Study of Reactive Metabolites of Food Additives and Contaminant (Bisphenol-A) Using Dansyl Glutathione, and the Correlation with Their Hepatotoxicity

by

Thitiwan Buranachokpaisan

A Dissertation submitted to

The Graduate School-New Brunswick

Rutgers, The State University of New Jersey

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Graduate Program in Pharmaceutical Sciences

written under the direction of

Dr. Paul E. Thomas

and approved by:

_________________________________________________________________

_________________________________________________________________

_________________________________________________________________

_________________________________________________________________

_________________________________________________________________

_________________________________________________________________

New Brunswick, New Jersey

January 2011
ABSTRACT OF THE DISSERTATION

Study of Reactive Metabolites of Food Additives and Contaminant (Bisphenol-A)
Using Dansyl Glutathione and the Correlation with Their Hepatotoxicity

By Thitiwan Buranachokpaisan

Dissertation Director:
Dr. Paul E. Thomas

Biologically reactive metabolites are known for their ability to bind to nucleophilic macromolecules (DNA, proteins, lipids) and their irreversible binding to hepatic proteins is known to lead to necrosis and liver damage. Dansyl glutathione (dGSH), a fluorescent surrogate of glutathione, was used to trap reactive metabolites formed during oxidative metabolism of food additives by rat liver microsomes. dGSH was not commercially available and had to be synthesized. Synthesis of dGSH was improved to achieve a higher yield by reaction in borate buffer. The method, using fluorescent-high performance liquid chromatography for detection/quantitation and mass spectrophotometer for identification, yielded dGSH adducts that are consistent with other studies. dGSH was also found to be a substrate of microsomal glutathione S-transferase enzymes.

To correlate the extent of adduct formation with their toxicity, selected hepatotoxic food additives were studied at low (0.1 mM) and high (1 mM)
concentrations and the rates of adduct formation (nmol/min/mg microsomal protein) were measured. Using the amount of dGSH adduct of acetaminophen formed under the same conditions as a reference, the adduct formation rates of anethole and pulegone correlated well with their toxicity profiles while those of eugenol and nordihydroguaiaretic acid did not. Examination of their structures suggested different roles that phase II conjugation could play: either as detoxification, in concurrent, hence, competing with phase I oxidation, or as facilitating excretion, during the metabolism depending on the functional group a compound possesses. Studies of reactive metabolites of a compound capable of undergoing such concurrent phase II conjugation in a system without phase II capability could result in a false positive. It is concluded that the extent of dGSH adduct, representing that of reactive metabolites, formed by rat liver microsomes correlate well with hepatotoxicity profile of a compound.

The method was utilized to examine reactive metabolites of bisphenol A, a food contaminant. Four bisphenol A-related adducts were identified, characterized, including their formation kinetics, and quantitated. The extent of the 3 stable adducts was in the range of 1.5 to 11 nmol/min/mg protein. This high adduct level, however, requires additional studies to further examine the potential toxicity of bisphenol A reactive metabolites.
ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Paul Thomas, for this thesis project, and the resources and support, in myriad ways, he gave during the course of the project. I would like to express my gratitude to my thesis committee members: Dr. Gisela Witz – whose contribution has become an essential part to the success of my thesis – for the so much time she spent for me, her exceptional insight, wisdom, and knowledge, and her first-rate mentoring; Dr. Stanley Stein – for his amazing grace, encouragement, and inspiration; Dr. Brian Buckley – for his invaluable comments and generous support, especially on the mass spectrometry work; and Dr. Nanjoo Suh – for her delightfully gracious support and comments.

The time during my thesis work has been so joyous because of the generous and kind support and friendship from many: Pei-Ming Chen, Dr. Anne Dombrowski, Dr. Bozena Winnik, Dr. Yi-Hua Jan, Wendy Wang, Dr. Vladimir Mishin, Hongmei Zhang, Rita Hahn, John Beloni, Bobbi Busch, Dr. Kavita Prasad, Myrna Trumbauer, and all other staffs in EOHSI and Chemical Biology department; each truly deserves an above and beyond award.

Last, but not least, I would like to thank my dearest family and close friends, for always being there, the place of no distance or lapse of time, for me.
TABLE OF CONTENTS

ABSTRACT OF THE DISSERTATION........................................................................... ii

ACKNOWLEDGEMENTS....................................................................................... iv

TABLE OF CONTENTS .......................................................................................... v

List of Tables ......................................................................................................... x

List of Figures ....................................................................................................... xi

List of Schemes ..................................................................................................... xiii

List of Abbreviations ............................................................................................ xiv

I. INTRODUCTION ............................................................................................... 1

   A. Liver and hepatotoxicity ............................................................................. 1

   B. Reactive metabolites ............................................................................... 2

   C. Glutathione and its role in detoxification .................................................. 3

   D. Xenobiotic metabolism ............................................................................ 5

   E. Analysis of reactive metabolites ............................................................... 6

   F. Reactive metabolites and toxicity screening ........................................... 8

II. RATIONALE, HYPOTHESIS, AND SPECIFIC AIMS ..................................... 16

   A. Rationale .................................................................................................... 16

   B. Hypothesis ................................................................................................. 19

   C. Specific aims ............................................................................................ 20

       1. Evaluation of dansyl glutathione to study the rate of formation of reactive
          metabolites .............................................................................................. 20
2. Study of reactive metabolites of food additives formed by rat liver microsomal enzymes using dansyl glutathione as a trapping agent and the correlation with their hepatotoxicity........................................................... 20

3. Study of reactive metabolites of a food contaminant, bisphenol-A, formed by rat liver microsomal enzymes and their toxicity potential........ 21

III. EVALUATION OF DANSYL GLUTATHIONE TO STUDY THE RATE OF FORMATION OF REACTIVE METABOLITES............................................. 23

A. Introduction.............................................................................................. 23

B. Materials and methods ............................................................................ 24

1. Materials.................................................................................................. 24

2. Synthesis of dansylated oxidized glutathione (d2GSSG) ..................... 24

3. Synthesis of dGSH ............................................................................... 25

4. HPLC analytical method......................................................................... 25

4a. Analytical HPLC method for detection of d2GSSG............................. 25

4b. Preparative HPLC method for d2GSSG and dGSH purification ....... 26

4c. Analytical HPLC method for dGSH adduct detection and quantitation 26

5. Infusion MS and LC-MS analysis.......................................................... 27

6. Microsomal incubation of APAP and pulegone................................. 27

7. HPLC-fluorescence spectra of dGSH and dGSH adducts.................... 28

C. Results and discussion............................................................................ 28

1. Synthesis of d2GSSG........................................................................... 28

2. Synthesis of dGSH............................................................................... 32
3. HPLC method development for analysis of reaction mixtures for dGSH synthesis ................................................................. 32

4. HPLC quantitation of dGSH adducts formed by APAP and pulegone in a microsomal system ................................................................................................................................. 33

D. Conclusions ................................................................................................................. 34

IV. STUDY OF REACTIVE METABOLITES OF FOOD ADDITIVES FORMED BY RAT LIVER MICROSOMAL ENZYMES USING DANSYL GLUTATHIONE AS A TRAPPING AGENT AND THE CORRELATION WITH THEIR HEPATOTOXICITY ................................................................. 47

A. Introduction ................................................................................................................. 47

B. Materials and methods ............................................................................................. 48

1. Materials .................................................................................................................... 48

2. Preparation of rat liver microsomes ........................................................................ 48

3. Microsomal incubation studies ................................................................................ 49

4. Analysis of dGSH adducts ....................................................................................... 50

C. Results ........................................................................................................................ 51

1. Identification and quantification of reactive metabolites of known hepatotoxic compounds ................................................................................................................................. 51

2. Identification and quantification of reactive metabolites of known non-hepatotoxic compounds ......................................................................................................................... 57

D. Discussion .................................................................................................................. 59

1. Significance of different rat liver microsomes ......................................................... 59
2. Dansyl GSH adducts of hepatotoxic compounds: a retrospective analysis ................................................................. 60

2a. APAP, the reference compound .................................................................................................................. 61

2ai. AMAP: a case of an unstable dGSH adduct ...................................................................................... 63

2b. Anethole ............................................................................................................................................. 64

2c. Pulegone ........................................................................................................................................... 65

2ci. Pulegone: a case of non-NADPH dependent metabolism and dGSH conjugation catalyzed by microsomal GST ................................................................. 67

2d. Eugenol ........................................................................................................................................... 70

2e. NDGA ............................................................................................................................................ 71

3. Dansyl GSH adduct of flavonoids: a case of electrophiles vs. free radicals ................................................................. 72

4. Assessment of toxicity potential of compounds: standard or general values vs. reference values .................................................................................................................. 76

5. Toxicity implications as derived from dGSH adduct studies of hepatotoxic dietary compounds ................................................................. 77

V. STUDY OF REACTIVE METABOLITES OF BISPHENOL-A, A FOOD CONTAMINANT, FORMED BY RAT LIVER MICROSONAL ENZYMES AND THEIR TOXICITY POTENTIAL ................................................................. 105

A. Introduction ............................................................................................................................................ 105

B. Materials and methods .......................................................................................................................... 108

1. Materials ............................................................................................................................................ 108

2. Synthesis of dGSH .............................................................................................................................. 108
3. Preparation of rat liver microsomes .................................................... 109
4. Microsomal incubation studies ........................................................... 109
5. Analysis of dGSH adducts ................................................................. 111

C. Results .............................................................................................. 112
1. Dansyl GSH-Bisphenol A (dGS-BPA) adduct formation ..................... 112
2. Inhibition study of dGS-BPA adduct formation ................................. 114
3. Kinetic parameters of dGS-BPA adducts formation ............................. 115
4. Dansyl GSH adduct formation of reference compounds .................... 116

D. Discussion ........................................................................................ 118
1. Reactive metabolites of BPA .............................................................. 118
2. Analysis of potential toxicity of BPA reactive metabolites ............... 121

E. Conclusion ......................................................................................... 126

VI. SUMMARY ...................................................................................... 140

VII. APPENDIX: NON SPECIFIC PROTEIN BINDING .......................... 145

VIII. BIBLIOGRAPHY ............................................................................ 150

IX. CURRICULUM VITAE ....................................................................... 166
List of Tables

Table 1: Summary of the differences of pharmaceutical vs. food and environmental contaminant compounds in terms of their nature of exposure and toxicity data available ................................................................. 22

Table 2: Summary of effect of different buffer systems on the yield of d2GSSG 36

Table 3: Characteristics of dGSH adducts formed by microsomal metabolism of selected hepatotoxic food additives ............................................................ 83

Table 4: Rate of metabolic activation, or dGSH formation, of hepatotoxic food additives .................................................................................................. 84

Table 5: Characteristics of dGSH adducts of flavonoids formed by microsomal metabolism ............................................................................................... 85

Table 6: Dansyl GSH adduct formation (nmol/min/mg protein) of flavonoids ..... 86

Table 7: Examples of substrates of human GST .......................................................... 87

Table 8: Summary of the extent of GSSG and dGSH adduct formed as a result of oxidation of phenol and catechol compounds b,c ........................................ 88

Table 9: Rate of dGSH adduct formation (nmol/min/mg protein) from incubations of 0.1 mM BPA with liver microsomes ..................................................... 127

Table 10: Kinetic parameters of adduct AB1 and AB4 formation from metabolism of 0.1 mM BPA by liver microsomes ......................................................... 128

Table 11: Summary of characteristics of dGSH conjugates of BPA ............... 129

Table 12: Formation of dGSH adducts (nmol/min/mg protein) of APAP and trans-anethole metabolites formed by liver microsomes ......................................................... 130

Table 13: Correction of the experimental apparent rate of dGS-NDGA adduct formation ....................................................................................................... 148
List of Figures

Figure 1: Catabolism of glutathione conjugates, or mercapturic acid biosynthesis pathway. ................................................................. 11

Figure 2: Synthesis, metabolism, and catabolism of glutathione. .................. 12

Figure 3: Role of glutathione as a reductant.............................................. 13

Figure 4: Correlation of microsomal protein covalent binding and hepatotoxicity studied by Obach et al. [2008]a .......................................................... 14

Figure 5: Correlation of thiol adducts and toxicity studied by Gan et al. [2009]. 15

Figure 6: A mass spectrum of d2GSSG ....................................................... 37

Figure 7: A representative HPLC-fluorescence chromatogram of a d2GSSG reaction mixture ........................................................................ 38

Figure 8: Kinetic profiles of d2GSSG synthesis in a borate buffer .................. 39

Figure 9: A representative HPLC-fluorescence chromatogram of a purified d2GSSG solution ................................................................. 40

Figure 10: Examples of mass and volume overload characteristics and a representative HPLC-fluorescence chromatogram of a d2GSSG synthesis reaction solution. ............................................................ 41

Figure 11: A mass spectrum of dGSH ................................................................ 42

Figure 12: A representative HPLC-fluorescence chromatogram showing peaks of dGSH adduct of APAP ................................................................. 43

Figure 13: A standard curve of dGSH ............................................................... 44

Figure 14: Structures of hepatotoxic food additives used in this study, and their proposed dGSH conjugates ............................................................ 89

Figure 15: HPLC-fluorescence chromatograms obtained from incubations of NDGA with rat liver microsomes ......................................................... 91

Figure 16: Formation rate of dGS-menthone adduct formed by incubation of 1 mM pulegone with different rat liver microsomes ................................. 92

Figure 17: Formation of dGS-menthone adduct from microsomal metabolism of pulegone in the presence of different mGST inhibitors .......................... 93

Figure 18: Flavonoid structures and their proposed dGSH adducts ............... 94
Figure 19: Metabolic pathways of APAP and AMAP in mice. ......................... 95
Figure 20: Metabolic pathways of anethole .................................................. 96
Figure 21: Metabolic pathways of pulegone ................................................ 97
Figure 22: An oxidation pathway of pulegone to menthofuran and its subsequent metabolic transformation to minilactone/isominlactone. .................... 98
Figure 23: Reactivity of \( \alpha,\beta \)-unsaturated ketone compounds with GSH. ....... 99
Figure 24: Metabolic pathways of eugenol .................................................. 100
Figure 25: Examples of compounds containing functional groups capable of forming glucuronide conjugates................................................................. 101
Figure 26: Chemical structures of compounds tested in Gan's study with detectable adducts but are not associated with drug-induced toxicity. ...... 102
Figure 27: Chemical structures of non-hepatotoxic compounds tested in Obach's study that covalently bound to protein when incubated with human liver microsomes. ............................................................... 103
Figure 28: A representative HPLC-fluorescence chromatogram of a BPA sample .................................................................................................................. 131
Figure 29: Mass chromatograms from LC-MS showing \( m/z \) 633, 675, 783, and 767 ........................................................................................................... 132
Figure 30: MS fragmentation pattern in positive ion mode of the mass \( m/z \) 783 (adduct AB3) in a BPA sample ................................................................. 133
Figure 31: MS\(^3\) fragmentation pattern in positive ion mode of a product ion \( m/z \) 664 of the parent ion \( m/z \) 767 (adduct AB4)............................................. 134
Figure 32: MS\(^3\) fragmentation pattern in positive ion mode of a product ion \( m/z \) 530 of the parent ion \( m/z \) 663 (adduct AB1)......................................................... 135
Figure 33: MS\(^3\) mass chromatograms from LC-MS of a BPA sample .......... 136
Figure 34: Inhibition of formation rate of dGSH adduct AB4 ......................... 137
Figure 35: Structures of the compounds studied and proposed structures of their dGSH adducts ......................................................................................... 138
Figure 36: Amount (expressed as peak area) of dGS-NDGA adduct formed at different microsomal protein concentration......................................... 149
List of Schemes

Scheme 1: Summary of preparative HPLC method development for purifying d2GSSG and dGSH ................................................................. 45

Scheme 2: A diagram showing differential kinetics between phase I oxidation and phase II conjugation. ......................................................... 104

Scheme 3: Proposed mechanism of formation of dGSH adduct AB1 and AB2 139
List of Abbreviations

\( \alpha \)-NF \( \alpha \)-Naphthoflavone
\( \beta \)-NF \( \beta \)-Naphthoflavone
AB1 dGSH adduct of BPA at RT 17.1
AB2 dGSH adduct of BPA at RT 21.5
AB3 dGSH adduct of BPA at RT 22
AB4 dGSH adduct of BPA at RT 25.7
ADR Adverse drug reaction
ALT Alanine aminotransferase
AMAP \( N \)-acetyl-\( m \)-aminophenol
APAP Paracetamol, acetaminophen, \( N \)-acetyl-para-aminophenol
BPA Bisphenol A
CDNB 1-Chloro-2,4-dinitrobenzene
COX Cyclooxygenase
d1GSSG or d1 Dansylated oxidized glutathione with 1 dansyl group
d2GSSG or d2 Dansylated oxidized glutathione with 2 dansyl groups
Da Daltons
dGSH Dansylglutathione
DILI Drug-induced liver injury
DTT Dithiothreitol
E° One electron oxidation potential
EDTA Ethylenediaminetetraacetic acid
ESI Electrospray ionization
Fe\(^{3+} \) Ferric ion
fu Fraction unbound
GGT \( \gamma \)-glutamyl transpeptidase
GPx Glutathione peroxidase
GRAS Generally recognized as safe
GSH Glutathione
GSSG Oxidized glutathione
GST Glutathione S-transferase
H Hydrogen
HPLC High-performance liquid chromatography
IC\(_{50} \) Half maximal inhibitory concentration
KCl Potassium chloride
K\(_m \) Michaelis constant
LC-MS Liquid chromatography – mass spectrometry
LC-MS/MS Liquid chromatography – tandem mass spectrometry
m/z Mass-to-charge ratio
MAb Monoclonal antibody
MAPEG Membrane-associated proteins in eicosanoid and glutathione
mGST Microsomal glutathione S-transferase
M\(_i \) Maximum loading mass
MS Mass spectrophotometry
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS²</td>
<td>Fragmentation of a parent ion</td>
</tr>
<tr>
<td>MS³</td>
<td>Fragmentation of a product ion</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>mx</td>
<td>Microsomes</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotine-adeninedinucleotidephosphate, reduced</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NAPQI</td>
<td>N-acetyl-p-benzoquinone imine</td>
</tr>
<tr>
<td>NDGA</td>
<td>Nordihydroguaiaretic acid</td>
</tr>
<tr>
<td>NOAEL</td>
<td>No observable adverse effect level</td>
</tr>
<tr>
<td>NOEL</td>
<td>No observed effect level</td>
</tr>
<tr>
<td>O</td>
<td>Oxygen</td>
</tr>
<tr>
<td>P450</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>PAb</td>
<td>Polyclonal antibody</td>
</tr>
<tr>
<td>PB</td>
<td>Phenobarbital</td>
</tr>
<tr>
<td>PBPK</td>
<td>Physiological-based pharmacokinetics</td>
</tr>
<tr>
<td>PCN</td>
<td>Pregnenolone-16α-carbonitrile</td>
</tr>
<tr>
<td>R₁</td>
<td>Compounds containing certain functional groups, see Scheme 2</td>
</tr>
<tr>
<td>R₂a</td>
<td>Compounds containing certain functional groups, see Scheme 2</td>
</tr>
<tr>
<td>R₂b</td>
<td>Compounds containing certain functional groups, see Scheme 2</td>
</tr>
<tr>
<td>RT</td>
<td>Retention time</td>
</tr>
<tr>
<td>S</td>
<td>Substrate</td>
</tr>
<tr>
<td>s.e.</td>
<td>Standard error</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine diphosphate</td>
</tr>
<tr>
<td>Vᵢ</td>
<td>Maximum loading volume</td>
</tr>
<tr>
<td>Vₘₐₓ</td>
<td>Maximum reaction rate</td>
</tr>
</tbody>
</table>
I. INTRODUCTION

A xenobiotic is defined as any exogenous chemical entity that the body is exposed to, actively/voluntarily or passively/involuntarily. Generally speaking, the two most important aspects in studying an impact of a xenobiotic to a host are its pharmacological effect and its toxicity. While an effect of a xenobiotic binding to a receptor is fundamental to its pharmacological action, the consequence of xenobiotic metabolism is often a key factor in its toxicity. The Millers discovered that many compounds were converted to reactive electrophiles during metabolism that were more toxic than the parent compound [Miller and Miller, 1977]. It could be said that the first step in understanding toxicity of a compound is to study its metabolism.

A. Liver and hepatotoxicity

Liver is the principal organ of metabolic transformation of xenobiotics, hence, making it prone to injury if metabolism yields intermediates more toxic than the parent compound. Hepatotoxicity is one of the major causes of new drug development failure during Phase I to III of the approval process. However, its occurrence remains frequent, second to cardiovascular toxicity, even after the launching, resulting in a drug withdrawal from the market [Redfern et al., 2010]. Unlike the early drug development reasons for failure, the later incidents are usually idiosyncratic cases, or Type B adverse drug reaction (ADR). This underlies the complexity of liver injury. Drug-induced liver injury (DILI), including that from natural food chemicals and environmental contaminants, is listed as the
leading cause of acute liver failure, observed by inflammation and hepatocellular necrosis. Investigation of such DILI cases revealed a link to the involvement of reactive metabolites [Caldwell and Yan, 2006; Kumar et al., 2008; Nassar and Lopez-Anaya, 2004].

B. Reactive metabolites

Reactive metabolites refers to metabolites derived from xenobiotics which undergo oxidation and become electrophiles and/or free radicals. Due to their reactivity, they are short-lived and are known to react with protein, DNA, and lipids, and/or cause oxidative stress, which then can result in cytotoxicity, genotoxicity, and/or triggering immunological responses to the “new” macromolecular entity [Caldwell and Yan, 2006; Kalgutkar et al., 2005a; Yang et al., 2006]. Unlike stable metabolites, reactive metabolites are not usually found in plasma. On an experimental level, reactive metabolites could be studied by two approaches.

The first approach is a chemical-oriented method. The reactive metabolites are reacted directly with small molecules that are efficient traps. Chemically, depending on their polarizability, electrophiles are considered to be either soft or hard, and will react with soft or hard nucleophiles, respectively. Examples of biologically relevant soft nucleophiles are thiol groups of glutathione and cysteine while those of hard nucleophiles are lysine, arginine, and amines of nucleic acids. Soft electrophiles such as quinone, quinone imine, quinone methide, epoxide, or arene oxide, can be trapped with glutathione (GSH) or N-acetyl cysteine. Hard electrophiles such as an aldehyde can be trapped with
amines such as semicarbazide, methoxylamine, or \( \alpha \)-acetyl lysine, while an iminium can be trapped with cyanide anion. Radicals are captured by spin-trap agents like 5,5-dimethylpyrrolidine-\( N \)-oxide.

Alternatively, reactive metabolites can be investigated by studying their biological consequences, e.g. covalent binding to proteins or DNA, time- and cofactor-dependent enzyme inhibition studies, or oxidative stress-related assays [Caldwell and Yan, 2006; Yan et al., 2007; Ma and Subramanian, 2006].

C. Glutathione and its role in detoxification

As a nucleophile, \( \gamma \)-L-glutamylcysteinylglycine, or GSH, reacts with reactive electrophiles either by addition or substitution forming a thio ether bond. GSH is found in liver and other tissues at relatively high levels, 1-10 mM, and is considered the most important detoxicant of electrophiles in the body. Although glutathione S-transferase (GST) is known to mediate this reaction, for many compounds the reaction rate of GSH conjugation in the absence vs. the presence of GST is found to be comparable [Nguyen et al., 2006; Frederick et al., 1992], indicating that the electrophiles can react with GSH spontaneously. The GSH conjugate is usually further metabolized by removal of glutamyl and glycyl groups and the remaining cysteiny1 residue is acetylated, becoming an \( N \)-acetylcysteine S-conjugate, or mercapturic acid. Several different species of a GSH conjugate can be found from its break down process in biliary space and small intestine, while mercapturic acids are the predominant form detected in urine (see Figure 1) [Hinchman and Ballatori, 1994]. Both GSH and mercapturic
acid forms are highly water soluble and are readily excreted. Nonetheless, in a few cases, there is evidence that both forms can be transported to other tissues and further metabolized to become reactive species, causing toxicity in kidney, nervous tissues, and the hematopoietic system [Monks and Lau, 1997; Bolton et al., 2000].

With its abundant supply and availability at the site of oxidative metabolism, GSH will react with reactive metabolites, preventing them from binding to other cellular macromolecules. In certain cases the reactive metabolite is so highly reactive that it alkylates the very enzyme (often cytochrome P450) that catalyzed its oxidation, and this can thereby inactivate the enzyme irreversibly [Zhou et al., 2005]. Changes in GSH level often correlate well with acute toxicity [Rinaldi et al., 2002]. Depletion of glutathione, especially in the mitochondrial pool, has shown to be an important mechanism of liver damage, demonstrated by acetaminophen and ethanol, the two most widely studied compounds for hepatotoxicity [Rodes et al., 2007]. Upon GSH depletion, electrophiles will bind to other cellular macromolecules. Studies of many compounds showed that the reaction rate of GSH conjugate formation resembles that of protein binding of the same compound [Masubuchi et al., 2007; Frederick et al., 1992]. However, the dynamics between this non-protein thiol vs. protein thiol still require much more research. In the case of acetaminophen (APAP), protein covalent binding of the reactive metabolite N-acetyl-p-benzoquinoneimine, NAPQI, occurred only at high doses and only when the GSH pool was depleted. But GSH was not able to completely prevent protein binding
of many other compounds even when tested at low concentrations [Obach et al., 2008].

