EFFECT OF GINSENG EXTRACTS ON THE GENE EXPRESSION OF HEPATIC DRUG-METABOLIZING ENZYMES IN RATS

by

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B.Sc., Taipei Medical College, Taipei, Taiwan, 2000

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We accept this thesis as conforming to the required standard

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ABSTRACT

The present study was conducted to investigate the effect of *Panax ginseng* and *Panax quinquefolius* extracts of known ginsenoside composition on rat hepatic cytochrome P450 (CYP) and microsomal epoxide hydrolase (mEH) gene expression. Adult male Sprague-Dawley rats (250-300 g) were administered by oral gavage *Panax ginseng* extract (30 or 100 mg/kg single dose), *Panax quinquefolius* extract (100 or 400 mg/kg once daily for 21 consecutive days), or an equivalent volume (2 ml/kg) of 0.9% NaCl (vehicle control). All the rats were terminated one day after the last dose. The administration of *Panax ginseng* extract or *Panax quinquefolius* extract did not affect: 1) body weight gain, 2) absolute or relative liver weight; 3) absolute or relative testes weight; 4) hepatic microsomal total CYP content; 5) hepatic CYP2B1, CYP3A23, CYP2C11, CYP1A2 and mEH mRNA expression, as determined by validated real-time PCR assays; 6) hepatic microsomal 7-benzyloxyresorufin O-dealkylation and 7-ethoxyresorufin O-dealkylation activities; or 7) hepatic CYP2C11 and mEH protein expression. However, results from the positive control experiments indicated that: 1) phenobarbital (80 mg/kg i.p. once daily for 4 consecutive days) increased hepatic CYP2B1 mRNA (47-fold), microsomal 7-benzyloxyresorufin O-dealkylation activity (37-fold), and hepatic mEH mRNA (17-fold) and protein (2-fold); 2) dexamethasone (100 mg/kg i.p. once daily for 3 consecutive days) increased hepatic CYP3A23 mRNA (75-fold); and 3) β-naphthoflavone (40 mg/kg i.p. once daily for 3 consecutive days) decreased hepatic CYP2C11 protein (85%) and increased hepatic CYP1A2 mRNA (20-fold) and microsomal 7-ethoxyresorufin O-dealkylation activity (19-fold). Therefore, the lack of an effect by the ginseng extracts on CYP and mEH expression was not due to the assay conditions. In another control experiment, the administration of albendazole (20 mg/kg once daily for 3 consecutive days) by oral gavage increased hepatic microsomal 7-ethoxyresorufin O-dealkylation activity by 21-fold, indicating that the lack of an effect by the ginseng extracts was not a consequence of improper technique in drug administration by oral gavage. In conclusion, *Panax ginseng* and *Panax quinquefolius* extracts, at the dosage regimens used in the present study, did not influence hepatic CYP2B1, CYP3A23, CYP2C11, CYP1A2, or mEH gene expression in adult male rats.
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A2 Effect of *Panax quinquefolius* extract on hepatic CYP2C11 protein expression
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<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolylphosphate</td>
</tr>
<tr>
<td>Bis</td>
<td>N,N'-methylene-bis-acrylamide</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>maximal concentration</td>
</tr>
<tr>
<td>CAR</td>
<td>constitutive androstane receptors</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<tr>
<td>CV</td>
<td>coefficient of variation</td>
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<tr>
<td>DDT</td>
<td>dichlorodiphenyltrichloroethane</td>
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<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>EH</td>
<td>epoxide hydrolase</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal injection</td>
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<td>kg</td>
<td>kilogram</td>
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<td>mEH</td>
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<tr>
<td>MOPS</td>
<td>4-morpholinepropanesulfonic acid</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate, reduced form</td>
</tr>
<tr>
<td>NBT</td>
<td>nitroblue tetrazolium chloride</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>CYP</td>
<td>cytochrome P450</td>
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<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PXR</td>
<td>pregnane X receptor</td>
</tr>
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<td>ribonuclease</td>
</tr>
<tr>
<td>RXR</td>
<td>retinoid X receptor</td>
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<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<td>SEM</td>
<td>standard error of the mean</td>
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<td>TCDD</td>
<td>2,3,7,8-tetrachlorodibenzo-p-dioxin</td>
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<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylenediamine</td>
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<tr>
<td>TRIS</td>
<td>tris [hydroxymethyl] aminomethane</td>
</tr>
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<td>UV</td>
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</tr>
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<td>V</td>
<td>voltage</td>
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CHAPTER 1. INTRODUCTION
Ginseng is one of the most commonly consumed herbs in Canada and is the focus of the present study. In North America, alternate therapies such as herbal remedies have become increasingly popular and the market for sales of herbal medicines was estimated to be $4 billion per year (Gruenwald, 2000). In Canada, the ten most commonly used herbs are Echinacea, garlic, Gingko biloba, St John’s wort, ginseng, evening primrose oil, Devil’s claw, lecithin, saw palmetto and valerian (The Nonprescription Drug Manufacturers Association of Canada, 1999). Of particular interest and given the suitable natural environment, British Columbia has become one of the most important locations for the cultivation of ginseng in North America (Soldati, 2000).

Evidence from case reports suggests that there may be interactions between ginseng and drug-metabolizing enzymes (Izzo and Ernst, 2001; Scott and Elmer, 2002;). However, this aspect has not been intensively investigated. The focus of this thesis is to investigate the effect of ginseng extracts on the expression of drug metabolizing-enzymes, such as cytochromes P450 (CYP) enzymes and microsomal epoxide hydrolase (mEH). Thus, a brief overview of the biological and adverse effects of various ginseng extracts will be discussed, with emphasis on the interaction with CYP enzymes.
1.1 HERBAL MEDICINE

Herbal medicines are isolated from plants and are used for their medicinal properties (Bateman et al., 1998). The market of herbal medicinal products has expanded rapidly in the past few decades. In 1999, the total sales of herbal medicinal products in the Canada were estimated to be more than $77.5 million dollars (The Nonprescription Drug Manufacturers Association of Canada, 1999). Consumers who use herbal products believe natural products can provide a certain level of benefit, either in preventing or alleviating the symptoms of illness (Bateman et al., 1998). However, warnings about known or potential adverse effects are not specifically required on these product labels. The general public usually assumes that natural products, unlike conventional medicines, are safe and have no side effects, even at high dosages. It has been reported that 31% of the patients who use herbal supplements do so in conjunction with prescribed drugs, and approximately 70% of patients who take herbs do not inform their physicians and pharmacists (Miller et al., 1998; Abebe, 2002). One of the biggest concerns regarding the concurrent ingestion of these products with conventional medicines is the potential risk of undesired consequences, including drug-drug interactions (Bateman et al., 1998). For instance, ginseng (Panax ginseng) has been found to decrease blood clotting time in patients taking warfarin concurrently (Janetzky and Morreale, 1997), and tremulousness and manic symptoms such as hallucinations have been described in some case reports when ginseng (Panax ginseng) and phenelzine were coadministered (Shader and Greenblatt, 1985).
1.2 GINSENG

Ginseng has been used in Asia for more than 3000 years in the treatment of various medical disorders. It was not well known to the Western world until the 18\textsuperscript{th} century (Huang, 1999). Since then, it has become one of the most commonly used herbal products in Europe and North America, and it is listed in the Swiss, German, Austrian and French pharmacopoeias (Liu \textit{et al.}, 1992; Attele \textit{et al.}, 1999; Soldati, 2000).

1.2.1 TYPES OF GINSENG

Several types of ginseng have been discovered to date, such as \textit{Panax}, \textit{Eleutherococcus} (or \textit{Acanthopanax}) \textit{senticus}, \textit{Lepidium meyenii} Walp., and \textit{Tetragonotheca helianthoides} \textit{L. ginseng}. The majority of these ginsengs are distributed in East Asia, Central Asia, and North America (Huang, 1999). Generally, the term ginseng refers to products prepared from the \textit{Panax} genus (Harkey \textit{et al.}, 2001). Among all the \textit{Panax} species that have been identified thus far, \textit{Panax ginseng} (Asian ginseng) and \textit{Panax quinquefolius} (American ginseng) are the most common species currently on the market (Morgan and Coop, 2000). Both species are low-growing perennial shade plants and belong to the family of \textit{Araliaceae}. The focus of this study is on the \textit{Panax ginseng} and \textit{Panax quinquefolius} extracts.

1.2.2 CHEMICAL CONSTITUENTS IN \textit{PANAX GINSENG} AND \textit{PANAX QUINQUEFOLIUS} EXTRACTS

Ginseng root is the main source of active constituents and is generally used to manufacture commercially available ginseng products, whereas other parts of the plant,
including leaves, stalks and stems, possess relatively lower concentrations of active constituents (Soldati, 2000). More than two hundred bioactive compounds have been isolated and identified in *Panax* genus, including ginsenosides, oligosaccharides, polysaccharides, oligopeptides, poly-acetylenes, phenols and fatty acids (Soldati, 2000). Among these compounds, ginsenosides are considered to be responsible for its various biological and pharmacological effects (Attele *et al.*, 1999). They are also commonly used as reference compounds to authenticate ginseng products produced by manufacturers (Soldati, 2000).

Ginsenosides are unique to the *Panax* species (Harkey *et al.*, 2001). Ginsenosides are glycosylated derivatives of aglycones that contain a 4 *trans*-ring rigid steroid skeleton with a modified side chain at the C-20 position, except for ginsenoside Ro, in which the steroid structure is absent (Attele *et al.*, 1999; Harkey *et al.*, 2001). Due to their chemical characteristics, ginsenosides have also been referred to as ginseng saponins, ginsenoside saponins, triterpenoid saponins, and dammarane derivatives (Attele *et al.*, 1999). The chemical structures of ginsenosides are illustrated in Figure 1. Based on their number, type, and location of the sugar moieties, ginsenosides can be categorized into three types: protopanaxadiols, protopanaxatriols and oleanic acid. In addition to the sugar moiety, ginsenosides also differ in stereochemistry at the C-20 position and the number and location of hydroxyl groups. The considerable structural variation may explain the diverse pharmacological effects of ginseng discussed in section 1.2.3 (Attele *et al.*, 1999).
Currently, more than twenty ginsenosides have been isolated from ginseng root, including \( \text{Rb}_1 \), \( \text{Rb}_2 \), \( \text{Rc} \), \( \text{Rd} \), \( \text{Re} \), \( \text{Rf} \), \( \text{Rg}_1 \), and \( \text{Rg}_2 \) (Attele et al., 1999). The distribution and proportion of ginsenosides vary among different Panax species and this may be used to differentiate between species (Harkey et al., 2001). For example, when compared to Panax ginseng, Panax quinquefolius has a relatively small amount of \( \text{Rb}_2 \); a greater amount of \( \text{Rb}_1 \), \( \text{Rc} \), \( \text{Rd} \) and \( \text{Re} \); a lower ratio of \( \text{Rg}_1 \) to \( \text{Rb}_1 \); and it lacks \( \text{Rf} \) (Morgan and Cupp, 2000; Harkey et al., 2001). Moreover, depending on the cultivation conditions, including harvest time, climate, and soil, the variability in ginsenoside content can be considerable (2% to 20%), even within the same species (Attele et al., 1999; Soldati, 2000; Harkey et al., 2001). This could explain the potency differences in pharmacological effects within the same species cultivated in different locations (Yuan et al., 1998; Attele et al., 1999).
Figure 1. Chemical structures of ginsenosides  Based on the chemical structures, ginsenosides can be
categorized into three groups: protopanaxdiols (A), protopanaxtriols (B) and oleanic acid (C).
(a. glc = glucose; b. arap = pyranose; c. araf = furanose; d. rha = rhamnose)
1.2.3 THERAPEUTIC USES OF PANAX GINSENG AND PANAX QUINQUEFOLIUS EXTRACTS

Panax ginseng and Panax quinquefolius are generally used to enlighten mood, enhance mental and physical abilities, boost energy and improve well-being (Attele, et al., 1999; Vogler et al., 1999; Coleman, et al., 2003). Several studies (Vogler et al., 1999; Coleman, et al., 2003) were conducted on participants taking ginseng alone or supplemented with other vitamins and minerals. Questionnaires were used to assess the effects of Panax ginseng and Panax quinquefolius extracts on the aspects indicated above and also on the improvement of the quality of life. However, the study was unable to directly correlate these beneficial effects with Panax ginseng or Panax quinquefolius extracts (Vogler et al., 1999; Coleman, et al., 2003).

1.2.4 ADVERSE EFFECTS OF PANAX GINSENG AND PANAX QUINQUEFOLIUS EXTRACTS

In animal studies conducted in both male and female beagle dogs administered Panax ginseng extract (Panax ginseng extract, G115\textsuperscript{®}, Pharmaton SA, Lugano, Switzerland) (1.5, 5, or 15 mg/kg, blended in the diet, for 90 days), no toxicological or treatment-related adverse effects were observed in the gross autopsy examination, urinary analysis (pH, glucose, total protein, occult blood, ketones, bile specific gravity and microscopic examination of the sediment) and hematology analysis (erythrocyte count, differential and total leucocyte counts, haematocrit, platelet count, reticulocyte count, sedimentation rate, levels of haemoglobin, glucose, urea nitrogen, total protein, bilirubin, sodium, chloride, potassium, and activities of serum glutamic-pyruvic
transaminase, serum glutamic-oxaloacetic transaminase and alkaline phosphatase (Hess et al., 1983). In a separate study that investigated the effect of ginseng on offspring development, both male and female Sprague-Dawley rats (F0 generation) were treated with *Panax ginseng* extract (*Panax ginseng* extract, G115®, Pharmaton SA, Lugano, Switzerland) (1.5, 5, or 15 mg/kg, blended in the diet, for three weeks). In this study, no toxicological or treatment-related adverse effects (including detailed hematology, urinary, and gross autopsy examinations as described above) were observed in the F0, F1, or F2 generations (Hess et al., 1982).

According to the *Food and Drug Act* from the Canadian Food Inspection Agency (CFIA), Health Canada, ginseng products currently marketed in Canada are categorized as food instead of therapeutic agents. Therefore, they are not regulated and manufacturers are not required to provide label warnings on the potential adverse effects or drug interactions (Canadian Food Inspection Agency, Health Canada, 2003). In the United States, ginseng is listed on the FDA's GRAS (generally recognized as safe) list. Adverse effects associated with ginseng have been observed in human subjects as outlined in Table 1. It should be noted that most of these case reports do not provide information regarding the preparations, manufacturers, or even the genus of the plants prepared for these ginseng products. Therefore, the reported observations should be evaluated objectively.
TABLE 1

Adverse effects of commercially available ginseng products

<table>
<thead>
<tr>
<th>Condition</th>
<th>Genus of ginseng</th>
<th>Product Name (Manufacture)</th>
<th>Other supplemented compounds</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Hammond and Whitworth, 1981</td>
</tr>
<tr>
<td>Vaginal bleeding</td>
<td>Panax ginseng</td>
<td>Geriatric® Pharmaton</td>
<td>minerals and vitamins</td>
<td>Greenspan 1983</td>
</tr>
<tr>
<td>Deteriorated psychiatric condition</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Gonzalez-Seijo et al., 1995</td>
</tr>
<tr>
<td>Diuretic resistance</td>
<td>N/A</td>
<td>Uncle Hsu's Korean ginseng</td>
<td>germanium</td>
<td>Becker et al., 1996</td>
</tr>
<tr>
<td>Gynaecomastia</td>
<td>Panax ginseng</td>
<td>Geriatric® Pharmaton</td>
<td>minerals and vitamins</td>
<td>Palop et al., 1999</td>
</tr>
<tr>
<td>Metrorrhagia</td>
<td>Panax ginseng</td>
<td>Pharmaton® Complex</td>
<td>dimethylaminoethanol bitartrate, vitamins, and minerals</td>
<td>Palop-Larrea et al., 2000</td>
</tr>
</tbody>
</table>

1.2.5 BIOLOGICAL EFFECTS OF PANAX GINSENG EXTRACT, PANAX QUINQUEFOLIUS EXTRACT, AND GINSENSIDES

Panax ginseng extract, Panax quinquefolius extract and individual ginsenosides have a wide range of biological effects involving the central nervous system, cardiovascular system and immunological system shown in Table 2. Furthermore, ginseng extracts have also been shown to prevent cancer, modulate metabolism, and augment antioxidant activity (Table 2).
TABLE 2
Summary of the main biological effects of *Panax ginseng* extract, *Panax quinquefolius* extract, and individual ginsenosides

<table>
<thead>
<tr>
<th>Biological effect</th>
<th>Type of Ginseng Extract /Individual Ginsenoside</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modulation of Metabolic State</td>
<td>Hypoglycemia</td>
<td><em>Panax ginseng</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Panax quinquefolius</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rb₁, Rb₂, Re, Rg₁, Rg₃</td>
</tr>
<tr>
<td>Hypolipidemia</td>
<td>Hypoglycemia</td>
<td><em>Panax ginseng</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rb₁, Rb₂, Rc, Rg₁</td>
</tr>
<tr>
<td>Modulation of Immune System</td>
<td>Modulation of immune response</td>
<td>Panax ginseng</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>-------------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Panax quinquefolius</td>
</tr>
<tr>
<td></td>
<td>Rg₁, Ro</td>
<td></td>
</tr>
<tr>
<td>Protection of Cardiovascular Disease</td>
<td>Decrease thrombin-induced endothelin release</td>
<td>Panax quinquefolius</td>
</tr>
<tr>
<td>Adapting cardiac contraction</td>
<td>Rb₁, Re</td>
<td></td>
</tr>
<tr>
<td>Decrease platelet aggregation</td>
<td>Panax ginseng</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rg₁, Rg₂, Rg₃, Ro</td>
<td></td>
</tr>
<tr>
<td>Enhancement of Antioxidant Activity</td>
<td>Protection of free radical-induced oxidation</td>
<td>Panax ginseng</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Panax quinquefolius</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rb₁, Rb₂, Rb₃, Rc, Rd, Re, Rg₁, Rh₂</td>
</tr>
<tr>
<td>Induction of antioxidant enzyme</td>
<td>Panax ginseng</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rb₂</td>
<td></td>
</tr>
<tr>
<td>CNS Effect</td>
<td>Antinarcotic effects</td>
<td>Panax ginseng</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>----------------------</td>
<td>---------------</td>
</tr>
<tr>
<td></td>
<td>Modulatory of neurotransmission</td>
<td>Panax quinquefolius</td>
</tr>
<tr>
<td></td>
<td>Enhance cognitive performance and learned memory</td>
<td>Panax ginseng</td>
</tr>
<tr>
<td>Moderation of Cancer</td>
<td>Antiproliferative effect</td>
<td>Panax ginseng</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Panax quinquefolius</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rg(_3), Rh(_1), Rh(_2)</td>
</tr>
<tr>
<td></td>
<td>Antimetastasis</td>
<td>Rb(_1), Rg(_3), Rh(_2), Rh(_3)</td>
</tr>
</tbody>
</table>
1.2.6 PHARMACOKINETICS OF INDIVIDUAL GINSENOSIDES

The pharmacokinetics of a few ginsenosides has been examined in detail. Chen et al. (1980) demonstrated a large difference in pharmacokinetic parameters between protopanaxdiol (Rb2) and protopanaxtriols (Rg1 and Re). Each ginsenoside was administered to rabbits at the same dosage (500 mg) by intraperitoneal injection. Compared to Rg1 and Re, the protopanaxdiol ginsenoside, Rb2, had a larger elimination half-life (412 minutes; compared to 24.7 and 69.9 minutes for Rg1 and Re, respectively), lower total body clearance (0.18 ml/min/kg; compared to 1.17 and 4.08 ml/min/kg for Rg1 and Re, respectively) and renal clearance (0.07 ml/min/kg; whereas it was 0.71 and 0.72 ml/min/kg for Rg1 and Re, respectively), and lower volume of distribution (115 ml/kg; whereas it was 134 and 246 ml/kg for Rg1 and Re, respectively).

Pharmacokinetic studies in rats focused on ginsenoside Rg1 (100 mg/kg, oral gavage, single dose) and findings indicated that Rg1 was metabolized in the gastrointestinal tract, but not in the liver (Odani et al., 1983). In this study, Rg1 reached $C_{\text{max}}$ (0.9 µg/ml) at 30 minutes, and was undetectable in the serum after six hours. The Rg1 concentrations in the liver and kidney ($C_{\text{max}} = 3.5 \pm 2.0$ and $2.6 \pm 1.5$ µg/g within 1.5 hours, respectively) were higher than other organs (below 1.5 µg/g at all times), including lung, heart, brain, and spleen. Approximately 15 minutes after administration, the recoveries of Rg1 in the stomach and small intestine were 42.3 ± 1.6% and 35.6 ± 4.3% of the dose, respectively. Within 24 hours, the cumulative urinary and fecal excretions of Rg1 were 0.40 ± 0.04% and 41.2 ± 2.6% of the dose. The significant portion of the dose that is mainly eliminated via the feces may be due to poor oral absorption.
The same research group also suggested a similar pattern of disposition of a representative protopanaxdiol, Rb₁ (Odani et al., 1983²). Approximately 15 minutes after the administration, the recoveries of ginsenoside Rb₁, (100 mg/kg, oral gavage, single dose) in the stomach and small intestine were 35 ± 8.5 and 43.0 ± 13.6% of the dose, respectively. Within 360 minutes post-administration, the concentrations of Rb₁, were less than 0.2 µg/ml in the serum and in various tissues (liver, kidney; heart, lung, spleen, and brain). The cumulative urinary and fecal excretions of Rb₁, were 0.05 ± 0.01% (within 48 hours) and 10.8 ± 1.5% (within 24 hours) of the dose, respectively. After the administration, the maximum excretion of Rb₁, into urine and feces occurred from 12 to 18 hours and 6 to 12 hours, respectively. The authors thus suggested that Rb₁, like Rg₁, is also biotransformed in the gastrointestinal tract.

Previous studies using rat models have shown that the orally administered ginsenosides (100 mg/kg, oral gavage, single dose), Rg₁ and Rb₁, were subjected to biotransformation in the gastrointestinal tract by bacteria and enzymatic hydrolysis (Odani et al., 1983³). Evidence supporting this finding was reported in both in vivo (Karikura et al., 1990; Karikura et al., 1991¹,²; Wakabayashi et al., 1997) and in vitro (Hasegawa et al., 1997) studies.

1.2.7 DRUG INTERACTIONS WITH PANAX GINSENG AND PANAX QUINQUEFOLIUS EXTRACTS

Interactions between conventional medicines and Panax ginseng or Panax quinquefolius extracts have not been extensively studied and are documented mostly as case reports. Also, there is only one reported clinical trial with ginseng.
Case report #1: Symptoms of headache and tremulousness were reported in a case in which a 64-year-old female patient took phenelzine (45 mg/day) with either ginseng tea or a California fad product, "Natrol High", containing *Panax ginseng* extract (Shader and Greenblatt, 1985; Shader and Greenblatt, 1988). However, in this case report, the history of phenelzine treatment, and detailed content information of these herbal products were not listed (Shader and Greenblatt, 1985; Shader and Greenblatt, 1988).

Case report #2: In another case, a 47-year-old male patient with a mechanical heart valve in the aortic position received warfarin (with a history of this treatment for nine months at 5 mg/day and 7.5 mg each Tuesday) and *Panax ginseng* extract capsules (Ginsana®, Pharmaton, Ridgefield, CT, USA) (standardized for eight ginsenosides, 100 mg/capsule, three times daily). His International Normalized Ratio (INR), which was stable for the nine months during warfarin therapy, had declined to a value of 1.5 (target 2.5-3.5) indicating reduced anticoagulant effect. The INR returned to the target range when the patient withdrew the ginseng supplement (Janetzky and Morreale, 1997). However, a rat study investigating the ginseng and warfarin interaction demonstrated that the pharmacological effect and pharmacokinetics of warfarin were not altered by the oral administration of ginseng extract (genus was not mentioned) (2 mg/kg, single dose; or 2 mg/kg/day for six days) in rats (Zhu et al., 1999).

Clinical trial: In an open and non-randomized clinical trial investigating the interaction of ginseng and alcohol, 14 healthy volunteers participated and each individual served as his/her own control. Forty minutes after co-administration of *Panax ginseng* extract (3
g/65 kg body weight) and alcohol (72 g/65 kg), 10 out of 14 subjects showed a 32% to 51% decrease in blood alcohol level when compared to the control values which was obtained when the test subject received alcohol alone (Lee et al., 1987). Evidence from animal studies suggested this reduced blood alcohol level was due to a delay in gastric emptying by ginsenosides (Lee et al., 1993; Koo et al., 1999).

