THE COMPARISON OF RAT AND HUMAN INTESTINAL AND HEPATIC GLUCURONIDATION OF ENTEROLACTONE DERIVED FROM FLAXSEED LIGNANS

A Thesis
Submitted to the College of Graduate Studies and Research
In partial Fulfillment of the Requirements
For the Degree of Master of Science
In the College of Pharmacy and Nutrition
University of Saskatchewan
Saskatoon

By
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ABSTRACT

The mammalian lignan, enterolactone (EL), is a gut microbe metabolite of plant lignan secoisolariciresinol diglucoside (SDG), which is most abundant in flaxseed. Numerous epidemiological, experimental and clinical studies suggest the protective effects of EL against various chronic diseases such as cancer, cardiovascular disease, and inflammation. However, EL’s oral bioavailability is low and highly variable due to extensive first-pass metabolism, especially glucuronidation, which results in the large amount of glucuronide metabolites but low levels of free EL in human plasma. Hepatocytes and enterocytes express UDP-glucuronosyltransferases (UGT), the enzymes responsible for the conjugation of glucuronic acid to EL. To better understand the contribution of liver and intestine to the first-pass glucuronidation, I conducted an in vitro enzyme kinetic analysis of EL glucuronidation using hepatic and intestinal microsomal fractions from both human and rat. An intrinsic clearance (CL_int) value was calculated using the substrate depletion approach. In addition to monitoring substrate depletion, high-pressure liquid chromatography (HPLC) analysis allowed detection of EL glucuronides, which were further substantiated by LC-MS. EL monoglucuronide was identified in rat and human intestinal and liver microsomes. Enzyme kinetic studies indicated the extent of hepatic microsomal glucuronidation exceeded intestinal glucuronidation in both human and rat, while the human liver CL_int value was slightly higher than that of rat liver. The CL_int value generated in human intestinal microsomes was only one third of the value of human liver, whereas, the CL_int of rat jejunum or colon was one-twentieth of rat liver, suggesting the human intestine makes a greater contribution to EL glucuronidation than rat intestine. These results suggest that both liver and intestine contribute to EL glucuronidation and the human intestine may exert a greater influence on the first-pass glucuronidation of EL than rat intestine, thereby significantly decreasing EL’s oral bioavailability. The rat might underestimate the extent of intestinal metabolism of EL relative to human.
ACKNOWLEDGEMENT

During my two and half year’s study in Canada, I benefit so much from my supervisors, committee members, my lab mates and friends here. I would sincerely thank my supervisors Dr. Jane Alcorn and Dr. Ed Krol for their academic guidance, generous support, kind understanding and trust. I’m really grateful of Dr. Alcorn’s guidance during my writing process; her comments, patient support and encouragement made me become an effective writer and researcher. I would like to thank my committee members, Dr. Andrew Olkowski and Dr. Fred Remillard for their helpful suggestions for my research work. The difficult questions asked by Dr. Andrew Olkowski before I submit my thesis challenged me, but he helped me to obtain a more clear idea of my research work and to improve my skills of answering questions. I also want to thank my external reader Dr. Lynn Weber from Department of Veterinary Biomedical Sciences for your valuable time and comments for my defense and thesis.

Sincerely thank Deborah Michel, Jennifer Billinsky, and Joshua Buse for their help on mass spectrometry during my research study. I also want to thank my lab mates Sabia Maini, Isaac Asiamah, Yunyun Di, Uma Manthena and Erica Ling for their assistance on my experimental work.

Special thanks are given to my dear friend Xue Yao and my parents for their generous support, encouragement, love and care during my master program.

I also greatly appreciate the technical help from Dorota Rogowski and Erling Madsen.

Lastly, I’m grateful of all my friends for their kindness and help during my stay in Canada.

Thanks the scholarships from University of Saskatchewan and Beijing Institute of Technology, and also the research funding from Natural Sciences and Engineering Research Council (NSERC).
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<th>Full Form</th>
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<tr>
<td>17β-HSD</td>
<td>17β-hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>4-MU</td>
<td>4-methylumbelliferone</td>
</tr>
<tr>
<td>ALA</td>
<td>α-linolenic acid</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under curve</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>Caco-2</td>
<td>Colon adenocarcinoma cell line</td>
</tr>
<tr>
<td>CAD</td>
<td>Collision activated dissociation</td>
</tr>
<tr>
<td>CE</td>
<td>Collision energy</td>
</tr>
<tr>
<td>CL_{int}</td>
<td>Intrinsic clearance</td>
</tr>
<tr>
<td>Cl_{s}</td>
<td>Systemic clearance</td>
</tr>
<tr>
<td>C_{max}</td>
<td>Maximum drug concentration in the blood</td>
</tr>
<tr>
<td>CRU</td>
<td>Curtain gas</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>CXP</td>
<td>Collision cell exit potential</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Double distilled water</td>
</tr>
<tr>
<td>DIN-HM</td>
<td>Homeopathic Medicine Number</td>
</tr>
<tr>
<td>DHT</td>
<td>³H-dihydrotestosterone</td>
</tr>
<tr>
<td>DMBA</td>
<td>9,10-dimethyl-1, 2-benzanthracene</td>
</tr>
<tr>
<td>DP</td>
<td>Declustering potential</td>
</tr>
<tr>
<td>E2</td>
<td>Estradiol</td>
</tr>
<tr>
<td>ED</td>
<td>Enterodiol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine-tetracetic acid</td>
</tr>
<tr>
<td>EL</td>
<td>Enterolactone</td>
</tr>
<tr>
<td>EL-Glu</td>
<td>Enterolactone glucuronide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>LQC</td>
<td>Low quality control</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass to charge ratio</td>
</tr>
<tr>
<td>MAT</td>
<td>Matairesinol</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>MNU</td>
<td>N-methyl-N-nitrosourea</td>
</tr>
<tr>
<td>MQC</td>
<td>Middle quality control</td>
</tr>
<tr>
<td>MRM</td>
<td>Multiple reaction monitoring</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>Disodium phosphate</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NDGA</td>
<td>Nordihydroguaiaretic</td>
</tr>
<tr>
<td>NHP</td>
<td>Natural Health Product</td>
</tr>
<tr>
<td>NHPD</td>
<td>Natural Health Products Directorate</td>
</tr>
<tr>
<td>NHPR</td>
<td>Natural Health Products Regulation</td>
</tr>
<tr>
<td>NPN</td>
<td>Natural Product Number</td>
</tr>
<tr>
<td>O₂⁻·</td>
<td>Superoxide</td>
</tr>
<tr>
<td>·OH</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>PINO</td>
<td>Pinoresinol</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonylfluoride</td>
</tr>
<tr>
<td>QC</td>
<td>Quality control</td>
</tr>
<tr>
<td>RCM</td>
<td>Rat colon microsomes</td>
</tr>
<tr>
<td>RDM</td>
<td>Rat duodenum microsomes</td>
</tr>
<tr>
<td>RJM</td>
<td>Rat jejunum microsomes</td>
</tr>
<tr>
<td>RLM</td>
<td>Rat liver microsomes</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RSD</td>
<td>Relative standard deviation</td>
</tr>
<tr>
<td>[S]</td>
<td>Substrate concentration</td>
</tr>
<tr>
<td>SBP</td>
<td>Sex steroid binding protein</td>
</tr>
<tr>
<td>SDG</td>
<td>Secoisolariciresinol diglucoside</td>
</tr>
<tr>
<td>SECO</td>
<td>Secoisolariciresinol</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>SHBG</td>
<td>Sex hormone binding globulin</td>
</tr>
<tr>
<td>$t_{1/2}$</td>
<td>Half life</td>
</tr>
<tr>
<td>TEM</td>
<td>Temperature</td>
</tr>
<tr>
<td>UDPGA</td>
<td>5'-diphosphogluconic acid</td>
</tr>
<tr>
<td>UGT</td>
<td>Uridine diphosphogluconosyl transferase</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>$V_d$</td>
<td>Volume of distribution</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>Maximum velocity</td>
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1. INTRODUCTION

Flaxseed is the richest source of dietary intake of the plant lignan secoisolariciresinol diglucoside (SDG). A growing body of evidence suggests that SDG and its metabolites, in particular enterolactone (EL), may exert protective effects against cancer and cardiovascular disease through diverse mechanisms. Epidemiological studies suggest that the lower risk of aforementioned chronic diseases is greatly attributed to the high circulating concentrations of EL. However, the systemic concentration of EL is highly variable. The low and varying bioavailability of EL might be greatly attributed to extensive first-pass metabolism, which is responsible for the low bioavailability of many xenobiotics. In human plasma and urine, the principal conjugates of EL are mainly glucuronides, suggesting a significant first-pass effect by glucuronidation occurring both in hepatocytes and enterocytes. Nevertheless, the exact contributions from liver and intestine are poorly understood.

Hence, a fundamental understanding of EL glucuronidation in the liver and intestine is essential to provide critical information regarding the oral absorption properties of EL, and also possibly to infer the potential for drug-lignan interactions. Therefore, in my project, in vitro enzyme kinetic studies using rat and human liver and intestinal microsomes with determination of intrinsic clearance (CL_{int}), which directly measures the efficacy of enzymes to metabolize substrates, allow the comparison of differences of EL glucuronidation between the two first-pass metabolism organs and among two species.

The literature review on flaxseed lignans contains several sections. The first two sections give an overview of the regulations as well as benefits and risks of Natural Health Products (NHPs) and the status of flaxseed and lignans as NHPs in Canada. It also provides a general framework of the production of EL. The third section demonstrates the health benefits of EL according to the evidence from both epidemiological and experimental animal studies, although the studies are not completely consistent. The next section discusses the various mechanisms responsible for the biological effects of EL. The fifth section essentially deals with the pharmacokinetics (absorption, metabolism, distribution and excretion) of plant and mammalian lignans with an emphasis on the first-pass glucuronidation of EL. The last two sections discuss the low systemic levels of EL and the factors possibly accounting for the low
bioavailability. The model system is also discussed to explain the significance of including both human and rat models into the study.
2. LITERATURE REVIEW

2.1. Natural Health Products

2.1.1. Introduction of Natural Health Products

In Canada, Natural Health Products (NHPs) are regulated under the Natural Health Products Regulations (NHPR), which is different from the regulations for drugs and food. The Natural Health Products Directorate (NHPD) in Canada defines NHPs as naturally occurring substances which include vitamins and minerals, herbal remedies, homeopathic medicines, traditional medicines like traditional Chinese and East Indian medicines, probiotics and other products like amino acids and essential fatty acids\(^1\). NHPs are widely used around the world, with increasing use in Western countries, to ensure health and wellness and/or for their putative medical benefits. A recent survey conducted in 2010 showed that 73% of Canadians were using NHPs with greater prevalence in older than younger populations\(^2\).

2.1.1.1. Regulation of Natural Health Products

Each NHP must receive market authorization by obtaining a product license based on evidence that the product is safe under the recommended conditions of use without a prescription, effective for the proposed claims, and of high quality\(^3\). NHPD requires evidence, which might vary depending on the product and type of health claim, to support the safety and efficacy of the NHP according to its recommended conditions of use prior to approval for sale in Canada. That evidence must come from human use; animal or \textit{in vitro} experimental evidence may be considered as additional supporting information but cannot be the basis for approval\(^4\). For health support claims implying treatment, cure, prevention, and risk reduction claims related to a major or serious condition, for example a health claim of a chemopreventive NHP for cancer prevention, the NHPD requires at least two Phase III trials (randomized, controlled, well-designed) and two prospective observational studies\(^5\). Other information packages that reflect the totality of evidence, including systematic literature review, various epidemiological studies, background information and safety data with respect to \textit{in vitro} experiments and animal studies are demanded in support of a requested claim\(^5\).
Once a product has been assessed the ingredients and claims to ensure safety, efficacy and a product of high quality, an eight-digit Natural Product Number (NPN) or Homeopathic Medicine Number (DIN-HM) will be granted, indicating that the product is reviewed and approved by Health Canada. Consumers can identify and/or search for licensed products for sale in Canada by looking for the NPN or DIN-HM on the label and/or Health Canada’s Licensed Natural Health Products Database. In addition to the product license, each NHP sold in Canada requires a site license in order to manufacture, package, label and import for sale, which should comply with the guidance of Good Manufacturing Practices (GMPs) specifically for NHPs. Since Health Canada has not yet evaluated all NHPs currently on the market, products with exemption numbers (EN) can also be sold in Canada. Except for the pre-market assessment, Health Canada also requires product license holders to report any serious adverse reactions and any serious unexpected adverse reaction to their products.

2.1.2. Benefits and Risks of Natural Health Products

NHPs are prevalently employed with drug therapies by consumers to promote health and treat diseases such as cold, inflammation, pain, heart diseases, liver cirrhosis, diabetes and central nervous system diseases. The principal reason for the progressive use of NHPs is mainly due to the belief that they are natural and, therefore, must be safe and nontoxic. While NHPs are generally safe and have few side effects, they are not free of risk. Life-threatening adverse reactions associated with NHPs have been discovered and reported with increasing incidence. Adverse effects can arise from an inherent toxicity of NHPs, manufacturing problems like contamination, incorrect ingredients or dosage levels, adulteration with pharmacologically active synthetic compounds, and interactions with prescription drugs or other NHPs which may lead to the loss of therapeutic efficacy, toxicity or even mortality.

2.1.2.1. Natural Health Product-drug interactions

An important risk of NHP usage is the potential for NHP-drug interactions. Numerous studies have documented the interactions of NHPs with Phase I enzymes, particularly the Cytochrome P450 (P450) enzymes. For instance, St. John’s wort, used worldwide in the
treatment of mild to moderate depression, induces CYP3A4, CYP2E1 as well as CYP2C19 and causes interactions with cyclosporine or antiretrovirals that are substrates of aforementioned P450 enzymes. Kava is another commonly used NHP in Western countries as a result of its anxiolytic and sedative properties. Recently, studies suggest that several kavalactones, the assumed active ingredients of kava extracts, are potent inhibitors of several enzymes of the P450 system (CYP1A2, 2C9, 2C19, 2D6, 3A4 and 4A9/11), which implies kava has a great potential to cause interactions with drugs that are mainly metabolized by the foregoing P450 enzymes.

Apart from the large numbers of P450 enzyme-mediated NHP-drug interactions, emerging evidence has suggested the high potential of Phase II enzymes, principally uridine diphosphoglucuronosyl transferases (UGT), to cause NHP-drug interactions. Valerian and valerian/hops, popular over-the-counter products for the treatment of sleep disturbances or anxiety, have the potential to cause NHP-drug interactions by inhibiting UGT. Significant inhibitory effects on the glucuronidation of acetaminophen, oestradiol, and morphines with both microsomes and expressed UGT in the presence of valerenic acid were observed, and marked reductions were found in UGT1A1 and UGT2B7 activities. Celastrol, a promising anti-tumor agent, exhibited strong inhibitory effects on UGT1A6 and UGT2B7-mediated 4-methylumbelliferone (4-MU) glucuronidation. The low inhibition kinetic parameters (Kᵢ of 0.49 and 0.045 µM for UGT1A6 and 2B7, respectively) indicate the possibility of celastrol-drug and/or celastrol-containing herb-drug interactions at the therapeutic concentration of celastrol for anti-tumor utilization. Other significant inhibitors of UGT enzymes include Epigallocatechin gallate (EGCG) for UGT1A4, milk thistle for both UGT1A6 and UGT1A9, saw palmetto (the extracts of its fruits for the treatment of benign prostatic hyperplasia) for UGT1A6, and cranberry for UGT1A9.

A growing concern exists among both consumers and healthcare practitioners due to the risk of NHP-drug interactions. Hence, the safety assessment of NHPs is of great importance to ensure the safe use of NHPs by consumers. However, the safety assessments of NHPs are not simple due to the compositional diversity of NHPs, their product quality as well as lack of standardization, and limited information is available on the pharmacological data, underlying mechanisms of actions and toxicities. More work is needed on clinical research and toxicity
studies of NHPs to gain a greater understanding of their pharmacokinetics, efficacy and safety data. Moreover, it is the consumer’s responsibility to consult and report the side effects to their healthcare professionals in order to minimize the risks of toxicity or NHP-drug interactions; meanwhile, healthcare professionals should be fully equipped with a thorough knowledge of NHPs to counsel their patients\textsuperscript{16}.