Glutathione plays a role in what considered to be the most important defense mechanism of the body. This is due to its versatility and abundance. Besides acting as a nucleophile, GSH can also act as a reductant or an antioxidant, where a thiyl radical, GS\(^{-}\), or the oxidized form, GSSG, is formed (see Figure 2). The free radical and/or change of GSH/GSSG ratio, hence, the redox state, could result in oxidative stress, where other defensive mechanisms will be called forth. It is speculated that several factors are involved in determining what role GSH will take in interacting with a certain compound, especially compounds with functional groups that could become either an electrophile or a radical such as a phenoxy group. Figure 3 shows the role of GSH in oxidative stress.

D. Xenobiotic metabolism

Xenobiotic metabolism can be conceptually divided into two distinct processes or phases. Phase I involves enzymatic oxidation, reduction, or hydrolysis reactions, that yield metabolites suitable for phase II which involves conjugation with a very water soluble molecule resulting in a glucuronide, sulfate, or glutathione conjugate. However, there are compounds known to be metabolized by either phase I or II alone or that are further metabolized after phase II conjugation [Deshpande, 2002]. The endothermic process of phase II conjugation can be divided into 2 types of conjugation: one of xenobiotic conjugates with activated cofactors, e.g. UDP-glucuronic acid and sulfate, and
another of activated xenobiotics, that is reactive metabolites, with nucleophiles such as GSH [Levens et al., 2005]. Hence, although GSH conjugation is categorized as a part of phase II metabolism, it is fundamentally distinct. The focal point of GSH conjugation study is to uncover reactive and therefore potentially toxic metabolites formed by oxidative metabolism during phase I. Although these reactions are grouped as phase II conjugations, conjugation with GSH, a nucleophile, serves more to reveal a potentially toxic pathway while glucuronidation and sulfation relate strictly to a detoxification pathway. The dynamics between these two processes plays a critical role in the final outcome.

GSH-related conjugates are not usually reported along with glucuronide and sulfate conjugates as a part of metabolism pathways of compounds during in vivo pharmacokinetic/metabolism studies, where “minor metabolites” (those found in relatively smaller quantities) are usually not characterized. Their reactivity is also not usually revealed during in vitro metabolite profiling, where every metabolite is characterized, because trapping agents are often not included. Unless screening for reactive metabolites is performed during toxicity assessment, their existence remaines undiscovered. The reactive metabolites of the antidepressant nefazodone, for example, was only reported recently in an attempt to understand the cause of its idiosyncratic hepatotoxicity [Kalgutkar et al., 2005b].

E. Analysis of reactive metabolites

Since reactive metabolite formation is often associated with adverse drug reactions, attempts are made during drug discovery and development to detect,
and, if possible, minimize their formation [Caldwell and Yan, 2006; Evans et al., 2004]. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is the method of choice for detection and characterization of adducts of reactive metabolites. It is used rather as a qualitative method since the efficiency of ionization and vaporization varies widely and cannot be reliably predicted. Therefore, there is a need to synthesize the proposed reactive-GSH adduct to use as a standard [Ma and Subramanian, 2006]. A quantitative method would be beneficial when rank ordering related compounds and as an aid in understanding the bioactivation mechanism. It is also needed to demonstrate the direct link of reactive metabolites to toxicity of a compound [Rao, 1977; Evans, 1983], although a model showing good correlation, hence predictive, of reactive metabolites and toxicity of compounds in general is yet to be established [Evans et al., 2004]. Radioactive labelled compounds have been used in both protein covalent adduct and GSH conjugation studies. However, special handling and the need of labelling make it an inconvenient and often an expensive method, especially when studying a group of compounds. Fluorescent dansyl glutathione (dGSH) was recently described as a promising reactant for quantitating reactive metabolites in toxicity assessment of compounds during drug discovery [Gan et al., 2005]. The reaction rates of dGSH and GSH were examined and found to be no different. dGSH, however, is not a cofactor of GST [Gan et al., 2005]. This is not necessarily a liability since the conjugation will simply reflect a chemical reactivity of the reactive metabolites. This reactivity would also represent a base value in a case a) that the conjugation were also catalyzed by GST and b) of
inter-individual variability due to the polymorphism of GST [Gan et al., 2005]. Nonetheless, since the publication of the method in 2005, there have been no other studies based on dGSH. Dansyl GSH is not commercially available and is needed in excess in the experiment at the level of 1-5 mM. It is speculated that synthesis of dGSH in a sufficient quantity could be a hurdle.

F. Reactive metabolites and toxicity screening

While there is a correlation between reactive metabolites binding to DNA and genotoxicity, the relationship of protein covalent binding and liver toxicity is found to be less well correlated [Liebler, 2008]. Nonetheless, a higher toxicity risk of compounds with high covalent protein binding is generally accepted [Evans et al., 2004; Yang et al., 2006]. The toxicity of a reactive metabolite, however, depends on many factors: dose or exposure, its pharmacokinetic profile, the extent of the reactive metabolite formed and its reactivity and stability, detoxification pathways, microenvironment effect, and the significance of its biological consequences [Chiba and Pang, 1995; Kumar et al., 2008; Yang et al., 2006]. Consequently, assessment of a compound’s toxicity requires both qualitative and quantitative measures. As a prime example, Evans et al. [2004] provided a decision tree to be exercised along with other “qualifying considerations,” if a compound will be advanced to the next phase of development when protein binding is found more than 50 pmol/mg protein.

Although reactive metabolites are known to be linked with liver toxicity, a good correlation model, hence predictive, is yet to be established. Obach et al. [2008] examined 18 drugs (9 hepatotoxic and 9 non-hepatotoxic) and their extent
of covalent protein binding in human liver microsomes, calculated as intrinsic clearance. The two groups were ranked with respect to their intrinsic clearances of total covalent binding (see Figure 4). The clearance values were also converted to estimated doses of covalent binding per day, calculated from the dose of each compound with an incorporation of the effect of GSH and glucuronic acid conjugation. The result was that both values (the intrinsic clearances and the estimated doses) of the non-hepatotoxic group could not be differentiated from the hepatotoxic group very well: some high binding values were found with non-hepatotoxic drugs and low binding values were found with hepatotoxic drugs. Another study performed by Gan et al. [2009] investigated the correlation of toxicity with dGSH adducts of 50 compounds, 10 of which are toxic drugs. The results showed dGSH adducts for 5 out of 10 toxic drugs (or 50% false negative). Using a cut-off of 0.2% (below which is considered negligible), a false positive of the non-toxic group was 8% (see Figure 5). Although there were false positives and false negatives, the authors concluded that there was a trend of higher thiol adduct formation with the group of hepatotoxic compounds. The daily burden of reactive metabolites was obtained by correcting the fraction of reactive metabolites with dose, fraction absorbed, and fraction of oxidative metabolism to total clearance. But these theoretical corrections did not make much significant change to the overall conclusion.

Due to their enrichment with P450 oxidative enzymes, liver microsomes are the most fundamental matrix used for in vitro metabolism studies, including studies of drug-drug interaction. However, due to the absence of detoxification
systems, the oxidative metabolism of liver microsomes may represent the worst case scenario, resulting in an over-estimate of the oxidative process. On the other hand, an under-estimate result is also possible if the activation mechanism does not involve the P450 enzymes. Non-specific binding to microsomal protein is also a well known factor which results in an under estimation [Obach, 1997; Kalvass et al., 2001; Austin et al., 2002]. It is a reversible non-covalent binding of the substrate to proteins due to physico-chemical properties of the compound, i.e. lipophilicity [Giuliano et al., 2005]. The fraction unbound, fu, representing the actual concentration that is available for any reaction, is measured and used to correct the in vitro data when it is needed for an in vivo analysis/correlation [Obach, 1997; Kalvass et al., 2001; Austin et al., 2002].

As the lack of good correlation between reactive metabolites and their potential toxicity was demonstrated above, it is a part of this thesis’s objective to further explore what other aspects may cause such a lack of correlation. Conceptually, the interaction of phase I and II is known to affect the final toxicity outcome. However, it is also important to understand how this dynamics is applied specifically in analyzing the correlation of the results.
Figure 1: Catabolism of glutathione conjugates, or mercapturic acid biosynthesis pathway. Glutathione conjugates of electrophiles are usually excreted as mercapturic acids. After a xenobiotic (represented by X) is metabolized and becomes an electrophile (E), it will form a conjugate with glutathione. The majority of this conjugate is transported to the kidney, where the glutamic acid and glycine moiety are removed by $\gamma$-glutamyltransferase ($\gamma$-GT) and dipeptidase, respectively. The cysteine S-conjugate is then N-acetylated by N-acetyl transferase (NAT) to N-acetyl cysteine S-conjugate, or mercapturic acid, and excreted in urine. Another portion of the glutathione conjugate is excreted into bile, catalyzed within the biliary tree, and reabsorbed back into liver for N-acetylation. The proportion of mercapturic acid excreted by kidney vs. through biliary tract is not yet well understood. [Hinchman et al., 1991; Hinchman and Ballatori, 1994]
Figure 2: Synthesis, metabolism, and catabolism of glutathione. Glutathione (GSH) can not enter the cell by itself; it is synthesized within the cell from γ-glutamyl-amino acid complex, utilizing 3 ATPs and glutamate-cysteine lygase (GCL) and glutathione synthase (GS) enzymes. GSH that reduces hydroperoxide (ROOH), catalyzed by glutathione peroxidase (GPx), to alcohol (ROH) and becomes glutathione disulfide (GSSG) can be regenerated by glutathione reductase (GR). Interaction of GSH with an electrophile (X) to a conjugate (GSX) can sometimes be catalyzed by glutathione S-transferase (GST). All GSH, GSSG, and GSX are transported outside the cell and broken down in a similar manner by γ-glutamyl transpeptidase (GGT) and dipeptidase, so that the free amino acids can be transported into the cell for the de novo synthesis. [Dickinson and Forman, 2002]

(excerpted from Dickinson and Forman [2002])
Figure 3: Role of glutathione as a reductant, as a part of a working network between cytoprotective enzymes and antioxidants in handling oxidative stress

(excerpted from Crinchton [2001])
Figure 4: Correlation of microsomal protein covalent binding and hepatotoxicity studied by Obach et al. [2008]

<table>
<thead>
<tr>
<th>Hepatotoxic group:</th>
<th>total CL_{int,cb}</th>
<th>Non-hepatotoxic group:</th>
<th>total CL_{int,cb}</th>
</tr>
</thead>
<tbody>
<tr>
<td>nefazodone</td>
<td>3.7</td>
<td>paroxetine</td>
<td>8.1</td>
</tr>
<tr>
<td>tiensilic acid</td>
<td>2.9</td>
<td>raloxifene</td>
<td>1.5</td>
</tr>
<tr>
<td>acetaminophen</td>
<td>0.12</td>
<td>propranolol</td>
<td>0.59</td>
</tr>
<tr>
<td>sudoxicam</td>
<td>0.056</td>
<td>buspirone</td>
<td>0.22</td>
</tr>
<tr>
<td>indomethacin</td>
<td>0.053</td>
<td>diphenhydramine</td>
<td>0.12</td>
</tr>
<tr>
<td>carbamazepine</td>
<td>0.039</td>
<td>meloxicam</td>
<td>0.095</td>
</tr>
<tr>
<td>diclofenac</td>
<td>0.035</td>
<td>simvastatin</td>
<td>0.067</td>
</tr>
<tr>
<td>benoxaprofen</td>
<td>ND</td>
<td>ibuprofen</td>
<td>ND</td>
</tr>
<tr>
<td>felbamate</td>
<td>ND</td>
<td>theophylline</td>
<td>ND</td>
</tr>
</tbody>
</table>

a Eighteen drugs (9 hepatotoxins, 9 non-hepatotoxins) were incubated with human liver microsomes and analyzed for their protein covalent binding. The results were presented in terms of clearance. The total covalent binding clearance (total CL_{int,cb}) (μL/min/mg protein) = CL_{int,cb,1} + CL_{int,cb,2}, where $CL_{int,cb,1} = \frac{V_{\text{max}}}{K_m}$, and $CL_{int,cb,2}$ was calculated from the equation:

$$
\nu = \frac{V_{\text{max}} \cdot [S]}{K_m + [S]} + CL_{int,cb,2} \cdot [S]
$$
Figure 5: Correlation of thiol adducts and toxicity studied by Gan et al. [2009]. Fifty drugs were tested for dGSH conjugates of reactive metabolites with human liver microsomes. Percent thiol adduct formation was compared between the group of drugs associated with drug-induced toxicity (DIT) and those without (non-DIT). Pulegone was used as a positive reference and also plotted in the DIT group.

(excerpted from Gan et al. [2009])
II. RATIONALE, HYPOTHESIS, AND SPECIFIC AIMS

A. Rationale

Although the role of reactive metabolites and the extent of reactive metabolite formation with toxicity is well recognized for individual compounds, this correlation for a group of compounds with different chemical classes has not been well demonstrated. This means there is not yet a standard measure that is generally accepted. There are a few deficiencies associated with different approaches presented by the three different groups: amounts of covalent binding/mg protein by Evans et al. [2004], rates of thiol adduct formation as a percentage of the starting substrate concentration by Gan et al. [2009], and clearance values of protein covalent binding by Obach et al. [2008]. A good comprehension of a compound’s toxicity would be limited by a study of only one concentration, as was done by the first two studies. It would be inefficient to use a result of a low concentration to understand their toxicity at high concentrations, and vice versa. The result of protein covalent bindings taken at 1 hr proposed by Evans is also likely to render an unfair comparison since some compounds may have reached the saturation while the others have not. While Obach obtained the rate of protein covalent adduct formations at different concentrations, these information were not accessible after they were converted to clearance values. These values are confusing since higher clearance, which, in general, means a compound is excreted faster, means higher toxicity in Obach’s context.
The correlation between reactive metabolites and toxicity has been a major interest in the pharmaceutical industry due to a need to find a good screening method to implement during drug discovery in order to minimize losses incurred developing a toxic compound. Therefore those studies were conducted to predict if a compound is relatively safe or non toxic. Some consider this unrealistic since a final outcome of toxicity depends on so many factors as mentioned in Chapter I. Additionally, although the result, e.g. clearance or percent adduct formation (shown in Fig. 4 and 5, respectively) is adjusted or corrected with other factors such as glucuronidation or fraction absorbed, this scientific operation does not necessarily make the results more accurate. Firstly, the values of those adjustments also possess statistical errors (especially those data from \textit{in vivo} studies) which were not captured by the simple mathematical adjustment. Secondly, interaction effects among those parameters were not addressed or incorporated. It is believed that a model with integrated statistical tools such as a physiologically-based pharmacokinetic (PBPK) modeling is needed in this type of analysis.

As only a certain level of correlation coefficient can be expected from any correlation or prediction method, false negatives and false positives are also expected with the use of GSH as a trapping agent to study the correlation of reactive metabolites of compounds and their toxicity. The false negatives in this case could occur due to the limitation of GSH as only a soft nucleophile as well as other activation mechanisms not present in the microsomal system that was
used. The false positives, however, are the cases where we would like to have a better understanding.

Since there has not been a correlation study performed on xenobiotics from food additives and contaminants, this thesis will further explore the correlation of reactive metabolites and toxicity with these chemicals instead. Several differences between pharmaceutical compounds vs. natural food chemicals and environmental contaminants are noted (see Table 1). It is of our interest to further explore the correlation of reactive metabolites and toxicity by a simple expression as rate of adduct formation at (at least) two different concentrations.

Through our diet, we consume both natural and synthetic chemicals that are both useful and toxic. These are in the form of natural nutrients, natural toxicants, food additives, pesticide residues, and environmental contaminants. Many natural food chemicals are introduced into food to enhance the flavor (i.e. food flavorings) or preserve its appearance (i.e. antioxidants). These food additives which have shown evidences of hepatotoxicity will be used in our studies as a positive group. They are: anethole, pulegone, eugenol, and nordihydroguaiaretic acid (NDGA). Flavonoids, which are food nutrients, will be studied as a negative group. A food contaminant, bisphenol A, whose metabolism-based toxicity is unknown, will be examined utilizing the same analysis approach as those of known toxicity.

Bisphenol A (BPA), a monomer used in making polycarbonate plastics and epoxy resins, has generated high public interest due to its potential toxicity,
especially when considering its potential impact due to its wide usage and very
large production volume of over 6 billion pounds per year [Vandenberg et al.,
2009; Roy et al., 2009]. The major route of exposure of BPA is by ingestion, due
to its application in food containers (e.g. can liners, water bottles), while skin
absorption is also a route of exposure since toxicity due to skin absorption has
also been reported [Qiu et al., 2004]. Since BPA has estrogenic activity, most of
the toxicity studies have focused on understanding this pharmacological effect.
Observation of BPA pharmacokinetics and metabolism fate has also focused
primarily on the parent BPA and its major glucuronide metabolites [Pottenger et
al., 2000; Volkel et al., 2002; Snyder et al., 2000; Kurebayashi et al., 2002].
However, “minor” metabolites may not be considered trivial, since they may be
highly toxic, especially those which are very reactive. BPA contains phenyl
moieties which are known to be bioactivated to reactive metabolites [Kalgutkar et
al., 2005a]. Metabolism-derived toxicity of BPA has been only briefly explored. A
few reactive metabolites of BPA have been reported, but their toxicity potential is
not well understood.

B. Hypothesis

Compounds that can be metabolized by rat liver microsomal enzymes to
electrophilic derivatives can react with the fluorescent derivative of GSH, dansyl
GSH. The amount of dansyl GSH adduct formed will correlate with the toxicity of
the parent compound.
C. Specific aims

Several previous investigations have shown a correlation between protein covalent binding of reactive metabolites and liver toxicity of a compound [Rao, 1977; Evans, 1983]. This thesis will focus on analyzing the extent of adduct formation at 2 concentrations, representing low and high doses, and identifying factors which help explain deviant results. Adduct formation of acetaminophen will be studied as a reference compound. Then a group of natural food hepatotoxins will be examined for their reactive metabolites as well as the correlation with their toxicity. Adduct formation of flavonoids will also be explored. Finally, the reactive metabolites of bisphenol-A will be examined for their reactivity with dGSH, a fluorescent derivative of GSH. Therefore, specific aims of this dissertation are:

1. Evaluation of dansyl glutathione to study the rate of formation of reactive metabolites
   
   1a. Synthesize dansyl glutathione
   
   1b. Validate an analytical method to detect dGSH adducts using acetaminophen and pulegone as testing compounds

2. Study of reactive metabolites of food additives formed by rat liver microsomal enzymes using dansyl glutathione as a trapping agent and the correlation with their hepatotoxicity
2a. Identify, characterize, and quantitate dGSH adduct formation of acetaminophen, as a reference compound, in rat liver microsomes, including its non-hepatotoxic isomer N-acetyl-m-aminophenol

2b. Identify, characterize, and quantitate dGSH adduct formation by rat liver microsomes of hepatotoxic food additives: anethole, pulegone, eugenol, and NDGA

2c. Identify, characterize, and quantitate dGSH adduct formation by rat liver microsomes of non-hepatotoxic food nutrients: the flavonoids, which are quercetin, apigenin, naringenin, diadzein, genistein, and chrysin

2d. Examine the correlation of the adduct formation and their toxicity profiles from retrospective analysis

3. **Study of reactive metabolites of a food contaminant, bisphenol-A, formed by rat liver microsomal enzymes and their toxicity potential**

   3a. Identify, characterize, and quantitate dGSH adduct formation by rat liver microsomes of bisphenol-A

   3b. Evaluate bisphenol-A reactive metabolites per their toxicity potential using a positive and negative reference compound
Table 1: Summary of the differences of pharmaceutical vs. food and environmental contaminant compounds in terms of their nature of exposure and toxicity data available

<table>
<thead>
<tr>
<th>Nature of exposure</th>
<th>Pharmaceutical compounds</th>
<th>Food and environmental contaminant compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>• Doses are specified/specific</td>
<td>• Uncontrolled exposure, usually low doses, could be high under special circumstances (for environmental cpds) or if used as nutritional supplement (for natural food chemicals)</td>
</tr>
<tr>
<td></td>
<td>• Limited duration; accumulation results in acute toxicity closely associated to that one compound</td>
<td>• Long-term, could be accumulative; accumulation results in chronic and complex symptoms and could be contributed by other factors, difficult to trace the cause</td>
</tr>
<tr>
<td></td>
<td>• Doses are decided from safety and efficacy studies</td>
<td>• Safety margin calculated from NOAEL and estimated exposure</td>
</tr>
<tr>
<td>Study of toxicity in human</td>
<td>• Clinical studies of intended doses with controlled population are available</td>
<td>• Limited data, mostly with uncontrolled population</td>
</tr>
<tr>
<td></td>
<td>• Background level is zero</td>
<td>• Negative control group may not exist</td>
</tr>
</tbody>
</table>
III. EVALUATION OF DANSYL GLUTATHIONE TO STUDY THE RATE OF FORMATION OF REACTIVE METABOLITES

A. Introduction

Reactive metabolites, formed from metabolism of xenobiotics or endobiotics, are unstable species that are electrophilic in nature and will bind to matching nucleophiles in their vicinity. Hence, using a trapping nucleophile, the conjugated product that is formed can be easily detected and identified by MS. On the other hand, quantitation of the conjugated product is generally performed with a radioactive labelling of either the studied substrates or the trapping/binding species. However, tagging of the substrates has a few drawbacks. The tagging position must be carefully placed not to be on a possible leaving group as a result of metabolism. This means the substrate’s metabolism pathway must be known or speculated before the study. The labelling must be synthesized for each substrate. This could be expensive when the study involves a comparison among several substrates. Labelling the trapping agent, hence, is more convenient. Recently, dansyl glutathione (dGSH), i.e. glutathione labeled with a fluorescent dansyl tag, has been reported to be potentially useful as a trapping agent [Gan et al., 2005]. However, dGSH has not been utilized by any other research group. Since dGSH is not commercially available, dGSH was synthesized and used as a trapping agent to study reactive metabolites formed by food additives and contaminants in a microsomal metabolizing system.
B. Materials and methods

1. Materials

Dansyl chloride was purchased from Fluka. Acetone was from JT Baker. Diethyl ether, acetaminophen (APAP), pulegone, sodium tetraborate decahydrate, sodium carbonate, and all other chemicals were purchased from Sigma-Aldrich. Solvents were HPLC grade.

2. Synthesis of dansylated oxidized glutathione (d2GSSG)

The synthesis of dGSH consisted of 2 steps, i.e. the synthesis d2GSSG, followed by a reduction to dGSH. The synthesis used the procedures described by Gan et al. [2005]. One hundred and forty milligrams of dansyl chloride (0.52 mmol) in 2 ml acetone was added to 150 mg of oxidized glutathione (GSSG) (0.24 mmol) in 1.2 ml of 1 M NaOH. After the mixture was stirred for 30 min at room temperature, it was washed with diethyl ether (2 x 10 ml). The bottom aqueous phase was then examined by high performance liquid chromatography (HPLC) using a fluorescent detector. The effluents of the observed fluorescent peaks at different retention times (RT) were collected and subjected to mass spectrometry (MS), by an infusion method, to detect the peak with the mass m/z 1079, i.e. d2GSSG. The target mass was then separated from the aqueous phase using preparative HPLC. The collected HPLC effluent was then lyophilized to obtain a white powder.

Since the yield of d2GSSG was lower than expected, the synthesis was also carried out using other buffer systems in place of the 1M NaOH to examine
whether the yield could be improved. The buffers tested were: 1 M Tris, 1 M carbonate, and 0.1 M borate buffers.

3. Synthesis of dGSH

To obtain dGSH, 285 mg of the d2GSSG powder from the previous step was dissolved in 0.1 M Tris buffer, pH 8. The solution was deoxygenated by bubbling nitrogen through the solution for 10 min. To this solution was added 77 mg dithiothreitol (DTT), continuously stirring under nitrogen for 30 min, and finally adjusting the pH to 4 with acetic acid. The solution was injected onto the HPLC in an analytical mode and a distinct fluorescent peak was collected. To confirm the identification the m/z of 541 was measured by direct infusion MS. The solution was then concentrated under nitrogen and purified by preparative HPLC. The HPLC effluent at RT 14 to 15 min was collected and lyophilized to obtain a white powder.

