to human liver microsomes can be predicted. Greatest binding, $f_{u(mic)} < 0.01$, occurs with highly lipophilic basic compounds (log P > 5.6), whereas hydrophilic compounds, irrespective of charge, do not bind to human liver microsomes.

For drugs with log P values in the range 0.2 – 5.6 the extent of microsomal binding is variable. However, a significant relationship between lipophilicity (as log P or log D,

denoted as log P/D) and non-specific microsomal binding (as log $(1\text{-}f_{u(inc)})/ f_{u(inc)}$) was reported for thirty-seven drugs (Austin et al. 2002). The parameter log $(1\text{-}f_{u(inc)}) / f_{u(inc)}$ was used as the descriptor for binding since it is similar to an equilibrium constant, and was thus considered appropriate for searching for free energy relationships. When log $(1\text{-}f_{u(inc)}) / f_{u(inc)}$ was plotted against log P (basic drugs) or log $D_{7.4}$ (acidic and neutral drugs) a significant linear relationship ($r^2 = 0.82$) was obtained (Austin et al. 2002). The relationship appeared to be strongest for compounds with log P/D values > 2. It was therefore concluded that $f_{u(inc)}$ may be predicted from log P/D, although different relationships may apply for bases, acids and neutral compounds.

The Austin et al. (2002) analysis included four bases with log P values < 3, and for these compounds the model developed tended not to predict $f_{u(inc)}$ accurately. Of note, Hallifax & Houston (2006a) described a non linear empirical quadratic relationship between $f_{u(inc)}$ and log P/D which was to be an considered improvement on the suggested bias predictions based on a linear relationship. In agreement with the data of Obach (1999), and data presented here in this chapter, Hallifax & Houston (2006a) suggest that neutral compounds are mostly moderately bound. While Austin et al (2002) and Hallifax & Houston (2006a) describe relationships between all drugs, log P/D and microsomal binding, Sykes et al (2006) separated drugs into acids, bases and neutral compounds and compared log P/D to binding. It was shown that log P methods for predictivity were only reliably productive for bases. It is clear that, despite the conclusions of Austin et al (2002), $f_{u(inc)}$ or $f_{u(mic)}$ is not solely dependent on log P. The observations reported in this chapter for lignocaine, bupivacaine and ropivacaine (log P 2.4 – 3.6) further demonstrate that non-specific binding is not determined by log P alone.

Permeability and absorption studies may provide direction for unraveling other factors that influence the non-specific microsomal binding of drugs. For example, specific descriptors such as dynamic surface properties (i.e. charge distribution) and polar surface area of a drug, rather than charge and consequent percent ionisation at pH 7.4, were used to predict drug membrane permeability and to correlate to drug absorption (Palm et al. 1996; Osterberg & Norinder 2000). These may also be relevant to drug-membrane interactions.

CHAPTER 4
DEVELOPMENT OF A FLUORESCENCE TECHNIQUE FOR MEASURING THE NON-SPECIFIC BINDING OF DRUGS TO HUMAN LIVER MICROSOMES

4.1 Introduction

From the equilibrium dialysis data presented in Chapter 3 and the subsequent discussion, it is apparent that physicochemical determinants other than log P, charge and extent of ionisation contribute to the non-specific microsomal binding of drugs. Elucidation of the physicochemical determinants of the human liver microsomal binding of drugs would be facilitated by datasets larger than those currently generated by equilibrium dialysis (and HPLC analysis), which is a laborious and time consuming procedure. Thus, a potentially high throughput fluorescence technique for assessing drug-microsome binding of a structurally and physicochemically diverse range of drugs was developed.

Fluorescent probes permit investigation of particular components of biomolecular assemblies, including cell membranes, with both sensitivity and selectivity (Haughland 1996). Membrane related fluorescence studies span fifty years (Radda & Vanderkooi 1972). Newton (1954) initially observed that 1-toluidinonaphthalene-8-sulphonate (TNS) showed a significant fluorescent enhancement when bound to a bacterial membrane, and that this effect could be used to monitor the damage produced in the membrane by the antibiotic, polymyxin B. Following this early report, fluorescence studies were directed more towards understanding the structure, interactions, and dynamics of proteins and enzymes (Weber 1960; Edelman & McClure 1967; Stryer 1968). However, a reemergence of fluorescence related membrane studies occurred when the binding of 8-anilinonaphthalene-1-sulfonate

(ANS) to haemoglobin-free rabbit erythrocyte membranes was demonstrated (Rubalcava, Martinez de Munoz & Gitler 1969). Following this observation, several groups reported that ANS fluorescence was enhanced in the presence of microsomes, and that the binding site of ANS was in a hydrophobic region of the microsomal membrane (Vanderkooi & Martonosi 1969; Diaugustine, Eling & Fouts 1970; Eling & Diaugustine 1971; Vanderkooi & Martonosi 1971; Dallner & Azzi 1972; Birkett 1974; Zierler & Rogus 1978).

It has been shown that there are two types of binding sites for ANS in biological membranes, membrane proteins and membrane lipids (Slavik 1982). The number of protein binding sites for ANS on biological membranes is lower than the number of lipid binding sites but their affinity for ANS is greater, thus at low ANS concentration the probe would first bind to proteins and then subsequently to lipids (Slavik 1982). At commonly used ANS concentrations (5-20 μM) most of the protein binding sites are fully occupied by ANS and the fluorescence of lipid bound ANS predominates in the total fluorescence simply because of the larger number of lipid-ANS binding sites. This is the reason why the fluorescence of membrane bound ANS often strongly resembles that of liposome bound ANS, especially at higher ANS concentrations (Slavik 1982).

A model has been proposed for the binding site of ANS in lipids (Haynes & Staerk 1974). Experimental evidence showed that the maximal binding of ANS was one ANS molecule per four phosphatidylcholine molecules. Furthermore, x-ray diffraction and nuclear magnetic resonance studies, as well as considerations of the quantum yield in the bound form, indicate that ANS is located in the polar head group region of membranes (Figure 4.1). Specifically, ANS is attracted

electrostatically to the quaternary ammonium head of phosphatidylcholine and then becomes bound in a hydrophobic pocket between the four polar head groups of phosphatidylcholine (Slavik 1982). The sulfonate group is assumed to align with the charged choline 'head'.

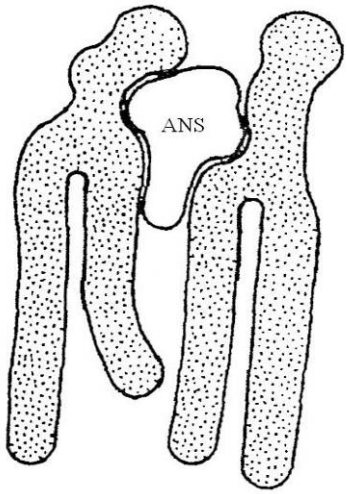


Figure 4.1: Proposed two dimensional space filling model of the packing of an ANS molecule in the hydrophobic pocket formed by four phosphatidylcholine molecules. Adapted from Slavik (1982).

The ANS binding reaction may thus be considered to arise from insertion of ANS between four phosphatidylcholine head groups with displacement of water from this region. Apart from the displacement of water there is no evidence that ANS binding alters any other membrane property (Haynes & Staerk 1974). Factors other than electrostatic interactions also appear to influence ANS binding since phosphatidylethanolamine binds little or no ANS. Phosphatidylserine and phosphatidylinositol also minimally bind ANS, while phosphatidic acid liposomes exhibit no ANS binding.

The number of ANS molecules bound to the membrane is strongly influenced by the surface charge of the membrane; it is inversely proportional to the negative membrane surface potential (Slavik 1982). The presence of cations or anions, or basic or acidic drugs, can affect the surface charge of the membrane and hence ANS binding and fluorescence. In particular, neutralisation of the negative charge associated with the phosphate head favours ANS binding.

The studies of Vanderkooi and Martonosi (1969), Vanderkooi and Martonosi (1971), and Dallner and Azzi (1972) showed fluorescence enhancement of microsomal membrane bound ANS after the addition of monovalent, divalent and trivalent cations. A range of positively charged drugs was additionally shown to increase the fluorescence of ANS in the presence of microsomes (Diaugustine, Eling & Fouts 1970; Hawkins & Freedman 1973; Birkett 1974). Conversely, acidic drugs and primary aliphatic alcohols were shown to decrease the fluorescence of ANS in the presence of microsomes (Diaugustine, Eling & Fouts 1970; Birkett 1974). No changes in fluorescence lifetime or the emission maximum of ANS were found and no significant changes in the quantum yield of ANS fluorescence occurred on addition of charged ions or molecules (Birkett 1974).

The changes in fluorescence were shown to result from the varying degree of ANS binding to microsomes (Birkett 1974). Cations and basic drugs caused an increase in the amount of ANS bound to microsomes whereas alcohols and acidic drugs caused a decrease in the amount of bound ANS. Neutral compounds have been shown to cause a decrease, like alcohols and acidic drugs, in the amount of bound ANS. The mechanism by which neutral compounds cause a decrement in ANS fluorescence (Birkett 1974) is yet to be described. The mechanisms proposed for the charged ion/drug induced changes of ANS fluorescence in microsomes are as follows:

- neutralization of microsomal surface charge by metal cations or basic drugs allows more ANS to bind to microsomes and fluoresce (Dallner & Azzi 1972; Slavik 1982)
- negatively charged species change the net charge on the membrane, either by exposure of negative charges or occlusion of positive charges, decreasing the amount of ANS which binds to microsomes and the fluorescence OR negatively charged species competitively displace ANS from phosphatidylcholine binding sites in microsomes (Birkett 1974; Slavik 1982).

As positively charged molecules enhance ANS fluorescence and negatively charged and neutral molecules decrease ANS fluorescence, it was hypothesised that drug induced shifts from baseline ANS fluorescence will provide a measure of the non-specific binding of drugs to human liver microsomes. The drugs characterised for binding to human liver microsomes using the ANS fluorescence technique developed in this chapter were those compounds investigated by equilibrium dialysis in Chapter 3.

4.2 Methods

4.2.1 Fluorescence procedure

The ANS fluorescence technique employed was modified from the procedure employed in previous studies (Diaugustine, Eling & Fouts 1970; Dallner & Azzi 1972; Birkett 1974). Human liver microsomes were prepared as described in Chapter 2, Section 2.2 and all fluorescence experiments were performed at 25°C.

4.2.2 ANS fluorescence protocol

Ten 10 μL aliquots of separate 20 mM aqueous stock solutions of imipramine, atenolol, bupivacaine, ropivacaine, lignocaine, and propranolol were added sequentially to 2 ml of PB containing ANS (10 μM) and human liver microsomes (0.25 mg/ml) in a 4.5 ml glass cuvette to produce drug concentrations in the range 100 – 1000 μM. In the case of diazepam, which was dissolved in methanol, four 5 μL aliquots of a 20 mM stock solution were added sequentially to 2 ml of PB containing ANS (10 μM) and human liver microsomes (0.25 mg/ml) in a 4.5 ml glass cuvette to produce drug concentrations in the range 50 – 200 μM. ANS fluorescence, recorded prior to and after addition of the drug, was measured using a Perkin Elmer 3000 Fluorescence Spectrometer. Excitation and emission wavelengths were 375 nm and 470 nm, respectively (Molecular Probes, Leiden, The Netherlands). All measurements were performed at least in duplicate.

4.2.2.1 ANS quenching control protocol

Fluorescence quenching reduces the fluorescence quantum yield without changing the fluorescence emission spectrum; it can result from transient excited state interactions (collisional quenching) or from formation of non-fluorescent ground state species (Haughland 1996).

A 100 μL aliquot of the drug solvent was added to 2 ml of an ethanolic solution of ANS (10 μM) in a 4.5ml glass cuvette. Readings were taken prior to and after the addition of the solvent. The cuvette was rinsed with ethanol and the procedure was repeated following the addition of 100 μL of a 20 mM solution of the drug in the solvent (i.e. water or methanol). Drugs that exhibited quenching were not investigated further. For the purposes of this thesis quenching was taken as a greater

than 15% decrement in fluorescence on addition of drug to the ethanolic solution of ANS.

4.2.2.2 Effect of solvents on ANS fluorescence in suspensions of human liver microsomes

Aliquots of solvent were added sequentially (10 × 10 μL for distilled water and 4 × 5 μL for methanol) to 2 mL of PB containing ANS (10 μM) and human liver microsomes (0.25 mg/ml). ANS fluorescence readings were recorded before and after the addition of the solvent.

4.2.2.3 Drug fluorescence in suspensions of human liver microsomes

Solutions of drugs (20 mM) were added sequentially (10 × 10 μL for drugs dissolved in distilled water and 4 × 5 μL for the methanolic solution of diazepam) to 2 ml PB containing human liver microsomes (0.25 mg/ml). Any fluorescence, at the excitation and emission wavelengths employed for ANS (Section 4.2.1), was recorded.

4.2.2.4 Calculation of fluorescence increment or decrement

The percentage fluorescence increment or decrement in ANS fluorescence due to added drug was calculated as described by Birkett (1974):

Equation 4.1

Percent ANS fluorescence increment/decrement

$$= \left[\frac{\text{fluorescence of ANS in presence of drug and matrix} - \text{drug fluorescence in matrix}}{\text{initial fluorescence of ANS in matrix}} \text{ x } 100 \right] - 100$$

4.3 Results

4.3.1 ANS fluorescence method validation

The initial fluorescence of ANS in the presence of human liver microsomes (0.25 mg/ml) was measured before the addition of drug. The coefficient of variation for the fluorescence of 10 μM ANS in the presence of 2 ml of 0.25 mg/ml human liver microsomes, measured on seventeen separate occasions, was 9%.

Compounds were analysed for quenching using ethanol as the hydrocarbon source (Section 4.2.1.1). None of the drugs investigated in this chapter exhibited quenching. Solvent effects on ANS fluorescence were also determined. Notably, inclusion of methanol above 1% v/v affected ANS fluorescence in human liver microsomes. Consequently, the maximum concentration of methanol present in fluorescence experiments was 1% v/v. The background fluorescence of drug in the experimental matrix was accounted for in the calculation of percent ANS fluorescence increment/decrement (Equation 4.1). It should be noted that percent ANS fluorescence increment/decrement was generally used as the measure of ANS fluorescence as this parameter represents the change from baseline (microsomes plus ANS) due to the effect of added drug. By using this parameter, ANS fluorescence measurements were unaffected by between-run differences in the output of the fluorescence spectrometer.

4.3.2 Effects of selected drugs on the fluorescence of ANS in the presence of human liver microsomes

Drugs characterised for non-specific microsomal binding by equilibrium dialysis in Chapter 3 were investigated using the ANS fluorescence technique described in Section 4.2.1. Results are shown in Table 4.1.

Table 4.1: Effect of test drugs on ANS fluorescence in the presence of human liver microsomes (drug concentration range 50 – 1000 μM)

Drug	Percent fluorescence increment/decrement at various concentrations (μM):											
	50	**100**	**150**	**200**	**300**	**400**	**500**	**600**	**700**	**800**	**900**	**1000**
Atenolol	-	-1	-	1	-2	-3	-4	2	-1	-4	-6	-5
Bupivacaine	-	0	-	1	1	2	3	4	4	4	6	6
Diazepam	-7	-19	-26	-35	-	-	-	-	-	-	-	-
Imipramine	-	46	-	77	107	133	154	176	192	213	225	241
Lignocaine	-	1	-	1	-1	2	-2	-3	-3	-3	-2	-2
Propranolol	-	27	-	47	65	77	86	107	118	131	133	136
Ropivacaine	-	1	-	1	2	4	1	1	1	3	4	3

Atenolol, bupivacaine, lignocaine and ropivacaine, in the drug concentration range 50 – 1000 μM, had little or no effect on ANS fluorescence in the presence of human liver microsomes. Imipramine and propranolol both enhanced ANS fluorescence. There was a hyperbolic relationship between the ANS fluorescence increment and added imipramine and propranolol concentrations (Figure 4.2). In contrast, diazepam decreased ANS fluorescence in a concentration dependent manner.

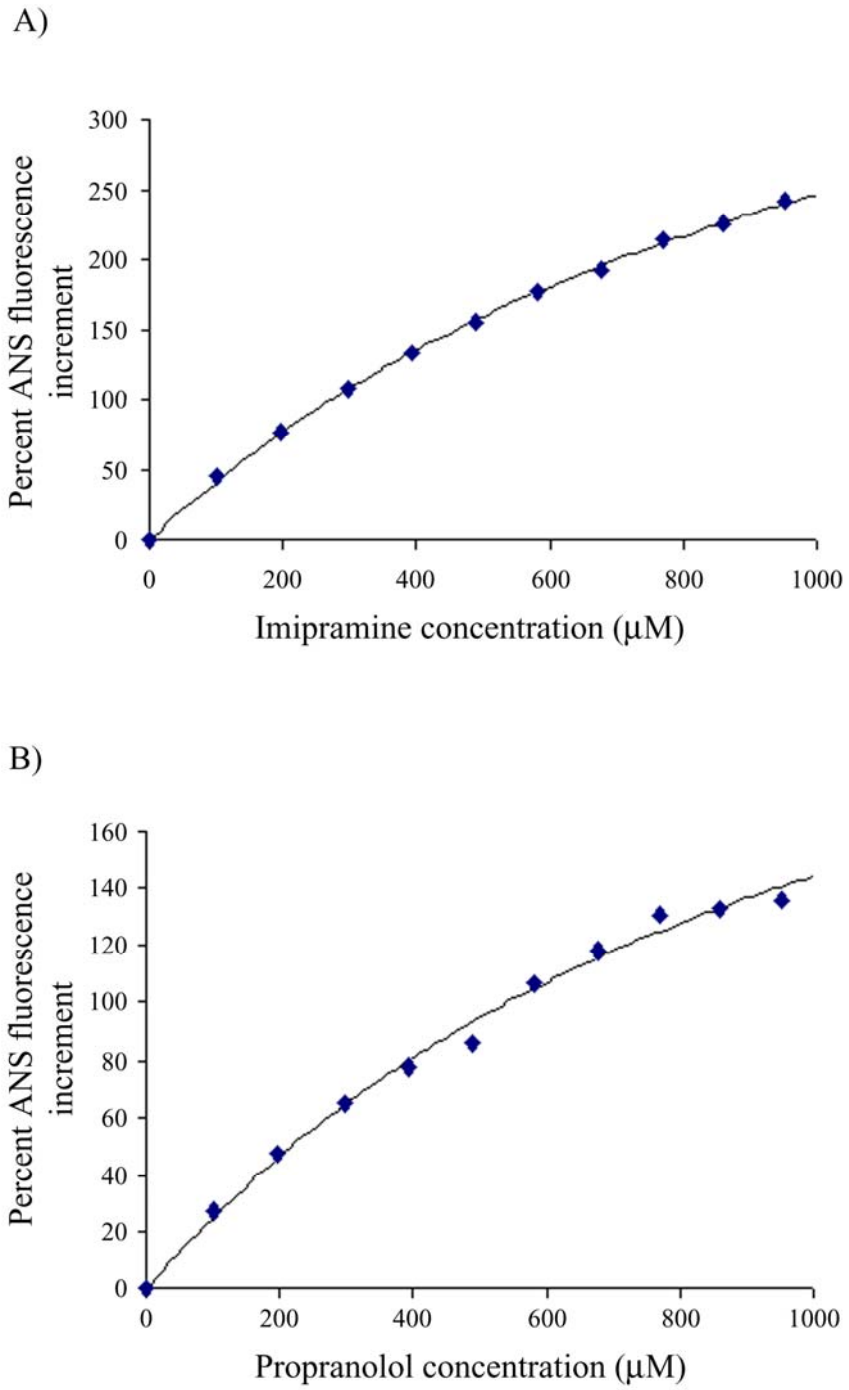


Figure 4.2: Relationship between percent ANS fluorescence increment and added A) imipramine and B) propranolol concentrations

Binding parameters (± SE of parameter fit) were calculated as described in Section 3.2.2. Derived K_D values were 1215 ± 60 and 1112 ± 170 μM for imipramine and propranolol, respectively.

4.3.2.1 Methodological comparison

4.3.2.1.1 Determination of the relationship between $f_{u(mic)}$ and percent ANS fluorescence

Since most drugs were investigated by equilibrium dialysis and ANS fluorescence at both 100 μM and 200 μM, these two concentrations were chosen for further analysis of the relationship between $f_{u(mic)}$ and percent ANS fluorescence increment/decrement. Compounds that bound significantly to human liver microsomes, as determined by equilibrium dialysis, were associated with a significant increment (the bases imipramine and propranolol) or decrement (the neutral compound diazepam) in ANS fluorescence whereas non-binding compounds (atenolol, bupivacaine, ropivacaine and lignocaine) showed minimal change from baseline ANS fluorescence (Table 4.2 and Table 4.3).

Table 4.2: Comparison of $f_{u(mic)}$ and percent ANS fluorescence increment/decrement of investigational drugs at 100 μM

Drug	**Equilibrium dialysis $f_{u(mic)}$**	**Percent ANS fluorescence increment/decrement**
Atenolol	1.00	-1
Bupivacaine[a]	0.98	0
Ropivacaine[a]	0.94	1
Lignocaine	0.92	1
Diazepam	0.75	-19
Propranolol	0.52	27
Imipramine	0.48	45

[a]Bupivacaine and ropivacaine $f_{u(mic)}$ values measured at 50 μM

Table 4.3: Comparison of $f_{u(mic)}$ and percent ANS fluorescence increment/decrement of investigational drugs at 200 μM

Drug	Equilibrium dialysis $f_{u(mic)}$	Percent ANS fluorescence increment/decrement
Lignocaine	1.00	1
Bupivacaine[b]	0.98	1
Atenolol	0.97	1
Ropivacaine[b]	0.97	1
Diazepam	0.77	-35
Propranolol	0.58	47
Imipramine	0.57	77

[b]Bupivacaine and ropivacaine $f_{u(mic)}$ values measured at 500 μM

The results shown in Tables 4.2 and 4.3 were plotted as fraction of unbound drug versus the modulus of the ANS fluorescence increment/decrement. There was a significant correlation ($r^2 = 0.92$) between parameters at both 100 μM and 200 μM (Figure 4.3).