1.3 CYTOCHROME P450

Cytochrome P450 is superfamily of heme-thiolate enzymes. These enzymes play an important role in xenobiotics biotransformation by catalyzing several types of chemical reactions, including hydroxylation, epoxidation, oxidation, dealkylation and dehydrogenation. The term "cytochrome P450" originates from its absorbance maxima at 450 nm when the reduced-hemeprotein forms a complex with carbon monoxide (Omura and Sato, 1964). CYP enzymes are present in almost all living organisms and are responsible for the biotransformation of many xenobiotics (e.g. drugs, dietary components, environmental toxins), and endogenous compounds, (e.g. fatty acids, steroids, prostaglandins and leukotrienes) (Kumar and Surapaneni, 2001; Guengerich, 2001).

Mammalian CYP drug-metabolizing enzymes are expressed in a tissue-dependent manner, primarily in the liver; however, they are also present in extrahepatic tissues, including intestine, testis, lungs, kidneys and brains (Nelson et al., 1996). For instance, CYP2B is expressed predominately in the liver, and it is present at much lower levels in extrahepatic tissues (Suwa et al., 1985). CYP enzymes are mainly found in the smooth endoplasmic reticulum, which can be isolated by differential ultracentrifugation.
(Ryan and Levin, 1990). The isolated smooth endoplasmic reticulum is referred to as microsomes.

CYP enzymes are classified into families and subfamilies based on their amino acid sequences. Enzymes with at least 40% similarity are included in the same family (e.g. CYP1, CYP2, CYP3), whereas those with at least 55% similarity belong in the same subfamily (e.g. CYP1A, CYP2B, CYP3A). Furthermore, each individual enzyme in the same subfamily is designated with an Arabic numeral (e.g. CYP2B1, CYP2B2) (Nelson, 1996).

CYP enzymes are regulated by a number of factors such as exposure to inducing agents. Enzyme induction is defined as an increase in protein synthesis. Many, but not all, of the CYP enzymes are subject to induction (Lin and Liu, 1998). The major inducible rat hepatic CYP enzymes and the enzyme-selective inducers are listed in Table 3. An interesting property of these enzymes is that different compounds can induce the same CYP enzyme, and a compound can induce several different CYP enzymes. Within the same family or subfamily, regulatory elements in each CYP gene vary, which could explain the differences in regulation and expression of closely related genes (Lewis, 1996).

Because each CYP enzyme metabolizes a number of substrates, induction of a CYP enzyme by a drug or other xenobiotic may alter the metabolic profile of a concurrently administrated drug. The effects of drug-drug interactions can sometimes be fatal. For example, rifampin is commonly used in the management of tuberculosis and leprosy and is also one of the most effective inducers for CYP3A. Subtherapeutic plasma concentrations of cyclosporin, an immunosuppressive agent that is metabolized
by CYP3A (Pichard et al., 1990), have frequently resulted in acute allograft rejection when cyclosporin is coadministered with rifampin (Daniels et al., 1984; Modry et al., 1985; Campana et al., 1996).

### TABLE 3
Major inducible rat CYP enzymes, enzyme-selective inducers and postulated induction mechanisms

<table>
<thead>
<tr>
<th>Rat Enzyme</th>
<th>Enzyme Selective Inducer</th>
<th>Receptor/Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A1; CYP1A2</td>
<td>BNF, TCDD, 3-MC</td>
<td>Aryl Hydrocarbon Receptor</td>
</tr>
<tr>
<td>CYP2B1; CYP2B2</td>
<td>PB</td>
<td>Constitutive Androstane Receptor</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glucocorticoid Receptor</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pregnan X Receptor</td>
</tr>
<tr>
<td>CYP3A9; CYP3A18; CYP3A23</td>
<td>Dexamethasone, PCN</td>
<td>Constitutive Androstane Receptor</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pregnan X Receptor</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>Ethanol, Isoniazid</td>
<td>mRNA Stabilization</td>
</tr>
</tbody>
</table>

The abbreviations used are: BNF: β-naphthoflavone; TCDD: 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin; PCN: pregnenolone 16α-carbonitrile; 3-MC: 3-methylcholanthrene; PB: phenobarbital. (Parkinson, 1992; Lewis, 1996; Mahnke et al., 1997; Whitlock et al., 1996; Waxman, 1999; Pascussi et al., 2003)
1.3.1 Rat CYP2B subfamily

To date, seven enzymes have been identified in the rat CYP2B subfamily (Nelson, 1996). In rats, CYP2B1 and CYP2B2 are the major enzymes expressed in the liver and phenobarbital is an effective inducer for these two enzymes (Wilson et al., 1984). In the constitutive state, CYP2B protein accounts for approximately 4% of the total cytochrome P450 content in the liver (Thomas et al., 1983; Suwa et al., 1985). However, CYP2B protein expression levels, especially CYP2B1, can be increased 80- to 300-fold by phenobarbital (Wilson et al., 1984). In addition to phenobarbital, CYP2B enzymes can also be induced by a group of structurally diverse chemicals, such as phenytoin (Conney et al., 1965), trans-stilbene oxide (Meijer et al., 1982), polychlorinated biphenyls (PCB) (Safe et al., 1985; Buchmann et al., 1986; Lubet et al., 1989) and dichlorodiphenyltrichloroethane (DDT) (Lubet et al., 1989). Although the amino acid sequence homology for CYP2B1 and CYP2B2 is approximately 97% identical, the CYP2B1 enzyme is constitutively expressed at a very low level and is highly inducible by phenobarbital as indicated above, whereas CYP2B2 is constitutively expressed at a relatively higher level and moderately inducible by phenobarbital (Wilson et al., 1984; Funae and Imaoka, 1993):

The constitutive androstane receptor (CAR) has been suggested to mediate the induction of CYP2B genes (Forman et al., 1998; Schuetz et al., 2001). CAR is an orphan nuclear receptor which is bound to endogenous inhibitory steroids (e.g. androstanol and androstenol) in an inactive state. The binding of ligand such as phenobarbital results in dissociation of CAR and the androstanes. Subsequently, CAR translocates to the nucleus, forms a heterodimer with the 9-cis retinoid X receptor, and
activates the phenobarbital-responsive enhancer module of CYP2B genes (Forman et al., 1998; Honkakoski et al., 1998; Sueyoshi and Negishi, 2001;).

Pentoxyresorufin O-dealkylation and testosterone 16β-hydroxylation activities are commonly used as markers for rats treated with phenobarbital or other CYP2B inducers (Lubet et al., 1985; Reik et al., 1985; Waxman et al., 1987; Dutton and Parkinson, 1989). However, these markers reflect mainly the enzyme activity of CYP2C11 in untreated rats (Chang et al., 1992; Nakajima et al., 1990). In contrast, 7-benzyloxyresorufin O-dealkylation activity is selective for CYP2B in liver microsomes isolated from untreated rats (Meehan et al., 1988; Burke et al., 1994; Wong and Bandiera, 1998). Non-planar molecules and relatively hydrophobic chemicals, such as barbiturates (Kaminsky et al., 1981; Kennedy et al., 1981) and cyclophosphamide (Ruzicka and Ruenitz, 1992), are typical substrates for CYP2B subfamily in mammals. In addition, procarcinogens, such as aminoanthracene and benzo[a]pyrene, are activated by CYP2B (Christou et al., 1992). Thus, induction of CYP2B enzymes has been correlated with tumor promotion and increased hepatocarcinogenesis (Soucek and Gut, 1992; Diwan et al., 1993; Rice et al., 1994).

1.3.2 RAT CYP3A SUBFAMILY

In mammals, the CYP3A subfamily consists of numerous enzymes that are responsible for metabolizing various xenobiotics and endobiotics with diverse structures. To date, approximately twenty-two different CYP3A isoforms have been discovered in several species, including humans, rabbits, macaques, hamsters, rats, mice and dogs (Nelson, 1996). The CYP3A enzymes are mostly present in liver and, to a lesser extent,
in extrahepatic tissues including the intestine, brain, kidney and leukocytes (Nelson et al., 1996).

In rats, five CYP3A enzymes have been identified, including CYP3A1, CYP3A2, CYP3A9, CYP3A18, and CYP3A23 (Nelson, 1996). However, Nagata et al. (1999) suggested that the CYP3A23 gene encodes a protein previously referred as CYP3A1. Regulation of CYP3A enzyme expression by steroidal compounds was first discovered and characterized using pregnenolone 16α-carbonitrile as the prototypical inducer (Whitlock, 1986; Guzelian, 1988). All hepatic CYP3A enzymes are inducible to varying degrees by endogenous glucocorticoid, testosterone, progesterone, synthetic steroids (e.g. PCN and dexamethasone), phenobarbital-like compounds (e.g. phenobarbital and phenytoin), and antibiotics (e.g. erythromycin and rifampin) (Schuetz et al., 1984; Guzelian, 1988). Kliewer et al. (1998) showed that a nuclear hormone receptor, pregnane X receptor (PXR), mediates the induction of CYP3A. After binding to a ligand, PXR heterodimerizes with the retinoid X receptor, transactivates the PXR response elements in the CYP3A promoters, and enhances the transcription of the CYP3A gene (Kliewer et al., 1998). In addition, there appears to be "cross-talk" between PXR and CAR in the regulation of CYP3A and CYP2B induction. Both PXR and CAR are regulated by several of the same compounds (Moore et al., 2000) and each receptor binds to the responsive element modules, forms a PXR heterodimer, and regulates both CYP3A and CYP2B genes (Xie et al., 2000; Smirlis et al., 2001).

In rats, CYP3A23 is the major glucocorticoid-responsive CYP3A gene. It is highly inducible by pregnenolone 16α-carbonitrile and steroid-like compounds such as dexamethasone, mifepristone (RU486), and spironolactone (Schuetz et al., 1984; Komori and Oda, 1994). The enzyme activity of CYP3A in induced rats is generally
assessed by the erythromycin N-demethylase and testosterone 6β-hydroxylase activities (Wrighton et al., 1985; Halvorson et al., 1990).

1.3.3 RAT CYP2C11

Several cytochrome P450 enzymes are constitutively expressed and sex-specific, such as rat CYP2C11 (Waxman et al., 1985). In untreated male rats, CYP2C11 is the main cytochrome P450 enzyme present in the liver. It is male-specific and catalyzes steroid 2α-hydroxylation (Waxman et al., 1985). Expression of hepatic CYP2C11 is regulated at the transcriptional level by androgen, which acts on the hypothalamic-pituitary axis to alter the secretion pattern of GH growth hormone (GH) (Jansson et al., 1985; Waxman and Chang, 1995). The pulsatile GH release stimulates CYP2C11 expression, whereas the continuous GH release suppresses the expression of CYP2C11 (Waxman et al., 1991). In neonatally castrated male rats, administration of testosterone in adulthood restores CYP2C11 expression to normal levels and the effects of androgen can be antagonized by estrogen (Waxman et al., 1985; Jansson and Frohman, 1987; Bandiera and Dworschak, 1992). Neonatal administration of estrogens suppresses the pulsatile GH release and elevates the continuous GH secretion in adult intact rats (Jansson et al., 1985) and CYP2C11 expression in both intact and castrated adult male rats (Dannan et al., 1986; Bandiera and Dworschak, 1992). Activation of the protein tyrosine kinase, Janus kinase 2 (Jak2), and phosphorylation of transcription factors, such as Stat5a and Stat5b, are suggested to contribute to the regulation of CYP2C11 by GH (Udy et al., 1997; Teglund et al., 1998).

The expression of CYP2C11 can also be downregulated by various physiological and pathological factors such as inflammation (Sewer et al., 1997), stress (Merrill et al.,
1992), fasting (Ma et al., 1989), diabetes (Favreau and Schenkman, 1988) and also xenobiotics, such as phenobarbital and dexamethasone (Morris and Davila, 1996).

In untreated adult male rats, testosterone 2α-hydroxylase has been used as an enzyme selective marker for CYP2C11 (Waxman et al., 1984; Chang et al., 1992). Various substrates can be metabolized by CYP2C11, including some frequently prescribed drugs such as S-mephenytoin (Yasumori et al., 1993) and other non-steroidal anti-inflammatory agents (Vage and Svensson, 1994).

1.3.4 RAT CYP1A SUBFAMILY

In the CYP1A subfamily, two enzymes, namely CYP1A1 and CYP1A2, have been discovered. These enzymes are studied because of their important roles in the bioactivation of procarcinogens. CYP1A1 and CYP1A2 are present in all classes of the animal kingdom and are highly conserved among mammalian species. The former is expressed mainly in extrahepatic tissues such as the placenta and lung, whereas the later is constitutively expressed in mammalian liver (Wilson et al., 1984; McKinnon et al., 1991; Schweikl et al., 1993).

Induction of both CYP1A1 and CYP1A2 is mediated by a ligand-activated transcription factor, the aryl hydrocarbon receptor (AhR) (Wiebel et al., 1971; Poland et al., 1976; Poland et al., 1982). After binding to the ligand, the AhR translocates to the nucleus, dimerizes with AhR nuclear translocator protein (Arnt) and binds to specific DNA target sequences to trigger the transcription (Whitlock et al., 1996). However, the precise mechanism of upregulation varies with the nature of the inducers. AhR-independent pathways have also been suggested for CYP1A induction. Ryu et al., (1996) reported induced CYP1A2 expression in AhR null mice treated with piperonyl
butoxide. Nevertheless, whether this finding is due to de novo induction or an increased constitutive expression still remains to be clarified. The commonly used inducers for CYP1A induction include 3-methylcholanthrene (3-MC) (Thomas et al., 1983), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Thomas et al., 1983), and β-naphthoflavone (Lau and Strobel, 1982).

Although CYP1A1 and CYP1A2 exhibit overlapping substrate specificities, CYP1A1 has a greater affinity for neutral polycyclic aromatic hydrocarbons, such as benzo[a]pyrene and nitropyrene, whereas CYP1A2 preferentially metabolizes polyaromatic and heterocyclic amines and amides, including 2-acetylaminofluorene, and aflatoxin B1 (Omiecinski et al., 1999). In induced animals, the 7-ethoxyresorufin O-dealkylation activity is an enzyme-selective marker for CYP1A (Rodrigues and Prough, 1991).

1.4 MICROSOMAL EPOXIDE HYDROLASE

The epoxide hydrolases (EH) are a group of enzymes that hydrolase simple epoxides to vicinal diols, or arene oxides to trans-dihydrodiols. EHs are often recognized as detoxifying enzymes for both endogenous and xenobiotic compounds (Omiecinski, 2000). Like cytochrome P450 enzymes, EHs are predominantly distributed in the liver and, to a lesser extent, in the brain, kidney, lung and adrenal gland (Simmons and Kasper, 1989). Based on the substrate specificities, molecular weights, and immunological properties, five different mammalian EHs have been identified: microsomal cholesterol 5,6-oxide hydrolase (Sevanian and McLeod, 1986), hepoxilin A₃ hydrolase (Pace-Aisciak and Lee, 1989), leukotriene A₄ hydrolase (Fu et al., 1989), cytosolic epoxide hydrolase (Beetham et al., 1993), and microsomal epoxide hydrolase
Among these five enzymes, mEH is considered to be the most important one in drug metabolism because of its significant role in the detoxification of epoxide intermediates (Omiecinski, 2000).

mEH is expressed ubiquitously, and as the name implies, it is predominantly distributed in the endoplasmic reticulum (Seidegard et al., 1997; Omiecinski, 2000). Compared to other EHs, substrates for mEH are relatively specific, such as epoxide-derivatives of procarcinogens (e.g. polycyclic aromatic hydrocarbons), toxic compounds (e.g. aflatoxin B₁, chrysene, nitropyrene, and benzene), and anticonvulsant drugs (e.g. phenytoin and carbamazepine) (Fretland and Omiecinski, 2000).

Benzo[a]pyrene-4,5-oxide, styrene oxide and cis-stilbene oxide are generally used as enzyme-specific markers to measure the enzyme activity of mEH (Moody and Hammock, 1987; Omiecinski et al. 1993; Fretland and Omiecinski, 2000). In rats, phenobarbital, 2(3)-tert-butyl-4-hydroxyanisole (BHA), and BNF are inducers of mEH (Hassett et al., 1998; Omiecinski, 2000). The mechanism of mEH induction has not been well elucidated (Omiecinski, 2000).

1.5 INHIBITION OF CYTOCHROME P450 CATALYTIC ACTIVITY BY PANAX GINSENG AND PANAX QUINQUEFOLIUS EXTRACTS

Panax ginseng and Panax quinquefolius extracts inhibit CYP enzyme activities in vitro (Kim et al., 1997; Chang et al., 2002). In a study with hepatic microsomes isolated from untreated adult male rats, several CYP enzyme assays were performed to characterize the inhibitory effect of ginseng. After the addition of Panax ginseng extract (500 and 1000 µg/ml) to hepatic microsomes isolated from rats, activities of ethoxyresorufin O-dealkylation, benzoyloxyresorufin O-dealkylation, erythromycin N-
demethylation, aminopyrine N-demethylation and p-nitrophenol hydroxylation were inhibited (Kim et al., 1997).

Chang et al. (2002) demonstrated inhibitory effects of Panax ginseng and Panax quinquefolius extracts, and individual ginsenosides (Rb1, Rb2, Rc, Rd, Re, Rf and Rg1) using human recombinant CYP1A1, CYP1A2 and CYP1B1 enzymes. The 7-ethoxyresorufin O-dealkylation assay was performed in the presence of ginseng extracts of known ginsenoside composition to assess the effect of ginseng extracts and individual ginsenosides on CYP1 enzyme activities. Panax ginseng and Panax quinquefolius extracts inhibited the catalytic activities of human CYP1A1, CYP1A2 and CYP1B1, but Panax quinquefolius extract was 45-fold more potent than Panax ginseng extract in inhibiting CYP1A2, based on the IC50 values. Ginsenosides Rb1 (4.56 μg/ml, Rb2 (0.31 μg/ml), Rc (1.09 μg/ml), Rd (1.43 μg/ml), Re (2.36 μg/ml), and Rg1 (0.24 μg/ml), either individually or as a mixture, did not affect the catalytic activities of the above enzymes. The concentrations of the above ginsenosides were reflective of the individual components present in an inhibitory concentration of 100 μg/ml Panax quinquefolius extract. However, Rb1, Rb2, Rc, Rd and Rf at the concentration of 50 μg/ml inhibited CYP1A1, CYP1A2, and CYP1B1 activities.

Henderson et al. (1999) suggested that individual ginsenosides interact with human CYP enzymes other than CYP1A1, CYP1A2 and CYP1B1. In this study, a panel of human recombinant CYP enzymes were incubated with individual ginsenosides and substrates {benzylether resorufin and 7-bezyloxy-4-trifluoromethylcoumarin for CYP3A4, 3-[2-(N,N-diethyl-N-methylamino)ethyl]-7-methoxy-4-methylcoumarin for CYP2D6, 7-methoxy-4-trifluoromethylcoumarin for CYP2C9, and 7-ethoxy-3-cyanocoumarin for CYP2C19}. Ginsenoside-Rd acted as a moderate inhibitor for CYP3A4 (IC50 = 74 and
58 μM for 7-benzyloxyresorufin and 7-benzyloxy-4-trifluoromethylcoumarin, respectively) and CYP2D6 (IC\textsubscript{50} = 76 μM), but the IC\textsubscript{50} values are orders of magnitude greater than ketoconazole (0.041 and 0.03 μM for benzylether resorufin and 7-bezyloxy-4-trifluoromethylcoumarin, respectively) and quinidine (0.008 μM). Based on the IC\textsubscript{50} values, ginsenoside Rd was a relatively weak inhibitor of CYP2C9 (154 μM) and CYP2C19 (134 μM) when compared to the positive controls sulfaphenazole (0.92 μM) and tranylcypromine (4.02 μM), respectively.

1.6 INDUCTION OF PANAX GINSENG EXTRACT ON CYTOCHROME P450 EXPRESSION

In an animal study, the administration of a single oral dose (10, 20 or 30 mg/kg) of Panax ginseng extract to adult male rats elevated hepatic microsomal aminopyrine N-demethylation activity by 18 to 24% (Lee \textit{et al.}, 1987\textsuperscript{2}). By employing benzo[a]pyrene as the substrate, the authors also claimed that the 30 mg/kg dosage significantly decreased the S-9 aryl hydrocarbon hydroxylase activity by 8%. It is known that hepatic microsomal aminopyrine \textit{N}-demethylation can be catalyzed by CYP2B, CYP2C11 and CYP3A (Imaoka \textit{et al.}, 1988), whereas CYP1A1 and CYP1A2 are known to catalyze aryl hydrocarbon hydroxylation (Wiebel \textit{et al.}, 1971). These results suggest selective effects of Panax ginseng on CYP enzyme activities.

1.7 EFFECT OF PANAX GINSENG EXTRACT ON MICROSONMAL EPOXIDE HYDROXYLASE ENZYME EXPRESSION

The effects of ginseng on microsomal epoxide hydrolase have not been extensively investigated. The administration of single oral doses (20 and 30 mg/kg) of
Panax ginseng extract increased hepatic microsomal epoxide hydrolase activity in adult male rats by 36% and 50%, respectively, which was determined in samples collected one day after the treatment using benzo[a]pyrene 4,5-oxide as a substrate (Lee et al., 1987). To date, there are no published studies with Panax quinquefolius.

1.8 HYPOTHESIS

Panax ginseng extract appears to have selective effects on hepatic CYP enzymes and epoxide hydrolase in rats (Lee et al., 1987). After the administration of Panax ginseng extract, hepatic microsomal aminopyrine N-demethylation activity (10, 20, and 30 mg/kg) was increased by 18 to 24%, whereas the microsomal epoxide hydrolase activity (20 and 30 mg/kg) was increased by 36 to 50%. Furthermore, the 30 mg/kg dosage significantly decreased the S-9 aryl hydrocarbon hydroxylase activity by 8%.

It is known that microsomal aminopyrine N-demethylation can be catalyzed by CYP2B, CYP2C11 and CYP3A (Imaoka et al., 1988), whereas hepatic S-9 aryl hydrocarbon hydroxylation activity can be used to assess CYP1A enzyme function (Wiebel et al., 1971; Wilson et al., 1984). Among the three CYP enzymes, namely CYP2B, CYP3A and CYP2C11, which can catalyze aminopyrine N-demethylation, only CYP2B and CYP3A are inducible (Imaoka et al., 1988; Okey, 1990). This suggests that the elevated activity of hepatic microsomal aminopyrine N-demethylase observed by Lee et al. (1987) could be due to induced CYP2B and/or CYP3A. It is known that CYP2C11 catalyzes aminopyrine N-demethylation (Imaoka et al., 1988) and the expression of this protein can be up regulated by androgen (Waxman and Chang, 1995) or down regulated by estrogen (Dannan et al., 1986; Bandiera and Dworschak, 1992). There is evidence that suggests that Panax ginseng and Panax quinquefolius extracts
both exhibit androgenic (Fahim et al., 1982) and estrogenic (Liu et al., 2001; Lee et al., 2003) activities, however, it is not known if these effects are mediated by a CYP enzymes-dependent mechanism.

Therefore, we hypothesized that ginseng extracts may selectively affect the hepatic expression of rat hepatic CYP enzymes and mEH.

1.9 OBJECTIVES

Objective #1 To validate real-time PCR assays for the quantification of CYP2B1, CYP3A23, CYP2C11, CYP1A2, mEH, and cyclophilin mRNA expression.

Objective #2 To determine the effect of a single oral dose of *Panax ginseng* extract of known ginsenoside composition on 1) hepatic CYP2B1, CYP3A23, CYP1A2, and mEH mRNA expression; 2) hepatic microsomal 7-benzyloxyresorufin O-dealkylation and 7-ethoxyresorufin O-dealkylation activities; and 3) hepatic CYP2C11 and mEH protein expression in adult male rats.

