Metabolism and Bioactivity Studies of Licorice Species and the Active Compounds

by

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THESIS
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This dissertation is dedicated to my parents, Jiefan Huang and Guowei Ye, as well as my
friends, for their support, encouragement and love.
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CONTRIBUTION OF AUTHORS STATEMENT

This dissertation includes previously published works: Chapter 2 contains information overlapping with reference (Huang et al. Anal. Chem. 2015), where K. Huang, L. Huang, and R.B. van Breemen proposed the idea, L. Huang and K. Huang conducted the experiment, K. Huang, L. Huang, and R.B. van Breemen contributed to the final version of the manuscript; Chapter 4 contains information overlapping with reference (Huang et al. Anal. Bioanal. Chem. 2017), where L. Huang, D. Nikolic and R.B. van Breemen proposed the idea, L. Huang conducted the experiment, L. Huang, and R.B. van Breemen contributed to the final version of the manuscript. For both publications, I am the co-author of those works, involving in the experiments design, conduction and manuscript written. I also have the permission from the copyright holders (attached in Appendices).

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<td>ADME</td>
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</tr>
<tr>
<td>CID</td>
<td>Collision-induced dissociation</td>
</tr>
<tr>
<td>CL\textsubscript{int}</td>
<td>Hepatic intrinsic clearance</td>
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<tr>
<td>CNL</td>
<td>Constant neutral loss</td>
</tr>
<tr>
<td>GG</td>
<td><em>Glycyrrhiza glabra</em> L</td>
</tr>
<tr>
<td>GI</td>
<td><em>Glycyrrhiza inflata</em> Bat</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GU</td>
<td><em>Glycyrrhiza uralensis</em> Fisch</td>
</tr>
<tr>
<td>HLM</td>
<td>Human liver microsomes</td>
</tr>
<tr>
<td>HRMS</td>
<td>High resolution mass spectrometry</td>
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<tr>
<td>ILG</td>
<td>Isoliquiritigenin</td>
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<tr>
<td>Keap1</td>
<td>Kelch-like ECH-associated protein 1</td>
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<tr>
<td>LC-MS</td>
<td>Liquid chromatography - mass spectrometry</td>
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<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography - tandem mass spectrometry</td>
</tr>
<tr>
<td>LicA</td>
<td>Licochalcone A</td>
</tr>
<tr>
<td>LicB</td>
<td>Licochalcone B</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-assisted laser desorption/ionization</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
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<tr>
<td>MDF</td>
<td>Mass defect filter</td>
</tr>
<tr>
<td>MRAs</td>
<td>Michael Reaction Acceptors</td>
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<tr>
<td>m/z</td>
<td>Mass-to-charge ratio</td>
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<td>β-Nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<td>Nrf2</td>
<td>NF-E2-related factor-2</td>
</tr>
<tr>
<td>NQO1</td>
<td>NAD(P)H quinone dehydrogenase 1</td>
</tr>
<tr>
<td>P450</td>
<td>Cytochromes P450</td>
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<tr>
<td>QqTOF</td>
<td>Quadrupole time-of-flight</td>
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<tr>
<td>SRM</td>
<td>Selected Reaction Monitoring</td>
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<tr>
<td>SULT</td>
<td>Sulfotransferase</td>
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<tr>
<td>TIC</td>
<td>Total ion chromatogram</td>
</tr>
<tr>
<td>TOF</td>
<td>Time-of-flight</td>
</tr>
<tr>
<td>UDPGA</td>
<td>Uridine diphosphate glucuronic acid</td>
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<tr>
<td>UGT</td>
<td>UDP-glucuronosyltransferase</td>
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SUMMARY

Botanical products have a long history of use as dietary supplements to maintain or improve health. Recently, the market for botanical dietary supplements has been growing steadily in United States. However, in the United States, dietary supplements can be marketed without proof of safety and efficacy under the U.S. Dietary Supplement and Health Education Act of 1994 (DSHEA). Considering the complexity of the herbal products, obtaining scientific evidence for their efficacy and safety is challenging but nevertheless important for customers.

Licorice root extracts are used in botanical dietary supplements all around the world. In recent years, licorice products have become popular among women seeking relief from premenstrual syndrome and menopausal symptoms. However, the bioactive constituents vary considerably between different licorice species, which will affect the bioactivity of licorice commercial products. Therefore, it is important to develop and apply both chemical and biological standardization methods for botanical dietary supplements.

This dissertation focused on the discovery of bioactive compounds from licorice and the investigation of their human metabolism. Some of these compounds may have the potential to cause drug-botanical interaction such as drug metabolizing enzyme inhibition or induction. To expedite these studies, a new screening assay based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a triple quadrupole mass spectrometer was developed for detecting reactive metabolites and electrophilic compounds from licorice extracts. In addition, several licorice chalcones have been reported to function as chemoprevention agents while at the same time potentially causing drug-botanical interactions such as induction or inhibition of drug metabolizing enzymes.
SUMMARY (Continued)

To confirm a mechanism of chemoprevention, a mass spectrometry-based approach based on MALDI-TOF was used to screen for licorice compounds, which can covalently modify the cytosolic protein Keap1, an essential protein involved in the regulation of the antioxidant response element, a chemoprevention pathway.

In addition to the screening of botanical supplements for bioactive compounds, another goal of this dissertation was to investigate the hepatic metabolism of these compounds. Specifically, licochalcone A (LicA), a licorice chalcone and potential chemoprevention agents from *Glycyrrhiza inflata*, was investigated with respect to its in vitro phase I and phase II metabolism. Recombinant enzymes were used to identify the specific enzymes responsible for the biotransformation of LicA. In vitro metabolic stability assays were also carried out as a means of estimating intrinsic clearance of this compound. Together, these data will facilitate the design of animal studies and clinical trials of LicA and licorice dietary supplements containing LicA.

In conclusion, it is useful to apply mass spectrometry-based screening and standardization assays to the study of safety and mechanisms of action of botanical dietary supplements. These assays are fast, highly selective and extremely sensitive. Liquid chromatography-mass spectrometry is also invaluable for studies of the metabolism and disposition of pharmacologically active agents occurring in these supplements.
CHAPTER 1:

BACKGROUND AND INTRODUCTION

1.1 Botanical Dietary Supplements

1.1.1 Botanical Dietary Supplements in the United States

As defined by the U.S. Food and Drug Administration (FDA), a dietary supplement is a product (other than tobacco) that is intended to supplement the diet; contains one or more of the dietary ingredients (including vitamins; minerals; herbs or other botanicals; amino acids; dietary substances for supplementing the diet by increasing the total dietary intake) or a concentrate, metabolite, constituent, extract, or combination of them; is intended to be ingested in pill, capsule, tablet, or liquid form; is not represented for use as a conventional food or as the sole item of a meal or diet; includes products such as an approved new drug, certified antibiotic, or licensed biologic that was marketed as a dietary supplement. Specifically, a dietary supplement contains ingredients to supplement the diet when taken by mouth, and the ingredients may include: vitamins, amino acids, minerals, herbs or other botanicals. Botanical dietary supplements, are usually used to refer plants or parts of plants that have medicinal or therapeutic value.

Herbal products are popular as alternative medicines worldwide and are widely used in United States. Since the enactment of the United States Dietary Supplement and Health Education Act of 1994 (DSHEA), use of botanical dietary supplements by American consumers has increased by 380%, and this market continues to grow steadily in U.S., reaching over $6.9 billion USD in 2015. Based on the survey conducted by National Health
and Nutrition Examination in 2011, 67% of respondents were taking dietary supplements, among whom, 53% were regular users, and 23% were using botanical dietary supplements. Women were also reported more likely than men to take dietary supplements. The most commonly reported reasons for using supplements were to improve (45%) or maintain (33%) overall health. Women used calcium products for bone health (36%), whereas men were more likely to report supplement use for heart health or to lower cholesterol (18%). Only 23% of products were used based on recommendations of a health care provider. These data showed that dietary supplements are widely used by American consumers.

Many consumers seem to assume that botanical dietary supplements are not only natural but safe. However, the safety of a botanical dietary supplement product depends on many things, for example, its chemical composition, its mechanism of action, manufacture’s preparation procedures, and dosage taken. However, in the United States, dietary supplements can be marketed without proof of safety and efficacy under DSHEA. The complexity of the herbal products and varies widely among species make it not easy to gain scientific evidence for their efficacy and safety. Some potential safety issues that are being avoided by implementing Good Manufacturing Practice (GMP) in the botanical dietary supplement industry as now required by the U.S. FDA include mis-identification or mislabeling of the plant species, adulteration with pharmaceutical agents, and contamination with pesticides, herbicides, heavy metals, or microbes. Herb-drug interactions via alteration of drug metabolizing enzyme activities can also result in toxicity. Therefore, to ensure the safety and efficacy of botanical dietary supplements, they should be developed in a manner similar with pharmaceuticals.
Since 1999, the UIC/NIH Center for Botanical Dietary Supplements Research has conducted several studies on botanical products to characterize and identify botanical species and their natural product constituents, evaluated biological activity, and established health benefits as well as risks of botanical dietary supplements by preclinical and clinical toxicity and efficacy studies. Those methods include the following: authentication of botanical ingredients through genetic fingerprinting and comparison with known botanical species by DNA barcoding; bioassay-guided fractionation screening for active constituents in botanical extract; characterization of absorption, distribution, metabolism, bioavailability, and pharmacokinetics of active constituents in botanicals extract; chemical standardization of botanical dietary supplements with the use of high performance liquid chromatography–tandem mass spectrometry (LC-MS/MS); potential herb-drug interaction; and conduct of clinical evaluations to determine safety and efficacy.

The UIC/NIH Center for Botanical Dietary Supplements Research focuses on the investigation of the potential benefits from botanical dietary supplements for women’s health, for the relief of menopausal symptoms. Traditionally, hormone therapy (HT) is a pharmacological treatment for menopausal and postmenopausal women. HT involves the use of medications designed to boost hormone levels such as estrogens, progesterone or progestins. The most commonly prescribed hormone therapy products are Premarin® and Prempro®. The decreased levels of circulating estrogen and progesterone hormones can cause menopausal discomfort, and HT is the most effective treatment for the short-term relief of menopausal symptoms (including 70-80% of hot flashes). However, a study conducted by Women's Health Initiative (WHI, funded by the National Institutes of Health) indicated that HT can slightly increase the incidence of breast cancer, heart attack, stroke, and pulmonary embolism.
The WHI recommended that women should take the lowest feasible dose of HT for the shortest possible time to minimize these risks. Thus, more and more women have used botanical dietary supplements or other alternative medicines for the relief of menopausal symptoms instead of using conventional HT.\textsuperscript{22} To find safe and effective botanical alternatives to HT for menopausal women, several herbs are being investigated at the UIC/NIH Center for Botanical Dietary Supplements Research, including red clover (\textit{Trifolium pratense}),\textsuperscript{23} black cohosh (\textit{Actaea racemosa}),\textsuperscript{24,25} hops (\textit{Humulus lupulus}),\textsuperscript{23,26,27} wild yam (\textit{Dioscorea villosa}),\textsuperscript{28} and licorice (\textit{Glycyrrhiza sp.}).\textsuperscript{29,30}

1.1.2 Chemopreventive Effects of Natural Products

Chemoprevention, a term coined by Michael Sporn in 1976, refers to the use of chemical agents that occur naturally in food or administrated as pharmaceuticals to inhibit or reverse the process of carcinogenesis.\textsuperscript{31} An ideal chemopreventive agent should have the following properties: 1) little or no toxicity, 2) high efficacy at multiple sites, 3) orally bioavailable, 4) known mechanisms of action, 5) low cost, and 6) human acceptance.\textsuperscript{32} Since 1976, a variety of natural products have been identified as candidates for preventing various diseases, including chronic diseases, diabetes and cancer.\textsuperscript{33,34,35} Considering the relatively low toxicity and chronic use of herb products, botanical dietary supplements are ideal to be developed as chemopreventive agents. Several widely consumed dietary constituents have been shown to have chemoprevention efficacy in experimental models of carcinogenesis, including resveratrol from grapes\textsuperscript{36} and epigallocatechin gallate (EGCG) from green tea.\textsuperscript{37} Many popular botanical dietary supplements such as ginger, garlic, milk thistle, soy products, and licorice
have also been reported to exhibit protective effects against cancer, and their mechanisms of action are summarized in Figure 1.\textsuperscript{34}

![Figure 1. Some of the chemopreventive natural products widely consumed in the United States and reported to have chemopreventive efficacy.](image)

Epidemiological studies have suggested that consumption of botanicals and botanical preparations can decrease the incidence of many cancers via up-regulation of cytoprotective factors, inducing detoxification enzymes like glutathione S-transferase P1 (GSTP1) and NAD(P)H:quinone oxidoreductase 1 (NQO1).\textsuperscript{38} Many carcinogens are activated via phase I metabolic reactions to electrophilic intermediates (carbocations, quinones, quinone methides, quinone imines, epoxides, etc.), which can attack biological nucleophiles such as proteins and DNA.\textsuperscript{39} These reactive metabolites can cause toxicity (especially at high doses) and but be deactivated through phase II conjugation reactions, such as conjugation with glutathione (GSH), sulfation or glucuronidation. Expressing high levels of these detoxification enzymes can protect cells from damage caused by electrophilic compounds.\textsuperscript{40} Because detoxification enzymes are not necessarily expressed or function at maximal capacity in cells, the
development of natural or synthetic compounds to induce the depletion of endogenous and environmental carcinogens through up-regulation of phase II detoxification enzymes has become a strategy for chemoprevention.\textsuperscript{41,42,43}

The activation of transcription factor, Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), has emerged as a key player in protecting cells against various intrinsic and extrinsic assaults.\textsuperscript{44} Nrf2 regulates more than 600 genes, including over one hundred that encode cytoprotective proteins,\textsuperscript{45} named for their ability to protect cells against oxidative stress, reactive electrophilic species, and other types of stress.\textsuperscript{46} Therefore, Nrf2 can lead to up-regulation of the antioxidant responsive element (ARE)-mediated expression of cytoprotective genes, which code for phase II detoxifying and anti-oxidative stress enzymes.\textsuperscript{47} In a cellular environment, Nrf2 is sequestered by the cytoplasmic actin-binding protein: Kelch-like ECH-associated protein 1 (Keap1).\textsuperscript{47,48,49,50} There are 27 cysteine residues in human Keap1 protein working as sensors for induction of the ARE totally.\textsuperscript{47,44,51} Covalent modification of one or more of these cysteine residues in human Keap1 can lead to nuclear accumulation of Nrf2, the up-regulation of cellular protective gene expression by the ARE, and prevention the process of carcinogenesis.\textsuperscript{52,53} Three cysteine residues (C151, C273, C288) in Keap1 have been reported to be import targets affecting Nrf2 nuclear accumulation and ARE signaling by electrophiles.\textsuperscript{54} In particular, C151 appears to be key target of alkylation on Keap1 by electrophilic compounds that function by triggering the ARE through Nrf2.\textsuperscript{55} Therefore, the alkylation of the sulfhydryl groups in the cysteine residues of human Keap1 protein (especially C151) will lead to elevated levels of Nrf2 both in the cytoplasm and the nucleus, which will trigger the ARE to express more detoxification enzymes. This is defined as the “Keap1-Nrf2-ARE” signaling pathway for
The signaling pathway leading to the induction of ARE by chemoprevention agents through the Keap1-Nrf2 system is shown in Figure 2.

Several flavonoids and chalcones occurring in botanical dietary supplement have been reported to exhibit potential anticancer or cancer chemoprevention activities. For example, xanthohumol, a major prenylated flavonoid from hops, was reported to induce the quinone reductase in vitro. The induction activity of the detoxification enzyme NAD(P)H: quinone oxidoreductase 1 (NQO1) corresponded with the alkylation of Keap1 protein by xanthohumol. Since modification of Keap1 is a mechanism of inducing the ARE via the Keap1-Nrf2 pathway, a Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) mass spectrometry-based screening assay was developed for the rapid detection of Keap1 alkylations.
discovery of xenobiotic agents that modify Keap1. This assay is faster than cell-based assays such as the quinone reductase induction assay. The molecular mass, elemental composition and structural information concerning Keap1-modifying compounds present in complex mixtures such as botanical extracts can be obtained using an LC-MS/MS assay following trapping active, electrophilic natural products as glutathione (GSH) conjugates.

1.2 Licorice Species

1.2.1 Medical History of Licorice

Licorice is one of the most popular herbal medicines in the world and has been consumed for over 4000 years. Licorice is derived from the dried roots of Glycyrrhiza species (Leguminosae family) and is the common name for these plants. It is widely used as flavoring additives by the food industry due to its sweet taste. In China, licorice is called “Gan-Cao”, which means “sweet weed”. The Glycyrrhiza genus contains more than 20 species and are widely distributed throughout the world. Three licorice species are commonly used in botanical dietary supplements as follows: Glycyrrhiza glabra L (G. glabra), Glycyrrhiza uralensis Fisch. (G. uralensis) and Glycyrrhiza inflata Bat (G. inflata). G. glabra is known as European licorice, whereas Chinese licorice mainly refers to G. uralensis but also includes G. inflata.

Licorice is recorded in the pharmacopoeias of many Asian and European countries. Licorice has been used in both Eastern and Western cultures as a medicinal herb to treat a variety of ailments. Licorice shows a variety of pharmacological activities, including anti-inflammatory, anti-allergic, anti-cancer, hepatoprotective, neuroprotective and memory enhancing activities. The dried roots of licorice are also used in botanical dietary
supplements to relieve premenstrual syndrome and menopause symptoms.\textsuperscript{75} Many menopausal dietary supplements sold in the United States contain licorice root extract, as licorice has been shown to have estrogenic activity.\textsuperscript{75,76} For example, Avlimil, a dietary supplement which is a mixture of eleven herbal components (including black cohosh, licorice, red raspberry, red clover and kudzu) was reported to stimulate the growth of estrogen-dependent breast tumors (MCF-7) in vivo at the recommended dose.\textsuperscript{77} These results suggested that Avlimil (containing several phenolic compounds) has the estrogenic potential for relieving menopausal symptoms and might help ameliorate female sexual dysfunction.\textsuperscript{77} In related experiments, the estrogenic activity of licorice species and hops were compared by induction of estrogen responsive element (ERE)-luciferase in MCF-7 cells. \textsuperscript{78,79} Liquiritigenin was found to be the principle phytoestrogen of the licorice extracts,\textsuperscript{78} and induced ER-\(\beta\) specific activity in transfection assays and competitive binding assays. However isoliquiritigen, the precursor chalcone of liquiritigenin, demonstrated significant estrogenic activities but was not selective for either ER subtype.\textsuperscript{79,78} Therefore, liquiritigenin and its precursor chalcone may account for the estrogenic activity of licorice extract.

1.2.2 Bioactive compounds from licorice species

Over 300 compounds have been identified and isolated from \textit{Glycyrrhiza} species, and the triterpene saponins and flavonoids are considered the main bioactive classes of compounds.\textsuperscript{80} Glycyrrhizin (or glycyrrhizinic acid) is the main triterpene saponin and the chief sweet-tasting constituent of licorice root. It is composed of a hydrophilic part, two glucuronic acids, and a hydrophobic fragment, glycyrrhetic acid, and is 30-50 times sweeter than sucrose.\textsuperscript{81,82} Licorice bioactive secondary metabolites include flavanones, flavanones, chalcones, isoflavans,
isoflavones, flavones, flavonols, isoflavones, and isoflavanones. Among these, flavanones and chalcones are the most abundant. The yellow color of licorice is due to the flavonoid content, and includes liquiritin, and isoliquiritin. An example of a bioactive secondary metabolite from licorice is glabrene, an isoflavene isolated from *G. glabra*, which showed estrogen receptor (ER) agonism in different tissues and antimicrobial activity against *Helicobacter pylori* in vitro.

The levels of secondary metabolites in licorice varies significantly among species (*G. uralensis*, *G. glabra*, or *G. inflata*), geographic sources, harvesting and processing, and thus affects the pharmacological effects. Liquiritigenin, liquiritin, isoliquiritigenin, and isoliquiritin are present in all three species. *G. glabra* and *G. inflata* produce similar amounts of glycyrrhizin, liquiritin, liquiritin apioside, isoliquiritin, isoliquiritin apioside, and liquiritigenin. However, *G. uralensis* differs from *G. glabra* or *G. inflata* by expressing higher contents of liquiritin, isoliquiritin and liquiritigenin. Some constituents are only present in one of the licorice species. For instance, glabridin is only present in *G. glabra*, while glycycoumarin occurs only in *G. uralensis*, and licochalcones A is found only in *G. inflata*. Therefore, quality control is critical to ensure the reproducible efficacy and safety of licorice dietary supplements.