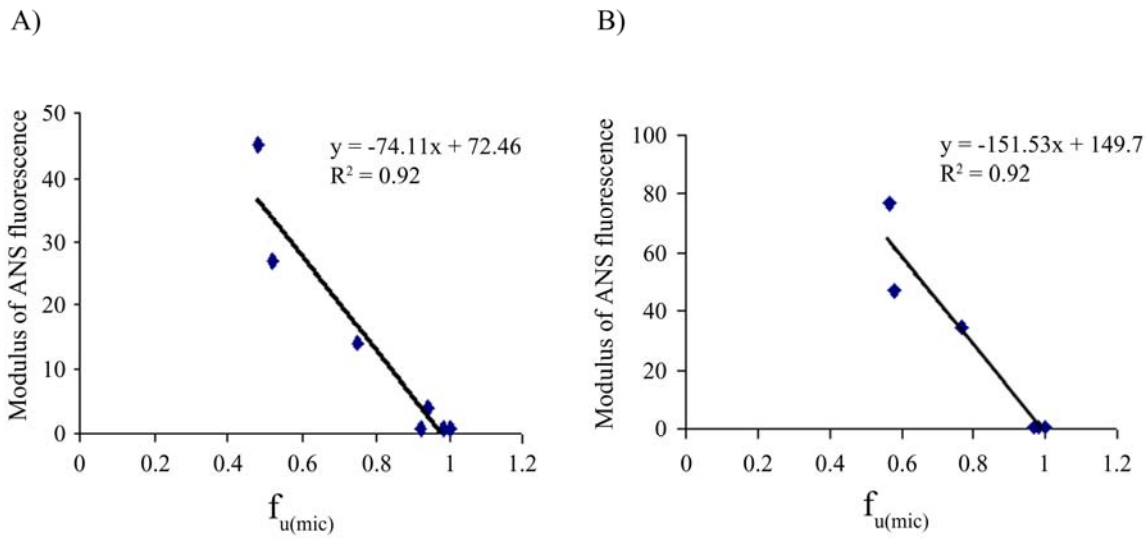


Figure 4.3: Relationship between the modulus of ANS fluorescence increment/decrement and $f_{u(mic)}$ at: A) 100 μM and B) 200 μM drug.

4.3.2.1.2 Relationship between bound drug concentration and percent ANS fluorescence increment

The concentration of imipramine and propranolol bound to microsomes (C_B) determined from equilibrium dialysis experiments (C_B = concentration of drug in the microsome side of the dialysis cell minus the concentration of drug in the buffer side of the dialysis cell) was plotted against the ANS fluorescence increment at the same added drug concentration. Results are shown in Figure 4.4.

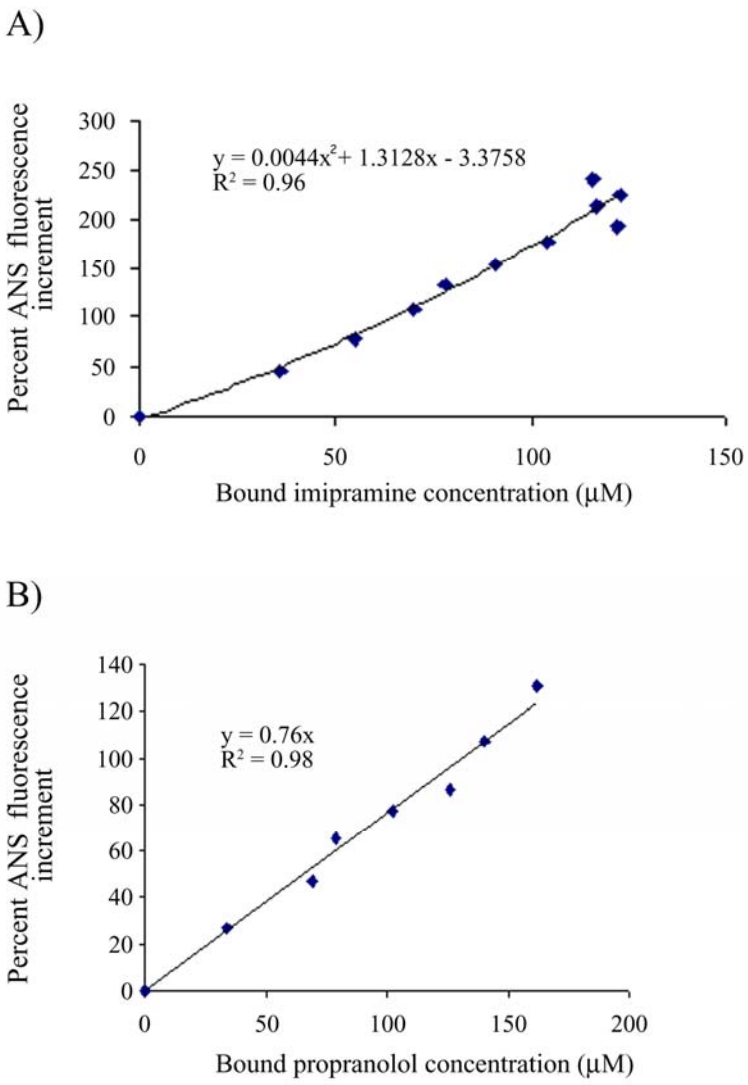


Figure 4.4: Plots of the relationship between the bound drug concentration and percent ANS fluorescence increment for: A) imipramine and B) propranolol

4.4 Discussion

4.4.1 Effect of investigational drugs on ANS fluorescence in the presence of human liver microsomes

The experiments performed in this chapter assessed, in a preliminary manner, changes in ANS fluorescence as a marker of the non-specific binding of drugs to human liver microsomes. As indicated previously, equilibrium dialysis is a time consuming process generally requiring several weeks to establish and validate an HPLC assay and to conduct dialyses. In contrast, the ANS fluorescence method is a potentially high throughput procedure since duplicate titrations per drug can be completed in fifteen minutes.

Atenolol, bupivacaine, lignocaine and ropivacaine caused little or no change in percent ANS fluorescence increment/decrement. Imipramine and propranolol had respective percent ANS fluorescence increment ranging from 45 to 241% and 27 to 136% over the concentration range (100 – 1000 μM) tested. Diazepam, the only neutral drug tested for an effect on ANS fluorescence in the presence of human liver microsomes, displayed an ANS fluorescence decrement ranging from –7% at 50 μM to –35% at 200 μM.

4.4.2 Comparison of ANS fluorescence and equilibrium dialysis methods

The validity of the ANS fluorescence method for measuring non-specific binding of drugs to microsomes was demonstrated, albeit with a small test set. Consistent with previous studies (Rubalcava, Martinez de Munoz & Gitler 1969; Vanderkooi & Martonosi 1969; Diaugustine, Eling & Fouts 1970; Dallner & Azzi 1972; Hawkins & Freedman 1973; Birkett 1974), basic compounds that bind to human liver microsomes, namely propranolol and imipramine, were shown to enhance ANS

fluorescence in the presence of microsomes. Using these two drugs, a relationship between bound drug concentration (from equilibrium dialysis experiments) and percent ANS fluorescence increment was apparent (Figure 4.4). The significant relationships observed here support the hypothesis that the concentration of drug bound to microsomes is directly related to the change from baseline ANS fluorescence (Diaugustine, Eling & Fouts 1970; Birkett 1974). Consistent with this notion, the non-binding compounds atenolol, bupivacaine, lignocaine and ropivacaine (Chapter 3) exhibited minimal effect on baseline ANS fluorescence in the presence of human liver microsomes (Table 4.1).

In comparing the binding parameters from the two techniques, order between the extent of microsomal binding of drugs from equilibrium dialysis and ANS fluorescence methods was observed (Table 4.2 and Table 4.3). Furthermore, there was a significant linear relationship ($r^2 = 0.92$) between $f_{u(mic)}$ and the modulus of percent ANS fluorescence for added drug concentrations 100 μM and 200 μM (Figure 4.3). Importantly, the gradient of the linear regression line of best fit at 200 μM (y = -151x) was twice that of the gradient of the linear regression line of best fit at 100 μM (y = -74x).

A hyperbolic relationship between percent ANS fluorescence increment and added drug concentrations (100 – 1000 μM), was observed for imipramine and propranolol (Figure 4.2). Derived dissociation constants (K_D) were 1215 ± 60 and 1112 ± 170 μM, respectively. The corresponding values from equilibrium dialysis experiments were 303 ± 52 and 200 ± 50 μM, respectively. Thus, an approximate 4- to 5- fold difference in 'apparent' K_D occurs between values determined using the different methods. The apparent lower affinity of both drugs for microsomes determined from

fluorescence experiments presumably reflects the more complex relationship between microsomally bound drug and ANS binding.

The effect of four of the investigational drugs on ANS fluorescence in the presence of microsomes is in agreement with a previously published study. Like lignocaine, bupivacaine and ropivacaine, the structurally related compounds procaine and mepivacaine have previously been shown to have no effect on ANS fluorescence in the presence of rat liver microsomes (Birkett 1974). The effect of imipramine on ANS fluorescence in the presence of hepatic microsomes was also consistent with a percent ANS fluorescence increment of 224 at 1000 μM added drug (present study) versus 241 reported by (Birkett 1974). These data suggest that there is little, if any difference, between results using human liver and rat liver microsomes.

4.4.3 Caveats of the ANS fluorescence method for measuring the non-specific binding of drugs to human liver microsomes

Concentrations of human liver microsomes differed between the equilibrium dialysis (1 mg/ml) and ANS fluorescence (0.25 mg/ml) methods. Increasing microsomal protein concentrations above 0.25 mg/ml using the ANS fluorescence method reduces accuracy of fluorescence measurement due to turbidity of the experimental matrix. Importantly, however, the ANS fluorescence and equilibrium dialysis data are in good agreement despite the difference in microsome concentrations.

Although there was a significant relationship between the bound drug concentration and ANS fluorescence increment (Figure 4.4), the correlation plots of imipramine (described by a quadratic expression), and propranolol (a linear regression) differed. While this may suggest that each individual drug differentially affects the interaction

between ANS and microsomes, the deviation from linearity of the quadratic plot fitted to the imipramine data was very small (x^2 constant (a) = 0.0044).

CHAPTER 5
VALIDATION OF AN ANS FLUORESCENCE TECHNIQUE FOR MEASURING THE NON-SPECIFIC BINDING OF DRUGS TO HUMAN LIVER MICROSOMES

5.1 Introduction

The validity of the ANS fluorescence technique for measuring the non-specific binding of drugs to human liver microsomes was assessed in a preliminary manner in Chapter 4. It was shown that microsomally bound compounds, as determined by equilibrium dialysis (Chapter 3), were associated with a fluorescence increment (the bases imipramine and propranolol) or decrement (the neutral compound diazepam), whereas non-binding compounds (atenolol, bupivacaine, lignocaine and ropivacaine) showed minimal or no change from baseline ANS fluorescence. However, because of the small test set used in Chapter 4, further validation of the ANS fluorescence technique was considered essential.

In the present study a selection of nine drugs, chosen on the basis of log P and charge, were tested for non-specific binding to human liver microsomes. Binding of the drugs was characterised using both equilibrium dialysis and ANS fluorescence to identify further the relationships between:

- $f_{u(mic)}$ and percent ANS fluorescence increment / decrement, and
- concentration of bound drug and the change in baseline ANS fluorescence

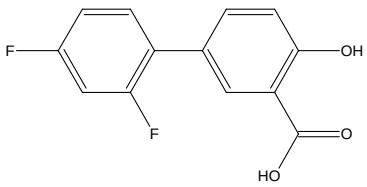
Since log P appeared to be an important (although not necessarily sole) determinant of binding, the acidic and basic drugs selected for investigation (Table 5.1) were all lipophilic with log P values ≥ 3.4.

Table 5.1: Physicochemical parameters of the investigational drugs

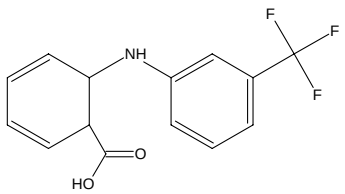
Compound	pK_a	% ionisation pH 7.4	log P	MM
Acidic drugs				
Diflunisal	2.9	100	4.3	250.2
Flufenamic acid	3.7	100	5.6	281.2
Meclofenamic acid	3.6	100	5.9	296.2
Basic drugs				
Bupropion	7.2	36.5	3.5	239.7
Chloroquine	8.4	90.9	4.7	319.9
Chlorpromazine	9.4	99.1	5.4	318.9
Mianserine	8.3	87.6	3.4	264.4
Trifluprom azine	7.1	33.9	5.2	479.5
Verapamil	8.9	96.9	4.9	454.6

Physicochemical parameters were determined using SciFinder Solaris, Advanced Chemistry Software V4.64.

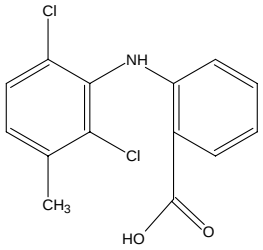
Structures of the test drugs listed in Table 5.1 are shown in Figure 5.1.



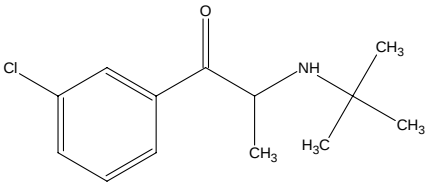
Diflunisal



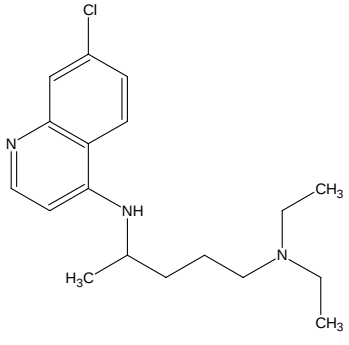
Flufenamic acid



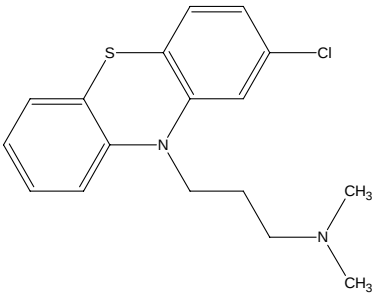
Meclofenamic acid



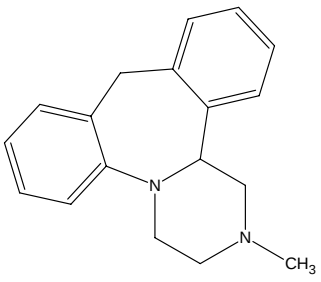
Bupropion



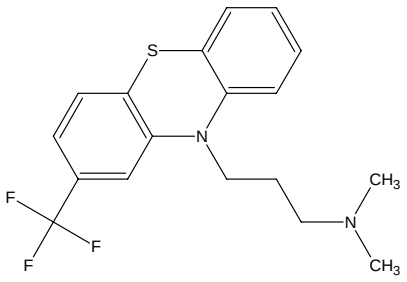
Chloroquine



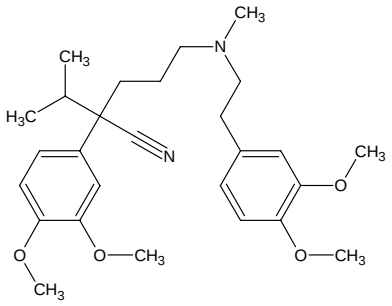
Chlorpromazine



Mianserine



Trifluprom azine



Verapamil

Figure 5.1: Chemical structures of the investigational compounds.

5.2 Methods

5.2.1 Equilibrium dialysis

5.2.1.1 Drug concentration range and drug solvents

Microsomal binding was investigated at three drug concentrations (100, 200 and 500 μM) according to the method described in Chapter 2, Section 2.3. Diflunisal and flufenamic acid were dissolved in methanol while the salts of bupropion, chloroquine, chlorpromazine, mianserine, trifluprom azine, and verapamil were dissolved in distilled water. A stoichiometric amount of NaOH was added to meclofenamic acid to facilitate dissolution. The concentrations of drug in the stock solutions (i.e. 10, 20 and 50 mM) were 100-fold higher than those required for the dialysis experiments since solutions were diluted 1:100 upon addition to the dialysis cells. As noted previously, experience in this laboratory indicates that 1% v/v

aqueous solutions of methanol (final diluted concentration) does not appreciably perturb non-specific binding of drugs to human liver microsomes.

5.2.2 ANS fluorescence procedure

Two separate 10 μL aliquots followed by a 30 μL aliquot of the 20 mM stock solutions of bupropion, chloroquine, chlorpromazine, meclofenamic acid, mianserine, triflupromazine, and verapamil, prepared as described in Section 5.2.2.1, were added sequentially to 2 ml of PB containing ANS (10 μM) and human liver microsomes (0.25 mg/ml) in a 4.5 ml glass cuvette to produce final drug concentrations of 100 μM (1 × 10 μL), 200 μM (plus 1 × 10 μL) and 500 μM (20 μL plus 30 μL). In the case of diflunisal and flufenamic acid, which were dissolved in methanol, two 10 μL aliquots of a 20 mM stock solution were added sequentially to 2 ml of PB containing ANS (10 μM) and human liver microsomes (0.25 mg/ml) in a 4.5 ml glass cuvette to produce drug concentrations 100 μM and 200 μM. It was not possible to prepare 500 μM solutions of these compounds while keeping the methanol concentration ≤ 1% v/v. ANS fluorescence was recorded prior to and after addition of the drug at excitation and emission wavelengths of 375 nm and 470 nm, respectively. Measurements were performed at least in duplicate.

In addition to the nine test drugs, five drugs previously characterised for microsomal binding in this laboratory, namely the bases amitriptyline and nortriptyline and the acids phenytoin, S-naproxen, and lamotrigine were used as a validation set for the ANS procedure (Section 5.4.5). These drugs were all dissolved in distilled water, with phenytoin and S-naproxen requiring a stoichiometric amount of NaOH to facilitate dissolution. The effects of the validation set drugs on ANS fluorescence in the presence of human liver microsomes were characterised at concentrations of 100,

200 and 500 μM using the procedure previously outlined for the test set compounds (Section 5.2.3).

5.3 Results

5.3.1 Equilibrium dialysis

5.3.1.1 HPLC conditions

Under the chromatographic conditions employed (Chapter 2, Table 2.3), chromatograms for bupropion, chloroquine, chlorpromazine, diflunisal, flufenamic acid, meclofenamic acid, mianserine, trifluprομazine, and verapamil were free from interfering peaks. Retention times for all analytes and internal standards were less than 10 min (Chapter 2, Table 2.3). Standard curves were linear ($r^2 > 0.990$) over the concentration ranges investigated.

5.3.1.2 Assay validation

Overall assay imprecision and inaccuracy for the individual assay procedures was determined from quadruplicate determinations of dialysis samples, as outlined in Chapter 2, Section 2.3.2.2. The assays developed and validated were suitably specific, accurate and precise for their experimental application (Table 5.2 and Table 5.3).

Table 5.2: Overall assay imprecision for drug standards prepared in a suspension of human liver microsomes in PB (1 mg/ml) and in PB alone

	Imprecision (%)					
Drug	**Human liver microsomes**			**Phosphate buffer**		
	Low	**Med**	**High**	**Low**	**Med**	**High**
Diflunisal	2.9	7.1	0.9	4.5	12.5	1.3
Flufenamic acid	3.7	1.8	2.8	0.0	1.1	0.5

Drug	Imprecision (%)					
	Human liver microsomes			Phosphate buffer		
	Low	Med	High	Low	Med	High
Meclofenamic acid	9.4	7.3	0.9	4.0	4.6	1.4
Bupropion	2.5	4.3	9.2	4.2	8.3	6.8
Chloroquine	3.0	0.9	5.3	6.2	2.4	2.6
Chlorpromazine	1.4	1.5	1.4	1.8	1.2	3.7
Mianserine	12.0	3.7	4.8	2.5	8.3	4.8
Trifluprom azine	4.2	1.9	1.6	1.0	1.7	2.6
Verapamil	2.7	3.0	6.0	1.6	3.5	3.5

Table 5.3: Overall assay inaccuracy for drug standards prepared in a suspension of human liver microsomes in PB (1 mg/ml) and in PB alone

Drug	Inaccuracy (%)					
	Human liver microsomes			Phosphate buffer		
	Low	Med	High	Low	Med	High
Diflunisal	4.0	5.6	11.9	14.8	7.3	0.7
Flufenamic acid	5.0	13.8	1.4	10.0	10.0	1.0
Meclofenamic acid	11.8	0.7	3.9	9.6	8.1	11.6
Bupropion	13.2	8.4	1.5	13.2	9.5	1.6
Chloroquine	9.6	12.9	5.1	12.0	6.0	2.3
Chlorpromazine	4.3	4.2	0.3	10.1	17.7	17.2
Mianserine	13.3	8.0	1.8	11.0	8.3	4.8
Trifluprom azine	8.9	9.2	6.4	6.4	0.8	0.7
Verapamil	0.8	1.4	7.7	9.2	8.4	5.4

5.3.1.3 Binding of test drugs to human liver microsomes

The non-specific binding of the three acidic and six basic test set drugs to human liver microsomes is shown in Table 5.4.

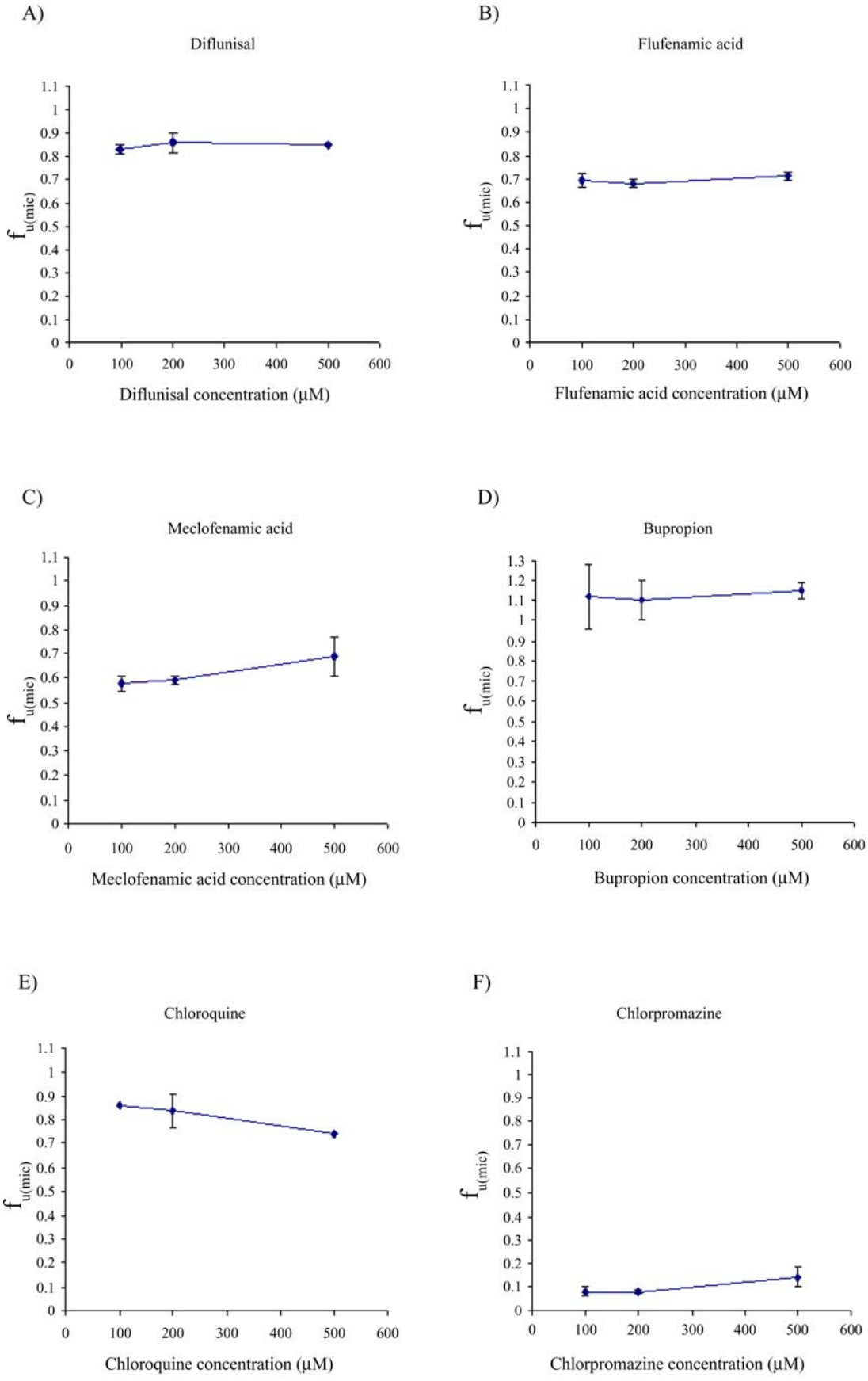
Table 5.4: Binding of investigational compounds to human liver microsomes. Free fraction of drug ($f_{u(mic)}$) determined from triplicate measurements is shown unless stated otherwise.