4. HPLC analytical method

4a. Analytical HPLC method for detection of d2GSSG

HPLC analysis was performed with a Shimadzu LC-6A binary HPLC system equipped with a SIL-6B auto-injector, a SCL-6B system controller, and a RF-10A spectrofluorometer detector (excitation wavelength set at 340 nm and emission at 525 nm). An APEC ODS C18 5μ (4.6 mm x 250 mm) column was used with a flow consisting of mobile phase A, 0.1% trifluoroacetic acid in HPLC grade water and mobile phase B, 0.1% trifluoroacetic acid in methanol. The
gradient program was 10% B equilibration for 3 min, increasing to 50% B within 20 min, then increasing to 90% B within 17 min, continuing at 90% B for 4 min, decreasing to 10% B within 1 min, and continuing at 10% B for 5 min (total = 50 min). The flow rate was 1 ml/min.

4b. Preparative HPLC method for d2GSSG and dGSH purification

The same HPLC system as described above was used with a Phenomenex AQUA C18 21.2 mm x 150 mm, 5 micron particle size column. The gradient program consisted of mobile phase A, 0.1% trifluoroacetic acid, and mobile phase B, 0.1% trifluoroacetic acid in methanol. The flow program was 10% B equilibration for 4 min, increasing to 37% B within 5 min, then increasing to 64% B within 15 min, continuing at 64% B for 3 min, decreasing to 10% B within 3 min, and continuing at 10% B for 4 min (total = 34 min). The flow rate was 9.5 ml/min.

4c. Analytical HPLC method for dGSH adduct detection and quantitation

The same HPLC system as described above was used. A Zorbax Eclipse XDB C18 4.6 mm x 150 mm, 3.5 micron particle size column, and a C18 guard column, was used with a gradient program (mobile phase A, 0.1% formic acid in HPLC grade water, and B, acetonitrile) of 20% B equilibration for 3 min, increasing to 56% B within 30 min, continuing at 56% B for 0.5 min, decreasing to 20% B within 0.5 min, and continuing at 20% B for 6 min (total = 40 min). The flow rate was 1 ml/min.
5. Infusion MS and LC-MS analysis

MS analysis of the HPLC isolated peaks (described above) was performed at EOHSI Analytical Core facility. A Finnigan LCQ Deca Ion Trap Mass Spectrometer equipped with an electrospray ionization (ESI) source, a Finnigan MAT Spectra system AS3000 Autosampler, and a P4000 pump system, with LCQ tune software for direct infusion MS. The dGSH solution was injected at 3 \( \mu \text{l}/\text{min} \) through a syringe pump to optimize the parameters for ionization, which was then saved as a tune file and used in the LC-MS assay. The ESI conditions were: capillary voltage 3.0 V, capillary temperature 282 °C, tube gate -30 V, maximum inject time 200 ms, and ion spray voltage 2 kV. The same column and flow program used in the HPLC analysis was used for the LC-MS assay with XCalibur software. The analysis was performed under a full scan positive ion mode in the mass range of 150-2000 Da at collision energy 30% and isolation width 2 \( m/z \).

6. Microsomal incubation of APAP and pulegone

APAP and pulegone were dissolved (separately) in acetonitrile and each compound was incubated with liver microsomes from rats treated with phenobarbital (prepared by Cooper et al. [1993]). The final reaction (total volume 0.5 ml) consisted of microsomal protein 1 mg/ml, 100 mM phosphate buffer pH 7.4, 1 mM NADPH, 100 \( \mu \text{M} \) substrate, and 1 mM dGSH. The mixture without a substrate was pre-incubated for 3 minutes at 37°C in a shaking water bath, and the reaction was initiated by addition of the substrate. After 30 min, two volumes
of ice-cold methanol containing 5 mM DTT were added to the mixture to stop the reaction. The mixture was centrifuged at 14,000 rpm for 10 min and 50 μl of the supernatant was injected onto the HPLC. The fluorescent adduct peak areas were quantitated against an external calibration curve of dGSH, which was in the range of 0.1 to 5 nmol/ml.

7. **HPLC-fluorescence spectra of dGSH and dGSH adducts**

HPLC-fluorescence spectra of dGSH and dGSH conjugates were obtained using the same HPLC system with the spectrofluorometer detector set up in a scan mode. The sample injection was initiated as normal but the time program was pre-set for the flow to stop at the RT of the compound of interest. The emission spectrum was then recorded using an excitation wavelength of 340 nm and a scan speed of 1 nm per second.

C. **Results and discussion**

1. **Synthesis of d2GSSG**

Following the synthesis of d2GSSG as described by Gan *et al.* [2005], a mass m/z 1079 (Fig. 6) was identified associated with a fluorescent peak at RT 22 (Fig. 7). However, an additional distinct peak was also observed at RT 16.5 (Fig. 7), where the mass was identified as m/z 846, corresponding to a mass of one dansyl group and GSSG (d1GSSG). In addition, there appeared to be several other peaks, most likely were impurities, which could account for the low percentage of the d2GSSG peak compared to total peak areas. After
lyophilization of the HPLC effluent collected at RT 22, the white powder obtained was calculated to account for less than a 5% yield of d2GSSG. Therefore, an effort was made in order to reduce the formation of d1GSSG and increase purity, hence, reaction efficiency, or yield of d2GSSG.

The synthesis of dGSH is summarized in the following equations:

\[
\text{2 dansyl chloride + GSSG} \rightarrow \text{d2GSSG + 2HCl} \quad (1)
\]

\[
\text{d2GSSG + DTT} \rightarrow \text{2 dGSH + oxidized DTT} \quad (2)
\]

In forming 1 mole of d2GSSG, the reaction (1) also results in the formation of 2 moles of hydrochloric acid. The original method used 1.2 ml of 1 M NaOH, which is equivalent to 1.2 mmol, which is 5 times molar equivalent to the starting amount of GSSG (150 mg, 0.24 mmol) or 2.5 times molar equivalent to the hydrochloric acid produced. It was believed that NaOH was needed to quench the HCl and to keep the reaction in a basic pH to facilitate the forward reaction. It was found that although the pH of the GSSG/NaOH solution before the reaction started was approximately 14, the pH during and at the end of the reaction was below 7. Therefore, different buffer systems were explored to investigate the effect of their buffer capacities on the pH of the mixture. Tris (1 M), carbonate (1 M), and borate (0.1 M, due to limited solubility) buffers were used to replace NaOH. The results of the synthesis using these buffers were compared with respect to d2GSSG yield, the ratio of d2GSSG to d1GSSG, and peak areas of d2GSSG and dGSSG to the total peak area. The results are summarized in Table 2.
When different reaction conditions for the synthesis of d2GSSG, i.e. batch# 2, 8, 9, and 10, are compared (Table 2), it is found that although TRIS, borate, and carbonate buffers are less basic than NaOH, they have the capacity to hold the pH at a basic level during and to the end of the reaction. Although the reaction time of different buffer trials were not exactly equal, carbonate buffer resulted in the highest ratio of d2GSSG to d1GSSG as well as yield of d2GSSG, while borate buffer showed the least impurity peaks (highest peak area of d2GSSG and dGSSG). Given the purity profile, the reaction in borate buffer was re-tested over night. The buffer volume was increased to be 2 fold molar equivalent to hydrochloric acid. The result (batch# 11, Table 2) shows a yield of d2GSSG ca. 12 fold higher than that obtained with NaOH. Hence, borate buffer was selected to further examine the rate of formation of d2GSSG. Figure 8 shows the peak area of d2GSSG, dGSSG, and the ratio of d2GSSG to d1GSSG as a function of time when the reaction was carried out in borate buffer. All three parameters are relatively comparable at 6.5, 24, and 42 hours. Based on these results, d2GSSG was synthesized using 0.1 M borate buffer and a reaction time of 6.5 hours. Figure 9 shows a fluorescent HPLC chromatogram of dGSSG after purification.

In purifying the d2GSSG using preparative HPLC, there were instrument-associated parameters which were found to affect the yield as well. Firstly, for each injection there was a 10% loss (i.e. 10 µl for each 100 µl) of sample due to a drawing excess. Secondly, each column can be different in its loading capacity, i.e. the maximum volume (V_i) or mass (M_i) of a sample that could be fed into a
column. Peak broadening which is the sign of overloading was observed in this study. Sample loading above its capacity could affect peak resolution, which could further compromise peak purity. Effect of a column capacity on the yield is explained below.

Loading capacity of a column depends on both fixed parameters, i.e. a diameter and a length of the column, and variable parameters, i.e. a separation ratio (between two solutes), and a capacity ratio of the target solute (experimentally determined by the distance between the dead volume peak and the peak maxima (of the target solute) / the distance between the injection point and the dead volume peak). This means that the loading limitation of a compound is a result of the innate property of the compound itself and the combination of column/mobile phases used. However, overloading is indeed a common technique used to increase the sample amount per injection in preparative HPLC (for separation or purification purposes) [Scott, 2003].

As there are volume and mass overloading effects (see Fig. 10), the broadening of the d2GSSG peak in this study was further examined. For a volume overload, typical injection volumes for different sizes of column could be used as a guide. A general guideline from ACE® showed a recommended maximum injection volume (expressed as ml) and an estimated sample capacity (expressed in μg) for a 4.6 x 150 mm and a 21.2 x 150 mm column as < 0.040 ml and 80-800 μg, and < 1 ml and 2-200 mg, respectively. In the preparative mode in this study with a 21.2 x 150 mm column, the injection volume was limited to 100 μl, due to a limited size of the injector syringe. Hence, the overload due to
the volume was unlikely. The peak shape observed in this study was also more consistent with that of a mass overloading peak, i.e. a tall tailing peak. Therefore, the peak broadening during the purification of d2GSSG was most likely due to a mass overloading.

Since the overloading effect will result in reducing peak resolution, the effluent was manually collected only for one minute duration, omitting the tailing portion, thus, decreasing the overall yield.

The overall yield of the d2GSSG solid was calculated to be approximately 60%.

2. Synthesis of dGSH

The process of disulfide bond reduction of d2GSSG to dGSH by DTT was found to be straightforward. The final overall yield of dGSH solid was calculated to be approximately 40-50%. A mass spectrum of dGSH is shown in Fig. 11.

3. HPLC method development for analysis of reaction mixtures for dGSH synthesis

A summary of the method development for the isolation of d2GSSG and dGSH by preparative HPLC is shown in Scheme 1. The HPLC method used for d2GSSG by Gan et al. [2005] (Method A) was first tested in an analytical mode at 1 ml/min with an APEC ODS 4.6 x 250 mm column to support the study of d2GSSG synthesis reaction. Since the chromatogram of d2GSSG synthesized using NaOH showed that the reaction solution contained many impurities, a method (Scheme 1, Method B) with a longer run and a higher organic solvent
content was developed to ensure a better peak resolution and a complete elution of all impurities. After different buffers were tested for the reaction efficiency and borate buffer was selected, a shorter method (Scheme 1, Method C) was developed to save time and resources. This method was relatively reproducible (a small shift of RT) when applied to a preparative column (Scheme 1, Method D). The peaks of d2GSSG and dGSH were collected at RT 18 to 19 and 14 to 15 min, respectively.

4. **HPLC quantitation of dGSH adducts formed by APAP and pulegones in a microsomal system**

Pulegones and APAP were selected as model compounds to develop an analytical method for dGSH adduct quantitation. Pulegones was shown as one of the compounds for which its dGSH adduct eluted late (RT 29 min) in the original method [Gan *et al.*, 2005] while APAP showed its dGSH adduct to be poorly resolved from the peak of dGSH itself. Samples from rat liver microsomal incubations of both compounds were found to contain the corresponding adduct peaks when analyzed by the method used in Gan *et al.*’s paper. The analytical method was then modified to be shorter, 40 min compared to 50 min, while still able to capture the pulegones adduct peak (RT 33.6 min, data not shown) and provide an adequate resolution of the APAP adduct peak (RT 13 min), shown in Figure 12. The APAP adduct peak area was quantitated to be ca. 0.2 % (of the substrate concentration), using a standard curve of dGSH prepared with concentrations from 0.1 to 5 μM (Fig. 13). In comparison, the APAP adduct
formed by human liver microsomes reported by Gan et al. [2005] was found at 0.5%.

Fluorescence spectra of dGSH and dGSH conjugating to BPA (the preparation is described in Chapter V) were examined in an attempt to evaluate if the spectrophotometric characteristic of dGSH may change as a result of the conjugation with a reactive metabolite. However, no difference was observed between the emission spectra of dGSH and dGSH conjugates (data not shown).

D. Conclusions

- The synthesis of d2GSSG was developed as follows. A solution of dansyl chloride (280 mg in 5 ml acetone) was added dropwise to a solution of GSSG (150 mg in 10 ml of 0.1M borate buffer, pH 9). After stirring for 6 hr, the solution was washed with diethyl ether (2 x 6 ml). The d2GSSG in the aqueous phase was concentrated under nitrogen and then purified by preparative HPLC. The HPLC effluent at RT 18 to 19 min was collected and lyophilized to obtain a white powder of d2GSSG.

- The synthesis of dGSH was accomplished as follows. Two hundred and eighty-five mg of d2GSSG powder from the previous step was dissolved in 0.1 M TRIS buffer, pH8. The solution was saturated with nitrogen for 10 min. To this solution was added 77 mg DTT, while continuously stirring under nitrogen for 30 min, and finally adjusting the pH to 4 with acetic acid. The solution was then concentrated under nitrogen and purified by preparative HPLC. The HPLC effluent at RT
14 to 15 min was collected and lyophilized to obtain a white powder of dGSH.

- Preparative HPLC method for purifying d2GSSG and dGSH was developed (Scheme 1, Method D), with 100 µl sample injected for each run.

- The final yield of dGSH synthesized by the method in this study was ca. 40-50%.

- An analytical HPLC method for detecting and quantifying dGSH adducts was developed (details under Section 4c), with a dGSH standard curve constructed in the concentration range of 0.1 to 5 nmol/ml at 50 µl injection volume.
Table 2: Summary of effect of different buffer systems on the yield of d2GSSG

<table>
<thead>
<tr>
<th>Batch#</th>
<th>Buffer</th>
<th>Buffer conc. (M)</th>
<th>Buffer vol (ml)</th>
<th>Buffer pH</th>
<th>pH during reaction</th>
<th>pH at end of reaction</th>
<th>Reaction time</th>
<th>Peak area of d2GSSG (a)</th>
<th>Peak area of d1GSSG</th>
<th>Ratio of d2/d1</th>
<th>d2+d1/total peak area</th>
<th>Final total volume collected (b) (ml)</th>
<th>Total amount* of d2GSSG obtained (yield) (arbitrary unit)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NaOH</td>
<td>1</td>
<td>1.2</td>
<td>14</td>
<td>below 7</td>
<td>below 7</td>
<td>0.5 hr</td>
<td>34.3</td>
<td>45</td>
<td>0.8</td>
<td>65.2</td>
<td>1.2</td>
<td>4,116</td>
</tr>
<tr>
<td></td>
<td>Tris</td>
<td>1</td>
<td>1.2</td>
<td>8.8</td>
<td>n/a</td>
<td>~ 8</td>
<td>0.5 hr</td>
<td>19.5</td>
<td>11</td>
<td>1.8</td>
<td>82.5</td>
<td>1.2</td>
<td>4,680</td>
</tr>
<tr>
<td></td>
<td>Borate</td>
<td>0.1</td>
<td>5</td>
<td>8.9</td>
<td>8.6</td>
<td>8.6</td>
<td>1 hr</td>
<td>1.5</td>
<td>3.1</td>
<td>0.5</td>
<td>97</td>
<td>8</td>
<td>2,400</td>
</tr>
<tr>
<td></td>
<td>Carbonate</td>
<td>1</td>
<td>1.2</td>
<td>9.3</td>
<td>n/a</td>
<td>8.6</td>
<td>1 hr</td>
<td>22</td>
<td>4.2</td>
<td>5.2</td>
<td>54</td>
<td>1.2</td>
<td>5,280</td>
</tr>
<tr>
<td></td>
<td>Borate</td>
<td>0.1</td>
<td>10</td>
<td>8.9</td>
<td>7.7</td>
<td>8.25</td>
<td>over night</td>
<td>21</td>
<td>4</td>
<td>5.3</td>
<td>90.5</td>
<td></td>
<td>50,400</td>
</tr>
</tbody>
</table>

* Total amount is calculated from (a) x (b) and adjusted (divided by) with the volume of injection injected to obtain (a).
Figure 6: A mass spectrum of d2GSSG, showing m/z 1079, of a peak collected from HPLC effluent at RT 22 of a reaction mixture from d2GSSG synthesis. The result was obtained by direct infusion MS in a positive ion mode.
Figure 7: A representative HPLC-fluorescence chromatogram of a d2GSSG reaction mixture showing a d2GSSG peak at RT 22, a d1GSSG peak at RT 16, and other impurity peaks. This chromatogram was obtained using Method B in an analytical mode.
Figure 8: Kinetic profiles of d2GSSG synthesis in a borate buffer showing the ratio of d2GSSG/d1GSSG (diamond), peak area of (d2GSSG+d1GSSG)/total peak area (%) (triangle), and peak area of d2GSSG (square) as a function of time at 0.5, 6.5, 24, and 42 hours. The peak area of d2GSSG is expressed in million units.
Figure 9: A representative HPLC-fluorescence chromatogram of a purified d2GSSG solution. The spectrum was obtained from a 10 µl of the fraction solution collected from the purification of d2GSSG using the method described in Section 4c.
Figure 10: Examples of mass and volume overload characteristics and a representative HPLC-fluorescence chromatogram of a d2GSSG synthesis reaction solution. Top panels: Peak characteristics of a sample with a mass, or concentration, (left panel) or a volume (right panel) overloading, a common phenomenon in preparative HPLC. Left panel: A sample of different concentrations of benzene was injected at a constant volume, resulting in a peak broadening becoming a triangular shape. The retention time is usually shifted forward. Right panel: A sample mixture of compound A, B, and C was placed on a column at increasing injection volumes, resulting in a peak broadening becoming a rectangular yet symmetrical shape. Peak heights of the overloading volumes will be constant. [Scott, 2003] Bottom panel: A representative HPLC-fluorescence chromatogram of a d2GSSG synthesis reaction solution obtained from preparative HPLC, showing d2GSSG at a retention time ~ 18 min. The peak signal was cut-off at 1.2 volts.

(excerpted from Scott [2003])
Figure 11: A mass spectrum of dGSH, m/z 541, of the powder after purification and lyophilization dissolving in water/methanol (1:1)
Figure 12: A representative HPLC-fluorescence chromatogram showing peaks of dGSH adduct of APAP (arrow pointing) obtained in this study (top) to that of Gan et al. [2005] (bottom). The big peak to the left is the dGSH peak. The sample was from a rat liver microsomal incubation, while that by Gan et al. was from human liver microsomes.
Figure 13: A standard curve of dGSH constructed with 0.1, 0.25, 1, and 5 nmol/ml of dGSH dissolved in a solvent consisting of 1 part of phosphate buffer pH 7.4 and 2 parts of methanol. The injection volume was 50 μl. The corresponding peak areas were quantitated from HPLC-fluorescence chromatograms.

\[ y = 960533x \]

\[ R^2 = 0.9988 \]
# Scheme 1: Summary of preparative HPLC method development for purifying d2GSSG and dGSH

## Method A*

<table>
<thead>
<tr>
<th>Column:</th>
<th>Phenomenex Luna C18 (21.2 x 250 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate:</td>
<td>20 ml/min</td>
</tr>
<tr>
<td>Flow program:</td>
<td>Mobile phase A: 90% water/10% methanol/0.1% TFA</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>20</td>
<td>33</td>
</tr>
</tbody>
</table>

*A method from Gan *et al.* [2005]

## Method B

<table>
<thead>
<tr>
<th>Column:</th>
<th>APEC ODS C18 5μ (4.6 X 250 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate:</td>
<td>1 ml/min</td>
</tr>
<tr>
<td>Flow program:</td>
<td>Mobile phase A: 0.1% TFA</td>
</tr>
<tr>
<td>0</td>
<td>90</td>
</tr>
<tr>
<td>3</td>
<td>90</td>
</tr>
<tr>
<td>23</td>
<td>50</td>
</tr>
<tr>
<td>40</td>
<td>10</td>
</tr>
<tr>
<td>44</td>
<td>10</td>
</tr>
<tr>
<td>45</td>
<td>90</td>
</tr>
<tr>
<td>50</td>
<td>90</td>
</tr>
</tbody>
</table>

## Method C

<table>
<thead>
<tr>
<th>Column:</th>
<th>APEC ODS C18 5μ (4.6 X 250 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate:</td>
<td>1 ml/min</td>
</tr>
<tr>
<td>Flow program:</td>
<td>Mobile phase A: 0.1% TFA</td>
</tr>
<tr>
<td>0</td>
<td>90</td>
</tr>
<tr>
<td>4</td>
<td>90</td>
</tr>
<tr>
<td>9</td>
<td>63</td>
</tr>
<tr>
<td>24</td>
<td>36</td>
</tr>
<tr>
<td>27</td>
<td>36</td>
</tr>
<tr>
<td>30</td>
<td>90</td>
</tr>
<tr>
<td>34</td>
<td>90</td>
</tr>
</tbody>
</table>
Method D

<table>
<thead>
<tr>
<th>Column:</th>
<th>Phenomenex AQUA C18 5μ (21.2 X 150 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate:</td>
<td>9.5 ml/min</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Flow program:</th>
<th>Mobile phase A: 0.1% TFA</th>
<th>Mobile phase B: 0.1% TFA in methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>9</td>
<td>63</td>
<td>37</td>
</tr>
<tr>
<td>24</td>
<td>36</td>
<td>64</td>
</tr>
<tr>
<td>27</td>
<td>36</td>
<td>64</td>
</tr>
<tr>
<td>30</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>34</td>
<td>90</td>
<td>10</td>
</tr>
</tbody>
</table>
IV. STUDY OF REACTIVE METABOLITES OF FOOD ADDITIVES FORMED BY RAT LIVER MICROSONAL ENZYMES USING DANSYL GLUTATHIONE AS A TRAPPING AGENT AND THE CORRELATION WITH THEIR HEPATOTOXICITY

A. Introduction

Reactive metabolites are known for their ability to bind to macromolecules. Their irreversible binding to hepatic proteins is also known to lead to necrosis and liver damage [Evans, 1983]. However, a correlation between reactive metabolites and hepatotoxicity is still not yet clear [Liebler, 2008]. Several studies were reported with pharmaceutical compounds (Chapter II, Section A), but none was with natural food compounds. In this chapter, the extent of dGSH adduct formation during NADPH supported microsomal metabolism of natural food chemicals, which have also been used as food additives, that are hepatotoxic will be examined at 2 substrate concentrations, representing nominal low and high doses. The results will be interpreted with respect to their metabolism and toxicity profile. Acetaminophen will be tested as a positive reference compound. N-acetyl-m-aminophenol, non-hepatotoxic isomer of acetaminophen will be included as a comparison. A few non-hepatotoxic food compounds will also be studied for their dGSH adduct formation.

It is of note that GSH plays an extremely important role in the cellular defense against electrophilic metabolites. The study of GSH levels and/or GSH adducts associated with hepatotoxicity [Evans, 1983; Reid and Krishna, 1973] demonstrated the role of GSH in protecting liver from reactive electrophiles. The
subject of toxicity arising from the GSH conjugate itself which serves as a transport form of the reactive metabolite to remote tissues/organs is not explored in this thesis.

B. Materials and methods

1. Materials

Acetaminophen (APAP), (R)-(+) pulegone, trans-anethole, eugenol, nordihydroguaiaretic acid (NDGA), and \( N\)-acetyl-\( m\)-aminophenol (AMAP) and all other chemicals were purchased from Sigma-Aldrich. Genistein and daidzein were purchased from Indofine. DC protein assay kit was purchased from Bio-Rad Laboratories. Solvents were HPLC grade. Mazola corn oil was purchased locally. dGSH was prepared by the method described in chapter III, section D.

2. Preparation of rat liver microsomes

Adult male and female Long-Evans rats (approx. 200 g body weight) were used in this study. Two rats each were treated with 10 mg/ml \( \beta\)-naphthoflavone (\( \beta\)-NF) in corn oil at 40 mg/kg, 18.8 mg/ml phenobarbital (PB) in water at 75 mg/kg, or 5 mg/ml pregnenolone-16\( \alpha\)-carbonitrile (PCN) in corn oil at 25 mg/kg. Corn oil (5 ml/kg) was used for the vehicle-treated (control) male and female groups. The rats were injected intraperitoneally once a day for three days, and sacrificed on the fourth day. The livers were removed and transferred to ice-cold saline. The liver was blotted to remove excess saline, cut into pieces, and homogenized with 3 volumes ice-cold homogenizing buffer (0.05 M TRIS HCl,
1.15% KCl, pH 7.4). The homogenate was centrifuged at 9,000 x g for 20 min. The supernatant (S9) portion was transferred to ultracentrifuge tubes and centrifuged at ca. 185,000 x g for 90 min in a Beckman 50.2 Ti rotor. The subsequent supernatant (cytosol) was removed; the pellet was homogenized with ice-cold buffer (1.15% KCl, 10mM EDTA, pH 7.4), and centrifuged at the same speed for 60 min. The pellet was resuspended and homogenized in half volume of 0.25 M sucrose. The resulting microsomes were assayed for protein content by BioRad DC protein assay kit using bovine serum albumin as a standard, and stored at -80°C.

