Complementary Products and Drug Interactions

Screening for the potential to cause pharmacokinetic interactions

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award. The content of the thesis is the result of work that has been carried out since the official commencement date of approved research program.

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Danielle Sevior

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Date
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Manuscripts


Conference Presentations


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<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ADME</td>
<td>Absorption, Distribution, Metabolism and Excretion</td>
</tr>
<tr>
<td>AGP</td>
<td>Alpha-1-acid glycoprotein</td>
</tr>
<tr>
<td>AhR</td>
<td>Aryl-hydrocarbon receptor</td>
</tr>
<tr>
<td>APPs</td>
<td>Acute phase proteins</td>
</tr>
<tr>
<td>ARNT</td>
<td>Aryl-hydrocarbon receptor nuclear translocator</td>
</tr>
<tr>
<td>ARTG</td>
<td>Australian Register of Therapeutic Goods</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAM</td>
<td>Complementary and alternative medicines</td>
</tr>
<tr>
<td>CAR</td>
<td>Constitutive androstane receptor</td>
</tr>
<tr>
<td>CCRP</td>
<td>Cytoplasmic CAR retention protein</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
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<tr>
<td>CEA</td>
<td>Carcinogenic embryonic antigen</td>
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<td>Con A</td>
<td>Concanavalin A</td>
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<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
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<tr>
<td>DA</td>
<td>Dansyl amide</td>
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<tr>
<td>DS</td>
<td>Dansyl sarcosine</td>
</tr>
<tr>
<td>EM</td>
<td>Extensive metaboliser</td>
</tr>
<tr>
<td>EMA</td>
<td>European Medical Agency</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FDA</td>
<td>U.S. Food and Drug Administration</td>
</tr>
<tr>
<td>FMO</td>
<td>Flavin monooxygenase</td>
</tr>
<tr>
<td>FXR</td>
<td>Farnesoid X receptor</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GMP</td>
<td>Good Manufacturing Practice</td>
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<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
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<tr>
<td>GST</td>
<td>Glutathione-S-transferase</td>
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<tr>
<td>HMPC</td>
<td>Committee on Herbal Medicinal Products</td>
</tr>
<tr>
<td>HNF</td>
<td>Hepatic nuclear factor</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>HSP</td>
<td>Heat shock protein</td>
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<td>LXR</td>
<td>Liver X receptor</td>
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<td>MDR</td>
<td>Multi-drug resistance</td>
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<td>MFO</td>
<td>Mixed function oxidase</td>
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<tr>
<td>Mit</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>MPPGL</td>
<td>Microsomal protein per gram of liver</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NBD</td>
<td>Nucleotide-binding domain</td>
</tr>
<tr>
<td>NBF</td>
<td>Nuclear binding fold</td>
</tr>
<tr>
<td>NICCAM</td>
<td>National Centre for Complementary and Alternative Medicine</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
</tr>
<tr>
<td>NR</td>
<td>Nuclear receptor</td>
</tr>
<tr>
<td>ORM</td>
<td>Orosomucoid</td>
</tr>
<tr>
<td>P-gp</td>
<td>Permeability glycoprotein</td>
</tr>
<tr>
<td>PAH</td>
<td>Polycyclic aromatic hydrocarbon</td>
</tr>
<tr>
<td>PAS</td>
<td>PER-ARNT-Sim</td>
</tr>
<tr>
<td>PM</td>
<td>Poor metaboliser</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptors</td>
</tr>
<tr>
<td>PXR</td>
<td>Pregnane X receptor</td>
</tr>
<tr>
<td>QR</td>
<td>Quinaldine red</td>
</tr>
<tr>
<td>QSAR</td>
<td>Quantitative structure-activity relationship</td>
</tr>
<tr>
<td>RAR</td>
<td>Retinoic acid receptors</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
</tr>
<tr>
<td>SLC</td>
<td>Solute carrier transporters</td>
</tr>
<tr>
<td>SNP</td>
<td>Short nucleotide polymorphism</td>
</tr>
<tr>
<td>TCDD</td>
<td>2,3,7,8-tetrachlorodibenzo-p-dioxin</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TGA</td>
<td>Therapeutic Goods Administration</td>
</tr>
<tr>
<td>TI</td>
<td>Therapeutic Index</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane binding domain</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TR</td>
<td>Thyroid hormone receptor</td>
</tr>
<tr>
<td>UGT</td>
<td>Uridine 5'-diphospho-gluuronosyltransferase</td>
</tr>
<tr>
<td>UM</td>
<td>Ultra-rapid metaboliser</td>
</tr>
</tbody>
</table>
Summary

The use of complementary products has increased dramatically over the last few decades. These products are not subjected to the same safety and toxicity testing that we demand of our conventional medicines, yet they are consumed without medical supervision or advice. The concurrent use of these products with conventional medicines raises the potential for drug interactions to occur. We investigated the potential for complementary products to interact with three key pharmacokinetic parameters. The displacement of drugs bound to plasma proteins; inhibition of the transporter p-glycoprotein; and inhibition of the quantitative most important class of drug metabolising enzymes, the cytochrome P450s.

Complementary products selected for investigation are likely to be concurrently used with a therapeutic drug for which, if an interaction was to occur, the outcome could be life threatening. This is based on the properties of the drug and the reported traditional therapeutic indication.

Site-specific fluorescent probes were employed for albumin and alpha-1-acid glycoprotein to determine the binding and displacement of previously bound compounds. Of the 5 products investigated significant binding ($K_d < 1\text{mg/ml}$) occurred at site I on human serum albumin for 1 extract (the methanolic extract of Goldenseal). A greater level of significant binding was seen at site II with 1 aqueous extract (CoEnzyme Q10) and 4 methanolic extracts (CoEnzyme Q10, Danshen, Ginkgo Biloba, Goldenseal) all binding with a dissociation constant less than 1 mg/ml. Investigations into binding to alpha-1-acid glycoprotein revealed that 1 product bund with significance (the methanolic extract of Echinacea).
Inhibition of the transcellular membrane pump p-glycoprotein was investigated with 5 products utilizing a rapid fluorescent assay and the data confirmed with the enzyme based ATPase assay. Significant inhibition (IC$_{50}$ <100 μg/ml) was seen for 4 extracts (the aqueous extract of Cordyceps, the methanolic extract of Milk Thistle and both aqueous and methanolic extracts for Slippery Elm).

The inhibition of cytochrome P450 was determined for 9 isoforms of the enzyme. Two methods were used; HPLC analysis combined with fluorometry with single substrates and their metabolites, and the N-in-one cocktail, which allows for the simultaneous monitoring of the 9 enzymes in a single incubation. The N-in-one assay was a more reliable assay with these complex products. Of the ten investigated products, Valerian was the most widely inhibitory as it inhibited all 9 of the isoenzymes to some degree. The inhibition of CYP3A4 by the methanolic extract was the most significant finding (IC$_{50}$ 3.5μg/ml). Reliable extrapolation of in vitro findings to in vivo situation is still an unsurmountable challenge.

Due to the large number of complementary products available, their variability in chemical composition and the requirement of ongoing monitoring, we have utilised assays that can provide rapid, cost-effective and reliable information with regards to the potential of these products to cause drug interaction. We have demonstrated the ability for rapid, accurate and reproducible assays to provide information on the potential for complementary products to cause drug interactions and presented a rationale basis for screening these products.
Chapter 1 - General Introduction:

Complementary products, the risk associated with use, current regulations and considerations for investigations.

1.1 Introduction

Complementary and alternative products are available to consumers without evaluation of their efficacy or safety, yet they are a widely used health care product. With a market value of $1.2 billion per year in Australia alone (Department of Innovation, 2011), it would reasonable to expect adequate safety evaluations of these products to be conducted. Our focus here is the potential for these products to cause drug interactions.

With self-administration of complementary products an increasingly common trend (Eisenberg et al., 1998, MacLennan et al., 2002), there is concern regarding simultaneous administration with therapeutic drugs. The concurrent administration of these products and prescription drugs or over-the-counter medications significantly increases the risk for clinically serious adverse reactions. Adding concern to this is the public's attitude regarding these products. Consumers feel that these products are natural and therefore safe and often do not report their use to medical professionals (Eisenberg et al., 1998).

Because of the variable nature of these products, it is not feasible to subject them to the same safety evaluation criteria as therapeutic drugs.
However, a rational basis can be formulated for essential studies to improve patient safety. With the large number of products available, prioritising safety studies becomes essential. The selection criteria for which product is to be investigated is best started considering the therapeutic drug for which interactions may be of clinical significance.

These therapeutic drugs have at least one of the following characteristics:

- Narrow therapeutic index;
- High potential for toxicity; and/or
- Life-threatening outcome from therapeutic failure.

The next step is to identify complementary products that are market leaders and on the basis of volume likely to be concurrently used with drugs that meet the above criteria. The products for investigation are then selected based on their particular traditional therapeutic indication.

### 1.2 Complementary and Alternative Products

The wide acceptance of these products and the advent of internet trading has contributed to their increase in use, reported to be as high as 18% of the adult population in the USA (Barnes and Bloom, 2008). Similar figures have been reported in Australia with 25% reportedly using complementary products (Xue et al., 2007), this figure rises to two-thirds of the population when therapies such as chiropractic and energy based modalities are included (NICM., 2009). In England, use of herbal products has been reported to be as high as 22% (Thomas et al., 2001).
The World Health Organization (WHO) reports that the use of traditional medicine accounts for 80-95% of primary health care in Africa and Asia, but this figure includes other forms of traditional medicine such as acupuncture and homeopathy (World Health Organization, 2008). The global market for these products was estimated at US$83 billion annually in 2008 (Robinson and Zhang, 2011).

Increasing the need for investigations into drug interactions is the use of complementary products in patient populations with chronic and/or terminal illness. There is a higher proportion of use of complementary products, 70-80%, in this patient group (Lee et al., 2006, Molassiotis et al., 2005). These patients are at a greater risk of drug interactions as not only do they often consume multiple therapeutic drugs (or conventional medicines) as part of their treatment, but also therapeutic failure could be life threatening.

1.2.1 Concerns Relating to Complementary Products

In the framework of safety, reliance on historical use of these products is no longer valid, as issues such as self-administration, concurrent use with therapeutic agents and a much more diverse genetic population being exposed all need to be considered. Traditionally many of these products were administered or prescribed by healers and physicians in the cultures where they were used but in today’s society these products are re-packaged into tablets and capsules and sold to consumers with no consultation.
Misidentification of plant material is also an area of concern. In 1993 over 100 cases of irreversible nephropathy were reported in young women attending a slimming clinic in Belgium. The nephrotoxicity was traced to the accidental substitution of *Stephania tetrandra* with the highly toxic *Aristolochia fangchi* (Vanherwghem, 1994). The misidentification of other *Aristolochia* species has occurred in the United Kingdom, China and France (Stengel and Jones, 1998, Cosyns et al., 1999).

Assessment of delayed toxicity including carcinogenesis is difficult, even with the strict monitoring of conventional medicines. Given the unregulated use of complementary products, any incidences of delayed toxicity including reproductive and developmental toxicity, would be extremely difficult to identify.

Deliberate substitution with other herbal and complementary products or illegal “fortification” with prescription medications is known to occur and may have serious consequences. The illegal and unlisted addition of corticosteroids in creams for the treatment of eczema (Kinsunthorn et al., 2011) and prescription medications such as sildenafil (Low et al., 2009), glibenclamide (Ching et al., 2011), and alprazolam (Rao et al., 2004) have all been reported in complementary products. These illegal additions could be responsible for pharmacodynamic interactions in patients already consuming similar medications, leading to synergistic effects, which may be toxic. These added medications may also cause allergic reactions in susceptible patients.
In the modern mass marketing of complementary products quality standards in manufacturing are important, as poor manufacturing is a health threat to the consumer. In January 2003, questions into the manufacturing and quality control processes were raised by the regulatory body in Australia with respect to a very large Australian manufacturer, Pan Pharmaceuticals. Investigations revealed a large number of irregular practices including:

- Misidentification of raw material, especially herbal materials, which could lead to severe organ damage, including renal and hepatic damage.
- Manipulation of assay results of finished products in order to comply with specifications.
- Fabrication of assay results of a finished vitamin product for export in order to comply with specifications.
- Cross-contamination or substitution of ingredients due to inadequate operating procedures and poor compliance with existing procedures which could lead to severe allergic reactions including anaphylaxis.
- Microbiological contamination through poor raw material sourcing and handling, poor cleaning practices, and inadequate operating procedures, potentially leading to infections.
- Substitution of shark cartilage for bovine cartilage, which could cause serious allergic reactions, in fish-protein sensitive individuals.
- Substitution of bovine cartilage for shark cartilage where the bovine cartilage has been sourced without any assurance that it is TSE-free, and the country of origin is unknown.
This investigation lead to a large-scale recall of medicines (both generic and complementary) involving as many as 219 products (Therapeutic Goods Administration, 2003). The manufacturing license of Pan Pharmaceuticals was subsequently suspended.

Heavy metal contamination of complementary products has been documented (Rao et al., 2011). The heavy metal content may rise above a safe level due to contaminated raw materials, from processing and manufacturing practices or it can be added deliberately. In the latter case it may be declared as a constituent of a complementary product. The Chinese Pharmacopoeia lists formulations for nearly fifty products that include heavy metals such as arsenic and mercury.

1.3 Investigating Complementary Products for Drug Interactions

Studying complementary products in the context of drug interactions presents a number of challenges, including the fact that these products are complex preparations of variable composition and multiple therapeutic targets. This variability in dose, strength and formulation makes the evaluation for safety in animal and/or clinical models both time and cost prohibitive.

Isolating individual components is one method that can be used to assist in the determination of the pharmacokinetic profiles of these products, though this is time consuming and in the end may not provide the answers required as these products are not taken as isolated components but as complex mixtures.
The many components of complementary products are not considered in isolation, instead the multiple components present are often considered integral to the action with “a concerted pharmacological intervention of multiple compounds interacting with multiple targets and possessing mutually interdependent activities that are required for an optimal effect” (Chan, 1995).

As variability is known to occur with these products between the manufacturers and even between the seasons and geographic locations of the raw materials, ongoing screening for potential drug interactions is required. By using a system that is rapid and reproducible, monitoring of the products can occur and fluctuations and variability in product composition can be detected.

Another challenge associated with complementary products is the choice of extraction method used for assays in the laboratory. Extractions may use a variety of solvents, heat, time, drying and crushing. Separation techniques may also utilize HPLC, GCMS and electrospray ionization.

1.4 Current Regulations

The regulation of complementary and alternative products differs from the regulation of therapeutic agents. Regional variation in the testing and marketing of these products also exists. It is important to understand how the regulations differ, as internet trading and unregulated or inconsistent export and import laws mean that these products are globally available. Consistent regulations would provide regulators, importers and manufacturers with clear guidelines and a regulated framework.
1.4.1 Europe

The European Medicines Agency (EMA) has within its body, the Committee on Herbal Medicinal Products (HMPC). Under EMA there are three classifications for complementary products:

- A herbal medicinal product is any medicinal product, exclusively containing as active ingredients one or more herbal substances or one or more herbal preparations, or one or more such herbal substances in combination with one or more such herbal preparations.

- A herbal substances is all mainly whole, fragmented or cut plants, plant parts, algae, fungi, lichen in an unprocessed, usually dried, form, but sometimes fresh. Certain exudates that have not been subjected to a specific treatment are also considered to be herbal substances. Herbal substances are precisely defined by the plant part used and the botanical name.

- Finally, herbal preparations are obtained by subjecting herbal substances to treatments such as extraction, distillation, expression, fractionation, purification, concentration or fermentation. These include comminuted or powdered herbal substances, tinctures, extracts, essential oils, expressed juices and processed exudates.

Under European medicines legislation (Directive 2004/24/EC), medicinal products containing herbal substances/preparations must fall within one of the following three categories to reach the market:

- A product can be classified under traditional medicinal use provisions (‘traditional use’) accepted on the basis of sufficient safety data and plausible efficacy: the product is granted a traditional use registration.
• A product can be classified under well-established medicinal use provisions ('well-established use'). This is demonstrated with sufficient safety and efficacy data. As a result the product is granted a marketing authorization.

• A product can be authorized after evaluation of a marketing authorization application consisting of only product-specific safety and efficacy data ('full dossier'). As a result the product is granted a marketing authorization.

1.4.2 United States of America

The National Centre for Complementary and Alternative Medicine (NCCAM), a centre within the National Institutes of Health (NIH) advises the U.S. Food and Drug Administration (FDA) on issues relating to complementary products. The regulation of these products is under the control of the FDA.

NCCAM classifies complementary and alternative products and therapies into four categories:

• Biologically-based practices;
• Energy therapies;
• Manipulative and body-based methods; and
• Mind-body medicine.

Many of the complementary products fall under the regulation of the Dietary Supplement Health and Education Act (DSHEA) of 1994. This act regulates products that are intended to supplement the diets and include vitamins, minerals, herbs or other botanicals, amino acids, and substances such as enzymes, organ tissues, glandular, and metabolites.
Under this act, the manufacturer is responsible for ensuring that the ingredient is safe before it is marketed, though products do not need to be registered with the FDA, or gain approval prior to selling.

Manufacturers must make sure that product label information is truthful and not misleading, and products are manufactured under Good Manufacturing Practices (GMP). The FDA is responsible for taking action against any unsafe dietary supplement product after it reaches the market.

1.4.3 Australia

In Australia the regulation of complementary and alternative products is the responsibility of the Therapeutic Goods Administration (TGA). Complementary products are often referred to as complementary medicines; this classification includes vitamins, minerals, herbal, aromatherapy and homeopathic products. These are all regulated under the Therapeutic Goods Act 1989.

The TGA defines a complementary medicine as a therapeutic good consisting wholly or principally of one or more designated active ingredients each of which has a clearly established identity and a traditional use.

Traditional use means use that is well documented, or otherwise established, according to the accumulated experience of many traditional healthcare practitioners over an extended period; and accords with well-established procedures of preparation, application and dosage.
All products imported into, supplied in or exported from Australia, must be listed on the Australian Register of Therapeutic Goods (ARTG). The legal responsibilities of sponsors who wish to register a product include the assessment of the product as low risk-, which means that the product is a Listed product, or higher risk-, which means that the product becomes a Registered product.

Listed products are restricted to claims relating to health maintenance, health enhancement or non-serious, self-limiting conditions. Importantly Listed medicines are not assessed individually for efficacy, though any direct therapeutic claims must have supporting evidence. Registered complementary medicines are assessed individually for quality, safety and efficacy.

All products imported and exported must be manufactured under the Good Manufacturing Practice (GMP). Post-marketing surveillance and reporting of adverse effects is the responsibility of the product sponsor.

1.5 Project Rationale

The advocates for these products raise obstacles and objections as to why they should not be subjected to the safety tests that we expect of our health-care products. We have addressed these claims with the aim of developing a basic framework for testing that can and should be undertaken. The obstacles raised and the responses to these obstacles are presented in Table 1-1.
This project approaches the field of drug interactions with complementary products from the basis of adverse interactions with therapeutic agents. A rational basis for the selection of products of products is presented and the potential for interactions assessed. The methods of assessment all allow for rapid screening of the products as complex mixtures to provide accurate and reproducible data. This data can be used to prioritise the products that require further investigation and as starting for monitoring the variation in the products.

Interaction potential based on three mechanisms were addressed:

- Protein binding (Chapter 2).
- P-glycoprotein inhibition (Chapter 3).
- CYP inhibition (Chapter 4).

The specific aims of each of these areas are detailed in each chapter. These individual areas have been targeted as clinical studies and case reports have identified these areas as key for interactions with herbs and conventional medicine (Chen et al., 2011, Shaojun and Ulrich, 2012).

Many attempts have been made to determine the frequency of adverse events that can be attributed to an interaction involving a complementary product but this is complicated not only because of self-medication and under-reported use of these products but also because of the wide variability (Farah et al., 2000). Additionally there is a lack of consistency in the reporting guidelines for adverse events.
Table 1-1: The proposed obstacles and objections to safety testing for complementary products and the response to discredit the validity of the claim.

<table>
<thead>
<tr>
<th>Proposed Obstacle</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>High cost of testing—this would cripple the industry.</td>
<td>The complementary products industry has an estimated value of US$83 billion annually. Safety testing could increase the market share as consumers who currently avoid these products due to concerns, may decide to start using them or be given permission by medical professionals to use them concurrently with conventional medicine, once the safety has been confirmed.</td>
</tr>
<tr>
<td>Complexity of products does not lend these products to standard safety testing.</td>
<td>Products do not need to be studied in isolation or as single components; they can be investigated in their complex form.</td>
</tr>
<tr>
<td>Historical use of these products demonstrates safety.</td>
<td>The use of these products and the population exposed has changed. More patients are now exposed, including patients with complex and chronic medical conditions and multiple drug therapies. Delayed toxicities (cancer and genotoxicity) are very difficult to study in users.</td>
</tr>
<tr>
<td>Practitioners are experienced and know which products may be toxic and how to “personalise” treatment for patients.</td>
<td>Consumers purchase these products off supermarket shelves and from the internet with no supervision or guidance. Practitioners may not have an understanding of western therapeutic drugs and the potential for interactions.</td>
</tr>
</tbody>
</table>
2.1 Introduction

Binding to plasma proteins can influence the distribution, metabolism and excretion behaviour of many endogenous and exogenous compounds. In vivo, drug molecules are either bound to proteins and lipids in plasma and tissues, or are free. These free drug molecules diffuse in the aqueous environment of the blood and tissues (Smith et al., 2010).

Because of their high molecular weights, plasma proteins and the compounds bound to them cannot cross capillary walls. Consequently, the fraction of the compound bound to plasma protein is not immediately available for distribution into the extra-vascular space or for filtration by the kidneys. In most cases, only the free drug (unbound) molecules interact with the therapeutic target to produce an effect. The unbound fraction is also important as it will affect the distribution, steady state concentration, rate of metabolism, and the rate of excretion of a drug (Noctor et al., 1993, Vallner, 1977).

Protein binding is important when predictions and investigations into the toxicity of a compound are undertaken, as typically the toxicity is manifested by the unbound fraction.
Therefore a compound with a high degree of plasma protein binding may not show
toxicity when compared to one that is less extensively bound to plasma proteins.
Paradoxically a high degree of protein binding increases the risk of adverse effects
resulting from displacement interactions with other compounds and the binding site. Due to the transient increase in the concentration as a result of one compound displacing the other (section 2.1.1).

Several key proteins found in human serum are capable of binding drugs; albumin (HSA) and alpha-1-acid glycoprotein (AGP) are of greatest importance (Fournier T et al., 2000). As a general rule acidic drugs bind to HSA and basic and neutral drugs bind to AGP. Protein-ligand interactions occur primarily as a result of hydrophobic forces, hydrogen bonding, and Van der Waals forces (Casarett L et al., 2008). This interaction is usually reversible and as unbound drug diffuses out of the capillaries, bound drug disassociates from the protein until the free fraction reaches equilibrium with the extra-vascular and the vascular space.

**2.1.1 Displacement from Protein**

The concentration of a drug or ligand bound to protein can alter significantly due to co-administered drugs/ligands. Thus the ability to bind to protein and to displace a previously bound compound from protein is an important consideration in drug development and in the prediction of drug interactions.

Simultaneous binding of drugs and/or endogenous ligands to protein can give rise to various potential interactions. The binding of the ligands can be independent; and therefore not influence the affinity of each other.
The binding may be cooperative; that is the binding of one ligand increases the affinity of the other. The binding may be anti-cooperative; where the binding of one ligand decreases the affinity of the other, and finally the binding may be competitive; where the two ligands bind to the same site, the competition determining a decrease of the affinity.

Determination of drug binding to different biomacromolecules, and particularly with specific plasma and tissue proteins, is mandatory in pharmacological and toxicological studies for therapeutic drugs. This is in order to predict nonlinear pharmacokinetic processes (Gillespie, 1993), stereoselective pharmacokinetics (Brocks, 2006), covalent binding of drug metabolites to different molecular structures (Nelson, 1982), drug displacement phenomena (MacKichan, 1989, Rolan, 1994), or inter-individual binding variability due to different physiological or pathological factors (age, disease, genetic aspects, etc.) (Eap and Baumann, 1989, Hervé et al., 1993, Zini et al., 1990b, Zini et al., 1990a, Oracová et al., 1996).

Whilst these studies are performed for therapeutic drugs before they enter clinical trial, let alone the market, in sharp contrast, this is not the case for complementary products. This is an alarming situation as these products are known to cause drug interactions involving protein binding and displacement (section 2.2.3.1 and 2.2.8.1).
2.2 Albumin

Human serum albumin (HSA), at a concentration of 40 mg/ml, is the most abundant protein in the blood, acting as a transport protein for numerous endogenous and exogenous compounds. It is synthesised in the liver and exported as a non-glycosylated protein (Peters T Jr, 1996). HSA has 585 amino acids and a mass of 66.5 kDa and is composed of three structurally similar α-helical domains, I, II and III (Thorarensen et al., 2007).

These subdomains are further divided; subdomain A containing 6 α-helices and subdomain B containing 4 α-helices, connected by flexible loops. The main binding sites for compounds are Site I, localised within domain II, referred to as the warfarin binding site, and Site II localised within domain III and referred to as the benzodiazepine binding site (Fournier T et al., 2000).

![The crystal structure of human serum albumin (HSA) at 2.5Å resolution.](http://www.rcsb.org/pdb/explore.do?structureId=1AO6)

**Figure 2-1:** The crystal structure of human serum albumin (HSA) at 2.5Å resolution. ([http://www.rcsb.org/pdb/explore.do?structureId=1AO6](http://www.rcsb.org/pdb/explore.do?structureId=1AO6))
2.2.1 **Biological Functions of Human Serum Albumin**

The abundance of HSA and its broad binding capabilities make it a key factor in the pharmacokinetic behaviour of many drugs, affecting their efficacy and rate of delivery. HSA may act as a reservoir for exogenous or endogenous ligands, or it can hold some ligands in a strained orientation, facilitating their metabolic modification and rendering potential toxins harmless.

Protein binding can result in an increased solubility in plasma, decreased toxicity (as the bound drug cannot cross cell membranes), and/or protection against oxidation of the bound ligand. However, binding can also have a significant impact on the pharmacokinetics of drugs in other ways. For example, binding to albumin can extend the in vivo half-life; bound drugs are unable to cross the renal epithelium and therefore are not excreted.

In addition to its binding and transport abilities, HSA also provides most of the acid/base buffering action of the plasma, and contributes to osmotic pressure. HSA accounts for most of the antioxidant capacity of human serum either directly or by binding and carrying radical scavengers or sequestering transitional metal ions with pro-oxidant activity. Additionally HSA acts as a depot and carrier for nitric oxide, leading to covalent modification of molecules (Fasano et al., 2005).

2.2.2 **Binding Properties of Human Serum Albumin**

HSA interacts reversibly with a broad group of compounds, though hydrophobic anionic compounds are most strongly bound. The binding capability of HSA is diverse; it contains multiple binding sites that vary in structure and polarity.
Additionally the binding of ligands may induce conformational changes in the HSA molecule, which alter the binding capabilities of the molecules.

Usually drugs bind to one or few high-affinity sites with typical association constants in the range of $10^4$-$10^6$ M (Kragh-Hansen et al., 2002). At high concentrations a single compound may populate multiple sites on albumin, binding at a primary site with high affinity and at secondary and tertiary site with lower affinity (Day and Myszka, 2002). The binding sites vary in size and polarity and allow drugs of differing structure and size to bind simultaneously.

Site I is a complex and large binding site. Evidence for this is shown in the binding of bilirubin, a large molecule with a molecular weight of 548 Daltons, and with ligands of very different chemical structures binding to the region with high affinity. This includes ligands as diverse as warfarin, tolbutamide and indomethacin (Yamasaki et al., 1996, Vallner, 1977, Sudlow et al., 1975).

Site I ligands tend to be dicarboxylic acids and/or bulky heterocyclic molecules with a negative charge localised in the middle of the molecule (Peters, 1985). Conformational changes in HSA post ligand binding have been proposed, changes could be anti-cooperative as in the case with warfarin and salicylate (Kragh-Hansen, 1985) or allosteric as seen with ibuprofen enantiomers and the stereoselective binding of 3-acyloxy-1,4-benzodiazepines (Fitos et al., 1999).

Site II ligands are often aromatic carboxylic acids with a negatively charged acidic group at one end of the molecule away from a hydrophobic centre (Peters, 1985).
Due to the size of the ligands that bind at site II, it is likely that this is a smaller binding site than site I. Binding to this site is also highly stereoselective, indicating that this region is not as flexible as site I. L-tryptophan which binds with an affinity 100 times greater than the D-isomer (McMenamy and Oncley, 1958), is an example of the highly stereoselective binding. Whilst binding to this site is more restricted there are several ligands that bind to site II with high affinity including ibuprofen, chlorothiazide and diazepam, (Ferrer et al., 2001, Sudlow et al., 1975, Takamura et al., 1994).

2.2.3 **Known Interactions Involving Human Serum Albumin**

Competition between two drugs for their binding to plasma protein can strongly affect the drug disposition of both drugs, with possible serious outcomes. This is of greater importance with compounds that are highly bound to HSA, i.e. over 90% (Peters, 1996, Kragh-Hansen et al., 2002). One well-studied interaction occurs between warfarin and phenylbutazone. Warfarin is an anti-coagulant that is over 90% bound to HSA. Co-administration with phenylbutazone leads to an increased bleeding risk in patients as the warfarin is displaced from the albumin, increasing its free concentration (Harder and Thürmann, 1996).

2.2.3.1 **Known Interaction with Complementary Products**

Several examples of plant and herbal products binding to HSA are known. Genistein, a major isoflavone present in soybeans binds at site I (warfarin binding site). The binding constant has been determined to be $1.0\pm0.2\times10^5$ M$^{-1}$ (Mahesha et al., 2006), using equilibrium dialysis (section 2.4.1.1).
Using micro-dialysis, two components of the traditional Chinese herb Rhizoma Chuanxiong: ferulic acid and 3-butylphthalide, were found to bind to HSA with 36.7 and 30.2% free compound detected, respectively (Guo et al., 2006). Fluorescence spectroscopy (section 2.4.3.2) was used to investigate the binding of Chinese medicinal herbs to HSA and bovine serum albumin (BSA). The anthraquinone, emodin, rhein, aloe-emodin and aloin, were all determined to have greater binding constants for HSA than BSA; 3.18-2.03x10^5, 2.20-1.16x10^5, 1.14x10^5-3.84 x 10^4, and 2.77-3.10 x 10^4 mol^-1, respectively (Bi et al., 2005).

Both Ginkgo and Ginger were suspected to alter the kinetics of warfarin by protein binding interactions but in vivo investigations using healthy human volunteers have found no alteration of the kinetics of warfarin when these herbal products are co-administered with warfarin (Jiang et al., 2005).

One of the potentially more significant examples of Chinese herbs binding to HSA is Danshen, a traditional Chinese medicine prepared from the root of *Salvia miltiorrhiza*. Studies have shown that Danshen is 50-70% bound to albumin, and can displace salicylate from its binding site (Gupta et al., 2002). Danshen is also of concern as it is suggested for patients with 'stagnation of blood flow' indicating that concurrent use with therapeutic agents such as warfarin are likely.

These studies all indicate that there is a potential for significant protein binding with complementary and alternative products and that their potential for displacement interactions involving HSA should be investigated.
2.2.4 Alpha-1-Acid Glycoprotein

Alpha-1-acid glycoprotein (AGP); also known as orosomucoid (ORM), was first described in 1950 concurrently by Karl Schmid and Richard J Winzler et al. (Schmid, 1950, Schmid, 1953, Weimer et al., 1950). It is a 41-43 kDa negatively charged (pI = 2.7-3.8), glycosylated acute phase protein that is 183 amino acids in length. AGP consists approximately of 45% carbohydrates (Schmid et al., 1977) attached in the form of five complex-type N-linked glycans (Yoshima H et al., 1981), (Figure 2-2). The negative charge of AGP is due to the salicylic acid residues which may number as many as 16, or 10-14% by weight (Kopecký V et al., 2003).

Five heteropolysaccharide groups are linked via an N-glycosidic bond to asparaginyl residues of the protein (Schmid et al., 1973a). AGP has a high β-sheet content (40%) and consists of eight anti-parallel β– strands which form the β-barrel with a central hydrophobic pocket (Breustedt et al., 2006, Albani, 2006, Kopecký et al., 2003). This protein also has an unusually high solubility in water and other polar organic solvents.
AGP is a member of the immunocalin family, a lipocalin subfamily. The lipocalin proteins are part of a larger group, the acute phase proteins (APPs). These APPs can be divided into two major classes depending on their response to cytokines. Type 1 APPs, including AGP, complement component 3, serum amyloid A, C-reactive protein, haptoglobin and hemopexin, are regulated by interleukin-1, interleukin-6 and glucocorticoids. Type 2 APPs including fibrinogen and several protease inhibitors are regulated by interleukin-6 type cytokines and glucocorticoids (Baumann et al., 1989, Baumann and Gauldie, 1994)
AGP is predominantly synthesised in the hepatocytes and parenchymal cells of the liver at a rate of 10 mg/kg (of body weight)/day (Lentner C, 1984), and secreted into the plasma at a mean concentration of 0.77 mg/ml with a range of 0.36-1.46 mg/ml in healthy volunteers (Blain et al., 1985), though the concentration of 1 mg/ml is most commonly reported (Fournier T et al., 2000, Zsila F, 2007, Schön A et al., 2003). Extra-hepatic synthesis of AGP also occurs in endothelial cells and alveolar type II cell macrophages (Sörensson et al., 1999; Fournier T, 1999). Protein synthesis and glycosylation of AGP is regulated by cytokines and glucocorticoids (MacKiewicz et al., 1987a, van Dijk et al., 1991).

A critically important characteristic of AGP is the change in plasma levels that occurs with conditions such as inflammation (e.g. arthritis) and chronic disease (e.g. cancer). In these patients, expression is increased 2-5 fold (Fournier et al., 2000). An increase in AGP also occurs following myocardial infarction (Johansson et al., 1972) and surgery (Voulgari et al., 1982), whilst lower levels are seen during pregnancy (Perucca E and Crema A, 1982), in thyroid disease (Feely J et al., 1981) and in patients with liver cirrhosis (Serbouce-Hougel et al., 1981). Such wild fluctuations must be considered when the potential for drug interactions are investigated with regards to AGP.

60% of AGP is found in the central compartment; the rest is in the extra-vascular space (Koj A, 1974, Lentner C, 1984). AGP is also distributed to other fluids including, but not limited to, gastric juice, cerebrospinal fluid, wound exudate, pleural and peritoneal effusions and synovial fluid (Lentner C, 1984).
This wide distribution gives AGP the potential to be involved in drug interactions and protein binding not just within the circulation, but also at the sites of drug action.

### 2.2.5 Structure and Variants of Alpha-1-Acid Glycoprotein

Each of the five N-glycosylation sites of AGP can express many different glycans. Though only 12-20 glycoforms of AGP can be detected in human serum, as site 1 never carries a tetra-antennary glycan, site 2 never carries glycans with fucose, site 4 never carries a di-antennary glycan, and only glycosylation sites 4 and 5 carry tetra-antennary glycans with more than one fucose (Fournier et al., 2000).

Glycosylation has been shown to differ with various inflammatory diseases, this micro-heterogeneity of AGP depends on the duration of the inflammatory process and not the aetiology (Fassbender et al., 1991). The differentiation between acute and chronic inflammatory states can be determined by crossed immuno-affinity electrophoresis on the basis of reactivity to concanavalin A (Con A) (Narasimhan et al., 1986, Bøg-Hansen, 1973, Hansen et al., 1984b).

A rise in reactivity to Con A has been reported in acute sepsis (Nicollet et al., 1981), pancreatitis (Raynes, 1982), and surgical trauma (Hansen et al., 1984a, Hansen et al., 1986). By contrast, in rheumatoid arthritis (MacKiewicz et al., 1987b), ankylosing spondylitis (MacKiewicz et al., 1989) and Crohn’s disease (Raynes, 1982, Hansen et al., 1986), a significantly decreased proportion of AGP reacts with Con A. Isoform differentiation can also be achieved by capillary electrophoresis with time-of-flight mass spectrometry (Ongay and Neusüss, 2010).
Polymorphism with AGP is due to a 22 amino acid difference existing between the variants. (Eap CB and Baumann P, 1989, Fournier T et al., 2000). The three genes: responsible AGP-A, AGP-B and AGP-B’ are located on chromosome 9. AGP-A (also known as ORM1) encodes for the major component of serum AGP, AGP-B and AGP-B’ (also known as ORM2) are identical and expressed 100-fold less than AGP-A and code for the variant of AGP (Dente et al., 1988).

Due to multiple allelic forms of the genes, there are 70-80 genetic variants of AGP in humans (Eap CB and Baumann P, 1989). The three main AGP variants are designated F1, S and A; based on their electrophoretic migration. In humans there are three main phenotypes, F1S/A, F1/A and S/A with respective frequencies of 50%, 35% and 15%. Although the majority of AGP ligands are not variant selective, some drugs exhibit preferential binding to the ‘A’ (e.g. disopyramide, methadone, amitriptyline) or ‘F1/S’ (e.g. warfarin, dipyridamole) variants (Hervé F et al., 1998). The relative abundance in commercial pooled AGP is reported to be ≈40, ≈30, and ≈30% for the F1, S and A variants respectively (Hervé et al., 1998).

### 2.2.6 Biological Functions of Alpha-1-Acid Glycoprotein

The pharmacological role of AGP is still under investigation but it has been suggested that AGP is probably the only high affinity carrier for basic drugs in the serum (Kremer et al., 1988). Additionally, AGP is considered as a natural anti-inflammatory and immunomodulatory agent (Williams et al., 1997). It also stimulates the secretion of tumour necrosis factor (TNFα) from mononuclear cells and macrophages (Su et al., 1999) and provides protection against the toxic effect of TNFα (Libert C, 1997).
The anti-neutrophil and anti-complement activity that has been reported is supported by in vitro and in vivo studies which show that AGP inhibits neutrophil activation (Vasson et al., 1994), increases the secretion of interleukin-1 inhibitor from macrophages (Bories et al., 1990) and modulates LPS-induced cytokine secretion by monocytes-macrophages (Boutten et al., 1992). This immunomodulation by AGP has been hypothesised to be a major problem in cancer patients because an impaired immune defence against the tumour may result in, and predispose to infections, and hinder immunotherapy treatment (Fournier et al., 2000, Tamura et al., 1981).

In the endothelial cells, AGP is an important component of the capillary barrier, which is essential for capillary charge selectivity (Sörensson et al., 1999). Other functions which are more likely to only be important under pathological conditions include the ability to bind toxic lectins (Frantz M et al., 2000), endotoxins and bacterial lipopolysaccharides (Moore D et al., 1997).

AGP has also been shown to inhibit the attachment of Mycoplasma pneumoniae to alveolar macrophages (Athamna A et al., 1996), and of human immunodeficiency virus type-1 envelope glycoprotein in CD4+ monocytic cells and macrophages (Rabehi L et al., 1995). AGP also inhibits rotavirus replication by directly acting on the virus due to the negative charge of the AGP (Superti et al., 1993). High levels of AGP have been associated with the development of gallstones (Thijs et al., 1999).
2.2.7 Binding Properties of Alpha-1-Acid Glycoprotein

AGP mainly binds basic and neutral compounds. Both from endogenous origins, including IgG3, heparin, serotonin (Schmid et al., 1973a), platelet activating factor (McNamara et al., 1986), melatonin (Morin et al., 1997) and histamine (Chachaj et al., 1980) and exogenous origins, including tamoxifen (Schmid et al., 1973, Schmid et al., 1973a) and propranolol (Albani et al., 1984).