LicA ((E)-3-(4-hydroxy-2-methoxy-5-(2-methylbut-3-en-2-yl)phenyl)-1-(4-hydroxy phenyl) prop -2-en-1-one; formula: C_{21}H_{22}O_{4}; molecular weight: 338.39; Figure 3) is a natural chalcone (Michael reaction acceptor, MRA) isolated from *G. inflata*, with about 8% w/w in root extract. LicA has been reported to show various bioactivities, such as antileishmanial, anticancer, antibacterial and antiviral (specifically against influenza neuraminidase) properties in vitro. As a chemoprevention agent, LicA can inhibit estrogen metabolism to
genotoxic catechols in MCF-10A cells and is likely responsible for the anti-inflammatory activity of *G. inflata*.\(^{30}\) *G. inflata* extract and LicA also showed induction of NAD(P)H:quinone oxidoreductase 1 (NQO1) activity in murine hepatoma (Hepa1c1c7) cells, which is a chemoprevention enzyme which plays a role in preventing diseases such as cancer, cardiovascular problems, and neurological disorders and is used as a marker for the ARE. Licorice extract can induce the ARE expression in human hepatoma cells, which indicated the involvement of the Keap1-Nrf2 pathway.\(^{89}\) LicA is an electrophilic chalcone, and its chemopreventive effects may come from the modification of Keap1 protein and upregulation of the detoxification enzymes via the ARE. In this dissertation, the alkylation of Keap1 protein by LicA (and other electrophiles from licorice extract) was tested, as well as the ability to modify other biologically important proteins and small cellular nucleophiles (*e.g.*, glutathione). Since absorption, distribution, metabolism, and excretion (ADME) can affect the bioactivity of botanical constituents in vivo, these factors should be investigated during the development of natural products as chemoprevention agents. Therefore, the hepatic metabolism of LicA - the main bioactive constituent from licorice species *G. inflata* – was investigated during this dissertation.

![Figure 3. Structure of licochalcone A](image_url)
1.3 Drug Metabolism in Natural Products Discovery and Development

The properties of absorption, distribution, metabolism, elimination, and toxicity (ADME/T) are important for the clinical success of a drug candidate. Drug metabolism is the process that lipophilic xenobiotics are chemically transformed, usually to more polar metabolites that exhibit increased water solubility, to facilitate their excretion in urine and bile, which is an essential physiological defense against toxic effects for xenobiotics. Drug metabolism connects many aspects of drug discovery and development including pharmacology, medicinal chemistry, candidate optimization, preclinical and clinical development, and toxicology. Most metabolites are inactive, but some of them are toxic or even active than parent compounds. For example, tamoxifen, which is a medicine that is used to prevent breast cancer, was found to increases the risk of uterine cancer by catechols metabolites, followed by metabolic oxidation to o-quinones. Therefore, the understanding of how metabolic processes terminate or limit the desired pharmacological effects (efficacy), and the possible biotransformation pathways which may lead to unintended consequences (safety) are essential for the success of a drug candidate.

The pharmaceutical industry has invested extensively in drug discovery and development in order to bring effective and safe medicinal agents to market while satisfying healthcare needs. The average cost of taking a drug from discovery to market to the market stage is more than $1 billion, with 8-12 years development time. The failures of many drug candidates (even if they had shown excellent properties in preclinical animal models) result from the poor bioavailability or toxicity, which can be related in part to metabolism. The importance of drug metabolism and pharmacokinetics (DMPK) is best illustrated by the observation that 40% of drug attrition during development before 1990 was due to undesirable
DMPK properties. With more awareness in early development and technology advances, DMPK-based drug failure during the clinical development stage has been reduced to less 1%, saving billions of dollars for the pharmaceutical industry and the healthcare system. Therefore, selecting drug candidates with good ADME properties is essential to ensure success in clinical trials.

Like drugs, the metabolic properties of active botanical constituents and the potential for drug-herbal interactions should also be considered during the development of herbal medicines or dietary supplements for human health. As with drugs, metabolism of natural products leads to the formation of pharmacologically less active metabolites, which are more polar and can be quickly excreted outside the body. Fast metabolism and elimination will limit the bioactivity of active components. For example, resveratrol which showed anticarcinogenic, antioxidative, phytoestrogenic, and cardioprotective activities in vitro, with a very short half-life. The most abundant resveratrol metabolites in rats were E-resveratrol-3-O-glucuronide and E-resveratrol-3-sulfate. Those phase II metabolites have been confirmed from human urine and serum, indicating absorption after oral administration followed by fast phase II elimination. Because of the extensive phase II metabolism via formation of glucuronides and sulfates, the oral bioavailability of resveratrol is negligible.

Less frequently, metabolism can produce compounds that are more active than their precursors, or even toxic species. Reactive metabolites can covalently modify DNA and proteins thereby causing toxicity, especially hepatotoxicity. Short-lived electrophilic metabolites can be trapped by glutathione, and then detected and characterize by using LC-MS/MS. A screening assay has been developed to detect the reactive metabolites from kava, red clover, licorice, and black cohosh. Drug-herb interaction is another type of
toxicity in which botanical dietary supplements cause inhibition or induction of drug metabolizing enzymes. Studies of hop extracts containing the chalcone xanthohumol have shown the potential for inhibition of the CYP2C family, which may affect the metabolism and bioactivity of some CYP2C substrate drugs when co-administered.\textsuperscript{17}

For these reasons, metabolic studies of active components of botanical dietary supplements should be performed at the early discovery stage. A “good” candidate compound should have moderate metabolic rate, no toxic metabolites, and predictable drug-herb interactions. Appropriate in vitro results can be applied to predict the metabolism of active constituents in vivo, as well as the potential efficacy and safety.

1.3.1 Hepatic Metabolism Reactions and Enzymes

Many organs in the body are involved in drug metabolism including kidneys, skin, lungs, and intestine. However, the liver is the most metabolically active tissue in which most drug metabolism takes place. After absorption, foreign compounds are converted to more polar metabolites by specific metabolic enzymes under the first pass hepatic metabolism, which increase the clearance of for xenobiotics.\textsuperscript{91}

The drug metabolism process is classified into two groups: phase I and phase II. Phase I reactions include oxidative, hydrolysis, and reductive metabolism of xenobiotics into more hydrophilic metabolites by introducing polar functional groups (-OH, SH, NH\textsubscript{2}, or COOH) to yield more polar derivatives. Cytochromes P450 (CYP450) are the predominant enzymes catalyzing phase I reactions, others include flavin-containing monoxygenase (FMO), peroxidases, monoamine oxidases (MAO), aldehyde oxidase, xanthine dehydrogenase, alcohol
dehydrogenases (ADH), aldehyde dehydrogenases (ALDH), esterases, hydrolase, and epoxide hydrolases. Phase II reactions result in the addition of a functional group either on the parent compound or on the metabolites from Phase I metabolism. They involve biomolecular conjugation with endogenous hydrophilic moieties, such as glutathione (GSH), glucuronic acid, and sulfate. The conjugative enzyme families include UDP-glucuronosyltransferases (UGTs), N-acetyltransferases, sulfotransferases (SULTs), methyltransferases and glutathione-S-transferases (GSTs). Most xenobiotics can undergo both phase I and then phase II reactions sequentially. Among the enzymes involved in the metabolism of drugs, the P450 enzymes are the most important ones (~75%), followed by UGTs and esterases (shown in Figure 4a). Together, these reactions account for ~95% of drug metabolism. The major hepatic metabolic enzymes and reactions are listed in Table I.
# TABLE I.
THE MAJOR HEPATIC METABOLIC ENZYMES AND REACTIONS

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</table>
1.3.1.1 Phase I Metabolism Enzymes

The most significant phase I metabolic enzymes are the cytochromes P450 (CYP) which are concentrated in liver in the smooth endoplasmic reticulum of hepatocytes. The cytochromes P450 are often partially purified as microsomes for in vitro metabolism studies. Other organs including the intestines, kidneys, lungs, and brain express low levels of the cytochromes P450. The cytochrome P450 can be classified into family, subfamily, and isoforms. The reactions mediated by P450 enzymes require co-factors, such as NADH and NADPH. Among the 57 functional P450 genes in humans, three families (CYP1, CYP2 and CYP3) predominate in the oxidative metabolism of more than 90% of clinical drugs, with about seven being dominant in drug metabolism (CYP1A2, 2A6, 2C9, 2C19, 2D6, 2E1 and 3A4(5)). The fractions of drugs that are P450 substrates metabolized by individual isoforms are shown in Figure 4b. Molecular biology studies have revealed that most CYP450 enzymes share a relatively large active site cavity that can accommodate a wide variety of substrates. Another aspect about P450 enzymes is the genetic polymorphisms, which may have the most impact on variations of the fate of therapeutic drugs in individuals. For example, individuals who are CYP2D6 poor metabolizers are comprise approximately 5-14% of Caucasians, 0-5% Africans, and 0-1% of Asians. Genetic polymorphisms within P450s can cause adverse effects and impact the efficacy of drugs that are metabolized by those particular enzymes.

Another phase I enzyme, flavin-monooxygenases (FMOs) have been implicated in the metabolism of a number of xenobiotics, including pesticides, pharmaceuticals and toxicants. FMO-catalyzed reactions are mixed-function oxidations and involve oxidation of heteroatoms, particularly nucleophilic atoms such as the nitrogen of amines. Among the five forms of
FMO occurring in humans, FMO3 is the most abundant form in human liver, and inactivation of FMO3 will lead to deficient metabolism of trimethylamine and a resulting “fish-odor syndrome.” Monoamine oxidases (MAOs) are flavoprotein oxidases and mostly located in the outer mitochondrial membranes of liver. There are two forms in MAOs: MAO-A and MAO-B. MAOs can oxidize a various compounds, especially amines, such as dopamine and tryptamine.

The major enzymes in the metabolism of ethanol in humans, alcohol dehydrogenases (ADHs) are a group of dehydrogenase enzymes that facilitate the inter-conversion between alcohols and aldehydes or ketones. Aldehyde dehydrogenases (ALDHs) are responsible for the oxidation of aldehydes to carboxylic acids. Aldo-keto reductases (AKRs) are mainly responsible for reduction of aldehydes and ketones. Epoxide hydrolase catalyzes the addition of water to epoxides and functions in detoxication during drug metabolism.

![Figure 4](image.png)

**Figure 4.** (a) Fractions of drugs metabolized by various enzyme systems; (b) Fractions of drugs that are P450 substrates metabolized by individual P450s.

1.3.1.2 Phase II Metabolism Enzymes

Unlike phase I metabolism, phase II reaction usually result in an increased molecular weight and are usually inactive metabolites. Glucuronidation is an important detoxification
pathway and the most frequent phase II conjugation reaction, which involves the addition of glucuronic acid to various functional groups. Glucuronidation is catalyzed by UDP-glucuronosyltransferases (UGT), which are a superfamily of membrane-bound enzymes, and requires the cofactor, uridine 5'-diphospho-glucuronic acid (UDPGA).\textsuperscript{114,115,116} Conjugation can occur on a large number of substrates, such as alcohols, phenols, or amines.\textsuperscript{115} After glucuronidation, compounds become more polar and have an increased molecular mass (+176 Da), following the excretion via kidney or via the bile into the small intestine.

Although liver is the major organ involved in glucuronidation, some UGT isoforms also exist at high levels in other organs, such as kidney and intestine.\textsuperscript{117} UGT 1A, UGT 2A and UGT 2B are the three major UGT gene families. Like the P450 gene family, the UGTs can be altered by induction, inhibition and genetic variability. Inducers or inhibitors of UGTs can affect half-lives and concentrations of compounds that are substrates for glucuronidation.\textsuperscript{115} The UGT enzymes have been reported to share genetic polymorphisms, such as UGT 1A1, 1A6, 2B15, 1A4, 1A9 and 2B7.\textsuperscript{118}

Occurring in most cells of the body and especially abundant in the liver, glutathione-S-transferases (GSTs) are a group of phase II protective enzymes. Glutathione (GSH) which is composed by glutamic acid, cysteine and glycine, normally from 0.1 to 10 mM in human tissues. Glutathione-S-transferases activate the thiol of GSH into the more reactive thiolate anion and thereby catalyze the reaction of glutathione with endogenous electrophiles ($\alpha,\beta$-unsaturated aldehydes, quinones, or epoxides). Reactive metabolites of xenobiotics formed via hepatic CYPs reactions can be substrates of GSTs.\textsuperscript{119} Genetic polymorphisms in cytosolic GSTs have been reported, which reduce enzymatic activity can increase susceptibility to carcinogenesis and inflammatory disease.\textsuperscript{120,121}
Sulfation is another common phase II reaction, which is mediated by the sulfotransferases (SULTs) superfamily. In humans, 13 different SULT genes have been identified, and many SULTs have overlapping substrate specificities. The human cytosolic SULTs catalyze the transfer of the cofactor 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to hydroxyl, amino, sulfhydryl, and N-oxide groups of their substrates.\textsuperscript{122,123} Usually a detoxification pathway, these sulfation (also called sulfonation) reactions render their substrates more water soluble and readily excreted from the body, resulting in inactivation of many steroid hormones, neurotransmitters and medicines. Several chemicals are known to inhibit SULTs, and this can cause drug–drug and food–drug interactions.\textsuperscript{124,125}

The phase II N-acetyltransferases (NATs) catalyze acetyl-CoA-dependent N-acetylation reactions,\textsuperscript{126} which are involved in the detoxification of various arylamine and hydrazine drugs.\textsuperscript{127} Human arylamine N-acetyltransferases are expressed as two polymorphic isoforms, NAT1 and NAT2, with sulfamethazine being selectively N-acetylated by NAT2 and with \textit{p}-aminobenzoic acid being a specific substrate for NAT1. Unlike NAT1, which is expressed in many other tissues, NAT2 is expressed mainly in the liver and intestine.\textsuperscript{128,129} Both NAT1 and NAT2 have been found to be polymorphic in human populations. Slow acetylator status has been associated with increased rates of adverse reactions to arylamine antibiotics, such as sulfamethoxazole.\textsuperscript{130}

1.3.2 \textit{In vitro Metabolic Models}

During drug discovery and development, nearly half of candidate drugs fail because of unacceptable ADME properties such as formation of toxic metabolites, drug-drug interactions
and/or poor metabolic stability. Therefore, reliable and predictive models for human metabolism have been established and implemented at an early stage, to speed up development and limit the number of late-stage failures of new chemical entities. In vitro drug metabolism studies including the evaluation of metabolic stability, identification of metabolic pathways, identification of drug metabolizing enzymes, and evaluation of drug-drug interactions have been effective in enhancing our understanding of metabolic pathways and interactions and predicting in vivo metabolism.

In vitro drug metabolism models include subcellular fractions of liver (microsomes, S9 and cytosolic fractions), liver slices, recombinant enzymes, and hepatocytes from animals and humans. Microsomal preparations, S9 fractions and cytosolic fractions are frequently used in vitro, but human liver microsomes (HLM) are the most popular in vitro model. HLM contain the majority of the phase I enzymes (P450s and FMOs, e.g.) and the phase II enzymes (UGTs), can provide a simple system with reproducible results. Both P450s and FMOs require NADPH as a cofactor to donate an electron for the metabolism reactions. For the evaluation of UGT mediated metabolism, the cofactor UDPGA is required to initiate the reaction. Therefore, HLM can be used for metabolic profiling, to evaluate metabolic stability and in vitro intrinsic clearance, to identify reaction phenotyping, and to evaluate drug candidates as inhibitors of P450 and UGT enzymes. However, due to the enriched concentrations of P450s and UGTs in the microsomal fractions and the absence of other enzymes and cofactors, liver microsome are not a completely evaluation model for in vivo metabolism than intact liver cells or tissues. These limitations make microsomes useful for qualitative but not quantitative prediction of in vivo human biotransformation.
Unlike microsomal enzymes such as P450s and UGTs, some soluble phase II metabolic enzymes, such as SULTs, NATs, and GST, are found in cytosolic fractions of hepatocytes. Those cytosolic enzymes involve in the metabolism of xenobiotics which are mostly metabolized by phase II soluble enzymes. Cytosolic enzymes can be obtained by differential centrifugation of whole-liver homogenates and need cofactors (acetyl coenzyme A for NATs; PAPS for SULT; and GSH for GST) for enzymatic activity.\textsuperscript{137}

The liver S9 fractions are prepared by homogenizing the liver, disrupting the cells and then centrifuging at 9000xg to obtain the supernatant. S9 fractions offer a more complete metabolic profile of drugs, because they contain both cytosolic and microsomal enzymes. Exogenous cofactors are needed to ensure reaction when conducting in vitro incubations. Due to lower enzyme levels than microsomes, the S9 fractions are not used as often as microsomes for hepatic metabolism studies.\textsuperscript{136}

Human hepatocytes, which are freshly isolated or cryopreserved from livers by a procedure called two-step collagenase digestion,\textsuperscript{138} are also commonly used in metabolic profiling studies. Compared with subcellular fractions (microsomes, S9 and cytosolic fractions), hepatocytes have an intact biological membrane and a complete set of all metabolic enzymes, receptors and cofactors needed for metabolic reactions (both phase I and phase II) at physiological concentrations.\textsuperscript{139} However, unlike subcellular fractions, hepatocyte experiments are more expensive, suffer from inter-individual variations from batch-to-batch, and cannot be used in automated assays.\textsuperscript{140,141}

High-precision cut liver slices with consistent dimensions are also used for in vitro metabolic studies.\textsuperscript{142} Unlike the cell fractions or isolated enzymes, this model can preserve the intact tissue architecture of the liver, therefore they can more closely mimic the in vivo enzyme
environment. This three-dimensional structure of the liver makes it possible to monic the drug-drug interactions in the cellular environment from an intact isolated tissue. However, due to inadequate penetration of the medium into the inner part of the slice, lack of optimal incubation methods and difficulties in storage and handling, the use of liver slices is not very common for drug metabolism studies.

Recently, cDNA-expressed human metabolic enzymes prepared from baculovirus-infected insect cells have become widely available and are being used increasingly to identify the enzymes metabolizing target compounds. The availability of specifically expressed recombinant enzymes such as human P450s, UGTs and NATs facilitates investigation of the contribution of individual metabolic enzymes to the overall biotransformation pathway. Another thing is, the use of recombinant enzymes can exclude enzyme interference present in microsomes or hepatocytes. Easy to use and well characterized preparations of all major isoforms are now commercially available. The identification of the isozyme-specific drug biotransformation facilitate identification of metabolic structure-activity relationships (SAR) and predictions of drug-drug interactions.

1.4 Application of Liquid Chromatography-Mass Spectrometry to Drug Discovery and Development

The combination of high-performance liquid chromatography and mass spectrometry (LC-MS) has become broadly used in all sectors in drug discovery and development over the past decades (Figure 5). Those analytical instruments with high sensitivity, selectivity,
reproducibility, and accessibility provide a powerful platform for bioanalysis. The application of LC-MS instruments in drug discovery and development will be discussed in next.

![Mass Spectrometer Diagram](image)

**Figure 5.** Schematic of a typical LC-MS based experiment.

1.4.1 **LC-MS**

High performance liquid chromatography (HPLC) has numerous applications in pharmaceutical research and development. Although widely used, HPLC is being supplanted by ultrahigh pressure liquid chromatography (UHPLC) for many applications. Typical characteristics of UHPLC include smaller particles (1.7 μm column particle size), fast flow rates (up to 2 mL/min), higher pressure limits (up to 15,000 psi instead of 6,000 psi for HPLC), and small column solvent volumes (100-200 μL), leading to higher throughput, higher resolution, higher sensitivity, and improved accuracy compared with HPLC.\(^{145}\)

Compatible with on-line HPLC or UHPLC separations, mass spectrometry (LC-MS or UHPLC-MS) adds another analytical dimension to drug discovery and development. Electrospray ionization (ESI), which is called as a “soft ionization” technique due to the low internal energies and in-source fragmentation of the ions, is widely used during LC-MS. Based on the measurement of mass-to-charge ratios \(m/z\) of ions, the most widely used types of mass analyzers for biomedical research are beam-type analyzers (magnetic sector and quadrupole mass spectrometers), pulsed analyzers (time-of-flight, TOF mass spectrometer) and ion-trapping mass analyzers (ion cyclotron resonance, orbitrap, ion trap mass spectrometers).\(^{146}\)
Ion trapping mass spectrometers offer limited scan range but more structural information through MS^n (multiple stages of tandem mass spectrometry) and ion cyclotron resonance. Tandem mass spectrometry is useful in proposing the fragmentation pathways and high resolution mass spectrometers, such as orbitrap or TOF, facilitate the determination of elemental compositions via accurate mass measurement. Hybrid quadrupole time-of-flight (QqTOF) mass spectrometers can provide collision-induced dissociation (CID) for more extensive fragmentation, as well as accurate mass measurements. Orbitrap is a kind of high resolution MS, which create the image current by detecting and converting the trapped ions into a mass spectrum using the Fourier transform of the frequency signal, they provide the ultrahigh resolving power for accurate mass measurement as well as MS^n capabilities and usually used most often in proteomics and metabolomics studies. In summary ion trap-TOF, QqTOF, and orbitrap instruments are high resolution tandem mass spectrometers extensively used in drug metabolism research due to their high resolution, accurate mass measurement and tandem capabilities that facilitate metabolite identification and structure elucidation.