2.1.3. Standardization of Natural Health Products

Since NHPs are exceptionally complicated and contain multiple active phytochemicals with distinct pharmacological efficacy and toxicological effects, the pharmacological studies and clinical trials involving the identification of the active ingredients, mechanisms of actions, NHP-drug interactions and toxicity are very challenging. A principal problem impeding this progress is the absence of standardization of NHPs (both on materials and analytical methods)\textsuperscript{17}. Recognition of the natural variation and proportions of the many active components in the raw material and correct identification of the main components responsible for the desired pharmacological effects are very critical in the standardization process, which ensures consistent quality and adequate levels of the key components for subsequent clinical investigation\textsuperscript{15,17}. Validated methods are vital for the evaluation of pharmacological, toxicological and clinical studies of major ingredients\textsuperscript{17}. Therefore, the standardization of NHPs is crucial to guarantee the reliability, consistency and reproducibility of the ingredients of NHPs for further clinical research.

2.2. Flaxseed Lignans

2.2.1. Flaxseed

Flax (\textit{Linum usitatissimum}) is abundant in fat, protein and dietary fiber. An analysis of brown Canadian flax averaged 41% fat, 20% protein, 28% total dietary fiber, 7.7% moisture and 3.4% ash\textsuperscript{18}. The composition of flax can vary with genetics, growing environment, seed processing and method of analysis\textsuperscript{19}. 
2.2.1.1. The status of flaxseed and lignans as Natural Health Product in Canada

Flax products such as oil, whole seeds or milled seeds are one of the most popular functional foods used by consumers due to the putative health benefits against degenerative diseases such as cardiovascular disease (CVD)\textsuperscript{20,21} and cancer\textsuperscript{22,23}. Various flaxseed oil softgels or capsules and a flax laxative granule have been granted an NPN by Health Canada, and are currently available on the market\textsuperscript{24}. Other flax products, for instance, golden flax seed and high lignan flax oil, can be purchased but are not approved by Health Canada.

Flaxseed is a rich source of soluble fiber, \(\alpha\)-linolenic acid (ALA) and plant lignan secoisolariciresinol diglucoside (SDG), the components of which are proposed to be responsible for the biological effects of flaxseed\textsuperscript{25}. However, researchers show a growing interest in lignans because of the significant health benefits associated with them. Though a large number of epidemiological studies and some \textit{in vitro} models as well as animal studies have shown flaxseed lignans play an important role in the prevention of CVD\textsuperscript{26-28}, diverse cancers\textsuperscript{29-31}, oxidative stress and inflammation\textsuperscript{32,33} and diabetes\textsuperscript{34,35}, flaxseed lignans or the purified lignans such as SDG have not been given an NPN, for clinical trials suggesting safety and efficacy data are insufficient and pharmacological properties (ADME), toxicities as well as drug interactions are not adequately studied.

2.2.2. Dietary Lignans

According to the International Union of Pure and Applied Chemistry (IUPAC), lignans are a large group of natural products derived from cinnamic acid residues, which are characterized by the coupling of two \(\text{C}_6\text{C}_3\) units as propylbenzene based on nomenclature purposes\textsuperscript{36} (Figure 2.1). Dietary plant lignans exist substantially in foods such as oilseeds, cereal grains, legumes, fruits, vegetables and beverages, but ground flaxseed is the richest source of the plant lignan secoisolariciresinol diglucoside (SDG), with pinoresinol (PINO) and matairesinol (MAT) as minor lignan components\textsuperscript{37-40}. In Canada, secoisolariciresinol (SECO), MAT, PINO, and lariciresinol (LARI) content in 121 foods were analyzed and data were presented on as is (wet) basis per 100 g and per serving\textsuperscript{40}. Based on the study, the richest sources of lignans were flaxseed (379 mg/100 g) and sesame seed (8 mg/100 g), followed by
cereals and breads (0.002-7.2 mg/100 g), legumes (0.002-1 mg/100 g), vegetables (0.001-0.6 mg/100 g), fruits (0.002-0.4 mg/100g), and nonalcoholic and alcoholic beverages (0.9-37.3 µg/100 g)\textsuperscript{40}.

![Cinnamic acid](image1.png)

\textbf{Cinnamic acid}

\textbf{C}_6\textbf{C}_3\text{ unit, propylbenzene}

Figure 2.1 The structural makeup of lignans

\textbf{2.2.3. Production of Enterolignans}

Following administration, dietary lignans undergo transformation by the intestinal bacteria before being absorbed into the systemic circulation. As the major plant precursor in flaxseed, the SDG complex and then SDG undergo hydrolysis in the gastrointestinal tract (GIT) to yield the aglycone plant lignan SECO. SECO then undergoes further metabolism to the mammalian lignans, ED and EL, and ED can be oxidized to form EL\textsuperscript{29,41}. As the minor lignan components in flaxseed, MAT, PINO and LARI can also be converted to EL in the GIT through one and several additional steps, respectively\textsuperscript{42} (Figure 2.2). A growing body of evidence suggests that SDG and its metabolites, in particular EL, may exert protective effects against cancers such as prostate, breast and colon cancer\textsuperscript{22,31,43,44}, CVD\textsuperscript{26,28}, inflammation\textsuperscript{45}, and oxidative damage through diverse mechanisms including phytoestrogenic\textsuperscript{46} and antioxidant effects\textsuperscript{47} from both \textit{in vitro} and \textit{in vivo} studies. Epidemiological studies also suggest that the lower risk of the aforementioned chronic diseases is greatly attributed to high circulating concentrations of EL\textsuperscript{48-51}. Hence, as the promising bioactive form of lignan, the health benefits, mechanisms of the biological effects as well as the pharmacokinetic properties of EL will be discussed below.
Figure 2.2 Pathway of the conversion from plant lignans to mammalian lignans ED and EL
2.3. **EL-Health Benefits**

Compelling data from epidemiological, experimental and clinical studies, suggests that lignans, particularly EL, are promising in the prevention of chronic diseases, although the results concerning the health benefits of lignans, especially from epidemiological studies, are not completely accordant due to the distinct diet habits in populations, varying lignan product quality and intake levels used for the research, lack of flaxseed product standardization and valid analytical methods and different experimental designs\(^52\).

2.3.1. **Epidemiological Studies**

Epidemiological studies have suggested the promising anti-cancer effects by large lignan consumption and/or high circulating EL levels in the plasma, although some controversial studies exist (Table 2.1). Many studies suggest high intake of lignan-rich foods, which contain plant precursors such as SECO, MAT, LARI, is significantly associated with reduction of breast cancer\(^53\)-\(^58\), while some studies are in opposition to this viewpoint\(^59\)-\(^61\). By measuring serum or urinary excretion of enterolignans, especially EL, numerous studies demonstrate high plasma concentrations (54 nM)/urinary excretion levels of EL are inversely correlated with breast\(^31\),\(^48\),\(^49\), colon\(^50\) and prostate cancer\(^51\) risk. However, Kilkkinen and coworkers conducted a nested case-control study in Finland, and their findings were against the hypothesis that high serum EL concentrations were associated with reduced breast cancer risk\(^62\). Tonkelaar and coworkers also found that higher urinary EL excretion was weakly and nonsignificantly associated with an increased breast cancer risk, but this study included a small number of cases (88)\(^63\). In another population-based case-referent study in Sweden, Hultén showed that very low plasma level of EL (2.9 nM) was associated with an increased breast cancer risk in all three cohorts, while very high EL plasma levels (58.2 nM) were also associated with an increased breast cancer risk in two cohorts\(^64\). The conflicting epidemiological results are in part due to inadequate databases used in intake estimation, dietary habits, lifestyles and lignan-converting bacteria in different populations\(^40\). It is notable that a clinical trial by Thompson and coworkers showed the potential to reduce tumor growth in patients with breast cancer supplemented with flaxseed, the richest dietary source of lignans\(^23\).
2.3.2. Experimental Animal Studies

Compared with epidemiological studies, the experimental results from animal models regarding health benefits of lignans are more consistent. In an early study, dietary supplementation with high intake of flaxseed (5%) or SDG reduced N-methyl-N-nitrosourea (MNU)-induced mammary tumor size and number in rats. Exposure to a diet with 10% flaxseed or SDG (equivalent to the amount in 10% flaxseed) during suckling suppressed 9,10-dimethyl-1, 2-benzanthracene (DMBA)-induced rat mammary tumorigenesis and enhanced mammary gland differentiation, suggesting that exposure to lignans at early stage of mammary gland development reduces susceptibility to mammary carcinogenesis later in life. Supplementation with 2.5 or 5% flaxseed or 2.5 or 5% defatted flaxseed increased cecal β-glucuronidase activity and significantly reduced the number of aberrant crypts per focus in the distal colon of rats, which implies the colon cancer protective effect of flaxseed may be associated with increased β-glucuronidase activity. In addition, a series of studies by Prasad and coworkers have demonstrated both flax lignan complex and SDG have beneficial effects in protecting against atherosclerosis.
<table>
<thead>
<tr>
<th>Study</th>
<th>Design and Location</th>
<th>Population (No. Cases/Total)</th>
<th>Lignan Studied</th>
<th>Adjusted factors Comments</th>
<th>OR, 95 CI and P</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pietinen 2001</td>
<td>Population based case-control study Finland</td>
<td>All women (194/402); Premenopausal (68/143); Postmenopausal (126/259)</td>
<td>Serum EL; EL concentration in the lowest quintile was 3.0 vs. 54.0 nM in the highest quintile</td>
<td>Age, area, age at menarche, age at first full-term pregnancy, OC, HRT, first-degree family history of BC, history of BBD, education, alcohol intake, smoking, physical activity, WHR, BMI</td>
<td>0.38 (0.18-0.77) P=0.03</td>
<td>Serum EL level was significantly inverse associated with BCR among both premenopausal and postmenopausal women</td>
</tr>
<tr>
<td>Linseisen 2004</td>
<td>Population based, case-control Germany</td>
<td>Premenopausal (278/944)</td>
<td>Plant lignans; SECO conc. in the lowest quintile was 275.2 vs. 1409.1 µg/day. MAT conc. in the lowest quintile was 19.5 vs. 37.6 µg/day. Enterolignans; ED conc. in the lowest quintile was 235.2 vs. 554.9 µg/day. EL conc. in the lowest quintile was 226.9 vs. 445.7 µg/day.</td>
<td>Family history of breast cancer, number of births, duration of breast feeding, total-energy intake, BMI, alcohol, education. FFQ</td>
<td>MAT 0.58 (0.37-0.94) ED 0.61 (0.39-0.98) EL 0.57 (0.35-0.92)</td>
<td>BCR significantly decreased with a high intake of the plant lignan MAT but not SECO or the sum of plant lignans. However, both estimated enterolignans (ED and EL) were inversely associated with BCR</td>
</tr>
<tr>
<td>McCann 2004</td>
<td>Population based, case-control US</td>
<td>All (1122/3158); Premenopausal (315/908); Postmenopausal (807/2250)</td>
<td>Plant lignans (sum of SECO and MAT) Lignan precursors conc. in the lowest quintile was &lt;329 vs. &gt;673 µg/day. Lignan precursors conc. in the lowest quintile was &lt;337 vs. &gt;713 µg/day.</td>
<td>Age, education, race, BMI, age at menarche, parity, age at first birth, history of BBD, family history of BC, smoking, total-energy intake. FFQ</td>
<td>0.66 (0.44-0.98) 0.93 (0.71-1.22)</td>
<td>Premenopausal women in the highest quartile of dietary lignan intake had reduced BCR, while no association was observed between lignan intakes and postmenopausal breast cancer.</td>
</tr>
</tbody>
</table>
| Study     | Design and Location | Population (No. Cases/Total) | Lignan Studied                                                                 | Adjusted factors Comments | OR, 95 CI and P | Results                                                                。
|-----------|----------------------|------------------------------|-------------------------------------------------------------------------------|---------------------------|---------------|-------------------------------------------------------------------------。
<p>| Cotterchio 2008 | Population based case-control study Canada | All (3063/6433); Premenopausal (930/2141); Postmenopausal (2067/4221) | Plant lignans (sum of SECO, PINO, LARI and MAT) Lignan precursors conc. in the lowest quintile was &lt;256 vs. &gt;5355 µg/day. Lignan precursors conc. in the lowest quintile was &lt;256 vs. &gt;5355 µg/day. Lignan precursors conc. in the lowest quintile was &lt;256 vs. &gt;5355 µg/day. | Age, family history of breast cancer, history of BBD, dietary fiber intake, age at first live birth, duration HRT (only for postmenopausal women) | 0.81 (0.65-0.99) 0.81 (0.58-1.12) 0.82 (0.63-1.07) | Lignan intake may be associated with reduced BCR among premenopausal women, and the data suggested BMI modifies this association. |
| McCann 2002 | Population-based case-control study US | Premenopausal (301/617); Postmenopausal (439/933) | Enterolignans; Enterolignans conc. in the lowest quintile was &lt;0.5 vs. &gt;0.69 mg/d in the highest quintile | Age, education, parity, BMI, history of BBD, family history of BC, age at menarche, total-energy intake FFQ | 0.49 (0.32-0.75) 0.72 (0.51-1.02) | Women in the highest tertile of dietary lignans have reduced BCR in both pre- and postmenopausal women |
| Ingram 1997 | Hospital-based case-control study Australia | All (144) | Urinary enterolignans; ED levels in the cases were 282.0 vs. 316.5 nM/24 h. EL levels in the cases were 1973.4 vs. 3097.7 nM/24 h in the controls. | Age at menarche, parity, alcohol intake and total fat intake | EL: 0.36 (0.15-0.86) P=0.013 | High excretion of EL was significantly associated with a substantial reduction in BCR |</p>
<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>49 Dai 2002</td>
<td>Population-based case-control study China</td>
<td>All (250/500); Premenopausal (132/264); Postmenopausal (118/236)</td>
<td>Urinary enterolignans; Median excretion rate of total lignans in cases was 1.77 vs. 4.16 nM/mg creatinine in controls</td>
<td>Age at first live birth, ever diagnosed with fibroadenoma, total meat intake and physical activity level</td>
<td>0.4 (0.24-0.64) P&lt;0.01</td>
<td>Urinary excretion of total enterolignans was substantially lower in breast cancer cases</td>
</tr>
<tr>
<td>50 Kuijsten 2006</td>
<td>Population-based case-control study Dutch</td>
<td>All (532/1035)</td>
<td>Plasma Enterolignans; ED conc. in the lowest quintile was &lt;0.7 vs. &gt;3.1 nM in the highest quintile. EL conc. in the lowest quintile was &lt;4.6 vs. 26.3 nM in the highest quintile</td>
<td>Age, sex, alcohol, nonsteroidal anti-inflammatory drug use, body mass index, physical activity, smoking, family history of colorectal cancer, antibiotic use and indication for endoscopy</td>
<td>ED: P=0.01 EL: P=0.05</td>
<td>A substantial reduction in colorectal adenoma risk among subjects with high plasma concentrations of enterolignans was observed.</td>
</tr>
<tr>
<td>51 Hedelin 2006</td>
<td>Population-based case-control study Sweden</td>
<td>All (1499/2629)</td>
<td>Serum EL; EL conc. in the lowest quintile was 9.1 vs. 55.4 nM in the highest quintile</td>
<td>Age, intake of antibiotics, zinc, animal fat, vegetable fat, vitamin A and protein during the last year and level of education</td>
<td>0.74 (0.41-1.32)</td>
<td>Intermediate serum levels of EL were associated with decreased PCR</td>
</tr>
</tbody>
</table>
The findings were against the hypothesis that high serum EL concentration was associated with reduced BCR. Higher urinary EL excretion was weakly and nonsignificantly associated with an increased BCR.

Very low plasma level of EL (2.9 nM) was associated with an increased BCR in all three cohorts, while very high plasma level (58.2 nM) was also associated with an increased BCR in two cohorts.