Disopyramide was the first drug described to bind to AGP (Kopitar and Weisenberger, 1971), to date over 300 drugs are known to bind to this protein. For some drugs, including cocaine and the protease inhibitors for HIV treatment, AGP is the major binding protein (Israel Z and Dayton P, 2001). AGP is also the major binding protein for steroids, including synthetic steroids such as RU486 (Grimaldi et al., 1989).

It is generally assumed that acidic drugs bind to HSA, but with the increase in AGP levels under certain physiological conditions (section 2.2.4) and/or when the HSA levels decrease (seen with liver failure), phenobarbital, an acidic drug will bind to AGP (Schley and Müller-Oerlinghausen, 1983). Additionally warfarin, which usually binds to HSA, has been shown to bind to AGP (Hervé et al., 1998).

Many groups have investigated the number and nature of binding sites on AGP. Initially it was proposed that AGP possessed one binding site located in the hydrophobic area of the protein (Müller, 1989), but seven binding sites with varying capacities and affinities have been described.
Whilst several binding sites have been characterised, it has been proposed, based on fluorescent probe and circular dichroism studies, that the binding sites significantly overlap and are influenced by each other (Maruyama et al., 1990), hence AGP is often referred to as having one wide and flexible binding area.

The binding of drugs to AGP has been mostly shown to be hydrophobic, though electrostatic interactions have also been reported, additionally an increase in the pH will increase binding (Ponganis and Stanski, 1985). In the case of basic drugs, stereoselective binding is also important.

### 2.2.8 Known Interactions Involving Alpha-1-Acid Glycoprotein

A clinically significant drug-interaction can occur with rifampicin which is 80% bound to AGP (Johnson and Smith, 2006) and used to treat *Mycobacterium tuberculosis*. If displaced by a competing ligand the metabolism of rifampicin will be increased, thus decreasing the therapeutic levels of the antibiotic, leading to therapeutic failure.

#### 2.2.8.1 Known Interaction with Complementary Products

As the levels of AGP are increased in chronic conditions including cancer, HIV and chronic inflammation the potential for significant interactions with complementary products is also increased. Use of complementary products amongst this patient group is extremely high with over 60% of cancer patients using herbal products (Richardson et al., 2000).
This potential for interactions is significant as chemotherapeutic drugs including tamoxifen (Paterson et al., 2003) and vinblastine (Steele et al., 1982) and the HIV anti-protease drugs saquinavir, indinavir, ritonavir and nelfinavir (Schön et al., 2003) all bind predominantly to AGP.

To date there are only very limited studies with complementary products and their ability to bind to AGP. The need for rapid and reliable testing for the potential of complementary products to bind and displace previously bound drugs from AGP is demonstrated by the increased risk of concurrent use with important therapeutic agents that bind to AGP, in particular those with a narrow therapeutic index.

2.3 Clinical Relevance of Displacement Interactions

Plasma protein binding and its impact on the in vivo efficacy of a drug is an ongoing area of research in pharmacokinetics. Many of the techniques that determine protein binding do so in an attempt to measure the amount of free drug. This is because of the accepted dogma that it is the free drug concentration in the plasma is responsible for the pharmacological activity. This is because it is the free drug that is available to passively partition into the site of action and interact with a molecular target. This concept is reflected by the pair of equilibrium equations given below:
Where $D$ is the free drug, $PP$ are plasma proteins, $DPP$ is drug bound to plasma proteins, $R$ is the molecular target at the site of action, and $DR$ is the drug bound to the target.

The concept of determining the free drug concentration has been proposed as misleading as it is the amount of free drug at the therapeutic target that determines the in vivo efficacy not the free drug in plasma (Smith et al., 2010). But generally concentrations in plasma and target sites are assumed to be in equilibrium.

The first adverse clinical outcomes attributed to protein-binding displacement interactions was the observation of an increase in prothrombin time in patients who were concurrently taking phenylbutazone and warfarin (Rowland and Tozer, 2010, Eisen, 1964). The hypoglycaemia observed in patients who were concurrently using sulphonamides and tolbutamide has also been attributed to displacement interactions (Christensen et al., 1963).

Whilst there are clearly clinically significant displacement interactions, it has been suggested that the role of displacement interactions have been overstated and a flowchart proposed to help determine which drugs are more likely to cause adverse reactions (Figure 2-3) (Rolan, 1994).
Figure 2-3: Flowchart for determining the clinical significance of potential protein binding displacement interactions (Rolan, 1994).

The flowchart allows for the various scenarios that may arise when two drugs (A and B) are concurrently administered. For example, if drug A, is displaced by drug B, an increase in the free concentration of drug A results, but this increase in free drug A may not correlate with an increase in drug A at the receptor site because it will also be available for redistribution to the rest of the body, any increase in free A following redistribution will also be available for elimination. For low clearance drugs, where intrinsic clearance of free drug is the only determinant of mean steady-state free drug concentration, free concentration for A will return to the pre-B level.
Thus any increase in the pharmacological effect of A will be transient and cannot be sustained. This flowchart also indicates that the interaction may become significant if the transient increase in the free drug is for a drug with a narrow therapeutic index.

Predicting if a clinically significant interaction will arise from in vitro data is further complicated by the potential for drug metabolites to bind to plasma proteins and potentially displace previous bound compounds. Binding of drug intermediates has been shown for several drugs including zomepirac (Smith et al., 1990) and tolmetin (Hyneck et al., 1988). These studies indicate that the parent compounds and the intermediates may be the cause of an adverse interaction and both require investigation.

2.4 Determination of Protein Binding

Protein binding is increasingly becoming important in the early stages of drug development. Whilst there are many methods to study the binding of drugs to proteins, each technique will depend on one of the following (Klotz, 1973):

- Separation of free and protein-bound fraction of ligand;
- Detection of a change in the physicochemical property of the complexed ligand;
- Detection of a change in a physicochemical behaviour of the binding protein.
The techniques used to determine protein binding can be generally divided into three main types: classical, chromatographic and spectrophotometric. Several of these techniques are discussed in more detail below and summarised in Table 2-1.

When investigating protein binding, purified proteins or samples of serum and plasma can be utilised. With the complex and undefined nature of complementary products, utilising purified proteins allows for a more targeted investigation of the key transport proteins (HSA and AGP) and specific study of the underlying cause of a pharmacokinetic interaction. Studies with whole serum can be employed and may provide additional information of binding to minor and variable proteins such as gelatin-binding protein, insulin-like growth factor binding protein and retinol-binding protein.

Assays with whole plasma often employ classical methods for the analysis of the free or bound drug (section 2.4.1) which require the ability to measure the test compound, a task which is often not possible with the unknown and variable composition of complementary products.

### 2.4.1 Classical Methods

Classical methods including equilibrium dialysis, ultrafiltration and gel separation, all involve the determination of the free or bound drug using a separation step. These techniques are all based on the separation of the free drug from the bound after equilibrium is reached. Therefore the common limitation of these techniques occurs when the drug is tightly bound to the protein. The concentration of the free drug should be, in these cases, very low, and therefore difficult to detect.
Additionally the results obtained differ for each of these methods, and whilst experimental factors may be the cause of these differing results the technique may be responsible. For example equilibrium dialysis indicated 23% plasma protein binding for fleroxacin but 47% by ultra-filtration (Zlotos et al., 1998). This same study also highlighted inter-laboratory variation with 20-40% for ciprofloxacin, 8-30% for ofloxacin, norfloxacin, and 30-50% for enoxacin.

Whilst these classical methods may provide information relating to a compounds ability to bind to albumin, they do not provide information relating to the specific binding site, which in the case of drug-drug interactions is key. With regards to complementary products these classical methods are limited in their use as these complex mixtures are often unknown in their composition with multiple components, therefore monitoring for “free drug” is not possible.

2.4.1.1 Equilibrium Dialysis

Equilibrium dialysis is based on the establishment of an equilibrium state between a protein compartment and buffer compartment separated by a membrane, which is permeable only for low-molecular weight ligands i.e., free or unbound drug. Equilibrium dialysis is the preferred method to determine the free drug fraction and is often the reference method where nonspecific binding of drug to the filtration membrane and other surfaces causes experimental artefact (Lin et al., 1987). Equilibrium dialysis is also amenable to high throughput screening (Kariv et al., 2001).
The major advantage of this technique is that the drug binding to plasma proteins is analysed at equilibrium, thus eliminating the effect of nonspecific binding. The disadvantage is the volume shift between the matrix and the buffer can disturb the equilibrium, though the addition of high molecular weight dextran can eliminate the difference in osmotic pressure (Lima et al., 1983).

Equilibrium dialysis has many disadvantages including the time required for the system to reach equilibrium (Kurz et al., 1977, Oracová et al., 1996, Bowers et al., 1984), volume shifts (Huang, 1983), Donnan effects (which hinder the passage of free ligand (Mapleson, 1987), nonspecific adsorption to dialysis apparatus (which is more problematic for highly lipophilic drugs i.e. cyclosporine (Henricsson, 1987, Fois and Ashley, 1991b, Fois and Ashley, 1991a), and difficulty in the control of experimental parameters i.e. pH of the medium (Henry et al., 1981, Brørs et al., 1984). Often the test compounds are radiolabeled, adding potential impurities to the experimental set-up.

2.4.1.2 Ultrafiltration

Ultrafiltration with semi-permeable membranes produces a separation of the free drug from macromolecules by employing a pressure gradient, which forces small molecules through the membrane. The advantages of this technique include that when compared to equilibrium dialysis the analysis time is shortened, and there is a lack of dilution effects and volume shifts.
When ultrafiltration is combined with Raman difference spectroscopy this allows for the label free detection and quantitation of protein-ligand binding, which yields both thermodynamic and structural information and requires low amounts of protein, approximately 10-100 $\mu$g (Xie et al., 2008).

Ultrafiltration, whilst quicker than dialysis, still requires the use of a labelled drug and/or additional analysis steps to determine the actual level of free drug by techniques such as immunoassay, gas chromatography or high-performance liquid chromatography. With the unknown composition of complementary products, ultrafiltration is not a suitable method to investigate protein binding.

2.4.1.3 Ultracentrifugation

This technique eliminates the problems associated with membrane effects seen in equilibrium dialysis and ultrafiltration and enables the separation of the free and protein bound fraction. Although there are discrepancies between the results obtained for ultracentrifugation from equilibrium dialysis and ultrafiltration. These discrepancies are most likely due to factors including sedimentation, viscosity and floating lipoprotein fractions (Kurz et al., 1977, Külpmann et al., 1984, Verbeeck and Cardinal, 1985, Barré et al., 1985).

2.4.1.4 Gel Filtration

Gel filtration is effective when separating bound drugs from unbound drugs when the molecular mass of the complex is considerably larger than the free drug. The technique was first used to measure the binding of bilirubin in neonates sera (Schiff et al., 1972).
Gel filtration columns are selected so that the protein of interest and the protein-ligand complex is in the mobile phase and the free ligand remains in the stationary phase. This technique is time consuming and complex so it is not commonly employed in laboratories.

However, gel filtration has been combined with radio ligand-binding assays to allow for a 96-well plate method (Liu et al., 2002). This method can be used to process a large number of samples but as it requires radiolabeling of the drug can alter the binding data.

### 2.4.2 Chromatographic techniques

Chromatographic techniques in general are more sensitive and reproducible than conventional techniques and are able to detect small differences in binding affinity (Oracová et al., 1996). The major advantage of these techniques is that the ligand in questions does not require labelling with fluorescent tags or radioactive material and does not involve separation steps.

#### 2.4.2.1 Affinity chromatography

Affinity chromatography combined with a protein stationary phase has many advantages including precision and reproducibility, this method also allows for enantioselective studies. Affinity chromatography is also useful in the investigation of drug interactions involving protein binding. Zonal elution with a known displacer at various concentrations can provide information on the ligand binding, including if the binding is co-operative, anti-cooperative or non-cooperative (Ascoli et al., 2006, Hage et al., 2009).
Unlike with HSA, binding studies with AGP are not reliable using affinity chromatography. Experiments have shown no correlation between the retention of compounds known to binds to AGP (Jewell et al., 1989) and the potential to displace (S)-propranolol which is a high affinity binding marker. It has been proposed that the high level of salicylic acid (section 2.2.4) is the reason for the lack of correlation (Schill et al., 1986).

2.4.2.2 Capillary electrophoresis

Capillary electrophoresis including affinity capillary electrophoresis and capillary affinity gel electrophoresis, have all been utilized for binding studies. Affinity capillary electrophoresis can be used as a rapid screening method of ligand-protein interaction and the evaluation of ligand-ligand interactions at the various binding sites (Birnbaum and Nilsson, 1992, Chu et al., 1993, Li and Lloyd, 1993).

The major disadvantage of capillary electrophoresis in the low sensitivity, with therapeutic concentration of drugs not being easily detected. Recent advances in coupling capillary electrophoresis with micro-fluidics has shown promise in particular with the detection of the binding of bilirubin to albumin in neonates (Sun et al., 2010) but at this stage this technology is not scaled to a high through put screening method.

2.4.3 Spectrophotometric Methods

Spectroscopic techniques have been used to study the interaction of drugs with macromolecules (Chignell, 1973, Chignell, 1969).
These techniques, including nuclear magnetic and electron spin resonance, optical rotary dispersion, circular dichroism and fluorescence all have the advantage over classical methods (see section 2.4.1) as they do not require separation, instead they measure a change in the physical property of the drug and/or protein upon binding.

2.4.3.1 Circular dichroism

Circular dichroism (CD) spectroscopy measures the difference in absorbance of right- and left-circularly polarized light producing characteristic bands—the Cotton effect. CD is often employed to investigate the secondary and tertiary structure of proteins but it can also be used in study of ligand-protein binding for both HSA and AGP as binding of a ligand to the protein will alter the Cotton effect. The spectral position, sign, shape, number and amplitude of the bands are used to determine the nature of the binding site and the mechanism of binding (Zsila et al., 2002, Beuckmann et al., 1999, Zsila et al., 2003).

Spectral data obtained from CD studies can be used to determine the binding stoichiometry and the association constant as demonstrated with bilirubin (Siligardi and Hussain, 1998, Knudsen et al., 1986). Competitive binding studies using CD can only be conducted providing that the ligand being investigated has little or no measurable CD absorption in the wavelength region of the induced CD spectrum.

The major limitation of CD for investigating protein binding is that UV inactive compounds cannot be investigated directly by this method and that not all ligand molecules with suitable chromophores give a measurable Cotton effect following interactions with proteins (Zsila et al., 2004).
2.4.3.2 Fluorescent Probes

HSA has two fluorophores, tryptophan (Trp-214) and tyrosine (Tyr) (He and Carter, 1992), whilst AGP has three (Trp-160, Trp-122 and Trp-25) (Schmid et al., 1973b, Hof et al., 1986, Schmid et al., 1973a). These residues can act as intrinsic fluorescence probes. If a ligand binds to the protein, there is a measurable decrease in the intrinsic fluorescence, known as quenching.

Fluorescence was first used to study the binding of warfarin to HSA, using enhancement of the warfarin fluorescence and the quenching of protein fluorescence as measure of bound drug (Chignell, 1970). Whilst fluorescence quenching is a rapid technique there are limitations that make this technique best for early stage screening and not for the detection of drug interactions.

The information from these studies does not indicate the specific site of ligand binding, only that binding occurs. As the binding site and the affinity are key in the probability for a drug interaction, quenching studies are limited in their application in drug binding and displacement studies. Additionally, interference from the compound is also problematic as compounds that absorb light at the excitation wavelength cause absorptive screening and if their absorption wavelengths are towards the red spectrum (>300nm) they quench the intrinsic tryptophan fluorescence (Epps et al., 1999).

By employing fluorescent probes as non-covalent competitive ligands for specific binding sites on HSA and AGP, information regarding the environment, number and affinity for each of the binding sites for a particular ligand can be determined.
Fluorescent probes may respond in nearly identical ways to a number of unrelated phenomena (Chance and Radda, 1971). A decrease in probe fluorescence may result from competitive displacement of the probe. Alternatively there may be non-competitive displacement of the probe or a decrease in the quantum yield of the bound probe as a result of ligand-induced conformational change in the protein.

Various fluorescent probes can be used in these displacement studies, for HSA: dansyl amide for site I and, dansyl sarcosine and dansyl glycine for site II (Muller et al., 1994) and coumarin derivatives (Goya et al., 1982). For AGP, auramine-O (Sugiyama et al., 1985), 7,chloro-2-(p-diethylaminophenyl)-2H-benzotriazolyl-5-amine (Narita et al., 1989) and 1-anilino-8-napthalene sulfonate (Johansen et al., 1992), have all been successfully used.

The main disadvantage for fluorescence studies is that coloured and concentrated protein solutions may cause difficulties. With probe displacement the main disadvantage is that several experiments need to run in order to investigate the ligand and its affinity for each of the binding sites, though this can be overcome somewhat by using multi-well plate techniques to increase the through-put of the assay.
Table 2-1: The key advantages and disadvantages of the common techniques to determine protein binding.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Method</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classical</td>
<td>Equilibrium dialysis</td>
<td>Amenable to high throughput screening. Drug binding to plasma proteins is analysed at equilibrium, eliminating the effect of nonspecific binding.</td>
<td>The volume shift between the matrix and the buffer can disturb the equilibrium. The system can take time to reach equilibrium and is affected by volume shifts, Donnan effects, nonspecific adsorption to dialysis apparatus. Difficulty in the control of experimental parameters i.e. pH of the medium. Radio-labelling of the drug will also add potential impurities to the experimental set-up.</td>
</tr>
<tr>
<td></td>
<td>Ultrasiltration</td>
<td>Shorter analysis time than equilibrium dialysis and lack of dilution effects and volume shifts. When combined with Raman difference spectroscopy allows for the label free detection and quantitation of binding.</td>
<td>Usually requires the use of a labeled drug and/or additional analysis steps to determine the actual level of free drug.</td>
</tr>
<tr>
<td></td>
<td>Ultracentrifugation</td>
<td>Eliminates problems associated with membrane effects of other classical methods.</td>
<td>Discrepancies in data with other methods.</td>
</tr>
<tr>
<td></td>
<td>Gel filtration</td>
<td>When combined with radio ligand-binding assay a 96-well plate method can be utilised.</td>
<td>Time consuming and complex. Radio-ligand labeling may alter binding parameters.</td>
</tr>
<tr>
<td>Classification</td>
<td>Method</td>
<td>Advantages</td>
<td>Disadvantages</td>
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<td>----------------------</td>
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<tr>
<td>Chromatography</td>
<td>Affinity chromatography</td>
<td>Precise and reproducible method that allows for enantioselective studies. Can indicate if the binding is co-operative, anti-cooperative or non-cooperative.</td>
<td>Cannot be used to investigate AGP.</td>
</tr>
<tr>
<td></td>
<td>Capillary electrophoresis</td>
<td>Can be used as a rapid screening method. Can be coupled with micro-fluidics to increase sensitivity.</td>
<td>Low sensitivity.</td>
</tr>
<tr>
<td>Spectroscopy</td>
<td>Circular dichroism</td>
<td>Can be used to determine the binding stoichiometry and the association constant.</td>
<td>Cannot be used with ligands that have little or no measurable CD absorption in the wavelength region of the induced CD spectrum of the protein. UV inactive compounds cannot be investigated directly.</td>
</tr>
<tr>
<td></td>
<td>Fluorescence</td>
<td>Fluorescence quenching is a rapid technique. Probe displacement can provide information on the environment, number and affinity for each of the binding sites for a particular ligand. Probe displacement is amenable to high-throughput screening.</td>
<td>The main disadvantage for fluorescence studies is that coloured and concentrated protein solutions cannot be used. Fluorescence quenching does not indicate the specific site of ligand binding; only that binding occurs. Interference from the ligand is often problematic due to absorptive screening. With probe displacement several experiments need to run in order to investigate the ligand and its affinity for each of the binding sites.</td>
</tr>
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</table>
2.5 Aims of this project

The general aim of this study was to determine the potential for complementary products to bind to albumin and alpha-1-acid glycoprotein, and determine the potential for these products to displace previously bound drugs from the proteins. Additionally the aim of this work was to use a rapid fluorometric method that can be easily adapted to high-throughput screening. The specific aims included:

- Establishing a standard extraction method that is easily reproducible.
- Determination of the disassociation constants from albumin (binding site I and II) for selected complementary products.
- Determination of the disassociation constants from alpha-1-acid glycoprotein for selected complementary products.
2.6 Materials and Methods

2.6.1 Chemicals

Albumin from human serum (HSA), dansyl amide (DA), dansyl sarcosine (DS), phenylbutazone, ibuprofen, alpha-1-acid glycoprotein (AGP), quinaldine red (QR) imipramine and chlorpromazine were all purchased from Sigma and were of the highest quality available. Phosphate buffered saline (PBS) was prepared in house and maintained at a pH of 7.4. Water was freshly prepared in-house with Milli-Q equipment and was ultra-pure grade (18.2MΩ). All other chemicals were from Sigma Chemical Co. (St Louis, MO, USA) and were of the highest purity available.

2.6.2 Herbal Samples

Danshen was a generous gift from Assoc. Prof. Chun Guang Li, Chinese Medicine Department, RMIT University, Melbourne, Australia. All other herbal products were purchased from local suppliers in Victoria, Australia and were all of commercial quality (Table 2-2). The methanolic and aqueous extraction method was modified from Unger et al., (Unger and Frank, 2004).

In brief, tablets were crushed, the contents of capsules emptied, extractions were either water or 80% methanol to allow for the aqueous and distinctly lipophilic components to be separated. Solvent volumes were adjusted so that standardised extracts at 100 mg of product/ml were obtained. This was based on the manufacturers’ stated concentration of the active or principal agent. The extracts were agitated in a shaking water bath at 37°C for 30 minutes then centrifuged at 2500 g for 10 minutes.
Standard extracts were diluted to final nominal concentrations of 0.1, 1 and 10 mg/ml. Methanol was evaporated to dryness under vacuum, the final volume of methanol did not exceed 1% (v:v). Extracts were kept in the dark and the shelf life set at 2 weeks.

2.6.3 Human Serum Albumin

2.6.3.1 Control Experiments

Stock solutions of human serum albumin (HSA) were prepared fresh on the day of use in PBS. Stock solutions of dansyl amide (DA) and dansyl sarcosine (DS) were prepared in methanol and PBS respectively. Immediately before use, stock solutions were dissolved in PBS. To determine the dissociation constant ($K_d$) of dansyl amide (DA) and dansyl sarcosine (DS) under our experimental conditions, the probes were titrated against known concentrations of HSA and the fluorescence recorded with a Perkin-Elmer Multi-label fluorometer with an excitation wavelength of 370nm and an emission wavelength of 475nm for DA and 350nm and 450nm for DS. All experiments were conducted in 96-well plates maintained at 37°C. $K_d$ values were calculated using non-linear regression with one site hyperbolic binding.

Positive controls were run with each experiment, phenylbutazone and ibuprofen were used as controls for sites I and II respectively. Stock solutions of phenylbutazone and ibuprofen were prepared in DMSO and immediately before use were diluted with PBS. The $K_d$ was determined for both binding sites with both controls. HSA was kept constant at 5 μM and the fluorescence recorded with increasing concentrations of the controls (0-1000 μM).
The fluorescence was recorded with a Perkin-Elmer Multi-label fluorometer with an excitation wavelength of 370nm and an excitation of 475nm for DA and 350 and 450nm for DS. All experiments were conducted using 96-well plates maintained at 37°C. $K_d$ values were calculated using non-linear regression with one site hyperbolic binding.

2.6.3.2 Displacement Studies

To ensure that the extracts did not cause significant changes in fluorescence in the absence of protein, each extract was assayed with the buffer and probes without the addition of HSA.

Displacement of the fluorescent probes dansyl amide (DA) and dansyl sarcosine (DS) from site I and II respectively was determined for each of the extracts. The final concentration of the fluorescent probes and the HSA was kept constant at 5 $\mu$M. The extract being investigated was added at various concentrations (0-2.5 mg/ml). Stock solutions were diluted with PBS to the required concentration.

Fluorescence was recorded with a Perkin-Elmer Multi-label fluorometer with an excitation wavelength of 370nm and an excitation of 475nm for DA and 350nm and 450nm for DS. All experiments were conducted using 96-well plates maintained at 37°C. $K_d$ values were calculated using non-linear regression with one site hyperbolic binding.
2.6.4 Alpha 1-acid Glycoprotein Studies

2.6.4.1 Control Experiments

Stock solutions of alpha-1-acid glycoprotein (AGP) were prepared fresh on the day of use in PBS. Stock solutions of quinaldine red (QR) were prepared in methanol and immediately before use were diluted with PBS. To determine the dissociation constant ($K_d$) for QR under our experimental conditions, the probe was titrated against known concentrations of AGP and the fluorescence recorded with a Perkin-Elmer Multi-label fluorometer with an excitation wavelength of 520nm and an emission wavelength of 610nm. All experiments were conducted using 96-well plates maintained at 37°C. $K_d$ values were calculated using non-linear regression with one site hyperbolic binding.

Imipramine and chlorpromazine were used as positive controls to investigate the basic and acidic drug binding to AGP, though these binding pockets are known to overlap (section 2.2.7). Stock solutions of imipramine and chlorpromazine were prepared in methanol and immediately before use were diluted with PBS. AGP was kept constant at 1 μM and the fluorescence recorded with increasing concentrations of the controls (0-1000 μM). The fluorescence was recorded with a Perkin-Elmer Multi-label fluorometer with an excitation wavelength of 520nm and an emission wavelength of 610nm. All experiments were conducted using 96-well plates maintained at 37°C. $K_d$ values were determined using non-linear regression with one site hyperbolic binding.
2.6.4.2 Displacement Studies

To ensure that the extracts did not cause significant changes in fluorescence in the absence of protein, each extract was assayed with the buffer and probes without the addition of AGP.

Displacement of the fluorescent probe quinaldine red (QR) was determined for each of the extracts. The concentration of the AGP was kept at 1 μM and the concentration of QR was kept at 20 μM. The extract being investigated was added at various concentrations (0-2.5 mg/ml). Stock solutions were diluted with PBS to the required concentration.

Fluorescence was recorded with a Perkin-Elmer Multi-label fluorometer with an excitation wavelength of 520nm and emission wavelength 610nm. All experiments were conducted using 96-well plates maintained at 37°C. K_d values were calculated using non-linear regression with one site hyperbolic binding.

2.6.5 Data Analysis

All assays were conducted in triplicate on five separate occasions. Analysis was conducted with Prism version 5.0. The dissociation constant (K_d) was determined by non-linear regression using one site binding. All K_d values reported are the average K_d ± SEM.
Table 2-2: Complementary products used to screen for their potential to cause displacement interactions with human serum albumin and alpha1-acid glycoprotein.

<table>
<thead>
<tr>
<th>Product</th>
<th>Principal/Active Component</th>
<th>[Principal component per tablet/capsule]</th>
<th>Manufacturer</th>
<th>Batch number</th>
<th>Reported benefit/Use</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human serum albumin studies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Co-Enzyme Q10</td>
<td>Ubidecarenone</td>
<td>50mg</td>
<td>Heron</td>
<td>57122</td>
<td>Reduces heart damage caused by free radicals and improves energy, stamina and endurance.</td>
</tr>
<tr>
<td>Danshen</td>
<td>Tanshinone IIA</td>
<td>80mg</td>
<td>*</td>
<td>N/A</td>
<td>Invigorates the blood to improve circulation. Treatment of liver disease including cirrhosis and hepatitis.</td>
</tr>
<tr>
<td>Dong Quai</td>
<td>Angelica polymorpha</td>
<td>520mg</td>
<td>Nature's Sunshine</td>
<td>01240714</td>
<td>Enrich the blood to promote circulation, regulate menstruation, calm nerves and soothe intestines.</td>
</tr>
<tr>
<td>Ginkgo Biloba</td>
<td>Ginkgo flavonglycosides</td>
<td>10.7mg</td>
<td>Herron</td>
<td>58751</td>
<td>Improve mental clarity and concentration. May also relieve the symptoms of vertigo and tinnitus.</td>
</tr>
<tr>
<td></td>
<td>Ginkgolides and Bilobalide</td>
<td>2.4mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Goldenseal</td>
<td>Hydrastis canadensis</td>
<td>500mg</td>
<td>Nature's Sunshine</td>
<td>01220333</td>
<td>Assist in the treatment of respiratory infections.</td>
</tr>
<tr>
<td><strong>Alpha 1-acid glycoprotein studies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Astragalus</td>
<td>Astragalus membranaceus</td>
<td>450mg</td>
<td>Nature's Sunshine</td>
<td>0128172</td>
<td>Helps to support the body’s immune system and in used as a “rejuvenating tonic”</td>
</tr>
<tr>
<td>Echinacea</td>
<td>Echinacea purpurea</td>
<td>410mg</td>
<td>Nature's Sunshine</td>
<td>0124471</td>
<td>Supports the immune defenses.</td>
</tr>
<tr>
<td>Ginkgo Biloba</td>
<td>Ginkgo flavonglycosides</td>
<td>10.7mg</td>
<td>Herron</td>
<td>56311</td>
<td>Helps to improve mental clarity and concentration and improves long and short term working memory. May also relieve the symptoms of vertigo and tinnitus.</td>
</tr>
<tr>
<td></td>
<td>Ginkgolides and Bilobalide</td>
<td>2.4mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gotu Kola</td>
<td>Centella asiatica</td>
<td>395mg</td>
<td>Nature's Sunshine</td>
<td>01223293</td>
<td>Eases anxiety and mood disorders.</td>
</tr>
<tr>
<td>Valerian</td>
<td>Valeriana officinalis</td>
<td>2250mg</td>
<td>Bio-Organics</td>
<td>301338</td>
<td>Antispasmodic and muscle relaxant. Relieve nervous tension, insomnia and anxiety.</td>
</tr>
</tbody>
</table>

*Product provided by the Chinese Medicine Research Group- RMIT University. Composition determined using HPLC (Li, Sheng et al. 2009). N/A- not applicable
2.7 Results

2.7.1 Human Serum Albumin

2.7.1.1 Dissociation of Site Specific Probes

To determine the dissociation constant (K\textsubscript{d}) of the site I and site II probes dansyl amide (DA) and dansyl sarcosine (DS) under our experimental conditions, probes were titrated against 5 µM of human serum albumin (HSA) and the fluorescence recorded (Figure 2-4). K\textsubscript{d} values were calculated using nonlinear regression with one site hyperbolic binding (Table 2-3).

![Graph showing fluorescence vs. probe concentration]

**Figure 2-4:** The relative fluorescence of human serum albumin (HSA) when bound with the site specific probes dansyl amide (DA), site I and dansyl sarcosine (DS), site II. Fluoresce recorded with an excitation wavelength of 370nm and an excitation of 475nm for DA and 350nm and 450nm for DS. Each point represents the mean of triplicate incubations for 5 replicate runs.

■ Dansyl amide  ▲ Dansyl sarcosine
Table 2-3: Dissociation constant ($K_d$) µM determined for dansyl amide (DA) and dansyl sarcosine (DS) binding to sites I and II on human serum albumin (HSA). $K_d$ values were calculated using non-linear regression with one site hyperbolic binding ($n = 5$).

<table>
<thead>
<tr>
<th></th>
<th>Dansyl amide (DA)</th>
<th>Dansyl sarcosine (DS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site I</td>
<td>6.84 ±0.42 µM</td>
<td>159.22 ±8.2 µM</td>
</tr>
<tr>
<td>Site II</td>
<td>136.91 ±11.36 µM</td>
<td>6.04 ±0.92 µM</td>
</tr>
</tbody>
</table>

2.7.1.2 Disassociation of Site Specific Controls

Phenylbutazone and ibuprofen were used as positive controls for sites I and II respectively. HSA (5 µM) was titrated against the controls (0-1000 µM) (Figure 2-5). $K_d$ values were determined using nonlinear regression with one site hyperbolic binding (Table 2-4). Each incubation was run in triplicate on 5 separate occasions.
**Figure 2-5:** % Dansyl amide (DA) and dansyl sarcosine (DS) displaced by phenylbutazone and ibuprofen from human serum albumin (HSA). DA is a specific probe for site I and DS for site II. Fluoresce recorded with an excitation wavelength of 370nm and an emission wavelength of 475nm for DA and 350nm and 450nm for DS. Each point represents the mean of triplicate incubations for 5 replicate runs ± SEM.

- ■ DA and Phenylbutazone ▲ DS and Phenylbutazone
- ▼ DA and Ibuprofen ◆ DS and Ibuprofen

**Table 2-4:** Dissociation constant (Kₐ) µM determined for sites I and II on human serum albumin for phenylbutazone and ibuprofen. Kₐ values were calculated using non-linear regression with one site hyperbolic binding (n = 5).

<table>
<thead>
<tr>
<th></th>
<th>Phenylbutazone (µM)</th>
<th>Ibuprofen (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site I</td>
<td>6.06 ±0.36</td>
<td>1222 ±96.2</td>
</tr>
<tr>
<td>Site II</td>
<td>102.5 ±11.36</td>
<td>8.35 ±0.41</td>
</tr>
</tbody>
</table>
2.7.1.3  *Displacement Studies with Complementary Products*

Displacement of the site-specific probes was investigated in the presence of the complementary product extracts. The total displacement of the probes is shown in Figure 2-6 and Figure 2-7. The $K_d$ was determined for each of the investigated products and is shown in Table 2-5.

No changes in fluorescence were seen when the extracts were incubated with the buffer and site specific probes in the absence of HSA.
Figure 2-6: Displacement of the site I probe dansyl amide (DA) from human serum albumin (HSA) for each of the products (a) the aqueous extracts, and (b) the methanolic extracts. Fluoresce recorded with an excitation wavelength of 370nm and an emission wavelength of 475nm. Data points represent the mean of triplicate incubations for 5 replicate runs ± SEM.

- CoEnzyme Q10
- Danshen
- Dong Quai
- Ginkgo Biloba
- Goldenseal

Chapter 2- Protein Binding
Figure 2-7: Displacement of the site II probe dansyl sarcosine (DS) from human serum albumin (HSA) for each of the products (a) the aqueous extracts, and (b) the methanolic extracts. Fluoresce recorded with an excitation wavelength of 350nm and an emission wavelength of 450nm Data points represent the mean of triplicate incubations for 5 replicate runs ± SEM.

- CoEnzyme Q10
- Danshen
- Dong Quai
- Ginkgo Biloba
- Goldenseal
Table 2-5: $K_d$ in mg/ml for each of the products investigated as determined by non-linear regression for site I and II on human serum albumin (HSA). $K_d$ values were calculated using non-linear regression with one site hyperbolic binding from the mean of 5 independent experiments ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>Site I</th>
<th></th>
<th>Site II</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aq</td>
<td>Meth</td>
<td>Aq</td>
<td>Meth</td>
</tr>
<tr>
<td>CoEnzyme Q10</td>
<td>*</td>
<td>4.4 ±0.30#</td>
<td>0.76 ±0.04</td>
<td>0.08 ±0.01</td>
</tr>
<tr>
<td>Danshen</td>
<td>1.73 ±0.06</td>
<td>7.39 ±0.42</td>
<td>3.15 ±0.30#</td>
<td>0.28 ±0.02</td>
</tr>
<tr>
<td>Dong Quai</td>
<td>21.27 ±2.56#</td>
<td>1.02 ±0.08</td>
<td>33.41 ±4.50#</td>
<td>13.36 ±1.09#</td>
</tr>
<tr>
<td>Ginkgo Biloba</td>
<td>14.43 ±1.37#</td>
<td>*</td>
<td>1.07 ±0.03</td>
<td>0.35 ±0.02</td>
</tr>
<tr>
<td>Goldenseal</td>
<td>5.78 ±0.25#</td>
<td>0.60 ±0.06</td>
<td>1.69 ±0.06</td>
<td>0.31 ±0.02</td>
</tr>
</tbody>
</table>

Aq- Aqueous extract.

Meth- Methanolic extract.

# $K_d$ determined was greater than the concentrations investigated.

* $K_d$ not determined.
2.7.2 Alpha-1-Acid Glycoprotein

2.7.2.1 Disassociation of Site Specific Probe

To determine the dissociation constant ($K_d$) of the probe quinaldine red (QR) under our experimental conditions, QR was titrated against 1µM of alpha-1-acid glycoprotein (AGP) and the fluorescence recorded (Figure 2-8). The $K_d$ value was calculated using nonlinear regression with one site hyperbolic binding and determined to be 3.3 ±0.43 µM.

![Graph showing relative fluorescence (RFU) of alpha-1-acid glycoprotein (AGP) when bound with the probe quinaldine red (QR). Fluorescence recorded with an excitation wavelength of 520nm and an emission wavelength of 610nm. Data points represent the mean of triplicate incubations for 5 replicate runs ± SEM.]

Figure 2-8: The relative fluorescence (RFU) of alpha-1-acid glycoprotein (AGP) when bound with the probe quinaldine red (QR). Fluorescence recorded with an excitation wavelength of 520nm and an emission wavelength of 610nm. Data points represent the mean of triplicate incubations for 5 replicate runs ± SEM.

2.7.2.2 Disassociation of Site Specific Control

Imipramine and chlorpromazine were used as positive controls to investigate the basic and acidic drug binding to AGP. AGP (1 µM) was titrated against the controls (0-1000 µM). $K_d$ values were determined using nonlinear regression with one site hyperbolic binding.
Under our experimental conditions the $K_d$ for imipramine and chlorpromazine was determined to be $10.22 \pm 0.78$ and $2.7 \pm 0.35 \mu M$, respectively. Each incubation was run in triplicate on 5 separate occasions.

**Figure 2-9:** % Quinaldine red (QR) displaced by imipramine and chlorpromazine from alpha-1-acid glycoprotein (AGP). Fluorescence recorded with an excitation wavelength of 520nm and an emission wavelength of 610nm. Data points represent the mean of triplicate incubations for 5 replicate runs ± SEM.

- ■ Imipramine
- ▲ Chlorpromazine

### 2.7.2.3 Displacement Studies with CAMs

Displacement of the probe (QR) was investigated in the presence of the complementary product extracts. The total displacement of the probes is shown in Figure 2-10. The $K_d$ was determined for each of the investigated products and is shown in Table 2-6.

No changes in fluorescence were seen when the extracts were incubated with the buffer and probe in the absence of AGP.
**Figure 2-10:** Displacement of the probe quinaldine red (QR) bound to alpha-1-acid glycoprotein (AGP) for (a) the aqueous extracts, and (b) the methanolic extracts. Fluorescence recorded with an excitation wavelength of 520nm and an emission wavelength of 610nm. Data points represent the mean of triplicate incubations for 5 replicate runs ± SEM.

- Astragalus
- Echinacea
- Ginkgo Biloba
- Gotu Kola
- Valerian
**Table 2-6**: $K_d$ in mg/ml for each of the products investigated for alpha-1-acid glycoprotein as determined by non-linear regression. $K_d$ values were calculated using non-linear regression with one site hyperbolic binding from the mean of 5 independent experiments ± SEM.

<table>
<thead>
<tr>
<th>Product</th>
<th>Aq</th>
<th>Meth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astragalus</td>
<td>1904 ±671.4&quot;</td>
<td>116.6 ±15.62&quot;</td>
</tr>
<tr>
<td>Echinacea</td>
<td>16.92 ±1.2&quot;</td>
<td>1.007 ±0.14</td>
</tr>
<tr>
<td>Ginkgo Biloba</td>
<td>12.09 ±0.6&quot;</td>
<td>2.043 ±0.17</td>
</tr>
<tr>
<td>Gotu Kola</td>
<td>50.14 ±3.97&quot;</td>
<td>19.22 ±0.72&quot;</td>
</tr>
<tr>
<td>Valerian</td>
<td>7.69 ±0.2&quot;</td>
<td>1.35 ±0.21</td>
</tr>
</tbody>
</table>

Aq- Aqueous extract.