Drug	**$f_{u(mic)}$ ± SD at :**		
	100 μM	**200 μM**	**500 μM**
Acidic drugs			
Diflunisal	0.83 ± 0.02	0.86 ± 0.04	0.85 ± 0.00
Flufenamic acid	0.69 ± 0.03	0.68 ± 0.02	0.71 ± 0.02
Meclofenamic acid	0.58 ± 0.03	0.59 ± 0.02	0.69 ± 0.08
Basic drugs			
Bupropion	1.12 ± 0.16	1.10 ± 0.10	1.15 ± 0.04
Chloroquine	0.86[a]	0.84 ± 0.07	0.74[a]
Chlorpromazine	0.08 ± 0.02	0.08 ± 0.01	0.14 ± 0.04
Mianserine	0.29 ± 0.11[b]	0.37 ± 0.01[b]	0.37 ± 0.08[b]
Triflupromazine	0.17 ± 0.01	0.27 ± 0.01	0.44 ± 0.03
Verapamil	0.75[a]	0.73 ± 0.05	0.70 ± 0.11

[a] Single measurement only

[b] Measurements were performed in duplicate

The $f_{u(mic)}$ values for the acids diflunisal, flufenamic acid, and meclofenamic acid ranged from 0.58 to 0.86. Of the bases, bupropion did not bind to microsomes, whereas chloroquine and verapamil bound to a moderate extent; 14 - 26% and 25 - 30%, respectively. Chlorpromazine, mianserine and triflupromazine bound extensively to human liver microsomes. The binding of triflupromazine was clearly concentration dependent, and there was a suggestion of concentration dependence with meclofenamic acid and chlorpromazine. Data are represented graphically in Figure 5.2.


A)
Diflunisal
$f_{u(mic)}$
Diflunisal concentration (µM)
B)
Flufenamic acid
$f_{u(mic)}$
Flufenamic acid concentration (µM)
C)
Meclofenamic acid
$f_{u(mic)}$
Meclofenamic acid concentration (µM)
D)
Bupropion
$f_{u(mic)}$
Bupropion concentration (µM)
E)
Chloroquine
$f_{u(mic)}$
Chloroquine concentration (µM)
F)
Chlorpromazine
$f_{u(mic)}$
Chlorpromazine concentration (µM)

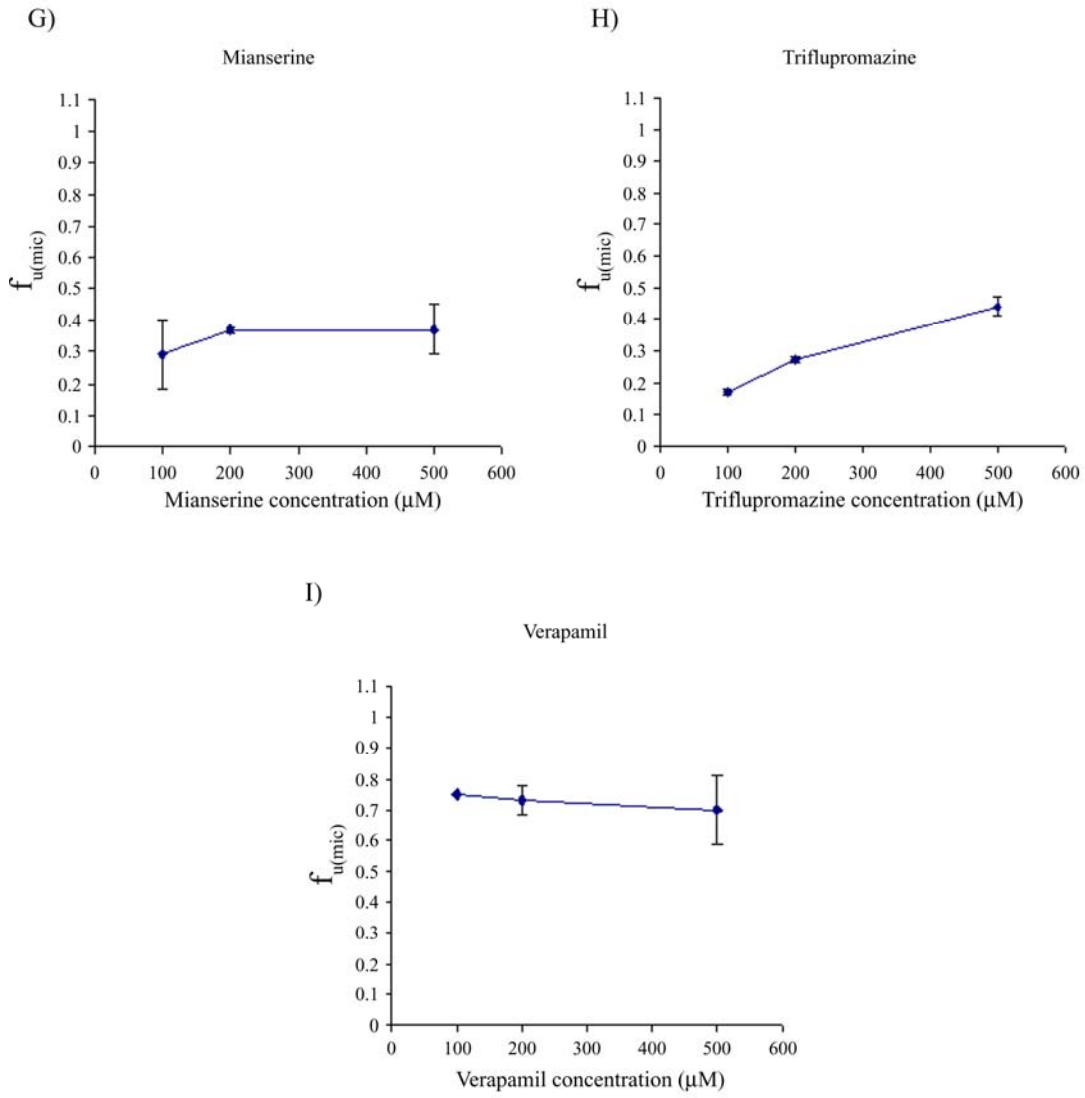


Figure 5.2: Non-specific binding to human liver microsomes of: A) diflunisal; B) flufenamic acid; C) meclofenamic acid; D) bupropion; E) chloroquine; F) chlorpromazine; G) mianserine; H) triflupromazine; and I) verapamil. Error bars represent the standard deviation.

5.3.2 ANS fluorescence

The percent ANS fluorescence increment/decrement was used as the measure of ANS fluorescence as this parameter represents the change from baseline (microsomes plus ANS) due to the effect of added drug (Equation 4.1, Chapter 4). The background fluorescence of drug in the experimental matrix was accounted for in the calculation of percent ANS fluorescence increment/decrement. None of the drugs investigated in this chapter exhibited quenching.

The drugs characterised for non-specific binding to human liver microsomes using equilibrium dialysis (Section 5.3.1) were investigated using the ANS fluorescence technique outlined in Section 5.2.3. Results are shown in Table 5.5.

Table 5.5: Effect of the investigational compounds on ANS fluorescence in the presence of human liver microsomes

Drug	**Percent ANS fluorescence increment/decrement at:**		
	100 μM	**200 μM**	**500 μM**
Acidic drugs			
Diflunisal	-8	-18	-
Flufenamic acid	-35	-43	-
Meclofenamic acid	-36	-62	-95
Basic drugs			
Bupropion	-11	-15	-24
Chloroquine	1	2	6
Chlorpromazine	107	179	353
Mianserine	45	77	158
Trifluprom azine	97	144	290
Verapamil	8	26	57

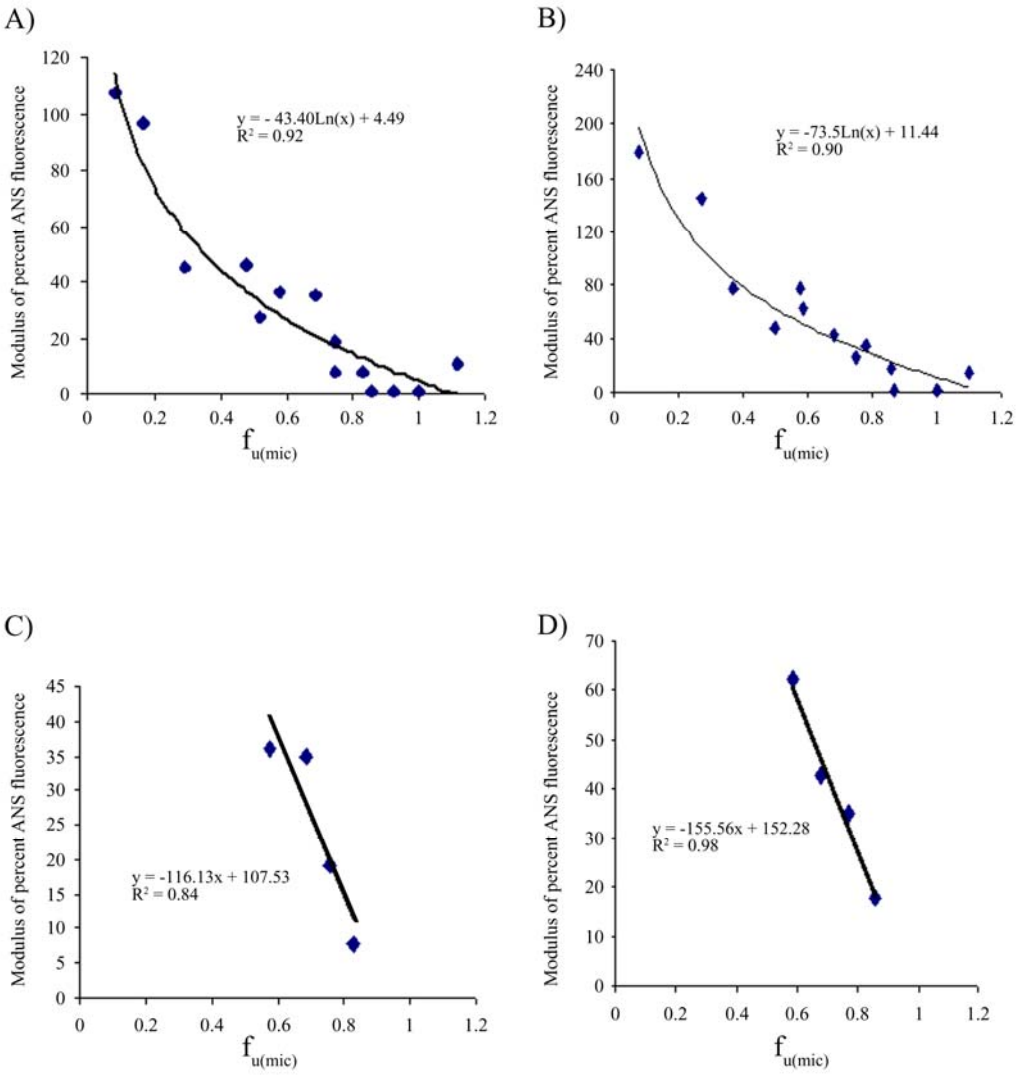
Diflunisal, flufenamic acid and meclofenamic acid produced a concentration dependent decrement in ANS fluorescence in the presence of human liver microsomes. Bupropion, also caused a concentration dependent decrement from baseline ANS fluorescence. In contrast, chloroquine, chlorpromazine, mianserine, trifluprom azine and verapamil all enhanced ANS fluorescence in a concentration dependent manner.

5.3.3 Relationship between $f_{u(mic)}$ and ANS fluorescence increment/decrement

Since all drugs investigated in this chapter were investigated at 100 μM and 200 μM, these results were pooled together with the binding results from Chapter 3 and Chapter 4 and were plotted as fraction of unbound drug ($f_{u(mic)}$) versus the modulus of ANS fluorescence increment/decrement. The analysis was grouped into three

classes that included all drugs, acidic/neutral drugs, and basic drugs alone (Figure 5.3).

A significant logarithmic relationship ($r^2 \geq 0.90$) was demonstrated between $f_{u(mic)}$ and the modulus of ANS fluorescence for all drugs and for basic drugs alone at concentrations of 100 μM and 200 μM (Figure 5.2 A, B, E, and F), while the acidic/neutral drugs showed a significant linear relationship ($r^2 \geq 0.84$) at these two concentrations (Figure 5.2 C and D). Bivariate Pearson correlations indicated statistical significance at the 0.01 level (2-tailed) for all data sets.



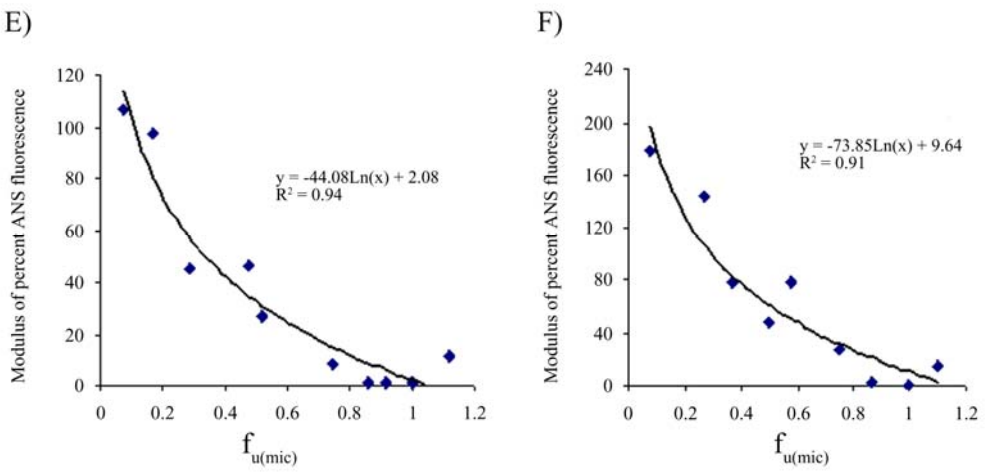


Figure 5.3: Relationships between the modulus of ANS fluorescence increment/ decrement and $f_{u(mic)}$ for: A) all drugs at 100 μM; B) all drugs at 200 μM; C) acid/neutral drugs at 100 μM; D) acid/neutral drugs at 200 μM; E) basic drugs at 100 μM; and F) basic drugs at 200 μM.

5.3.4 Relationship between bound drug concentration and ANS fluorescence increment/decrement

The concentration of drug bound to human liver microsomes, as determined from the equilibrium dialysis experiments, was plotted against the modulus of ANS fluorescence increment/decrement for the full range of added drug concentrations investigated (50 to 1000 μM) as reported in Chapters 3, 4 and 5. This analysis provided 60 data points in total. Once again, the analysis was grouped into three classes that included all drugs, acidic/neutral drugs, and basic drugs alone. Significant linear relationships were demonstrated with coefficients of determination (r^2) of 0.85 for all drugs and for basic drugs, and 0.97 for acidic/neutral drugs (Figure 5.4). Bivariate Pearson correlations indicated statistical significance at the 0.01 level (2-tailed) for all datasets.

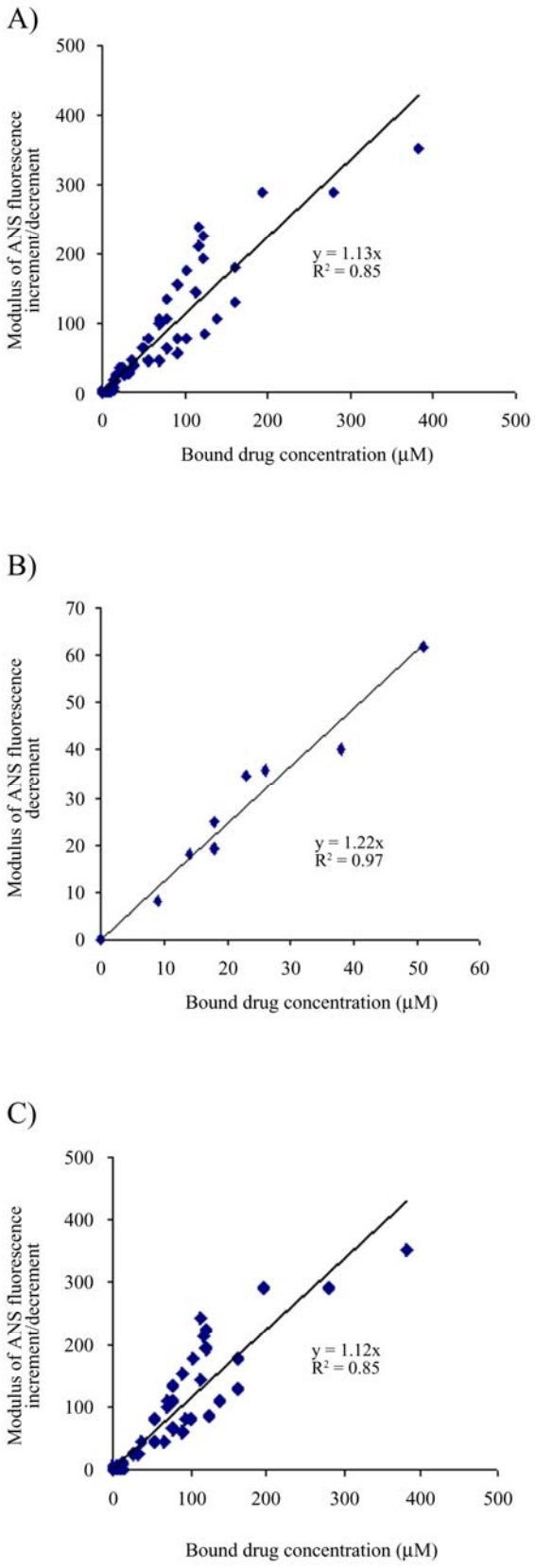


Figure 5.4: Relationships between bound drug concentration and the modulus of ANS fluorescence increment/decrement for: A) all drugs; B) acidic/neutral drugs; and C) basic drugs.

5.4 Discussion

5.4.1 Human liver microsomal binding of investigational drugs using equilibrium dialysis

The studies reported in this chapter aimed to further validate the ANS fluorescence method for measuring the non-specific binding of drugs to human liver microsomes. In order to achieve this, the characterisation of microsomal binding using equilibrium dialysis required the development and validation of nine HPLC assays.

The organic acids diflunisal, flufenamic acid and meclofenamic acid are all completely ionised at the experimental pH (7.4) and exhibit log P values of 4.3, 5.6 and 5.9 respectively (Table 5.1). The binding of diflunisal was independent of concentration and relatively low (15% on average). The more lipophilic compounds flufenamic acid and meclofenamic acid bound to microsomes to a greater extent, with $f_{u(mic)}$ values ≤ 0.71 across the concentration range tested. In contrast to previous studies, where acidic drugs were reported to bind minimally to human liver microsomes (Obach 1997, 1999; McLure, Miners & Birkett 2000), the more lipophilic flufenamic acid and meclofenamic acid bound appreciably.

The lack of binding observed for the lipophilic base bupropion was similar to the results obtained for lignocaine, bupivacaine, and ropivacaine. Interestingly, bupropion contains an 'internalised' carbonyl group, as do the local anaesthetics, as part of the amide function (Figure 5.1 and Figure 3.1). Chloroquine and verapamil bound to microsomes with respective $f_{u(mic)}$ values ranging from 0.74 – 0.86 and 0.70 – 0.75 across the 100 – 500 μM concentration range. The most extensive binding was observed with chlorpromazine ($f_{u(mic)}$ range 0.08 - 0.14), which was in agreement with a previous report using rat liver microsomes (Obach 1999), and with mianserine ($f_{u(mic)}$ range 0.29 – 0.37) and triflupromazine ($f_{u(mic)}$ range 0.17 – 0.44).

5.4.2 Effect of investigational drugs on ANS fluorescence in the presence of human liver microsomes

In agreement with previous studies (Diaugustine, Eling & Fouts 1970; Birkett 1974), microsomally bound acidic compounds, in this instance diflunisal, flufenamic acid and meclofenamic acid, caused a decrement in ANS fluorescence. Surprisingly, the basic compound bupropion also caused an ANS fluorescence decrement, ranging from –11 to –24 % (Table 5.3). This result was clearly an exception to the general effect basic compounds have on ANS fluorescence in the presence of human liver microsomes, as will be demonstrated in Chapter 6. Consistent with previous publications (Rubalcava, Martinez de Munoz & Gitler 1969; Vanderkooi & Martonosi 1969; Diaugustine, Eling & Fouts 1970; Dallner & Azzi 1972; Hawkins & Freedman 1973; Birkett 1974) and with results presented in Chapter 4, the other bases (chloroquine, chlorpromazine, mianserine, trifluprom azine and verapamil) caused an enhancement in ANS fluorescence in the presence of hepatic microsomes. The effects of chloroquine and verapamil were minor compared to mianserine, trifluprom azine and chlorpromazine, despite similar log P values (Table 5.1 and Table 5.5).

The equilibrium dialysis and ANS fluorescence data for the bases investigated in this chapter further suggest that factors other than lipophilicity (measured as log P) influence binding to human liver microsomes.

5.4.3 Relationship between $f_{u(mic)}$ and modulus of ANS fluorescence increment/decrement

The relationship between $f_{u(mic)}$ and the modulus of ANS fluorescence increment/decrement was characterised by a logarithmic function ($r^2 \geq 0.90$) for all drugs and basic drugs alone at 100 and 200 μM (Figure 5.3 A, B, E, & F). The

coefficient of the logarithmic function at 200 μM was approximately twice that of the 100 μM for all and basic drug groups. Given the logarithmic relationship, changes in $f_{u(mic)}$ in the 'highly' bound range (i.e. $f_{u(mic)} \leq 0.2$) are associated with comparatively larger changes in ANS fluorescence increment compared to the same change in $f_{u(mic)}$ for a compound that binds human liver microsomes to a lesser extent. However, despite the highly significant relationship there appeared to be some 'noise' in the data that was more prevalent for compounds that bound to a lesser extent. For example chloroquine and verapamil exhibited similar $f_{u(mic)}$ values but ANS fluorescence increment differed markedly. Nevertheless, the ANS fluorescence increment/decrement clearly differentiates compounds that bind extensively to microsomes from those that exhibit low or no non-specific binding.