Triple quadrupole mass spectrometers are a type of tandem mass spectrometer that are ideal for quantitative analysis of both small molecules and peptides due to their exceptional sensitivity when used in selected reaction monitoring (SRM) mode. Quantitative analyses of a parent drug or its metabolites are usually applied on metabolic stability, drug-drug interaction and enzyme kinetic studies during drug discovery and development process. Triple quadrupole instruments are also capable of unique scan modes such as constant neutral loss (CNL) scanning or precursor ion scanning that are particularly useful when screening biological samples for specific classes of metabolites. Hybrid Q-trap instruments are able to do SRM quantitation like triple quadrupole instruments, as well as MS3 for further structure
However, triple quadrupole and Q-trap mass spectrometers cannot do accurate mass measurements because of relatively low resolution.\textsuperscript{153}

Matrix-assisted laser desorption/ionization (MALDI) uses a pulsed laser and a laser light absorbing matrix to simultaneous form and desorb ions (including ions of large molecules) with minimal fragmentation.\textsuperscript{154} Compared with electrospray ionization technique, MALDI is useful for the analysis of biomolecules, such as DNA, small molecules, peptides and proteins, but typically produces far fewer multiply charged ions of large molecules.\textsuperscript{155} During MALDI analysis, the sample is first mixed with a suitable matrix material (2,5-dihydroxy benzoic acid; sinapinic acid; α-cyano-4-hydroxycinnamic acid etc.) and applied to a metal target. Then, a pulsed laser irradiates the sample, resulting in desorption of the matrix and sample with simultaneous ionization of the analyte (usually protonation or deprotonation) in the hot plume of ablated gases. The ions are then accelerated into mass spectrometer for analysis.\textsuperscript{156} A pulsed analyzer, TOF mass spectrometers are often equipped with MALDI because it is a pulsed ionization technique.\textsuperscript{156}

1.4.2 LC-MS Applications in Drug Discovery and Development

During discovery and preclinical stages of drug development, metabolic stability and biotransformation pathways of the lead compounds are studied to identify the metabolic soft spots for compound optimization, with an optimal pharmacokinetics (PK) profiles. Metabolite elucidation is mainly based on prior knowledge of the likely routes of biotransformation, predicted losses or gains in molecular mass.\textsuperscript{157} Common drug biotransformations and associated mass changes are listed in Table II. High resolution mass spectrometry, such as
QqTOF, or orbitrap are widely used for metabolite characterization and identification. Other methods of identification include comparison of unknown metabolites with HPLC retention times and tandem mass spectra of standards, and structural analysis using NMR.\textsuperscript{158}

**TABLE II.**

COMMON DRUG METABOLISM BIOTRANSFORMATIONS AND THEIR CORRESPONDING ELEMENTAL COMPOSITIONS AND MASS CHANGES

<table>
<thead>
<tr>
<th>Metabolic reaction</th>
<th>Molecular formula change</th>
<th>(m/z) change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Debenzylation</td>
<td>-C(_7)H(_6)</td>
<td>-90.0468</td>
</tr>
<tr>
<td>tert-Butyl dealkylation</td>
<td>-C(_4)H(_8)</td>
<td>-56.0623</td>
</tr>
<tr>
<td>Decarboxylation</td>
<td>-CO(_2)</td>
<td>-43.9898</td>
</tr>
<tr>
<td>Isopropyl dealkylation</td>
<td>-C(_3)H(_6)</td>
<td>-42.0468</td>
</tr>
<tr>
<td>Hydroxymethylene loss</td>
<td>-CH(_2)O</td>
<td>-30.0106</td>
</tr>
<tr>
<td>Deethylation</td>
<td>-C(_2)H(_4)</td>
<td>-28.0312</td>
</tr>
<tr>
<td>Decarboxylation</td>
<td>-CO</td>
<td>-27.9949</td>
</tr>
<tr>
<td>Dehydration</td>
<td>-H(_2)O</td>
<td>-18.0105</td>
</tr>
<tr>
<td>Demethylation</td>
<td>-CH(_2)</td>
<td>-14.0157</td>
</tr>
<tr>
<td>Hydroxylation+dehydration</td>
<td>-H(_2)</td>
<td>-2.0157</td>
</tr>
<tr>
<td>Alcohols to aldehyde/ketone</td>
<td>-H(_2)</td>
<td>-2.0157</td>
</tr>
<tr>
<td>Desaturation</td>
<td>-H(_2)</td>
<td>-2.0157</td>
</tr>
<tr>
<td>Ketone to alcohol</td>
<td>+H(_2)</td>
<td>2.0157</td>
</tr>
<tr>
<td>N, O, S methylation</td>
<td>+CH(_2)</td>
<td>14.0157</td>
</tr>
<tr>
<td>Hydroxylation</td>
<td>+O</td>
<td>15.9949</td>
</tr>
<tr>
<td>Hydration, hydrolysis</td>
<td>+H(_2)O</td>
<td>18.0105</td>
</tr>
<tr>
<td>Hydroxylation and methylation</td>
<td>+CH(_2)O</td>
<td>30.0105</td>
</tr>
<tr>
<td>2×Hydroxylation</td>
<td>+O(_2)</td>
<td>31.9898</td>
</tr>
<tr>
<td>Acetylation</td>
<td>+C(_2)H(_2)O</td>
<td>42.0106</td>
</tr>
<tr>
<td>3×Hydroxylation</td>
<td>+O(_3)</td>
<td>47.9847</td>
</tr>
<tr>
<td>Glycine conjugation</td>
<td>+C(_2)H(_3)NO</td>
<td>57.0215</td>
</tr>
<tr>
<td>Sulfation</td>
<td>+SO(_3)</td>
<td>79.9568</td>
</tr>
<tr>
<td>Cysteine conjugation</td>
<td>+C(_3)H(_2)NOS</td>
<td>103.0092</td>
</tr>
<tr>
<td>Decarboxylation and glucuronidation</td>
<td>+C(_5)H(_6)O(_5)</td>
<td>148.0372</td>
</tr>
<tr>
<td>Glucuronic acid conjugation</td>
<td>+C(_6)H(_6)O(_6)</td>
<td>176.0321</td>
</tr>
<tr>
<td>GSH conjugation</td>
<td>+C(<em>{10})H(</em>{17})N(_3)O(_6)S</td>
<td>307.0839</td>
</tr>
<tr>
<td>2×Glucuronic acid conjugation</td>
<td>+C(<em>{12})H(</em>{16})O(_{12})</td>
<td>352.0642</td>
</tr>
</tbody>
</table>
When a xenobiotic compound and its metabolites share a common substructure that may be lost during fragmentation in a tandem mass spectrometer, the use of precursor ion scanning or constant neutral loss (CNL) scanning of all molecular species in a metabolic reaction mixture may be used for the selective detection of these structurally related compounds. In particular, phase II conjugated metabolites may be easily identified using this approach. For example, CNL of 176, 129 and 80 Da are typical neutrals eliminated glucuronides, glutathione conjugates, and sulfates, respectively, during MS/MS with CID.\textsuperscript{159} Compared to stable metabolites, reactive metabolites have short half-lives and are therefore challenging to isolate or even to detect. Therefore, in vitro chemical trapping has been used to form stable products that may be analyzed using mass spectrometry and/or nuclear magnetic resonance.\textsuperscript{160,161} For example, chemical trapping using the endogenous nucleophile GSH has been reported followed by MS/MS selective detection.\textsuperscript{162} The trapping experiments typically include the incubation of the test agent with liver microsomes, NADPH and thiol trapping agents such as GSH or N-acetylcysteine. GSH is present in all mammalian tissues, highly concentrated (~10 mM) in liver tissue, and serves as a natural scavenger for reactive metabolites by reacting with a broad range of electrophiles including epoxides, quinones, and Michael addition acceptors.\textsuperscript{163,164}

Fragmentation of GSH conjugates during MS/MS with collision-induced dissociation (CID) shows characteristic patterns similar to the dissociation of peptides (Figure 6).\textsuperscript{165,166} GSH conjugates generally undergo a neutral loss of 129 Da (corresponding to pyroglutamatic acid) during positive ion tandem mass spectrometry, which enables GSH conjugates to be detected during MS/MS using CNL scanning of 129 Da in positive mode. During negative ion MS/MS product ion scanning of the deprotonated molecule, GSH conjugates form a common
fragment anion of \( m/z \) 272 (deprotonated \( \gamma \)-glutamyl-dehydroalanyl-glycine).\(^{167,161}\) This facilitates the detection of GSH conjugates in negative mode during MS/MS using precursor ion (PI) scanning of 272 Da.

Figure 6. Characteristic fragment ions of glutathione conjugates under collision induced dissociation (CID).

LC-MS/MS based quantitative analysis of drugs and drug-related substances in support of pharmacokinetics is intensely used during pre-clinical and clinical stages of development due to its high sensitivity and selectivity. For example, in metabolic stability studies, LC-MS/MS has been used to evaluate the rate of disappearance of the compound of interest by detecting its concentration before and after incubation with hepatic enzymes.\(^{168}\) Quantitative LC-MS/MS assays generally involve four steps: sample preparation, assay calibration, sample analysis, and data management. Quantitative analysis is used most often with triple quadrupole mass spectrometers in SRM mode. During SRM, the first and third quadrupoles are static and set to transmit pairs of preselected precursor ions and (after fragmentation in the middle quadrupole) the preselected product ions. Triple quadrupole mass spectrometers provide a high
degree of sensitivity, selectivity and better limits of quantitation (LOQ) than scan modes for the analysis of complex mixtures.\textsuperscript{169}

Drug metabolism involves addition of polar groups, for example, -OH, -SH, -NH\textsubscript{2}, or -COOH, to the parent compound. Taking advantage of the active hydrogen in these functional groups, hydrogen/deuterium (H/D) exchange techniques are also broadly used in structure characterization by mass spectrometry.\textsuperscript{170} Stable isotope labeling (\textsuperscript{2}H, \textsuperscript{13}C, \textsuperscript{15}N, \textsuperscript{18}O, \textsuperscript{34}S, etc) is also often used to facilitate identification of drug metabolites by providing a diagnostic isotope pattern to understand their disposition in complex mixtures during mass spectrometric analysis.\textsuperscript{171} Accurate mass measurements by QqTOF, ion trap-TOF-TOF, Fourier transform ion cyclotron resonance, orbitrap, or magnetic sector mass spectrometers can provide high mass resolving power and high mass accuracy for elemental composition determination during the structure identification of metabolites from both in vivo and in vitro studies.\textsuperscript{149}

Another MS approach used to detect and characterize drug metabolites is mass defect filtering (MDF) which was introduced for drug metabolism studies in 2003. MDF is based on the mass defects (the difference between the exact molecular mass and nominal molecular mass) of common metabolites. For example, within a 50 mDa window for common drug metabolites, mass defect changes for hydroxylation of a compound are -5 mDa, dehydrogenation -16 mDa, demethylation -23 mDa, glucuronidation +32 mDa, sulfation -43 mDa, and GSH conjugation +68 mDa. High mass accuracy mass spectrometers (\textit{e.g.}, QqTOF) with a mass defect window setting of ± 50 mDa can remove the majority of interfering ions and noise while allowing selective detection of metabolites structurally related to their precursor drug. This approach is particularly useful for detecting novel biotransformation pathways or unexpected metabolites.\textsuperscript{172} Computer based algorithms enable mass spectrometers
to adjust the type of measurements being made while acquiring data in real time. For example, data-dependent acquisition during a survey scan (normal scan, precursor ion scan, or constant neutral scan), can provide information that signals the mass spectrometer system to switch from one type of scan (such as MS scan mode) to MS/MS mode (or from one type of MS/MS mode to another) when pre-defined criteria are met, thereby maximizing mass spectrometric information within a single run.¹⁷³
CHAPTER 2:

DEVELOPMENT OF A UHPLC-MS/MS METHOD FOR DETECTING REACTIVE METABOLITES IN BOTANICAL EXTRACTS

This chapter contains information overlapping with reference\textsuperscript{62} (Huang et al. Anal. Chem. 2015), which is a peer-reviewed manuscript resulting from this dissertation.

2.1 Background and Introduction

Although xenobiotic metabolism is usually a detoxification process rendering drugs and similar molecules more polar and more rapidly excreted, metabolic transformation may result in electrophilic products that are potentially more toxic than their precursors. There are many examples of metabolic activation of drugs to electrophiles that covalently bind to macromolecules causing cell damage, and this process is responsible for 60\% of instances where drugs had to be withdrawn from the U.S. market or had black box warnings added to their packaging due to hepatotoxicity.\textsuperscript{174} To address this safety concern, lead compounds are typically evaluated early during drug discovery and development for the formation of reactive metabolites and then chemically modified or abandoned to avoid this potentially toxic pathway.\textsuperscript{167} Since reactive metabolites have short half-lives and are therefore challenging to isolate or even to detect, in vitro chemical trapping has been used to form stable products that may be analyzed using mass spectrometry and/or nuclear magnetic resonance.\textsuperscript{160,161,175} These experiments typically include the incubation of liver microsomes with NADPH and thiol trapping agents such as glutathione (GSH) or N-acetylcysteine. GSH is present in all
mammalian tissues and serves as a natural scavenger for reactive metabolites,\textsuperscript{166} by reacting with a broad range of electrophiles including epoxides, arene oxides, alkylhalides, quinones, and Michael addition acceptors.\textsuperscript{164} Tandem mass spectrometric fragmentation of GSH conjugates during CID shows characteristic peptide product ions.\textsuperscript{176,165,162} For example, GSH conjugates generally undergo a neutral loss of pyroglutamic acid (weighing 129 Da) during positive ion electrospray tandem mass spectrometry (MS/MS), which enables GSH conjugates to be detected with considerable selectivity using constant neutral loss (CNL) scanning of 129 Da (Figure 7).\textsuperscript{162,177} Unfortunately, not all GSH conjugates form positive ions, and some that do ionize in positive mode do not produce a neutral loss of 129 Da during CID.\textsuperscript{178} Another major drawback of this approach is false positive results due to interference from endogenous compounds. To overcome such false positives, Yan and Caldwell used an equimolar mixture of GSH and isotope labeled GSH ([\textsuperscript{13}C\textsubscript{2},\textsuperscript{15}N]-glycine) (Figure 7) to trap reactive drug metabolites from liver microsomal incubations for detection using HPLC-MS/MS with CNL scanning.\textsuperscript{179} This method produced unambiguous doublet isotopic peaks with a mass difference of 3 Da for GSH conjugates that formed positive ions during electrospray, but those conjugates that did form positive ions or eliminate 129 Da remained undetected.

When deprotonated GSH conjugates are formed, negative ion MS/MS product ion scanning usually shows fragment ions of \textit{m/z} 272 corresponding to deprotonated \(\gamma\)-glutamyldehydroalananyl-glycine (Figure 7). Therefore, precursor ion scanning of \textit{m/z} 272 in negative ion mode is an approach to detect GSH conjugates that do not form positive ions while being less prone to false positive results.\textsuperscript{178} To reduce false negative results and to characterize the detected GSH conjugates, Wen \textit{et al.} used polarity switching during HPLC-MS/MS to combine PI scanning of \textit{m/z} 272 of GSH conjugates in negative ion mode with
positive ion MS/MS product ion scanning.\textsuperscript{180} This approach to the detection and characterization of GSH conjugates worked well for the subset of GSH conjugates forming both product ions of \( m/z \) 272 during negative ion electrospray as well as protonated molecules in positive ion mode.

Another MS-based approach for detecting GSH conjugates is mass defect filtering (MDF).\textsuperscript{181,182} Using high resolution mass spectrometry with accurate mass measurement, GSH conjugates can be detected by applying MDF, which takes into account the unique fractional mass (the mass to the right of the decimal point) of a GSH conjugate. In its original form, this approach requires that the approximate elemental composition of the GSH conjugate be known in advance. Then, a mass defect range could be calculated and applied to the high resolution mass spectra such that those ions with mass defects within the predicted range are determined to be GSH conjugates. Recognizing that MDF cannot be used to screen for GSH conjugates of unknown compounds (as in the case of botanical dietary supplements), Zhu revised the approach to test for the negative ion electrospray GSH fragment ion of exact mass \( m/z \) 272.0888.\textsuperscript{183} Of course, MDF that is limited to negatively charged GSH conjugates will miss those that form only positive ions. Furthermore, MDF requires access to high resolution accurate mass spectrometry.

In the present study, selected features from several previous methods for GSH conjugate detection were combined with new approaches that included CNL MS/MS scanning,\textsuperscript{165} stable isotope labeled GSH,\textsuperscript{179} polarity switching,\textsuperscript{180} precursor ion MS/MS scanning,\textsuperscript{178} and the new features of ultrahigh pressure liquid chromatography (UHPLC) and fast-scanning triple quadrupole mass spectrometry. False positive results were avoided by using a 1:1 ratio of GSH and stable isotope labeled GSH to trap reactive metabolites. Fast
positive ion CNL MS/MS scanning of 129 Da was alternated online with negative ion
precursor ion MS/MS scanning of m/z 272 (unlabeled GSH) and m/z 275 (labeled GSH) for
the unambiguous detection of GSH conjugates that formed either positive or negative ions
(Figure 7). Compared with our previous GSH screening assay based on HPLC-MS/MS,\textsuperscript{180}
UHPLC-MS/MS reduced analysis time from 30 min to less than 8 min.

The utility of this new approach was verified using seven compounds known to form
reactive metabolites (acetaminophen, ticlopidine, diclofenac, p-cresol, 4-ethylphenol,
amodiaquine, and 17α-ethinylestradiol) and four compounds that do not form reactive
metabolites as negative controls (dextromethorphan, testosterone, midazolam, and
tolbutamide; Figure 8). Finally, the method was used to study the bioactivation of a licorice
extract from \textit{Glycyrrhiza glabra}, which was found to form multiple GSH conjugates, including
previously reported GSH conjugates with the chalcone isoliquiritigenin as well as new
conjugates with the isoflavan glabridin (Figure 8).\textsuperscript{184} Glabridin is known to inactivate
cytochrome P450 3A4 and CYP2B6 in a time-dependent manner, although no reactive
metabolites have yet been reported.\textsuperscript{185}
Figure 7. UHPLC-MS/MS approach to screening for GSH conjugates.
2.2 Experimental Section

2.2.1 Materials

Acetaminophen, diclofenac, \( p \)-cresol, ticlopidine, amodiaquine, \( 17\alpha \)-ethinyl estradiol, dextromethorphan, testosterone, midazolam, tolbutamide, reduced GSH, \( ([^{13}\text{C}_2,^{15}\text{N}] \)-glycine) GSH (Figures 8), \( \beta \)-nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt (NADPH), glabridin, isoliquiritigenin, and trichloroacetic acid were purchased from Sigma-Aldrich (St. Louis, MO). A methanolic extract of botanically authenticated \textit{Glycyrrhiza glabra} roots was prepared as described previously.\(^{78}\) Pooled human liver microsomes (20 mg/mL, 150 donors) were purchased from BD Biosciences (Woburn, MA). Bond Elute C\(_{18}\) solid phase extraction cartridges (3 mL, 200 mg sorbent) were purchased from Agilent Technologies.
(Santa Clara, CA), and HPLC-grade solvents were purchased from Thermo Fisher (Pittsburgh, PA).

2.2.2 **Microsomal Incubations**

Human liver microsomes (0.5 mg/mL) and 1 mM GSH (equimolar unlabeled GSH and labeled GSH) were incubated separately with each test compound (10 to 50 μM) or extract (500 μg/mL) in 100 mM phosphate buffer (pH 7.4) at 37 °C in a final volume of 1 mL. NADPH (1 mM) was added to initiate oxidative metabolism after a 5 min preincubation. Control experiments were carried out without microsomes, NADPH, test compound, or GSH. After 60 min, the reactions were terminated by the addition of 100 μL of trichloroacetic acid (10%) followed by 15 min centrifugation at 12 000xg and 4 °C. Supernatants (1 mL) of each were removed and loaded onto solid phase extraction cartridges.

2.2.3 **Solid Phase Extraction**

Sample preparation was carried out using C₁₈ solid phase extraction cartridges that were prewashed with 1 mL of methanol and then conditioned with 1 mL of water. After loading a supernatant from a microsomal incubation, the cartridge was washed with 2 mL of water and then eluted with 2 mL of methanol. The methanol elute was evaporated to dryness under a stream of nitrogen and reconstituted in 100 μL of acetonitrile/water (10:90, v/v) before analysis using UHPLC-MS/MS.
2.2.4 **Mass Spectrometry**

A Shimadzu (Kyoto, Japan) LCMS-8050 triple quadrupole mass spectrometer equipped with a Shimadzu Nexera UHPLC system was used with a Waters (Milford, MA) Acquity UPLC BEH Shield RP$_{18}$, 2.1 × 50 mm, 1.7 μm column for UHPLC-MS/MS analyses. The UHPLC mobile phase A was 5 mM ammonium acetate in water containing 0.1% formic acid, and mobile phase B was acetonitrile containing 0.1% formic acid. The gradient elution profile consisted of a 3.5 min linear gradient from 5% B to 100% B. The column was re-equilibrated with 5% B for at least 1 min between analyses. The UHPLC flow rate was 0.4 mL/min. The column temperature was 40 °C, and the injection volume was 10 μL.

Electrospray was used for ionization during UHPLC-MS/MS while the following cycle of MS/MS scans was carried out: negative ion precursor ion scanning for $m/z$ 272 (unlabeled GSH conjugates), negative ion precursor ion scanning for $m/z$ 275 (labeled GSH conjugates), and positive ion CNL scanning for a loss of 129 Da (labeled and unlabeled GSH conjugates). The scan range was $m/z$ 400–700 at unit resolving power. The polarity switching speed was 5 ms, and each scan was recorded over 0.12 s. Additional mass spectrometer parameters included an electrospray voltage of 4.0 kV (positive ion) and −4.5 kV (negative ion), a Q3 voltage of −17 V (positive ion) and 13 V (negative ion), a collision energy of −13 V (positive ion) and 12 V (negative ion), a nebulizing gas flow of 2 L/min, a heating gas flow of 10 L/min, a drying gas flow of 10 L/min, an interface temperature of 300 °C, a desolvation line temperature of 250 °C, and a heat block temperature of 400 °C.