Meth- Methanolic extract.

# $K_d$ determined was greater than the concentrations investigated.
2.8 Discussion

2.8.1 Product Selection

The products selected for these investigations were based on the potential for concurrent use with therapeutic agents that are known to bind to albumin or alpha-1-acid glycoprotein, based on the traditional claimed therapeutic benefit.

In addition to the therapeutic claims overlapping with the conventional medicine, products were selected if the scientific literature indicated that there may be an interaction, i.e. Dong Quai, which has been suspected of interacting with the highly protein bound warfarin (Table 2-2).

2.8.2 Selection of Method to Screen CAMs

Each method for determining protein binding has its own advantages and disadvantages (Table 2-1). With so many products on the market and very little information regarding these products there is a need for a rapid and reproducible method to screen for protein binding and potential drug interactions due to displacement of previously bound drugs. As the composition of complementary products is often unknown and widely variable traditional methods such as equilibrium dialysis, are not suitable.

Probe displacement studies can provide information for the key binding sites on both HSA and AGP and can be scaled to a high-throughput method. These studies are also rapid and cost efficient.
The potential for interference by the products at the excitation and emission wavelengths must be taken into consideration but can be determined by assaying the products with the buffer and probes without the addition of the protein.

Spectroscopy techniques have been shown to have good correlation with separation methods (Sugiyama et al., 1985, Baumann and Eap, 1988, Rahman et al., 1993), though this is mainly for high-affinity binding sites; with low affinity interactions the sensitivity decreases. Whilst there is a risk that low affinity reactions may not be identified in these studies, it is far more likely that high affinity binding will be involved in adverse interactions and therefore are the important to identify (Figure 2-3).

### 2.8.3 General Discussion

Previous investigations into the ability for complementary products to displace bound drugs from human serum albumin (HSA) and alpha-1-acid glycoprotein (AGP) are very limited. This study utilises a rapid fluorometric method to investigate the potential for these products to bind to plasma proteins and potentially displace previously bound drugs.

Investigations into protein binding should be conducted with human protein as species differences in binding properties are known (Benke et al., 2009, Brown et al., 1979, Son et al., 1998). Parameters including temperature and pH need to be controlled as small changes can alter protein binding, increasing the free-fraction of drugs (Christensen, 1989, Kodama et al., 2001).
The concentration of the protein used may alter the results obtained in these studies, in order to relate the in vitro data more closely to the in vivo situation the concentration of protein was set at physiological levels, 5μM for HSA and 1μM for AGP.

Studying complementary products provides many challenges including the variable composition and effect of the extraction method selected. Our choice was made on the basis of the relevance of the extract to the patient use of the product. As these products are generally administered as tablets or infusions ('teas'), aqueous and alcohol (methanol) extracts were prepared for these studies. Methanol was selected to extract the distinctly more lipid soluble components.

2.8.4 **Displacement Studies- Human Serum Albumin**

2.8.4.1 **Site Specific Probe Binding**

To confirm that the probes, dansyl amide (DA) and dansyl sarcosine (DS) were site specific they were titrated with HSA under our experimental conditions. $K_d$ values were determined using non-linear regression with one site hyperbolic binding. For site I the $K_d$ for DA and DS was $6.84 \pm 0.42$ and $159.22 \pm 8.2 \mu M$ respectively, and for site II $136.91 \pm 11.36$ and $6.04 \pm 0.92 \mu M$ respectively (Table 2-3).

This data is in line with previous studies. The $K_d$ for DA at site I was previously determined to be 5.6-7.5 μM and 6-6.06 μM for DS at site II (Sudlow et al., 1975, Epps et al., 1995). The specificity of the probe binding is confirmed by the high $K_d$ for DA at site II ($136.91 \pm 11.36 \mu M$ and for DS at site I ($159.22 \pm 8.2 \mu M$).
2.8.4.2 Site Specific Positive Controls

Phenylbutazone and ibuprofen were used as positive controls for sites I and II respectively (Figure 2-5). $K_d$ values were determined using non-linear regression with one site hyperbolic binding. $K_d$ values were determined to be 6.06 ±0.36 µM and 8.35 ±0.41 µM, for phenylbutazone and ibuprofen respectively (Table 2-4). The values are in line with previously reported data.

Previous studies of phenylbutazone for site I have reported $K_d$ values of 1.9±0.3 µM (Epps et al., 1995) and 8.4 ±1.7 µM (Parikh et al., 2000). For ibuprofen the $K_d$ has been previously determined as 2.7 ±1.2 µM (Epps et al., 1995) and 11.6 µM (Wanwimolruk et al., 1983). The specificity of the probes is confirmed with the high $K_d$ values for phenylbutazone at site II and ibuprofen at site I, 102.5 ±11.36 and 1222 ±96.2 µM respectively.

2.8.5 Displacement Studies with Complementary Products

Whilst site I is referred to as the more flexible binding site on HSA, in general, displacement from site II was more significant in our experiments. For 6 of the extracts a $K_d < 1$mg/ml for site II was determined. Whilst only one extract (the methanolic extract of Goldenseal) had a $K_d <1$ mg/ml for site I (Table 2-5). The lowest $K_d$ was for the methanolic extract of CoEnzyme Q10 at site II with a value of 0.08 ±0.01 mg/ml.

It is known that alkaloids bind to site II (Zhang et al., 2011) and that many plant and herbal products contain alkaloids, which may account for the more significant binding at this site.
In general the methanolic extracts caused more displacement of the bound probes than the aqueous extracts. The alkaloids that are the most likely cause of the binding would be present in the methanolic rather than the aqueous extracts.

2.8.5.1 CoEnzyme Q10

CoEnzyme Q10, also known as ubiquinone, is proposed as treatment for heart failure and to lower blood pressure. CoEnzyme Q10 is the only lipid soluble antioxidant synthesised endogenously and is present in all cellular membranes. It plays a role in cellular metabolism, acting as an electron carrier and also protects membranes and lipoproteins from protein oxidation and lipid peroxidation (Villalba et al., 2010, Crane, 2001, Stocker et al., 1991).

Plasma CoEnzyme Q10 levels range from 0.40-1.91 µmol/L (Bhagavan and Chopra, 2006). It is estimated that the daily dietary intake is 3-5 mg per day, and that diet supplementation does not increase tissue levels above this point of saturation (Crane, 2001, Beal, 1999, Weber et al., 1997). Despite this, concern for drug interactions involving CoEnzyme Q10 exist as this product is often consumed by patients diagnosed with conditions including Parkinson's and cardiovascular disease. Entacapone and clofibrate (and other fibrates) used in the treatment of Parkinson's and high cholesterol respectively are bound to HSA. Entacapone is >98% bound to human serum albumin (Casarett et al., 2008) and the fibrates are >80% bound to albumin (Miller and Spence, 1998).
Previous in vivo studies have shown no interaction between CoEnzyme Q10 and warfarin (Gage and Milligan, 2005), whilst binding to site I of albumin (the binding site of warfarin) was determined, the $K_d$ was high, $4.4 \pm 0.30$ mg/ml, and therefore not clinically likely to occur. This highlights the importance of correct extrapolation of in vitro data to the in vivo situation when the potential for drug interactions are being investigated.

In vitro studies using human blood have shown that quercetin a flavonol found in Coenzyme Q10 binds to albumin (Kaldas et al., 2005). This binding is thought to be most likely at site II. These experiments determined, binding to site II was more significant for the methanolic extract ($K_d$ 0.08 $\pm 0.01$ mg/ml) than the aqueous extract ($K_d$ 0.76 $\pm 0.04$) (Table 2-5). This data supports the conclusion that the flavonols including quercetin are responsible for the binding seen as the flavonols are best extracted in the methanolic solvent.

### 2.8.5.2 Danshen

Danshen (*Salvia miltiorrhiza*) is of concern with regards to herb-drug interactions as it is used traditionally as a ‘blood thinning’ herb and therefore the potential for concurrent use of this product with anti-coagulant therapy such as warfarin (a coumarin derivative that is bound to site I on albumin) is significant, additionally Danshen is known to be 50-70% bound to albumin (Liu et al., 2008).

Dialysis studies into components of Danshen have demonstrated binding to albumin, though this work was conducted with bovine albumin which has only a 76% sequenced identity with human albumin (Peters, 1985).
Using dialysis sampling coupled with high-performance liquid chromatography, danshensu (a component of Danshen) was shown to have a strong association with bovine albumin ($9.68 \pm 1.23 \times 10^3 \text{K/L mol}^{-1}$) (Wang et al., 2011).

Previous studies have shown that the interaction between Danshen and warfarin is due to tanshonine II sulfonate, a component of Danshen, displacing warfarin from its binding site on albumin (Liu et al., 2008). Danshen is also known to displace salicylate from HSA (Gupta et al., 2002). In this study, the displacement of salicylate by Danshen was increased at the higher concentrations of salicylate. Whilst salicylates bind to site I, at the higher concentrations investigated in this study salicylate would be bound to site I and II on albumin. This displacement of salicylate being more significant at the higher concentrations can be explained by the binding of Danshen to site II.

These experiments concluded that Danshen is able to bind to both sites I and II on albumin, though there was more significant displacement with the site II probe. In particular the methanolic extract produced significant displacement with a $K_d$ of $0.28 \pm 0.02 \text{mg/ml}$. Displacement of dansyl amide, the probe for site I did occur in our studies with Danshen (Figure 2-7) but the $K_d$ values were high, $1.73 \pm 0.06$ and $7.39 \pm 0.42 \text{mg/ml}$ for the aqueous and methanolic extracts respectively.

### 2.8.5.3 Dong Quai

Dong Quai (*Angelica sinensis*) is reported to be beneficial in the treatment of menopause, in particular in easing the symptoms of hot flushes.
It is known to contain coumarin (Zhao et al., 2003) and has been suspected of being involved in adverse interactions based on pharmacodynamics with warfarin (Hu et al., 2005).

The methanolic extract of Dong Quai bound to site I on albumin with a $K_d$ of 1.02 ±0.08 mg/ml, whilst this is the most significant $K_d$ determined for Dong Quai in our studies, it may not be at a level to be clinically significant. As can be seen in Figure 2-6, at the highest investigated concentration of the methanolic extract of Dong Quai, 65% of the probe was displaced from its binding site, at 1mg/ml 50% of the probe was still bound to site I.

As with Danshen and Ginkgo Biloba the pharmacodynamic interaction between Dong Quai and warfarin may be of greater clinical significance than the pharmacokinetic interaction. That is the coumarins potentiate the effect of the warfarin.

### 2.8.5.4 Ginkgo Biloba

Ginkgo Biloba is known to contain flavonoids, glycosides and terpenoids (ginkgolides, bilobaldides). It has been suspected of clinical interactions with warfarin due to its anti-platelet activity (Diamond et al., 2000). However, a randomized, single-dose crossover study found no significant alteration of the pharmacokinetic or pharmacodynamic profile of ticlopidine (an antiplatelet agent) with a single administration of Ginkgo Biloba (Kim et al., 2010).
The $K_d$ determined for the binding at site I for the aqueous extract was above the range investigated and could not be determined for the methanolic extract. The displacement that occurred with the site II probe is not likely to cause a clinically significant interaction. Ginkgo Biloba is claimed to have a therapeutic benefit in the treatment of dementia and Alzheimer's disease and as such it may be taken concurrently with medications for these conditions including galantamine and rivastigmine which are both alkaloids likely to bind to site II on albumin, but the binding of these agents to albumin is relatively low therefore reducing the likelihood of a clinically significant interaction.

### 2.8.5.5 Goldenseal

Goldenseal (*Hydrastis canadensis*) contains several isoquinalone alkaloids including berberine and hydrastine (Douglas et al., 2010). Isoquinalone alkaloids are known to bind to site II of albumin (Khan et al., 2012, Cheng et al., 2009). Both the aqueous and methanolic extracts were able to displace dansyl sarcosine, the site II probe with $K_d$ values of 1.07 ±0.03 and 0.35±0.02 mg/ml respectively (Table 2-5).

Goldenseal is claimed to be useful in the treatment of respiratory infections. A potential interaction can arise with the concurrent use of penicillin's, which also bind to site II. This interaction would increase the plasma concentration of the penicillin potentially increasing the occurrence and severity of adverse effects including hypersensitivity, nausea and neurotoxicity in patients susceptible to these side effects. However, clinically inhibition of renal extraction of penicillin is more likely to be the cause of adverse drug interactions.
2.9 Displacement Studies- Alpha-1-Acid Glycoprotein

2.9.1 Site Specific Probe Binding

Quinaldine red (QR) is known to bind to AGP, under our experimental conditions the $K_d$, determined using non-linear regression with one site hyperbolic binding, was $3.3 \pm 0.43 \mu M$ (Figure 2-8). Previous studies have demonstrated variability in the $K_d$ for QR with AGP with figures ranging from 1.3 $\mu M$ (Imamura et al., 1994) to 40 $\mu M$ (Hazai et al., 2006). The differences in these values can be explained by the experimental conditions. All experiments were conducted in this study were at 37°C, whilst the other studies were conducted at 23°C and room temperature respectively, temperature is known to alter the binding properties of proteins (Kodama et al., 2001, Christensen, 1989).

2.9.2 Site Specific Positive Controls

Imipramine and chlorpromazine were used as positive controls. Imipramine is a basic tricyclic drug that is known to bind to AGP (Kremer et al., 1988) but with lower affinity to the F1/S variant (Hervé et al., 1993) whilst chlorpromazine has a preference for the A variant (Hervé et al., 1996).

In line with previous reported data, $K_d$ for imipramine and chlorpromazine was determined to be $10.22 \pm 0.78$ and $2.7 \pm 0.35 \mu M$, respectively, under our experimental conditions. Imipramine has been reported to have a $K_d$ of 2.23 - 11.4 $\mu M$ (Zsila and Iwao, 2007, van der Sluijs and Meijer, 1985) and chlorpromazine a $K_d$ of 2.32, 2.6 and 2.8 $\mu M$ (Fitos et al., 2010, Berry et al., 2009, Matsumoto et al., 2002).
2.10 Displacement Studies with Complementary Products

Investigations into the displacement of previously bound drugs from alpha-1-acid glycoprotein (AGP) are very limited, even more limited are published reports involving the interaction between complementary products with AGP. This is the first study to combine dye displacement method with AGP interactions for complementary products.

As with the investigations into displacement by complementary products from albumin, more significant displacement was seen with the methanolic extracts than the aqueous extracts (Table 2-6).

2.10.1 Astragalus

Astragalus (*Astragalus membranaceus*) is native to the temperate regions of the Northern Hemisphere. Previous studies with Astragalus have focused on the potential for treatment of type II diabetes and its cardio-protective effects. Astragaloside IV, a saponin isolated from the *Astragalus membranaceus* plant is proposed to improve the sarcoplasmic reticulum Ca$^{2+}$ pump function in myocardial injury (Xu et al., 2007). Pharmacokinetic studies using rat and dog plasma demonstrated that astragaloside IV is 83% bound to plasma protein (Zhang et al., 2006), though the specific plasma protein was not identified.

The experiments conducted have shown no significant binding to AGP for the aqueous or methanolic extract of Astragalus (Figure 2-10). Therefore the binding to plasma protein by astragaloside IV is most likely to a different plasma protein, potentially albumin.
Though it should be noted that the study that determined binding to protein did so in animal models and not human.

### 2.10.2 Echinacea

Echinacea (*Echinacea purpurea*) is a genus of flowering plants belonging to the Asteraceae family. There are nine members of the species, all endemic to North America but *Echinacea purpurea* is most commonly used in complementary and alternative products. Echinacea has gained popularity as it is reported to support and boost the immune system. This is of concern as several antibiotics and drugs used in the treatment of HIV/AIDS also bind to AGP including rifampicin (Johnson and Smith, 2006) and the protease inhibitors (Schön et al., 2003). Additionally, levels of AGP increase in patients with tuberculosis (Ebersole and Cappelli, 2000) and HIV/AIDS (Kremer et al., 1988).

Most significantly, interactions with HIV/AIDS medication via displacement of the bound drug to AGP can reduce the therapeutic efficacy of the treatment, contributing to the emergence of drug resistant viral strains (Finzi et al., 1997, Williams and Sinko, 1999).

The most significant displacement of QR for the investigated extracts occurred with the methanolic extract of Echinacea ($K_d = 1.01 \pm 0.14$ mg/ml). The aqueous extract did not cause significant displacement ($K_d = 16.92 \pm 1.18$ mg/ml) (Figure 2-10). This displacement highlights the need to prioritise further investigations into Echinacea in the context of drug interactions.
2.10.3 Ginkgo Biloba

As Ginkgo Biloba is used to aid in the treatment of memory loss it has gained popularity and is now one of the most commonly used herbal products (Xue et al., 2007, Eisenberg et al., 1998). Ginkgo has predominantly been investigated for its interaction with the cytochrome P450 enzymes and for its increased anti-coagulant effect, most likely due to the coumarin in the product, when combined with warfarin (Izzo, 2004, Matthews, 1998).

The experiments conducted did not show significant displacement of QR with the aqueous extract ($K_d = 12.09 \pm 0.56 \text{ mg/ml}$) or the methanolic extract ($K_d = 2.04 \pm 0.17 \text{ mg/ml}$). Whilst this is the first study to investigate the binding of Ginkgo Biloba to AGP, Chen et al., determined the binding of ginkgolide B (a major component of Ginkgo Biloba) to human plasma to be 17.99-21.49% using equilibrium dialysis (Chen et al., 2007). These limited studies suggest that protein binding is not likely to be a cause of adverse interactions with Ginkgo Biloba.

2.10.4 Gotu Kola

Gotu Kola (*Centella asiatica*) is a small plant native to several countries including India, Sri Lanka, Indonesia and Iran. It is recommended in the treatment of bacteria and viral infections and also for any inflammatory conditions. As with Echinacea, there is an increased risk of concurrent use with antibiotics and antiviral drugs that are known to bind to AGP.
No significant displacement of QR was detected with the aqueous ($K_d = 50.14 \pm 3.97 \text{ mg/ml}$) or methanolic ($K_d = 19.22 \pm 2.73 \text{ mg/ml}$) extracts, therefore these studies indicate that displacement of drugs bound to AGP is not likely to occur with Gotu Kola. This negative finding is important as Gotu Kola is used in the treatment of anxiety and depression and therefore may be concurrently used with anti-anxiety medication that is known to bind with high affinity to AGP including chlorpromazine (90% bound with affinity constants ($K_a$) of $3.4 \times 10^5$-$3.9 \times 10^6$) (Verbeeck and Cardinal, 1985, Piafsky et al., 1978, Wright et al., 1988) and diazepam (60% bound with affinity constants ($K_a$) of $4 \times 10^4$-$2.5 \times 10^5$) (Schley and Müller-Oerlinghausen, 1983, Muruyama et al., 1992).

2.10.5 Valerian

Valerian (*Valeriana officinalis*) is native to Europe and Asia, and is used as a muscle relaxant and in the treatment of anxiety and insomnia. Valerenic acid and valerenol, components of Valerian, are known to bind to GABA(A) receptors (Benke et al., 2009). Valerian binding to plasma proteins to date has not been reported but the increased use of this product highlights the need for investigations such as these.

The methanolic extract was determined to have a $K_d$ of $1.35 \pm 0.20 \text{ mg/ml}$; this was the second greatest displacement of all the extracts investigated with AGP. The potential for adverse interactions whilst not significant may warrant further investigations or caution in patients concurrently taking Valerian and therapeutic agents with a narrow therapeutic index such as phenobarbital, which is bound to AGP (Lai et al., 1995).
2.11 Conclusions

The binding of complementary and alternative products was more significant with human serum albumin than with alpha-1-acid glycoprotein. Six of the investigated extracts were determined to have a $K_d < 1\text{mg/ml}$ with albumin whilst none of the extracts produced a $K_d < 1\text{mg/ml}$ with alpha-1-acid glycoprotein.

Specifically with regards to albumin binding, the displacement of the site II bound probe (dansyl sarcosine) was more significant than the site I probe (dansyl amide). Site II is known to be the binding site for carboxylic acids which are present in high levels in complementary and herbal products (Fournier et al., 2000).

The lowest $K_d$ was for the methanolic extract of CoEnzyme Q10 ($0.08 \pm 0.01 \text{mg/ml}$). The displacement of dansyl amide, the site I probe by Dong Quai is potentially the most significant interaction as it suggests that there is a pharmacokinetic interaction between this product and warfarin, which is also bound to site I.

With alpha-1-acid glycoprotein, the most significant binding was seen with the methanolic extract of Echinacea ($K_d = 1.01 \pm 0.14 \text{mg/ml}$). Importantly the plasma concentration of alpha-1-acid glycoprotein can vary dramatically, up to 2-5 fold (Huang et al., 2010). This variation may contribute to adverse interactions, and must be considered when in vitro data is extrapolated to in vivo.
The displacement of previously bound compounds from proteins in the serum can be significant in the context of drug interactions, though caution should be used when placing significance on these interactions (Rolan, 1994). As highlighted in section 2.3, binding to protein is only one criterion to consider. In these studies not only does the binding of components to the protein have to be significant but also there has to be a risk of displacing another significantly bound compound, for which displacement would cause therapeutic failure or toxicity. The interactions observed in these experiments are of greatest significance for products that could potentially displace warfarin due to the narrow therapeutic index and likely concurrent use of complementary products.

Interactions with protein binding can alter the kinetic profile of a drug which can lead to adverse interactions or a decrease in the therapeutic effect. We have proposed a rapid screening method that will allow for the potential displacement interactions to be identified from both HSA and AGP.
Chapter 3 - P-glycoprotein:

Inhibition of the transmembrane transporter P-glycoprotein

3.1 Overview of Drug Transporters

Transporters are membrane proteins that facilitate the flux of molecules into and out of cells. They control the intake and efflux of endogenous substrates including amino acids, sugars and inorganic ions. Many foreign compounds including complementary products are also recognised by transporters, and as a consequence, transporters play a role in determining the bioavailability, elimination, and therapeutic efficacy of many endogenous and exogenous compounds.

Transporters can be induced or inhibited by a wide variety of compounds; additionally transporters can alter the transport of endogenous substrates by altering homeostasis. The pharmacokinetic behaviour of drugs that are substrates for transporters can be influenced by concurrently administered compounds including conventional medicines, foods and complementary products, that function as inhibitors or inducers of transporter function.

Advances in the identification of carrier-mediated transport systems for drugs have been made in recent years. In total, it is estimated that at least 5% of all human genes are transporter related, indicative of the importance of the transport function in normal biological and toxicological outcomes (Hediger et al., 2004).
Transporters can be divided into two categories; passive transporters which allow molecules to move across cell membranes down their electrochemical gradients, and facilitated transporters which typically move molecules against their electrochemical gradients, requiring energy.

Transporters are classified based on their molecular structure or their mechanism of action, as either members of the adenosine triphosphate (ATP) binding cassette (ABC) family or the solute carrier (SLC) transporter family. ABC transporters are a family of membrane transport proteins that require ATP hydrolysis for moving the substrates across membranes. Therefore, ABC transporters are primarily active transporters. SLC transporters utilize the electrochemical potential difference in the substance transported and are therefore classified as facilitated transporters. Most drug transporters belong to the SLC family.

3.2 ABC Transporter Family

The ABC (ATP-binding cassette) proteins form one of the largest protein families, with members found in all living organisms from microbes to plants and mammals. The widespread presence of these proteins with a relatively conserved structure and function suggests a fundamental role. Members of the ABC superfamily are responsible for the active transport of compounds across biological membranes, including phospholipids, ions, peptides, steroids, polysaccharides, amino acids, organic anions, bile acids, drugs and xenobiotics (Klein I et al., 1999).
ABC transporters bind ATP and use the energy from ATP hydrolysis to transport various compounds across cell membranes as well as intracellular membranes of the endoplasmic reticulum, peroxisome and mitochondria (Higgins C, 1992). The functional protein contains two ATP-binding domains or nuclear binding folds (NBF) and two transmembrane (TM) domains.

ABC genes are organized as either full transporters containing two TM and two NBF or as half transporters containing one of each domain (Hyde et al., 1990). The half transporters can form functional transporters by combining as either heterodimers or homodimers.

In humans, 48 ABC transporters have been identified to date, and classified on the basis of phylogenetic analysis into 7 subfamilies (A-G) (Dean M and Allikmets R, 2001, Klein I et al., 1999). ABCA (12 members; previously ABC1), ABCB (11 members; previously MDR/TAP), ABCC (12 members; previously MRP/CFTR), ABCD (4 members; previously ALD), ABCE (1 member; previously OABP), ABCF (3 members; previously GCN20) and ABCG (5 members; previously White) (Dean M et al., 2001). The subfamily designation is not based on functionality; genes in different subfamilies may have more similarity in substrate recognition than genes in the same subfamily.

Members of three ABC subfamilies have been recognised to play a key role in drug efflux from cells: the MDR1 P-glycoprotein (ABCB1) of the ABCB subfamily, the breast cancer resistance protein (BCRP, ABCG2) of the ABCG subfamily and the multidrug resistance proteins (MRPs) of the ABCC subfamily.
3.3 P-Glycoprotein

Permeability glycoprotein (P-gp) is a 170-180 kDa transmembrane glycoprotein first described in 1976 (Juliano RL and Ling V, 1976). It is composed of two homologous and symmetrical cassettes; each contains six transmembrane domains that are separated by an intracellular flexible linker-polypeptide loop with an ATP binding motif (Higgins C, 1992, Juliano RL and Ling V, 1976).

P-gp is expressed at low levels in most tissues but is found in higher levels in epithelial cells with excretory roles. The location of P-gp is tissue-specific, it is localized on the canalicular surface of hepatocytes, the apical surface of renal tubular epithelial cells, the apical surface of epithelial cells in the intestine and placenta and the luminal surface of the capillary endothelial cells in the brain (Theibaut F et al., 1987, Cordon-Cardo C et al., 1990). P-gp is synthesised in the endoplasmic reticulum and subsequently modified in the Golgi apparatus (Loo and Clarke, 1999).

Whilst the physiological function of P-gp is still not fully understood, it is known to extrude a wide variety of structurally and chemically unrelated compounds out of cells. Due to its localization on tissues, P-gp is thought to have a protective effect for organs such as the testes and brain (Fromm M, 2004a). This is because P-gp extrudes toxic drugs and metabolites. Other functional roles include the secretion of metabolites into bile, urine and the lumen of the gastrointestinal tract (Leung and Bendayan, 1999a, Fromm M, 2004b, Kullak-Ublick and Becker, 2003, Leung and Bendayan, 1999b) and also the transport of hormones from the adrenal gland (van Kalken et al., 1993) and uterine epithelium (Axiotis et al., 1991).
Surprisingly when the broad range of substrates and diverse roles of P-gp are considered, P-gp may not be essential to life. This is demonstrated by transgenic knockout mice being fertile, viable and phenotypically indistinguishable from wild type mice (Schinkel et al., 1994). Though to date no human null alleles have been reported (You G and Morris M, 2007).

### 3.3.1 P-glycoprotein Isoforms

P-gp is the product of several genes. Two members of the P-gp gene family (MDR1 and MDR3) occur in humans, whilst three members of this family (mdr1a, mdr1b and mdr2) are found in mice (Gottesman and Pastan, 1993). The P-gp encoded by human MDR1 and mouse mdr1a/1b genes function as a drug efflux transporter, whilst human MDR3 and mouse mdr2 encoded P-gp are believed to transport phospholipids (van Helvoort et al., 1996, Ruetz and Gros, 1994). The isoforms have a 70% sequence homology.

### 3.3.2 P-Glycoprotein Structure

P-gp consists of two tandem repeats of approximately 1280 amino acids joined by a linker region of approximately 60 amino acids (Chen et al., 1986). Each repeat consists of an NH2-transmembrane (TM) domain (TMD) containing six potential TM segments, followed by a hydrophilic region containing a nucleotide biding domain (NBD) (Loo et al., 2010) (Figure 3-1).
Figure 3-1: Schematic representation of the primary structure of P-gp as embedded in the membrane. The molecule contains 1280 amino acids spanning 28 exons (each exon sequence shown in a different color). Black dots show the location of some of the identified SNPs. Modified from Ambudkar et al. (Ambudkar et al., 2003).

The secondary structure of P-gp has been established using Fourier transform attenuated reflective infrared spectroscopy and circular dichroism. P-gp approximately consists of 2-43% α-helix, 16-26% β-sheet, 15-29% β-turn and 13-26% unordered structure (Sonveaux et al., 1996; Dong M, 1998 #259). What is known is that the nucleotide-binding domains are not required for interaction with substrates or for trafficking of P-gp to the cell surface (Loo and Clark, 1999).
The binding pocket of P-gp has been extensively studied but to date there is no high-resolution crystal structure. Radio-ligand binding and functional transport studies indicate that there are anywhere from two (van Veen et al., 1998, Homolya et al., 1993, Loo and Clark, 1999, Shapiro et al., 1999) to at least four (Martin et al., 2000) substrate-binding sites. These sites fall into two categories:

- Transport: at which translocation of drug across the membrane can occur, and,
- Regulatory sites: which modify P-gp function.

Multiple substrates can bind with P-gp. Using cysteine-scanning mutagenesis of the TM segments, P-gp was demonstrated to have overlapping binding for colchicine and calcein, but not verapamil (Loo et al., 2003). Other data indicates that substrate-binding sites may overlap or be allosterically coupled (Dey et al., 1997, Ayesh et al., 1996), leading to the possibility that there is only a single common binding site (Borgnia et al., 1996).

### 3.3.3 Regulation of Expression of P-Glycoprotein

The human MDR1 promoter region is atypical as it does not contain a TATA promoter sequence, but it does have multiple response elements (Hennessy and Spiers, 2007). P-gp is constitutively expressed in a cell and tissue-specific manner (section 3.3.5), but can be induced by heat shock, cytokines, chemotherapeutic agents, UV radiation, oxygen free radicals and tumour suppression genes (Miyazaki et al., 1992a, Chaudhary and Roninson, 1993, Hu et al., 2000, Wartenberg et al., 2005).
In many cell lines, P-gp expression is increased through the up-regulation of MDR1 mRNA levels (Thorgeirsson et al., 1987, Abolhoda et al., 1999, van Kalken et al., 1992). The tumour suppressor protein p53 can either positively or negatively regulate MDR1 transcription. The p53 gene modulates the expression activity of MDR1 gene promoter in a promoter-CAT system (Li et al., 1997). The up-regulation of MDR1 expression has been shown to be a two-step process: mRNA stabilization and translational initiation (Yague et al., 2003). Post-transcriptional regulation occurs with heat shock factor 1 (Tchénio et al., 2006).

### 3.3.4 Genetic Variability of P-Glycoprotein

The MDR1 gene is located on chromosome 7 and consists of 28 exons. Evidence of structural variability in the MDR1 locus was first described in 1998 (Mickley et al., 1998) and to date more than 100 mutations have been identified. 15 single nucleotide polymorphisms (SNPs) were identified when the 28 exons were investigated (Hoffmeyer et al., 2000). Nine mutations alter the amino acid sequence of P-gp. Complete P-gp deficiency has not been reported for any of these polymorphisms, though the SNP at position C3435 in exon 26 does influence the expression level of intestinal P-gp (Lin J, 2003, Hoffmeyer et al., 2000).

#### 3.3.4.1 Genetic Variability and Drug Disposition

Several other polymorphisms are known to influence drug disposition, including the disposition of digoxin (Hoffmeyer et al., 2000, Cascorbi et al., 2001, Sakaeda et al., 2001), phenytoin (Kerb et al., 2001) and cyclosporine (von Ahsen et al., 2001).
3.3.5  *Tissue distribution of P-glycoprotein*

P-gp is expressed in tumour cells and in cells of many normal tissues. The cellular localization differs for each tissue. Thus transporter activity changes in any location can modify drug disposition in the body leading to an increased exposure and inducing specific tissue toxicity issues. This information is summarised, Table 3-1.

3.3.5.1  *Gastrointestinal Tract*

P-gp is expressed at the apical surface of the epithelial cells lining the intestine (Thiebaut et al., 1987, Maliepaard et al., 2001), with levels increasing from the duodenum to the colon (Dietrich et al., 2003). The role of P-gp in the gastrointestinal tract is to prevent and/or modulate the passage of certain xenobiotics or their metabolites from the gut intro circulation. Murine studies have indicated a second potential role, the prevention of accumulation of inflammation-inducing bacteria and bacterial products (Panwala et al., 1998).

3.3.5.2  *Lung*

P-gp is localized on the apical surface of bronchus and bronchiolar epithelium and at the plasma membrane of alveolar macrophages, it has been suggested that it removes environmental compounds to the lung lumen (Scheffer et al., 2002). Though this protective role has not yet been demonstrated in vivo.

3.3.5.3  *Central Nervous System*

High levels of P-gp are found in the brain, on the apical side of capillary endothelial cells, taking part in the blood-brain barrier (Cordon-Cardo et al., 1989, Thiebaut et al., 1987, Thiebaut et al., 1989).
In the brain P-gp is expressed in many cell types including choroid plexus, astrocytes, microglia and capillary endothelium. P-gp is also located on the luminal plasma membrane of the capillary endothelium, where it prevents the passage of drugs and toxins (Fricker and Miller, 2004).

### 3.3.5.4 Testis

The structure of the blood-testes barrier is similar to the blood-brain barrier and likewise it provides a protective function. Immunohistochemical studies have shown that P-gp is observed at the luminal side of the capillary endothelial cells (Thiebaut et al., 1989, Cordon-Cardo C et al., 1990).

### 3.3.5.5 Placenta

P-gp is expressed at relatively high levels in the syncytiotrophoblast cells (Atkinson et al., 2003), the role of this is to protect the foetus (Lankas et al., 1997).

### 3.3.5.6 Liver

In the liver, several ABC and other transporters are important for the elimination of drugs and toxins across the canalicular or sinusoidal hepatocyte membrane into bile or systemic circulation (Leslie E et al., 2005).

### 3.3.5.7 Kidney

P-gp is found on the apical surface of the epithelial cells of the proximal tubules (Schaub et al., 1999, Thiebaut et al., 1987). The role of P-gp in the kidney is to mediate the export of certain drugs (including uncharged and cationic drugs) from, blood into urine (Fricker et al., 1999, Gutmann et al., 2000, Schaub et al., 1999).
Table 3-1: The cellular localisation of P-glycoprotein in tissues and the effect on drug disposition. Material from various resources (Fromm, 2002, Cordon-Cardo C et al., 1990, Thiebaut et al., 1987).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Localization</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastrointestinal tract</td>
<td>Apical membrane of epithelial cells</td>
<td>Secretion of drugs into gut lumen</td>
</tr>
<tr>
<td>Lung</td>
<td>Apical surface of bronchial and bronchiolar epithelium</td>
<td>Remove environmental compounds/particulates to the lung lumen</td>
</tr>
<tr>
<td>Central nervous system</td>
<td>Apical side of capillary endothelial cell</td>
<td>Prevents the passage of drugs and toxins</td>
</tr>
<tr>
<td>Testis</td>
<td>Luminal side of the capillary endothelial cells</td>
<td>Protective function, blood-testis barrier</td>
</tr>
<tr>
<td>Placenta</td>
<td>Trophoblasts</td>
<td>Protection of the foetus from xenobiotics</td>
</tr>
<tr>
<td>Kidney</td>
<td>Apical membrane of epithelial cells of proximal tubules</td>
<td>Secretion of drugs into tubules lumen</td>
</tr>
<tr>
<td>Liver</td>
<td>Canalicicular membrane of hepatocytes</td>
<td>Secretion of drugs into bile</td>
</tr>
</tbody>
</table>

3.4 P-glycoprotein Binding and Efflux

Two to three molecules of ATP are hydrolysed per molecule of substrate transported (Eytan et al., 1996, Ambudkar et al., 1997, Shapiro A and Ling V, 1998, Delannoy et al., 2005). In the absence of a substrate, both NBDs are occupied with either ATP or ADP (Qu et al., 2003). Two potential models have been proposed in order to explain the sequence of events on the efflux cycle (Figure 3-2):

- An ATP hydrolysis driven efflux pump (Ambudkar et al., 2006).
Chapter 3 – P-glycoprotein Inhibition
Figure 3-2: Summary of the two models proposed for the translocation process for P-gp. a) The nucleotide switch model in which dimerization of the nucleotide-binding domains drives the pump. b) Model in which ATP hydrolysis drives the pump. Both models encompass four distinct stages: (i) Loading of P-gp with drug and nucleotide. (ii) Reorientation of the drug-binding sites from high to low affinity. (iii) Nucleotide hydrolysis. (iv) Resetting phase. Modified from (Hennessy M and Spiers JP, 2007, Callaghan et al., 2006, Hennessy and Spiers, 2007).
In general, transport by P-gp is saturable, osmotically sensitive, requires ATP hydrolysis, and generates a drug concentration gradient (Sharom, 1997). P-gp exhibits a high level of basal ATPase activity in the absence of a substrate (Shapiro and Ling, 1994, Sharom et al., 1995, Hennessy M and Spiers JP, 2007, Hennessy and Spiers, 2007). Upon substrate binding the level of ATPase activity in increased 3-4 fold (Senior et al., 1995, Martin et al., 1997), though increases of 20-fold have also been reported (Ambudkar et al., 1992).

### 3.5 P-Glycoprotein Substrates and Inhibitors

P-gp is a highly promiscuous transporter, able to recognize and transport an amazing number of diverse substrates that differ in chemical structure and pharmacological action, this includes many clinically important drugs, for example, doxorubicin, vinblastine, cortisol, indinavir, colchicine and rifampicin (Hennessy and Spiers, 2007). A typical substrate is large (M, >400), hydrophobic, amphipathic, with a planar ring system, and often carries a positive charge at physiological pH (Pearce et al., 1989), though this is not always true.

Dissociation constant (Kd) values for P-gp substrates cover a 1000-fold range, indicating that P-gp is able to discriminate between substrates (Sharom, 1997). Key physico-chemical properties for substrates and inhibitors have been identified, including lipid solubility, cationic charge and molecular refractivity, additionally hydrogen bonding potential, presence of an amine group, molecular weight, size, surface area and the presence of aromatic ring structures (Wang et al., 2003, Zamora et al., 1988, Ueda et al., 1997b, Stouch and Gudmundsson, 2002, Ueda et al., 1997a).
The presence of a hydrogen bond acceptor (or electron donor) moiety with a defined spatial separation is key (Seelig, 1998, Seelig et al., 2000). This spatial separation pattern is defined as type I, with two electron donor groups separated by 2.5 ± 0.3Å, or type II, with two electron donor groups separated by 4.6 ±0.6Å. A prerequisite for substrate binding is either a type I or II pattern (Seelig et al., 2000).

3.6 P-Glycoprotein Induction

The mechanism of transcription of the MDR1 gene is complex and not yet fully understood. Induction of P-gp/MDR1 expression does not occur via direct drug binding to P-gp, it is regulated by nuclear factors at the transcriptional level (Kuwano et al., 2004). As described in section 3.3.5, P-gp is constitutively expressed in a cell and tissue-specific manner, but it may be induced by environmental factors such as heat shock, cytokines, chemotherapeutics agents, UV radiation, oxygen free radicals and tumour suppressor genes (Chin et al., 1990a, Miyazaki et al., 1992b, Chaudhary and Roninson, 1993, Hu et al., 2000, Thottassery et al., 1997, Wartenberg et al., 2005, Chin et al., 1990b).