<table>
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<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>62Kilkkinen 2004</td>
<td>Cross-sectional population surveys nested case-control study</td>
<td>All (206/421) Premenopausal (69/unknown); Postmenopausal (137/unknown)</td>
<td>Serum EL; Mean EL conc. was 25.2 vs. 24.9 nM between cases and controls</td>
<td>Alcohol, smoking, education, BMI, physical activity</td>
<td>1.3 (0.73-2.31) 0.73 (0.34-1.59) 1.22 (0.69-2.16)</td>
<td>The findings were against the hypothesis that high serum EL concentration was associated with reduced BCR.</td>
</tr>
<tr>
<td>63-den Tonkelaar 2001</td>
<td>Prospective cohort, nested case-control study</td>
<td>Postmenopausal (88/356)</td>
<td>Urinary EL; EL conc. in the lowest quintile was 235.6 vs. 969.9 µM/M creatinine in the highest quintile</td>
<td>BMI, age at menarche, age at first birth, age at menopause, batch</td>
<td>1.43 (0.79-2.59) P=0.25</td>
<td>Higher urinary EL excretion was weakly and nonsignificantly associated with an increased BCR</td>
</tr>
<tr>
<td>64-Hultén 2002</td>
<td>Population-based case-referent study</td>
<td>All (248/740)</td>
<td>Plasma EL; Mean EL conc. in the low quintile was 2.9 nM vs. 58.2 nM in the highest quintile</td>
<td>Smoking, BMI, menopausal status</td>
<td>1.10 (0.7-1.7)</td>
<td>Very low plasma level of EL (2.9 nM) was associated with an increased BCR in all three cohorts, while very high plasma level (58.2 nM) was also associated with an increased BCR in two cohorts</td>
</tr>
</tbody>
</table>
2.4. **EL-Mechanisms for Bioactivities**

The underlying mechanisms responsible for the bioactivities of either naturally occurring lignans or their metabolites generally pertain to the antioxidant activity, the influence of lignans on hormone mechanisms, the estrogenic and anti-estrogenic effects, and an impact on gene expression and/or enzyme activity\(^{69}\). Based on numerous studies, the enterolignans, particularly EL, essentially account for these functions.

2.4.1. **Antioxidant Activity**

Oxidant by-products reactive oxygen species (ROS) such as superoxide (\(O_2^-\)), hydrogen peroxide (\(H_2O_2\)), and hydroxyl radical (\(\cdot OH\)) of normal metabolism often cause oxidative damage to DNA, proteins and lipids which give rise to degenerative diseases of aging such as cancers, CVD, immune-system decline and brain dysfunctions\(^{70}\). A number of studies provide evidence that plant lignans are capable of enhancing antioxidant defense mechanisms\(^{33,47,71-73}\). The antioxidant activities of the flaxseed lignan, SDG, and its mammalian lignans, enterodiol (ED) and enterolactone (EL) were evaluated in both lipid and aqueous in vitro model systems\(^ {33}\). All three lignans significantly (\(p \leq 0.05\)) inhibited linoleic acid peroxidation at both 10 and 100 µM and DNA scissions in a concentration-dependent manner. In addition, SDG demonstrated the weakest activity at scavenging \(\cdot OH\) compared to ED and EL at both 10 and 100 µM\(^ {33}\). A study comparing the antioxidant activity of SECO, ED and EL against SDG and vitamin E showed that the antioxidant activity was highest with SECO and ED and lowest with vitamin E, and SECO, ED, and EL were respectively 3.82, 3.95, and 3.43 more potent than SDG, which may suggest the effectiveness of SDG in hypercholesterolemic atherosclerosis, diabetes, and endotoxic shock is generally due to its metabolites\(^ {73}\).

2.4.2. **Estrogenic and Anti-estrogenic Effects**

The steroid hormone estrogen influences the growth, differentiation, and functioning of many target tissues including female and male reproductive systems such as mammary gland, uterus, vagina, ovary, testes, epididymis and prostate\(^ {74,75}\). Given the widespread role for estrogen in human physiology, estrogen is involved in the development or progression of
many diseases such as cancer (breast, ovarian, colorectal, prostate, endometrial), osteoporosis, neurodegenerative diseases, CVD and obesity, in some of which estrogen mediates its effects through binding the estrogen receptor (ER)\textsuperscript{76}. ER, existing in 2 main forms ER\textalpha{} and ER\textbeta{}, has distinct tissue expression patterns in both humans and rodents\textsuperscript{77}. ER\textalpha{} and ER\textbeta{} have high sequence homologies in the DNA binding domain (> 90\%) and in the ligand binding domain (~60\%)\textsuperscript{78}. ER contains two transcriptional activation domains: the autonomous transcriptional activation domain AF-1, which is located at the N-terminus, and the lignan-dependent activation domain AF-2, located at the C-terminus; however, the primary sequence of AF-2 differs significantly between ER\textalpha{} and ER\textbeta{}. This gives rise to different agonist/antagonist features of various chemicals\textsuperscript{69}.

Xenobiotics such as environmental or industrial chemicals and phytoestrogens that can interfere with the hormonal or endocrine system can mimic estrogen by binding to ER and induce (agonize) steroid response to cause estrogenic effects, or inhibit (antagonize) to prevent estrogenic activity, resulting in antiestrogenic effects\textsuperscript{79,80}. The estrogenic or antiestrogenic effects at the cellular and molecular level are affected by many factors such as concentration dependency, receptor status, presence or absence of endogenous estrogens, and the type of target organ or cell\textsuperscript{81}.

Lignans have received considerable attention as a source for phytoestrogens. Based on a study examining the mechanism to interfere with ER by which enterolignans exert estrogenic and/or antiestrogenic effects, it indicated that enterolignans had distinct properties for transactivation of ER\textalpha{} and ER\textbeta{}. ED, as estradiol (E2), induced ER\textalpha{} transcriptional activation through transactivation functions AF-1 and AF-2, while EL was less efficient in inducing AF-1 but acted predominantly through AF-2\textsuperscript{82}.

The estrogenic effects of enterolignans were evaluated by some studies. In an early study, Sathyamoorphy found that EL at 1 \textmu{}M was able to produce a weak estrogenic response, while ED at 1 \textmu{}M was inactive. In addition, MCF-7 cell proliferation in the presence of 1 \textmu{}M EL was 92\% compared with cells treated with 0.1 nM E2, whereas the addition of ED at the same concentration did not show growth stimulation\textsuperscript{83}. In another study, E2 (1 nM) and EL (0.5-2 \textmu{}M) separately stimulated the proliferation of MCF-7 cells, but their combination had no stimulatory effect compared to control\textsuperscript{84}, which might result from the competition between EL
and E2 on binding to the ER.

An antiestrogenic effect was also observed by some studies. Saarinen proposed that EL potently down regulated E2-stimulated angiogenic factors derived both from the stroma and the cancer cells. Higher concentrations above 10 µM of EL inhibited MCF-7 cells significantly. EL was evaluated for its effects on DNA synthesis in MCF-7 cells. Based on the analysis, EL was found to have a biphasic effect on DNA synthesis, exhibiting induction at 10-50 µM (with a peak value of 210% at 10 µM) and inhibition at high concentrations, with an IC₅₀ (half maximal inhibitory concentration) of 82.0 µM.

According to the above analysis, EL is likely essential for the estrogenic and antiestrogenic activities of flaxseed, suggesting the significance of the transformation from plant lignans to enterolignans by the intestinal bacteria. However, the serum concentration of EL in humans is normally < 10 µM and most often < 1 µM. Therefore, a stimulatory effect on cell growth rather than inhibition is more likely to happen. The agonistic properties of EL might apply for conventional hormone replacement therapy in postmenopausal women. Nevertheless, the potential cell growth simulation and the ability to induce estrogen-responsive genes of EL need to be paid particular attention when used on hormone-dependent breast cancer patients.

2.4.3. Binding to Some Proteins (SHBG and SBP)

Sex hormone binding globulin (SHBG) and sex steroid binding protein (SBP), glycoproteins in human plasma, have high affinity towards endogenous estrogens. Lignans are proposed to potentially increase plasma SHBG and SBP levels, which then bind a large proportion of endogenous estrogens, thus decrease the free plasma estrogen and testosterone levels available to the cancer cells. One study evaluated the correlation between intake of various fibers, lignan excretion and plasma levels of estrogens, free testosterone and SHBG in women and demonstrated a significant positive correlation between fiber intake and urinary excretion of lignans, and the concentration of plasma SHBG. In addition, EL excretion correlated negatively with plasma percentage free estradiol and testosterone suggesting that fiber-rich foods containing lignans might stimulate SHBG and in this way to reduce levels of...
free hormone in plasma. In an in vitro study EL stimulated the synthesis of SHBG by HepG2 liver cancer cells in culture acting synergistically with estradiol, which implied that lignans may affect uptake and metabolism of sex hormones by participating in the regulation of plasma SHBG levels. Furthermore, Martin and coworkers studied the interactions of SBP and the lignans EL, ED, Nordihydroguaiaretic (NDGA) as well as isoflavonoid phytoestrogens (equol, diazein, and genistein). This researcher found that the phytoestrogens had different dose-dependent inhibitory effects on steroid binding by SBP, with relative efficiencies $\text{EL} \geq \text{NDGA}=\text{equol}>\text{genistein}$ for displacing E2, but ED and diazein were much less active.

2.4.4. Influence on Enzyme Activity

The mammalian lignans, especially EL, can inhibit several steroid metabolizing enzymes including aromatase, 5α-reductase, 7α-hydroxylase and 17β-hydroxysteroid dehydrogenase (17β-HSD). Aromatase, also named estrogen synthetase, is a cytochrome P450 enzyme responsible for catalyzing the conversion of androgens to estrogens. EL was a moderate inhibitor of aromatase, while ED was a relative weak inhibitor in a human preadipose cell culture system. Such activity suggests that the high concentration of lignans in vegetarians, via inhibiting aromatase in peripheral and/or cancer cells and lowering estrogen levels, may play a protective role against estrogen-dependent cancers.

The inhibition of 5α-reductase and 17β-HSD by lignans in human genital skin fibroblast monolayers and homogenates, and in benign prostatic hyperplasia tissue homogenates was reported. EL inhibited both 5α-reductase and 17β-HSD in genital skin fibroblasts, and EL was the most potent inhibitor of 5α-reductase. EL inhibited E2 production via inhibition of the 17β-HSD type 1 pathway. Additionally, the reduction in E2 production by EL was significantly related to a reduction in MCF-7 cell proliferation.

2.5. Pharmacokinetics of Plant and Mammalian lignans

The pharmacokinetic properties of plant as well as mammalian lignans are rather complex and not thoroughly characterized. This section will summarize the absorption,
metabolism, distribution and excretion of lignans, with emphasis on the conjugative glucuronidation of EL.

2.5.1. Absorption

2.5.1.1. The absorbable form of lignans

An oligomer composed of five SDG residues interconnected by four 3-hydroxy-3-methyl glutaric acid (HMGA) residues is identified as the main lignan of flaxseed\textsuperscript{95}. SDG oligomers in the flaxseed extract are often hydrolyzed to break the ester linkages for the release of SDG and the glycosidic bonds for the release of SECO\textsuperscript{96}. No studies specifically investigated whether SDG or the aglycone SECO is the absorbable form of lignans. Nevertheless, some indirect evidence suggests that the aglycone form SECO is primarily absorbed. Caco-2 cells derived from human colon adenocarcinoma were used as a model to investigate the bidirectional permeation (100 µM SDG, SECO, ED and EL) in our lab (manuscript under review). The results showed that the apical-to-basolateral and basolateral-to-apical permeation rates for SDG could not be calculated as SDG levels were below 50 ng/mL (0.0728 µM) under the level of quantification of our developed HPLC assay. However, permeation of SECO, ED and EL occurred in both of the apical-to-basolateral as well as basolateral-to-apical directions.

In another study, the uptake of EL and ED by human colon epithelial cells was observed as the intracellular levels of conjugated EL and ED in HT29 cells rose immediately after starting the exposure, suggesting EL and ED are likely to permeate the colon epithelial cells and undergo phase II metabolism\textsuperscript{97}.

Most of the lignan metabolites other than SDG were found in their conjugated forms in the portal vein after oral administration of flaxseed in rats\textsuperscript{98}. An investigation showed that only in 5% of the 150 diphenolic fractions extracted from the urine of women plant lignans including LARI, isolariciresinol, MAT and SECO were identified, but SDG was not found in the urine\textsuperscript{99}. When female rats were given a gavage of \textsuperscript{3}H-SDG (3.7 kBq/g body), ED, EL and SECO accounted for 75-80% of urine radioactivity, while SDG was not detected in the urine\textsuperscript{100}. The aforementioned data suggest that no measurable SDG levels in plasma and/or urine are found after flaxseed or pure SDG supplementation. Additionally, several distinct
forms of aglycone were found after anaerobic incubation of SDG with a human fecal suspension, which demonstrates intestinal microflora is capable of transforming SDG into other aglycone forms\textsuperscript{101}. Therefore, SDG is converted to its aglycone form, SECO, in the small intestine, which is subsequently absorbed or undergoes further conversion to the enterolignans.

2.5.1.2. Pharmacokinetic parameters of lignans

A pharmacokinetic study in rats following a single oral bolus dose of pure lignans (SDG, SECO, ED and EL was 40, 40, 10 and 5 mg/kg, respectively) and an intravenous bolus dose of lignans (SDG, SECO, ED and EL was 20, 20, 5 and 1 mg/kg, respectively) indicated that the serum concentration of SDG following oral administration was below the limit of quantification (0.0728 µM) at all time points, whereas SDG level following intravenous dosing was detectable up to 4 h (manuscript under review). The concentrations of SECO in intravenous and oral serum samples were detected up to 8 h and 4 h, respectively. The oral pharmacokinetic profile of SECO exhibited a very rapid absorption such that $C_{\text{max}}$ (the maximum drug concentration in the blood) was achieved at 5 min. Quantifiable levels of ED were obtained up to 4 h post dose following both oral and intravenous administration. Besides, SECO exhibited the highest bioavailability of ~25% in rats while SDG and ED showed poor oral bioavailability. Another pharmacokinetic analysis of SECO was performed in rats following a bolus intravenous injection (SECO was 20 mg/kg)\textsuperscript{102}. The systemic clearance ($Cl_s$) for SECO was $7.3 \pm 1.1 \text{ L/h kg}$, elimination rate constant (k) was $0.15 \pm 0.01 \text{h}^{-1}$ and half life ($t_{1/2}$) was $4.5 \pm 0.4 \text{ h}$. Volume of distribution ($V_d$) for SECO was $47.4 \pm 10.9 \text{ L/kg}$ and area under the curve (AUC$_{0-\infty}$) was $2.79 \pm 0.41 \text {h*\mu g/mL}$.\textsuperscript{102}

In twelve healthy men and women enterolignans appeared in plasma 8-10 h after ingestion of a single dose of purified SDG (1.31 µM/kg body wt.)\textsuperscript{103}. ED reached its maximum plasma concentration 14.8 \pm 5.1 h after ingestion of SDG, whereas EL reached its maximum 19.7 \pm 6.2 h after ingestion. The mean elimination half-life of ED (4.4 \pm 1.3 h) was shorter than that of EL (12.6 \pm 5.6 h). The mean residence time for ED was 20.6 \pm 5.9 h and that for EL was 35.8 \pm 10.6 h\textsuperscript{103}. In another seven volunteers EL plasma levels and urinary
excretion were measured after the administration of a single dose of strawberries (500 g) equivalent to 11.7 mg of SECO and 0.61 mg of MAT\textsuperscript{104}. EL appeared in plasma 8 h after the post meal, and reached the maximum at 24 h. Maximum EL excretion was observed in 25-36 h urine samples\textsuperscript{104}.