The linear relationship between $f_{u(mic)}$ and modulus of ANS fluorescence decrement for the acidic/neutral group of compounds at both 100 and 200 μM presumably reflects the lower extent of binding of acidic and neutral compounds (i.e. binding occurs only in the linear range; Figure 5.3 C and D).

5.4.4 Relationship between bound drug and modulus of ANS fluorescence increment/decrement

Fundamentally the most important relationship for the validation of the ANS fluorescence technique for measuring the non-specific binding of drugs to microsomes was between bound drug concentration and modulus of ANS fluorescence increment/decrement. Expressions generated from the linear regression analysis of all drugs ($y = 1.13x$, $r^2 = 0.85$; Figure 5.4 A), acidic/neutral drugs ($y = 1.22x$, $r^2 = 0.97$; Figure 5.4 B) and basic drugs ($y = 1.12x$, $r^2 = 0.85$; Figure 5.4 C) were similar and were shown to be statistically significant. Thus, these results confirm the hypothesis that the concentration of drug bound to microsomes is

directly related to the change from baseline ANS fluorescence. Importantly, the free fraction of drug in a microsomal incubation ($f_{u(mic)}$) can be predicted from ANS fluorescence measurements using the expressions outlined in Figure 5.3 and Figure 5.4. An example showing the ANS fluorescence increment/decrement to $f_{u(mic)}$ conversion using Figure 5.4A is shown in Example 5.1.

Example 5.1

Modulus of ANS fluorescence for 200 μM added drug = 100 %

Step 1: Using the expression from Figure 5.4 A (y = 1.13x), the concentration of drug bound to microsomes is calculated:

100 = 1.13x

x = 88 μM

Thus, in this instance, the concentration of drug bound to microsomes = 88 μM.

Step 2: Calculating an $f_{u(mic)}$ value requires some basic theory from equilibrium dialysis experiments whereby:

$C_{Total\ added} = C_{bound} + 2C_{free}$

200 μM = 88 μM + $2C_{Free}$

112 μM = $2C_{Free}$

C_{Free} = 56 μM

And as $f_{u(mic)} = \frac{C_{free}}{C_{free} + C_{bound}}$

$f_{u(mic)} = \frac{56\,\mu M}{56\,\mu M + 88\,\mu M}$

$f_{u(mic)}$ = 0.39

5.4.5 Validation set

Five drugs previously characterised for microsomal binding using the equilibrium dialysis method (McLure, Miners & Birkett 2000; Rowland et al. 2006) were chosen as the validation set. The effect of amitriptyline, nortriptyline, phenytoin, S-naproxen, and lamotrigine on ANS fluorescence in the presence of human liver microsomes was determined in duplicate (Table 5.6).

Table 5.6: Effect of amitriptyline, nortriptyline, phenytoin, S-naproxen and lamotrigine on ANS fluorescence in the presence of human liver microsomes

Drug	Percent ANS fluorescence increment/decrement at:		
	100 μM	200 μM	500 μM
Amitriptyline	66	106	196
Nortriptyline	54	91	173
Phenytoin	-7	-13	-21
S-Naproxen	3	-3	-8
Lamotrigine	4	6	6

The two bases amitriptyline and nortriptyline showed a concentration dependent increase in ANS fluorescence. In contrast, the acidic compound phenytoin exhibited a concentration dependent decrement, whereas S-naproxen and lamotrigine caused minimal change to baseline ANS fluorescence. Values of $f_{u(mic)}$ were then predicted from the percent ANS fluorescence increment/decrement using the method outlined in Example 5.1 and compared to the observed $f_{u(mic)}$ values (Table 5.7).

Table 5.7: Predicted (from ANS fluorescence) versus observed $f_{u(mic)}$ values for validation set

Drug	Predicted $f_{u(mic)}$ and Observed $f_{u(mic)}$ at:					
	100 μM		200 μM		500 μM	
	Predicted	Observed	Predicted	Observed	Predicted	Observed
Amitriptyline	0.26	0.44	0.36	-	0.48	0.55
Nortriptyline	0.35	0.46	0.43	0.40	0.53	0.61
Phenytoin	0.89	0.83	0.89	0.89	0.93	-
S-Naproxen	0.95	0.99	0.97	0.99	0.97	0.97
Lamotrigine	-	0.93	0.95	1.0	0.98	0.96

With the exception of amitriptyline at the lowest concentration (100 μM), $f_{u(mic)}$ values predicted from ANS fluorescence data differed from observed fractions unbound by ≤ 24 %. Importantly, the method differentiated between low (phenytoin, S-naproxen and lamotrigine) and moderately extensively (amitriptyline and nortriptyline) bound drugs.

CHAPTER 6
RELATIONSHIPS BETWEEN SELECTED PHYSICOCHEMICAL CHARACTERISTICS AND THE NON-SPECIFIC BINDING OF DRUGS TO HUMAN LIVER MICROSOMES

6.1 Introduction

There is a body of evidence suggesting that lipophilicity and charge are the key determinants of the non-specific binding of drugs to human liver microsomes and, indeed, other biological membranes (Bickel & Steele 1974; Bickel et al. 1975; Schuster, Fleschurz & Helm 1975; Di Francesco & Bickel 1977; Obach 1997; Krämer et al. 1998; Obach 1999; McLure, Miners & Birkett 2000; Austin et al. 2002). However, the systematic evaluation of these two determinants described in Chapter 3 indicates that lipophilicity (as log P) and charge (which effects extent of binding; bases > neutrals and acids) are major, but not the sole determinants of non-specific binding. Notably, the non-binding of the lipophilic basic compounds bupivacaine, ropivacaine, lignocaine (Chapter 3), and bupropion (Chapter 5), implicates physicochemical characteristics other than lipophilicity and charge as important.

The progression of non-specific binding research parallels that of permeability research whereby lipophilicity and charge were originally identified as key determinants (Taylor, Pownall & Burke 1985; Artursson 1989; von Geldern et al. 1996; Yamashita et al. 1997). However, it is now well established that additional physicochemical characteristics have an effect on permeability (Camenisch, Folkers & van de Waterbeemd 1996; Palm et al. 1996; Lipinski et al. 1997; Winiwarter, Lanzner & Muller 1998; Osterberg & Norinder 2000; Burton et al. 2002). The most

extensive absorption/permeability related study (Lipinski et al. 1997) involved the computational analysis of experimental data from over 2200 compounds to develop 'Lipinski's rule of 5' which predicts poor absorption and/or permeability when a drug contains more than 5 hydrogen bond donors (HBD), more than 10 hydrogen bond acceptors (HBA), has a molecular mass greater than 500, or a calculated log P (as ClogP > 5 or MlogP > 4.15). Thus, an initial evaluation of the relationship between the number of HBD, and HBA, molecular mass, log P, and pK_a and the non-specific binding of drugs to human liver microsomes was undertaken here.

The ANS fluorescence method described in Chapter 4 provided the basis for evaluating the relationships between physicochemical parameters and non-specific microsomal binding. Using the ANS fluorescence procedure, the non-specific binding of ninety-nine physicochemically diverse drugs was investigated. Test drugs, along with their physicochemical characteristics, are listed in Table 6.1 (including compounds from Chapters 4 and 5).

Acids, bases and neutrals were classified by their ionisation state at pH 7.4. Molecules containing a negatively charged functional group at pH 7.4 were defined as acids, while compounds with a positively charged functional group were defined as bases. Un-ionised compounds were classified as 'neutral', while compounds with more than one functional group were classified according to their dominant ionisation state at pH 7.4.

HBA and HBD were defined according to Lipinski et al. (1997); that is HBDs are expressed as the sum of OH and NH groups, whereas HBAs are expressed as the sum of N and O atoms in a molecule. All physicochemical characteristics were generated using the program SciFinder Scholar (American Chemical Society, 2004; calculated

using Advanced Chemistry Development (ACD) Software Solaris V4.67), except for sodium salicylate, which used Marvin 4.0.1 (copyright 1998 - 2005, ChemAxon Ltd, http://www.chemaxon.com/marvin) to generate pK_a and log P values.

Table 6.1: Physicochemical characteristics of investigational compounds

Drug	pK_a	log P	MM (Da)	H donors	H acceptors
Acids					
Alclofenac	4.3	2.8	226.7	1	3
Caffeine	0.7	-0.1	194.2	0	6
Cicloprofen	4.3	3.8	238.3	1	2
Diclofenac	4.2	4.1	296.2	2	3
Diflunisal	2.9	4.4	250.2	2	3
Fenoprofen	4.2	3.8	242.3	1	3
Flufenamic acid	3.7	5.6	281.2	2	3
Flurbiprofen	4.1	4.1	244.3	1	2
Gliclazide	3.9	1.6	323.4	2	6
Ibuprofen	4.4	3.7	206.3	1	2
Isoniazid	3.8	-0.9	137.1	3	4
Indomethacin	4.0	3.1	357.8	1	5
Lamotrigine	5.4	-0.2	256.1	4	5
Meclofenamic acid	3.6	6.7	296.2	2	3
Mefenamic acid	3.7	5.3	241.3	2	3
Naproxen	4.8	3.0	230.3	1	3
Niflumic acid	4.7	4.9	282.2	2	4
Phenacetin	1.4	1.6	179.2	1	3
Phenelzine	8.1	1.1	136.2	3	2
Phenytoin	8.3	2.5	252.2	2	4
Probenecid	3.7	3.3	285.4	1	5
Salicylic acid	2.8	1.9	138.1	2	3
Tolbutamide	5.1	2.3	270.4	2	5
Warfarin	4.5	3.4	308.3	1	4
Bases					
1-Acetyl-2-phenylhydrazine	12.8	0.8	150.2	2	3
Albendazole	11.4	3.1	265.3	2	5
Amiodarone	9.4	8.9	645.3	0	4
Amitriptyline	9.2	4.9	277.4	0	1
Amodiaquine	9.4	4.8	355.9	2	4
Antazoline	10.3	4.4	265.4	1	3
Atenolol	9.2	0.1	266.3	4	5
Benzydamine	9.3	3.8	309.4	0	4
Benzylamine	9.4	1.1	107.2	2	1
N-Benzylmethylamine	9.8	1.5	121.2	1	1
S-(-)-N-Benzyl-α-methylbenzylamine	8.8	3.8	211.3	1	1
Bupivacaine	8.2	3.6	288.4	1	3
Bupropion	7.2	3.5	239.7	1	2
Carbazole	17.0	3.7	167.2	1	1
Chloroquine	10.5	4.7	319.9	1	3
Chlorpheniramine	9.3	3.4	274.8	0	2

Drug	pK_a	log P	MM (Da)	H donors	H acceptors
Chlorphentermine	9.8	2.8	183.7	2	1
Chlorpromazine	9.4	5.2	318.9	0	2
Cinnoline	3.0	0.9	130.2	0	2
Clonidine	8.1	1.4	230.1	2	3
Clozapine	7.1	3.5	326.8	1	4
Debrisoqiune	13.2	0.1	175.2	3	3
Desipramine	10.4	4.1	266.4	1	2
Desmethylnortriptyline	9.5	6.4	249.4	2	1
Dextropropoxyphene	0.2	5.4	339.5	0	3
N-Didesmethylimipramine	9.9	3.7	252.4	2	0
Diltiazem	8.9	3.6	414.5	0	6
N-N-Dimethylbenzylamine	8.8	2.0	135.2	0	2
Diphenhydramine	8.8	3.7	255.4	0	2
4-4' - Dipyridyl	3.8	1.2	156.2	0	2
Disopyramide	10.1	2.9	339.5	2	4
Doxepin	9.2	3.9	279.4	0	2
Econazole	6.7	5.3	381.7	0	3
Fluoxetine	10.1	4.1	309.3	1	2
Fluphenazine	6.9	4.8	437.5	1	4
Fluvoxamine	9.4	3.1	318.3	2	4
3-Hydroxytyramine	9.4	0.1	153.2	4	3
Imipramine	9.5	4.8	280.4	0	2
Itraconazole	6.5	4.3	705.6	0	12
Labetolol	8.2	2.3	328.4	5	5
Lignocaine	8.5	2.4	234.3	1	3
Mephentermine	10.4	2.3	163.3	1	1
Mianserine	8.3	3.4	264.4	0	2
Nicardipine	7.3	5.1	479.5	1	9
Nortriptyline	10.0	5.7	263.4	1	1
Perhexiline	11.2	7.0	277.5	1	1
Phentolamine	9.5	3.6	281.4	2	4
4-Phenylbutylamine	10.7	2.4	149.2	2	1
β-Phenylethylamine	9.9	1.5	121.2	2	1
Phenylpropanolamine	8.5	0.8	151.2	3	2
4-Phenylpyridine	5.4	2.6	155.2	0	1
Propranolol	9.1	3.1	259.3	2	3
Protriptyline	10.6	5.1	263.4	1	1
Quinine	9.3	3.4	324.4	1	4
Quipazine	8.9	1.6	213.3	1	3
Ropivacaine	8.2	3.1	274.4	1	3
Spermidine	10.5	-0.84	145.3	5	3
Spermine	10.9	-1.0	202.3	6	4
Terbutaline	9.1	0.5	225.3	4	4
Tetrahydrozoline	10.4	3.3	200.3	1	2
Thioridazine	9.6	6.1	370.6	0	2
Thiothixene	7.7	3.9	443.6	0	5
Tranylcypromine	8.8	1.3	133.2	2	1
Trifluoperazine	8.2	5.1	407.5	0	3
Triflupromazine	9.4	5.7	352.4	0	2
Verapamil	9.0	3.9	454.6	0	6

Drug	pK_a	log P	MM (Da)	H donors	H acceptors
Neutrals					
Budesonide	12.9	3.1	430.5	2	6
Carbamazepine	13.9	2.7	236.3	2	3
Clonazepam	11.2	2.3	315.7	1	6
Diazepam	3.4	3.0	284.7	0	3
9-Ethylcarbazole	-	4.5	195.3	0	1
Hydralazine	5.0	0.9	160.2	3	4
Metyrapone	4.6	1.2	226.3	0	3
Nitrazepam	11.4	2.2	281.3	1	6
Propofol	11.0	4.2	178.3	1	1

6.2 Methods

Drugs were tested for microsomal binding according to the method described in Chapter 5, Section 5.2.3. Measurement of the non-specific binding of compounds dissolved in distilled water was performed at three drug concentrations (100, 200 and 500 μM), whereas compounds dissolved in methanol or acetonitrile were measured at only two concentrations (100 and 200 μM) to prevent a solvent concentration greater than 1 % v/v in the experimental mixture. The initial parameter for the measurement of microsomal binding was percent ANS fluorescence increment/decrement (as described in Chapter 4, Equation 4.1). Fluorescence data were subsequently converted to $f_{u(mic)}$ values using the ANS fluorescence relationships derived in Chapter 5 (Figure 5.3 and Figure 5.4).

6.2.1 Solvents and drugs used in fluorescence experiments

Test drugs were solubilised in distilled water, methanol, or acetonitrile as described below.

Distilled water: 1-acetyl-2-phenylhydrazine, amitriptyline, amodiaquine, antazoline, atenolol, benzydamine, benzylamine, N-benzylmethylamine, (S)-(-)-N-benzyl-∝-methylbenzylamine (solubilised with the aid of orthophosphoric acid), bupivacaine, bupropion, caffeine, chloroquine, chlorpheniramine, chlorpromazine (solubilised with the aid of glacial acetic acid), cinnoline, cloazpine, clonidine, clorphentermine,

desipramine, desmethyl nortriptyline, dextropropoxyphene, N-didesmethy-imipramine, diclofenac, diltiazem, diphenhydramine, 4-4'-dipyridyl, disopyramide (solubilised with the aid of orthophosphoric acid), doxepin, fluoxetine, fluphenazine, flurbiprofen, hydralazine, 3-hydroxytyramine, ibuprofen, imipramine, isoniazid, labetolol, lamotrigine (solubilised with the aid of orthophosphoric acid), lignocaine, memphentermine, mianserine, naproxen (solubilised with the aid of 1M NaOH), nicardipine, nortriptyline, phenelzine, phentolamine, 4-phenylbutylamine, β-phenylethylamine, phenylpropanolamine, 4-phenylpyridine (solubilised with the aid of orthophosphoric acid), phenytoin (solubilised with the aid of 1M NaOH), propranolol, protriptyline, quinine (solubilised with the aid of glacial acetic acid), quipazine, ropivacaine, salicylic acid, spermidine, spermine, terbutaline, tetrahydrozoline, thioridazine, thiothixene, tranylcypromine, trifluoperazine, triflupromazine, and verapamil.

Methanol: albendazole, alclofenac, amiodarone, budesonide, carbamazepine, carbazole, cicloprofen, clonazepam, debrisoquine, diazepam, diflunisal, N,N dimethylbenzylamine, econazole, 9-ethylcarbazole, fenoprofen, flufenamic acid, fluvoxamine, meclofenamic acid, mefenamic acid (solubilised with the aid of orthophosphoric acid), metyrapone, niflumic acid, nitrazepam, perhexiline, phenacetin, probenecid, propofol, tolbutamide, and warfarin.

Acetonitrile: gliclazide and itraconazole.

6.3 Results

6.3.1 ANS fluorescence

All compounds were analysed for quenching using ethanol as the solvent (Section 4.2.1.1). Quenching was observed with amiodarone, amodiaquine, clonazepam, clozapine, hydralazine, indomethacin, mefenamic acid, nicardipine, nitrazepam and trifluoperazine. Consequently, the effect of these compounds on ANS fluorescence in the presence of human liver microsomes (extent of microsomal binding) was not characterised.

Minor background fluorescence of compounds in the experimental matrix was accounted for in the calculation of percent ANS fluorescence increment/decrement (Equation 4.1). Notably, 9-ethylcarbazole fluoresced extensively in the presence of human liver microsomes and was not studied further. The remaining compounds (listed in Table 6.1) were characterised for binding to human liver microsomes using the ANS fluorescence technique described in Section 4.2.1. All experiments were performed at least in duplicate. Mean results are shown in Table 6.2.

Table 6.2: ANS fluorescence increment/decrement in the presence of human liver microsomes and derived $f_{u(mic)}$ values (shown in brackets) for the investigational compounds

Drug	**Percent ANS fluorescence increment/decrement and (derived $f_{u(mic)}$)**		
	100 μM	**200 μM**	**500 μM**
Acids			
Alclofenac	-3 (0.95)	-4 (0.97)	-
Caffeine	0 (1.00)	-1 (0.99)	-3 (0.99)
Cicloprofen	-4 (0.93)	-10 (0.92)	-
Diclofenac	-7 (0.88)	-25 (0.80)	-40 (0.87)
Diflunisal	-8 (0.87)	-18 (0.85)	-
Fenoprofen	4 (0.93)	-3 (0.97)	-
Flufenamic acid	-35 (0.53)	-43 (0.68)	-
Flurbiprofen	-6 (0.90)	-10 (0.92)	-23 (0.92)
Gliclazide	0 (1.00)	-2 (0.98)	-
Ibuprofen	-2 (0.97)	-5 (0.96)	-14 (0.95)
Isoniazid	-4 (0.93)	-7 (0.94)	-10 (0.97)
Lamotrigine	4 (0.93)	6 (0.95)	6 (0.98)
Meclofenamic acid	-36 (0.52)	-62 (0.57)	-
Naproxen	3 (0.95)	-3 (0.97)	-8 (0.97)
Niflumic acid	-17 (0.74)	-27 (0.79)	-
Phenacetin	-1 (0.98)	-6 (0.95)	-
Phenelzine	3 (0.95)	2 (0.98)	2 (0.99)
Phenytoin	-7 (0.88)	-13 (0.89)	-21 (0.93)
Probenecid	-4 (0.93)	-7 (0.94)	-
Sodium Salicylate	2 (0.97)	4 (0.97)	1 (1.00)
Tolbutamide	-2 (0.97)	-6 (0.95)	-
Warfarin	-5 (0.92)	-8 (0.93)	-
Bases			
1-Acetyl-2-phenylhydrazine	0 (1.00)	1 (0.99)	2 (0.99)
Albendazole	-2 (0.97)	-1 (0.99)	-
Amitriptyline	66 (0.26)	106 (0.36)	196 (0.58)
Antazoline	16 (0.75)	24 (0.81)	50 (0.84)
Atenolol	-1 (0.98)	1 (0.99)	-4 (0.98)
Benzydamine	21 (0.69)	36 (0.73)	72 (0.78)
Benzylamine	3 (0.95)	0 (1.00)	-1 (1.00)
N-Benzylmethylamine	-2 (0.97)	-4 (0.97)	-6 (0.98)
S-(-)-N-benzyl-α-methylbenzylamine	26 (0.63)	40 (0.70)	77 (0.76)