Nuclear receptors (NR) relevant for the expression of the ABC transporters are liver X receptor (LXR), farnesoid X receptor (FXR), pregnane X receptor (PXR), and peroxisome proliferator-activated receptors α and γ (PPARα and PPARγ) (Borst and Elferink, 2002). The NRs are also known to be involved in the induction of the cytochrome P450 enzymes (section 4.7).
The up-regulation of MDR1 mRNA levels, may involve several transcription factors including GC, HSF1, Sp1, AP-1, NF-Il6, NF-Y, EGR1, YB-1 and MED-1 (Chaudhary and Roninson, 1993, Thorgeirsson et al., 1987, van Kalken et al., 1992). The tumour suppressor protein p53 can either positively or negatively regulate the MDR1 transcription (Thottassery et al., 1997, Li et al., 1997).

3.7 Methods for studying P-Glycoprotein

Transporter interactions can be assessed by several methods. These assays can classify compounds as inhibitors and/or substrates of P-gp (Sharom, 1997, Seelig, 1998). Combinatorial chemistry and advances in synthetic chemistry have led to the development of many new compounds in the area of drug discovery and this has driven the development of screening assays for permeability. Ideally screening assays will have high efficiency, reproducibility, time, cost and space effectiveness and capability for high throughput.

3.7.1 In vitro Assays

There are many in vitro assays available to investigate the interaction of a test compound with P-gp, each with advantages and disadvantages.

3.7.1.1 Transcellular Transport

Transport studies can identify P-gp substrates, modulators or inhibitors. With monolayer efflux assays, where the ratio or basolateral-to-apical (B→A) permeability versus apical-to-basolateral (A→B) permeability is regarded as the standard for identifying P-gp substrates as it measures the efflux of drugs in the most direct manner (Polli. J et al., 2001a, Rautio et al., 2006).
The major disadvantage of these assays is they are labour intensive due to cell culture and analytical techniques, limiting throughput.

In brief, cells are seeded on a membrane surface and the test compound is added to the apical and/or basolateral compartment. At pre-determined time points the concentration of the compound is determined in each compartment. In the case of P-gp which is apically located, the basolateral to apical flux will dominate (Szakács et al., 2001). As this technique requires identification of the compound, it is not suitable for complementary products as the composition is often unknown and is highly variable.

In these studies different cell types may be employed including human colonic adenocarcinoma (Caco-2), Madin-Darby canine kidney (MDCK) and porcine kidney epithelial (LLC-PK1) cells. Caco-2 cells resemble intestinal epithelium, and are the most extensively characterized model for examining the permeability of drugs (Balimane et al., 2006, Elsby et al., 2008b, Elsby et al., 2008a).

Membranes isolated from the cells mentioned above, can be isolated. They contain high levels of transporters suitable for the characterization. Membrane-based assays characterization of the transporter may follow (i) the catalytic activity, (ii) the binding of the compound to the transporter, (iii) the actual substrate transport. An assay based on inside-out membrane vesicles is a clinically relevant method (Kharasch et al., 2005).
3.7.1.2 ATPase Expression

As P-gp is ATP dependent (see section 3.3), monitoring ATPase activity is a useful tool to determine P-gp interactions. In the absence of a test compound, P-gp will express a basal ATPase activity level. If a test compound binds to P-gp then an increase in ATPase activity will be detected. Whilst monitoring ATPase activity can be scaled to a high-throughput assay (Garrigues et al., 2002), these studies can not determine if the test compound is acting as an inhibitor, inducer or substrate for the transporter.

One important advantage of ATPase assays that is key when working with unknown tests compounds such as complementary products is that these assays do not require analytical techniques to identify the product. ATPase assays have been developed for a variety of systems including enriched and crude plasma membranes (Kokubu et al., 1997, Garrigues A et al., 2002, Garrigues et al., 2002), and enriched microsomal membranes (Litman et al., 1997). Purified P-gp can also be used (Ambudkar et al., 1997, Lu et al., 2001).

3.7.1.3 Cellular Accumulation

The accumulation of fluorescent dyes within the cell can be used to identify inhibition of P-gp. These assays are easily adapted for high-throughput screening, though identification of inhibitor or substrates can be difficult as there are multiple binding sites on P-gp (Schwab D et al., 2003a).
Measurement of the cellular accumulation of these dyes in the presence of a test compound may be done via flow cytometry, as in the case of Rhodamine 123 (a cationic dye) or by standard fluorometric measurements as with Calcein. Calcein AM is a non-fluorescent, highly lipid soluble dye that rapidly penetrates the plasma membrane of cells. In addition to this, Calcein AM is a good substrate for the efflux carriers for both P-gp and multidrug resistance protein (MRP). When Calcein AM enters the cells it is metabolized by the cytosolic esterase's into calcein, which is highly hydrophilic and fluorescent (Figure 3-3).

**Figure 3-3:** Schematic representation of the calcein AM extrusion assay. a) The lipid soluble calcein AM is able to cross the cell membrane. b) In the presence of esterase's the calcein AM is hydrolyses to the fluorescent calcein which is also hydrophilic. If inhibition of the P-gp pump occurs then calcein cannot be extruded and the fluorescence is recorded.
3.7.2  In Vivo Assays

In vivo mouse models can be used to evaluate the interaction of compounds with P-gp. The use of mdr1 knockout mice is useful in the identification of the role of P-gp in drug absorption and disposition (Bain and LeBlanc, 1996, Schinkel et al., 1994), though these mice have two members of drug-transporting P-gp (mdr1a (brain and intestine) and mdr1b (liver and kidney) expressed in a tissue specific manner (Croop et al., 1989).

The double knockout mdr1a/b mice have more recently been developed and can be used to study P-gp interactions (Schinkel et al., 1997). These mice display a 100-fold increase in the sensitivity to the centrally acting neurotoxic pesticide ivermectin, and to the carcinostatic drug vinblastine (3-fold). In addition to P-gp alterations, both single and double knockout mice display an increased expression of hepatic CYP2B and CYP3A proteins (Schinkel et al., 1997), which must be taken into consideration when pharmacokinetic studies are conducted using these animals.

3.7.3  In Silico

In silico studies have been utilised to predict the potential interaction of a drug with P-gp. One such study was used to predict the ability of drugs to form H-bonds, which as shown in section 3.5 are key for an interaction with the receptor to occur (Seelig and Landwojtowicz, 2000), lipophilicity and molecular weight have also been correlated with binding (Bain et al., 1997). A multi-tier approach has been utilised to classify compounds into three classes, substrates, inhibitors or non-interaction, with 82, 72 and 89% accuracy (Bain and LeBlanc, 1996).
With complementary products, the composition is often unknown and complex; this limits the use of in silico techniques with these products. In the future, in silico modelling may have a role in predicting the fate of these products when more information is available and potentially classes or family of products are found to behave in a similar pattern. At this stage the technology cannot provide predictions on the metabolic outcomes and kinetic behaviour of these products.

**Table 3-2:** The major advantages and disadvantages of the systems used in the investigation of P-glycoprotein interactions.

<table>
<thead>
<tr>
<th>System</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transcellular transport</td>
<td>Can identify substrates, modulators and inhibitors.</td>
<td>Labour intensive with limited throughput.</td>
</tr>
<tr>
<td>ATPase activity</td>
<td>Rapid and amenable to high throughput. Can identify substrates, modulators and inhibitors.</td>
<td>Cannot distinguish between substrates modulators and inhibitors.</td>
</tr>
<tr>
<td>Cellular accumulation</td>
<td>Rapid and cost efficient.</td>
<td>Indicate inhibition of P-gp, not induction.</td>
</tr>
<tr>
<td>In vivo</td>
<td>Knockout mice are available.</td>
<td>Not rapid or cost efficient for screening studies.</td>
</tr>
<tr>
<td>In silico</td>
<td>No reagents or drug required.</td>
<td>Molecular and structural knowledge of the product being investigated is required.</td>
</tr>
</tbody>
</table>

### 3.8 Interactions Caused by Herbal Products

To date several complementary and alternative products have been identified as substrates, inhibitors and/or inducers of P-gp. One of the most clinically significant examples is St. John’s Wort (*Hypericum perforatum*), which is commonly used to treat mild depression.
Studies have indicated that St. John’s Wort induces intestinal P-gp in vitro and in vivo (Hennessy et al., 2002, Perloff et al., 2001). These findings have been consistent in healthy volunteers who consumed the product for 14 days, with a subsequent 1.4-1.5 fold increase in their expression of P-gp (Dürr et al., 2000).

Silymarin, a component of the herb Milk Thistle has been studied due to concerns of adverse interactions between this herb and indinavir which is a known substrate for P-gp used in the treatment of AIDS. In healthy volunteers, treatment with Milk Thistle (175mg) for 3-weeks caused a 9-36% reduction in the AUC of indinavir.

3.9 Aims of the Present Study

The general aim of this study was to investigate complementary products that have the potential to alter the kinetic behaviour of therapeutic agents via the inhibition of P-glycoprotein. The specific goals included:

- Establishing a standard extraction method that is easily reproducible.
- To determine the potential for two assay types, the ATPase activity and cellular accumulation assay can be used in the context of complex mixtures such as complementary products.
- To determine if these methods produce comparable results when using complex mixtures.
- To determine if these methods can be used as high throughput screening assays for investigating the interaction of complementary and alternative products with P-glycoprotein.
3.10 Materials and Methods

3.10.1 Chemicals

Verapamil, calcein, Hank’s balanced salt solution (HBSS), MgATP, peroxidase-conjugated goat anti-mouse IgG and anti-mouse β-actin antibody were purchased from Sigma-Aldrich, Australia. Sodium orthovanadate was purchased from Merck. Advanced RPMI-1640 media from Gibco Technologies. K562 and K562-MDR cells were a generous donation from Dr Dodie Pouniotis, Cancer and Tissue Repair Research Group, RMIT University. Human p-glycoprotein membranes were purchased from BD Gentest. (US). Nitrocellulose membrane (Geneworks, SA, Australia) and 0.2-μm pore size Clear Blot Membrane-P (Atto Corp., Tokyo, Japan). Anti-P Glycoprotein antibody (JSB-1) was purchased from Abcam Ltd. UK.

Water was freshly prepared in-house with Simplicity 185 (Millipore S.A., Molsheim, France) water purification system and was ultra pure grade (18.2MΩ). All other chemicals were from Sigma Chemical Co. (St Louis, MO, USA) and were of the highest purity available.

3.10.2 Preparation of Herbal Samples

Herbal products were purchased from local suppliers in Victoria, Australia and were all of commercial quality (Table 3-3). The methanolic and aqueous extraction method was modified from that previously described (Unger and Frank, 2004). Tablets were crushed and the contents of capsules emptied, extractions were either water or 80% methanol to allow for the aqueous and distinctly lipophilic components to be separated.
Solvent volumes were adjusted so that standardised extracts at 100 mg of product/ml were obtained. This was based on the manufacturers’ stated concentration of the active or principle component.

Extracts were agitated in a shaking water bath at 37°C for 30 minutes then centrifuged at 2500 g for 10 minutes. Standard extracts were diluted to final nominal concentrations of 10, 100 and 1000 μg/ml. Methanol was evaporated to dryness under vacuum and the final volume of methanol did not exceed 1% (v:v). Extracts were kept in the dark and shelf life set at 2 weeks.

### 3.10.3 Cell culture

The human erythromyeloblastoid leukaemia cell line K562 and its over expressing variant K562-MDR cells were maintained in Advanced RPMI 1640 supplemented with 10% (v:v) heat-inactivated foetal bovine serum, 2mM L-glutamine, 100 μg/ml penicillin-streptomycin and were grown under standard conditions (5% CO₂, 37°C).
**Table 3-3:** Complementary products screened for their potential to interact with P-glycoprotein.

<table>
<thead>
<tr>
<th>Product</th>
<th>Active or Principle Component</th>
<th>[Active or principle component] in each tablet/capsule</th>
<th>Manufacturer</th>
<th>Batch Number</th>
<th>Reported benefit/Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cordyceps</td>
<td>Cordyceps sinensis</td>
<td>530mg</td>
<td>Nature’s Sunshine</td>
<td>0170794</td>
<td>For general wellbeing and aphrodisiac. Assist in the treatment of inflammatory conditions and fatigue.</td>
</tr>
<tr>
<td>Echinacea</td>
<td>Echinacea purpurea</td>
<td>410mg</td>
<td>Nature’s Sunshine</td>
<td>0124471</td>
<td>Supports the body's own immune defences.</td>
</tr>
<tr>
<td>Milk Thistle</td>
<td>Silybum marianum</td>
<td>84mg</td>
<td>Nature’s Own</td>
<td>302090</td>
<td>Assist the liver in detoxification.</td>
</tr>
<tr>
<td>Slippery Elm</td>
<td>Ulmus rubra</td>
<td>400mg</td>
<td>Nature’s Own</td>
<td>314087</td>
<td>Sooth digestive disturbance and enhance overall digestive health.</td>
</tr>
<tr>
<td>Valerian</td>
<td>Valeriana officinalis</td>
<td>2250mg</td>
<td>Bio-Organics</td>
<td>301338</td>
<td>Antispasmodic and muscle relaxant. Relieve nervous tension and anxiety.</td>
</tr>
</tbody>
</table>
3.10.4 Immunoblotting Analysis

Immunoblotting analysis for P-gp was undertaken as previously described with minor modifications (Auora et al., 2005). K562 and K562-MDR cells were centrifuged, and suspended in lysis buffer containing 2 mM EDTA, 25 mM Tris-phosphate, pH 7.8, 1% Triton X-100, and 10% protease inhibitor cocktail. Protein concentration was determined using the method of Bradford (Bradford, 1976).

Equal amounts of protein lysates were separated on SDS/7% (w/v)-polyacrylamide gels and then transferred to nitrocellulose in glycine transfer buffer. The blots were blocked overnight with 3% non-fat dry milk and probed with anti P-glycoprotein antibody (JSB-1) (0.5 μg/ml). Immunoblots were detected by horseradish peroxidase-conjugate anti-mouse IgG using chromogen 3,3’-diaminobenzidine tetrahydrochloride (dilution 1:1500). Anti mouse β-actin antibody was used at a 1:10,000 dilution as an internal load control. Immunoblots were scanned and band intensities quantitatively measure using Metamorph version 4.01 imaging software (Universal Imaging Corporation, USA).

3.10.5 Calcein AM Assay

Calcein AM assay was modified from previously described (Liminga G et al., 1994, Polli. J et al., 2001b, Polli. J et al., 2001a). Calcein AM was dissolved in dimethyl sulfoxide (DMSO) to a stock solution of 1 mg/ml and kept at -20°C until use. Accumulation of the calcein AM dye in K562-MDR cells was determined using a Perkin-Elmer Multi-label fluorometer, with an excitation of 485nm and an emission of 538nm. 1 x 10⁵ cells were seeded in 100 μl Hank’s balanced salt solution (HBSS), pH 7.4, in 96-well black plates with clear bottoms.
3.10.5.1 Control Experiments

Increasing concentrations of verapamil, a known P-gp inhibitor was incubated with K562-MDR cells for 15 minutes before the addition of calcein AM at a final concentration of 250 nM. Fluorescence intensities were recorded for 10 minutes at 30-second intervals, with an excitation of 485nm and an emission of 538nm in a Perkin-Elmer Multi-label fluorometer. Experiments were run in triplicate on three separate occasions.

3.10.5.2 Inhibition Studies

The aqueous and methanolic extracts of the complementary products were added to cells seeded at a density of $1 \times 10^5$ cells HBSS, pH 7.4 and pre-incubated at 37°C for 15 minutes. Calcein was added at a final concentration of 250 nM. Fluorescence intensities were recorded for 10 minutes at 30-second intervals with an excitation of 485nm and an emission of 538nm in a Perkin-Elmer Multi-label fluorometer. Positive control measurements to determine 100% inhibition were carried out in the presence of 50 μM verapamil. Experiments were run in triplicate on three separate occasions.

3.10.5.3 Calculations

From the time dependent increase of cellular fluorescence the initial rate of fluorescence generation (IRF) was determined. Quantification of P-gp inhibition was determined with the following equation:

$$\text{% Inhibition} = \frac{(IRF(\text{test compound}) - IRF(\text{background}))}{(IRF(\text{verapamil}) - IRF(\text{background}))} \times 100$$
Where IRF (control) was the average of three reading with verapamil 50 \( \mu \text{M} \) used as the positive control, and IRF (background) was the IRF in the absence of test compound. A compound was determined to inhibit P-gp when an inhibition \( \geq 50\% \) was reached.

The IC\(_{50}\) values for the extracts (concentration causing 50% reduction of control activity) were determined from the mean of the triplicate incubations run on three separate occasions by non-linear regression analysis (GraphPad Prism, v5.0).

### 3.10.6 ATPase Assay

The ATPase activity of human P-gp membranes was determined by measuring the inorganic phosphate released from ATP. The ATPase activity measured in the presence of 100 \( \mu \text{M} \) sodium orthovanadate represents non P-gp ATPase activity and was subtracted from the activity generated without sodium orthovanadate to determine basal ATPase activity.

All incubations were carried out in reaction buffer containing 50 mM MES, 2 mM EGTA, 2 mM dithiothreitol, 50 mM potassium chloride, 5 mM sodium azide (pH adjusted to 6.8 with Tris) and 1000 \( \mu \text{M} \) ATP magnesium salt. Incubations were conducted at 37°C for 20 minutes and stopped by the addition of 0.03 ml of 10% SDS.

At the end of the incubation, detection reagent (7 mM ammonium molybdate, 3 mM zinc acetate, and 16% ascorbic acid, pH 5.0) was added to each well. The colour reaction was incubated for 20 minutes at 37°C.
The plate was read immediately for endpoint absorbance at 850 nm using a Perkin-
Elmer Multi-label spectrophotometer. A potassium phosphate standard curve was
used to extrapolate nanomoles of phosphate from the absorbance readings.

3.10.6.1 Control Experiments

P-gp membranes (25 μg) were incubated at 37°C for 20 minutes in the presence of
increasing concentrations of verapamil as a positive control, DMSO (1%) was used
as a negative control. Assays were run in 96-wells in triplicate on three separate
occasions.

3.10.6.2 Complementary Products

Aqueous and methanolic extracts of the complementary products were incubated at
increasing concentrations (0-500 μg/ml) with P-gp membranes (25 μg) at 37°C for
20 minutes in the presence of verapamil. Assays were run in 96-wells in triplicate
on three separate occasions.

3.10.6.3 Calculations

The drug stimulated ATPase activity (nmol/min/mg of protein) was determined as
the difference between the amount of inorganic phosphate released from ATP in
the absence and presence of sodium orthovanadate for each of the extracts.
Phosphate standards were prepared in each plate and verapamil was used as a
positive control. The ATPase activity in verapamil stimulated cells was determined
as a % of the control (50 μM verapamil). The results are expressed as the mean
±SEM of triplicate replications run on three separate occasions.
The IC_{50} values for the extracts (concentration causing 50% reduction of control activity) were determined from the mean of the triplicate incubations run on three separate occasions by non-linear regression analysis (GraphPad Prism, v5.0).
3.11 Results

3.11.1 Immunoblotting Analysis

K562 and K562-MDR cells were subjected to immunoblotting to confirm the presence of P-gp. Anti P-glycoprotein antibody (JSB-1) is specific for the MDR1 isoform of P-gp and does not cross-react with MDR3. K562-MDR but not K562 cells were shown to express P-gp (Figure 3-4).

![Immunoblot analysis](image)

Figure 3-4: P-glycoprotein levels determined with immunoblot analysis. a) K562 and K652-MDR cells were probed with anti P-glycoprotein monoclonal antibody C219 anti-mdr1. Immunoblots were detected by horseradish peroxidase-conjugate anti-mouse IgG using chromogen 3,3’-diaminobenzidine tetrahydrochloride (dilution 1:1500). b) Bands were quantitatively measured by scanning densitometry and the density compared to the loading control anti mouse β-actin antibody. Data points represent the mean protein level ± SEM for 3 independent assays each run in triplicate.
3.11.2 Calcein AM

Calcein AM is a non-fluorescent highly lipid soluble dye that rapidly penetrates the plasma membrane of cells. It is a substrate for P-gp and multiple resistance protein (MRP) (Essodaigui M et al., 1998). Inside the cell, calcein AM is hydrolysed by the cytosolic esterases into the hydrophilic and fluorescent calcein. In the presence of a P-gp inhibitor, the fluorescent calcein molecules remain in the cell.

3.11.2.1 Positive Control Experiments

Verapamil, a known inhibitor of P-gp was used as a positive control. Under our experimental conditions maximum inhibition of P-gp was seen at 50 μM of verapamil (Figure 3-5).

![Figure 3-5](image)

Figure 3-5: Inhibition of calcein AM extrusion from K562-MDR cells in the presence of increasing concentrations of verapamil. Cells were pre-incubated with verapamil for 15 minutes prior to the addition of 250 nM calcein. Fluorescence was recorded for 10 minutes at 485nm excitation and 538nm emission. Data points represent the mean % inhibition ± SEM for 3 independent assays each ran in triplicate.
3.11.2.2 Inhibition of Calcein Extrusion by Complementary Products

K562-MDR cells were incubated with increasing concentrations of the aqueous and methanolic extracts of the complementary product extracts for 15 minutes. Calcein AM (250 nM) was added to the incubation and the fluorescence recorded. The % inhibition of P-gp calcein retention was determined for each of the tested extracts (Figure 3-6) as per the method previously described (section 3.1.12).
Figure 3-6: Inhibition of calcein AM extrusion in the presence of (a) aqueous and (b) methanolic extracts of complementary products, in K562-MDR cells. Cells were incubated with the extracts for 15 minutes prior to the addition of 250 nM of calcein. Fluorescence was recorded for 10 minutes at 485nm excitation and 538nm emission. Data points represent the mean % inhibition ± SEM for 5 independent assays ran in triplicate.

- Cordyceps
- Echinacea
- Milk Thistle
- Slippery Elm
- Valerian
The IC\textsubscript{50} for P-gp inhibition was determined from the mean of triplicate experiments ran on 3 separate occasions by nonlinear regression analysis as per the method previously described (section 3.1.12).

**Table 3-4:** The IC\textsubscript{50} (µg/ml) for products with the Calcein AM assay (n = 3).

<table>
<thead>
<tr>
<th>Product</th>
<th>Extract</th>
<th>IC\textsubscript{50} µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cordyceps</td>
<td>Aq</td>
<td>90.58 ±10.1</td>
</tr>
<tr>
<td>Cordyceps</td>
<td>Meth</td>
<td>#</td>
</tr>
<tr>
<td>Echinacea</td>
<td>Aq</td>
<td>#</td>
</tr>
<tr>
<td>Echinacea</td>
<td>Meth</td>
<td>#</td>
</tr>
<tr>
<td>Milk Thistle</td>
<td>Aq</td>
<td>#</td>
</tr>
<tr>
<td>Milk Thistle</td>
<td>Meth</td>
<td>96.93 ±13.7</td>
</tr>
<tr>
<td>Slippery Elm</td>
<td>Aq</td>
<td>69.95 ±8.44</td>
</tr>
<tr>
<td>Slippery Elm</td>
<td>Meth</td>
<td>45.71 ±5.04</td>
</tr>
<tr>
<td>Valerian</td>
<td>Aq</td>
<td>#</td>
</tr>
<tr>
<td>Valerian</td>
<td>Meth</td>
<td>353.1 ±27.4</td>
</tr>
</tbody>
</table>

\textsuperscript{a}IC\textsubscript{50} was greater than the concentration investigated and could not be determined.

Aq = Aqueous extract

Meth = Methanolic extract
3.11.3 ATPase Assay

ATPase activity is increased above the basal level when compounds bind to P-gp. Basal ATPase activity is determined by measuring the ATPase activity in the presence of orthovanadate, this represents non P-gp ATPase activity and can be subtracted from the activity generated without orthovanadate to yield vanadate-sensitive ATPase activity.

3.11.3.1 Control Experiments

Verapamil was incubated at increasing concentration with P-gp membranes (25 μg) (Figure 3-7). The biphasic response seen with verapamil in the assay which is consistent with previous reports (Sharom et al., 1995, Borgnia et al., 1996). As DMSO is known to stimulate ATPase activity (Lehotský et al., 1992), increasing concentrations were added to ensure that the 1% used in the assays did not affect the incubation. At the 1% level used there is no affect of the DMSO on ATPase activity (Figure 3-8). For each control experiment, ATPase activity measured in the presence of sodium orthovanadate was subtracted from the activity generated without sodium orthovanadate. Reactions were stopped by the addition of SDS (3%) and were run in triplicate on three separate occasions.
Figure 3-7: Concentration dependent stimulation of ATPase activity in K562-MDR cells by verapamil. Each point represents the mean of three incubations ±SEM ran in triplicate.

Figure 3-8: Concentration dependent stimulation of ATPase activity in K562-MDR cells by DMSO. Each point represents the mean of three incubations ±SEM ran in triplicate.
3.11.3.2 ATPase Activity with Complementary Products

To determine the potential for the complementary products to inhibit the verapamil stimulated ATPase activity of P-gp, human P-gp membranes (25 μg) were incubated at 37°C for 20 minutes in the presence of 50 μM verapamil. The ATPase activity in the presence of verapamil was shown to increase 2 fold above the basal level at this concentration (Figure 3-7). Increasing concentrations of extracts of the complementary products (0-500 μg/ml) were added to the incubation and the ATPase activity determined as described (section 3.11.3).
**Figure 3-9:** Inhibition of verapamil stimulated ATPase activity in K562-MDR cells by a) aqueous and b) methanolic extracts of complementary products. ATPase activity determined in the presence and absence of sodium orthovanadate, fluorescence recorded at 850nm. Data expressed as the mean ±SEM of three independent experiments ran in triplicate.

- **Cordyceps** ▲
- **Echinacea** ▲
- **Milk Thistle** ▲
- **Slippery Elm** ▲
- **Valerian** ▲

Chapter 3 – P-glycoprotein Inhibition
The IC_{50} for inhibition of the ATPase activity was determined from the mean of triplicate experiments ran on 3 separate occasions by nonlinear regression analysis as per the method previously described (section 3.10.6.3).

**Table 3-5:** The IC_{50} (μg/ml) for products in the verapamil stimulated ATPase assay with K563-MDR cells (n = 3).

<table>
<thead>
<tr>
<th>Product</th>
<th>Extract</th>
<th>IC_{50} μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cordyceps</td>
<td>Aq</td>
<td>138.5 ±14.42</td>
</tr>
<tr>
<td>Cordyceps</td>
<td>Meth</td>
<td>#</td>
</tr>
<tr>
<td>Echinacea</td>
<td>Aq</td>
<td>#</td>
</tr>
<tr>
<td>Echinacea</td>
<td>Meth</td>
<td>#</td>
</tr>
<tr>
<td>Milk Thistle</td>
<td>Aq</td>
<td>#</td>
</tr>
<tr>
<td>Milk Thistle</td>
<td>Meth</td>
<td>135.55 ±15.13</td>
</tr>
<tr>
<td>Slippery Elm</td>
<td>Aq</td>
<td>51.17 ±11.81</td>
</tr>
<tr>
<td>Slippery Elm</td>
<td>Meth</td>
<td>28.48 ±4.55</td>
</tr>
<tr>
<td>Valerian</td>
<td>Aq</td>
<td>#</td>
</tr>
<tr>
<td>Valerian</td>
<td>Meth</td>
<td>467.1 ±17.31</td>
</tr>
</tbody>
</table>

^{*}IC_{50} was greater than the concentration investigated and could not be determined.

Aq = Aqueous extract

Meth = Methanolic extract
3.12 Discussion

3.12.1 Product Selection

Products were selected for investigation based on the risk of concurrent use with therapeutic agents that are known to inhibit P-gp (Table 3-3). As many conventional medications are substrates, inducers and/or inhibitors of P-gp, the focus in this project was on products that have a broad therapeutic claim with particular focus on those that would likely to be concurrently consumed by patients also taking drugs for which failure would be a critical outcome i.e.: the protease inhibitors for HIV/AIDS treatment.

3.12.2 Method Selection

The inhibition of P-gp can be determined by several assays (Table 3-2). Calcein AM was selected as this assay is amenable to high-throughput screening and can provide information relating specifically to the inhibition of P-gp. The ATPase assays have been used extensively and like the calcein AM assay can be relatively high-throughout.

The major advantage with these assays in the context of complementary products is that neither requires the identification of the product for the analysis, both have an end point that is determined without directly measuring the product being investigated.
3.12.3 Immunoblotting Assays

To confirm the expression of P-gp, K562-MDR cells were stained with the specific MDR1 anti P-glycoprotein antibody (JSB-1). K562 cells, which do not express P-gp, were used as a negative control. Immunoblotting confirmed the presence of P-gp on the K562-MDR cells but not the K562 cells (Figure 3-4). This data confirms that the K562-MDR cells were a viable cell line to use in these studies.

3.12.4 Calcein AM Assay

Calcein AM is a non-fluorescent, highly lipid soluble dye that rapidly penetrates the plasma membrane of cells. In addition to this, Calcein AM is a good substrate for the efflux carriers for both P-gp and multidrug resistance protein (MRP). When Calcein AM enters the cells it is metabolized by the cytosolic esterases into calcein, which is highly hydrophilic and fluorescent product.

In our study 5 of the 10 tested extracts produced and IC\(_{50}\) <100 µg/ml (Table 3-4), with the methanolic extract of Slippery elm being the most significant (45.71 ±5.04 µg/ml).

3.12.5 ATPase Assay

ATPase activity is increased above the basal level when compounds interact with P-gp, though these studies cannot determine if the test compound is acting as an inhibitor, inducer or substrate for the transporter. By investigating the ATPase activity in the presence of verapamil-stimulated cells, the data indicates which of the extracts is more likely to be acting as an inhibitor.
Of the 10 extracts investigated, the 5 that were positive in the calcein AM assay ($IC_{50} < 100 \, \mu g/ml$) also inhibited the release of ATPase from the K5632-MDR verapamil stimulated cells.

### 3.12.6 Comparison of the Assays

As shown in Table 3-6, the $IC_{50}$s determined are comparable between the two methods. For some of the extracts the Calcein AM assay was more sensitive (the aqueous extract of Cordyceps and the methanolic extract of Milk Thistle) for the remaining extracts, the ATPase assay was more sensitive. Significantly no extract was positive in one assay and not the other.

#### Table 3-6: $IC_{50}$ ($\mu g/ml$) for the extracts that showed inhibition in the Calcein AM and ATPase assays (n = 3).

<table>
<thead>
<tr>
<th>Product</th>
<th>Extract</th>
<th>Calcein AM Assay</th>
<th>ATPase Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cordyceps</td>
<td>Aq</td>
<td>90.58 ±10.1</td>
<td>138.5 ±14.42</td>
</tr>
<tr>
<td>Milk thistle</td>
<td>Meth</td>
<td>96.93 ±13.7</td>
<td>135.55 ±15.13</td>
</tr>
<tr>
<td>Slippery elm</td>
<td>Aq</td>
<td>69.95 ±8.44</td>
<td>51.17 ±11.81</td>
</tr>
<tr>
<td>Slippery elm</td>
<td>Meth</td>
<td>45.71 ±5.04</td>
<td>28.48 ±4.55</td>
</tr>
<tr>
<td>Valerian</td>
<td>Meth</td>
<td>353.1 ±27.4</td>
<td>467.1 ±17.31</td>
</tr>
</tbody>
</table>

Aq = Aqueous extract  
Meth = Methanolic extract

Several other studies have concluded that multiple assays should be utilised to determined interactions of compounds with P-gp including those used in these studies and additional assays such as transcellular transport assays (Schwab D et al., 2003b, Seelig et al., 2000).
3.12.7 Herbal Products

3.12.7.1 Cordyceps

Cordyceps (Cordyceps sinensis) is from the genus of ascomycete fungi that originates in Tibet. Traditional use in Tibetan and Chinese medicine is as an aphrodisiac and as treatment for fatigue, inflammatory conditions, ageing, anti-viral, anti-fungal, cancer and to enhance well being (Nogueira Neto et al., 2011). Research to date has focused on isolating the many active components of this mushroom including, cordycepin (3’-de-oxyadenosine), ergosterol and peptides including alpha-aminoisobutyric acid, which have been proposed to be responsible for the therapeutic claims (Ng and Wang, 2005). This is the first report of investigations into extracts of Cordyceps and the potential for inhibition of P-gp.

Inhibition of P-gp was determined with the calcein AM assay for the aqueous extract (IC$_{50}$ 90.58 ±10.1 μg/ml). The ATPase assay also confirmed that the aqueous but not the methanolic extract of Cordyceps can inhibit P-gp (IC$_{50}$ 138.5 ±14.42). This inhibition is of note as Cordyceps is a product with a very broad therapeutic claim.

Concurrent use of this product with conventional medicines used in the treatment of infections and chronic inflammatory conditions such as several of the macrolide antibiotics including clarithromycin and roxithromycin which are also known to inhibit P-gp (Hughes and Crowe, 2010), could result in a significant inhibition of P-gp. Protease inhibitors used in the treatment of HIV, including nelfinavir and ritonavir are also inhibitors of P-gp. With the reported benefits of Cordyceps there is a likelihood of concurrent use with the protease inhibitors.
3.12.7.2 Echinacea

Echinacea (*Echinacea species*) is a genus of herbaceous plants that belongs to the Asteraceae family, endemic to eastern and central North America. Medicinal species of the Echinacea genus includes *E. purpurea*, *E. angustifolia*, and *E. pallida*, which vary in their chemical composition (Greene et al., 2007). This is of concern as products labelled as Echinacea may contain a wide variety of components. These studies utilised *E. purpurea*. This product is commonly taken as an immune system boost, for colds and flu, and to increase immune function. For this reason it is commonly consumed by patients diagnosed with HIV/AIDS and those receiving chemotherapeutic treatment.

With the high risk of concurrent use of Echinacea with anti-viral and/or chemotherapeutic treatment, Echinacea is an important product to study in regards to its interaction with P-gp. As substrates for P-gp include the protease inhibitors indinavir and nelfinavir (Kim et al., 1998), and the chemotherapeutics doxorubicin and vinblastine (Zhou S et al., 2008).

In our experiments, no significant inhibition was detected with any of the assays conducted. These findings are consistent with previous studies indicating that the assays used in our experiments do not overestimate the inhibition of P-gp. Studies with Caco-2 cells and the transcellular transport of radiolabelled digoxin (a known substrate for P-gp) also determined that there was no inhibition of P-gp with ethanol extracts of *E. purpurea* (Hellum and Nilsen, 2008).
This in vitro study is supported further by a human in vivo trial. Echinacea (267mg) was administered three times a day for two weeks to healthy volunteers, this supplementation did not alter the pharmacokinetics of digoxin (Gurley B et al., 2008).

### 3.12.7.3 Milk Thistle

Milk Thistle (*Silybum marianum*) is a flowering plant that is native to the Mediterranean regions of Europe, North Africa and the Middle East. It is commonly used as a liver tonic.

The aqueous extract showed no inhibition in either assay. This is consistent with a previous study that determined no interaction with P-gp with the aqueous extract in the Caco-2 cell transport assay (Budzinski et al., 2007). The methanolic extract showed inhibition in both assays with an IC₅₀ of 96.93 ± 13.7 μg/ml in the Calcein assay and 135.55 ± 15.13 μg/ml in the ATPase assay.

In agreement with the experimental data (Table 3-6), studies using 50 μM silymarin (a lipid soluble component of Milk Thistle) produced a significant accumulation of digoxin in transport assay with Caco-2 cells (Zhang and Morris, 2003). This inhibition was most significant for the apical to basal transport, mean transport ratio $P_{\text{app B-A}}/P_{\text{app A-B}}$ 1.62 and 4.48, respectively. Indicating that silymarin is a substrate for P-gp and can inhibit the P-gp mediated efflux. As silymarin is lipid soluble it is possible that this compound is responsible for the inhibition seen.
In vivo studies with healthy human volunteers determined no statistically significant effect on digoxin pharmacokinetics with Milk Thistle (900mg a day) (Gurley et al., 2006a). This lack of correlation may be explained as the in vitro studies conducted by Zhang and Morris (Zhang and Morris, 2003) did not include investigations with known P-gp inhibitors. As other transporters including MRP2 and BCRP are also present in Caco-2 cells, the more significant transport seen in this bi-directional assay may have been due to transporters other than P-gp. Conducting these studies in the presence of selecting P-gp inhibitors can confirm that the results are due to P-gp based efflux transport.

3.12.7.4 Slippery Elm

Slippery Elm (Ulmus rubra) is native to eastern North America, the bark is traditionally used to neutralize stomach acids, soothe digestive inflammation and alleviate digestive pain. To date limited studies have been conducted into slippery elm, several have investigated its use in the treatment of irritable bowel syndrome (Zhou S et al., 2004), but to date there are no reports relating to the pharmacokinetic characteristics of the herb.

With the proposed use of Slippery Elm as aiding in digestive disturbances there is an increased risk of concurrent use of this product with patients receiving treatment for cancer and HIV/AIDS as these treatments are known to cause digestive disturbances and nausea. Slippery elm has also been investigated for its use in the treatment of neoplasia (Mazzio and Soliman, 2009).
This is of concern as P-gp is an important measurement in determining the dose and type of chemotherapy prescribed (Gasparini et al., 1993) and any alteration in the measurement of P-gp activity may lead to incorrect dosage of chemotherapeutic agents.

These studies demonstrated that both the aqueous and methanolic extracts of Slippery Elm are inhibitors of P-gp. The methanolic extract of Slippery Elm was a more potent inhibitor (IC\(_{50}\) 45.71 ± 5.04 and 28.48 ± 4.55 \(\mu\)g/ml in the calcein and ATPase assay respectively). Though the levels of inhibition by the aqueous extracts is also significant (IC\(_{50}\) 69.95 ± 8.44 and 51.17 ± 11.81 \(\mu\)g/ml in the calcein and ATPase assay respectively). This IC\(_{50}\) is comparable to the known inhibitor fluoxetine (IC\(_{50}\) 64.0 ± 16.8 \(\mu\)g/ml) (Weiss et al., 2003), and therefore should be considered significant and investigated further.

3.12.7.5 Valerian

Valerian (Valeriana officinalis) is a flowering plant that is native to Europe and Asia and is used in the treatment of insomnia. The use of Valerian is increasing and therefore the risk of interactions is increasing.

The methanolic extract acted as a weak P-gp inhibitor (IC\(_{50}\) 353.1 ± 27.4 and 467.1 ± 17.31 \(\mu\)g/ml in the calcein and ATPase assay respectively). In a previous study the IC\(_{50}\) could not be determined in the Caco-2 cell transport assay, but the IC\(_{25}\) was shown to be 1777 \(\mu\)g/ml (Hellum and Nilsen, 2008). The discrepancy in this data may not only be due to the different assays used but also the extraction technique for the herbal products.
In the studies conducted by Hellum and Nilsen (Hellum and Nilsen, 2008), ethanol was used as the extraction solvent, this would extract different components than the methanol used in these experiments.

### 3.13 Conclusions

These studies have shown that several products can act as inhibitors of P-glycoprotein and that this inhibition may be significant for several of the extracts tested. Significant inhibition ($IC_{50} < 100 \mu g/ml$) was determined for the aqueous and methanolic extracts of Cordyceps and Milk Thistle respectively and all the extracts of Slippery Elm. The methanolic extract of Slippery Elm was responsible for the most significant inhibition of P-glycoprotein with an $IC_{50}$ of $45.71 \pm 5.04 \mu g/ml$ with the calcein AM assay and $28.48 \pm 4.55 \mu g/ml$ with the ATPase assay.