2.5.1.3. Conversion of lignans in human gastrointestinal tract

\textit{In vitro} conversion of plant lignans by human fecal flora\textsuperscript{101,105,106}, germ-free rats\textsuperscript{98} and humans taking antimicrobials\textsuperscript{107} demonstrates intestinal bacteria play a crucial role in the production of enterolignans. Intestinal bacteria metabolize plant lignans by a series of processes including deglycosylation, ring cleavage, demethylation, dehydroxylation and oxidation\textsuperscript{69,101}. Strains of \textit{Bacteroides} and \textit{Clostridium} genera deglycosylate SDG to form SECO\textsuperscript{108}. It is suggested that the conversion occurs between terminal ileum and caecum in pigs\textsuperscript{109}. Similarly, Eeckhaut and his coworkers proposed SDG was only hydrolyzed into SECO through microbial action in the ascending colon\textsuperscript{110}, which is supported by an early study in eight ileostomy patients having low plasma and urine lignan levels\textsuperscript{111}. Subsequently, SECO is partially absorbed into systemic circulation and/or is biotransformed into mammalian lignans\textsuperscript{112,113}. SECO undergoes dehydroxylation and demethylation catalyzing by strains of \textit{Ruminococcus} products and \textit{Eggerthella lenta} to yield ED, which can be further oxidized to form EL by \textit{Lactonifactor longoviformis}\textsuperscript{108}. As the minor lignan components in flaxseed, MAT, PINO and LARI can also be converted to EL by the bacterial flora through one and several additional steps, respectively\textsuperscript{42}. Figure 2.3 outlines the bioconversion from plant lignans to mammalian lignans by the intestinal microflora in the gastrointestinal tract. Apparently, diverse bacteria communities are favorable to the transformation of plant lignans to mammalian lignans; thus, the variation of intestinal bacterial may result in large inter-individual difference on EL levels.
Figure 2.3 Diagram of the intestinal microflora mediated conversion of lignans in human gastrointestinal tract.
2.5.2. Metabolism

Once mammalian lignans are absorbed, they undergo primarily phase II metabolism prior to systemic circulation, with the predominant formation of conjugative metabolites, particularly EL glucuronides.\(^{97,114,115}\)

2.5.2.1. Oxidative metabolism

Oxidative metabolism of enterolignans is principally mediated by hepatic P450 enzymes. Human and rat hepatic microsomes biotransform EL to oxidative metabolites with an additional hydroxyl group either at the aromatic or aliphatic moiety.\(^{116}\) A study evaluating the metabolic fate of EL and ED by ingesting flaxseed to humans showed that monohydroxylated EL and ED metabolites were identified at the para- and ortho-positions on either aromatic ring, which indicated the lignan was oxidatively metabolized \textit{in vivo}.\(^{117}\) This is in agreement with the results found in rats where several hydroxylated metabolites of EL and ED were found in bile and urine after feeding a diet containing 5% flaxseed\(^{114}\) (Figure 2.4 and Figure 2.5). Nevertheless, the phase I metabolites of enterolignans represent a minor percent when compared with phase II metabolites.\(^{115,117}\)

Figure 2.4 Oxidative metabolites of ED (Adapted from Alister D. Muir\(^{118}\))

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2.5.2.2. Conjugative metabolism

2.5.2.2.1. UDP-glucuronosyltransferases

The UDP-glucuronosyltransferases (UGT) are a superfamily of endoplasmic reticulum membrane-bound enzymes, utilizing UDP-glucuronic acid as a cosubstrate for the formation of glucuronides from substrates such as steroids, bile acids, hormones and thousands of xenobiotics that include drugs, environmental toxicants and carcinogens, the process of which is known as glucuronidation\(^\text{119}\). Glucuronidation transforms lipophilic substrates into hydrophilic glucuronides, more water-soluble compounds that can be subsequently eliminated through the bile and urine. Thus, the glucuronidation pathway is primarily regarded as a detoxification reaction\(^\text{120}\). The highest UGT activities frequently occur in the microsomal fractions extracted from the liver, kidney and intestine, and various factors such as age, gender, hormonal status, genetic factors as well as environmental exposures will exert great influence.
on the exact composition of UGT in tissues\textsuperscript{120}.

In humans, four UGT families have been identified including UGT1, UGT2, UGT3, UGT8, in which UGT1 and UGT2 mainly use UDP-glucuronic acid to glucuronidate endogenous substances and xenobiotics including lignans, flavonoids and isoflavones that are not accessible for UGT3 and UGT8 families\textsuperscript{121}. Up to now, UGT1 family includes 9 functional isoforms in humans, and 7 in rat; UGT2 include 6 members in humans and 7 in rat\textsuperscript{119-121}. Since the expression of UGT in the same and/or different organs between humans and rats is different, species difference in glucuronidation can be expected.

2.5.2.2. Enterolignan glucuronidation

ED and EL undergo hepatic phase II reactions, primarily glucuronidation, with minor contributions to phase II metabolism by sulfotransferases\textsuperscript{122,123}. ED and EL were incubated with rabbit liver microsomal UGT and the lignan glucuronides were purified. The monoglucuronide conjugates of each lignan were confirmed, which indicated the hepatic microsomal UGT system conjugated EL to either phenolic hydroxyls and ED to either non-phenolic or phenolic hydroxyl groups\textsuperscript{124} (Figure 2.6). Dean and coworkers also substantiated that EL underwent extensive glucuronidation with rhesus liver microsomes to form O-glucuronides at both phenolic hydroxyl groups\textsuperscript{115} (Figure 2.7). Additionally, incubations of EL with human and rhesus hepatocytes resulted in a large percent of glucuronic acid conjugates, with minor amounts of the sulfate conjugates and monohydroxylated products\textsuperscript{115}.

In addition to the hepatic glucuronidation, several studies have demonstrated the intestinal glucuronidation of enterolignans. Human colon epithelial cells (HT29 and Caco-2) can take up, metabolize and excrete the enterolignans EL and ED, and these cell lines produce conjugation products, which exist as EL monoglucuronide, EL sulfate and ED glucuronide. Moreover, more than 90\% of the EL in the medium was present in its conjugated form within 10 h, however in vivo a significant amount of EL is probably conjugated by the intestinal cells before entering the circulation\textsuperscript{97}. The extent of glucuronidation and sulfation of lignans (SDG, SECO, ED and EL) in the Caco-2 monolayer was also assessed in our own lab (manuscript...
under review). Limited conjugation of SDG was observed up to 48 h of incubation with Caco-2 cells, but SECO, ED and EL exhibited significant conjugation. Hydrolysis of cell lysate and supernatant media with β-glucuronidase/sulfatase enzyme showed less than 3% SDG conjugation. SECO and ED presented 95% and 90% conjugation, respectively, while EL was completely conjugated within 8 h. This implies that lignans undergo extensive and significant first-pass metabolism prior to systemic circulation.

Subsequently, conjugated lignans are excreted back to the digestive tract via bile, reabsorbed from the intestine, and eventually are excreted in the urine as glucuronide conjugates, a process known as enterohepatic circulation \(^{98}\) (Figure 2.8). This process enhances the exposure of enterolignans to intestine and liver, resulting in further substantial conjugation reactions.

Most of the ED and EL detected in the portal vein of rats were present principally as glucuronide conjugates \(^{98}\). In a plasma analysis of enterolignans in 27 pre- and postmenopausal women approximately 80% total enterolignans were present as monoglucuronides, with minor a percent of diglucuronides and sulfoglucuronides \(^{123}\). The excretion of enterolignans in urine and bile was largely as glucuronides, while mono- and disulphates were also found \(^{98}\). In human urine, 92% of ED and 98% of EL in the glucuronide fraction were observed, in which 84.5% monoglucuronide of ED and 94% monoglucuronide of EL occurred in the glucuronide fractions \(^{125}\).

Thus, most of the absorbed dose of lignans predominantly exists as monoglucuronide conjugates. These findings raise some important questions regarding the bioactive lignan form, i.e. whether the plant lignans or their conjugates, or the mammalian lignans or their conjugates mediate the positive health benefits of flaxseed consumption. Traditionally, glucuronidation is considered as a reaction that results in the loss of pharmacological activity of xenobiotics and rapid excretion. However, several glucuronide conjugates are known to possess biological effects. For instance, a widely used opioid analgesic 6-O-glucuronide of morphine contributes to the pharmacological activity in addition to the parent compounds \(^{120}\). Given the high concentrations of enterolignans glucuronides, the pharmacological activity of these glucuronides needs further study.
Figure 2.6 Conjugative glucuronidation metabolites of ED

Figure 2.7 Conjugative glucuronidation metabolites of EL
2.5.3. Distribution

Despite the biological forms of lignans that are beneficial to human health, the efficient delivery of drugs from the site of administration to the target organs, tissues or receptors is required. However, little is known about the lignan distribution and the concentrations in the tissues, which are essential to understand their pharmacological effects and underlying mechanisms of pharmacological action. Some general information observed in tissue distribution studies is presented below.

In rats, a dose-related increase in the tissue distribution of ED and EL was observed in rat liver, testes, prostate and lung after administration of SDG, where the liver and prostate achieved the highest concentrations of ED (1.844 and 1.899 pmol/mg, respectively) of all tissues examined at the 60 mg/kg SDG, a significant increase over the levels obtained in the controls and 15 mg/kg dose group\textsuperscript{127}. In addition, lung exhibited a significant 2.8-fold increase...
in EL concentration with the dosage of SDG increase from 15 to 60 mg/kg\(^{127}\). Lignans, but only in the form of EL, were also detected in the tissue of colon, liver, breast and brain at a much higher level with rye than wheat when high-fiber wheat and high-fiber rye bread administered to pigs for 58-67 d\(^{112}\). Tissue accumulation of lignans in human prostate and/or breast cyst fluids was observed in several studies, suggesting an anticarcinogenic effect towards prostate and breast cancer. In the semen of 6 men, EL existed in both conjugated and unconjugated forms at the mean concentration of 183 nM, which was much greater than 73 nM in the blood\(^{128}\). In the semen of 4 bulls, the mean concentration of total conjugated and unconjugated EL was 3200 nM, an order of magnitude higher than that was in the blood (228 nM)\(^{128}\). In 191 women, the median intracystic level of EL (63 nM) was approximate four times higher than the median value of serum EL (17 nM)\(^{129}\). Therefore, the accumulation of EL in prostate and/or breast cyst fluids might be, in part, responsible for decreasing the prostate and/or breast cancer risk.

Chronic or prolonged administration of SDG might alter lignan distribution. \(^3\)H-SDG disposition in female rats with acute or chronic SDG treatment over 48 h (3.7 kBq/g body weight \(^3\)H-SDG versus 1.5 mg unlabeled SDG/d) was evaluated by the Thompson group. The results found that tissue radioactivity was highest (by 0.5 to 176-fold) in the cecum, and levels in the liver, kidney and uterus were 0.2- to 7.5-fold higher than in other nongastrointestinal tissues\(^{130}\). Also, cecal content, liver and adipose radioactivity were one-to-threefold greater in rats with chronic SDG exposure as compared with acute exposure\(^{130}\). In a later study by the same research group \(^3\)H-SDG administration (3.7 kBq/g body weight) resulted in an increased radioactivity in all tissues (liver, kidneys, bladder, spleen, lungs, brain, thymus, heart, muscle, adipose, mammary gland, ovaries, vagina, uterus, testis, seminal vesicles, coagulating glands and ventral prostate) examined in both male and female rats, and the levels were further increased after prolonged SDG exposure, while liver contained the majority of the tissue lignans (48-56%) in both sexes after 1 d or 7 d exposure regimens\(^{131}\). After prolonged exposure, females had higher lignan concentrations in heart and thymus, demonstrating sex-related differences in lignan tissue distribution\(^{131}\). Hence, the foregoing data suggest lignans mainly accumulate in the liver, prostate, kidney and intestine. However, it is noteworthy that the tissue distribution of lignans was mostly investigated through animal
models such as rats; thus, careful attention should be paid when extrapolating the distribution pattern from animals to humans.

Since only the unbound form of a compound can exert an effect, the extent to which lignans bind to serum protein is of importance. However, limited information about the plasma protein binding data of flaxseed lignans is available in the literature. SDG, SECO, ED and EL (each 50 µg/mL) were evaluated for their serum protein binding capacity in rat serum by our developed HPLC method (manuscript under review). The results showed that SDG was not bound to serum protein, while SECO, ED and EL displayed increasing serum protein binding characteristics in an ascending order. This similar trend of the binding affinities of SECO, ED and EL was also observed with steroid hormone binding globulins (SHBG) that SECO and EL had higher binding affinity than ED\textsuperscript{132}. Besides, SECO and EL displaced 60 ± 7% and 55 ± 7% of $^3$H-dihydrotestosterone (DHT) from the binding site, respectively, whereas ED exhibited only 16 ± 6%\textsuperscript{133}.

2.5.4. Excretion

Urinary excretion is one of the primary routes of excretion of lignan metabolites. The excretion of enterolignans in urine and bile were mainly as glucuronides, while small amounts of mono- and disulphates were also found\textsuperscript{98}. In human urine, 92% of ED and 98% of EL in the glucuronide fractions were observed\textsuperscript{125}. A very small percent of plant lignans was also excreted in the urine, which might result from an insufficient metabolic capacity of intestinal bacteria\textsuperscript{99}. The urinary excretion of EL varied from 1 to 4.2 µM/24 h\textsuperscript{134,135}, and the urinary excretion of enterolignans is significantly related to lignan intake. Rickard and coworkers conducted a study in rats fed ground flaxseed (2.5, 5 or 10 g/100 g) or SDG (1.1, 2.2 or 4.4 mM), and found that urinary lignan excretion increased linearly with doses from 0-5% flaxseed and 0-2.2 mM SDG/d\textsuperscript{136}. In postmenopausal women consuming 5 or 10 g of ground flaxseed/d, urinary excretion of ED was increased by 1,009 and 2867 nM/d, while urinary excretion of EL was enhanced by 21,242 and 52,826 nM/d\textsuperscript{137}.

Fecal excretion is another major route of excretion of lignan metabolites. Fecal excretion of the lignans ED, EL and MAT increased significantly with flax consumption, from 80 to
2560, 640 to 10300 and 7.33 to 11.9 nM/d\textsuperscript{138}. Excretion in feces of pigs, predominantly in the form of EL, represented 46% of the intake when pigs were fed rye diet containing high percent of lignans\textsuperscript{139}. This is somewhat higher than that which was found in humans, where 34-35% (mean) of the mammalian lignans were excreted through the fecal route\textsuperscript{139}. When rats were given a single gavage of \(^3\)H-SDG, >80% of the recovered dose was excreted in both feces (> 50%) and urine (28-32%) after 48 h\textsuperscript{130}. The high fecal excretion of enterolignans may be due to the incomplete absorption or enterohepatic circulation.

2.6. Bioavailability of EL

The proposed health benefits of lignans have promoted its consumption by individuals suffering from various chronic diseases. However, systemic levels of EL are highly variable in the population. Several factors are crucially responsible for the varying circulating EL concentrations, which are discussed below.

2.6.1. Dietary and Lifestyle Determinants

Dietary and lifestyle determinants are important factors that influence circulating EL concentrations. Dietary components such as whole-grain products, vegetables, fiber and coffee showed positive associations with plasma EL concentration, while negative associations were observed among body mass index (BMI), smoking and frequency of bowel movements with plasma EL concentration\textsuperscript{140-142}.

2.6.2. Interindividual Variation of Gut Microflora

Interindividual variations in bacteria lead to significant differences of lignan metabolism in the population, which can be separated into high, moderate and low mammalian lignan producers\textsuperscript{143}. \textit{In vitro} SECO incubation with human fecal samples showed that higher proportions of \textit{Peptostreptococcus} products and related species, as well as bacteria belonging to the \textit{Atopobium} group were typical for moderate to high concentrations of EL-producing communities\textsuperscript{106}, while ED production correlated negatively with \textit{Clostridium coccoides-Eubacterium rectale} counts\textsuperscript{143}. Furthermore, since gut bacteria are critical in the
formation of EL from plant precursors, factors that affect lignan-converting bacteria will potentially influence the absorbable level of EL. As enterolignans precursors usually occur in fiber-rich foods, dietary fiber might play a profound role on the number and diversity of bacteria that inhabit the large intestine. The presence of fermentable dietary fiber in lignan-rich foods is likely to increase microfloral bacteria level, resulting in an enhanced level of EL. Another influential factor on intestinal bacteria is antibiotics. The impact of antibiotics on EL concentration has been reported by several studies, which suggested that the concentration of EL in blood was reduced by antibiotics among those who had used antibiotics during the preceding year. Consequently, interindividual variation in the density and diversity of gut microflora may significantly contribute to varying levels of EL.

2.6.3. First-pass Glucuronidation

Another critical factor causing the variable systemic concentrations of EL is its low and varying bioavailability owing to the extensive first-pass glucuronidation. In human plasma and urine, the principal conjugates of EL were mainly monoglucuronides, whereas very low levels of free EL were found. High circulating levels of EL glucuronide with concomitant low levels of EL suggest a significant first-pass effect by glucuronidation. Glucuronidation occurs both in hepatocytes and enterocytes. Extensive glucuronidation results in variable and significant reductions in the oral bioavailability of EL, which may limit the biological properties of EL. However, the exact contributions from liver and intestine are poorly understood.