Drug	Percent ANS fluorescence increment/decrement and (derived $f_{u(mic)}$)		
	100 μM	**200 μM**	**500 μM**
Bupropion	-11 (0.82)	-15 (0.88)	-24 (0.91)
Carbazole	20 (0.70)	22 (0.82)	-
Chloroquine	1 (0.98)	2 (0.98)	6 (0.98)
Chlorpheniramine	19 (0.71)	24 (0.81)	43 (0.86)
Chlorphentermine	13 (0.79)	21 (0.83)	36 (0.88)
Chlorpromazine	107 (0.03)	179 (0.12)	354 (0.23)
Cinnoline	1 (0.98)	-8 (0.93)	-12 (0.96)
Clonidine	7 (0.88)	5 (0.96)	11 (0.96)
Debrisoquine	17 (0.74)	50 (0.64)	-
Desipramine	57 (0.33)	86 (0.45)	139 (0.61)
Desmethylnortriptyline	63 (0.28)	110 (0.35)	188 (0.50)
Dextropropoxyphene	12 (0.81)	12 (0.90)	34 (0.89)
N-Didesmethylimipramine	55 (0.35)	91 (0.43)	166 (0.55)
Diltiazem	12 (0.81)	23 (0.82)	37 (0.88)
N-N-Dimethylbenzylamine	2 (0.97)	-5 (0.96)	-
Diphenhydramine	10 (0.84)	21 (0.83)	35 (0.88)
4-4' Dipyridyl	8 (0.87)	-2 (0.98)	-5 (0.98)
Disopyramide	8 (0.87)	7 (0.94)	11 (0.96)
Doxepin	27 (0.61)	55 (0.61)	114 (0.66)
Econazole	172 (0.02)	451 (0.003)	-
Fluoxetine	53 (0.36)	79 (0.48)	140 (0.60)
Fluphenazine	203 (0.01)	356 (0.009)	624 (-)
Fluvoxamine	18 (0.73)	27 (0.79)	-
3-Hydroxytyramine	2 (0.97)	-2 (0.98)	-5 (0.98)
Imipramine	46 (0.42)	77 (0.42)	154 (0.57)
Itraconazole	138 (0.05)	345 (0.01)	-
Labetolol	7 (0.88)	12 (0.90)	22 (0.93)
Lignocaine	1 (0.98)	1 (0.99)	-2 (0.99)
Mephentermine	-2 (0.97)	-3 (0.97)	5 (0.98)
Mianserine	45 (0.43)	77 (0.49)	158 (0.56)
Nortriptyline	54 (0.35)	91 (0.43)	173 (0.53)
Perhexiline	301 (0.001)	676 (0.0001)	-
Phentolamine	6 (0.90)	38 (0.71)	79 (0.75)
4-Phenylbutylamine	8 (0.87)	13 (0.89)	22 (0.93)
β-Phenylethylamine	-3 (0.95)	-4 (0.97)	-7 (0.97)
Phenylpropanolamine	0 (1.00)	-5 (0.96)	-6 (0.98)
4-Phenylpyridine	0 (1.00)	-2 (0.98)	-4 (0.99)
Propranolol	27 (0.61)	47 (0.66)	86 (0.74)
Protriptyline	59 (0.31)	92 (0.42)	160 (0.56)
Quinine	15 (0.77)	21 (0.83)	53 (0.83)
Quipazine	8 (0.87)	11 (0.91)	26 (0.91)
Ropivacaine	1 (0.98)	1 (0.99)	1 (1.00)
Spermidine	-2 (0.97)	-1 (0.99)	-2 (0.99)
Spermine	3 (0.95)	5 (0.96)	3 (0.99)
Terbutaline	2 (0.97)	5 (0.96)	1 (1.00)
Tetrahydrozoline	1 (0.98)	-1 (0.99)	9 (0.97)
Thioridazine	156 (0.03)	267 (0.03)	785 (-)
Thiothixene	108 (0.02)	174 (0.13)	371 (0.21)
Tranylcypromine	4 (0.93)	7 (0.94)	9 (0.97)
Triflupromazine	97 (0.08)	144 (0.22)	290 (0.32)
Verapamil	8 (0.87)	26 (0.79)	57 (0.82)

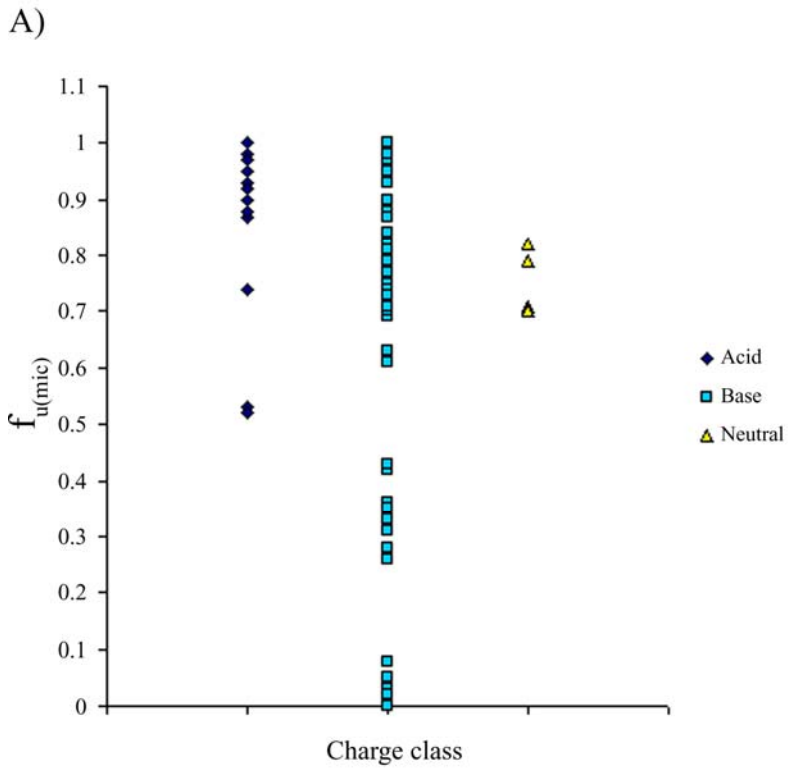
Drug	Percent ANS fluorescence increment/decrement and (derived $f_{u(mic)}$)		
	100 μM	200 μM	500 μM
Budesonide	-13 (0.79)	-28 (0.78)	-
Carbamazepine	-13 (0.79)	-15 (0.88)	-
Diazepam	-19 (0.71)	-35 (0.73)	-
Metyrapone	-11 (0.82)	-14 (0.88)	-
Propofol	-20 (0.70)	-31 (0.76)	-

ANS fluorescence data for fluphenazine and thioridazine (at 500 μM) were unable to be converted into $f_{u(mic)}$ values as these two readings exceeded the relationships derived in Section 5.3.3 and Section 5.3.4.

6.3.2 Relationship between non-specific microsomal binding and the charge of test compounds

6.3.2.1 Charge classification

Microsomal binding data for each compound was plotted according to charge state; acid, base or neutral (Figure 6.1).



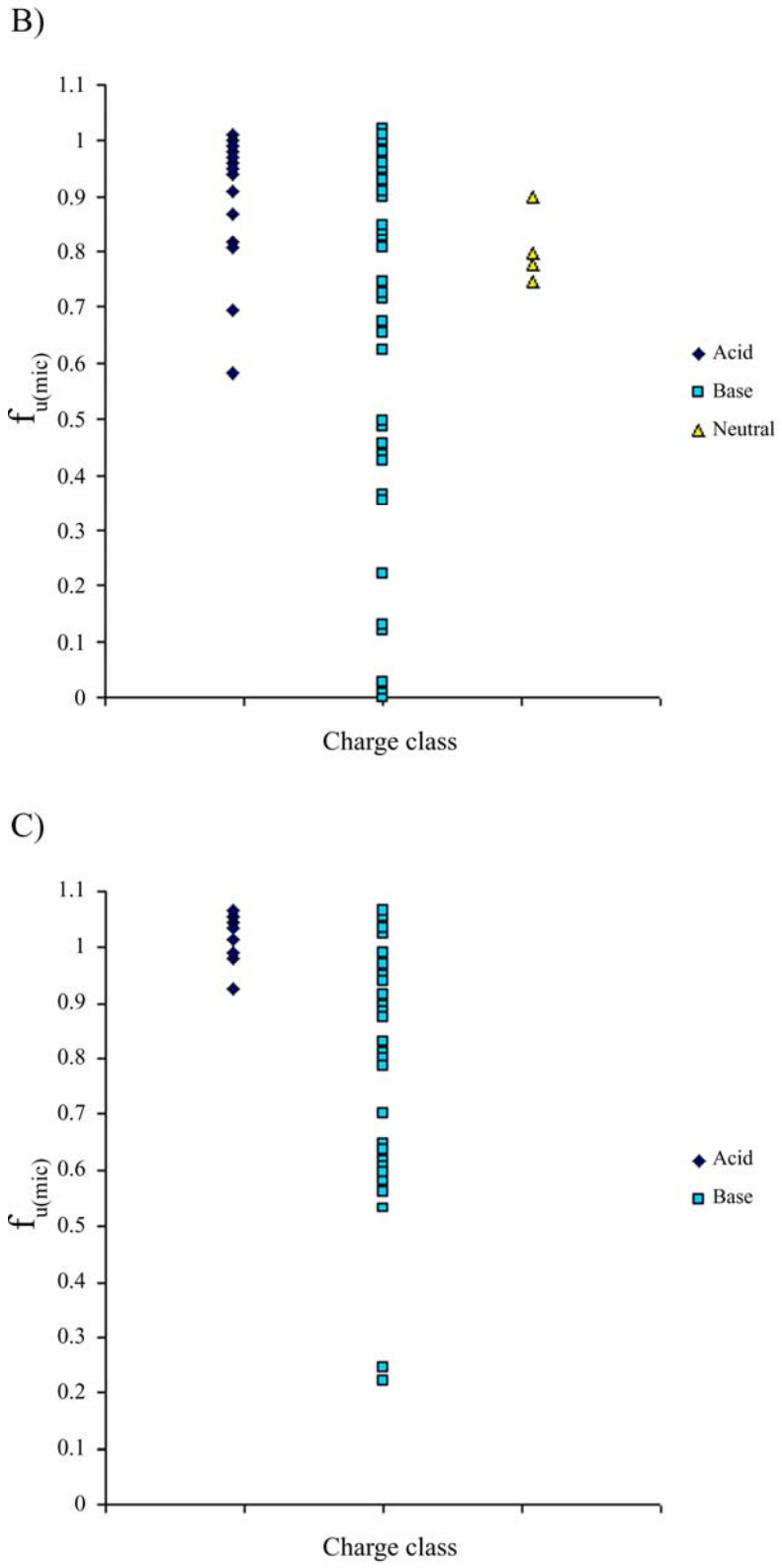


Figure 6.1: Relationship between $f_{u(mic)}$ and the charge of test compounds at concentrations of: A) 100 µM; B) 200 µM; and C) 500 µM.

As expected, basic compounds generally show a far wider range of non-specific binding than acidic and neutral compounds (Figure 6.1 A, B, and C). Acids and neutrals, with the exception of flufenamic acid and meclofenamic acid at 100 and 200 μM (Table 6.2), were shown to bind to human liver microsomes with free fractions ≥ 0.70. Furthermore, almost 80% of compounds in these two classes exhibited $f_{u(mic)}$ values > 0.85.

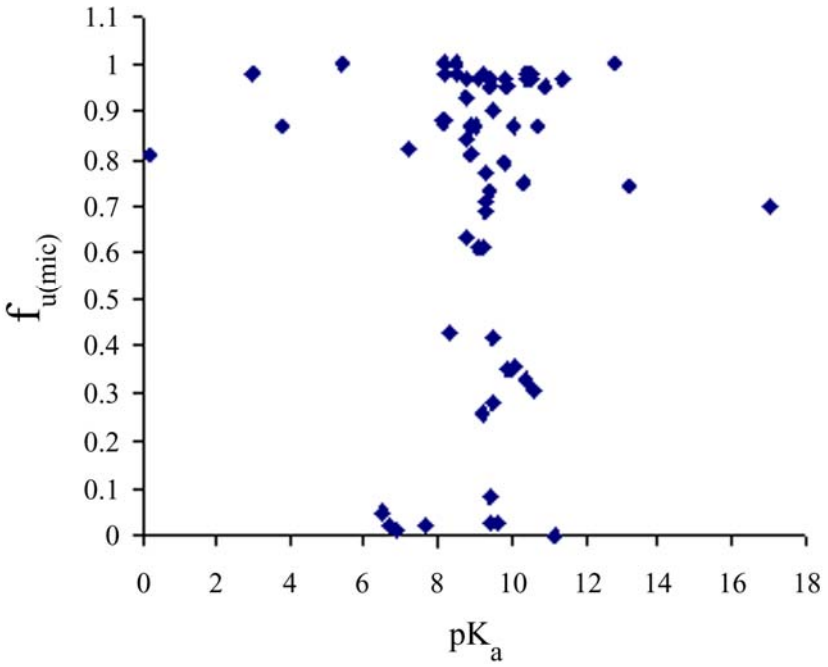
Given the minor binding of acids and neutrals no further analysis of these classes was undertaken. Moreover, $f_{u(mic)}$ values ≥ 0.5 are considered as 'minor' because the most significant effect of binding on clearance and inhibition prediction occurs for compounds that display $f_{u(mic)}$ values < 0.5 (Austin et al. 2002; Sykes, Sorich & Miners 2006).

6.3.3 Relationships between the non-specific microsomal binding of test bases and selected physicochemical characteristics

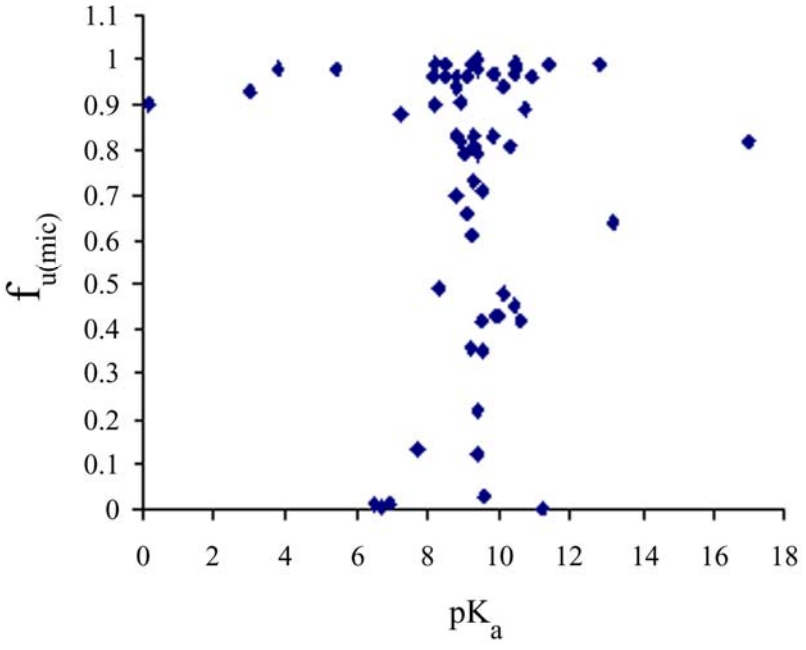
6.3.3.1 pK_a

The pK_a values of basic compounds characterised for non-specific binding to human liver microsomes were plotted against the derived $f_{u(mic)}$ for drug concentrations of 100, 200 and 500 μM (Figure 6.2).

A)


1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
$f_{u(mic)}$
0 2 4 6 8 10 12 14 16 18
pK_a

B)


1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
$f_{u(mic)}$
0 2 4 6 8 10 12 14 16 18
pK_a

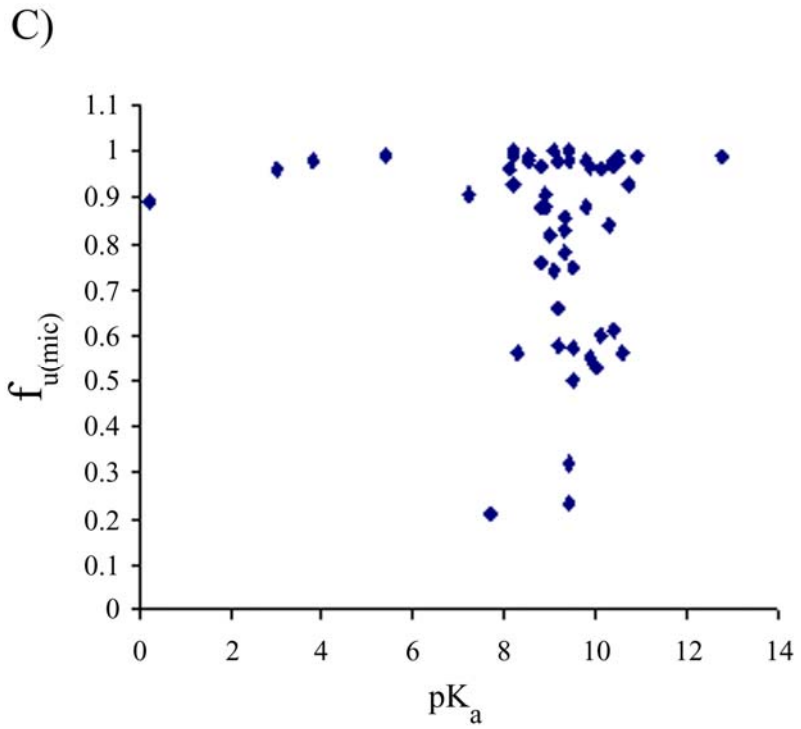
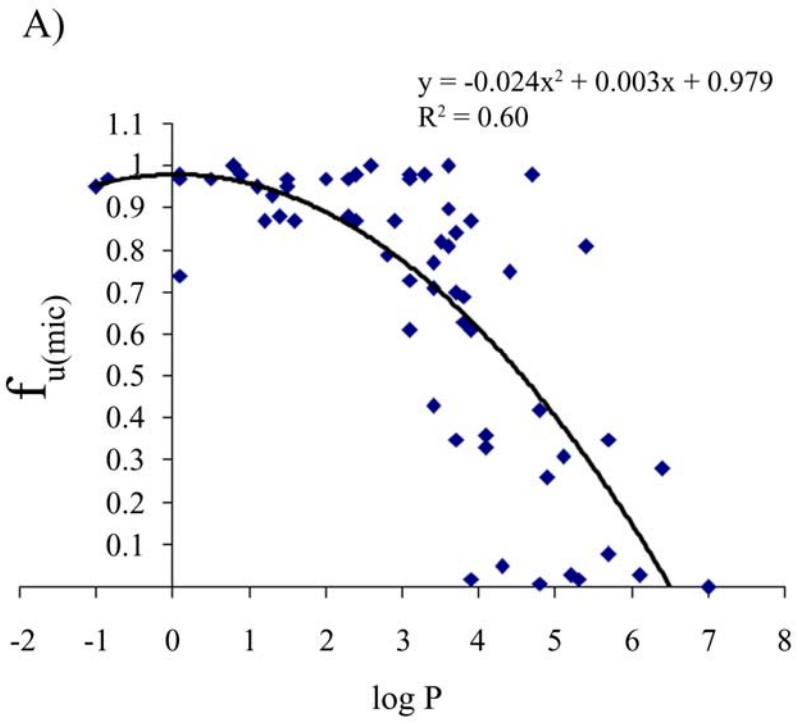


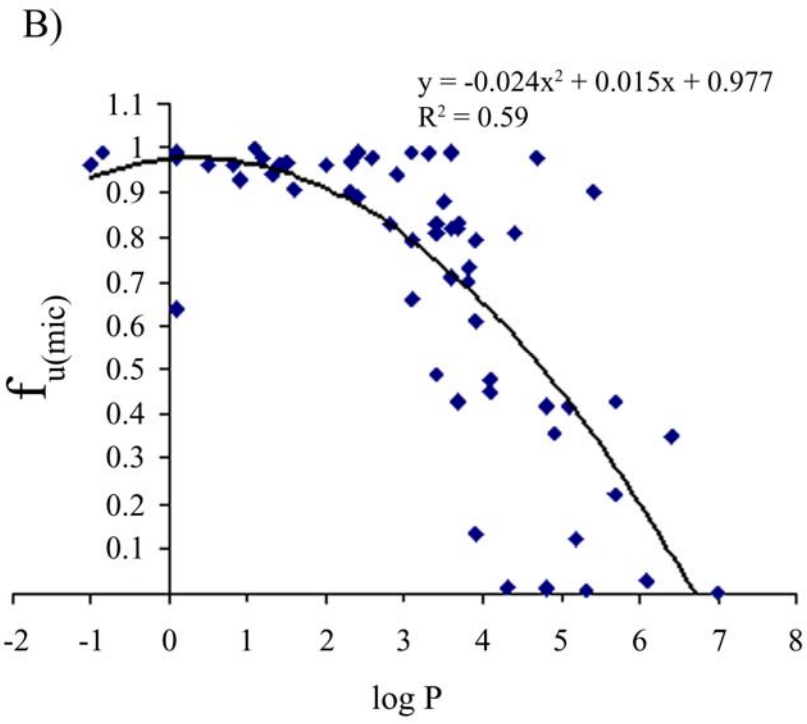
Figure 6.2: Relationship between pK_a and the non-specific binding of basic compounds to human liver microsomes at concentrations of: A) 100 μM; B) 200 μM; and C) 500 μM.

The majority of compounds tested had pK_a values ranging from 6 – 11. The highest binding compounds were within this range, while there was also a wide range of binding shown across the range. Compounds with lower or higher values than in this range tended to bind minimally or not at all to human liver microsomes (Figure 6.2 A, B, and C).

6.3.3.2 log P

The lipophilicity (as log P) of each base characterised for non-specific binding to human liver microsomes was plotted against the derived $f_{u(mic)}$ for drug concentrations of 100, 200 and 500 μM. Results are shown in Figure 6.3.


A)
y = -0.024x² + 0.003x + 0.979
R² = 0.60
f u(mic)
log P


B)
y = -0.024x² + 0.015x + 0.977
R² = 0.59
f u(mic)
log P

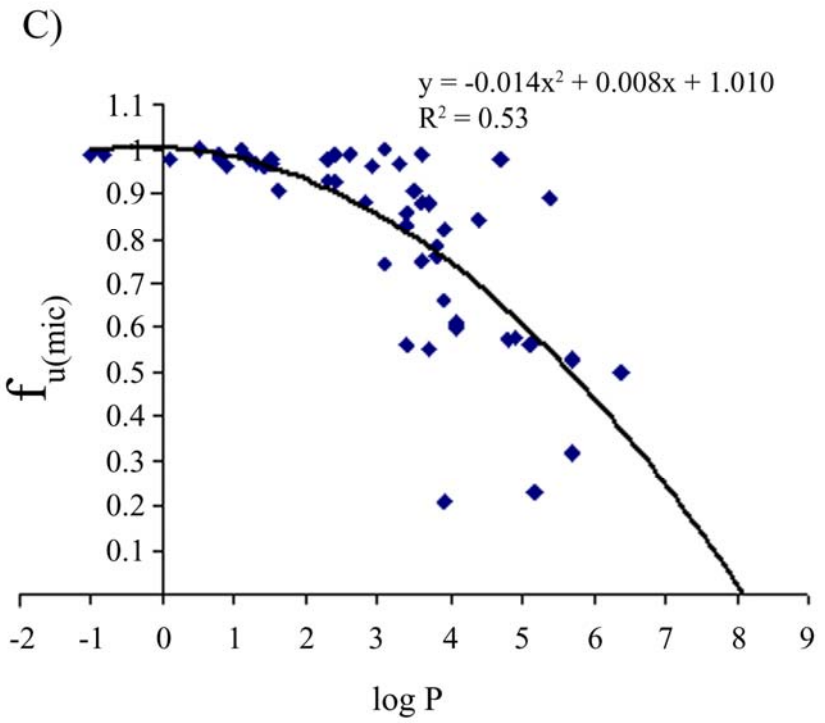


Figure 6.3: Relationship between log P and the non-specific binding of basic compounds to human liver microsomes at concentrations of: A) 100 μM; B) 200 μM; and C) 500 μM.

The relationship between non-specific microsomal binding and the log P of basic compounds at all three drug concentrations tested was best described by a second order polynomial (Figure 6.3 A, B, and C). R^2 values were 0.60, 0.59, and 0.53 for added concentrations of 100, 200, and 500 μM, respectively.