We have also demonstrated that the data between the two assay methods utilised-the fluorescent based calcein AM assay and the enzyme based ATPase assay are comparable when being used with this limited number of complementary products. Previous studies have shown that these assays are often comparable (Schwab D et al., 2003), though difference are known to occur. Compounds that have high esterase activity are known to cause false positives in the calcein assay and several known sub stares for P-gp do not stimulate ATPase activity (colchicine, vincristine and methotrexate) (Litman et al., 1997). The potential for false positives or false negatives at the early screening stage is a risk that can be avoided by employing both the ATPase and the calcein AM assay simultaneously.
Chapter 4 - Cytochrome P450:

Inhibition of Human Cytochrome P450 Enzymes by Complementary Products

4.1 Introduction - Mixed Function Oxidases

The mixed function oxidases (MFOs) were initially characterised in 1955 concurrently by two groups (Hayaishi et al., 1955, Mason et al., 1955), and require both an oxidant and a reductant. Reactions catalysed by the MFOs cause one atom of molecular oxygen to be reduced to water whilst the other is incorporated into an enzyme substrate.

4.2 Cytochrome P450 Monooxygenases

The cytochrome P450 (CYP) system is comprised of a family of haem-containing (haemoprotein) enzymes with closely related isoforms, belonging to the MFOs. This enzymatic system is found in almost all organisms, including mammals, bacteria and plants; being crucial for the oxidative, per-oxidative, and reductive metabolism of exogenous and endogenous compounds. More than >11,000 CYPs have been identified, and new CYPs are continuously being added (Nelson, 2009).

The most common reaction catalysed by the CYP system is a monooxygenase reaction, shown in the equation below:

\[ \text{NADPH} + H^+ + O_2 + RH \xrightarrow{\text{Cytochrome P450}} \text{NADP} + H_2O + ROH \]

where RH represents an oxidisable drug substrate and ROH the hydroxylated metabolite, NADPH-nicotinamide adenine dinucleotide phosphate.
CYPs are composed of approximately 500 amino acids and have an iron protoporphyrin IX as the prosthetic group, shown in Figure 4-1.

**Figure 4-1**: Structure of iron protoporphyrin IX, the prosthetic group of cytochrome P450 (Gibson and Skett, 2001).

Evidence for the existence of CYPs first emerged in the 1950s; studies revealed an enzyme system in liver that converted drugs and aromatic compounds into more polar metabolites and required NADPH (Axelrod, 1955, Brodie et al., 1955). An understanding of the biochemical nature of CYPs was gained from work initially involving liver pigments. It was observed in liver microsomes that a red-brown pigment when reduced by NADPH could be detected complexed with carbon monoxide due to its characteristic absorption peak at 450 nm (Garfinkel, 1958, Klingenberg, 1958).
This peak, from which CYPs gain their name, is due to the iron contained in the haeme prosthetic group, which is normally in the oxidized state, this absorbs light at a maximum wavelength of 418 nm but when reduced produces the peak at 450 nm. With the use of detergent solubilisation of microsomes, and interaction studies with isocyanide ligands, this pigment was ultimately characterized by Omura and Sato (Omura and Sato, 1964a, Omura and Sato, 1964b). It was shown that the pigment was a cytochrome with typical $\alpha$, $\beta$ and Soret absorption bands, Figure 4-2.

![Image of absorption spectra](image.png)

**Figure 4-2:** Typical absorption spectra for cytochrome P450. Carbon monoxide and ethyl isocyanide difference spectra of liver microsomes (Omura and Sato, 1962).
4.3  Cytochrome P450 Superfamily

4.3.1  CYP Families

The CYP enzymes have been subdivided into families and subfamilies based on similarities in their amino acid sequence. Isoenzymes with 40% similarity are grouped in families denoted by a number i.e. CYP1 or CYP2. Isoenzymes within a family that have greater than 55% sequence similarity are grouped in a subfamily designated by a capital letter i.e. groups of family CYP2 are known as CYP2C, CYP2D, CYP2E. Individual isoenzymes that have been specifically identified are given a further number i.e. CYP2E1.

To date there have been 107 genes identified encoding for CYPs in humans, with 18 families and 45 subfamilies. Of the genes identified, 57 have been isolated, identified and characterised, most appear to be expressed primarily in the endoplasmic reticulum. Table 4-1 summarises information for the known human CYP genes.
Table 4-1: Human cytochrome P450 genes. The 57 CYPs isolated in humans are shaded based on catalytic activities. Information collated from various sources: Number of amino acids, substrates, inducers and inhibitors (Cytochrome P450 Knowledgebase, 2006), tissue sites, subcellular location and typical reactions (Guengerich, 2005), with additional typical reactions from (Weizmann Institute of Science, 2009) and the classification (Klaassen, 2008).

<table>
<thead>
<tr>
<th>CYP</th>
<th>Amino acids</th>
<th>Tissue sites</th>
<th>Subcellular location*</th>
<th>Classification</th>
<th>Typical reaction^b</th>
<th>Substrates</th>
<th>Inhibitors</th>
<th>Inducers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A1</td>
<td>512</td>
<td>Lung, extra-hepatic sites. Peripheral blood cells.</td>
<td>ER</td>
<td>Xenobiotics</td>
<td>Benzo[α]pyrene 3-hydroxylation</td>
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<td>Caffeine N3-demethylation</td>
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<td>17β-oestradiol 4-hydroxylation</td>
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*a* ER- endoplasmic reticulum (microsomal), Mit = mitochondria

*b* If known

*c* Also involved in fatty acid and eicosanoid metabolism

*d* Mainly endoplasmic reticulum, some detected in the mitochondria

*e* Also involved in bile acid synthesis

*f* Thromboxane A synthase (TBXAS1)

*g* Prostaglandin I2 (prostacyclin) synthase (PTGIS)

*h* Also involved in retinoic acid metabolism

*i* Also involved in vitamin D metabolism

*j* Also involved in cholesterol biosynthesis

*k* Information from (Weizmann Institute of Science, 2009).
4.4 Function of CYPs

Of the CYPs with known catalytic activities, 14 are involved in steroidogenesis, 4 in aspects of metabolism of vitamins, 5 in eicosanoid metabolism, 4 have fatty acids as their substrates and 15 catalyse transformation of xenobiotic chemicals (Table 4-1). Those classified as xenobiotic transforming are mainly in the families 1-3 and discussed in greater detail (section 4.9, 4.10 and 4.11 respectively).

The CYP catalytic cycle leading to substrate oxidation is complex and may not necessarily take place in the linear fashion shown in Figure 4-3. Substrate binding, which is shown (in this figure) to occur prior to the reduction of the haeme iron may actually occur at other steps of the catalytic cycle (Yun et al., 2005, Isin and Guengerich, 2006).
Figure 4-3: The catalytic cycle, adapted from Gibson and Skett (Gibson and Skett, 2001). Where RH represents the drug substrate and ROH the corresponding hydroxylated metabolite.
4.4.1 Drug Metabolism

It has been shown that ≈75% of all drugs can be metabolised by 3 CYPs (CYP3A4, CYP2D6 and CYP2C9) (Rendic, 2002), and a set of 6-7 CYPs (CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 CYP2E1 and CYP3A4) will account for 90-95% of all drug metabolism (Evans and Relling, 1999) (Figure 4-4). CYPs are involved in the metabolism of 78% of the 200 top selling prescription medications in the United States (Zanger et al., 2008, Evans and Relling, 1999).

The relative contributions of these xenobiotic-metabolising CYPs are, to some extent, a function of the relative abundance, although there are some important exceptions. The patterns reported for human liver do not necessarily apply in other tissues and/or species.
Figure 4-4: Estimated contribution of individual human cytochrome P450 isoforms to the metabolism of the 200 top selling drugs. Adapted from Evans and Relling (Evans and Relling, 1999).
4.5 Regulation of CYPs

The regulation of CYP enzymes is controlled by several factors including age, hormones (sex steroids, growth hormones, thyroid hormones), diet, nutritional and disease status.

4.5.1 Factors that Regulate CYPs

4.5.1.1 Age

Microsomal protein content changes with age, neonates have an average of 26 mg/g liver (Hines, 2008), the average for a 30 year old is 40 mg/g liver with a decline by the age of 60 to an average of 31 mg/g liver (Barter et al., 2007b).

4.5.1.2 Hormonal Factors

The hormones of the pituitary, adrenal, testes and ovaries are predominantly involved in the developmental control and sexual dimorphism of the CYPs. The differing patterns of growth hormones are known to cause the induction (section 4.7) or repression of some CYP isoenzymes.

For example continuous low levels of growth hormone in women causes a reduction in CYP2C1 and an increase in CYP2C12 by altering gene transcription (Gibson and Skett, 2001). As pregnancy alters the hormone status, in particular the increase in progesterone (and its metabolites) in the mother's circulation and the breast milk may inhibit drug metabolism. During pregnancy, elimination of drugs metabolised by CYP2A6, CYP3A4, CYP2D6 and CYP2C9 is increased, whilst elimination of CYP1A2 and CYP2C19 substrates is decreased (Dempsey et al., 2002, Anderson, 2005, Hodge and Tracy, 2007).
4.5.1.3 Diseases

As the liver contains the greatest concentration of CYPs, diseases affecting the liver including cirrhosis, alcoholic liver disease, hepatitis and hepatocarcinoma all alter the level and activities of the CYPs and therefore drug metabolism. CYP activity may also be decreased due to altered hepatic blood flow and hypoalbuminaemia. Infection and/or inflammation can alter the activity and expression levels of CYPs in the liver as well as extra-hepatic tissues such as the kidney and brain (Renton, 2005). Known agents that can alter CYP levels include viruses (Hepatitis A, Influenza A and B), bacteria (*Helicobacter pylori, Listeria monocytogenes*) and inflammatory agents (vaccines and tissue injury) (Devchand et al., 1996).

Generally this infection/inflammation results in an inhibition of activity (section 4.8) but in some circumstances may lead to induction (section 4.7). The latter is particularly seen with the CYP4A family. Under inflammatory conditions this enzyme family which is predominantly involved in fatty acid hydroxylation (Table 4-1), is induced, it has been proposed that this is an important step in the termination of the inflammatory eicosanoids (Morgan, 1997).

4.5.1.4 Diet and Nutrition

The CYPs are highly responsive to nutrient (protein, carbohydrates, fat, vitamins and minerals), and non-nutrient factors (phytochemicals, antioxidants, flavours and agents including tobacco smoke and pesticides) present in the diet (Gibson and Skett, 2001, Parke and Ioannides, 1994).
In rodents, the activities of hepatic MFOs are known to increase up to 30% when the dietary protein content is increased (Kato et al., 1980), conversely rats fed a low-protein diet are known to have decreased drug metabolism capability (Gibson and Skett, 2001). Vitamin A and its metabolites are known to affect drug metabolism, ligands that bind to the retinoid X receptor stimulate CYP2B1, CYP2C11, CYP3A and CYP4A, but ligands for the retinoic acid receptor decrease CYP1A2 (Howell et al., 1998, Gibson and Skett, 2001).

### 4.5.1.5 Environmental Factors

Lead has been shown to decrease CYP1A1 activity in isolated human hepatocytes (Vakharia et al., 2001a), and mercury is a known potent inhibitor of CYP1A2 (Vakharia et al., 2001b). Inhibition of CYP1A1 by heavy metal contamination either from diet, environmental exposure and even as components of complementary products may therefore cause adverse drug interactions with therapeutic agents that are metabolised by the CYP1 family including the anti-cancer agents 5-fluorouracil (Yoshisue et al., 2001), and the anti-malarial drug quinidine and quinine (Granvil et al., 2002). As heavy metals can accumulate over time, cumulative exposure becomes important to consider.

TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) a polycyclic herbicide is known to affect both phase I and II metabolism, it causes induction of the metabolism of polycyclic hydrocarbons and the enzymes UDP-glucuronosyltransferase and glutathione-S-transferase (GST) (Gibson and Skett, 2001). TCDD is known to induce CYP1A1 via activation of the aryl-hydrocarbon receptor (Nebert et al., 2000).
4.6 Genetic Polymorphism

Genetic polymorphism is a common phenomenon in the CYP superfamily, and is a major cause of inter-individual and ethnic differences in the rates (which can vary by more than 100-fold) at which individuals metabolise drugs. The occurrence of different gene variants translates into four major phenotypes:

- **Poor metabolisers (PMs):** Carrying two defect alleles and therefore completely lacking in enzyme activity.
- **Intermediate metabolisers (IMs):** Heterozygous for a defect allele or carrying two alleles resulting in enzyme with decreased activity.
- **Extensive metabolisers (EMs):** Carrying two functioning alleles.
- **Ultrarapid metabolisers (UMs):** Carrying more than 2 active gene copies.

To date alleles have been described for the CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2A13, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP2F1, CYP2J2, CYP2R1, CYP2S1, CYP2W1, CYP3A4, CYP3A5, CYP3A7, CYP3A43, CYP4A11, CYP4A22, CYP4B1, CYP4F2, CYP5A1, CYP8A1, CYP19A1, CYP21A2, and CYP26A1 genes (http://www.cypalleles.ki.se). The different polymorphic variants are designated by the addition of an asterisk followed by a number for example, CYP2D6*1.

Toxicity can arise depending on the phenotype. With PMs, a xenobiotic may accumulate, or in the case of a pro-drug, therapeutic efficacy may be compromised due to a lack of activation. Alternatively toxicity may arise in the UMs due to the accumulation of an active or reactive metabolite or therapeutic efficacy may be decreased due to the rapid clearance of a xenobiotic.
The number of alleles varies for each CYPs and is shown in Figure 4-5. CYP2D6 and 21A2 have the greatest level of polymorphism.

**Figure 4-5:** Frequency of alleles identified for human cytochrome P450s. The number of alleles includes haplotypes, normal variants, gene deletions, pseudo gene-derived mutations, combinations of pseudo-derived mutations and rare mutations and combinations. Additionally many single nucleotide polymorphisms (SNPs) have been identified for which the haplotype has yet to be identified. Information is based on the homepage of the human cytochrome P450 (CYP) allele nomenclature committee (http://www.cypalleles.ki.se).
4.7 Induction

The ability for foreign chemicals to induce CYPs was first described in 1954 (Brown et al., 1954). Subsequent work demonstrated that PAHs and barbiturates increase enzyme activity by inducing the synthesis of enzyme protein (Conney et al., 1957, Conney et al., 1961). Many CYPs in humans are susceptible to induction including CYP1A, CYP2A, CYP2B, CYP2C, CYP2E and CYP3A. To date induction of CYP2D6 has not been identified.

The induction of CYPs can have a major impact on drug metabolism, pharmacokinetic properties, and drug-drug interactions, on the toxicity and carcinogenicity of foreign chemicals, and on the activity and disposition of endogenous hormones (Conney, 1982). It is now known that a wide range of chemicals are capable of causing induction including therapeutic agents, pesticides, food additives, industrial chemicals, diet, natural products and environmental pollutants (Pelkonen et al., 2008).

Induction can arise as a consequence of increased synthesis, decreased degradation, activation of pre-existing components or a combination of these processes. Induction is said to occur when there is an increase in the amount and activity of enzyme. The time for induction is not only dependent on the half-life of the inducing agent but also the time course for enzyme degradation production. The two main mechanisms by which CYP induction occurs are:

1) Nuclear receptor-mediated induction. Transcriptional gene activation is the most common mechanism for CYP induction (section 4.7.1.1).
2) Stabilization of the mRNA or enzyme. For example, troleandomycin induces rat CYP3A by decreasing the rate of CYP3A protein degradation with no increase in the rate of protein synthesis (Watkins et al., 1986).

Though induction of CYPs is highly conserved, species differences are known (Dickins, 2004, Hewitt et al., 2007). The transcription factors encoded for by the nuclear receptors are the molecular basis of species differences.

### 4.7.1 Nuclear-Receptor Family

The nuclear receptor (NR) superfamily codes for transcription factors that transform extra-cellular and intra-cellular signals into cellular responses by triggering the transcription of target genes, including the expression of phase I and II enzymes and transporters.

The NR super-family consists of a large group of transcription factors, including the aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR, NR1I3), pregnane X receptor (PXR or the human orthologue SXR; NR1I2), bile-activated farnesoid X receptor (FXR, NR1H4), peroxisome proliferator-activated receptor (PPAR), cholesterol-sensing liver X receptor (LXR, NR1H2/3), thyroid hormone receptors (TR), the retinoic acid receptors (RAR), hepatic nuclear factor (HNF) family members, glucocorticoid receptor (GR), and the CCAAT/enhancer-binding proteins (C/EBPs) (Tompkins and Wallace, 2007, Pelkonen et al., 2008, Honkakoski and Negishi, 2000).
CYP genes are able to respond to both endogenous and exogenous signals by changes in CYP gene expression, and also to modulate the strength and duration of these signals and even to form new signalling molecules through CYP-mediated metabolism. These signalling molecules may then exert their function via the ligand-dependent NRs. (Honkakoski and Negishi, 2000). Not all of the NRs listed above are involved in CYP induction. AhR, PXR/ SXR, and CAR have been identified as the xenosensors; the others are not primarily activated by xenobiotics.

4.7.1.1 Nuclear-Receptor Mediated Induction

The NRs, AhR and CAR are localized in the cytoplasm of hepatocytes and once activated are translocated to the nucleus, whilst PXR has been identified in the cytoplasm but is mainly located in the nucleus (Richter et al., 2001, Kawana et al., 2003, Squires et al., 2004, Amacher, 2010). In the absence of a ligand, the NRs are associated with co-repressor complexes, conferring a basal level of transcription (Figure 4-6).

With ligand binding to the ligand-binding domain of the NRs, conformational changes occur leading to the release of co-repressors and recruitment of co-activators. Recruitment of co-activators and a dimerization partner (retinoid X receptor, RXR, for CAR/PXR and the AhR nuclear translocator, ARNT, for AhR) leads to chromatin remodelling and subsequent transcriptional activation. Regulation of gene transcription is achieved through binding of the nuclear receptor DNA binding domain to respective DNA response elements present in the promoter region of the target CYP gene (Amacher, 2010).
4.7.1.2 AhR

The aryl hydrocarbon receptor (AhR) is a basic helix-loop-helix protein that belongs to the family of PAS (PER- Drosophila period clock protein, ARNT-AhR nuclear translocator, SIM- Drosophila single-minded protein) domain transcription factors which binds dioxins and aromatic hydrocarbons (Gu et al., 2000, Poland and Glover, 1976).
The AhR and the AhR nuclear translocator, both present in high levels in placental tissue, (Hakkola et al., 1997, Manchester et al., 1987), control expression and activity of the isoenzymes belonging to the CYP1 family.

### 4.7.1.3 CAR

The constitutive active/androstane receptor (CAR) is located in the cytosol and when activated by phenobarbital induces CYP2B. It is now known that activated CAR induces many enzymes and transporters, including those that encode for the expression of CYP2B, 2C, 3A, NADPH-cytochrome P450 reductase, sulfotransferases, glucoronsyltransferases and glutathione-S-transferase, and transporters such as Mrp2 and Mrp4 (Ueda et al., 2002, Kast et al., 2002, Assem et al., 2004).

### 4.7.1.4 PXR

The pregnane X receptor (PXR) also known as PAR, SXR and NR1I2 activates CYP3A genes in response to diverse chemicals including natural and synthetic steroids (Zhou et al., 2009a, Lehmann et al., 1998, LeCluyse, 2001, Istrate et al., 2010). Unlike the other nuclear receptors, PXR has more divergence across the species with the ligand binding domain having only 70-80% homology (LeCluyse, 2001, Lehmann et al., 1998) which accounts for the species differences seen, for example rifampicin is an inducer of CYP3A in humans and rabbits but not rodents (Waxman, 1999).
4.7.2 Post-Transcriptional Induction

Post-transcriptional regulation is known to occur, with CYP2E1 being mainly regulated at this level. Xenobiotics responsible for the regulation of CYP2E1 include ethanol, acetone and isoniazid. It has been proposed that these agents work by enzyme stabilization and therefore prevention of the degradation of the CYP2E1 enzyme (Song et al., 1989, Carroccio et al., 1994).

4.8 Inhibition

Inhibition of metabolism may in general mean an acute decrease of metabolism of a particular substrate by another simultaneous present chemical (concurrent administration) or a time-dependent decrease in the amount of drug metabolizing enzyme by several factors such as chemical injury or a disease process. Specifically, inhibition of CYPs can occur at several stages of the catalytic cycle (Figure 4-3), this may be at the point of substrate binding; the reduction of the ferrous form by NADPH-cytochrome P450 reductase; the binding of molecular oxygen; the point of substrate oxidation; interference with the electron supply (from P450 reductase and/or cytochrome b₅); the point of product release.

The inhibition of any CYP isoenzyme may be clinically significant; the degree of significance and the potential for significance is determined by several factors:

- Therapeutic index (TI) of the drug/xenobiotic.
  - Drugs with a narrow TI have an increased risk of adverse reactions.
- Proportion of metabolism of the drug conducted by the inhibited pathway.
  - Many drugs are capable of being metabolised by an alternate and/or secondary pathway(s).
• Affinity constant (Ki) of the inhibitor relative to the affinity constant (Km) of the drug.
  o An inhibitor may have a much greater affinity for a CYP catalytic site than the drug/xenobiotic of interest.
• Whether or not the drug is metabolised to a pharmacologically active or toxic metabolite by the inhibited enzyme.
  • Liver damage.
    o Whilst this is not truly an inhibition of CYP enzymes, destruction of hepatic tissue causes a decrease in CYP enzymes available.

As CYPs have broad substrate specificity, competition between substrates for the same enzyme occurs. Such competition depends on their relative affinities and inhibitory potencies. Additionally inhibition is dose related; at low concentrations the inhibitor may be relatively selective for a single CYP, whilst at high concentrations, the inhibitor may be relatively non-selective and several CYP enzymes may be inhibited. The inhibition of CYPs can be broadly divided into two categories; irreversible and reversible. The distinction between these two groups is relative and can be difficult to determine if the inhibitor binds tightly to the enzyme and is slowly released (Wienkers and Heath, 2005).

### 4.8.1 Irreversible Inhibition

Irreversible inhibition occurs with competitive/non-competitive co-substrates. There are two basic processes that lead to irreversible inhibition:
• Autocatalytic inactivation- (or suicide inhibition) occurs when a reactive drug metabolite binds to CYP and alters the structure of the CYP irreversibly. The inhibition of CYP1A2 by furafylline is a classical autocatalytic inactivation reaction. (Ortiz de Montellano et al., 1979, Yin et al., 2000, Kunze and Trager, 1993). Additionally drugs that contain terminal olefinic or acetylinic substituent’s are likely to be suicide substrates of CYPs (Hasler et al., 1999).

• Complexation of reactive intermediates occurs when drug metabolites are formed that bind tightly to the CYP haem moiety. After formation, the complex between the CYP and the metabolite intermediate is quite stable. The insecticide piperonyl butoxide is a classic example of this (Hasler et al., 1999, Murray and Reidy, 1990). The most significant class of drugs that form metabolite-intermediates is the alkylamines (Hasler et al., 1999).

The major distinction between autocatalytic inactivation and complexation of metabolic intermediate, is that in the latter case the haemeprotein is rendered catalytically inert, but not destroyed. As the CYP must be reactivated or re-synthesized to restore activity, irreversible inhibition is said to be long lasting (Pelkonen et al., 2008, Halpert, 1995, Kent et al., 2001).

4.8.2 Reversible Inhibition

Reversible inhibitors (or direct inhibitors) differ from irreversible inhibitors in that they act fast and do not permanently destroy the enzyme (Lin and Lu, 1998, Hollenberg, 2002, Pelkonen et al., 2008).
They bind to the active site with varying affinities and compete with substrates (Pelkonen et al., 2008). This includes agents that bind to the hydrophobic regions of the active site, or coordinate to the haeme iron atom or, enter into specific hydrogen bonding or ionic interactions with active-site-residues (Correia and Ortiz de Montellano, 2005, Lewis and Ito, 2010, Murray and Reidy, 1990).

Reversible inhibition can be further subdivided into the following categories:

- **Competitive inhibition**
  - competition between a substrate and the inhibitor to bind to the same position on the active site of the enzyme.

- **Non-competitive inhibition**
  - the active binding site of the substrate and inhibitor is different from each other.

- **Uncompetitive inhibition**
  - the inhibitor binds to the enzyme-substrate complex, but not to the free enzyme entity.

- **Mixed type inhibition**
  - both competitive and non-competitive inhibition is observed. This is the most frequently observed type of inhibition (Madan et al., 2002).

### 4.9 CYP1 Family

This family has been identified in 41 species and in humans contains 2 subfamilies (CYP1A and 1B) and is encoded by 4 genes (Cytochrome P450 Knowledgebase, 2006, Nelson, 2009). The human CYP1 family consists of three enzymes, CYP1A1, CYP1A2 and CYP1B1, although all three enzymes are not expressed in all tissues.
All mammalian species possess two inducible CYP1A enzymes, CYP1A1 and CYP1A2, which share approximately 70% amino acid sequence identity. CYP1A1 is readily detectable in the lung (Shimada et al., 1992), intestine and skin (Shimada et al., 1996), lymphocytes (Vanden Heuvel et al., 1993) and placenta (Fujino et al., 1984), particularly from cigarette smokers. It is also expressed in foetal liver (Kitada et al., 1991) but not adult liver. It can be induced in primary human hepatocyte cultures (Liu et al., 2006).

4.9.1  CYP1A2

CYP1A2 accounts for approximately 5-10% of drug metabolism, (Figure 4-4), and 10-15% of total CYP content, with individual levels varying approximately 40 fold (Guengerich, 2005). Variation in the elimination of drugs metabolised by CYP1A2 has been attributed to both genetic and environmental factors.

4.9.1.1 Sites of Expression

CYP1A2 is almost exclusively expressed at low levels in the liver. CYP1A2 is not expressed in extra-hepatic tissues; therefore, in humans, CYP1A2 and CYP1A1 can be considered hepatic and extra-hepatic enzymes, respectively.

4.9.1.2 Regulation and Polymorphism

The gene for CYP1A2 is located on chromosome 15 at location 15q24.1 and 36 allelic variants are known (Figure 4-5). CYP1A2 is induced through AhR-mediated transactivation following ligand binding and nuclear translocation, and is induced by PAHs and TCDD (Nebert et al., 2000). Consumption of charcoal-broiled meat for 7 days can induce CYP1A2 (Fontana et al., 1999).
In human liver microsomes, a large (10- to 80-fold) inter-individual variability in the activity of CYP1A2 has been observed using various probes (Gunes and Dahl, 2008). These findings may reflect a genetically determined difference in constitutive and/or inducible CYP1A2 gene expression. Of the polymorphic alleles showing variability in the promoter region, only CYP1A2*1K has been associated with altered enzyme activity, with 40% lower inducibility (Aklillu et al., 2003).

4.9.1.3 Substrates and Reactions

Model substrates and their reactions include caffeine (caffeine N3-demethylation), theophylline (theophylline N-demethylation) and phenacetin (phenacetin O-deethylation). The CYP1 family is important in the catalysis of carcinogen bioactivation reactions, such as PAH epoxidation and aromatic/heterocyclic amine N-hydroxylation (Nebert et al., 2004). Importantly, all members of the CYP1 family are active in the metabolism of PAHs to intermediates that can bind to DNA, if the damage is unrepaired, this may produce mutations involved in neoplastic transformation (Baird et al., 2005).

4.9.1.4 Inhibitors

Several CYP1A2 inhibitors are known from clinical work, including furafylline (Ortiz de Montellano et al., 1979, Kunze and Trager, 1993) and isoniazid (Wen et al., 2002). α-Naphthoflavone is a strong inhibitor as are several of the polycyclic acetylenes (Shimada et al., 1998).
4.9.1.5 Clinical Issues

Drug interactions involving CYP1A2 have been reported. High levels of CYP1A2 activity have been associated with failure of theophylline therapy in the treatment of asthma (Kappas et al., 1978). Clinical studies have shown that smoking can increase the clearance and reduce the plasma concentrations of many therapeutic drugs including, theophylline, melatonin, clozapine, riluzole, lidocaine, verapamil, erlotinib, fluvoxamine, and ropivacaine (Zhou et al., 2010).

Since CYP1A2 (and CYP1A1) catalyses the metabolic activation of PAHs and heterocyclic aromatic amines/amides to ultimate carcinogens, it is expected that induction of the enzyme is detrimental in humans exposed to high levels of PAHs and heterocyclic aromatic amines/amides such as by cigarette smoking.

4.10 CYP2 Family

This family has been identified in 41 species and in humans contains 13 subfamilies and is encoded by 36 genes (Cytochrome P450 Knowledgebase, 2006, Nelson, 2009). It is the largest family in terms of the numbers of isoenzymes; and is represented by five major subfamilies, CYP2A, CYP2B, CYP2C, CYP2D and CYP2E.

4.10.1 CYP2A6

CYP2A6 represents less than 1% of the total enzyme in the liver (Figure 4-4) but accounts for approximately 5% of drug metabolism. It was first purified as coumarin 7-hydroxylase and is the major enzyme responsible for the metabolism of nicotine (Yun et al., 1991, Nakajima et al., 1996).
4.10.1.1 Sites of Expression

CYP2A6 is expressed primarily in the liver, but has also been detected in the nasal mucosa, trachea, lung (Ding and Kaminsky, 2003) and oesophageal mucosa (Lechevrel et al., 1999).

4.10.1.2 Regulation and Polymorphism

The CYP2A6 gene spans a region of approximately 6kb pairs consisting of 9 exons and has been mapped to the long arm of chromosome 19 (between 19q12 and 19q13.2). CYP2A6 is subject to genetic polymorphism, with four allelic variants having been described and 1% of the population having an inactive variant (Brockmöller et al., 2000). Polymorphisms are the major factor contributing to the inter-individual differences in activity and expression, but dietary or environmental factors as well as endogenous factors such as steroid hormones are also involved. Studies have suggested that this variation in activity affects smoking behaviour and cancer susceptibility (Kamataki et al., 2005).

CYP2A6 is regulated by PXR, oestrogen receptors and the glucocorticoid receptors (Onica et al., 2008). Induction is known to occur with phenobarbitone. CYP2A6 is responsible for approximately 80% of hepatic metabolism of nicotine (Benowitz et al., 1994) and as smokers need to maintain levels of nicotine, increased activity of CYP2A6 has been linked to an increase in smoking. Tobacco is a source of cadmium, which is an inducer of CYP2A6 and therefore may be one of the mechanisms that increase smoking behaviour (Satarug et al., 2004).
4.10.1.3 Substrates and Reactions

The most characteristic reaction for CYP2A6 is coumarin 7-hydroxylation (Yun et al., 2005). In addition to nicotine metabolism, it also metabolically activates tobacco-specific nitrosamines such as 4-methylnitrosoamino-1-(3-pyridyl)-1-butane and N-nitrosonornicotine (Tiano et al., 1993).

4.10.1.4 Inhibitors

A number of potent inhibitors with variable selectivity against CYP2A6 have been characterised. The most widely used in vitro inhibitors include tranylcypromine and methoxsalen (8-methoxypsoralen) (Pelkonen et al., 2008).

4.10.1.5 Clinical Issues

Clinical issues are often due to polymorphic variation. This variation alters the metabolism of substrates including therapeutic agents (halothane, valproic acid), toxins (nicotine, cotinine) and pro-carcinogens (Raunio et al., 2001).

4.10.2 CYP2B6

Until specific probes were developed, CYP2B6 was thought to be a minor CYP, but expression levels with 25-250 fold differences are now known (Aleksa et al., 2005, Hesse et al., 2000, Wang and Tompkins, 2008). CYP2B6 accounts for 2-10% of the total P450 content in human liver (Figure 4-4) (Wang and Tompkins, 2008, Shimada et al., 2004, Stresser and Kupfer, 1999) contributes to approximately 2% of drug metabolism but values of 8-10% have also been quoted (Turpeinen, 2006). Importantly 25-30% of CYP3A4 substrates are also substrates for CYP2B6 (Walsky et al., 2006, Kharasch et al., 2008).
4.10.2.1 Sites of Expression

CYP2B6 is expressed in the liver, though it differs from other CYPs in that it is located diffusely in the hepatic lobules and not in the centrilobular region (Murray and Reidy, 1990).

CYP2B6 is also expressed in extra-hepatic sites including the brain, lung, kidney, intestine, endometrium, bronchoalveolar macrophages, peripheral lymphocytes and skin (Gervot et al., 1999, Janmohamed et al., 2001, Ding and Kaminsky, 2003).

4.10.2.2 Regulation and Polymorphism

Hepatic CYP2B genes represent the most inducible CYP isoforms by phenobarbital-type compounds in mammalian species (Wang and Tompkins, 2008). The regulation of CYP2B6 transcription is thought to be one of the major underlying reasons for the large inter-individual variability seen.

CYP2B6 is highly polymorphic with over 40 alleles identified (Figure 4-5), with various phenotypes. CYP2B6*6 is a clinically significant variant associated the slow metabolism of methadone, leading to increased concentrations of the drug and subsequent fatalities (Bunten et al., 2010).

4.10.2.3 Substrates and Reactions

CYP2B6 acts as an alternate pathway for 25% of CYP3A4 substrates. It is the predominant isoenzyme for the metabolism of several clinically important drugs including bupropion, artemisinin, propofol, methadone and pethidine (Hesse et al., 2000, Faucette et al., 2000, Wang and Tompkins, 2008).
It has also been shown that CYP2B6 is able to metabolise drugs via different reaction pathways to other CYPs. The antiepileptic agent mepobarbital is metabolised by the CYP2C’s to the 4-hydroxylated metabolite but CYP2B6 catalyses the N-demethylation of this compound (Kobayashi et al., 1999, Kobayashi et al., 2001).

**4.10.2.4 Inhibitors**

Several environmental chemicals are known to be potent inhibitors of CYP2B6, including the organophosphate chlorpyrifos (Abass et al., 2009). Therapeutic agents including the anti-platelet agents clopidogrel and ticlopidine are very potent inhibitors with IC$_{50}$ values of 0.5 and 0.2 μM, respectively (Walsky et al., 2006).

**4.10.2.5 Clinical Issues**

As CYP2B6 is the major isoenzyme for the metabolism of several drugs with a narrow therapeutic index, the antiretroviral efavirenz for example, clinical issues can be life threatening. Elevated plasma levels of efavirenz can lead to CNS side effects and therapeutic failure (Rakhmanina and van den Anker, 2010).

The anticancer agent cyclophosphamide requires activation by CYP2B6, increased activity increases the therapeutic effectiveness of this drug and induction of CYP2B6 may be used to enhance the effectiveness (Jounaidi and Waxman, 2004). Conversely it is logical to assume that inhibition or polymorphic variation causing decreased CYP2B6 activity would decrease therapeutic effectiveness.
4.10.3 CYP2C8

CYP2C8 makes up approximately 7% of total microsomal human hepatic CYP (Figure 4-4) (Shimada et al., 1994, Rendic, 2002, Lai et al., 2009), and is responsible for approximately 1% of drug metabolism though values as high as 5% have been quoted (Totah and Rettie, 2005). Like all members of the CYP2C family, the gene for CYP2C8 is located on chromosome 10.

4.10.3.1 Sites of Expression

Expression of CYP2C8 is one of the highest in the kidney but it is expressed at low levels in the liver (Guengerich, 2005) Other extra-hepatic sites of CYP2C8 includes the adrenal glands, brain, uterus, lung, mammary gland and ovaries (Klose et al., 1999, Guengerich, 2005).

4.10.3.2 Regulation and Polymorphism

The levels of CYP2C8 can vary 20 fold in human hepatic tissue (Bahadur et al., 2002), and is induced by rifampicin (Rae et al., 2001). To date 14 different alleles have been identified (Figure 4-5). The most significant allelic variant is CYP2C8*3 which occurs with high frequency and can account for a decrease in taxol metabolism. The level of decrease varies from 90% (Dai et al., 2001) to 25% (Soyama et al., 2001, Bahadur et al., 2002). CYP2C8*5 to CYP2C8*14 have only been detected in the Japanese population (Jiang et al., 2011).

4.10.3.3 Substrates and Reactions

The active site of CYP2C8 is a large site that can accommodate large substrates (Schoch et al., 2004). Therapeutic drugs metabolised by CYP2C8 include the...
anticancer drugs taxol and paclitaxel (Rahman et al., 1994, Sonnichsen et al., 1995), amiodarone (an antiarrhythmic drug) (Ohyama et al., 2000), troglitazone (an anti-diabetes drug) (Yamazaki et al., 1999), and carbamazepine (an antiepileptic drug) (Kerr et al., 1994). Additionally, CYP2C8 generally has low catalytic activity towards the known substrates for CYP2C9 and CYP2C19 (Guengerich, 2005).

4.10.3.4 Inhibitors

Inhibition of CYP2C8 is known to occur with high concentrations of furanocoumarins, which are present in plant products and complementary products. Gemfibrozil, a lipid lowering drug has also been identified as an inhibitor, furthermore this inhibition may be the cause for the interaction between gemfibrozil and the cholesterol lowering drug cerivastatin (Wang et al., 2002).

4.10.3.5 Clinical Issues

The disposition of taxol and paclitaxel may be one of the more significant clinical issues arising with CYP2C8. A potential interaction between the antimicrobial agent trimethoprim and the glucose lowering drug repaglinide has also been identified. Trimethoprim raises the plasma concentrations of repaglinide by inhibiting its CYP2C8-mediated biotransformation, this interaction increases the risk of hypoglycaemia (Niemi et al., 2003).

4.10.4 CYP2C9

CYP2C9 accounts for approximately 20% of total hepatic CYP content and approximately 15% of drug metabolism (Figure 4-4).
4.10.4.1 Sites of Expression

CYP2C9 is considered primarily a hepatic CYP and is second to CYP3A4 in levels of abundance. This isoenzyme has also been detected in the small intestine, though variability as high as 18-fold have been reported (Ding and Kaminsky, 2003).

4.10.4.2 Regulation and Polymorphism

CYP2C9 is the only member of the CYP2C family that can be detected at significant levels in untreated human hepatocytes. Expression is induced by rifampicin, dexamethasone, phenobarbital (Raucy et al., 2002, Gerbal-Chaloin et al., 2001) and phenytoin (Miners and Birkett, 1998).

CYP2C9 like other members of the CYP2C family is highly polymorphic, with over 30 alleles identified to date (Figure 4-5). It is so polymorphic that up to 40% of the Caucasian population are carriers that encode partially defective enzymes (Rettie and Jones, 2005). The CYP2C9*2 and *3 alleles (Rettie et al., 1994, Sullivan-Klose et al., 1996) cause reduced drug metabolic capacity. CYP2C9*5 also causes reduced metabolic activity, this allele has a higher frequency in the African population (Dickmann et al., 2001).

4.10.4.3 Substrates and Reactions

Many of the drugs metabolised by CYP2C9 have a narrow therapeutic index, including (S)-warfarin, tolbutamide and phenytoin (Rendic, 2002). Many of the anti-inflammatory agents including diclofenac and ibuprofen are also substrates for CYP2C9 (Rettie and Jones, 2005).
Using in vitro studies, CYP2C9 has also been shown to metabolise endogenous compounds including 5-hydroxytryptamine and linoleic acid (Fradette et al., 2004).

### 4.10.4.4 Inhibitors

As drugs with a narrow therapeutic index are metabolised by CY2C9, inhibition of this isoenzyme can cause adverse drug interactions. Many inhibitors are acidic, for example arylacetic acid. But neutral compounds may also bind with a high affinity (Miners and Birkett, 1998, Locuson et al., 2004). Sulfaphenazole is recognised as a highly selective competitive inhibitor of CYP2C9 (Veronese et al., 1990) with poor affinity for the other members of the CYP2C family (Ha-Duong et al., 2001). Tienilic acid is a mechanism-based inactivator (Beaune et al., 1987).

### 4.10.4.5 Clinical Issues

Clinical issues arising from CYP2C9 are common due to the high amount of allelic variance with this isoenzyme and the many substrates with a narrow therapeutic index that it is responsible for the metabolism of. Several NSAIDS are metabolised by CYP2C9, and variant alleles are more frequent in patients with NSAID-induced acute gastric bleedings (Martínez et al., 2004).