3. Microsomal incubation studies

All substrates were dissolved in acetonitrile, which was kept at less than or equal to 1.0% of the total incubation volume, i.e. 2 μl per 0.2 ml. Each substrate was incubated with NADPH and five different rat liver microsomes known to be enriched with different P450s. For these studies, the final reaction (total volume 0.2 ml) consisted of microsomal protein 0.25 mg/ml, 100 mM phosphate buffer pH 7.4, 1 mM NADPH, 100 μM or 1 mM substrate, and 1 mM or 5 mM dGSH, respectively. The mixture without substrate was pre-incubated for 3 minutes at 37°C in a shaking water bath, and the reaction was initiated by addition of the substrate. After 30 min, two volumes of ice-cold methanol containing 5 mM DTT were added to the mixture to stop the reaction. The mixture was centrifuged at 14,000 rpm (or 9800 x g) for 10 min, and the supernatant was analyzed by HPLC with fluorescence detection and then by LC-MS. Two control samples were
regularly included: one without the substrate and one without NADPH. The control without the substrate was used as a comparison to ensure that the peaks detected were substrate-associated adducts. The control without NADPH helped determine that the fluorescent peaks of interest were not formed by an auto-oxidation or a non-NADPH dependent metabolism.

In inhibition studies of pulegone, selected microsomal glutathione S-transferase (GST) inhibitors were pre-incubated with the reaction mixture without the substrate for 15 min before initiating the reaction. The inhibitors used were: 1-chloro-2,4-dinitrobenzene (CDNB) at 2 mM, naringenin at 200 μM, ellagic acid at 500 μM, quercetin at 500 μM [Yu, 2002], and the system of 50 μM FeCl₃/1 mM ascorbate [Letelier et al., 2010]. The concentrations were chosen at 10 times their IC₅₀.

4. Analysis of dGSH adducts

HPLC analysis was performed with a Shimadzu LC-6A binary HPLC system equipped with a SIL-6B auto-injector, a SCL-6B system controller, and a RF-10A spectrofluorometer (excitation 340 nm, emission 525 nm). A Zorbax Eclipse XDB C18 4.6 mm x 150 mm with 3.5 micron particle size column, and a C18 guard column, was used with a flow program (mobile phase A: 0.1% formic acid and B: acetonitrile) of 20% B equilibration for 3 min, increasing to 56% B within 30 min, continuing at 56% B for 0.5 min, decreasing to 20% B within 0.5 min, and continuing at 20% B for 6 min. The flow rate was 1 ml/min. The adduct
peak areas were quantitated against an external calibration curve of dGSH, in the range of 0.1 to 5 nmol/ml.

LC-MS analysis of the HPLC adduct peaks was performed at EOHSI Analytical Core facility: a Finnigan LCQ Deca Ion Trap Mass Spectrometer equipped with an electrospray ionization (ESI) source, a Finnigan MAT Spectra system AS3000 Autosampler, a P4000 pump system, and an XCalibur software. The same column and flow program used in the HPLC analysis was used for the LC-MS assay. The analysis was performed under a full scan positive ion mode in the mass range of 150-2000 Da. The ESI conditions of each substrate were tuned from a direct infusion of the substrates using LCQ tune software. A negative ion mode was also performed to confirm the parent ions (of the adducts) found in the positive ion mode. In some cases, the parent masses were further fragmented in MS$^2$ and MS$^3$ modes, with collision energy 30 % and an isolation width 2 m/z, to obtain conjugation site information.

C. Results

1. Identification and quantification of reactive metabolites of known hepatotoxic compounds

A group of hepatotoxic compounds was selected to study the reaction of their reactive metabolites with dGSH. trans-Anethole, pulegone, eugenol, and NDGA are food additives with evidence of reported hepatotoxicity (see references in Section D under their corresponding subheadings). APAP was selected as a reference/model compound for its wealth of information on its
metabolism to the reactive metabolite \(N\)-acetyl-\(p\)-benzoquinone imine (NAPQI) and its liver toxicity. AMAP was also included for its non-hepatotoxic isomer of APAP.

Dansyl GSH conjugates of the six compounds were identified and characterized. Their retention times, molecular masses, and the corresponding chemical compositions are summarized in Table 3. Their proposed structures, along with the parent molecules, are shown in Figure 14. Dansyl GSH adducts of APAP (dGS-APAP) and AMAP (dGS-AMAP) were eluted at the same RT, with a mass of 689 and 705, respectively. The mass 689 of the dGS-APAP adduct corresponds to a known GSH adduct, 3-(glutathion-\(S\)-yl)-APAP, reported earlier, where a GSH is conjugated to an ortho- position to the hydroxyl group through the reactive metabolite NAPQI [Nelson and Gordon, 1982; Potter and Hinson, 1987]. The mass 705 of the dGS-AMAP corresponds to a dGSH conjugate of a hydroxylated AMAP. Only one adduct peak of AMAP was found with rat liver microsomes in this study. However, in a study with mouse microsomes, two adduct peaks of mono GSH conjugates of AMAP were found [Rashed and Nelson, 1989], where all are structural isomers: dimers of 2-acetamidohydroquinones and 3’,4’-dihydroxyacetanilide. Additional studies are needed to determine whether the dGSH adduct of AMAP in this study is one of the hydroquinone or one of the o-quinone.

One dGSH adduct was found with trans-anethole. The mass of 704 is equivalent to a conjugation with an addition of an oxygen without a loss of two hydrogens. This suggests that dGSH most likely conjugated to the side chain
double bond, possibly via an epoxide precursor. This is consistent with Sangster's study [Sangster et al., 1984b], in which a mercapturate species of 2-hydroxy-1-methylthio-1-(4′-methoxyphenyl)propane was detected in their anethole metabolism study in rats and mice.

Two dGSH conjugates were found with pulegone. The mass of 688 is postulated to be a dGSH conjugate of menthofuran, one of pulegone's metabolites. The conjugation is proposed to be formed through an epoxidation [Khojasteh-Bakht et al., 1999]. The mass of 692, which is equivalent to an addition of dGSH to pulegone without a usual loss of two hydrogens in forming a new conjugated bond, suggests a 1,4-Michael Addition to the C8 (through the \( \alpha,\beta \)-unsaturated keto group). This conjugate is consistent with a S-conjugate metabolite (which was further hydroxylated) characterized by Chen [Chen et al., 2001]. This C8-S conjugate is indeed a dGSH adduct of a menthone.

A dGSH conjugate was found with eugenol; the MW of 702 is proposed to be an addition of dGSH to the allylic side chain, consistent with a finding by Thompson [Thompson et al., 1990]. The suggested regio-isomers of \( 1' \) and \( 3' \)-adducts were demonstrated characteristically in this study by a double-peak (not a split peak) in both fluorescence and MS chromatograms.

Two adducts were found with NDGA. The mass 840 is speculated to be a ring conjugation of dGSH. This adduct was also found in the absence of NADPH (to a much lesser extent), reflecting the reactivity of the parent molecule to undergo an autoxidation. The second adduct is a bis-conjugate of dGSH which exhibits a regio-isomer characteristic (shown as double peaks) (see Figure 15),
in a similar manner as those of eugenol. These two adducts of NDGA have never been reported previously.

The dGSH adducts of these compounds formed in rat liver microsomes incubation with 0.1 mM, a nominal value for low concentration, and 1 mM, a nominal value for high concentration, substrate concentration were quantitated and summarized in Table 4. Five different rat liver microsomes were used for metabolism studies of the substrates. They were prepared from adult rats treated with β-NF (NF-mx, male), PB (PB-mx, male), PCN (PCN-mx, female), and corn oil (as a control) (CM-mx for male and CF-mx for female). Since the amount of NDGA adducts formed was high, more than 10% of the initial substrate concentration at 0.1 mM in some of the microsomes tested, the incubation was not performed with the high concentration.

The dGSH adduct of APAP was formed the most by PCN-mx, at 0.5 and 0.8 nmol/min/mg protein at low and high concentration respectively, followed by NF-mx and PB-mx for both concentrations. The adduct formed at 1 mM APAP was ca. 1.6 to 2.7 fold that at 0.1 mM. Those formed with CM-mx and CF-mx was below 0.1 nmol/min/mg protein and relatively unchanged at both substrate concentrations.

Cytochrome P450 3A1 and 1A2 are known to be induced by PCN and β-NF respectively [Jan et al., 2006; Thomas et al., 1983], and these microsomes are known to catalyze the oxidation of APAP resulting in higher GSH adducts [Patten et al., 1993; Bessems and Vermeulen, 2001]. The effect of PB on hepatic GSH levels in rats has also been shown [van Bree et al., 1989]; pretreatment
with PB (followed by APAP administration) further reduced the GSH level to ca. 25% of those of the vehicle-control rats compared to the effect of APAP alone (ca. 65% of those of the control). Hence, the relative amounts of dGSH adduct of APAP formed by different microsomes in our study are consistent with different P450 isoforms known to play a role in its toxicity.

The dGSH adduct of AMAP was found to be unstable. Generally, the quantitation of a peak area was presented as an average of two injections of the same sample, where the two values were mostly within a 5% difference. However, the adduct peak observed in the first injection of AMAP was absent in the second injection (6-12 hrs apart). Therefore, the peak area of only the first injection is presented in parenthesis. These values are speculated to be an under-estimate.

The highest rate of anethole metabolism and dGSH adduct formation was by PCN-mx at ca. 0.1 nmol/min/mg protein at 0.1 mM, and increased to 0.3 nmol/min/mg protein at 1 mM substrate. With other microsomes, the adduct was negligible at 0.1 mM. At 1 mM anethole, the adduct formed with other microsomes at ca. 0.04 to 0.06 nmol/min/mg protein, and was absent with the CF-mx incubation. The role of P450 3A1, the major isoform expressed in microsomes from PCN-induced rats [Jan et al., 2006], as one of the major isoforms catalyzing the reactive metabolite of anethole has not been reported previously.

Microsomal metabolism of 0.1 mM pulegone in the presence of dGSH yielded two adducts but at very low levels; ca. 0.03 nmol/min/mg protein or
below. At 1 mM, however, a marked increased of 10 fold or more was observed with the menthone adduct formation (mass 692); 0.25 and 0.2 nmol/min/mg protein with PCN-mx and CM-mx, respectively, compared to 0.02 and 0.01 nmol/min/mg protein at 0.1 mM concentration. Formation of the menthofuran adduct (mass 688) at the high concentration also increased but to a much lesser extent, approx. 3 to 5 fold compared to those at the low concentration.

While adduct formation of the dGS-menthofuran was NADPH-dependent, that of the dGS-menthone was not; it was also found in the control sample without NADPH. Further investigation demonstrated that, however, the menthone adduct formation was catalyzed by other enzymes present in the microsomes since the adduct was absent in boiled microsomes or without the microsomes, shown in Figure 16. When chemicals which are known to be substrates or inhibitors of microsomal GST were pre-incubated with the PCN-mx, formation of the menthone adduct was inhibited the most by CDNB (85%), followed by quercetin (82%) and the system of Fe$^{3+}$/ascorbate (52%) (Figure 17). Ellagic acid and naringenin had only a minor effect.

The dGSH adduct of eugenol was formed at ca. 1 nmol/min/mg protein (with CF-mx) to as high as ca. 7 nmol/min/mg protein (with NF-mx). The adduct formation by all five microsomes was relatively unchanged at the two concentrations (Table 4).

The mono dGSH conjugate of NDGA was formed at very high levels with all microsomes tested, between 8-14 nmol/min/mg protein. Of this amount, approximately 1 nmol/min/mg protein of this conjugate was found in the control,
i.e. NADPH-independent. The bisconjugates was also formed at significant levels, between 0.4 to 5 nmol/min/mg protein.

2. Identification and quantification of reactive metabolites of known non-hepatotoxic compounds

Reactive metabolites of flavonoids, dietary compounds which have no report of hepatotoxicity, were examined with rat liver microsomal metabolism. Quercetin, genistein, daidzein, naringenin, apigenin, and chrysin (structures shown in Fig. 18) were incubated with the microsomes under the same conditions as those used for the hepatotoxic compounds. Characteristics and chemical compositions of the dGSH adducts found are summarized in Table 5, and the proposed structures of the conjugates are also shown in Figure 18.

Quercetin is a dietary flavonoid which has been studied most extensively for its antioxidant property. GSH conjugates of quercetin have also been examined extensively. Awad et al. [2002] found that quercetin formed GSH adducts at the 6- and 8- position. Given that the B ring of quercetin contains a catechol, the GSH conjugation on the A ring was proposed to occur through quinone methide intermediates [Awad et al., 2000]. The adducts were found in several cellular systems, i.e. mouse melanoma cancer cells B16F-10 [Awad et al., 2002; van der Woude et al., 2005]; human intestinal Caco-2 and hepatic Hep G2 cells [Walle et al., 2003]; and human promyelotic leukemia HL60 cell line [van der Woude et al., 2005]. However, Spencer found that GSH could also conjugate to the 2'- position, which was demonstrated in human dermal fibroblast cells [Spencer et al., 2003]. Spencer also suggested that the occurrence of the 6- and
8- conjugates vs the 2’- conjugate was condition-specific; the prior would be obtained by a reaction in a low pH environment while the 2’- conjugate would be obtained at a higher pH [Spencer et al., 2003]. However, the A ring conjugates, formed in an isomeric form, appeared to be unstable [Awad et al., 2003] while the B ring conjugate (2’- glutathionyl quercetin) appeared to be stable. Since dGSH conjugates of quercetin in this study showed the characteristics of both being an isomer (the double peaks) and unstable, we anticipated that they are of the 6- and 8- conjugates.

Genistein, daidzein, naringenin, and apigenin are flavonoids with a phenoxy B ring. Their dGSH conjugates, all of which have never been reported previously, are all in the hydroxylated forms. It is most likely that they are oxidized from a phenol to a catechol before conjugating to dGSH. In a rat liver microsomes study, the 3’,4’-dihydroxylated compound was also reported to be the major metabolite of flavonoids through their two main metabolic pathways: the hydroxylation and 4’-O-demethylation [Nielsen et al., 1998]. Both dGSH conjugates of genistein and naringenin in this study showed isomerisms.

Chrysin was the only flavonoid tested that did not show any dGSH adduct peak, although it is structurally related to apigenin. Studies of chrysin metabolism have shown conflicting results. In one study, chrysin was quickly hydroxylated to become apigenin in rat liver microsomes [Nielsen et al., 1998]. However, in other two studies, no oxidation product of chrysin was detected in either Caco-2 and Hep G2 cells [Galijatovic et al., 1999] (where a glucuronide and a sulfate conjugate were found) or in human liver microsomes [Otake and Walle, 2002].
in vivo, apigenin, the 4'-hydroxylated chrysin, was found in urine of male Wistar rats orally administering chrysin [Griffiths and Smith, 1972].

The amounts of dGSH adducts formed with different flavonoids at 0.1 mM substrate concentration with the rat liver microsomes are summarized in Table 6. As mentioned previously, dGS-quercetin was unstable (shown in parenthesis). Considering that these values would be under-estimation due to its instability, the results suggest that reactivity of quercetin with dGSH is much higher than that of other flavonoids tested, and that of APAP. The dGSH adducts of genistein, daidzein, and apigenin were formed below 0.03 nmol/min/mg protein. Naringenin was the flavonoid with the highest (stable) dGSH adduct formation, found at between 0.05 to 0.2 nmol/min/mg protein. In a study with rat liver microsomes, a hydroxylation of flavonoids was found to be mainly catalyzed by enzyme CYP1A [Nielsen et al., 1998]. However, in our study, flavonoid adducts were formed to a similar level by all different microsomes tested.

D. Discussion

1. Significance of different rat liver microsomes

Enzymatic activation is usually considered the first step toward drug or xenobiotic induced cell injury, and cytochromes P450 are responsible for most bioactivation reactions. Liver microsomes are a rich source of these enzymes, and the rat has proven a reliable species for toxicological studies. Rats are also known to exhibit marked sexual dimorphism among several cytochromes P450, which provides an advantage in examining xenobiotic toxicity differences
between males and females. Additionally, liver microsomes from rats treated with diverse inducers are known to be enriched with different P450s and such treatment may markedly modulate the toxicity of xenobiotics.

Five rat liver microsomes from both genders known to be enriched with different P450 enzymes were selected: adult vehicle-treated males (CM-mx) and females (CF-mx), adult males treated with β-NF (NF-mx) or PB (PB-mx), and adult females treated with PCN (PCN-mx). CYP2C11, 2C13, and 3A2 [Jan et al., 2006; Ghosal et al., 1996] are male specific enzymes, hence, known to express in CM-mx, while those expressed in CF-mx include CYP2C12 [Sundseth et al., 1992] and 2C7 [Bandiera et al., 1986]. NF-mx and PB-mx are highly enriched in CYP1A1/2 and 2B1/2, respectively [Thomas et al., 1983]; while treatment of female rats with PCN leads to a marked induction of P4503A1 [Jan et al., 2006].

2. **Dansyl GSH adducts of hepatotoxic compounds: a retrospective analysis**

In order to assess the significance of dGSH adduct formation obtained in this study, a retrospective analysis of the compounds’ metabolic pathway and toxicity profile/data pertinent to reactive metabolites was pursued.

Several factors are to be taken into consideration in this analysis and assessment. First, two substrate concentrations of 0.1 and 1 mM were selected for the conjugation studies of all hepatotoxic compounds in this thesis. This nominal low and high dose was chosen based mainly on the most common concentrations found in several studies. Examining the results of different
compounds at the same concentration allows for a fair comparison. However, more appropriate low and high doses of each compound could be quite different between a potent vs. a non-potent compound. A more realistic low dose, intended for the exposure dose, is also usually more challenging, compared to a high or toxic dose, in relation to a sensitivity of an analytical method and an experimental design. Second, there are roles of other P450 isoforms which are not included in our studies. This also includes the difference between human and rat/animal isoforms.

2a. APAP, the reference compound

APAP could be considered a model compound to be used as a reference with respect to the role of reactive metabolites in hepatotoxicity; a large number of studies have been published. Although APAP is a hepatotoxic compound, it is considered relatively safe, especially at the therapeutic dose. Thus, the measured endpoints at a low and a high dose of APAP could be particularly useful as a ballpark for a level that may not be toxic vs. one that maybe potentially toxic, respectively.

At a therapeutic dose in humans, APAP undergoes extensive glucuronide and sulfate conjugation, with less than 5% oxidation to the reactive metabolite NAPQI (see more detailed metabolic pathway in Figure 20). At higher toxic doses, however, the oxidation increases to 7-15% [Court et al., 2001]. Increased serum alanine aminotransferase (ALT) and decreased hepatic GSH, especially those in mitochondria, which are common parameters indicating liver damage,
were observed (in various lab animals and human) at the toxic doses of APAP [Bessems and Vermeulen, 2001].

In mice at a toxic dose of 250 mg/kg, GSH conjugates found in urine were present at 23% [Rashed et al., 1990]. Rashed et al. [1990] also found hepatic protein covalent binding at ~ 0.8 nmol/mg protein at 1 hour and 1.2 nmol/mg protein at 3 hours, while Myers et al. [1995] found it to be 1.82 nmol/mg protein at 2 hours. Administration of 500 mg/kg to mice found GSH adducts of APAP at 518 nmol/g liver, which is equivalent to about 0.2 nmol/min/mg protein (1 hour study, 45 mg microsomal protein/g liver), with an additional 9480 nmol/ml in bile [Dai et al., 2005]. In in vitro studies, Masubuchi et al. [2007] found the GSH conjugate at approx. 0.2 pmol/min/mg protein in both human and rat liver microsomes at 10 μM substrate, while Gan et al. [2005] found 0.5% dGSH conjugate with human liver microsomes at 50 μM substrate, which is equivalent to 8.7 pmol/min/mg protein (30 min incubation, 1 mg/ml protein).

Our studies showed dGS-APAP adduct to be formed in the range of less than 0.5 and less than 0.8 nmol/min/mg protein at 0.1 and 1 mM, respectively. There was approximately 1.6 to 2.7 fold increase in adduct formation at the high dose with three microsomes that formed the adduct the most: PCN-mx, PB-mx, and β-NF-mx. Specifically for APAP, the 0.1 mM substrate concentration is considered at a high end of its therapeutic dose [Laine et al., 2009], while the 1 mM concentration is considered at a low end of the toxic dose. In addition, effect of CYP2E1 which is the most active isoform at high dose [Bessems and Vermeulen, 2001; Nelson, 1995] was not examined in our studies. Hence, our
results appear to be reasonably consistent with others and thus may be used as a sensible reference value for comparing with other hepatotoxic and non-hepatotoxic compounds in this thesis.

2ai. **AMAP: a case of an unstable dGSH adduct**

AMAP is a non-hepatotoxic compound. At a toxic dose of 600 mg/kg in mice, it caused respiratory failure with no effect on plasma ALT levels. *In vivo*, the relative contribution of glutathione-related conjugates vs. glucuronide/sulfate conjugates was 5% vs. 84%, compared to 23% vs. 64% of APAP (at a dose of 250 mg/kg) (see Figure 19) [Rashed *et al.*, 1990]. However, a study of protein covalent binding did not show the difference to be of a similar extent; protein binding was found at 0.63 nmol/hr/mg protein compared to 0.91 nmol/hr/mg protein of APAP, for the dose of 600 mg/kg and 250 mg/kg, respectively [Myers *et al.*, 1995].

Interestingly, we found that the amount of the dGSH adduct formed by AMAP was much lower than that of APAP, although this result could be an under-estimate due to the instability of the adduct. However, the unstable adduct itself could be an explanation for its non-hepatotoxicity. The unstable GSH adduct could translate into an unstable covalent protein adduct. The instability of AMAP-protein adducts was observed by Myers *et al.* [1995] and was suggested as one of the reasons supporting its non-hepatotoxicity. The difference in critical and non-critical proteins to which reactive metabolites bind has been generally well-recognized in understanding the complex mechanism in toxicity [Bessems and Vermeulen, 2001], and especially in explaining the difference in toxicity.
outcome when the measured parameters were not significantly different. On the other hand, unstable GSH adducts are often linked with toxicity at distant tissues from liver [Monks and Lau, 1994].

2b. Anethole

trans-Anethole is a flavoring agent found as a major constituent in anise, fennel, and star anise with a temporary acceptable daily intake of 0-0.6 mg/kg [Vavasour, 1999]. Both anethole and eugenol (to be discussed in the next section) are allylbenzenes which have been shown to be hepatotoxic, while their structurally related compounds, i.e. safrole, estragole, and methyleugenol are hepatocarcinogenic [Tsai et al., 1994]. The 1'-hydroxylation metabolite of the latter group, which was further sulfate conjugated to become the genotoxic species [Rietjens et al., 2005], was not reported with trans-anethole and eugenol. Anethole has a no-observed-effect level (NOEL) value of 125 mg/kg based on a one year study in rats and is on the GRAS (Generally Recognized As Safe) list. Hence, although anethole is hepatotoxic, it is considered a relatively safe compound.

Anethole’s hepatotoxicity was observed in a 90-day rat study only at very high doses (600 and 900 mg/kg daily) and the 1’,2’-oxide was found to be the cytotoxic metabolite [Vavasour, 1999]. Studies in rats by Sangster et al. [1984b] at 0.05, 5, 50, and 1500 mg/kg dose range caused reduction (from 56% to 32%) of O-demethylation, considered a detoxification pathway, as dose increased; while 1’,2’-epoxidation, the toxification pathway, increased from 2 to 15% and 0.6 to 3.2%, detected as a 1’,2’-diol and a S-conjugate, respectively. The metabolism
pathway of anethole is summarized in Figure 20. Marshall and Caldwell [1996] found that cytotoxicity (expressed as % lactate dehydrogenase leakage), as well as depletion of glutathione, in rat hepatocytes was pronounced only at 5 mM and above, but not at 1 mM and below, even in the presence of cytosolic epoxide hydrolase inhibitor. Our results (Table 4) showed a low level of adduct formation, formed primarily by PCN-mx. The marginal increase of 0.1 to 0.3 nmol/min/mg protein adduct formation at 0.1 mM and 1 mM is also consistent with Sangster’s study in that the significant shift of metabolic pathways was only observed over the concentration range of a 30,000 fold difference. As a flavoring agent, exposure to anethole is expected to be at a very low level. Compared with the reference values of APAP, anethole is also considered “safer,” which is consistent with the current ADI and its safety profile.