As part of the method validation (although not required for routine GSH conjugate screening), each sample was also analyzed using high resolution accurate mass measurement with data-dependent product ion MS/MS on a Shimadzu LCMS-ITTOF hybrid mass
spectrometer equipped with a Shimadzu Prominence UFLC-XR HPLC system. Separations were obtained using a Waters X-Terra C18, 2.1 × 100 mm, 3.5 μm HPLC column. The mobile phase consisted of a 20 min linear gradient from 2 mM ammonium acetate in water containing 0.1% formic acid to acetonitrile containing 0.1% formic acid. The flow rate was 0.3 mL/min, and the column temperature was 30 °C. In the electrospray source, the nitrogen nebulizing gas flow was 1.5 L/min, the heating gas flow 10 L/min, the interface temperature was 300 °C, the desolvation line 200 °C, and the heat block was 300 °C. Positive ion electrospray mass spectra and product ion tandem mass spectra were recorded from m/z 100 to 700. Product ion mass spectra were recorded in 0.411 s using unit resolution selection in the ion trap and a resolving power of 14 000 in the time-of-flight (TOF) sector. Ion accumulation times in the ion trap were 0.02 s for MS and 0.015 s for MS/MS. During collision-induced dissociation in the ion trap, the collision energy was set to 80%, the collision gas was 80%, and the frequency was 0.251 (45 kHz). The detector voltage was 1.65 kV.

2.3 Results and Discussion

2.3.1 Screening GSH Conjugates of Tested Compounds

The MS/MS conditions for GSH conjugate detection were optimized using 1 μM GSH prior to the analysis of the GSH conjugates. By varying the concentration of formic acid from 0 to 0.2%, it was determined that 0.1% formic acid was optimum for the formation of abundant protonated GSH during positive ion electrospray as well as abundant deprotonated GSH during negative ion electrospray. The optimum CE values for MS/MS (−13 V for positive ion and 12 V for negative ion) that were determined using GSH were confirmed to be valid for GSH
conjugates such as those of acetaminophen. Even though the triple quadrupole mass spectrometer that was used was capable of 0.01 s per scan over the selected mass range, scans of 0.10 to 0.15 s produced tandem mass spectra with superior signal-to-noise in both CNL and precursor ion scanning modes (data not shown). Subsequently, 0.12 s per scan was used for all GSH conjugate screening.

**Acetaminophen.** Cytochrome P450 enzymes in the human liver catalyze the oxidation of acetaminophen to several metabolites including the electrophile $N$-acetyl-$p$-benzoquinone imine.\(^{160}\) Figure 9 shows CNL and precursor ion MS/MS chromatograms of acetaminophen metabolites after incubation with human liver microsomes, cofactor NADPH, unlabeled GSH, and $[^{13}C_2,^{15}N]$-GSH. Two peaks were detected eluting at 1.75 and 1.86 min in all three chromatograms using our new approach, indicating that these were GSH conjugates of acetaminophen.

The positive ion electrospray CNL (129 Da) MS/MS chromatogram of the acetaminophen GSH conjugate eluting at 1.75 min showed two protonated molecules of equal abundance at $m/z$ 473 and $m/z$ 476 (Table III), corresponding to conjugation with unlabeled and labeled GSH, respectively, and confirming that this peak was not a false positive result. The corresponding deprotonated molecules of $m/z$ 471 and $m/z$ 474 at equal abundance observed during negative precursor ion scanning (Table III) also indicated that this peak was a GSH conjugate. This compound is consistent with a previously reported monoxygenated acetaminophen quinone imine that forms a GSH conjugate (acetaminophen + $O-2H$ + GSH).\(^{180}\) The most intense peak (retention time 1.86 min; Figure 9) showed protonated molecules of $m/z$ 457 and $m/z$ 460 at equal abundance during positive ion CNL scanning and also formed the corresponding deprotonated molecules of $m/z$ 455 and $m/z$ 458 at
approximately equal abundance during negative precursor ion scanning of $m/z$ 272 (Table III). These masses are consistent with the reaction of GSH with an N-acetyl-p-benzoquinone imine metabolite of acetaminophen (acetaminophen−2H+GSH) as reported previously.$^{179,181}$

![Figure 9. UHPLC-MS/MS chromatograms of GSH conjugates formed during incubation of acetaminophen with human liver microsomes, NADPH, GSH, and $[^{13}C_2,^{15}N]$-GSH. A) Positive ion electrospray neutral loss MS/MS scanning of 129 Da; B) negative ion electrospray MS/MS scanning for precursors of $m/z$ 272 (unlabeled GSH); and C) negative ion electrospray MS/MS precursor ion scanning for $m/z$ 275 (labeled GSH).](figure)

For method validation, positive ion electrospray HPLC-MS/MS analyses with product ion scanning of the ions of $m/z$ 473, $m/z$ 476, $m/z$ 457, and $m/z$ 460 were carried out using a high resolution hybrid mass spectrometer. For example, accurate mass measurements of the ions of $m/z$ 457 and $m/z$ 460 were within 5 ppm of expected formula $C_{18}H_{24}N_4O_8S$, and both conjugates shared a common fragment ion of $m/z$ 382 (Figure 10), which represented the loss of labeled/unlabeled glycine from the GSH moiety. Another common fragment ion of $m/z$ 182
corresponded to cleavage of the S-peptide bond with a loss of the GSH residue and retention of the positive charge on the unlabeled acetaminophen metabolite. Other abundant fragment ions of \( m/z \) 328 (unlabeled) and \( m/z \) 331 (labeled) originated from the loss of a GSH-characteristic pyroglutamate (129 Da) group, and ions of \( m/z \) 311 and \( m/z \) 314 corresponded to a loss of unlabeled amino-pyroglutamate (146 Da) from the protonated molecules (Figure 10). Therefore, this new screening assay correctly detected and characterized the known quinoid GSH conjugates of acetaminophen oxidative metabolites.

![Figure 10](image)

**Figure 10.** High resolution product ion MS/MS spectra of protonated molecules of acetaminophen GSH conjugates eluting at a retention time of 1.86 min in Figure 9 containing A) unlabeled GSH (\( m/z \) 457); and B) isotope-labeled GSH (\( m/z \) 460).

A minor chromatographic peak was observed at a retention time of 2.95 min only in the negative precursor ion scanning chromatogram (Figure 9). The corresponding precursor ion scans indicated ions of \( m/z \) 443.0 and \( m/z \) 445.5 (\( \Delta M = 2 \); Table III) which are inconsistent with GSH conjugates differing by 3 u. Also, the relative abundances of these two signals were 40\% and 100\%, respectively (Table III), which are also inconsistent with the expected equal
abundances of conjugates formed by reaction of an acetaminophen metabolite with equimolar GSH and labeled GSH. Therefore, the signal detected at 2.95 min may be dismissed as a false positive without the need for any additional experimentation.

**4-Ethylphenol.** UHPLC-MS/MS analysis of metabolites of 4-ethylphenol after incubation with human liver microsomes, NADPH, and labeled and unlabeled GSH showed five peaks (Figure 11), and each of these five peaks showed signals separated by 3 u corresponding to the isotope signatures for labeled and unlabeled GSH (Table III). The peak eluting at 3.31 min corresponded to a previously reported quinone methide metabolite of 4-ethylphenol that eliminated 2H atoms before reacting with GSH (4-ethylphenol − 2H + GSH). The most abundant GSH conjugate (retention time 3.85 min) has also been reported previously and consisted of a catechol metabolite of 4-ethylphenol that was oxidized to an orthoquinone before reacting with GSH (4-ethylphenol + GSH − 2H + O). The GSH conjugate eluting at 4.14 min (Table III) was a previously unreported minor isomer of the ortho-quinone conjugate.181

The peaks eluting at 2.18 and 2.38 min (Figure 11) corresponded to previously unreported isomeric GSH conjugates of 4-ethylphenol metabolites which had gained two oxygen atoms and lost two hydrogen atoms (4-ethylphenol +2O − 2H + GSH). These two peaks were only observed in negative PI scan mode, which emphasizes the need for measuring negative ions and not just positively charged GSH conjugates. A peak was detected at 5.24 min during positive ion CNL screening but not during negative precursor ion screening, and without any additional experimentation, it could be determined that this peak was not a GSH conjugate. Although the positive ion CNL scanning indicated ions of both m/z 458.3 and m/z 461.2 (ΔM = 3), the relative abundances of these two signals were 100% and 48%, respectively.
(Table III), which is inconsistent with the equal abundances of conjugates that would be formed by reaction of an electrophilic metabolite with equimolar GSH and labeled GSH.

**Figure 11.** UHPLC-MS/MS chromatograms of GSH conjugates formed during incubation of 4-ethyl phenol with human liver microsomes, NADPH, GSH, and \([^{13}\text{C}_2^{15}\text{N}]\)-GSH. A) Positive ion electrospray neutral loss MS/MS scanning of 129 Da; B) negative ion electrospray MS/MS scanning for precursors of \(m/z\) 272 (unlabeled GSH); and C) negative ion electrospray MS/MS precursor ion scanning for precursors of \(m/z\) 275 (labeled GSH).

**17α-Ethynyl Estradiol.** Five GSH conjugates were detected during positive ion CNL and/or negative precursor ion MS/MS scanning of the liver microsomal metabolites of 17α-ethynyl estradiol (Figure 12; Table III). Each of these peaks was confirmed as a GSH conjugate by the presence of ions differing by 3 u that corresponded to the isotope signatures for labeled and unlabeled GSH (Table III). The precursor ions for the peaks eluting at 3.82, 4.00, and 4.13 min were \(m/z\) 618/621 in positive ion mode (CNL) and \(m/z\) 616/619 in negative mode (precursor ion), which corresponded to an unlabeled molecular mass of 617 (17α-ethynyl
estradiol + O − 2H + GSH). This mass is consistent with the known ortho-quinone metabolite of 17-α-ethinyl estradiol.\textsuperscript{186}

The precursor ions of the peaks eluting at 3.47 and 3.68 min (Figure 12) showed doublet ions of equal intensity at $m/z$ 634 and $m/z$ 637 during positive ion CNL scanning and at $m/z$ 632 and $m/z$ 635 during negative precursor ion scanning (Table III), which indicated that these peaks corresponded to GSH conjugates. Previously unreported GSH conjugates of 17α-ethinyl estradiol, these isomers corresponded to the addition of two oxygen atoms and a loss of two hydrogen atoms before conjugation with GSH (17α-ethinyl estradiol + 2O − 2H + GSH). Although a peak was observed at 3.29 min during negative precursor ion scanning, it was inconsistent with a GSH conjugate since the precursor ions of $m/z$ 632.1 and $m/z$ 636.0 differed by 4 u instead of 3 u, as would be expected for conjugates containing unlabeled and labeled GSH, respectively (Table III).
Diclofenac. After incubation of diclofenac with human liver microsomes, NADPH, and GSH, seven peaks were detected during UHPLC-MS/MS with positive ion CNL and negative precursor ion scanning (Figure 13; Table III). The peak eluting at 4.00 min was confirmed as a GSH conjugate by the precursor ions of equal abundance at m/z 583 and m/z 586 during positive ion CNL scanning and the corresponding doublet of m/z 581 and m/z 584 observed during negative precursor ion scanning (Table III). This GSH conjugate corresponds to a previously reported metabolite of molecular mass 582 formed by monooxygenation of diclofenac, the loss of HCl, and the addition of GSH (diclofenac + O − HCl + GSH). Peaks eluting at 4.14, 4.27, and 4.60 min during UHPLC-MS/MS produced protonated molecules of equal abundance of m/z 617/620 during positive ion CNL scanning and of m/z 615/618 during
negative precursor ion scanning (Table III), which confirmed that they were isomeric
diclofenac GSH conjugates (diclofenac + O -2H + GSH) as had been reported
previously.\textsuperscript{180,181,183,187}

\textbf{Figure 13.} UHPLC-MS/MS chromatograms of GSH conjugates formed during incubation of diclofenac with
human liver microsomes, NADPH, GSH, and \[^{13}C_2,^{15}N\]-GSH. A) Positive ion electrospray neutral loss MS/MS
scanning of 129 Da; B) negative ion electrospray MS/MS scanning for precursors of m/z 272 (unlabeled GSH);
and C) negative ion electrospray MS/MS precursor ion scanning for precursors of m/z 275 (labeled GSH).

\textbf{p-Cresol.} Three GSH conjugates of \textit{p}-cresol metabolites were detected during
UHPLC-MS/MS at retention times of 2.93, 3.21, and 3.39 min (Figure 14; Table III). The peak
eluting at 2.93 min formed a pair of protonated molecules of \textit{m/z} 414/417 of equal abundance
during positive ion CNL MS/MS and a pair of deprotonated molecules of \textit{m/z} 412/415 during
negative precursor ion scanning MS/MS, which confirmed that this was a GSH conjugate. The
molecular mass of this compound (413 u) corresponded to a known quinone methide of p-cresol (p-cresol− 2H + GSH). The peaks eluting at 3.21 and 3.39 min were isomeric GSH conjugates as indicated by the pairs of protonated and deprotonated molecules of equal relative abundance at \( m/z \) 430/433 and \( m/z \) 428/431, respectively (Table III). With a molecular mass of 429 u, these two isomers corresponded to GSH conjugates of known catechol metabolites of p-cresol (p-cresol + O − 2H + GSH).

**Figure 14.** UHPLC-MS/MS chromatograms of GSH conjugates formed during incubation of p-cresol with human liver microsomes, NADPH, GSH, and \( [^{13}C_2, ^{15}N] \)-GSH. A) Positive ion electrospray neutral loss MS/MS scanning of 129 Da; B) negative ion electrospray MS/MS scanning for precursors of \( m/z \) 272 (unlabeled GSH); and C) negative ion electrospray MS/MS precursor ion scanning for precursors of \( m/z \) 275 (labeled GSH).

**Ticlopidine.** During analysis of the ticlopidine incubation mixture, two peaks were detected at retention times of 1.83 and 2.02 min in the UHPLC-MS/MS chromatograms (Figure
Pairs of precursor ions (ΔM = 3) of equal abundance were detected in both positive mode (m/z 587/590) and negative mode (m/z 585/588) for each peak corresponding to conjugates with light and heavy GSH, respectively. These isomeric compounds were formed by monooxygenation of ticlopidine followed by conjugation with GSH (ticlopidine + O + GSH). GSH conjugates of monooxygenated ticlopidine have been reported previously.

Figure 15. UHPLC-MS/MS chromatograms of GSH conjugates formed during incubation of ticlopidine with human liver microsomes, NADPH, GSH, and [13C2, 15N]-GSH. A) Positive ion electrospray neutral loss MS/MS scanning of 129 Da; B) negative ion electrospray MS/MS scanning for precursors of m/z 272 (unlabeled GSH); and C) negative ion electrospray MS/MS precursor ion scanning for precursors of m/z 275 (labeled GSH).

Amodiaquine. As reported previously, two GSH conjugates of electrophilic metabolites of amodiaquine (retention times 3.63 and 3.84 min) were detected during negative ion electrospray UHPLC-MS/MS precursor ion scanning, and one peak eluting at 3.84 min was detected during positive ion UHPLC-MS/MS CNL scanning (Figure 16; Table III). The
peak eluting at 3.63 min corresponded to a GSH conjugate of amodiaquine that had undergone oxidative N-deethylation (amodiaquine − C₂H₆ + GSH). The presence of a pair of ions of equal abundance that differed by 3 u (m/z 632/635) confirmed that this peak was a GSH conjugate (Table III). The abundant peak eluting at 3.84 min corresponded to an amodiaquine quinonimine that had reacted with GSH (amodiaquine − 2H + GSH). Pairs of ions of equal abundance differing by 3 u in positive mode (m/z 662/665) and in negative mode (m/z 660/663) confirmed that this compound was a GSH conjugate (Table III). Although the peak observed at 5.69 min formed an ion of m/z 576.4/579.4 during positive ion CNL MS/MS and negative ions of m/z 574.0/577.5 during precursor ion MS/MS, the relative abundances of each pair of ions were unequal. Therefore, this compound was not a GSH conjugate. Finally, the peak detected at 6.12 min during positive ion CNL MS/MS was not a GSH conjugate, since its precursor ions did not differ by 3 u or show equal abundances (Table III).
Figure 16. UHPLC-MS/MS chromatograms of GSH conjugates formed during incubation of amodiaquine with human liver microsomes, NADPH, GSH, and \[^{13}\text{C}_2,^{15}\text{N}\]-GSH. A) Positive ion electrospray neutral loss MS/MS scanning of 129 Da; B) negative ion electrospray MS/MS scanning for precursors of m/z 272 (unlabeled GSH); and C) negative ion electrospray MS/MS precursor ion scanning for precursors of m/z 275 (labeled GSH).

**Dextromethorphan, Midazolam, Testosterone, and Tolbutamide.** Since dextromethorphan, midazolam, testosterone, and tolbutamide have not been reported to form electrophilic metabolites or GSH conjugates,\(^{179}\) these compounds were used as negative controls to validate the UHPLC-MS/MS method. As expected, no GSH conjugates were detected using either positive ion CNL scanning or negative precursor ion scanning, indicating that the new GSH conjugate screening approach is highly accurate at preventing false positive results (data not shown).
2.3.2 Licorice

As a general approach, GSH screening using UHPLC-MS/MS requires no advanced knowledge of the compounds that might form GSH conjugates. To demonstrate its suitability for testing complex botanical extracts, a methanolic extract of the botanical dietary supplement licorice root (*Glycyrrhiza glabra*) was tested. Licorice is used as a dietary supplements as well as a flavoring agent and has been reported to inhibit the drug metabolizing enzyme CYP3A4. A large number of peaks were detected during UHPLC-MS/MS analysis (Figure 17; Table III), and all were GSH conjugates except for the peak eluting at 3.0 min, which showed ions of $m/z$ 511/514 that were not of equal abundance (100% and 68%, respectively).

![Figure 17. UHPLC-MS/MS chromatograms of GSH conjugates formed during incubation of licorice extract (*G. glabra*) with human liver microsomes, NADPH, GSH, and $^{13}$C$_2$-$^{15}$N-GSH. A) Positive ion electrospray neutral loss MS/MS scanning of 129 Da; B) negative ion MS/MS scanning for precursors of $m/z$ 272 (unlabeled GSH); and C) negative ion MS/MS scanning for precursors of $m/z$ 275 (labeled GSH).]
Based on dereplication of the measured masses using NAPRALERT, SciFinder, and Reaxys, we hypothesized that at least some of the conjugates were formed through reaction of GSH with metabolites of the isoflavan glabridin and with the chalcone isoliquiritigen and its metabolites. These peaks were observed at retention times of 2.38, 2.65, 2.95, 3.05, 3.55, and 3.62 min (Figure 17, Table III). The other GSH conjugates are still under investigation. An electrophilic α,β-unsaturated ketone, isoliquiritigenin (Figure 18), has been reported to form a GSH adduct without metabolic activation, and a peak of $m/z$ 564/567 corresponding to unchanged isoliquiritigenin plus GSH was observed at 2.38 min in all incubations including control incubations of licorice extract without liver microsomes or without NADPH (data not shown). The peak eluting at 2.65 min was a GSH conjugate of isoliquiritigenin after monooxygenation and a loss of two hydrogen atoms (isoliquiritigenin + O − 2H + GSH) and was observed only in the presence of GSH, liver microsomes, and NADPH. Separate incubation of purified isoliquiritigenin confirmed the formation of the GSH conjugates eluting at both 2.38 and 2.65 min (Figure 18; Table III).
Figure 18. UHPLC-MS/MS chromatograms of GSH conjugates formed during incubation of isoliquiritigenin with human liver microsomes, NADPH, GSH, and \[^{13}\text{C}_2^{15}\text{N}]\text{-GSH. A) Positive ion electrospray neutral loss MS/MS scanning of 129 Da; B) negative ion MS/MS scanning for precursors of m/z 272 (unlabeled GSH); and C) negative ion MS/MS scanning for precursors of m/z 275 (labeled GSH).}

The natural product glabridin from licorice can irreversibly inhibit CYP3A4, although no reactive metabolites of glabridin have yet been reported.\(^{185}\) UHPLC-MS/MS analysis of a glabridin incubation mixture after incubation with human liver microsomes showed peaks at retention times of 2.95 and 3.05 min only in the positive ion CNL MS/MS chromatogram (Figure 19; Table III). The precursor ions of each peak corresponded to a pair of ions of equal abundance at m/z 630 and m/z 633 (ΔM = 3; Table III). Therefore, these peaks, which were also present in the licorice extract incubation (Figure 17), were confirmed as isomeric GSH conjugates. Accurate mass measurements of these two peaks were within 5 ppm of the
elemental composition C_{30}H_{35}N_{3}O_{10}S, which is consistent with a quinone methide of glabridin that had reacted with GSH to form two isomers (glabridin−2H+GSH). Note that these glabridin GSH conjugates were observed only during positive ion MS/MS scanning, which emphasizes the need for measuring positive ions as well as negative ions.

