EFFECT OF GINKGO BILOBA ON ACETAMINOPHEN-INDUCED HEPATOTOXICITY IN MALE LONG-EVANS RATS

by

CATHERINE YUEN SHAN CHEUNG

B.Sc.(Hons.), The University of British Columbia, 1999
B.Sc.(Pharm.), The University of British Columbia, 2003

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Pharmaceutical Sciences)

THE UNIVERSITY OF BRITISH COLUMBIA

April 2006

© Catherine Yuen Shan Cheung, 2006
ABSTRACT

Acetaminophen (APAP) is commonly used as an analgesic and an antipyretic. This drug is considered safe, but it may cause severe hepatotoxicity and even fatality in certain situations. Ginkgo biloba extract (GBE), which is a popular herbal medicine used mainly to improve memory, induces rat hepatic cytochrome P450 (CYP) enzymes, including CYP1A2, CYP2E1, and CYP3A. APAP is bioactivated by these enzymes to the hepatotoxic metabolite, N-acetyl-p-benzoquinoneimine. Hence, GBE may modulate APAP-induced hepatotoxicity. The current study investigated the influence of GBE (containing 6.2% w/w terpene trilactones and 21% w/w flavonol glycosides) on APAP-induced hepatotoxicity in male Long-Evans rats, as assessed by liver histological analysis and plasma alanine aminotransferase (ALT) levels. The validated plasma ALT assay was accurate, precise, and reproducible, although it had poor sensitivity and a narrow dynamic range. Control experiments indicated that 20% Tween 80 in 0.9% NaCl (the vehicle used to suspend APAP) did not increase plasma ALT levels, fasting was required to elicit APAP-induced hepatotoxicity, and maximal plasma ALT levels were obtained with APAP at a dosage of 1000 mg/kg. GBE (500 mg/kg ip once daily for 8 days) did not increase plasma ALT levels, when compared to the 0.9% NaCl-treated control group, although histological analysis of hepatic tissues from GBE-treated rats showed that four of the five samples exhibited steatosis, necrosis, capsular inflammation, or sinusoidal dilatation. By comparison, GBE pretreatment (500 mg/kg ip once daily for 9 days) prevented the increase in plasma ALT levels in rats administered APAP (1000 mg/kg ip). The plasma ALT levels were 41 ± 3 U/L (mean ± SEM), 114 ± 22 U/L, and 53 ± 7 U/L in the vehicle-treated control group, APAP-treated group, and GBE and APAP-treated group, respectively. In contrast, GBE was not
able to attenuate the occurrence of steatosis, necrosis, capsular inflammation, or sinusoidal dilatation. These effects were accompanied by an increase (5-fold) in hepatic CYP3A23 mRNA expression, but no change in UDP-glucuronosyltransferase 1A6 mRNA expression. In summary, based on histological assessment, GBE exacerbated APAP-induced hepatotoxicity in male Long-Evans rats, and this might be related to increased bioactivation rather than reduced glucuronidation of APAP by GBE.
## TABLE OF CONTENTS

Abstract .................................................................................................................. ii
Table of Contents .................................................................................................. iv
List of Tables ......................................................................................................... vii
List of Figures ....................................................................................................... viii
List of Abbreviations ............................................................................................. ix
Acknowledgements ............................................................................................... x

1. INTRODUCTION ............................................................................................ 1
   1.1 Herbal Medicine ......................................................................................... 1
       1.1.1 Definitions ........................................................................................ 1
       1.1.2 Prevalence of Usage .......................................................................... 1
       1.1.3 Reasons for Use ................................................................................ 2
   1.2 Ginkgo biloba ............................................................................................. 2
       1.2.1 Clinical Uses ...................................................................................... 2
       1.2.2 Chemical Constituents ....................................................................... 3
       1.2.3 Pharmacokinetics of Some Terpene Trilactones and Flavonols ....... 3
           1.2.3.1 Absorption and Bioavailability ................................................... 4
           1.2.3.2 Distribution ................................................................................ 5
           1.2.3.3 Metabolism ............................................................................... 5
           1.2.3.4 Elimination ............................................................................... 8
           1.2.3.5 Effect of Dosage Form on Pharmacokinetic Parameters .......... 9
       1.2.4 Adverse Effects ................................................................................... 9
       1.2.5 Drug Interactions .............................................................................. 9
       1.2.6 Inhibition of Drug-Metabolizing Enzymes ....................................... 13
       1.2.7 Induction of Drug-Metabolizing Enzymes ....................................... 14
   1.3 Acetaminophen ........................................................................................... 15
       1.3.1 Clinical Uses ...................................................................................... 15
       1.3.2 Mechanism of Therapeutic Action .................................................... 16
       1.3.3 Pharmacokinetics ............................................................................. 16
           1.3.3.1 Absorption and Bioavailability ................................................... 16
           1.3.3.2 Distribution ................................................................................ 17
           1.3.3.3 Metabolism ............................................................................... 17
           1.3.3.4 Elimination ............................................................................... 19
       1.3.4 Adverse Effects ................................................................................... 19
       1.3.5 Hepatotoxicity ................................................................................... 20
           1.3.5.1 Mechanism of Hepatotoxic Action .............................................. 20
           1.3.5.2 Effect of Fasting ......................................................................... 22
           1.3.5.3 Strain Differences ....................................................................... 23
           1.3.5.4 Role of Nuclear Receptors in APAP-Induced Hepatotoxicity .... 24
           1.3.5.5 Other Modulators of APAP-Induced Hepatotoxicity ............... 26
   1.4 Rationale .................................................................................................... 29
   1.5 Research Hypothesis ............................................................................... 30
   1.6 Experimental Hypothesis ........................................................................... 30
   1.7 Specific Aims ............................................................................................. 30
2. MATERIALS AND METHODS .................................................................32
  2.1 Chemicals and Reagents ..........................................................32
  2.2 Animals .................................................................................33
  2.3 Treatment .................................................................................33
    2.3.1 Positive Control Experiment ................................................33
    2.3.2 Effect of Fasting and APAP ..................................................34
    2.3.3 Effect of 20% Tween 80 in 0.9% NaCl (the vehicle for APAP) ...34
    2.3.4 Effect of the Dose of APAP ..................................................34
    2.3.5 Effect of GBE ......................................................................35
    2.3.6 Effect of the Combination of GBE and APAP .......................35
  2.4 Termination of Animals, Blood Collection, and Preparation of Plasma ...36
  2.5 Alanine Aminotransferase (ALT) Assay ......................................36
    2.5.1 Principle of the Assay .........................................................36
    2.5.2 Methodology ....................................................................38
  2.6 Validation of the ALT Assay ......................................................39
    2.6.1 Lower Limit of Linearity ......................................................39
    2.6.2 Limit of Quantitation ..........................................................39
    2.6.3 Upper Limit of Linearity ......................................................39
    2.6.4 Dynamic Range ..................................................................40
    2.6.5 Accuracy and Precision .......................................................40
    2.6.6 Intra-day and Inter-day Variabilities ....................................40
    2.6.7 Inter-laboratory Comparison of ALT Values .......................41
  2.7 Histological Assessment of Liver Tissues .....................................41
  2.8 Data Analysis .........................................................................41

3. RESULTS .......................................................................................43
  3.1 Validation of the ALT Assay ......................................................43
    3.1.1 Lower Limit of Linearity ......................................................43
    3.1.2 Limit of Quantitation ..........................................................44
    3.1.3 Upper Limit of Linearity ......................................................46
    3.1.4 A Representation Standard Curve ........................................47
    3.1.5 Accuracy and Precision .......................................................48
    3.1.6 Intra-day and Inter-day Variabilities ....................................49
    3.1.7 Positive Control Experiment ................................................52
    3.1.8 Inter-laboratory Comparison of ALT Values .......................54
  3.2 Control Experiments with APAP .................................................57
    3.2.1 Effect of Fasting on Plasma ALT Levels in Rats Administered APAP...57
    3.2.2 Effect of the Vehicle on Plasma ALT Levels in Rats ................59
    3.2.3 Effect of the Dose of APAP on Plasma ALT Levels in Rats ..........61
  3.3 Experiments with GBE ...............................................................63
    3.3.1 Effect of GBE on Plasma ALT Levels in Rats .........................63
    3.3.2 Effect of GBE on Liver Histology in Rats ...............................65
  3.4 Experiments with GBE and APAP .............................................66
    3.4.1 Effect of Pretreatment with GBE on Plasma ALT Levels in Rats ...66
                                  Administered APAP
3.4.2 Effect of Pretreatment with GBE on Liver Histology in Rats

Administered APAP

4. DISCUSSION

4.1 Validation of the ALT Assay

4.1.1 Accuracy

4.1.2 Precision

4.1.3 Reproducibility

4.1.4 Dynamic Range

4.1.5 Sensitivity

4.1.6 Summary

4.2 Control Experiments with APAP

4.2.1 Effect of Fasting on Plasma ALT Levels in Rats Administered APAP

4.2.2 Effect of the Vehicle on Plasma ALT Levels in Rats

4.2.3 Effect of the Dose of APAP on Plasma ALT Levels in Rats

4.3 Effect of GBE on Liver Toxicity in Rats

4.3.1 Effect of GBE on Plasma ALT Levels in Rats

4.3.2 Effect of GBE on Liver Histology in Rats

4.4 Effect of GBE Pretreatment on Liver Toxicity in Rats treated with APAP

4.4.1 Effect of GBE on Plasma ALT Levels in Rats treated with APAP

4.4.2 Effect of other chemicals in GBE on Plasma ALT Levels in Mice treated with APAP

4.4.3 Effect of GBE on Liver Histology in Rats treated with APAP

4.4.4 Conflicting Results between ALT data and Histology data

4.4.5 Potential Mechanisms by which GBE Exacerbates APAP-Induced Hepatotoxicity

4.4.5.1 Increased Bioactivation of APAP by GBE

4.4.5.2 Decreased Detoxification of APAP by GBE

4.4.5.3 Combination of the Effects

4.5 Comparison of Current Study to Published Studies

4.6 Limitations of the Study

4.7 Future Studies

5. SUMMARY AND CONCLUSIONS

6. REFERENCES

7. APPENDICES

7.1 Appendix 1: The amount of ginkgolides, bilobalide, and flavonols in the GBE used in the study

7.2 Appendix 2: Effect of pretreatment with GBE on CYP3A23 mRNA expression in rats administered APAP

7.3 Appendix 3: Effect of pretreatment with GBE on UGT1A6 mRNA expression in rats administered APAP
LIST OF TABLES

Table 3.1. Accuracy and precision of the ALT assay ...........................................48
Table 3.2. Intra-day variability of the ALT assay .................................................50
Table 3.3. Inter-day variability of the ALT assay ................................................51
Table 3.4. Inter-laboratory comparison of ALT values ........................................55
Table 3.5. Comparison of ALT levels obtained from the ALT assay performed in our lab and those obtained from the Central Lab for Veterinarians ..........56
Table 3.6. Effect of the vehicle on plasma ALT levels in rats ..............................60
Table 3.7. Effect of GBE on liver histology scores in rats ..................................65
Table 3.8. Effect of GBE on liver histology scores in rats administered APAP ....69
Table A1. The amount of ginkgolides, bilobalide, and flavonols in the GBE .........107
LIST OF FIGURES

Figure 1.1. A simplified scheme of the bioactivation and detoxification pathways of APAP .............................................19

Figure 1.2. Mechanisms of APAP-induced hepatotoxicity ..................................................................................22

Figure 1.3. Dose-response curve for hepatic necrosis in Long-Evans and Sprague-Dawley rats administered APAP ..............................................................................................................24

Figure 2.1. Principle of the ALT assay (the first step in the reaction) ..........................................................37

Figure 2.2. The second step in the ALT assay .................................................................................................37

Figure 3.1. Determination of the lower limit of linearity for the ALT assay ...........................................43

Figure 3.2. Determination of the limit of quantitation of the ALT assay ...............................................45

Figure 3.3. Determination of the upper limit of linearity for the ALT assay ...........................................46

Figure 3.4. A representative standard curve for the ALT assay ..............................................................47

Figure 3.5. Effect of CCl4 on plasma ALT levels in rats ..............................................................................53

Figure 3.6. Effect of fasting on plasma ALT levels in rats treated with APAP or the vehicle ................58

Figure 3.7. Effect of the dose of APAP on plasma ALT levels in rats ..........................................................62

Figure 3.8. Effect of GBE on plasma ALT levels in rats ............................................................................64

Figure 3.9. Effect of pretreatment with GBE on plasma ALT levels in rats administered APAP ..........67

Figure 3.10. Summary of scores for steatosis in rat liver tissues ...............................................................70

Figure 3.11. Summary of scores for necrosis in rat liver tissues ...............................................................71

Figure 3.12. Summary of scores for capsular inflammation in rat liver tissues ....................................72

Figure 3.13. Summary of scores for sinusoidal dilatation in rat liver tissues .........................................73

Figure A1. Effect of pretreatment with GBE on CYP3A23 mRNA expression in rats administered APAP .109

Figure A2. Effect of pretreatment with GBE on UGT1A6 mRNA expression in rats administered APAP .110
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT</td>
<td>alanine aminotransferase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>APAP</td>
<td>acetaminophen</td>
</tr>
<tr>
<td>AST</td>
<td>aspartate aminotransferase</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>area under curve</td>
</tr>
<tr>
<td>CAM</td>
<td>complementary and alternative medicine</td>
</tr>
<tr>
<td>CAR</td>
<td>constitutive androstane receptor</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CV</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>CYP</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double-stranded deoxyribonucleic acid</td>
</tr>
<tr>
<td>GBE</td>
<td><em>Ginkgo biloba</em> extract</td>
</tr>
<tr>
<td>ip</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>LOQ</td>
<td>limit of quantitation</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>NAPQI</td>
<td><em>N</em>-acetyl-/?-benzoquinoneimine</td>
</tr>
<tr>
<td>NSAIDS</td>
<td>non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PXR</td>
<td>pregnane X receptor</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SULT</td>
<td>sulfotransferase</td>
</tr>
<tr>
<td>UGT</td>
<td>uridine diphosphoglucuronosyltransferase or UDP-glucuronosyltransferase</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I would like to sincerely thank my supervisor, Dr. Thomas Chang, for his continued patience and guidance throughout my undergraduate and graduate research experience. Thank you for not losing faith in me. Also, thanks to all my committee members, Dr. Stelvio Bandiera, Dr. Wayne Riggs, and Dr. Zhaoming Xu. Their support and helpful advice are greatly appreciated. Special thanks go to Dr. Ron Reid for chairing all my committee meetings, and Dr. Frank Abbott for being the external examiner.

In terms of financial support, I want to thank Merck Frosst Canada Inc. for the James E. Frosst Postgraduate Pharmacy Fellowship, the Faculty of Pharmaceutical Sciences at the University of British Columbia for the Kam Li Ma Scholarship in Pharmaceutical Sciences, Merck Research Laboratories (U.S.A.) for a partial M.Sc. traineeship, and the University of British Columbia for teaching assistantships. I would also like to acknowledge Canadian Institutes of Health Research (CIHR) for funding this research project. The Ginkgo biloba extract was generously provided by Indena S.A. (Tours, France).

This study would not be made possible without the generous assistance from Dr. Ganesh Rajaraman, the postdoctoral fellow in our lab. Without his generous help in the animal experiments and continuous support, this project would not have run as smoothly. Thanks to our knowledgeable and hard-working technician, Jessie Chen, for her continued support and help in gathering all the mRNA expressions data.
1. INTRODUCTION

1.1 Herbal Medicine

1.1.1 Definitions

Complementary and alternative medicine (CAM) is defined by the National Center for Complementary and Alternative Medicine as "a group of diverse medical and health care systems, practices, and products that are not presently considered to be part of conventional medicine" (Barnes et al., 2004). "Complementary medicine is used together with conventional medicine", whereas "alternative medicine is used in place of conventional medicine" (Barnes et al., 2004). Herbal products belong to the "biologically based therapies" category, which is one of the five categories in CAM (Barnes et al., 2004). The World Health Organization has defined "traditional medicine (including herbal drugs) as comprising therapeutic practices that have been in existence, often for hundreds of years, before the development and spread of modern medicine and are still in use today. Traditional preparations comprise medicinal plants, minerals and organic matter etc. Herbal drugs constitute only those traditional medicines which primarily use medicinal plant preparations for therapy" (Pal and Shukla, 2003).

1.1.2 Prevalence of Usage

Various factors such as place of residence, ethnicity, educational level, gender, age, health and socio-economical status can affect the prevalence of usage of CAM among the public (Barnes et al., 2004). Kamboj (2000) estimated that about 75 to 80% of the people in the developing countries use herbal medicine as their primary health remedies. Numerous surveys were performed at a national level in the United States to more closely portray the use of CAM by the public in the United States, but the results of the surveys have been
inconsistent (Barnes et al., 2004). However, a more complete and comprehensive survey conducted by the National Center for Health Statistics in 2002 indicated that about 36% of respondents (N = 31044) used some form of CAM in the past 12 months (Barnes et al., 2004). Among these surveyed users of CAM throughout the United States, about 19% of them have used natural products in the past 12 months (Barnes et al., 2004). About 21% of these natural product users have used Ginkgo biloba in 2002, and this made Ginkgo biloba the third most popular natural product in the United States in that year according to this survey (Barnes et al., 2004).

1.1.3 Reasons for Use

Different factors can affect why an individual would use herbal medicine (Ernst and Pittler, 2002). Some potential reasons for trying herbal medicine include perceived safety, self-control over treatment, easier accessibility, affluence, rejection of established science and technology, desperation and dissatisfaction with conventional medicine due to ineffective conventional therapies, or experience of adverse effects from conventional drug treatments (Ernst and Pittler, 2002). Advertising may also be one of the reasons. It was also suggested that the general public tend to use herbal medicine for treating diseases and conditions that are relatively not severe and can heal by themselves (Barnes et al., 2003).