Objective #3 To determine the effect of multiple oral doses of *Panax quinquefolius* extract of known ginsenoside composition on 1) hepatic CYP2B1, CYP3A23, CYP2C11, CYP1A2, and mEH mRNA expression; 2) hepatic microsomal 7-benzyloxyresorufin O-dealkylation and 7-ethoxyresorufin O-dealkylation activities; and 3) hepatic CYP2C11 and mEH protein expression in adult male rats.
CHAPTER 2. MATERIALS AND METHODS
### 2.1 CHEMICALS AND REAGENTS

<table>
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<tr>
<th>CHEMICAL/REAGENT</th>
<th>SUPPLIER</th>
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<tbody>
<tr>
<td>acrylamide 99.9%; <em>N,N</em>-methylene-bis-acrylamide (BIS)</td>
<td>BioRad Laboratories Inc. (Mississauga, Ontario, Canada)</td>
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<td>agarose</td>
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<td>albendazole</td>
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<td>alkaline phophatase conjugated, goat F(ab')&lt;sub&gt;2&lt;/sub&gt; anti-rabbit IgG, gamma and light chain specific, affinity purified</td>
<td>BioSource International (Camarillo, California, U.S.A)</td>
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<td>ammonium persulfate</td>
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<td>monospecific rabbit anti-rat microsomal epoxide hydrolase polyclonal IgG</td>
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<td>*Panax quinquefolius extract, 10% total ginsenosides w/w</td>
<td>Canadian Phytopharmaceuticals Corp. (Richmond, British Columbia, Canada)</td>
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* Content of individual ginsenosides in the *Panax ginseng* and *Panax quinquefolius* extracts were quantified by high performance liquid chromatography (HPLC) by the suppliers and is shown in Figure 2.
Figure 2. Content of individual ginsenosides in a *Panax ginseng* extract and a *Panax quinquefolius* extract. Shown is the content (% w/w) of individual ginsenosides in a *Panax ginseng* extract and a *Panax quinquefolius* extract. The amount of individual ginsenosides was determined by HPLC and the data were provided by the manufacturers of the *Panax ginseng* extract (Pharmaton S.A., Lugano, Switzerland) and the *Panax quinquefolius* extract (Canadian Phytopharmaceuticals Corp., Richmond, BC, Canada).
<table>
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<td>Aldrich Chemical Company Inc. (Milwaukee, Wisconsin, USA)</td>
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<tr>
<td>RiboGreen™ RNA Quantitation Kit</td>
<td>Molecular Probes Inc. (Eugene, OR, USA)</td>
</tr>
<tr>
<td>skim milk powder</td>
<td>Carnation Inc. (Toronto, Ontario, Canada)</td>
</tr>
<tr>
<td>sodium acetate buffer solution, 3M: 0.2 µm filtered (molecular biology grade)</td>
<td>Sigma Chemical Co. (St. Louis, Missouri, U.S.A.)</td>
</tr>
<tr>
<td>sodium chloride</td>
<td>Fisher Scientific Ltd. (Vancouver, British Columbia, Canada)</td>
</tr>
<tr>
<td>sodium dithionite</td>
<td>J. T. Baker Chemical Co. (Phillipburg, New Jersey, USA)</td>
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<tr>
<td>sodium lauryl sulfate (SDS)</td>
<td>Fisher Scientific Ltd. (Vancouver, British Columbia, Canada)</td>
</tr>
<tr>
<td>sodium phosphate dibasic</td>
<td>Sigma Chemical Co. (St. Louis, Missouri, U.S.A.)</td>
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<tr>
<td>sodium phosphate monobasic</td>
<td>Sigma Chemical Co. (St. Louis, Missouri, U.S.A.)</td>
</tr>
<tr>
<td>sucrose</td>
<td>Sigma Chemical Co. (St. Louis, Missouri, U.S.A.)</td>
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### CHEMICAL/REAGENT |

<table>
<thead>
<tr>
<th>CHEMICAL/REAGENT</th>
<th>SUPPLIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>SuperScript™ II RNase H' reverse transcriptase</td>
<td>Invitrogen Canada Inc. (Burlington, Ontario, Canada)</td>
</tr>
<tr>
<td>SYBR Green™ I dye</td>
<td>Sigma Chemical Co. (St. Louis, Missouri, U.S.A.)</td>
</tr>
<tr>
<td>N,N,N',N'-tetramethylethylenediamine (TEMED)</td>
<td>BioRad Laboratories Inc. (Mississauga, Ontario, Canada)</td>
</tr>
<tr>
<td>tris(hydroxymethyl)aminomethane (Trizma base) (biotechnology performance certified)</td>
<td>Sigma Chemical Co. (St. Louis, Missouri, U.S.A.)</td>
</tr>
<tr>
<td>tris(hydroxymethyl)aminomethane hydrochloride (Trizma HCl)</td>
<td>Sigma Chemical Co. (St. Louis, Missouri, U.S.A.)</td>
</tr>
<tr>
<td>TRIZOL™ reagent</td>
<td>Invitrogen Canada Inc. (Burlington, Ontario, Canada)</td>
</tr>
<tr>
<td>Tween 20 (polyoxyethylene sorbitan monolarurate)</td>
<td>Sigma Chemical Co. (St. Louis, Missouri, U.S.A.)</td>
</tr>
<tr>
<td>water (molecular biology grade)</td>
<td>Sigma Chemical Co. (St. Louis, Missouri, U.S.A.)</td>
</tr>
</tbody>
</table>

2.2 ANIMALS

Adult male Sprague-Dawley rats (250-300 g body weight) were purchased from Charles River Laboratories Inc., (Montreal, Quebec, Canada). They were housed on corn cob bedding under conditions of controlled temperature (20-23°C) and a 12-hour dark-light cycle (7:00 am on and 7:00 pm off). Rat chow (LabDiet® 5001 Rodent Diet) (PMI® Nutrition International Inc., Brentwood, MO, USA) and tap water were provided ad libitum. The animals were acclimatized in our animal care facility for at least one week before initiation of treatment.
2.2.1 TREATMENTS OF ANIMALS

Treatment, dosage, corresponding vehicle control, injection volume, route of administration, duration and the number of rats in each group are listed in Table 4. The content of individual ginsenosides in the ginseng extracts is shown in Figure 2. All rats were decapitated one day after the last injection. Livers were immediately excised, one portion was snap-frozen in liquid nitrogen and stored at -70°C for subsequent RNA analysis. The remaining portion was used immediately to prepare microsomes.

2.3 ISOLATION OF RNA

Total liver RNA was isolated using TRIZOL™ reagent according to the manufacturer's instructions. The protocol is based on a previously published method (Chomczynski and Sacchi, 1987). A piece of frozen liver sample was ground into fine powder and incubated with 1 ml of TRIZOL™ reagent for 5 minutes. The sample was extracted with 200 µl of chloroform: isoamyl alcohol (49:1 v/v) and then spun at 10,500 X g for 3 minutes and incubated for another 3 minutes at room temperature, followed by another spin at 10,500 X g for 15 minutes at 4°C. To precipitate RNA, the aqueous phase was transferred to a clean sterile microcentrifuge tube containing 0.5 ml ice-cold isopropanol (99+%), spun at 10,500 X g for 1 minute, kept at -70°C for 30 minutes, and spun again at 10,500 X g for 15 minutes at 4°C. The supernatant was discarded and the RNA pellet was washed with 1 ml of ice-cold 75% ethanol, followed by centrifugation at 6,610 X g for 5 minutes at 4°C. The supernatant was carefully removed without disturbing the pellet using a pipette and the pellet was air-dried for 45 minutes. The RNA pellet was suspended in sterile 50 µl of 1X TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at -70°C for subsequent analysis.
TABLE 4
Treatment of animals

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dosage (mg/kg/day)</th>
<th>Vehicle control</th>
<th>Volume administered (ml/kg)</th>
<th>Route of administration</th>
<th>Duration of treatment (day)</th>
<th>Number of rats in each group</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experimental treatment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Panax ginseng</em> extract</td>
<td>30 or 100</td>
<td>0.9% NaCl</td>
<td>2</td>
<td>oral gavage</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td><em>Panax quinquefolius</em> extract</td>
<td>100 or 400</td>
<td>0.9% NaCl</td>
<td>2</td>
<td>oral gavage</td>
<td>21</td>
<td>4 or 5</td>
</tr>
<tr>
<td><strong>Control treatment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>albendazole</td>
<td>20</td>
<td>corn oil</td>
<td>2</td>
<td>oral gavage</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>β-naphthoflavone</td>
<td>40</td>
<td>corn oil</td>
<td>1</td>
<td>intraperitoneal</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>dexamethasone</td>
<td>100</td>
<td>2% Tween 80</td>
<td>1</td>
<td>intraperitoneal</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>phenobarbital</td>
<td>80</td>
<td>0.9% NaCl</td>
<td>1</td>
<td>intraperitoneal</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>
2.4 QUALITY ASSESSMENT OF THE ISOLATED RNA SAMPLES

The purity of each isolated RNA sample was assessed by measuring the absorbance at 260 nm and 280 nm and calculating the ratio of the absorbance at 260 nm and 280 nm. The absorbance was determined by using a GeneSpec® spectrophotometer (Hitachi Software Engineering Co., Ltd.). The blank sample contained 1X TE buffer (pH 8.0). The net absorbance for each unknown sample was calculated by subtracting the absorbance reading of the blank sample from that of the unknown sample. The $A_{260}/A_{280}$ ratios of all the RNA samples prepared in this study were at least 1.85.

Gel electrophoresis was conducted to assess the integrity of each RNA sample. Each RNA sample (2 μl) was mixed with 1 μl ethidium bromide (0.08 μg/ml) and 9 μl of a solution containing 50% (v/v) formamide, 7% (v/v) formaldehyde, 20 mM MOPS solution (pH 7.0, containing 2 mM EDTA and 10 mM sodium acetate), and 0.1% (w/v) bromophenol blue in diethylpyrocarbonate (DEPC)-treated water. To denature the RNA, the mixture was incubated at 65°C for 10 minutes and then immediately chilled on ice. The 37% (v/v) formaldehyde/1.5% (w/v) agarose solution was prepared by dissolving 1.5 g agarose in 84.6 ml DEPC-treated water in a microwave, and mixing with 10 ml of 200 mM MOPS solution (pH 7.0, containing 20 mM EDTA and 100 M sodium acetate) and 5.4 ml of 37% (v/v) formaldehyde (12.3 M). The solution was poured onto an electrophoresis cassette until solidified. After the gel was hardened, a 20 mM MOPS solution (pH 7.0, containing 2 mM EDTA and 10 mM sodium acetate) was added to completely cover the gel and the denatured RNA sample mixtures were loaded onto the gel and then electrophoresed at a constant voltage of 75 V for one hour. A representative photograph of an ethidium bromide-stained agarose gel electrophoresis
is shown in Figure 3. Two distinct bands, 28S rRNA and 18S rRNA, were detected in each sample, suggesting that the quality of the RNAs were not compromised.

![Figure 3. Representative formaldehyde agarose gel electrophoresis of RNA samples.](image)

2.5 DETERMINATION OF TOTAL RNA CONCENTRATION

Total RNA concentration was determined spectrofluorometrically by using the RiboGreen™ RNA Quantitation Kit according to the manufacturer’s instructions (Jones et al., 1998). To remove any DNA present in the RNA samples, each 2 μl of RNA sample was incubated with 5 units of deoxyribonuclease I and 1 μl of a 10X digestion buffer (200 mM Tris-HCl, pH 7.5 containing 100 mM MgCl₂ and 20 mM CaCl₂) in 90 μl of 1X TE buffer (pH 8.0) at 37°C for 90 minutes. The diluted RNA sample mixture was prepared by adding 3 μl of the RNA sample mixture to 147 μl of 1X TE buffer (pH 8.0). Each 100 μl of the RiboGreen™ reagent stock solution (included in the kit; stock
concentration was not provided by the manufacturer) was diluted in 19.9 ml 1X TE buffer (pH 8.0). Each 100 μl diluted RNA sample mixture or known concentrations of ribosomal RNAs (16S and 23S ribosomal RNA isolated from *E. Coli* (included in the kit) were incubated in the diluted RiboGreen™ reagent in a 96-well microplate in the dark for 2–5 minutes at room temperature. Spectrofluorometric measurements were taken by using a CytoFluor® 4000 fluorescence microplate reader (Millipore, Bedford, MA, USA) at an excitation wavelength of 485 nm (20 nM), an emission wavelength of 530 nm (20 nM), and a gain setting at 50. A blank sample, which contained RiboGreen™ reagent and 1X TE buffer (pH 8.0), was included in the assay. The net fluorescence readings for the unknown samples and ribosomal RNAs were calculated by subtracting the fluorescence reading of the blank sample from that of the unknown and standard samples. The standard curve was constructed by plotting the net fluorescence readings against known concentrations (0.25, 0.5, 1, and 5 μg/μl) of ribosomal RNAs. Linear regression analysis was conducted and the equation was obtained. The amount of RNA present in the unknown samples was determined by using the linear regression equation.

2.6 REVERSE TRANSCRIPTION

Reverse transcription was conducted by incubating 2 μl of RNA samples (1 μg/μl) with 1 μl of oligo(dT) primer (0.5 μg/μl) in 6 μl of DEPC-treated water at 65°C for 10 minutes, followed by immediately chilling on ice. Subsequently, each RNA sample was incubated with a 10 μl mixture [1 mM dNTP mix, 10 mM MgCl₂, 0.01 M DTT and 0.2 unit of deoxyribonuclease I in PCR buffer (200 mM Tris-HCl, pH 8.4, containing 500 mM KCl)] at 37°C for 30 minutes. The RNA sample was incubated again at 75°C for 5
minutes to denature deoxyribonuclease I, followed immediately by chilling on ice. To synthesize cDNA, each of the RNA sample mixtures was incubated with 200 units of Superscript II reverse transcriptase at 42°C for 20 minutes. The samples were then stored at -20°C until subsequent analysis.

2.7 DETERMINATION OF TOTAL cDNA CONCENTRATION

Total cDNA concentrations were determined by using the PicoGreen™ dsDNA Quantification Kit according to the manufacturer’s instructions (Singer et al., 1997). Each 2.5 μl of the cDNA sample was diluted in 247.5 μl of 1X TE buffer (pH 8.0). Each 100 μl of the PicoGreen™ reagent stock solution (800 mM, included in the kit) was diluted in 19.9 ml 1X TE buffer (pH 8.0). Subsequently, each of the diluted cDNA samples (100 μl) or known concentrations of the λ-DNA standards (100 μl) (included in the kit) was incubated with the diluted PicoGreen™ reagent (100 μl) a 96-well microplate in the dark for 2–5 minutes at room temperature. Spectrofluorometric measurements were taken by using a CytoFluor® 4000 fluorescence microplate reader at an excitation wavelength of 485 nm (20 nM), an emission wavelength of 530 nm (20 nM), and a gain setting at 60. A blank sample, which contained the diluted PicoGreen™ reagent, was included in the assay. The net fluorescence readings for the unknown samples and λ-DNA standards were calculated by subtracting the fluorescence reading of the blank sample from that of the unknown and standard samples. The standard curve was constructed by plotting the net fluorescence readings against known concentrations of λ-DNA standards (2, 10, 20, 100, 200, and 400 ng/ml). Linear regression analysis was conducted and an equation was obtained. The amount of dsDNA present in the unknown samples was determined by linear regression.
2.8 REAL-TIME POLYMERASE CHAIN REACTION (PCR) ASSAYS

Gene expression analyses for rat hepatic CYP2B1, CYP3A23, CYP2C11, CYP1A2, microsomal epoxide hydrolase and cyclophilin were determined by using a real-time DNA thermal cycler (LightCycler, Roche Diagnostics, Laval, Quebec, Canada). Each PCR assay was validated and the assay conditions, such as MgCl\textsubscript{2} and primer concentrations, primer concentration, cycling condition, were optimized prior to performing these PCR assays.

In this study, gene expression is expressed as copies per ng of total dsDNA. Based on the size of the amplicons and Avogadro's constant (6.022 X 10\textsuperscript{23} copies/mole), the amount of cDNA can be expressed in copies. For example, when the designated CYP2B1 primers (Table 5) were used, the expected size of the amplicons generated in the real-time CYP2B1 PCR assay was 201 base pair (bp). It is known that the average molecular weigh of each bp is 660 g, therefore, the molecular weight (g/mole) for the CYP2B1 amplicon is 201 X 660 = 132660 g. Based on the PicoGreen\textsuperscript{TM} dsDNA Quantification assay, the concentration of the purified CYP2B1 amplicon was 8.53 X 10\textsuperscript{-9} g/\mu{l}. Therefore, the quantity of the purified CYP2B1 amplicon can be converted from g/\mu{l} to copies/\mu{l} using the following calculation:

\[
\frac{8.35 \times 10^{-9} \times 6 \times 10^{23}}{201 \times 660} = 3.8 \times 10^{10}
\]

2.8.1 PCR PRIMERS

Primer sequences are shown in Table 5. The specificity of the primers was confirmed by conducting sequencing analysis of the segments amplified with each primer set. Sequencing analysis was performed by using Applied Biosystems 377 DNA
sequencer (Applied Biosystems Inc., Foster City, CA, USA) in the Nucleic Acid and Protein Service Unit, University of British Columbia (Vancouver, British Columbia, Canada). The primers used to amplify CYP2B1 do not amplify CYP2B2, CYP2B3, or the CYP2B pseudogenes, CYP2B14P and CYP2B16P (Trottier et al., 1996). CYP3A2, CYP3A9, and CYP3A18, are not amplified by the primers used to amplify CYP3A23, and CYP1A1 is not amplified by the primers used to amplify CYP1A2. The primers used to amplify rat microsomal epoxide hydrolase do not amplify cytosolic epoxide hydrolase.

2.8.2 PCR REACTION MIXTURE

Based on the concentration of the cDNA sample determined using the PicoGreen™ dsDNA Quantification Kit, a constant amount (1 ng) of cDNA for each sample was amplified. A master mix solution was prepared by adding the reagents to 1X PCR buffer (200 mM Tris-HCl, pH 8.0, containing 500 mM KCl) in the following order: 3 mM MgCl$_2$ (except for microsomal epoxide hydrolase PCR assay, where 4 mM MgCl$_2$ was used), 0.25 mg/ml bovine serum albumin, 0.2 mM dNTP, 0.2 μM of each primer, 1:3000 SYBR Green™ I dye, and 0.2 unit of Platinum® Taq polymerase. For each PCR reaction, 5 μl of the cDNA sample was added to a 15 μl master mixture solution. In each PCR assay, two negative control samples were included: a no-template control and a no-primer control. As the name implies, the “no-template” negative control sample did not contain cDNA samples, and the “no-primer” negative control sample did not contain PCR primers.

2.8.3 PCR CYCLING CONDITIONS

The PCR cycling conditions for each assay are listed in Table 6.
**TABLE 5**

**PCR Primers**

<table>
<thead>
<tr>
<th>Gene (GenBank accession no.)</th>
<th>Forward Primer (5'-3')</th>
<th>Reverse Primer (5'-3')</th>
<th>Amplicon Size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>microsomal epoxide hydrolase (M26125)</td>
<td>CAC-ATC-CAA-GCC-ACC-AAG-CC</td>
<td>CAG-GCC-TCC-ATC-CTC-CAG</td>
<td>135</td>
<td>Schilter et al., 2000</td>
</tr>
</tbody>
</table>
TABLE 6
Cycling conditions for the quantitative real-time PCR assays

<table>
<thead>
<tr>
<th>Program</th>
<th>Temperature (°C)</th>
<th>Reaction time (second)</th>
<th>Temperature transition (°C/second)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95</td>
<td>300</td>
<td>20</td>
</tr>
<tr>
<td>(denaturation)</td>
<td>95</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>PCR (annealing)*</td>
<td>60</td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>(elongation)</td>
<td>72</td>
<td>12</td>
<td>20</td>
</tr>
</tbody>
</table>

*The annealing temperature for the real-time CYP2B1 and CYP2C11 was 56°C.

2.8.4 STANDARD CURVE

A standard curve was used to determine the level of gene expression of unknown samples. It was constructed by plotting the cross points against the corresponding known amounts of purified cDNA amplicon. At least five different amounts of purified cDNA amplicons were included in each standard curve.

2.8.5 GEL-PURIFICATION OF PCR AMPLICON

All cDNA standards used in the real-time PCR assays were gel-purified. They were initially amplified by real-time PCR assays and the amplicons were subjected to agarose gel (1.5%, w/v) electrophoresis at a constant voltage of 75 V for 2 hours. After the gel electrophoresis was completed, DNA fragments corresponding to the appropriate size were excised from the agarose gel and purified using QIAquick® Gel Extraction Kit according to manufacturer’s instructions. Each gel slice was mixed and incubated in QG Buffer (300 μl per 100 mg gel slice) (proprietary compound mixture
which contains 50-100% guanidinium thiocyanate; included in the kit) at 50°C for 10 minutes or until the slices were completely dissolved. Subsequently, isopropanol (99+%) (100 µl per 100 mg gel slice) was mixed with the dissolved gel. The mixture was passed thorough the QIAquick® column (included in the kit) by spinning at 16,000 X g for 1 minute at room temperature and the filtrate was discarded. To wash off the chaotropic agent, guanidinium thiocyanate, 750 µl of PE Buffer (proprietary solution which contains approximately 80% ethanol; included in the kit) was passed through the QIAquick® column by spinning at 16,000 X g for 1 minute at room temperature and the filtrate was removed. The purified cDNA amplicon was eluted from the column by passing 50 µl of Tris-Cl buffer (10 mM, pH 8.5) through the column and spinning the column at 16,000 X g for 1 minute at room temperature.

2.8.6 VALIDATION OF PCR ASSAYS

Accuracy and Precision

The accuracy and precision of the real-time PCR assays were determined by amplifying purified cDNA amplicons of 2 different amounts in quadruplicate. These amounts represent the range of mRNA levels expected in the unknown samples. The accuracy and precision of the real-time PCR methods for the quantification of gene expression are expressed as % bias (Equation 1) and % coefficient of variation (c.v.) (Equation 2), respectively.

% bias = [(mean observed value - nominal value) / nominal value] X 100% (Equation 1)
% c.v. = (standard deviation / mean of four observed values) X 100% (Equation 2)
Intra-day and Inter-day assay variabilities

The level of mRNA expression of the samples included in each assay was determined in quadruplicate in one experiment and the same experiment was repeated on four different days. The intra-day assay variability was expressed as the % c.v. of the quadruplicate determinations obtained in one experiment. The inter-day assay variability was expressed as the % c.v. of the quadruplicate determinations obtained on the four different days.

2.8.7 QUALITY ASSESSMENT OF PCR AMPLICON

In each real-time PCR assay, melting curve analysis was conducted to assess the presence nonspecific amplification such as the formation of primer-dimers. The melting curve analysis was performed by increasing the temperature up to 95°C by using the thermal cycling program in the LightCycler™ software. High temperature denatures the PCR amplicons to single strands that can no longer bind to SYBR Green I Dye, and consequently, the fluorescence signal starts to decrease. Changes in the fluorescence signals are illustrated in a plot of the negative derivative of fluorescence with respect to temperature (−dF/dT) against temperature (e.g. Figure 6). When the fluorescence transition is expressed as −dF/dT, the fluorescence signal can be visualized as a melting peak. Multiple peaks in cDNA samples or any peak in negative controls (i.e. no template control and no primer control) indicate nonspecific amplification, such as the formation of primer-dimers.

The presence of nonspecific amplification such as the formation of primer-dimers was also assessed by conducting agarose gel electrophoresis. After the PCR amplification was completed, 10 µl of the PCR amplicons were mixed with 2 µl of gel
loading solution (6X concentrate) and subjected to 1.5% (w/v) agarose gel electrophoresis. The 1.5% (w/v) agarose gel was prepared by dissolving 1.5 g agarose powder in 100 ml of 1X Tris-borate-EDTA buffer (0.09 M Tris-borate, pH 8.3, containing 0.004 M EDTA,) in a microwave, and the gel mixture was subsequently poured onto a electrophoresis cassette until solidified. After the gel was hardened, 1X Tris-borate-EDTA buffer (pH 8.3) was poured into electrophoresis cassette to completely cover the gel. The amplification products were loaded into the wells and then electrophoresed at a constant voltage of 75 V for two hours. A 5 µl of nucleic acid marker (Ready-Load™ 100 bp DNA Ladder) was diluted in 15 µl of DNase-free and RNase-free, autoclaved water and 12 µl of the mixture was subjected to the 1.5% (w/v) agarose gel for electrophoresis. Multiple bands in lanes containing PCR amplicons or any band in the negative controls (i.e. no template and no primer controls) reflect the presence of nonspecific PCR amplicons or primer-dimers.