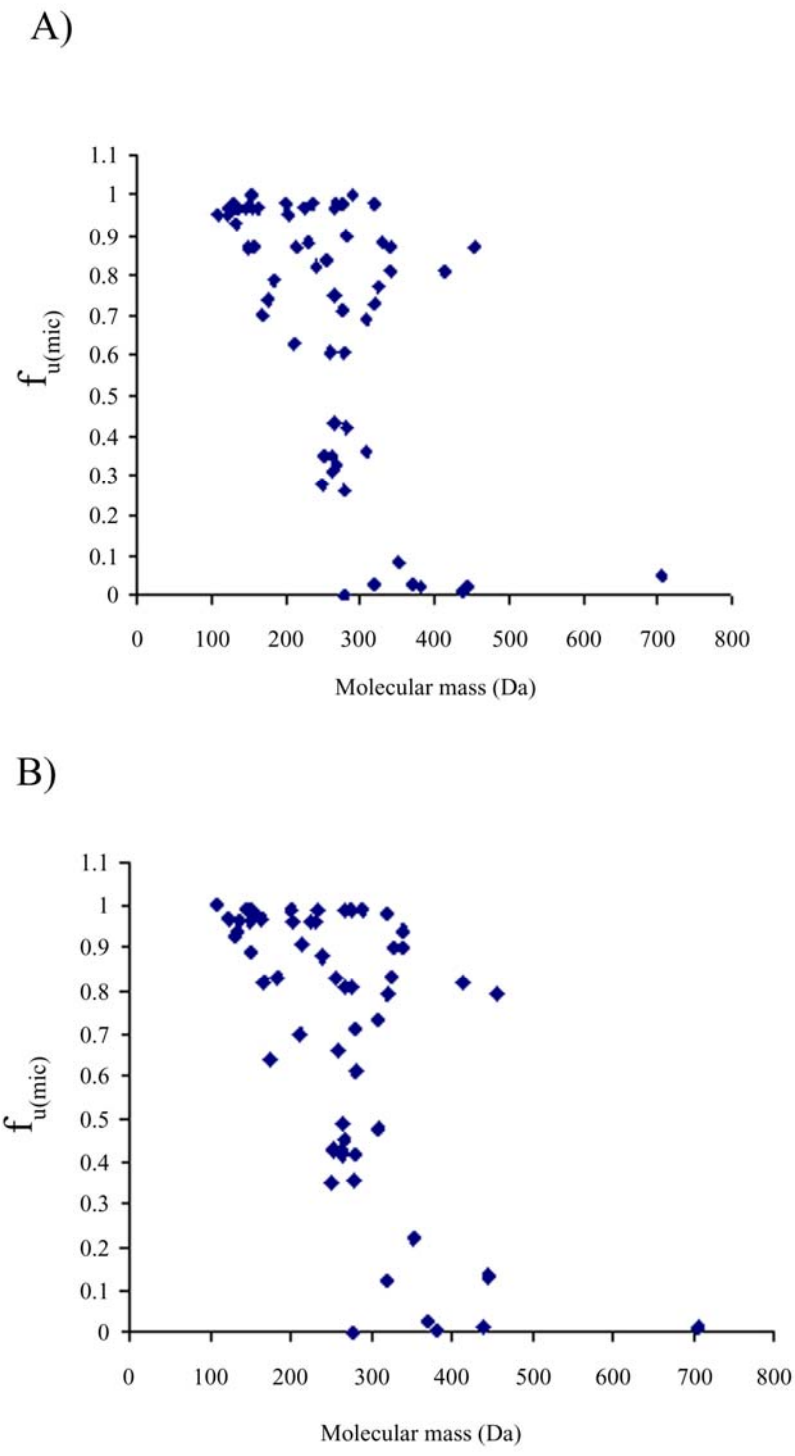
The derived $f_{u(mic)}$ values of bases were further stratified according to log P range. Results are shown in Table 6.3.

Table 6.3: Derived $f_{u(mic)}$ values for bases (determined at 100 μM) stratified for log P

log P	**Proportion of bases in each $f_{u(mic)}$ range**			
	0.90 – 1.0	**0.50 – 0.89**	**0.10 – 0.49**	**< 0.10**
-2 – 1	8/9	1/9	0/9	0/9
1.1 - 3	8/15	7/15	0/15	0/15
3.1 - 4	5/20	12/20	2/20	1/20
4.1 - 5	1/8	1/8	4/8	2/8
≥ 5.1	0/9	1/9	3/9	5/9

6.3.3.3 Molecular mass

The molecular mass of basic compounds characterised for non-specific binding was plotted against the derived $f_{u(mic)}$ at concentrations of 100, 200 and 500 μM. Results are shown in Figure 6.4.



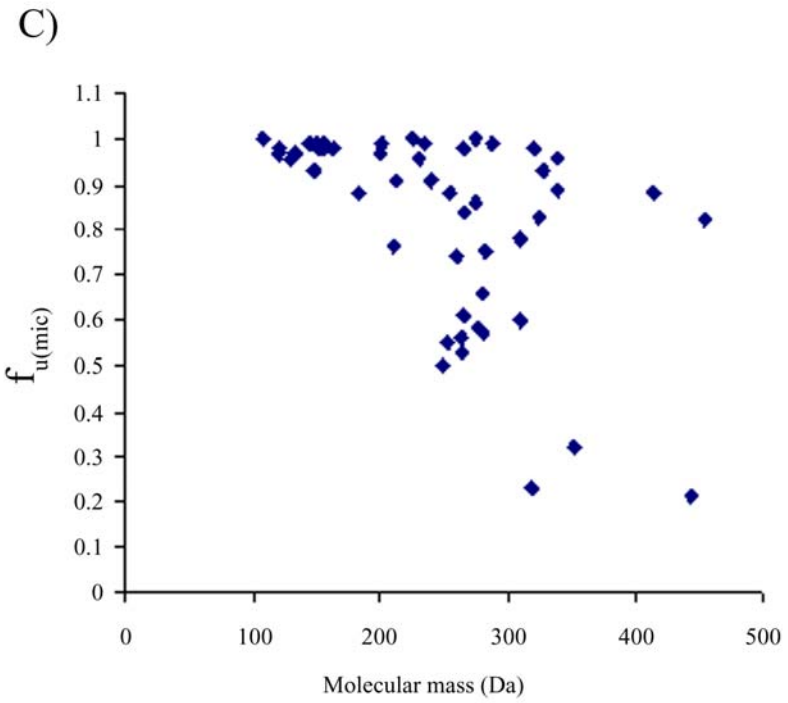


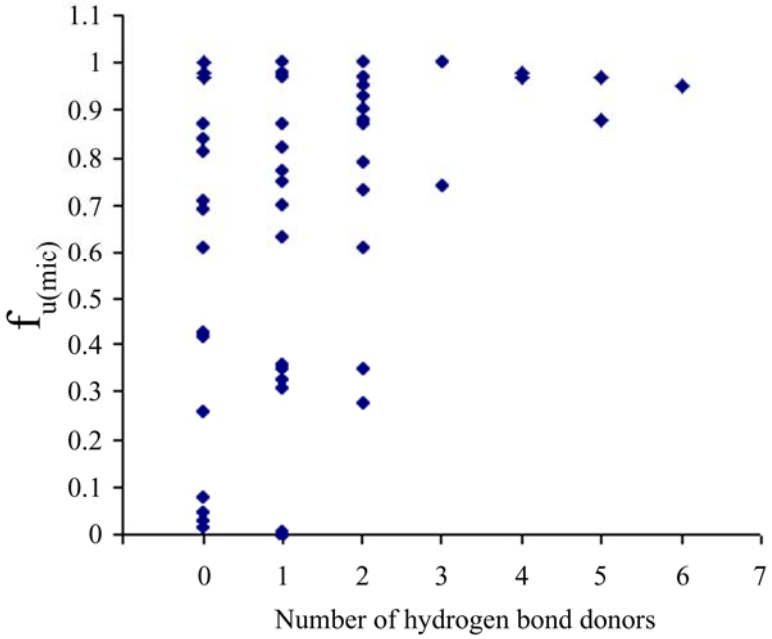
Figure 6.4: Relationship between molecular mass and the non-specific binding of basic compounds to human liver microsomes at concentrations of: A) 100 μM; B) 200 μM; and C) 500 μM.

Data were separated into two groups; basic compounds with molecular mass ≥ 250 Da and compounds of molecular mass < 250 Da and $f_{u(mic)}$ values were compared using the Mann-Whitney test. The mean $f_{u(mic)}$ (± standard deviation) values of compounds with molecular mass ≥ 250 Da were significantly lower at added drug concentrations of 100 μM (0.53 ± 0.35 vs 0.88 ± 0.16; p = 0.005), 200 μM (0.57 ± 0.34 vs 0.90 ± 0.14; p = 0.005) and 500 μM (0.71 ± 0.23 vs 0.94 ± 0.11; p = 0.008).

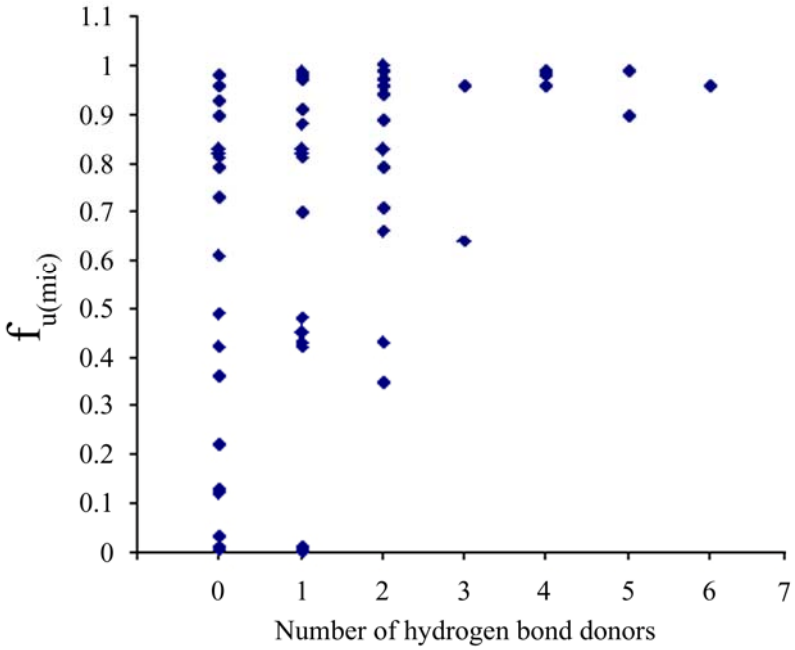
6.3.3.4 Hydrogen bond donors

The number of hydrogen bond donors present in each basic compound was plotted against derived $f_{u(mic)}$ at concentrations of 100, 200 and 500 μM. Results are shown in Figure 6.5.

A)


1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
$f_{u(mic)}$
0 1 2 3 4 5 6 7
Number of hydrogen bond donors

B)


1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
$f_{u(mic)}$
0 1 2 3 4 5 6 7
Number of hydrogen bond donors

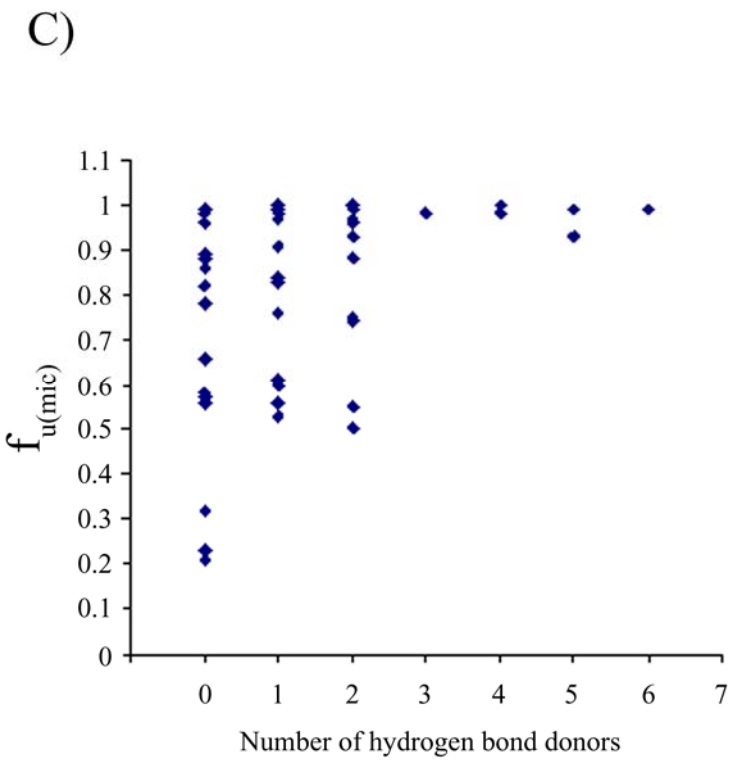
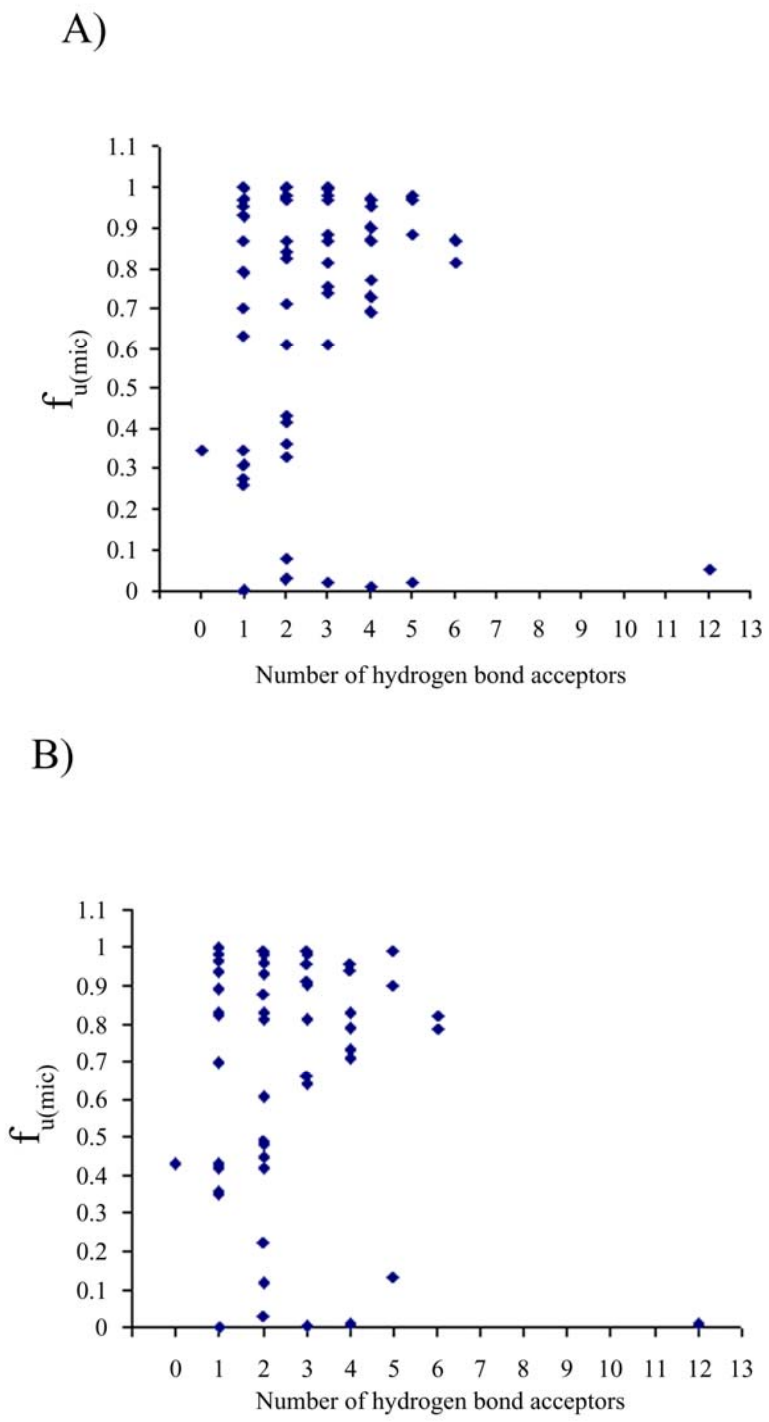


Figure 6.5: Relationship between the number of hydrogen bond donors present in each base and the non-specific binding of basic compounds to human liver microsomes at concentrations of: A) 100 μM; B) 200 μM; and C) 500 μM.

Trends in non-specific binding were similar at 100 μM (Figure 6.5 A), 200 μM (Figure 6.5 B), and 500 μM (Figure 6.5 C) with extensive ranges of binding for bases containing zero, one, or two hydrogen bond donors, while relatively minor binding was observed for those bases containing three, four, five, or six hydrogen bond donors. The $f_{u(mic)}$ values of compounds that contain 0, 1, or 2 hydrogen bond donors were compared to those which contain ≥ 3 hydrogen bond donors using the Mann-Whitney test. The mean $f_{u(mic)}$ (± standard deviation) values for compounds with 0-2 hydrogen bond donors were significantly lower than the mean $f_{u(mic)}$ values of compounds with 3-6 hydrogen bond donors at all three drug concentrations investigated: 100 μM, 0.64 ± 0.34 vs 0.93 ± 0.09 ($p = 0.003$); 200 μM, 0.68 ± 0.33 vs 0.92 ± 0.12 ($p = 0.014$); and 500 μM 0.79 ± 0.21 vs 0.98 ± 0.02 ($p = 0.001$).

6.3.3.5 Hydrogen bond acceptors

The number of hydrogen bond acceptors present in each basic compound characterised for microsomal binding was plotted against the derived $f_{u(mic)}$ at drug concentrations of 100, 200 and 500 μM. Results are shown in Figure 6.6.



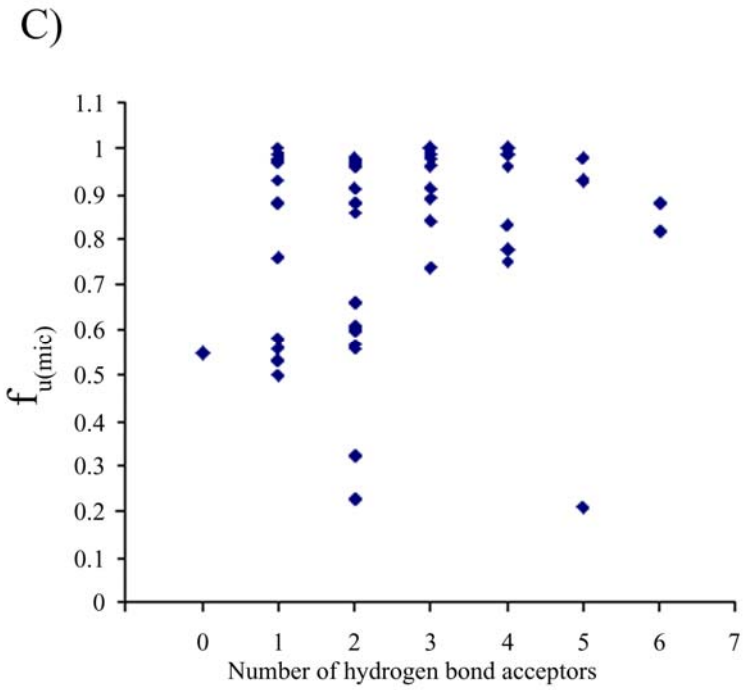


Figure 6.6: Relationship between the non-specific binding of basic compounds to human liver microsomes and the number of hydrogen bond acceptors present in each base at concentrations of: A) 100 μM; B) 200 μM; and C) 500 μM.

As with the hydrogen bond donors, compounds were separated into two groups, those compounds that contain 0, 1, or 2 hydrogen bond acceptors and those which contain ≥ 3 hydrogen acceptors for statistical analysis (Mann Whitney test). The mean $f_{u(mic)}$ (± standard deviation) values for compounds with 0-2 hydrogen bond acceptors were significantly lower then mean $f_{u(mic)}$ values of compounds with ≥ 3 hydrogen bond acceptors at 100 μM, (0.64 ± 0.33 vs 0.76 ± 0.32; $p = 0.046$) and at 500 μM (0.76 ± 0.23 vs 0.89 ± 0.17; $p = 0.014$), while there was no significant difference ($p > 0.05$) between mean values at 200 μM (0.66 ± 0.32 vs 0.77 ± 0.32).

6.4 Discussion

Of the ninety-nine investigational compounds listed (Table 6.1), eighty-eight, comprised of twenty-two acids, sixty-one bases and five neutral drugs, were characterised for non-specific binding to human liver microsomes (Table 6.2).

Eleven compounds were not characterised; ten due to ANS fluorescence quenching and one which fluoresced extensively in the presence of human liver microsomes (Section 6.3.1).

ANS fluorescence data were in agreement with previous reports and with the results presented in Chapters 4 and 5. That is, basic compounds that bind to human liver microsomes generally cause an increment in ANS fluorescence whereas neutral and acidic drugs that bind generally cause a decrement in ANS fluorescence. The ANS fluorescence change ranged from 4 to –62 and 6 to –35 percent for acidic and neutral drugs, respectively. ANS fluorescence changes for bases ranged from –24 (the anomalous ANS fluorescence decrement observed for bupropion) to 785 percent ANS fluorescence increment over the three drug concentrations tested.

6.4.1 Microsomal binding of the investigational compounds

6.4.1.1 Acidic compounds

Of the twenty-two acids tested for non-specific binding flufenamic acid, meclofenamic acid, and niflumic acid exhibited the highest binding of their class (Table 6.2). All three drugs are lipophilic compounds with log P values $\geq$ 4.9 (Table 6.2). Interestingly, flufenamic acid and niflumic acid contain a trifluoromethyl (-CF_3) group, while meclofenamic acid has two chlorine atoms. Available data indicates that the presence of halogens enhances membrane binding (Gerebtzoff et al. 2004). The remaining nineteen acids were bound minimally or not at all, displaying $f_{u(mic)}$ values > 0.8 (Table 6.2).

The negligible binding of caffeine, naproxen and tolbutamide (Table 6.2) based on ANS fluorescence data was consistent with previous observations from equilibrium dialysis experiments performed in this laboratory (McLure, Miners & Birkett 2000).

Furthermore, the –5% ANS fluorescence decrement observed for warfarin at 100 μM, which equates to an $f_{u(mic)}$ of 0.92 using the ANS fluorescence to $f_{u(mic)}$ conversion described in Figure 5.4 A, was in agreement with the $f_{u(mic)}$ value of 0.95 reported by (Obach 1997).