Hence, a fundamental understanding of EL glucuronidation in the intestine and liver in vitro is essential to provide critical information regarding the oral absorption properties of EL, and also possibly to infer the potential for interactions. The in vitro evaluations of EL glucuronidation are obtained by the determination of intrinsic clearance (CL\text{int}), which directly measures the efficacy of enzymes to metabolize substrates. Therefore, the in vitro evaluations of EL glucuronidation can provide a great understanding as to what extent EL undergoes intestinal and hepatic glucuronidation, which conjugation metabolites would be produced, and also the contributions from intestine and liver to EL glucuronidation.
2.7. Model System

2.7.1. Microsomes

Two basic strategies are often used for in vitro investigation of the metabolic profile of a drug: incubation with subcellular fractions, for example, microsomes, and incubation with differentiated cellular models such as primary cultures, encapsulated hepatocytes or tissue slices. With intact cells, the plasma membrane, metabolic pathways, levels of physiological cofactors and coenzymes and active gene expression are reasonably well maintained for several hours/days in culture, which makes hepatocytes the closest model for in vivo studies. However, microsomes are often the simple, affordable and best-characterized in vitro model, allowing an easy comparison of the metabolic profile of a compound across animal species. Microsomes are easily prepared from tissues by homogenization and centrifugation, which contain almost only P450 and UGT enzymes. Microsomes can be stored at -80°C for years with little or no loss of enzyme activities. As well, microsomes from different animal species are commercially available and are well standardized. The main drawback of microsomes is that the results obtained in vitro might be different from those obtained in vivo due to the enriched enzymes in microsomal fractions and the absence of other competing enzymes, but some equations exist to extrapolate in vitro data to in vivo pharmacokinetics.

2.7.1.1. Intrinsic clearance

Intrinsic clearance (CL\textsubscript{int}) is the ability of each organ to remove drugs from the blood, which is independent of physiological factors such as blood flow and protein binding. CL\textsubscript{int} directly measures the efficacy of enzyme to metabolize substrate. Hence, determination of CL\textsubscript{int} in vitro will identify the contribution of the enzymes of each organ to the metabolism of the xenobiotics in vivo. Since CL\textsubscript{int} is the cornerstone to in vitro-in vivo extrapolation of metabolic data, the comparison of intestinal CL\textsubscript{int} with hepatic CL\textsubscript{int} by the enzyme kinetic studies in vitro will, to a large degree, inform us of the first-pass glucuronidation of EL as well as the contribution of each organ to the first-pass metabolism of EL in vivo.
2.7.2. Human versus Rat Models

Although humans are the most relevant system for evaluation, animal models are commonly used in the preclinical development of new drugs to predict the metabolic profile of new compounds in human. In comparison with other animal models, rat offers many advantages and is an excellent model for human diseases because of the well-characterized physiology that is more like the corresponding human conditions\textsuperscript{150}. A volume of data has developed and numerous experimental protocols are well established, which enables an easier way to obtain information to design experiments, make comparisons between studies, and to extrapolate from in vitro to in vivo and from animals to humans. Furthermore, rat is frequently used as an experimental model to explore and evaluate the pharmacokinetic activities of lignans. Thus, conducting intestinal and hepatic glucuronidation of EL in rats is important to human health. The results will be considerably valuable for further future study and extrapolating to humans.
2.8. Hypothesis and Objectives

SDG and its metabolites, especially EL, have received increasing interest for their putative health benefits. Once generated in the intestine, EL permeates the gastrointestinal barrier and undergoes conjugation with principally glucuronic acid before entering the systemic circulation. In human plasma and urine, the predominant conjugates of EL are mainly glucuronides, while very low levels of free EL were found. This suggests a significant first-pass glucuronidation occurring both in hepatocytes and enterocytes, which, to a large extent, results in variable and significant reductions in the oral bioavailability of EL. However, the exact contributions from liver and intestine are poorly understood. Hence, the purpose of the study is to provide an estimate of the relative contribution of the intestine and liver to presystemic glucuronidation of EL, which will provide a fundamental understanding regarding the oral absorption properties of EL. In vitro enzyme kinetic studies using intestinal and hepatic microsomal fractions from human and rat with determination of CL\text{int} allow the comparison of differences of EL glucuronidation between different organs and species. The aims of this project are accomplished by the following hypothesis and objectives:

2.8.1. Hypothesis

The intrinsic clearance (CL\text{int}) values from rat and human liver microsomes are greater than the values from intestinal microsomes.

2.8.2. Objective 1

Enzymatically generate EL glucuronides in rat liver microsomes, then purify and verify the glucuronides.

2.8.3. Objective 2

Validate the existing HPLC assay to allow for the quantitative determination of EL.

2.8.4. Objective 3

Conduct enzyme kinetic studies using rat and human hepatic and intestinal microsomal
fractions to determine and compare $\text{CL}_{\text{int}}$ of EL glucuronidation from each organ system.
3. MATERIALS AND METHODS

3.1. Chemicals

Enterolactone (EL), uridine 5’-diphosphoglucuronic acid trisodium salt, D-saccharic acid 1,4-lactone monohydrate, Trizma base, bovine serum albumin (BSA) and internal standard (umbelliferone) were obtained from Sigma-Aldrich Canada Ltd (Oakville, ON). A MilliQ Synthesis (Millipore, Bedford, MA) Water Purification system provided purified deionized water. All other chemicals used were analytical grade. Human liver and intestinal microsomes were purchased from XenoTech, LLC (Lenexa, KS).

3.2. Experiment one: EL Glucuronide Synthesis and Characterization

3.2.1. Preparation of Rat Liver and Intestinal Microsomes

Male Wistar rats (N=6, weight range 250-300 g and age range 7-9 weeks) were obtained from the Animal Resources Centre (ARC), University of Saskatchewan. This work was approved by the University of Saskatchewan’s Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

3.2.1.1. Preparation of rat liver microsomes

Rat liver and intestinal microsomes were prepared according to established protocols in our lab. Rat liver was removed promptly following isoflurane anesthesia of the rat and rinsed twice in ice water to remove excess blood. The liver was immediately immersed into liquid nitrogen and stored at -80°C until preparation. Three grams of rat liver from three individuals were homogenized in 12 mL buffer (pH 7.4) containing 50 mM Tris buffer, 150 mM KCl, 0.1 mM dithiothreitol, 1 mM ethylenediamine-tetraacetic acid (EDTA), 20% glycerol, 0.1 mM phenylmethylsulfonylflouride (PMSF) and double distilled water (ddH2O). The liver homogenate was then ultracentrifuged at 9,184 × g for 30 min. The supernatant was transferred and ultracentrifuged at 112,504 × g for 30 min. The microsomal pellet was washed with 150 mM KCl in ddH2O, and then ultracentrifuged at 112,504 × g for another 30 min. Microsomal pellets were resuspended in 0.25 M sucrose solution. The rat liver microsomes
were stored at -80°C. The total protein concentration was determined by BioRad RC DC Protein Assay Kit (Bio-Rad Laboratories Ltd., Mississauga, ON) using bovine serum albumin (BSA) as a standard.

3.2.1.2. **Preparation of rat intestinal microsomes**

To compare the metabolic activities of different sections of rat small intestine, intestinal microsomes extracted from duodenum, jejunum and colon were prepared. Tissue consisting of the proximal duodenum, jejunum and colon were removed from anaesthetized rats (n=2). The intestinal sections were instantly placed in ice-cold buffer solution A (KCl 1.5 mM, NaCl 96 mM, sodium citrate 27 mM, KH₂PO₄ 8 mM, Na₂HPO₄ 5.6 mM, pH to 7.4)¹⁵¹ to flush out intestinal contents. The remaining intestinal sections were dissected longitudinally and spreaded on an ice-cold glass plate with the mucosal side upwards. The mucosa was then gently scraped using a microscope slide. The scraped mucosa was homogenized in solution B (histidine 5 mM pH 7.0, sucrose 0.25 M, NaEDTA 0.5 mM, pH to 7.4) by sonication, 7 cycles, 10 seconds each (wattage=4). The homogenate was then centrifuged at 15,000 × g for 10 min. The supernatant was collected, and 1.25 mL of 52 mM CaCl₂ for each 7 mL supernatant was added. The tubes containing microsomes were gently shaken for 10 s and then allowed to stand for 15 min on ice. Fractions were then centrifuged at 25,000 × g for 10 min¹⁵². Pellets were resuspended in 0.25 M sucrose/0.02 M Tris buffer. The rat liver and intestinal microsomes were stored at -80°C. The total protein concentration was determined by BioRad RC DC Protein Assay Kit using BSA as a standard.

3.2.2. **Enterolactone Glucuronide Synthesis**

EL glucuronide (EL-Glu) was enzymatically synthesized by incubating with pooled human and rat liver and intestinal microsomes, respectively, at room temperature for 22 h. The incubation experiment was conducted basically as described in a previous study¹²⁴, while some experimental conditions were changed. Briefly, the incubation mixture (1 mL) consisted of 5 mM uridine 5’-diphosphoglucuronic acid trisodium salt, 5 mM MgCl₂, 5 mM d-saccharic acid 1,4-lactone monohydrate, 100 mM Trizma base (adjusted with HCl to pH 7.4), 2 mM EL
and microsomal protein (3.1 mg/mL). The reaction was initiated by adding uridine 5’-diphosphogluconic acid trisodium salt. At the end of the incubation methanol (2 volumes) was added to terminate the reaction and precipitate proteins. After centrifugation of the mixture for 10 min at 10,000 × g in an Eppendorf microcentrifuge (Model 5417C, Brinkmann Instruments, Westbury, NY), the supernatant was concentrated by rotary evaporation and made up to its original volume.

3.2.3. Purification of EL Glucuronide Conjugates

The mixture of EL-Glu generated from rat liver microsomes was purified by HPLC Waters 600 system. The HPLC system consisted of Waters Model 600 solvent delivery system, Model 2996 photodiode Array Detector with the fixed wavelength at 280 nm, Model 717 plus autosampler. All chromatographic separations were carried out on a reversed-phase semi-preparative column (Allsphere ODS-2 300×10 mm I.D., 5 µm particle size). The analytes were eluted under gradient mode with mobile phase consisting of water with 0.1% formic acid (component A) and acetonitrile with 0.1% formic acid (component B) in different ratios delivered at a flow rate of 3 mL/min. The gradient consisted of 85:15 component A: component B from 0 to 12 min, an increasing gradient from 15% to 50% component B and from 50% to 90% between 12 and 14 min and then 90% between 14 and 18 min, with a decreasing gradient from 90% to 15% component B from 18 to 20 min and a return to 85:15 component A: component B between 20 and 25 min. Fractions containing glucuronide at the desired retention time were collected automatically by Waters Fraction Collector, and solvents were removed by rotary evaporation.

3.2.4. Characterization of EL Glucuronide

Liquid chromatography-mass spectrometry (LC-MS) was used to verify and confirm the formation and structure of the purified fractions containing EL glucuronide. The LC-MS/MS analysis was conducted on a Hybrid Triple Quadrupole/Linear Ion Trap mass spectrometer (AB Sciex 4000 QTRAP, MS/MS system, Applied Biosystems Inc., Foster City, CA) fitted with Turbo V electrospray ionization (ESI) source in the negative mode, coupled with Agilent
Technologies 1100 Series LC which consisted of a binary pump and autosampler. The chromatographic separation was performed on a Waters Symmetry Reverse-Phase C18 column (150×4.6 mm I.D., 5 µm particle size). The gradient was the same as described above. The flow rate was delivered at 1 mL/min. The MS parameters were optimized for EL-Glu as follows: declustering potential (DP) -90 V, collision energy (CE) -36 V, Collision Cell Exit Potential (CXP) -23 V. The ion source parameters were curtain gas (CRU) 10 V; ion spray voltage (IS) -4500 V; temperature (TEM) 400; ion source gas 1 (GS1), 40 psi; ion source gas 2 (GS2), 40 psi; collision activated dissociation (CAD) medium; entrance potential (EP) -10 V. The fragmentation transitions for multiple reaction monitoring (MRM) were mass to charge ratio (m/z) 473.0→297.0 and 473.0→175.0.

3.3. Experiment Two: HPLC Method

A reliable and simple HPLC method for the quantitative determination of flaxseed lignans was established in our lab\textsuperscript{102}. Hence, a partial HPLC method validation was conducted to allow for the quantitative determination of EL in this study.

3.3.1. Instrumentation and Chromatographic Conditions

The HPLC (Agilent Technologies, Mississauga, ON) system consisted of a Series 1200 quaternary pump (G1311A) with online degasser (G1322A), autosampler (G1329A) and fluorescence detector (G1321A). Excitation wavelength was set at 277 nm and emission wavelength at 617 nm. Processed samples (50 µL) were injected onto a Waters Symmetry Reverse-Phase C18 column (150×4.6 mm I.D., 5 µm particle size). The analytes were eluted under gradient mode with mobile phase consisting of water with 0.1% formic acid (component A) and acetonitrile with 0.1% formic acid (component B) in different ratios delivered at a flow rate of 1 mL/min. The gradient consisted of 85:15 component A: component B from 0 to 12 min, an increasing gradient from 15% to 50% component B and from 50% to 90% between 12 and 14 min and then 90% between 14 and 18 min, with a decreasing gradient from 90% to 15% component B from 18 to 20 min and a return to 85:15 component A: component B between 20 and 25 min.
3.3.2. Validation of HPLC Method

HPLC method validation procedures were performed based on FDA guidelines. Specificity was assessed by analyzing pooled human liver microsomes (HLM), human intestinal microsomes (HIM), rat duodenum microsomes (RDM), rat jejunum microsomes (RJM), rat colon microsomes (RCM) and six different rat liver microsomes (RLM), whose preparations were supplemented only with internal standard (umbelliferone) to determine the absence of endogenous substances with similar retention time as EL and/or EL-Glu.

The sensitivity of the method was evaluated by determination of the limit detection (LOD) and the lowest limit of quantification (LLOQ). LOD was defined as the lowest detectable concentration, considering the signal-to-noise ratio of 3. The lowest limit of quantification (LLOQ) was the lowest concentration of the standard curve that gave acceptable accuracy and precision. Accuracy (%) was expressed as [(calculated amount/predicted amount)*100] with acceptable limits at 15% except at LLOQ, where it should not deviate by more than 20%. Precision was expressed as % relative standard deviation (RSD) with acceptable level at 15% of the coefficient of variation (CV) except at LLOQ, where it should not exceed 20% of CV. The intra-day accuracy and precision were evaluated by analyzing six replicates of quality control (QC) samples (low, medium and high concentrations of quality controls) on a single day. The inter-day accuracy and precision were determined from the same QC samples on three different days.

The linearity of the method was assessed by processing a 6-point calibration curve on several different days. The ratios of the peak areas of analytes and the internal standard were plotted against the nominal concentrations of the analytes. A linear least-squares regression analysis using $1/X^2$ as weighting factor was conducted to determine slope, intercept and coefficient of determination ($r^2$) to demonstrate linearity of the method.

3.4. Experiment Three: Enzyme Kinetic Analysis

Enzyme kinetics were conducted using pooled HLM, HIM, RLM, RJM and RCM, respectively. Pooled human liver (pool of 50) and intestinal microsomes (pool of 13) from
mixed gender were purchased from XenoTech, LLC (Lenexa, KS). Human intestinal microsomes were prepared from duodenal and jejunal tissues. Pooled rat liver (pool of 3) and jejunum and colon (pool of 2) from male gender were extracted based on established protocols in our lab. Comparison of EL glucuronidation between species and tissues was assessed by determination of intrinsic clearance (CL$_{int}$) in intestinal and liver microsomes. CL$_{int}$ (as a measure of the maximal efficacy of enzyme to metabolize substrate) was determined by the substrate depletion method, in which the depletion of substrate was monitored as a function of time$^{153}$.