2c. Pulegone

Pulegone has been used as a food flavoring agent, present at 1-2% in peppermint oil [WHO Food Additives Series 46]. The pennyroyal oil (essential oil of a plant in the mint genus), in which it constitutes 80-90% [Barceloux, 2008], is used as an insect repellent, as well as an abortifacient and emmenagogue in folk medicine [Goldfrank, 2006]. Pulegone and one of its metabolites, menthofuran, are known to be hepatotoxic. Evaluated as a flavoring agent, the NOEL value of pulegone is 440 μg/kg, with the estimated daily intake of 0.034 μg/kg/day and it is considered to be of no safety concern [WHO Food Additives Series 46]. On the other hand, consumption of 15-30 ml of pennyroyal oil can cause death, where hepatic necrosis was observed [Barceloux, 2008]. Metabolism of pulegone is
quite complex and more than 30 metabolites have been identified [Thomassen et al., 1991]. Of 14 metabolites from a F344 rat study (oral doses of 0.8, 8, and 80 mg/kg) [Chen et al., 2001] and another 14 metabolites from a IISc male rat study (oral dose of 250 mg/kg) [Madyastha and Raj, 1993], only 2 metabolites were common to both studies. Chen’s study found three species of a C8-S conjugate (see Figure 21), but the menthofuran metabolite was not detected, while that of Madyastha found the latter but not the former metabolites. Thomassen found that pulegone-induced liver toxicity was closely correlated with glutathione depletion, but that neither pulegone nor menthofuran caused this effect directly [Thomassen et al., 1990]. Hence, it was believed that a γ-ketoenol, 8-pulegone aldehyde, which was evidently trapped with semicarbazide, was instead the reactive metabolite more responsible for pulegone toxicity (see Figure 22) [Khojasteh-Bakht et al., 1999].

In our studies, the two dGSH conjugates of less than 0.03 and 0.3 nmol/min/mg protein at low and high dose, respectively, were mostly formed by PCN-mx and CM-mx. The low level at low dose seems to support its safety profile as a flavoring agent. Based on the toxicity reports in human [Anderson et al., 1996], it appears that the dose of pulegone that can cause hepatotoxicity is very high. The ten times increase at high dose suggests that pulegone could deplete GSH reservoir when the dose is increased. Thus, although the toxicity of 8-pulegone aldehyde may not be discounted, the role of the pulegone itself, as opposed to any other reactive intermediate, as a significant contributor to its hepatotoxicity might be worth consideration.
2ci. Pulegone: a case of non-NADPH dependent metabolism and dGSH conjugation catalyzed by microsomal GST

In *in vitro* metabolism studies, P450 enzymes, whether as a recombinant enzyme or in a sub-cellular fraction like microsomes, require NADPH as a co-factor for the oxidation process. In general, a control without NADPH is also tested to ensure that the catalytic reaction is NADPH dependent. A metabolite (observed as an HPLC peak) that is also present in this incubation mixture means it could be formed either by autoxidation or NADPH-independent enzymes and is usually disregarded.

The dGSH adduct of pulegone eluting at RT 27, or the menthone adduct, was found to be NADPH-independent (Fig. 16). However, due to the different amounts formed by different microsomes, this adduct was not likely to be formed by an autooxidation. Transformation of a substrate through an autooxidation would occur to a similar extent regardless of different types of microsomes. Formation of the menthone adduct was further suggested to be enzymatically catalyzed by its absence when tested without microsomes or with boiled microsomes (Fig. 16). Hence, possible NADPH-independent enzymes were investigated. One such enzyme is cyclooxygenase (COX), as it is known to co-oxidize many xenobiotics. However, COX is not generally localized in liver microsomes [Hodgson, 2010]. Another two NADPH-independent oxidation enzymes, i.e. aldehyde oxidase and monoamine oxidase, catalyze the oxidation of certain functional groups which, however, are not present in pulegone. Aldehyde oxidases reacts mainly with aldehydes and nitrogen-containing heterocyclic compounds, and also locate
mostly in cytosol [Obach et al., 2004]. Monoamine oxidases is a mitochondrial enzymes involving only primary, secondary, and tertiary amines [Beedham, 1997].

As a possible mechanism of dGS-menthone formation was examined, it was found that dGSH could react with pulegone directly since pulegone possesses an $\alpha,\beta$–unsaturated ketone moiety. $\alpha,\beta$–Unsaturated carbonyl compounds are known to react with glutathione either with or without the GST enzymes [Janzowski et al., 2003; Boyland and Chasseaud, 1970; Schultz et al., 2005; Bohme et al., 2009]. Schultz et al. [2005] showed that both steric and electronic factors however may play a role in the reactivity of a compound (see Figure 23). Since pulegone has two methyl groups on the $\beta$–carbon of the $\alpha,\beta$–unsaturated carbonyl system, this could result in reducing its reactivity towards GSH. Thus, it is not surprising that conjugation with GSH would be facilitated by catalyzing GST enzymes.

Along with the versatility of GSH and its role as a universal indicator of cell viability [Kranner et al., 2006], GST is considered one of the most important enzymes in oxidative stress response [Gong et al., 2006]. GST enzymes are categorized into 3 families (see Table 7). Those that are found in cytosol and mitochondria are soluble, while those found in microsomes are membrane-bound [Hayes et al., 2005]. Microsomal GSTs, mGST, now also known as Membrane-associated Proteins in Eicosanoid and Glutathione (MPEG), constitutes up to 3% of the microsomal protein [Zhang et al., 2004]. The best known substrate for GST enzymes in general is 1-chloro-2,4-dinitrobenzene (CDNB), however, arene
oxides, quinones, and $\alpha,\beta$–unsaturated carbonyls are also good substrates [Hayes et al., 2005].

In confirming the involvement of mGST for the menthone adduct formation, we have utilized CDNB as substrate, as well as known inhibitors of rat liver microsomal GST (the system of Fe3+/ascorbate) [Letelier et al., 2010] or inhibitors of GST in microsomes of Fall Armyworm (quercetin, naringenin, and ellagic acid) [Yu, 2002]. Based on the results (Fig. 17), it could be said that CDNB (85% inhibition) had a better affinity to mGST than pulegone while quercetin (83% inhibition) was also a good inhibitor of rat mGST, in addition to that of Fall Armyworm. Interestingly, the inhibition activity of Fe3+/ascorbate that was shown to be maximized at 65%, tested with Sprague-Dawley rats [Letelier et al., 2010] was found to be comparable, 52%, in our study as well. It is worth noting that dGSH was previously reported as not a substrate to GST [Gan et al., 2005].

Since the dGS-menthone adduct showed a steeper increase at high concentration than dGS-menthofuran, it would be interesting to study the correlation of this mGST-facilitating process with pulegone hepatotoxicity. In fact, the role of a menthone had been suspected, and it was studied and concluded to be non-toxic [McClanahan et al., 1989]. However, McClanahan et al. [1989] studied the protein covalent binding of a menthone compound directly. This does not represent the potential toxicity of the dGS-menthone adduct found in this study since the menthone does not possess a reactive $\alpha,\beta$–unsaturated ketone group.
2d. Eugenol

Eugenol is a major constituent in clove oil. It has been used as a flavoring agent with a temporary ADI 0-2.5 mg/kg. Use as a dental analgesic is also reported [Eugenol, WHO Food Additives Series 17]. Eugenol is extensively metabolized. In a study in human volunteers taking the dose of 150 mg, eugenol was found at less than 0.1% of the dose in urine; 50% was found as glucuronide and sulfate conjugates while 11% was a thiophenol (see Figure 24) [Fischer et al., 1990]. Hepatotoxicity of eugenol is closely related with GSH depletion [Thompson et al., 1991].

Several studies have shown eugenol to be a relatively safe compound, especially at low doses. In a 10-day rat study, oral doses given up to 1000 mg/kg resulted in no sign of hepatotoxicity [Sipes and Mattia, 2006]. In another study with up to 1% eugenol in diets for 19 weeks, there was no sign of any adverse effect. At higher doses: 1400 to 4000 mg/kg, considerable mortality including a change in liver size was observed in a 34 day study [Eugenol, WHO Food Additives Series 17]. \textit{In vitro} data, however, showed eugenol to be quite highly cytotoxic. Hutzler \textit{et al.} [2008] reported 13% and 60% cytotoxicity of freshly isolated rat hepatocytes at 0.3 and 1 mM eugenol after 4 hours. Approximately 0.5-0.7 nmol/min/mg protein GSH conjugate was formed in male rat liver microsomes at 1 mM eugenol [Thompson \textit{et al.}, 1990]. Thompson \textit{et al.} [1998] also found that eugenol reduced GSH levels significantly starting from a concentration as low as 10 μM and up to 1 mM, using cultured rat liver cells Clone 9. Although our results, showing high levels of dGS-eugenol adducts at
both low and high concentrations, are consistent with other \textit{in vitro} results, the discrepancy with \textit{in vivo} data requires further examination.

\textbf{2e. NDGA}

NDGA (Fig. 14) is a lignan, found as a main component in creosote bush, greasewood, and chaparral tea. In a pure form, NDGA has been used as an antioxidant of fats and oils but was removed from the GRAS list in 1968, due to lymph node and kidney toxicity in rat studies [Maga and Tu, 1995]. However, use of its natural forms remains and is perhaps becoming more popular. Creosote bush has been used for more than 40 indications in traditional medicine [Arteaga \textit{et al.}, 2005]. As chaparral has been increasingly used as a dietary supplement for its anti-inflammatory effect, reports of associated hepatic injuries have increased [Arteaga \textit{et al.}, 2005]. The ADI has not been set and there has been no study on metabolism of NDGA either in \textit{in vitro} or in animals. In \textit{in vitro} studies, NDGA was found to be highly toxic. At 0.1 mM, NDGA caused 80% loss of mouse hepatocyte viability [Lambert \textit{et al.}, 2002]. In a study with rat hepatocytes, the LC$_{50}$ was found to be 150 $\mu$M [Moridani \textit{et al.}, 2002]. However, with regards to its hepatotoxicity, the potency found in male CD-1 mice, measured as ALT levels, was in the order of gallic acid $<$ NDGA $<$ tannic acid $<$ epigallocatechin-3-gallate (a polyphenolic in green tea) [Galati \textit{et al.}, 2006]. Hence, the relationship of our results of NDGA and its published data appears to be similar to that of eugenol, i.e. our \textit{in vitro} results are consistent with other \textit{in vitro} results but in conflict with the \textit{in vivo} data.
3. Dansyl GSH adduct of flavonoids: a case of electrophiles vs. free radicals

Flavonoids are plants polyphenolic compounds. They are categorized into many subgroups according to the substitutions and the connection between benzene ring A and B (Fig. 18) [Heim et al., 2002]. There are more than 1000 flavonoid compounds and much research is still needed to understand their pharmacokinetics and pharmacological effects. However, flavonoids are considered to be very safe compounds [Galati and O'Brien, 2004]. In the context of this thesis, no hepatotoxicity has ever been reported with a group of flavones (chrysin, apigenin), flavonols (quercetin), flavanones (naringenin) or isoflavones (genistein, daidzein). Although flavonoids are well-known for their anti-oxidant activity, lack of direct clinical evidence on health benefits, in addition to many reports of their pro-oxidant effects [Galati and O'Brien, 2004], suggests that extensive research regarding their mechanism of action is still needed.

Due to the large number of compounds, the diversity among flavonoids of their properties and characteristics, hence, absorption and bioavailability, is vast. The pharmacokinetics of each flavonoid may differ when ingested from different sources of food. However, collectively speaking, flavonoids exist in food as O-glycoside forms. Upon ingestion, intestinal absorption of both glycosides and aglycone (free form) has been observed. Absorption as high as 52% of quercetin in the glycoside forms has been reported [Ross and Kasum, 2002]. Their extensive biotransformation includes hydrolysis of the glycosides to aglycones.
and degradation of the aglycones to phenolic acids by enterocytes, and phase II conjunction in the liver and enterocytes [Heim et al., 2002].

From our results, all 4 flavonoids studied, i.e. genistein, daidzein, naringenin, and apigenin (quercetin is excluded for its unstable adducts), showed low levels of dGSH adducts (Table 6) which is consistent with their safety profiles. The adduct quantity of naringenin, which is the highest of the 4 compounds, is less than or equal to that of APAP (Table 4) at the same concentration. Although this level is still considered safe, it is 3-10 fold higher than that of the other 3 flavonoids. This prompted us to investigate if the higher adduct level of naringenin may be related to a difference in its anti-/pro- oxidant properties compared with those of the others. This is especially relevant since GSH has an ability to be either a nucleophile, reacting with an electrophile, or a reductant, resulting in itself becoming oxidized and form GSSG [Rosen et al., 1984].

The dynamics between the 2 roles of GSH can be demonstrated as following. In acting as an anti-oxidant scavenging a free radical, a hydroxyl group (which for flavonoids is the one on the B ring) is the functional group providing an electron in this reaction [Heim et al., 2002]:

$$\text{F-OH} + \text{R}^* \rightarrow \text{F-O}^* + \text{RH}$$

(1)

The F-O' radical could react with GSH to form GS' (equation (2)) and the latter can form GSSG and superoxide anion radical, O$_2$' - , a reactive oxygen species (ROS), as shown in equations (3) and (4). Flavonoids may redox cycle
as indicated by equations (1) and (2), and thus produce ROS in the process, equation (3) and (4) [Galati et al., 1999].

\[ \text{F-O}^- + \text{GSH} \rightarrow \text{GS}^- + \text{F-OH} \]  

(2)

\[ \text{GS}^- + \text{GS}^- \leftrightarrow \text{GSSG}^- \]  

(3)

\[ \text{GSSG}^- + \text{O}_2 \leftrightarrow \text{GSSG} + \text{O}_2^- \]  

(4)

Galati et al. [1999] suggested that only a compound that possesses a higher one electron oxidation potential \( (E^\circ) \) than that of GSH will react with GSH in this manner. Hence, phenoxyln compounds which possess higher \( E^\circ \) values than that of GSH will oxidize GSH to GSSG, which would result in oxygen consumption in the process (equation (4)). On the other hand, catechol compounds which possess lower \( E^\circ \) will deplete GSH through the conjugation instead [Galati et al., 1999]. In the study by Galati et al. [1999], only GSSG levels and oxygen consumption were measured. An attempt to detect GSH conjugation was not successful. Therefore, to facilitate further understanding, data of the \( E^\circ \) values and extent of GSSG reported in the study by Galati et al. [Galati et al., 1999] and GSH conjugates (as dGSH) from our study of selected phenolic and catechol compounds are tabulated together in Table 8. It can be seen that an oxidation of apigenin, a phenol with higher \( E^\circ \) than GSH, resulted in relatively high level of GSSG and low level of dGSH adduct while that of quercetin, a catechol with lower \( E^\circ \) than GSH, yielded the opposite. However, naringenin with a phenolic B ring has a lower \( E^\circ \) value than that of GSH and showed relatively high levels of both GSSG and dGSH adducts during its oxidation. Although this is
a small set of examples, it appears that $E^\circ$ values may not be a good indicator of whether a compound will oxidize GSH or form a GSH adduct. As a matter of fact, the two processes are often shown as going through different mechanisms; the former is a one electron process, as shown by equation (1) to (4), while the latter is a two-electrons process, as illustrated by equation (5):

\[
\begin{align*}
\text{H}_3\text{C} & \text{-} \overset{\delta^-}{\text{O}} \overset{\delta^-}{\text{Cl}} \rightarrow \text{H}_3\text{C} & \text{-} \overset{\delta^-}{\text{C}} \overset{\delta^-}{\text{Cl}} & \rightarrow \text{H}_3\text{C} & \text{-} \overset{\delta^-}{\text{C}} \overset{\delta^-}{\text{Nu}} + \text{HCl} \\
\text{Nu-H} & \text{Nu-H} & \text{Nu-H}
\end{align*}
\]

(5)

Depending on the physico-chemical properties of a compound, and perhaps other factors, some compounds may have a tendency to become either a radical or an electrophile, while for some other compounds the result may be influenced by the environment. The latter can be demonstrated for eugenol. Microsomal incubation of eugenol in the presence of NADPH resulted in a considerable amount of GSH adduct (0.5-0.7 nmol/min/mg protein), as also shown in our study (as a dGSH adduct), and protein covalent binding (196 pmol/min/mg protein), as a result of two electron oxidation through a quinone methide [Thompson et al., 1990]. However, when eugenol was oxidized to be a phenoxy radicals by a horseradish peroxidase/H$_2$O$_2$ system, which facilitates a one electron oxidation, the process resulted in 65% of the GSH pool being converted to GSSG while only 1% was found in a GSH conjugate form [Thompson et al., 1989]. Therefore, it appeared that the dynamics between the
processes of generating GSSG vs. GSH adduct formation of antioxidant compounds are much more complicated than dependence on their $E^\circ$ values alone.

Another aspect which may contribute to the complexity of effects and interactions of a phenolic antioxidant compound is that in a biological environment it could also be hydroxylated to a catechol, which could possess different preferential metabolic pathways than its parent phenol. For apigenin, as well as daidzein and genistein, it could be that the hydroxylation was minimal and apigenin exerts its action through the phenoxy radical, while a hydroxylation of naringenin could be more extensive and its hydroxylated form may contribute more to the overall pharmacological/toxicological effect. Therefore, it is speculated that the higher adduct formation of naringenin compared to other flavonoids tested in this study might be a result of it higher hydroxylation.

4. Assessment of toxicity potential of compounds: standard or general values vs. reference values

To understand the relationship between reactive metabolites and their toxic effects, the quantitative measurements were performed consisting mainly of either protein covalent binding or glutathione conjugation [Ma and Subramanian, 2006]. Attempts to understand the predictability of these results for toxicity using different approaches was discussed earlier. Although the correlation has not been established, a so-called cautionary level was suggested if protein covalent binding (at 10 $\mu$M substrate with human liver microsomes) was found to be 50
pmol/hr/mg protein [Evans et al., 2004] or 200 pmol/hr/mg protein of thiol adduct (at 50 μM substrate with human liver microsomes) [Gan et al., 2009]. For a comparison with the present studies, the suggestions were 0.83 pmol protein covalent binding/min/mg protein and 3.33 pmol thiol adduct/min/mg protein, respectively.

However, in practice it is difficult to apply so-called “standard or general values” to other studies due to different experimental conditions, e.g. different substrate concentrations used, different sources of P450 enzymes (microsomes vs. hepatocytes; human vs. animals; etc.), etc. As a closest comparison, the dGS-APAP adduct was found at 8.7 pmol/min/mg protein at 50 μM with human liver microsomes [Gan et al., 2005], compared to 50-500 pmol/min/mg protein at 100 μM with rat liver microsomes (this study). Therefore, it would be more appropriate for each study to obtain its own reference values using a selected model compound, such as APAP, to compare with their compounds of interest. The amounts of dGS-APAP adduct obtained at both concentrations in this study have shown to be a good reference of toxicity potential when compared with other known toxic and non-toxic compounds.

5. Toxicity implications as derived from dGSH adduct studies of hepatotoxic dietary compounds

For hepatotoxic compounds, the results at low and high concentrations of APAP, anethole, and pulegone appear to correlate with their toxicity profiles. However, the high levels of eugenol and NDGA adducts suggested that both
compounds are highly toxic, which are not consistent with their in vivo data. Therefore, our in vitro results of eugenol and NDGA are considered as false positives.

On further analysis of the chemical structures of these compounds, it appears that they could be divided into two groups: a group which formed high dGSH adduct levels, consisting of APAP, eugenol, and NDGA, and a group which formed low adduct levels, consisting of anethole and pulegone. The chemical structures of the first group contain a phenoxy group, i.e. a hydroxylated benzene ring, while those of the latter group do not. A phenoxy group is a functional group that could either be oxidized by P450 enzymes or peroxidases, or directly undergo a phase II conjugation (see Figure 25) [Deshpande, 2002]. When comparing the metabolism pathways of APAP (Fig. 19) and eugenol (Fig. 24) with those of anethole (Fig. 20) and pulegone (Fig. 21), it is observed that the prior group has a phase II conjugation pathway concurrent with phase I oxidation while a phase II conjugation of the latter group occurred only after phase I oxidation. This difference is important for 2 reasons. First, when a glucuronidation, or a phase II conjugation, occurs after a functional group has been introduced to the parent molecule (i.e. so-called a subsequent conjugation) the role of this conjugation is essentially to facilitate an excretion of the metabolite. On the other hand, when a glucuronidation occurs in parallel (i.e. so-called a direct conjugation) to another oxidative pathway which results in a reactive metabolite, this conjugation could compete with the oxidation and is indeed playing a detoxification role. Second, as a result, an in vitro metabolism
study of a compound with a phase II conjugation as a detoxification will be more affected by the presence (or absence) of a phase II enzyme than that of a compound with a phase II conjugation as an excretion mediator.

To find more compounds which would support this observation, the compound set in the correlation study by Gan et al. [2009] was examined. The group of false positive, non toxic compounds with the thiol adduct values above 0.2%, consists of paroxetine, losartan, and raloxifene. As shown in Figure 26, all these three compounds contain the hydroxyl functional group which undergoes glucuronidation in \textit{in vivo}, concomitant with other oxidative pathways [Zhao et al., 2007; Alonen et al., 2008; Jeong et al., 2005]. The false positive results of these compounds may be changed to negative if phase II enzymes were present. A study of losartan by Stearns et al. [1992] further demonstrates the effect of phase II enzymes on this type of compounds. When metabolism of losartan in liver slices of rats was compared with those in monkeys and human, the ratios of metabolic products obtained by oxidation vs. glucuronidation pathways were different due to the different abundance between the phase I and phase II catalyzing enzymes in each species [Stearns et al., 1992].

Regarding the correlation study of protein binding and toxicity by Obach et al. [2008], since the results were expressed as a clearance, ranking of the compounds based on the extent of adduct formation was not feasible. This means that in the group of false negatives, compounds which may form insignificant levels of adducts are not known. Therefore, an analysis can not be performed, in addition to the fact that the end point is protein covalent binding
and not a direct trap of reactive metabolites as in the present study. However, strictly speaking in terms of the chemical structures, of the seven false positive compounds in the non-hepatotoxic group (see Fig. 4 and 27), only two compounds did not agree with this hypothesis: buspirone, a compound which does not contain a group that is readily conjugated by phase II reactions, and meloxicam, a compound which does contain a hydroxyl group but did not undergo a phase II conjugation [Davies and Skjodt, 1999]. Of the other 5 compounds, besides paroxetine and raloxifene, which were discussed during the analysis of Gan’s study above, diphenhydramine, propranolol, and simvastatin all undergo direct glucuronide conjugation [Breyer-Pfaff et al., 1997; Buch and Barr, 1998; Prueksaritanont et al., 2002].

It is interesting to note that paroxetine, which was tested in both studies, showed the highest covalent binding clearance value in Obach’s study [Obach et al., 2008] but showed the lowest dGSH conjugate formation in Gan’s study [Gan et al., 2009]. One possibility to explain this difference might be related to non-specific protein binding. Considering that Obach measured the covalent binding via radioactivity of the substrate, the non-specific binding of the substrate itself to the protein would add an artifact to the radioactivity of the reactive metabolites that covalently bind to the protein, hence, resulting in an over-estimate (as opposed to an under-estimate as mentioned in chapter I, section F) when no blank subtraction was performed.

Although the role of phase II conjugation as a detoxification pathway is well-known, it is proposed that only when it occurs as a direct conjugation,
concurrently with other phase I metabolism, that the conjugation could be considered as such. The phase II conjugation that occurs as a subsequent process after phase I metabolism is more considered as facilitating the excretion. Therefore, a diagram in Scheme 2 is proposed, demonstrating the situations where phase II conjugation behaves as either excretion facilitation (for R₁ compound type) or detoxification (for R₂ compound type). It is speculated that physico-chemical properties of a compound could be one of the factors which determine whether an R₂ compound will preferentially undergo an oxidation or a phase II conjugation. In an R₂b case, an in vitro study in the absence of phase II conjugation enzymes could result in a higher contribution of a phase I oxidation than that occurring in in vivo. Contribution of phase II conjugation is often cited, rather as a general principle, to explain the discrepancy of in vitro and in vivo results. When most compounds are excreted in phase II conjugated forms anyway, this explanation sometimes seems over simplified, considering R₁ compounds also undergo phase II conjugation. This analysis demonstrated that results from in vitro studies may not correlate with those from in vivo studies for certain compounds which exhibit high levels of phase I oxidation in vitro in the absence of phase II conjugation enzymes. Compounds with certain functional groups like a hydroxyl might have competing metabolic pathways between phase I oxidation and phase II conjugation, while others compounds without such functional groups would not.

This analysis helps understanding the high adduct levels of eugenol and NDGA since both compounds contain a hydroxyl group; their results at low and
high concentrations are probably higher than expected in the microsomal system. Therefore, it is concluded that studies with different substrate concentrations could provide additional insight regarding the potential toxicity of a compound. The data of both the extent of adduct formation and the difference between low and high concentrations provide results which appear to correlate with a compound's toxicity profile.