GSH conjugates of mono-oxygenated glabridin were detected at 3.55 and 3.62 min during both positive ion CNL and negative precursor ion MS/MS scanning of the glabridin incubation mixture as well as the licorice extract incubation mixture (Figure 19; Figure 17). Pairs of precursor ions of approximately equal abundance were observed for both peaks,
confirming that they were GSH conjugates (Table III). With a molecular mass of 645 Da and an elemental composition of $C_{30}H_{35}N_3O_{11}S$ (ΔM = 4.05 ppm), the compound eluting at 3.55 min corresponded to a conjugate of GSH with either a mono-oxygenated glabridin quinone methide or a monooxygenated glabridin ortho-quinone (glabridin + O − 2H + GSH). The compound eluting at 3.62 min (Figure 17) had a molecular mass of 643 Da and an elemental composition of $C_{30}H_{33}N_3O_{11}S$ (ΔM = 3.35 ppm), which corresponded to a GSH conjugate of mono-oxygenated glabridin that had eliminated 4H atoms (glabridin + O − 4H + GSH). The observation of four GSH conjugates formed from at least three different glabridin metabolites supports the hypothesis that electrophilic metabolites of glabridin might be responsible for irreversible inactivation of CYP3A4. Confirmation of the structures of these glabridin GSH conjugates and other licorice GSH conjugates is in progress. Finally, the peak eluting at 3.00 min (Figure 19; Figure 17) in the glabridin and licorice UHPLC-MS/MS chromatograms was not a GSH conjugate based on the absence of a pair of ions (ΔM = 3) of equal abundance (Table III).
### TABLE III
GSH CONJUGATES AND CORRESPONDING PRECURSOR IONS DETECTED DURING UHPLC-MS/MS WITH POSITIVE ION ELECTROSPRAY CONSTANT NEUTRAL LOSS (CNL) SCANNING AND WITH NEGATIVE ION ELECTROSPRAY PRECURSOR ION (PI) SCANNING

<table>
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<tr>
<th>Compound</th>
<th>GSH conjugate</th>
<th>Retention time (min)</th>
<th>CNL 129 Da GSH</th>
<th>PI m/z 275/278 GSH</th>
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<tr>
<td></td>
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<td></td>
<td>[13C₂, 15N]-GSH</td>
<td>[13C₂, 15N]-GSH</td>
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*a* apparent m/z value of precursor ion  

*b* (relative abundance)

### 2.4 Conclusions

To validate this new GSH screening assay, seven compounds known to form electrophilic intermediates and GSH conjugates and four compounds that do not form such metabolites were tested. All of these positive control and negative control compounds produced the expected results. In addition to the detection of known metabolites, three new GSH conjugates of 4-ethyl phenol and two new GSH conjugates of 17α-ethinyl estradiol were detected. This indicated that the new GSH screening assay is extremely sensitive. Licorice, which had been reported to inactivate CYP3A4 but had not been reported previously to form electrophilic metabolites, was tested to demonstrate how the new method may be applied to complex mixtures without advanced knowledge of elemental compositions of possible conjugates. The licorice chalcone isoliquiritigenin and one of its metabolites were found to form GSH conjugates as did metabolites of glabridin, which confirmed that licorice contains compounds capable of forming electrophilic metabolites that react with biological nucleophiles.
Since some GSH conjugates might produce only positive ion signals while others might form only negation ions, our approach to measuring GSH conjugates using both positive ion and negative ion MS/MS in a single assay both provides higher throughput and consumes less of the sample than two separate analyses. This approach also detects more GSH conjugates than would UHPLC-MS/MS screening using only positive ion or only negative ion electrospray. For example, GSH conjugates of 17α-ethinyl estradiol eluting at 3.47 min and amodiaquine eluting at 3.63 min (Table III) were detected only when using negative ion electrospray precursor ion scanning MS/MS, while GSH conjugates of glabridin eluting at 2.95 and 3.05 min (Table III) were observed only during positive ion electrospray CNL MS/MS.

In addition to the 2-fold enhancement of throughput by combining negative ion and positive ion MS/MS into a single analysis, the use of UHPLC instead of HPLC increases the throughput of the assay from ~30 min to <8 min per injection. The incorporation of labeled and unlabeled GSH eliminates the need for subsequent measurements to determine if a peak is actually a GSH conjugate or a false positive result and thereby enhances throughput at least another 2-fold. Therefore, by using fast polarity switching, fast MS/MS, and UHPLC instead of HPLC, this new approach increases the throughput of GSH screening >10-fold.
CHAPTER 3:

MS-BASED SCREENING OF LICORICE EXTRACTS FOR
CHEMOPREVENTIVE AGENTS THAT COVALENTLY MODIFY
KEAP1 PROTEIN, A REGULATOR OF THE ANTIOXIDANT
RESPONSE ELEMENT

3.1 Background and Introduction

Natural products are important sources of cancer chemopreventive agents, which are used for the induction of phase II chemopreventive enzymes, such as glutathione S-transferases (GSTs), NAD(P)H quinone oxidoreductase 1 (NQO1) or heme oxygenase-1 (HO-1). The expression of detoxification enzymes can protect cells against the toxic and neoplastic effects of carcinogens. Activation of the Keap1-Nrf2 signaling pathway can regulate the antioxidant response element (ARE), which is located in the promoter region of multiple cytoprotective genes. Under basal conditions, Nrf2 is bound to a Keap1 dimer in the cytosol. In response to oxidative species and exposure to xenobiotic electrophiles, the cytosolic and nuclear concentrations of Nrf2 increase, upregulating the ARE genes and inducing the expression of these detoxification enzymes. One hypothesis for this process is that ubiquitination and proteosome-mediated degradation of Nrf2 in the cytoplasm decrease, when cysteine residues on Keap1 are covalently alkylated by xenobiotic electrophiles, and result in higher Nrf2 levels both in the cytoplasm and in the nucleus. Therefore, the up-regulation of ARE will stimulate the transcription of cytoprotective genes and the expression of chemopreventive enzymes, which is believed to be an adaptive response for reactive oxygen and xenobiotic electrophiles.
Many constituents of natural products have been identified as ARE activators, such as xanthohumol from hops, sulforaphane from broccoli sprouts, isoliquiritinigenin from licorice, and quercetin from green and black tea. After comparing the structures among these inducers, Talalay et al. found that most are electrophiles have the preference on sulfhydryl groups. Alkylation cysteines in Keap1 by electrophilic compounds intends to modulate the Keap1–Nrf2 pathway, as well as ARE activation. Since the significant role of Keap1 in chemoprevention, screening of electrophiles that modify this protein will be a promising way to discover the chemopreventive agents.

In studies of botanical dietary supplements for the alleviation of menopausal symptoms in women, licorice extracts and their constituents, isoliquiritinigenin (ILG) and LicA have been found to induce NQO1 in murine hepatoma (Hepa1c1c7) cells in vitro, which is among the enzymes regulated by the ARE. ILG and LicA are both Michael reaction acceptors (MRAs), similar to the known chemopreventive compounds such as xanthohumol from hops. Induction of the ARE luciferase in human hepatoma (HepG2-ARE-C8) cells by licorice extracts and compounds suggest involvement of the Keap1-Nrf2 pathway. These data suggest that the chemopreventive potential of licorice species could come from ILG and LicA. In vivo rat studies indicated that extracts of the licorice species G. glabra induced NQO1 activity in the mammary tissue but not in the liver. Administration with ILG for rats didn’t induce NQO1 in vivo most likely because of its intramolecular cyclization to form liquiritigenin (which was also found in vivo), extensive metabolism, and its low bioavailability in vivo. Since LicA cannot undergo intramolecular cyclization like ILG and has high content in G. inflata, in vivo induction experiments of G. inflata and LicA should be more promising than G. glabra and ILG.
These NQO1 data showed the chemopreventive potential of licorice species in vitro could be due to the MRAs ILG and LicA. We hypothesize that there might be other MRAs in licorice extracts and that different species will contain unique profiles of compounds that will produce different activities. In this chapter, three main licorice species were screened, *Glycyrrhiza uralensis* (GU), *G. glabra* (GG) and *G. inflata* (GI), which are the most frequently used species in licorice dietary supplements. A fast UHPLC-MS/MS screening method was used to detect and identify electrophilic compounds in licorice extracts by trapping them as GSH conjugates. Four main electrophilic compounds were found in licorice: licochalcone A (LicA), licochalcone B (LicB), echinatin, and isoliquiritigenin (ILG) (Figure 20). The different screening results for each licorice species emphasize the standardization of botanicals used as dietary supplements are highly recommended.

![Chemical structures of licorice chalcones](image)

**Figure 20.** Chemical structures of licorice chalcones.
3.2 **Experimental Section**

3.2.1 **Materials and Reagents**

Methanol fractions of *G. glabra* [GG (TO1)], *G. uralensis* [GU (BC716)] and *G. inflata* [GI (BC711)] and the isolated licorice compound echinatin were prepared by Dr. Yang Yu and Dr. Charlotte Simmler at the University of Illinois at Chicago as follows. Each licorice species was botanically authenticated through a series of microscopic analyses and by comparison to voucher specimens at the Field Museum of Natural History (Chicago, IL). Then, plant materials were extracted using ethanol/isopropanol/water (90:5:5). Each extract was loaded onto XAD-2 resin and eluted with water, water/methanol (50:50), methanol, and acetone, respectively, to obtain 4 sub-fractions for each botanical. The methanol fraction of each species was used for testing.

LicA, isoliquiritigenin (ILG), tris(2-carboxyethyl)phosphine hydrochloride (TCEP), triethyl ammonium bicarbonate (TEAB), glutathione (GSH), ([13C2,15N]-glycine)-GSH, *N*-acetyl cysteine, cysteine, deoxyguanosine (dGMP), deoxyadenosine monophosphate (dAMP), *N*-acetyl lysine, carnosine, sinapinic acid, bovine serum albumin (BSA), formic acid, and trifluoroacetic acid were purchased from Sigma–Aldrich (St. Louis, MO). Licochalcone B (LicB, purity > 98%) was purchased from Chengdu Push Biotechnology (Chengdu, China). Recombinant human Keap1 protein containing a histidine tag was a gift from Dr. Aimee L. Eggler of Villanova University. The protein was expressed in and purified from *Escherichia coli* as a previous reported.199 Keap1 protein (142 µM) was stored in 50 mM Tris–HCl buffer (pH 8.0), 2 mM tris[2-carboxyethyl]phosphine hydrochloride, 250 mM sodium chloride, and 20% glycerol (v/v). Trypsin Protease and 10 KDa centrifugal spin filters were purchased from EMD Millipore (Billerica, MA).
3.2.2 GSH Conjugates Screening Assay

The experimental procedure for screening licorice for electrophilic compounds that react with GSH was similar to the GSH conjugates screening assay described in Chapter 2, except that metabolic activation was not used. Therefore, human liver microsomes and NADPH were not used in the new assay. Licorice extracts (prepared from botanically authenticated GU, GG and GI; 1 mg/mL) or pure licorice compounds (LicA, LicB, echinatin, and ILG; 10 µM) were separately incubated with GSH and isotopically labeled GSH (1:1) in 100 mM phosphate buffer (pH 7.4) at 37 °C in a final volume of 1 mL. Control experiments were carried out without GSH. After 30 min, the reactions were stopped by addition 100 µL of trichloroacetic acid (10%), and the samples were centrifuged for 15 min at 12 000xg and 4 °C. Supernatants were removed, loaded onto C<sub>18</sub> solid-phase extraction cartridges, extracted as the described method in Chapter 2, evaporated to dryness, and reconstituted in 100 µL of acetonitrile/water (10:90, v/v) before analysis using UHPLC-MS/MS.

A Shimadzu (Kyoto, Japan) LCMS-8050 with a Shimadzu Nexera UHPLC system was used with a Waters (Milford, MA) Acquity UPLC BEH Shield RP<sub>18</sub>, 2.1 × 50 mm, 1.7 µm column for GSH conjugate screening. Electrospray was used for ionization while the following cycle of MS/MS scans was carried out: negative precursor ion scanning for m/z 272 (unlabeled GSH conjugates), negative precursor ionscanning for m/z 275 (labeled GSH conjugates), and positive ion CNL scanning for a loss of 129 Da (labeled and unlabeled GSH conjugates). The scan ranges were m/z 400–700 at unit resolving power. The polarity switching speed was 5 ms, and each scan was recorded over 0.12 s. The details for other parameters were reported in our published UHPLC-MS/MS method.62
The GSH conjugates of each licorice compound were also characterized using accurate mass measurement HPLC-MS/MS on a Waters (Milford, MA) hybrid QqTOF Synapt G1 mass spectrometer. Leucine enkephalin was added post-column as a lock mass for accurate mass measurement. Mass spectra and tandem mass spectra were acquired from \( m/z \) 50 – 800 using positive ion electrospray. Product ion tandem mass spectra were obtained using CID with argon collision gas at a collision energy of 15 eV. Data collection and processing were carried out using Waters MassLynx 4.1 software.

3.2.3 Kinetics Measurements

LicA, LicB, echinatin, and ILG (10 \( \mu \)M) were incubated separately with 10 mM nucleophiles, including thiols (GSH, N-acetyl cysteine, and cysteine), nucleosides (dGMP and dAMP) and amino acids (N-acetyl lysine and carnosine) in phosphate buffer (pH 7.4) at room temperature, the total volume was 1 mL. The kinetics of the reactions were monitored by absorbance of UV/vis at 370 nm (Beckman Coulter DU 800) for short-term (3 hours) and long term (2 days). Solvent blanks (pure water) were used to zero the instrument before every measurement. The control groups were identical but omitted nucleophilic agents.

3.2.4 Covalent Modification of Keap1 by Licorice

Keap1 protein (final concentration 20 \( \mu \)M for MALDI-TOF MS measurements) was dissolved in 10 mM triethyl ammonium bicarbonate buffer and 200 \( \mu \)M tris(2-carboxyethyl)phosphine (pH 8) to reduce disulfide bonds at 37 °C for 30 min. The protein was
then incubated for 2 hours at 37 °C separately with licorice extracts (2 mg/mL) or purified compounds LicA, LicB, echinatin, and ILG (200 μM, 1:10 molar ratio).

Positive ion MALDI-TOF mass spectra of intact Keap1 protein were acquired using a Bruker (Billerica, MA) AutoFlex MALDI-TOF mass spectrometer, and 0.1 μg/mL BSA (molecular weight: 66000 kDa) was served as calibration standard before each analysis. A 1 μL aliquot of the intact Keap1 or “Keap1-botanicals” reaction solution was mixed with 1 μL matrix solution which contained sinapinic acid (10 mg/mL) in acetonitrile/water/trifluoroacetic acid (69.9:30:0.1, v/v). A 1 μL aliquot of the mixture was then spotted on a MALDI-TOF sample stage and air-dried before analysis. For each sample, mass spectra from 800 laser shots were acquired in linear mode (LP) and signal averaged over the range m/z 55000 to m/z 90000. After time-delayed extraction, the ions were accelerated to 19.2 kV for MALDI-TOF mass spectrometric analysis.

3.3 Results and Discussion

3.3.1 Discovery of Electrophilic Compounds in Licorice

After incubation with GSH, UHPLC-MS/MS screening of all three licorice extracts (G. glabra, G. uralensis and G. inflata) indicated formation of GSH conjugates (Figure 21). Glutathione conjugate selective UHPLC-MS/MS screening of the G. glabra mixture indicated two peaks at retention times of 4.18 and 4.37 min (Figure 21A). Pairs of precursor ions (ΔM = 3) of equal abundance, corresponding to conjugates with light and heavy GSH, were detected for both peaks using positive ion CNL scanning (m/z 578/581; m/z 564/567) and negative mode precursor ion scanning (m/z 576/579; m/z 562/565), respectively (Table IV). Identical GSH
conjugate peaks were detected in the other two licorice extracts, *G. uralensis* and *G. inflata* (Figure 21B and 21C; Table IV). In addition, a new peak was detected in the *G. uralensis* and *G. inflata* UHPLC-MS/MS chromatograms eluting at 3.81 min with positive ions of *m/z* 594/597 and negative ions *m/z* 592/595 confirming that this was a GSH conjugate (Figure 21B &C; Table IV). A unique peak that was detected only in the *G. inflata* incubation mixture was observed at a retention time of 5.72 min with precursor ions of *m/z* 646/649 in positive mode with CNL scanning and *m/z* 644/647 in negative mode with precursor ion scanning, again confirming that this was a GSH conjugate (Figure 21C; Table IV). No GSH conjugates were detected in the range *m/z* 700-1000 for all three licorice species (data not shown). Based on dereplication of the measured masses using the natural products databases SciFinder and Reaxys, all of the GSH conjugates appeared to be formed from known licorice chalcones (LicA, LicB, echinatin, and isoliquiritigenin), probably through Michael addition reaction with GSH.
Figure 21. UHPLC-MS/MS chromatograms of GSH conjugates formed during incubation of licorice extract with GSH, and \[ ^{13}C_2^{15}N \]-GSH.  A) GSH conjugates of \textit{G. glabra} extract; B) GSH conjugates of \textit{G. uralensis} extract; and C) GSH conjugates of \textit{G. inflata} extract.

Figure 22. UHPLC-MS/MS chromatograms of GSH conjugates formed during incubation of licorice compounds with GSH, and \[ ^{13}C_2^{15}N \]-GSH.  A) GSH conjugates of licochalcone A; B) GSH conjugates of licochalcone B; C) GSH conjugates of echinatin; and D) GSH conjugates of isoliquiritigenin.
For additional structural characterization of the GSH conjugates, positive ion electrospray HPLC-MS/MS analyses with product ion scanning were carried out for each of the GSH conjugates using a high resolution QqTOF mass spectrometer (Figure 23 and Figure 24). Accurate mass measurements of these ions were within 5 ppm of expected formula as follows: LicA-GSH, m/z 646.2435 (theoretical 646.2434, ΔM -0.15 ppm); LicB-GSH, m/z 594.1773 (theoretical 594.1757, ΔM -2.69 ppm); echinatin-GSH, m/z 578.1801 (theoretical 578.1808, ΔM 1.21 ppm); ILG-GSH, m/z 564.1643 (theoretical 564.1652, ΔM 1.59 ppm). The product ion of m/z 308 in each of these tandem mass spectra (Figure 23 and Figure 24) was formed by loss of the chalcone with the positive charge being retained on the GSH moiety. The related product ion of m/z 179 in all four mass spectra was formed by loss of the pyroglutamyl group (129 Da) from the GSH ion of m/z 308. Other GSH-characteristic fragmentation included the ion of m/z 339 (LicA); m/z 287 (LicB); m/z 271 (echinatin); m/z 257 (ILG), which resulted from the neutral loss of the GSH moiety (Figure 23 and Figure 24). Therefore, the peaks eluting at retention times of 3.81, 4.18, 4.37, and 5.72 min were confirmed to be GSH conjugates of licorice compounds.

Identification of the peaks in Figures 23 and 24 as GSH conjugates of LicA (5.72 min), LicB (3.81 min), echinatin (4.18 min), and ILG (4.37 min) was carried out by incubating each isolated compound with GSH and then analyzed them using UHPLC-MS/MS with CNL and precursor ion scanning. As shown in Figure 22 and Table IV, the masses and UHPLC retention times of the standard GSH conjugates were identical with those found after incubating the licorice extracts with GSH (Figures 23 and 24; Table IV).
Figure 23. Positive ion electrospray CID product ion mass spectra of GSH conjugates of licorice chalcones. A) Licochalcone A GSH conjugate; B) Licochalcone B GSH conjugate. GSH reacted with the α,β-unsaturated ketones of the chalcones by Michael addition.
Figure 24. Positive ion electrospray CID product ion mass spectra of GSH conjugates of licorice chalcones. A) Echinatin GSH conjugate; B) Isoliquiritigenin B GSH conjugate. GSH reacted with the α,β-unsaturated ketones of the chalcones by Michael addition.
TABLE IV.

GSH conjugates resulting from incubation of GSH with licorice extracts or isolated licorice compounds analyzed using UHPLC-MS/MS with positive ion electrospray constant neutral loss (CNL) scanning and with negative ion electrospray precursor ion (PI) scanning.