The incubation mixture consisted of (at their final concentrations): microsomes (0.5 mg/mL), 5 mM MgCl$_2$, 5 mM d-saccharic acid 1,4-lactone monohydrate, 100 mM Trizma base (adjusted with HCl to pH 7.4). A range of final EL concentrations (10-500 µM) was incubated with HLM and RLM, respectively, while EL concentrations (10-300µM) were incubated with HIM. The total incubation volume for all of three incubations was 1.5 mL. EL concentrations (1.5-100 µM) were used when incubating with RJM and RCM with a total volume of 1.0 mL. Trizma base (pH 7.4) containing uridine 5’-diphosphoglucuronic acid trisodium salt (5 mM final concentration) preincubated at 37ºC for 5 min in a shaking water bath was used to initiate the reaction. At multiple incubation time points, aliquots (0.2 mL) in the incubations containing HLM, HIM and RLM were withdrawn and 20 µL internal standard (1.23 mM umbelliferone) in acetonitrile was added to the aliquots. At equivalent incubation time points, aliquots (130 µL) in the incubations containing RJM and RCM were withdrawn and 13 µL of internal standard in acetonitrile was added. Subsequently, the aliquots were immersed into the liquid nitrogen immediately to terminate the reaction. After thawing on the ice, the mixture was centrifuged at 10,000 × g for 10 min in an Eppendorf microcentrifuge (Model 5417C, Brinkmann Instruments, Westbury, NY). 50 µL of the supernatant (triplicate) for HLM, HIM and RLM incubations as well as duplicates (50 µL) for RJM and RCM incubations was injected on the Waters Symmetry Reverse-Phase C18 column (150×4.6 mm I.D., 5 µm particle size) for the immediate HPLC analysis of EL. Heated microsomes, no EL and no uridine 5’-diphosphoglucuronic acid trisodium salt incubation reactions were used as negative controls.
3.5. Data Analysis

To determine CL\textsubscript{int}, kinetic parameters in microsomal preparations were estimated with the methods reported by both Obach and Reed-Hangen and Komura and Iwaki\cite{154,155}. Analyte/internal standard peak area were determined and normalized to the ratio obtained at time, t=0, which represents 100%. A plot of natural log EL percent remaining versus time was constructed to calculate the substrate depletion rate constant (\(k\text{\_dep}\)) (the slope of each linear line) for each EL concentration. If substrate decline demonstrated nonlinearity on log percentage remaining versus time curves, only those initial time points wherein log linearity was observed were used to determine depletion rate constants. The \(K_m\) and \(k\text{\_dep([S]→0)}\) values were determined by plotting depletion rate constants (\(k\text{\_dep}\)) versus various initial EL concentrations by a nonlinear least squares regression analysis with Prism 4.0 software (GraphPad Prism, San Diego, CA, USA) using the following equation:

\[
k\text{\_dep} = k\text{\_dep([S]→0)} \cdot \left(1 - \frac{[S]}{[S] + K_m}\right)
\]

where \(k\text{\_dep}\) is the apparent first-order rate constant of substrate depletion, \([S]\) is the substrate concentration and \(k\text{\_dep([S]→0)}\) is the theoretical maximum consumption rate constant at an infinitesimally low substrate concentration and \(K_m\) is the Michaelis-Menten constant. The intrinsic clearance at an infinitesimally low substrate concentration (CL\textsubscript{int, app([S]=0)}) was calculated by dividing \(k\text{\_dep([S]→0)}\) by the microsomal protein concentration used. \(V_{\text{max}}\) was derived from generated \(K_m\) and CL\textsubscript{int, app([S]=0)}. 
4. RESULTS

4.1. EL Glucuronide Synthesis with Rat and Human Microsomes

EL glucuronide was enzymatically generated by incubation with rat liver and intestinal (duodenum, jejunum and colon) microsomes, human liver and intestinal microsomes, respectively, at room temperature for 22 h as most of the EL was converted to EL glucuronide in this time frame. HPLC (Waters 600 system, semi-preparative column) analysis of EL glucuronide from rat liver microsomes (RLM) resulted in the appearance of a single product peak. The retention time for EL glucuronide (EL-Glu) was 14.1 min, and some EL was unreacted and observed in the incubation mixtures (Figure 4.1A). After purification of EL-Glu from RLM by semi-preparative HPLC, three milligrams of EL-Glu was collected and no EL was detected in this sample (Figure 4.1B). However, I found that the peak shape of purified EL-Glu was not symmetrical, indicating the presence of more than one form of EL-Glu. Accordingly, LC-MS/MS was used to identify and confirm the formation and structures of the purified fractions containing EL-Glu. MS analysis suggested that the glucuronide for EL from RLM predominantly occurred as the monoglucuronide with an m/z of 473. The optimized fragments for EL monoglucuronide were 297 and 175, which corresponded with the cleavage of the bond between EL and glucuronic acid (Figure 4.2).

Multiple Reaction Monitoring (MRM) was also employed to further oversee the formation of EL monoglucuronide from each tissue and species. According to two peaks observed with very close retention times around 9 min, the data suggested the formation of two monoglucuronides for EL incubated with RLM (Figure 4.3). Interestingly, only one peak was observed by MRM for EL-Glu from rat intestinal microsomes (Figure 4.4A-C), human liver and intestinal microsomes (Figure 4.4D-E). In addition, preliminary microsomal incubation results showed that colon made a greater contribution to EL glucuronidation than jejunum, but both tissues were dramatically greater than that of duodenum in rat intestine. Hence, only colon and jejunum were used to further explore the enzyme kinetics for EL glucuronidation in rat intestine. Human liver produced EL-Glu more efficiently than human intestine.
Figure 4.1 Representative HPLC chromatograms of unpurified EL glucuronide (A) and purified EL glucuronide (B) generated from rat liver microsomes. Conditions: Waters 600 HPLC system; reversed-phase semi-preparative column (300 × 10 mm I.D., 5 µm particle size); component A: water with 0.1% formic acid, component B: acetonitrile with 0.1% formic acid; detection wavelength at 280 nm.
Figure 4.2 LC-MS analysis of proposed EL glucuronide (m/z 473) formed from rat liver microsomes obtained in negative electrospray ionization (ESI) mode.
Figure 4.3 Multiple reaction monitoring chromatograms of unpurified EL glucuronide (A) and purified EL glucuronide (B) generated from rat liver microsomes (RLM)
XIC of -MRM (4 pairs): 473.158/297.000 Da ID: EL-Gluc from Sample 1 (Duo scrap ELGlu) of 2011-09-16 Anal -RISM ELGlu 2011-09-

Max. 6633.3 cps.

Mono EL-Glu from RDM

Unreacted EL 12.27 min

A

XIC of -MRM (4 pairs): 473.158/297.000 Da ID: EL-Gluc from Sample 2 (Jeju scrap ELGlu) of 2011-09-16 Anal -RISM ELGlu 2011-09-

Max. 1.2e6 cps.

Mono EL-Glu from RJM

Unreacted EL 12.30 min

B
XIC of -MRM (4 pairs): 473.158/297.000 Da ID: EL-Gluc from Sample 3 (Colon scrap ELGlu) of 2011-09-16 Anal-HPLC(EIC)2011-09-16...

Max. 3.2e6 cps.

XIC of -MRM (4 pairs): 473.158/297.000 Da ID: EL-Gluc from Sample 2 (HLM ELGlu) of 2011-10-13 HLM...HLM(EIC)2011-10-13HLM...

Max. 3.9e6 cps.

Mono EL-Glu from RCM

Unreacted EL 12.25 min

Mono EL-Glu from HLM

Unreacted EL 12.03 min
Figure 4.4 Multiple reaction monitoring analysis of EL glucuronide incubated with rat duodenum microsomes (RDM) (A), rat jejunum microsomes (RJM) (B), rat colon microsomes (RCM) (C), human liver microsomes (HLM) (D) and human intestinal microsomes (HIM) (E)
4.2. HPLC Method Validation for EL

A HPLC method was validated for the quantitative determination of EL in RLM. Figure 4.5 represented HPLC chromatograms for blank rat liver microsomes and EL, and its internal standard (umbelliferone) in RLM. No endogenous substances were found in RLM, RCM, HLM and HIM that interfered with EL. The chromatographic conditions gave retention times for internal standard (umbelliferone) and EL as 6.8 and 12.0 min, respectively. The recovery of EL was 104.9, 110.6 and 109.8% at LQC, MQC and HQC, respectively. The lower limit of quantification (LLOQ) for EL was 41.7 ng/mL. Table 4.1 and Table 4.2 summarize the intra- and interday precision and accuracy data. Overall, the intra- and interday precision and accuracy for EL of this HPLC method were ≤ 10%, suggesting the method was both precise and accurate to quantitatively determine EL in rat and human liver and intestinal microsomes.
Figure 4.5 Representative HPLC chromatograms of blank rat liver microsomes (A) and rat liver microsomes spiked with internal standard umbelliferone (100µg/mL, 6.8 min) and EL (4 µg/mL, 12.0 min) (B). Conditions: Agilent 1200 HPLC system; Waters Symmetry reversed-phase C18 analytical column (150 × 4.6 mm I.D., 5 µm particle size); component A: water with 0.1% formic acid, component B: acetonitrile with 0.1% formic acid; excitation wavelength at 277 nm and emission wavelength at 617 nm.
Table 4.1 Intraday assay precision and accuracy for EL in rat liver microsomes (N=6)

<table>
<thead>
<tr>
<th>QC levels</th>
<th>Precision</th>
<th>Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LLOQ</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>2.7</td>
<td>92.7</td>
</tr>
<tr>
<td>Day 2</td>
<td>1.6</td>
<td>101.4</td>
</tr>
<tr>
<td>Day 3</td>
<td>4.8</td>
<td>101.1</td>
</tr>
<tr>
<td><strong>LQC</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>4.4</td>
<td>90.0</td>
</tr>
<tr>
<td>Day 2</td>
<td>4.3</td>
<td>100.6</td>
</tr>
<tr>
<td>Day 3</td>
<td>2.8</td>
<td>95.4</td>
</tr>
<tr>
<td><strong>MQC</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>3.3</td>
<td>94.7</td>
</tr>
<tr>
<td>Day 2</td>
<td>2.2</td>
<td>106.5</td>
</tr>
<tr>
<td>Day 3</td>
<td>4.0</td>
<td>100.2</td>
</tr>
<tr>
<td><strong>HQC</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>1.5</td>
<td>93.9</td>
</tr>
<tr>
<td>Day 2</td>
<td>2.8</td>
<td>102.3</td>
</tr>
<tr>
<td>Day 3</td>
<td>3.1</td>
<td>97.0</td>
</tr>
</tbody>
</table>

* Precision is expressed as CV% = $\frac{\text{standard deviation}}{\text{mean}} \times 100\%$

* Accuracy is calculated as $\frac{\text{mean determined concentration}}{\text{actual concentration}} \times 100\%$

* LLOQ for EL is 41.7 ng/mL; LQC for EL is 150 ng/mL; MQC is 4000 ng/mL and HQC is 8000 ng/mL

Table 4.2 Interday assay precision and accuracy for EL in rat liver microsomes (N=6)

<table>
<thead>
<tr>
<th>QC levels</th>
<th>Precision</th>
<th>Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LLOQ</strong></td>
<td>3.9</td>
<td>98.4</td>
</tr>
<tr>
<td><strong>LQC</strong></td>
<td>5.0</td>
<td>95.4</td>
</tr>
<tr>
<td><strong>MQC</strong></td>
<td>4.9</td>
<td>100.5</td>
</tr>
<tr>
<td><strong>HQC</strong></td>
<td>3.4</td>
<td>97.8</td>
</tr>
</tbody>
</table>

* Precision is expressed as CV% = $\frac{\text{standard deviation}}{\text{mean}} \times 100\%$

* Accuracy is calculated as $\frac{\text{mean determined concentration}}{\text{actual concentration}} \times 100\%$

* LLOQ for EL is 41.7 ng/mL; LQC for EL is 150 ng/mL; MQC is 4000 ng/mL and HQC is 8000 ng/mL
4.3. *In Vitro* Hepatic and Intestinal Microsomal Intrinsic Clearance of EL

The *in vitro* CL_{int} values of EL glucuronidation were examined using RLM, RJM and RCM extracted in our lab and commercial sources of HLM and HIM (XenoTech, LLC) by the substrate depletion approach^{154} (Table 4.3). Control assays conducted with heated microsomes and without microsomes and uridine 5’-diphosphoglucuronic acid trisodium salt exhibited no substrate depletion over time. The relationships between the first-order constants for substrate depletion (termed $k_{dep}$) and EL concentrations for EL glucuronidation were shown in Figure 4.6. Typical examples of the process to generate $k_{dep}$ value were exhibited in Appendix I. $k_{dep}$ values for each substrate concentration for rat and human liver and intestinal microsomes were summarized in Appendix I. The *in vitro* CL_{int} values for EL glucuronidation determined with RLM and HLM were much greater than those for intestinal microsomes, and the HLM (1.18 mL/min/mg) gave the highest value. RJM and RCM gave comparable CL_{int} values; on the other hand, they were considerably lower than HIM (Figure 4.7A). Similar trends also applied to $V_{max}$ values, nonetheless, RLM showed the highest $V_{max}$ value (Figure 4.7B). The $K_m$ values for EL glucuronidation evaluated using rat microsomes were significantly larger than human microsomes among which RCM had the highest $K_m$ value as 45.9 µM. Rat intestinal microsomes generated greater $K_m$ values than rat liver microsomes, the results of which were in agreement with that of human (Figure 4.7C).
Table 4.3  \( CL_{int} \) and Michaelis-Menten constants of EL glucuronidation by rat and human liver and intestinal microsomes

<table>
<thead>
<tr>
<th>Microsomes</th>
<th>( CL_{int} ) ( \text{mL/min/mg} )</th>
<th>95% CI for ( CL_{int} )</th>
<th>( K_m ) ( \mu M )</th>
<th>95% CI for ( K_m )</th>
<th>( V_{max} ) ( \text{nmol/min/mg} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>RLM</td>
<td>1.00 ± 0.1</td>
<td>0.77 - 1.24</td>
<td>25.6 ± 5.07</td>
<td>13.2 - 38.0</td>
<td>25.7</td>
</tr>
<tr>
<td>RJM</td>
<td>0.05 ± 0.003</td>
<td>0.042 - 0.058</td>
<td>31.6 ± 7.72</td>
<td>12.7 - 50.4</td>
<td>1.58</td>
</tr>
<tr>
<td>RCM</td>
<td>0.058 ± 0.003</td>
<td>0.050 - 0.066</td>
<td>45.9 ± 11.4</td>
<td>16.6 - 75.1</td>
<td>2.65</td>
</tr>
<tr>
<td>HLM</td>
<td>1.18 ± 0.09</td>
<td>0.97 - 1.40</td>
<td>8.91 ± 1.07</td>
<td>6.44 - 11.4</td>
<td>10.6</td>
</tr>
<tr>
<td>HIM</td>
<td>0.31 ± 0.027</td>
<td>0.25 - 0.37</td>
<td>12.6 ± 1.84</td>
<td>8.36 - 16.9</td>
<td>3.90</td>
</tr>
</tbody>
</table>

Different range of EL concentrations were incubated with liver and intestinal microsomes from human and rat (0.5 mg/mL) for 30 min. \( CL_{int} \), intrinsic clearance; \( K_m \), the substrate concentration at half maximum velocity; \( V_{max} \), maximum velocity; RLM, rat liver microsomes (n=3); RJM, rat jejunum microsomes (n=2); RCM, rat colon microsomes (n=2); HLM, human liver microsomes (n=50); HIM, human intestinal microsomes (mixture of duodenal and jejunal tissues, n=13); ±, standard error of the calculated kinetic parameters; CI, confidence intervals.
Figure 4.6 Plots of \textit{in vitro} depletion rate constants \((k_{dep})\) \((\text{min}^{-1})\) versus EL concentrations \((\mu\text{M})\) for EL glucuronidation by rat liver microsomes (A), rat jejunum microsomes (B), rat colon microsomes (C), human liver microsomes (D), and human intestinal microsomes (E).

Substrate depletion rate constant \((k_{dep})\) for each substrate concentration was equal to the slope of the linear line from a plot of ln analyte peak area percentage remaining (normalized to the initial peak area obtained at \(t=0\)) versus time. Refer to Appendix I for all \(k_{dep}\) values for various EL concentrations for different organs from different species.
Figure 4.7 The $\text{CL}_{\text{int}}$ (A), and Michaelis-Menten constants, $V_{\text{max}}$ (B) and $K_{\text{m}}$ (C), for EL glucuronidation by rat liver microsomes (RLM, n=3), rat jejunum microsomes (RJM, n=2), rat colon microsomes (RCM, n=2), commercial human liver microsomes (HLM, n=50) and commercial intestinal microsomes (HIM, n=13)
5. DISCUSSION

The aim of the project was to quantitatively make a comparison of the intestinal and hepatic glucuronidation of enterolactone (EL) derived from flaxseed lignans by the determination of intrinsic clearance (CLint) using the substrate depletion approach. Human and rat were employed to define the interspecies differences of EL glucuronidation to reduce the uncertainties of extrapolating pharmacokinetic data from rat studies to humans. The characterization of EL pharmacokinetics (e.g. metabolism) is critical to evaluate the exposure dose, susceptibility of low-dose exposure caused by first-pass metabolism, as well as drug-lignan interactions resulting from coadministration of flaxseed lignans with drugs and/or other Natural Health Products (NHPs). To accomplish this purpose, three experiments were carried out for this research project.