As the (S)-enantiomer of warfarin is metabolised by CYP2C9 and this enantiomer is predominantly responsible for the anticoagulant effect of the drug, excess bleeding is of concern. In particular patients with CYP2C9*2 and *3 alleles have a significant warfarin reduction clearance and are more susceptible to bleeding (Kirchheiner and Brockmöller, 2005).
4.10.5 CYP2C19

CYP2C19 accounts for approximately 5% of the total hepatic content, and this isoenzyme accounts for approximately 3-5% of drug metabolism (Figure 4-4).

4.10.5.1 Sites of Expression

Significant expression of CYP2C19 has been found in the liver (Guengerich, 2005) but low levels have also been detected in the small intestine, with variation up to 17-fold known (Ding and Kaminsky, 2003).

4.10.5.2 Regulation and Polymorphism

CYP2C19 is induced by several drugs including rifampicin, dexamethasone and phenobarbital (Raucy et al., 2002). To date more than 30 allelic variants have been described (Figure 4-5) several of which are clinically important. CYP2C19*2 and *3 (De Morais et al., 1994), have a reduced drug metabolic capacity for therapeutics such as mephenytoin and omeprazole. An increase in enzyme activity is seen with CYP2C19*17 (Sim et al., 2006).

Attention on CYP2C19 polymorphisms has increased due to the pro-drug clopidogrel which requires activation by CYP2C19 (Kazui et al., 2010). Patients with the CYP2C19*17 allele have an improved protective effect of clopidogrel treatment (Tiroch et al., 2010), but this also increases the risk of bleeding (Sibbing et al., 2010). Decreased effectiveness of clopidogrel is seen in patients with the CYP2C19*2 and *3 alleles (Sibbing et al., 2010, Mega et al., 2010).

CYP2C19 is absent in approximately 5% of the Caucasian population and 20% of the Asian population (Wrighton and Stevens, 1992).
4.10.5.3 Substrates and Reactions

Drugs metabolised by CYP2C19 are usually amides or weak bases (Lewis, 2004). The classic reaction is hydroxylation of the S-isomer of mephenytoin and omeprazole 5-hydroxylation, the later commonly employed as a probe substrate. CYP2C19 is responsible for the metabolism of many drugs including diazepam, amitriptyline, phenytoin and omeprazole (Pelkonen et al., 2008). CYP2C19 also oxidizes steroids, including progesterone 21-hydroxylation and testosterone 17-oxidation (Yamazaki and Shimada, 1997), additionally it inactivates the organophosphate insecticide diazinon (Kappers et al., 2001).

4.10.5.4 Inhibitors

Potent and selective inhibitors of CYP2C19 are not currently known. Omeprazole and tranylcypromine are used in the laboratory as inhibitors, but these compounds also inhibit CYP3A4 and CYP2A6, respectively (Lampen et al., 1995, Draper et al., 1997). CYP2C19 is also inhibited by isoniazid and ticlopidine resulting in clinically relevant interactions (Donahue et al., 1997, Tateishi et al., 1999, Salminen et al., 2011, Venkatakrishnan and Obach, 2007).

4.10.5.5 Clinical Issues

Most clinical issues arise due to the polymorphic variation. In addition to clopidogrel (section 4.10.5.2), omeprazole a known substrate for CYP2C19 is more effective for the treatment of ulcers in individuals with low enzyme activity (Chiba et al., 1993, Karam et al., 1996).
4.10.6 CYP2D6

CYP2D6 accounts for approximately 4% of the total CYP in liver (Zuber et al., 2002) (Figure 4-4), and for approximately 15% of drug metabolism, though figures as high as 30% have been reported (Zuber et al., 2002, Evans and Relling, 1999). The CYP2D locus is located at chromosome 22q13.1 (Kimura et al., 1989).

4.10.6.1 Sites of Expression

CYP2D6 is mainly expressed in the liver (Correia and Ortiz de Montellano, 2005), and in several extra-hepatic sites including the brain (with higher levels in alcoholics) (Zanger et al., 2004), and then long (low levels) (Lo Guidice et al., 1997). Levels of CYP2D6 are low in the gastrointestinal tract, but are known to vary at this site as much as they do in hepatic tissue (Madani et al., 1999).

4.10.6.2 Regulation and Polymorphism

To date no inducers have been identified though more than 85 allelic variants of CYP2D6 have been classified (Figure 4-5), though not all of these have a clinical impact. The CYP2D6 protein and enzymatic activity is completely absent in less than 1% of Asian people and in up to 10% of Caucasians. This absence in activity is due to two null alleles, which do not encode a functional P450 protein product (Zanger et al., 2004). The most frequent polymorphic variant in Caucasians is CYP2D6*4 which occurs with a frequency of 20-25% (Zanger et al., 2004).

CYP2D6*9 is characterized as an allele associated with decreased enzyme activity (Broly and Meyer, 1993). Increased enzyme activity is seen in patients with multiple copies of functional CYP2D6 (Zanger et al., 2004).
The most significant variants of CYP2D6 are CYP2D6*2xn, CYP2D6*4, CYP2D6*5, CYP2D6*10, CYP2D6*17 (Johansson and Ingelman-Sundberg, 2010).

4.10.6.3 Substrates and Reactions

Substrates for CYP2D6 have a common characteristic, a basic nitrogen at a distance of 5-7 Å from the site of oxidation (Wolff et al., 1985). CYP2D6 contributes to the metabolism of dextromethorphan, debrisoquine, and bufuralol, which all have been used as model substrates (Pelkonen et al., 2008).

CYP2D6 metabolises a number of drugs that mainly target the central nervous and cardiovascular systems (Michalets, 1998, Rendic, 2002, Zhou et al., 2009b), amongst which are many drugs with a narrow therapeutic index. This includes the tricyclic antidepressants, antipsychotics, opioids (codeine and tramadol) and amphetamines (Wang et al., 2009).

4.10.6.4 Inhibitors

Many of the inhibitors for CYP2D6 are also substrates for the isoenzyme. Traditionally the anti-arrhythmic drug quinidine (which is not a substrate) has been utilised as a highly selective CYP2D6 inhibitor. Paroxetine and cimetidine are both mechanism based inhibitors, with the latter also being a reversible inhibitor (Bertelsen et al., 2003, Madeira et al., 2004). Antipsychotics including chlorpromazine and haloperidol are metabolised by CYP2D6 and also competitively inhibit the enzyme (Shin et al., 1999).
4.10.6.5 Clinical Issues

For some drugs, the role of CYP2D6 is dominant and clinical manifestations of genetic polymorphisms are important and even deadly (Idle et al., 1978, Köhnke et al., 2002). CYP2D6 was suspected to be involved in the development of Alzheimer’s and Parkinson’s disease but to date no clear evidence of this link has been found (Gołab-Janowska et al., 2007).

4.10.7 CYP2E1

CYP2E1 is found predominantly in the liver and accounts for approximately 4% of drug metabolism (Figure 4-4). CYP2E1 was first discovered in 1965 (Orme-Johnson and Ziegler, 1965) and the human gene was purified in 1988 (Umeno et al., 1988).

4.10.7.1 Sites of Expression

In addition to hepatic expression, CYP2E1 is also expressed in the lung (Shimada et al., 1996) and oesophagus (Huang et al., 1992). It is not present in foetal liver, but appears within hours of birth (Vieira et al., 1996).

4.10.7.2 Regulation and Polymorphism

The regulation of CYP2E1 expression depends on transcriptional, post-transcriptional, and post-translational factors. The human CYP2E1 gene has been fully sequenced and lies on chromosome 10. Several genetic variants have been described. These variants may account for the enhanced gene transcription that can lead to an increased metabolic activation of carcinogens and consequent initiation of malignancies (Hayashi et al., 1991, Uematsu et al., 1991).
CYP2E1 is unlike other CYPs in terms of regulation of protein levels and enzyme activity. Whilst the main control over CYPs is at the level of transcriptional activation, CYP2E1 activity appears to be most affected by substrate binding and stabilisation of the protein or other factors that stabilise its mRNA and decrease protein degradation (Koop and Tierney, 1990). These post-transcriptional and post-translational levels of control may explain why polymorphisms shown to modulate enzyme inducibility in vitro may not have predictable consequences in vivo.

4.10.7.3 Substrates and Reactions

CYP2E1 exhibits very broad substrate specificity, with over 80 substrates known (Ioannides, 2008). The main function of CYP2E1 is contributing to the metabolism of those xenobiotics that are metabolised primarily by other CYPs. It acts as an alternative demethylation pathway for a variety of substrates metabolised by CYP3A4. The substrates of CYP2E1 usually consist of hydrophobic and low molecular weight compounds, such as paracetamol, chlorzoxazone, benzene, ethanol, carbon tetrachloride, chloroform, vinyl chloride and anaesthetics (enflurane, halothane, methoxyflurane) (Gonzalez, 2005).

This CYP is inducible by several of it own substrates (e.g. ethanol, acetone, imidazole, benzene and isopropanol). The expression of the enzyme is also altered in response to many different conditions, including gender, circadian rhythm (Klingenberg, 1958), nutrition (e.g. starvation and obesity), metabolic and endocrine disorders (e.g. diabetes), inflammation (in response to cytokines), viral infections (e.g. hepatitis C) and hepatocellular carcinoma (Ioannides, 2008).
4.10.7.4 Inhibitors

Several substrates for CYP2E1 also act as inhibitors for the isoenzyme, in particular the low molecular weight solvents (Guengerich, 2005).

The therapeutic drug disulfiram, used in the treatment of alcohol abuse, was found to inhibit CYP2E1. Inhibition of CYP2E1 is associated with reduced hepatotoxicity from chloroform, carbon tetrachloride, acetaminophen, and NDMA in rats (Brady et al., 1991).

4.10.7.5 Clinical Issues

The major clinical issues involve the role of CYP2E1 in the oxidation of certain drugs, alcoholism, oxidative stress, and risk from cancer (Guengerich, 2005).

Levels of CYP2E1 are highest in the centrilobular zone of the liver (Ingelman-Sundberg et al., 1998) which is also the region of the liver that is destroyed by toxins such as ethanol, acetaminophen, nitrosamines and carbon tetrachloride.

Paracetamol overdose is a common cause of poisoning, being the leading cause of acute liver failure in the United States and the United Kingdom (Chun et al., 2009). Studies with CYP2E1 knockout mice indicated that CYP2E1 is a major determinant of acetaminophen toxicity, as the toxicity was considerably attenuated in null animals (Lee et al., 1996).

4.11 CYP3 Family

This family has been identified in 30 species and in humans contains 1 subfamily and is encoded by 10 genes (Cytochrome P450 Knowledgebase, 2006, Nelson, 2009). The CYP3 family is represented in the liver by only one subfamily, CYP3A.
CYP3A is the most prominent CYP enzyme in human liver and intestine and is involved in the metabolism of a large number of therapeutic agents.

4.11 CYP3A4

CYP3A4 is the most abundant CYP in humans and as can be seen in Figure 4-4, is the major CYP involved in drug metabolism, 48%. The gene is located at chromosome 7q22.1 (Inoue et al., 1992).

4.11.1 Sites of Expression

Levels of CYP3A4 are greatest in the liver, but it is also the most abundant CYP in the small intestine (Guengerich, 2005). Additionally CYP3A4 is also expressed in the lung, stomach, colon (Ding and Kaminsky, 2003, Raunio et al., 2005) and the adrenal glands (Koch et al., 2002).

4.11.2 Regulation and Polymorphism

CYP3A4 is degraded by a ubiquitin linked pathway (Murray and Correia, 2001) and regulated by PXR (see section 4.7.1.4). The extent of induction is highly variable between individuals with reports of more than 10-fold differences in the induction of CYP3A4 protein and mRNA in liver and intestine (Lin and Lu, 2001).

Polymorphic variations are known for CYP3A4 but to date none have been found to be clinically important (Johansson and Ingelman-Sundberg, 2010). CYP3A4*1B has been reported as increasing the risk of prostate cancer (Tayeb et al., 2003), though other studies have failed to find a statistically significant correlation (Sarma et al., 2008).
4.11.1.3 Substrates and Reactions

The crystal structure of CYP3A4 reveals a large active site, consistent with the broad range of substrates and endogenous substances catalysed by this enzyme (Guengerich, 2005, Rendic, 2002). To date more than 50% of the therapeutic drugs on the market are metabolised by CYP3A4 (Rendic, 2002). The active site can accommodate a large variety of molecules based on their size, from metyrapone (MW- 226 daltons) to cyclosporine (MW- 1203 daltons) (Zhou, 2008).

Importantly due to its size and flexibility, multiple small molecules are able to be present simultaneously in the active site. CYP3A4 displays a high degree of region- and stereo-selectivity in may reactions, for example the hydroxylation of testosterone (Krauser and Guengerich, 2005). One of the classic and fastest reactions catalysed by CYP3A4 is 6β-testosterone hydroxylation (Guengerich et al., 1986). Bioactivation of some procarcinogens such as aflatoxin B1 (Aoyama et al., 1990) and PAHs (Hecht, 1999) are also mediated partially via CYP3A4.

4.11.1.4 Inhibitors

Inhibition of CYP3A4 is a major issue in the pharmaceutical industry. Inhibitors of CYP3A4 vary widely in structure and can act in a competitive or mechanism-based way (Zhou, 2008, Pelkonen et al., 2008). Clinically important inhibitors include azole antifungals (ketoconazole and itraconazole) (Back and Tjia, 1991), macrolide antibiotics (clarithromycin and erythromycin) (Jones et al., 2007), antidepressants (fluoxetine) (Mayhew et al., 2000), calcium channel blockers (verapamil and diltiazem) (Yeo and Yeo, 2001, Ma et al., 2000), and steroids (gestodene and mifepristone) (Jones et al., 2007).
4.11.1.5 Clinical Issues

Clinical issues involving CYP3A4 arise due to the large number of therapeutic agents that are metabolised by this isoenzyme. High levels of enzyme activity toward a drug will reduce bioavailability and variations in levels of CYP3A4 can cause problems when the therapeutic window is narrow.

A major interaction occurs with cyclosporine, which is used to prevent organ rejection post-transplant. Cyclosporine is a substrate for CYP3A4 and will not reach therapeutic levels in patients with increased CYP3A4 activity (Kronbach et al., 1989).

As CYP3A4 is also present in the small intestine, oral administration of an inhibitor causes a direct and high level of exposure in the intestine. Therefore, even low doses of a CYP3A4 inhibitor may cause drug interactions in the intestine rather than in the liver (Tachibana et al., 2009).

4.12 Species Differences in Cytochrome P450

Differences between species are known not only in the distribution of the various CYP isoenzymes and substrate specificity, but also in total CYP content. Importantly a high degree of sequence identity does not automatically mean similar catalytic activity (Guengerich, 1997), and a single amino acid substitution may give changes in substrate specificity (Lindberg and Negishi, 1989). Therefore orthologous enzymes are not always comparable for catalytic specificity (Nelson et al., 1993). These differences complicate the extrapolation from animal studies to humans.
With regards to species extrapolation the four points below have been proposed (Guengerich, 1997); subsequent work has supported these statements (Bogaards et al., 2000, Guengerich, 1997).

1- CYP subfamilies in which species extrapolation appears to hold well:
   2E1.

2- CYP subfamilies in which some caution is required in extrapolation:
   1A1, 1A2 and 4A.

3- CYP subfamilies in which more caution is advised in extrapolation:
   2D and 3A.

4- CYP subfamilies for which major problems in extrapolation exist:
   2A, 2B and 2C.

With these differences in mind, investigations into CYP activity ideally should utilise human tissue and/or cells to avoid discrepancies with the data due to species differences.

4.13 Methods for Investigating Cytochrome P450 Inhibition

As shown in Figure 4-3, there are many steps in the CYP catalytic cycle that can be monitored to give an indication of inhibition, though inhibition of substrate metabolism is the most common method used. There are several technologies for measuring the inhibition of substrate metabolism and the generation of metabolites, which themselves may cause inhibition.
4.13.1 In Vitro Methods

In vitro systems are commonly used to study human drug metabolism to identify principal metabolites preferably prior to the human ADME studies (Brandon et al., 2003, Pelkonen and Raunio, 2005, Pelkonen et al., 2005).

Whilst this review focuses on the in vitro methods used in the investigation of CYP inhibition it is important to note that in vitro investigations may address several metabolic issues, involving CYP and the other enzymes that may be involved in metabolism including:

- Drug metabolic stability and metabolic profile.
- Metabolite identification and structure clarification.
- Prediction of in vivo pharmacokinetic parameters from in vitro data.
- Identification of the enzymes involved in metabolism.
- Species comparison to aid in the selection of species for pre-clinical studies.
- Drug interactions due to enzyme induction/inhibition.
- Drug toxicity associated with metabolism.

There are many systems that can be used as part of the in vitro screening for CYP inhibition including cDNA expression systems, microsomes, S9 fraction, cell lines (including transgenic cell lines) hepatocytes, liver slices, liver perfusions; each with their own advantages and disadvantages. Several of these systems are discussed below and summarised in Table 4-2.
4.13.1.1 Expression of recombinant CYPs

Recombinant CYPs or baculovirus-mediated expression of mammalian CYPs (baculosomes or supersomes) were first produced in 1989 (Asseffa et al., 1989). In these preparations, cytochrome P450 reductase is also co-expressed, and therefore does not have to be added exogenously (Ong et al., 1998, Chen et al., 1997). Some preparations also have cytochrome b5 expressed, providing the full complement of CYP redox partner proteins.

However, exogenous NADPH or an NADPH regenerating system (e.g., glucose-6-phosphate dehydrogenase conversion of NADP+ to NADPH in the presence of the substrate glucose-6-phosphate) must be added if it is not present in the preparation (Taavitsainen et al., 2000).

A major limitation inherent to recombinant models are that concentrations of CYPs are far in excess of their relative amount in the human liver and that secondary metabolism cannot be identified. Additionally only a single isoenzyme is expressed, therefore the contribution of other involved enzymes (including other CYP isoenzymes) is not represented.

The metabolic activities of expressed microsomal CYPs and human liver microsomes using probe substrates have been determined to be a reasonably accurate model of the in vivo situation with respect to $K_m$ values, although considerable variability was observed with respect to $V_{max}$ values (McGinnity et al., 1999). $IC_{50}$ values of reference inhibitors are also comparable between baculosomes and microsomes (Turpeinen et al., 2006).
Pharmacogenetic variability may contribute greatly to individual variety in drug metabolism, this genetic variation can be investigated with baculosomes that are commercially available (i.e. CYP2C9*1, CYP2C9*2, CYP2D6*1 and CYP2D6*2).

### 4.13.1.2 S9 Fraction

The S9 fraction is obtained as the supernatant from the centrifugation of the liver homogenate at 9000g, for 20-30 minutes, and contains both microsomal and cytosolic fractions. Compared to microsomes, human liver S9 fractions offer a more complete representation of the in vivo situation, as they contain a wide variety of oxidative (also known as Phase I) and conjugative (also known as Phase II) enzymes including CYPs, flavin monooxygenases (FMOs), carboxylesterases, epoxide hydrolase, UDP glucuronosyltransferases (UGTs), sulfotransferases, methyltransferases, acetyltransferases, glutathione- S -transferases and other drug-metabolizing enzymes (Brandon et al., 2003).

Although this in vitro system is more complete in terms of the number of biotransformation enzymes present, some metabolites may remain unnoticed due to reduced enzyme activity. Compared to either microsome or supersome preparations, S9 fractions exhibit 20–25% lower enzyme activity (Kramer and Tracy, 2008).

### 4.13.1.3 Liver Microsomes

Human liver microsomes are the most popular in vitro model for CYP kinetic based studies. Microsomes consist of vesicles of the hepatocyte endoplasmic reticulum and are prepared by differential centrifugation (Pelkonen et al., 1974).
Microsomal preparations contain only enzymes localised to the endoplasmic reticulum, which include CYPs, FMOs and UGTs.

As with other preparations from human tissue the activity of microsomes can vary substantially between individuals (Gómez-Lechón et al., 2004). This problem, however, can be successfully solved by the application of pooled (from multiple donors) microsomes, which results in a representative enzyme activity (Araya and Wikvall, 1999). Besides the relatively cheap costs and the ease of use, the major advantage of microsomes is that they allow the influence of specific isoenzymes to be studied in the presence of specific inhibitors (Birkett et al., 1993).

The major disadvantage of microsomes is that they cannot be used for quantitative estimations of in vivo human biotransformation, because CYPs and UGTs are enriched in the microsomal fraction and there is no competition with other enzymes.

This results in higher biotransformation rates in microsomes compared to the human in vivo situation, but also compared to primary hepatocytes and liver slices (Sidelmann et al., 1996). Additionally, the absence of other enzymes (e.g., NAT, GST, and ST) and cytosolic cofactors can leave metabolites formed in intact liver cells unnoticed (Crommentuyn et al., 1998).

4.13.1.4 Primary Hepatocytes

Hepatocytes can be held in suspension, in which case they remain viable for only a few hours, or maintained in monolayer culture for a maximum of 4 weeks.
Cryopreserved hepatocytes have been shown to retain the activity of most phase I and phase II enzymes (Annaert et al., 2001, Silva et al., 1999).

Human hepatocytes in culture show active levels of major CYPs that are involved in drug metabolism (Rodríguez-Antona et al., 2002, Donato et al., 1999), though differences amongst preparations are found due to different donors (Donato et al., 1995, Gómez-Lechón et al., 2007, LeCluyse, 2001). As mentioned in section 4.5 there are many factors that can alter the expression and activity of the CYPs but additional changes occur in cultures of primary hepatocytes due to the microenvironment that the cells are cultured in. These limitations can be quantified but not overcome by characterising the cells.

The expression of CYPs declines during culture and cells differentiate and become more similar to hepatoma cell lines. There is an initial rapid loss in mRNA (20-40%) at 4-6 hours after culturing, this loss in mRNA precedes the decrease in CYP protein and activity (Gómez-Lechón et al., 2004, Rodríguez-Antona et al., 2002). Importantly not all CYPs degrade at the same rate due to stability of the individual enzymes (Gómez-Lechón et al., 2007). For some CYPs this loss becomes evident after a few days (CYP2E1 and CYP3A4), whilst others remain unaffected (CYP1A2 and CYP2C9) (George et al., 1997).

A comparison of cultured hepatocytes and microsomes prepared from the same donor showed good correlation, although the activity in microsomes was higher (Gómez-Lechón et al., 2004). A disadvantage in using primary hepatocytes is the lack of liver non-hepatocyte cells.
Although hepatocytes account for the vast majority of the liver volume (about 80%), other cells such as Kupffer cells are necessary for cofactor supply (Brandon et al., 2003).

4.13.1.5 Immortalized Cell Lines

Different human and animal cell lines are available (http://www.lgcpromochem-atcc.com). Because of interspecies differences, animal cell lines cannot accurately predict human biotransformation (section 4.12). Human liver cell lines can be isolated from primary tumours of the liver parenchyma, developed after chronic hepatitis or cirrhosis (Crommelin and Sindelar, 1997). The most common human cell lines for biotransformation studies are HepG2 (hepatocellular carcinoma) (Galijatovic et al., 1999, Urani et al., 1998, Walle et al., 2000, Yoshitomi et al., 2001), BC2 (hepatoma) (Gómez-Lechón et al., 2001) and the lung-derived line A549 (Hukkanen et al., 2000).

Recently a new human hepatoma cell line, HepaRG, was derived from a hepatocellular carcinoma. The HepaRG cells express a large panel of liver-specific genes including several CYP enzymes such as CYP1A2, CYP2B6, CYP2C9, CYP2E1 and CYP3A4, which is in contrast to the other hepatoma cell lines such as HepG2. In addition to CYP enzymes, HepaRG cells have a stable expression of phase II enzymes, transporters and nuclear transcription factors over a time period of six weeks in culture (Aninat et al., 2006, Anthérieu et al., 2010, Kanebratt and Andersson, 2008). The major disadvantage of these cell lines is that they are derived from a single donor and therefore are very limited in the genetic variability.
4.13.1.6 Liver Slices

Liver slices that are prepared as ultrathin sections provide the advantage of intact cellular interactions and normal spatial arrangements. They are a multicellular three-dimensional in vitro model. Potassium, protein and ATP-levels are well maintained for 8-24 hours (de Kanter et al., 2002, Vandenbranden et al., 1998), indicating the length of time that liver slices may be viable for.

As with hepatocytes, CYP activity decreases by approximately 20% in 4 hours and by 65% at 24 hours with no detectable activity after 96 hours (Vandenbranden et al., 1998). This limitation is the biggest disadvantage of using liver slices. Advances in cryopreservation may prolong the life of the slices enabling them to be used more extensively.
Table 4-2: Comparison of human in vitro enzyme sources used for inhibition studies with cytochrome P450- modified from (Pelkonen and Turpeinen, 2007).

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinant CYP Enzymes</td>
<td>Can be used in a high-throughput screen.</td>
<td>Single enzyme studies in isolation.</td>
</tr>
<tr>
<td></td>
<td>The role of individual CYPS can be studied.</td>
<td>Metabolites need to be tested separately to rule out the reaction with a second CYP (Parkinson et al., 2010).</td>
</tr>
<tr>
<td></td>
<td>Data can be normalized (Rodrigues, 1999).</td>
<td>Loss of architecture.</td>
</tr>
<tr>
<td></td>
<td>Able to generate useable amounts of metabolic products (Friedberg et al., 1999, Moody et al., 1999).</td>
<td>Requires co-factor addition.</td>
</tr>
<tr>
<td></td>
<td>Useful for when the enzyme being investigated is not present in high levels.</td>
<td>Contains only phase I enzymes and UGT.</td>
</tr>
<tr>
<td></td>
<td>Polymorphic variants can be studied.</td>
<td>UGT levels decline over time (Ogilvie et al., 2008).</td>
</tr>
<tr>
<td></td>
<td>IC_{50} values are higher than those obtained with microsomes (Ogilvie et al., 2008).</td>
<td>Requires co-factor addition.</td>
</tr>
<tr>
<td>S9 fraction</td>
<td>Contains basically all hepatic enzymes.</td>
<td>Substrates utilized must be specific or antibodies are required.</td>
</tr>
<tr>
<td></td>
<td>Can be easily prepared, pooled from multiple donors and stored.</td>
<td></td>
</tr>
<tr>
<td>Microsomes</td>
<td>Can be easily prepared, pooled from multiple donors and stored.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Contain levels of cytochrome b5 and cytochrome P450-reductase similar to in vivo (Foti et al., 2010).</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Allows for the investigation of intermediates and metabolites.</td>
<td></td>
</tr>
<tr>
<td>Hepatocytes</td>
<td>Cellular architecture maintained.</td>
<td>Difficult to pool.</td>
</tr>
<tr>
<td></td>
<td>Best in vitro system to determine the generation of metabolites (Dalvie et al., 2009).</td>
<td>Enzyme levels decrease over the time of culture.</td>
</tr>
<tr>
<td></td>
<td>Can detect metabolism by non-CYP enzymes (Parkinson et al., 2010).</td>
<td></td>
</tr>
<tr>
<td>Immortalized cell lines</td>
<td>Cells express a large panel of liver-specific genes including several phase I, phase II and transporters.</td>
<td>Derived from single source so polymorphic variation is not present.</td>
</tr>
<tr>
<td>Liver Slices</td>
<td>Contains the whole complement of drug metabolising enzymes.</td>
<td>Limited viability.</td>
</tr>
<tr>
<td></td>
<td>Cell-to-cell architecture maintained.</td>
<td>Requires healthy donors.</td>
</tr>
<tr>
<td></td>
<td>Allows for the study of induction.</td>
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</tbody>
</table>
4.13.2 Humanized In Vivo Modelling

Transgenic mice with human CYPs have been developed and can be used for CYP investigations, though these mice usually express only one humanised isoenzyme, for example CYP2D6 or CYP3A4 (Corchero et al., 2001, Robertson et al., 2003, Zhang et al., 2003).

Chimeric mice with humanised livers can be used to predict the human in vivo interaction of a chemical with the CYPs and the metabolites generated. The first reports of substantial repopulation of a mouse liver with human hepatocytes were published in 2001 (Dandri et al., 2001, Mercer et al., 2001). Investigations with these chimeric models have identified expression of human-specific mRNA for CYP1A1, CYP1A2, CYP2C9, CYP2D6 and CYP3A4 (Katoh et al., 2004).

In vivo studies with probe substrates (debrisoquine and warfarin) have shown significantly higher concentrations of their human metabolites in chimeric mice than in control mice, though this finding was not consistent for all investigated substrates (amosulalol was lower than expected) (Kamimura et al., 2010). Chimeric mice are also of interest as they express Phase II enzymes and the transporters (Strom et al., 2010) This would allow for a more detailed metabolic profile and therefore more realistic extrapolation to human in vivo studies.
4.13.3 In Silico Modelling

In silico modelling is a rapidly growing area in all aspects of ADME predictions. These approaches can be classified as ligand-based (pharmacophore and quantitative structure-activity relationship (QSAR)), protein-based (crystallographic protein and homology models), and ligand-protein interactions models (ligand-protein docking) (Sun and Scott, 2010, de Graaf et al., 2005).

In silico modelling systems are capable of predicting potential sites of metabolism in drug molecules and provide metabolic trees and estimate the likelihood of metabolite production, and potential for inhibition (Turpeinen, 2006, Pelkonen and Raunio, 2005).

Whilst these techniques are cost efficient, rapid and allow for the exploration of minor structural changes, they rely on knowledge of the chemical/xenobiotic being investigated. In the case of complementary products, the composition is often unknown and complex. In silico modelling may have a role in predicting the fate of specific constituents of complementary products when more information is available, but at this stage in silico modelling can not provide predictions on the metabolic outcomes and kinetic behaviour of these products.

4.14 In Vitro – In Vivo Extrapolation for CYP Inhibition

In vitro studies are often used as the basis for extrapolation for in vivo predictions, known as in vitro to in vivo extrapolation (IVIVE). IVIVE focuses on two major areas, the prediction of metabolic clearance and the prediction of drug interactions.
Whilst there are many factors that are involved in IVIVE this section will focus on those that are relevant to CYP inhibition.

In vitro systems utilised for CYP studies each have their own advantages and limitations (Table 4-2) with regards to extrapolation of data to the in vivo situation. In vitro studies are used to determine the metabolism profile of a drug including the metabolite profile and the time for the drug to be metabolised. From this the metabolic fate in vivo is predicted.

The extrapolation of in vitro data to in vivo requires scaling. This scaling consists of several factors, including the intrinsic clearance and the prediction of hepatic clearance, which is then used to calculate the oral bioavailability, total body clearance and half-life (Pelkonen and Turpeinen, 2007). Each of the in vitro systems used require different scaling factors. This requires the assumption of factors including protein binding, distribution to other tissues, hepatic blood flow and extra-hepatic routes of clearance.

For investigations using hepatic microsomes, values are usually reported as millilitres per minute per milligram of microsomal protein and scaling further to a rate per gram of liver requires a factor known as milligrams of microsomal protein per gram of liver (MPPGL). A value of 32 mg g-1 (Barter et al., 2007a, Pelkonen and Turpeinen, 2007) and 40 mg g-1 (Hakooz et al., 2006) for human liver MPPGL has been proposed.
4.15 **Known Inhibitions by Complementary Products**

As CYP inhibition is a major cause of adverse drug reactions, it is important to investigate if complementary products are able to cause inhibition. Studies investigating complementary products interacting with CYPs are often conflicting though on further investigation the source of this conflict can usually be found. Most commonly the products used vary (manufacturer, formulation, composition) and/or the concentration and preparation of extracts for in vitro tests differs.

As complementary products are often complex they contain many potentially pharmacologically active components including essential oils, tannins, flavonoids, anthraquinones, alkaloids and polyphenols (Liu et al., 2006, Zhou et al., 2004, Zhou et al., 2005, Izzo and Ernst, 2009). Several complementary products have been proven to interact with CYPs, St John’s Wort (*Hypericum perforatum*), garlic (*Allium sativum*), liquorice (*Glycyrrhiza glabra*) and ginseng (*Panax ginseng*), though not all of these are inhibitors of the CYPs. St John’s Wort is known to induce CYP3A4 (Henderson et al., 2002)

Furanocoumarins isolated from grapefruit juice are mechanism-based inhibitors of CYP3A4 (Tassaneeyakul et al., 2000) and resveratrol has been shown to be mechanism-based inhibitor of CYP1A2 and CYP3A4 (Chan and Delucchi, 2000). Analysis of 30 herbal plants from Indonesia demonstrated their ability to inhibit CYP2D6 and CYP3A4 (Subehan et al., 2006). With an increase in the incubation time, five plants showed more than a 30% increase in CYP3A4 mechanism-based inhibition, and three showed a 30% increase in CYP2D6 mechanism-based inhibition.
CYP2E1 inhibitors from natural sources include compounds found in garlic (diallyl sulfide, diallyl sulfoxide, and diallyl sulfone) that were shown to competitively inhibit CYP2E1 (Gurley et al., 2005a, Brady et al., 1991). Two clinical trials implied that garlic oil may selectively inhibit CYP2E1, as revealed by the decreased 6-hydroxyclohexoxazone/chlorhexoxazone serum ratios (Gurley et al., 2005a). Ginseng has been shown to inhibit CYP2D6 (Gurley et al., 2008).

With evidence that complementary products are capable of causing CYP inhibition there is a need to identify those products that are most likely to cause inhibition and therefore be responsible for drug interactions. A rapid screening method is required to investigate the large number of products and the variability of the products, for this reason and the advantages outline in Table 4-2, microsomes are the ideal testing system.

4.16 Aims of the Present Study

The general aims of this study was to investigate 10 complementary and herbal products that have the potential to interact with therapeutic agents in relation to their ability to inhibit the CYPs involved in drug metabolism. The specific goals include:

- Establishing a standard extraction method that is easily reproducible.
- Comparison of two methods for screening for potential CYP inhibition.
- To determine if the more rapid N-in-one cocktail method can provide accurate results with complex mixtures such as extracts of complementary products.
4.17 Materials and Methods

4.17.1 Chemicals

Bupropion and hydroxybupropion were a generous donation from GlaxoSmithKline (Research Triangle, NC, USA), midazolam and 1-hydroxymidazolam were obtained from F. Hoffmann-La Roche (Basle, Switzerland), and omeprazole, omeprazole sulphone and 5-hydroxyomeprazole were obtained from Astra Zeneca (Mölndal, Sweden). The metabolite standards 6-hydroxychlorzoxazone, desethyl amodiaquine, hydroxytolbutamide, 6β-hydroxytestosterone and dextrorphan were purchased from BD Biosciences Discovery Labware (Bedford, MA, USA). Formic acid and LichroSol GG acetonitrile were obtained from Merck KGaA (Darmstadt, Germany). Itraconazole, ticlopidine, montelukast, amodiaquine, tolbutamide, sulphaphenzole, dextromethorphan, quinidine, chlorzoxazone, pyridine and fluconazole were all purchased from Sigma Chemical Co. (St Louis, MO, USA). Water was in-house freshly prepared with Simplicity 185 (Millipore S.A., Molsheim, France) water purification system and was ultra pure grade (18.2MΩ). All other chemicals were from Sigma Chemical Co. (St Louis, MO, USA) and were of the highest purity available.

4.17.2 Preparation of Herbal Samples

Herbal products were purchased from local suppliers in Victoria, Australia and were all of commercial quality Table 4-3. The methanolic and aqueous extraction method was modified from that previously described (Unger and Frank, 2004). Tablets were crushed and the contents of capsules emptied, extractions were either water or 80% methanol to allow for the aqueous and distinctly lipophilic components to be separated.
Solvent volumes were adjusted so that standardised extracts at 100 mg of product/ml were obtained. This was based on the manufacturers’ stated concentration of the active or principle component.

Extracts were agitated in a shaking water bath at 37°C for 30 minutes then centrifuged at 2500 g for 10 minutes. Standard extracts were diluted to final nominal concentrations of 20, 100 and 500 µg/ml. Methanol was evaporated to dryness under vacuum and the final volume of methanol did not exceed 1% (v:v). Extracts were kept in the dark and shelf life set at 2 weeks.

### 4.17.3 Liver Samples and Microsome Preparation

Human liver samples were obtained from the University Hospital of Oulu as surplus from cadaver kidney transplantation donors. The collection of surplus tissue was approved by the Ethics Committee of the Medical Faculty, University of Oulu, Finland. All donors were Caucasians ranging in age from 21 to 62, four were female and six were male. Detailed information is shown in Table 4-4. Samples were transferred to ice immediately after surgical excision, dissected into pieces of approximately 1cm³ in size, and snap frozen in liquid nitrogen at -80°C.

Microsomes were prepared by standard differential ultra-centrifugation as described previously (Pelkonen et al., 1974). A microsomal pool of extensively characterised microsomes was used for all incubations. Previous studies have shown sufficient model activities, no known polymorphisms, expected effects of model inhibitors and quantitation of CYPs by Western blotting.
The final pellet was suspended in 0.1M phosphate buffer pH 7.4. Protein content was determined by the method of Bradford (Bradford, 1976).
Table 4-3: Complementary products used to screen for the potential inhibition of cytochrome P450 enzymes in the single substrate and N-in-one cocktail assays.

<table>
<thead>
<tr>
<th>Product</th>
<th>Active or Principal Component</th>
<th>Concentration of active or principal component in each tablet/capsule</th>
<th>Manufacturer</th>
<th>Batch Number</th>
<th>Reported benefit/Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black Cohosh</td>
<td>Cimicifuga racemosa</td>
<td>500mg</td>
<td>Nature's Own</td>
<td>302858</td>
<td>Eases the symptoms of menopause.</td>
</tr>
<tr>
<td>Dong Quai</td>
<td>Angelica polymorpha</td>
<td>520mg</td>
<td>Nature's Sunshine</td>
<td>00356532</td>
<td>Assist with menstrual cramps and the management of menstrual irregularity, fatigue and high blood pressure.</td>
</tr>
<tr>
<td>Goldenseal</td>
<td>Hydrastis Canadensis</td>
<td>500mg</td>
<td>Nature's Sunshine</td>
<td>01220333</td>
<td>Assist in the treatment of respiratory infections, anti-inflammatory, and as laxative.</td>
</tr>
<tr>
<td>Gotu Kola</td>
<td>Centella asiatica</td>
<td>395mg</td>
<td>Nature's Sunshine</td>
<td>01223293</td>
<td>Eases anxiety and mood disorders.</td>
</tr>
<tr>
<td>Horse Chestnut</td>
<td>Aesculus hippocastanum</td>
<td>2000mg</td>
<td>NutraLife</td>
<td>B4159</td>
<td>Treatment for blood stagnation.</td>
</tr>
<tr>
<td>Horsetail</td>
<td>Equisetum arvense</td>
<td>360mg</td>
<td>Nature's Sunshine</td>
<td>00243503</td>
<td>Used as a diuretic and in the treatment of urinary tract infections.</td>
</tr>
<tr>
<td>Liquorice Root</td>
<td>Glycyrrhiza glabra</td>
<td>425mg</td>
<td>Nature's Sunshine</td>
<td>00238614</td>
<td>Used as an expectorant anti-inflammatory agent. Primary adrenal tonic.</td>
</tr>
<tr>
<td>Milk Thistle</td>
<td>Silybum marianum</td>
<td>84mg</td>
<td>Nature's Own</td>
<td>302090</td>
<td>Assist the liver in detoxification.</td>
</tr>
<tr>
<td>Saw Palmetto</td>
<td>Serenoa serrulata</td>
<td>550mg</td>
<td>Nature's Sunshine</td>
<td>00455693</td>
<td>Treatment of prostate hyperplasia.</td>
</tr>
<tr>
<td>Valerian</td>
<td>Valeriana officinalis</td>
<td>2250mg</td>
<td>Bio-Organics</td>
<td>301338</td>
<td>Antispasmodic and muscle relaxant. Relieve nervous tension and anxiety.</td>
</tr>
</tbody>
</table>
Table 4-4: Characteristics of human liver samples used for the cytochrome P450 inhibition studies.