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<td>597.3 (100)</td>
</tr>
<tr>
<td></td>
<td>Y</td>
<td>4.18</td>
<td>578.3 (100)</td>
<td>581.4 (98)</td>
</tr>
<tr>
<td></td>
<td>Y</td>
<td>4.37</td>
<td>564.3 (100)</td>
<td>567.3 (95)</td>
</tr>
<tr>
<td></td>
<td>Y</td>
<td>5.72</td>
<td>646.3 (94)</td>
<td>649.4 (100)</td>
</tr>
<tr>
<td>Licochalcone A</td>
<td>Y</td>
<td>5.72</td>
<td>646.3 (94)</td>
<td>649.4 (100)</td>
</tr>
<tr>
<td>Licochalcone B</td>
<td>Y</td>
<td>3.81</td>
<td>594.2 (100)</td>
<td>597.3 (100)</td>
</tr>
<tr>
<td>Echinatin</td>
<td>Y</td>
<td>4.18</td>
<td>578.3 (100)</td>
<td>581.4 (98)</td>
</tr>
<tr>
<td>Isoliquiritigenin</td>
<td>Y</td>
<td>4.37</td>
<td>564.3 (100)</td>
<td>567.3 (95)</td>
</tr>
</tbody>
</table>

\(^a\) apparent m/z value of precursor ion
\(^b\) (relative abundance)

Since these four licorice chalcones are all Michael reaction acceptors (MRAs), they can react with nucleophiles such as GSH to form covalent adducts as indicated in Figure 25. Chalcones exhibit several absorbance bands in the UV/visible spectrum and have absorbance maxima around 370 nm (for example, \(\lambda_{\text{max}} = 370\) nm for xanthohumol from hops).\(^{200}\) This strong UV absorbance arises from the conjugated system involving the \(\alpha,\beta\)-unsaturated ketone and the aromatic B-ring (Figure 25). When this conjugated system becomes disrupted through degradation to other compounds or by Michael addition of nucleophiles to the \(\alpha,\beta\)-unsaturated ketone (Figure 25), chalcones lose this strong absorbance at 370 nm. Therefore, the UV absorbance at 370 nm can be used to monitor the stability of this conjugated system.
and in this dissertation, was used to monitor the kinetics of reaction of licorice chalcones with nucleophilic agents.

Figure 25. The conjugated system in chalcones, which allows the absorbance at UV 370 nm. The loss of absorbance will come from the disappearance of the conjugated system. The licorice chalcone, licochalcone A, is used as an example.

Nucleophilic agents including the thiols GSH, N-acetyl cysteine, and cysteine, the nucleosides dGMP and dAMP, and the amino acids N-acetyl lysine and carnosine were incubated separately with licorice chalcones LicA, LicB, echinatin, and ILG. Control incubations containing no nucleophiles were also carried out. The nucleophiles (10 mM) were present in overwhelming excess relative to the chalcones (10 μM), and the UV absorbance of the chalcones at 370 nm was monitored during the reaction. Because the nucleophiles were in overwhelming abundance, the reaction followed pseudo first-order kinetics according to the following equation:

$$A_T = A_B + (A_I - A_B)e^{-kT}$$

where T is the reaction time (min), $A_T$ is the real time absorbance, $A_B$ is the final absorbance, $A_I$ is the initial absorbance, and $k$ is the apparent first-order rate constant.
Loss of absorbance at 370 nm due to formation of chalcone conjugates was observed during incubation with the thiols: GSH, N-acetyl cysteine (NAC), and cysteine, but not with nucleosides or amino acids (data not shown). Reactions of licorice electrophiles with 10 mM GSH was shown in Figure 26. Using a linear plot of real time absorbance versus reaction time (ln[\(A_T\)) vs. T), the apparent rate constant (k) for each chalcone was calculated from the slope of each equation as follows: LicA, k = 0.0049 /min; LicB k = 0.0126 /min; echinatin k = 0.0134 /min; and ILG k = 0.0244 /min. Therefore, the reaction rate order was determined to be ILG > echinatin ≈ LicB > LicA (Figure 27). The calculated apparent rate constants (k) for each chalcone with GSH, NAC and cysteine were summarized in Table V, following the reaction rate: cysteine>GSH>NAC.

**Figure 26.** Reactions of licorice electrophiles with 10 mM GSH and disappearance of the α,β-conjugated Michael acceptor were monitored at 370 nm. When similar incubations were carried out with amine nucleophiles such as amino acids and nucleosides, no reaction was observed indicating that those compounds have a preference for thiol nucleophiles.
Figure 27. After linear plot to determine $k$ ($\ln[AT]$ v. s. $T$), the apparent rate constant ($k$) for each chalcone is calculated as the slope of each equation. Therefore, reaction rate order is: ILG>Echi>LicB>LicA.

<table>
<thead>
<tr>
<th></th>
<th>NAC</th>
<th>GSH</th>
<th>cysteine</th>
</tr>
</thead>
<tbody>
<tr>
<td>LicA</td>
<td>0.0021</td>
<td>0.0049</td>
<td>0.0091</td>
</tr>
<tr>
<td>LicB</td>
<td>0.0055</td>
<td>0.0126</td>
<td>0.0365</td>
</tr>
<tr>
<td>Echi</td>
<td>0.0053</td>
<td>0.0134</td>
<td>0.0422</td>
</tr>
<tr>
<td>ILG</td>
<td>0.0125</td>
<td>0.0244</td>
<td>0.0864</td>
</tr>
</tbody>
</table>

This in vitro kinetics data indicate that licorice chalcones are weak electrophiles that can react with strong biological nucleophiles such as the thiol group of GSH, NAC, or cysteine but cannot react with amino groups. These results suggest that licorice chalcones should be able to react with cysteine sulfhydryl groups on Keap1 and thereby activate the antioxidant
response element via the Keap1/Nrf2 chemoprevention pathway. Therefore, reaction of these licorice chalcones with Keap1 protein was investigated next.

### 3.3.2 Alkylation of Keap1 Protein

In the cytoplasm, all 27 of the cysteine residues in human Keap1 are in reduced form and possibly available to react with electrophiles. To confirm the licorice compounds could covalently modify Keap1, extracts of three licorice species (G. glabra, G. uralensis and G. inflata) were incubated separately with Keap1 in buffer and then analyzed using positive ion MALDI-TOF mass spectrometry. After incubation with buffer only, the mass of Keap1 (m/z 71805) was essentially unmodified at m/z 71817 indicating that Keap1 remained stable and unchanged under the incubation conditions (Figure 28A). Incubation of Keap1 with extracts of the licorice species G. glabra, G. uralensis or G. inflata increased its mass to m/z 72345, m/z 72455, or m/z 73195, respectively. The increase in mass of Keap1 following incubation with licorice extracts indicated covalent modification due to attachment of electrophilic natural products to cysteine residues of Keap1.
Figure 28. Positive ion MALDI-ToF mass spectra of Keap1 incubated without (black line) or with (colored lines) (A) buffer; (B) GG extract; (C) GU extract; or (D) GI extract. The increase in mass of Keap1 following incubation with licorice extracts indicates covalent modification due to attachment of electrosphilic natural products to cysteine residues of Keap1.

After detecting Keap1 modified by the licorice extracts using MALDI-TOF mass spectrometry, the same approach was applied to determine the relative reactivity of licorice chalcones toward Keap1. Pure licorice chalcones (LicA, LicB, echinatin, ILG) were incubated separately with Keap1 at a molar ratio of 10:1 (chalcone:protein). As shown in Figure 29, all four licorice chalcones covalently modified Keap1. The mass increase of Keap1 (+1029 Da to m/z 72784) was greatest after incubation with LicA, which indicated that an average of 3 LicA molecules covalently bound to each molecule of Keap1 (Figure 29A). Incubation with
echinatin increased the mass of Keap1 by 675 Da \((m/z \, 72430)\), corresponding to covalent attachment of an average of 2.5 molecules of this chalcone to each molecule of Keap1. ILG increased the mass of Keap1 to \(m/z \, 72334\), which is an increase of 579 Da or an average of 2.3 ILG molecules to each molecule of Keap1. Finally, incubation with LicB increased the mass of Keap1 by 531 Da \((m/z \, 72286)\) which corresponded to only 1.9 molecules of chalcone to each Keap1 molecule.

Figure 29. Positive ion MALDI-TOF mass spectra of Keap1 incubated without (black lines) or with licorice chalcones (A) LicA, (B) LicB, (C) Echi, or (D) ILG. Based on increase in mass, all 4 licorice chalcones covalently modified Keap1.
The relative reactivity of licorice chalcones toward Keap1 was LicA > echinatin > ILG > LicB. Therefore, LicA was determined to be the most potent chemopreventive chalcone in licorice. Although the concentrations of electrophilic chalcones as well as their relative electrophilicities will affect the extent of Keap1 modification, the G. inflata preparation containing not only ILG but exclusively LicA and LicB was found to be the most effective licorice species with respect to covalently modifying Keap1. Consequently, G. inflata is predicted to be the most effective licorice species at upregulating the ARE as a mechanism of chemoprevention.

3.4 Conclusions

A mass spectrometry based screening assay originally developed for the detection of reactive metabolites was repurposed to test botanical extracts like licorice for the presence of weak electrophiles such as chalcones, which might function as natural chemoprevention agents. This assay is based on rapid MS/MS screening for GSH conjugates formed in botanical extracts after incubation with GSH. The GSH screening assay was found to be particularly sensitive for detecting chalcones, which are Michael reaction acceptors. These chalcones were then shown using another MS-based assay to covalently modify Keap1, which is a chemoprevention target in the Keap1/Nrf2 pathway that regulates the ARE. Therefore, the licorice chalcones LicA, LicB, ILG, and echinatin are predicted to be chemoprevention agents.

With a long history of use in Traditional Chinese Medicine as well as a flavoring agent in Western medicines, licorice contains phytoestrogens and is finding new applications in botanical dietary supplements for the management of menopausal symptoms in women. In
addition to estrogenic properties, licorice also appears to have potential chemopreventive properties. In vitro and in vivo experiments by others have demonstrated induction of the chemopreventive enzyme, NQO1, due to treatment with licorice extracts and isolated licorice compounds. This dissertation supports these observations by confirming that four abundant licorice chalcones (LicA, LicB, echinatin, and ILG) are present in licorice species used in dietary supplements and can react with biological thiols. The lack of reactivity of these licorice chalcones toward amine-containing biological nucleophiles suggests that these compounds should not cause cytotoxicity or genotoxicity.

Because LicA and LicB are found primarily in *G. inflata*, not all licorice dietary supplements should be expected to contain high levels of all four of these chalcones. Therefore, chemical standardization of licorice dietary supplements with respect to these chalcones will be essential to ensure their chemopreventive activities.
CHAPTER 4:

HEPATIC METABOLISM OF LICOCHALCONE A, A POTENTIAL CHEMOPREVENTIVE CHALCONE FROM LICORICE

(*Glycyrrhiza inflata*)

This chapter contains information overlapping with reference 201 (Huang et al. Anal. Bioanal. Chem. 2017), which is a peer-reviewed manuscript resulting from this dissertation.

4.1 *Background and Introduction*

Ensuring the safety of botanical dietary supplements is a high priority for a market that continues to grow worldwide.7,202 As in drug development, studies of the absorption, distribution, metabolism, excretion, and toxicity (ADMET) of active compounds from botanical dietary supplements can provide essential data for understanding the effects of these products. This information may then be used to design appropriate dosages and dosage forms for clinical trials of safety and efficacy.203 Too often, clinical trials or animal studies of botanical dietary supplements and active compounds from these products result in negative outcomes due to lack of appropriate ADMET studies or the understanding of the bioavailabilities of the active constituents.204

Among the oldest and most popular herbal medicines in the world,63 licorice (“Gan-Cao” in Chinese) is recorded in the pharmacopoeias of many Asian and Western countries. The roots of three licorice species are commonly used in botanical dietary supplements and as medicinal plants, namely *Glycyrrhiza glabra* L., *Glycyrrhiza uralensis* Fisch. and *Glycyrrhiza inflata* Bat.65 Licochalcone A (LicA) ((E)-3-(4-hydroxy-2-methoxy-5-(2-methylbut-3-en-2-


yl)phenyl)-1-(4-hydroxyphenyl)prop-2-en-1-one, Figure 3), is a Michael acceptor occurring in *Glycyrrhiza inflata* root extracts, at levels of approximately 8% (w/w).16

As a chemoprevention agent, LicA has been reported to inhibit the formation of genotoxic estrogen catechols30 and to induce detoxification enzymes regulated by the antioxidant response element such as NAD(P)H:quinone oxidoreductase 1 via the Keap1/Nrf2 pathway.89 In addition, LicA has antimalarial,86 anticancer,87 antibacterial,205 and antiviral properties.88 Although a promising chemoprevention agent, there has been no comprehensive evaluation of the phase I and phase II metabolism of LicA.

Like pharmaceutical agents, natural products can be substrates for phase I and II metabolic enzymes, which include the phase I cytochromes P450 (CYP) family (a family of heme-monooxygenases located in most tissues and concentrated in the liver, not needed for DMD), and the phase II 5’-diphosphoglucuronyl transferases (UGTs) and sulfotransferases. Phase I metabolism might form products with new pharmacological activities or enhanced toxicity, while phase II conjugation reactions are more likely to form metabolites which are less active and become excreted more rapidly into urine or bile. Phase I and phase II studies of licochalcone A metabolism were carried out using human liver microsomes, recombinant human metabolic enzymes, and human hepatocytes, and the metabolites predicted by these model systems were confirmed in vivo using the ACI rat model. Metabolic stability assays were also carried out as a means of estimating intrinsic clearance of LicA. Finally, the enzymes responsible for metabolic biotransformation of LicA were identified. Together, these data may be used to facilitate the design of animal studies and clinical trials of LicA and licorice dietary supplements containing LicA.
4.2 Experimental Section

4.2.1 Materials and Reagents

LicA (E-97%, Z-3% by qNMR, Figure 30) and β-nicotinamide adenine dinucleotide phosphate, reduced sodium salt (NADPH), uridine 5’-diphosphoglucuronic acid triammonium salt (UDPGA), sulfatase, β-glucuronidase, sulfur trioxide-pyridine complex, glutathione (GSH) and naringenin were purchased from Sigma-Aldrich (St. Louis, MO). Pooled human liver microsomes (20 mg/mL, 150 donors), cDNA-expressed human cytochrome P450 1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, and 3A4 (0.5 nmol of P450 in 0.5 mL) and cDNA-expressed recombinant human UGTs including UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17 (5 mg protein/mL), were purchased from Corning Life Sciences (Tewksbury, MA). HPLC-grade solvents were purchased from ThermoFisher (Pittsburgh, PA). Cryopreserved human hepatocytes (50 donors pooled) and manufacturer-specified cell culture media were purchased from ThermoFisher Scientific (Waltham, MA).
Figure 30. Proton NMR (600 MHz, DMSO-$d_6$) analysis of licochalcone A showing the E to Z isomerization during 8-hour exposure to laboratory light. A1) Sample protected from light and containing only trans-licochalcone A; and A2) sample incubated for 8-hour under light, thus containing 3% cis isomer (Simmler et al, 2017).

4.2.2 Microsomal Incubations

Incubations of human liver microsomes consisted of 0.5 mg/mL of microsomal protein and either 20 μM LicA and 100 mM phosphate buffer (pH 7.4) in a total volume of 0.3 mL for phase I studies, or 10 μM LicA, 5 mM saccharic acid, 8 mM MgCl₂, 25 μg/mL alamethicin, 100 mM phosphate buffer (pH 7.4) in a total volume of 0.4 mL for phase II studies. The phase I reactions were initiated by adding NADPH (1 mM) after a 5-min pre-incubation at 37 °C,
and the phase II glucuronidation reactions were initiated by adding UDPGA (2 mM). After 60 min, each phase I incubation was terminated by adding 1 mL of ice-cold acetonitrile and chilling the resulting mixture on ice. Each phase II incubation was terminated in the same manner after 30 min. After centrifugation to remove precipitated proteins, the supernatants were removed and evaporated to dryness under a stream of dry nitrogen. Each residue was reconstituted in 200 µL of acetonitrile/water (30:70, v/v) immediately before analysis using high resolution HPLC-MS/MS or HPLC-UV.

4.2.3 Identification of Cytochrome P450 Isozymes

The cytochrome P450 enzymes involved in the formation of the most abundant mono-oxygenated LicA metabolites (M1, M2 and M3, Figure 31) were determined using cDNA-expressed human recombinant P450 1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, and 3A4 (stock solutions of 0.5 nmol of P450 in 0.5 mL). A 5 µM solution of LicA in 0.2 mL of phosphate buffer (pH 7.4) was incubated with 5 pmol of each P450 enzyme and 1 mM NADPH for 30 min at 37°C. Each incubation was terminated by the addition of 0.8 mL ice-cold acetonitrile to precipitate proteins followed by 15 min centrifugation at 13000 x g, the supernatants were analyzed directly using LC-MS/MS. Control experiment without enzymes was also conducted. All incubations were carried out at least three times, and the mean values of the metabolites were compared using one-way ANOVA with Tukey's test, p < 0.01.
4.2.4 **Hepatocyte Incubations**

Cryopreserved human hepatocytes were thawed according to the supplier's instructions, and approximately $1 \times 10^6$ cells in a 1-mL suspension were incubated with LicA (10 µM) per well of a 6-well plate. Control experiments were identical except for the use of heat-inactivated hepatocytes. The plate was placed in an incubator at 37 °C with 5% CO$_2$ and 90% relative humidity and gently shaken at 120 rpm for 4 h. Incubations were terminated by addition of 3 mL of ice-cold acetonitrile. The cell suspensions were centrifuged, and the supernatants were removed and evaporated to dryness under a stream of nitrogen. After reconstitution in 400 µL of water/acetonitrile (70:30, v/v), each residue was analyzed using HPLC-MS/MS.

4.2.5 **Deconjugation by β-glucuronidase and Sulfatase**

A 200 µL aliquot of each sample containing phase II conjugates was evaporated to dryness and reconstituted in 200 µL ammonium acetate buffer (10 mM, pH 5.0) containing a combination of β-glucuronidase (400 units) and sulfatase (40 units). The deconjugation reactions were carried out at 37 °C for 4 h and terminated by the addition of 600 µL ice-cold acetonitrile. After centrifugation, supernatants of each sample were analyzed using HPLC-MS/MS. Aliquots of samples not treated with hydrolytic enzymes were used as controls.

4.2.6 **Synthesis of Licochalcone A Monosulfate Isomers**

To determine the sites of sulfate conjugate on LicA, both the monosulfate isomers were synthesized as previously described with some modifications.$^{95}$ LicA (1.7 mg, 5 µmol) was mixed with sulfur trioxide-pyridine complex (8 mg, 50 µmol; molar ratio, 1:10) in 0.5 mL dry
pyridine and reacted at 60 °C for 3 h. The reaction was stopped by adding 0.5 mL 100 mM NaHCO₃ then sample was clean by solid phase extraction. After reconstituted with 20% aqueous acetonitrile, sample was diluted at appropriate concentration before running in LC-MS/MS and LC-UV.

4.2.7 Synthesis of Licochalcone A GSH Conjugate

LicA (10 μM) was incubated with 1 mg/mL human liver microsomes, 2 mM NADPH, and 5 mM GSH in 100 mM phosphate buffer (pH 7.4) with a total volume of 0.5 mL. After terminating reaction at 1 h, samples were partially purified by using C₁₈ solid phase extraction. The GSH conjugate of LicA was detected using a previously published UHPLC-MS/MS GSH adduct screening method. The retention time and tandem mass spectrum were also compared with potential GSH conjugates of LicA in the hepatocyte and rat samples.

4.2.8 Identification of UGTs

cDNA-expressed human UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, or UGT2B17 (stock solutions of 5 mg of protein/mL) were incubated with 10 μM LicA. Briefly, each incubation contained 0.25 mg UGT protein/mL, 5 mM saccharic acid, 8 mM MgCl₂, and 25 μg/mL alamethicin, 100 mM phosphate buffer (pH 7.4) in total volume of 0.4 mL at 37 °C. After 3 min pre-incubation, enzymatic reactions were initiated by adding UDPGA (2 mM final concentration in a total volume of 400 μL). Incubations were terminated after 20 min and prepared for UHPLC-MS/MS as described above for the microsomal incubations. All
incubations were carried out at least three times, and the mean values of LicA monoglucuronides were compared using one-way ANOVA with Tukey's test, p < 0.01.

4.2.9 Metabolic Stability

LicA (2 μM) was pre-incubated for 5 min at 37 °C with human liver microsomes (1 mg/mL) in phosphate buffer (pH 7.4, 100 mM) in a final volume of 500 μL. Phase I or phase II reactions were initiated by adding NADPH (1 mM) or UDPGA (2 mM). Aliquots (50 μL each) were removed at 0, 5, 10, 20, 30, 40, 50 and 60 min, and mixed with 300 μL ice-cold acetonitrile (containing 250 μM naringenin as internal standard). After centrifugation at 13,000xg for 15 min, the supernatants were analyzed immediately using UHPLC-MS/MS. Negative control incubations containing no microsomes were carried out in parallel under the same conditions.