1.2 Ginkgo biloba

1.2.1 Clinical Uses

Patients use Ginkgo biloba for many different reasons. Its purported uses include anxiety, asthma, bronchitis, cardiovascular diseases, circulatory disorders, hearing loss, memory loss, Raynaud's disease, sexual dysfunction, stress, and tinnitus (SK Cancer Center,
2004). Other additional uses include age-related macular degeneration, vertigo, and altitude sickness (Ang-Lee et al., 2001). *Ginkgo biloba* is mainly used in the United States to help patients "enhance mental focus" in diseases such as dementia and Alzheimer's disease (Kudolo, 2001). In Europe, *Ginkgo biloba* is widely sold to treat "early-stage Alzheimer's disease, vascular dementia, peripheral claudication, and tinnitus of vascular origin" (Sierpina et al., 2003).

1.2.2 Chemical Constituents

There are standardized *Ginkgo biloba* products available on the market. These products are extracts from the leaves of *Ginkgo biloba* trees, and are standardized to the contents of flavonol glycosides (24.0% w/w on average, range of 22-27% w/w) and terpene trilactones (6.0% w/w on average, range of 5-7% w/w) (van Beek, 2002; Kressmann et al., 2002). Most of the flavonol glycosides in these standardized products are glycosides of quercetin, kaempferol, and isorhamnetin, and the terpene trilactones include ginkgolides A, B, C, and J and bilobalide (van Beek, 2002; Kressmann et al., 2002; Drago et al., 2002). Other chemical compounds such as proanthocyanidins, simple phenolic acids, glucose, rhamnose, and a minute amount of alkylphenols (ginkgolic acid) are also present in these *Ginkgo biloba* products (Ahlemeyer and Krieglstein, 2003). One of the commonly used standardized products of *Ginkgo biloba* is called EGb 761.

1.2.3 Pharmacokinetics of Some Terpene Trilactones and Flavonols

Dietary quercetin and other flavonoids are absorbed in the intestine and undergo intestinal metabolism by enterocytes before reaching the liver, entering the circulation, and ultimately excreted in the urine (Ross and Kasum, 2002; Murota and Terao, 2003). Most of
the terpene trilactones (ginkgolides A and B and bilobalide) are absorbed and excreted unchanged in the urine (Kleijnen and Knipschild, 1992).

1.2.3.1 Absorption and Bioavailability

In a study of 12 volunteers (6 males, 6 females), each of them received an intravenous infusion of 100 mg of EGB 761 for 15 min, followed by an oral ingestion of 120 mg of EGB 761 solution. The bioavailability of ginkgolide A, ginkgolide B, and bilobalide were 80%, 88%, and 79% respectively, whereas ginkgolide C was reported to be not bioavailable (Biber, 2003). A single oral dose of 120 mg of EGB 761 in capsule formulation was ingested by each of the 12 healthy male volunteers in a study by Kressmann et al. (2002). AUC values were reported to be 121.35, 59.88, and 217.24 ng h/mL for ginkgolide A, ginkgolide B, and bilobalide, respectively. C<sub>max</sub> values were reported to be 22.22, 8.27, and 54.42 ng/mL for ginkgolide A, ginkgolide B, and bilobalide, respectively. Similar T<sub>max</sub> values (ranged from 1.17 to 1.54 h) were reported for all three chemicals (Kressmann et al., 2002).

In a recent controlled, randomized, and crossover study of 12 males (age 18 to 35), the volunteers ingested a standardized GBE for 7 days (Drago et al., 2002). The regimen of 40 mg twice daily (vs. 80 mg once daily) led to a greater AUC, and longer half-life and mean residence time of ginkgolides A and B and bilobalide (Drago et al., 2002). However, the 80 mg once daily regimen led to a greater maximum peak concentration (C<sub>max</sub>) of these three compounds. With both dosage regimens, time to maximum concentration (T<sub>max</sub>) of all three compounds was reached after 2 to 3 h of oral administration (Drago et al., 2002).

A study of ten adult volunteers in which each of them was given a single oral dose of 6 tablets of GBE reported absorption half-lives of quercetin and kaempferol to be 1.51 h and 1.56 h, respectively, whereas the T<sub>max</sub> of quercetin and kaempferol were reported as 2.30 h
and 2.68 h, respectively. In the same study, bioavailability of both quercetin and kaempferol was reported to be low (< 1%) in humans (Wang et al., 2003).

Biber and Koch (1999) performed a pharmacokinetic study using male Sprague-Dawley rats by giving each of them an oral dose of the standardized GBE EGb 761 at one of the three doses (30, 55, and 100 mg/kg body weight). At the 30 mg/kg dose of EGb 761, the mean \( C_{\text{max}} \) values were reported to be 68, 40, and 159 ng/mL for ginkgolide A, B and bilobalide, respectively. The clearance values of ginkgolide A, B and bilobalide were very similar to each other, and were reported be 30.1, 29.0, and 29.2 mL/min/kg, respectively (Biber and Koch, 1999).

1.2.3.2 Distribution

Kleijnen and Knipschild (1992) studied the pharmacokinetics of ginkgolide A, ginkgolide B, and bilobalide in humans administered GBE. In that study, the volume of distribution was reported to be 40 – 60 L for ginkgolide A and 60 – 100 L for ginkgolide B, whereas the volume of distribution for bilobalide was 170 L. According to the same study, the degree of protein binding was not known for the individual components of *Ginkgo biloba* (Kleijnen and Knipschild, 1992).

1.2.3.3 Metabolism

In a study performed using female Wistar rats by Pietta et al. (1995), rats were fed with an extract of *Ginkgo biloba* leaves at 4 g/kg body weight. Biological samples including urine, feces, and blood were collected from the rats for 5 days. Within 24 h, the flavonoids in the GBE were metabolized to seven different phenylalkyl acids: 3,4-dihydroxyphenyl-acetic acid, hippuric acid, 3-hydroxy-phenylacetic acid, homovanillic acid, benzoic acid, 3-(4-
hydrophenyl)propionic acid, and 3-(3-hydrophenyl)propionic acid. No glycosides or aglycones were detected in any of the biological samples (Pietta et al., 1995). Moon et al. (2001) treated rats orally with quercetin aglycone and they were the first group to isolate a glucuronide metabolite of quercetin (quercetin 3-O-β-glucuronide) in rat plasma.

In a separate study performed by Pietta et al. (1997), the flavonol metabolites identified after oral administration of an extract of Ginkgo biloba leaves to humans were different from those in rats. Substituted benzoic acids were detected in urine samples of volunteers – 4-hydroxybenzoic acid conjugate, 4-hydroxyhippuric acid, 3-methoxy-4-hydroxyhippuric acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid and 3-methoxy-4-hydroxybenzoic acid (vanillic acid). Also, no phenylacetic acid or phenylpropionic acid derivatives were found in the urine samples.

Murota et al. (2000) have reported enhanced diffusion of quercetin aglycone, compared to its glycosides, across the Caco-2 cell monolayer. In addition, they suggested that quercetin glycosides would be converted to its aglycone forms by enterobacteria before conjugation occurred, producing sulfation and glucuronidation metabolites. For those glycosides that were not converted to aglycones but absorbed, it was suggested that they would mostly be conjugated to form metabolites before entering the circulation (Murota et al., 2000), except that in vivo studies in humans have reported the detection of low levels of flavonoids glycosides in plasma following ingestion of Ginkgo biloba tablets by four volunteers (Oliveira et al., 2002). In vitro studies using HepG2 cells yielded similar results as in studies using Caco-2 cells (Galijatovic et al., 1999).

Wang et al. (2003) studied the disposition of quercetin and kaempferol in human subjects. Each adult human in the study was given an oral dose of six tablets of GBE (each
tablet containing 1.134 mg quercetin and 1.233 mg kaempferol). Both quercetin and kaempferol underwent sulfation and glucuronidation, and they were mainly excreted as glucuronides in the urine (Wang et al., 2003). This is in accordance with the studies performed in rats. Oliveira et al. (2002) studied the metabolism of quercetin and kaempferol in rat hepatocytes. Four monoglucuronides of quercetin and two monoglucuronides of kaempferol (glucuronide metabolites not identified) were detected after 40 min incubation of quercetin and kaempferol with the rat hepatocytes. The glucuronide metabolites were reported to be the same ones formed after glucuronidation by UGT1A9 (Oliveira et al., 2002).

It is generally believed that in both rats and human, quercetin and other flavonoids such as kaempferol and isorhamnetin undergo sulfate conjugation by phenol sulfotransferase, glucurononyl conjugation by UDP-glucuronosyltransferase, and methylation by catechol-O-methyltransferase in the intestine before being absorbed into the circulation, with glucuronidation being the major metabolism pathway (Murota and Terao, 2003; Oliveira et al., 2002). A quercetin metabolite in rat plasma, quercetin 3-O-β-D-glucuronide, was successfully isolated after oral administration of quercetin aglycone (Moon et al., 2001). In humans, a sulfate metabolite of quercetin in plasma was identified as quercetin 3'-O-sulfate (Day et al., 2001).

In addition to conjugation reactions, flavonoids are metabolized by the hepatic cytochrome P450 (CYP) enzymes. An in vitro study performed by Breinholt et al. (2002) using human recombinant CYP enzymes has shown 3'-hydroxylation of kaempferol to quercetin being mediated by CYP1A2. It has been reported that 70% of ginkgolide A, 50% of ginkgolide B, and 30% and bilobalide were excreted unchanged in the urine following oral
ingestion of GBE in humans, which indicated that some of the terpene trilactones in the GBE were not biotransformed after entering the human body (Kleijnen and Knipschild, 1992).

1.2.3.4 Elimination

Biber (2003) reported the elimination half-lives for ginkgolide A, B, and bilobalide to be 4.5 h, 10.6 h, and 3.2 h, respectively, following an IV infusion of 100 mg of EGb 761 for 15 min in 12 volunteers. Kressmann et al. (2002) reported the terminal half-lives of elimination of ginkgolide A, ginkgolide B, and bilobalide to be 3.93 h, 6.04 h, and 3.19 h, respectively, in 12 healthy male volunteers. The results of these human studies are in contrast to the study performed in rats in which the elimination half-lives of ginkgolide A, ginkgolide B, and bilobalide were reported to be 1.7 h, 2.0 h, and 2.2 h, respectively (Biber and Koch, 1999). The elimination half-lives of these three chemicals in rats appeared to be shorter than those in human.

Elimination half-lives of quercetin and kaempferol were reported to be 2.17 h and 2.76 h, respectively, after studying the urine of ten volunteers who ingested a single oral dose of 6 tablets of GBE (Wang et al., 2003).

One of the Ginkgo biloba flavonol metabolite called benzoic acids were detected only in urine samples, but not in blood samples, of human volunteers after oral ingestion of GBE (Pietta et al., 1997). No phenylacetic acid or phenylpropionic acid derivatives were detected in human urine, whereas these metabolites were detected in rats. The metabolites detected in human urine contributed to only less than 30% of the flavonoids given which was similar as in rats (Pietta et al., 1997).
1.2.3.5 Effect of Dosage Form on Pharmacokinetic Parameters

A study performed in 18 healthy volunteers compared three different dosage forms (capsules, drops, and tablets) of *Ginkgo biloba*. After administration of a single oral dose, the $T_{\text{max}}$ values of quercetin, kaempferol and isorhamnetin were reported to be prolonged in the capsules formulation when compared with the drops and tablets formulation (Wojcicki *et al.*, 1995). The use of phospholipid complex (Ginkgoselect Phytosome) form vs. the free (Ginkgoselect) form prolonged the $T_{\text{max}}$ values of ginkgolides A and B and bilobalide from 120 min to 180-240 min in a study of volunteers who were administered a single oral dose of GBE (Mauri *et al.*, 2001).

1.2.4 Adverse Effects

Patients considered *Ginkgo biloba* to be well tolerated in most clinical trials in that its "adverse event profile was not different from that of the placebo" (Le Bars and Kastelan, 2000). Alkylphenols (ginkgolic acid) is always limited to less than 5 ppm of the products of *Ginkgo biloba* due to its potential toxicity in causing cell death and apoptosis (Ahlemeyer and Krieglstein, 2003). Occasionally, *Ginkgo biloba* may cause headache, nausea, gastric symptoms, diarrhea, or allergic skin reactions (Commission E Monograph). The Commission E Monograph listed "very seldom stomach or intestinal upsets, headaches, or allergic skin reaction" as the side effects of *Ginkgo biloba* (Commission E Monograph).

1.2.5 Drug Interactions

In terms of pharmacodynamic drug interactions in human, there have been case reports of patients taking recommended doses of *Ginkgo biloba* and experiencing spontaneous hyphema upon taking warfarin (Rosenblatt and Mindel, 1997) or intracerebral
haemorrhage upon taking aspirin (Matthews, 1998) as these drugs may intensify the platelet antagonist effect of *Ginkgo biloba* in humans. However, in a crossover randomized study performed in 12 healthy male subjects in which each ingested a single dose of 25 mg of warfarin after a pretreatment with 2 tablets of a standardized GBE (each tablet standardized to 9.6 mg of ginkgo flavonglycosides and 2.4 mg of ginkgolides and bilobalide) three times a day for 7 days, no significant changes in platelet aggregation and international normalized ratio (INR) of prothrombin time, compared to the parameters measured when they did not receive the pretreatment, were reported (Jiang *et al.*, 2005). Also, *Ginkgo biloba* may increase blood pressure in humans when combined with a thiazide diuretic such as hydrochlorothiazide (Fugh-Berman, 2000).

Yin *et al.* (2004) investigated the pharmacokinetic drug interaction of *Ginkgo biloba* with omeprazole in 18 healthy Chinese volunteers. *Ginkgo biloba* was administered orally to each volunteer at a dose of 140 mg twice daily for 12 days. It was reported to induce omeprazole hydroxylation and reduce the AUC of omeprazole. According to the results of this study, *Ginkgo biloba* may reduce the effect of omeprazole to a significant level by increasing the clearance of omeprazole.

In a crossover randomized study performed in 12 healthy male subjects in which each ingested a single dose of 25 mg of warfarin after a pretreatment with 2 tablets of a standardized GBE (each tablet standardized to 9.6 mg of ginkgo flavonglycosides and 2.4 mg of ginkgolides and bilobalide) three times a day for 7 days, no significant changes in warfarin enantiomer protein binding, warfarin enantiomer concentrations in plasma, as well as S-7-hydroxywarfarin concentration in urine, compared to the parameters measured when they did not receive the pretreatment, were reported (Jiang *et al.*, 2005).
Markowitz et al. (2003) investigated whether oral ingestion of 120 mg of the GBE called EGb 761 twice daily for 14 days in 12 humans would affect the pharmacokinetic parameters of dextromethorphan (a CYP2D6 substrate) and alprazolam (a CYP3A4 substrate). The mean AUC of alprazolam among all 12 subjects was found to have significantly decreased by 17% when compared to the baseline AUC before Ginkgo biloba treatment. The mean $C_{\text{max}}$ of alprazolam was not significantly decreased after Ginkgo biloba treatment, although a significant decrease of 32% in $C_{\text{max}}$ was reported only in the six male subjects. Mean half-life of elimination was not significantly decreased after Ginkgo biloba treatment, despite a significant decrease of 19% reported only in the six female subjects. No significant changes were reported in $T_{\text{max}}$ and apparent oral clearance in all subjects and in either sex. Although the investigators concluded that the GBE was not likely to cause clinically significant drug interactions with medications metabolized by hepatic CYP2D6 and CYP3A4 based on the insignificant changes in apparent oral clearance, the results of this study suggested the potential for the GBE to significantly shorten half-life of alprazolam in female subjects and decrease AUC of alprazolam in male subjects (Markowitz et al., 2003).

Considering the data from all the subjects, a reduction in AUC without shortening the elimination half-life of alprazolam suggested the effect of Ginkgo biloba on reduced oral absorption of alprazolam, either through induction of intestinal CYP enzymes and/or inhibition of carrier-mediated transport, or through other mechanisms.

Gurley et al. (2002) investigated the effect of Ginkgo biloba on CYP2E1, CYP3A4, CYP2D6, and CYP1A2 drug metabolisms in 12 healthy volunteers. It was reported that Ginkgo biloba did not produce any significant effect on these four specific CYP activities using chlorzoxazone, midazolam, dextromethorphan, and caffeine as the CYP substrate for
CYP2E1, CYP3A4, CYP2D6, and CYP1A2, respectively.

In terms of pharmacodynamic drug interaction studies in animals, Sugiyama et al. (2004b) have reported a potential drug interaction between GBE and tolbutamide in rats. Rats were either pretreated with a 0.1% w/w Ginkgo biloba diet for 5 days before they were orally administered tolbutamide at 40 mg/kg body weight, or they were given the tolbutamide dose simultaneously with 100 mg/kg body weight of Ginkgo biloba orally. Under both conditions, Ginkgo biloba was reported to affect the hypoglycemic action of tolbutamide, and this effect was more prominent in aged rats than in young rats (Sugiyama et al., 2004b).

A study using a mouse model investigated the effect of Ginkgo biloba on barbital-induced sleeping time (Brochet et al., 1999). It was reported that single injections of the GBE called EGB 761 (at 25 and 50 mg/kg body weight) decreased barbital-induced sleeping time when given 1 h before sodium barbital. Ginkgolide B and bilobalide were reported to be responsible for this interaction (Brochet et al., 1999). The results of this study were in accordance with a pharmacokinetic drug interaction study using a rat model. Kubota et al. (2004) fed rats a 0.1% w/w, 0.5% w/w, or 1.0% w/w Ginkgo biloba diet for 2 weeks, and they reported a reduction in not only the $C_{\text{max}}$ and AUC of phenobarbital, but also a reduction in the phenobarbital-induced sleeping time in rats.

In another study using rats to investigate the pharmacokinetic interaction between GBE and nicardipine (a calcium channel blocker), Kubota et al. (2003) reported that a 0.5% w/w Ginkgo biloba diet to rats for 14 days decreased the $C_{\text{max}}$ and AUC of nicardipine (30 mg/kg body weight administered orally to rats after 2 weeks). This was in contrast to the conclusion Yoshioka et al. (2004) reached after they co-administered GBE and nifedipine (another calcium channel blocker) in rats. Yoshioka et al. (2004) reported the oral co-
administration of nifedipine at 5 mg/kg body weight and GBE at 20 mg/kg body weight caused a significant increase in $C_{\text{max}}$, AUC, and absolute bioavailability of nifedipine due to the ability of *Ginkgo biloba* to inhibit CYP3A. In rats, a GBE called Ginkgolon-24 increased the bioavailability of diltiazem (Ohnishi *et al.*, 2003).