5.1. EL Glucuronide Synthesis and Characterization

My first objective was to enzymatically synthesize EL glucuronides (EL-Glu) in vitro and verify the structures by LC-MS/MS to ensure the formation of EL glucuronides, which was the foundation of enzyme kinetic studies for EL glucuronidation. Based on the literature, the suggested incubation time for EL and ED were 8 h and 24 h at 25°C, respectively. In order to confirm 8 h was sufficient to convert EL to EL-Glu, I made a comparison of the incubation reaction for EL at 8 h, 22 h and 24 h, respectively, and found 22 h was long enough for the biotransformation of EL into EL-Glu, while the reaction was not complete at 8 h (data not shown). As 37°C, representing the body temperature, is often used as the temperature for enzyme incubation, a comparison was made between 25 and 37°C to assess the appropriate temperature for the incubation. No significant difference existed between these two temperatures; while 25°C was chosen considering that this condition was easier to control.

After incubation of EL with rat liver microsomes (RLM) and purification of the generated glucuronides, a single peak at 14.1 min was observed by semi-preparative chromatography (4.1B). The asymmetrical peak suggested that more than one form of glucuronide was possibly produced. This was further substantiated by LC-MS/MS. Two peaks
whose retention time were close to 9 min, were determined by multiple reaction monitoring (MRM) mode, indicating two monoglucuronides were generated for EL incubating with RLM. As EL is not a symmetric molecule, glucuronidation of the different phenol groups would be expected to produce isomers. This result was consistent with the glucuronidation of EL in rabbit and/or rhesus monkeys liver microsomes, suggesting that EL was conjugated to either phenolic hydroxyl and was able to form two phenol β-O-glucuronides\textsuperscript{115,124}. Hence, the generated EL glucuronides from RLM were actually a mixture of two monoglucuronides, which can barely be separated by HPLC\textsuperscript{124} or the LC-MS in my project. It was interesting that a single peak was detected by MRM for EL-Glu generated from rat intestinal microsomes, human liver and intestinal microsomes, implying only one or a major monoglucuronide was produced by those microsomes, which was different from the results from RLM. This might be due to the interspecies (rat versus human) as well as interorgan (liver versus intestine) differences, of which the types and content of UGT might be distinct, resulting in the varying levels of EL glucuronidation.

5.2. Substrate Depletion Approach

Traditionally, the kinetic behavior of an enzyme is often characterized by monitoring product formation rates at various substrate concentrations\textsuperscript{153}. To fulfill this, metabolite concentrations in \textit{in vitro} matrices have to be measured by analytical methods, requiring that metabolites have been definitively identified and authentic standards prepared to construct calibration curves\textsuperscript{154}. However, these are not always readily and/or feasibly obtained. In my experiment, EL-Glu generated from RLM have been identified by LC-MS/MS and purified by HPLC system. Nevertheless, the purity of the fractions cannot be exactly determined to ensure highly purified EL-Glu to be used as authentic standards. Furthermore, the enzymatic synthesis method was costly both in time and money to accumulate sufficient amounts of purified EL-Glu to be used as standards because of the complex and time-consuming production process and the expensive incubation materials (EL and uridine 5′-diphosphoglucuronic acid trisodium salt). Therefore, the substrate depletion approach that monitors depletion of a substrate as a function of time was used to determine the kinetic
parameters of the UGT. Nath and his coworkers have demonstrated that kinetic parameters obtained from the substrate depletion approach can be meaningfully compared with those obtained by measuring product formation\textsuperscript{153}.

It has to be noted that the substrate depletion approach also possesses some practical limitations. One of the significant shortcomings of the substrate depletion approach is that the enzyme kinetics of formation of individual products are possibly missed, resulting in the overestimation of the individual intrinsic clearance (\(\text{CL}_{\text{int}}\)) and/or \(K_m\) values. The obtained values represent a conglomeration of all \(\text{CL}_{\text{int}}\) or \(K_m\) values for individual metabolic pathways\textsuperscript{154}. For EL glucuronidation, EL was incubated with microsomes that primarily contained P450 enzymes and UGT; however, only cofactor uridine 5’-diphosphoglucuronic acid trisodium salt was added into the incubation system for the conjugative reaction, which theoretically should yield only EL-Glu. This supposition was corroborated by the experimental results with HPLC analysis, which showed the appearance of a single product peak; control assays conducted without cofactor uridine 5’-diphosphoglucuronic acid trisodium salt demonstrated no product formation. Additionally, LC-MS confirmed the formation as well as the structure of EL-Glu. Therefore, the substrate depletion approach can be used to estimate the enzyme kinetic parameters for EL glucuronidation.

An important constraint when using the substrate depletion approach is the use of time points for calculation of \(k_{\text{dep}}\) where no more than 10\% of the substrate has been consumed; otherwise, the estimated \(k_{\text{dep}}\) values will be associated with significant error, which in turn results in \(K_m\) and \(V_{\text{max}}\) values with significant error\textsuperscript{153}. However, a simulation study indicated only 15\% error in the estimated kinetic parameters when the data used to obtain \(k_{\text{dep}}\) included time points where 50\% of the substrate has been consumed\textsuperscript{153}. In my study, we had to include time points with ~50\% (and sometimes greater) EL depletion to generate parameter estimates following the nonlinear regression analysis of the data. Hence, our calculated \(k_{\text{dep}}\) values and reported \(K_m\) values have error associated with their determination (> 15\%), and the calculated \(\text{CL}_{\text{int}}\) and \(V_{\text{max}}\) values are likely greater than their true values\textsuperscript{153}. Some experimental conditions should be carefully considered to improve the substrate depletion approach in the future. As a large portion of substrate (~50\%) is potentially depleted, the microsomal protein content and the incubation time should be carefully optimized to decrease the rate of substrate
consumption, thus to minimize the errors of calculated kinetic parameters due to the over depletion of substrate.

5.3. Interorgan Differences on EL Glucuronidation

5.3.1. Human Liver versus Human Intestine

Organ and species differences in $CL_{\text{int}}$ and Michaelis-Menten parameters ($V_{\text{max}}$ and $K_{m}$) for in vitro glucuronidation of EL were investigated (Table 4.3). The results were consistent with the reports by the other, for which the microsomal UGT activities in the small intestine and/or colon were lower than that in the liver\textsuperscript{156}. My study showed that all the hepatic $CL_{\text{int}}$ values as well as glucuronidation rates were greater than that of the intestine, suggesting liver is predominant and more efficiently transforms EL into glucuronides as compared with the intestine. An understanding of the level of expressed UGT in the liver and intestine is necessary to further understand the relationship between hepatic and intestinal enzyme levels and glucuronidation\textsuperscript{157}.

Currently, 15 human enzymes and 14 rat enzymes are known to exhibit significant conjugative activity towards endogenous and xenobiotic compounds\textsuperscript{119,156}. A significant number of studies have reported the expression and localization of UGT mostly by utilizing reverse transcription-polymerase chain reaction (RT-PCR) or Northern blotting analysis\textsuperscript{156,158-162}. Ten UGT are extensively expressed in human liver with large interindividual variability, including 5 UGT1 isoforms (UGT1A1, 1A3, 1A4, 1A6 and 1A9) and 5 UGT2 isoforms (UGT2B4, 2B7, 2B10, 2B15 and 2B17), and the sum of the UGT2B mRNA levels was higher than that of UGT1A mRNA levels\textsuperscript{160} (Table 5.1). An extremely high expression of UGT2B4, 2B10, 2B15, 2B7 was found, followed by some UGT1 members including UGT1A1, 1A6 and 1A9\textsuperscript{121,158-160}.

Expression of UGTs in the human small intestine and colon is less abundant relative to the liver. Several UGT isoforms such as UGT1A1, 1A5, 1A10, 2B7, 2B15 and 2B17 are expressed, whereas UGT1A10 and 2B17 are found primarily in the intestine\textsuperscript{158}. UGT1A8 is expressed in the intestine and colon but not liver\textsuperscript{158,163}. Hence, the various types and considerable content of UGT1A and UGT2B families in human liver help explain why hepatic
glucuronidation of EL was significantly larger than that in the human intestine. In addition to the UGT activity, the concentration of its significant cofactor 5'-diphosphoglucuronic acid (UDPGA) has been measured in human liver and intestinal mucosa, where it was found that the concentration of UDPGA in the liver was 279 nmol/g, over 14 times higher than that in the intestinal mucosa (19.3 nmol/g)\textsuperscript{164}. This evidence further demonstrates the overall hepatic glucuronidation activity is much greater than that in the intestine.

However, it is noteworthy the CL\textsubscript{int} value of human intestine (CL\textsubscript{int}=0.309) was approximately one third of the value of human liver (CL\textsubscript{int}=1.18), suggesting human intestine makes an important contribution to EL glucuronidation and may exert a significant influence on the first-pass metabolism of EL. In a study evaluating the glucuronidation of EL at 100 µM with 9 expressed human UGT isoforms (UGT1A1, 1A3, 1A4, 1A6, 1A8, 1A9, 1A10, 2B7, 2B15), only human UGT1A4 and UGT1A6 failed to catalyze EL glucuronidation, while UGT2B7 and UGT1A9 showed the highest catalytic activities, followed by 1A8 and 1A10, with minor contributions from UGT1A1, 1A3 and 2B15\textsuperscript{115}. From the foregoing reports, it is known UGT1A9 and UGT2B7 are substantially expressed in the liver, whereas UGT1A8 and UGT1A10 are specific for intestine, which implies liver, as expected, is the primary organ to transform EL into glucuronides; however, the UGT isoforms such as UGT1A8 and UGT1A10 in the intestine also play a crucial role in EL glucuronidation.

The human intestinal microsomes used in my project were prepared from duodenal and jejunal tissues. The distribution of UGT is not well characterized along the small intestine in the literature. Nevertheless, based on the catalytic UGT activities examined in the small intestine and liver using 18 substrates, it was suggested that the catalytic activities were greatest in the jejunum, and the proximally located duodenum showed higher catalytic activities than the distally located ileum in 16 substrates\textsuperscript{161}. Therefore, the combination of duodenum and jejunum is able to largely represent the general UGT activity in human small intestine. Furthermore, the finding that the catalytic activities using 18 substrates in the jejunum were universally greater than those found in liver suggested that extrahepatic glucuronidation in small intestine can function to complement hepatic glucuronidation\textsuperscript{161}. Although the liver has been long considered as the major organ responsible for the xenobiotic glucuronidation, the small intestine, as the first site of oral exposure, could possibly alter
first-pass metabolism and affect bioavailability in humans. Thus, omitting the role of human intestinal glucuronidation will underestimate overall metabolic clearance\textsuperscript{165}.

5.3.2. Rat Liver versus Rat Intestine

In rat, 7 functional members of UGT1 family and 7 UGT2 isoforms have been identified\textsuperscript{119,166} (Table 5.2). However, the tissue distribution of rat UGT1 and UGT2 isoforms has not been thoroughly examined, especially UGT2 family. Similar to human liver, several UGT2B members are mainly expressed in rat liver including UGT2B1, 2B2, 2B3, 2B6 and 2B12\textsuperscript{167,168}. In contrast to rat UGT2B family, of the seven members of the UGT1A family, only UGT1A1, 1A5 and 1A6 are detected at an appreciable level in rat liver\textsuperscript{167,169}. In my project, the estimated hepatic CL\textsubscript{int} was ten times higher than that of rat intestinal microsomes including both rat jejunum and colon microsomes. The substantial expression of UGT in rat liver may explain the large amount of glucuronidation by the liver. Another study that evaluated the glucuronidation of several aglycones in small intestinal microsomes of rats and compared these with glucuronidation in liver microsomes also suggested the intestinal activities were much lower than in liver, representing 5-15\% of hepatic activities\textsuperscript{170}, which was consistent with my results. Moreover, the UDPGA concentrations in rat liver and small intestine were 400 and 100 nmol/g tissue, respectively\textsuperscript{171}, providing supportive evidence that rat liver is more competent to mediate glucuronidation than small intestine.

Multiple UGT1 family members are expressed in intestine, with UGT1A2, 1A3 and 1A7 as the predominant isoforms\textsuperscript{167,169} (Table 5.2). Unlike with the UGT1 family, few UGT2B subfamily members are detected, while only UGT2B8 is the principal isoform expressed in duodenum\textsuperscript{167,168}. Quantitative evaluation of RNA levels by Northern blot revealed gradients of expression, with highest UGT1A mRNA levels found in duodenum and decreasing levels in the distal small and large intestine\textsuperscript{169}. The aforementioned data suggest rat duodenum contributes more to UGT activities than other sections in intestine. However, this is somewhat against my finding that rat duodenum made the least contribution to EL glucuronidation. This discrepancy might be due to the differences in tissue collection. Alternatively, the isoforms responsible for EL glucuronidation might demonstrate higher expression in the colon. The
CL_{int} for EL glucuronidation was comparable between rat jejunum and colon (0.0500 and 0.0578, respectively), which suggests similar UGT activity for EL glucuronidation might exist between rat jejunum and colon.
<table>
<thead>
<tr>
<th>Tissue</th>
<th>Liver (GAPDH 94.8 ± 0.0)</th>
<th>Small intestine (GAPDH 124 ± 0.7)</th>
<th>Colon (GAPDH 59.4 ± 1.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A1</td>
<td>1430 ± 26.4</td>
<td>582 ± 9.7</td>
<td>372 ± 9.4</td>
</tr>
<tr>
<td>1A3</td>
<td>131 ± 5.4</td>
<td>24.4 ± 1.5</td>
<td>N.D.</td>
</tr>
<tr>
<td>1A4</td>
<td>618 ± 3.8</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>1A5</td>
<td>8.59 ± 2.99</td>
<td>71.6 ± 4.1</td>
<td>45.6 ± 13.4</td>
</tr>
<tr>
<td>1A6</td>
<td>468 ± 5.4</td>
<td>94.5 ± 2.7</td>
<td>N.D.</td>
</tr>
<tr>
<td>1A7</td>
<td>5.07 ± 4.00</td>
<td>26.3 ± 1.0</td>
<td>25.1 ± 4.0</td>
</tr>
<tr>
<td>1A8</td>
<td>N.D.</td>
<td>70.0 ± 3.5</td>
<td>117 ± 8.4</td>
</tr>
<tr>
<td>1A9</td>
<td>1210 ± 9.7</td>
<td>38.3 ± 4.4</td>
<td>50.0 ± 2.7</td>
</tr>
<tr>
<td>1A10</td>
<td>N.D.</td>
<td>968 ± 28.4</td>
<td>754 ± 32.4</td>
</tr>
<tr>
<td>2B4</td>
<td>37,900 ± 711</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>2B7</td>
<td>4220 ± 12.7</td>
<td>1930 ± 7.4</td>
<td>2210 ± 30.3</td>
</tr>
<tr>
<td>2B10</td>
<td>3380 ± 92.7</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>2B11</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>2B15</td>
<td>18,500 ± 285</td>
<td>738 ± 168.5</td>
<td>543 ± 132.8</td>
</tr>
<tr>
<td>2B17</td>
<td>197 ± 12.6</td>
<td>2680 ± 2.8</td>
<td>3960 ± 20.2</td>
</tr>
</tbody>
</table>

Values shown are copy number \( \times 10^4 \) normalized with GAPDH (glyceraldehyde-3-phosphate dehydrogenase), mean ± S.D.