2.9 ISOLATION OF RAT HEPATIC MICROSONES

Liver tissue sample was quickly homogenized in 20 ml of ice-cold 0.05 M Tris/KCl buffer, pH 7.5. The homogenate was centrifuged at 9,000 X g in a Beckman Model J2-21 centrifuge (Beckman Instruments Inc., Palo Alto, CA, USA) for 20 minutes at 4°C. The supernatants were then filtered through 4 layers of cheese cloth and spun at 100,000 X g in a Beckman Model L5-50 ultracentrifuge for 60 minutes at 4°C. Each pellet was suspended in ice-cold 1.15% KCl/10 mM EDTA (pH 7.4). The mixture was spun at 100,000 X g in a Beckman Model L5-50 ultracentrifuge for 60 minutes at 4°C. Each pellet was suspended in ice-cold 0.25 M sucrose and stored at -70°C freezer until further analysis.
2.10 DETERMINATION OF MICROSOMAL PROTEIN CONTENT

Rat hepatic microsomal protein content was determined by using the BioRad® Protein Assay Kit, according to the manufacturer's protocol, which is based on the method of Bradford (1976). Each 10 μl of the microsome sample was diluted in 1.98 ml of distilled water, whereas each 5 ml of the BioRad® Protein Assay Dye reagent (stock concentration was not provided by the manufacturer) was diluted in 200 ml of distilled water. Bovine serum albumin was used as the protein standard. Each 100 μl of the diluted microsome sample or known concentrations of the protein standard was incubated in 5 ml of diluted BioRad® Protein Assay Dye Reagent in the dark for 30 minutes at room temperature. The absorbance at 595 nm was determined using a Beckman model DU-64 spectrophotometer (Beckman Instruments Inc.) and a Protein Assay Soft-Pac module. The absorbance measurements were taken at a wavelength of 595 nm. The spectrophotometric measurement of the blank sample, which contained the diluted BioRad® Protein Assay Dye reagent, was determined. The net absorbance readings for the unknown samples and protein standards were calculated by subtracting the measurement of the blank sample from that of the unknown and standard samples. The standard curve was constructed by plotting the net absorbance readings against known concentrations (70, 140, 210, and 280 μg/ml) of protein standards. Linear regression analysis was conducted and an equation was obtained. The amount of protein present in the unknown samples was determined by using the linear regression equation.
2.11 DETERMINATION OF TOTAL CYP CONTENT

Hepatic microsomal total cytochrome P450 content was determined by the sodium dithionite-reduced, carbon monoxide difference method (Omura and Sato, 1964). Microsome samples (200 μl) were diluted in 3.8 ml of 100 mM sodium phosphate buffer, pH 7.4, containing 0.1 mM EDTA and 20% (v/v) glycerol, in reference and sample cuvettes. Both the reference and sample cuvettes were added with sodium dithionite and the cuvette containing the sample was saturated with carbon monoxide for 1 minute (one bubble per second). This allows binding of carbon monoxide to the reduced heme in cytochrome P450. The spectrum was scanned from a wavelength of 325 nm to 625 nm using a SLM-AMINCO DW-2 scanning spectrophotometer (SLM Instruments Inc. Illinois, USA).

2.12 7-BENZYLOXYRESORUFIN O-DEALKYLATION AND 7-ETHOXYRESORUFIN O-DEALKYLATION ASSAYS

The 7-benzyloxyresorufin O-dealkylation assay was conducted based on the method developed by Burke et al. (1985). Preliminary experiments were performed to determine that the assay was linear with respect to reaction time and the amount of microsomal protein. The assay was performed at 37°C. Each 2 ml reaction mixture contained 0.1 mM HEPES buffer, pH 7.8, 5 mM MgCl₂, 5 μM 7-benzyloxyresorufin and microsomes (75 μg protein). The fluorescence of the standard samples was determined as described above, except the NADPH and the sample microsomes were not included in the reaction mixture. Instead, known concentrations of resorufin and heat-inactivated microsomes were added to the cuvette. The 7-benzyloxyresorufin O-dealkylation assay was determined using a Shimadzu spectrophotometer RF-540 (Shimadzu America/ Illinois, USA).
Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) at an excitation wavelength of 530 nm (5 mm slit width) and an emission wavelength of 582 nm (5 mm slit width). Fluorescence readings of each standard were taken before and after the addition of resorufin. The difference between these two readings was expressed as the net fluorescence reading. The standard curve was plotted as the net fluorescence readings versus the corresponding known concentrations of resorufin. Linear regression analysis was conducted and the equation was obtained.

For each unknown sample, background fluorescence reading was recorded prior to the addition of NADPH. After NADPH was added to initiate the reaction, fluorescence readings were recorded every 30 seconds for 2 minutes. The net fluorescence readings at each time point were calculated by subtracting the measurement of the background from that of the unknown samples. Subsequently, the net fluorescence readings were converted to the amount of resorufin product using the linear regression equation obtained from the standard curve. All fluorescence readings of each sample were within the linear range of the standard curve and all the readings taken in 2 minutes were within a liner reaction period.

For each sample, a product formation curve was constructed by plotting the amount of product formation versus the reaction time. Linear regression analysis was conducted. The slope of the equation is equal to the rate of product formation. The 7-benzyloxyresorufin O-dealkylation activity for each sample was calculated by dividing the rate of product formation (nmol/min) dividing by the amount (mg) of microsomal protein.
The 7-ethoxyresorufin O-dealkylation assay was conducted using the same protocol as the 7-benzyloxyresorufin O-dealkylation assay, except that 7-ethoxyresorufin was the substrate and the amount of microsomal protein was 50 μg in each reaction.

2.13 SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Hepatic microsomal protein was separated by discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) using a Hoefer SE 600 vertical slab gel unit (Hoefer Scientific Instruments, San Francisco, CA, U.S.A). The SDS-polyacrylamide gel consisted of a stacking gel (1.0 cm long x 0.75 mm thick) and a separating gel (12.5 cm long x 0.75 mm thick). The stacking gel was prepared with 3% (w/v) BIS, 0.125 M Tris-HCl (pH 6.8), 0.1% (w/v) of SDS, 0.08% (v/v) of ammonium phosphate, and 0.05% (v/v) TEMED. The separating gel consisted of 7.5% (w/v) BIS, 0.375 M Tris-HCl (pH 8.8), 0.1% (w/v) SDS, 0.042% (v/v) ammonium phosphate, and 0.03% (v/v) TEMED. Microsome samples were diluted to 0.03-0.25 mg/ml (Table 7) in sample dilution buffer containing 0.062 M Tris-HCl (pH 6.8), 1% SDS, 0.01% bromophenol blue, 10% glycerol, and 5% beta-mercaptoethanol, followed by denaturing in boiling water for 2 minutes. The denatured microsome samples were loaded onto the gel in a total volume of 20 μl. The gel was subjected to electrophoresis in a cooling system at a constant current setting at 12 mA per gel for approximately 1 hour or until the dye front migrated through the stacking gel, and then at a constant current setting at 24 mA per gel for 2 hours.
<table>
<thead>
<tr>
<th>Sample*</th>
<th>Protein (µg/lane)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CYP2C11 immunoquantitation</strong></td>
<td></td>
</tr>
<tr>
<td>β-naphthoflavone-treated rats</td>
<td>0.8 or 1</td>
</tr>
<tr>
<td>corn oil-treated rats</td>
<td>4 or 5</td>
</tr>
<tr>
<td><em>Panax ginseng</em> extract- or the corresponding vehicle control-treated rats</td>
<td>0.6-1</td>
</tr>
<tr>
<td><em>Panax quinquefolius</em> extract- or the corresponding vehicle control-treated rats</td>
<td>0.6-1</td>
</tr>
<tr>
<td><strong>Microsomal epoxide hydrolase immunoquantitation</strong></td>
<td></td>
</tr>
<tr>
<td>phenobarbital-treated rats</td>
<td>2 or 4</td>
</tr>
<tr>
<td>0.9% NaCl-treated rats</td>
<td>2 or 4</td>
</tr>
<tr>
<td><em>Panax ginseng</em> extract- or the corresponding vehicle control-treated rats</td>
<td>4</td>
</tr>
<tr>
<td><em>Panax quinquefolius</em> extract- or the corresponding vehicle control-treated rats</td>
<td>4</td>
</tr>
</tbody>
</table>

*The treatments for the rats are described in detail in Table 4.*

2.14 **PURIFICATION OF ANTIBODIES**

The primary antibodies (monospecific rabbit anti-rat CYP2C11 polyclonal IgG and monospecific rabbit anti-rat microsomal epoxide hydrolase polyclonal IgG) were provided by Dr. Stelvio Bandiera (Faculty of Pharmaceutical Sciences, The University of British Columbia).
2.15 PURIFICATION OF CYP2C11 AND MICROsomAL EPOXIDE HYDROLASE PROTEINS

Purified rat hepatic CYP2C11 and microsomal epoxide hydrolase protein standards were provided by Dr. Stelvio Bandiera (Faculty of Pharmaceutical Sciences, The University of British Columbia).

2.16 IMMUNOBLOT ASSAY

The proteins resolved on each separating gel were transferred electrophoretically onto a nitrocellulose membrane according to the method developed by Towbin et al. (1979) using a Hoefer TE52 Transport Unit with power lid. The proteins were transferred in transfer buffer at a constant current of 0.4 A for 2 hours at 4°C. The transfer buffer contained 0.025 M Tris base, pH 8.3, 0.192 M glycine, 0.01% (w/v) SDS and 20% (v/v) methanol. Subsequently, each nitrocellulose membrane was incubated in blocking buffer containing 1% (w/v) bovine serum albumin, 3% (w/v) skim milk powder in modified phosphate buffered saline (PBS), pH 6.9, overnight at 4°C. The modified PBS consisted of 0.137 M NaCl, 2.6 mM KCl, 8.1 mM sodium phosphate, 0.15 M potassium phosphate, and 0.2 mM EDTA. The blocking buffer was discarded the next day. The primary antibody [monospecific rabbit anti-rat CYP2C11 polyclonal IgG (16 μg/ml) or monospecific rabbit anti-rat microsomal epoxide hydrolase polyclonal IgG (5 μg/ml)] was initially diluted in antibody solution containing 1% (w/v) BSA, 3% (w/v) skim milk powder, and 0.05% (v/v) Tween 20 in modified PBS. The membrane was then incubated in the primary antibody solution for two hours at 37 °C with shaking. The membrane was
washed by conducting three 10 minute-incubations at 37°C with shaking using the washing buffer, which contained 0.05% (v/v) Tween 20 in modified PBS. A total 50 ml of washing buffer was used in each washing step. The membrane was subsequently incubated with the secondary antibody (alkaline phosphatase conjugated, goat F(ab')2 anti-rabbit IgG, 1:3000 dilution) in an antibody dilution buffer [0.05% (v/v) Tween 20, 1% (w/v) BSA, 3% (w/v) skim milk powder in modified PBS] for 2 hours at 37°C with shaking. The washing steps were repeated two more times. Each membrane was rinsed with distilled water several times to remove the washing buffer, followed by incubating in substrate solution, 0.03% (w/v) 4-nitro blue tetrazolium (NBT) and 0.015% (w/v) 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 0.1 M Tris-HCl containing 0.5 mM MgCl2, pH 9.5, at room temperature for 2-3 minutes under dim light. The reaction was terminated by rinsing off the substrate solution with distilled water. Each sample was determined in duplicate or triplicate.

2.17 QUANTIFICATION OF IMMUNOBLOTS

Densitometric quantification of immunoblots was performed by using a pdi 420oe™ densitometer (PDI Inc., New York, NY, U.S.A) linked to an AGFA Arcus II scanner using the pdi Quantity One® version 3.0 software program. The quantification for each sample was performed by determining the contour quantity (OD X mm2), which was calculated by multiplying the optical density by the contour area. The relative contour quantity was determined by dividing by the contour quantity of the internal standard sample. The internal standard sample was 0.25 pmol of purified CYP2C11 protein or 0.05 mg of purified microsomal epoxide hydrolase protein. The standard curve was constructed by plotting the net contour quantity of each protein standard
staining bands against the known amounts of protein per lane (0.125, 0.25, 0.375 and 0.5 pmol for CYP2C11 and 0.01, 0.05, 0.1, 0.25 µg for microsomal epoxide hydrolase). Linear regression was conducted and an equation was obtained. The amount of protein in each liver microsomal sample was calculated using the linear regression equation.

2.18 STATISTICAL ANALYSIS

Data were analyzed using Student's t-test when comparing two groups. Whereas when comparing more than two groups, data were analyzed using one-way analysis of variance (ANOVA) and, if appropriate, was followed by the Student Newman-Keuls test. Differences between means were considered statistically significant when the p value was less than 0.05. All data were analyzed using SigmaStat (Jandell Scientific, San Rafael, CA, USA).
CHAPTER 3. RESULTS
3.1 VALIDATION OF REAL-TIME PCR ASSAYS

The real-time PCR assays used to quantify CYP2B1, CYP3A23, CYP2C11, CYP1A2, microsomal epoxide hydrolase and cyclophilin mRNA expression were validated to assess the reliability and reproducibility of the data generated. In this section, linearity of standard curves, reproducibility (precision; intra-day and inter-day variabilities), and accuracy (bias) were evaluated. In addition, melting curve analyses and gel electrophoresis were performed to detect the presence of the PCR amplicons and the formation of primer-dimers.

3.1.1 CYP2B1

The progress curve for the amplification of purified CYP2B1 amplicons is shown in Figure 4. The progress curve was plotted as the fluorescence signal against the cycle number. During amplification, the fluorescence signal increases as the SYBR Green™ I Dye binds to the amplicons. The cycle number, or the cross point, was determined by adjusting the baseline so it was in the log-linear portion of the progress curve. In the present study, the baseline was set at a fixed level (F = 0.01) of fluorescence signal where it is in the log-linear portion of all the progress curves included in each real-time PCR assay. The cross points for the amplification of 100, 300, 1000, 3000, 10000, 30000, 100000 and 300000 copies of purified CYP2B1 amplicon per reaction were 27.69, 25.17, 23.29, 21.54, 19.73, 17.93, 16.33 and 14.82, respectively (Figure 4).

A standard curve for the amplification of purified CYP2B1 amplicon is constructed by plotting the cross point against the amount of purified CYP2B1 amplicon (Figure 5). A log-linear relationship between the cross point and the quantity of these amplicons was obtained between 100 and 300,000 copies.
The accuracy and precision of the real-time CYP2B1 PCR assay were assessed by analyzing the amplification of purified CYP2B1 amplicons of known amounts (1,000 and 100,000 copies per reaction). These amounts represent the range of mRNA levels expected in the unknown samples. The accuracy (% bias) of the 1,000 and 100,000 copies were -3% and -1%, respectively (Table 8). The precision (% coefficient of variation) of the 1,000 and 100,000 copies were 15% and 4%, respectively (Table 8).

The intra-day variability and inter-day variability of the real-time CYP2B1 PCR assay were assessed by amplifying two cDNA samples (induced and control). The cDNA samples were obtained from a rat injected intraperitoneally with sodium phenobarbital (80 mg/kg) (induced sample) or an equivalent volume of 0.9% NaCl (1 ml/kg) (control sample) once daily for four consecutive days. As shown in Table 9, the intra-day and inter-day variabilities (% c.v.) for the induced sample were 2% and 13%, respectively, whereas the intra-day and inter-day variabilities for the control sample were 7% and 30%, respectively.

As shown by the melting curve analysis, a peak was present in the CYP2B1 amplicon (cDNA from a phenobarbital-treated rat) and no peak was detected in the negative controls (i.e. no-template control and no-primer control) (Figure 6). Figure 7 is a representative ethidium bromide-stained agarose gel illustrating the presence of CYP2B1 amplicons. The expected size of the CYP2B1 amplicon is 201 bp. A single band was detected in lanes containing purified CYP2B1 amplicon (lanes 8-9), the induced sample (cDNA obtained from the phenobarbital-treated rat) (lanes 6-7), and the control sample (cDNA obtained from the 0.9% NaCl-treated rat) (lanes 4-5). No band was detected in the negative controls (lanes 2-3). The results from the melting curve
analysis and agarose gel electrophoresis indicate a lack of primer-dimer formation and the absence of multiple amplicons.

Figure 4. A representative progress curve for the real-time amplification of purified CYP2B1 amplicons. Shown is a plot of increase in fluorescence versus cycle number for the amplification of known amounts of purified CYP2B1 amplicons, the quantity of which was determined by the PicoGreen™ dsDNA Quantitation Assay Kit.
**Figure 5.** A representative standard curve for the real-time amplification of purified CYP2B1 amplicons. Shown is a plot of the cross point versus known amounts of purified CYP2B1 amplicons. The cross point is the cycle number at which the fluorescence signal is greater than a defined threshold, one in which all the reactions are in the logarithmic phase of amplification. Each point represents a single determination.
An adult male rat was injected intraperitoneally with sodium phenobarbital (80 mg/kg) dissolved in 0.9% NaCl (1 ml/kg) once daily for four consecutive days. The rat was sacrificed one day after the last injection. The liver was excised and snap-frozen in liquid nitrogen. Total RNA was isolated and reverse transcribed. CYP2B1 cDNA was amplified by real-time PCR, purified using QIAquick™ Gel Extraction Kit, and quantified using the PicoGreen™ Assay Kit. Known amounts (1,000 and 100,000 copies) of purified CYP2B1 amplicons were amplified by real-time PCR in quadruplicate. Data are expressed as % bias (accuracy) and % c.v. (precision).

<table>
<thead>
<tr>
<th>Amount of purified CYP2B1 amplicons (copies)</th>
<th>Accuracy (% bias)</th>
<th>Precision (% c.v.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,000</td>
<td>-3</td>
<td>15</td>
</tr>
<tr>
<td>100,000</td>
<td>-1</td>
<td>4</td>
</tr>
</tbody>
</table>


**TABLE 9**

Intra-day and inter-day variabilities of a real-time PCR method for the quantification of CYP2B1 mRNA expression

<table>
<thead>
<tr>
<th>Sample</th>
<th>Intra-day Variability (% c.v.)</th>
<th>Inter-day Variability (% c.v.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Induced Sample</td>
<td>2</td>
<td>13</td>
</tr>
<tr>
<td>Control Sample</td>
<td>7</td>
<td>30</td>
</tr>
</tbody>
</table>

An adult male rat was injected intraperitoneally with sodium phenobarbital (induced sample) (80 mg/kg) or an equivalent volume of 0.9% NaCl (vehicle control) (1 ml/kg) once daily for four consecutive days. Both rats were sacrificed one day after the last injection. Livers were excised and snap-frozen in liquid nitrogen. Total RNA was isolated and reverse transcribed. CYP2B1 cDNA was amplified by real-time PCR. The levels of CYP2B1 mRNA expression for the induced sample (cDNA from the phenobarbital-treated rat) and the control sample (cDNA from the 0.9% NaCl-treated rat) were determined in quadruplicate in one experiment and the same experiment was repeated on four different days. The intra-day assay variability was expressed as the % c.v. of the quadruplicate determinations obtained in one experiment. The inter-day assay variability was expressed as the % c.v. of the quadruplicate determinations performed on four different days.
Figure 6. A representative melting curve for the analysis of CYP2B1 amplicon. Shown is a plot of the rate of change in fluorescence versus temperature for a CYP2B1 amplicon (cDNA from a phenobarbital-treated rat) amplified by real-time PCR. A single peak was present in the CYP2B1 amplicon (panel A). No peak was obtained in the no-template and no-primer negative controls (panels B and C, respectively). This suggests the absence of primer-dimer formation and the lack of contamination with nonspecific amplicons.
(A). CYP2B1 amplicon (cDNA from the phenobarbital-treated rat)

(B). No-template negative control

(C). No-primer negative control
Figure 7. Agarose gel electrophoresis of CYP2B1 amplicons. An adult male rat was injected intraperitoneally with sodium phenobarbital (80 mg/kg) or an equivalent volume of 0.9% NaCl (1 ml/kg) once daily for four consecutive days. Both rats were sacrificed one day after the last injection. Livers were excised and snap-frozen in liquid nitrogen. Total RNAs were isolated and reverse transcribed. CYP2B1 cDNAs were amplified by real-time PCR (36 cycles) and the amplicons were subjected to agarose (3%, w/v) gel electrophoresis. Shown is an ethidium bromide-stained agarose gel. The expected size of the CYP2B1 amplicon is 201 bp. Lane 1, DNA ladder; lane 2, no template control; lane 3, no primer control; lanes 4 and 5, control sample (cDNA from the 0.9% NaCl-treated rat); lanes 6 and 7, induced sample (cDNA from the phenobarbital-treated rat); lanes 8 and 9, purified CYP2B1 amplicon.
3.1.2 CYP3A23

The progress curve for the amplification of purified CYP3A23 amplicons is shown in Figure 8. The cross points for the amplification of 1000, 3000, 10000, 30000, 100000, and 300000 copies of purified CYP3A23 amplicon per reaction were 14.68, 16.48, 18.34, 19.90, 21.92, and 23.41, respectively.

Shown in Figure 9 is a standard curve for the real-time amplification of purified CYP3A23 amplicons. A log-linear relationship between the cross point and the quantity of these amplicons was obtained between 1,000 to 300,000 copies.

The accuracy and precision of the real-time CYP3A23 PCR assay were assessed by analyzing the amplification of purified CYP3A23 amplicons of known amounts (10,000 and 100,000 copies per reaction). The accuracy (% bias) of the amplification of 10,000 and 100,000 copies were 2% and 1%, respectively (Table 10). The precision (% c.v.) of the amplification of 10,000 and 100,000 copies were 6% and 3%, respectively (Table 10).

The intra-day variability and inter-day variability of the real-time CYP3A23 PCR assay were assessed by amplifying two cDNA samples (induced and control). The cDNA samples were obtained from a rat injected intraperitoneally with dexamethasone (100 mg/kg) (induced sample) or an equivalent volume of 2% Tween 80 (1 ml/kg) (control sample) once daily for three consecutive days. The intra-day and inter-day variabilities (% c.v.) for the induced sample were 8% and 14%, respectively, whereas the intra-day and inter-day variabilities for the control sample were 4% and 22%, respectively (Table 11).

As shown by the melting curve analysis, a peak was present in the CYP3A23 amplicon (cDNA from a dexamethasone-treated rat) and no peak was detected in the
negative controls (i.e. no-template control and no-primer control) (Figure 10). Figure 11 is a representative ethidium bromide-stained agarose gel illustrating the presence of CYP3A23 amplicons. The expected size of the CYP3A23 amplicon is 328 bp. A single band was detected in lanes containing purified CYP3A23 amplicon (lanes 8-9), the induced sample (cDNA from the dexamethasone-treated rat) (lanes 6-7), and the control sample (cDNA from the 2% Tween 80-treated rat) (lanes 4-5). No band was detected in the negative controls (lanes 2-3). Results from the melting curve analysis and agarose gel electrophoresis indicate a lack of primer-dimer formation and the absence of multiple amplicons.
Figure 8. A representative progress curve for the real-time amplification of purified CYP3A23 amplicons. Shown is a plot of increase in fluorescence versus cycle number for the amplification of known amounts of purified CYP3A23 amplicons, the quantity of which was determined by the PicoGreen™ dsDNA Quantitation Assay Kit.
Figure 9. A representative standard curve for the real-time amplification of purified CYP3A23 amplicons. Shown is a plot of the cross point versus known amounts of purified CYP3A23 amplicons. The cross point is the cycle number at which the fluorescence signal is greater than a defined threshold, one in which all the reactions are in the logarithmic phase of amplification. Each point represents a single determination.
TABLE 10

Accuracy and precision of a real-time PCR method for the quantification of CYP3A23 mRNA expression

<table>
<thead>
<tr>
<th>Amount of purified CYP3A23 amplicons (copies)</th>
<th>Accuracy (% bias)</th>
<th>Precision (% c.v.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10,000</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>100,000</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

An adult male rat was injected intraperitoneally with dexamethasone (100 mg/kg) dissolved in 2% Tween 80 (1 ml/kg) once daily for three consecutive days. The rat was sacrificed one day after the last injection. The liver was excised and snap-frozen in liquid nitrogen. Total RNA was isolated and reverse transcribed. CYP3A23 cDNA was amplified by real-time PCR, purified using QIAquick™ Gel Extraction Kit, and quantified using the PicoGreen™ Assay Kit. Known amounts (10,000 and 100,000 copies) of purified CYP3A23 amplicons were amplified by real-time PCR in quadruplicate. Data are expressed as % bias (accuracy) and % c.v. (precision).
TABLE 11
Intra-day and inter-day variabilities of a real-time PCR method for the quantification of CYP3A23 mRNA expression

<table>
<thead>
<tr>
<th>Sample</th>
<th>Intra-day Variability (% c.v.)</th>
<th>Inter-day Variability (% c.v.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Induced Sample</td>
<td>8</td>
<td>14</td>
</tr>
<tr>
<td>Control Sample</td>
<td>4</td>
<td>22</td>
</tr>
</tbody>
</table>

An adult male rat was injected intraperitoneally with dexamethasone (100 mg/kg) or an equivalent volume of 2% Tween 80 (vehicle control) (1 ml/kg) once daily for three consecutive days. Both rats were sacrificed one day after the last injection. Livers were excised and snap-frozen in liquid nitrogen. Total RNA was isolated and reverse transcribed. CYP3A23 cDNA was amplified by real-time PCR. The levels of CYP3A23 mRNA expression for the induced sample (cDNA from the dexamethasone-treated rat) and the control sample (cDNA from the 2% Tween 80-treated rat) were determined in quadruplicate in one experiment and the same experiment was repeated on four different days. The intra-day assay variability was expressed as the % c.v. of the quadruplicate determinations obtained in one experiment. The inter-day assay variability was expressed as the % c.v. of the quadruplicate determinations performed on four different days.
Figure 10. A representative melting curve for the analysis of CYP3A23 amplicon. Shown is a plot of the rate of change in fluorescence versus temperature for a CYP3A23 amplicon (cDNA from a dexamethasone-treated rat) amplified by real-time PCR. A single peak was present in the CYP3A23 amplicon (panel A). No peak was obtained in the no-template and no-primer negative controls (panels B and C, respectively). This suggests the absence of primer-dimer formation and the lack of contamination with nonspecific amplicons.
(A). CYP3A23 amplicon (cDNA from a dexamethasone-treated rat)

(B). No-template negative control

(C). No-primer negative control
Figure 11. Agarose gel electrophoresis of CYP3A23 amplicons. An adult male rat was injected intraperitoneally with dexamethasone (100 mg/kg) or an equivalent volume of 2% Tween 80 (1 ml/kg) once daily for three consecutive days. Both rats were sacrificed one day after the last injection. Livers were excised and snap-frozen in liquid nitrogen. Total RNAs were isolated and reverse transcribed. CYP3A23 cDNAs were amplified by real-time PCR (36 cycles) and the amplicons were subjected to agarose (1.7%, w/v) gel electrophoresis. Shown is an ethidium bromide-stained agarose gel. The expected size of the CYP3A23 amplicon is 328 bp. Lane 1, DNA ladder; lane 2, no template control; lane 3, no primer control; lanes 4 and 5, control sample (cDNA from the 2% Tween 80-treated rat); lanes 6 and 7, induced sample (cDNA from the dexamethasone-treated rat); lanes 8 and 9, purified CYP3A23 amplicon.
3.1.3 CYP2C11

Shown in Figure 12 is the progress curve for the amplification of purified CYP2C11 amplicons. The cross points for the amplification of 1000, 3000, 10000, 30000, 100000, and 300000 copies of purified CYP2C11 amplicon per reaction were 24.57, 22.90, 20.83, 19.00, 17.14, and 15.35, respectively.