1.2.6 Inhibition of Drug-Metabolizing Enzymes

GBE was recently reported to competitively inhibit 7-ethoxyresorufin O-dealkylation in hepatic microsomes prepared from rats whose hepatic CYP1A had been induced by once daily intraperitoneal (ip) injections with β-naphthoflavone for 3 days (Kuo *et al.*, 2004). However, *in vitro* inhibition of CYP1A activity in rats by GBE was not caused by the diglycosides of kaempferol, quercetin, or isorhamnetin, ginkgolides A, B, C, or J, or bilobalide. The aglycones of kaempferol, quercetin, and isorhamnetin were capable of inhibiting rat hepatic CYP1A activity (Kuo *et al.*, 2004). They were found to inhibit 7-ethoxyresorufin O-dealkylation activity in human liver microsomes as well (Chang *et al.*, in press). Kaempferol, quercetin, and isorhamnetin were reported to inhibit human recombinant CYP1A1 and CYP1A2 *in vitro*, as shown by their relatively low $K_i$ values (Chang *et al.*, in press). One speculation was that the aglycones could be responsible for CYP1A inhibition by the GBE *in vivo*, as flavonol glycosides can be biotransformed to their corresponding aglycones in the gastrointestinal tract (Walle, 2004).

Gaudineau *et al.* (2004) were the first group to report CYP2C9 and CYP2E1 inhibition by GBE (EGb 761) using microsomes prepared from human B-lymphoblastoid cells expressing individual human CYP. Their group demonstrated that the flavonoid fraction of EGb 761 was responsible for inhibition of human recombinant CYP2C9, CYP1A2, CYP2E1, and CYP3A4, and the terpenoid fraction of EGb761 was responsible for
inhibition of human recombinant CYP2C9 (Gaudineau et al., 2004). *In vitro* capability of quercetin to significantly inhibit tolbutamide 4-methylhydroxylation (IC$_{50}$ of 35 μM) and testosterone 6β-hydroxylation (IC$_{50}$ of 38 μM) has been reported in human liver microsomes (He and Edeki, 2004). In the same study, ginkgolide A was reported to inhibit tolbutamide 4-methylhydroxylation with an IC$_{50}$ of 200 μM.

1.2.7 Induction of Drug-Metabolizing Enzymes

Rats that have been fed *Ginkgo biloba* were found to have their hepatic CYPs induced. Sugiyama et al. (2004a) incorporated 0.5% w/w of a standardized GBE in the diet of male Wistar rats for 1 week, and they monitored the CYP activities for another 1, 2, or 3 weeks. After 1 week of oral treatment of GBE, the liver weight, the total CYP content in the liver microsomes, glutathione S-transferases activity, and six hepatic CYP marker enzyme activities (ethoxyresorufin O-deethylation, methoxyresorufin O-demethylation, pentoxyresorufin O-dealkylation, S-warfarin 7-hydroxylation, p-nitrophenol hydroxylation, and testosterone 6β-hydroxylation) were increased significantly. The total CYP content and the six hepatic CYP marker enzyme activities in the liver microsomes returned to basal level after discontinuing GBE for 1 week, whereas the glutathione S-transferases activity returned to basal levels after discontinuing the extract for 3 weeks (Sugiyama et al., 2004a).

In another study performed by Umegaki et al. (2002), rats were given GBE at 1, 10, 100, or 1000 mg/kg body weight daily by intragastric gavage for 5 days. The content of cytochrome P450 increased significantly compared to control starting at a dose of 10 mg/kg body weight of GBE. The activity of glutathione S-transferases increased significantly starting at a dose of 1 mg/kg body weight of GBE. Activities of ethoxyresorufin O-deethylase, methoxyresorufin O-demethylase, pentoxyresorufin O-dealkylase, p-nitrophenol...
hydroxylase, testosterone 6β-hydroxylase, and S-warfarin 7-hydroxylase were all reported to increase significantly compared to control at 100 and 1000 mg/kg body weight of GBE treatment. The increase in pentoxyresorufin O-dealkylase activity was substantial (65-fold compared to control level).

Hepatic mRNA expression of CYP2B1/2, CYP3A1, and CYP3A2 (but not that of CYP1A1/2, CYP2E1, CYP2C11, or CYP4A1) were markedly increased in male rats on a diet containing 0.5% w/w GBE for 4 weeks (Shinozuka et al., 2002). In another study performed by the same group, rats were given GBE at 1000 mg/kg body weight intragastrically for 5 days. Western blot analysis using anti-rat CYP2B1/2B2 antibody showed increased expression of CYP2B1 and CYP2B2 proteins in liver microsomes prepared from these rats treated with Ginkgo biloba (Umegaki et al., 2002).

Yang et al. (2003) investigated the effect of Ginkgo biloba on CYP activities when it was administered to rats by oral gavage at 100 mg/kg or 200 mg/kg body weight for 10 days. At the Ginkgo biloba dose of 200 mg/kg body weight, methoxyresorufin O-demethylase activity, ethoxyresorufin O-deethylase activity, pentoxyresorufin O-dealkylase activity, aminopyrine N-demethylase activity, and aniline hydroxylase activity in rat liver microsomes all increased significantly compared to the control group. At the dose of 100 mg/kg body weight, all but the ethoxyresorufin O-deethylase and the aniline hydroxylase activities in rat liver microsomes increased significantly.

1.3 Acetaminophen

1.3.1 Clinical Uses

APAP is a commonly used analgesic and antipyretic that is available without a prescription in North America. APAP has little or no side effects if used as directed but it
may cause liver damage during an overdose. A survey conducted in 308 patients in the United States with acute liver failure by Ostapowicz et al. (2002) indicated that APAP overdose accounted for 39% of all cases. Hepatotoxicity is more frequently observed in overdose of APAP and in patients with alcoholism (Thummel et al., 2000).

1.3.2 Mechanism of Therapeutic Action

APAP has antipyretic and analgesic properties similar to non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin and ibuprofen. However, in contrast to NSAIDs, APAP does not have anti-inflammatory and anticoagulation activities as it is only a weak inhibitor of cyclo-oxygenase-1 and cyclo-oxygenase-2 enzymes (Warner et al., 1999). The mechanism of action of APAP at the molecular level is not clear. Its pharmacological action is thought to be mediated through central (instead of peripheral in NSAIDs) inhibition of prostaglandin synthesis at the spinal cord and the hypothalamus (Muth-Selbach et al., 1999).

1.3.3 Pharmacokinetics

1.3.3.1 Absorption and Bioavailability

APAP is rapidly absorbed (absorption half-life of 30 min) upon oral administration and its elimination half-life is approximately 2 h (Rumack, 2004). Its volume of distribution is estimated to be 1 L/kg (Rumack, 2004) and its oral bioavailability is dose-dependent, ranging from 70% to 90% (Forrest et al., 1982).
1.3.3.2 Distribution

APAP is not known to bind to any plasma proteins. APAP was found to distribute to brain regions including the dorsal spinal cord and the cerebellum in rats (Courad et al., 2001). Volume of distribution in human was reported to be 66 L (Anderson et al., 2002).

1.3.3.3 Metabolism

Data from human and animal studies have revealed that APAP may undergo one of the three metabolic pathways upon oral administration. As shown in Figure 1.1, it may conjugate with sulfate, conjugate with glucuronide, or it may be metabolized by cytochrome P450 (CYP) enzymes (Bartlett, 2004). In humans, majority (96%) of APAP undergoes glucuronidation or sulfation to form non-toxic conjugated metabolites, whereas CYP2E1 is the main CYP enzyme that is responsible for the other 4% of the metabolism of APAP to form the hepatotoxic metabolite – N-acetyl-p-benzoquinoneimine (NAPQI) (Hersh and Moore, 2004; Rumack, 2002; Manyike et al., 2000). Other CYP enzymes such as CYP3A4, CYP1A2, CYP2A6 have also been suggested to be involved in the formation of the hepatotoxic metabolite (Bartlett, 2004). The hepatotoxic metabolite is then eliminated by binding to glutathione to form non-toxic metabolites in hepatocytes, a process that is catalyzed by glutathione S-transferases (Kaplowitz, 2004).

Overdose of APAP may result in severe hepatic necrosis in humans and in various laboratory animals. Among laboratory animals, hamsters and mice are more sensitive, whereas rats, rabbits, and guinea pigs are less sensitive to APAP-induced hepatotoxicity (Bessems and Vermeulen, 2001). In adult male rats treated with APAP alone, a 46% and a 41% inhibition of APAP oxidation was observed when the liver microsomes were incubated with CYP2E1 and CYP1A2 antibodies, respectively, at an APAP concentration of 1 mM,
suggesting the important roles of these two CYP enzymes in the metabolism of APAP to form the hepatotoxic metabolite (Patten et al., 1993). This concentration of APAP (1 mM) may not be relevant in in vivo rat studies because APAP has been reported to reach a level of 1043 ng/mL (or 0.007 mM) in rat plasma 4 h after oral administration of APAP at 5 mg/kg body weight (Abu-Qare and Abou-Donia, 2001). CYP3A1/2 antibodies showed a slight inhibition (10%), suggesting the involvement of CYP3A1/2 in the metabolism of APAP, but to a lesser extent as compared to CYP2E1 and CYP1A2 (Patten et al., 1993). The 3-hydroxylation of APAP in rats was reported to exceed the formation of the hepatotoxic metabolite rendering the relative insensitivity of APAP-induced hepatotoxicity in rats (Bessems and Vermeulen, 2001).

In terms of Phase II biotransformation reactions, APAP is metabolized by sulfotransferase (SULT) enzymes and UDP-glucuronosyltransferase (UGT) enzymes to non-toxic metabolites for elimination. Kessler et al. (2002) suggested the UGT1A6 enzyme plays a key role in glucuronidation of APAP as Gunn rats, which have nonfunctional UGT1A6, showed reduced rate of APAP glucuronidation and increased APAP-induced hepatotoxicity. The members of the SULT1 family of sulfotransferases were suggested to be responsible for the sulfation of APAP (Duffel, 1997).
Figure 1.1. A simplified scheme of the bioactivation and detoxification pathways of APAP. APAP can undergo sulfation in the presence of SULT1 (pathway A) or glucuronidation in the presence of UGT1A6 (pathway B) to form non-toxic conjugated metabolites. APAP can also be metabolized by CYP2E1, CYP1A2, and CYP3A to form the hepatotoxic metabolite, NAPQI (pathway C). In the presence of glutathione, NAPQI can be converted into non-toxic metabolites through the action of glutathione S-transferases (pathway D).

1.3.3.4 Elimination

The sulfate and glucuronide metabolites of APAP are more water-soluble than the other metabolites and are eliminated mainly by urine. A small amount (glucuronide of APAP) is eliminated by bile. The hepatotoxic metabolite NAPQI, formed by the cytochrome P450 enzymes, can bind to and form a conjugate with glutathione. This conjugate can further be converted to mercapturic acid for elimination in the urine (Bessems and Vermeulen, 2001).

1.3.4 Adverse Effects

In a study done in 66 human volunteers who each underwent a surgical dental procedure, 1000 mg of APAP was given to each volunteer after the procedure and about 17% of the subjects reported adverse effects (Olson et al., 2001). About 2% of the subjects
reported fever, 6% of them reported headache, 3% of them experienced nausea, and 4.5% of them reported somnolence as adverse drug reactions. However, no difference in adverse effects was reported between APAP (1000 mg twice daily) and placebo in a randomized, single blind, placebo-controlled study of 72 healthy volunteers treated for 2.5 days (Jerussi et al., 1998). In another randomized, double blind, cross-over study of 24 healthy volunteers, there was no difference in occurrence of gastrointestinal lesions between APAP (1000 mg four times a day) and placebo (Lanza et al., 1998). Nonetheless, when APAP is ingested at a toxic dose of more than 150 mg/kg by a human adult, it can potentially cause acute liver toxicity (Wallace et al., 2002; Tenenbein, 2004).

*N*-Acetylcysteine is a clinically useful antidote for APAP-induced poisoning, if given within a few hours after ingestion of toxic doses of APAP (Thomas, 1993). Glutathione is a tripeptide that is poorly absorbed by cells. *N*-acetylcysteine can act as a precursor for the synthesis of glutathione.

### 1.3.5 Hepatotoxicity

#### 1.3.5.1 Mechanism of Hepatotoxic Action

Many mechanisms leading to APAP-induced hepatotoxicity have been proposed. It is generally believed that the CYP-mediated formation of NAPQI from APAP (pathway C in Figure 1.1) is a key event in causing the hepatotoxicity. At hepatotoxic doses of APAP, the store of glutathione can be depleted, leading to increased accumulation of the hepatotoxic metabolite, NAPQI. This reactive metabolite of APAP can potentially bind covalently to DNA and critical cellular protein(s), resulting in loss of function of the protein(s) and ultimately to cell death. These proteins have been postulated to be mitochondrial proteins and/or proteins responsible for cellular ion control (Nelson, 1990). Recently, it has been
hypothesized that APAP toxicity occurs when there is increased oxygen and/or nitrogen stress in the system, and this increased oxidative/nitrosative stress can lead to mitochondrial permeability transition (Hinson et al., 2004). Mitochondrial permeability transition is “an abrupt increase in the permeability of the inner mitochondrial membrane to small molecular weight solutes” (Hinson et al., 2004). It has been hypothesized that this process can be lethal to the cells as it occurs with an increased release of superoxides. Superoxides are toxic to the cells because they can lead to peroxynitrite and tyrosine nitration (Kim et al., 2003).

The intracellular signaling mechanisms leading to liver cell death during APAP-induced hepatotoxicity were summarized in detail in a recent review by Jaeschke and Bajt (2006). The hepatotoxic metabolite of APAP (NAPQI) “first depletes cellular glutathione and subsequently covalently bind to cellular proteins. These initiating events lead to disturbances of the cellular Ca$^{2+}$ homeostasis, with increase of the cytosolic Ca$^{2+}$ levels, Bax and Bid translocation to the mitochondria, and a mitochondrial oxidative stress and peroxynitrite formation. The Bcl-2 family members form pores in the outer mitochondrial membrane and release cytochrome c, Smac (the second mitochondria-derived activator of caspases), apoptosis-inducing factor, and endonuclease G from the mitochondrial intermembrane space. Reactive oxygen species and peroxynitrite induce the membrane permeability transition, which causes the collapse of the mitochondrial membrane potential, eliminates ATP synthesis, and causes further release of mitochondrial proteins.” “Apoptosis-inducing factor and endonuclease G translocate to the nucleus and induce DNA fragmentation.” “The massive nuclear DNA damage and the rapid elimination of functional mitochondria, together with activation of intracellular proteases (calpains), lead to cell membrane failure and oncotic necrosis of the hepatocytes” (Jaeschke and Bajt, 2006).
1.3.5.2 Effect of Fasting

Price et al. (1987) have reported the effect of fasting on APAP-induced hepatotoxicity in male Long-Evans rats. The rats experienced an overnight fast before receiving ip injections of APAP. Fasting not only decreased the hepatic levels of glutathione, but also decreased clearance (due to reduced glucuronidation and sulfation) of APAP. The depression in glucuronidation and sulfation hence increased the formation of the hepatotoxic metabolite NAPQI, rendering fasted-rats more susceptible to APAP-induced hepatotoxicity (Price et al., 1987).
1.3.5.3 Strain Differences

Strain differences in APAP-induced hepatotoxicity became evident in rats as early as 1986, when the susceptibility to APAP-induced hepatotoxicity was compared between Sprague-Dawley and Long-Evans rats (Price and Jollow, 1986). Both strains of rats were fasted for 24 h prior to ip injections of APAP at 800 mg/kg. The Sprague-Dawley rats showed no evidence of liver injury, whereas 90% of the Long-Evans rats showed liver necrosis 48 h after the dose of APAP. In the same study, both strains of rats were fasted and received ip injections of APAP at 400, 600, 800, and 1200 mg/kg body weight, and were terminated 48 h after the APAP dose. There was an obvious left shift in the dose-response curve of the Long-Evans rats compared to that of the Sprague-Dawley rats, indicating the Long-Evans strain of rats was more susceptible to APAP-induced hepatotoxicity (Figure 1.3).
Figure 1.3. Dose-response curve for hepatic necrosis in Long-Evans and Sprague-Dawley rats administered APAP. The effect of various intraperitoneal doses of APAP on liver necrosis in two strains of rats 48 h after administration of APAP (original data from study done by Price and Jollow, 1986).

1.3.5.4 Role of Nuclear Receptors in APAP-induced Hepatotoxicity

Recently, the pregnane X receptor (PXR) was suggested to play an important role in APAP-induced hepatotoxicity in mice (Wolf et al., 2005). PXR is a nuclear receptor that is abundant in the liver and the intestine. Upon ligand binding to PXR, a heterodimer will be formed between PXR and retinoid X receptor. This is followed by activation of “ER6 (everted repeat with a 6 nucleotide spacer) elements” upstream to the target CYP genes, leading to increased transcription (Waxman, 1999). The PXR(-/-) mice were reported to be less susceptible, compared to wild-type mice, to hepatotoxicity induced by oral ingestion of
Wild-type mice were also found to have greater hepatic CYP1A2 protein levels than PXR(-/-) mice. CYP1A2 was hence suggested to be critical in modulating APAP hepatotoxicity in the wild-type mice, as caffeine (a CYP1A2 inhibitor) was able to protect these mice against the hepatotoxicity caused by APAP. The same study also suggested the effect of PXR on intestinal APAP-transporting proteins, as wild-type mice absorbed APAP from the intestine to a greater degree than PXR(-/-) mice. The absence of PXR in the PXR(-/-) mice may lead to a decreased expression of these transporter proteins, leading to a decrease absorption of APAP and hence a decreased susceptibility of APAP-induced hepatotoxicity in these PXR(-/-) mice.