Since false positive results of GSH adduct formation could occur due to certain functional groups that can directly undergo phase II conjugation, and false negative result could also occur due to the limitation of oxidative enzymes present in microsomes, GSH conjugation studies may not serve as a quick prediction tool. However, this does not detract from an understanding of the potential toxicity of a compound.
Table 3: Characteristics of dGSH adducts formed by microsomal metabolism of selected hepatotoxic food additives, including those of APAP and AMAP

<table>
<thead>
<tr>
<th>Compounds</th>
<th>MW (Da)</th>
<th>Mass of the adduct found$^a$ (Da)</th>
<th>RT$^b$ (min)</th>
<th>Corresponding chemical composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>APAP</td>
<td>151</td>
<td>689</td>
<td>13.0</td>
<td>S+dGSH-2H</td>
</tr>
<tr>
<td>AMAP</td>
<td>151</td>
<td>705</td>
<td>13.0</td>
<td>S+dGSH-2H+O</td>
</tr>
<tr>
<td>t-Anethole</td>
<td>148</td>
<td>704</td>
<td>19.4</td>
<td>S+dGSH+O</td>
</tr>
<tr>
<td>Pulegone$^c$</td>
<td>152</td>
<td>692$^z$</td>
<td>27.3</td>
<td>S+dGSH</td>
</tr>
<tr>
<td></td>
<td></td>
<td>688</td>
<td>33.6</td>
<td>S+dGSH-4H</td>
</tr>
<tr>
<td>Eugenol</td>
<td>164</td>
<td>702</td>
<td>20.4/21$^*$</td>
<td>S+dGSH-2H</td>
</tr>
<tr>
<td>NDGA$^{#}$</td>
<td>302</td>
<td>840$^z$</td>
<td>26.3</td>
<td>S+dGSH-2H</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1378$^\wedge$</td>
<td>27/27.6$^*$</td>
<td>S+2dGSH-4H</td>
</tr>
</tbody>
</table>

$^a$ a double peak, representing regio-isomers, see text for details
$^z$ also found in the absence of NADPH
$^\wedge$ m/z in positive mode is 690
$^\#$ GSH adducts were not reported before
$^c$ first-time the two reactive metabolites reported together
$^a$ mass of the adduct was obtained from LC-MS; mass of dGSH is 540 Da
$^b$ an adduct was identified as an additional peak in an HPLC chromatogram of a sample obtained from an incubation of a substrate with rat liver microsomes in phosphate buffer pH 7, in the presence of NADPH and dGSH, compared to that without a substrate
Table 4: Rate of metabolic activation, or dGSH formation, of hepatotoxic food additives, including those of APAP and AMAP, using liver microsomes from rats treated with prototypical inducers of cytochrome P450. See details of experimental conditions and quantitation under section B.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Mass of the adduct (Da)</th>
<th>NF-mx*</th>
<th>PB-mx*</th>
<th>CM-mx*</th>
<th>PCN-mx*</th>
<th>CF-mx*</th>
</tr>
</thead>
<tbody>
<tr>
<td>at 0.1 mM substrate (nmol/min/mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APAP</td>
<td>689</td>
<td>0.18</td>
<td>0.19</td>
<td>0.08</td>
<td>0.49</td>
<td>0.05</td>
</tr>
<tr>
<td>AMAP</td>
<td>705</td>
<td>n.d.</td>
<td>(0.008)</td>
<td>n.d.</td>
<td>(0.015)</td>
<td>n.d.</td>
</tr>
<tr>
<td>Anethole</td>
<td>704</td>
<td>n.d.</td>
<td>0.007</td>
<td>0.007</td>
<td>0.111</td>
<td>n.d.</td>
</tr>
<tr>
<td>Pulegone</td>
<td>692</td>
<td>0.003</td>
<td>n.d.</td>
<td>0.010</td>
<td>0.017</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>688</td>
<td>0.011</td>
<td>0.027</td>
<td>0.013</td>
<td>0.003</td>
<td>0.004</td>
</tr>
<tr>
<td>Eugenol</td>
<td>702</td>
<td>6.67</td>
<td>3.50</td>
<td>2.15</td>
<td>2.32</td>
<td>1.53</td>
</tr>
<tr>
<td>NDGA</td>
<td>840</td>
<td>12.62</td>
<td>9.96</td>
<td>14.40</td>
<td>11.60</td>
<td>8.23</td>
</tr>
<tr>
<td></td>
<td>1378</td>
<td>2.71</td>
<td>5.38</td>
<td>1.53</td>
<td>4.79</td>
<td>0.43</td>
</tr>
<tr>
<td>at 1 mM substrate (nmol/min/mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APAP</td>
<td>689</td>
<td>0.48</td>
<td>0.33</td>
<td>0.10</td>
<td>0.79</td>
<td>0.07</td>
</tr>
<tr>
<td>AMAP</td>
<td>705</td>
<td>(0.04)^</td>
<td>(0.01)</td>
<td>n.d.</td>
<td>(0.09)</td>
<td>n.d.</td>
</tr>
<tr>
<td>t-Anethole</td>
<td>704</td>
<td>0.045</td>
<td>0.061</td>
<td>0.040</td>
<td>0.335</td>
<td>n.d.</td>
</tr>
<tr>
<td>Pulegone</td>
<td>692</td>
<td>0.015</td>
<td>0.027</td>
<td>0.200</td>
<td>0.249</td>
<td>0.090</td>
</tr>
<tr>
<td></td>
<td>688</td>
<td>0.043</td>
<td>0.037</td>
<td>0.130</td>
<td>0.044</td>
<td>0.051</td>
</tr>
<tr>
<td>Eugenol</td>
<td>702</td>
<td>7.19</td>
<td>3.43</td>
<td>1.97</td>
<td>2.14</td>
<td>0.99</td>
</tr>
<tr>
<td>NDGA</td>
<td>(not tested since very high levels were already detected at 0.1 mM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n.d. - not detected
^ values in parenthesis mean the adduct is unstable (see text for more details)
* NF-mx, PB-mx, and CM-mx represent liver microsomes from male rats treated with β-naphthoflavone, phenobarbital, and corn oil, respectively. PCN-mx and CF-mx represent liver microsomes from female rats treated with pregnenolone-16α-carbonitrile and corn oil, respectively.
Table 5: Characteristics of dGSH adducts of flavonoids formed by microsomal metabolism

<table>
<thead>
<tr>
<th>Compounds</th>
<th>MW (Da)</th>
<th>Mass of the adduct found(^a) (Da)</th>
<th>RT(^b) (min)</th>
<th>Corresponding chemical composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin</td>
<td>302</td>
<td>840</td>
<td>19.2/19.6(^*)</td>
<td>S+dGSH-2H</td>
</tr>
<tr>
<td>Genistein(^#)</td>
<td>270</td>
<td>824</td>
<td>19.1/19.4(^*)</td>
<td>S+dGSH-2H+O</td>
</tr>
<tr>
<td>Daidzein(^#)</td>
<td>254</td>
<td>808</td>
<td>17.1</td>
<td>S+dGSH-2H+O</td>
</tr>
<tr>
<td>Naringenin(^#)</td>
<td>272</td>
<td>826</td>
<td>20.3/21.2(^*)</td>
<td>S+dGSH-2H+O</td>
</tr>
<tr>
<td>Apigenin(^#)</td>
<td>270</td>
<td>824</td>
<td>21.0</td>
<td>S+dGSH-2H+O</td>
</tr>
<tr>
<td>Chrysin</td>
<td>254</td>
<td>n.d.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n.d. - not detected

\(^a\) a double peak, representing regio-isomers, see text for more details
\(^b\) GSH adducts were not reported before
\(^a\) mass of the adducts was obtained from LC-MS; mass of dGSH is 540 Da
\(^b\) an adduct is identified as an additional peak in an HPLC chromatogram of a sample obtained from an incubation of a substrate with rat liver microsomes in phosphate buffer pH 7, in the presence of NADPH and dGSH, compared to that without a substrate
Table 6: Dansyl GSH adduct formation (nmol/min/mg protein) of flavonoids by liver microsomes from rats treated with prototypical inducers of cytochrome P450 at 0.1 mM substrates. See details of experimental conditions and quantitation under section B.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Mass (Da)</th>
<th>NF-mx*</th>
<th>PB-mx*</th>
<th>CM-mx*</th>
<th>PCN-mx*</th>
<th>CF-mx*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin</td>
<td>840</td>
<td>(0.140)^a</td>
<td>(0.256)</td>
<td>(0.153)</td>
<td>(0.451)</td>
<td>(0.118)</td>
</tr>
<tr>
<td>Genistein</td>
<td>824</td>
<td>0.012</td>
<td>0.002</td>
<td>0.017</td>
<td>0.006</td>
<td>0.007</td>
</tr>
<tr>
<td>Daidzein</td>
<td>808</td>
<td>0.009</td>
<td>0.001</td>
<td>0.004</td>
<td>0.004</td>
<td>0.002</td>
</tr>
<tr>
<td>Naringenin</td>
<td>826</td>
<td>0.106</td>
<td>0.182</td>
<td>0.089</td>
<td>0.091</td>
<td>0.052</td>
</tr>
<tr>
<td>Apigenin</td>
<td>824</td>
<td>0.031</td>
<td>0.030</td>
<td>0.010</td>
<td>0.010</td>
<td>0.008</td>
</tr>
</tbody>
</table>

^ values in parenthesis mean the adduct is unstable (see text for more details)
* NF-mx, PB-mx, and CM-mx represent liver microsomes from male rats treated with β-naphthoflavone, phenobarbital, and corn oil, respectively. PCN-mx and CF-mx represent liver microsomes from female rats treated with pregnenolone-16α-carbonitrile and corn oil, respectively.
Table 7: Examples of substrates of human GST

<table>
<thead>
<tr>
<th>Family</th>
<th>Class, enzyme</th>
<th>Example of substrates or reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosolic</td>
<td>Alpha</td>
<td>benzo[a]pyrene diol epoxide, busulfan, chlorambucil, ethacrynic acid, 4-hydroxynonenal</td>
</tr>
<tr>
<td></td>
<td>Mu</td>
<td>styrene-7,8-oxide, 1-chloro-2,4-dinitrobenzene (CDNB), Prostaglandin H₂ (PGH₂) → PGE₂</td>
</tr>
<tr>
<td></td>
<td>Pi</td>
<td>acrolein, chlorambucil, ethacrynic acid</td>
</tr>
<tr>
<td></td>
<td>Sigma</td>
<td>PGH₂ → PGD₂</td>
</tr>
<tr>
<td></td>
<td>Theta</td>
<td>ethylene oxide, menaphthyl sulfate</td>
</tr>
<tr>
<td></td>
<td>Zeta</td>
<td>dichloroacetate, 2-chloropropionate</td>
</tr>
<tr>
<td></td>
<td>Omega</td>
<td>dehydroascorbic acid</td>
</tr>
<tr>
<td>Mitochondrial</td>
<td>Kappa</td>
<td>CDNB, Leukotriene A₄ (LTA₄) → LTC₄</td>
</tr>
<tr>
<td>Microsomal (MAPEG)</td>
<td>gp I, MGST2</td>
<td>CDNB, LTA₄ → LTC₄</td>
</tr>
<tr>
<td></td>
<td>gp I, FLAP</td>
<td>non enzymatic binding of arachidonic acid</td>
</tr>
<tr>
<td></td>
<td>gp I, LTC4S</td>
<td>LTA₄ → LTC₄</td>
</tr>
<tr>
<td></td>
<td>gp II, MGST3</td>
<td>CDNB, LTA₄ → LTC₄</td>
</tr>
<tr>
<td></td>
<td>gp IV, MGST1</td>
<td>CDNB, hexachlorobuta-1,3-diene</td>
</tr>
<tr>
<td></td>
<td>gp IV, PGES1</td>
<td>PGH₂ → PGE₂</td>
</tr>
</tbody>
</table>

(Modified from Hayes et al. [2005])
Table 8: Summary of the extent of GSSG and dGSH adduct formed as a result of oxidation of phenol and catechol compounds

<table>
<thead>
<tr>
<th>Compounds</th>
<th>One-electron redox potential $E^\circ$ (mV) at pH 7 (PhO$^-$/PhO$^{-}$)</th>
<th>GSSG formed ($\mu$M GSH equivalent)$^{a,c}$</th>
<th>dGSH adduct formed (nmol/min/mg protein)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compounds with phenolic structure:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenol</td>
<td>860</td>
<td>96±21</td>
<td></td>
</tr>
<tr>
<td>Apigenin</td>
<td>&gt; 1000</td>
<td>143±15</td>
<td>0.01</td>
</tr>
<tr>
<td>Naringenin</td>
<td>600</td>
<td>155±16</td>
<td>0.089</td>
</tr>
<tr>
<td>Compounds with catechol structure:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Catechol</td>
<td></td>
<td>4±1</td>
<td></td>
</tr>
<tr>
<td>Quercetin</td>
<td>398</td>
<td>5±2</td>
<td>(0.153)$^e$</td>
</tr>
</tbody>
</table>

Data collected from $^a$ Galati et al. [1999] and $^b$ current study (using the values of control male microsomes).

$^b$ oxidation system for dGSH adduct: male rat liver microsomes in phosphate buffer pH 7.4 at 37°C, 30 min, 100 $\mu$M substrate, 1 mM dGSH

$^c$ oxidation system for GSSG formation: horseradish peroxidase/H$_2$O$_2$ in Tris buffer pH 7.4 at room temperature, 30 min, 25 $\mu$M substrate, 400 $\mu$M GSH. [Galati et al., 1999]

$^d$ $E^\circ$ of GSH is 850 mV

$^e$ values in parenthesis mean the adduct is unstable (see text for more details)
Figure 14: Structures of hepatotoxic food additives used in this study, and their proposed dGSH conjugates, together with APAP and AMAP.

- Acetaminophen, APAP
- 3-Acetamidophenol, AMAP
- Trans-anethole
- Pulegone
- Menthone
- Menthofuran
Figure 15: HPLC-fluorescence chromatograms obtained from incubations of NDGA with rat liver microsomes. The mono dGSH adduct of NDGA peak at RT 26.3 min is also present, but to a lesser extent, in the samples without NADPH (the lower chromatogram), compared to that with NADPH (the upper chromatogram). The bis-conjugate double peaks are those shown at RT 27 and 27.6 min. The mass of the peak at RT 25 min was characterized by MS to be 932 Da. See details of experimental condition under section B.
Figure 16: Formation rate of dGS-menthone adduct formed by incubation of 1 mM pulegone with different rat liver microsomes in the presence (dark bars) or absence (light bars) of NADPH. The adduct was absent when the incubation was performed without microsomes (no microsomes) or negligible with boiled PCN-mx (PCN-mx, boiled). NF-mx, PB-mx, and CM-mx represent liver microsomes from male rats treated with β-naphthoflavone, phenobarbital, and corn oil, respectively. PCN-mx and CF-mx represent liver microsomes from female rats treated with pregnenolone-16α-carbonitrile and corn oil, respectively. See details of experimental conditions and quantitation under section B.
Figure 17: Formation of dGS-menthone adduct from microsomal metabolism of pulegone in the presence of different mGST inhibitors. Formation of dGS-menthone adduct, in the absence of NADPH, when liver microsomes (from female rats treated with pregnenolone-16α-carbonitrile) incubated with pulegone were preincubated with the different mGST inhibitors for 15 min. The rates of formation are expressed as % control, which is that of 1 mM pulegone incubation without the inhibitors. See details of experimental conditions and quantitation under section B.
Figure 18: Flavonoid structures and their proposed dGSH adducts
Figure 19: Metabolic pathways of APAP and AMAP in mice. Metabolism profiles of APAP and AMAP derived from metabolites found in urine of mice administered at a toxic dose of 250 mg/kg and 600 mg/kg, respectively. APAP was found as glucuronidate and sulfate conjugates (64%), as well as thioether conjugates (23%). Although its catechol and methoxy metabolites could become reactive, NAPQI is considered its major electrophile. Glucuronidation and sulfation of AMAP and its hydroxylate and methoxylate metabolites was found at 84%. The reactive metabolites of AMAP that could react with GSH (found at 5%) or proteins were formed only after a hydroxylation, in comparison to NAPQI of APAP which was directly oxidized from the parent molecule. [Rashed et al., 1990]
Figure 20: Metabolic pathways of anethole, proposed by Sangster obtained from a study with female albino Wistar rats and male CD-1 mice, administered at 50 mg/kg orally and intraperitoneally, respectively. Eleven metabolites were identified. Three major pathways are: 1) O-demethylation which gave rise to 4-hydroxyprophenylbenzene and CO₂, which accounted for 42% of the dose in rats and 47% in mice. 2) Side chain epoxidation resulting in a thiol conjugate of 2-hydroxy-1-methylthio-1-(4′-methoxyphenyl)propane and other metabolites. 3) Side chain hydroxylation. The major metabolites found in urine were the two isomers of 1,2-diol, hippuric acid, and the thiol metabolites. The majority of 4-hydroxyprophenylbenzene, 4-methoxycinnamyl alcohol, 4-methoxylacetophenone, 4-methoxyhippuric acid, 1,2-diol, and the thiol metabolites were found as glucuronide conjugated forms. [Sangster et al., 1984b]

(excerpted from Sangster et al. [1984b])
Figure 21: Metabolic pathways of pulegone derived from metabolites found in urine obtained from F344 rats orally administered up to 80 mg/kg pulegone. The pathway a is a hydroxylation of pulegone at different positions followed by glucuronidation. The pathway b is a reduction of the carbon-carbon double bond to become menthone/isomenthone which were further hydroxylated and glucuronidated. The pathway c is Michael addition of GSH to S-conjugates of menthone/isomenthone. Hydroxypulegone glucuronides and hydroxy menthone/isomenthone glucuronides were the major metabolites, while the S-conjugates were found at 9-13%. [Chen et al., 2001]
Figure 22: An oxidation pathway of pulegone to menthofuran and its subsequent metabolic transformation to minlactone/isominlactone. A hydroxylation of C9 yields a menthofuran which could further oxidized to minlactone/isominlactone. Glutathione reacts with menthofuran through the epoxide intermediate. The proposed intermediate γ-ketoenal was confirmed by a trapping agent semicarbazide.

(modified from Khojasteh-Bakht et al. [1999], Madyastha and Raj [1993], and [WHO Food Additives Series 46])
Reactivity of $\alpha,\beta$-unsaturated ketone compounds with GSH. $\alpha,\beta$-Unsaturated ketones can react with GSH through a Michael addition. Reactivity of ketone compounds with GSH was studied by incubating different concentrations of the ketones with 1.375 M GSH in a phosphate buffer, pH 7.4 at room temperature. At the end of 2 hrs, the free thiol groups was quantitated by reacting with 5,5'-dithio-bis(2-nitrobenzoic acid), and measuring the absorbance at 412 nm. Series A shows a compound with the C-C double bond located at the terminal is much more reactive that that of the internal placement, 4-hexen-3-one, which is much more reactive than 5-hexen-2-one (which the C-C double bond is not an $\alpha,\beta$-unsaturated bond). Series B shows reactivity of 4-hexen-3-one was significantly reduced with a methyl substitute acting as a steric hindrance. The substitution has more effect (becoming less reactive) on the $\beta$-position (4-methyl-3-penten-2-one) since it is the site of reacting with GSH. [Schultz et al., 2005]

**Series A**

![Chemical structures](image)

**Series B**

![Chemical structures](image)
Figure 24: Metabolic pathways of eugenol, studied in human volunteers by Fischer et al. [1990] taking 150 mg oral dose. The major pathway was glucuronide/sulfate conjugation. The epoxide-diol pathway, glutathione conjugation, and propionic acid were found at 13%, 10%, and 5% of the dose, respectively. Other minor metabolites were double bond migration and reduction products.

(modified from Fischer et al. [1990])
Figure 25: Examples of compounds containing functional groups capable of forming glucuronide conjugates

- Benzyl glucuronide (alcohol type)
- Benzoyle glucuronide (carboxylic acid type)
- Phenyl glucuronide (phenol type)
- Thiophenol glucuronide (thiol type)
- 4-Hydroxycoumarin glucuronide (enol type)
- Aniline glucuronide (amine type)
- Sulfathiazole-N-glucuronide (sulfonamide type)
- N-Hydroxy-2-acetaminofluorene glucuronide (hydroxylamine type)
- Meprobamate glucuronide (carbamate type)

(excerpted from Deshpande [2002])
Figure 26: Chemical structures of compounds tested in Gan’s study with detectable adducts but are not associated with drug-induced toxicity. Of 40 drugs with no toxicity background, 10 drugs were found positive for dGSH adducts (false positive) (above LOQ). Of these 10 compounds, adduct formation of risperidone, duloxetine, simvastatin, and rosiglitazone was considered negligible (below 0.2%). Three (omeprazole, lansoprazole, montelukast) out of the six compounds that showed significant adduct formation was excluded from the final analysis since their adducts were also formed without NADPH. Paroxetine, raloxefine, and losartan contain functional groups that undergo concurrent phase I oxidation and phase II conjugation.

(modified from Gan et al. [2009])
Figure 27: Chemical structures of non-hepatotoxic compounds tested in Obach's study that covalently bound to protein when incubated with human liver microsomes. Of the 9 non-hepatotoxic drugs, ibuprofen and theophylline gave negative results. With the exclusion of simvastation (showed negligible thiol adduct (see Fig. 26)) and meloxicam (no phase II conjugation), 4 out of the rest 5 compounds contain functional groups that undergo concurrent phase I oxidation and phase II conjugation.

(modified from Obach et al. [2008])
Scheme 2: A diagram showing differential kinetics between phase I oxidation and phase II conjugation. Xenobiotics, themselves, and their metabolites could be found circulating in the body or excreted as phase II conjugates. However, it is observed that the conjugation plays a slightly different role for different compounds. In this proposal, compounds are categorized into 2 groups: those with functional groups that could conjugate directly to phase II (type R$_2$) and those that do not (type R$_1$). In order for a R$_1$ compound to undergo a phase II conjugation, it must be oxidized and a polar functional group, e.g. a hydroxyl, is introduced. This “subsequent” conjugation makes it non-competing to the phase I oxidation step. On the other hand, a R$_2$ compound contains already the functional group which could undergo phase II conjugation in simultaneous to phase I oxidation. Thus, both processes could become competing. Based on the chemical structure and other factors, a compound may have a preferential for phase I oxidation (R$_{2a}$) or phase II conjugation (R$_{2b}$). For a R$_{2b}$ compound, the absence of phase II enzymes could yield a misleading result with respect to the extent of phase I oxidation.
V. STUDY OF REACTIVE METABOLITES OF BISPHENOL-A, A FOOD CONTAMINANT, FORMED BY RAT LIVER MICROSOMAL ENZYMES AND THEIR TOXICITY POTENTIAL

A. Introduction

Reactive metabolites, such as electrophiles and free radicals, are known to react with cellular macromolecules such as proteins, DNA, and lipids, and/or produce oxidative stress. This results in cytotoxicity, genotoxicity, and/or formation of neoantigens which trigger immunological responses [Yang et al., 2006; Kalgutkar et al., 2005a; Caldwell and Yan, 2006]. These metabolites are often not detected in blood or urine samples, but their formation can be followed \emph{in vitro} by trapping agents, protein covalent binding studies, enzyme inhibition studies, or oxidative stress related events [Caldwell and Yan, 2006; Nassar and Lopez-Anaya, 2004]. Although a relationship between the amount of protein-covalent adduct formation and the extent of organ toxicity is not always correlated since not all protein covalent binding leads to toxicity [Yang et al., 2006; Evans et al., 2004; Obach et al., 2008], nevertheless, it is well accepted the higher the extent of covalent binding the greater the potential risk for toxicity [Obach et al., 2008; Evans et al., 2004].

The liver, because of its central role in xenobiotic metabolism, is the target organ most commonly linked with toxicity from reactive metabolites [Caldwell and Yan, 2006; Zhou et al., 2007]. Hepatotoxicity is often reported along with reduction of glutathione (GSH) levels, especially in mitochondria [Bessems and
Vermeulen, 2001]. The toxicological outcome of the reactive metabolite, however, depends on many factors: (1) dose or exposure; (2) pharmacokinetic profile; (3) the reactivity, stability, and extent of the reactive metabolite formed; (4) detoxification pathways; (5) microenvironment effect; and (6) significance of its biochemical consequences [Kumar et al., 2008; Yang et al., 2006; Chiba and Pang, 1995]. As a result, assessment of a compound's toxicity requires both qualitative and quantitative measures.

Glutathione is an endogenous thiol-containing peptide, whose conjugation with electrophiles is considered a major detoxification pathway, preventing them from binding to biological macromolecules [Evans et al., 2004; Rashed et al., 1990]. The conjugation products, often present in mercapturic acid form, are found both in bile [Baillie and Kassahun, 1994] and urine. The extent of reactive metabolite formation was studied by measurement of GSH conjugation or protein covalent binding [Masubuchi et al., 2007]. While quantitation of protein adducts is mainly possible with the use of radioactive chemicals, a non-radioactive quantitative method for GSH conjugation was evaluated by Gan et al. [2005], utilizing a dansyl group as a fluorescent tag on GSH. Correlation of GSH conjugation rate and protein covalent binding rate was previously shown [Masubuchi et al., 2007].