As illustrated in the standard curve for the real-time amplification of purified and unpurified CYP2C11 amplicon, a log-linear relationship between the cross point and the quantity of these amplicons was obtained between 1,000 to 300,000 copies (Figure 13).

The accuracy and precision of the real-time CYP2C11 PCR assay were assessed by analyzing the amplification of purified CYP2C11 amplicons of known amount (100,000 copies per reaction). As shown in Table 12 the accuracy (% bias) was < 1% and the precision (% c.v.) was 2%, respectively.

The intra-day variability and inter-day variability of the real-time CYP2C11 PCR assay were assessed by amplifying one cDNA sample obtained from a rat injected intraperitoneally with 0.9% NaCl (1 ml/kg) once daily for four consecutive days. The intra-day and inter-day variabilities (% c.v.) for the real-time CYP2C11 PCR assay were 3% and 17%, respectively (Table 13).

As shown by the melting curve analysis, a peak was present in the CYP2C11 amplicon (cDNA from a 0.9% NaCl-treated rat) and no peak was detected in the negative controls (i.e. no-template control and no primer control) (Figure 14). Figure 15 is a representative ethidium bromide-stained agarose gel illustrating the presence of CYP2C11 amplicons. The expected size of the CYP2C11 amplicon is 248 bp. A single band was detected in lanes containing purified CYP2C11 amplicon (lanes 6-7) and the cDNA sample (obtained from the 0.9% NaCl-treated rat) (lanes 4-5), and no band was
detected in the negative controls (lanes 2-3). The results from the melting curve analysis and agarose gel electrophoresis indicate a lack of primer-dimer formation and the absence of multiple amplicons.

**Figure 12.** A representative progress curve for the real-time amplification of purified CYP2C11 amplicons. Shown is a plot of increase in fluorescence versus cycle number for the amplification of known amounts of purified CYP2C11 amplicons, the quantity of which was determined by the PicoGreen™ dsDNA Quantitation Assay Kit.
Figure 13. A representative standard curve for the real-time amplification of purified CYP2C11 amplicons. Shown is a plot of the cross point versus known amounts of purified CYP2C11 amplicons. The cross point is the cycle number at which the fluorescence signal is greater than a defined threshold, one in which all the reactions are in the logarithmic phase of amplification. Each point represents a single determination.

\[ Y = -3.74 \log X + 35.82 \]
### TABLE 12

Accuracy and precision of a real-time PCR method for the quantification of CYP2C11 mRNA expression

<table>
<thead>
<tr>
<th>Amount of purified CYP2C11 amplicons (copies)</th>
<th>Accuracy (% bias)</th>
<th>Precision (% c.v.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100,000</td>
<td>&lt; 1</td>
<td>2</td>
</tr>
</tbody>
</table>

An adult male rat was injected intraperitoneally with 0.9% saline (1 ml/kg) once daily for four consecutive days. The rat was sacrificed one day after the last injection. The liver was excised and snap-frozen in liquid nitrogen. Total RNA was isolated and reverse transcribed. CYP2C11 cDNA was amplified by real-time PCR, purified using QIAquick™ Gel Extraction Kit, and quantified using the PicoGreen™ Assay Kit. A known amount (100,000 copies) of purified CYP2C11 amplicon was amplified by real-time PCR in quadruplicate. Data are expressed as % bias (accuracy) and % c.v. (precision).
An adult male rat was injected intraperitoneally with 0.9% NaCl (1 m/kg) once daily for four consecutive days. The rat was sacrificed one day after the last injection. The liver was excised and snap-frozen in liquid nitrogen. Total RNA was isolated and reverse transcribed. CYP2C11 cDNA was amplified by real-time PCR. The level of CYP2C11 mRNA expression for the cDNA sample was determined in quadruplicate in one experiment and the same experiment was repeated on four different days. The intra-day assay variability was expressed as the % c.v. of the quadruplicate determinations obtained in one experiment. The inter-day assay variability was expressed as the % c.v. of the quadruplicate determinations performed on four different days.
Figure 14. A representative melting curve for analysis of CYP2C11 amplicon. Shown is a plot of the rate of change in fluorescence versus temperature for a CYP2C11 amplicon (cDNA from a 0.9% NaCl-treated rat) amplified by real-time PCR. A single peak was present in the CYP2C11 amplicon (panel A). No peak was observed in the no-template and no-primer negative controls (panels B and C, respectively). This suggests the absence of primer-dimer formation and the lack of contamination with nonspecific amplicons.
A). CYP2C11 amplicon (cDNA from a 0.9% NaCl-treated rat)

(B). No-template negative control

(C). No-primer negative control
Figure 15. Agarose gel electrophoresis of CYP2C11 amplicons. An adult male rat was injected intraperitoneally with 0.9% NaCl (1 ml/kg) once daily for four consecutive days. The rat was sacrificed one day after the last injection. The liver was excised and snap-frozen in liquid nitrogen. Total RNA was isolated and reverse transcribed. CYP2C11 cDNA was amplified by real-time PCR (36 cycles) and the amplicons were subjected to agarose (3%, w/v) gel electrophoresis. Shown is an ethidium bromide-stained agarose gel. The expected size of the CYP2C11 amplicon is 248 bp. Lane 1, DNA ladder; lane 2, no template control; lane 3, no primer control; lanes 4 and 5, cDNA sample from the 0.9% NaCl-treated rat; lanes 6 and 7, purified CYP2C11 amplicon.
3.1.4 CYP1A2

The progress curve for the amplification of purified CYP1A2 amplicons is shown in Figure 16. The cross points for the amplification of 10000, 30000, 100000, 300000, and 1000000 copies of purified CYP1A2 amplicons per reaction were 22.54, 20.69, 18.83, 17.14, and 15.23 respectively.

Shown in Figure 17 is a standard curve for the real-time amplification of purified CYP1A2 amplicons. A log-linear relationship between the cross point and the quantity of these amplicons was obtained between 10,000 and 1,000,000 copies.

In the real-time CYP1A2 PCR assay, the accuracy and precision were assessed by analyzing the amplification of purified CYP1A2 amplicons of known amounts (30,000 and 300,000 copies per reaction). These amounts represent the range of mRNA levels expected in the unknown samples. The accuracy (% bias) of the 30,000 and 300,000 copies were 5% and 3% (Table 14), respectively. The precision (% c.v.) of the 30,000 and 300,000 copies were -19% and 15%, respectively (Table 14).

The intra-day variability and inter-day variability for the real-time CYP1A2 PCR assay were assessed by amplifying two cDNA samples (induced and control). The cDNA samples were obtained from a rat injected intraperitoneally with β-naphthoflavone 40 mg/kg (induced sample) or an equivalent volume of corn oil (1 ml/kg) (control sample) once daily for three consecutive days. The intra-day and inter-day variabilities (% c.v.) for the induced sample were 3% and 12%, respectively, whereas the intra-day and inter-day variabilities for the control sample were 4% and 13%, respectively (Table 15).

As shown by the melting curve, a peak was present in the CYP1A2 amplicon (cDNA from a β-naphthoflavone-treated rat) and no peak was detected in the negative
controls (i.e. no-template control and no-primer control) (Figure 18). Figure 19 is a representative ethidium bromide-stained agarose gel illustrating the presence of CYP1A2 amplicons. The expected size of the CYP1A2 amplicon is 236 bp. A single band was detected in lanes containing purified CYP1A2 amplicon (lanes 8-9), the induced sample (cDNA obtained from the β-naphthoflavone-treated rat) (lanes 6-7), and the control sample (cDNA from the corn oil-treated rat) (lanes 4-5). No band was detected in the negative controls (lanes 2-3). The results from the melting curve analysis and agarose gel electrophoresis indicate a lack of primer-dimer formation and the absence of multiple amplicons.
Figure 16. A representative progress curve for the real-time amplification of purified CYP1A2 amplicons. Shown is a plot of increase in fluorescence versus cycle number for the amplification of known amounts of purified CYP1A2 amplicons, the quantity of which was determined by the PicoGreen™ dsDNA Quantitation Assay Kit.
Figure 17. A representative standard curve for the real-time amplification of purified CYP1A2 amplicons. Shown is a plot of the cross point versus known amounts of purified CYP1A2 amplicons. The cross point is the cycle number at which the fluorescence signal is greater than a defined threshold, one in which all the reactions are in the logarithmic phase of amplification. Each point represents a single determination.

\[ Y = -3.63 \log X + 37.03 \]
TABLE 14

Accuracy and precision of a real-time PCR method for the quantification of CYP1A2 mRNA expression

<table>
<thead>
<tr>
<th>Amount of purified CYP1A2 amplicons (copies)</th>
<th>Accuracy (% bias)</th>
<th>Precision (% c.v.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30,000</td>
<td>5</td>
<td>-19</td>
</tr>
<tr>
<td>300,000</td>
<td>3</td>
<td>15</td>
</tr>
</tbody>
</table>

An adult male rat was injected intraperitoneally with β-naphthoflavone (40 mg/kg) suspended in corn oil (1 ml/kg) once daily for three consecutive days. The rat was sacrificed one day after the last injection. The liver was excised and snap-frozen in liquid nitrogen. Total RNA was isolated and reverse transcribed. CYP1A2 cDNA was amplified by real-time PCR, purified using QIAquick™ Gel Extraction Kit, and quantified using the PicoGreen™ Assay Kit. Known amounts (30,000 and 300,000 copies) of purified CYP1A2 amplicons were amplified by real-time PCR in quadruplicate. Data are expressed as % bias (accuracy) and % c.v. (precision).
<table>
<thead>
<tr>
<th>Sample</th>
<th>Intra-day Variability (% c.v.)</th>
<th>Inter-day Variability (% c.v.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Induced Sample</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>Control Sample</td>
<td>4</td>
<td>13</td>
</tr>
</tbody>
</table>

An adult male rat was injected intraperitoneally with β-naphthoflavone (40 mg/kg) or an equivalent volume of corn oil (vehicle control) (1 m/kg) once daily for three consecutive days. Both rats were sacrificed one day after the last injection. Livers were excised and snap-frozen in liquid nitrogen. Total RNA was isolated and reverse transcribed. CYP1A2 cDNA was amplified by real-time PCR. The levels of CYP1A2 mRNA expression for the induced sample (cDNA from the β-naphthoflavone-treated rat) and the control sample (cDNA from the corn oil-treated rat) were determined in quadruplicate in one experiment and the same experiment was repeated on four different days. The intra-day assay variability was expressed as the % c.v. of the quadruplicate determinations obtained in one experiment. The inter-day assay variability was expressed as the % c.v. of the quadruplicate determinations performed on four different days.
Figure 18. A representative melting curve for analysis of CYP1A2 amplicon. Shown is a plot of the rate of change in fluorescence versus temperature for a CYP1A2 amplicon (cDNA from a β-naphthoflavone-treated rat) amplified by real-time PCR. A single peak was present in the CYP1A2 amplicon (panel A). No peak was observed in the no-template and no-primer negative controls (panels B and C, respectively). This suggests the absence of primer-dimer formation and the lack of contamination with nonspecific amplicons.
(A). CYP1A2 amplicon (cDNA from a β-naphthoflavone-treated rat)

(B). No-template negative control

(C). No-primer negative control
Figure 19. Agarose gel electrophoresis of CYP1A2 amplicons. An adult male rat was injected intraperitoneally with β-naphthoflavone (40 mg/kg) or an equivalent volume of corn oil (1 ml/kg) once daily for three consecutive days. Both rats were sacrificed one day after the last injection. Livers were excised and snap-frozen in liquid nitrogen. Total RNAs were isolated and reverse transcribed. CYP1A2 cDNAs were amplified by real-time PCR (36 cycles) and the amplicons were subjected to agarose (3%, w/v) gel electrophoresis. Shown is an ethidium bromide-stained agarose gel. The expected size of the CYP1A2 amplicon is 236 bp. Lane 1, DNA ladder; lane 2, no template control; lane 3, no primer control; lanes 4 and 5, control sample (cDNA from the corn oil-treated rat); lanes 6 and 7, induced sample (cDNA from the β-naphthoflavone-treated rat); lanes 8 and 9, purified CYP1A2 amplicon.
3.1.5 MICROSOMAL EPOXIDE HYDROLASE

The progress curve for the amplification of purified microsomal epoxide hydrolase amplicons is shown in Figure 20. The cross points for the amplification of 1000, 3000, 10000, 30000, 100000 and 300000 copies of purified microsomal epoxide hydrolase amplicons per reaction were 24.29, 22.65, 20.32, 18.43, 16.32 and 14.45, respectively.

A standard curve for the amplification of purified microsomal epoxide hydrolase amplicon is shown in Figure 21. A log-linear relationship between the cross point and the quantity of these amplicons was obtained between 1,000 and 300,000 copies.

The accuracy and precision of the real-time microsomal epoxide hydrolase PCR assay were assessed by analyzing the amplification of purified microsomal epoxide hydrolase amplicons of known amounts (10,000 and 100,000 copies per reaction). The accuracy (% bias) of the amplification of 10,000 and 100,000 copies were -2% and 4%, respectively (Table 16). The precision (% c.v.) of the amplification of 10,000 and 100,000 copies were 8% and 4%, respectively (Table 16).

The intra-day variability and inter-day variability of the real-time microsomal epoxide hydrolase PCR assay were assessed by amplifying two cDNA samples (induced and control). The cDNA samples were obtained from a rat injected intraperitoneally with sodium phenobarbital (80 mg/kg) (induced sample) or an equivalent volume of 0.9% NaCl (1 ml/kg) (control sample) once daily for four consecutive days. The intra-day and inter-day variabilities (% c.v.) for the induced sample were 3% and 11%, respectively, whereas the intra-day and inter-day variabilities for the control sample were 4% and 13%, respectively (Table 17).
The melting curve analysis is presented in Figure 22. As shown in the melting curve, a peak was present in the microsomal epoxide hydrolase amplicon (cDNA from a phenobarbital-treated rat) and no peak was detected in the negative controls (i.e. no-template control and no-primer control). Figure 23 is a representative ethidium bromide-stained agarose gel illustrating the presence of microsomal epoxide hydrolase amplicons. The expected size of the microsomal epoxide hydrolase amplicon is 135 bp. A single band was detected in lanes containing purified microsomal epoxide hydrolase amplicon (lanes 8-9), the induced sample (cDNA obtained from the phenobarbital-treated rat) (lanes 6-7), and the control sample (cDNA obtained from 0.9% NaCl-treated rat) (lanes 4-5). No band was detected in the negative controls (lanes 2-3). The results from the melting curve analysis and agarose gel electrophoresis indicate a lack of primer-dimer formation and the absence of multiple amplicons.
Figure 20. A representative progress curve for the real-time amplification of purified microsomal epoxide hydrolase amplicons. Shown is a plot of increase in fluorescence versus cycle number for the amplification of known amounts of purified microsomal epoxide hydrolase amplicons, the quantity of which was determined by the PicoGreen™ dsDNA Quantitation Assay Kit.
Figure 21. A representative standard curve for the real-time amplification of purified microsomal epoxide hydrolase (mEH) amplicons. Shown is a plot of the cross point versus known amounts of purified microsomal epoxide hydrolase amplicons. The cross point is the cycle number at which the fluorescence signal is greater than a defined threshold, one in which all the reactions are in the logarithmic phase of amplification. Each point represents a single determination.
TABLE 16

Accuracy and precision of a real-time PCR method for the quantification of microsomal epoxide hydrolase mRNA expression

<table>
<thead>
<tr>
<th>Amount of purified microsomal epoxide hydrolase amplicons (copies)</th>
<th>Accuracy (% bias)</th>
<th>Precision (% c.v.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10,000</td>
<td>-2</td>
<td>8</td>
</tr>
<tr>
<td>100,000</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

An adult male rat was injected intraperitoneally with sodium phenobarbital (80 mg/kg) dissolved in 0.9% NaCl (1 ml/kg) once daily for four consecutive days. The rat was sacrificed one day after the last injection. The liver was excised and snap-frozen in liquid nitrogen. Total RNA was isolated and reverse transcribed. Microsomal epoxide hydrolase cDNA was amplified by real-time PCR, purified using QIAquick™ Gel Extraction Kit, and quantified using the PicoGreen™ Assay Kit. Known amounts (1,000 and 100,000 copies) of purified microsomal epoxide hydrolase amplicons were amplified by real-time PCR in quadruplicate. Data are expressed as % bias (accuracy) and % c.v. (precision).
### TABLE 17

Intra-day and inter-day variabilities of a real-time PCR method for the quantification of microsomal epoxide hydrolase mRNA expression

<table>
<thead>
<tr>
<th>Sample</th>
<th>Intra-day Variability (% c.v.)</th>
<th>Inter-day Variability (% c.v.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Induced Sample</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>Control Sample</td>
<td>4</td>
<td>13</td>
</tr>
</tbody>
</table>

An adult male rats was injected intraperitoneally with sodium phenobarbital (80 mg/kg) or an equivalent volume of 0.9% NaCl (vehicle control) (1 ml/kg) once daily for four consecutive days. Both rats were sacrificed one day after the last injection. Livers were excised and snap-frozen in liquid nitrogen. Total RNA was isolated and reverse transcribed. Microsomal epoxide hydrolase cDNA was amplified by real-time PCR. The levels of microsomal epoxide hydrolase mRNA expression for the induced sample (cDNA from the phenobarbital-treated rat) and the control sample (cDNA from the 0.9% NaCl-treated rat) were determined in quadruplicate in one experiment and the same experiment was repeated on four different days. The intra-day assay variability was expressed as the % c.v. of the quadruplicate determinations obtained in one experiment. The inter-day assay variability was expressed as the % c.v. of the quadruplicate determinations performed on four different days.
Figure 22. A representative melting curve for analysis of microsomal epoxide hydrolase (mEH) amplicon. Shown is a plot of the rate of change in fluorescence versus temperature for a microsomal epoxide hydrolase amplicon (cDNA from a phenobarbital-treated rat) amplified by real-time PCR. A single peak was present in the microsomal epoxide hydrolase amplicon (panel A). No peak was observed in the no-template and no-primer negative controls (panels B and C, respectively). This suggests the absence of primer-dimer formation and the lack of contamination with nonspecific amplicons.
(A). Microsomal epoxide hydrolase amplicon (cDNA from a phenobarbital-treated rat)

(B). No-template negative control

(C). No-primer negative control
Figure 23. Agarose gel electrophoresis of microsomal epoxide hydrolase amplicons. An adult male rat was injected intraperitoneally with sodium phenobarbital (80 mg/kg) or an equivalent volume of 0.9% NaCl (1 ml/kg) once daily for four consecutive days. Both rats were sacrificed one day after the last injection. Livers were excised and snap-frozen in liquid nitrogen. Total RNAs were isolated and reverse transcribed. Microsomal epoxide hydrolase cDNAs were amplified by real-time PCR (36 cycles) and the amplicons were subjected to agarose (1.7%) gel electrophoresis. Shown is an ethidium bromide-stained agarose gel. The expected size of the microsomal epoxide hydrolase is 135 bp. Lane 1, DNA ladder; lane 2, no template control; lane 3, no primer control; lanes 4 and 5, control sample (cDNA from the 0.9% NaCl-treated rat); lanes 6 and 7, induced sample (cDNA from the sodium phenobarbital-treated rat); lanes 8 and 9, purified microsomal epoxide hydrolase amplicon.
3.1.6 CYCLOPHILIN

The progress curve for the amplification of purified cyclophilin amplicons is shown in Figure 4. The cross points for the amplification of 3000, 10000, 30000, 100000, 300000, 1000000 copies of purified cyclophilin amplicon per reaction were 22.66, 20.81, 18.92, 16.88, 15.09, and 13.28, respectively (Figure 24).

A standard curve for the amplification of purified cyclophilin amplicon is constructed by plotting the cross point against the amount of purified cyclophilin amplicon (Figure 25). A log-linear relationship between the cross point and the quantity of these amplicons was obtained between 3000 and 1000000 copies.

The accuracy and precision of the real-time cyclophilin PCR assay were assessed by analyzing the amplification of purified cyclophilin amplicons of known amount (100,000 copies per reaction). As shown in Table 18 the accuracy (% bias) was 4% and the precision (% c.v.) was 4%.

The intra-day variability and inter-day variability of the real-time cyclophilin PCR assay were assessed by amplifying one cDNA sample obtained from a rat injected intraperitoneally with 0.9% NaCl (1 ml/kg) once daily for four consecutive days. The intra-day and inter-day variabilities (% c.v.) for the real-time cyclophilin PCR assay were 4% and 8%, respectively (Table 19).

As shown by the melting curve analysis, a peak was present in the cyclophilin amplicon (cDNA from a 0.9% NaCl-treated rat) and no peak was detected in the negative controls (i.e. no-template control and no primer control) (Figure 26). Figure 27 is a representative ethidium bromide-stained agarose gel illustrating the presence of CYP2C11 amplicons. The expected size of the cyclophilin amplicon is 265 bp. A single band was detected in lanes containing purified cyclophilin amplicon (lanes 6-7) and the
cDNA sample (obtained from the 0.9% NaCl-treated rat) (lanes 4-5), and no band was detected in the negative controls (lanes 2-3). The results from the melting curve analysis and agarose gel electrophoresis indicate a lack of primer-dimer formation and the absence of multiple amplicons.