In contrast, Guo et al. (2004) reported enhanced hepatotoxicity, as shown by elevated serum ALT levels and worse hepatocyte necrosis, in PXR-null mice at 24 h after ip injection of APAP. This enhanced APAP-induced hepatotoxicity in PXR-null mice was attributed to an increase in CYP3A11 mRNA expression and a decrease in glutathione S-transferases -Pi mRNA expression (both by northern blot analysis) in PXR-null mice. Also, there was a lower mRNA expression of CYP1A2 in PXR-null mice, although the CYP2E1 mRNA expression were similar in both PXR-null and wild-type mice. However, enhanced APAP-induced hepatotoxicity was reported in wild-type mice upon treatment with a PXR activator, pregnenolone 16α-carbonitrile. Enhanced APAP-induced hepatotoxicity was not seen in PXR-null mice, whose CYP3A11 expression and NAPQI formation were both lower, upon treatment with the same activator, suggesting the important role of PXR in APAP-induced hepatotoxicity.

Both studies that investigated the effect of PXR in APAP-induced hepatotoxicity in mice found an increase in CYP3A expression and a decrease in CYP1A2 expression in PXR-
null mice, when compared to wild-type mice. CYP2E1 expression in both PXR-null mice and wild-type mice were similar. However, APAP-induced hepatotoxicity was enhanced in wild-type mice in the study by Wolf et al. (2005), whereas it was enhanced in PXR-null mice in the study by Guo et al. (2004).

On the other hand, constitutive androstane receptor (CAR) was suggested to be another key regulator in APAP-induced hepatotoxicity (Zhang et al., 2002). CAR(-/-) mice showed significantly lower ALT levels than wild-type mice at 5 h and 24 h after given 500 or 800 mg/kg dose of APAP. Also, as early as 2 h after the dose of APAP at 500 mg/kg, CYP1A2, CYP3A11, and glutathione S-transferases -Pi mRNA expression were all increased significantly in wild-type mice, but not in CAR-null mice, by 2.8-, 4.4-, and 3.9-fold, respectively. In the same study, both wild-type and CAR-null mice were pretreated with a CAR activator or agonist, either phenobarbital or 1,4-bis[2-(3,5-dichloropyridyloxy)]-benzene, before dosed with a non-hepatotoxic dose APAP at 250 mg/kg body weight. Elevated ALT levels and liver necrosis were observed only in wild-type mice, but not in CAR-null mice, at 24 h after dosing with APAP.

1.3.5.5 Other Modulators of APAP-Induced Hepatotoxicity

**Enhancement of APAP-induced hepatotoxicity**

A few compounds have been reported to enhance APAP-induced hepatotoxicity in laboratory models by modulation of Phase I or II drug-metabolizing enzymes. Drugs such as acarbose and troglitazone may potentiate the hepatotoxicity effect of APAP by inducing the expression of CYP2E1 or CYP3A in rats (Wang et al., 1999; Li et al., 2002). Acarbose, when included in a 3-week-diet of rats at 40 or 80 mg /100g body weight diet, was reported to increase the levels of ALT and the levels of aspartate aminotransferase (AST) by 1.5
(40 mg) and 2 (80 mg) times relative to APAP control (APAP only) after ip injection of APAP at 750 mg/kg body weight (Wang et al., 1999).

In another study, rats were fed a troglitazone (0, 100, or 500 mg/kg body weight/day) diet for 3 weeks starting at an age of 10 weeks, followed by ip injection of APAP at 0.75 g/kg body weight in a 0.9% saline solution (10 mL/kg) (Li et al., 2002). In the plasma, the levels of ALT, AST, and α-glutathione S-transferases in the group pretreated with troglitazone at 500 mg/kg/day (700 mg drug per 100 g food) prior to APAP vs. control (APAP without troglitazone) were increased by 1.6-fold, 1.3-fold, and 2.3-fold, respectively, suggesting the increased risk of APAP-induced toxicity when troglitazone was given in the diet (Li et al., 2002). At a troglitazone dose of 500 mg/kg/day (700 mg drug per 100 g food), the glutathione level was observed to be significantly less (83% of APAP control level) compared to the APAP control (Li et al., 2002).

Ethanol can induce CYP2E1 leading to increased formation of the hepatotoxic metabolite, thereby increasing the risk of hepatotoxicity (Zand et al., 1993; Thummel et al., 2000). In the study by Thummel et al. (2000), each of the ten healthy human volunteers received an intravenous infusion of ethanol, a CYP2E1 inducer, for 6 h. Each volunteer received an oral dose of APAP at 500 mg 8 h after the end of the infusion. The formation of NAPQI was reported to be significantly increased by 22% in the group that received ethanol prior to APAP, compared to the control group that received 5% dextrose in water infusion (Thummel et al., 2000). In cultured human hepatocytes, both phenobarbital (2 mM) and phenytoin (0.2 mM) have been reported to increase APAP (5 mM) hepatotoxicity by inhibition of UGT1A6, UGT1A9, and UGT2B15 (Kostrubsky et al., 2005).
Protection against APAP-induced hepatotoxicity

Many compounds have been investigated for their protective potential against APAP-induced hepatotoxicity. For instance, chemicals such as stiripentol may have a protective effect on APAP-induced hepatotoxicity in rats (Tran et al., 2001). Stiripentol (200 mg/kg body weight ip in corn oil), an *in vitro* inhibitor of hydroxylation of naphthalene by CYPs in cerebral tissues of rats (Mesnil et al., 1988), administered 30 min before an overdose of APAP (two doses at 500 mg/kg body weight ip at 2.5 h apart) and 5 h after the first administration at 100 mg/kg body weight ip, lowered mortality rate of the rats from 63% (0.9% saline and corn oil) to 0% by inhibiting the formation of the hepatotoxic metabolite of APAP by CYP inhibition (Tran et al., 2001). Disulfiram pretreatment (500 mg orally at bedtime received by each volunteer 10 hour before a 500 mg oral dose of APAP was administered) was reported to decrease formation of the hepatotoxic metabolite by 74% in humans (Manyike et al., 2000). Isoniazid is a CYP2E1 inhibitor upon a single dose (O'Shea et al., 1997). It was reported to inhibit the formation of APAP thioether metabolites when given orally to ten volunteers as a single dose of 300 mg daily for 7 days (Zand et al., 1993).

There was only one study in the literature that investigated the effect of GBE on APAP-induced hepatotoxicity in rats (Shenoy et al., 2002). At an oral dose of APAP at 2000 mg/kg once daily for 3 days and in the absence of any GBE treatment (Group II), the mean ± SEM plasma ALT level was 256 ± 25 IU/L. When the same APAP treatment was followed by a vehicle treatment using 2% gum acacia for 7 days (Group III), plasma ALT level decreased slightly to 213 ± 19 IU/L. When GBE was given as an ip dose at 50 mg/kg simultaneously with an oral dose of APAP at 2000 mg/kg once daily for 3 days (Group IV), plasma ALT level decreased significantly to 43 ± 7 IU/L (when compared to Group II).
similar plasma ALT level (44 ± 6 IU/L) was obtained when GBE was given for 7 days after
the same regimen (oral dose of APAP at 2000 mg/kg once daily for 3 days) of APAP (Group
V). This was significantly less than that in Group III. Both the plasma ALT data and the
liver histology data in this study by Shenoy et al. (2002) suggested a protective effect of
GBE when it is given together with or after a hepatotoxic dose of APAP in rats. However,
Shenoy et al. (2002) failed to investigate the effect of a pretreatment with GBE to the rats
before APAP is given. Their study only addressed the effect of GBE on APAP-induced
hepatotoxicity when GBE was given concurrently or after the treatment with APAP.

1.4 Rationale

APAP is a widely used over-the-counter antipyretic and analgesic drug. Although
generally regarded as a well-tolerated medication, APAP can cause liver toxicity. The
majority of APAP is converted to non-toxic sulfate and glucuronide conjugates of APAP for
excretion from the body (Thomas, 1993). APAP-induced hepatotoxicity can result when the
conjugation pathways are saturated. During oxidative stress and in conditions where the
level of hepatic glutathione is reduced (e.g. malnourishment), the risk of APAP-induced
hepatotoxicity can also be increased (Vendemiale et al., 1996).

The accumulation of the hepatotoxic metabolite NAPQI, potentially leading to
oxidative stress and covalent binding to functional proteins rendering cellular damage, is
directly associated with the hepatotoxicity caused by APAP (Mitchell et al., 1973). This
hepatotoxic metabolite is formed by a cytochrome P450 oxidation pathway mediated mainly
by CYP2E1, CYP1A2, and CYP3A (Patten et al., 1993; Thummel et al., 1993). The
capacity of the glutathione S-transferases to detoxify the hepatotoxic metabolite also plays a
crucial role in alleviating the hepatotoxicity caused by APAP.
GBE has been shown to significantly induce methoxyresorufin $O$-demethylase, $p$-nitrophenol hydroxylase, and testosterone $6\beta$-hydroxylase activities, which are thought to represent the activities of CYP1A2, CYP2E1, and CYP3A, respectively, in rats (Umegaki et al., 2002). Hence, it is plausible that GBE can exacerbate APAP-induced hepatotoxicity by increasing formation of NAPQI as a result of CYP1A2, CYP2E1, and CYP3A induction by GBE. GBE was shown to be hepatoprotective in rats pretreated or co-treated with APAP (Shenoy et al., 2002). However, that study did not include a pretreatment regimen of GBE prior to APAP. GBE is a supplement that people consume on a daily basis. It would be invaluable to investigate whether pretreatment of rats with GBE can modulate APAP-induced hepatotoxicity. Both APAP and GBE are popular medications that are available over-the-counter without a prescription. The results obtained from the current study may have important clinical implications.

1.5 Research Hypothesis

GBE will modify the degree of APAP-induced hepatotoxicity.

1.6 Experimental Hypothesis

GBE pretreatment will modulate APAP-induced hepatotoxicity in male Long-Evans rats, as assessed by the plasma alanine aminotransferase (ALT) assay and histological analysis.

1.7 Specific Aims

(1) To validate the plasma ALT assay

(2) To perform control experiments to verify some conditions (e.g. dose of APAP, fasting) required to increase plasma ALT levels in male Long-Evans rats
(3) To determine the effect of GBE pretreatment on plasma ALT levels and liver histology (steatosis, necrosis, capsular inflammation, and sinusoidal dilatation) in male Long-Evans rats administered APAP
2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Chemicals used in the study were purchased from the following:

BDH Inc. (Toronto, Ontario, Canada)

Sodium chloride powder

Best Foods Canada Inc. (Etobicoke, Ontario, Canada)

100% pure Mazola Corn oil

Biotron Diagnostics Inc. (Hemet, California, USA)

ALT Kit containing:

ALT Reagent: 0.15 mg/L \( \alpha \)-ketoglutaric acid, 8.9 mg/L dl-alanine in phosphate buffer and preservative

Color Developer A: 0.02% w/v 2,4-dinitrophenyl-hydrazine, 8.6% hydrochloric acid, and preservative

Color Developer B: 1.6% w/v sodium hydroxide

Abnormal Clinical Chemistry Control – lyophilized standard

Fisher Scientific (Fair Lawn, New Jersey, USA)

Tween 80 (Polysorbate 80)

Indena S.A. (Milan, Italy)

*Ginkgo biloba* extract (Lot no. 1306A) [the levels of terpene trilactone and flavonols are listed in Table A1 in the appendix]

MTC Pharmaceuticals (Cambridge, Ontario, Canada)

Somnotol (Sodium pentobarbital for injection)

Sigma-Aldrich Inc. (St. Louis, Missouri, USA)

Acetaminophen SigmaUltra, minimum 99.0%
10% Formalin solution
Carbon tetrachloride
Heparin sodium salt

2.2 Animals

Male and female Long-Evans rats were purchased from Charles River Canada Inc. (Montreal, Quebec, Canada). The animals were housed in pairs in polycarbonate cages with corncob bedding for at least 5 days before any experiment was performed. Food (Lab Diet, Purina Mills, Inc.) and water were available ad libitum (except during fasting condition), and their room was under controlled temperature (20 °C) and lighting (lights on from 7 a.m. to 7 p.m.) during the course of each experiment. All animals weighed 100 – 150 g and were 30 – 37 days of age before the start of each experiment.

Long-Evans rats were used because they are more susceptible to APAP-induced hepatotoxicity when compared to another strain of rats [Sprague-Dawley] (Price and Jollow, 1986). Mice are more susceptible to APAP-induced hepatotoxicity compared to rats (Bessems and Vermeulen, 2001) but their blood volume is too small to have sufficient amount of plasma isolated for repeated analyses of ALT levels in a single plasma sample.

2.3 Treatment

2.3.1 Positive Control Experiment

Carbon tetrachloride (CCl₄) served as a positive control. It was diluted with corn oil to a 1:1 v/v mixture for intraperitoneal (ip) injection at a dose of 2 ml/kg body weight. Animals were terminated 24 h after the injection. In the control group, animals were injected
with 4 ml/kg body weight of corn oil ip (this was used accidentally as the dose for corn oil
instead of 2 ml/kg).

2.3.2 Effect of Fasting and APAP

Half of the animals were fasted overnight (from 6 pm to 8 am with food but not water
withheld), while the other half was provided free access to food. On the next day, each
animal in the fasted group was given either an ip injection of APAP at 1000 mg/kg body
weight or an equivalent volume (injection volume of 3 ml/kg) of 20% Tween 80 in 0.9%
NaCl, the vehicle used to suspend APAP (Gardner et al., 1998). In the non-fasted group,
each animal was given an ip injection of APAP at 1000 mg/kg body weight or the vehicle (at
the same injection volume of 3 ml/kg body weight). Food was returned to the fasted animals
after the injections. Animals were terminated the next day at 24 h (Price et al., 1987) after
injections of APAP or the vehicle.

2.3.3 Effect of 20% Tween 80 in 0.9% NaCl (the vehicle for APAP)

Animals were fasted overnight and injected ip with either 0.9% NaCl (at an injection
volume of 3 ml/kg body weight) or an equal volume (3 ml/kg body weight) of 20% Tween
80 in 0.9% NaCl (the vehicle used to suspend APAP). Food was returned to the cages and
the animals were terminated the next day at 24 h after the injections.

2.3.4 Effect of the Dose of APAP

In the dose response experiment, animals were fasted overnight and injected with an
ip dose of either 700, 1000, or 1300 mg/kg body weight of APAP suspended in the 20%
Tween 80 in 0.9% NaCl. In the control group, each animal was given an ip injection of the
20% Tween 80 in 0.9% NaCl (the vehicle used to suspend APAP). Animals continued to be
fasted after the injections. Animals were sacrificed 8 h after the dose of APAP or the vehicle. Animals in this experiment and subsequent experiments with APAP were sacrificed at 8 h instead of 24 h. This was because the fold-increases in plasma ALT levels at 1000 mg/kg ip of APAP (vs. respective control groups) at both 8 h and 24 h after dosing were similar, according to my preliminary APAP experiments (data not shown).

2.3.5 Effect of GBE

Animals were injected with either GBE at a dose of 500 mg/kg ip (dissolved in 0.9% NaCl) or an equivalent volume of 0.9% NaCl (injection volume of 2 ml/kg) once daily for 8 consecutive days. They were fasted overnight and terminated on Day 9 at about 24 hours after the last injections.

2.3.6 Effect of the Combination of GBE and APAP

The animals were separated into three groups. One group received ip injections of GBE at a dose of 500 mg/kg (dissolved in 0.9% NaCl) once daily for 8 consecutive days. The other two groups received ip injections of 0.9% NaCl (at the same injection volume of 2 ml/kg) once daily for 8 consecutive days. All animals were fasted overnight, and on the 9th day, each animal in the GBE-treated group received an additional dose of GBE at 500 mg/kg, while each animal in the other two groups received an additional dose of 0.9% NaCl. About 30 min after the last dose, each animal in the GBE-treated group and in one of the 0.9% NaCl-treated groups received an ip injection of APAP at the dose of 1000 mg/kg (injection volume of 4 ml/kg). The animals in the other 0.9% NaCl-treated group each received an equivalent volume (4 ml/kg ip injection) of the vehicle for APAP. Food was returned to the cages after the injections. Each animal was terminated about 8 hours after the last injection.
2.4 Termination of Animals, Blood Collection, and Preparation of Plasma

Animals were anesthetized by ip injection of sodium pentobarbital at 65 mg/kg body weight (Wawryko et al., 2004) at 8 or 24 h after the ip dose of APAP (or carbon tetrachloride in the case of the positive control experiment) was given the previous day. The injection volume for sodium pentobarbital was 1 ml/kg body weight. Blood was then collected from the inferior vena cava using heparinized 3 ml syringe (pre-filled with 300 units of heparin diluted with 0.9% NaCl). Each animal was terminated by decapitation after blood collection was completed. Our protocol used for termination of the animals was in accordance with the guidelines of the Canadian Council on Animal Care.

Immediately after blood was collected from each rat into a syringe, the needle was inserted into a BD vacutainer (PLUS-with K$_2$ EDTA 7.2 mg). After the blood was transferred to the vacutainer, the tube was put on ice immediately. All tubes of whole blood were centrifuged at 1,000×g for 10 min in a Beckman Model J-6B centrifuge with a JS 4.2 motor within 15 min of collection. Immediately after centrifugation, the supernatant (plasma) was transferred to a clean Eppendorf tube. All samples were put on ice before further analysis. Plasma samples were put into a −20 °C freezer when not used within the same day.

2.5 Alanine Aminotransferase (ALT) Assay

2.5.1 Principle of the Assay

The ALT assay is a modified method by Reitman and Frankel (1957). The ALT Reagent contains alanine and α-ketoglutarate. ALT catalyzes the conversion of alanine to glutamate and α-ketoglutarate to pyruvate.
Upon addition of 2,4-dinitrophenylhydrazine (Developer A), hydrazones of the pyruvate (2,4-dinitrophenylhydrazone of pyruvate) are formed, in the presence of NaOH (Developer B). These colored complexes formed from the reaction can be quantified by a spectrophotometer at the wavelength of 540 nm.