Bisphenol A (BPA) (see Fig. 35 for its structure), a monomer used in making polycarbonate plastics and epoxy resins, has generated high public interest due to its potential toxicity, especially when one considers the impact of over 6 billion pounds produced per year [Vandenberg et al., 2009; Roy et al.,
The major route of exposure to BPA is by ingestion, due to its application in food containers (e.g. can liners, water bottles), while toxicity via skin absorption was also reported [Qiu et al., 2004]. A BPA toxicokinetic study in humans at the dose of 5 mg/person demonstrated that the major metabolite in plasma and urine was the glucuronide conjugate [Volkel et al., 2002]. In rats, however, both Snyder et al. [2000] and Pottenger et al. [2000] found feces to be the primary route of excretion with free BPA as the major species, while the glucuronide was the major metabolite in urine. Pottenger et al. [2000] also concluded that the low bioavailability of BPA via the oral route was due to first pass metabolism, not poor absorption. Based on the estrogenic activity of BPA, most of the toxicity studies have been focused on understanding this pharmacological effect [Vandenberg et al., 2009]. Studies of BPA pharmacokinetics and metabolic fate focused primarily on the parent compound and its major glucuronide metabolites [Pottenger et al., 2000; Volkel et al., 2002; Snyder et al., 2000; Kurebayashi et al., 2002]. However, “minor” metabolites may not be considered trivial, since they could be quite toxic. BPA contains phenyl moieties which are known to be bioactivated to reactive moieties [Kalgutkar et al., 2005a] but metabolism-derived toxicity of BPA has been only briefly explored. A few reactive metabolites of BPA have been reported [Jaeg et al., 2004], but their toxicity potential is not well understood. In our studies of BPA metabolism using rat liver microsomes, there was a focus on detecting, characterizing, and quantifying reactive metabolites, using fluorescent dansyl glutathione (dGSH).
The P450 enzymes responsible for forming the reactive metabolites were also examined.

B. Materials and methods

1. Materials

Bisphenol A (BPA), acetaminophen (APAP), and all other chemicals were purchased from Sigma-Aldrich. DC protein assay kit was purchased from Bio-Rad Laboratories. Solvents were HPLC grade. Mazola corn oil was purchased locally. MAb C8, MAb B46, and PAb were prepared as described by Thomas et al. [1984], Reik et al. [1985], and Cooper et al. [1993], respectively.

2. Synthesis of dGSH

The synthesis of dGSH was adapted from Gan et al. [2005] as described previously (chapter III, section D). Briefly, in the first step of synthesizing dansylated oxidized glutathione (d2GSSG), a solution of dansyl chloride (280 mg in 5 ml acetone) was added dropwise to a solution of oxidized glutathione (GSSG) (150 mg in 10 ml of 0.1M borate buffer). After stirring for 6 hours, the solution was washed with diethyl ether (2 x 6 ml). The d2GSSG in the aqueous phase was concentrated under nitrogen and then purified by preparative high performance liquid chromatography (HPLC). The HPLC effluent at retention time (RT) 18 to 19 min was collected, analyzed by MS (positive mode) to confirm the mass of m/z 1079, and lyophilized to obtain a white powder of d2GSSG. In the second step of reducing d2GSSG to dGSH, a solution of d2GSSG (285 mg in 10
ml 0.1M Tris buffer, pH 8) was saturated with nitrogen for 10 min. To this solution was added 77 mg dithiothreitol (DTT), while continuously stirring under nitrogen for 30 min, and finally adjusting to pH 4 with acetic acid. The solution was concentrated under nitrogen and then purified by preparative HPLC. The HPLC effluent at RT 14 to 15 min was collected, analyzed by MS to confirm the mass of m/z 541, and lyophilized to obtain dGSH white powder.

Preparative HPLC was performed with a Shimadzu LC-6A binary HPLC system equipped with a SIL-6B auto-injector, a SCL-6B system controller, and a RF-10A spectrofluorometer for fluorescent detection with excitation wavelength 340 nm and emission wavelength 525 nm. A Phenomenex AQUA 21.2 mm x 150 mm with a 5 micron particle size column was used with a flow program (mobile phase A: 0.1% trifluoroacetic acid, and B: 0.1% trifluoroacetic acid in methanol) of 10% B equilibration for 4 min, increasing to 37% B within 5 min, then increasing to 64% B within 15 min, continuing at 64% B for 3 min, decreasing to 10% B within 3 min, and continuing at 10% B for 4 min. The flow rate was 9.5 ml/min.

3. Preparation of rat liver microsomes

Microsomes previously prepared from adult male and female Long-Evans rats (see Chapter IV, Section B2) were used in the present studies.

4. Microsomal incubation studies

The substrates were dissolved in acetonitrile, which was kept at less than or equal to 1.0% of the total incubation volume, i.e. 2 μl per 0.2 ml. Each
substrate was incubated with NADPH and five different rat liver microsomes known to be enriched with different P450s. The dGSH adduct peaks were then detected and quantitated by HPLC with fluorescence detection. For these studies, the final reaction (total volume 0.2 ml) consisted of microsomal protein 0.25 mg/ml, 100 mM phosphate buffer pH 7.4, 1 mM NADPH, 100 μM substrate, and 1 mM dGSH. The mixture without substrate was pre-incubated for 3 minutes at 37°C in a shaking water bath, and the reaction was initiated by addition of the substrate. After 30 min, two volumes of ice-cold methanol containing 5 mM DTT were added to the mixture to stop the reaction. The mixture was centrifuged at 14,000 rpm for 10 min, and the supernatant was analyzed by HPLC with fluorescence detection and then by LC-MS. Two control samples were regularly included: one without the substrate and one without NADPH. The control without the substrate was used as a comparison to the original samples, so as to detect the substrate-associated peaks; the control without NADPH helped determine that the fluorescent peaks of interest were not due to auto-oxidation or non-NADPH dependent metabolism.

In kinetic studies, BPA concentrations from 10 to 200 μM were used, while dGSH concentrations were added at 5 fold higher than the BPA concentrations. A screening study was performed to be certain the reaction was linear for 15 min with 0.25 mg/ml microsomal protein. In BPA inhibition studies, the inhibitors were pre-incubated with the reaction mixture without the substrate for 15 min before initiating the reaction. The chemical inhibitors alpha-naphthoflavone (α-NF) for P450 1A1/2 [Chang et al., 1994], secobarbital for P450 2B1 [He et al., 1996],
diclofenac sodium for P450 2C11 [Masubuchi et al., 2001; Shen et al., 1997], and clotrimazole for P450 3A [Turan et al., 2001] were used at final concentrations of 10 μM, 125 μM, 10 μM, and 600 nM, respectively. The antibody inhibitors MAb C8 (anti-P450 1A1 [Thomas et al., 1984]), MAb B46 (anti-P450 2B1/2 [Reik et al., 1985]), and PAb (anti-P450 3A1/2 [Cooper et al., 1993]) were used at 1 mg, 1 mg, and 5 mg per mg microsomal protein, respectively.

5. Analysis of dGSH adducts

HPLC analysis was performed with a Shimadzu LC-6A binary HPLC system equipped with a SIL-6B auto-injector, a SCL-6B system controller, and a RF-10A spectrofluorometer (excitation 340 nm, emission 525 nm). A Zorbax Eclipse XDB C18 4.6 mm x 150 mm with a 3.5 micron particle size column, and a C18 guard column, was used with a gradient elution program (mobile phase A: 0.1% formic acid and B: acetonitrile) of 20% B equilibration for 3 min, increasing to 56% B within 30 min, continuing at 56% B for 0.5 min, decreasing to 20% B within 0.5 min, and continuing at 20% B for 6 min. The flow rate was 1 ml/min. The adduct peak areas were quantitated against an external calibration curve of dGSH, in the range of 0.1 to 5 nmol/ml.

LC-MS analysis of the HPLC adduct peaks was performed with a Finnigan LCQ Deca Ion Trap Mass Spectrometer equipped with an electrospray ionization (ESI) source, a Finnigan MAT Spectra system AS3000 Autosampler, P4000 pump system, and XCalibur software. The same column and flow program used in the HPLC analysis was used for LC-MS analysis of the adducts. The analysis
was performed under full scan positive ion mode. Negative ion mode was also performed to confirm the parent ions found in the positive ion mode. In some cases, the targeted masses were further fragmented using MS² and MS³ modes, with normalized collision energy 30 % and an isolation width of 2 m/z, to elucidate structural information. The flow program of LC during MS³ analysis was modified to 20% B equilibration for 3 min, increasing to 32% B within 2 min, increasing to 45% B within 20 min, increasing to 56% B within 8 min, continuing at 56% B for 0.5 min, decreasing to 20% B within 0.5 min, and continuing at 20% B for 6 min (total = 40 min).

C. Results

1. Dansyl GSH-Bisphenol A (dGS-BPA) adduct formation

HPLC analysis of the solutions from liver microsomal incubations showed four dGSH adduct peaks that are associated with BPA at RT 17.1 (AB1), 21.5 (AB2), 22 (AB3), and 25.7 min (AB4) (Figure 28). Quantitation of the amount of formation of each adduct formed after 15 min of incubation (Table 9) demonstrated that adduct AB4 was formed in the greatest amount, followed by AB1, AB2, and AB3. The values of AB3 are shown in parenthesis for comparison with the other adducts since this adduct was unstable, and for that reason the values could be an under-estimate. The quantitation of peak area was an average of two injections of the same sample, where the two values were mostly within 5% of each other. However, the AB3 peak observed in the first injection was absent in the second injection. Therefore, the peak area of only the first
injection is presented in parenthesis. The total of the three stable dGSH adducts formed depended on the microsomal sample and ranged from 1.5 to 11 nmol/min/mg protein at 100 μM BPA concentration. These three adducts were preferentially formed by liver microsomes from male rats that were PB-treated, followed by vehicle-treated males, PCN-treated females, β-NF-treated males, and vehicle-treated females. The formation of all four dGS-BPA adducts was dependent on the presence of NADPH.

MS analysis in positive ion mode of AB1, AB2, AB3, and AB4 yielded the parent masses of \( m/z \) 633, 675, 783, and 767, respectively. Figure 29 shows the MS chromatograms corresponding to the peaks in the fluorescence HPLC chromatogram. The corresponding masses of the four adducts in negative ion mode were also obtained (data not shown). Interestingly, integration of the peak areas from LC-MS resulted in the same rank order as those of HPLC, i.e. AB4 (98.8 million units) > AB1 (92.1 million units) > AB2 (41.4 million units) > AB3 (5.2 million units). The parent ions were further analyzed in MS\(^2\) and MS\(^3\) levels in both positive and negative ion modes.

Figure 30 shows MS\(^2\)/MS\(^3\) fragmentation in the positive ion mode of AB3. The transition of \( m/z \) 783 to 680 to 546 supported the proposed structure of the dGSH conjugated to the catechol ring. Figure 31 and 32 show MS\(^3\) fragmentation of one of the product ions of AB4, \( m/z \) 644, and AB1, \( m/z \) 530, suggesting a ring conjugation of dGSH by the mass ion of \( m/z \) 530 and 363, respectively. There is a similar fragmentation pattern in MS\(^3\) of \( m/z \) 680 (Figure 30B) and 664 (Figure 31) for AB3 and AB4, respectively. The loss of a mass 94 (\( m/z \) 680 to 586 of AB3
and m/z 664 to 570 of AB4), which is the phenol moiety, means a hydrogen atom is taken from the remaining structure, resulting in a quinone methide structure. Hence, the subsequent loss of C₃H₆, having the mass of 42 (leaving behind the catechol ring, in the case of AB3, and the phenol ring, in the case of AB4) results in a net loss of 40 instead, due to addition of two hydrogens onto the remaining structure. No informative mass spectrum could be retrieved from MS² and MS³ fragmentation of m/z 675 (AB2). This was unexpected, given that the MS¹ intensity of AB2 was stronger than that of AB3. MS³ chromatograms of the four adducts are shown in Figure 33.

2. Inhibition study of dGS-BPA adduct formation

Formation of the two most abundant adducts AB4 and AB1 was measured after pre-incubation of microsomes with chemical inhibitors and antibodies specific for certain cytochrome P450 enzymes. Inhibition profiles of the two adducts were almost identical indicating the same P450 enzymes catalyzed the activation of both. The results for AB4 are shown in Figure 34. Treatment of rats with β-NF is an effective inducer of P450 1A1 and 1A2. α-NF, which inhibits both P450 1A1 and 1A2, reduced the adduct formation by 70% in microsomes from male rats treated with β-NF; however, MAb C8, which inhibits only P450 1A1, showed no marked effect. This indicates that 70% of the catalytic activity of microsomes from β-NF-treated rats was due to P450 1A2 only and not 1A1. Secobarbital, which inhibits only P450 2B1, and MAb B46, which inhibits both P450 2B1 and 2B2 reduced the formation by 42% and 79%, respectively. These
results indicate that both P450 2B1 and 2B2 contributed to the activity in microsomes from male rats treated with PB accounts for at least 80% of the activity of these microsomes. Diclofenac is an inhibitor of the male specific P450 2C11 and when used with microsomes from vehicle-treated male rats it resulted in 50% inhibition indicating at least 50% of the activity of those microsomes is due to P450 2C11. Since male rat liver is known to also express P450 3A2 [Jan et al., 2006] and 2C13 [Bandiera et al., 1986], these enzymes may account for the 50% not inhibited by diclofenac. PCN treatment of female rats resulted in high levels of P450 3A1 but minimal levels of other 3As, i.e. 3A2, 3A9, and 3A18 [Jan et al., 2006]. Clotrimazole was found to be a relatively specific inhibitor of P450 3A. Moreover, anti-P450 3A1 antibody is also a specific inhibitor of all four members of the P450 3A family. Use of clotrimazole and anti-P450 3A1 reduced adduct formation by 75% and 86%, respectively, showing that the major catalyst of dGS-BPA adducts in microsomes from PCN-treated rats is likely P450 3A1.

Liver microsomes from vehicle-treated female rats had the lowest activity of the five liver microsomes and no attempt to determine the relative contribution of activating enzymes was made. Microsomes from female rats are known to express female specific P450 2C12 and female predominant P450 2A1 and 2C7 [Pampori and Shapiro, 1999; Bandiera et al., 1986]. In conclusion, the activity of the P450 enzymes in these liver microsomes which catalyze the dGS-BPA adduct formation, ranking from high to low, are: 2B1/2 > 2C11 > 3A1 ~ 1A2.

3. Kinetic parameters of dGS-BPA adducts formation
Data from the rates of dGSH adduct formation in the first 15 minutes of incubation at 10, 25, 50, 75, 100, or 200 μM of BPA were fitted to the Michaelis-Menten equation (GraphPad Prism software) to calculate the kinetic parameters. The apparent \( K_m \) and \( V_{\text{max}} \) for the formation of adducts AB1 and AB4, the two most abundant, are shown in Table 10. The highest affinity, lowest \( K_m \), for both AB1 and AB4, was obtained with control female microsomes, which also showed the lowest \( V_{\text{max}} \). The highest capacity, or the highest \( V_{\text{max}} \), was found with microsomes from rats treated with PB. It was not possible to obtain the kinetics of adduct AB3 (unstable) or adduct AB2 (data did not fit well into the Michaelis-Menten equation).

4. **Dansyl GSH adduct formation of reference compounds**

APAP and anethole (structures shown in Fig. 35) were chosen as positive and negative reference compounds for comparison. They were tested under the same conditions used in the BPA studies. One adduct was found for each compound. Molecular masses of all the adducts and their corresponding chemical compositions are summarized in Table 11. The mass \( m/z \) 690 of APAP adduct corresponds to the known GSH adduct, 3-(glutathion-S-yl)-APAP, reported earlier, where GSH is conjugated to an ortho- position of the hydroxyl group through the reactive metabolite \( N \)-acetyl-\( p \)-benzoquinone imine (NAPQI) [Nelson and Gordon, 1982; Potter and Hinson, 1987]. For anethole, the mass of \( m/z \) 704 can be accounted for by an addition of one oxygen without a loss of two hydrogens, suggesting that the dGSH most likely conjugated to one of the
carbons of the double bond on the side chain via reaction with an epoxide intermediate. This was in agreement with Sangster et al. [1984b], in which a mercapturate species of 2-hydroxy-1-methylthio-1-(4′-methoxyphenyl)propane was detected in their anethole metabolism study in rats and mice.

The quantitation of APAP and anethole adducts is shown in Table 12. Liver microsomes from rats treated with PCN were most active in catalyzing dGSH adduct formation with APAP, ca. 0.5 nmol/min/mg protein, which is approximately 2.5 fold higher than that formed by microsomes from β-NF- and PB- administered rats.

Cytochrome P450 3A1 and 1A2 are known to be induced by PCN and β-NF respectively, and these enzymes are known to catalyze the oxidation of APAP resulting in higher GSH adduct formed [Patten et al., 1993; Bessem and Vermeulen, 2001]. The effect of PB on hepatic GSH levels in rats has also been shown [van Bree et al., 1989]; pretreatment with PB (followed by APAP administration) further reduced the GSH level to ca. 25% of those of the vehicle-control rats compared to the effect of APAP alone (ca. 65% of those of the control). Hence, our dGSH results from microsomal metabolism of APAP are consistent with the known role of P450 enzymes in forming its electrophilic intermediates.

For anethole, the dGSH adduct was predominantly formed at ca. 0.1 nmol/min/mg protein by microsomes from rats treated with PCN. The role of P450 3A1, the major isoform expressed in microsomes from PCN-treated rats
[Jan et al., 2006], as one of the major isoforms catalyzing the reactive metabolite of anethole has not been reported previously.

The proposed structures of the dGSH conjugates detected in this study along with the parent compounds are shown in Figure 35.

D. Discussion

1. Reactive metabolites of BPA

Enzymatic activation is usually considered the first step toward drug or xenobiotic induced cell injury and cytochromes P450 are responsible for most bioactivation reactions. Liver microsomes are a rich source of these enzymes and the rat has proven a reliable species for toxicological studies. Besides the extensive literature on the rat, the rat is known to exhibit marked sexual dimorphism among several cytochromes P450. This sexual dimorphism provides an advantage in examining xenobiotic toxicity differences between males and females. Additionally, liver microsomes from rats treated with diverse inducers are known to be enriched with different P450s and such treatment may markedly modulate the toxicity of xenobiotics.

Five rat liver microsomes from both genders and known to be enriched with different P450 enzymes, i.e. liver microsomes from adult vehicle-treated males and females, adult males treated with β-NF or PB, and adult females treated with PCN were selected. P450 2C11, 2C13, and 3A2 [Jan et al., 2006; Ghosal et al., 1996] are male specific enzymes, while those expressed in female rats include P450 2C12, 2A1, and 2C7 [Bandiera et al., 1986; Pampori and
Male rats treated with β-NF or PB are highly enriched in P450 1A1/2 and 2B1/2, respectively [Thomas et al., 1983]; while treatment of female rats with PCN leads to marked induction of P450 3A1 [Jan et al., 2006].

The rate of BPA metabolite formation was dependent on the type of microsomes used and on the presence of NADPH. Four dGSH adducts were found (Fig. 28) but one of them, AB3, was not stable. Formation of BPA adducts by the different types of microsomes was also examined in the presence of the corresponding chemical inhibitors and antibodies to confirm the expression of the above mentioned isoforms. The results from different microsomes (Table 9) together with those of the inhibition studies (Fig. 34) indicate that P450 isoforms that catalyzed the BPA adduct formation, from high to low, are: 2B1/2 > 2C11 > 3A1 ~ 1A2. The kinetic studies showed microsomes from rats treated with PB and control male microsomes formed dGSH adducts with high capacity, re-affirming the significant effect of P450 2B1/2, 2C11, and 3A1 activities.

MS analysis of the dGSH-BPA adducts showed parent ion masses corresponding to dGSH conjugated with a phenol, a 4-isopropyl phenol, 3-hydroxy BPA, and BPA moieties for AB1, AB2, AB3, and AB4, respectively (see Fig. 35 for the proposed structures). The equivalent masses of GSH conjugation with a phenol, a 4-isopropyl phenol, and BPA were reported earlier [Jaeg et al., 2004], while that of the 3-hydroxy BPA-GSH adduct was only demonstrated previously through chemical synthesis [Atkinson and Roy, 1995; Qiu et al., 2004]. The method used in our study was able to capture the formation of this conjugate
in rat liver microsomes. The instability of this adduct might explain why it was not detected previously.

Further MS analysis of the adduct AB3 showed dGSH conjugated to the catechol ring (m/z 546, Fig. 30B). The conjugation could occur through a mechanism of an enzymatic oxidation of the BPA to a catechol 3-hydroxy metabolite, which is further oxidized to the reactive o-quinone [Roy et al., 1997; Kalgutkar et al., 2005a], which subsequently reacts with dGSH to form AB3. For AB4, the conjugation of dGSH to the phenyl ring (m/z 530, Fig. 31) may be mediated through an arene oxide intermediate [Chang et al., 2006]. Dansyl GSH conjugation to the phenyl ring in the case of AB1 is evident from the mass m/z 530 (Fig. 32). However, no mass information was obtained (see Fig. 33) from MS² and MS³ fragmentation of AB2, m/z 675, where its MS¹ intensity was stronger than that of AB3 (detected as forming the least of the four adducts).

The finding of phenol together with 4-isopropyl phenol adducts, especially in an approximately 1:1 ratio, suggested that AB1 and AB2 could be derived from BPA via cleavage of the phenyl ring from the adjacent aliphatic carbon. In order to account for the concurrent formation of the two dGSH adducts, the mechanism, shown in Scheme 3 is proposed. During microsomal incubation BPA initially undergoes a one-electron oxidation to A. The unpaired electron is delocalized over the aromatic ring and the reaction with a thiyl radical results in B. Subsequent one-electron reduction results in the radical anion species C, which might undergo carbon-carbon bond breakage via a β-scission, forming the adduct AB1 and the radical species D. One-electron oxidation of D leads to a
quinone methide E which reacts with GSH to form the adduct AB2. GSH conjugation via addition of a thyl radical was demonstrated with benzo[a]pyrene-7,8-dihydrodiol by Foureman and Eling [1989], who suggested the thyl formation by peroxidase or by way of the phenoxyl radical. The evidence of thyl radical formation through the phenoxyl radical was shown by Thompson et al. [1989]. From this proposed mechanism, AB2 is the only adduct where dGSH is not conjugated to the aromatic ring. Its distinct MS behavior from the other three adducts might be the result of this difference.

2. Analysis of potential toxicity of BPA reactive metabolites

The total of the three stable dGSH adducts formed by microsomal metabolism at 100 μM BPA is in the range of 1.5 – 11 nmol/min/mg protein. To put the amount of BPA adduct formation in perspective, two additional chemicals were examined using the same liver microsomes and dGSH method. Anethole was chosen as a potential negative reference compound and APAP as a positive reference compound.

APAP could be considered a model and reference compound with respect to the role of reactive metabolites in hepatotoxicity. At a therapeutic dose in humans (500 mg), APAP undergoes extensive glucuronide and sulfate conjugation, with less than 5% oxidation to the reactive metabolite NAPQI; however, this increases to 7-15% at higher doses which are toxic [Court et al., 2001]. In mice at a toxic dose of APAP of 250 mg/kg, the GSH conjugate was present at 23% [Rashed et al., 1990]. Rashed et al. [1990] also found hepatic
protein covalent binding at ~ 0.8 nmol/mg protein at 1 hour and 1.2 nmol/mg protein at 3 hours, while Myers et al. [1995] noted it to be 1.82 nmol/mg protein at 2 hours. Administration of 500 mg/kg to mice yielded GSH adduct of APAP at 518 nmol/g liver, which is equivalent to about 0.2 nmol/min/mg protein (1 hour study, 45 mg microsomal protein/g liver), with an additional 9480 nmol/ml in bile [Dai et al., 2005]. In in vitro studies, Masubuchi et al. [2007] found the GSH conjugate at approx. 0.2 pmol/min/mg protein in both human and rat microsomes at 10 μM substrate, while Gan et al. [2005] found 0.5% of dGSH conjugate with human liver microsomes at 50 μM substrate, which is equivalent to 8.7 pmol/min/mg protein (30 min incubation, 1 mg/ml protein).

In our study the dGSH adduct of APAP was found in the range of less than 0.5 nmol/min/mg protein (Table 12). Several factors are to be kept in mind in considering these results. First, the 0.1 mM APAP concentration is considered at the high end of its therapeutic dose [Laine et al., 2009]. Secondly, in an in vivo situation, total adduct formation would be the additive effect of the catalyzing role of various P450 isoforms. However, their expressions at “induced” levels would not occur concurrently. Lastly, there are roles of other P450 isoforms which are not included in our studies. This also includes the difference between human and rat/animal isoforms. For APAP, an effect of P450 2E1 which is the most active isoform at high dose [Bessems and Vermeulen, 2001; Nelson, 1995] was not examined in our studies. Hence, the results of 0.05 to 0.5 nmol/min/mg protein (Table 12) appear to be reasonably consistent with others and thus a sensible positive reference values. Therefore, in comparison, the sum of 1.5-11
nmol/min/mg protein of three dGSH conjugates at 0.1 mM BPA is suggested as potentially more toxic.