Table 5.2 The expression of UGT in rat tissues at the detection levels\(^{167,169}\)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>UGT</th>
<th>UGT1</th>
<th>UGT2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>1A1, 1A2, 1A3, 1A5, 1A6, 1A7, 1A8</td>
<td>2A1, 2B1, 2B2, 2B3, 2B6, 2B8, 2B12</td>
<td></td>
</tr>
<tr>
<td>Rat liver</td>
<td>1A1, 1A5, 1A6, 1A8</td>
<td>2B1, 2B2, 2B3, 2B6, 2B12</td>
<td></td>
</tr>
<tr>
<td>Rat small intestine</td>
<td>1A1, 1A2, 1A3, 1A6, 1A7</td>
<td>2B3, 2B8, 2B12</td>
<td></td>
</tr>
<tr>
<td>Rat colon</td>
<td>1A1, 1A2, 1A3, 1A6, 1A7</td>
<td>2B12</td>
<td></td>
</tr>
</tbody>
</table>
5.4. Interspecies Differences on EL Glucuronidation

Interspecies differences in EL glucuronidation were also observed in my study. Rat is frequently used as an experimental model to evaluate the pharmacokinetic properties of xenobiotics including lignans, the data of which is significant to explore species differences to reduce the uncertainties related to data extrapolation from animals to humans. In my study, quantitative determination of EL glucuronidation was performed to determine the suitability of rat as a model to evaluate EL metabolism in humans. My evaluations suggested that some species discrepancies existed, especially with regard to intestinal metabolism. Based on the data, CL\textsubscript{int} value of human liver was only slightly higher than the value from rat liver, while the human intestinal CL\textsubscript{int} was much greater than the value either from RJM or RCM. Though the commercial human intestinal microsomes were a mixture of duodenum and jejunum tissues, the relatively great differences in CL\textsubscript{int} values imply human intestinal glucuronidation of EL was higher than that of rat intestine. These data suggests the human intestine might have a greater influence on the first-pass metabolism of EL, thereby decreasing EL’s oral bioavailability. Therefore, rat can be used as a reasonable model to evaluate the hepatic glucuronidation of EL occurring in humans, while the extent of intestinal glucuronidation of EL in humans might be underestimated when experiments are carried out on rats.

5.5. The Significance of $K_m$ Value

$K_m$ is defined as the substrate concentration that yields half the theoretical maximal velocity at infinite substrate concentration. The pharmacokinetic parameters $K_m$, $V_{max}$ and $CL\textsubscript{int}$, play an important role in drug discovery and development, which aids to understand the importance of xenobiotic metabolizing enzymes in the clearance of new drug entity, thereby enabling the assessment of potential drug-drug interactions, the influence of genetic polymorphisms, and also possible non-linear dose-exposure$^{172}$. Non-linear dose exposure is not solely dependent on but is significantly related to $K_m$$^{173}$. $K_m$ reflects the potential of a drug’s ability to saturate, or partially saturate, a specific enzyme in metabolism. The lower the $K_m$ value, the greater the affinity of the substrate to the enzyme, which might cause saturation of a metabolic clearance pathway at low drug concentrations depending upon enzyme capacity.
(i.e. \( V_{\text{max}} \))\textsuperscript{173}. This can be problematic with non-linear dose-exposure relationships, particular for drugs with low-therapeutic indices. Examples include cyclophosphamide, 5-fluorouracil, fluvoxamine, indinavir, and nefazodone, where a small increase in dose might cause toxicity as drug concentration \textit{in vivo} will increase to a greater extent than expected\textsuperscript{154}. The \( K_m \) values for HLM and HIM are 8.91 and 12.6 \( \mu \text{M} \), respectively, which are at least 2-fold smaller when compared with the values from RLM or rat intestinal microsomes (Table 4.3), implying EL has a higher affinity towards UGT enzymes in human liver and intestine than in rat tissues. Alternatively, this could reflect simple experimental variation. The higher affinity of EL towards UGT might result in drug-drug interactions with glucuronide drugs such as morphine. More than one UGT enzyme such as UGT2B7, 1A1, and 1A8 are suggested to metabolize morphine into the more potent and active metabolite morphine-6-glucuronide and inactive morphine-3-glucuronide, with a \( K_m \) value over 60 \( \mu \text{M} \textsuperscript{174-176} \). Thus, the higher affinity of EL towards UGT might cause saturation of UGT enzymes or competitive inhibition, resulting in the decreased analgesic effects of morphine. Consequently, lignans or the initial dose of lignans should be regarded cautiously when coadministered with glucuronide drugs where the principal metabolic pathway involves UGT enzymes. Given only a two-fold difference in \( K_m \) values, the rat might be a suitable model to estimate the interactions between EL and a coadministered compound.
6. CONCLUSIONS AND FUTURE WORK

The purpose for this study is to provide an estimate of the relative contribution of the intestine and liver to presystemic glucuronidation of enterolactone (EL), thereby understanding the critical information regarding the oral absorption properties of EL. To fulfill this goal, *in vitro* enzyme kinetic studies using intestinal and liver microsomes from human and rat with determination of intrinsic clearance (CL$_{int}$) using the substrate depletion approach was conducted to allow the comparison of differences of EL glucuronidation between the two first-pass metabolism organs and among these two species. Prior to carrying out the enzyme kinetic studies, it was necessary to make sure EL glucuronidation was able to occur in both human and rat intestinal and liver microsomes. In addition, an analytical method required validation to quantify EL in microsomes. Therefore, three objectives were established for my project.

For my first objective, EL glucuronide (EL-Glu) was enzymatically generated by incubating EL with intestinal and liver microsomes from both human and rat. The formation and molecular identity of EL-Glu were substantiated by liquid chromatography-mass spectrometry (LC-MS). MS analysis suggested that two monoglucuronides were formed for EL incubated with rat liver microsomes (RLM), while only a single monoglucuronide was found for rat intestinal, human liver and intestinal microsomes.

For my second objective, a simple and reliable HPLC method, which was developed in our lab, was employed for the quantitative determination of EL in RLM. The specificity was determined in the matrices including rat and human intestinal and liver microsomes. The results showed no interfering endogenous substances existed. The intra- and interday precision and accuracy for EL were $\leq 10\%$, suggesting this HPLC method was precise and accurate to quantify EL in rat and human liver and intestinal microsomes.

For my final objective and the aim for my project, the *in vitro* CL$_{int}$ values of EL glucuronidation were examined using RLM, rat jejunum microsomes (RJM) and rat colon microsomes (RCM) extracted by myself in the lab and commercial sources of human liver microsomes (HLM) and human intestinal microsomes (HIM) by the substrate depletion approach. Overall, EL hepatic glucuronidation was greater than intestinal glucuronidation in
both human and rat, indicating liver is the predominant organ responsible for EL glucuronidation as expected. The in vitro CL_{int} value of HLM was slightly higher than that of RLM. CL_{int} value of human intestine was one third of human liver, suggesting the human intestine is likely to make a great contribution to the first-pass glucuronidation of EL, which might largely reduce the oral bioavailability of EL. The hepatic CL_{int} value of rat was twenty times higher than that of the RJM or RCM, respectively. RJM and RCM gave comparable CL_{int} values but were much lower than that of HIM. In brief, these results indicate rat can be used as models to evaluate hepatic glucuronidation of EL in humans, while experiments on rats may underestimate the extent of intestinal glucuronidation of EL that occurs in humans.

In future, the UGT isoforms and their abundance responsible for EL glucuronidation in human liver and intestine need to be identified. This will provide insight into the importance of specific UGT enzymes to the pharmacokinetic properties of EL. Furthermore, the identification of specific UGT isoforms responsible for EL glucuronidation will potentially help avoid drug-drug interactions when lignans are coadministred with drugs or other Natural Health Products (NPHs) that are predominantly metabolized by the same types of UGT. Pharmacokinetic information of EL-Glu needs to be investigated, as EL-Glu is the principal forms in human plasma. The determination of biological properties of EL-Glu will assist us to understand the lignan forms mediating the health beneficial effects. Additional pharmacokinetic information of EL needs to be thoroughly examined so that lignans can be consumed safely and efficiently.
7. REFERENCES


14. Mohamed ME, Frye RF. Inhibitory effects of commonly used herbal extracts on UDP-glucuronosyltransferase 1A4, 1A6, and 1A9 enzyme activities. *Drug Metab Dispos*. 2011;39(9):1522-1528.


## APPENDIX I

Table A.1 The natural log percent remaining for EL at multiple time points when a low EL concentration was incubated with rat liver microsomes

<table>
<thead>
<tr>
<th>Rat liver microsomes</th>
<th>The value of peak area ratios at multiple time points was normalized to t=0</th>
<th>Percent</th>
<th>Ln percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak area ratios of analyte/IS at time t=0 min</td>
<td>1.32</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Peak area ratios of analyte/IS at time t=2.5 min</td>
<td>0.72</td>
<td>0.541</td>
<td>54.1</td>
</tr>
<tr>
<td>Peak area ratios of analyte/IS at time t=5 min</td>
<td>0.47</td>
<td>0.355</td>
<td>35.5</td>
</tr>
<tr>
<td>Peak area ratios of analyte/IS at time t=7.5 min</td>
<td>0.28</td>
<td>0.209</td>
<td>20.9</td>
</tr>
<tr>
<td>Peak area ratios of analyte/IS at time t=10 min</td>
<td>0.14</td>
<td>0.109</td>
<td>10.9</td>
</tr>
<tr>
<td>Peak area ratios of analyte/IS at time t=12.5 min</td>
<td>0.07</td>
<td>0.054</td>
<td>5.4</td>
</tr>
<tr>
<td>Peak area ratios of analyte/IS at time t=15 min</td>
<td>0.03</td>
<td>0.025</td>
<td>2.5</td>
</tr>
</tbody>
</table>

A low EL concentration (25 µM) was chosen as an example to show the process of generating ln percent remaining, then to further plot a linear line to obtain $k_{dep}$ value for each EL concentration.

![Figure A.1 A plot of natural log percent remaining versus time](image)

$$y = -0.2408x + 4.6913$$

$R^2 = 0.99165$

$k_{dep} = -0.2408$
Table A.2 The natural log percent remaining for EL at multiple time points when a high EL concentration was incubated with rat liver microsomes

<table>
<thead>
<tr>
<th>Rat liver microsomes</th>
<th>The value of peak area ratios at multiple time points is normalized to t=0</th>
<th>Percent</th>
<th>Ln percent</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>300 µM</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak area ratios of analyte/IS at time t=0 min</td>
<td>14.97</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Peak area ratios of analyte/IS at time t=5 min</td>
<td>11.80</td>
<td>0.788</td>
<td>78.8</td>
</tr>
<tr>
<td>Peak area ratios of analyte/IS at time t=10 min</td>
<td>10.70</td>
<td>0.715</td>
<td>71.5</td>
</tr>
<tr>
<td>Peak area ratios of analyte/IS at time t=15 min</td>
<td>9.62</td>
<td>0.643</td>
<td>64.3</td>
</tr>
<tr>
<td>Peak area ratios of analyte/IS at time t=20 min</td>
<td>8.49</td>
<td>0.567</td>
<td>56.7</td>
</tr>
<tr>
<td>Peak area ratios of analyte/IS at time t=25 min</td>
<td>7.21</td>
<td>0.482</td>
<td>48.2</td>
</tr>
<tr>
<td>Peak area ratios of analyte/IS at time t=30 min</td>
<td>5.77</td>
<td>0.385</td>
<td>38.5</td>
</tr>
</tbody>
</table>

A high EL concentration (300 µM) was chosen as an example to show the process of generating ln percent remaining, then to further plot a linear line to obtain $k_{dep}$ value for each EL concentration.

![Figure A.2 A plot of natural log percent remaining versus time](image)

\[ y = -0.0291x + 4.5754 \]

\[ R^2 = 0.9817 \]

\[ k_{dep} = -0.0291 \]
Table A.3 Various EL concentrations (10 - 500µM) versus percent remaining for rat liver microsomal kinetic study

<table>
<thead>
<tr>
<th>EL Concentration (µM)</th>
<th>Final percent remaining</th>
<th>(k_{dep}) (min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>2.6</td>
<td>0.362</td>
</tr>
<tr>
<td>25</td>
<td>2.5</td>
<td>0.241</td>
</tr>
<tr>
<td>50</td>
<td>1.7</td>
<td>0.198</td>
</tr>
<tr>
<td>75</td>
<td>5.5</td>
<td>0.141</td>
</tr>
<tr>
<td>100</td>
<td>5.4</td>
<td>0.0916</td>
</tr>
<tr>
<td>200</td>
<td>35.6</td>
<td>0.0335</td>
</tr>
<tr>
<td>300</td>
<td>38.5</td>
<td>0.0291</td>
</tr>
<tr>
<td>500</td>
<td>70.8</td>
<td>0.0110</td>
</tr>
</tbody>
</table>

Table A.4 Various EL concentrations (1.5 - 100µM) versus percent remaining for rat jejunum microsomal kinetic study

<table>
<thead>
<tr>
<th>EL Concentration (µM)</th>
<th>Final percent remaining</th>
<th>(k_{dep}) (min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>42.6</td>
<td>0.0273</td>
</tr>
<tr>
<td>3</td>
<td>58.2</td>
<td>0.0208</td>
</tr>
<tr>
<td>6</td>
<td>55.3</td>
<td>0.0195</td>
</tr>
<tr>
<td>10</td>
<td>57.7</td>
<td>0.0177</td>
</tr>
<tr>
<td>20</td>
<td>60.1</td>
<td>0.0159</td>
</tr>
<tr>
<td>30</td>
<td>64.5</td>
<td>0.0139</td>
</tr>
<tr>
<td>50</td>
<td>78.1</td>
<td>0.0086</td>
</tr>
<tr>
<td>100</td>
<td>78.3</td>
<td>0.0077</td>
</tr>
</tbody>
</table>

Table A.5 Various EL concentrations (1.5 - 100µM) versus percent remaining for rat colon microsomal kinetic study

<table>
<thead>
<tr>
<th>EL Concentration (µM)</th>
<th>Final percent remaining</th>
<th>(k_{dep}) (min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>45.8</td>
<td>0.0309</td>
</tr>
<tr>
<td>4.5</td>
<td>45</td>
<td>0.0248</td>
</tr>
<tr>
<td>6</td>
<td>44.7</td>
<td>0.0239</td>
</tr>
<tr>
<td>10</td>
<td>48.4</td>
<td>0.0229</td>
</tr>
<tr>
<td>20</td>
<td>52.5</td>
<td>0.0201</td>
</tr>
<tr>
<td>50</td>
<td>58</td>
<td>0.0164</td>
</tr>
<tr>
<td>100</td>
<td>77.9</td>
<td>0.0077</td>
</tr>
</tbody>
</table>
Table A.6 Various EL concentrations (10 - 500µM) versus percent remaining for human liver microsomal kinetic study

<table>
<thead>
<tr>
<th>EL Concentration (µM)</th>
<th>Final percent remaining</th>
<th>$k_{dep}$ (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1.5</td>
<td>0.276</td>
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<tr>
<td>25</td>
<td>1.3</td>
<td>0.165</td>
</tr>
<tr>
<td>37.5</td>
<td>2.3</td>
<td>0.117</td>
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<tr>
<td>50</td>
<td>5</td>
<td>0.0917</td>
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<tr>
<td>75</td>
<td>16.2</td>
<td>0.0556</td>
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<tr>
<td>100</td>
<td>27.5</td>
<td>0.0402</td>
</tr>
<tr>
<td>150</td>
<td>42.3</td>
<td>0.0277</td>
</tr>
<tr>
<td>200</td>
<td>45.3</td>
<td>0.0238</td>
</tr>
<tr>
<td>300</td>
<td>66.5</td>
<td>0.0127</td>
</tr>
<tr>
<td>500</td>
<td>80.7</td>
<td>0.0070</td>
</tr>
</tbody>
</table>

Table A.7 Various EL concentrations (10 - 300µM) versus percent remaining for human intestinal microsomal kinetic study

<table>
<thead>
<tr>
<th>EL Concentration (µM)</th>
<th>Final percent remaining</th>
<th>$k_{dep}$ (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>6.4</td>
<td>0.0871</td>
</tr>
<tr>
<td>17.5</td>
<td>14.6</td>
<td>0.0601</td>
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<tr>
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<td>17.4</td>
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<td>25.4</td>
<td>0.0414</td>
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<tr>
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<td>0.0317</td>
</tr>
<tr>
<td>75</td>
<td>50.5</td>
<td>0.0223</td>
</tr>
<tr>
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<td>0.0138</td>
</tr>
<tr>
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<td>0.0119</td>
</tr>
<tr>
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<td>79</td>
<td>0.0077</td>
</tr>
<tr>
<td>300</td>
<td>84.9</td>
<td>0.0051</td>
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</tbody>
</table>