Figure 2.2. The second step in the ALT assay. Pyruvate formed from the first step reacts with 2,4-dinitrophenylhydrazine (Developer A) to produce the colored complex (2,4-dinitrophenylhydrazone of pyruvate) in the presence of NaOH. (Chemical structures shown here were obtained from: http://www.steve.gb.com/science/molecules.html)
2.5.2 Methodology

A 0.8 ml aliquot of ALT Reagent was pipetted into each empty glass tube. Exactly 5 min after each tube was put into a 37 °C water bath, 100 µl of each sample (standard or plasma) was pipetted into each tube. In the blank tubes, 100 µl of distilled water was added in place of the standard or plasma. Each tube was then incubated for exactly 30 min before the addition a 0.5 ml aliquot of Developer A. After being left at room temperature for 20 min, a 2.0 ml aliquot of Developer B was added into each tube. Following the addition of each reagent, each tube was vortexed for at least 5 sec. Absorbance measurement did not start for at least 5 min after the addition of Developer B for color development at room temperature. Measurement was completed within 30 min of the addition of Developer B. Absorbance values were measured at 540 nm with a glass cuvette in a Beckman DU®64 Spectrophotometer. Incubation of samples, addition of reagents, and measurement of absorbance values were carried out under subdued light due to the light sensitive nature of the ALT reagents. Net absorbance for each sample was obtained by subtracting the absorbance of the blank from the absorbance of the sample. The ALT level for each sample was then calculated using the equation of the standard curve generated by the linear regression method according to each corresponding net absorbance. Plasma samples with ALT levels greater than the upper limit of linearity of the standard curve were diluted with 0.9% NaCl to be in the dynamic range of the standard curve and were re-assayed on the same day. A standard curve was performed on each day of the ALT assays. Each unknown sample was done in duplicate measurement.
2.6 Validation of the ALT Assay

2.6.1 Lower Limit of Linearity

The standard (at an ALT level of 76 U/L) was diluted with distilled water to the desired ALT levels (i.e. 15, 20, 25, and 30 U/L). Six determinations at each ALT level were carried out using the ALT assay. The net absorbance at 540 nm of each ALT level was plotted against its respective ALT level (15, 20, 25, and 30 U/L). The lower limit of linearity was determined to be the lowest ALT level that would still fall within the linear region of the standard curve.

2.6.2 Limit of Quantitation

The same data set from the lower limit of linearity experiment was used for the determination of the limit of quantitation. Mean, standard deviation, and coefficient of variation (CV) were determined for each ALT level (15, 20, 25, and 30 U/L). The LOQ was set as the lowest concentration where the precision of the assay (CV) was still acceptable (less than 15%) (Shah et al., 1992).

2.6.3 Upper Limit of Linearity

The standard was diluted with distilled water to make 25, 30, 35, 40, 60 U/L. An appropriate volume of the standard was used to reach a final ALT level of 80, 100, 120, 130, and 140 U/L in each sample during the ALT assay. Four trials of ALT assays were carried out at each of the ALT levels. The mean net absorbance at 540 nm for each ALT level was plotted against its respective ALT level. Linear regression analysis was performed. The upper limit of linearity was the greatest ALT level included in the linear regression analysis that would produce a coefficient of determination as close to 1 as possible.
2.6.4 Dynamic Range

Dynamic range is the range between the lower and the upper limits of linearity in which the assay is linear and able to quantify the amount of an analyte reliably. A standard curve that contains that dynamic range was performed prior to analysis of unknown plasma samples for the ALT levels. The standard was diluted using distilled water to 20, 40, and 60 U/L, and an appropriate volume of the standard was used to reach 80 U/L. ALT assays were carried out in duplicate (two determinations for the absorbance at 540 nm) at each of these ALT levels. There were three determinations for the absorbance of the blank (distilled water instead of standard in each assay) at 540 nm.

2.6.5 Accuracy and Precision

To determine the accuracy (measured value reflects the true value in the sample) and the precision (results are reproducible), ALT assays in quadruplicate were carried out using the standard (at an ALT level of 76 U/L) and the diluted standard (at an ALT level of 30 U/L). Accuracy is presented as a percentage difference \[\frac{(\text{measured value} - \text{expected value})}{\text{expected value}} \times 100\%\] compared to the expected level. Precision is presented as CV at each ALT level. The two ALT levels were chosen to represent the lower and the higher end of the dynamic range of the ALT assay.

2.6.6 Intra-day and Inter-day Variabilities

Three female Long-Evans rats (weighed 300 – 350 g) were terminated and plasma was prepared as described above. Rat plasma was pooled into one sample and was divided into 5 aliquots and stored at -20 °C prior to ALT assays. For intra-day variability, 6 determinations of ALT level for the pooled plasma sample and the standard curve were done
on the same day. For inter-day variability, an ALT assay was done each day using the pooled plasma sample (6 replicates) and standards (4 levels in duplicate as described above for construction of the standard curve) for 5 consecutive days.

2.6.7 Inter-laboratory Comparison of ALT Values

ALT levels of the rat plasma samples in the positive control experiment were obtained using the plasma ALT assay in our lab. An aliquot of each plasma sample was sent to the Central Lab for Veterinarians (Langley, B.C.) for ALT analysis. The ALT values obtained from our lab for each sample were then compared with those reported by the Central Lab for Veterinarians.

2.7 Histological Assessment of Liver Tissues

Liver tissue sections were fixed in 10% v/v formalin solution and stained with hematoxylin-eosin (HE). Histological examination of the liver tissues was performed by Wax-it Histology Services (Vancouver, B.C., Canada). The pathologist who analyzed the liver tissues was blinded to the identity of the samples. Assessments of severity of liver damage were presented in scores. To grade steatosis, liver tissues were scored as follows: 0, negative; 1 – 6, steatosis. To grade necrosis, liver tissues were scored as follows: 0, negative; 1, single cell necrosis, 2 – 5, coagulative necrosis. In terms of capsular inflammation and sinusoidal inflammation, scoring was as follows: 0, negative; MINIMUM, very scant amount and is less than mild; 1, mild; 2, moderate; 3, severe.

2.8 Data Analysis

All data were presented as mean ± standard error of the mean (SEM). Data were analyzed using the one-tailed, unpaired Student’s t-test (StudyResult 1.0), one-way ANOVA.
with Student Newman-Keuls multiple comparison test, or two-way ANOVA (SigmaPlot). Results were considered to be statistically significant when the p-value (p) was less than 0.05.
3. RESULTS

3.1 Validation of the ALT Assay

3.1.1 Lower Limit of Linearity

The lower limit of linearity was the lowest ALT level at which the standard curve was still linear. The standard was used to prepare the ALT levels to be tested (15, 20, 25, and 30 U/L). Figure 3.1 shows the net absorbance (at 540 nm) at each ALT level being tested. The standard curve was linear at an ALT level as low as 15 U/L. Therefore, the lower limit of linearity for the ALT assay was at least 15 U/L.

Figure 3.1. Determination of the lower limit of linearity for the ALT assay. The standard was used to prepare the ALT levels (15, 20, 25, and 30 U/L). The ALT assay was performed in six replicates at each ALT level. Mean net absorbance at 540 nm with SEM was plotted against each ALT level.
3.1.2 Limit of Quantitation

The limit of quantitation (LOQ) was set to be at the lowest analyte concentration or level where the coefficient of variation (CV), or precision of the assay, was still less than 15% (Shah et al., 1992). For the ALT assay, the LOQ was the lowest ALT level at which the assay still had acceptable accuracy and precision. Several ALT levels prepared using the standard were used to determine the LOQ. The coefficients of variation for the net absorbance at 540 nm for 15, 20, 25, and 30 U/L were 23%, 5%, 4%, and 5%, respectively. The LOQ was determined to be at least 20 U/L as the CV increased dramatically from 5% to 23% when the ALT level decreased from 20 U/L to 15 U/L. A coefficient of variation of more than 15% was considered to be unacceptable at the LOQ for an assay (Shah et al., 1992). Figure 3.2 shows the coefficient of variation at each ALT level.
Figure 3.2. Determination of the limit of quantitation of the ALT assay. The standard was diluted with distilled water to ALT levels of 15, 20, 25, and 30 U/L. ALT assay was done in six replicates at each ALT level. This was the same data set that was used in the determination of the lower limit of linearity. Net absorbance at 540 nm was calculated using the absorbance of the blank. Mean, standard deviation, and CV of the net absorbance at each level were calculated. CV was plotted for each ALT level.
3.1.3 Upper Limit of Linearity

The upper limit of linearity was the highest ALT level at which the standard curve deviates from linearity. For each ALT assay trial, the standard was used to prepare the ALT levels to be tested (25, 30, 35, 40, 60, 80, 100, 120, 130, and 140 U/L). Four trials were performed to determine the upper limit of linearity for the ALT assay. The net absorbance at 540 nm for each level was determined. The mean and SEM for each level were calculated and plotted in Figure 3.3. The curve started to deviate from linearity at 100 U/L. Therefore, 80 U/L was determined to be the upper limit of linearity for the ALT assay.

Figure 3.3. Determination of the upper limit of linearity for the ALT assay. The standard was used to prepare the following ALT levels: 25, 30, 35, 40, 60, 80, 100, 120, 130, and 140 U/L. Four trials were performed using these levels. Net absorbance at 540 nm was calculated, and the mean net absorbance with SEM was plotted for each level.
3.1.4 A Representative Standard Curve

Based on the information from the limit of quantitation (LOQ), and the lower and the upper limits of linearity for the assay, the dynamic range was determined to be from 20 to 80 U/L. Shown below is a representative standard curve for the ALT assay.

Figure 3.4. A representative standard curve for the ALT assay. The net absorbance was measured in duplicate at 540 nm for four ALT levels (20, 40, 60, and 80 U/L). Each point represents the mean net absorbance at 540 nm at that ALT level.
3.1.5 Accuracy and Precision

The mean ± SD of plasma ALT level for the quality control sample (ALT = 76 U/L) was 80 ± 3 U/L, with a CV of 5% (Table 3.1). The mean plasma ALT level was 5% greater than the expected ALT level of the quality control sample.

The quality control sample was diluted to an ALT level of 30 U/L. The mean plasma ALT level was 29 ± 6 U/L, with a CV of 20%. The mean plasma ALT level was 3% less than the expected level of 30 U/L.

Table 3.1. Accuracy and precision of the ALT assay. The assay was performed in quadruplicate at ALT levels of 30 and 76 U/L. Accuracy was measured as the percentage difference between the measured and the expected ALT level. Precision is presented as CV.

<table>
<thead>
<tr>
<th>Expected ALT Level (U/L)</th>
<th>Measured ALT Level (U/L)</th>
<th>Accuracy (% Difference)</th>
<th>Precision (CV in %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>34</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>29</td>
<td>-3%</td>
<td>20%</td>
</tr>
<tr>
<td>SD</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>76</td>
<td>80</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>78</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>76</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>80</td>
<td>+5%</td>
<td>5%</td>
</tr>
<tr>
<td>SD</td>
<td>3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.1.6 Intra-day and Inter-day Variabilities

Three female Long-Evans rats were sacrificed and a pooled plasma sample was collected for the intra-day and inter-day variability studies. The pooled plasma sample was divided into five aliquots. In the intra-day study, a standard curve was run together with six replicates of the plasma sample on the same day. The ALT level for each replicate was then calculated using the linear regression method. The mean ± SD for the ALT level of the pooled plasma sample on the same day was 68 ± 2 U/L. The coefficient of variation was 3% (Table 3.2).

In the inter-day study, a standard curve was run together with six replicates of the plasma sample once daily for five consecutive days. Table 3.3 shows the mean ALT level obtained on each day using the standard curve. The mean ± SD for the ALT level of the plasma sample was 67 ± 2 U/L. The coefficient of variation in the inter-day study was 2%.
Table 3.2. Intra-day variability of the ALT assay. The assay was performed in six replicates for a pooled plasma sample prepared from three female Long-Evans rats. This table shows the ALT level for each replicate, together with the mean, SD, and CV of the six replicates.

<table>
<thead>
<tr>
<th>Replicate No.</th>
<th>ALT level (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>70</td>
</tr>
<tr>
<td>2</td>
<td>65</td>
</tr>
<tr>
<td>3</td>
<td>69</td>
</tr>
<tr>
<td>4</td>
<td>67</td>
</tr>
<tr>
<td>5</td>
<td>71</td>
</tr>
<tr>
<td>6</td>
<td>67</td>
</tr>
<tr>
<td>Mean</td>
<td>68</td>
</tr>
<tr>
<td>SD</td>
<td>2</td>
</tr>
<tr>
<td>CV</td>
<td>3%</td>
</tr>
</tbody>
</table>
Table 3.3. Inter-day variability of the ALT assay. The assay was performed in six replicates using the pooled plasma sample for five consecutive days. This table shows the average ALT level obtained from each day, together with the mean, SD, and CV of the averages of the five days.

<table>
<thead>
<tr>
<th>Day</th>
<th>Average ALT level (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>68</td>
</tr>
<tr>
<td>2</td>
<td>65</td>
</tr>
<tr>
<td>3</td>
<td>66</td>
</tr>
<tr>
<td>4</td>
<td>66</td>
</tr>
<tr>
<td>5</td>
<td>69</td>
</tr>
<tr>
<td>Mean</td>
<td>67</td>
</tr>
<tr>
<td>SD</td>
<td>2</td>
</tr>
<tr>
<td>CV</td>
<td>2%</td>
</tr>
</tbody>
</table>
3.1.7 Positive Control Experiment

A positive control experiment using carbon tetrachloride (CCl₄) was used to ensure the ALT assay could detect increased ALT levels in the samples. CCl₄ was diluted with corn oil to a 1:1 v/v mixture for ip injection to four male Long-Evans rats at a dose of 2 ml/kg body weight. In the control group, 4 ml/kg of corn oil was injected to another four male Long-Evans rats. All eight animals were terminated 24 h after dosing. Figure 3.5 shows the mean ALT level of the plasma obtained from each treatment group. The mean ± SEM for the CCl₄-treated group was 311 ± 127 U/L, with a CV of 81%. The mean ± SEM for the control group was 42 ± 2 U/L, with a CV of 9%. The mean ALT value for the CCl₄-treated group was significantly greater than that for the control group (p = 0.039, unpaired one-tailed Student’s t-test). The mean ALT level of the plasma in the CCl₄-treated group was 7.5 times greater than that in the control group.
Figure 3.5. Effect of CCl₄ on plasma ALT levels in rats. Four rats were treated with 2 ml/kg CCl₄ ip and were terminated 24 h after dosing. The four rats in the control group were treated with 4 ml/kg of corn oil. Plasma from all rats was prepared and plasma ALT assay was performed using each plasma sample. This figure shows the mean and SEM of ALT level for each treatment group.

* Significantly different from corn oil-treated group (p = 0.039)
3.1.8 Inter-laboratory Comparison of ALT Values

ALT levels of the samples in the carbon tetrachloride experiment obtained through the ALT assay were compared with those obtained from the Central Lab for Veterinarians. The mean ± SEM for the CCl₄-treated group was 313 ± 118 U/L with a CV of 75%, which was not significantly different from the value obtained by us (311 ± 127 U/L, p = 0.99, unpaired two-tailed Student's t-test). The mean ± SEM for the control group was 56 ± 2 U/L, with a CV of 7%, which was significantly different from the value obtained by us (42 ± 2 U/L, p = 0.001, unpaired two-tailed Student's t-test). The mean ALT level in the CCl₄-treated group was significantly greater than that in the control group (p = 0.036, unpaired one-tailed Student's t-test). These ALT values obtained from the Central Lab for Veterinarians were compared with those obtained from the ALT assay performed in our lab. The ALT levels determined in each laboratory are listed in Table 3.4. The mean plasma ALT level in the CCl₄-treated group was 5.5 times greater than that in the control group, according to the independent laboratory assessment by the Central Lab for Veterinarians, whereas this fold-increase was 7.5 times according to the ALT assay performed in our lab. Table 3.5 shows the percentage differences in the ALT levels obtained using the ALT assay in our lab as compared to those obtained from the Central Lab for Veterinarians. On average, the percentage differences in the ALT levels in the CCl₄-treated group was −6%, whereas the percentage difference of the ALT levels in the control group was −25%.
Table 3.4. Inter-laboratory comparison of ALT values. Plasma samples were prepared from rats treated with CCl₄ and corn oil. The plasma ALT values obtained from our lab were compared with those from the Central Lab for Veterinarians. The mean, SEM, and CV for each group are shown in this table.

<table>
<thead>
<tr>
<th>Treatment / Animal No.</th>
<th>ALT Level (U/L)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Our Lab</td>
<td>Central Lab for Veterinarians</td>
<td></td>
</tr>
<tr>
<td>CCl₄:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>76</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>565</td>
<td>557</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>111</td>
<td>131</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>492</td>
<td>473</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>311*</td>
<td>313*</td>
<td></td>
</tr>
<tr>
<td>SEM</td>
<td>127</td>
<td>118</td>
<td></td>
</tr>
<tr>
<td>CV</td>
<td>81%</td>
<td>75%</td>
<td></td>
</tr>
<tr>
<td>Corn oil:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>43</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>38</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>46</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>40</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>42 #</td>
<td>56 #</td>
<td></td>
</tr>
<tr>
<td>SEM</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>CV</td>
<td>9%</td>
<td>7%</td>
<td></td>
</tr>
</tbody>
</table>

Fold-Increase in ALT Level with CCl₄ treatment

- *Significantly different from the corn oil-treated group (p < 0.05)
- # Significantly different from each other (p = 0.001)
Table 3.5. Comparison of ALT levels obtained from the ALT assay performed in our lab and those obtained from the Central Lab for Veterinarians. Percentage difference was calculated as (value obtained from our lab – value obtained from Central Lab for Veterinarians)/ value obtained from Central Lab for Veterinarians × 100%.