<table>
<thead>
<tr>
<th>Liver</th>
<th>Age</th>
<th>Sex</th>
<th>Cause of death</th>
<th>Drug history</th>
<th>Liver pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL20</td>
<td>54</td>
<td>M</td>
<td>ICH</td>
<td>Diazepam&lt;sup&gt;a&lt;/sup&gt;</td>
<td>None</td>
</tr>
<tr>
<td>HL21</td>
<td>44</td>
<td>M</td>
<td>ICH</td>
<td>Phenytoin&lt;sup&gt;a&lt;/sup&gt;, alcohol abuse</td>
<td>Cirrhotic</td>
</tr>
<tr>
<td>HL22</td>
<td>40</td>
<td>F</td>
<td>ICH</td>
<td>Dexamethasone&lt;sup&gt;a&lt;/sup&gt;, nizatidine&lt;sup&gt;a&lt;/sup&gt;, phenytoin&lt;sup&gt;a&lt;/sup&gt;</td>
<td>None</td>
</tr>
<tr>
<td>HL23</td>
<td>43</td>
<td>M</td>
<td>ICH</td>
<td>Diazepam&lt;sup&gt;a&lt;/sup&gt;, smoker</td>
<td>None</td>
</tr>
<tr>
<td>HL24</td>
<td>47</td>
<td>M</td>
<td>ICH</td>
<td>No medication, smoker</td>
<td>None</td>
</tr>
<tr>
<td>HL28</td>
<td>21</td>
<td>M</td>
<td>Stroke</td>
<td>Dexamethasone&lt;sup&gt;a&lt;/sup&gt;, smoker</td>
<td>None</td>
</tr>
<tr>
<td>HL29</td>
<td>39</td>
<td>F</td>
<td>ICH, SAH</td>
<td>Dexamethasone&lt;sup&gt;a&lt;/sup&gt;</td>
<td>None</td>
</tr>
<tr>
<td>HL30</td>
<td>53</td>
<td>F</td>
<td>ICH, SAH</td>
<td>No medication</td>
<td>Steatosis</td>
</tr>
<tr>
<td>HL31</td>
<td>44</td>
<td>F</td>
<td>ICH, SAH</td>
<td>No medication</td>
<td>Steatosis</td>
</tr>
<tr>
<td>HL32</td>
<td>62</td>
<td>M</td>
<td>ICH, SDH</td>
<td>Metformin, alcohol abuse, smoker</td>
<td>None</td>
</tr>
</tbody>
</table>

<sup>a</sup> Drugs were administered only during the 24 hours before death; M = male; F = female; ICH = intracerebral haemorrhage; SAH = subarachnoid haemorrhage; SDH = subdural haematoma
4.17.4 **CYP Inhibition Assays: Single Substrate Incubations**

The incubation and analysis conditions used in the single substrate assays are summarised in Table 4-5. The isoenzymes investigated were: CYP1A1/2 (ethoxyresorufin O-deethylation), CYP2A6 (coumarin 7-hydroxylation), CYP2B6 (bupropion hydroxylation), CYP2C8 (amodiaquine deethylation), CYP2C9 (tolbutamide hydroxylation), CYP2C19 (omeprazole 5-hydroxylation), CYP2D6 (dextromethorphan O-demethylation), CYP2E1 (chlorzoxazone 6-hydroxylation) and CYP3A4 (midazolam \( \alpha \)-hydroxylation and omeprazole sulphoxidation).

Detailed information on the methodology can be found in the original papers (Table 4-5). The probe substrates and their metabolites are shown in Figure 4-7 and Figure 4-8. Known specific inhibitors were used for each of the isoenzymes investigated, Figure 4-9. As midazolam hydroxylation and omeprazole sulphoxidation both monitor CYP3A4 inhibition, the designations of (M)3A4 and (OS)3A4 (respectively) were used.

For each assay, pooled microsomes were diluted to the required protein concentration (Table 4-5) with 0.1M phosphate buffer (pH = 7.4), substrate or inhibitor and test product were all pre-incubated for 2 minutes at 37°C. The reaction was started with the addition of \( \beta \)-nicotinamide adenine dinucleotide phosphate (NADPH) 10mM (CYP2B6, CYP2C8, CYP2C19 and CYP3A4) or cofactor mixture, containing 10mM MgCl\(_2\), 0.2M KCl, NADP (1.25 mM) glucose-6-phosphate (6 mM), and 100 units of glucose-6-phosphate dehydrogenase, (CYP1A1/2, CYP2A6, CYP2C9, CYP2D6 and CYP2E1).
Incubations were maintained at 37°C for the appropriate time (Table 4-5) in a shaking incubator block (Eppendorf Thermomixer 5436, Hamburg, Germany), and stopped with the appropriate stop reagent: methanol (CYP1A1/2, CYP2C9, CYP2E1, CYP3A4) acetonitrile (CYP2B6, CYP2C8, CYP2C19) hydrochloric acid (CYP2D6) and trichloroacetic acid (CYP2A6).

Once the reaction was stopped, incubations were kept on ice for a minimum of 5 minutes then centrifuged for 15 minutes at 10,000 g. Supernatants were collected into clean tubes and stored at -20°C until analysis. For analysis, incubations were thawed at room temperature, shaken and centrifuged for 10 minutes at 10,000 g.

HPLC analysis was with a Shimadzu VP series HPLC system with Waters Symmetry C18 column (3.9x150mm, 5μm, Waters Corp, Milford, MA) and Lichrospher 100 RP-18 guard column (4.0x4.0 mm, E. Merck, Darmstadt, Germany). Ethoxyresorufin O-deethylation (EROD) and coumarin 7-hydroxylation (ECOD) assays were read immediately following the incubations. The metabolites of ethoxyresorufin and coumarin were determined using a Hitachi F-1040 fluorescence spectrophotometer.
Table 4-5: Summary of the single substrate in vitro assay parameters for the cytochrome P450 inhibition studies.

<table>
<thead>
<tr>
<th>CYP</th>
<th>Substrate</th>
<th>Reaction</th>
<th>Inhibitor</th>
<th>[S]/Km</th>
<th>[Protein] mg</th>
<th>Cofactor System</th>
<th>Incubation time (min)</th>
<th>Assay method and Eluent</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A1/2</td>
<td>7-Ethoxyresorufin Ethoxyresorufin O-deethylation</td>
<td>2.5</td>
<td>0.2</td>
<td>NADPH regenerating</td>
<td>5</td>
<td>Fluorometric (530/585nm)</td>
<td>(Burke et al., 1977)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2A6</td>
<td>Coumarin Coumarin 7-hydroxylation</td>
<td>10</td>
<td>0.1</td>
<td>NADPH regenerating</td>
<td>10</td>
<td>Fluorometric (365/454nm)</td>
<td>(Aitio, 1978)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Raunio et al., 1988)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2B6</td>
<td>Bupropion Bupropion hydroxylation</td>
<td>Ticlopidine</td>
<td>0.6</td>
<td>0.4</td>
<td>NADPH</td>
<td>15</td>
<td>UV-HPLC (204/214nm) 75% 50 mM o-phosphoric acid-buffer: 25% acetonitrile</td>
<td>(Hesse et al., 2000)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Faucette et al., 2000)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2C8</td>
<td>Amodiaquine Amodiaquine deethylation</td>
<td>Montelukast</td>
<td>12</td>
<td>0.1</td>
<td>NADPH</td>
<td>20</td>
<td>UV-HPLC (342nm) 70% 50 mM o-phosphoric acid-buffer: 30% acetonitrile</td>
<td>(Li et al., 2002)</td>
<td></td>
</tr>
<tr>
<td>2C9</td>
<td>Tolbutamide Tolbutamide hydroxylation</td>
<td>Sulfaphenazole</td>
<td>0.5</td>
<td>0.15</td>
<td>NADPH regenerating</td>
<td>20</td>
<td>UV-HPLC (236nm) 70% 50 mM o-phosphoric acid-buffer: 30% acetonitrile</td>
<td>(Knodell et al., 1987)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Sullivan-Klose et al., 1996)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP</td>
<td>Substrate</td>
<td>Reaction</td>
<td>Inhibitor</td>
<td>[S]/Km</td>
<td>[Protein] mg</td>
<td>Cofactor System</td>
<td>Incubation time (min)</td>
<td>Assay method and Eluent</td>
<td>Reference</td>
</tr>
<tr>
<td>-----</td>
<td>-----------</td>
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<td>-------------</td>
<td>-----------------</td>
<td>----------------------</td>
<td>-----------------------</td>
<td>----------</td>
</tr>
<tr>
<td>2C19</td>
<td>Omeprazole</td>
<td>Omeprazole 5-hydroxylation</td>
<td>Fluconazole</td>
<td>2</td>
<td>0.1</td>
<td>NADPH</td>
<td>20</td>
<td>UV-HPLC (204/304nm) 25 mM α-phosphoric acid-buffer (A) and acetonitrile (B)</td>
<td>(Åbelö et al., 2000)</td>
</tr>
<tr>
<td>2D6</td>
<td>Dextromethorphan</td>
<td>Dextromethorphan O-demethylation</td>
<td>Quinidine</td>
<td>2</td>
<td>0.1</td>
<td>NADPH regenerating</td>
<td>20</td>
<td>UV-HPLC (204/280nm) 75% 50 mM α-phosphoric acid-buffer: 25% acetonitrile</td>
<td>(Park et al., 1984) (Kronbach et al., 1987)</td>
</tr>
<tr>
<td>2E1</td>
<td>Chlorzoxazone</td>
<td>Chlorzoxazone 6-hydroxylation</td>
<td>Pyridine</td>
<td>2.5</td>
<td>0.1</td>
<td>NADPH regenerating</td>
<td>20</td>
<td>UV-HPLC (282nm) 70% 50 mM α-phosphoric acid-buffer: 30% acetonitrile</td>
<td>(Peter et al., 1990)</td>
</tr>
<tr>
<td>3A4</td>
<td>Midazolam</td>
<td>Midazolam α-hydroxylation</td>
<td>Itraconazole</td>
<td>2</td>
<td>0.1</td>
<td>NADPH regenerating</td>
<td>5</td>
<td>UV-HPLC (245nm) 60% water: 40% acetonitrile</td>
<td>(Kronbach et al., 1989)</td>
</tr>
<tr>
<td>3A4</td>
<td>Omeprazole</td>
<td>Omeprazole sulphoxidation</td>
<td>Ketoconazole</td>
<td>0.4</td>
<td>0.1</td>
<td>NADPH</td>
<td>20</td>
<td>UV-HPLC, (304/204nm) 25 mM α-phosphoric acid-buffer (A) and acetonitrile (B)</td>
<td>(Åbelö et al., 2000)</td>
</tr>
</tbody>
</table>
4.17.5 CYP Inhibition Assays: N-In-One-Cocktail Incubations

The incubation conditions and instrumentation used for the enzyme inhibition assays have all been previously described in detail covering the following CYP isoenzymes: CYP1A1/2 (ethoxyresorufin O-deethylation), CYP2A6 (coumarin 7-hydroxylation), CYP2C9 (tolbutamide hydroxylation), CYP2D6 (dextromethorphan O-demethylation), CYP2E1 (chlorzoxazone 6-hydroxylation) CYP3A4/5 (midazolam α-hydroxylation) (Taavitsainen et al., 2001). The assays for CYP2B6 (bupropion hydroxylation), CYP2C8 (amodiaquine N-deethylation) and CYP2C19 and CYP3A4 (omeprazole 5-hydroxylation and sulphoxidation) have all been described previously (Turpeinen et al., 2004, Turpeinen et al., 2006).

The N-in one cocktail method was performed as previously described (Tolonen et al., 2007, Turpeinen et al., 2005) and is summarised in Table 4-6. The probe substrates and their metabolites are shown in Figures 4-7 and 4-8. As omeprazole-5-hydroxylation and omeprazole demethylation both monitor CYP2C19 inhibition, the designations of (5)2C19 and (D)2C19 (respectively) were used. For CYP3A4, midazolam hydroxylation, omeprazole sulfoxidation, omeprazole-3-hydroxylation and 6β-testosterone hydroxylation, were monitored: and denoted (M)3A4, (OS)3A4, (3OH)3A4 and (T)3A4.

Each incubation contained 0.5 mg microsomal protein/ml, 100mM phosphate buffer (pH7.4), 1mM NADPH and all ten-probe substrates. Substrates and their final concentrations for the incubations were: melatonin (4 µM), coumarin (2 µM), bupropion (1 µM), amodiaquine (2 µM), tolbutamide (4 µM), omeprazole (2 µM), dextromethorphan (0.2 µM), chlorzoxazone (6 µM), midazolam (0.4 µM) and
testosterone (1 µM). Reaction mixture (final volume 200 µl) was pre-incubated for 2 minutes at +37°C in a shaking incubator block (Eppendorf Thermomixer 5436, Hamburg, Germany) before the addition of NADPH to initiate the reaction.

Each reaction was stopped after 20 minutes by the addition of 100 µl of acetonitrile containing phenacetin (0.5 µM) (Figure 4-9) as an internal standard. Proteins were precipitated by cooling the samples in an ice bath for a minimum of 5 minutes then centrifuged for 15 minutes at 10,000 g. The supernatant was collected into clean tubes and stored at -20°C until analysis. All incubations were run in duplicate on two separate occasions. For analysis, the incubations were thawed at room temperature, shaken and centrifuged for 10 min at 10,000 g. The supernatants were transferred to a Waters Total Recovery vial (waters Corporation, Milford, Massachusetts, USA) for LC/MSMS analysis.
Figure 4-7: Structures of the substrates utilised in the single substrate (#) and N-in-one cocktail (*) cytochrome P450 inhibition enzyme assays, with the relevant metabolising CYP.
Figure 4-8: Structures of the metabolites detected in the single substrate (#) and N-in-one cocktail (*) cytochrome P450 inhibition enzyme assays, with the relevant metabolising CYP.
Figure 4-9: Structures of the inhibitors utilised in the single substrate enzyme assays and the internal standard for the N-in-one cocktail cytochrome P450 inhibition enzymes assays, with the relevant metabolising CYP.
Table 4-6: Summary of the N-in-one cocktail in vitro assays parameters for the cytochrome P450 inhibition studies, adapted from Turpeinen et al. (Turpeinen et al., 2004). The Km values (Pelkonen et al., 1998), except for amodiaquine, (Li et al., 2002) bupropion (Hesse et al., 2000) and melatonin (Härtter et al., 2001).

<table>
<thead>
<tr>
<th>CYP</th>
<th>Substrate</th>
<th>Reaction</th>
<th>Metabolite</th>
<th>[Substrate] (μM)</th>
<th>Km (μm)</th>
<th>Metabolite retention time (min)</th>
<th>Extracted ions (m/z)</th>
</tr>
</thead>
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4.17.6 Data and Statistical Analysis

4.17.6.1 Enzyme Assays- Single Substrate

The concentration of the metabolite was calculated from peak height ratios of the UV chromatograms on the basis of standard calibration curves of authentic metabolites. The enzyme activity in the presence of the extracts was compared with control incubations into which only solvent was added.

The IC$_{50}$ values for the extracts (concentration causing 50% reduction of control activity) were determined from duplicate incubations run on two separate occasions by non-linear regression analysis (GraphPad Prism, v5.0). In order to interpret the results, the IC$_{50}$ values were converted to reflect the volume one dose unit (one tablet or capsule) should be dissolved in to obtain the corresponding concentration as per the method described previously (Strandell et al., 2004).

Metabolites of ethoxyresorufin and ethoxycoumarin were analysed fluorometrically and the inhibition determined from standard calculations:

EROD

\[
\frac{1000 \text{ pmol} \times \text{FU}}{\text{Standard} \times 5 \text{ min} \times 0.2 \text{mg(protein)}} = \text{pmol/min/mg(protein)}
\]

ECOD:

\[
\frac{1250 \text{ pmol} \times \text{FU}}{\text{Standard} \times 10 \text{ min} \times 0.1 \text{mg(protein)}} = \text{pmol/min/mg(protein)}
\]

Where FU = fluorescence units.
4.17.6.2 Enzyme Assays- N-In-One-Cocktail

Data is presented as the % activity of control for each of the products; each point is the average of duplicate incubations, conducted on two independent occasions where possible an IC\textsubscript{50} was calculated using a non-linear regression analysis program (GraphPad Prism, v5.0). In order to interpret the results, the IC\textsubscript{50} values were converted to reflect the volume one dose unit (one tablet or capsule) should be dissolved in to obtain the corresponding concentration as per the method described previously (Strandell et al., 2004).
4.18 Results

4.18.1 Single Substrate Incubations

4.18.1.1 General Comments

The single substrate assays were all run in duplicate on two separate occasions. The inhibition for each of the extracts was determined from peak height ratios on the basis of standard calibrations and compared to control incubations. The variability between the two runs was negligible but was greater than the variability seen with the N-in-one cocktail assay (Appendix A). Between the individual incubations the variability did not exceed 15%.

The inhibition potential for several extracts could not be determined due to limitations of the method; the metabolite peaks could not be resolved from the HPLC as the extracts themselves produced a peak adjacent to the metabolite peak. This interference with the peaks was seen most predominantly with Liquorice Root, with six of the aqueous extracts (CYP1A2, CYP2A6, CYP2C8, CYP2D6, CYP2E1 and CYP3A4-using midazolam hydroxylation as the probe reaction), and 5 of the methanolic extracts (CYP2B6, CYP2C9, CYP2C19, and both CYP3A4 incubations) not being able to be determined.

4.18.2 CYP Inhibition

Significant inhibition for at least two of the isoenzymes was seen for each of the products investigated. Inhibition was dose dependent; with the methanolic extracts causing more significant inhibition than the aqueous extracts, with higher levels of inhibition recorded and more isoenzymes being inhibited (Figure 4-10 - Figure 4-19).
With the aqueous extracts, inhibition >50% of the control did not occur for any of the isoenzymes investigated for Black Cohosh (Figure 4-10), Dong Quai (Figure 4-11), and Liquorice Root (Figure 4-16). Though in all cases, inhibition could not be determined for each of the isoenzymes investigated due to interference. For CYP2C9 and CYP2C8, inhibition >50% only occurred with Goldenseal and Saw Palmetto respectively (Figure 4-12, Figure 4-18). CYP3A4 was not inhibited >50% by any of the aqueous extracts for either of the reactions monitored. CYP2C8 was the most significantly inhibited isoenzyme for the aqueous extracts with four of the products causing >50% inhibition, Horsetail, Milk Thistle, Saw Palmetto and Valerian. Valerian was the most potent aqueous extract causing the inhibition of five isoenzymes at a level >50%, CYP2A6, CYP2B6, CYP2C8, CYP2D6 and CYP2E1 (Figure 4-19).

The methanolic extracts all caused inhibition >50% of at least one of the investigated isoenzymes. As with the aqueous extracts, the most potent methanolic extract was Valerian, which caused inhibition of all the investigated isoenzymes except CYP2C9, which due to the problems of interference could not be determined (Figure 4-19). CYP2C19 was the isoenzyme most inhibited, with all 9 extracts except for Liquorice Root (which could not be determined due to interference problems) causing >50% inhibition.
4.18.3 Inhibition Constants (IC$_{50}$)

The IC$_{50}$ values were determined by non-linear regression and those that produced an IC$_{50}$ < 100 µg/ml are shown in Table 4-7. The most potent inhibition for the aqueous extract was seen with Horse Chestnut, with an IC$_{50}$ of 18.23 µg/ml for CYP2E1. The methanolic extracts caused the most potent inhibition of CYP2C19 with all except Liquorice Root, which could not be determined, producing an IC$_{50}$ < 100 µg/ml. The most potent was the methanolic extract of Goldenseal, which was determined to have an IC$_{50}$ of 5.7 µg/ml with the IC$_{50}$ for Saw Palmetto being 5.9 µg/ml for CYP2B6.

In order to interpret the results, the IC$_{50}$ values were converted to reflect the volume one dose unit (one tablet or capsule) should be dissolved in to obtain the corresponding concentration as per the method described previously (Strandell et al., 2004), with results shown in Table 4-8. A converted IC$_{50}$ value of 5 litres/dose unit indicates that one dose unit diluted to approximately the blood volume (of an average person) would result in a concentration of inhibitory substances equivalent to the recorded IC$_{50}$ of CYP enzyme inhibition. Whilst this produces a conservative figure it is useful to identify potentially clinically relevant CYP inhibition. The most potent aqueous extract using this method was Horse Chestnut, which produced an inhibition in L/dose units of 109.7. The most potent methanolic extract was Valerian, which produced an inhibition of 286 L/dose units for CYP2B6 and 232.5 L/dose units for CYP2C19.
Figure 4-10: % Inhibition of control for the aqueous (Aq) and methanolic (Meth) extracts of Black Cohosh for the cytochrome P450 isoforms investigated with the single substrate incubations. Results are the average of replicate incubations ran on two separate occasions.
Figure 4-11: % Inhibition of control for the aqueous (Aq) and methanolic (Meth) extracts of Dong Quai for the cytochrome P450 isoforms investigated with the single substrate incubations. Results are the average of replicate incubations ran on two separate occasions.
**Figure 4-12:** % Inhibition of control for aqueous (Aq) and methanolic (Meth) extracts of Goldenseal for each of the cytochrome P450 isoforms investigated with the single substrate incubations. Results are the average of replicate incubations ran on two separate occasions.
Figure 4-13: % Inhibition of control for the aqueous (Aq) and methanolic (Meth) extracts of Gotu Kola for each of the cytochrome P450 isoforms investigated with the single substrate incubations. Results are the average of replicate incubations ran on two separate occasions.
Figure 4-14: % Inhibition of control for the aqueous (Aq) and methanolic (Meth) extracts of Horse Chestnut for each of the cytochrome P450 isoforms investigated with the single substrate incubations. Results are the average of replicate incubations ran on two separate occasions.
Figure 4-15: % Inhibition of control for the aqueous (Aq) and methanolic (Meth) extracts of Horsetail for each of the cytochrome P450 isoforms investigated with the single substrate incubations. Results are the average of replicate incubations ran on two separate occasions.
Figure 4-16: % Inhibition of control for the aqueous (Aq) and methanolic (Meth) extracts of Liquorice Root for each of the cytochrome P450 isoforms investigated with the single substrate incubations. Results are the average of replicate incubations ran on two separate occasions.
Figure 4-17: % Inhibition of control for the aqueous (Aq) and methanolic (Meth) extracts of Milk Thistle for each of the cytochrome P450 isoforms investigated with the single substrate incubations. Results are the average of replicate incubations ran on two separate occasions.
Figure 4-18: % Inhibition of control for the aqueous (Aq) and methanolic (Meth) extracts of Saw Palmetto for each of the cytochrome P450 isoforms investigated with the single substrate incubations. Results are the average of replicate incubations ran on two separate occasions.
Figure 4-19: % Inhibition of control for the aqueous (Aq) and methanolic (Meth) extracts of Valerian for each of the cytochrome P450 isoforms investigated with the single substrate incubations. Results are the average of replicate incubations ran on two separate occasions.
**Table 4-7:** Estimated IC<sub>50</sub> (μg/ml) for the products investigated for CYP inhibition using the single substrate assay. IC<sub>50</sub> calculated using a non-linear regression analysis program (GraphPad Prism, v5.0).

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<th>2B6</th>
<th>2C8</th>
<th>2C9</th>
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Aq = aqueous extracts, Meth = methanolic extracts. * Inhibition could not be determined.
Table 4-8: Inhibition of CYP isoenzymes by extracts that produced an IC<sub>50</sub> < 100 µg/ml in the single substrate assay. IC<sub>50</sub> values are expressed as litre/dose unit.

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Aq = aqueous extracts, Meth = methanolic extracts. * Inhibition could not be determined.
4.19  N-in-one Cocktail Assays

4.19.1 General Comments

The N-in-one cocktail assays were all run in duplicate on two separate occasions. The inhibition for each of the extracts was determined against the control and was shown to be dose dependant for the extracts investigated (Figure 4-20 - Figure 4-29). The variability between the two runs was negligible and less than the variability seen in the single substrate assay with the variability between individual incubations not exceeding 10% (Appendix A).

Unlike the single substrate assays, the N-in-one cocktail assay did not have the same problems of interference. As the N-in-one cocktail method utilises time of flight mass spectrometry which is a more sensitive and discriminatory (i.e. resolution is superior) method than the HPLC the substrates and metabolites could be determined for all of the investigated extracts, as the detection of ions is based on the exact mass/charge (m/z) ratio.

4.19.2 CYP Inhibition

As with the single substrate incubations, the methanolic extracts caused more significant inhibition than the aqueous extracts, with higher levels of inhibition recorded and more isoenzymes being inhibited. Inhibition was dose dependant for all of the extracts investigated (Figure 4-20 - Figure 4-29).

With regards to the aqueous extracts, inhibition >50% did not occur for any of the isoenzymes investigated for Black Cohosh (Figure 4-20) and Dong Quai (Figure 4-21), this pattern was also seen with the single substrate incubations.
CYP2B6 and CYP2C9 were only significantly inhibited by Valerian (Figure 4-29) and Milk Thistle (Figure 4-27), respectively. The most potent aqueous extract was Valerian which caused >50% inhibition at the highest investigated concentration of 6 isoenzymes (Figure 4-29).

CYP2D6 and CYP2E1 were both significantly inhibited by 3 products, whilst CYP2A6 and CYP2C8 were both significantly inhibited by 4 products. CYP3A4 inhibition varied depending on the reaction being monitored. No significant inhibition was seen for any of the aqueous extracts using midazolam hydroxylation ((M)3A4) or omeprazole sulfoxidation ((OS)3A4), but significant inhibition was detected using the omeprazole 3-hydroxylation ((3OH)3A4) and testosterone hydroxylation ((T)3A4).

The methanolic extract of Valerian significantly inhibited all of the isoenzymes investigated (Figure 4-29). CYP2C19 was the most significantly inhibited isoenzyme with all of the methanolic extracts inhibiting this isoenzyme using both probe reactions (omeprazole 5-hydroxylation and omeprazole demethylation). CYP2E1 was inhibited by all of the extracts except Milk Thistle. CYP1A2 and CYP2D6 were both inhibited significantly by 3 of the methanolic extracts. CYP2A6 was only significantly inhibited by 2 of the methanolic extracts, Dong Quai and Valerian. CYP2C8 and CYP2C9 were significantly inhibited by 4 and 5 of the methanolic extracts respectively. CYP3A4 inhibition varied as it did with the aqueous extracts. Goldenseal, Saw Palmetto and Valerian all caused significant inhibition of CYP3A4 with all 4 probe reactions.
4.19.3 Inhibition Constants (IC$_{50}$)

Estimated IC$_{50}$s were determined for the extracts that produced significant inhibition (<100 µg/ml) and are shown in Table 4-9. The most potent aqueous extract was Goldenseal with an IC$_{50}$ of 6.7 µg/ml for CYP2D6. Valerian produced an IC$_{50}$ <100 µg/ml for all of the isoenzymes except CYP2A6. The most potent methanolic extract was Milk Thistle, which produced an IC$_{50}$ of 2.9 µg/ml for CYP2C9.

In order to interpret the results, the IC$_{50}$ values were converted to reflect the volume one dose unit (one tablet or capsule) should be dissolved in to obtain the corresponding concentration as per the method described previously (Strandell et al., 2004), Table 4-10. Using this method to represent the data the methanolic extract of Valerian is the most potent inhibitor of the CYP isoenzymes investigated, with 5 CYPs (CYP2B6, CYP2C8, CYP2C9, CYP2C19 and CYP2E1) having an inhibition in L/dose units over 100 L. The methanolic extracts of Goldenseal, Horse Chestnut, Liquorice Root and Saw Palmetto all produced an inhibition >100 L/dose units for four isoenzymes. With the aqueous extracts, only Horse Chestnut produced an inhibition >100 L/dose units for both CYP2A6 and CYP2E1 with values of 112.4 and 119 L/dose units respectively.
Figure 4-20: % Inhibition of control for the aqueous (Aq) and methanolic (Meth) extracts of Black Cohosh for each of the cytochrome P450 isoforms investigated with the N-in-one cocktail incubation. Results are the average of replicate incubations ran on two separate occasions.
Figure 4-21: % Inhibition of control for the aqueous (Aq) and methanolic (Meth) extracts of Dong Quai for each of the cytochrome P450 isoforms investigated with the N-in-one cocktail incubation. Results are the average of replicate incubations ran on two separate occasions.
Figure 4-22: % Inhibition of control for the aqueous (Aq) and methanolic (Meth) extracts of Goldenseal for each of the cytochrome P450 isoforms investigated with the N-in-one cocktail incubation. Results are the average of replicate incubations ran on two separate occasions.
**Figure 4-23:** % Inhibition of control for the aqueous (Aq) and methanolic (Meth) extracts of Gotu Kola for each of the cytochrome P450 isoforms investigated with the N-in-one cocktail incubation. Results are the average of replicate incubations ran on two separate occasions.
Figure 4-24: % Inhibition of control for the aqueous (Aq) and methanolic (Meth) extracts of Horsetail for each of the cytochrome P450 isoforms investigated with the N-in-one cocktail incubation. Results are the average of replicate incubations ran on two separate occasions.
Figure 4-25: % Inhibition of control for the aqueous (Aq) and methanolic (Meth) extracts of Horse Chestnut for each of the CYP isoform assays using the N-in-one cocktail incubation. Results are the average of replicate incubations ran on two separate occasions.
Figure 4-26: % Inhibition of control for the aqueous (Aq) and methanolic (Meth) extracts of Liquorice Root for each of the cytochrome P450 isoforms investigated with the N-in-one cocktail incubation. Results are the average of replicate incubations ran on two separate occasions.
Figure 4-27: % Inhibition of control for the aqueous (Aq) and methanolic (Meth) extracts of Milk Thistle for each of the cytochrome P450 isoforms investigated with the N-in-one cocktail incubation. Results are the average of replicate incubations ran on two separate occasions.
Figure 4-28: % Inhibition of control for the aqueous (Aq) and methanolic (Meth) extracts of Saw Palmetto at for each of the cytochrome P450 isoforms investigated with the N-in-one cocktail incubation. Results are the average of replicate incubations ran on two separate occasions.
**Figure 4-29:** % Inhibition of control for the aqueous (Aq) and methanolic (Meth) extracts of Valerian for each of the cytochrome P450 isoforms investigated with the N-in-one cocktail incubation. Results are the average of replicate incubations ran on two separate occasions.
Table 4-9: Estimated IC\textsubscript{50} (µg/ml) for the products investigated for CYP inhibition using the N-in-one cocktail assay. IC\textsubscript{50} calculated using a non-linear regression analysis program (GraphPad Prism, v5.0).

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Aq = aqueous extracts, Meth = methanolic extracts.
Table 4-10: Inhibition of CYP isoenzymes by extracts that produced an IC$_{50}$ <100 μg/ml in the N-in-one cocktail assay. IC$_{50}$ values are expressed as litre/dose unit.

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Aq = aqueous extracts, Meth = methanolic extracts.
4.20 Discussion and Conclusions

4.20.1 Product Selection

The products selected for this study are amongst the top selling products in Australia (Table 4-3). As the cytochrome P450 enzymes are the main drug metabolising enzymes in the body and responsible for the metabolism of the majority of therapeutic drugs then selecting products based on product market share is a rational starting point.

Several products selected were done so on the basis of interaction potential with therapeutic drugs based on the traditional reported use of the product (Table 4-3). Horse chestnut is reported to be useful in the treatment of “blood stagnation” based on this therapeutic indication there is a risk of concurrent use with anti-coagulant drugs such as warfarin. As warfarin has a narrow therapeutic index, any drug interaction resulting in increased or deceased bioavailability of warfarin could be life threatening.

4.20.2 Selection of Inhibition Assay Method

Using pooled microsomes to investigate CYP inhibition allows for multiple isoenzymes to be investigated from multiple donors, this gives a better representation of the in vivo situation.

The single substrate assays employs HPLC and fluorescence detection of CYP specific substrates and their metabolites. These substrates are well characterised and routinely used to investigate interactions with CYPs. The disadvantage of this technique is that only CYP can be monitored at once.
To increase the throughput of these assays and reduce the amount of human tissue and labour required, the N-in-one cocktail assay can be used. With this assay the simultaneous monitoring of multiple CYPs in the one reaction can be undertaken. The substrates are specific for a CYP or multiple CYPs with different metabolites generated for the different CYP. The assay requires LC/MSMS analysis to identify the 10 substrates and 13 metabolites.

### 4.20.3 General Discussion of Results

The fact that CYPs are critical for the disposition of a large percentage of clinically important drugs is well established and consequently any interference of this enzyme system can lead to unexpected and adverse events. It has been estimated that the majority of 200 important drugs are dependent on drug metabolism for their elimination and CYPs played a dominant role in this metabolism (Williams et al., 2004, Evans and Relling, 1999, Zanger et al., 2008). Members of the CYP3A family contributed to the metabolism of 37% of the drugs, followed by CYP2C9 (17%), CYP2D6 (15%), CYP2C19 (10%), CYP1A2 (9%), and CYP2C8 (6%). CYP2B6 and other CYP isoforms (CYP2A6 and CYP2E1) participated in the metabolism of 4% and 2% of the drugs, respectively (Zanger et al., 2008). In light of this, complementary products need to be investigated for their potential to interact with and inhibit CYP enzymes.

Studying complementary products in this context presents a number of challenges, including the fact that these products are complex preparations of variable composition.
Isolating individual components is one method that can be used to assist in the determination of the kinetic profiles of these products but this is time consuming and in the end may not provide the answers required as these products are not taken as isolated components but as complex mixtures.

As variability is known to occur with these products between the manufacturers and even between seasons and geographic locations of the raw materials (Song et al., 2010, Strandell et al., 2004), ongoing screening is required. Screening for CYP inhibition rather than simply testing for isolated components is more clinically significant as components often interact to produce an effect and any single component responsible for CYP inhibition may not be the components being isolated and quantified.

Representing the IC$_{50}$ in litres/dose gives some insight into the possibility of reaching in vivo concentrations that may be significant. For example, an IC$_{50}$ of 5-litres/dose units indicates that one recommended dose unit diluted into a volume equivalent to the approximate blood volume of the average person would result in a concentration of inhibitory substances equivalent to the IC$_{50}$ of CYP enzyme inhibition. Obviously the likelihood of a notable inhibition being associated with a product that has IC$_{50}$s reaching into the range of 100 L or more is much greater. Strandell et al. used litres/dose to present their data and concluded that IC$_{50}$ of less than 0.88 L/dose would not be inhibitory (Strandell et al., 2004). This method of representing the data whilst producing a conservative figure is an attempt to provide information that will aid in prioritising the products that require more urgent investigation.
Another challenge associated with complementary products is the choice of extraction method. For these studies, the choice was made on the basis of the relevance of the extract to patient use of these products. The products are generally administered as tablets or water infusions (‘teas’). Therefore aqueous and alcohol (methanol) extracts were prepared, methanol was selected to extract distinctly more lipid soluble components.

4.21 Black Cohosh

Black Cohosh (*Cimicifuga racemosa*) is a shrub like plant native to the Eastern forests of North America. Because of reports proposing benefit in alleviating symptoms of menopause and the subsequent reports of hepatotoxicity, Black Cohosh is one of the most investigated herbal products. Although several in vitro and in vivo studies on some CYP activities have been conducted (Gurley et al., 2005b, Gurley et al., 2006b, Gurley et al., 2008), this is the first report of Black Cohosh being screened for inhibition potential against a full panel of CYPs and also using an N-in-one cocktail approach.

Whilst none of the aqueous extracts resulted in an IC$_{50}$ <100 µg/ml, three isoenzymes recorded an IC$_{50}$ below 100 µg/ml with the methanolic extracts, CYP2B6, CYP2C19 and CYP2E1 with values of 24.5, 32.8 and 11.38 µg/ml respectively, using the single substrate assay method and 49.2, 23.9-36.3 and 11.5 µg/ml respectively, using the N-in-one cocktail method.

Previous studies with Black Cohosh have highlighted the problems with data interpretation that arise when these complex and variable products are assessed.
In vivo studies in humans initially found that Black Cohosh may weakly inhibit CYP2D6 to the value of 7% (Gurley et al., 2005b), but later studies conducted by the same group concluded that Black Cohosh had no inhibitory effect (Gurley et al., 2008). In both these studies the method for determining inhibition is the same but the product investigated was different. The 2005 study used a Black Cohosh that was standardized to 0.2% triterpene glycosides per tablet, whilst the 2008 study used a product that was standardized to 2.5% triterpene glycosides per tablet. This finding suggests that whilst the triterpene glycosides are often reported as the principle component of these preparations it may not be this component that causes CYP inhibition and that other components in these preparations may be capable of causing inhibition.

Human in vivo studies have also found that there is no inhibition of CYP3A4 with Black Cohosh (Gurley et al., 2006b, Gurley et al., 2005b) and our data is in agreement with this. In vitro studies using human liver microsomes also found no significant inhibition of CYP3A4 but did demonstrate the difference in inhibition patterns that arises with various manufacturers products (Wanwimolruk et al., 2009). Induction of CYP3A4 has been investigated, CYP3A11 in mice which is the homolog of CYP3A4 was shown to be up-regulated specifically in the liver but this may not be mediated by PXR (Houston and Galetin, 2010).

The inhibition of CYP2B6 may be significant as several important pharmaceutical drugs including cyclophosphamide and verapamil are substrates for this isoenzyme (Turpeinen et al., 2006). Polymorphic variation in CYP2B6 has also been shown to be an important factor in drug metabolism (Xie et al., 2003).
4.22 Dong Quai

Dong Quai (*Angelica sinensis*), also known as female ginseng, is indigenous to China. Traditionally it has been used to treat gynaecological ailments, fatigue, anaemia and high blood pressure. Previously it has been shown to induce CYP2D6 and CYP2A4 in rodent species (Tang et al., 2006). To date there have been no reports of inhibition of CYP enzymes with Dong Quai though other species of Angelica appear to inhibit CYP2A6 (Yoo et al., 2007).

As with Black Cohosh, none of the aqueous extracts resulted in an IC$_{50}$ <100 µg/ml. The methanolic extracts caused significant inhibition of three isoenzymes: CYP2A6, CYP2B6 and CYP2C19 with IC$_{50}$'s of 93.8, 17.7 and 19.7 µg/ml for the single substrate assays and 94.7, 11.4 and 13.7-14.3 µg/ml respectively for the N-in-one cocktail assays. Inhibition by Dong Quai may be significant as inhibition by other CYP2C19 inhibitors including isoniazid ($K_i = 25.4 \mu$M) are known to cause adverse clinical interactions when administered concurrently with diazepam or phenytoin (both are substrates for CYP2C19) (Kay et al., 1985, Ochs et al., 1981).

Inhibition of CYP2B6 with the methanolic extract of Dong Quai was the most significant, IC$_{50}$ of 45.6 L/dose units (from the N-in-one cocktail assay), which is greater than the 5 L/dose unitss that would approximate blood volume of the average person. This inhibition along with the potential for interaction with CYP2C19 (36.4 L/dose units from the N-in-one cocktail assay) inhibition helps to prioritise Dong Quai as a product that requires further investigation.
Using the formation of 1-OH midazolam as the probe reaction, other species of Angelica have been shown to inhibit CYP3A4 weakly (unpublished data from our laboratory). These investigations did not show significant inhibition of CYP3A4. Using testosterone as the probe substrate, the aqueous 500 µg/ml extract caused 43% inhibition of control (Figure 4-12).