6.4.1.2 Basic compounds

Less than a third of the bases characterised for non-specific binding exhibited $f_{u(mic)}$ values < 0.5. Nine of the sixty-one bases exhibited $f_{u(mic)}$ values in the range 0.10 - 0.49; amitriptyline, desipramine, desmethylnortriptyline, N-didesmethylimipramine, fluoxetine, imipramine, mianserine, nortriptyline, and protriptyline. All nine drugs are lipophilic ($3.4 \leq \log P \leq 6.4$), with a molecular mass greater than 249 Da (range 249.4 - 309.3 Da) and possess zero, one or two hydrogen bond donors. Eight compounds were shown to bind very highly to human liver microsomes ($f_{u(mic)} < 0.1$); chlorpromazine, econazole, fluphenazine, itraconazole, perhexiline, thioridazine, thiothixene, and trifluprom azine (Table 6.2). The outstanding physicochemical features of these molecules is that they too are lipophilic ($3.9 \leq \log P \leq 7.0$), have relatively high molecular mass (288 - 706 Da) and contain either zero or one hydrogen bond donor. Furthermore, in agreement with Gerebtzoff (2004) and results presented for the binding of acids, the presence of a halogen enhances membrane binding; chlorpromazine, econazole, fluphenazine, itraconazole and trifluprom azine all contain one or more chlorine atoms or a trifluoromethyl group. The remaining forty-four bases exhibited low non-specific binding to human liver microsomes ($f_{u(mic)} \geq 0.5$), with twenty-two of these having negligible or no microsomal binding ($0.90 \leq f_{u(mic)} \leq 1$; Table 6.2). These compounds (1-acetyl-2-phenylhydrazine, albendazole, atenolol, benzylamine, N-benzylmethylamine, bupivacaine, chloroquine, cinnoline, N-N-dimethylbenzylamine, 3-hydroxytyramine, lignocaine,

mephentermine, phentolamine, β-phenylethylamine, phenylpropanolamine, 4-phenylpyridine, ropivacaine, spermidine, spermine, terbutaline, tetrahydrozoline, and tranyl-cypromine) contained one or more of the following physicochemical characteristics; a low log P value (log P < 2), low molecular mass (< 200 Da), and three or more hydrogen bond acceptors.

Chlorpromazine, amitriptyline, nortriptyline, and imipramine (at a concentration of 1000 μM) were previously investigated for their effect on ANS fluorescence in the presence of rat liver microsomes (0.4 mg/ml). Birkett (1974) reported respective percent ANS fluorescence increments of 390, 295, 290 and 224 percent for these compounds.

6.4.1.3 Neutral compounds

Budesonide, carbamazepine, diazepam, metyrapone, and propofol all showed low to minimal binding ($0.70 \leq f_{u(mic)} \leq 0.88$) over the drug concentration range tested. Apart from metyrapone all of the neutral compounds are reasonably lipophilic with $\log P \geq 2.7$.

6.4.2 Relationships between selected physicochemical characteristics and the non-specific binding of compounds to human liver microsomes

The relationship between the charge state of the investigational compounds and microsomal binding was initially evaluated (Figure 6.1). With the exception of two acidic compounds (meclofenamic acid and flufenamic acid), the acid and neutral classes of compounds were shown to bind to a low or negligible extent, or not at all (Figure 6.1 A, B, and C and Table 6.2). Therefore, these two classes of compounds were defined as weak binders and as such were not subjected to any further analysis. In contrast, bases spanned close to the full $f_{u(mic)}$ range (Figure 6.1 A, B, and C) and were therefore further investigated for relationships between non-specific binding

and pK_a, log P, molecular mass, number of hydrogen bond donors, and number of hydrogen bond acceptors per molecule.

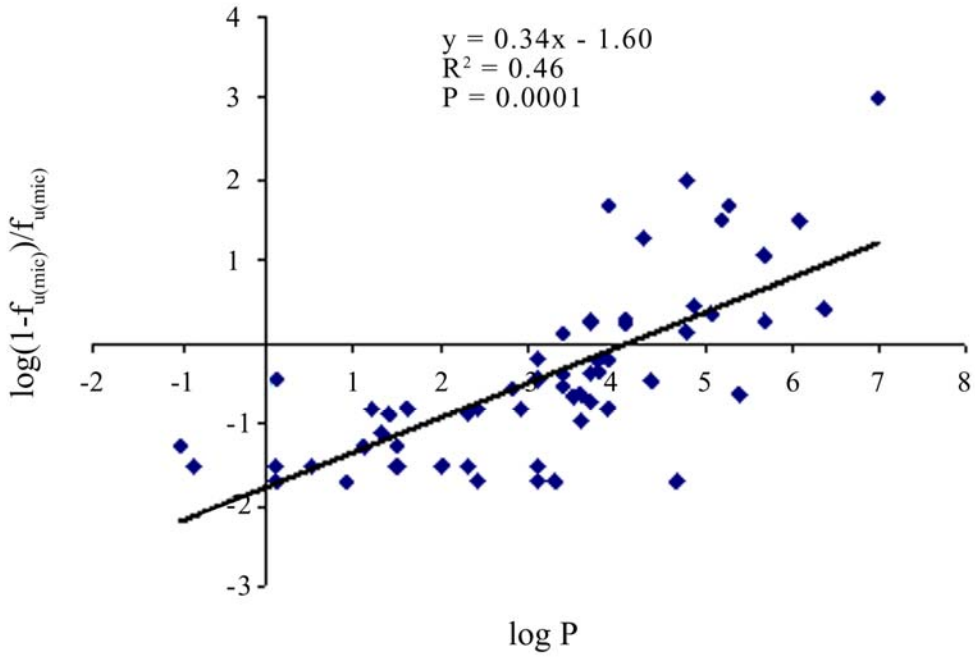
6.4.2.1 pK_a

Although there was no clear relationship between pK_a and non-specific microsomal binding (Figure 6.2 A, B, and C), the most significant binding ($f_{u(mic)} \leq 0.49$) was shown by bases with pK_a values between 6.3 - 10.7. However, there were also many compounds within this range that bound moderately, minimally or not at all. The majority of bases with pK_a values in the range 6.8 - 7.7 showed minimal to no binding. It should be noted that relatively few compounds with pK_a values between 7.8 – 8.7 and 10.8 – 11.7 were tested here for non-specific binding to human liver microsomes.

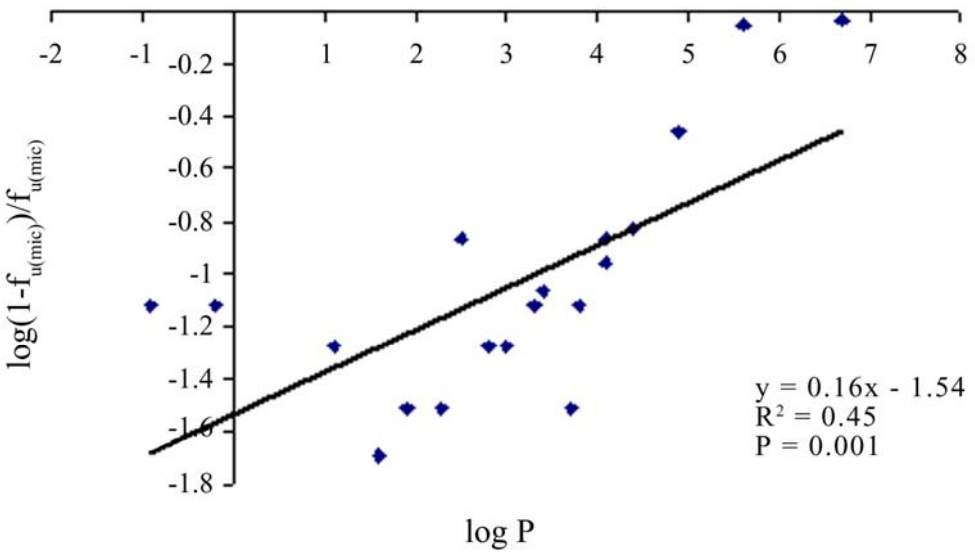
6.4.2.2 log P

The relationship between log P and microsomal binding of bases was best described by a second order polynomial (Figure 6.3 A, B, and C). Recent studies (Austin et al. 2002; Sykes, Sorich & Miners 2006) have explored the relationship between log P and microsomal binding as the logarithmic transformation log $(1-f_{u(mic)})/ f_{u(mic)}$, which is similar to an equilibrium constant and hence appropriate for considering linear free energy relationships (Austin et al. 2002). Thus, data generated at a drug concentration of 100 μM were re-evaluated as log P versus log $(1-f_{u(mic)}) / f_{u(mic)}$ (Figure 6.7).

A)


y = 0.34x - 1.60
R² = 0.46
P = 0.0001
log(1-f u(mic))/f u(mic)
log P

B)


y = 0.16x - 1.54
R² = 0.45
P = 0.001
log(1-f u(mic))/f u(mic)
log P

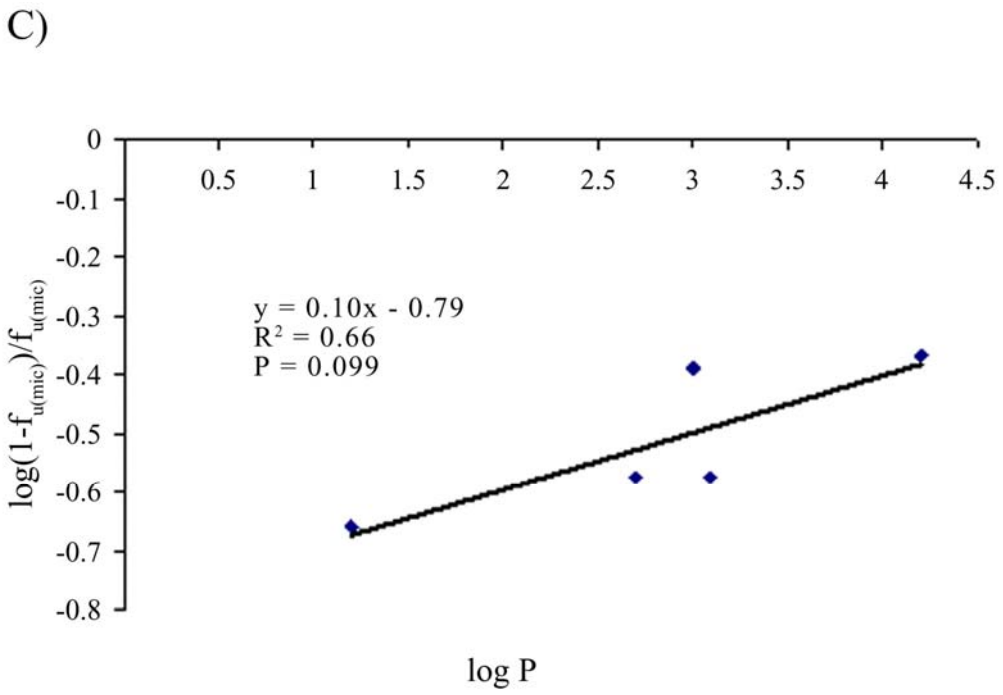


Figure 6.7: Plots of log $(1\text{-}f_{u(mic)}) / f_{u(mic)}$ versus log P for: A) bases, B) acids, and C) neutral compounds at an added drug concentration of 100 µM.

In agreement with Sykes et al. (2006) and Austin et al. (2002) a significant linear relationship was observed between log P and log $(1\text{-}f_{u(mic)}) / f_{u(mic)}$. Austin et al. (2002) showed a linear relationship for all drugs whereas Sykes et al. (2006) analysed data according to charge state; that is for bases, acids, and neutrals. In agreement with Sykes et al. (2006) each class of drug showed a linear relationship with the bases showing the most statistically significant relationship ($p = 0.0001$) when analysed using a linear regression.

In general, non-specific binding of the bases increased with lipophilicity (Table 6.3). There were, however, many exceptions to the trend. There were eight compounds which have log P values $\geq$ 3.1 which bound minimally or not at all to human liver microsomes. Those compounds were albendazole, bupivacaine, chloroquine, dextropropoxyphene, phentolamine, ropivacaine, tetrahydrozoline, and verapamil (Table 6.2). Notably, with the exception of tetrahydrozoline, all contain $\geq$ 3

hydrogen bond acceptors, a determinant associated with lower non-specific microsomal binding (Section 6.3.3.5). Interestingly, all compounds contain 0-2 hydrogen bond donors which was described as a determinant of extensive binding in Section 6.3.3.4. This once again shows that there are exceptions to the general rules described. It should also be noted that choroquine and verapamil were investigated for non-specific binding using equilibrium dialysis in Chapter 5, and exhibited $f_{u(mic)}$ values lower than those derived from the ANS fluorescence protocol. The reason for this is unknown.

6.4.2.3 Molecular mass

There was a statistically significant difference in the binding of compounds with molecular mass < 250 Da and compounds with molecular mass ≥ 250 Da. Higher microsomal binding was observed for compounds with a molecular mass ≥ 250 Da (Figure 6.4 A, B, and C). A notable exception was desmethylnortriptyline (molecular mass 249.4), which exhibits an $f_{u(mic)}$ value of 0.28 at 100 μM.

6.4.2.4 Hydrogen bond donors

A relationship was demonstrated between the number of hydrogen bond donors present in each base and microsomal binding (Figure 6.5 A, B, and C). Extensively bound bases contained either zero, one or two hydrogen bond donors. Bases with three, four, five, and six hydrogen bond donors showed comparatively minimal binding.

6.4.2.5 Hydrogen bond acceptors

Higher binding was shown to be more likely for compounds containing 0, 1, or 2 hydrogen bond acceptors compared to compounds containing ≥ 3 hydrogen bond acceptors. A notable exception was itraconazole, which has 12 hydrogen bond

donors but binds extensively to human liver microsomes. Moreover, there were several compounds with < 3 hydrogen bond acceptors which exhibited minimal binding.

6.4.3 Comparison between physicochemical characteristics associated with non-specific binding and membrane permeability

Permeability was the process chosen for comparative purposes as partitioning of a compound into the membrane necessarily occurs as part of the process by which a drug traverses the membrane (Oldendorf 1974; Conradi, Burton & Borchard 1996; Malkia et al. 2004). In agreement with four of the predictive relationships described between physicochemical characteristics and extent of non-specific binding, Burton et al. (2002) indicates solute size, lipophilicity, hydrogen bond potential and charge characteristics as being important for the membrane partitioning of drugs. Data described in this chapter should not be compared directly to the results of Lipinski et al. (1997) since the 'rule of five' relates to both the solubility and permeability.

The presented results indicate that acidic and neutral compounds bind human liver microsomes to a 'minor' extent while bases bind over a wide range. Statistical analysis of data indicates clear relationships between the extent of binding of bases and the physicochemical characteristics molecular mass, number of hydrogen bond donors, and number of hydrogen bond acceptors per molecule and extent of non-specific binding to human liver microsomes. Furthermore, a general trend of increasing lipophilicity (as log P) of bases is associated with higher binding, although there are exceptions. High binding was also shown by bases with pK_a values in the range 6.3 – 10.7, although there were also many bases in this category that bound moderately, minimally or not at all. It is for the exceptions to general rules and even to statistically significant data that a molecular modeling path will be

taken to attempt to identify particular molecular structures which are found to be important for the non-specific binding of drugs to human liver microsomes.

CHAPTER 7
IN SILICO MODELING OF THE NON-SPECIFIC BINDING OF DRUGS TO HUMAN LIVER MICROSOMES

7.1 Introduction

The prediction of pharmacokinetic parameters *in vivo* has become an integral part of the preclinical drug development process, as early as possible, to eliminate candidate compounds with poor disposition and to progress better candidates into the clinical development phase (Yu & Adedoyin 2003). Traditionally, *in vitro* and animal *in vivo* data have been used to investigate drug kinetic behaviour. However, *in silico* (computational) techniques are becoming more widely utilized to explore ADMET properties of new chemical entities (Darvas et al. 2002; Lombardo et al. 2002). In particular, Quantitative Structure-Activity Relationships (QSAR) and other computational designs (pharmacophores, molecular similarity techniques, and quantum chemical approaches) are available for modeling a biological outcome (Palm et al. 1996; Cruciani, Pastor & Guba 2000).

The disposition of a drug *in vitro* or *in vivo* can be closely linked to its structure and physicochemical characteristics (von Geldern et al. 1996; Ekins & Obach 2000; Osterberg & Norinder 2000; Kulkarni, Han & Hopfinger 2002). The effect of lipophilicity and pK_a, while maintaining molecular mass constant, on the non-specific binding of drugs to human liver microsomes were initially investigated (Chapter 3) in this thesis. Subsequently, the relationships between selected physicochemical characteristics identified as important for the permeability of a compound, charge, pK_a, log P, molecular mass, and the number of hydrogen bond donors and acceptors per molecule (Figure 6.1 – Figure 6.6), were explored.

Statistically significant relationships were observed between $f_{u(mic)}$ of bases and log P, charge, molecular mass, and the number of hydrogen bond donors and acceptors per molecule. This Chapter reports computational modeling of the ANS fluorescence data generated in Chapter 6. The approach adopted is based on a previous modeling study conducted in this laboratory (Sykes, Sorich & Miners 2006) which employed the program ROCS (Rapid Overlay of Chemical Structures) to analyse the non-specific binding of a smaller dataset of drugs.

Virtual high throughput screening is a computational technique used in drug discovery research. It involves assessment of large libraries of chemical structures in order to guide the selection of likely drug candidates. To shorten the time spent in the drug development phase and to minimise the high rate of attrition of active compounds, drug researchers are beginning to incorporate structure-permeation, structure-distribution, structure-metabolism, and structure-toxicity relations into drug design. Thus, biological, physicochemical, and computational approaches are being developed whose objectives are to increase the clinical relevance of drug discovery, and to eliminate as soon as possible compounds with unfavourable physicochemical and pharmacokinetic properties.

7.2 Methods

The eighty-eight compounds (twenty-two acids, sixty-one bases, and five neutrals) characterized for non-specific binding in Chapter 6 were taken through a three step process which aimed to differentiate 'high' binders ($f_{u(mic)} < 0.5$) from compounds with 'low' binding ($f_{u(mic)} \geq 0.5$). The basis for this approach is a fundamental principle of chemoinformatics, the similarity property principle (Sykes, Sorich &

Miners 2006). This notion is based upon the assumption that structurally similar molecules will have similar biological activity.

The three step process undertaken was as follows:

1. *Defining the structure of the molecule*

   A one dimensional structure of each molecule was generated using SMILES (Weininger 1988). This is known as a 'SMILES string'. SMILES is a simple yet comprehensive chemical nomenclature. SMILES strings can be obtained from numerous chemical databases; the two programs used for this study were ChemBank (http://chembank.broad.harvard.edu/) and DrugBank (Wishart et al. 2006).

2. *3-Dimensional structure and conformer generation*

   Omega Version 2.2.1 (Omega 2007) was used to generate 3-dimensional structures and conformers from the SMILES string. Omega comprises two main components; model building and torsion driving. Omega builds initial models of structures by assembling fragment 'libraries', generated from fragmentation of rotable bonds. The torsion-driving stage then generates conformers within predefined limits based on energy and ensemble size. Energy ranking is performed using the mmff94s forcefield (Halgren 1996). Omega has been shown to perform well with respect to producing conformations of protein-bound ligands (Bostrom 2001; Bostrom, Greenwood & Gottfries 2003), and in recent studies in this laboratory (Sykes, Sorich & Miners 2006; Sykes, McKinnon & Miners 2008).

3. *Overlays*

The program ROCS (2007) was used to overlay one chemical structure on another. All conformers generated were overlaid onto the query molecule, chlorpromazine, and the best match by color score selected. Chlorpromazine was chosen as the query molecule from a previous analysis of molecules that discriminated between high and low binders using a smaller data set (Sykes, Sorich & Miners 2006).

ROCS maximises the overlap between the volumes of the query molecule and the database molecule of interest using Gaussian functions (Grant, Gallardo & Pickup 1996). The overlay is quantified by a shape tanimoto. A maximum score of 1.0 is given for shape similarity. The closer the shape tanimoto is to 1.0, the better the match (i.e. the more similar the molecules). A color forcefield can also be used as a representation of the chemical features of the molecule. The color forcefield assesses chemical complementarity and refines the shape overlays on the basis of chemical features. Molecules are sorted into chemical types with respect to hydrogen bond donors, hydrogen bond acceptors, rings, anions, cations, hydrophobic and hydrophilic regions, etc and the color forcefield is applied to the initial shape overlay. A maximum score of 1.0 is given for a color score.

Thus, there are three options for quantification using ROCS; shape, color, and their combination (shape plus color). A perfect combination (or ‘combo’) score would be 2.00. However Sykes et al. (2006) found that ‘high’ binders could be differentiated from ‘low’ binders using the color score alone. Nevertheless, the importance of the molecular shape should not be underestimated, as the initial

ROCS alignment makes use of shape. In addition shape can be employed to visualize differences between 'high' and 'low' binders.

7.3 Results

Table 7.1 shows the results of the ROCS analysis ranking the compounds by their color scores. In each case the best match by color score of each multiconformer database member is shown. 'High' binding molecules are highlighted. A previous study in this laboratory, based on a smaller database (Sykes, Sorich & Miners 2006), showed that a color score of ≥ 0.6 identified 94% of the high binders.

Table 7.1: Results from the ROCS analysis ranked in order of color score. High binding compounds are coloured in blue.