Figure 24. A representative progress curve for the real-time amplification of purified cyclophilin amplicons. Shown is a plot of increase in fluorescence versus cycle number for the amplification of known amounts of purified cyclophilin amplicons, the quantity of which was determined by the PicoGreen™ dsDNA Quantitation Assay Kit.
Figure 25. A representative calibration curve for the real-time amplification of purified cyclophilin amplicons. Shown is a plot of the cross point versus known amounts of purified cyclophilin amplicons. The cross point is the cycle number at which the fluorescence signal is greater than a defined threshold, one in which all the reactions are in the logarithmic phase of amplification. Each point represents a single determination.
### TABLE 18

Accuracy and precision of a real-time PCR method for the quantification of cyclophilin mRNA expression

<table>
<thead>
<tr>
<th>Amount of purified cyclophilin amplicon (copies)</th>
<th>Accuracy (% bias)</th>
<th>Precision (% c.v.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100,000</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

An adult male rat was injected intraperitoneally with 0.9% NaCl (1 ml/kg) once daily for four consecutive days. The rat was sacrificed one day after the last injection. The liver was excised and snap-frozen in liquid nitrogen. Total RNA was isolated and reverse transcribed. Cyclophilin was amplified by real-time PCR, purified using QIAquick™ Gel Extraction Kit, and quantified using the PicoGreen™ Assay Kit. A known amount (100,000 copies) of purified cyclophilin amplicon was amplified by real-time PCR in quadruplicate. Data are expressed as % bias (accuracy) and % c.v. (precision).
TABLE 19

Intra-day and inter-day variabilities of a real-time PCR method for the quantification of cyclophilin mRNA expression

<table>
<thead>
<tr>
<th>Intra-day Variability (% c.v.)</th>
<th>Inter-day Variability (% c.v.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>8</td>
</tr>
</tbody>
</table>

An adult male rat was injected intraperitoneally with 0.9% NaCl (1 m/kg) once daily for four consecutive days. The rat was sacrificed one day after the last injection. The liver was excised and snap-frozen in liquid nitrogen. Total RNA was isolated and reverse transcribed. Cyclophilin cDNA was amplified by real-time PCR. The level of cyclophilin mRNA expression for the cDNA sample was determined in quadruplicate in one experiment and the same experiment was repeated on four different days. The intra-day assay variability was expressed as the % c.v. of the quadruplicate determinations obtained in one experiment. The inter-day assay variability was expressed as the % c.v. of the quadruplicate determinations performed on four different days.
Figure 26. A representative melting curve for the analysis of cyclophilin amplicon. Shown is a plot of the rate of change in fluorescence versus temperature for a cyclophilin amplicon (cDNA from a 0.9% NaCl-treated rat) amplified by real-time PCR. A single peak was present in the cyclophilin amplicon (panel A). No peak was observed in the no-template and no-primer negative controls (panels B and C, respectively). This suggests the absence of primer-dimer formation and the lack of contamination with nonspecific amplicons.
(A) Cyclophilin amplicon (cDNA from a 0.9% NaCl-treated rat)

(B) No-template negative control

(C) No-primer negative control
Figure 27. Agarose gel electrophoresis of cyclophilin amplicons. An adult male rat was injected intraperitoneally with 0.9% saline (1 ml/kg) once daily for four consecutive days. The rat was sacrificed one day after the last injection. The liver was excised and snap-frozen in liquid nitrogen. Total RNA was isolated and reverse transcribed. Cyclophilin cDNA was amplified by real-time PCR (36 cycles) and the amplicons were subjected to agarose (3%, w/v) gel electrophoresis. Shown is an ethidium bromide-stained agarose gel. The expected size of the cyclophilin amplicon is 265 bp. Lane 1, DNA ladder; lane 2, no template control; lane 3, no primer control; lanes 4 and 5, cDNA sample from the 0.9% NaCl-treated rat; lanes 6 and 7, purified cyclophilin amplicon.
3.2 TREATMENT OF ADULT MALE RATS WITH PANAX GINSENG EXTRACT

In the present study, adult male Sprague-Dawley rats were administered a single dose of Panax ginseng extract (30 or 100 mg/kg) of known ginsenoside composition (Figure 1) or an equivalent volume (2 ml/kg) of 0.9% NaCl (vehicle control) by oral gavage. All rats were decapitated one day after the treatment. The following dependent variables were determined: body weight gain; absolute and relative liver weight; absolute and relative testes weight; hepatic CYP2B1, CYP3A23, CYP1A2, and microsomal epoxide hydrolase mRNA levels; hepatic CYP2C11 and microsomal epoxide hydrolase protein levels; and hepatic microsomal 7-benzyloxyresorufin O-dealkylation and 7-ethoxyresorufin O-dealkylation activities.

3.2.1 BODY WEIGHT GAIN, LIVER WEIGHT AND TESTES WEIGHT

The effects of Panax ginseng extract on body weight gain, liver weight and testes weight are summarized in Table 20. A single oral dose of a Panax ginseng extract (30 or 100 mg/kg) did not affect body weight gain, liver weight or testes weight, when expressed as absolute or relative values.

3.2.2 TOTAL HEPATIC MICROSONAL CYTOCHROME P450 CONTENT

As shown in Figure 28, a single oral dose of a Panax ginseng extract (30 or 100 mg/kg) did not affect hepatic microsomal total cytochrome P450 content.
3.2.3 HEPA CYP2B EXPRESSION

3.2.3.1 CYP2B1 mRNA

Lee et al. (1987)\textsuperscript{2} reported an increased hepatic microsomal aminopyrine N-demethylation activity in rats treated by oral gavage with a single dose (10, 20, or 30 mg/kg) of Panax ginseng extract. CYP2B enzymes catalyze aminopyrine N-demethylation (Imaoka et al., 1988) and CYP2B1 is a major inducible CYP2B enzyme in rats (Thomas et al., 1983). To determine whether the increased hepatic microsomal aminopyrine N-demethylation activity was due to an increase in CYP2B enzyme expression, hepatic CYP2B1 mRNA expression was measured in rats that received Panax ginseng extract. A single dose (30 or 100 mg/kg) of Panax ginseng administered by oral gavage did not alter hepatic CYP2B1 mRNA expression, regardless of whether the data were expressed as copies per ng total dsDNA (Figure 29) or as a ratio of CYP2B1 mRNA over cyclophilin mRNA expression (Table A1, see Appendix).

3.2.3.2 MICROSOMAL 7-BENZYLOXYRESORUFIN O-DEALKYLATION ACTIVITY

To determine the effect of Panax ginseng extract on CYP2B enzyme activity in rat liver microsomes, the 7-benzyloxyresorufin O-dealkylation assay was performed. As shown in Figure 30, a single dose (30 or 100 mg/kg) of Panax ginseng extract administered by oral gavage did not affect hepatic microsomal 7-benzyloxyresorufin O-dealkylation activity.

3.2.3.3 POSITIVE CONTROL EXPERIMENTS WITH PHENOBARBITAL

To verify that the absence of an effect by the Panax ginseng extract was not due to the assay conditions, positive control experiments were conducted. Under the same
PCR conditions, a 47-fold (p < 0.05) increase in CYP2B1 mRNA level was obtained in the phenobarbital-treated group (80 mg/kg, intraperitoneal injection, once daily for four consecutive days), when compared to the 0.9% NaCl-treated group (Figure 29). Furthermore, another positive control experiment was included in the 7-benzoyloxyresorufin O-dealkylation assay. Adult male rats were injected intraperitoneally with phenobarbital (80 mg/kg) or an equivalent volume of 0.9% NaCl (1 ml/kg) (vehicle control) for four consecutive days. As shown in Figure 31, under the same assay conditions, the phenobarbital-treated group had an approximately 37-fold increase in hepatic microsomal 7-benzoyloxyresorufin O-dealkylation activity, when compared to the corresponding vehicle control-treated group (p < 0.05).

3.2.4 HEPATIC CYP3A23 EXPRESSION

3.2.4.1 HEPATIC CYP3A23 mRNA

Another explanation for the elevated levels of hepatic microsomal aminopyrine N-demethylation activity in rats treated with a single dose of Panax ginseng extract (Lee et al, 1987) could be an increase in CYP3A enzyme expression. In rat liver, CYP3A23 is the major CYP3A inducible enzyme (Komori and Oda, 1994; Mahnke et al, 1997). To investigate whether Panax ginseng extract increases hepatic CYP3A23 mRNA expression, a real-time PCR assay was performed. As illustrated in Figure 32, the administration by oral gavage with a single dose (30 or 100 mg/kg) of Panax ginseng extract did not alter hepatic CYP3A23 mRNA expression, regardless of whether the data were expressed as copies per ng total dsDNA (Figure 32) or as a ratio of CYP3A23 mRNA over cyclophilin mRNA expression (Table A1, see Appendix).
3.2.4.2 POSITIVE CONTROL EXPERIMENT WITH DEXAMETHASONE

A positive control experiment was performed to determine the effect of a known inducer of CYP3A23 mRNA expression, as analyzed by a real-time PCR assay. Adult male rats were injected intraperitoneally with dexamethasone (100 mg/kg) once daily for three consecutive days. The dexamethasone increased the CYP3A23 mRNA expression by 75-fold (p < 0.05), when compared to the group treated by oral gavage with a single dose of 0.9% NaCl (1 ml/kg) (Figure 32).

3.2.5 HEPATIC CYP2C11 EXPRESSION

3.2.5.1 CYP2C11 PROTEIN

Based on previous study (Lee et al., 1987), the elevated activity of hepatic microsomal aminopyrine N-demethylation reported by Lee et al. (1987)² could be due to induced CYP2B, CYP3A or a combination of induced CYP2B, CYP3A, and/or a moderately decreased CYP2C11. It is known that CYP2C11 can also catalyze aminopyrine N-demethylation (Imaoka et al., 1988) and it can be upregulated by androgen (Waxman and Chang, 1995) or downregulated by estrogen (Dannan et al., 1986; Bandiera and Dworschak, 1992). Furthermore, there is evidence to suggest both androgenic (Fahim et al., 1982) and estrogenic (Bespakov et al. 2001, Liu et al., 2001; Amato et al., 2002; Lee et al., 2003) activities of Panax ginseng extract.

The effect of Panax ginseng extract on CYP2C11 protein expression in hepatic microsomes was determined. Figure 33 (panel A) illustrates a representative immunoblot probed with monospecific anti-CYP2C11 polyclonal IgG. A single dose (30 or 100 mg/kg) of Panax ginseng extract administered by oral gavage did not affect hepatic microsomal CYP2C11 protein expression, expressed as pmol/mg hepatic
microsomal protein (Figure 33, panel B) or as pmol/nmol hepatic microsomal total cytochrome P450 content (Figure A1, see Appendix).

3.2.5.2 POSITIVE CONTROL EXPERIMENT WITH β-NAPHTHOFLAVONE

Microsome samples were prepared from rats injected intraperitoneally with β-naphthoflavone (40 mg/kg/day) or an equivalent volume of corn oil (1 ml/kg) (vehicle control) once daily for three consecutive days. Under the same assay conditions, β-naphthoflavone decreased the CYP2C11 protein expression by 85%, when compared to the corresponding vehicle control-treated group (p < 0.05) (Figure 34).

3.2.6 HEPATIC CYP1A EXPRESSION

3.2.6.1 CYP1A2 mRNA

CYP1A1 and CYP1A2 are known to catalyze aryl hydrocarbon hydroxylation (Wiebel et al., 1971), and CYP1A2 is the major hepatic enzyme expressed in rat liver (Wilson et al., 1984). According to Lee et al. (1987), hepatic S-9 aryl hydrocarbon hydroxylase activity was significantly decreased (8%) after a single oral dose of Panax ginseng extract in rats, suggesting that Panax ginseng may affect hepatic CYP1A2 expression in rats. Therefore, hepatic CYP1A2 mRNA expression was measured in the present study. As illustrated in Figure 35, the administration by oral gavage with a single dose (30 or 100 mg/kg) of Panax ginseng extract did not affect hepatic CYP1A2 mRNA expression, regardless of whether the data were expressed as copies per ng total ds DNA (Figure 35) or as a ratio of CYP1A2 mRNA over cyclophilin mRNA expression (Table A1, see Appendix).
3.2.6.2 MICROSONAL 7-ETHOXYRESORUFIN O-DEALKYLATION ACTIVITY

To determine whether *Panax ginseng* extract affects hepatic microsomal CYP1A2 enzyme activity, the 7-ethoxyresorufin O-dealkylation assay was performed. As shown in Figure 36, a single dose (30 or 100 mg/kg) of *Panax ginseng* extract administered by oral gavage did not affect hepatic microsomal 7-ethoxyresorufin O-dealkylation activity.

3.2.6.3 POSITIVE CONTROL EXPERIMENTS WITH β-NAPHTHOFLAVONE

Positive control experiments were conducted to determine the effect of a known inducer of hepatic CYP1A2 mRNA expression and enzyme activity, as measured by a real-time PCR assay and the 7-ethoxyresorufin O-dealkylation assay, respectively. Under the same PCR condition, the β-naphthoflavone-treated rats had a 20-fold increase (p < 0.05) in CYP1A2 mRNA expression when compared to the group treated by oral gavage with a single dose of 0.9% NaCl (1 ml/kg) (Figure 35). Furthermore, in another control experiment, adult male rats were injected intraperitoneally with β-naphthoflavone (40 mg/kg) or an equivalent volume of corn oil (1 ml/kg) (vehicle control) once daily for three consecutive days. The rats were terminated one day after the last injection. As shown in Figure 37, under the same assay condition, β-naphthoflavone increased the hepatic microsomal 7-ethoxyresorufin O-dealkylation activity by 19-fold (p < 0.05), when compared to the corn oil-treated group.
3.2.7 HEPATIC MICROSONMAL EPOXIDE HYDROLASE EXPRESSION

3.2.7.1 MICROSOMAL EPOXIDE HYDROLASE mRNA

As indicated earlier, Lee et al. (1987)\(^2\) also found an increase in microsomal epoxide hydrolase activity in rats treated by oral gavage with a single dose (20 or 30 mg/kg) of Panax ginseng extract. To determine whether Panax ginseng extract affects microsomal epoxide hydrolase mRNA expression, a real-time PCR assay was performed. The administration by oral gavage of a single dose (30 or 100 mg/kg) of Panax ginseng extract did not affect microsomal epoxide hydrolase mRNA expression, regardless of whether the data were expressed as copies per ng total ds DNA (Figure 38) or as a ratio of microsomal epoxide hydrolase mRNA over cyclophilin mRNA expression (Table A1, see Appendix).

3.2.7.2 MICROSOMAL EPOXIDE HYDROLASE PROTEIN

The effect of Panax ginseng extract in hepatic microsomal epoxide hydrolase protein expression was assessed. Figure 39 (panel A) illustrates a representative immunoblot probed with monospecific anti-microsomal epoxide hydrolase polyclonal IgG. A single dose (30 or 100 mg/kg) of Panax ginseng extract administered by oral gavage did not affect microsomal epoxide hydrolase protein expression (Figure 39, panel B).

3.2.7.3 POSITIVE CONTROL EXPERIMENT WITH PHENOBARBITAL

Positive control experiments were conducted to determine the effect of phenobarbital on hepatic microsomal epoxide hydrolase mRNA and protein expression. Under the same PCR condition, phenobarbital-treated rats had an approximately 17-fold increase (p < 0.05) in the hepatic microsomal epoxide hydrolase mRNA expression.
when compared to the group treated by oral gavage with a single dose of 0.9% NaCl (1 ml/kg) (Figure 38). In another control experiment, adult male rats were injected intraperitoneally with phenobarbital (80 mg/kg) or an equivalent volume of 0.9% NaCl (1 ml/kg) (vehicle control) once daily for four consecutive days. As shown in Figure 40, phenobarbital increased hepatic microsomal epoxide hydrolase protein levels by 2-fold, when compared to the vehicle control-treated group (p < 0.05).

3.2.8 CONTROL EXPERIMENT WITH ALBENDAZOLE

To determine whether the lack of effect of oral administration of Panax ginseng on hepatic drug-metabolizing enzymes was due to improper oral gavage administration technique, a control experiment was conducted. Adult male rats were administered by oral gavage with albendazole, a known inducer for CYP1A in rat liver (Souhaili-el Amri et al., 1988; Asteinza et al., 2000). The effect of albendazole on hepatic microsomal CYP1A2 catalytic activity was assessed by the 7-ethoxyresorufin O-dealkylation assay. As shown in Figure 41, the oral treatment of albendazole (20 mg/kg, once daily for three consecutive days) increased hepatic microsomal 7-ethoxyresorufin O-dealkylation activity by 21-fold.
<table>
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<th>100 mg/kg</th>
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<td><strong>Initial Body Weight</strong> (g)</td>
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<td>277 ± 3</td>
<td>281 ± 5</td>
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<tr>
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<td><strong>Body Weight Gain</strong> (g)</td>
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<td>4 ± 3</td>
<td>10 ± 1</td>
</tr>
<tr>
<td><strong>Absolute Liver Weight</strong> (g)</td>
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<td>11.6 ± 0.7</td>
</tr>
<tr>
<td><strong>Relative Liver Weight</strong> (% of final body weight)</td>
<td>4.0 ± 0.1</td>
<td>4.0 ± 0.1</td>
<td>4.0 ± 0.2</td>
</tr>
<tr>
<td><strong>Absolute Testes Weight</strong> (g)</td>
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<td>2.9 ± 0.1</td>
<td>3.2 ± 0.1</td>
</tr>
<tr>
<td><strong>Relative Testes Weight</strong> (% of final body weight)</td>
<td>1.0 ± 0.06</td>
<td>1.0 ± 0.01</td>
<td>1.1 ± 0.02</td>
</tr>
</tbody>
</table>

Adult male rats were administered by oral gavage with a single dose of *Panax ginseng* extract (30 or 100 mg/kg) or an equivalent volume (2 ml/kg) of 0.9% NaCl (vehicle control). All rats were sacrificed the following day. Liver and testes were excised, rinsed in buffer (0.05 M Tris/1.15% KCl, pH 7.5), blotted dry, and weighed. Body weight was determined daily. Data are expressed as mean ± S.E.M. for four individual rats per treatment group. No significant differences were obtained.
Figure 28. Effect of *Panax ginseng* extract on hepatic microsomal cytochrome total P450 content in adult male rats. Adult male rats were administered by oral gavage with a single dose of *Panax ginseng* extract (30 or 100 mg/kg) or an equivalent volume (2 ml/kg) of 0.9% NaCl (vehicle control). All rats were sacrificed the following day. Livers were excised and hepatic microsomes were prepared by differential ultracentrifugation. Hepatic microsomal cytochrome total P450 concentration was determined spectrophotometrically (Omura and Sato, 1964). Data are expressed as mean ± S.E.M. for four individual rats per treatment group. No significant differences were obtained.
Figure 29. Effect of *Panax ginseng* extract on hepatic CYP2B1 mRNA expression in adult male rats. Adult male rats were administered by oral gavage with a single dose of *Panax ginseng* extract (30 or 100 mg/kg) or an equivalent volume (2 ml/kg) of 0.9% NaCl (vehicle control) and sacrificed the following day. As a positive control, another group of rats was injected intraperitoneally with sodium phenobarbital (80 mg/kg) once daily for four consecutive days and sacrificed one day after the last dose. Livers were excised and snap-frozen in liquid nitrogen. Total RNA was isolated and reverse transcribed. CYP2B1 cDNA was amplified by real-time PCR. Data are expressed as the mean ± S.E.M. for four individual rats per treatment group. *Significantly different from the vehicle control-treated group (p < 0.05).
Figure 30. Effect of Panax ginseng extract on 7-benzyloxyresorufin O-dealkylation activity in hepatic microsomes isolated from adult male rats. Adult male rats were administered by oral gavage with a single dose of Panax ginseng extract (30 or 100 mg/kg) or an equivalent volume (2 ml/kg) of 0.9% NaCl (vehicle control). All rats were sacrificed the following day. Livers were excised and hepatic microsomes were prepared by differential ultracentrifugation. The 7-benzyloxyresorufin O-dealkylation activity was determined spectrofluorometrically (Burke et al., 1985). Data are expressed as mean ± S.E.M. for four individual rats per treatment group. No significant differences were obtained.
Figure 31. Effect of phenobarbital on 7-benzyloxyresorufin O-dealkylation activity in hepatic microsomes isolated from adult male rats. Adult male rats were injected intraperitoneally with sodium phenobarbital (80 mg/kg) or an equivalent volume (1 ml/kg) of 0.9% NaCl (vehicle control) once daily for four consecutive days. All rats were sacrificed one day after the last dose. Livers were excised and hepatic microsomes were prepared by differential ultracentrifugation. The 7-benzyloxyresorufin O-dealkylation activity was determined spectrofluorometrically (Burke et al., 1985). Data are expressed as mean ± S.E.M. for four individual rats per treatment group. *Significant different from the 0.9% NaCl-treated group (p < 0.05).
Figure 32. Effect of *Panax ginseng* extract on hepatic CYP3A23 mRNA expression in adult male rats. Adult male rats were administered by oral gavage with a single dose of *Panax ginseng* extract (30 or 100 mg/kg) or an equivalent volume (2 ml/kg) of 0.9% NaCl (vehicle control) and sacrificed the following day. As a positive control, another group of rats was injected intraperitoneally with dexamethasone (DEX) (100 mg/kg) once daily for three consecutive days and sacrificed one day after the last dose. Livers were excised and snap-frozen in liquid nitrogen. Total RNA was isolated and reverse transcribed. CYP3A23 cDNA was amplified by real-time PCR. Data are expressed as the mean ± S.E.M. for four individual rats per treatment group. *Significantly different from the 0.9% NaCl-treated group (p < 0.05).*
Figure 33. Effect of *Panax ginseng* extract on hepatic microsomal CYP2C11 protein expression in adult male rats. Adult male rats were administered by oral gavage with a single dose of *Panax ginseng* extract (30 or 100 mg/kg) or an equivalent volume (2 ml/kg) of 0.9% NaCl (vehicle control). All rats were sacrificed one day later. Livers were excised and hepatic microsomes were prepared by differential ultracentrifugation. Microsomal proteins were resolved by SDS-PAGE and transferred electrophoretically onto a nitrocellulose membrane. Shown in panel A is the representative immunoblot probed with monospecific rabbit anti-rat CYP2C11 polyclonal IgG (16 μg/ml) as the primary antibody followed by alkaline phosphatase conjugated, goat F(ab')2 anti-rabbit IgG (1:3000 dilution) as the secondary antibody. Immunoreactive CYP2C11 protein was detected using BCIP/NBT as a colorimetric substrate for alkaline phosphatase. Lanes 1 to 4 contain 0.125, 0.25, 0.375 and 0.5 pmol of purified CYP2C11 protein standards, respectively. Lanes 5 to 6 contain hepatic microsomes isolated from rats treated with 0.9% NaCl. Lanes 7-10 contain microsomes isolated from rats treated with *Panax ginseng* extract at the dosage of 30 mg/kg. Lanes 11-14 contain microsomes isolated from rats treated with *Panax ginseng* extract at the dosage of 100 mg/kg. Shown in panel B is the densitometric quantitation of hepatic microsomal CYP2C11 protein expression. Data are expressed as mean ± S.E.M. for four individual rats per treatment group. Hepatic microsomal CYP2C11 protein expression was not significantly different between the various groups.
(A) Standards 0.9% NaCl

(B) Panax ginseng extract

Panax ginseng extract (mg/kg)
Figure 34. Effect of β-naphthoflavone on hepatic CYP2C11 protein expression

Adult male rats were injected intraperitoneally with β-naphthoflavone (40 mg/kg) or an equivalent volume (1 ml/kg) of corn oil (vehicle control) once daily for three consecutive days. All rats were sacrificed one day after the last dose. Livers were excised and hepatic microsomes were prepared by differential ultracentrifugation. Microsomal proteins were resolved by SDS-PAGE and then transferred electrophoretically onto a nitrocellulose membrane. The primary antibody used was monospecific rabbit anti-rat CYP2C11 polyclonal IgG at the concentration of 16 μg/ml. The secondary antibody used was alkaline phosphatase conjugated, goat F(ab')2 anti-rabbit IgG at 1:3000 dilution. The intensity of CYP2C11 protein bands was determined densitometrically using a standard curve constructed by CYP2C11 protein standards of known concentrations. Data are expressed as mean ± S.E.M. for four individual rats per treatment group. *Significant different from the corn oil-treated group (p < 0.05).
Figure 35. Effect of *Panax ginseng* extract on hepatic CYP1A2 mRNA expression in adult male rats. Adult male rats were administered by oral gavage with a single dose of *Panax ginseng* extract (30 or 100 mg/kg) or an equivalent volume (2 ml/kg) of 0.9% NaCl (vehicle control) and sacrificed the following day. As a positive control, another group of rats was injected intraperitoneally with β-naphthoflavone (BNF) (40 mg/kg) once daily for three consecutive days and sacrificed one day after the last dose. Livers were excised and snap-frozen in liquid nitrogen. Total RNA was isolated and reverse transcribed. CYP1A2 cDNA was amplified by real-time PCR. Data are expressed as the mean ± S.E.M. for four individual rats per treatment group. *Significantly different from the 0.9% NaCl-treated group (p < 0.05).*
Figure 36. Effect of *Panax ginseng* extract on 7-ethoxyresorufin O-dealkylation activity in hepatic microsomes isolated from adult male rats. Adult male rats were administered by oral gavage with a single dose of *Panax ginseng* extract (30 or 100 mg/kg) or an equivalent volume (2 ml/kg) of 0.9% NaCl (vehicle control). All rats were sacrificed the following day. Livers were excised and hepatic microsomes were prepared by differential ultracentrifugation. The 7-ethoxyresorufin O-dealkylation activity was determined spectrofluorometrically (Burke et al., 1985). Data are expressed as mean ± S.E.M. for four individual rats per treatment group. No significant differences were obtained.
Figure 37. Effect of β-naphthoflavone on 7-ethoxyresorufin O-dealkylation activity in hepatic microsomes isolated from adult male rats. Adult male rats were injected intraperitoneally with β-naphthoflavone (40 mg/kg) or an equivalent volume (1 ml/kg) of corn oil (vehicle control) once daily for three consecutive days. All rats were sacrificed one day after the last dose. Livers were excised and hepatic microsomes were prepared by differential ultracentrifugation. The 7-ethoxyresorufin O-dealkylation activity was determined spectrofluorometrically (Burke et al., 1985). Data are expressed as mean ± S.E.M. for four individual rats per treatment group. *Significant different from the corn oil-treated group (p < 0.05).
Figure 38. Effect of Panax ginseng extract on hepatic microsomal epoxide hydrolase (mEH) mRNA expression in adult male rats. Adult male rats were administered by oral gavage with a single dose of Panax ginseng extract (30 or 100 mg/kg) or an equivalent volume (2 ml/kg) of 0.9% NaCl (vehicle control) and sacrificed the following day. As a positive control, another group of rats was injected intraperitoneally with sodium phenobarbital (80 mg/kg) once daily for four consecutive days and sacrificed one day after the last dose. Livers were excised and snap-frozen in liquid nitrogen. Total RNA was isolated and reverse transcribed. Microsomal epoxide hydrolase cDNA was amplified by real-time PCR. Data are expressed as the mean ± S.E.M. for four individual rats per treatment group. *Significantly different from the 0.9% NaCl-treated group (p < 0.05).
Figure 39. Effect of *Panax ginseng* extract on hepatic microsomal epoxide hydrolase (mEH) protein expression in adult male rats. Adult male rats were administered by oral gavage with a single dose of *Panax ginseng* extract (30 or 100 mg/kg) or an equivalent volume (2 ml/kg) of 0.9% NaCl (vehicle control). All rats were sacrificed one day later. Livers were excised and hepatic microsomes were prepared by differential ultracentrifugation. Microsomal proteins were resolved by SDS-PAGE and then transferred electrophoretically onto a nitrocellulose membrane. Shown in panel A is the representative immunoblot probed with monospecific rabbit anti-rat microsomal epoxide hydrolase polyclonal IgG (5 µg/ml) as the primary antibody followed by alkaline phosphatase conjugated, goat F(ab')2 anti-rabbit IgG (1:3000 dilution) as the secondary antibody. Immunoreactive microsomal epoxide hydrolase protein was detected using BCIP/NBT as a colorimetric substrate for alkaline phosphatase. Lanes 1 to 4 contain 0.01, 0.05, 0.1 and 0.25 pmol of purified microsomal epoxide hydrolase protein standards, respectively. Lanes 5 to 8 contain hepatic microsomes isolated from rats treated with 0.9% NaCl. Lanes 9-12 contain microsomes isolated from rats treated with *Panax ginseng* extract at the dosage of 30 mg/kg. Lanes 13-16 contain microsomes isolated from rats treated with *Panax ginseng* extract at the dosage of 100 mg/kg. Shown in panel B is the densitometric quantitation of hepatic microsomal epoxide hydrolase protein expression. Data are expressed as mean ± S.E.M. for four individual rats per treatment group. Hepatic microsomal epoxide hydrolase protein expression was not significantly different between the various groups.
Panax ginseng extract