<table>
<thead>
<tr>
<th>Treatment / Animal No.</th>
<th>% Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCl₄:</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-19%</td>
</tr>
<tr>
<td>2</td>
<td>+2%</td>
</tr>
<tr>
<td>3</td>
<td>-15%</td>
</tr>
<tr>
<td>4</td>
<td>+4%</td>
</tr>
<tr>
<td>Mean % difference</td>
<td>-6%</td>
</tr>
<tr>
<td>Corn oil:</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>-27%</td>
</tr>
<tr>
<td>6</td>
<td>-26%</td>
</tr>
<tr>
<td>7</td>
<td>-20%</td>
</tr>
<tr>
<td>8</td>
<td>-33%</td>
</tr>
<tr>
<td>Mean % difference</td>
<td>-25%</td>
</tr>
</tbody>
</table>
3.2 Control Experiments with APAP

3.2.1 Effect of Fasting on Plasma ALT Levels in Rats Administered APAP

The purpose of this experiment was to determine the effect of fasting on plasma ALT levels in rats injected with APAP. Seventeen adult male rats were divided into four groups (four rats in the non-fasted/vehicle group, four rats in the non-fasted/APAP group, four rats in the fasted/vehicle group, and five rats in the fasted/APAP group). Rats were either fasted or not fasted overnight and received single injection of either 20% Tween 80 in 0.9% NaCl (the vehicle used to suspend APAP) or APAP at 1000 mg/kg the next morning, followed by termination at 24 h after the last dose. The mean ± SEM of the plasma ALT level in the non-fasted/vehicle group was 38 ± 4 U/L, which was not significantly different from that in the non-fasted/APAP group (41 ± 4 U/L) (Figure 3.6). In the fasted groups, however, the mean plasma ALT level in the fasted/APAP group was 245 ± 106 U/L, which was 4.5 times greater than that in the fasted/vehicle group (55 ± 1 U/L), in which the animals were also fasted but were injected with the vehicle instead of APAP. The CV in the fasted group treated with APAP was very large (97%) compared to the rest of the groups (ranged from 2% to 21%). There was not a statistical significant interaction between fasting and treatment (p = 0.167, two-way ANOVA).
Figure 3.6. Effect of fasting on plasma ALT levels in rats treated with APAP or the vehicle. Animals were fasted overnight (F) or had free access to food (NF). They were then injected with either APAP at 1000 mg/kg ip or 20% Tween 80 in 0.9% NaCl (vehicle) the next day. All animals were terminated 24 after the last injection. This figure shows mean and SEM of plasma ALT level for each group (N = 4 in the NF/Vehicle group; N = 4 in the NF/APAP group; N = 4 in the F/Vehicle group; N = 5 in the F/APAP group).
3.2.2 Effect of the Vehicle on Plasma ALT Levels in Rats

The purpose of this experiment was to investigate if the vehicle used to suspend APAP (20% Tween 80 in 0.9% NaCl) would affect the plasma ALT levels in rats. Seven adult male rats were assigned into two groups (four rats in the 0.9% saline-treated group and three rats in the vehicle-treated group). Rats in both groups were fasted overnight before each was injected with either 0.9% NaCl (saline group) or 20% Tween 80 in 0.9% NaCl (vehicle group). They were terminated at 24 h after the last dose. The mean ± SEM of the plasma ALT level in the rats treated with the vehicle was 63 ± 10 U/L (CV = 27%), which was not significantly different (p = 0.73, unpaired two-tailed Student’s t-test) from the mean plasma ALT level in the rats treated with 0.9% NaCl (66 ± 3 U/L, with CV = 10%) (Table 3.6).
Table 3.6. Effect of the vehicle on plasma ALT levels in rats. This table shows individual plasma ALT level for each animal, together with the mean, SEM, and CV for each group.

<table>
<thead>
<tr>
<th>Treatment / Animal No.</th>
<th>ALT Level (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% NaCl:</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>75</td>
</tr>
<tr>
<td>2</td>
<td>66</td>
</tr>
<tr>
<td>3</td>
<td>63</td>
</tr>
<tr>
<td>4</td>
<td>61</td>
</tr>
<tr>
<td>Mean</td>
<td>66</td>
</tr>
<tr>
<td>SEM</td>
<td>3</td>
</tr>
<tr>
<td>CV</td>
<td>10%</td>
</tr>
<tr>
<td>20% Tween 80 in 0.9% NaCl (vehicle):</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>81</td>
</tr>
<tr>
<td>6</td>
<td>47</td>
</tr>
<tr>
<td>7</td>
<td>61</td>
</tr>
<tr>
<td>Mean</td>
<td>63</td>
</tr>
<tr>
<td>SEM</td>
<td>10</td>
</tr>
<tr>
<td>CV</td>
<td>27%</td>
</tr>
</tbody>
</table>
3.2.3 Effect of the Dose of APAP on Plasma ALT Levels in Rats

To study the effect of the dose of APAP on the plasma ALT levels in rats, the following experiment was performed. Twenty adult male rats were assigned into four groups (five rats in the control group, five rats in the 700 mg/kg group, four rats in the 1000 mg/kg group, and six rats in the 1300 mg/kg group). All rats were fasted overnight and rats in the different treatment groups were injected ip with various doses (700, 1000, and 1300 mg/kg body weight) of APAP. In the control group, animals were injected with the vehicle. All rats were terminated at 8 h after the last injection. The mean ± SEM of the plasma ALT level was 30 ± 3 U/L (CV = 22%) in the control group. In the treatment groups, the mean plasma ALT level was 65 ± 11 U/L (CV = 37%), 137 ± 34 U/L (CV = 50%), and 136 ± 55 U/L (CV = 99%), in the 700 mg/kg, 1000 mg/kg, and 1300 mg/kg groups, respectively (Figure 3.7). The mean plasma ALT level in the 1300 mg/kg group was 4.5 times greater than the mean plasma ALT level in the control group (30 U/L). In the 700 mg/kg and the 1000 mg/kg groups, the mean plasma ALT level was twice and 4.6 times greater than the control group, respectively. The mean plasma ALT level of the 1000 mg/kg group was similar to that of the 1300 mg/kg group. However, the differences in the mean plasma ALT levels among the four groups were not statistically significant (p = 0.14, one-way ANOVA).
Figure 3.7. Effect of the dose of APAP on plasma ALT levels in rats. Animals were fasted overnight and injected ip with the vehicle (for the control group, N = 5) or different doses of APAP (700 mg/kg for five rats, 1000 mg/kg for four rats, or 1300 mg/kg for six rats) the next day. Fasting continued and they were terminated 8 h after the last injection. The mean ± SEM of the plasma ALT level for the treatment group is shown in this figure. The mean ± SEM of the plasma ALT value for the vehicle-treated control group was 30 ± 3 U/L.
3.3 Experiments with GBE

3.3.1 Effect of GBE on Plasma ALT Levels in Rats

To determine whether GBE has any effect on the plasma ALT levels in rats, the following study was performed. Five adult male rats were injected with 500 mg/kg of GBE ip (treatment group), whereas another five adult male rats were injected with 0.9% NaCl (control group) once daily for 8 consecutive days. Animals were sacrificed 24 h from the last injection after an overnight fast. The mean ± SEM of the plasma ALT level for the treatment group was 50 ± 3 U/L (CV = 14%), which was not significantly different from the mean plasma ALT level for the control group (54 ± 6 U/L, CV = 25%) (p = 0.59, unpaired two-tailed Student’s t-test) (Figure 3.8).
Figure 3.8. Effect of GBE on plasma ALT levels in rats. Animals were injected ip with either 0.9% NaCl (N = 5) or GBE (N = 5) at 500 mg/kg once daily for 8 days. Animals were terminated 24 h after the last dose. Plasma from each animal was prepared and the ALT level was determined for each plasma sample. The means and SEM for the plasma ALT levels of both groups were plotted in this figure.
3.3.2 Effect of GBE on Liver Histology in Rats

In addition to plasma samples prepared from the rats as described in Section 3.3.1, hepatic tissues were also excised from these rats and stored in 10% formalin immediately after termination. Histological analysis was performed on each piece of tissue and a score was assigned to each animal’s liver in terms of steatosis, necrosis, capsular inflammation, and sinusoidal dilatation around the central vein (Table 3.7). GBE did not cause severe steatosis or necrosis to hepatic tissues as compared to the vehicle (with the exception of Animal D7, in which severe necrosis and moderate steatosis and capsule inflammation were reported). Severe capsular inflammation was reported for Animal D4.

Table 3.7. Effect of GBE on liver histology scores in rats. Rats were treated as described in the legend to Figure 3.8. Liver tissues from each animal were assessed and a score was assigned for each animal’s liver histology in terms of steatosis, necrosis, capsular inflammation, and sinusoidal dilatation.

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Treatment</th>
<th>Steatosis</th>
<th>Necrosis</th>
<th>Capsular Inflammation</th>
<th>Sinusoidal dilatation (Hepatocyte collapse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D4</td>
<td></td>
<td>0-1</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>D5</td>
<td></td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>D6</td>
<td>GBE</td>
<td>0</td>
<td>1</td>
<td>MINIMUM</td>
<td>MINIMUM</td>
</tr>
<tr>
<td>D7</td>
<td></td>
<td>1-2</td>
<td>3</td>
<td>2</td>
<td>MINIMUM</td>
</tr>
<tr>
<td>D8</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>F1</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>F2</td>
<td></td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0-1</td>
</tr>
<tr>
<td>F3</td>
<td>0.9% NaCl</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>F4</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>F5</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Steatosis: 0 = Negative, 1-6 = Steatosis
Necrosis: 0 = Negative, 1 = Single cell necrosis, 2-5 = coagulative necrosis
Capsular inflammation: 1 = Mild, 2 = Moderate, 3 = Severe
Sinusoidal dilatation around central vein: 1 = Mild, 2 = Moderate, 3 = Severe
MINIMUM = very scant amount and is less than mild

65
3.4 Experiments with GBE and APAP

3.4.1 Effect of Pretreatment with GBE on Plasma ALT Levels in Rats Administered APAP

The purpose of this study was to investigate whether GBE pretreatment could modulate APAP-induced hepatotoxicity in rats. Twenty-two adult male rats were divided into three groups – five in the 0.9% NaCl/20% Tween 80 (vehicle-treated group) group, eight in the 0.9% NaCl/APAP (APAP-treated) group, and nine in the combination group (GBE and APAP). During the pretreatment period, rats in the vehicle-treated control group and the APAP-treated group were injected with 0.9% NaCl ip, whereas the rats in the combination group were injected with GBE at 500 mg/kg ip once daily for 9 consecutive days. Thirty minutes after the last dose, the rats in the vehicle-treated control group were injected with the vehicle ip, whereas those in the APAP-treated group and the combination group were injected with 1000 mg/kg APAP ip. All rats were terminated 8 h after the last dose was given. As shown in Figure 3.9, the mean ± SEM of the plasma ALT level for the vehicle-treated control group, the APAP-treated group, and the combination group, was 41 ± 3 U/L (CV = 16%), 114 ± 22 U/L (CV = 55%), and 53 ± 7 U/L (CV = 37%), respectively. There was a significant difference in the mean plasma ALT levels among the three groups (p = 0.008, one-way ANOVA). Moreover, the mean plasma ALT level for the APAP-treated group was shown to be significantly greater than both the combination group (p = 0.008, Student Newman-Keuls (SNK) multiple comparison test) and the vehicle-treated control group (p = 0.016, SNK multiple comparison test). The mean plasma ALT levels for the combination group and the vehicle-treated control group were not significantly different from each other (p = 0.624, SNK multiple comparison test).
Figure 3.9. Effect of pretreatment with GBE on plasma ALT levels in rats administered APAP. Animals received either GBE (500 mg/kg) or 0.9% NaCl ip once daily for 9 consecutive days (an overnight fast before the 9th dose). Thirty minutes after the last dose, they were injected ip with either APAP (1000 mg/kg) or the vehicle. Animals were terminated 8 h thereafter. The mean and SEM of plasma ALT level for each group (vehicle, N = 5; APAP, N = 8; GBE/APAP, N = 8 because 1 rat died before plasma could be prepared) are shown in this figure.

* Significantly different from both the vehicle and the GBE/APAP groups (p < 0.05).
3.4.2 Effect of Pretreatment with GBE on Liver Histology in Rats Administered APAP

In addition to plasma samples prepared from the twenty-two rats as described in Section 3.4.1, hepatic tissues were also isolated from these rats and stored in 10% v/v formalin immediately after termination. Histological analysis was performed on each piece of tissue and a score was assigned to each animal's liver in terms of steatosis, necrosis, capsular inflammation, and sinusoidal dilatation around the central vein (Table 3.8). Hepatic tissues in all five animals in the vehicle-treated group did not show any steatosis, necrosis, capsular inflammation, or sinusoidal dilatation. The number of animals in each treatment group with a particular histology score for each category has been summarized in Figures 3.10, 3.11, 3.12, and 3.13. In terms of steatosis, two out of the eight rats in the APAP-treated group, while none of the rats in the combination group, scored 6 out of 6. However, three out of the nine rats in the combination group, while only one out of the eight rats in the APAP-treated group, scored 5 out of 6. In terms of hepatic necrosis, all eight rats in the APAP-treated group scored 2 (out of 5) or lower, whereas three out of the nine rats in the combination group scored 3 (out of 5) or higher. The slightly worse hepatic tissue necrosis reported from the combination group was in accordance with the higher capsular inflammation scores in the group. Two rats in the combination group showed severe (vs. none in the APAP-treated group) and five rats showed moderate (only one in the APAP-treated group) capsular inflammation. Also, more rats in the combination group (four out of the nine vs. two out of the eight in the APAP-treated group) showed moderate sinusoidal dilatation in the liver, although both groups had two rats that showed severe sinusoidal dilatation.
Table 3.8. Effect of GBE on liver histology scores in rats administered APAP. Rats were treated as described in the legend to Figure 3.9. All rats were terminated 8 h after last dose. Scores were assessed for each animal’s liver histology in terms of steatosis, necrosis, capsular inflammation, and sinusoidal dilatation.

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Treatment</th>
<th>Steatosis</th>
<th>Necrosis</th>
<th>Capsular Inflammation</th>
<th>Sinusoidal dilatation (Hepatocyte collapse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C2</td>
<td>0.9% NaCl/Vehicle</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C3</td>
<td>Vehicle</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C4</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C5</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B1</td>
<td></td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>B2</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>MINIMUM</td>
</tr>
<tr>
<td>B3</td>
<td></td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>B4</td>
<td>0.9% NaCl/APAP</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>MINIMUM</td>
</tr>
<tr>
<td>B5</td>
<td>APAP</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>B6</td>
<td></td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>B7</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E1</td>
<td></td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>A1</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A2</td>
<td></td>
<td>0</td>
<td>5</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>A3</td>
<td></td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>A4</td>
<td>GBE/APAP</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>A6</td>
<td>APAP</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>A7</td>
<td></td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>D1</td>
<td></td>
<td>5</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>D2</td>
<td></td>
<td>5</td>
<td>1</td>
<td>MINIMUM</td>
<td>3</td>
</tr>
<tr>
<td>D3</td>
<td></td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

Steatosis: 0 = Negative, 1-6 = Steatosis
Necrosis: 0 = Negative, 1 = Single cell necrosis, 2-5 = Coagulative necrosis
Capsular inflammation: 1 = Mild, 2 = Moderate, 3 = Severe
Sinusoidal dilatation around central vein: 1 = Mild, 2 = Moderate, 3 = Severe
MINIMUM = very scant amount and is less than mild
Figure 3.10. Summary of scores for steatosis in rat liver tissues. Rats were treated as described in the legend to Figure 3.9. The percentage of rats in the two key treatment groups (0.9% NaCl/APAP and GBE/APAP) having a specific score is shown in this figure (0 = Negative, 1-6 = Steatosis).
Figure 3.11. Summary of scores for necrosis in rat liver tissues. Rats were treated as described in the legend to Figure 3.9. The percentage of rats in each treatment group having a specific score is shown in this figure (0 = Negative, 1 = Single cell necrosis, 2-5 = Coagulative necrosis).
Figure 3.12. Summary of scores for capsular inflammation in rat liver tissues. Rats were treated as described in the legend to Figure 3.9. The percentage of rats in each treatment group having a specific score is shown in this figure (1 = Mild, 2 = Moderate, 3 = Severe, MINIMUM (MIN) = very scant amount and is less than mild).
Figure 3.13. Summary of scores for sinusoidal dilatation in rat liver tissues. Rats were treated as described in the legend to Figure 3.9. The percentage of rats in each treatment group having a specific score is shown in this figure (1 = Mild, 2 = Moderate, 3 = Severe, MINIMUM (MIN) = very scant amount and is less than mild).
4. DISCUSSION

4.1 Validation of the ALT Assay

Assay validation is essential to ensure the accuracy and precision of an assay. ALT is an enzyme that is produced in hepatocytes. The hepatotoxic metabolite of APAP, NAPQI, can cause hepatocellular death and centrilobular liver necrosis, which can lead to “leaky membranes” of hepatocytes, resulting in leakage of ALT from cytosol of hepatocytes into the circulation, and hence leading to elevation of ALT level in the plasma. Another marker of liver damage is aspartate aminotransferase (AST). However, AST is a less specific marker compared to ALT because AST exists in tissues other than liver, such as in heart and skeletal muscles (Mason, 2004). In this study, only the plasma ALT assay was used because of problems in setting-up the AST assay.

4.1.1 Accuracy

Accuracy is defined as the “closeness of agreement between the value that is accepted either as a conventional true value or an accepted reference value and the value found” (Lundblad, 2001). A sample (in which nominal level was known) from the plasma ALT kit was used for determination of accuracy of the ALT assay. The percentage differences between the measured ALT level and the true ALT level in the quality control sample were –3% and +5% at 30 U/L and 76 U/L, respectively (Table 3.1), which were within the acceptable level of 15% (Shah et al., 1992). Therefore, the assay was considered to be accurate.
4.1.2 Precision

Precision is "the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under prescribed conditions" (Lundblad, 2001). At 76 U/L, the CV was less than 15% (Table 3.1), and hence was considered to be acceptable (Shah et al., 1992). However, the CV at 30 U/L was 20% (more than 15%). The assay was precise at the greater ALT level of 76 U/L. At 30 U/L, the assay was not as precise. The greater variability at the lower ALT level could be explained by how the CV (SD/Mean x 100%) is calculated. A smaller mean value would result in a greater CV.

4.1.3 Reproducibility

To know whether the assay would be reproducible on the same day and from a day-to-day basis, intra-day and inter-day variabilities of the plasma ALT assay were investigated. The results of the study were reported in Section 3.1.6. As shown in Table 3.2 and Table 3.3, the relatively small intra-day and inter-day CVs (2% and 3%, respectively) were considered to be acceptable as they were both less than 15% (Shah et al., 1992).