Estimates of intrinsic clearance of LicA were based on substrate disappearance during 60 min incubations with human liver microsomes after addition of NADPH or UDPGA. The slope of the linear regression curve (-k) from log percentage remaining of LicA vs. incubation time was used to compute half-life using the following equation: $t_{1/2} = \frac{0.693}{k_e}$. Hepatic intrinsic clearance (CLint, mL/min/kg) was calculated by using the following equation: $^{115}$

$$CL_{int} = \frac{0.693}{t_{1/2}} \frac{SF}{SF} = \left(\frac{G_{microsomes}}{W_t \text{ liver}}\right)\left(\frac{W_t \text{ liver}}{W_t \text{ body}}\right)\left(\frac{C_{protein}}{SF}ight),$$

SF, scaling factor (mL/kg); Gmicrosomes, the average quantity of microsomal protein in the liver (mg), Wt liver, liver weight (g); Wt body, body weight (kg); and Cprotein, protein
concentration in the reaction mixture (mg/mL). Gmicrosomes/Wt liver is usually considered to be approximately 45 mg/g, while Wt liver/Wt body is approximately 20 g/kg in humans. The Cprotein in our experiment is 1 mg/mL, and substituting those numbers into this equation gives the following:

\[ \text{CLint} = 45 \times 20 \times 0.693/ (t_{1/2} \times 1) = 900 \times 0.693/ t_{1/2}, \text{ in units of mL/min/kg.} \]

4.2.10 Rat Metabolism of Licochalcone A

For the in vivo study, LicA (>95% [w/w] by qHNMR) was purified by high-speed countercurrent chromatography (300 mL HSCCC, Pharma-Tech, Baltimore, MD) from a LicA enriched Glycyrrhiza inflata extract (purity of LicA > 50%) that was obtained from Qinghai Lake Medicinal (Xining, China). Serum, mammary tissue, and liver tissue from rats administered LicA as part of a separate estrogen metabolism study were obtained to evaluate absorption and metabolism of LicA in vivo. Female ACI rats were purchased at 5 weeks of age and acclimated for 1 week with a phytoestrogen-free diet (AIN-76A from Harlan Laboratories). The rats were randomly divided into 3 groups with 6 rats per group as follows: vehicle only (sesame oil), estradiol-benzoate (1 mg/kg/day), and estradiol-benzoate (1 mg/kg/day) plus LicA (80 mg/kg/day). Treatments were administered subcutaneously daily for 4 days at 6 weeks of age. At day 5, the rats were sacrificed by CO₂ asphyxiation. Blood was collected, and serum was prepared immediately. Mammary and liver tissues were collected, snap frozen in liquid nitrogen and stored in -80 °C until analysis. The animal protocol complied with the Guide for the Care and Use of Laboratory Animals and all procedures were approved by UIC Institutional Animal Care and Use Committee (Protocol No.13-047 and 16-033).
After thawing at room temperature, serum (50 µL) was mixed with ice-cold acetonitrile (300 µL) to precipitate proteins. Tissues were weighed accurately (500-800 mg for liver and 50-100 mg for mammary) and homogenized with 70% aqueous methanol containing 0.1 % formic acid (adding 5 mL for liver and 1 mL for mammary). A 200 µL aliquot of each homogenate was mixed with 800 µL of ice-cold acetonitrile. Then, serum/tissue homogenate mixtures were vortexed and then centrifuged for 15 min at 13000×g at 4 °C. The supernatant was evaporated to dryness and reconstituted with 100 µL of 20% aqueous acetonitrile. An aliquot (5 µL) of each serum/tissue extract was analyzed using UHPLC-MS/MS.

4.2.11 Quantitation Measurement of Licochalcone A in vivo

The same rat samples from section 4.2.10 were analyzed for levels of LicA using UHPLC-MS/MS. After thawing at room temperature, serum (50 µL) was transferred to a 1.5 mL Eppendorf tube and mixed with 10 µL of acetonitrile containing naringenin (500 nM) as an internal standard (IS). Ice-cold acetonitrile (200 µL) was added for protein precipitation, and the mixture was then centrifuged for 15 min at 13000×g at 4 °C. After centrifugation, 200 µL of the supernatant was transferred to a new Eppendorf tube and evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 100 µL of 20% acetonitrile, and 5 µL was injected onto the UHPLC-MS/MS for analysis.

Rat liver and mammary tissues were homogenized in 70% aqueous methanol containing 0.1 % formic acid, and 200 µL aliquots were removed and mixed with the internal standard naringenin at a final concentration of 500 nM. Ice-cold acetonitrile (600 µL) was added to each homogenate for protein precipitation. Mixtures were then centrifuged for 15 min.
at 13000xg at 4 °C. After centrifugation, 400 µL of each supernatant was transferred to a new Eppendorf tube and evaporated to dryness under a stream of nitrogen. Each residue was reconstituted in 100 µL of 20% acetonitrile, and 5 µL aliquots were injected onto the UHPLC-MS/MS for analysis.

LicA stock solution was prepared in methanol at a final concentration of 100 µM and stored in amber glass vials. Working standards were made by serial dilution from stock solutions using blank serum and homogenate (from rats not treated with LicA) as diluent. Quality control (QC) stock solutions were prepared from a separate weighings of the reference standards and stored at 4 °C. For serum, 8-point plasma standard curves consisted of points for 2, 5, 10, 25, 50, 100, 250, and 500 nM LicA; for liver, 7-point homogenate tissue standard curves consisted of points at 2, 5, 10, 25, 50, 100, and 250 nM LicA; for mammary tissue, 8-point homogenate tissue standard curves consisted of 1, 2, 5, 10, 25, 50, 100, and 250 nM LicA. For all serum and tissue measurement, QC samples were prepared at 8 nM (low), 40 nM (medium) and 200 nM (high) LicA. The sample extraction method was same as describe above.

4.2.12 HPLC-UV, HPLC-MS/MS and UHPLC-MS/MS Analyses

LicA metabolites were characterized using accurate mass measurement HPLC-MS and HPLC-MS/MS on a Waters (Milford, MA) hybrid QqTOF Synapt G1 mass spectrometer. Chromatographic separations were carried out on a Waters Xterra MS C\textsubscript{18} 2.1x100 mm, 3.5 µm column. The mobile phase consisted of 0.1 % formic acid in water and acetonitrile with a 20-min or a 30-min gradient from 15 to 95 % acetonitrile. The flow rate was 0.2 mL/min, the column temperature was 30 °C, and the injection volume was 5 µL. Leucine enkephalin was
added post-column as a lock mass for accurate mass measurement. Mass spectra and tandem mass spectra were acquired from \( m/z \) 50 – 800 using positive ion electrospray. Product ion tandem mass spectra were obtained using collision-induced dissociation (CID) with argon collision gas at a collision energy of 18 – 22 eV. Data collection and processing were carried out using Waters MassLynx 4.1 software.

For quantitative analysis, UHPLC-MS/MS with CID and selected reaction monitoring (SRM) were used on a Shimadzu (Kyoto, Japan) LCMS-8050 or LCMS-8060 triple quadrupole mass spectrometer equipped with a Shimadzu Nexera UHPLC system and Waters Acquity UPLC BEH Shield RP \(_{18}\), 2.1x50 mm, 1.7 μm column. The UHPLC mobile phase A was water containing 0.1% formic acid, and mobile phase B was acetonitrile containing 0.1% formic acid. The flow rate was 0.6 mL/min, and the gradient was as follows: 0–1 min, hold at 20% B; 1–3.5 min, 20% to 45% B; 3.5-4.5 min, 45% to 95% B; hold at 95% B for 1.5 min, and then re-equilibrate at 20% B for 2 min. The column temperature was 40 °C, and the injection volume was 2 μL. During UHPLC-MS/MS, electrospray was used with polarity switching (5 µsec) and a nebulizing gas flow of 2.5 L/min, a heating gas flow was 10 L/min, an interface temperature of 300 °C, a desolvation line temperature of 250 °C, a heating block temperature of 400 °C, and a drying gas flow rate of 10 L/min. The SRM transitions (quantifier and qualifier) were \( m/z \) 339 to 121 and \( m/z \) 339 to 297 for LicA, and \( m/z \) 273 to 153 and \( m/z \) 273 to 147 for the internal standard naringenin. The SRM dwell time was 15 msec per transition. The SRM transitions for each phase I and phase II metabolites were established based on their tandem mass spectra during metabolites characterization using Q-ToF as stated above. For LC-UV analysis of LicA glucuronides and sulfate isomers, UV spectra from 210 - 400 nm were acquired using a Shimadzu photodiode array (PDA) HPLC detector.
For comparison of the in vivo results with those from human hepatocytes and liver microsomes, the Waters Xterra column and HPLC gradient mobile phase used for accurate mass measurement was transferred to a Shimadzu LCMS-8060 triple quadrupole mass spectrometer. Based on data-dependent UHPLC-MS/MS analyses in scan mode, the positive ion electrospray SRM transitions for each analyte were established as \( m/z \) 515 to 339 for LicA monoglucuronides, \( m/z \) 646 to 339 for the glutathione conjugate of LicA, \( m/z \) 419 to 339 for LicA sulfate, \( m/z \) 531 to 339 for monooxygenated LicA glucuronides, and \( m/z \) 545 to 339 for catechol-\( O \)-methylated LicA glucuronides.

For the quantitative measurement of LicA in rat samples, method validation was carried out in accordance with the US Food and Drug Administration (FDA) guidelines for bioanalytical method validation. Calibration curves were constructed by plotting the peak area ratio for LicA and naringenin (IS) versus the corresponding concentration and fitting a linear regression equation to the data (weighting factor of \( 1/c \)). Shimadzu Labsolution software and Microsoft Excel were applied for data analysis.

4.3 Results and Discussion

4.3.1 Phase I Metabolism of Licochalcone A

After incubation of LicA with human liver microsomes and NADPH, three major (M1, M2 and M3) and two minor phase I metabolites (M4 and M5) were detected using high resolution HPLC-MS (Figure 31). When microsomes or NADPH were omitted from the incubation, no metabolites were detected (data not shown). Eluting at 10.6, 11.2 and 14.5 min, respectively, M1, M2 and M3 were determined to be mono-oxygenated metabolites of LicA,
based on accurate mass measurement of their protonated molecules of \textit{m/z} 355.1558 (M1), \textit{m/z} 355.1562 (M2) and \textit{m/z} 355.1555 (M3) which corresponded to an elemental composition of C$_{21}$H$_{22}$O$_5$ (ΔM 3.66 ppm, M1; ΔM 4.79 ppm, M2; and ΔM 2.82 ppm, M3).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure31.png}
\caption{Positive ion electrospray high resolution HPLC-MS total ion chromatogram (TIC) and computer-reconstructed mass chromatograms showing licochalcone A and its phase I metabolites (M1 – M5) following incubation with human liver microsomes and NADPH.}
\end{figure}

The high resolution tandem mass spectra of protonated M1 and M2 were identical (Figure 32), and like LicA, showed an abundant fragment ion of \textit{m/z} 121 corresponding to an unchanged A-ring. During positive ion MS/MS with CID, chalcone compounds have been reported to form a product ion via Nazarov cyclization (loss of ketene, C$_2$H$_2$O, -42 units)
An ortho substituent on the B-ring, an α,β-unsaturated ketone and a free hydrogen at the ortho position (no substituent) of the A-ring are required for Nazarov cyclization of chalcones and the formation of ketene-loss ion. In the tandem mass spectra of M1 and M2, the unchanged A-ring ion at m/z 121 and the loss of a ketene fragment observed at m/z 313 (Figure 32) localized the addition of oxygen on or near the B-ring. Interestingly, M1 and M2 co-elute during UHPLC-MS/MS with two newly described dihydrobenzofuran congeners of LicA isolated from G. inflata (Figure. 33). These licorice compounds have been identified as E- and Z-isomers of 3-(2-(hydroxymethyl)-6-methoxy-3,3-dimethyl-2,3-dihydrobenzofuran-5-yl)-1-(4-hydroxyphenyl)prop-2-en-1-one, (LicAF1), and exhibited identical elemental composition and product ion tandem mass spectra compared to M1 and M2 (Figure 32). Thus, M1 and M2 were identified as the E- and Z-isomers of LicAF1. Phase I metabolites M1 and M2 might have been formed through epoxidation of the prenyl group followed by intramolecular attack by the neighboring hydroxyl group to form a five-member ring.
Figure 32. Positive ion electrospray product tandem mass spectra of A) licochalcone A; and B) mono-oxygenated licochalcone phase I metabolites M1 and M2 formed using human liver microsomes. Note that M1 and M2 produced identical tandem mass spectra.
Figure 33. A) UHPLC-MS/MS chromatograms of licochalcone A phase I metabolites (top) and compounds in a G. inflata fraction showing identical retention times for M1 and M1* as well as M2 and M2*. B) Proton NMR analysis (900 MHz, DMSO-d6) of E to Z isomers (M1* and M2*) of G. inflata secondary metabolite 3-(2-(hydroxymethyl)-6-methoxy-3,3-dimethyl-2,3-dihydrobenzofuran-5-yl)-1-(4-hydroxyphenyl)prop-2-en-1-one, which are identical to the phase I licochalcone A metabolites M1 and M2 (Simmler et al, 2017).

The high resolution product ion tandem mass spectrum of protonated M3 (Figure 35) showed an abundant ion of \( m/z \) 137.0235 corresponding to an elemental composition of C_7H_5O_3 (ΔM -2.19 ppm) and indicating hydroxylation on the ortho or meta position of A-ring.
Because the base peak of \( m/z \) 313.1440 (C_{19}H_{21}O_{4}, \Delta M < 0.001 \) ppm was formed by loss of a ketene via the Nazarov cyclization, there could be no substituent at the ortho position of the A-ring. If the hydroxylation occurred on the ortho position of A-ring, then the Nazarov cyclization could not happen, and no loss of ketene ion would have been observed.\(^{208}\) Therefore, phase I metabolite M3 was a catechol formed by hydroxylation of LicA at the meta position of the A-ring (Figure 34).

Minor metabolites M4 and M5 (retention times 8.7 and 9.3 min, respectively, Figure 31) were determined to be di-oxygenated LicA metabolites with an elemental composition of C_{21}H_{22}O_{6} (measured M4 \( m/z \) 371.1474, \( \Delta M \) -5.39 ppm; and M5 \( m/z \) 371.1509, \( \Delta M \) 4.04 ppm). M4 and M5 produced identical product ion tandem mass spectra (Figure 34) showing a Nazarov cyclization ion of \( m/z \) 329 (LicA plus 2 oxygen atoms) and an A-ring ion of \( m/z \) 137 (plus one oxygen atom). Because these product ions indicated mono-hydroxylation at the ortho position of the A-ring plus mono-hydroxylation on the B-ring, M4 and M5 were probably mono-oxygenated derivatives from M1, M2, and M3 (Figure 34).
To investigate the roles of individual human P450 enzymes in the metabolism of LicA, incubations were carried out using cDNA-expressed recombinant enzymes. The formation of metabolites obtained from individual incubations with each P450 enzyme were multiplied by the mean specific content of the corresponding P450 enzymes in human liver microsomes to normalize the contribution. After normalization, CYP 1A2, CYP 2C8, CYP 2C9 and CYP 3A4 showed the most activity for the formation of epoxide related metabolites (>50% contribution) M1 and M2, which indicated that they shared a similar biotransformation pathway. M3, the aromatic hydroxylated metabolite, was primarily metabolized by CYP 1A2 and CYP 3A4, followed by CYP 2E1 and 2C family enzymes (Figure 36). No specific cytochrome P450 enzymes that might contribute to the formation of M4 or M5 were identified, instead, all the enzymes tested appeared to contribute slightly (data not shown).
Figure 35. Positive ion electrospray product tandem mass spectrum of mono-oxygenated licochalcone A phase I metabolites, A) M3; and B) M4 and M5. Note that M4 and M5 produced identical tandem mass spectra.
Figure 36. Relative formation of licochalcone A phase I metabolites by recombinant cytochrome P450 enzymes (5 pmol each). Each recombinant enzyme was incubated with licochalcone A and NADPH, and formation of licochalcone A metabolites M1, M2 and M3 were compared using UHPLC-MS/MS. Data were multiplied by the mean specific content of corresponding cytochrome P450 enzymes in human liver microsomes to normalize their contributions. (Mean ± S.E.; n=3).
4.3.2 Phase II Metabolism of Licochalcone A

HPLC-MS accurate mass measurement analysis of the human hepatocyte metabolites of LicA (Figure 37) indicated 3 abundant glucuronides, MG1, MG2, and MG3 (retention times 12.3, 12.9 and 15.4 min, respectively) that weighed 176 Da more than LicA, 6 minor oxygenated LicA glucuronides (MG4 - MG9) and one monosulfate metabolite (retention time 20.7 min). None of these metabolites were detected in control incubations using heat-inactivated hepatocytes. After treatment with β-glucuronidase and sulfatase, all these conjugates disappeared confirming that they were glucuronides and sulfates, respectively.

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**Figure 37.** Positive ion electrospray LC-MS high resolution mass chromatograms of human hepatocyte metabolites of licochalcone A. Phase II conjugates of licochalcone A and its major phase I metabolites were detected.
Accurate mass measurements of protonated MG1, MG2 and MG3 ($m/z$ of 515.1934 MG1, $m/z$ 515.1915 MG2 and $m/z$ 515.1945 MG3) were within 6.2 ppm of the formula of C$_{27}$H$_{30}$O$_{10}$ corresponding to LicA monoglucuronides. The product ion tandem mass spectra of MG1 and MG2 were identical, and although the tandem mass spectrum of the less abundant MG3 was slightly different, all showed loss of dehydrated glucuronic acid at $m/z$ 339 from the protonated molecules, further confirming that these metabolites were glucuronides (Figure 38). Characteristic LicA fragment ions were also observed for MG1, MG2 and MG3 including the A-ring fragment ion of $m/z$ 121 and loss of ketene at $m/z$ 297 (Figure 38).
Figure 38. Positive ion electrospray product ion tandem mass spectra of licochalcone A glucuronides MG1-MG6 formed during incubation with human hepatocytes. A) MG1 and MG2; B) MG3; and C) MG4, MG5 and MG6. Note that the product ion tandem mass spectra of MG1 and MG2 were identical, as well as those of MG4, MG5 and MG6.
Glucuronidation at either one of the phenolic oxygens of LicA should have formed only two monoglucuronides (MG1 and MG2). Therefore, the third less abundant glucuronide M3 probably corresponded to the Z-isomer of LicA, which might have formed from Z-isomer by photoisomerization in solution.\textsuperscript{210} NMR analysis of the LicA used in the incubation indicated that it was 97\% (\textit{E})-LicA (w/w) and 3\% (\textit{Z})-LicA (w/w) (Figure 30).

UV spectroscopy has been used to determine sites of substitution on flavonoids and chalcones.\textsuperscript{211, 212, 213} In particular, the UV spectra of chalcones exhibit two major absorption peaks in the region from 200 nm to 400 nm. Band I (Ia, 340-380 nm; Ib, 300-330 nm) is associated with the B-ring, and Band II (240-280 nm) corresponds to the A-ring.\textsuperscript{214} The conjugated system of the B-ring is stabilized by delocalization of unpaired electrons from the 4-OH oxygen into the aromatic ring. Glucuronidation at the 4-OH position would interrupt this stability, resulting in a hypsochromic shift of Band I and reduced intensity compared with unconjugated LicA. In contrast, glucuronidation at the 4’-OH position of the A-ring would have little effect on UV absorption wavelength or intensity.

The UV spectrum of LicA (Figure 39A) showed an intense Band Ia (374 nm), a less intense Band Ib (312 nm) and a Band II (260 nm). In the UV spectrum of MG1 (Figure 39B), Band Ia (358 nm) was less intense and shows a large hypsochromic shift (16 nm) compared with that of LicA, which is consistent with glucuronidation at the 4-OH position. Therefore, MG1 was identified as (\textit{E})-LicA-4-glucuronide. In contrast, the UV spectrum of MG2 (Figure 39C) is almost identical to that of LicA, which indicates conjugation at the 4’-OH position.

The less abundant glucuronide, MG3, which probably corresponds to the Z-isomer, showed a shift of Band Ia (362 nm) with lower intensity, which is consistent with glucuronidation on the 4-OH position of the B-ring (Figure 39D). Thus, the three most abundant monoglucuronides
were identified as (E)-LicA-4-glucuronide (MG1), (E)-LicA-4\'-glucuronide (MG2), and (Z)-LicA-4-glucuronide (MG3). These structures have been reported previously by Nadelmann, et al.,\textsuperscript{215,216} who identified monoglucuronides of E and Z-LicA formed by rabbit and pig liver microsomes.

![UV spectra of licochalcone A and its monoglucuronides](image)

**Figure 39.** UV spectra of A) licochalcone A; and its monoglucuronides; B) MG1; C) MG2; and D) MG3. Band I (300-380 nm) and Band II (240-280 nm) are associated with the B-ring and A-ring, respectively.

Based on accurate mass measurements, tandem mass spectrometry and deconjugation by \(\beta\)-glucuronidase, minor metabolites MG4, MG5 and MG6 (retention times 12.3, 13.5 and 15.9 min, respectively; Figure 37) were determined to be glucuronides of the catechol-\(O\)-methyl metabolite of LicA (Figure 34). Accurate mass measurements of MG4 (m/z 545.2027), MG5 (m/z 545.2037) and MG6 (m/z 545.2035), were within 2.6 ppm of the elemental composition C\textsubscript{28}H\textsubscript{32}O\textsubscript{11} (LicA catechol+CH\textsubscript{2}+glucuronic acid). After treatment with \(\beta\)-glucuronidase, the HPLC-MS/MS peaks corresponding to MG4, MG5 and MG6 were
eliminated. Product ion tandem mass spectra of all three metabolites were similar (Figure 38C) and showed a base peak of \( m/z \) 369, corresponding the loss of glucuronic acid, \([\text{MH}-176]^+\), and fragment ions of \( m/z \) 327 (formed by Nazarov cyclization) and \( m/z \) 151 (A-ring plus OCH\(_2\)), indicating an \( O \)-methyl catechol metabolite of LicA.