Potential toxicity of BPA has been observed in several studies. A study in male Wistar rats, fed BPA orally at 25 mg/kg/day for 45 days, found a reduction of GSH and an increase in lipid peroxidation levels in the brain [Aydogan et al., 2008]. Further, Bindhumol et al. [2003] showed that oxidative stress in male Wistar rat liver was observed even at the oral dose as low as 0.2 mg/kg/day, dosing for 30 days. Potential toxicity resulting from reactivity of BPA and its metabolite 3-hydroxy, i.e. adduct AB4 and AB3, was demonstrated by Roy et al. [1997] for their capability to form DNA adducts. Since adduct AB3 is not stable, the extent of its formation is probably under-estimated. The implication of reactive metabolites that form unstable adducts has not been thoroughly studied or fully understood. The concern with an unstable adduct is that it could exert its toxicity in another organ besides the one in which it is formed. 3-Acetamidophenol (AMAP), a regioisomer of APAP, is not hepatotoxic but produces respiratory failure at high doses. This toxicity may be associated with unstable adducts of reactive metabolites since it appeared that the protein adducts extracted from livers of mice treated with AMAP were not stable [Myers et al., 1995]. The dGSH conjugate of a reactive metabolite of AMAP was also found to be unstable in our study (Table 4).

Further insight regarding a toxic potential of BPA reactive metabolites is obtained by examining the metabolism of anethole, a hepatotoxin but considered a relatively safe compound. trans-Anethole is a flavoring agent found as a major
constituent in anise, fennel, and star anise, with a temporary acceptable daily intake (ADI) of 0-0.6 mg/kg [Vavasour, 1999]. It has a NOEL value of 125 mg/kg from a one year study in rats and is on the GRAS (Generally Recognized As Safe) list. Its hepatotoxicity was observed in a 90-day rat study only at high doses (600 and 900 mg/kg) and the 1',2'-oxide was found to be the cytotoxic metabolite [Vavasour, 1999]. Studies in rats by Sangster et al. [1984a] at a dose range of 0.05, 5, 50, or 1500 mg/kg produced reduction (from 56 to 32%) of O-demethylation, considered a detoxification pathway (as CO₂ excretion), as the dose increased; while 1',2'-epoxidation, the toxification pathway, rose from 2 to 15% and 0.6 to 3.2%, detected as a 1',2'-diol and a S-conjugate, respectively. Marshall and Caldwell [1996] found that cytotoxicity (expressed as % lactate dehydrogenase leakage), as well as depletion of GSH, in rat hepatocytes was pronounced only at 5 mM and above, but not at 1 mM and below, even in the presence of cytosolic epoxide hydrolase inhibitor. Our results (Table 4) showing a low level of adduct formation of anethole at 0.1 mM, primarily by microsomes from rats treated with PCN only, appear to be consistent with its safety profile. As a flavoring agent, exposure to anethole is at low levels. This provides a better side-by-side comparison when dose is taken into consideration. The reference tolerated dose of BPA of 16 μg/kg bw/day [Willhite et al., 2008] is approximately 40 times lower than that of anethole, but the extent of reactive metabolite formation is about 1000 fold higher, thus, considerable potential for toxicity is apparent.
On a last note, regarding the above brief details of data gathered from *in vitro* and *in vivo* studies on reactive metabolites of different compounds, it can be seen that different *in vitro* studies yield quite different end point values. Different studies may select different substrate concentrations, for different reasons and objectives, which would result in varying results. As a closest comparison between two studies: the dGSH adduct of APAP was ca. 8.7 pmol/min/mg protein at 50 μM substrate in human liver microsomes from Gan *et al.* [2005] vs. 50 to 500 pmol/min/mg protein at 100 μM substrate using different rat liver microsomes in our studies. Furthermore, studies with different substrate concentrations might provide additional insight regarding the potential toxicity of a compound. Measurement of adduct formation was developed as a screening method to quickly identify a safer drug candidate for further development. Evans *et al.* [2004] proposed 50 pmol protein covalent binding/hr/mg protein (performed at 10 μM substrate) while Gan *et al.* [2009] suggested 200 pmol thiol adduct/hr/mg protein (performed with 50 μM substrate) as a precautionary level. However, in practice, in many cases, a more thorough assessment, hence a better understanding, is always needed, except the case of a direct comparison of derivative compounds of the same chemical class. Considering that there has not been a good understanding of *in vitro-in vivo* correlation of reactive metabolites, it would be difficult to use a “general” threshold value for a compound whether or not it was to be considered toxic. Our methodology of assessing a compound’s toxicity potential by comparing it to the result of a
reference hepatotoxic compound in a side by side comparison provides reliable qualitative data on a compound’s relative toxicity potential.

E. Conclusion

In conclusion, data showed that the trapping of reactive metabolites by fluorescent dGSH is a useful and sensitive quantitative method to detect electrophilic metabolites formed by liver microsomes. BPA metabolism by rat liver microsomes yielded four electrophilic metabolites which formed dGSH adducts. Of the five rat liver microsomes used those from male rats treated with PB were the most active while those from vehicle treated female rats were the least active. The activities of these microsomes were as much as 90 fold more active in forming dGSH metabolites of BPA than of APAP, and more than 1000 fold compared to anethole. The marked ability of BPA to form reactive electrophilic metabolites may be an important factor in understanding its potential for adverse effects.
Table 9: Rate of dGSH adduct formation (nmol/min/mg protein) from incubations of 0.1 mM BPA with liver microsomes from rats treated with different inducers in phosphate buffer pH 7.4, in the presence of NADPH and dGSH for 15 min

| Adduct: | Microsomes from rats treated with: |  
|---------|----------------------------------|---|
|         | β-NF, male^ | PB, male^ | Vehicle, male^ | PCN, female^ | Vehicle, female^ |  
| AB1     | 0.82        | 3.25      | 2.38         | 1.35        | 0.47          |  
| AB2     | 0.67        | 3.11      | 1.77         | 1.66        | 0.34          |  
| AB3     | (0.27)      | (0.05)    | (0.15)       | (0.37)      | (0.17)        |  
| AB4     | 1.12        | 4.57      | 3.01         | 1.79        | 0.68          |  
| Total   | 2.61        | 10.93     | 7.16         | 4.80        | 1.49          |  

*Values in parentheses mean unstable adduct, see text for explanation

^β-NF, male; PB, male; and Vehicle, male represent liver microsomes from male rats treated with β-naphthoflavone, phenobarbital, and corn oil, respectively. PCN, female and Vehicle, female represent liver microsomes from female rats treated with pregnenolone-16α-carbonitrile and corn oil, respectively.
Table 10: Kinetic parameters of adduct AB1 and AB4 formation from metabolism of 0.1 mM BPA by liver microsomes from rats administered different P450 inducers in phosphate buffer pH 7.4, in the presence of NADPH and dGSH for 15 min

<table>
<thead>
<tr>
<th>Microsomes from rats treated with:</th>
<th>Adduct AB1</th>
<th>Adduct AB4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ $^a$ (± s.e.)</td>
<td>$V_{max}$ $^b$ (± s.e.)</td>
</tr>
<tr>
<td>$\beta$-NF, male $^c$</td>
<td>139 ± 50</td>
<td>755 ± 152</td>
</tr>
<tr>
<td>PB, male $^d$</td>
<td>116 ± 41</td>
<td>2151 ± 394</td>
</tr>
<tr>
<td>Vehicle, male $^e$</td>
<td>85 ± 28</td>
<td>1472 ± 225</td>
</tr>
<tr>
<td>PCN, female $^f$</td>
<td>60 ± 23</td>
<td>695 ± 110</td>
</tr>
<tr>
<td>Vehicle, female $^c$</td>
<td>31 ± 16</td>
<td>189 ± 30</td>
</tr>
</tbody>
</table>

$^a$ $K_m$ values in $\mu$M

$^b$ $V_{max}$ values in pmol/min/mg protein

$^c$ $\beta$-NF, male; PB, male; and Vehicle, male represent liver microsomes from male rats treated with $\beta$-naphthoflavone, phenobarbital, and corn oil, respectively. PCN, female and Vehicle, female represent liver microsomes from female rats treated with pregnenolone-16$\alpha$-carbonitrile and corn oil, respectively.
Table 11: Summary of characteristics of dGSH conjugates of BPA, together with those of APAP and trans-anethole, formed by rat liver microsomes

<table>
<thead>
<tr>
<th>dGSH adducts:</th>
<th>MW of substrate (Da)</th>
<th>Mass of the adduct found (Da)</th>
<th>Corresponding chemical composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB1 of BPA</td>
<td>228</td>
<td>632</td>
<td>Phenol+dGSH-2H</td>
</tr>
<tr>
<td>AB2 of BPA</td>
<td>674</td>
<td>4-Isopropyl phenol+dGSH-2H</td>
<td></td>
</tr>
<tr>
<td>AB3 of BPA</td>
<td>782</td>
<td>BPA+dGSH-2H+O</td>
<td></td>
</tr>
<tr>
<td>AB4 of BPA</td>
<td>766</td>
<td>BPA+dGSH-2H</td>
<td></td>
</tr>
<tr>
<td>APAP</td>
<td>151</td>
<td>689</td>
<td>APAP+dGSH-2H</td>
</tr>
<tr>
<td>trans-Anethole</td>
<td>148</td>
<td>704</td>
<td>Anethole+dGSH+O</td>
</tr>
</tbody>
</table>
Table 12: Formation of dGSH adducts (nmol/min/mg protein) of APAP and trans-anethole metabolites formed by liver microsomes from rats administered different P450 inducers incubated in phosphate buffer pH 7.4 in the presence of NADPH and dGSH for 15 min with 0.1 mM substrates

<table>
<thead>
<tr>
<th>Compound</th>
<th>/β-NF, male$^\wedge$</th>
<th>PB, male$^\wedge$</th>
<th>Vehicle, male$^\wedge$</th>
<th>PCN, female$^\wedge$</th>
<th>Vehicle, female$^\wedge$</th>
</tr>
</thead>
<tbody>
<tr>
<td>APAP</td>
<td>0.18</td>
<td>0.19</td>
<td>0.08</td>
<td>0.49</td>
<td>0.05</td>
</tr>
<tr>
<td>trans-Anethole</td>
<td>n.d.</td>
<td>0.007</td>
<td>0.007</td>
<td>0.111</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

n.d.: not detected

$^\wedge$/β-NF, male; PB, male; and Vehicle, male represent liver microsomes from male rats treated with β-naphthoflavone, phenobarbital, and corn oil, respectively. PCN, female and Vehicle, female represent liver microsomes from female rats treated with pregnenolone-16α-carbonitrile and corn oil, respectively.
Figure 28: A representative HPLC-fluorescence chromatogram of a BPA sample incubated with rat liver microsomes, in the presence of NADPH and dGSH, compared to a control sample incubated without NADPH
Figure 29: Mass chromatograms from LC-MS showing m/z 633, 675, 783, and 767 of AB1, AB2, AB3, and AB4 peak, respectively, of a BPA sample incubated with rat liver microsomes.
Figure 30: MS fragmentation pattern in positive ion mode of the mass \(m/z\) 783 (adduct AB3) in a BPA sample incubated with rat liver microsomes

A) MS\(^2\) of the parent ion \(m/z\) 783

B) MS\(^3\) of the product ion \(m/z\) 680.
Figure 31: MS$^3$ fragmentation pattern in positive ion mode of a product ion m/z 664 of the parent ion m/z 767 (adduct AB4), formed with a BPA sample incubated with rat liver microsomes. The product ions from MS$^2$ of AB4 are m/z 692, 664, and 380 (data not shown).
Figure 32: MS$^3$ fragmentation pattern in positive ion mode of a product ion $m/z$ 530 of the parent ion $m/z$ 663 (adduct AB1), formed with a BPA sample incubated with rat liver microsomes. The product ions from MS$^2$ of AB1 are $m/z$ 558, 530, and 380 (data not shown).
Figure 33: MS$^3$ mass chromatograms from LC-MS of a BPA sample incubated with rat liver microsomes showing good signals of product ions of the parent mass $m/z$ 633 (AB1), 783 (AB3), and 767 (AB4), but not that of $m/z$ 675 (AB2)
Figure 34: Inhibition of formation rate of dGSH adduct AB4 formed by rat liver microsomes by chemical inhibitors (shaded bars) and antibodies (dotted bars) compared to those of control (no inhibitors, dark bars). $\beta$-NF, male mx; PB, male mx; and Vehicle, male mx represent liver microsomes from male rats treated with $\beta$-naphthoflavone, phenobarbital, and corn oil, respectively. PCN, female mx represents liver microsomes from female rats treated with pregnenolone-16$\alpha$-carbonitrile and corn oil. See details of experimental condition and quantitation under section B.
Figure 35: Structures of the compounds studied and proposed structures of their dGSH adducts

- Acetaminophen
- dGSH adduct of APAP
- trans-Anethole
- dGSH adduct of anethole
- Bisphenol A
- BPA adduct AB1
- BPA adduct AB2
- BPA adduct AB3
- BPA adduct AB4
Scheme 3: Proposed mechanism of formation of dGSH adduct AB1 and AB2
VI. SUMMARY

Glutathione adducts of reactive metabolites of dietary compounds were examined in this thesis through trapping using dGSH. Synthesis of dGSH was developed to achieve a sufficient yield by a reaction in a borate buffer. HPLC using fluorescence for detection and quantification, coupled with LC-MS, for identification, yielded results that are consistent with other studies. In detecting and identifying the dGSH adducts of 11 compounds, those of APAP, AMAP, anethole, pulegone, eugenol, and quercetin matched with previous reports. It is the first time that 2 adducts of pulegone were reported together in microsomes. Adducts of NDGA, genistein, daidzein, apigenin, and naringenin detected in the present studies are also the first to be identified. The method was also able to detect unstable adducts of AMAP and quercetin, the instability of which had previously been reported earlier by others. The method was also able to separate regio-isomers of eugenol and NDGA, showing that dGSH can react in a similar fashion as GSH. Low levels of flavonoids' adducts also demonstrated the sensitivity of this fluorescent method.

Incubation of the substrates with different microsomes enriched with different isoforms of P450 also provided valuable information. The amount of dGS-APAP adduct was highest with PCN-mx, followed by NF-mx and PB-mx, and lowest with CM-mx and CF-mx. These results correlate well with other in vivo and in vitro findings, showing the significance of isoforms P450 3A1, 1A2, and 2B. Consequently, this served as a good reference to deduce the effect of P450 isoforms from different microsomes metabolism of the other 9 compounds.
The dGSH adduct of anethole was predominantly formed by PCN-mx, while those of pulegone were found in PCN-mx and CM-mx. High levels of eugenol adduct were formed by all microsomes but that by NF-mx was 2-7 fold higher than by other microsomes. NDGA adducts were also highly formed by all microsomes, although the bis-conjugates were preferentially formed by PB-mx and PCN-mx. The extent of adduct formation of flavonoids was low and showed no significant difference among different microsomes.

The amount of adducts formed was presented as the rate of formation (in nmol/min/mg protein) at low (0.1 mM) and high (1 mM) substrate concentrations. The ca. 2 fold increase of dGS-APAP adduct at high concentration was consistent with its wide safety margin. Using the amount of dGS-APAP adduct found as a reference, the extent of adduct formation at a low dose and the increment at a high dose of anethole and pulegone fit with their reported safety profile. However, our results suggested eugenol and NDGA might be considered highly toxic. Although these results were consistent with other in vitro studies, it did not correlate well with in vivo data. When their metabolic pathways were examined, it was found that the structures of eugenol and NDGA, which contain a phenoxy group, allow for direct glucuronidation while glucuronidation of anethole and pulegone can only occur as a subsequent step after a phase I oxidation. In phase I metabolism studies of compounds like eugenol and NDGA in the absence of phase II enzymes, the extent of reactive metabolites trapped with GSH, or dGSH as in the present studies, could be unrealistically high. Furthermore, when groups of false positive compounds from Gan’s and Obach’s
studies (studies of reactive metabolites by dGSH adduct formation and protein covalent binding, respectively) were examined, our analysis of their results confirmed this hypothesis of the preferential kinetics between phase I vs. phase II as a result of certain functional groups.

When a group of non-hepatotoxic flavonoids was investigated, dGSH adduct formation of genistein, daidzein, and apigenin was considered negligible, while that of chrysin was negative. Although the amount of dGSH adduct of narigenin formed was lower than that of APAP, it was higher than that of anethole and pulegone. Analysis was made of antioxidant compounds in general regarding their interaction with GSH as an electrophile, resulting in a GSH conjugate, vs acting as antioxidants, resulting in radical formation and GSSG. It was found that the redox potential of a compound could not predict the preferred pathway. There have not been many studies on the dynamics between the two pathways and much more research is needed.

Therefore, it is concluded that dGSH reacts with reactive metabolites in a similar fashion to that of GSH and the amount of dGSH adducts formed by rat liver microsomes appears to correlate with toxicity profile of a compound. False positives, obtained with compounds possessing certain functional groups, or false negatives, due to limitation of GSH as a soft nucleophile or other activation mechanisms not present in microsomes, could occur and only suggest that the result obtained could not be used to quickly predict potential toxicity of a compound. The failure of doing so only reflects the complexity of toxicity process, rather than questions about reliability of the method. Thus, a study of GSH
adduct formation is encouraged both as a part of screening efforts and in the process of toxicity assessment.

The method was then utilized to examine reactive metabolites of BPA, the molecule of which contains the phenol moieties which could become reactive yet its mechanism-based toxicity has not been explored. Four BPA-related adducts were identified, characterized, and quantitated. A dGSH adduct conjugating directly to BPA was the most abundant, followed by ca. equal amounts of phenol and 4-isopropyl phenol adducts, while that of hydroxylated BPA adduct was unstable. The formation of these adducts was catalyzed (in decreasing order) by P450 2B1/2, 2C11, 3A1, and 1A2. A mechanism was proposed for formation of dGSH adducts of phenol and 4-isopropyl phenol as results suggested the two adducts may be splitting parts of a BPA molecule. The extent of the 3 stable adducts was in the range of 1.5 to 11 nmol/min/mg protein. These high levels suggested additional studies are encouraged to further examine the potential toxicity of BPA metabolites. One of the next studies could be an in vivo protein covalent binding study.

Another important finding was that associated with pulegone. One of the pulegone adducts, a dGS-menthone, appeared to be catalyzed by mGSTs. This means dGSH is capable of being a substrate of GST enzymes. By the same token, this showed that pulegone itself could be the toxic species responsible for its hepatotoxicity. As metabolism of pulegone is rather complex; a toxic metabolite of pulegone was initially known as menthofuran, and later acknowledged to be 8-pulegone aldehyde. It would be interesting to further
examine the contribution of this menthone adduct, i.e. the reactivity of pulegone itself, as well as the contribution of mGST enzymes, as a toxic species of pulegone.
VII. APPENDIX: NON SPECIFIC PROTEIN BINDING

Since liver is the primary site of metabolism, liver microsomes have been used as one of the *in vitro* tools to study anything metabolism-related: metabolic stability, clearance, drug-drug interaction, Michaelis constant ($K_m$), etc. These results are known to be dependent on microsomal protein concentration, where increase of the protein concentration does not necessarily result in proportionally increase of the measured parameters due to more enzyme. This is known to be an effect of nonspecific binding of the substrate to microsomal protein [Zhang *et al.*, 2010]. A fraction unbound ($fu$) is needed to correct the experimental apparent values which will also result in a better *in vivo* prediction. Experimentally, $fu$ is determined by equilibrium dialysis, ultracentrifugation, or ultrafiltration method. Due to the time consuming of these methods, recently a calculation of corrected values from the experimental apparent values at different microsomal protein concentration was demonstrated [Giuliano *et al.*, 2005].

In obtaining a corrected value, the experimental apparent value must be corrected with $fu$ which was obtained at the same microsomal protein concentration. Therefore, it is important that the experimental apparent values or $fu$ values are reported specifying the microsomal protein concentration at which they were generated. If the two values were generated at different microsomal protein concentration, the $fu$ at the concentration corresponding to that of the apparent values can be calculated by the following equation [Austin *et al.*, 2002]:
In our microsomal incubation of NDGA, the dGSH adduct of NDGA was formed either with or without NADPH. However, adduct formation in the absence of NADPH was much lower than that with NADPH. Based on the chemical structure, it was speculated that NDGA could undergo an autooxidation. This was supported by results which showed that the adduct formation with NADPH was between 8-14 nmol/min/mg protein in different microsomes, while that without NADPH was approximately around 2.8 nmol/min/mg protein in all microsomes. Adduct formation of NDGA was then studied at different microsomal protein concentrations, 0, 0.25 and 2 mg/ml, in the presence and absence of NADPH, shown in Figure 36. The decrease of the adduct formation due to autooxidation at higher protein concentrations reflected reduction of available substrate due to more non specific binding of the substrate to the protein as protein concentrations increased. The ratio of this adduct formation (due to autooxidation) at different protein concentrations to that without microsomes (absence of non-specific protein binding) could then be utilized to calculate the fraction unbound (fu) of NDGA at each protein concentration. This presents a unique method in determining fu in order to correct the experimental apparent values.

In our study, fu obtained from the adduct formation in the absence of NADPH was used to correct adduct formation in the presence of NADPH. Table 13 demonstrates the steps that the experimental apparent values are corrected

\[
fu_2 = \frac{1}{\frac{C_2(1 - fu_1)}{C_1 fu_1} + 1}
\]
and shows how the corrected amounts of adduct formed at different protein concentrations are equal and higher than those apparent values.

The estimation of fu at the second protein concentration using equation (1) above was also carried out with our data. A calculation using 0.25 mg/ml concentration as concentration 1 yields fu of 2 mg/ml at 0.31, while using 2 mg/ml as concentration 1 yields fu of 0.25 mg/ml at 0.62. This shows the agreement of our results (Table 13) with the calculation and that the equation (1) gives a good estimation of a second fu from the existing fu.

In conclusion, this study and analysis showed 1) how non specific protein binding, hence, protein concentration, affected the experimental apparent parameters and 2) how, in this case, the non-NADPH oxidation, or autooxidation of the substrate, could be used to obtain fu values enabling correction of its own NADPH oxidation results.
Table 13: Correction of the experimental apparent rate of dGS-NDGA adduct formation, formed by rat liver microsomes, with fraction unbound at different protein concentrations

<table>
<thead>
<tr>
<th>Rate of dGS-NDGA adduct formation at different microsomal protein concentrations (data of control male rat liver microsomes):</th>
<th>Microsomal protein concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>By metabolism (with NADPH) (µM/min)</td>
<td>0.25</td>
</tr>
<tr>
<td>By autooxidation (without NADPH) (µM/min)</td>
<td>0.72</td>
</tr>
<tr>
<td>By metabolism, adjusted (subtracted autooxidation) (µM/min)</td>
<td>1.80</td>
</tr>
<tr>
<td>By metabolism, normalized with protein concentration (nmol/min/mg protein)</td>
<td>7.20</td>
</tr>
<tr>
<td>Fraction unbound, fu</td>
<td>0.78</td>
</tr>
<tr>
<td>By metabolism, corrected with fu (nmol/min/mg protein)</td>
<td>9.27</td>
</tr>
</tbody>
</table>
Figure 36: Amount (expressed as peak area) of dGS-NDGA adduct formed at different microsomal protein concentration, after incubated for 30 min at 0.1 mM NDGA, with (square) and without (diamond) NADPH.
VIII. BIBLIOGRAPHY

Drug-induced liver toxicity.
http://www.fda.gov/Drugs/scienceresearch/researchareas/ucm071471.htm


http://www.library4science.com.eula.html


IX. CURRICULUM VITAE

Education:

Ph.D. in Pharmaceutical Sciences, Rutgers University, Jan 2011

M.S. in Pharmaceutical Sciences, Rutgers University, May 1998

Work experience:

Novartis Pharmaceuticals Corp. 2001 – 2009

Carter-Wallace, Inc. 1999 – 2001

Lavipharm Laboratories, Inc. 1998 – 1999

Patents:


Chen, LH; Pfister, WR; Renn, DW; Buranachokpaisan, T; Osborne, J; Tan, HS; and Tao, L. Compositions and methods for mucosal delivery. US Patent# US6552024 April 22, 2003.

Manuscript:

Wang, Q; Bhardwaj, RK; Herrera-Ruiz, D; Hanna, NN; Hanna, IT; Gudmundsson, OS; Buranachokpaisan, T; Hidalgo IJ; and Knipp, GT. Expression of multiple drug resistance conferring proteins in normal Chinese and Caucasian small and large intestinal tissue samples. Mol Pharm 2004; 1 (6): 447-54.