To investigate whether the plasma ALT assay could measure an elevated ALT level in rats with liver damage, a positive control experiment was performed using a known hepatotoxin, CCl4. The statistical significant elevation in plasma ALT level in the CCl4-treated group (as shown in Figure 3.5) revealed that the plasma ALT assay used in this study was able to quantify elevated plasma ALT levels as caused by liver damage. When compared to literature values, Janakat and Al-Merie (2002) also reported a statistical increase in plasma ALT levels in male albino Wistar rats at 24 h after CCl4 administration (2 ml/kg ip in olive oil). This was the same treatment regimen used in the present study. The mean ±
SEM of plasma ALT levels was 686 ± 9 U/L at 24 h after the dose, which was a 7-fold increase compared to its control group (104 ± 4 U/L). Although the mean plasma ALT level in the CCl₄-treated group as quantified by our assay method (311 U/L) did not agree with the value (686 ± 9 U/L) reported from Janakat and Al-Merie (2002), the fold-increases compared to the control group were similar (7.5-fold increase from our assay vs. 7-fold increase from Janakat and Al-Merie, 2002).

As part of validation, it was necessary to ensure the plasma ALT levels obtained from our plasma ALT assay were comparable to those obtained from an independent laboratory. In fact, inter-laboratory comparison of the plasma ALT values obtained from the carbon tetrachloride study showed that our plasma ALT assay was able to quantify similar plasma ALT levels as another laboratory (Table 3.4, Table 3.5). In both the CCl₄-treated and the control group, both our mean plasma ALT levels and the variabilities (CV) were similar to those from the other laboratory. The fold-increase in plasma ALT level in the CCl₄-treated group when compared to the control group was 7.5, according to our plasma ALT assay. This fold-increase was 5.5, as reported from another laboratory. This difference in fold-increase could be explained by the greater plasma ALT level in the control group as reported by another laboratory (56 ± 2 U/L was significantly greater than 42 ± 2 U/L, p = 0.001, unpaired two-tailed Student's t-test). Although the fold-increase obtained from our plasma ALT assay data was different from that reported from another laboratory, both laboratories were able to quantify a statistical significant increase in mean plasma ALT level in the CCl₄-treated group when compared to the control group.
4.1.4 Dynamic Range

A standard curve was necessary for quantification of ALT level in unknown plasma samples. Considering the lower and upper limit of linearity, together with the LOQ, the standard curve was constructed using ALT levels between 20 U/L to 80 U/L (Figure 3.4). The plasma ALT assay used in the current study has a narrow dynamic range. Most of the samples with minimally elevated plasma ALT levels were beyond the upper limit of the dynamic range. This is a major limitation of the assay, as plasma samples suspected to have elevated ALT levels would have to be diluted. The dilution had to be done so that the diluted plasma sample would have an ALT level within the dynamic range.

4.1.5 Sensitivity

Sensitivity is defined as “the slope of the calibration curve” and is commonly misinterpreted as limit of detection (Karnes et al., 1991). An assay is sensitive “if small changes in concentration cause larger changes in analytical response” (Karnes et al., 1991). In the current study, the standard curve has a slope of 0.002 absorbance unit per unit of ALT per liter of plasma. Therefore, it is not a very sensitive assay for quantification of ALT levels, as it makes differentiation between samples with control levels very difficult.

4.1.6 Summary

The plasma ALT assay used in this study was considered to be accurate, precise, and reproducible, although it had a limited dynamic range and was not sensitive.
4.2 Control Experiments with APAP

4.2.1 Effect of Fasting on Plasma ALT Levels in Rats Administered APAP

Fasting was necessary to achieve a greater plasma ALT level in the APAP-treated group compared to the control group. As described in Section 3.2.1 and as illustrated in Figure 3.6, in the two non-fasted groups of rats, the mean plasma ALT levels in the group of rats given APAP was not significantly different from those in rats that were given the vehicle used to suspend APAP (20% Tween 80 in 0.9% NaCl). However, upon an overnight fast, the mean plasma ALT levels in rats dosed with APAP became greater than those in non-fasted rats dosed with APAP. Fasting *per se* did not cause an elevation in plasma ALT levels because the mean plasma ALT levels were not elevated in fasted rats dosed with the vehicle when compared to fasted rats dosed with 0.9% NaCl. Although the mean ALT plasma levels among the four groups in that study were not statistically different from each other, the results of this control experiment provided us with the insight that fasting the rats before dosing with APAP was necessary to obtain an increase in plasma ALT levels. The lack of statistical significance in the results can be explained by the insufficient power of the study. The number of rats used in the study should be increased to counter the highly variable plasma ALT levels observed in the fasted rats treated with APAP. However, the experiment was not repeated for ethnical reasons. This was only a control experiment to verify the need for fasting in subsequent experiments. The results of this experiment clearly suggested the need for fasting to have elevated plasma ALT levels in rats injected with APAP.

The finding of this experiment verified the results of many rodent studies that showed fasting was required to increase the susceptibility of the animals to APAP-induced
hepatotoxicity. In some studies using Sprague-Dawley and albino Wistar rats, an overnight fast was required before ip injection (750, 800, or 1000 mg/kg) or oral administration (2000 mg/kg) of APAP (Dehpour et al., 1999; Kukongviriyapan et al., 2003; Bauer et al., 2000; Brennan et al., 1994; Kucukardali et al., 2002). In one of the studies using male Long-Evans rats, animals were fasted 20 h before and for 2 h after ip injection with APAP at 650 mg/kg (Lim et al., 1995). Investigators fasted mice from 16 to 24 h prior to oral (300 or 800 mg/kg) or ip (300 or 500 mg/kg) injection of APAP in some studies (Thomsen et al., 1995; Gamal el-din et al., 2003; Manautou et al., 1996; Hewawasam et al., 2003).

There were a few studies that did not require fasting (or fasting was not mentioned) but the investigators were able to report a statistical significant elevation in plasma ALT levels in rats after treatment of APAP. For instance, Shenoy et al. (2002) reported an 8-fold increase compared to control in plasma ALT levels in adult male albino Wistar rats after receiving APAP at 2000 mg/kg body weight orally once daily for 3 days. Gilani and Janbaz (1995) reported a 19-fold increase in plasma ALT levels in adult male albino Wistar rats at 24 h after a single oral dose of APAP at 640 mg/kg body weight. Ahmed and Khater (2001) reported a 6-fold increase in plasma ALT levels in adult male Sprague-Dawley rats at 24 h after a single oral dose of APAP at 640 mg/kg body weight. Porcherzhian and Ansari (2005) reported a similar 6-fold increase at 48 h after an oral dose of APAP at 3000 mg/kg body weight.

The mechanism through which fasting potentiates APAP-induced hepatotoxicity were investigated by Price et al. (1987). An overnight fast was initiated in Long-Evans Hooded rats 24 h prior to ip administration of various doses of APAP from 150 mg/kg to 1000 mg/kg. As compared to fed rats, significantly greater plasma glutamic-oxaloacetic transaminase
(former name for AST) levels were reported in fasted rats 24 h after APAP doses of 500 mg/kg or greater. Also, both the incidence and severity of liver necrosis (at 48 h after APAP) were increased in rats received APAP doses of 300 mg/kg or greater. Hepatic glutathione and hepatic glycogen levels were significantly lower in fasted rats at 0, 2, 4, and 6 h after ip administration of APAP at a dosage of 700 mg/kg. In addition, the elimination half-life of APAP was reported to be significantly longer (1.98 h) in fasted rats than in fed rats (1.52 h). Hence, it was suggested that acute fasting increased susceptibility of rats to APAP-induced hepatotoxicity by reducing hepatic glutathione levels and hepatic glycogen levels, leading to impairment of both the detoxification pathway of NAPQI and glucuronidation of APAP elimination, respectively, in fasted rats (Price et al., 1987). Reduced levels of hepatic glutathione in fasted rats could potentially lead to accumulation of the hepatotoxic metabolite NAPQI as binding of glutathione to NAPQI is essential for its elimination. A reduced level of hepatic glycogen could lead to a lower supply of glucose for synthesis of UDP-glucuronic acid, which is a co-substrate for UGT. Hence, a reduced hepatic glycogen level upon fasting could potentially lead to a lower rate of glucuronidation, a lower rate of elimination of APAP, and ultimately an accumulation of NAPQI (Price et al., 1987).

The current study used a fasted animal model to conduct the drug-induced hepatotoxicity experiment. Hence, it would be important to address the clinical relevance of using a fasted model. Clinical data also suggested increased risk of APAP-induced hepatotoxicity in patients who had been fasted (Whitcomb and Block, 1994). An anorexic patient had severe liver failure after ingestion of APAP, although the effect could have been due to the concurrent administration of carbamazepine (Young and Mazure, 1998). Also, when a patient is sick (e.g. fever), appetite will likely decrease.
In summary, many studies revealed that overnight fasting prior to APAP dosing was necessary to increase the susceptibility of rats and mice to APAP-induced hepatotoxicity. This is in agreement with the results found in the current study.

4.2.2 Effect of the Vehicle on Plasma ALT Levels in Rats

APAP has low water solubility. In the process of identifying a suitable suspending vehicle for APAP, it was essential to ensure the vehicle itself would not cause liver toxicity. Hence, it was necessary to investigate the effect of the vehicle used in this study (20% Tween 80 in 0.9% NaCl, Gardener et al., 1998) on plasma ALT levels in rats. As discussed in Section 3.2.2, 24 h after the injection, the mean plasma ALT levels in rats injected with the vehicle was not significantly different from those in rats injected with 0.9% NaCl. Therefore, it was concluded that 20% Tween 80 in 0.9% NaCl was a suitable vehicle to suspend APAP because it did not cause an increase in plasma ALT levels.

The majority of studies published in the literature involved using a vehicle for APAP for oral administration or ip injection. Many different kinds of vehicle such as 0.2% tragacanth gum (Jorgensen et al., 1988; Poulsen et al., 1985), 20% Tween 80 in 0.9% NaCl (Gardner et al., 1998; Kamanaka et al., 2003), 1% methylcellulose (Janbaz and Gilani, 2000; Ahmed and Khater, 2001), 50% propylene glycol (Devi et al., 2004), and dimethyl sulfoxide (Nakae et al., 1988), have been used in published studies involving APAP. The vehicle used to suspend APAP used in our study was 20% Tween 80 in 0.9% NaCl with reference to the studies performed by Gardner et al. (1998) and Kamanaka et al. (2003) who also used the same APAP dose (1000 mg/kg ip) as in the current study. In the current study, 20% Tween 80 in 0.9% NaCl did not cause an elevation in plasma ALT levels.
4.2.3 Effect of the Dose of APAP on Plasma ALT Levels in Rats

The similar plasma ALT levels for both the 1000 mg/kg and the 1300 mg/kg groups indicated that the dose-response curve (Figure 3.7) had reached a plateau at the dose of 1000 mg/kg. The variability (CV) of the mean plasma ALT levels in the current study increased with the dose of APAP used. The CV in mean plasma ALT level gradually increased from 22% in the control group, to 37%, 50%, and 99%, in the 700 mg/kg, 1000 mg/kg, and 1300 mg/kg, respectively.

There are no published data available that involve plasma ALT measurements in fasted male Long-Evans rats at 8 h after an APAP dose of 1000 mg/kg ip. However, there was one study in which fasted female Sprague-Dawley rats were used (Bauer et al., 2000). The mean plasma ALT levels at 8 h after an APAP dose of 1000 mg/kg ip was estimated (data were shown in a figure) to be at 165 U/L, which was about 5.5-fold greater than control level (estimated to be at 30 U/L). The fold-increase at this particular ip dose of APAP and time-point was comparable to ours (4.6-fold, see Section 4.2.3), considering that a different strain of rats (Sprague-Dawley) and a different vehicle for APAP (37 °C warm 0.9% NaCl with pH adjusted to 10 with 0.1 N NaOH) were used in the study by Bauer et al. (2000).

In this study, the CVs ranged from 22% to 99% among the four groups. The large inter-animal variability in plasma ALT levels was not caused by technical problems in the ALT assay. Intra-day and inter-day variability data (CV) from the validation study ranged from 2% to 3% and hence provided direct evidence to support the reproducibility of the ALT assay. Also, CV was large (81%) for the CCl₄-treated group in the carbon tetrachloride study. The CV was similarly large (75%) for the CCl₄-treated group as determined by another laboratory in the inter-laboratory comparison of the data. The large variability
observed in the APAP-treated rats could be due to biological variability in the susceptibility of rats to APAP-induced hepatotoxicity. Different rats have different basal glutathione levels and glutathione level has to reach below a certain threshold before toxicity is observed in the animals. Hence, the large inter-animal variability can be potentially explained by the differences in susceptibility of each rat to APAP-induced hepatotoxicity.

There are no published data on the variability of plasma ALT levels in Long-Evans rats at 8 h after being injected with an APAP dose of 1000 mg/kg ip. However, plasma AST data are available from one study and it was reported that mean ± SEM of the plasma AST level in male Long-Evans rats at 24 h after APAP dose at 1000 mg/kg was 3858 ± 590 U/L (Price et al., 1987). This represents a CV of 48%, which was comparable to both the CV for the 1000 mg/kg group in the dose-response study (50%, see Section 3.2.3) and that in the APAP group in the combination study (55%, see Section 3.4.1), although that the plasma ALT levels were measured at 8 h after the APAP dose.

Although the differences in mean plasma ALT levels among the four groups were not statistically significant, the results of this study confirmed the use of 1000 mg/kg as the ip dose in elevating plasma ALT levels by APAP. The lack of statistical significance in the study was caused by lack of power in the study. Increasing the number of subjects used in each group could potentially reduce the variability and hence increased the likelihood of a statistical significant result. Again, more animals were not used due to ethical reasons and the fact that this was only one of the control experiments to verify the dose of APAP being used in subsequent studies.

Overall, results from our APAP dose-response control experiment did agree with the results published by other groups (Price and Jollow, 1986; Price et al., 1987; Bauer et al.,
2000). This ip dose of APAP at 1000 mg/kg was used because it has been shown to cause hepatotoxicity in fasted Long-Evans rats up to 24 h after the APAP dose (Price and Jollow, 1986; Price et al., 1987).

4.3 Effect of GBE on Liver Toxicity in Rats

4.3.1 Effect of GBE on Plasma ALT Levels in Rats

Treatment of rats with GBE at an ip dose of 500 mg/kg once daily for 8 consecutive days did not increase plasma ALT levels. The mean plasma ALT level in rats treated with GBE did not differ significantly from the control group (treated with a once daily ip injection of 0.9% NaCl for 8 consecutive days) (Figure 3.8). Therefore, GBE (using the current treatment regimen) did not have any effect on the plasma ALT levels in rats.

There were no other studies that used an ip dose of GBE at 500 mg/kg which made direction comparison of results difficult. Sener et al. (2005) injected male albino Wistar rats at 50 mg/kg/day ip once daily for 28 days. The average plasma ALT level in rats dosed with GBE was not significantly different from the control group. Shinozuka et al. (2002) fed a 0.5% w/w GBE diet to male Wistar rats for 4 weeks and no increase in ALT was observed compared to the control group. In the studies performed by Sener et al. (2005) and Shinozuka et al. (2002), the time between the last dose of GBE and the time of termination was not mentioned. Albino Wistar rats of both sexes were fasted 12 h prior to receiving two ip doses of GBE at 50 mg/kg/day (12 h apart) (Sakarcan et al., 2005). Average plasma ALT levels in rats injected with GBE were not significantly different from that in the control group at 24 h after termination (same time from GBE dose to termination as in our study).
4.3.2 Effect of GBE on Liver Histology in Rats

In contrast to the plasma ALT data, the histology data indicated that GBE given at 500 mg/kg ip caused damage in the liver tissues. As shown in Table 3.7, saline treatment did not cause any effect in the liver tissues because all scores reported were “0” (normal) for all five animals in each of the four categories (steatosis, necrosis, capsular inflammation, and sinusoidal dilatation). However, mild to severe necrosis, capsular inflammation, and steatosis were observed in some of the liver tissues from the GBE group (Table 3.7), which indicated some effects of GBE on rats at an ip dose of 500 mg/kg once daily for 8 consecutive days.

4.4 Effect of GBE Pretreatment on Liver Toxicity in Rats treated with APAP

4.4.1 Effect of GBE on Plasma ALT Levels in Rats treated with APAP

In the combination study where pretreatment of rats with GBE for 9 days was followed by a hepatotoxic dose of APAP, the same GBE dose of 500 mg/kg was used. The results from the plasma ALT data suggested that GBE was blocking the APAP-induced hepatotoxicity. The mean plasma ALT level in the combination group was not significantly different from the control group in which the rats were pretreated with 0.9% NaCl. Without the GBE pretreatment, the mean plasma ALT level was significantly elevated only in the APAP-treated group. There is no similar study published in the literature for comparison of the results involving GBE (at 500 mg/kg ip once daily for 9 days) and APAP.

4.4.2 Effect of other chemicals in GBE on Plasma ALT Levels in Mice treated with APAP

Janbaz et al. (2002) reported a protective effect by a chemical (rutin) in GBE on APAP-induced hepatotoxicity in Swiss male mice using plasma ALT levels as a marker for
liver toxicity. Rutin is a diglycoside form of quercetin. This compound was quantified in the GBE used in the current study (Table A1). Assuming that all diglycosides present in the GBE used in the current study are rutin, a 500 mg/kg dose of GBE would be equivalent to 20 mg/kg of rutin. However, rutin was given orally, instead of intraperitoneally, to the rats in the study by Janbaz et al. (2002). The study by Janbaz et al. (2002) did show that pretreatment with four oral doses of rutin at 20 mg/kg (12 hours apart) was able to prevent an increase in plasma ALT levels caused by APAP at an oral dose of 640 mg/kg. With the pretreatment of rutin, the mean ± SEM of the plasma ALT level was 61 ± 15 U/L, which was significantly lower than that for the APAP-only group (686 ± 219 U/L), although the time of termination (time between the APAP dose and termination) was not mentioned. However, the plasma ALT levels were significantly less in the rats pretreated with rutin before dosing with APAP when compared to those without the rutin pretreatment. This particular finding was consistent with the plasma ALT results our current study using GBE. Also, this study by Janbaz et al. (2002) only used plasma ALT level as a liver toxicity marker and no histological assessment was performed on the rat livers.