### 4.23 Goldenseal

Goldenseal (*Hydrastis canadensis*) is a perennial herb that is native to south-eastern Canada and north-eastern United States. It is used as an antimicrobial and a laxative. In vitro studies using human microsomes have shown inhibition of CYP2D6 with two components of Goldenseal, berberine and hydrastine, $IC_{50} = 45$ µM and 350µM respectively (Chatterjee and Franklin, 2003). In vivo studies conducted in healthy human subjects also concluded that inhibition of CYP2D6 and CYP3A4 occurs with Goldenseal (Gurley et al., 2008).

In vitro investigations have shown Goldenseal to be an inhibitor of CYP2C9, CYP2D6 and CYP3A4 with $IC_{50}$ values of 0.98, 0.66 and 0.18% of control respectively (Chatterjee and Franklin, 2003). In addition to these isoenzymes, CYP2C19 has been shown to be inhibited by Goldenseal in vitro up to 80% of control (Taavitsainen, 2001).

The data indicates that aqueous extracts of Goldenseal causes significant inhibition of CYP2D6 ($IC_{50} = 6.7$ µg/ml, 74.6 L/dose units), though no inhibition was detected using the single substrate assays.
Inhibition of CYP2D6 may be clinically significant as dextromethorphan, a substrate of CYP2D6 and used to treat coughs, may be used concurrently with Goldenseal.

The methanolic extract caused significant inhibition with 5 of the isoenzymes CYP2B6, CYP2C19, CYP3A4, CYP2D6 and CYP2E1. This is the first report of inhibition of CYP2B6 and CYP2E1 with Goldenseal. The most potent inhibition was seen with CYP3A4 with and IC$_{50}$ of 35.32 and 26.7 µg/ml for the single substrate and N-in-one cocktail respectively. This data was obtained using the midazolam hydroxylation reaction; lower levels of inhibition were detected using the other probe reactions. This variability in the inhibition of CYP3A4 highlights the need for multiple probe reactions to be monitored for CYP3A4 investigations in order to gain a true representation of the inhibition potential of the compound being investigated.

4.24 Gotu Kola

Gotu Kola (Centella asiatica) is a small plant native to several countries including India, Sri Lanka, Indonesia and Iran. It is used in the treatment of stress, anxiety and depression. Previous in vitro studies determined that tea made from Gotu Kola at a concentration of 25 mg/ml caused a weak to moderate inhibition of CYP2C9 (24.8%), CYP2C19 (42.2%), CYP2D6 (23.9%) and CYP3A4 (51.5%) (Taavitsainen, 2001).
No significant inhibition was seen with the aqueous extracts in our studies. Whilst it did not reach significant levels, inhibition of CYP1A2 with the aqueous extract was moderate with inhibition reaching 75 and 78% of control, for the single substrate and the N-in-one cocktail assays respectively, at the highest concentration (500 µg/ml) investigated (Figure 4-13 and Figure 4-23). This finding is of concern as many antidepressants including the tricyclics are substrates for CYP1A2. The potential for herb-drug interactions is therefore possible in patients who are taking antidepressants and concurrently taking Gotu Kola (which claims to help in the treatment of anxiety and mood disorders) to aid in their condition.

Using cDNA expressed isoforms, CYP2C9 was shown to be inhibited by the ethanolic and dichloromethane extracts of Gotu kola with a $K_i$ of 39.1 and 26.6 µg/ml, with S-mephenytoin as the probe substrate (Pan et al., 2010). Whilst in our studies inhibition of CYP2C9 was not detected (using tolbutamide as the probe substrate), previous work has shown that the IC$_{50}$ values of model inhibitors differs dependent on the models and methods used (Turpeinen et al., 2006). Additionally CYP2C9 has been reported to be more catalytically active in cDNA expressed enzymes than human microsomes (Venkatakrishnan et al., 2000).

The methanolic extracts of Gotu Kola caused significant inhibition of CYP2B6, CYP2C19, CYP2E1 and CYP3A4 with IC$_{50}$ values of 14, 7.8-8.3, 9.5 and 58.4 µg/ml respectively, with the N-in-one cocktail assays, though CYP2B6 inhibition was not detected using the single substrate assays.
Importantly the inhibition of CYP3A4 was detected only with the 6β-testosterone hydroxylation reaction, which once again demonstrates the importance of using multiple probe reactions when investigations into this isoenzyme are undertaken.

The IC$_{50}$ in litre/dose unit indicate that the greatest potential for inhibition and therefore interactions occurs with CYP2C19 with values of 34.5 and 47.6 -50.6 L/dose units for the single substrate and the N-in-one cocktail respectively. CYP2C19 is responsible for the metabolism of omeprazole (Äbelö et al., 2000), phenytoin (Guengerich, 2005) and amitriptyline (Venkatakrishnan et al., 1998), therefore inhibition of CYP2C19 may cause adverse drug interactions due to increased concentrations of phenytoin leading to toxicity.

### 4.25 Horse Chestnut

Horse Chestnut (*Aesculus hippocastanum*) is a large tree that is native to the Balkans and temperate regions of Europe. It is used for chronic venous insufficiency and haemorrhoids. To date very few investigations have been carried out into the potential for Horse Chestnut to inhibit CYP enzymes with most studies focusing on its anti-platelet activity (Felixsson et al., 2010, Wu et al., 2012) and the toxicity associated with the alkaloids present in the plant. The induction potential of Horse Chestnut has been assessed in cultured human hepatocytes and was found to be negative for CYP2C19 (Hellum et al., 2009).

In vitro studies using a cDNA baculovirus system concluded that inhibition of CYP3A4 did occur but a high IC$_{50}$ was determined (1173 μg/ml) using the 6β-testosterone hydroxylation as the probe reaction (Hellum and Nilsen, 2008).
These investigations have also shown inhibition of CYP3A4, this was dependent on the specific probe reaction used with the 3-OH-omeprazole probe reaction being the most sensitive with the aqueous and methanolic extracts having an IC\textsubscript{50} of 66.1 and 4.4 µg/ml, respectively, for the N-in-one cocktail assay. Inhibition was not detected using the single substrate assays. Not being able to detect inhibition with the single substrate assays may have been caused by interference from the products with the analytical method.

CYP2B6 was significantly inhibited by the methanolic extract with IC\textsubscript{50} value of 18.0 µg/ml, though this was only with the N-in-one cocktail assay as the data from the single substrate assay could not be obtained due to interference from the product with the HPLC peak detection (see section 4.18.1.1). The methanolic extract also caused significant inhibition of CYP2C19 with IC\textsubscript{50} of 26.07 µg/ml for the single substrate assay and 20.0-21.3 µg/ml for the N-in-one cocktail assay.

The inhibition of CYP2C19 is of concern as there is a potential for concurrent use of Horse Chestnut which is taken to prevent blood stagnation, and the anti-platelet drug clopidogrel which is a pro-drug that is converted to its active metabolite by CYP2C19. This potential for an interaction to occur is not only strengthened by the potential for concurrent use of these products but also the potential for Horse Chestnut to reach a concentration that may be significant as demonstrated by the high IC\textsubscript{50} in L/dose units of 111.
Our results also indicate that the Horse Chestnut can significantly inhibit CYP2E1. The aqueous and methanolic extracts having IC$_{50}$ of 18.2 and 9.59 µg/ml for the single substrate and 16.8 and 8.6 µg/ml for the N-in-one cocktail assays. To date no clinically significant interactions have been reported with regards to CYP2E1 inhibition, but inhibition of this isoenzyme may alter the disposition of alcohol, benzene and carcinogens such as azoxymethane, which are all, metabolised by CYP2E1.

Inhibition of CYP2A6 was seen with aqueous extract, IC$_{50}$ of 17.8-53.52 µg/ml. Inhibition of CYP2A6 is potentially significant because of the role this isoenzyme plays in the metabolism of several drugs with a narrow therapeutic index including valproic acid.

### 4.26 Horsetail

Horsetail (*Equisetum arvense*) is a bushy plant that is native to the Northern hemisphere and is used to aid in the treatment of urinary tract infections, cystitis and prostate problems. In vitro studies have previously indicated that ethanol extracts can cause inhibition of CYP3A4 (22% of control) (Scott et al., 2006) using a fluorescent probe substrate (dibenzylfluorescein). Though our studies did not detect any inhibition of CYP3A4 this may be either due to differences in the herbal preparations or the difference is the components isolated with the different extraction solvents.
The aqueous extracts significantly inhibited CYP2A6 and CYP2C8 with IC\textsubscript{50} values 80.0 and 93.6 µg/ml for the single substrate assays, and 18.3 and 93.0 µg/ml for the N-in-one cocktail assays, respectively. The methanolic extracts also caused significant inhibition of CYP1A2, CYP2B6 and CYP2C19 with IC\textsubscript{50}'s of 14.8, 21.2 and 22.6 µg/ml respectively, for the single substrate assays and 12.6, 11.3 and 7.9-15.5 µg/ml respectively, for the N-in-one cocktail assays. Inhibition of these isoenzymes has not been previously reported.

Clinically the inhibition of CYP2C8 may be significant; this is despite the low IC\textsubscript{50} litre/dose unit of 3.9 as many of the loop diuretics are substrates for CYP2C8. Adverse reactions may arise in patients with urinary tract infections and cystitis who may use Horsetail concurrently with antibiotics such as trimethoprim, which is also an inhibitor of CYP2C8. This potential interaction may lead to increased concentrations of trimethoprim in the serum, which can lead to adverse effects such as rashes, GIT disturbances and folate deficiency.

4.27 Liquorice Root

Liquorice Root (Glycyrrhiza glabra) is a plant that is native to southern Europe and parts of Asia and is one of the most potent CYP inhibitors investigated in this study. It is used in the treatment of acute viral hepatitis, to relieve cough and in autoimmune diseases. It is a known inhibitor of CYP2B6, CYP2C9 and CYP3A4 (Kent et al., 2002). Constituents of liquorice have been well studied and the furanocoumarins including bergamottin and the isoflavone glabridin are known to be the major constituents causing CYP inhibition.
Extracts of Liquorice Root caused the most interference with the single substrate assays (section 4.18.1.1). The inhibition for the aqueous extracts could not be determined for CYP1A2, 2A6, 2C8, 2D6, 2E1 and (M)3A4. For the methanolic extracts CYP2B6, 2C9, 2C19 and both of the 3A4 assays the inhibition could not be determined.

The aqueous extract caused significant inhibition of CYP2A6, CYP2D6 and CYP2E1 with IC₅₀’s of 41.8, 97.2 and 8.8 μg/ml with the N-in-one cocktail assays. Due to the interference problems mentioned, inhibition could not be detected with the single substrate assays for these isoenzymes.

The methanolic extracts significantly inhibited all isoenzymes except CYP2A6. The inhibition of CYP3A4 varied depending on the probe substrate being used. The data indicates that the formations of 3-OH-omeprazole and OH-midazolam were the most and least sensitive assays, respectively. Inhibition was not detected using the 6β-hydroxylation of testosterone as the probe reaction.

The inhibition caused by Liquorice Root is not only extensive but also potent as four of the isoenzymes have an IC₅₀ >100 L/dose units, i.e. CYP2B6, CYP2C9, CYP2C19 and CYP3A4 with values of 101.2, 118.1, 141.7 and 118.1 L/dose units.
This high value indicates that the potential for one dose unit to cause significant inhibition is possible. As Liquorice Root is used to treat a wide variety of conditions as well as a product added to many herbal remedies to “harmonize” the ingredients in the formula and “regulate the meridians” (Bensky et al., 2004) it is a product that certainly should be investigated in more detail for its ability to inhibit CYPs.

4.28 Milk Thistle

Milk Thistle (Silybum marianum) is a flowering plant that is native to the Mediterranean regions of Europe, North Africa and the Middle East. It is commonly used as a liver tonic. For this reason this product presents a unique problem in that many of the patients consuming this may have pre-existing liver damage and therefore a decreased capacity to metabolise xenobiotics. Several in vivo and in vitro investigations have been conducted with Milk Thistle. No clinically significant inhibition of CYP1A2, CYP2D6, CYP3A4 and CYP2E1 was determined in healthy human subjects (Gurley et al., 2004, Gurley et al., 2006b, Gurley et al., 2008).

Our work indicates that inhibition of CYP2C9 occurs with the aqueous extract, with an IC<sub>50</sub> of 61.7 µg/ml (1.36 L/dose units) and 64.2 µg/ml (1.3 L/dose units) for the single substrate and the N-in-one cocktail assays respectively (Figure 4-17 and Figure 4-27). Inhibition of CYP2C9 may warrant further consideration and investigation even though there is a low risk with the IC<sub>50</sub> in L/dose units being <5, as warfarin, a vitamin K antagonist, has a narrow therapeutic index and the S-enantiomer is metabolised by CYP2C9 (Yamazaki et al., 1998).
Inhibiting CYP2C9 increases the biological availability of warfarin and may cause increased risk in bleeding.

The methanolic extract caused significant inhibition of CYP2B6 ($IC_{50}$ 7.7 μg/ml), CYP2C9 ($IC_{50}$ 2.9 μg/ml), CYP2C19 ($IC_{50}$ 4.2-4.8 μg/ml) and CYP3A4 (with only the omeprazole-3-hydroxylation reaction, $IC_{50}$ 15.5 μg/ml) in the N-in-one cocktail assays (Table 4-9). This potential for CYP inhibition should be considered in patients with decreased liver function who may be taking Milk Thistle to aid in their condition concurrently with therapeutic agents that are metabolised by these CYPs including warfarin and phenytoin.

4.29 Saw Palmetto

Saw palmetto (*Serenoa repens*) is a fruit extract that is used in the treatment of urinary and reproductive disorders. Investigations into Saw Palmetto are increasingly urgent as the use of this product is increasing and as reports of benefit in the treatment of benign prostatic hyperplasia continue to emerge (Tacklind et al., 2009).

In vitro studies concluded that Saw Palmetto was a potent inhibitor of CYP3A4 with an $IC_{50}$ of 7.41 μg/ml (Budzinski et al., 2000), though this was not supported by in vivo studies. In vivo studies have shown no inhibition of CYP1A2, CYP2D6, CYP2E1 and CYP3A4 using the dose recommended by the manufacturer (Gurley et al., 2004, Markowitz et al., 2003).
None of the aqueous extracts caused significant inhibition of any of the isoenzymes investigated. The methanolic extracts caused significant inhibition of CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP3A4 and CYP2E1 with the N-in-one cocktail assays (Table 4-9). With the single substrate assays, CYP2B6, CYP2C8, CYP2C19 were the only three significantly inhibited isoenzymes (Table 4-7). When the IC$_{50}$ is determined in L/dose units, 4 isoenzymes are significantly inhibited. The isoenzymes inhibited were CYP2B6, CYP2C9, CYP2C19 and CYP3A4 and the respective IC$_{50}$ values in L/dose units’ were 141.0, 134.1, 110 and 157 (Table 4-10).

The IC$_{50}$ for CYP2C9 was 4.1 µg/ml with the N-in-one cocktail assay, but inhibition was not detected with the single substrate assay. As both of these methods use the same probe reaction, tolbutamide hydroxylation, the discrepancy may be due to the greater sensitivity of the N-in-one cocktail method or interference from the product with the HPLC results.

### 4.30 Valerian

Valerian (*Valeriana officinalis*) is a flowering plant that is native to Europe and Asia and is used in the treatment of insomnia. Previous studies in vitro have reported that Valerian caused inhibition of CYP3A4 (Lefebvre and Foster, 2004, Hellum and Nilsen, 2008b) but investigations in vivo did not correlate with these findings (Gurley et al., 2005b).
None of the CYP isoenzymes investigated were significantly inhibited by the aqueous extracts of Valerian with either of the assay methods used. Inhibition of CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP3A4, CYP2D6 and CYP2E1 was seen with the methanolic extracts with both the single substrate and the N-in-one cocktail assays (Table 4-7 and Table 4-9). This wide variety of CYPs inhibited by Valerian and the many reported uses of the product including sleep disorders, anxiety and muscle relaxant has the potential to lead to adverse reactions.

When the IC$_{50}$ is converted to L/dose units we see that not only does Valerian inhibit the most isoenzymes, but it is also the most potent inhibitor with CYP2C8 producing an IC$_{50}$ of 523.3 L/dose units. This is particularly important when the therapeutic agents metabolised by CYP2C8, including paclitaxel, cerivastatin, repaglinide, rosiglitazone and pioglitazone (Jaakkola et al., 2006, Wang et al., 2002, Rahman et al., 1994) are considered. Increases in the plasma level of paclitaxel which occurs when metabolism is inhibited may lead to hypersensitivity and hypotension (Panchagnula, 1998).

4.31  Comparison Between the Methods

Whilst these studies have highlighted potential important inhibition of CYPs with commonly used complementary products, it has also shown that the N-in-one cocktail method can be used to screen these complex mixtures. By using the N-in-one cocktail method the problems of interference with peak detection with HPLC are avoided. Additionally the variability in the estimated inhibition is lower with the N-in-one cocktail than with the single substrate assay (Appendix A).
The inhibition of CYP3A4 is of key importance as it is the most abundant CYP in the liver and accounts for the metabolism of over 50% of therapeutic agents (Evans and Relling, 1999, Zanger et al., 2008). Inhibition of CYP3A4 can be determined using a variety of probe substrates. As multiple binding sites are known on the enzyme, and binding may occur simultaneously at multiple sites, it has been proposed that inhibition of CYP3A4 should be investigated using multiple probe substrates (Kenworthy et al., 1999).

Midazolam and testosterone have previously been described as substrates that belong to two distinct groups, the benzodiazepines and the large molecular weight groups respectively. These studies highlights that of the 4 probe reaction monitored these two follow a pattern with similar levels of inhibition, for example the aqueous extract of Black Cohosh recording an inhibition of 76% and 71% at the highest tested concentration for midazolam and testosterone respectively (Figure 4-20).

Omeprazole, a pyridine, is a probe substrate for CYP2C19 but can also be used as a substrate for CYP3A4. Omeprazole hydroxylation was the most sensitive probe reaction investigated in our studies, though it does not correlate with omeprazole sulfoxidation. This highlights the importance of using multiple probe substrates when investigating the potential inhibition of CYP3A4 (Table 4-7 and Table 4-9).

The IC_{50}'s are comparable between the two methods, though in general the IC_{50} was higher with the single substrate assays than with the N-in-one cocktail assay (Table 4-7 and Table 4-9).
One of the most significant differences between the methods is with CYP3A4 using omeprazole sulfoxidation as the probe reaction for the methanolic extract of Horse Chestnut. The IC$_{50}$ using the single substrate assay was 69 $\mu$g/ml but was >100 $\mu$g/ml for the N-in-one cocktail method (108 $\mu$g/ml), both assay methods were negative for inhibition when midazolam was used as the probe substrate.

### 4.32 Conclusions

There is sufficient reason to be aware of the real possibility of complementary products inhibiting CYP enzymes. The consequences of adverse clinical reactions resulting from such inhibition are of concern and should receive adequate attention. The difficulties of conducting systematic studies of these products with respect to their potential CYP inhibition are the number of products available and the inherent variability in these products. Quantitative evaluation of CYP inhibition is essential to detect clinically significant interactions with herbal products. However, screening of this nature serves as an alert for clinicians.

The multiple forms of CYPs playing a major role in drug metabolism, is an additional challenge. However, the N-in-one cocktail method allows for the rapid screening for CYP inhibition by these products with data comparable to the more commonly used single substrate method. The N-in-one method also allows for multiple probe reactions for CYP3A4 inhibition to be run simultaneously.
Those interactions which are deemed to be significant from a clinical perspective should be prioritised for further monitoring and study based on the likelihood of being used concurrently with medications with a narrow therapeutic index and with those that have a high IC\textsubscript{50} in L/dose units (Table 4-8 and Table 4-10).

Extraction efficiency is always a vexing question when dealing with poorly characterised plant or animal extracts. Until a thorough analysis of the extracts exists, one needs to rely on rigid adherence to an adopted extraction method. These studies have shown that the extraction method selected is reproducible as the data from the incubations is derived from extractions on separate occasions (Appendix A). One of the important points of this study is that it permits the screening of unresolved complex mixtures. The next stage of these investigations would be to fractionate the products showing significant inhibition. The fractionation would permit detailed study the inhibition potency by the individual components.

The N-in-one cocktail is a rapid and reliable method that can be used as an initial screen to prioritise which products require further investigation and also can be applied to monitor the variation that can arise in these products.
Chapter 5 - Concluding Discussion:

The primary objective of this project was to identify reliable and reproducible methods that can be employed to detect key pharmacokinetic interactions with complementary products and conventional medications.

5.1 Interaction Potential

The inescapable reality is that majority of patients resorting to the use of complementary products are likely to be treated with prescription or over the counter drugs (MacLennan et al., 2002, Eisenberg et al., 1998).

Additionally these patients see multiple therapists along with medical practitioners. In combination this is a basis for significant therapeutic problems. The therapeutic problems are primarily product interactions with prescription and over the counter medications. The pharmacokinetic interactions can be grouped into four major areas Figure 5-1.
Figure 5-1: Pharmacokinetic interactions based on absorption, protein binding, metabolism and excretion (Baxter, 2010).
The experimental work presented in this thesis addresses pharmacokinetic interaction issues that relate to absorption, protein binding and metabolism. Studying these with pure compounds is relatively uncomplicated however complementary products presents a number difficult problems which must be solved in order to conduct any meaningful studies with them. These problems include:

- Product selection.
- Extraction method.
- Constituent concentration.

We have addressed these for the purposes of the experimental work presented here and as possible criteria for regulatory studies that we recommend.

### 5.1.1 Product Selection

Products are initially selected for testing based on the likelihood for concurrent use with therapeutic agents with a high risk. This high risk may be due to a narrow therapeutic index (i.e.: digoxin, zidovudine, warfarin and phenytoin) or because of a potential synergistic effect between the complementary product and the therapeutic drug (i.e.: the anticoagulant warfarin and the herbal treatment for blood stagnation Danshen). In the case of a specific investigation i.e.: binding to albumin, a product may be selected for testing if there is likely to be concurrent use with a therapeutic agent known to have significant binding to albumin.
The product selection for the experimental purposes here also serves as a guideline for practitioners of complementary products in their practice. They need to be vigilant when products are concurrently used with selected conventional medicines.

5.1.2 Extraction Method

Another challenge associated with complementary products is the choice of extraction method. For these studies, the choice was made on the basis of the relevance of the extract to the patient use of the product. As these products are generally administered as tablets or infusions (‘teas’) an aqueous and alcohol (methanol) extract was prepared. Methanol was selected to extract the distinctly more lipid soluble components. There was never an intention to fractionate these products into their individual constituents as they are consumed as complex mixtures and therefore should be studied in this form.

5.1.3 Constituent Concentration

In interaction studies with pure substances molar concentrations are easily calculated. With complementary products, which are complex mixtures this is not possible. A different approach is needed to achieve meaningful results.

The chemical composition of these products is not always known and even in cases where it is known, it may be highly variable. A reference point that can be used is the recommended dose and the manufacturers labelling as it reflects what the patients will consume. It will be reflective of plasma concentrations of the various active and inactive constituents.
As the products used were manufactured in Australia under GMP, the information contained on the product label was assumed to be accurate. In absence of chemical profiling, routine studies with these products can be based on this criterion. Whilst it is possible to determine the concentration of the labelled ingredient this was not conducted with these studies as the labelled ingredient may not be responsible for the activity seen in the assays. A better approach would be to determine if a product requires further investigation and from that point, fractionate and identify the component responsible for the activity seen.

5.1.4 Metabolites

The absorption, distribution, metabolism and elimination (ADME) of a xenobiotic are often considered distinct from each other but a series of complex interactions occurs between these processes and the xenobiotic (Figure 5-2).
As most complementary products are taken orally, the bioavailability is low or reduced due to incomplete absorption. Orally absorbed products may be subjected to extensive metabolism, most commonly in the intestine and/or the liver. These metabolites can be the cause of a pharmacokinetic interactions or responsible for direct toxicity. The complexity of these products may give rise to multiple metabolites.

Ginkgo biloba is a herbal product that is known to have key metabolites that have their own kinetic profile. Several of the parent ginsenosides are converted to metabolites by gut microbiota. The metabolite Compound K, possess significantly stronger cancer chemoprevention activity than the parent compounds.
Pharmacokinetically, the active metabolites of ginseng differ in distribution and clearance from that of the parent compound (Qi et al., 2011).

The example of Ginkgo biloba highlights the need to investigate the potential for metabolites of complementary products to cause adverse interactions. These studies would involve the generation of the product metabolites by incubating the parent product with the major metabolising systems of the body: the gastrointestinal tract and the liver.

5.2 Conclusions From the Experimental Work

With the experimental work, 24 of the 34 extracts tested were found to result in pharmacokinetic interactions (Table 5-1).

The interactions involve protein binding, transport protein and CYP inhibition. Detailed results for each of the parameters were previously detailed; protein binding (Table 2-5 and Table 2-6), P-glycoprotein inhibition (Table 3-4 and Table 3-5) and CYP inhibition (Table 4-7 and Table 4-9).
Table 5-1: Summary of the experimental work for the pharmacokinetic parameters investigated with the extracts of complementary products.

<table>
<thead>
<tr>
<th>Product</th>
<th>Extract</th>
<th>HSA</th>
<th>AGP</th>
<th>P-gp</th>
<th>CYP450</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Site I</td>
<td>Site II</td>
<td>Calcein AM</td>
<td>ATPase</td>
</tr>
<tr>
<td>Astragalus</td>
<td>Aq</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Astragalus</td>
<td>Meth</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Black cohosh</td>
<td>Aq</td>
<td></td>
<td></td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Black cohosh</td>
<td>Meth</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CoEnzyme Q10</td>
<td>Aq</td>
<td>X</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CoEnzyme Q10</td>
<td>Meth</td>
<td>X</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cordyceps</td>
<td>Aq</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cordyceps</td>
<td>Meth</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Danshen</td>
<td>Aq</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Danshen</td>
<td>Meth</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dong quai</td>
<td>Aq</td>
<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Dong quai</td>
<td>Meth</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Echinacea</td>
<td>Aq</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Echinacea</td>
<td>Meth</td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Ginkgo Biloba</td>
<td>Aq</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Ginkgo Biloba</td>
<td>Meth</td>
<td>X</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Goldenseal</td>
<td>Aq</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Goldenseal</td>
<td>Meth</td>
<td>*</td>
<td></td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Gotu Kola</td>
<td>Aq</td>
<td></td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Gotu Kola</td>
<td>Meth</td>
<td></td>
<td>X</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>Horse Chestnut</td>
<td>Aq</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Horse Chestnut</td>
<td>Meth</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Horsetail</td>
<td>Aq</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Horsetail</td>
<td>Meth</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liquorice Root</td>
<td>Aq</td>
<td></td>
<td>X</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>Liquorice Root</td>
<td>Meth</td>
<td></td>
<td></td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>Milk Thistle</td>
<td>Aq</td>
<td></td>
<td>X</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>Milk Thistle</td>
<td>Meth</td>
<td></td>
<td></td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>Saw Palmetto</td>
<td>Aq</td>
<td></td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Saw Palmetto</td>
<td>Meth</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slippery Elm</td>
<td>Aq</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slippery Elm</td>
<td>Meth</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Valerian</td>
<td>Aq</td>
<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Valerian</td>
<td>Meth</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Aq - Aqueous extracts  Meth - Methanolic extracts
* - Significant interaction  X - No significant interaction
HSA - Binding to human serum albumin, site I and II. Significance at $K_d < 1$mg/ml.
AGP - Binding to alpha-1-acid glycoprotein. Significance at \( \text{K}_d < 1 \text{mg/ml} \).
P-gp inhibition - Calcein AM extrusion. Significance at IC\(_{50} < 100 \mu\text{g/ml}\).
P-gp inhibition - ATPase activity. Significance at IC\(_{50} < 100 \mu\text{g/ml}\).
CYP450 inhibition - Single substrate assays. Significance at IC\(_{50} < 100 \mu\text{g/ml}\).
CYP450 inhibition - N-in-one cocktail assays. Significance at IC\(_{50} < 100 \mu\text{g/ml}\).
5.3 General Recommendations

In addition to recognising the interactions discovered here that deserve vigilance in clinical use, the following general recommendations to improve the safety of complementary products are proposed based on the literature.

Due to the complex nature of these products and the specific challenges associated with them, pharmacovigilance needs to be expanded from the categories that are currently used with conventional medicines. Additional categories required include the origin of the product, presence of contaminant and adulterants and the name attributed to the product (traditional, botanical, common). If this information was available to regulators worldwide then consistent risk assessments could be carried out and the risk to patients dramatically decreased.

Table 5.2, summarises the information that should be provided to regulatory authorities, these parameters have been recommended based on the required information to decrease patient risk and ensure product consistency for consumers.
Table 5-2: Parameters recommended for inclusion in the information submitted to regulators and the basis of the recommendations.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Basis</th>
<th>Recommendation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive product identification</td>
<td>Misidentification of raw material has lead to toxicity, <em>Aristolochia fangchi</em>, which is nephrotoxic, was mistakenly identified as the safe <em>Stephania tetrandra</em>.</td>
<td>Identification should be made by a qualified botanist and confirmed with genetic identification.</td>
</tr>
<tr>
<td>Source of the raw material</td>
<td>Geographical location can alter the composition of the raw material.</td>
<td>Confirmation of collection site, location and date of raw material to be provided.</td>
</tr>
<tr>
<td>Plant parts used</td>
<td>Chemical composition varies with the part of the plant i.e.: root, leaves, flowers, to be used.</td>
<td>Correct identification of which part of the plant, or in the case of multiple parts how much of each part, to be provided.</td>
</tr>
<tr>
<td>Constituent composition</td>
<td>Composition of the raw material is altered due to many factors including, geographical location, season, and soil conditions.</td>
<td>Chemical identification to be provided, marker compounds may be used if well characterized.</td>
</tr>
<tr>
<td>Historical use</td>
<td>The history of the use of the product may give an indication of toxicity and will also provide information on the patient group most likely to consume this product.</td>
<td>Full history from the relevant pharmacopeia to be provided.</td>
</tr>
<tr>
<td>Intended use</td>
<td>Intended use of the product will give an indication of the patient group likely to consume this product. The risk of concurrent use with therapeutic agents can be established.</td>
<td>Therapeutic claims and the condition/disease for which the product is to be clearly stated. Evidence for therapeutic claims must be provided.</td>
</tr>
<tr>
<td>Quality control in manufacturing</td>
<td>Lack of good manufacturing practice resulted in widespread withdrawal of substandard products and eventual closure of Pan Pharmaceuticals.</td>
<td>Therapeutic agents and foods are all required to manufactured under GMP. Complementary products to be subjected to the same manufacturing regulations.</td>
</tr>
<tr>
<td>Microbial content</td>
<td>Contamination may arise during processing or storage with bacterial and fungal elements.</td>
<td>Testing for microbial contamination and their toxic secondary metabolites i.e. mycotoxins.</td>
</tr>
<tr>
<td>Heavy metal content</td>
<td>Heavy metals including lead and arsenic are known toxic contaminants of particularly Ayurvedic products.</td>
<td>Testing to ensure that heavy metals are at a safe level for consumers.</td>
</tr>
<tr>
<td>Adulterant detection</td>
<td>Adulterants particularly therapeutic agents (steroids, analgesics, sedatives and erectile dysfunction drugs) are frequently found in herbal products to enhance the therapeutic claims.</td>
<td>Testing to be conducted to ensure adulterants are not present or if they are added appropriate regulatory approval must be sought.</td>
</tr>
</tbody>
</table>
Toxicological information
A number of example of direct toxicity have been reported over many years including Aristolochia and Danshen.
In absence of unequivocal historical evidence of safety, standard pre-clinical safety evaluation as well as ADME properties should be conducted.

Pharmacovigilance
Both direct toxicity and interactions of low frequency can be only detected by observing substantial patient populations
A harmonized reporting system for these products to allow rapid identification adverse events.
5.4 Future Directions

This work provides clear directions for investigating pharmacokinetically based interactions involving complementary products. Specifically, plasma protein binding and displacement interactions, inhibition of cytochrome P450 and inhibition of P-glycoprotein. The obvious extension of this work should include potential drug interactions due to other pharmacokinetic parameters:

- CYP induction.
- Induction of p-glycoprotein.
- Inhibition and induction of other drug transporters including the organic anion and cation transporters.
- Inhibition and induction of phase II metabolism enzymes including sulfotransferases and glutathione S-transferase.
- The interactions caused by the metabolites of the products.

As many of these pharmacokinetic parameters are connected there is also work that can be conducted on the interplay that is possible in the in vivo situation.

There is adequate justification to extend this line of research to a range of complementary products.
**Figure 5-3:** Schematic representation of the interplay of transporters and enzymes in the hepatocyte. Cytochrome P450 (CYP) and other hepatic enzymes such as epoxide hydrolase (EH), conjugating enzymes, UDP glucuronyl transferase (UGT), sulphotransferase (SULT) or glutathione S-transferase (GST) are important in metabolic handling of xenobiotics. The enzymes depend on molecular oxygen and NADPH (CYP), UDPG (UGT), PAPS (SULT) and reduced glutathione (GSH) (GST). The cellular membrane has many transporters, OATS, ABC transporters and OCTS, which are important for the transmembrane flux of many xenobiotics including the products of the conjugating enzymes (Sevior et al., 2012).
Another approach can be developed based on the plant families. By working with families of plants, it may be possible to identify broader classifications of raw material that have similar drug interactions. This should focus the research to the products most likely to cause adverse drug interactions.

Finally, there are the so-called ‘omics’ technologies that may provide unexpected advances in the identification and ongoing monitoring of these products. In particular the NMR and LC-MS based metabonomics can track biomarkers that could reveal both adverse effects and interactions in vivo (Ouedraogo et al., 2012, Liang et al., 2010). Additionally these technologies have applications in identifying the components of these products and chemical variability (Yang et al., 2012, Sheridan et al., 2012).

Evidence for using gene based ‘omics’ technologies with interesting outcomes has involved products such as kava-kava. In these studies kava-kava was administered to rats and subsequent genome wide gene-expression revealed differences in the expression of drug metabolizing enzymes from the control group, and alterations in the mitochondrial function and oxidative stress response pathways (Guo et al., 2010b, Guo et al., 2009). Microarrays have also been used to identify gene expression changes. Ginkgo Biloba has been shown to alter a number of genes in a dose dependent manner (Guo et al., 2010a).
This work has outlined approaches to study complex and variable mixtures such as complementary products and made recommendations for improving the patient safety in face of a bewildering array of herbal and other complementary products which have a total market value of some $83 billion per year. The dollar value makes it entirely possible to implement most of these recommendations.
Chapter 6 - Appendix A:

This appendix contains all the data relating to the inhibition of cytochrome P450 for the aqueous and methanolic extracts for the 10 complementary products (Table 4-3). The raw data is shown for the single substrate and the N-in-one cocktail incubations.
Figure 6-1: % Inhibition of control for individual incubations for the extracts of Black Cohosh at 20, 100 and 500 µg/ml for each of the CYP isoform assays using the single substrate incubations.

(●) Aqueous extracts (▲) Methanolic extracts
Figure 6-2: % Inhibition of control for individual incubations for the extracts of Dong Quai at 20, 100 and 500 µg/ml for each of the CYP isoform assays using the single substrate incubations.

(●) Aqueous extracts (▲) Methanolic extracts
Figure 6-3: % Inhibition of control for individual incubations for the extracts of Goldenseal at 20, 100 and 500 µg/ml for each of the CYP isoform assays using the single substrate incubations.

(●) Aqueous extracts (▲) Methanolic extracts
Figure 6-4: % Inhibition of control for individual incubations for the extracts of Gotu Kola at 20, 100 and 500 µg/ml for each of the CYP isoform assays using the single substrate incubations.

(●) Aqueous extracts (▲) Methanolic extracts
Figure 6-5: % Inhibition of control for individual incubations for the extracts of Horse Chestnut at 20, 100 and 500 μg/ml for each of the CYP isoform assays using the single substrate incubations.

(●) Aqueous extracts (▲) Methanolic extracts
Figure 6-6: % Inhibition of control for individual incubations for the extracts of Horsetail at 20, 100 and 500 µg/ml for each of the CYP isoform assays using the single substrate incubations.

(●) Aqueous extracts (▲) Methanolic extracts
Figure 6-7: % Inhibition of control for individual incubations for the extracts of Liquorice Root at 20, 100 and 500 µg/ml for each of the CYP isoform assays using the single substrate incubations.

(●) Aqueous extracts (▲) Methanolic extracts
Figure 6-8: % Inhibition of control for individual incubations for the extracts of Milk Thistle at 20, 100 and 500 µg/ml for each of the CYP isoform assays using the single substrate incubations.

(●) Aqueous extracts (▲) Methanolic extracts
Figure 6-9: % Inhibition of control for individual incubations for the extracts of Saw Palmetto at 20, 100 and 500 μg/ml for each of the CYP isoform assays using the single substrate incubations.

(●) Aqueous extracts (▲) Methanolic extracts
Figure 6-10: % Inhibition of control for individual incubations for the extracts of Valerian at 20, 100 and 500 µg/ml for each of the CYP isoform assays using the single substrate incubations.

(●) Aqueous extracts (▲) Methanolic extracts
Figure 6-11: % Inhibition of control for individual incubations for the extracts of Black Cohosh at 20, 100 and 500 µg/ml for each of the CYP isoform assays using the N-in-one cocktail assay.

(●) Aqueous extracts (▲) Methanolic extracts
Figure 6-12: % Inhibition of control for individual incubations for the extracts of Dong Quai at 20, 100 and 500 µg/ml for each of the CYP isoform assays using the N-in-one cocktail assay.

(●) Aqueous extracts (▲) Methanolic extracts
Figure 6-13: % Inhibition of control for individual incubations for the extracts of Goldenseal at 20, 100 and 500 µg/ml for each of the CYP isoform assays using the N-in-one cocktail assay.

(●) Aqueous extracts (▲) Methanolic extracts
Figure 6-14: % Inhibition of control for individual incubations for the extracts of Gotu Kola at 20, 100 and 500 μg/ml for each of the CYP isoform assays using the N-in-one cocktail assay.

(●) Aqueous extracts (▲) Methanolic extracts
Figure 6-15: % Inhibition of control for individual incubations for the extracts of Horse Chestnut at 20, 100 and 500 µg/ml for each of the CYP isoform assays using the N-in-one cocktail assay.

(●) Aqueous extracts (▲) Methanolic extracts
Figure 6-16: % Inhibition of control for individual incubations for the extracts of Horsetail at 20, 100 and 500 μg/ml for each of the CYP isoform assays using the N-in-one cocktail assay.

(●) Aqueous extracts (▲) Methanolic extracts
Figure 6-17: % Inhibition of control for individual incubations for the extracts of Liquorice Root at 20, 100 and 500 μg/ml for each of the CYP isoform assays using the N-in-one cocktail assay.

(●) Aqueous extracts (▲) Methanolic extracts
Figure 6-18: % Inhibition of control for individual incubations for the extracts of Milk Thistle at 20, 100 and 500 μg/ml for each of the CYP isoform assays using the N-in-one cocktail assay.

(●) Aqueous extracts (▲) Methanolic extracts
Figure 6-19: % Inhibition of control for individual incubations for the extracts of Saw Palmetto at 20, 100 and 500 μg/ml for each of the CYP isoform assays using the N-in-one cocktail assay.

(○) Aqueous extracts (▲) Methanolic extracts
Figure 6-20: % Inhibition of control for individual incubations for the extracts of Valerian at 20, 100 and 500 µg/ml for each of the CYP isoform assays using the N-in-one cocktail assay.

(○) Aqueous extracts (▲) Methanolic extracts
Chapter 7 – References:


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