(A)

(B)

Panax ginseng extract

standards 0.9% NaCl 30 mg/kg 100 mg/kg
Figure 40. Effect of phenobarbital on hepatic microsomal epoxide hydrolase (mEH) protein expression. Adult male rats were injected intraperitoneally with phenobarbital (80 mg/kg) or an equivalent volume (1 ml/kg) of 0.9% NaCl (vehicle control) once daily for four consecutive days. All rats were sacrificed one day after the last dose. Livers were excised and hepatic microsomes were prepared by differential ultracentrifugation. Microsomal proteins were resolved by SDS-PAGE and then transferred electrophoretically onto a nitrocellulose membrane. The primary antibody used was monospecific rabbit anti-rat microsomal epoxide hydrolase polyclonal IgG at the concentration of 5 µg/ml. The secondary antibody used was alkaline phosphatase conjugated, goat F(ab')₂ anti-rabbit IgG at 1:3000 dilution. The intensity of the microsomal epoxide hydrolase protein bands was determined densitometrically. Data are expressed as mean ± S.E.M. for four individual rats per treatment group. *Significant different from the 0.9% NaCl-treated group (p < 0.05).
Figure 41. Effect of albendazole on 7-ethoxyresorufin O-dealkylation activity in hepatic microsomes isolated from adult male rats. Adult male rats were administered by oral gavage with albendazole (20 mg/kg) or an equivalent volume (2 ml/kg) of corn oil (vehicle control) once daily for three consecutive days. All rats were sacrificed one day after the last dose. Livers were excised and hepatic microsomes were prepared by differential ultracentrifugation. The 7-ethoxyresorufin O-dealkylation activity was determined spectrofluorometrically (Burke et al., 1985). Data are expressed as mean ± S.E.M. for four individual rats per treatment group. *Significantly different from the vehicle control-treated group (p < 0.05).
3.3 TREATMENT OF ADULT MALE RATS WITH *PANAX QUINQUEFOLIUS* EXTRACT

Adult male Sprague-Dawley rats were administered by oral gavage with *Panax quinquefolius* extract (100 or 400 mg/kg) of known ginsenoside composition (Figure 1) or an equivalent volume (2 ml/kg) of 0.9% NaCl (vehicle control) once daily for 21 consecutive days. All rats were terminated one day after the last dose. The following dependent variables were determined: body weight gain; absolute and relative liver weight; absolute and relative testes weight; hepatic CYP2B1, CYP3A23, CYP2C11, CYP1A2, and microsomal epoxide hydrolase mRNA levels; hepatic CYP2C11 and microsomal epoxide hydrolase protein levels; and hepatic microsomal 7-benzyloxyresorufin O-dealkylation and 7-ethoxyresorufin O-dealkylation activities.

3.3.1 BODY WEIGHT GAIN, LIVER WEIGHT AND TESTES WEIGHT

The effects of *Panax quinquefolius* extract on body weight gain, liver weight and testes weight are summarized in Table 21. The administration by oral gavage with *Panax quinquefolius* extract (100 or 400 mg/kg, once daily for 21 consecutive days) did not affect body weight gain, liver weight or testes weight, when expressed as absolute or relative values.

3.3.2 TOTAL HEPATIC MICROSOMAL CYTOCHROME P450 CONTENT

As shown in Figure 42, the administration by oral gavage with *Panax quinquefolius* extract (100 or 400 mg/kg, once daily for 21 consecutive days) did not affect hepatic microsomal total cytochrome P450 content.
3.3.3 HEPATIC CYP2B EXPRESSION

3.3.3.1 CYP2B1 mRNA

The administration by oral gavage of Panax quinquefolius extract (100 or 400 mg/kg, once daily for 21 consecutive days) did not affect hepatic CYP2B1 mRNA expression, regardless of whether the data were expressed as copies per ng total ds DNA (Figure 43) or as a ratio of CYP2B1 mRNA over cyclophilin mRNA expression (Table A2, see Appendix).

3.3.3.2 MICROsomAL 7-BENZYLOXYRESORUFIN O-DEALKylation ACTIVITY

To determine whether Panax quinquefolius extract affects hepatic microsomal CYP2B enzyme activity, the 7-benzyloxyresorufin O-dealkylation assay was performed. The administration by oral gavage with Panax quinquefolius extract (100 or 400 mg/kg, once daily for 21 consecutive days) did not alter hepatic microsomal 7-benzyloxyresorufin O-dealkylation activity (Figure 44).

3.3.4 HEPATIC CYP3A23 EXPRESSION

3.3.4.1 HEPATIC CYP3A23 mRNA EXPRESSION

As illustrated in Figure 45, the administration by oral gavage with Panax quinquefolius extract (100 or 400 mg/kg, once daily for 21 consecutive days) did not alter the hepatic CYP3A23 mRNA expression in rats, regardless of whether the data were expressed as copies per ng total dsDNA (Figure 45) or as a ratio of CYP3A23 mRNA over cyclophilin mRNA expression (Table A2, see Appendix).
3.3.5 HEPATIC CYP2C11 EXPRESSION

3.3.5.1 CYP2C11 mRNA

As shown in Figure 46, the administration by oral gavage with Panax quinquefolius extract (100 or 400 mg/kg, once daily for 21 consecutive days) did not suppress the expression of hepatic CYP2C11 mRNA expression in rats, regardless of whether the data were expressed as copies per ng total dsDNA (Figure 46) or as a ratio of CYP2C11 mRNA over cyclophilin mRNA expression (Table A2, see Appendix).

3.3.5.2 CYP2C11 PROTEIN

To further characterize the effect of Panax quinquefolius extract on hepatic CYP2C11 protein expression, immunoblot assay was performed. Figure 47 (panel A) illustrates a representative immunoblot probed with monospecific anti-CYP2C11 polyclonal IgG. The administration by oral gavage with Panax quinquefolius extract (100 or 400 mg/kg, once daily for 21 consecutive days) did not affect hepatic CYP2C11 protein level, expressed as pmol/mg hepatic microsomal protein (Figure 47, panel B) or as pmol/nmol hepatic microsomal total cytochrome P450 content (Figure A2, see Appendix).

3.3.6 HEPATIC CYP1A EXPRESSION

3.3.6.1 CYP1A2 mRNA

As illustrated in Figure 48, the administration by oral gavage with Panax quinquefolius extract (100 or 400 mg/kg, once daily for 21 consecutive days) did not affect the hepatic CYP1A2 mRNA expression in rats, regardless of whether the data
were expressed as copies per total ng dsDNA (Figure 48) or as a ratio of CYP1A2 mRNA over cyclophilin mRNA expression (Table A2, see Appendix).

3.3.6.2 MICROSOMAL 7-ETHOXYRESORUFIN O-DEALKYLATION ACTIVITY

To determine whether Panax quinquefolius extract affects hepatic microsomal CYP1A2 enzyme activity, the 7-ethoxyresorufin O-dealkylation assay was performed. As shown in Figure 49, the administration by oral gavage with Panax quinquefolius extract (100 or 400 mg/kg, once daily for 21 consecutive days) did not affect hepatic microsomal 7-ethoxyresorufin O-dealkylation activity.

3.3.7 HEPATIC MICROSOMAL EPOXIDE HYDROLASE EXPRESSION

3.3.7.1 MICROSOMAL EPOXIDE HYDROLASE mRNA

The administration by oral gavage with Panax quinquefolius extract (100 or 400 mg/kg, once daily for 21 consecutive days) did not affect hepatic microsomal epoxide hydrolase mRNA expression, regardless of whether the data were expressed as copies per ng dsDNA (Figure 50) or as a ratio of microsomal epoxide hydrolase mRNA over cyclophilin mRNA expression (Table A2, see Appendix).

3.3.7.2 MICROSOMAL EPOXIDE HYDROLASE PROTEIN

To further characterize the effect of Panax quinquefolius extract on microsomal epoxide hydrolase protein expression, immunoblot assay was performed. Figure 51 (panel A) illustrates a representative immunoblot probed with monospecific anti-microsomal epoxide hydrolase polyclonal IgG. The administration by oral gavage with
Panax quinquefolius extract (100 or 400 mg/kg, once daily for 21 consecutive days) did not affect microsomal epoxide hydrolase protein expression (Figure 51, panel B).
TABLE 21

Body weight gain, liver weight and testes weight in adult male rats treated with Panax quinquefolius extract

<table>
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<tr>
<th></th>
<th>0.9% NaCl (N = 4)</th>
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<th>400 mg/kg/day (N = 5)</th>
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<tr>
<td><strong>Initial Body Weight (g)</strong></td>
<td>286 ± 7</td>
<td>283 ± 6</td>
<td>282 ± 3</td>
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<tr>
<td><strong>Final Body Weight (g)</strong></td>
<td>381 ± 19</td>
<td>381 ± 10</td>
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<td><strong>Absolute Liver Weight (g)</strong></td>
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</tr>
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<td><strong>Relative Liver Weight (% of final body weight)</strong></td>
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</table>

Adult male rats were administered by oral gavage with Panax quinquefolius extract (100 or 400 mg/kg) or an equivalent volume (2 ml/kg) of 0.9% NaCl (vehicle control) once daily for 21 consecutive days. All rats were sacrificed one day after the last dose. Livers and testes were excised, rinsed in buffer (0.05 M Tris/1.5% KCl, pH 7.5), blotted dry, and weighed. Body weight was determined daily during the treatment period and prior to sacrifice. Data are expressed as mean ± S.E.M. for four or five individual rats per treatment group. No significant differences were obtained.
Figure 42. Effect of *Panax quinquefolius* extract on hepatic microsomal total cytochrome P450 content in adult male rats. Adult male rats were administered by oral gavage with *Panax quinquefolius* extract (100 or 400 mg/kg) or an equivalent volume (2 ml/kg) of 0.9% NaCl (vehicle control) once daily for 21 consecutive days. All rats were sacrificed one day after the last dose. Livers were excised and hepatic microsomes were prepared by differential ultracentrifugation. Hepatic microsomal total cytochrome P450 content was determined spectrophotometrically (Omura and Sato, 1964). Data are expressed as mean ± S.E.M. for four or five individual rats per treatment group. No significant differences were obtained.
Figure 43. Effect of *Panax quinquefolius* extract on hepatic CYP2B1 mRNA expression in adult male rats. Adult male rats were administered by oral gavage with *Panax quinquefolius* extract (100 or 400 mg/kg) or an equivalent volume (2 ml/kg) of 0.9% NaCl (vehicle control) once daily for 21 consecutive days. All rats were sacrificed one day after the last dose. Livers were excised and snap-frozen in liquid nitrogen. Total RNA was isolated and reverse transcribed. CYP2B1 cDNA was amplified by real-time PCR. Data are expressed as the mean ± S.E.M. for four or five individual rats per treatment group. No significant differences were obtained.
Figure 44. Effect of *Panax quinquefolius* extract on 7-benzyloxyresorufin O-dealkylation activity in hepatic microsomes isolated from adult male rats. Adult male rats were administered by oral gavage with *Panax quinquefolius* extract (100 or 400 mg/kg) or an equivalent volume (2 ml/kg) of 0.9% NaCl (vehicle control) once daily for 21 consecutive days. All rats were sacrificed one day after the last dose. Livers were excised and hepatic microsomes were prepared by differential ultracentrifugation. The 7-benzyloxyresorufin O-dealkylation activity was determined spectrofluorometrically (Burke *et al.*, 1985). Data are expressed as mean ± S.E.M. for four or five individual rats per treatment group. No significant differences were obtained.
Figure 45. Effect of *Panax quinquefolius* extract on hepatic CYP3A23 mRNA expression in adult male rats. Adult male rats were administered by oral gavage with *Panax quinquefolius* extract (100 or 400 mg/kg) or an equivalent volume (2 ml/kg) of 0.9% NaCl (vehicle control) once daily for 21 consecutive days. All rats were sacrificed one day after the last dose. Livers were excised and snap-frozen in liquid nitrogen. Total RNA was isolated and reverse transcribed. CYP3A23 cDNA was amplified by real-time PCR. Data are expressed as the mean ± S.E.M. for four or five individual rats per treatment group. No significant differences were obtained.
Figure 46. Effect of *Panax quinquefolius* extract on hepatic 2C11 mRNA expression in adult male rats. Adult male rats were administered by oral gavage with *Panax quinquefolius* extract (100 or 400 mg/kg) or an equivalent volume (2 ml/kg) of 0.9% NaCl (vehicle control). All rats were sacrificed one day after the last dose. Livers were excised and snap-frozen in liquid nitrogen. Total RNA was isolated and reverse transcribed. CYP2C11 cDNA was amplified by real-time PCR. Data are expressed as the mean ± S.E.M. for four or five individual rats per treatment group. No significant differences were obtained.
Figure 47. Effect of *Panax quinquefolius* extract on hepatic microsomal CYP2C11 protein expression in adult male rats. Adult male rats were administered by oral gavage with *Panax quinquefolius* extract (100 or 400 mg/kg) or an equivalent volume (2 ml/kg) of 0.9% NaCl (vehicle control) once daily for 21 consecutive days. All rats were sacrificed one day after the last dose. Livers were excised and hepatic microsomes were prepared by differential ultracentrifugation. Microsomal proteins were resolved by SDS-PAGE and then transferred electrophoretically onto a nitrocellulose membrane. Shown in panel A is the representative immunoblot probed with monospecific rabbit anti-rat CYP2C11 polyclonal IgG (16 μg/ml) as the primary antibody followed by alkaline phosphatase conjugated, goat F(ab')2 anti-rabbit IgG (1:3000 dilution) as the secondary antibody. Immunoreactive CYP2C11 protein was detected using BCIP/NBT as a colorimetric substrate for alkaline phosphatase. Lanes 1 to 4 contain 0.125, 0.25, 0.375 and 0.5 pmol of purified CYP2C11 standards, respectively. Lanes 5 to 8 contain hepatic microsomes isolated from rats treated with 0.9% NaCl. Lanes 9-12 contain microsomes isolated from rats treated with *Panax quinquefolius* extract at the dosage of 100 mg/kg. Lanes 13-17 contain microsomes isolated from rats treated with *Panax quinquefolius* extract at the dosage of 400 mg/kg. Shown in panel B is the densitometric quantification of hepatic microsomal CYP2C11 protein expression. Data are expressed as mean ± S.E.M. for four or five individual rats per treatment group. Hepatic microsomal CYP2C11 protein expression was not significantly different between the various groups.
A: CYP2C11

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<td>Standards</td>
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Panax quinquefolius extract

B: CYP2C11 PROTEIN CONTENT (pmol/mg microsomal protein)

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<th>0.9% NaCl</th>
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Panax quinquefolius extract (mg/kg/day)
Figure 48. Effect of *Panax quinquefolius* extract on hepatic CYP1A2 mRNA expression in adult male rats. Adult male rats were administered by oral gavage with *Panax quinquefolius* extract (100 or 400 mg/kg) or an equivalent volume (2 ml/kg) of 0.9% NaCl (vehicle control) once daily for 21 consecutive days. All rats were sacrificed one day after the last dose. Livers were excised and snap-frozen in liquid nitrogen. Total RNA was isolated and reverse transcribed. CYP1A2 cDNA was amplified by real-time PCR. Data are expressed as the mean ± S.E.M. for four or five individual rats per treatment group. No significant differences were obtained.
Figure 49. Effect of *Panax quinquefolius* extract on 7-ethoxyresorufin O-dealkylation activity in hepatic microsomes isolated from adult male rats. Adult male rats were administered by oral gavage with *Panax quinquefolius* extract (100 or 400 mg/kg) or an equivalent volume (2 ml/kg) of 0.9% NaCl (vehicle control) once daily for 21 consecutive days. All rats were sacrificed one day after the last dose. Livers were excised and hepatic microsomes were prepared by differential ultracentrifugation. The 7-ethoxyresorufin O-dealkylation activity was determined spectrofluorometrically (Burke et al., 1985). Data are expressed as mean ± S.E.M. for four or five individual rats per treatment group. No significant differences were obtained.
Figure 50. Effect of *Panax quinquefolius* extract on hepatic microsomal epoxide hydrolase (mEH) mRNA expression in adult male rats. Adult male rats were administered by oral gavage with *Panax quinquefolius* extract (100 or 400 mg/kg) or an equivalent volume (2 ml/kg) of 0.9% NaCl (vehicle control) once daily for 21 consecutive days. All rats were sacrificed one day after the last dose. Livers were excised and snap-frozen in liquid nitrogen. Total RNA was isolated and reverse transcribed. Microsomal epoxide hydrolase cDNA was amplified by real-time PCR. Data are expressed as the mean ± S.E.M. for four or five individual rats per treatment group. No significant differences were obtained.
Figure 51. Effect of *Panax quinquefolius* extract on hepatic microsomal epoxide hydrolase (mEH) protein expression in adult male rats. Adult male rats were administered by oral gavage with *Panax quinquefolius* extract (100 or 400 mg/kg) or an equivalent volume (2 ml/kg) of 0.9% NaCl (vehicle control) once daily for 21 consecutive days. All rats were terminated one day after the last dose. Livers were excised and hepatic microsomes were prepared by differential ultracentrifugation. Microsomal proteins were resolved by SDS-PAGE and then transferred electrophoretically onto a nitrocellulose membrane. Shown in panel A is the representative immunoblot probed with monospecific rabbit anti-rat microsomal epoxide hydrolase polyclonal IgG (5 μg/ml) as the primary antibody followed by alkaline phosphatase conjugated, goat F(ab')2 anti-rabbit IgG (1:3000 dilution) as the secondary antibody. Immunoreactive microsomal epoxide hydrolase protein was detected using BCIP/NBT as a colorimetric substrate for alkaline phosphatase. Lanes 1 to 4 contain 0.01, 0.05, 0.1 and 0.25 pmol of purified microsomal epoxide hydrolase protein standards, respectively. Lanes 5 to 8 contain hepatic microsomes isolated from rats treated with 0.9% NaCl. Lanes 9-12 contain microsomes isolated from rats treated with *Panax quinquefolius* extract at the dosage of 100 mg/kg. Lanes 13-17 contain microsomes isolated from rats treated with *Panax quinquefolius* extract at the dosage of 400 mg/kg. Shown in panel B is the densitometric quantitation of hepatic microsomal epoxide hydrolase protein expression. Data are expressed as mean ± S.E.M. for four or five individual rats per treatment group. Hepatic microsomal epoxide hydrolase protein expression was not significantly different between the various groups.
Panax quinquefolius extract

(A)

Standards 0.9% NaCl 100 mg/kg/day 400 mg/kg/day

Panax quinquefolius extract

(B)

Panax quinquefolius extract (mg/kg/day)

mEH PROTEIN CONTENT (µg/mg microsomal protein)

0.9% NaCl 100 400
CHAPTER 4. DISCUSSION
4.1 REAL-TIME PCR QUANTIFICATION OF GENE EXPRESSION

In the present study, the quantification of mRNA expression of CYP genes (CYP2B1, CYP3A23, CYP2C11, CYP1A2) and mEH was conducted using real-time PCR assays. Real-time PCR was the method of choice for this investigation for several reasons. Other conventional methods such as northern blotting analysis, RNase protection assay, in situ hybridization, and conventional PCR are less sensitive, and more labour intensive compared to real-time PCR (Melton et al., 1984; Bustin, 2000). Another advantage that real-time PCR has over conventional PCR is the ability to continuously monitor and quantitate the nucleic acid at the log-linear phase of the amplification. Conventional PCR is only semi-quantitative because it is difficult to predict the log-linear phase, therefore measurements may be taken at the plateau phase of the amplification, which results in inaccurate determinations.

The real-time PCR assays for the quantification of the mRNA expression of CYP2B1, CYP3A23, CYP2C11, CYP1A2 and mEH were validated. The accuracy (% bias) and precision (% c.v.) were determined by amplifying purified amplicons of known amounts, whereas the intra- and inter-day variabilities were determined by amplifying the cDNA samples obtained from rats treated with inducing agents or the vehicle controls. The accuracy (% bias) was within ± 5 % of the nominal amounts and the precision did not exceed 20% c.v. for all real-time PCR assays in the present study. The intra- and inter-day variabilities (% c.v.) ranged from 2% to 8% and 11% to 30%, respectively. In conventional quantitative assays, the variabilities (% c.v.) should not exceed than 15% (Shah et al., 1992). However, it may not be appropriate to apply this conventional standard to the quantitative real-time PCR assay where trivial variations are amplified exponentially as each PCR cycle continues (Wu et al., 1991).
The mRNA expression of all samples was quantified by analyzing the calibration curve and back calculating the cycle numbers for the unknown samples from the slope of the standard curve. The mRNA expression of all experimental samples fell within the linear range of the standard curve. However, in the real-time PCR assays for CYP3A23 and mEH, the level of mRNA expression for the positive control samples were above the values for the highest standard. As a result, the mRNA expression of the positive control was not accurately determined; nevertheless, a marked increase in these control samples indicated that the above assays were functioning.

House-keeping genes are generally used as internal controls and have moderately abundant and constant mRNA expression that should not be altered by any treatment included in the experiment (Bustin et al. 2000). Expression of the house-keeping genes can be used to correct the variability of samples by normalizing the expression to one of the target genes (Karge et al., 1998). In the present study, the mRNA expression of cyclophilin, a commonly used house-keeping gene (Haendler et al., 1987), of each sample was measured and was not altered by any of the treatments. All mRNA data were expressed in two different ways (i.e. normalized to the amount of dsDNA and cyclophilin mRNA levels). Results from these two methods were consistent with each other.

4.2 EFFECTS OF A SINGLE ORAL DOSE OF PANAX GINSENG EXTRACT ON HEPATIC CYP ENZYMES AND mEH

Previous evidence suggests that ginseng extract may have an effect on hepatic CYP enzymes and mEH. One key study has shown the effects of Panax ginseng extract on hepatic CYP enzymes and mEH in adult male rats (Lee et al., 1987²). In that
study, the oral administration of a single dose of *Panax ginseng* (10, 20 and 30 mg/kg) extract increased hepatic microsomal aminopyrine \textit{N}-demethylase activity and the activity of mEH on benzo[a]pyrene 4,5-oxide. Furthermore, 30 mg/kg of *Panax ginseng* extract significantly decreased the S-9 aryl hydrocarbon hydroxylase activity on benzo[a]pyrene by 8\% (Lee \textit{et al.}, 1987\textsuperscript{2}). As indicated earlier, aminopyrine \textit{N}-demethylation is catalyzed by two inducible enzymes, primarily CYP2B, and to a lesser extent, CYP3A (Imaoka \textit{et al.}, 1988), whereas aryl hydrocarbon hydroxylation can be catalyzed by CYP1A.