#	Drug	Shape	Color	ComboScore	$f_{u(mic)}$
1	Chlorpromazine	1.00	1.00	2	0.03
2	Triflupromazine	0.93	1.00	1.93	0.08
3	Fluphenazine	0.77	1.00	1.77	0.01
4	Thioridazine	0.76	1.00	1.76	0.03
5	N-Didesmethylimipramine	0.78	0.97	1.75	0.35
6	Desipramine	0.82	0.97	1.78	0.33
7	Imipramine	0.82	0.97	1.79	0.42
8	Doxepin	0.82	0.95	1.77	0.61
9	Nortriptyline	0.78	0.93	1.70	0.35
10	Thiothixene	0.62	0.92	1.54	0.02
11	Amitriptyline	0.77	0.91	1.67	0.26
12	Desmethylnortriptyline	0.74	0.91	1.65	0.28
13	Protriptyline	0.57	0.86	1.44	0.31
14	Propranolol	0.67	0.75	1.42	0.61
15	Chloroquine	0.58	0.75	1.32	0.98
16	Diphenhydramine	0.71	0.74	1.45	0.61
17	Benzydamine	0.47	0.73	1.21	0.69
18	Disopyramide	0.59	0.72	1.31	0.87
19	Chlorpheniramine	0.68	0.72	1.40	0.71
20	Diltiazem	0.41	0.72	1.13	0.81
21	Fluoxetine	0.62	0.72	1.34	0.36
22	Quinine	0.60	0.70	1.30	0.77
23	Mianserine	0.77	0.70	1.46	0.43
24	Perhexiline	0.57	0.69	1.25	0.001
25	Carbamazepine	0.67	0.68	1.35	0.79
26	Dextropropoxyphene	0.55	0.68	1.22	0.81
27	Antazoline	0.64	0.68	1.31	0.75
28	Econazole	0.65	0.65	1.30	0.02
29	Cicloprofen	0.43	0.64	1.08	0.93
30	Carbazole	0.64	0.63	1.27	0.70
31	Quipazine	0.57	0.57	1.14	0.87

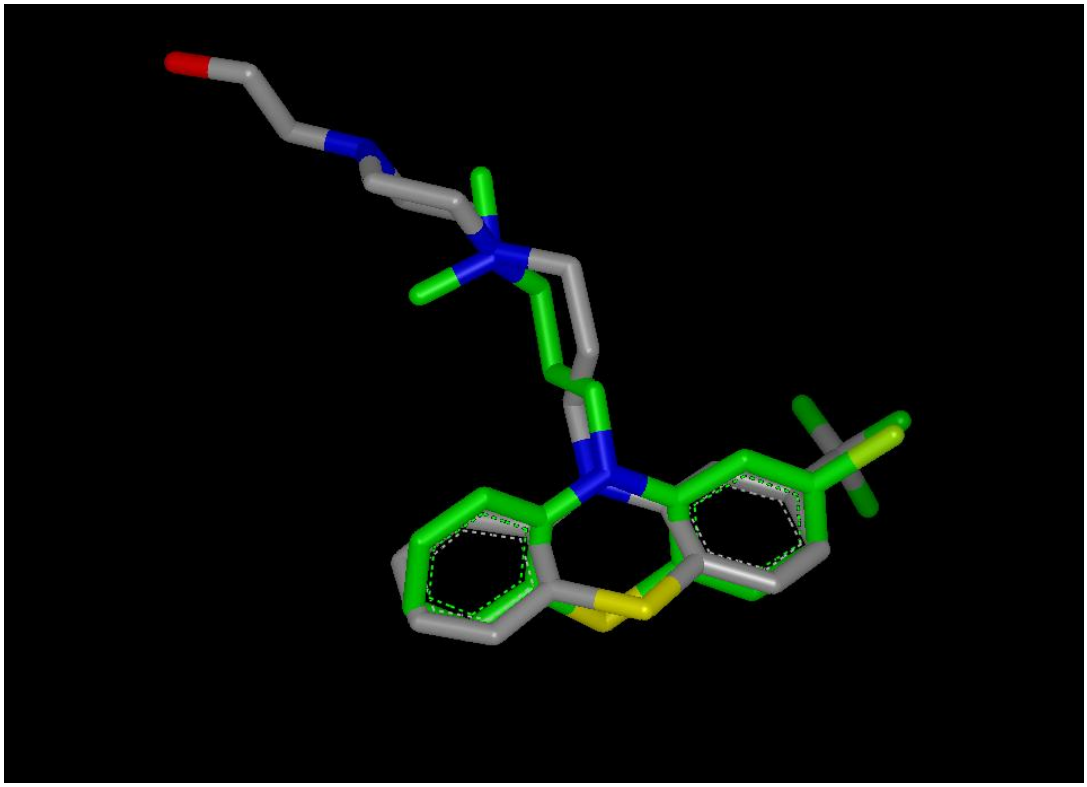
#	Drug	Shape	Color	ComboScore	$f_{u(mic)}$
32	Budesonide	0.39	0.57	0.96	0.79
33	Labetalol	0.48	0.53	1.02	0.88
34	Phentolamine	0.62	0.51	1.13	0.9
35	Verapamil	0.43	0.51	0.93	0.87
36	Lignocaine	0.42	0.50	0.92	0.98
37	4-Phenylbutylamine	0.50	0.50	1.00	0.87
38	Atenolol	0.54	0.50	1.04	0.98
39	Fluvoxamine	0.40	0.50	0.90	0.73
40	Niflumic acid	0.49	0.50	0.99	0.74
41	Bupivacaine	0.36	0.50	0.86	1.00
42	Ropivacaine	0.60	0.50	1.10	0.98
43	Diazepam	0.62	0.50	1.12	0.71
44	Tetrahydrozoline	0.44	0.50	0.94	0.98
45	Meclofenamic acid	0.50	0.50	1.00	0.52
46	Flufenamic acid	0.51	0.50	1.01	0.53
47	Itraconazole	0.23	0.50	0.73	0.05
48	Diclofenac	0.51	0.50	1.01	0.88
49	Debrisoquine	0.32	0.50	0.82	0.74
50	Warfarin	0.55	0.50	1.05	0.92
51	Naproxen	0.63	0.50	1.13	0.95
52	Cinnoline	0.54	0.50	1.04	0.98
53	Phenytoin	0.63	0.50	1.13	0.88
54	Fenoprofen	0.50	0.49	0.99	0.93
55	Albendazole	0.52	0.48	1.00	0.97
56	Caffeine	0.31	0.48	0.79	1.00
57	Metyrapone	0.68	0.47	1.15	0.82
58	Clonidine	0.54	0.47	1.00	0.88
59	Chlorphentermine	0.44	0.47	0.90	0.79
60	Mephentermine	0.45	0.47	0.91	0.97
61	β-Phenylethylamine	0.40	0.46	0.86	0.95
62	Tranylcypromine	0.38	0.46	0.84	0.93
63	3-Hydroxytyramine	0.44	0.46	0.89	0.97
64	Terbutaline	0.49	0.46	0.95	0.97
65	Phenylpropanolamine	0.45	0.45	0.90	1.00
66	Bupropion	0.45	0.44	0.89	0.82
67	Gliclazide	0.40	0.44	0.84	1.00
68	Diflunisal	0.56	0.43	0.99	0.87
69	Flurbiprofen	0.53	0.43	0.95	0.90
70	4-Phenylpyradine	0.54	0.43	0.97	1.00
71	4-4'-Dipyridyl	0.46	0.43	0.89	0.87
72	Lamotrigine	0.57	0.42	0.99	0.93
73	S(-)-N-benzyl-α-methylbenzylamine	0.51	0.38	0.89	0.63
74	N-N-Dimethylbenzylamine	0.35	0.25	0.60	0.97
75	N-Benzylmethylamine	0.48	0.25	0.73	0.97
76	Benzylamine	0.34	0.25	0.60	0.95
77	Tolbutamide	0.50	0.25	0.75	0.97
78	Alclofenac	0.62	0.25	0.87	0.95
79	Sodium salicylate	0.50	0.25	0.76	0.97
80	Probenecid	0.57	0.25	0.82	0.93
81	Phenelzine	0.43	0.25	0.68	0.95
82	Ibuprofen	0.44	0.25	0.69	0.97
83	Isoniazid	0.49	0.25	0.74	0.93
84	1-Acetyl-2-phenylhydrazine	0.51	0.25	0.76	1.00

#	Drug	Shape	Color	ComboScore	$f_{u(mic)}$
85	Propofol	0.59	0.25	0.84	0.70
86	Phenacetin	0.48	0.25	0.73	0.98
87	Spermine	0.38	0.25	0.63	0.95
88	Spermidine	0.46	0.25	0.71	0.97

Sixteen of the seventeen high binding molecules were within the ≥ 0.6 color cutoff score. The one high binding molecule outside of the cutoff score was itraconazole. However, 14 compounds were incorrectly classified as 'high' binders based on this cutoff score.

Figure 7.1 shows the combined shape/color overlay of two compounds, fluphenazine (high binder; Figure 7.1 A) and warfarin (low binder; Figure 7.1 B). As observed in the ROCS modeling study of Sykes et al. (2006), high binders such as chlorpromazine and fluphenazine have an amino group on a conformationally flexible side chain. The amino group is located approximately 4-5 Å from a hydrophobic ring system. Warfarin lacks a side-chain amino group and the tricyclic ring characteristic of chlorpromazine.

A)



B)

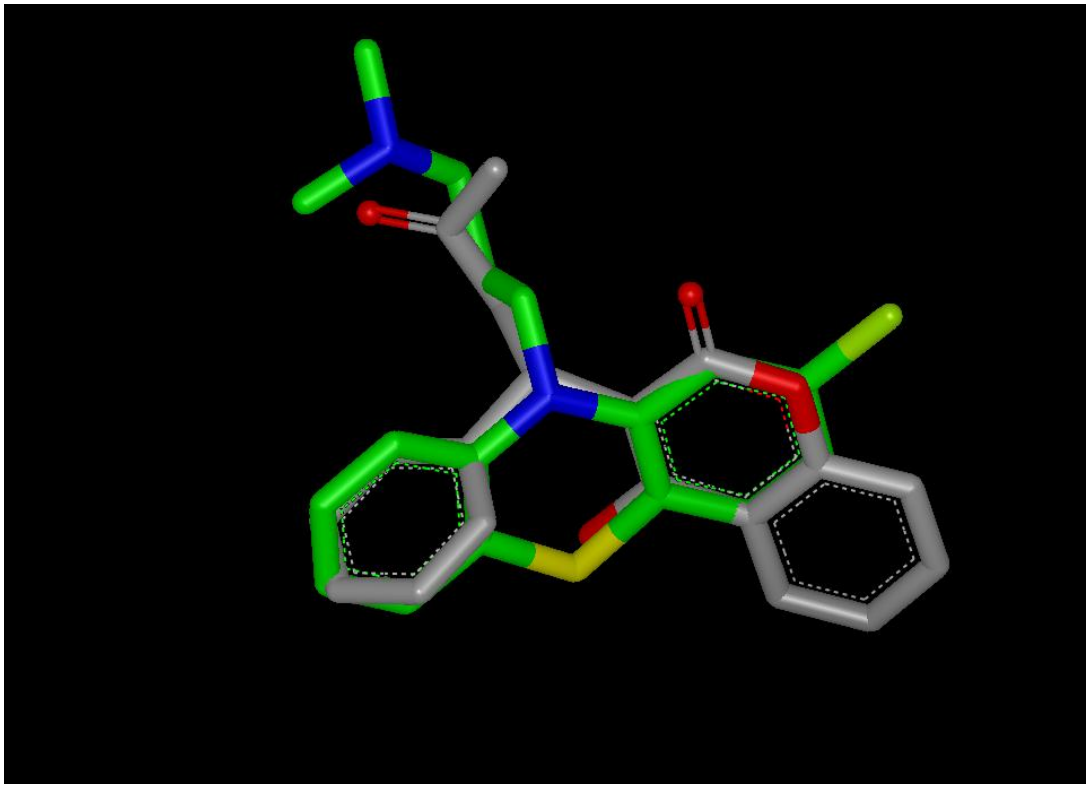


Figure 7.1: Combined shape/color overlay from the ROCS program of: A) fluphenazine (color score 1) and B) warfarin (color score 0.5). In each case the query molecule, chlopromazine, is shown in green.

7.4 Discussion

A previous study from this laboratory (Sykes, Sorich & Miners 2006) applied ROCS to a database of 56 drugs for which non-specific microsomal binding had been determined (Austin et al. 2002). The Austin et al. (2002) database included 17 acids, 19 neutrals, and 20 bases. Experimental data were generated in both the authors' laboratory and by Obach (1997 and 1999) with either human or rat liver microsomes. Sykes et al. (2006) found that use of chlorpromazine as the query molecule more effectively discriminated between 'high' and 'low' binding molecules compared to all other compounds in the Austin et al. (2002) database. The 18 high binders in the dataset were grouped in the top 22 'hits' from ROCS.

This Chapter reports a preliminary modeling study of the database generated in Chapter 6 using the ANS fluorescence technique. All but one high binder was predicted using a color score of $\geq$ 0.6 as the cut-off. As noted in Results, a color score $\geq$ 0.60 identified 94% of high binders in the study of Sykes et al. (2006). The one high binder below this color score was itraconazole. Itraconazole is a large compound (molecular mass 706), with seven rings. The large difference in size and shape between itraconazole and the query compound could potentially cause problems for the ROCS algorithm. Another azole antifungal, econazole, was correctly predicted as a high binder. Econazole is somewhat smaller than itraconazole, with a molecular mass of 382 and only three rings.

Although a color cut-off score of $\geq$ 0.60 correctly predicted all but one high binder, there were 14 false positives, compared to just 2 in the study of Sykes et al. (2006). Use of a cut-off score of $\geq$ 0.80 would correctly predict 12 high binders with just 1 false positive, but there would be 6 false negatives (i.e. 67% prediction accuracy). Similarly, use of a combo score cut-off $\geq$ 1.25 would discriminate all high binders

(except itraconazole), but exclude just four of the 14 false positives. Nevertheless, the color score cut-off of 0.60 excluded several compounds that might be predicted to be high binders on the basis of simple analyses, such as the use of log P alone (e.g. bupropion and bupivacaine).

The database modeled here is larger than that of Sykes et al. (2006), and has greater structural diversity. It is possible that more than one query molecule needs to be employed to correctly identify all high binders. Inspection of Figure 7.1 shows that compounds with structural features in common to chlorpromazine, such as a side-chain amino group and tricyclic ring system, overlay well. Thus, all tricyclic antidepressants are predicted to be high binders, even though doxepin is not in this category. Similarly, other tricyclic structures (e.g. carbamazepine) and compounds with an amino containing side-chain (e.g. propranolol) have high color and combo scores.

The color (and combo) score decreases with decreasing structural similarity to the query molecule. In this regard, Sykes et al. (2008) reported that the combo score for a series of cytochrome P450 2C9 (CYP2C9) substrates based on flurbiprofen as the query molecule failed to identify known neutral (e.g. safrole) and basic (e.g. fluoxetine) substrates of this enzyme. Further inclusion of fluoxetine as a query molecule successfully identified ‘atypical’ amine substrates of CYP2C9. Thus, the use of multiple query molecules may permit higher color- or combo- score cut-offs that successfully identify high binders while excluding low binders.

CHAPTER 8
CONCLUDING COMMENTS

For more than forty years *in vitro* studies utilising a range of experimental systems have been employed to predict *in vivo* drug metabolism and disposition parameters prior to clinical trials in humans. Varied success has been documented for quantitative prediction of hepatic clearance and drug–drug interaction potential (Rane, Wilkinson & Shand 1977; Houston 1994; Iwatsubo et al. 1996; Zomorodi & Houston 1996; Iwatsubo et al. 1997a; Ito et al. 1998a; Naritomi et al. 2001; Soars, Burchell & Riley 2002; Yao & Levy 2002; Miners et al. 2006). An important cause of erroneous predictions is sub-optimal experimental conditions and failure to account for the many variables associated with quantitative *in vitro – in vivo* extrapolation.

Factors that impact on the prediction of *in vivo* hepatic clearance include:

- Incubation conditions. Enzyme activities can vary with buffer pH, type and ionic strength, and in the case of glucuronidation, the presence of activators (Miners et al. 2006).
- Differences between enzyme sources (human liver microsomes, hepatocytes, recombinant enzymes).
- The occurrence of atypical enzyme kinetics *in vitro*, and hence the *in vitro* kinetic parameter used for extrapolation (Houston & Kenworthy 2000).
- Physiological scaling factors, particularly microsome yield and hepatocellularity (Barter et al. 2007).

- Use of the unbound fraction of drug in the plasma, rather than in blood, in mathematical models of hepatic clearance such as the well-stirred model (Yang et al. 2006).
- The potential role of transporters in the hepatic uptake of drugs. However, (Hallifax & Houston 2006b) have shown recently that hepatic uptake is not rate-limiting in the clearance of lipophilic drugs by isolated hepatocytes.
- Non-specific binding of the drug to the microsomal membrane.

Many of these variables can be accommodated by good experimental design and the use of appropriate scaling factors. In the case of non-specific binding, however, it is necessary to know the extent of membrane binding, which requires measurement of this parameter.

The non-specific binding of drugs to the *in vitro* matrix was first documented in 1963 (Gillette 1963). Twenty-three years passed before this type of binding was accounted for in the calculation of *in vitro* intrinsic clearance (Baarnhielm, Dahlback & Skanberg 1986). Numerous papers since have corrected for non-specific binding in calculations of intrinsic clearance and inhibition constant, leading to a significant improvement in the prediction accuracy of CL_{int} *in vivo* and the magnitude of inhibitory interactions (Obach 1997; Carlile et al. 1999; Obach 1999, 2000; Venkatakrishnan et al. 2000; Austin et al. 2002; Tran et al. 2002; Yao & Levy 2002; Margolis & Obach 2003). As a result of this improvement, investigation of the nature of the drug-membrane interactions has slowed. Several publications have documented the location of particular drugs within biomembranes and synthetic phospholipid bilayers using increasingly sophisticated physical techniques, such as atomic force microscopy (Schuster, Fleschurz & Helm 1975; Herbette, Katz & Sturtevant 1983; Herbette, Chester & Rhodes 1986; Austin, Davis & Manners 1995;

Hanakam et al. 1996; Krämer et al. 1998; Schreier, Malheiros & de Paula 2000; Nussio et al. 2007). Furthermore, some studies have described the lateral movement of drug along phospholipid bilayers, a route which enables access of drug to membrane bound receptors (Rhodes, Sarmiento & Herbette 1985; Mason & Chester 1989; Seydel & Wiese 2002). Despite the wider importance of drug-membrane interactions, however, few studies have investigated the contribution of physicochemical factors other than lipophilicity and charge as determinants of the non-specific binding of drugs and other chemicals to membranes.

The non-specific binding of drugs to human liver microsomes was well-recognised at the commencement of this thesis and, as noted above, log P and charge were considered the main, if not sole, determinants of binding. Initial experiments were conducted to systematically investigate the relationships between log P and pK_a (at constant molecular mass) and the non-specific binding of drugs to human liver microsomes. The lack of binding observed for lignocaine, bupivacaine, and ropivacaine indicated that physicochemical characteristics other than lipophilicity and charge may be important for binding. This was confirmed by observations, for example the non-binding of bupropion, in later experiments.

It was considered that elucidation of the physicochemical characteristics of drugs that determined the non-specific of drugs to human liver microsomes would be facilitated by datasets larger than those currently able to be generated by labour- and time-intensive techniques such as equilibrium dialysis. Thus, a potentially high throughput method for assessing drug-microsome binding was developed (Chapter 4). ANS was selected as the fluorescent probe for ‘reporting’ drug binding to microsomes. Changes in ANS fluorescence due to drug binding to the microsomal matrix had previously been demonstrated (Diaugustine, Eling & Fouts 1970;

Hawkins & Freedman 1973; Birkett 1974), and it was considered that this approach could be applied more widely to the measurement of non-specific binding.

After the preliminary assessment of the utility of ANS binding reported in Chapter 4, systematic validation of the ANS fluorescence method was undertaken (Chapter 5). Nine drugs chosen on the basis of log P and charge were characterized for their non-specific binding to human liver microsomes using equilibrium dialysis and ANS fluorescence. Significant relationships between $f_{u(mic)}$ and percent ANS fluorescence increment/decrement were demonstrated for bases, acids, and neutrals. The ANS fluorescence technique was subsequently employed to generate microsomal binding for eighty-eight compounds.

The progression of non-specific binding research parallels that of permeability research, whereby lipophilicity and charge were both originally identified as key determinants. However, it has been shown that other physicochemical properties also have an effect on permeability, and the database generated by the ANS fluorescence technique provided a basis for exploring the effects of additional properties on the non-specific binding of drugs to human liver microsomes. Relationships between a range of physicochemical properties (Figure 6.1 - Figure 6.6), and $f_{u(mic)}$ were determined for eighty-eight drugs. Acidic and neutral drugs were generally found to bind to microsomes to a 'low' extent ($f_{u(mic)} \geq 0.5$). In contrast, bases exhibited a wide range of $f_{u(mic)}$ values, from 0.0001 – 1. Statistically significant relationships were found between $f_{u(mic)}$ and log P, number of hydrogen bond donors, number of hydrogen bond acceptors, and molecular mass of bases. However, exceptions to these relationships were also observed. For example, antazoline is a base that has a log P = 4.4 and an $f_{u(mic)}$ 0.84 at 500 μM, phentolamine has two hydrogen bond

donors and an $f_{u(mic)}$ = 0.9 at 100 μM, fluphenazine has four hydrogen bond acceptors and an $f_{u(mic)}$ = 0.01 at 100 μM, and disopyramide which has a molecular mass of 339.5 has an $f_{u(mic)}$ = 0.96 at 500 μM. Notably, the presence of halogen(s) appears to enhance the binding of acids and bases (e.g. meclofenamic acid, flufenamic acid, trifluprom azine, chlorpromazine).

Preliminary computational modeling of the microsomal binding database generated by the ANS fluorescence technique was undertaken in Chapter 7 using the program ROCS. All but one (itraconazole) of the high binding bases were discriminated using chlorpromazine as the query molecule and a color score cut-off of ≥ 0.60. However, there were 14 false positives, resulting in relatively low prediction accuracy overall. Studies are currently underway to determine whether the use of multiple query molecules will improve the prediction accuracy of the ROCS approach.

This thesis focused largely on the physicochemical characteristics of drugs that confer binding to human liver microsomes. Work conducted here and elsewhere demonstrates that many compounds bind extensively to microsomes, and indeed other membranes. A relatively unexplored area is the perturbation of the membrane structure and properties by drugs and other chemicals, and possible effects on membrane-bound receptors, ion channels, etc. This appears to be the ‘next frontier’ for drug-membrane research.

In conclusion:

1. A drug is more likely to bind extensively ($f_{u(mic)} \leq 0.5$) to human liver microsomes when it is a base with the following properties:

- log P > 3
- 0,1, or 2 hydrogen bond donors
- 0,1, or 2 hydrogen bond acceptors
- Molecular mass ≥ 250
- A ROCS color score ≥ 0.6
- Contains a halogen atom(s)

This is demonstrated in Table 8.1. All high binders filled the classification with respect to log P and number of hydrogen bond donors, and all bar one for molecular mass, and ROCS color score.

Table 8.1: Physicochemical characteristics of high binding compounds.

Drug	log P	MM	HBD	HBA	Color	Halogen(s)	$f_{u(mic)}$ (100μM)
Amitriptyline	4.9	277.4	0	1	0.91	-	0.26
Chlorpromazine	5.2	318.9	0	2	1.00	Cl	0.03
Desipramine	4.1	266.4	1	2	0.97	-	0.33
Desmethylnortriptyline	6.4	249.4	2	1	0.91	-	0.28
Econazole	5.3	381.7	0	3	0.65	Cl (3)	0.02
Fluoxetine	4.1	309.3	1	2	0.72	F (3)	0.36
Fluphenazine	4.8	437.5	1	4	1.00	F (3)	0.01
Imipramine	4.8	280.4	0	2	0.97	-	0.42
Itraconazole	4.3	705.6	0	12	0.50	Cl (2)	0.05
Mianserine	3.4	264.4	0	2	0.70	-	0.43
N-Didesmethylimipramine	3.7	252.4	2	0	0.97	-	0.35
Nortriptyline	5.7	263.4	1	1	0.93	-	0.35
Perhexiline	7.0	277.5	1	1	0.69	-	0.001
Protriptyline	5.1	263.4	1	1	0.86	-	0.31
Thioridazine	6.1	370.6	0	2	1.00	-	0.03
Thiothixene	3.9	443.6	0	5	0.92	-	0.02
Triflupromazine	5.7	352.4	0	2	1.00	F (3)	0.08

2. A drug is more likely to be a ‘low’ binder ($f_{u(mic)} \geq 0.5$) to human liver microsomes when it is an acid, a neutral, or a base with the following properties:

- log P ≤ 3
- Hydrogen bond donors ≥ 3
- Hydrogen bond acceptors ≥ 3
- Molecular mass < 250

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