4.4.3 Effect of GBE on Liver Histology in Rats treated with APAP

In contrast to the plasma ALT data, the histology data (Figure 3.10 to Figure 3.13) from the current study suggested that GBE at 500 mg/kg for 9 days seemed to have a negative impact on liver histology in the presence of APAP, especially in terms of necrosis (Figure 3.11) and capsular inflammation (Figure 3.12). The relatively higher histology scores obtained from the liver tissues in GBE/APAP group were unlikely caused by mishandling of tissues prior to the histology assessment because the liver tissues in the control group were completely normal.
4.4.4 Conflicting Results between ALT data and Histology data

The effect of GBE on worsening of liver damage caused by APAP when compared to APAP alone was not reflected in our ALT data. The histology data are more reliable. The histology data are direct evidence showing damage to liver tissues, whereas plasma ALT level is only an indirect marker of liver toxicity. Also, histological assessment was performed independently by another laboratory and the pathologist was blinded to the identity of the samples so any potential bias could be prevented.

GBE contains many individual chemicals and one or more of these chemicals might have the potential to inhibit plasma ALT. The lower ALT level in the GBE/APAP group, as compared to the APAP group, could potentially be explained by ALT enzyme inhibition. If the plasma ALT enzyme was inhibited, it would fail to convert alanine to pyruvate (Figure 2.1), and hence the formation of 2,4-dinitrophenylhydrazone of pyruvate (Figure 2.2) would be inhibited as well. Inhibition of ALT by GBE is plausible, although there was 8.5 h between the last GBE dose and blood collection. Inhibition of ALT has been shown to occur. For example, Maekawa et al. (2002) reported the presence of an immunoglobulin G (IgG) inhibitor in a patient's serum, causing significantly reduced serum ALT activity. Further experiments will be required to investigate whether GBE inhibits ALT. Moreover, the plasma ALT level, although a commonly used liver toxicity marker, may not reflect the extent of hepatic necrosis (Zhao et al., 1998). A single ip injection of diallyl disulfide at 200 mg/kg to \( \beta \)-naphthoflavone-pretreated mice 2 h before an ip injection of APAP at 350 mg/kg was able to prevent the increase in the plasma ALT level by APAP, but not able to improve the histological assessment or mortality. Without the treatment of diallyl disulfide, severe necrosis was observed in all the mice pretreated with \( \beta \)-naphthoflavone at 4 h after APAP.
4.4.5 Potential Mechanisms by which GBE Exacerbates APAP-Induced Hepatotoxicity

4.4.5.1 Increased Bioactivation of APAP by GBE

GBE might have increased the bioactivation of APAP. Increased bioactivation of APAP could lead to increased accumulation of the hepatotoxic metabolite NAPQI, leading to liver toxicity. A mechanism by which this could occur is induction of CYP3A23 by GBE (pathway C in Figure 1.1), as shown by the CYP3A23 mRNA expression data from the current study (Figure A1). A pretreatment regimen of GBE at 500 mg/kg ip once daily for 9 days, followed by an ip dose of APAP at 1000 mg/kg 30 min after the last GBE dose in rats, was able to cause a statistical significant 5-fold increase in hepatic CYP3A23 mRNA expression, when compared to the APAP group (Figure A1).

The inductive effect of GBE on CYP3A23 could potentially be mediated through PXR or CAR. PXR and CAR have been hypothesized as key regulators for CYP3A and other CYP gene expressions (Waxman, 1999). One or more of the chemicals present in GBE may activate PXR or CAR, leading to increased transcription of the CYP3A23 gene. There is no published evidence that GBE can increase CYP3A gene expression through activation of PXR or CAR. However, it has been reported that quercetin (a chemical present in GBE) increased CYP3A4 mRNA expression in human hepatocytes in culture (Raucy, 2003), but it was suggested that the mechanism by which quercetin induced CYP3A might not involve activation of PXR.

Inhibition of UGT enzymes by GBE might be another potential mechanism whereby GBE enhances APAP-induced hepatotoxicity. Currently, there are no published data on the effect of GBE on the catalytic activity of UGT enzymes. Kostrubsky et al. (2005) reported that some compounds such as phenobarbital and phenytoin enhanced APAP-induced
hepatotoxicity in both rat liver microsomes and cultured human hepatocytes by inhibiting UGT enzymes. Hepatotoxicity caused by APAP can be worsened when the UGT enzymes are inhibited, potentially leading to decreased glucuronidation and hence increased bioactivation of APAP to the hepatotoxic metabolite NAPQI (pathway B in Figure 1.1). Exacerbation of APAP-induced hepatotoxicity by GBE through UGT inhibition is therefore possible but it will require further investigation.

Another potential mechanism of how GBE can exacerbate APAP-induced hepatotoxicity might be inhibition of SULT1. An enzyme(s) in the SULT1 family plays a major role in transformation of APAP to non-toxic metabolites (Duffel, 1997). If GBE can significantly inhibit the catalytic activity of SULT1 (pathway A in Figure 1.1), the extent of elimination of APAP will decrease and hence more NAPQI will be produced from biotransformation of APAP. The risk of APAP-induced hepatotoxicity may increase when the amount of NAPQI increases and the body is not able to eliminate the excess NAPQI produced in time. However, there is currently no published information on whether GBE inhibits the catalytic activity of SULT enzymes.

4.4.5.2 Decreased Detoxification of APAP by GBE

GBE might have exacerbated APAP-induced hepatotoxicity by suppressing the expression of UGT1A6 and/or SULT1 enzymes responsible for detoxifying APAP. GBE might have decreased the rate of APAP elimination by glucuronidation (pathway B in Figure 1.1) or sulfation (pathway A in Figure 1.1), leading to decreased elimination of APAP and hence increased formation of the hepatotoxic metabolite (NAPQI) in the liver. This mechanism was not likely as the hepatic UGT1A6 mRNA expression was neither increased
nor decreased by GBE in the current study (Figure A2). There is no published information on whether GBE can increase or decrease the expression of SULT1.

Glutathione S-transferase is a conjugation enzyme that is directly responsible for the detoxification of the hepatotoxic metabolite of APAP, NAPQI. Hence, decreased levels of glutathione S-transferase enzyme expression can potentially lead to reduced conjugation of glutathione with NAPQI, ultimately leading to increased risk of APAP-induced hepatotoxicity (pathway D in Figure 1.1). However, this mechanism is not likely, as several research groups have reported increased liver glutathione S-transferase activity in rats fed a GBE diet. Sugiyama et al. (2004b) reported a statistical significant 2-fold increase in liver glutathione S-transferase activity in rats that were fed a 0.1% w/w GBE diet for just 5 days. Similarly, glutathione S-transferase activity in livers of male Wistar rats was significantly increased by 2.7 times compared to control group after being fed orally with a 0.5% w/w GBE diet for 1 week (Sugiyama et al., 2004a). For as long as a 4-week duration, rats were fed a 0.5% w/w GBE diet and their liver glutathione S-transferase activity was increased significant by 2.6 times as compared to control (Shinozuka et al., 2002).

4.4.5.3 Combination of the Effects

Combination of any of the effects as discussed in Section 4.4.5.1 and 4.4.5.2 is possible. For instance, GBE is known to induce CYP3A (in rats) and GBE may have an inhibitory effect on UGT1A6. Induction of CYP3A per se can lead to increased formation of NAPQI from APAP. UGT1A6 inhibition may lead to increased accumulation of APAP and hence formation of NAPQI. Therefore, when both events are happening simultaneously the resultant detrimental effect on the liver can be worse than the toxic effect caused by either event alone.
4.5 Comparison of Current Study to Published Studies

The study by Shenoy et al. (2002) used albino Wistar rats and the ip dose of GBE used in that study was only at 50 mg/kg/day, a dose that is ten times lower than the dose used in the current study (500 mg/kg/day). Instead of a GBE pretreatment, the GBE was given to the rats either simultaneously with the APAP or after the APAP dose. The respective order of the GBE and the APAP treatment may make a drastic difference in the results. Any potential CYP enzyme induction effect caused by GBE will take more than a few days to happen. Shenoy et al. suggested a protective effect of GBE when it is given together with or after a hepatotoxic dose of APAP in rats, based on their assessment of plasma ALT and liver histology data. A problem with the study performed by Shenoy et al. (2002) was that there was no information regarding the amounts of terpene trilactones and flavonol glycosides in the GBE used in their study. Recent evidence revealed that ginkgolide A is a chemical that contributes to CYP3A induction by GBE (Chang et al., 2006). It is possible that ginkgolide A was not present in that extract or was present at a level too low to result in CYP3A induction. This could have explained the lack of enhanced hepatotoxicity in rats treated with GBE and APAP in the study by Shenoy et al. (2002).

Sener et al. (2006) suggested a protective effect of GBE against APAP-induced toxicity in mice. A problem with this study is that only a low dosage of GBE (50 mg/kg ip) was used. This GBE dosage may not be sufficient to elicit any CYP induction. CYP induction is crucial for increased NAPQI formation from APAP. Absence of CYP induction might have prevented the investigators from finding an exacerbation of APAP-induced hepatotoxicity by GBE. Also, GBE was given to the mice after APAP injection. The effect
of pretreatment with GBE on APAP-induced hepatotoxicity was not addressed in this study by Sener et al. (2006).

4.6 Limitations of the Study

The degree of CYP induction by GBE may vary from one species to the next. Hence, the results of this study in rats may or may not represent the situation in humans.

In the current study, the plasma ALT assay did not agree with the liver histology data. Hence, the data could only be explained in terms of either the plasma ALT levels or the liver histology, but not both. Liver histological analysis is more reliable because it is a direct way of assessing liver toxicity. Plasma ALT only served as a biochemical marker in assessing liver toxicity. It would be better if there were more than one way of assessing liver toxicity.

Only one dosage of GBE was used in the current study. This relatively high dosage was used because it induces CYP3A activity. However, smaller dosages of GBE could have been used in the study to determine the range of dosages that do not modulate APAP-induced hepatotoxicity.

GBE was given as a pretreatment to rats for 8 or 9 days. We did not investigate whether GBE given as a shorter pretreatment period would have similar effects on liver histology or plasma ALT levels.

4.7 Future Studies

In the present study, there was enhanced APAP-induced hepatotoxicity by GBE. It will be important to investigate which chemical(s) in GBE is(are) responsible for this observed effect.
Some of the experiments can be repeated in the future with more animals in each group to increase the power of the highly variable data. This is necessary to confirm the effect of GBE pretreatment on APAP-induced hepatotoxicity using histology for assessment of liver damage in rats.

It would be important to investigate whether the order of administering GBE and APAP would affect APAP-induced hepatotoxicity. Published data suggested administering GBE together with or after APAP prevented APAP-induced hepatotoxicity (Shenoy et al., 2002; Sener et al., 2006). Repeating the same published studies will allow us to know the effect of GBE co- and post-treatment with APAP on APAP-induced hepatotoxicity.

The lack of an increase in plasma ALT levels could be explained by a potential inhibition on the ALT enzyme by GBE. It will be interesting to investigate whether GBE inhibits plasma ALT enzyme and to identify the component(s) of GBE responsible for this inhibition.

Smaller dosages of GBE should be used in future studies to investigate whether the increase in APAP-induced hepatotoxicity by GBE, as observed in the current study, will occur with smaller dosages. Different durations of pretreatment with GBE, either administered orally or intraperitoneally, can be studied to investigate whether it will play any role in modulating APAP-induced hepatotoxicity. The time between the last GBE dose and the time of termination can be another crucial factor.

The effect of GBE pretreatment on expression and catalytic activities of certain drug metabolizing enzymes (e.g. CYP3A, CYP1A2, CYP2E1, SULT1, UGT1A6) in rats administered APAP should be investigated in future studies to help elucidate the mechanism by which GBE modulates APAP-induced hepatotoxicity. In the current study, there was an
increase in CYP3A23 mRNA expression in rats treated with GBE and APAP. It will be important to find out the individual chemical(s) in GBE that is responsible for any enzyme induction and inhibition effects. Also, quantifying the NAPQI metabolite may be helpful in explaining the modulation of APAP-induced hepatotoxicity by GBE.
5. SUMMARY AND CONCLUSIONS

- The plasma ALT assay used in this study was successfully validated. The assay had a dynamic range of 20 to 80 U/L, with a LOQ of 20 U/L. The inter-day and intra-day variabilities were acceptable based on published guidelines (Shah et al., 1992). The assay was accurate, precise, and reproducibility, although it had a narrow dynamic range and was not very sensitive.

- Fasting was required to elicit APAP-induced hepatotoxicity.

- 20% Tween 80 in 0.9% NaCl (the vehicle used to suspend APAP) did not elevate the plasma ALT levels in rats.

- Maximal plasma ALT levels were obtained with APAP at a dosage of 1000 mg/kg.

- GBE (500 mg/kg ip once daily for 8 days) did not increase plasma ALT levels, when compared to the 0.9% NaCl-treated control group, although histological analysis of hepatic tissues from GBE-treated rats showed that four of the five samples exhibited steatosis, necrosis, capsular inflammation, or sinusoidal dilatation.

- GBE at 500 mg/kg ip once daily for 9 days prevented the increase in plasma ALT levels in male Long-Evans rats, when assessed at 8 h after a single ip dose (1000 mg/kg) of APAP.

- In contrast, pretreatment of male Long-Evans rats with GBE at 500 mg/kg ip once daily for 9 days did not attenuate the occurrence of hepatic steatosis, necrosis, capsular inflammation, or sinusoidal dilatation, when assessed at 8 h after a single ip dose (1000 mg/kg) of APAP.

- Caution should be exercised when using plasma ALT levels as a marker to assess hepatotoxicity, especially in experiments involving GBE.
6. REFERENCES


### 7. APPENDICES

#### 7.1 Appendix 1

Table A1. The amount of ginkgolides, bilobalide, and flavonols in the GBE used in the study.

<table>
<thead>
<tr>
<th></th>
<th>Ginkgo biloba Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amount (% w/w)</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Diterpene</strong></td>
<td></td>
</tr>
<tr>
<td>Ginkgolide A</td>
<td>1.1</td>
</tr>
<tr>
<td>Ginkgolide B</td>
<td>0.3</td>
</tr>
<tr>
<td>Ginkgolide C</td>
<td>1.4</td>
</tr>
<tr>
<td>Ginkgolide J</td>
<td>0.6</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>3.4</td>
</tr>
<tr>
<td><strong>Sesquiterpene</strong></td>
<td></td>
</tr>
<tr>
<td>Bilobalide</td>
<td>2.8</td>
</tr>
<tr>
<td><strong>Total Terpene Trilactones</strong></td>
<td>6.2</td>
</tr>
<tr>
<td><strong>Flavonol and its Glycosides</strong></td>
<td></td>
</tr>
<tr>
<td>Kaempferol (aglycone)</td>
<td>none</td>
</tr>
<tr>
<td>Kaempferol (diglycosides)</td>
<td>1.9</td>
</tr>
<tr>
<td>Kaempferol (other glycosides)</td>
<td>4.4</td>
</tr>
<tr>
<td>Kaempferol (sum of aglycone and glycosides)</td>
<td>6.3</td>
</tr>
<tr>
<td>Quercetin (aglycone)</td>
<td>none</td>
</tr>
<tr>
<td>Flavonol Glycosides</td>
<td>Amount</td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>Quercetin (diglycosides)</td>
<td>4.0</td>
</tr>
<tr>
<td>Quercetin (other glycosides)</td>
<td>6.6</td>
</tr>
<tr>
<td>Qercetin (sum of aglycone and</td>
<td>10.6</td>
</tr>
<tr>
<td>Glycosides)</td>
<td></td>
</tr>
<tr>
<td>Isorhamnetin (aglycone)</td>
<td>none</td>
</tr>
<tr>
<td>Isorhamnetin (3-O-rutinoside)</td>
<td>0.6</td>
</tr>
<tr>
<td>Isorhamnetin (other glycosides)</td>
<td>3.5</td>
</tr>
<tr>
<td>Isorhamnetin (sum of aglycone and</td>
<td>4.1</td>
</tr>
<tr>
<td>glycosides)</td>
<td></td>
</tr>
</tbody>
</table>

*Total Flavonol Glycosides* 21

The amount of terpenes in *Ginkgo biloba* extract (lot no. 1306A) was quantified by gas chromatography (Indena S.A., Milan, Italy) and the amount of flavonols in the extract was quantified by liquid chromatography – mass spectrometry (ChromaDex, Inc., Santa Ana, CA).
Figure A1. Effect of pretreatment with GBE on CYP3A23 mRNA expression in rats administered APAP. Rats were treated as described in the legend to Figure 3.9. CYP3A23 mRNA was quantified by real-time PCR and the mean and SEM of the CYP3A23 mRNA expression are shown here for each treatment group. CYP3A23 mRNA expression was 6083 ± 1021 copies per 1 ng of dsDNA for the group treated with both GBE and APAP (combination group). This amount was significantly greater than the CYP3A23 mRNA expression for both the APAP-treated group (1272 ± 275 copies per 1 ng of dsDNA) and the vehicle-treated group (359 ± 149 copies per 1 ng of dsDNA) (p < 0.001, one-way ANOVA and SNK multiple comparison test). The CYP3A23 mRNA expression of the APAP-treated group was not significantly different from the vehicle-treated group, according to the SNK multiple comparison test. The assay was performed by Jessie Chen.

* Significantly different from the APAP-treated group and the vehicle-treated group (p < 0.001).
Figure A2. Effect of pretreatment with GBE on UGT1A6 mRNA expression in rats administered APAP. Rats were treated as described in the legend to Figure 3.9. Mean and SEM of the UGT1A6 mRNA expression for each treatment group are presented in this figure. The pretreatment of GBE did not appear to affect hepatic UGT1A6 mRNA expression in rats administered APAP. UGT1A6 mRNA expression was 82 ± 11 copies per 1 ng of dsDNA for the vehicle-treated group. The mean UGT1A6 mRNA expression for the APAP-treated group (107 ± 21 copies per 1 ng of dsDNA) and the combination group (96 ± 17 copies per 1 ng of dsDNA) were not significantly different from the vehicle-treated group (p = 0.69, one-way ANOVA and and SNK multiple comparison test). The assay was performed by Jessie Chen.