MG7, MG8, and MG9 (retention times 8.9, 10.4, and 14.7 min, respectively; Figure 37) were determined to be glucuronides of the monooxygenated LicA metabolites, based on their accurate mass measurements, susceptibility to hydrolysis by \( \beta \)-glucuronidase and tandem mass spectra. Accurate mass measurements of all three metabolites were within 3.58 ppm of the elemental composition \( \text{C}_{27}\text{H}_{30}\text{O}_{11} \) (\( m/z \) 531.1885, \( m/z \) 531.1878 and \( m/z \) 531.1855 for MG7, MG8 and MG9, respectively). Furthermore, all three compounds could be hydrolyzed by \( \beta \)-glucuronidase, which confirmed that they were \( \beta \)-D-glucuronides. The product ion tandem mass spectra of MG7 and MG8 were identical (Figure 40A) and consisted of a base peak of \( m/z \) 355 (corresponding the loss of a glucuronic acid moiety, \([\text{MH}-176]^+\), from monooxygenated LicA), and ions of \( m/z \) 313 (formed by Nazarov cyclization after loss of glucuronic acid) and \( m/z \) 121 (corresponding to an unchanged A-ring of LicA). The ion of \( m/z \) 121 indicated that MG7 and MG8 were probably formed by glucuronidation of M1 and M2, which had been oxygenated on the B-ring of LicA. The tandem spectrum of MG 9 (Figure 40B) is consistent with glucuronidation of the LicA chalcone based on the ion of \( m/z \) 355 (formed by loss of glucuronic acid from the protonated molecule) followed by subsequent loss of ketene (\( m/z \) 313), and the fragment ion of \( m/z \) 137 (corresponding to the mono-oxygenated A-ring of M3). Note that the peak eluting at 15.4 min in Figure 37 was an impurity from the cell culture medium that appeared also in the negative control and had an exact mass inconsistent with \( \text{C}_{27}\text{H}_{30}\text{O}_{11} \) (data not shown).
Figure 40. Positive ion electrospray product tandem mass spectra of protonated licochalcone A phase II metabolites (A) MG7 and MG8; (B) MG9; and (C) licochalcone A-sulfate. Note that MG7 and MG8 produced identical tandem mass spectra.
To identify the UGT enzymes responsible for the formation of the three abundant monoglucuronides of LicA (MG1, MG2, and MG3), LicA was incubated with the cofactor UDPGA and the recombinant human UGT enzymes UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17, which represent the most abundant hepatic UGT isoforms.\textsuperscript{117} UGT1A9 showed the greatest catalytic activity in the formation of MG1 \((E)-\text{LicA-4'-glucuronide})\), followed by UGT 1A1, 1A7, 1A8, and 1A10 (Figure 41). In the formation of MG2 \((E)-\text{LicA-4-glcucuronide})\), UGT1A9 was again the most active enzyme followed by UGT 1A1, 1A3, 1A7 and 1A8. UGT 1A1, 1A3, and 1A10 were the most important enzymes for the formation of the minor metabolite MG3 \((Z)-\text{LicA-4-glucuronide})\). Note that the relative amount of MG3 was only \(\sim7\%\) that of MG1 and \(\sim9.3\%\) that of MG2.
Figure 41. Relative formation of licochalcone A glucuronides by recombinant UGT enzymes (0.5 mg of protein/mL). Each recombinant UGT was incubated with licochalcone A and UDPGA, and formation of licochalcone A metabolites MG1, MG2 and MG3 were compared using UHPLC-MS/MS. The control incubations contained no enzyme. Data were normalized to the yield of MG1 (100%) catalyzed by UGT1A9. (mean±S.E.; n=3).
One LicA sulfate conjugate was observed at a retention time of 20.8 min during the analysis of the human hepatocyte metabolites (Figure 37). Accurate mass measurement of the protonated molecule of \( m/z \) 419.1185 indicated a formula of \( \text{C}_{21}\text{H}_{23}\text{O}_{7}\text{S} \) (\( \Delta M \) 4.77 ppm). The base peak of the product ion tandem mass spectrum (Figure 40C) was observed at \( m/z \) 339 and corresponded to loss of \( \text{SO}_3^- \), \( [\text{MH}-80]^+ \), from the protonated molecule. Treatment with sulfatase resulted in the hydrolysis of this metabolite and the formation of LicA, which confirmed its identification as a sulfate conjugate. To determine the structure of this sulfate metabolite, LicA monosulfate isomers were synthesized and tested on LC-MS-UV with the identical HPLC condition. From the UV data, the peak at 15.4 min was confirmed to correspond to sulfation of LicA at 4-OH position (Band Ia shifts at 361 nm with lower intensity); the peak at 19.6 min was confirmed as LicA with sulfation at the 4’-OH position, since its spectrum was almost identical with LicA, which means that 4’-OH sulfation had little effect on its UV absorption (Figure 42). Because the synthetic LicA-4’-OH-sulfate co-eluted with the hepatic incubation sample (data not shown) as well as having identical elemental composition and tandem mass spectra, it was identified as the human sulfate metabolite of LicA.
Although too minor to be detected during high resolution HPLC-MS, a glutathione conjugate of LicA (MW 645) was observed eluting at 8.9 min during the UHPLC-MS/MS analysis of the hepatocyte incubation with LicA (Figure 43). The formation of this LicA glutathione conjugate was confirmed by comparing its retention time and tandem mass spectrum with those from incubation of LicA with glutathione (Data not shown). A summary of all phase I and II metabolites of LicA formed by human liver microsomes and hepatocytes is shown in Figure 34 and Table VI.
The metabolic stability of LicA was evaluated with respect to phase I as well as phase II metabolism. Phase I metabolic stability was estimated by incubating LicA with human liver microsomes and the cofactor NADPH. Phase II metabolic stability was assessed by incubating LicA with liver microsomes and UDPGA. LicA was slowly metabolized by phase I enzymes, decreasing 15.8% in 60 min, but was metabolized much more rapidly by phase II glucuronidation, decreasing 76.1% in 60 min (Figure 44). For phase I metabolism of LicA, the elimination rate constant \( k \) was 0.0028 with a half-life \( t_{1/2} \) of 247 min, and an intrinsic clearance \( \text{CL}_{\text{int}} \) of 2.52 mL/min/kg. In contrast, phase II glucuronidation of LicA by human liver microsomes was much faster with a rate constant \( k \) of 0.0227, a half-life \( t_{1/2} \) of 30.5 min, and an intrinsic clearance \( \text{CL}_{\text{int}} \) of 20.43 mL/min/kg. These data indicate that the
contribution of phase I metabolism to the elimination of LicA will be much lower than phase II glucuronidation.

Figure 44. Metabolic stability of licochalcone A during incubation with human liver microsomes and (A) NADPH for phase I metabolism; and (B) UDPGA for phase II glucuronidation. The amount of licochalcone A remaining at each time point was determined by LC-MS/MS. Incubation without microsomes was used as a negative control. Data are expressed as “Mean±S.D.”
### TABLE VI.

LICOCHALCONE A METABOLITES OBSERVED FOLLOWING INCUBATIONS WITH HUMAN LIVER MICROSOMES AND HUMAN HEPATOCYTES OR FOLLOWING SUBCUTANEOUS ADMINISTRATION TO RATS

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√√ = major metabolites; √ = minor metabolites; N.D. = not detected

4.3.3 Detection of Licochalcone A and its Metabolites in vivo

Serum (Figure 45), liver (Figure 46) and mammary tissue (Figure 47) from treated ACI rats were analyzed for LicA metabolites using UHPLC-MS/MS, and the results are summarized in Figure 34 and Table VI. All of the LicA metabolites that were predicted using
in vitro models were observed in the rat except that the phase I metabolites were observed only as subsequent phase II conjugates (Table VI). The predominance of phase II conjugation reactions was consistent with the metabolic stability studies described above. With respect to specific tissues, all LicA metabolites were observed in rat serum, liver and mammary tissue with four exceptions. First, LicA sulfate and MG9 were below the limit of detection in mammary tissue (Table VI; Figure 47); and second, MG4, MG5 and MG9 were not observed in rat liver (Table VI; Figure 46). Different results of human hepatocytes and rat tissues be a result of species differences. After treatment of serum and tissue homogenate with β-glucuronidase/sulfatase, all the phase II conjugates disappeared from the UHPLC-MS/MS chromatograms, and the peak heights of the corresponding aglycones increased (data not shown).

![Figure 45. Positive ion UHPLC-MS/MS chromatograms of licochalcone A metabolites formed in rat serum.](image-url)
Figure 46. Positive ion UHPLC-MS/MS chromatograms of licochalcone A metabolites formed in rat liver.

Figure 47. Positive ion UHPLC-MS/MS chromatograms of licochalcone A metabolites formed in rat mammary.
The calibration curves and equations for serum, liver, and mammary tissues are shown in Figure 48. The limit of detection (LOD, S/N ratio of 3) and LLOQ for LicA were 0.5 nM and 1 nM for both serum and tissue homogenates. The calibration curves were linear over the ranges of 2-500 nM (rat serum); 2-250 nM (rat liver); and 1-250 nM (rat mammary), (r² > 0.99). No chromatographic interference was observed while analyzing blank serum and blank tissue homogenate (data not shown).

Figure 48. Standard calibration curves for quantitative analysis of licochalcone A in: A) rat serum; B) rat liver homogenate; C) rat mammary homogenate.
Intra-day assay accuracy and precision were determined by measuring 5 replicates at low, medium, and high concentrations, and inter-day precision was measured by comparing 3 sets of QC samples analyzed on 3 consecutive days. The results are summarized in Table VII.

**TABLE VII.**

**INTER-DAY AND INTRA-DAY REPRODUCIBILITY OF UHPLC–MS–MS QUANTITATION OF LicA IN RAT SERUM, LIVER AND MAMMARY TISSUE**

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*Intra-day accuracy and CV were determined on 3 different days, and representative data from day 1 are shown.

LicA concentrations were determined for each rat (N = 6) and expressed as mean±SD of 3 replicates. The concentrations in tissues are expressed as the weight of “LicA/tissue”. (For LicA, 1 nM = 0.338 ng/mL). The results are shown in Table VIII.
After subcutaneous injection of LicA (80 mg/kg/day) to ACI rats for 4 days, the average LicA levels in tissues of all 6 animals were 258.5 ± 44.46 nM in serum, 64.74 ± 15.57 ng/g in liver, and 18.62 ± 3.20 ng/g in mammary tissue. These data show that following subcutaneous administration, a small amount of unmetabolized LicA can be detected in serum and tissues.

4.4 Conclusions

The hepatic metabolism of LicA was investigated using both in vitro and in vivo models. Incubation of LicA with human liver microsomes was useful in determining the relative contributions of cytochrome P450 enzymes in phase I metabolism. Five phase I metabolites were detected using high resolution mass spectrometry, including three major mono-oxygenated metabolites (M1, M2 and M3) and two minor di-oxygenated metabolites (M4 and M5). M1 and M2 were identical to two plant secondary metabolites of *G. inflata*, and were the cis- and trans- isomers of an intramolecular cyclization product on B-ring of LicA.
CYP 1A2, 2C8, 2C9 and 3A4 were catalyzed the formation of M1 and M2, which probably involved epoxidation followed by non-enzymatic ring closure. A similar P450 related epoxide reaction of the prenyl group followed by an intramolecular ring closure had been reported previously for 8-prenylnaringenin, a phytoestrogen from hops. The formation of both cis and trans isomers may come from the photoisomerization of LicA in solution.

The most abundant phase I metabolite of LicA, M3 was identified as a catechol on the A-ring, based in part on a Nazarov cyclization fragment ion in the tandem mass spectrum. Additional evidence for catechol formation was detection of COMT reaction in vivo indicated an existence of catechol product. The recombinant P450 isozymes experiment clarified that CYP1A2 and CYP3A4 contributed most to M3 formation. In the literature, CYP1A2 was reported to play a major role in the biotransformation of trans-resveratrol into its catechol metabolite - piceatannol. In the enzymatic pathways involved in estradiol aromatic hydroxylation metabolism, CYP1A2 and CYP3A4 are also involved in the formation of its major catechol metabolites, 2-hydroxyestradiol in the liver. Hydroxychalcones with phenolic groups on both the A and B rings like M3 are generally more cytotoxic and more effective as cancer therapeutic agents than those containing just one hydroxyl group only on the A ring.

The minor phase I di-oxygenated metabolites, M4 and M5, share a catechol structure on the A-ring and oxygenation on the B-ring or B-side, thus they are probably derivatives of the mono-oxygenated metabolites (M1, M2 and M3). Due to their low level of formation, no specific cytochrome P450 enzymes that might contribute to the formation of M4 or M5 were identified.
Incubation of LicA with human hepatocytes formed 3 abundant mono-glucuronides (MG1, MG2, and MG3), 6 minor oxygenated glucuronides (MG4-MG9; probably derivatives of phase I metabolites), one monosulfate metabolite and one GSH conjugate. No phase I metabolites without additional phase II glucuronidation were detected in the human hepatocyte incubations. Overall, glucuronidation was the most important metabolic pathway for LicA.

Chalcones can undergo E to Z transformation in solution when light exposure available.\textsuperscript{210} It has been reported that the absence of a free hydroxyl group at the 4-position of chalcones inhibits photoisomerization between the E and Z-isomers.\textsuperscript{220} A free hydroxyl group at the 4-position enables keto-enol tautomerization and free rotation around the \(\alpha,\beta\)-bond. Therefore, (\(E\))-LicA and (\(E\))-LicA-4'-glucuronide (MG2) can undergo photoisomerization to the Z-isomers and back again to the E-isomers. However, once (\(Z\))-LicA becomes conjugated to form (\(Z\))-LicA-4-glucuronide, it cannot readily convert back to the more stable E-isomer. Hence, (\(Z\))-LicA-4-glucuronide (MG3) was the only (\(Z\))-LicA-glucuronide detected. Another hypothesis to explain the formation of Z-isomer glucuronides and the isomerization of phase I metabolites, M1 and M2, is that trans- and cis- isomerization may be induced by cytochrome P450 enzymes, which was reported as an uncommon P450 reaction in the biotransformation of tamoxifen.\textsuperscript{221,222}

The experiments with cDNA-expressed UGTs showed that the mono-glucuronidation of LicA was catalyzed primarily by UGT1A1, UGT 1A7, UGT 1A8, UGT 1A9 and 1A10. Most UGTs are expressed in the liver, but some isoforms, such as UGT1A7, UGT1A8, and UGT1A10, are expressed predominantly in the gastrointestinal tract.\textsuperscript{223} For the two major glucuronides (MG1 and MG2), UGT1A9 showed the highest activities, which is distributed mainly in the liver, colon, and kidney.\textsuperscript{224} Therefore, the UGT1A9 in human kidney and colon
and UGT1A7, UGT1A8 and UGT1A10 in the GI tract should contribute significantly to the extrahepatic clearance of LicA. The in vitro intrinsic clearance \( CL_{int} \) due to glucuronidation was calculated to be 20.43 mL/min/kg, which was almost 10-fold faster than the phase I clearance rate (2.52 mL/min/kg). Thus, glucuronidation should be the most important clearance route for in vivo metabolism of LicA.

Besides those major phase I metabolites and glucuronides, the GSH metabolite was proposed as a product via Michael addition reaction after comparing its retention time and tandem mass spectrum with the one from incubation of LicA with glutathione. This conjugate was also confirmed using the previously published UHPLC-MS/MS GSH adducts screening method.\(^{62}\) The sulfate metabolite was confirmed as sulfation on 4’-OH position on A-ring based on LC-UV-MS. Please note that, only one GSH conjugate and one sulfate were detected in vitro incubation, the abundance were quite lower than the major glucuronidation metabolites. The similar results were observed in vivo metabolites screening from rat serum, liver and mammary tissues as well. As an initial in vivo screening model, LicA was administered to rats, and all of the metabolites predicted in vitro by the hepatocyte model were observed, and the predominance of phase II conjugation reactions over phase I oxygenation that had been predicted was confirmed.

In summary, metabolism of LicA involves both phase I oxygenation and phase II conjugation. Although the phase I metabolites might have more potent or different pharmacological or toxic activities than LicA, phase II glucuronidation will dominate the metabolism of LicA.
CHAPTER 5:

CONCLUSIONS AND FUTURE DIRECTIONS

Dietary supplements, which include vitamins, amino acids, minerals, and herbs or other botanicals, are mainly taken orally. Botanical dietary supplements, which include plants or parts of plants that have medicinal or therapeutic value, have been consumed by people all over the world for many generations. The total market for botanical dietary supplements has been growing in the United States recently. A major step in ensuring the safety of botanical dietary supplements has been the requirement by the U.S. FDA that current Good Manufacturing Practices (cGMP) be used by the botanical dietary supplement industry to help prevent botanical misidentification, product mislabeling, or contamination (pesticides, heavy metals, etc.). However, there are no regulatory requirements that these products be tested for safety or efficacy. As with pharmaceuticals, consumer health would be better protected if botanical dietary supplements were also evaluated for mechanisms of action and active constituents such that chemical standardization could be carried out based on the active compounds, and biological standardization could be performed based on pharmacological activity. Botanical dietary supplements should also be evaluated for their safety and efficacy, such as drug–botanical interactions, metabolism of active compounds, and finally, human clinical studies.

Licorice has been consumed as a medicinal herb for the thousands of years, which is also widely used in the United States. Three licorice species are mainly used as commercial resource, *Glycyrrhiza glabra* L (*G. glabra*), *Glycyrrhiza uralensis* Fisch. (*G. uralensis*) and *Glycyrrhiza inflata* Bat (*G. inflata*). A current topic within the UIC Botanical Center is the
investigation of potential benefits of licorice dietary supplements for women’s health. The data in this dissertation show that licorice constituents such as chalcones have potential anticancer or cancer chemoprevention activities. In related studies, licorice extracts and their constituents isoliquiritigenin (ILG) and licochalcone A (LicA) have been found to induce NQO1 in murine hepatoma (Hepa1c1c7) cells in vitro, which is among the enzymes regulated by the ARE. This suggests the involvement of the Keap1-Nrf2 pathway, which is a cellular chemopreventive pathway.

A refined UHPLC-MS/MS assay was developed to detect electrophilic compounds such as reactive metabolites formed via hepatic metabolism of complex mixtures such as botanical extracts by trapping them as GSH conjugates. This MS-based approach combines the use of a 1:1 ratio of unlabeled GSH and stable isotope labeled GSH (3 Da difference) to avoid false positives; applying positive constant neutral loss scanning of 129 as well as negative precursor ion scanning of 272 (275 for isotope labeled GSH) to detect GSH conjugates in both positive and negative channels; and using UHPLC instead of HPLC to shorten the analysis time. The screening assay was validated by several testing compounds which were previously reported to form reactive metabolites via hepatic metabolism, and applied to the detection of reactive metabolites of glabridin (a unique constituent from *G. glabra*) in a licorice extract. By omitting metabolizing enzymes and the cofactor NADPH, this assay was shown to be useful for detection of electrophilic compounds already present in botanical mixtures. Four abundant chalcones (LicA, LicB, echinatin, and ILG) were identified in licorice extracts using this screening approach. These licorice chalcones were shown to react preferentially with biological thiols including cysteine residues on Keap1 protein. These data indicated a
mechanism through which licorice compounds can activate cytoprotective ARE enzymes via the Keap1-Nrf2 pathway.

The metabolism of the chemoprevention agent LicA, which is a chalcone found in abundance in the licorice species Glycyrrhiza inflata, was investigated using human liver microsomes, human hepatocytes, and in rats. Five oxygenated phase I metabolites of LicA were formed by human liver microsomes, including a catechol on the A-ring, two intramolecular cyclization products following epoxidation of the exocyclic alkene at position 5 of the B-ring, and two dioxygenated products. Nine phase II monoglucuronides of LicA and its oxygenated phase I metabolites were formed during incubation with human hepatocytes. These included \((E)\)-LicA-4-glucuronide, \((E)\)-LicA-4’-glucuronide, \((Z)\)-LicA-4-glucuronide, glucuronic acid conjugates of all of the monooxygenated phase I metabolites, and glucuronides of the licochalcone catechol after methylation by catechol-O-methyl transferase. In addition, human hepatocytes formed one sulfate conjugate and one glutathione conjugate of LicA. Following subcutaneous administration of LicA to rats, all of the major metabolites formed by human hepatocytes were observed in rat serum, liver, and mammary tissues. The structures of major metabolites were determined, and the cytochrome P450 enzymes and UDP-glucuronosyltransferases responsible for their formation were identified. Based on in vitro hepatic clearance calculations, LicA is predicted to be primarily metabolized primarily by phase II conjugation reactions, which is consistent with the in vivo observations. Therefore, to achieve pharmacological effects in vivo, higher dosages might be required. However, the possibility of forming of phase I metabolites at higher dosage, such as catechols which may have potential toxicity, must also be considered.
In summary, advanced analytical tools, especially the use of LC-MS/MS are facilitating safety and efficacy studies of natural products. In this dissertation, a new and rapid UHPLC-MS/MS screening method was used to detect reactive metabolites via hepatic metabolism or electrophilic compounds trapping as GSH from botanical mixture. Mass spectrometry-based screening assays involving Keap1 protein were used to detect the potential chemopreventive compounds in licorice extracts, which indicated that LicA is among the most promising. In vitro and in vivo metabolism studies were carried out for LicA, a unique chalcone in the licorice species *G. inflata*. Using licorice as an example, all of these studies contribute new approaches and information regarding that may be applied to studies of botanical dietary supplement safety and efficacy.
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