In our study, the effect of *Panax ginseng* extract (30 or 100 mg/kg, oral gavage, single dose) on CYP2B1, CYP3A23, CYP2C11, CYP1A2, and mEH expression in adult male rats was investigated. The results suggested that *Panax ginseng* extract did not influence hepatic CYP2B1 mRNA expression or microsomal 7-benzyloxyresorufin O-dealkylation activity, while phenobarbital (positive control) resulted in a marked increase in both the two above dependent variables. The microsomal 7-benzyloxyresorufin O-dealkylation assay is more specific in determining CYP2B enzyme activity as compared to the microsomal aminopyrine \textit{N}-demethylation assay used by Lee \textit{et al.} (1987)\textsuperscript{2}, because the latter is considered to be a non-selective enzyme marker for CYP2B, CYP3A and CYP2C11 (Imaoka \textit{et al.}, 1988).

Our study also examined CYP3A23 mRNA expression with the rationale that CYP3A may be involved in the increased aminopyrine \textit{N}-demethylation activity observed by Lee \textit{et al.} (1987)\textsuperscript{2}. Results from our study also indicated that a single oral dose of *Panax ginseng* extract had no effect on CYP3A23 mRNA expression, while dexamethasone (positive control) resulted in a marked increase in the expression. Other supporting evidence also indicated that *Panax ginseng* extract did not increase
the mRNA expression of the orthologous gene, CYP3A4, in humans (Gurley et al., 2002). On the other hand, there is evidence from in vitro studies that ginseng may exhibit glucocorticoid-like activities (Kang et al., 1994; Kim et al., 1994; Lee et al., 1997), and this may have an effect on CYP3A gene expression. The mechanism by which glucocorticoid-activating ligands initiate gene transcription is by the formation of a ligand-glucocorticoid receptor (GR) complex that binds to the glucocorticoid responsive element (McEwan et al., 1994). Lee et al (1997) reported that ginsenoside Rg1 is a functional ligand of the GR and, in their in vitro study, Rg1 competitively inhibited the binding of dexamethasone, a synthetic glucocorticoid, to GR and activated a GRE-containing luciferase reporter gene (Lee et al., 1997). The above studies were all conducted in vitro and the relevance of their findings to CYP3A gene expression in the whole animal has yet to be determined.

As shown in section 3.2, following the oral administration of a single dose of Panax ginseng extract, significant differences were not observed in CYP2C11 protein expression between the treatment and the vehicle control groups. CYP2C11 gene expression has been shown to be up-regulated by testosterone (Waxman and Chang, 1995) and down-regulated by estrogen (Dannan et al., 1986; Bandiera and Dworschak, 1992). Previous studies suggested that Panax ginseng extract increases plasma testosterone level in humans (Youl et al., 2002; Salvati et al., 1996) and in Holtzman strain rats (Fahim et al., 1982). In contrast, in vitro evidence suggests that Panax ginseng (Liu et al., 2001) and ginsenosides (Lee et al., 2003) exhibit estrogenic activities. Despite the above observations described by other investigators, our studies did not demonstrate an effect on CYP2C11 protein expression by Panax ginseng extract,
whereas the administration of β-naphthoflavone (positive control) suppressed CYP2C11 protein expression.

According to the previous study (Lee et al., 1987²), Panax ginseng extract had a minor inhibitory effect on S-9 aryl hydrocarbon hydroxylation activity (8% decrease as compared to control). In order to determine if the observed inhibition of S-9 aryl hydrocarbon hydroxylation activity was mediated by Panax ginseng extract on CYP1A2, we investigated the effect of Panax ginseng extract on hepatic CYP1A2 mRNA expression and CYP1A catalytic activity. Our findings indicated that Panax ginseng extract did not influence CYP1A2 mRNA expression or activity compared to the control group, while β-naphthoflavone resulted in an increase in both CYP1A2 expression and CYP1A catalytic activity. Furthermore, there is also evidence supporting our findings that Panax ginseng extract did not increase CYP1A2 mRNA expression in human (Gurley et al., 2002).

We also demonstrated that a single oral dose of Panax ginseng extract did not affect hepatic mEH mRNA or protein expression when compared to the control group, whereas phenobarbital (positive control) elevated both mRNA and protein expression of mEH. However, our results do not support the findings from the study of Lee et al. (1987)² in which Panax ginseng extract increased mEH catalytic activity.

The effects of ginseng extracts on CYP and mEH expression have not been extensively investigated. This project was based on the findings from only one study investigating the effects of Panax ginseng extract of biotransformation enzymes (Lee et al., 1987²). However, results from the present study cannot be directly compared with ones from that study, in which several non-specific and non-selective enzyme assays were used and the source of ginseng extract was not provided. We examined the
effects of *Panax ginseng* extracts on the expression of CYP enzymes and mEH with more specific methods, including validated real-time PCR methods, 7-benzyloxyresorufin and 7-ethoxyresorufin O-dealkylation assays. The *Panax ginseng* extract used in present study is standardized to 4% (w/w) total ginsenosides; whereas this information was not specified by Lee *et al.* (1987). To the best of my knowledge, this is the first study investigating the effect of a single dose of *Panax ginseng* extract on the expression of CYP2B1, CYP3A23, CYP1A2, CYP2C11 and mEH. Although it is not known if the above biotransformation enzymes are induced following a single dose of ginseng, other compounds have been shown to induce these enzymes with a single dose (Kim *et al.*, 1995; Bandiera and Wong, 1998; Meridith *et al.*, 2003). For example, a single dose of phenobarbital (60 mg/kg or 120 mg/kg, intraperitoneal injection) increases the protein expression and catalytic activity of rat hepatic CYP2B by 25- and 22-fold, respectively; the same treatment also increased the above dependent variables of rat hepatic CYP3A by 8- and 1.6-fold, respectively (Bandiera and Wong, 1998). According to Kim *et al.* (1995), the administration of a single dose of clotrimazole (50 mg/kg, oral gavage) elevated the hepatic mEH mRNA expression by 18-fold in rats. Also, a single dose of Aroclor 1254 (500 mg/kg, intraperitoneal injection) increased the mRNA levels of CYP1A2, CYP2B1, and CYP3A1 (CYP3A23) by 52-, 5480-, and 47-fold, respectively (Meridith *et al.*, 2003).

4.3 EFFECT OF MULTIPLE ORAL DOSES OF *PANAX QUINQUEFOLIUS* EXTRACT ON RAT HEPATIC CYP ENZYMES AND mEH

Due to the unavailability of *Panax ginseng* extract, *Panax quinquefolius* extract was used to further characterize the effects of multiple oral doses of ginseng extract on
rat hepatic CYP enzymes and mEH. The difference between the two extracts is that *Panax quinquefolius* contains a different composition of ginsenosides, and a pseudoginsenoside known as F₁₁ (Chan et al., 2000; Li et al., 2000; Morgan and Cupp, 2000; Harkey et al., 2001). Our results suggested that the administration of *Panax quinquefolius* extract (100 or 400 mg/kg/day, oral gavage, for 21 days) did not have any effects on the following dependent variables: (1) mRNA expression of hepatic CYP2B1, CYP3A23, CYP2C11, CYP1A2; and mEH (2) hepatic microsomal 7-benzyloxyresorufin and 7-ethoxyresorufin O-dealkylation activities; and (3) protein expression of hepatic CYP2C11 and mEH; in adult male rats. As mentioned earlier, previous *in vitro* studies suggested the glucocorticoid-like activities of ginsenosides (Kang et al., 1994; Kim et al., 1994; Lee et al., 1996; Lee et al., 1997) and this may influence CYP3A expression. Moreover, the *in vitro* estrogenic effects of *Panax quinquefolius* (Duda et al., 1996; Duda et al., 1999; Liu et al., 2001) and ginsenosides (Lee et al., 2003) may also have an impact on CYP2C11 expression. However, the *in vivo* relevance of the above studies is still to be verified.

In summary, our results may be interpreted in different ways. Either *Panax ginseng* and *Panax quinquefolius* extracts have no effects on the above dependent variables, or if they do, there may be other confounding factors that produced false negative results (which will be discussed later in this section).

Results from the positive controls indicated that the absence of effects of the *Panax ginseng* and *Panax quinquefolius* extracts is unlikely due to assay conditions. The positive control treatments (i.e. phenobarbital, dexamethasone, and β-naphthoflavone) resulted in significant increases or decreases in all the dependent
variables analyzed in our study, and this suggests these assays were functioning. Phenobarbital (positive control) elevated CYP2B1 and mEH mRNA expression, 7-benzyloxyresorufin O-dealkylation activities, and mEH protein expression. Dexamethasone (positive control) elevated CYP3A23 mRNA expression. The β-naphthoflavone treatment increased CYP1A2 mRNA expression and catalytic activity, and also reduced CYP2C11 mRNA and protein expression. We also examined the oral administration technique by conducting a positive control experiment, in which rats were administered with a well-known CYP1A inducer, albendazole (Souhaili-el Amri et al., 1988; Asteinza et al., 2000) by oral gavage. The increased hepatic microsomal 7-ethoxyresorufin O-dealkylation activity in the albendazole-treated group suggested that the lack of an observed effect by our ginseng extracts was not due to inappropriate oral administration.

4.4 CONFOUNDING FACTORS CONTRIBUTING TO THE LACK OF EFFECTS OF GINSENG EXTRACTS

**Inadequate Absorption**

One of the possibilities for the absence of effects of our ginseng extracts may have been poor absorption of the bioactive compounds from the gastrointestinal tract. There is supporting evidence from one study (Kim et al., 1990) using the same *Panax ginseng extract* as the one used in our study. In their study, a single oral dose of *Panax ginseng* extract (100 mg/kg) resulted in CNS effects in rats (Kim et al., 1990). This indicates that the active compound(s) present in the *Panax ginseng* extract were absorbed. However, because ginseng extract is a mixture of numerous unknown compounds at unknown concentrations, the plasma concentration of the responsible
active ingredient(s) that elicited the CNS effect may be different from the one that is hypothesized to interact with CYP enzymes and mEH.

Previous studies investigating the metabolism and pharmacokinetics of ginseng extracts were mostly conducted using ginsenosides. Based on the pharmacokinetic profile of both protopanaxdiol- and protopanaxtriol-type of ginsenosides, Odani et al. (1983\textsuperscript{2,3}) found that a relatively low ratio of the orally administered ginsenosides was recovered in plasma and urine, compared to the recovery in feces. This implies that there was poor absorption of these ginsenosides from the gastrointestinal tract. Previous studies using rat models have shown that the orally administered ginsenosides were subjected to biotransformation in the gastrointestinal tract by bacteria and enzymatic hydrolysis (Odani et al., 1983\textsuperscript{2,3}; Karikura et al., 1991\textsuperscript{1}; Wakabayashi et al., 1997). However, it should be noted that the above pharmacokinetic studies only characterized a few minor constituents of the overall dose, and thus very little is known about the absorption, distribution, metabolism, and excretion of ginseng extracts as a whole.

As indicated earlier, ginsenosides are considered to be responsible for various biological and pharmacological effects of ginseng (Attele et al., 1999), and they are generally used as reference compounds in standardizing ginseng products. An important and over-looked factors influencing ginsenoside content is the cultivation condition (Attele et al., 1999; Soldati, 2000; Harkey et al, 2001), which may contribute to possible differences in potency of its content of the extracts (Yuan et al., 1998; Attele et al., 1999). Previous in vitro studies have shown evidence for interactions between ginsenosides and hepatic CYP enzymes (Henderson et al., 1999; and Chang et al., 2001), however; the in vivo relevance of these findings was not determined.
Furthermore, other bioactive constituents are also present in ginseng extracts (Attele et al., 1999) and they may interact with hepatic CYP enzymes and mEH (Ahn et al., 1996; Feng et al., 2002; Huynh and Teel et al., 2002; Stupans et al., 2002; Szaefer et al., 2003). Therefore, depending on the purposes of the study, ginsenosides may not necessarily be the best reference compounds.

Effects of various routes of administration of ginseng extracts have not been investigated. Different results obtained from different routes of administration of ginseng extracts were reported in previous studies (Dey et al., 2002; Xie et al., 2002). For example, after the administration of Panax quinquefolius berry extract (150 mg/kg/day for 5 days) by intraperitoneal injection, anti-hyperglycemic and anti-obesity activities were observed in ob/ob mice (Xie et al., 2002), however, the same research group also reported that similar results were not obtained when the extract (300 mg/kg/day for 5 days) was administered by oral gavage (Dey et al., 2002). This suggests poor bioavailability that may due to low intestinal absorption and/or high pre-systemic metabolism. Although the ginseng extract used in the above study is different than ones used in our study, the above observation demonstrates the importance of considering the different routes of administration.

Effects of the constituents present in the Panax ginseng extract

According to our suppliers, the total ginsenosides accounted for 4% (w/w) and 10% (w/w) of Panax ginseng and Panax quinquefolius extracts, respectively. As mentioned above, there are numerous other compounds present in ginseng, such as polysaccharides, oligopeptides, poly-acetylenes, fatty acids and phenolic compounds (Attele et al., 1999; Soldati, 2000) that may contribute to the biological and
pharmacological effects of ginseng, including interactions with hepatic CYP enzymes and mEH. For example, several studies have demonstrated effects of plant-derived phenolics on CYP- or mEH-mediated enzyme activities (Ahn et al., 1996; Feng et al., 2002; Huynh and Teel et al., 2002; Stupans et al., 2002; Szaefer et al., 2003). Also, orally administered plant phenolics, such as protocatechuic acid and ellagic acid, inhibit hepatic microsomal 7-ethoxyresorufin, 7-methoxyresorufin, and 7-pentoxyresorufin dealkylation activities in rats (Szaefer et al., 2003).

Effects of contaminations present in the Panax ginseng extract

Results obtained from ginseng studies are difficult to interpret due to the presence of an unknown variety of contaminants. The analysis of 21 different commercially available Panax ginseng extracts (Khan et al., 2001) reported the presence of heavy metals and chlorinated pesticides, including dichlorodiphenyltrichloroethane (DDT) and its metabolites, quinotiozone (PCNB), β-hexachlorocyclohexane (β-HCH), hexachlorobenzene (HCB), γ-hexachlorocyclohexane (lidane), α- and trans-chlordane, aldrin and dieldrin. Depending on the type, amount, and potency, these contaminant may interact with and/or alter the expression of CYP enzymes and/or mEH to various levels (Nims and Lubert, 1995).

4.5 LIMITATIONS OF THE PROJECT

Inherent to studies using ginseng extracts is the fundamental problem that the extracts contain mostly unspecified constituents of unknown concentrations. Moreover, other factors such as batch-to-batch variability and potential contaminants may also
result in variable effects of the ginseng extracts. Thus, our negative findings may reflect the combined hybrid effects of all the unknown compounds in our ginseng extracts.

If the ginseng extracts used in our study do have effects on the CYP enzymes and mEH expression, one possibility for the false-negative results may have been insufficient power of our statistical tests to detect small differences among each group. The power of the test is the ability to statistically distinguish a difference when the difference truly exists, i.e. not due to chance. The statistical power can be influenced by several factors, including the expected experimental effects, the sample size, the P-value, and also the probability of the occurrence of false-negative results. Results from previous study (Lee et al., 1987) suggested that Panax ginseng extract has only moderate effects on the catalytic activities of the hepatic drug-metabolizing enzymes. Therefore, we did not expect substantial changes in the dependent variables analyzed in our study from the ginseng extract treatments. However, it should be noted that the level of catalytic activities and the mRNA expression of these enzymes may not be linear-correlated. Furthermore, assuming ginseng extracts used in our study do have certain level of effects on the hepatic drug-metabolizing enzymes, the insufficient statistical power may have been the small sample size (i.e. N = 4 or 5 individual rats in each group) used in our study. For example, there was certain level of changes in the mRNA expression of CYP2B1 (Figure 43) and CYP2C11 (Figure 46) in the Panax quinquefolius-treated groups as compared to the vehicle control-treated group, however, the changes was not statistically significant. Therefore, the above results may have been either the lack of effects of Panax quinquefolius extract, or insufficient statistical power in our experiment design.
As discussed earlier, due to the high sensitivity and the nature of exponential amplification, the variability of real-time PCR assay may be higher than other conventional quantitative methods. And depending on the transcription level of the target genes, sensitivity of the assay may vary (Freeman et al., 1999). A method validation guideline for determining the acceptable range of precision and accuracy has yet to be established for quantitative PCR.

4.6 FUTURE STUDIES

One of the possibilities to rationalize the different conclusions between this present study and the one by Lee et al. (1987)² could be the allosteric phenomenon resulting in enzyme activation. Constituent(s) present in the Panax ginseng extract used in the study by Lee et al. (1987)² might modify the catalytic binding sites of the enzymes and hence, elevate the catalytic activity. To resolve this issue, enzyme kinetic studies could be conducted in vitro by incubating purified recombinant CYP enzymes and microsomal epoxide hydrolase with ginseng extracts.

Secondly, due to an incomplete pharmacokinetic profile of ginseng extracts, absorption rate of the ginseng extracts is not known. Experiments of different routes of administration, including intravascular injection, intraperitoneal injection, and oral gavage, could be conducted in parallel in exploring this area.

As indicated earlier, cultivation conditions play an important role in the variability in ginsenosides content (Attele et al., 1999; Soldati, 2000; Harkey et al., 2001) and in the potency in pharmacological effects (Yuan et al., 1998; Attele et al., 1999). A previous study demonstrated a wide variation between the reported amounts of ginsenosides by the supplier and the actual amounts (Harkey et al., 2001). Given the assumption that
ginsenosides are the appropriate reference compounds, experiments in this area could include ginsenosides content and analysis of batch-to-batch differences.

4.7 SUMMARY AND CONCLUSIONS

1. Under the experimental conditions used in the present study, the administration of Panax ginseng extract did not affect: 1) body weight gain, 2) absolute or relative liver weight; 3) absolute or relative testes weight; 4) hepatic microsomal total CYP content; 5) hepatic CYP2B1, CYP3A23, CYP1A2 and mEH mRNA expression, as determined by validated real-time PCR assays; 6) hepatic microsomal 7-benzyloxyresorufin O-dealkylation and 7-ethoxyresorufin O-dealkylation activities; or 7) hepatic CYP2C11 and mEH protein expression.

2. Under the experimental conditions used in the present study, the administration of Panax quinquefolius extract did not affect: 1) body weight gain, 2) absolute or relative liver weight; 3) absolute or relative testes weight; 4) hepatic microsomal total CYP content; 5) hepatic CYP2B1, CYP3A23, CYP2C11, CYP1A2, and mEH mRNA expression, as determined by validated real-time PCR assays; 6) hepatic microsomal 7-benzyloxyresorufin O-dealkylation and 7-ethoxyresorufin O-dealkylation activities; or 7) hepatic CYP2C11 and mEH protein expression.
3. Results from the positive control experiments suggest that the lack of effects of ginseng extracts were unlikely due to assay conditions and/or an improper oral administration technique.

4. Due to batch-to-batch variations, and the impact of other unknown constituents and contaminations present in our ginseng extracts, our results may not represent the effects of all *Panax ginseng* and/or *Panax quinquefolius* extracts, but only the ones used in the present study.
CHAPTER 5. REFERENCES
REFERENCES


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APPENDIX
Figure A1. Effect of *Panax ginseng* extract on hepatic CYP2C11 protein expression. Adult male rats were administered by oral gavage with a single dose of *Panax ginseng* extract (30 or 100 mg/kg) or an equivalent volume (2 ml/kg) of 0.9% NaCl (vehicle control). All rats were sacrificed one day after the last dose. Livers were excised and hepatic microsomes were prepared by differential ultracentrifugation. Microsomal proteins were resolved by SDS-PAGE and then transferred electrophoretically onto a nitrocellulose membrane. The primary antibody used was monospecific rabbit anti-rat CYP2C11 polyclonal IgG at a concentration of 16 pg/ml. The secondary antibody used was alkaline phosphatase conjugated, goat F(ab')2 anti-rabbit IgG at 1:3000 dilution. The intensity of the CYP2C11 protein bands was determined densitometrically. Data are expressed as mean ± S.E.M. for four individual rats per treatment group. Hepatic microsomal CYP2C11 protein expression was not significantly different between the various groups.
Figure A2. Effect of *Panax quinquefolius* extract on hepatic CYP2C11 protein expression. Adult male rats were orally administered with *Panax quinquefolius* extract (100 or 400 mg/kg) or an equivalent volume (2 ml/kg) of 0.9% NaCl (vehicle control). All rats were sacrificed one day after the last dose. Livers were excised and hepatic microsomes were prepared by differential ultracentrifugation. Microsomal proteins were resolved by SDS-PAGE and then transferred electrophoretically onto a nitrocellulose membrane. The primary antibody used was monospecific rabbit anti-rat CYP2C11 polyclonal IgG at a concentration of 16 µg/ml. The secondary antibody used was alkaline phosphatase conjugated, goat F(ab')₂ anti-rabbit IgG at 1:3000 dilution. The intensity of CYP2C11 protein bands was determined densitometrically. Data are expressed as mean ± S.E.M. for four or five individual rats per treatment group. Hepatic microsomal CYP2C11 protein expression was not significantly different between the various groups.
**TABLE A1**

Relative mRNA expression in adult male rats treated with *Panax ginseng* extract

<table>
<thead>
<tr>
<th>Panax ginseng extract (mg/kg)</th>
<th>CYP2B1</th>
<th>CYP3A23</th>
<th>CYP1A2</th>
<th>Microsomal epoxide hydrolase</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% NaCl</td>
<td>3.2 ± 0.6</td>
<td>65 ± 7</td>
<td>21 ± 2</td>
<td>28 ± 3</td>
</tr>
<tr>
<td>30</td>
<td>2.7 ± 0.7</td>
<td>60 ± 8</td>
<td>25 ± 3</td>
<td>24 ± 5</td>
</tr>
<tr>
<td>100</td>
<td>2.3 ± 0.4</td>
<td>78 ± 12</td>
<td>22 ± 4</td>
<td>35 ± 2</td>
</tr>
</tbody>
</table>

Adult male rats were administered a single oral dose (30 or 100 mg/kg) of *Panax ginseng* extract or an equivalent volume (2 ml/kg) of 0.9% NaCl (vehicle control). All rats were sacrificed the following day. Livers were excised and snap-frozen in liquid nitrogen. Total RNA was isolated and reverse transcribed. Individual cytochrome P450, microsomal epoxide hydrolase, and cyclophilin cDNAs were amplified by real-time PCR. Data are expressed as mean ± S.E.M. for four individual rats per treatment group. Relative mRNA expression was calculated by dividing the level of mRNA of interest to that of cyclophilin and multiplying the value by 100. No significant differences were obtained.
Relative mRNA expression in adult male rats treated with *Panax quinquefolius* extract

TABLE A2

<table>
<thead>
<tr>
<th><em>Panax quinquefolius</em> extract (mg/kg/day)</th>
<th>CYP2B1 (X 10^2)</th>
<th>CYP3A23</th>
<th>CYP2C11</th>
<th>CYP1A2</th>
<th>Microsomal epoxide hydrolase</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% NaCl</td>
<td>0.8 ± 0.3</td>
<td>76 ± 9</td>
<td>84 ± 37</td>
<td>22 ± 3</td>
<td>21 ± 6</td>
</tr>
<tr>
<td>100</td>
<td>0.8 ± 0.1</td>
<td>101 ± 16</td>
<td>65 ± 11</td>
<td>26 ± 4</td>
<td>30 ± 2</td>
</tr>
<tr>
<td>400</td>
<td>0.8 ± 0.2</td>
<td>105 ± 21</td>
<td>64 ± 12</td>
<td>28 ± 2</td>
<td>37 ± 4</td>
</tr>
</tbody>
</table>

Adult male rats were administered by oral gavage with *Panax quinquefolius* extract (100 or 400 mg/kg) or an equivalent volume (2 ml/kg) of 0.9% NaCl (vehicle control) once daily for twenty one consecutive days. All rats were sacrificed one day after the last dose. Livers were excised and snap-frozen in liquid nitrogen. Total RNA was isolated and reverse transcribed. Individual cytochrome P450, microsomal epoxide hydrolase, and cyclophilin cDNAs were amplified by real-time PCR. Data are expressed as mean ± S.E.M. for four or five individual rats per treatment group. Relative mRNA expression was calculated by dividing the level of mRNA of interest to that of cyclophilin and multiplying the value by 100. No significant differences were obtained.